New insights into the association between carotenoids, polyphenols and vitamin A, and highly prevalent diseases

María Marhuenda Muñoz

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

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MARÍA MARHUENDA MUÑOZ

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Dra. Rosa M. Lamuela-Raventós i Dra. Miriam Martínez-Huélamo (directores)

María Marhuenda-Muñoz (doctoranda)

MARÍA MARHUENDA MUÑOZ

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Abstract

The present dissertation aims at broadening current knowledge regarding the importance of bodily or tissue status of bioactive compounds that are highly abundant in the Mediterranean diet (carotenoids and polyphenols) and vitamin A on chronic and communicable disease. The current literature does not extensively speak to the association between carotenoid intake and fat intake on plasma concentrations of these bioactive compounds. In addition, the association of dietary carotenoids and polyphenols with health outcomes have mainly been investigated based on intake, and less on actual circulating or excreted concentrations. Lastly, vitamin A cell biology has been thoroughly studied within the liver but is less well studied in the lungs, where it is important for mounting responses to external injuries.

We investigated the relationship between intake and plasma concentrations of carotenoids in a cross-sectional study of a senior free-living population at high risk of developing cardiovascular disease and with no dietary restrictions. High consumption of fruits and vegetables was associated with higher systemic levels of total carotenoids, particularly when fat intake was low-to-moderate, but contrary to what we were expecting, not when fat intake was very high. In this same population, circulating carotenoid concentrations correlated with lower plasma triglycerides and fitter fatty acid profile (higher polyunsaturated fatty acids and lower saturated fatty acids). In women, carotenoid concentrations were also correlated with higher plasma HDL-cholesterol and in men with lower fasting plasma glucose. In a similar population, we found that urinary hydroxybenzoic acid glucuronide was associated with a lower likelihood of developing type 2 diabetes when baseline plasma glucose was not considered. Following the COVID-19 outbreak, we performed experiments in mice to understand the cell biology of vitamin A within the lung, where it might have a role in the maintenance of lung functions when the tissue is injured. We discovered that local, but not systemic, retinoid stores in the lung are critical in the metabolic maintenance of this tissue during acute lung injury. Based on their transcriptional signatures, we also identified ten distinct cell-types that store retinoids. In addition, we established that dietary retinol supplementation during acute lung injury reduces lethality.

The results of the studies presented in this work suggest that carotenoids, polyphenols, and vitamin A may be early markers of disease risk or prognosis.

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Abbreviations

ALDH1A: Aldehyde dehydrogenase

ALI: Acute lung injury

AMAC: Ammonium acetate

ANOVA: Analysis of variance

APCI: Atmospheric pressure chemical ionization

BAL: Bronchoalveolar

BCO1: β-carotene-15,15'-oxygenase

CBG: Cytosolic β-glucosidase

CCA: Canonical correlation analysis

CD36: Cluster Determinant 36

CE: Cholesterol ester

CI: Confidence interval

CM: Chylomicron

CRP: C-reactive protein

CYP26: Cytochrome P450, type 26

DASH: Dietary Approaches to Stop Hypertension

DEG: Differential gene expression

DGAT1: Diacylglycerol acyltransferase 1

DMEM: Dulbecco's Modified Eagle Medium

ELISA: Enzyme linked immune assay

ESI: Electro-spray ionization

EVLW: Extravascular lung water

EVOO: Extra virgin olive oil

F&V: Fruit and vegetable

FACS: Fluorescence activated cell sorting

FBS: Fetal bovine serum

FSC: Forward scatter

FTMS: Fourier transformed mass spectrometry

GLUT4: Glucose transporter 4

H&E: Hematoxylin/eosin

HBSS: Hanks' balanced salt solution

HDL: High-density lipoprotein

HPLC: High pressure liquid chromatography

KNN: K-nearest neighbor

LDL: Low-density lipoprotein

LoD: Limit of detection

LoQ: Limit of quantitation

LPH: Lactase-phlorizin hydrolase

LPL: Lipoprotein lipase

LPS: Lipopolysaccharide

LRAT: Lecithin:retinol acyltransferase

MET: Metabolic task equivalents

MNN: Mutual nearest neighbors

MPM: Microbial phenolic metabolite

MRM: Multiple reaction monitoring

MS: Mass spectrometry

MS/MS or MS2: Tandem mass spectrometry

MTBE: Methyl tert-butyl ether

MUFA: Monounsaturated fatty acids

NPC1L1: Niemann-Pick C1-Like 1

OR: Odds ratio

PBS: Phosphate-buffered saline

PCA: Principal component analysis

PCR: Polymerase chain reaction

PREDIMED: Prevención con Dieta Mediterránea

PUFA: Polyunsaturated fatty acids

PVDF: Polyvinylidene fluoride

qRT-PCR: Quantitative polymerase chain reaction

RalR: Retinaldehyde reductase

RAR: Retinoic acid receptor

RBP4: Retinol-binding protein 4

RDH: Retinol dehydrogenase

RE: Retinyl ester

REH: Retinyl ester hydrolase

ROH: Retinol

scRNA-seq: Single cell RNA sequencing

SD: Standard deviation

SDS PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFA: Saturated fatty acids

SGLT1: Sodium-dependent glucose transporter 1

SR-BI: Scavenger Receptor-class B type I

SSC: Side scatter

STRA6: Stimulated by retinoic acid 6

UMAP: Uniform manifold approximation and projection

UMI: Unique molecular identifier

UPLC: Ultra performance liquid chromatography

Introduction

Global burden of disease

The concept of health burden is one of the simplest ways to assess the health status of a population. It provides a measure of what people die from, their mortality, and also provides information regarding illness(es) that cause them suffering while they are alive, a concept known as morbidity (1). Nowadays there are three categories of health burden: communicable diseases, that spread from one individual to another; non-communicable diseases, also known as chronic diseases; and injuries, caused by an external force.

Many diseases are preventable or can, at least, be alleviated to reduce the burden they pose. Studying the underlying causes of disease and addressing modifiable risk factors is the most powerful tool in the hands of health professionals for preventing the health burden from growing worse.

Cardiovascular disease and type 2 diabetes

The last Global Burden of Disease report, published in 2019, showed that more than 65 % of the global burden was due to non-communicable diseases, with cardiovascular disease being the leading one by far (2). In addition, following a significant increase of 70 % since the year 2000, type 2 diabetes entered the list of the top 10 causes of death (3).

Cardiovascular disease is actually a cluster of diseases that affect the heart and blood vessels. The most common forms of this disease, coronary heart disease and stroke, are caused by a lack of blood flowing into the heart and brain, leading to an insufficiency of oxygen supply to those essential organs (4–6). This situation is usually caused by a vessel blockage arising from the deposit and accumulation of fat in the inner walls of the vessels, a process known as atherosclerosis (7,8). Bleeding from the vessels or heart malfunction are other common reasons of oxygen insufficiency in a tissue (9).

Poverty, stress, and hereditary factors are underlying determinants in the development of cardiovascular diseases, but the major recognized risk factors are behavioral, related to the diet, substance abuse and physical activity (4). In the Global Burden of Disease 2019 report, high blood pressure, elevated fasting plasma glucose, obesity and overweight were also highlighted as risk factors which may increase the risk of

death by weakening the immune system and increasing the risk of inflammation. This process is seen as a quiet pandemic that poses a great threat to future health progress (10,11). Indeed, high blood pressure and elevated plasma glucose, among others, are known as intermediate risk factors because they are often the clinical manifestation of unhealthy behaviors that contribute to the development of atherosclerosis and vessel leakage.

High systolic blood pressure has long been known to be the risk factor that accounts for almost half of the healthy years of life lost worldwide (11). Related to this, vascular inflammation (12), endothelial disfunction (13) and osmotic imbalance related to the renin-angiotensin system (12,14) are different mechanisms that have been proposed to account for the rise in peripheral resistance. Obesity and overweight are themselves inflammatory states that can also contribute to these conditions (15–18).

Disturbances in the blood lipid profile can also play a role in the development of cardiovascular diseases. Elevated circulating triglycerides and low-density lipoprotein (LDL)-cholesterol contribute to the progression of atherosclerosis, infiltrating into the intima layer of arteries and accumulating there (19,20). In the intima, they become oxidized and create a proinflammatory environment that can result in the generation of a necrotic core and the narrowing of the lumen of the vessel (7).

Elevated fasting plasma glucose is both a key risk factor of cardiovascular disease and a risk factor and marker for a separate disease directly related to glucose: diabetes. Like obesity and overweight, uncontrolled diabetes creates an inflammatory environment that weighs in the development of cardiovascular disease (21).

Diabetes is defined as the impairment of glucose metabolism due to a malfunctioning of insulin, the hormone that regulates blood glucose levels. This defect leads to an increase in circulating glucose that, when prolonged in time, increases the susceptibility of cells to oxidative stress, which creates a proinflammatory and prothrombotic environment (22,23). There are different types of diabetes depending on where the problem in insulin action occurs: when the body is unable of synthetizing it, the disease is known as type 1, while type 2 diabetes is the condition where the body cannot respond to insulin effectively. There is also a gestational form of diabetes that appears during pregnancy because of hormonal alterations that block the binding of insulin to its receptor, as well as other minor forms known as secondary diabetes (24).

Type 2 diabetes is, by far, the diabetes form with the highest incidence worldwide (25). Although the precise causes of this disease are not yet fully understood, both a number of genetic variations as well as impaired molecular mechanisms have been proposed to be associated with a decrease in insulin responsiveness (26–28). Interestingly, this type of diabetes is the only one that largely arises from poor behavioral practices (25).

Acute lung injury

Returning to the global health burden, more than 25 % is due to communicable diseases. Neonatal disorders were the most frequent communicable diseases, closely followed by lower respiratory tract infections that were, in fact, the first cause of death in this category (2). In early 2020, though, the world was shaken by SARS-CoV-2, a new coronavirus (29). Which, although there is still much data to process, will likely be the leading communicable disease in that year (30,31). This is due to globalization but also because of the lack of preparation and coordination of all health-care systems worldwide, independent of national public health strategies (32).

The acute lung injury and respiratory distress elicited by lower respiratory tract infections are usually the cause of death related to these communicable diseases (33), the bacterial genus *Streptococcus pneumoniae* being the infectious organism that causes the highest morbidity and mortality worldwide. Another bacterium (*Haemophilus influenzae*) and the viruses that cause the flu also play an important role (34). In addition, although minor up until now, some species from the coronavirus family, which is mostly involved in the development of the common cold, have proven their lethality by targeting the lower respiratory tract (35,36). The last and most lethal outbreak of these kind of viruses, SARS-CoV-2, is still placing a great burden on humankind, for more than two years now (37,38).

The lower respiratory tract, specifically, the alveoli -a structure composed of a mixture of cell types that perform different functions (39)- is the area of the respiratory system where gas exchange between air and blood takes place. **Figure 1** depicts the cellular diversity of alveolar cell types adapted and simplified from Travaglini et al. (40). Endothelial cells line the blood capillaries, and their basement membranes are fused for maximum efficiency of gas exchange with the ones from epithelial type 1 cells, which line most of the alveolar surface. Type 2 cells, also located in the alveolar

surface, produce surfactant, a mix of phospholipids and proteins that reduce the surface tension in the alveoli, thus preventing them from collapsing. Fibroblasts reside in the interstitium between capillaries and alveoli; macrophages and immune cells can be found in the alveolar space (40,41).

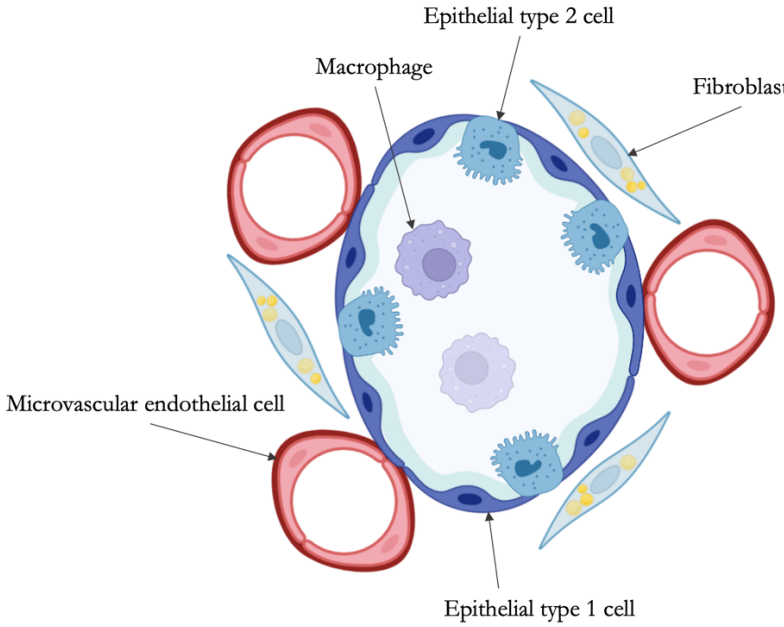

Figure 1. Diagram of a section of the alveolus (adapted and simplified from Travaglini et al. 2020 (40)).

When the lower respiratory tract is insulted, either by one of the aforementioned infective species or by other physical insults, the cells release inflammatory mediators. These signal the immune system, which responds with a migration of immune cells to the tissue, an event that contributes to the amplification of the phenomenon, that is, both the immune cascade and the injury. If the insult is not reversed or eliminated in a timely manner, breathing is compromised resulting in a ubiquitous oxygen deficiency that can result in death (42).

Although great part of the risk for developing and dying from acute lung injury is due to environmental factors and behavioral habits unrelated to diet, malnutrition still accounts for a percentage of the burden (43). In addition, nutrition in an intensive care patient can impact their prognosis, since a high nutritional risk has been seen to

be associated with a significantly higher mortality in cases of acute respiratory distress (44,45).

When the COVID-19 pandemic started, it was treated as a comorbidity, together with non-communicable diseases. Rather, COVID-19 is, in fact, a syndemic; where both previously mentioned types of diseases are combined: a viral communicable infection and an array of non-communicable diseases (46,47). Their adverse effects are combined to become one, so tackling the associated non-communicable diseases is a prerequisite for containing the effects of the virus in a successful way.

Preventive nutrition: the Mediterranean diet

For a long time, all efforts in health management have been put into diagnosing and finding cures for diseases, affairs for which medicine is the spearhead. Every year, immense resources are utilized to alleviate or to cure a range of diseases. In the second half of the 19th century the contributions of Pasteur, Lister and others led to the use of surgical masks to prevent infections, profoundly changing global health burden causes (48). Soon after, the emergence of clinical epidemiology in the 20th century shifted the paradigm, smoking was associated with lung cancer (49) and investigators from large cohorts like the Framingham Study determined a set of factors that were associated with a higher risk of developing heart disease (50). Growing out of these early epidemiological studies, interest in identifying and addressing such factors before they were strong enough to produce disease increased exponentially.

Some risk factors, namely age, gender, or genetics are beyond our control, hence they are called non-modifiable risk factors. On the other hand, nutrition is one of the largest behaviorally modifiable risk factor that contributes to the development and prognosis of non-communicable and communicable diseases, respectively, together with tobacco and alcohol abuse and physical activity (11). While substance abuse is always detrimental (51–53) and physical activity mainly favorable (54,55), diet can act both as friend or foe, depending on its composition. For instance, an unhealthy diet rich in trans and saturated fats and sugar has been associated with the development of metabolic syndrome, cardiovascular disease, type 2 diabetes, and many more non-communicable diseases (56,57). Conversely, diets rich in fruits and vegetables and polyunsaturated fats have shown to have the completely opposite effects, preventing these diseases (58–60). Consequently, similar to political strategies like the regulation

of tobacco usage which has proven to ameliorate the chances of developing non-communicable diseases arising from use of this substance (61), the promotion of a healthy and balanced diet is also central to maintaining good health throughout the lifespan.

Diets such as the Dietary Approaches to Stop Hypertension (DASH), Traditional Asian Diet, Nordic Diet and the Mediterranean Diet differ from the widespread Western diet in that they are largely plant based with some, albeit low, animal protein and fat content and have been associated with positive health outcomes (58,62–64). All share a similar macronutrient distribution, the difference comes, mainly, from the food sources available in the different geographical regions. Coupled to this, the consumption of in-season and local provisions is being promoted as a way for taking care of both health and the environment at the same time, by reducing the carbon emissions associated with long distance shipping and monocultures (65).

For years, the Mediterranean Diet has been recognized as a healthy approach for nourishing the inhabitants of the Mediterranean basin (66). Shown in **Figure 2**, are the updated recommendations for this diet which transcend the nutrient content and introduce social and environmental elements, thus becoming a lifestyle proposal.

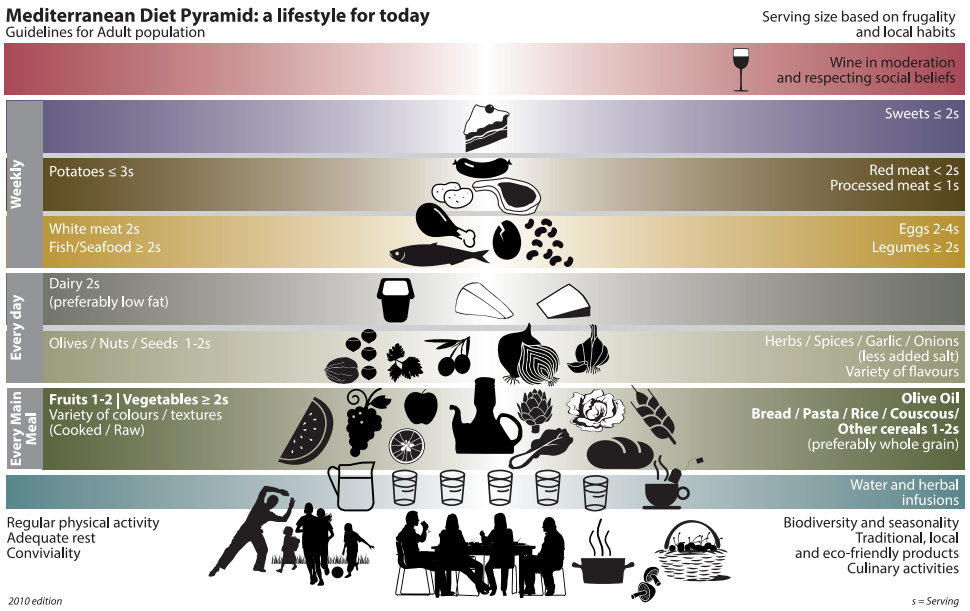

Figure 2. Mediterranean diet pyramid.

These dietary recommendations propose a diet rich in fruits and vegetables and the famed virgin olive oil as its main source of healthy fat. All these dietary components are also rich in bioactive non-nutrients, some of them at highly variable concentrations depending on the climate and stress to which the plants are subject and to the culinary techniques applied to the foods prior to their ingestion (67–70).

Experimental approaches in human health and nutrition research

Advances in understanding human metabolism, physiology and pathophysiology have been traditionally achieved from a combination of different types of studies. Numerous experiments have been undertaken in animals and human beings, prior to the institution of national and international regulations that were instituted to protect the wellbeing of the experimental subjects (71,72). Even though new technologies and approaches are being developed in order to reduce and rationalize the use of animals in research and development, animal models are still a reliable source of knowledge regarding molecular mechanisms in the context of a whole organism and hence scientific progress depends on their use in research.

In vitro and animal model studies

Known as basic or molecular biology, this area of investigation, based on *in vitro* cell culturing and animal model usage, comprises the study of the precise mechanisms by which different molecules are internalized by the body, taken up by the different tissues and how they induce a response at the cellular level. This is possible because of the tight control that the researcher has over the study: animals are housed in a closed space and cells cultured in defined media. What is more, in both cases, the individuals share their genetic background to a large degree (73). Once the experiment is finished, all types of samples can be collected, extracted, and analyzed. For this reason, most variables can be controlled when performing experiments and interpreting results.

The development of genetic engineering in the 1970s allowed for the generation of transgenic animal models, with modifications in one or several genes. Newer techniques, like the Cre-loxP system, broadened these possibilities by enabling the control of these genetic modifications in space and time (74). Nowadays, scientists worldwide

can generate and use genetically modified animal models with alterations in specific tissues, cell types, and even starting from a precise timepoint in life.

When using animal models, the genetically modified animals are compared with their wild type counterparts (often littermates) in order to understand the responses to specific treatments. In the field of nutrition, animal models are usually either subject to a control (usually chow) diet in order to study the role of a gene of interest, or fed particular diets in order to gain understanding of specific molecules, including of their absorption or to assess their bioactivity (75,76). The animal models can also be subject to disease-mimicking insults that can affect their well-being and survival depending on the genetic modification (77).

From these animals, one can also obtain cells and specific cell types from specific tissues for cell culture studies. These, which closely resemble the original cell *in situ,* can be used to ascertain mechanisms that are difficult to study within the whole body. This is referred to as primary culture, as opposed to the more conventional study of immortalized cell lines.

The most common techniques used in basic research are to study gene expression by the polymerase chain reaction (PCR), quantitation of proteins by Western blot, and characterization of metabolic responses such as plasma insulin and fatty acids levels by enzyme linked immune assay (ELISA) or other traditional colorimetry methods after subjecting the animals to a glucose charge (78).

Aside from for culturing, cells can also be sorted according to selected characteristics and undergo processing by new methodologies that allow for the precise characterization of each individual cell. With single cell transcriptomics, which is one of these modern technologies, single cells are tagged and all of their genetic expression (transcriptome) is obtained for study (40,79,80). This information can also be used to create clusters of cells that share certain traits and to assess if they may come from a common progenitor.

Human interventions: the PREDIMED and PREDIMED Plus clinical trials

Unlike in basic biology, it is nearly impossible to control all variables in large interventions in humans. In the field of nutrition, study individuals are, most of the time, volunteers under free living conditions. Even when they are kept under observation in a closed facility, there is a high variability in their genetics and environmental input since birth. Besides, not all types of samples can be obtained from a living person, the main reason why animal models are still very much needed. Nevertheless, as new techniques are developed, more and more information may be gathered from easily obtainable samples like blood and urine (81,82).

There are two broad possibilities when examining the effects of a particular molecule (be it a macronutrient, a micronutrient, or a non-nutrient), food, or even dietary patterns in the general population. One is to simply observe the whole sample. This is known as descriptive epidemiology and is based on the calculation of frequencies (83). These studies assess the incidence of diseases or other events and are useful for detecting trends in the population and developing prevention programs. They may also lead to the formulation of hypotheses that can be tested in analytic studies where the assessment is performed with the presence of a predetermined exposure variable and the population is subject to monitoring with the use of questionnaires and sample data collection. This second possibility in epidemiology has two procedural modes: observational and interventional studies. In observational studies the exposure variable is random or present in the environment, and the researcher follows the population, assessing its response to the exposure variable by the analysis of events and biological samples (84,85). Interventional studies, involve administration of an exposure variable by the investigator in a population with the intent of proving causality of the intervention (86).

In 2003, after some observational studies proposed the hypothesis that a Mediterranean dietary pattern was beneficial for good cardiovascular health (87), investigators from different parts of Spain started a large interventional trial for the primary prevention of cardiovascular events, the PREDIMED Study (Prevención con Dieta Mediterránea). The population of the study, 7447 people aged 55 – 80, disease free but at high risk of developing cardiovascular disease, were randomized into three

groups: a Mediterranean diet supplemented with extra virgin olive oil, a Mediterranean diet supplemented with nuts or a low-fat diet. The final results of this trial, republished in 2018 after some corrections, showed that both Mediterranean supplemented diet groups had a significantly lower incidence of major cardiovascular events (88). The success of this intervention led to the design of the PREDIMED-Plus study, that started in 2013, in which the intervention group implemented a whole healthy Mediterranean lifestyle adding physical activity and behavioral changes to an energy-restricted Mediterranean diet. This intervention, still ongoing, was designed to evaluate the effect of weight loss on cardiovascular health in comparison with a regular Mediterranean diet (89). For this trial, 6874 people between 55 and 75 years old with similar risk conditions as the previous one were recruited. The inclusion criteria for both interventional trials are summarized below (**Table 1**). All risk factors of cardiovascular disease listed in the introduction and the established limit values for these interventions can be seen in the following table.

Table 1. Inclusion criteria of the PREDIMED and PREDIMED-Plus trials.

		PREDIMED	PREDIMED-Plus
Age	Women	60-80	60-75
	Men	55-80	55-75
Risk factors		Type 2 diabetes or at least three of the following:	Overweight or obesity and at least three of the following:
Smoking habit		> 1 cigarette/day in the last month	-
Blood pressure		systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg or antihypertensive medication	systolic ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication
Plasma cholesterol		HDL-cholesterol ≤ 50 mg/dL in women or ≤ 40 mg/dL in men; LDL-cholesterol ≥ 160 mg/dL, or lipid-lowering therapy	HDL-cholesterol < 50 mg/dL in women or < 40 mg/dL in men, or drug treatment for low HDL -cholesterol
Plasma triglycerides		-	≥ 150 mg/dL or drug treatment for elevated triglycerides
Fasting plasma glucose		-	≥ 100 mg/dL or drug treatment for hyperglycemia
Waist circumference		-	≥ 88 cm in women and ≥ 102 cm in men
Body mass index		≥ 25 Kg/m^2	≥ 27 and < 40 Kg/m^2
Family history of premature cardiovascular history		Coronary heart disease	-

In both trials, participants received advice for following their assigned diet and were periodically asked to fill out several forms, under the supervision of a professional, to monitor their socioeconomic situation, physical activity, and dietary habits (89,90). To assess the latter, two questionnaires were used: a Mediterranean diet adherence questionnaire composed of 14 items (91) and a food frequency questionnaire containing 137 food items in the PREDIMED Study (92); and extended versions of these questionnaires comprising 17 items (93) and 143 food items in the PREDIMED-Plus (89). Trained personnel also took anthropometric and blood pressure measurements, and collected blood, urine and nail samples for future analyses (89,90).

The biological samples obtained from these large interventional studies are very valuable because the molecules that they contain are compounds that are (or have been) present inside the body. These molecules can potentially exert a biological activity different from simply serving as energy sources (94). When they present this trait, they are known as bioactive compounds (95). The measurement of substances reflects tissue exposure in a more accurate and objective way than inference based on questionnaires and hence allows for the association with health outcomes. If an association is proven, the bioactive compound can then be used as a biomarker of risk for a certain disease or health status (96). Some biomarkers of risk are recognized risk factors, like blood pressure or cholesterol levels. These are not only associated with cardiovascular disease development (biomarkers of risk) but they also participate in the causal pathway of the disease development (risk factors) (97).

Sometimes, aside from their potential bioactivity, the molecules present in biological samples can be associated specifically with the intake of different foods, and they are called food biomarkers or biomarkers of intake (98). Because of the variety of bioactive compounds in foods, it is not easy to find distinct biomarkers of intake of specific foods. For this reason, food biomarkers work as complementary tools for traditional food intake assessments and not as a replacement for the traditional assessments.

Genes, glucose, lipid profile and different metabolites present in blood or urine from the PREDIMED, and the PREDIMED-Plus trials have been successfully linked to outcomes that range from dietary intake of different foods to disease risk (99–103). When both exposure and outcome variables are from the same timepoint, these associations are cross-sectional, with no possibility of inferring causality. When the outcome comes after the exposure, the association can have predictive value.

Bioactive compounds abundant in the Mediterranean Diet (carotenoids and polyphenols) and vitamin A

As stated before, the Mediterranean Diet is not only interesting for its macro and micronutrient value but also because of its non-nutrient composition. Non-nutrients or bioactive compounds are substances that are not essential for maintaining good health, although they can be sources of essential nutrients, like provitamin A carotenoids, or they may have a positive effect on health due to their transcriptional, antioxidant or yet to be discovered biological activities (104,105).

Carotenoids and vitamin A

Carotenoids are lipophilic compounds synthetized by plants and other autotroph organisms that, because of their ability to absorb light in the visible part of the spectrum, give them their yellow to reddish color (106). When ingested and absorbed, these pigments can have different effects on human health. Some of them have provitamin A activity, which means that the product of their centric cleavage is retinaldehyde, a form of vitamin A, which is an essential micronutrient (107). Some others do not have this trait but have been proven to be very important for eye health due to their antioxidant activity (108), which has been their main recognized trait for a while now, together with their anti-inflammatory action (109). Carotenoids can also be classified based on their chemical composition: xanthophylls contain oxygen whereas carotenes are exclusively hydrocarbon chains (110). **Figure 3** shows these two classifications for the main carotenoids found in human plasma.

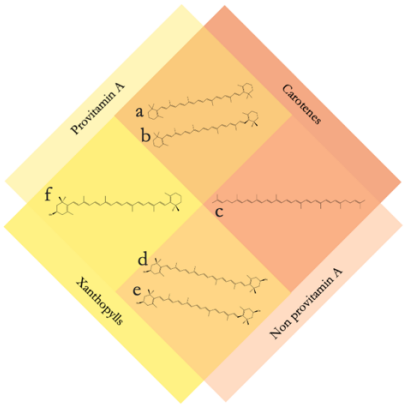

Figure 3. Main carotenoids found in plasma and their two classifications.

a. β-carotene, b. α-carotene, c. lycopene, d. zeaxanthin, e. lutein and f. β-cryptoxanthin.

Because humans cannot synthetize them, carotenoid appearance in plasma has been used as biomarker of consumption of the foods they come from: either from the direct sources, the photosynthetic organisms that synthetize them, or from animals that have ingested and accumulated them. In this way, α-carotene has traditionally been used as a carrot ingestion biomarker (111,112), lycopene has been defined as a biomarker for tomato (113) and watermelon consumption (114), cryptoxanthin for orange and grapefruit consumption (112), and lutein for green leafy vegetables (113). Astaxanthin is quite common in fish and crustaceans because it is mainly synthetized by certain types of marine algae (115). Canthaxanthin, on the other hand, comes mainly from fungi, cyanobacteria and green algae and is usually ingested as a supplement or because of its addition as a food additive or food colorant (116), rather than as a component of a regular human diet where it is present at only very low concentrations. Nevertheless, because they are all present in a wide variety of foodstuffs, most of these molecules are specific biomarkers when other sources of the same carotenoid are absent.

Carotenoids must be released from the food matrix to be absorbed. At this point being in crystalline form within crystalloid chromoplasts, where they are found in photosynthetic organisms, makes them more difficult to release than when they are in a lipid-dissolved form, as usually is the case in animal-based foods. The latter has been described for the more studied carotene group rather than the xanthophyll group (117). Being embedded within the vegetal fibrous structure or sometimes attached to proteins is also a hindering factor for their release and uptake following ingestion. Simple culinary methods such as cutting, homogenization and cooking with oils improve carotenoid availability from the food matrix (70).

Once released from the food matrix, carotenoids are solubilized into oil droplets and further integrated into mixed micelles, which are composed by dietary fats and bile salts released by the liver in response to the increase of fats in the intestine (118). In these, there are also lipases that hydrolyze phospholipids, triglycerides, and esters -all three cholesterol, retinoid, and carotenoid- into their free forms. **Figure 4** depicts the processes that take place from the moment these micelles reach the apical surface of the enterocyte where they interact with brush border proteins. There, the carotenoids can be absorbed in two ways: by active uptake or by passive diffusion, the latter is believed to happen only in supra-physiological concentrations. The membrane

transporters that are responsible for the active internalization of carotenoids are the Scavenger Receptor-class B type I (SR-BI), Niemann-Pick C1-Like 1 (NPC1L1) and the Cluster Determinant 36 (CD36), all three being fat transporters (119).

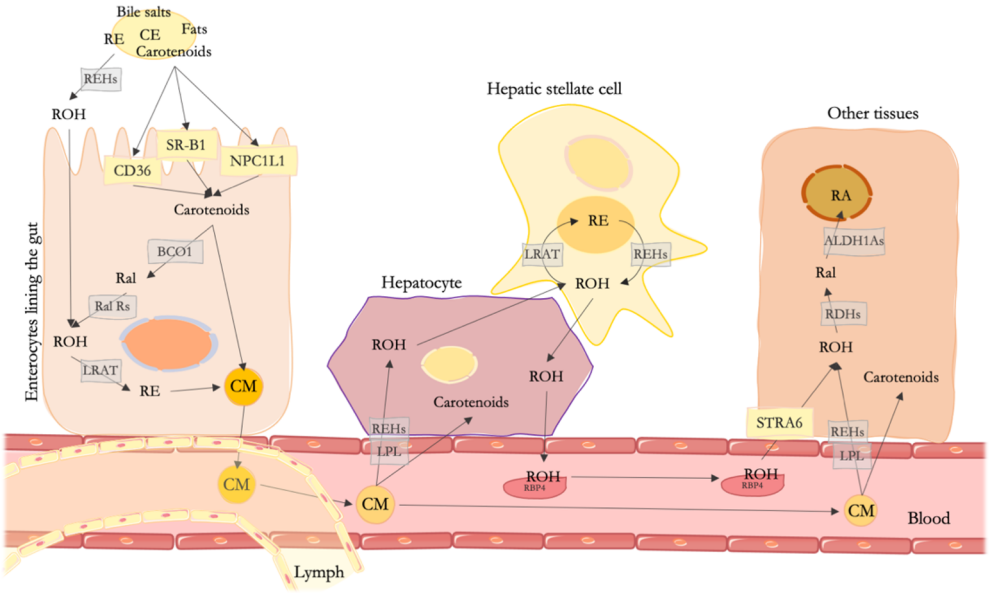

Figure 4. Known absorption and distribution pathways of carotenoids and vitamin A.

CE: cholesterol ester; RE: Retinyl ester; REH: retinyl ester hydrolase; ROH: retinol; CD36: Cluster Determinant 36; SR-B1: Scavenger Receptor-class B type I; NPC1L1: Niemann-Pick C1-Like 1; BCO1: β-carotene-15,15'-oxygenase; RalR: retinaldehyde reductase; LRAT: lecithin:retinol acyltransferase; CM: chylomicron; LPL: lipoprotein lipase, RBP4: retinol-binding protein 4; STRA6: stimulated by retinoic acid 6; RDH: retinol dehydrogenase; ALDH1A: aldehyde dehydrogenase.

Immediately after absorption, carotenoids and vitamin A circulate in the lymph and blood in chylomicrons, from where they are distributed throughout the body both for performing their biological actions but also for storage; more hydrophilic dietary carotenoids like the apocarotenals can move directly to the portal blood system (106).

Due to their lipophilic nature, co-ingestion with fat has been proposed to increase their intestinal absorption, however studies investigating this have mainly been performed *in vitro* and with rather low fat quantities (120–122). Some studies have compared different populations and described their differences in plasma concentrations of carotenoids, mainly due to study subjects' dietary habits (123,124). Nevertheless,

similar to other dietary components, there is great inter-individual variability in carotenoid plasma concentrations even after the same type of meal. Overall, two types of factors influence the appearance of carotenoids in plasma. One is bioaccesibility, the facility with which carotenoids are released from the food matrix, mixed with other food and bolus components and attainable for uptake. The main factors involved in this regard are dietary habits and seasonality; culinary techniques and processing of the foodstuffs; the composition of the food (fat, dietary fiber, proteins and even the mixture of carotenoids) and the gastric and intestinal environment. The second factor is bioavailability, the facility with which carotenoids are taken up and reach the circulation. This is mainly affected by intrinsic host factors such as health and nutritional status, genetic polymorphic variants, and the gut microbial population. In the following chapter the current state of the art regarding these different factors that affect plasma levels of carotenoids in different populations and even within the same population are considered (125).

A review of factors that affect carotenoid concentrations in human plasma: differences between Mediterranean and Northern diets

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REVIEW ARTICLE

A review of factors that affect carotenoid concentrations in human plasma: differences between Mediterranean and Northern diets

María Marhuenda-Muñoz[1,2,3] · Sara Hurtado-Barroso[1,2,3] · Anna Tresserra-Rimbau[3,4] · Rosa Maria Lamuela-Raventós[1,2,3]

Abstract
Carotenoids are naturally occurring pigments of autotroph organisms that have been related to many health benefits and this is not only because some of them are precursors of vitamin A. Individual or whole carotenoid consumption has been associated with a lower risk of developing cancer, cardiovascular and metabolic diseases among others. However, the blood levels of carotenoids vary largely from person to person due to different factors. Diet is the most important one because of the dietary patterns that different populations follow, the time of the year of consumption or the personal preferences. Nevertheless, the intrinsic host factors such as the absorption, distribution, metabolism and excretion genetic polymorphisms, the volume of distribution and the person's microbiota and others such as carotenoid interactions are also inducing this so called inter-individual variability. Besides, culinary methods and processing produce changes in the foods that directly affect carotenoid content and hence their blood profile. Different types of studies have been performed to understand the between-subject variation of the carotenoid profile in human plasma. This research is focused on this matter as levels of carotenoids in human plasma could be useful for the prediction of some diseases. The Mediterranean diet is probably the most carotenoid rich diet stemming from its high proportion of fruits and vegetables. Its differences with other diets and the effect on the carotenoid blood profile of the consumers are currently a very interesting topic of study.

Introduction

Carotenoids are a numerous class of naturally occurring pigments synthesized by autotrophs (plants, algae and photosynthetic bacteria) that are associated with the yellow, orange and red colors of many plants [1]. They are mainly present in vegetable products but might be also found in animal origin foods depending on the livestock diet [2]. There are more than 700 carotenoids in nature; however, only a few, from fruits and vegetables, are ingested in sufficient quantity to be detected in human plasma; the most abundant being lycopene, β-carotene, lutein, α-carotene, β-cryptoxanthin, and zeaxanthin, along with their more common *cis*-isomers and some degradation products. Fig. 1 shows the chemical structures of the major carotenoids that are present in human plasma (see Fig. 1). Approximately 10% of them can be converted by the body to retinol, provitamin A, mainly α-carotene and β-carotene and some xanthophylls such as β-cryptoxanthin; and some apo-carotenoids are provitamin A, having β-carotene the greatest vitamin A activity [2]. Other carotenoids cannot produce retinoids (i.e., zeaxanthin and lycopene). However, they all have in common a long carbon chain terminated at each end by an ionone ring, with the exception of lycopene, that has not the terminal rings [3].

Consumption of carotenoid-rich foods is important since vitamin A deficiency is associated with blindness, reduced immune function [4] and increased risk of mortality [5].

✉ Rosa Maria Lamuela-Raventós
lamuela@ub.edu

[1] Department of Nutrition, Food Sciences, and Gastronomy, School of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain

[2] Biomedical Research Networking Centres in Physiopathology of Obesity and Nutrition (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain

[3] Nutrition and Food Safety Research Institute (INSA-UB), University of Barcelona, Barcelona, Spain

[4] Human Nutrition Unit, Faculty of Medicine and Health Sciences, Department of Biochemistry and Biotechnology, Pere Virgili Health Research Center, University Hospital of Sant Joan de Reus, Universitat Rovira i Virgili, Reus, Spain

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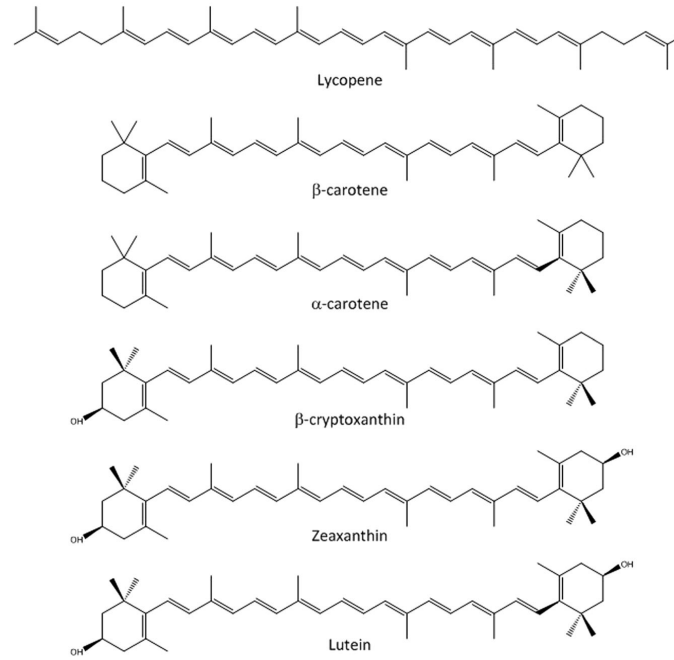

Fig. 1 Chemical structures of the major carotenoids present in human plasma

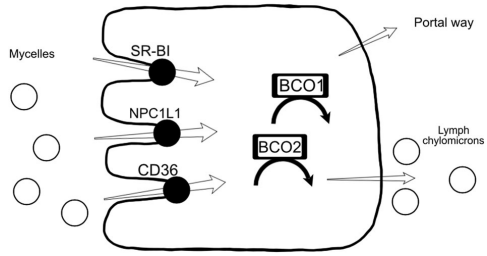

Fig. 2 Proteins involved in carotenoid transport across the human enterocyte

Through other mechanisms, zeaxanthin and lutein also contribute to maintain eye health and they can prevent age-related macular degeneration [6]. Besides the provitamin classification, carotenoids can be also divided into two groups: carotenes and xanthophylls. Xanthophylls, but not carotenes, have oxygen atoms in their molecular structure and so, they are more polar [7]. Most carotenoids are found in food in all-*trans* form; however, processing and cooking can result in the formation of other isomers. Due to their association with proteins in the plant matrix, their bioavailability is relatively low. It can be increased, though, by common culinary methods such as chopping, homogenizing and cooking [8–10]. Their lipophilic profile is also a determinant on the bioavailability, which is why using oil when cooking is also very positive.

Carotenoids are absorbed intestinally after their incorporation into mixed micelles that are composed by bile salts secreted by the liver and several types of lipids coming from the meal. The rate and extent of the absorption appear to be influenced not only by the type and amount of carotenoids but also by the type of fat (medium-chain vs. long-chain triglycerides) and the presence of soluble fiber [11]. The absorption takes place by passive diffusion and also by active uptake by the Scavenger Receptor-class B type I (SR-BI), Niemann-Pick C1-Like 1 (NPC1L1) and the Cluster Determinant 36 (CD36), the three of them being fat transporters [12].

Within the enterocytes, carotenoids can be cleaved by oxygenase enzymes: β-carotene 15,15′-oxygenase 1 (BCO1), which shows a higher affinity for provitamin A carotenoids, and β-carotene 9′,10′-oxygenase 2 (BCO2), whose affinity is higher toward non provitamin A carotenoids [1]. The products of the cleavage and the remaining uncleaved carotenoids are incorporated in the chylomicrons, the lipoproteins that distribute dietary fat and lipophilic vitamins to the different tissues. All of them reach the liver in the chylomicron remnants but more hydrophilic molecules such as apocarotenal can travel directly through the portal blood system. In the liver, they can undergo

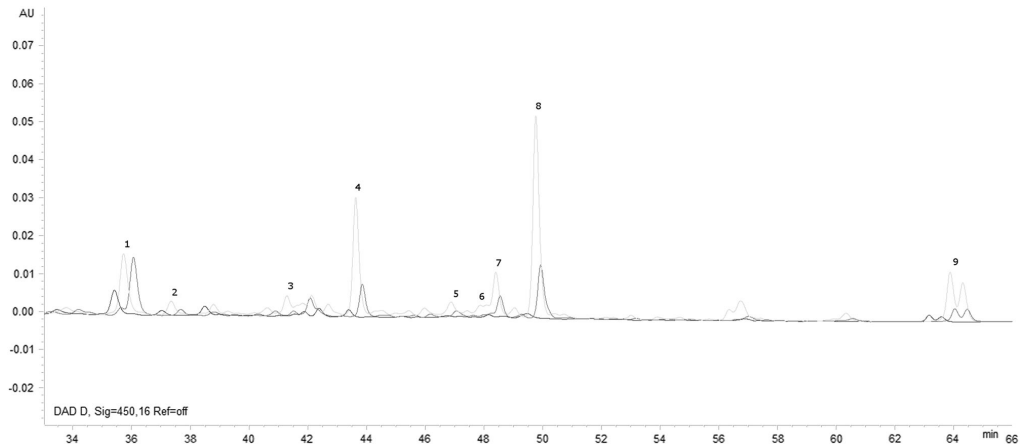

Fig. 3 HPLC chromatograms of random samples of carotenoids extracted from human plasma. 1–Astaxanthin; 2–Zeaxanthin; 3–*E*-*β*-apo-8′-carotenal; 4– Cryptoxanthin; 5–13-*Z*-*β*-carotene; 6–*α*-carotene; 7–*β*-carotene; 8–9-*Z*-*β*-carotene; 9–Lycopene

further or new cleavage and then are distributed to extra-hepatic tissues [3]. Figure 2 shows a very schematic representation of these mechanisms (see Fig. 2).

Provitamin A carotenoid uptake and conversion to retinol —the active form of vitamin A—is, in part, controlled by the intestine-specific homeobox (ISX) transcription factor, which is under the control of retinoic acid receptor (RAR)-dependent mechanisms [1]. Thus, when vitamin A stores are high, ISX is activated and represses the expression of SR-BI and BCO1, and the contrary occurs when the stores are low.

Genetic differences among individuals are supposed to be the cause of the inter-individual variations in concentrations of carotenoids in blood and tissues. Three chromatograms demonstrating great differences among carotenoid peak areas between the individuals are represented in Fig. 3 (see Fig. 3). These chromatograms were obtained from the carotenoid plasma extraction of healthy volunteers (*non-published results*), the carotenoids were separated by HPLC and detected at 450 nm, using the validated method developed by our research group [13]. A number of single nucleotide polymorphisms (SNPs) have been identified in genes that code for proteins that are involved in carotenoid intestinal uptake, transport and metabolism [14, 15]. The lipophilic profile of the carotenoids makes their volume of distribution in the body quite high and so, only to some extent will plasma concentrations reflect tissue levels [14].

In the last few years, several studies and systematic reviews have been focused on the effect that carotenoids have on human health. Antioxidant activity and capacity of carotenoids have been described and related with reduced risk of some types of cancer and enhancement of the immune system [16, 17]. But also evidences indicate that carotenoids are inhibitors of pro-inflammatory and pro-thrombotic factors and can reduce the risk of cardiovascular and other chronic diseases [18–20]. Because of their anti-oxidant and anti-inflammatory activities, carotenoids could also reduce the risk of metabolic syndrome [21]. Carotenoid consumption has been associated with the prevention and treatment of Type 2 Diabetes Mellitus and some of its complications such as nephropathy, retinopathy and neuropathy [22]. This effect is thought to be achieved by the antioxidant capacity of these compounds, that reduces the oxidative stress and inflammation involved in the triggering and progression of the complications [22]. This reduction in reactive oxygen species (ROS) and reactive nitrogen species (RNS), and probably the modulation of inflammation might, as well, be the explanation for the carotenoid ability of reducing the risk for cardiovascular diseases [18, 19]. Carotenoids and carotenoid conversion products inhibit adipogenesis and fat storage capacity by suppressing PPARɣ. Lower serum levels of carotenoids have been found in overweight and obese individuals [23]. Also obese subjects could have a reduced capacity of conversion from carotenoids to retinoids [24]. A prospective study suggests a positive effect on children adiposity and BMI concomitant with carotenoid supplementation [25]. Lifestyle of subjects and antioxidant activity from carotenoids could explain this fact, but also, it is important to highlight that adipose tissue is one of the main storages of carotenoids and retinoids [26]. There are other studies that focus on particular compounds, a cardioprotective profile has been delineated for lycopene and some potential mechanisms have been described for its

anti-atherogenic effect [20] and it has also been shown to be inversely associated with the positive prostate cancer risk [27]. Cerhan et al. showed a lower risk of rheumatoid arthritis when levels of β-cryptoxanthin in body were higher [28]; however, this relationship was not found by other authors [29]. Moreover, other conditions related with oxidative stress like Alzheimer disease have been associated with low concentration of carotenoids in subjects [30, 31]. Although some results are discordant or require further research, the positive health effect of high carotenoid intake through the diet is clearly demonstrated [21].

In this review, we aim to go through the different factors that influence the high inter-individual variability of carotenoid levels in human plasma.

Matherials and methods

Literature search and selection

A comprehensive literature search about plasma carotenoid level variability was performed between May and August 2017 through the database Pubmed. The search terms included "carotenoid" in combination with "variability", "plasma levels", "Mediterranean diet" or "Northern diet". After the removal of duplicates and articles with only abstract in English available the articles were selected by title and through links of related articles and references. Then, studies with no relevant outcome or data were eliminated as well.

Results

Factors influencing carotenoid levels in human plasma

The concentration of carotenoids found in human plasma after the same meal may not be equal in different subjects. There is a high inter-individual variability that might be due to very different causes (see Table 1).

Not only diet, but also biological activity depends on bioavailability that is mainly determined by bioaccesibility.

Table 1 Causes for plasma carotnoid inter-individual variability

Carotenoid interactions	
Culinary factors and processing	
Dietary habits	
Intrinsic host factors	ADME
	Volume of distribution
	Microbiota

ADME absorption, distribution, metabolism and excretion

Food matrix, dietary fat, dietary fiber, interaction between carotenoids and interaction between factors affect bioaccesibility, while passive diffusion and facilitated transport of carotenoids determine their bioavailability.

Mediterranean diet

When talking about inter-individual variability, the main cause is, certainly, the diet. It is difficult, in large studies where volunteers are asked to follow a particular diet, to control dietary habits of the participants to the extent of measuring the exact amount of carotenoids or carotenoid containing foods. In this way, although the participants may not change significantly their habits from one intervention to the other, very different practices from one person to another might occur and these differences could be crucial.

Globally, differences between populations are explained through diet. Mediterranean and Northern diet are typical European diets. Mediterranean diet is the typical diet from Mediterranean countries (Spain, Greece, France, Italy, Portugal…), whereas the countries from northern Europe, like UK and Republic of Ireland have another diet commonly known as Northern diet. The Mediterranean diet is probably the most carotenoid bearing diet for its richness in fruits and vegetables [32]. Because of this, it would be understandable that people that follow this type of diet are more likely to have higher content of carotenoids in their plasma.

A good example of this is an exchange list diet study performed in the United States in 2009. A group of healthy women were asked to follow a Greek-Mediterranean diet for 6 months after which, plasma carotenoids and fatty acids among other parameters were measured. As a result of the intervention the carotenoids in plasma were doubled, reflecting the larger fruit and vegetable consumption [33].

Other studies that have assessed the properties of the Mediterranean diet show that the consumption of this diet leads to an increase of the carotenoid content in plasma [34, 35].

A report from the European Prospective Investigation into Cancer and Nutrition was able to differentiate people from different European regions according to their carotenoid profile in plasma. Total carotenoids were higher in Southern regions, same as individual carotenoids, with the exception of carotenes, that showed no clear north-south difference [36]. Mediterranean population have higher amount of carotenoids present in plasma than anglo saxon population, especially for lycopene [37]. This fact can be explained by the large intake of tomatoes in the Mediterranean diet [38]. O'Neill et al. carried a study in which five European countries participated (Spain, The Netherlands, Finland, France, Republic of Ireland). With 80 subjects per country higher levels of lycopene and lutein and two-to-three-fold more β-cryptoxanthin in Spain, regarding

to other European countries, were found. There was, though, a similar concentration of α-carotene and β-carotene [38].

Another study described blood concentrations of different biomarkers of fruit and vegetables intake, namely carotenoids, tocopherols, ascorbic acid and retinol in young and healthy people from five European countries (France, UK (Northern Ireland), Republic of Ireland, The Netherlands and Spain). The serum concentrations of the cited biomarkers were considered "reference values" for these populations due to the study design. The authors found differences in the xanthophylls to carotenes ratio, being double in Spain compared to the northern countries (Northern Ireland and Republic of Ireland). The Spanish cohort also had higher levels of lutein, zeaxanthin and β-cryptoxanthin. The study concluded that some carotenoids (lutein, zeaxanthin, β-cryptoxanthin) and total xanthophylls in human plasma could be markers of the Mediterranean diet adherence [37].

Moreover, changes within Mediterranean diet consumers can be explained by seasonal variations [37–39]. For example, in Spanish diet higher β-cryptoxanthin and lycopene levels were found in winter and summer, respectively, because of the citrus fruits being more consumed in winter and tomatoes and watermelon in summer [39, 40]. Also, differences in some carotenoids and serum concentrations can take place because of the geographic, timing, demographic and cultural factors. In general, European countries from Mediterranean (southern) areas consume greater amounts of vegetables and fruits than northern countries [41, 42].

Intrinsic host factors

The distribution profile of carotenoids in the body is a more difficult aspect to assess due to the immense number of factors related not only to the food or carotenoid itself but maybe to the influence of host factors.

Earlier this year, Bohn et al. reviewed inter-individual discrepancies in host factors that might be affecting plasma carotenoid levels. Apart from dietary habits, health status including viral infections, micronutrient status, blood lipid profile, respiratory conditions and thyroid disorders are the main cause of variability according to, mostly, observational studies [14]. Other conditions related to the intestine length, permeability and function are obviously affecting overall absorption, not only carotenoid one. Other observational studies suggested other lifestyle habits such as smoking, alcohol consumption and physical activity also as sources of variability, as well as gender, age, weight, and ethnicity [14].

The volume of distribution of carotenoids is, as mentioned before, quite large in the body due to their lipophilic profile [14]. Because of this, and although it should not change intra-individually depending on the levels absorbed, the carotenoids could remain more or less constant in plasma but be higher in the different target tissues, accumulating in the adipose tissue, the skin, the liver and maybe other tissues. Besides, volume of distribution might not be the same in all individuals and can change according to health status. Plasma is one of the easiest samples that can be obtained in humans whereas tissue biopsies are quite invasive. A more expensive option would be to use isotopically labeled carotenoids and measure them in tissue [43].

In this matter, another reason that can make the profiles to vary is whether the volunteers are, or not, deficient in carotenoids (provitamin A or non provitamin A carotenoids). An intervention trial performed by Record et al. consistent in consumption of diets high or low in fruit and vegetables or following dietary supplementation with an antioxidant mixture detected a significant increase in α- and β-carotene, lutein and zeaxanthin [44]. When the studies are designed with a depletion period before the intervention, the absorption might be enhanced in order to replenish the body and not because of a different content of carotenoids in the foods.

Carotenoid absorption, distribution, metabolism and excretion (ADME), as every nutrient, non-nutrient or drug, can be affected by many reasons. In this case, some physiological parameters could be affecting carotenoid bioaccessibility. Some in vitro studies have been performed in order to assess them. Biehler et al. described that a low gastric pH would be degrading some carotenoids and thus, reducing their accessibility and absorption [45]. Periago et al. also found that high pepsin concentration facilitates the breakdown of protein-lycopene complexes increasing their availability [46]. In a study by Garret et al. and a posterior paper by Biehler et al. suggested that the concentrations of bile salts and pancreatic enzymes is critical for the micellization of carotenoids [47, 48]. A rather new approach to nutrition and metabolism, nutrigenomics, is starting to ease the explanation of ADME inter-individual variability following intervention trials. Two reviews have described a number of genes involved in the ADME processes whose different polymorphic variants could be the cause of the different outcomes [14, 15]. The genes mentioned vary from transport to –SR-BI, CD36, NPC1L1–, from –ATP binding cassette (ABC) proteins—and within the intestinal cells—fatty acid binding protein (FABP)–; to cleavage in intestinal and non-intestinal cells –BCO1 and 2–; transport in the circulatory system—the different apolipoproteins and the cholesterol ester transfer protein (CETP)—and elimination enzymes –cytochrome P450.

Lastly, although not much has been made clear in the area, the microbiota might have an influence on carotenoid

assimilation. Even though it seems that carotenoids are not very much absorbed in the large intestine, Karlsson et al. stated that the bacterial genus *Collinsella* was enriched in obese patients with atherosclerosis and that these same patients presented lower ß-carotene levels [49]. The authors suggested that the genome of this genus might not be favorable for the production of this carotenoid. These findings, though probably insufficient to state that microbiota has a critical function in carotenoid absorption, may hint an interesting role of the microbiome balance.

Interaction between carotenoids

Some studies have shown a competition of carotenoids to be micellized or absorbed [50]. There are evidences about the decrease of lutein when it is consumed along with lycopene or β-carotene [51–53] and of α-carotene and lycopene after β-carotene uptake [54], inversely to described by an Australian study [55].

Culinary factors and processing

Carotenoids are characterized by their high instability. During processing, due to their antioxidant capacities, some of the carotenoids are oxidized and degraded; however, isomerizations also occur. This degradation is induced by heat, light, oxygen, acids, transition metals, or interactions with radical species. Thermal processing causes the breakdown of the cellular matrix of the plant material and may also induce *trans* to *cis* isomerization due to the heating, increase in surface area, and agitation processes involved. The *cis* isoforms that are originated are more bioavailable because it seems that the packed structure of *cis*-isomers is more soluble in bile acid micelles and may be preferentially incorporated into chylomicrons. So, in processed foods and in human plasma and tissues, higher quantities of *cis*-isomers are found [56].

Some mechanisms of carotenoid degradation in food have been studied. Vallverdú-Queralt et al. and Rinaldi de Alvarenga et al. [9, 10] recently described the effect that cooking time and ingredient synergism have on carotenoid levels and isomerization. An adequate processing time and temperature and the addition of extra virgin olive oil or onion to the mix improved the bioavailability of carotenoids. Small variations in this aspect could be, to some extent, responsible for the inter-individual variability of carotenoid content in plasma.

Conclusion

In general, it is known that carotenoids have an important role in prevention of several diseases. However, the absorption and transformation of these compounds are essential for these health benefits to take place. Different types of studies have been performed to decipher the causes of the variation of the carotenoid profile in human plasma from different subjects after the same type of meal. Factors such as age, region, diet and intrinsic factors from the host have been suggested as possible causes for this variability but some of them are not fully understood. The levels of carotenoids in human plasma could be useful for the prediction of some diseases if we knew exactly how and why they vary from person to person. Some research is already taking place in this matter; however, more studies are needed in order to use these molecules as predictors.

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Compliance with ethical standards

Conflict of interest AT-R received grant funding from The European Foundation for Alcohol Research (ERAB); RLR received lecture fees from ERAB, Beer and Health o Centro de Información Cerveza y Salud (CICS) and grant support from the Ministry of Economy, Industry and Competitivity, Catalonia Government. The remaining authors declare that they have no conflict of interest.

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The discovery of vitamin A as a dietary factor that is required for proper growth and development was achieved after a succession of experiments that spanned the last decades of the 19th century to the mid-1930s (126), more or less at the same time of the description of the nutritional value of carotenoids (127). Retinol is the circulating form of vitamin A, whose active forms depend on their mode of action: in the eye, one of the forms of retinaldehyde (11-*cis*-retinaldehyde) is the chromophore of rhodopsin, whereas all-*trans*-retinoic acid is a potent transcriptional regulator involved in the regulation of more than 500 genes engaged in the development and maintenance of all sorts of cells (104,128). Retinol and all its metabolites are known as retinoids, and this name is now interchangeably used with the more common term in nutrition: vitamin A. Below, **Figure 5** is a schematization on vitamin A metabolism within cells.

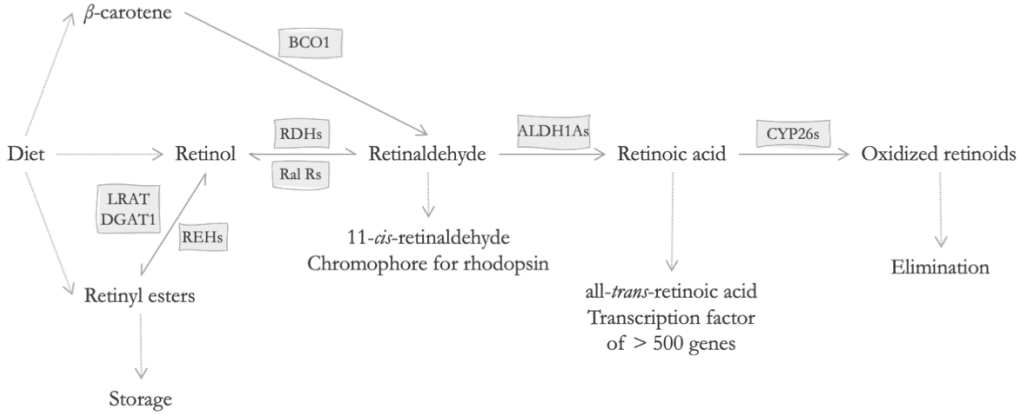


Figure 5. Forms of vitamin A within the human body, their metabolism, and roles of the active forms (adapted from Blaner, 2019 (129)).

LRAT: lecithin:retinol acyltransferase; DGAT1: diacylglycerol acyltransferase 1; REH: retinyl ester hydrolase; BCO1: β-carotene-15,15'-oxygenase; RDH: retinol dehydrogenase; RalR: retinaldehyde reductase; ALDH1A: aldehyde dehydrogenase; CYP26: cytochrome P450, type 26.

Aside from provitamin A carotenoids which can be cleaved into retinaldehyde by β-carotene-15,15'-oxygenase (BCO1), we can obtain this micronutrient in two different forms: esterified with long chain fatty acids or as retinol. These forms are mainly present in animal foodstuffs. Retinol is taken up directly by the enterocyte while the esters need to be hydrolyzed by a luminal retinyl ester hydrolase (REH) in order for retinol to be internalized (130).

As displayed in **Figure 4** (page 34), once inside the enterocyte, lecithin:retinol acyltransferase (LRAT) primarily, but also diacylglycerol acyltransferase 1 (DGAT1) in some instances, re-esterify retinol allowing for it to be incorporated into chylomicrons, together with carotenoids and other dietary fats, for uptake and distribution throughout the body. The chylomicron remnant ultimately reaches the liver, where it is taken up and the vitamin A is stored. Several retinyl ester hydrolases (REHs) within the liver hydrolyze stored vitamin A esters (retinyl esters) to retinol for distribution to tissues from hepatic storage in times of dietary vitamin A insufficiency. In the body, retinol dehydrogenase 10 (RDH10) oxidizes retinol to retinaldehyde and a number of retinaldehyde reductases (Ral Rs) are able to catalyze the reverse reaction in order to obtain retinol. The production of the transcriptionally active retinoic acid from retinaldehyde by the action of one of three aldehyde dehydrogenases (ALDH1A1, ALDH1A2, and ALDH1A3) is irreversible. Retinoic acid, usually found within cells in the low nM range, is ultimately catabolized by a number of cytochrome P450 (CYPs) species and eliminated (129).

Vitamin A has long been known to be stored in the liver and, to some extent, in adipose tissue. Thus, the body does not need to depend solely or routinely on dietary intake of vitamin A. The extensive research on this matter led to the discovery and description of the hepatic stellate cells, the primary cellular site of vitamin A storage, within the liver. These cells contain lipid droplets that are highly specialized in vitamin A storage involving retinyl esters synthesized exclusively by LRAT. From the liver, retinol is released and represents the major circulating form of vitamin A in the fasting circulation. Retinol is found in plasma in the low μM range, and it is secreted from the liver and distributed throughout the body bound to retinol-binding protein 4 (RBP4). Retinol bound to RBP4 reaches peripheral tissues and is, in some cases, taken up thanks to a membrane receptor known as STRA6 (stimulated by retinoic acid 6), although this is not the case for all tissues. Nor is retinol-RBP4 the only pathway for retinol delivery within the body, as postprandial lipoprotein transport is also an important route for distribution in the postprandial state (104).

Polyphenols

Polyphenols are a group of secondary plant metabolites involved in defense mechanisms within these organisms that had been considered anti-nutrients because of their ability to bind macronutrients and make them precipitate (131). Nevertheless, after

having been recognized for their antioxidant properties, they have been considered by researchers to have important bioactive properties for the last few decades. (132,133).

These molecules are characterized by the presence of at least one phenol group in their structure, they can be as simple as bearing only one of these groups and as complex as being highly polymerized structures (134). Their classification is primarily based on the number and organization of these phenol groups (**Figure 6**): the fundamental structure of flavonoids is a diphenylpropane, two unsaturated aromatic rings bound by three carbon atoms that form an hemiacetal; whereas non-flavonoids are usually less complex, with one or two rings that are united by simple hydrocarbon chains.

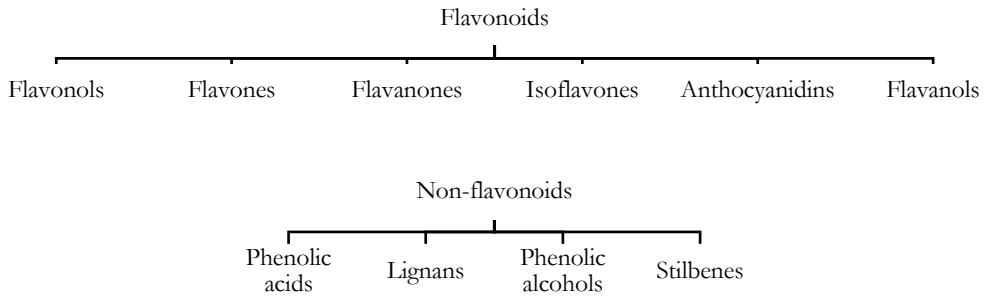


Figure 6. Classification of polyphenols.

Because the sole source of polyphenols are vegetable products, the Mediterranean diet is rich in flavonoids and stilbenes thanks to the abundance of these food sources (fruits, olive oil and red wine), whereas some non-Mediterranean cultures show a high intake of phenolic acids, mainly due to the high consumption of tea and coffee. Other well-known sources of dietary polyphenols are legumes for isoflavones, red berries for anthocyanidins and some seeds for lignans (135,136).

Figure 7 shows the absorption and metabolism of polyphenols within the human body. Even though the chyme spends a shorter time there than in the colon, the small intestine is the main location of absorption of polyphenols. These molecules are often bound to a glycoside group, which needs to be hydrolyzed by either the lactase-phlorizin hydrolase (LPH), at the edge of the enterocyte, or the cytosolic β-glucosidase (CBG) prior to entering circulation (134). Additionally, some polyphenols can

reach the colon, where the chyme resides for a longer time, and the resident microbial community can hydrolyze the glycosidic bond and transform the polyphenols into new molecules which are, then, absorbed (137). These new molecules are known as microbial phenolic metabolites (MPMs) and they can reach target organs as well as their parent compounds. Diversity or changes in the microbiota could, then, be responsible for the variability in production of different MPMs and hence on, their ultimate effect on the human body (94,138). Factors like the type of delivery at birth, the kind of milk when nursing (breast *vs.* formula), the dietary habits and lifestyle and some diseases can lead to alterations in microbial communities or to the development of dysbiosis, if the balance between communities is lost (139).

Polyphenols are modified in the liver by phase II enzymes that add methyl, sulphate, or glucuronide groups to their structure making them even more water soluble enabling them to leave the circulation and be eliminated through the urinary system. Some other polyphenols that undergo entero-hepatic circulation, when not reabsorbed, are excreted through the feces, together with the non-absorbed ones (134,140). We provided an overview of studies that detected and/or quantified MPMs in Marhuenda-Muñoz et al. 2019 (94).

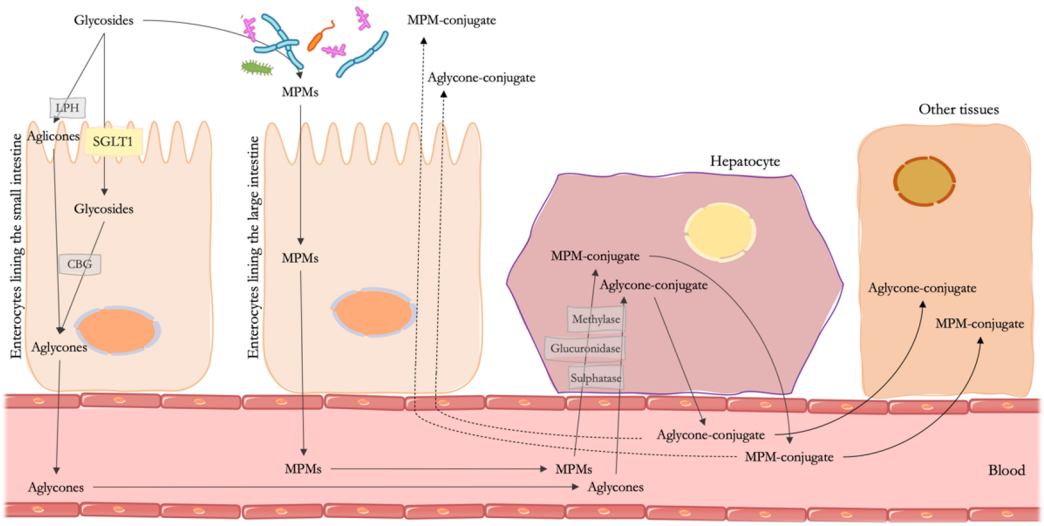


Figure 7. Absorption and distribution pathways of polyphenols and their MPMs (adapted from Marhuenda-Muñoz et al. 2019 (94)).

LPH: lactase-phlorizin hydrolase; SGLT1: sodium-dependent glucose transporter 1; CBG: cytosolic β-glucosidase; MPM: microbial phenolic metabolite.

Analysis of bioactive compounds in human samples

To be able to associate the concentrations of bioactive compounds in biological samples with health outcomes, these molecules need to be extracted from the tissue matrix where they are present and quantified using appropriate methodologies.

Analytical techniques

Because of their liposolubility, carotenoids are easily isolated from plasma samples using liquid-liquid extraction (141). Water can be used to differentiate a more polar environment, ethanol precipitates the abundant proteins and hexane, supplemented with an antioxidant such as butylhydroxytoluene, is a proper organic solvent for desolving and extracting carotenoids. Likewise, retinoids can be extracted from biological samples using this absolute ethanol/hexane-based extraction method (142).

The polarity of MPMs makes them suitable for solid phase extraction from urine, where they bind a sorbent that retains small polar metabolites and lets the rest of the compounds that are present in the urine samples to flow through. Using this approach, the sample is fractionated/cleaned, and the compounds of interest can be eluted in a small volume of solvent allowing their concentration (143).

After extraction, the extracted samples are usually dried under a nitrogen stream and redissolved in a suitable medium for separation and detection (141–143).

Because of the wide array of molecular structures in both groups of compounds, the best methodology for detection is one that can identify several molecules from the same extract in a relatively small amount of sample and time. In the last decades, one of the most widely used approach is the separation via high pressure liquid chromatography (HPLC), because of the easy separation through gradient creation, and detection with mass spectrometry (MS) thanks to the accurate identification that is achieved by fragmenting the molecules (144).

C18 and C30 reversed-phase columns have been used for separating carotenoids by liquid chromatography. The latter long chain sorbent is very specific when separating *cis/trans* carotenoid isomers. Separations can be achieved by creating a non-linear gradient consisting of methanol and methyl-tert-butyl ether (141,145). Retinoids can be easily separated with C18 reversed-phase columns using a linear flow of acetonitrile, methanol, and methylene chloride (142). The C18 reversed-phase columns have been

traditionally used for polyphenol separation (144) but new phenol sorbents also work well for these means. A non-linear gradient composed of water and acetonitrile is successful and achieves a good separation of polyphenols (146). In all cases, the compounds will elute depending on their polarity, the most polar first, followed by the more non-polar (147).

For carotenoids, an ultraviolet detector set at 450 nm (the wavelength at which most carotenoids present their maximum absorption) is the most commonly used technique. In addition, mass spectrometry allows for a higher sensibility and detection of molecules. The coupling of the two techniques will make possible the precise identification and quantitation of all carotenoids including lycopene, which is not easily ionized (145). Similarly, retinoids can be quantified by mass spectrometry and have a maximum absorption around 325 nm, which allows for their quantitation in comparison with available standards.

Regarding MPMs, several methods have been described for quantitation, from UV and fluorescence to mass spectrometry detection, being the latter the most widely used (144). Nowadays, high resolution mass spectrometry is starting to be used for these means as well, for this reason, we developed a new detection approach utilizing a high-resolution mass spectrometer that detects the exact mass of compounds with an accuracy of up to 4 decimals (146).

Statistical analyses

After detection, compounds need to be quantified to allow for assessment of possible differences in the levels of each compound of interest by statistical analysis. For this purpose, regression analyses adjusted for confounding variables can be performed to associate the biological levels with health outcomes.

However, analytical methodologies have limitations when the concentration of an analyte is very low. Precise quantitation of molecules below certain limits is not possible. Those limits are known as the limits of detection (LoD) and quantitation (LoQ). The LoD is the lowest quantity of an analyte that can be accurately distinguished from its absence in a sample, while the LoQ is the lowest quantity that can be quantified with a certain accuracy and precision (148,149).

Figure 8 displays the different possibilities that an observation can have. A value might be lower than the LoD, lie between the LoD and the LoQ, or be quantified exactly. Any value that lies below the LoQ is known as a censored observation.

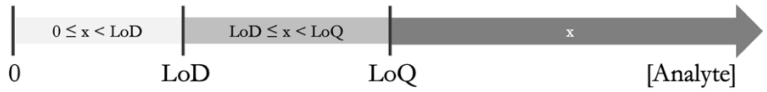


Figure 8. Possible observations of a compound quantified by HPLC-MS.

Different approaches have been employed in order to be able to use all the data in regression analyses (150). When censored data are treated as the response variable, the most accurate statistical approach has already existed for a long time for other purposes in epidemiology: survival analyses consider all possibilities within an interval, whether it is a time point or a concentration, does not matter (151).

Alternatively, when censored data are the exposure variable the analyses have involved different strategies and, until the present, there has been no consensus for an accurate use of such data. At first, investigators would simply eliminate those values, leading to losses of valuable information that could have consequences for interpreting results (151). One common approach nowadays is to replace the unknown values with the midpoint between the LoD and the LoQ, or half the LoD. Other similar approaches have also been proposed -where it is also possible to create quantiles (150). Unfortunately, these approaches reduce the variability, and the statistical effect of this loss varies greatly depending on the number of observations that are censored.

An extension of a mathematical approach for the accurate use of these data when it is used as the exposure variable is described in "Regression analysis with interval-censored covariates. Application to liquid chromatography." Gómez Melis G., Marhuenda-Muñoz M. and Langohr K. in Emerging Topics in Modeling interval-censored Survival Data. Sun and Chen (Eds.). Springer Nature. 2022. In press. This extended method considers both exactly determined values and the unknown values, treated as interval-censored random variables whose limits are given by the LoD and LoQ. Unknown observations can be the interval from zero to the LoD or from the LoD to the LoQ. In some cases, determinations from individual compounds from a given family can be statistically analyzed together, giving rise to composite variables. The sum variable is created by multiple overlapping, but not identical, intervals, because each sample contains different concentrations of the individual compounds.

Hypotheses and aims

Research on nutrition as a preventive and prognostic tool was first based on macronutrients, later on micronutrients and more recently on other minor non-essential dietary components. The global hypothesis of this work is that bioactive compounds present at high levels in the Mediterranean diet, like carotenoids and polyphenols, and vitamin A status could be early markers of disease risk or prognosis. The present dissertation aims at broadening current knowledge about the importance of these compounds and their body status on human health.

Fruits and vegetables are established as the main source of carotenoids in the diet. The existing literature highlights the importance of the presence of fat for carotenoid absorption in the gut but there is no evidence of this on the plasma concentrations of these compounds in a free-living population, where there is a wide range of fat intake. Our hypothesis was that a very high intake of fat, together with a high intake of fruits and vegetables would correlate with an elevated presence of carotenoids in plasma. For this reason, the first objective was:

1. To investigate the cross-sectional relationship between carotenoid and fat intake and the levels of carotenoids in plasma in a free-living population with no dietary restrictions.

The ingestion of carotenoids and polyphenols has been associated with a better health. Nevertheless, research on the actual circulating or excreted concentrations of these compounds or their metabolites, which more accurately reflect tissue exposure, is scarce. Our hypotheses were that plasma carotenoids are associated with a better profile of cardiovascular risk markers and that urinary microbial phenolic metabolites are associated with a lower type 2 diabetes risk. To test these hypotheses, the second and third objectives were:

2. To assess the cross-sectional association between circulating carotenoids and early markers of cardiovascular disease and,

3. To study the association between microbial phenolic metabolite concentration in urine and type 2 diabetes development.

Vitamin A cell biology within the liver, its main storage location within the body, is extensive, unlike in the lung, a tissue that is an important target for external injury. The hypothesis raised by this gap in knowledge was that local lung stores of vitamin A have a critical role in the maintenance of lung function when the tissue is injured. In order to assess this presumption, the fourth objective was:

4. To understand the cell biology of vitamin A storage within the lung and whether these lung stores are needed to protect the lungs upon acute injury.

Materials and methods

Carotenoids

Study design

The first and second objectives were investigated making use of the baseline K3-EDTA plasma samples of the PREDIMED-Plus trial, which has been described in the introduction (**Table 1**), and whose protocol was more detailed elsewhere (89). The study was carried out according to the guidelines of the Declaration of Helsinki, approved by the Institutional Review Boards of participating centres and registered in the ISRCTN of London, England (89898870). All participants signed written informed consent.

Using the validated food frequency questionnaires of the year prior to inclusion the population was divided into deciles of fruit and vegetable (F&V) consumption and into quartiles of fat intake. The first and tenth decile of F&V consumption and the first and fourth quartile of fat intake were used to create four groups from which 60 samples from participants with similar characteristics were selected (**Figure 9**).

The four resulting groups were:

1. Low-to-moderate fat intake and low F&V consumption
2. Low-to-moderate fat intake and high F&V consumption
3. Very high fat intake and low F&V consumption
4. Very high fat intake and high F&V consumption

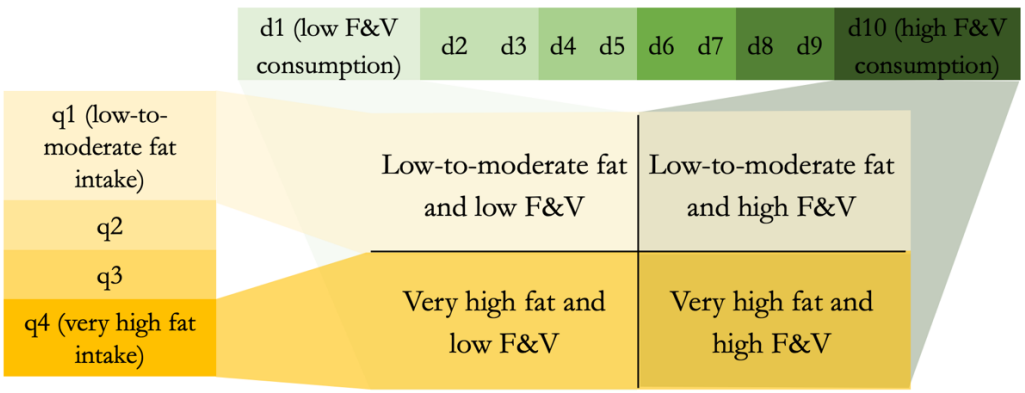


Figure 9. Grouping strategy. F&V: fruits and vegetables

The sample size allowed ≥ 80 % statistical power to detect significant differences of 0.42 μg/mL of total carotenoids in plasma between groups with a 2-sided type I error of 0.05 and 5 % of loss rate. This was based on the differences of total plasma carotenoids in middle-aged Spanish adults (Standard deviation (SD) = 0.80) (123).

Analytical determinations

Carotenoid extraction and analysis

Plasma samples that had been stored at - 80 °C and calibration curves prepared with stock solution of blank human plasma (Sigma-Aldrich) spiked with carotenoid standards were subject to liquid-liquid extraction under cool conditions and filtered light to avoid oxidation and isomerizing.

Carotenoid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): retinol, astaxanthin, canthaxanthin, *E*-β-apo-8'-carotenal, α-carotene, β-carotene, fucoxanthin, and lycopene; Cayman Chemical (Ann Arbor, MI, USA): lutein; Extrasynthese (Genay, Lyon, France): zeaxanthin and β-cryptoxanthin; and Carbosynth (Newbury, Berkshire, UK): 13-*Z*-β-carotene and 9-*Z*-β-carotene. They were all stored in powder form at - 20 °C and protected from light until use.

The extraction was carried out using a method previously developed by the group with minor modifications (141). 450 µL of thawed sample were mixed with 800 µL of ethanol (Sigma-Aldrich), 500 µL of ultrapure water generated by a Millipore system (Bedford, MA, USA) and 2 mL of n-hexane/butylated hydroxytoluene (100 mg/L) (Sigma-Aldrich). 100 µL of fucoxanthin at 1 mg/mL were also added for its use as internal standard. The mix was vortexed for 1 minute, centrifuged at 2070 × g for 5 minutes at 4 °C, and the upper nonpolar layer was separated and reserved. The lower aqueous phase was re-extracted with 2 more milliliters of n-hexane/butylated hydroxytoluene (100 mg/L) and the same procedure described above. The upper nonpolar layer was combined with the first one and underwent evaporation to dryness by a sample concentrator under nitrogen gas at room temperature. The evaporate was reconstituted with 100 µL of methanol and stored in glass amber vials with inserts at - 80 °C until the day of analysis.

Following a method validated by the group, a YMC Carotenoid S-5 µm, 250 × 4.6 mm (Waters, Milford, MA, USA) chromatography column maintained at 40 °C was used for separation, and it was coupled to a UV-VIS detector set at 450 nm and a triple quadrupole mass spectrometer QTRAP4000 (Sciex, Foster City, CA, USA) equipped with atmospheric pressure chemical ionization (APCI) source and controlled by Analyst v.1.6.2 software (Sciex) for detection (145). 20 µL of the sample were injected into the system and the separation was achieved by gradient generation with two mobile phases at a flow rate of 0.6 mL/min:

A. Methanol (Sigma-Aldrich), 0.7 g/L ammonium acetate (AMAC) Panreac Quimica SLU (Barcelona, Spain) and 0.1 % acetic acid Panreac Quimica SLU.

B. Methyl tert-butyl ether (MTBE) (Sigma-Aldrich) and methanol (80:20, v/v), 0.7 g/L AMAC and 0.1 % of acetic acid.

The gradient conditions were (t (min), % A): (0.0, 90); (10.0, 75); (20.0, 50); (25.0, 30); (35.0, 10); (37.0, 6); (39.0, 90); (50.0, 90). Total run time of analysis was 50 minutes.

The concentrations of carotenoids in plasma were calculated from their interpolation into the calibration curves using MultiQuant software version 3.0.1 (Sciex) by the internal standard method, which was implemented for these analyses. The *Z*-lycopene standard labile profile prevented its use, and this carotenoid was quantified in *E*-lycopene equivalents. Due to technical difficulties, retinol was under the LoQ in all cases, but we were able to compare the concentrations by calculating the area under the curve and correcting it by the area under the curve of the internal standard.

The quantitation of the concentrations of the individual carotenoids allowed for the grouping into the two classification groups of carotenoids: carotenes (α-carotene, β-carotene, *E*-lycopene and *Z*-lycopene) and xanthophylls (astaxanthin, lutein, canthaxanthin and β-cryptoxanthin); and provitamin A (α-carotene, β-carotene and β-cryptoxanthin) and non-provitamin A (*E*-lycopene and *Z*-lycopene, astaxanthin, lutein and canthaxanthin); as well as for the calculation of the total sum of carotenoids. Due to the limits of detection and quantitation of the method, the concentrations were interval-censored.

Anthropometric, body composition variables and blood pressure

Trained personnel took anthropometric measurements following the study protocol. Calibrated scales were used to record weight with barefoot participants wearing light clothing; and wall-mounted stadiometers were used for measuring height. Body mass index was calculated as weight (Kg) divided by the squared height (m^2). Waist circumference was measured using non-stretchable measuring tapes midway between the lowest rib and the iliac crest with subjects standing at the end of gentle expiration. Semiautomatic oscillometers (Omron HEM-705CP) were used to measure systolic and diastolic blood pressure, and heart rate in triplicate.

Biochemical parameters

Glucose (mg/dL), glycated hemoglobin (%), triglycerides (mg/dL), total cholesterol and High-density lipoprotein (HDL)-cholesterol (mg/dL) were determined by standard laboratory enzymatic methods.

Plasma fatty acid profile

Following a validated method, plasma samples that had been stored at - 80 °C were subject to extraction (152). 100 μL of sample were mixed with 20 μL of the internal

standard tridecanoic acid methyl ester (C13:0) (Sigma-Aldrich) and 1 mL of sodium methylate (0.5 % w/v) (Sigma-Aldrich) and heated for 15 minutes to 100 °C. After cooling, samples were esterified at the same conditions as before with 1 mL of boron trifluoride-methanol reagent (Sigma-Aldrich). Fatty acids methyl esters were isolated when the mix was cool by adding 500 μL of n-hexane (Sigma-Aldrich). After vortexing for 1 minute, 1 mL of a saturated sodium chloride solution (Panreac Quimica SLU) was added, and the mix was centrifuged for 10 minutes at 3000 rpm at room temperature. The extract was then dried with anhydrous sodium sulphate (Schalab, Barcelona, Spain), and the clear top layer was transferred into an automatic injector vial equipped with a volume adapter of 300 μL.

A 40 m × 0.18 mm I.D. x 0.1 μm film thickness capillary column coated with an RTX-2330 stationary phase of 10 % cyanopropyl phenyl – 90 % byscyanopropyl polysiloxane from Restek (Bellefonte, USA) was used to separate fatty acids methyl esters on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. 1 μL of sample was injected with a split-splitness injector used in split mode with a split ratio of 1:50 and maintained at 250 °C. The program initial temperature was 110 °C, increased at 52 °C/min to 195 °C and held at this temperature for 6 minutes, then it was increased at 25 °C/min to 230 °C and held for 6.5 minutes for a total run time of 16.03 minutes. The carrier gas was hydrogen at a constant pressure of 26 psi, linear velocity of 40 cm/s at 110 °C. The detector was maintained at 300 °C. Data acquisition and processing were performed with the Shimadzu-Chemstation software for GC systems and Supelco 37 component fatty acids methyl ester mix and PUFA No.2 (Animal source), both from Merck (Darmstadt, Germany), were used for peak identification. The results were expressed as relative percentages of total fatty acids.

Other covariates

Baseline information regarding age, sex, sociodemographic and lifestyle habits (like physical activity and smoking habit), individual and family medical history, medical conditions (such as prevalence of diabetes, hypercholesterolemia, and hypertension), and medication use were collected by self-reported questionnaires with the support of trained personnel (89). Consumption in g/day of alcohol and energy intake in Kcal/day was estimated from the validated food frequency questionnaire. Dietary quality was estimated based on the 17-item energy-reduced Mediterranean diet adherence score: the higher the value of the score obtained, the higher the overall quality of the diet (153). When assessing the relationship between F&V consumption and fat intake, and carotenoid plasma concentration we estimated the overall quality of diet regardless of the consumption of fruits, vegetables, dietary fats and alcohol (a

modified version that was obtained using the 9 questions that were independent from the consumption of these items).

Statistical analyses

Descriptive characteristics of the participants are presented as means and SD for continuous variables and percentages for categorical data. One-way analysis of variance (ANOVA) for continuous variables and χ^2-test for categorical variables were used to determine possible differences between F&V and fat intake groups. Nine samples were excluded from the analyses for they had implausible energy intakes reported (>3500 Kcal/day for women and >4000 Kcal/day for men) (92).

First, we determined whether there was a significant interaction between the groups according to F&V and fat intake and carotenoid concentrations by adapting survival regression models where the plasma carotenoid concentration was the dependent variable and applying a likelihood ratio test between the nested models with and without an interaction product-term of "F&V intake group × fat intake group". Any *p*-value < 0.1 for the interaction was considered as significant (154,155). Then, intergroup differences were determined by using adapted survival regression models (151) using the 'survival' package in R Software. Three regression models of increasing complexity were used adjusting for age (continuous), sex (discrete), physical activity (continuous), total energy intake (continuous), the modified Mediterranean diet adherence score (continuous) and alcohol consumption (continuous). Statistical analyses were performed for individual carotenoids and also for the two classification groups of carotenoids: carotenes and xanthophylls; and provitamin A and non-provitamin A, for the sum of lycopenes and for the total sum of measured carotenoids.

For the cross-sectional correlation of plasma carotenoids with cardiovascular risk factors, linear regression models specifically prepared to consider the interval-censored nature of the data were used (156,157). This trait prevented from the possibility of creating quantiles without making imputation, since the intervals overlap. The model assumptions of homoscedasticity and normality were graphically checked by means of residual plots. The analyses were stratified by sex (158) and increasingly adjusted for age, body mass index, leisure time physical activity, total energy intake and adherence to the Mediterranean diet, all continuous variables, unless the variable was being tested. Blood pressure was additionally adjusted for anti-hypertensive agents and serum triglycerides for cholesterol-lowering agents.

In both cases, the analyses were performed with the statistical software R, version 4.1.0 (Vienna, Austria; https://www.r-project.org/) and statistical significance was set at 0.05.

Polyphenols

Study design

The third objective was investigated making use of the baseline and one-year urine samples of the PREDIMED trial, detailed in the introduction (**Table 1**), and whose protocol was detailed elsewhere (90). The study was carried out according to the guidelines of the Declaration of Helsinki, approved by the Institutional Review Boards of participating centers and registered in the ISRCTN of London, England (35739639). All participants signed written informed consent.

Out of the 3541 participants that were free of type 2 diabetes at baseline, 273 developed the disease during 4.1 years of follow-up. From these, a case-control study with 46 cases and 126 resemblant controls randomly picked from the participants that had available urine samples at baseline and one-year visit was designed.

Information on type 2 diabetes status was registered yearly during the trial. New onset of the disease was diagnosed in accordance with the American Diabetes Association criteria (> 7.0 mmol/L fasting plasma glucose or > 11.1 mmol/L 2-h plasma glucose after a 75 g oral glucose overload) (159) by the PREDIMED Clinical Event Committee, which was blinded to intervention group. Only confirmed cases were included in the analyses.

Analytical techniques

Microbial phenolic metabolites extraction and analysis

Morning spot urine samples from the baseline and 1-year visits that had been stored at - 80 °C and calibration curves prepared by spiking synthetic urine (160) with increasing concentrations of a mixture of phenolic standards were subject to solid phase extraction under filtered light and under cool conditions to prevent phenolic oxidation.

Standards were purchased from Sigma-Aldrich: 3-hydroxytyrosol, protocatechuic acid, 4-hydroxybenzoic acid, 3,4-dihydroxyphenylpropionic acid, 3'-hydroxyphenylacetic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, enterodiol, urolithin-A, and urolithin-B; Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA): 3'-hydroxytyrosol-3'-glucuronide, dihydroresveratrol, (+)*cis, trans*-abscisic acid d_6; and Fluka (St. Louis, MO, USA): 3-hydroxybenzoic acid, vanillic acid, syringic acid and enterolactone. They were stored in powder form and protected from light.

50 µL of thawed urine were diluted with ultrapure water bringing them to 1 mL, acidified with 2 µL of formic acid and centrifuged at 15000 x g at 4 °C for 4 minutes.

Reversed phase Waters Oasis HLB 96-well plates 30 μm (30 mg) (MA, USA) that had been activated with 1 mL of methanol and 1 mL of 1.5 M formic acid, added consecutively, were used for extraction. 1 mL of sample was loaded into the plates together with 100 μL of abscisic acid d_6 as an internal standard. 500 μL of 1.5 M formic acid and 0.5 % methanol were used for sample clean-up, and elution was achieved using 1 mL of methanol acidified with 0.1 % of formic acid. The eluted fraction was evaporated to dryness under a nitrogen stream at room temperature and the evaporate was reconstituted with 100 μL of 0.05 % formic acid in water. The reconstituted and concentrated sample was vortexed for 20 minutes and filtered with 0.22 μm polytetrafluoroethylene 96-well plate filters from Millipore (MA, USA) prior to injection into the chromatograph.

A Kinetex F5 100Å (50 x 4.6 mm i.d., 2.6 μm) (Phenomenex, Torrance, CA, USA) column was used for separation, and detection was achieved with an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an electro-spray ionization (ESI) source working in negative mode (146). 5 μL of sample, that were maintained at 4 °C in the autosampler, were injected into the reversed phase chromatographic column, that was kept at 40 °C, and gradient elution was performed with two phases at a constant flow rate of 0.5 mL/min:

A. Water, 0.05 % formic acid (Panreac Quimica)
B. Acetonitrile (Sigma-Aldrich), 0.05 % formic acid

The gradient conditions were (t (min), % A): (0.0, 98); (1.0, 98); (2.5, 92); (7.0, 80); (9.0, 70); (11.0, 50); (12.0, 30); (15.0, 0); (16.0, 0); (16.5, 98); (21.5, 98). Total run time of analysis was 21.5 min.

Mass spectra were acquired in profile mode with a setting of 30,000 resolution at m/z 400, the mass range was from m/z 100 to 2000. Source voltage was 5 kV; sheath gas, 50 units; auxiliary gas, 20 units; sweep gas, 2 units; and capillary temperature, 375 °C. Data acquisition was performed by Fourier transformed mass spectrometry (FTMS) mode (scan range m/z 100–1000) in combination with product ion scan experiments (MS2) (resolution range 15000 – 30000 FWHM).

Aglycones were identified by comparison with the retention time of the standards and phase II metabolites by comparison with tandem mass spectrometry (MS/MS) spectra found in the literature for the accurate mass with an error of 5 ppm. Trace Finder software version 4.1 (Thermo Fisher Scientific, San Jose, CA) was used for chromatographic analysis, in which phase II metabolites were quantified in their respective aglycone equivalents.

MPM concentrations were expressed as μg MPM/g creatinine because the concentrations of creatinine in spot urine samples can be used to assess urinary excretion of compounds in the absence of disease (161–164). Creatinine was measured by an adapted Jaffé alkaline picrate method for 96-well plates (165).

Due to the high variability of MPM identified in participants, probably derived from metabolic and metabotype differences, only metabolites that had less than 20 % of values below the LoQ were considered for statistical analyses. Values below the LoD were replaced by half the limit, and values below the LoQ were replaced by the midpoint between the LoD and the LoQ.

Covariates

Information regarding age, sex, sociodemographic and lifestyle habits (like physical activity and smoking habit), individual and family medical history, medical conditions (such as hypercholesterolemia and hypertension), and medication use were collected by self-reported questionnaires with the support of trained personnel (90). Anthropometric measurements were also taken by trained staff. Energy intake in Kcal/day was estimated from the validated food frequency questionnaire. Dietary quality was estimated based on the 14-item Mediterranean diet adherence score: the higher the value of the score obtained, the higher the overall quality of the diet (91). Participants reporting implausible energy intakes (<500 or >3500 Kcal/day for women and <800 or >4000 Kcal/day for men) were eliminated from the selection (92).

Statistical analyses

Baseline characteristics are described as the mean and standard deviation for continuous variables and percentages for categorical variables. Significant differences between controls and cases were calculated by analysis of t-test for continuous variables and the χ^2-test for categorical variables.

MPM concentrations were transformed with a rank-based inverse normal method to approximate a normal distribution (166) and their 1-year changes (difference between 1-year and baseline) were analyzed as both continuous variables and tertiles. To examine the association between changes of MPM with type 2 diabetes, multivariable logistic regression models were conducted, estimating odds ratios (OR) and their 95 % confidence intervals. All analyses were adjusted in an increasing complexity manner for sex (discrete), age (continuous) and intervention group (discrete, 3 categories), body mass index (continuous), physical activity (continuous), smoking status (discrete, 3 categories: never, current, or past smoker), education level (discrete, 2 categories: primary education or secondary /higher education), baseline hypertension (discrete), dyslipidemia (discrete) and energy intake (continuous) and baseline fasting plasma glucose (discrete, 3 categories: < 100 mg/dL, 100 – 125 mg/dL, or > 126

mg/dL). We used robust variance estimators to account for recruitment center in all models. The Simes procedure was used to account for multiple testing, by adjusting the *p*-values of the multivariable-adjusted associations between 1-year changes and type 2 diabetes risk (167).

The analyses were performed with Stata software, version 16.0 (Stata Corp.) and significance testing was considered for $p < 0.05$.

Vitamin A

Study design

Groups of three-month-old (10-12 weeks-old) male mice were used in all studies. Depending on the experiment, 5-14 animals per group were employed. Lecithin:retinol acyltransferase-deficient mice (*Lrat*-deficient, $Lrat^{-/-}$ mice (168)) on a C57BL/6 genetic background and age-matched littermates (wild type, $Lrat^{+/+}$ mice) and retinol-binding protein 4-deficient mice on a C57BL/6J/129 SV mixed genetic background (*Rbp4*-deficient, $Rbp4^{-/-}$ mice (169)) and age-matched littermate controls (wild type, $Rbp4^{+/+}$ mice) were used in the experiments.

To induce acute lung injury (ALI), anesthetized mice from each genotype group were given a dose of lipopolysaccharide (LPS) in sterile phosphate-buffered saline (PBS) via intranasal instillation. LPS concentration was 25 mg/Kg body weight (lethal dose) for the ALI survival experiments and 5 mg/Kg body weight (nonlethal dose) for early phase ALI experiments. Control animals were instilled with an equal volume of sterile PBS (170).

In the ALI survival experiments, mice from each of the four genotype groups were monitored for physical parameters and survival over a 7-day period. In the vitamin A-supplementation experiment, separate groups of either LPS or vehicle (PBS) treated $Lrat^{+/+}$ and $Lrat^{-/-}$ mice additionally received 300 IU of retinol in the form of retinyl acetate each day for 7 days. The mice were sacrificed 7 days after the instillation. At the time of sacrifice, lungs, liver, and blood were collected.

For the early phase ALI experiments, groups of $Lrat^{+/+}$ and $Lrat^{-/-}$ mice were sacrificed 24 hours after 5 mg/Kg LPS instillation. Bronchoalveolar (BAL) fluid, lungs, liver, and blood were collected at the time of sacrifice. For BAL fluid collection, the lungs of anesthetized mice were lavaged 5-times with ice-cold PBS without calcium and magnesium (1 mL each time) through a tracheal cannula. All dissected tissues were quickly weighed, frozen in liquid N_2, and stored at - 80 °C until analysis.

All experiments involving mice were carried out with the approval of the Institutional Animal Care and Use Committee of Columbia University according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences (171).

Analytical techniques

Acute lung injury assessment

Assessment of ALI development in mouse models was performed regarding parameters that are in accordance with the American Thoracic Society recommendations (172). BAL fluids collected from each mouse were centrifuged for 10 minutes at 350 x g and 4 °C.

For cell counts, pellets were resuspended in 1 mL of PBS and stained with Turk's solution and counting was performed using a hemocytometer (Hausser Scientific). The supernatant was analysed for protein concentration using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.). BAL and plasma TNFα concentration were assessed using a mouse TNFα ELISA kit (Enzo Life Sciences, Inc.) and plasma C-reactive protein concentrations were assessed using a mouse C-reactive protein/CRP ELISA kit (R&D Systems) all according to the manufacturer's protocol. For surfactant phospholipid analyses, BAL fluid supernatants were further centrifuged for 30 minutes at 13,000 x g at 4 °C to pellet cell debris. The resultant supernatants were used for further lipidomics analysis by ultra-high-performance liquid chromatography (UPLC)/MS/MS (see next page for details).

For histological analysis, the lungs were inflated to total lung capacity with neutral buffered formalin and then processed into paraffin blocks according to standard protocols (173). The embedded tissues were cut into 5 μm slices, mounted on charged adhesive slides, and dried overnight at 50 °C. Slides were then deparaffinized in xylene and rehydrated in graded alcohol and distilled water. Representative histological sections of each specimen were stained with hematoxylin/eosin (H&E) according to standard staining methods.

Alveolar capillary barrier integrity was assessed by measuring extravascular lung water (EVLW) content based on the wet-dry ratio of the lung homogenate, corrected for blood water content (170).

Retinol and retinyl ester extraction and analysis

Plasma, liver, lung, and lung lipofibroblasts that had been stored at - 80 °C were subject to extraction under dim yellow safety light and under cool conditions to prevent oxidation.

Following a previously described methodology (142), livers and lungs were homogenized in 10 vol of PBS (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride)

using a tissue tearer. A 200 μL aliquot of plasma, tissue homogenate or cell suspension was then treated with an equal volume of absolute ethanol from Thermo Fisher Scientific (Pittsburgh, PA) containing a known amount of retinyl acetate as an internal standard. Retinoids present in either plasma, tissue homogenates, or cell suspensions were extracted into hexane (Thermo Fisher Scientific). The hexane extract was dried under a stream of nitrogen and redissolved in benzene (Thermo Fisher Scientific).

A 4.6 × 250 mm Symmetry C18 Column (Waters, Milford, MA, USA) was used for separation of tissue and plasma retinol and retinyl esters. Separation was achieved by a running solvent consisting of acetonitrile/methanol/methylene chloride (70:15:15 v/v) all from Thermo Fisher Scientific flowing at 1.8 mL/min. A Waters HPLC system (Waters 717 Autosampler, Waters 515 HPLC Pump, and Waters 2996 Photodiode Array Detector) controlled by Empower version 2 software was employed for these analyses. Retinol and retinyl esters were detected at 325 nm and quantitation was based on comparisons of the area under the peaks and spectra for unknown samples to those of known amounts of standards.

Lipidomic profiling of bronchoalveolar fluid

Lipid extracts were prepared from BAL fluid samples using a modified Bligh and Dyer extraction (174), spiked with appropriate internal standards and analyzed on a platform comprising Agilent 1260 Infinity HPLC integrated to Agilent 6490A QQQ mass spectrometer controlled by Masshunter v 7.0 (Agilent Technologies, Santa Clara, CA).

A normal phase Agilent Zorbax Rx-Sil column (2.1 x 100 mm, 1.8 μm) maintained at 25 °C was used for glycerophospholipids and sphingolipids separation (175) using the following mobile phases:

A. Chloroform:methanol:ammonium hydroxide, 89.9:10:0.1, v/v
B. Chloroform:methanol:water:ammonium hydroxide, 55:39:5.9:0.1, v/v

The gradient conditions were (t (min), % A): (0.0, 95); (2.0, 95); (20.0, 30); (23.0, 25); (25.0, 95); (31.0, 95). Total run time of analysis was 31 minutes.

A reversed phase Agilent Zorbax Eclipse XDB-C18 column (4.6 x 100 mm, 3.5 μm) was used for separation of sterols and glycerolipids using an isocratic mobile phase, chloroform, methanol, 0.1 M AMAC (25:25:1) at a flow rate of 300 μL/min.

Quantitation of lipid species was accomplished by using multiple reaction monitoring (MRM) transitions (175–177) under both positive and negative ionization modes and referencing of appropriate internal standards: PA 14:0/14:0, PC 14:0/14:0, PE 14:0/14:0, PG 15:0/15:0, PI 17:0/20:4, PS 14:0/14:0, BMP 14:0/14:0, APG 14:0/14:0, LPC 17:0, LPE 14:0, LPI 13:0, Cer d18:1/17:0, SM d18:1/12:0, dhSM

d18:0/12:0, GalCer d18:1/12:0, GluCer d18:1/12:0, LacCer d18:1/12:0, D7-cholesterol, CE 17:0, MG 17:0, 4ME 16:0 diether DG, D5-TG 16:0/18:0/16:0 (Avanti Polar Lipids, Alabaster, AL). Lipid concentrations for each sample were calculated and final data are presented as nmol/μg of protein. In addition, lipid levels for each sample were calculated by summing the total number of moles of all lipid species measured by all three LC-MS methodologies and then normalizing the total as mol %.

Pulmonary UV-positive cell isolation and culture

The lungs of anesthetized wild type C57BL/6 mice (*Lrat*$^{+/+}$ mice) were first perfused through the right ventricle with calcium- and magnesium-free Hanks' balanced salt solution (HBSS). Then the lungs were perfused *in situ* with HBSS containing calcium, magnesium, and Dispase II (Sigma-Aldrich) followed by a second perfusion with HBSS containing calcium, magnesium, and type IV collagenase (Worthington). The lungs were removed, rinsed and minced into small pieces. The minced lung tissue was incubated in HBSS containing Dispase II (Sigma-Aldrich), type IV collagenase (Worthington), and DNase I (Sigma-Aldrich) on a rotating shaker maintained at 37 °C for 60 minutes. At each of three 20-minute intervals, the minced tissue was then passed 10-times through a 10-mL pipette to dissociate cells. The resultant cell suspension was next passed through a 100 μm strainer to collect single cells and an equal volume of cold (4 °C) complete medium containing Dulbecco's Modified Eagle Medium (DMEM), 10 % fetal bovine serum (FBS), penicillin and streptomycin (10,000 units/100 mL) was added. Cells were pelleted by centrifugation at 500 x g for 10 minutes and resuspended in DMEM containing 10 % FBS.

Fluorescence activated cell sorting (FACS) was employed to sort live, individual retinoid-containing cells based on their emission at 455 nm (UV-positive cells) (178). Singlet discrimination was sequentially performed using plots for forward scatter (FSC-A versus FSC-H) and side scatter (SSC-W versus SSC-H). Dead cells were excluded by scatter characteristics and staining with propidium iodide. All FACS isolations were performed on a FACSAria cell sorter (Becton Dickinson). FACS data were analyzed using FlowJo software version 10 (TreeStar, Ashland, OR).

After isolation, a fraction of cells was either collected for analysis (referred to as freshly isolated or day 0 cells) or plated on 6-well plastic plates at a density 2×10^5 cells/well followed by culture in DMEM (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10 % FBS (Peak Serum, Wellington, CO, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies Corporation, Carlsbad, CA, USA) in a humidified 5 % CO_2 incubator at 37 °C. Culture medium was first changed after overnight (12 hours) culture and subsequently every 2 days.

For analysis of cultured cells, attached cells were washed with PBS, treated with 0.25 % trypsin-EDTA solution (Life Technologies Corporation, Carlsbad, CA, USA) and then collected by centrifugation. Cells were harvested after 12 hours culture (day 0.5

time point), and 3-day (72 hours) culture (day 3 time point). The harvested cells were pelleted and snap frozen in liquid nitrogen and stored at - 80 °C until analysis. The DNA concentration of each cell sample was assessed employing a High Sensitivity Quant-iT™ dsDNA Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Single cell RNA-sequencing

Freshly isolated UV-positive cells with viability > 70 % were submitted for single cell RNA sequencing (scRNA-seq) at The Single Cell Analysis Core at JP Sulzberger Columbia Genome Center. Single cell libraries were prepared using the Chromium Single Cell 3' Reagent Kits v3.1 (10x Genomics) according to the manufacturer's instructions followed by their sequencing on a NovaSeq 6000 (Illumina) in a 2 x 100 bp configuration. Initial data processing, including demultiplexing reads and converting raw base call files into the fastq format, was performed using Cell Ranger version 5.0.1 (10X Genomics). Expression counts for each gene in all samples were collapsed and normalized to unique molecular identifier (UMI) counts using Cell Ranger 5.0.1 (10X Genomics). The result is a large digital expression matrix with cell barcodes as rows and gene identities as columns. Filtered feature matrix outputs from Cell Ranger were used for all downstream analyses.

RNA preparation and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from frozen tissues or cell preparations employing TRIzol Reagent (Ambion, Foster City, CA, USA) and isolated using the E.Z.N.A Total RNA Kit II (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. RNA was quantitated at 260 nm using a Nanodrop spectrophotometer. cDNA synthesis was performed using 0.5 μg of total RNA (in a final volume of 20 μL) and was carried out for 10 minutes at 25 °C followed by 120 minutes at 37 °C employing reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The reaction was stopped at 85 °C for 5 minutes, using a thermal cycler (Eppendorf, Westbury, NY, USA). The primers employed for qRT-PCR analyses of target gene expression are provided in the Annex (**Supplementary Table 1**). 18S RNA was employed as the reference housekeeping gene used to normalize mRNA expression.

qRT-PCR was performed in a total volume of 20 μL, including 40 ng of cDNA template, forward and reverse primers (100 nM each), and PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA) using a LightCycler 480 instrument controlled by LightCycler 480 software version 1.5.0.39 (Roche, Branchburg, NJ, USA). After initial denaturation and enzyme activation (95 °C for 10 minutes), 40 cycles (94 °C for 10 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds) were performed for the annealing/extension steps, and fluorescence was measured. A dissociation curve program was performed after each cycle. Expression levels of target genes were calculated based on the efficiency of each reaction and the crossing point deviation of each

sample versus control and are expressed as fold differences in comparison with the reference gene.

Western blotting

Hepatic proteins were extracted using RIPA lysis and extraction buffer (Thermo Scientific, Rockford, IL) containing protease inhibitor cocktail (Millipore Sigma, Burlington, MA). For tissue samples, 20 μg of total homogenate protein was analyzed. For plasma protein analysis, 2 μL of plasma were employed. The proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) in 12 % gels followed by a transfer onto polyvinylidene fluoride (PVDF) membrane. Immunoblotting was performed using rabbit polyclonal antibody against mouse retinol-binding protein 4 (RBP4, 1:3000). Protein loading and relative quantitation of protein expression were normalized using β-actin as a reference protein (1:10,000; Millipore Sigma, St. Louis, MO). Protein bands were visualized using Super Signal West Pico PLUS chemiluminescence system (Thermo Scientific, Rockford, IL), membranes were digitally scanned using Odyssey XF Infrared Imager controlled by a LI-COR Acquisition Software version 1.0.19 (LI-COR, Inc., Lincoln, NE) and analyzed using ImageJ version 1.53a (National Institutes of Health) to generate quantitative relative protein expression data (179).

Statistical and bioinformatic analyses

All data are presented as means ± SD. Student's t-test was used to analyze differences between genotype groups. Statistical comparisons involving more than two groups were first analyzed by a one-way ANOVA followed by multiple comparisons employing Tukey's HSD post hoc test. Survival rates were analyzed by the Kaplan-Meier log rank test. *P*-values below 0.05 were considered significant.

scRNA-seq post-processing analysis was performed using the 'Seurat' package version 4.0.4 in R Software version 4.1.1 (Vienna, Austria; https://www.r-project.org/). Expression profiles of cells from different samples were first analyzed and clustered separately. Before the analysis, quality control on each individual sample was undertaken on the number of genes detected in each cell (nFeature_RNA), number of transcripts detected in each cell (nCount_RNA), and percentage of mitochondrial (percent_mito) and ribosomal (percent_ribo) genes in each cell. These metrics were further used to exclude outliers, low quality cells with low gene numbers, or/and transcripts detected per cell and/or cells with a high number of transcripts mapped to mitochondrial genes. Cells were filtered using the *Subset* function to exclude all cells with fewer than 200 detected genes or more than 10 % mitochondrial genes. The *NormalizeData* function was used to normalize and log scale the data. A subset of features that exhibit high cell-to-cell variation in the dataset was next calculated using the *FindVariableFeatures* function. The *ScaleData* function was used to regress

out heterogeneity associated with cell cycle stage or mitochondrial contamination. To integrate the samples, integration anchors were identified using the *FindIntegrationAnchors* and the list of samples (individual seurat objects) were integrated using *IntegrateData* function. Canonical correlation analysis (CCA) and mutual nearest neighbors (MNN) detection functions in 'Seurat' were used to remove batch-associated effects. Principal component analysis (PCA) on the scaled data was performed for dimension reduction using the *RunPCA* function. The optimum dimensionality of the dataset for downstream clustering was determined using both the *JackStraw* and *ElbowPlot* methods. K-nearest neighbor (KNN) graph was constructed based on the Euclidean distance in PCA space, and the edge weights between any two cells based on the shared overlap in their local neighborhoods (Jaccard similarity) were refined using the *FindNeighbors* function. To cluster the cells, the Louvain algorithm was applied by using the *FindClusters* function at low resolution (resolution = 0.2). The cluster resolution value was determined using the *Clustree* function and biologically relevant cell clusters were defined as clusters that have at least 10 unique differentially expressed genes. Visualization of the clusters on a 2D map was performed with uniform manifold approximation and projection (UMAP) using *RunUMAP* (dims = 1:15). Differentially expressed genes of each cluster were identified using the Wilcoxon rank-sum test with the *FindAllMarkers* function.

A combination of supervised and unsupervised approaches to determine cell type annotation was employed. To identify major cell type subsets (stromal, epithelial, endothelial, and myeloid), we assessed the expression of the canonical markers *Col1a1*, *Epcam*, *Pecam 1*, and *Ptprc*. The *FindAllMarkers* function was used to identify a list of top genes that was unique to each cell cluster. To obtain the most sensitive and specific differentially expressed genes for each cluster, we identified genes with a *p*-value of less than 10^{-5} and an average log fold-change greater than 1. Preliminary annotation was undertaken by comparing this list to previously published conserved gene lists from scRNA-seq data in mouse lung tissue (40,180–183). Top conserved markers of final annotated cell types were visualized again with *FeaturePlot* and *ViolinPlot*. The stromal clusters were separated for further analysis using the *Subset* function.

Results

Relationship between fruit and vegetable consumption and fat intake, and carotenoid plasma concentrations

As explained in the previous section, the first objective, to investigate the cross-sectional relationship between carotenoid and fat intake and the levels of carotenoids in plasma, was performed by the comparison of four groups of combined extreme F&V consumption and fat intake.

Part of the following results were published in Marhuenda-Muñoz M. et al. **High fruit and vegetable consumption and moderate fat intake are associated with higher carotenoid concentration in human plasma.** *Antioxidants*, 2021, 10, 473. DOI: 10.3390/antiox10030473 (184).

Table 2 shows the baseline characteristics of the four groups. Inter-group differences in food and nutrient intake are detailed in **Table 3**.

Table 2. Participant characteristics by Fruit and Vegetable and Fat intake groups.

Characteristics	All	Low F&V		High F&V		*p*-value
		Low-to-Moderate Fat	Very High Fat	Low-to-Moderate Fat	Very High Fat	
No. of subjects	230	59	58	60	53	
Age, years	66.1 ± 4.40	65.9 ± 4.46	66.3 ± 3.61	65.8 ± 5.07	66.2 ± 4.25	0.935
Women, *n* (%)	106 (46.1)	26 (44.1)	26 (44.8)	32 (53.3)	22 (41.5)	0.604
Type-2 diabetes, *n* (%)	55 (23.6)	11 (18.6)	11 (19.0)	21 (35.0)	11 (20.8)	0.135
Hypercholesterolemia, *n* (%)	155 (67.4)	39 (66.1)	42 (72.4)	40 (66.7)	34 (64.2)	0.811
Hypertension, *n* (%)	200 (87.0)	50 (84.7)	53 (91.4)	53 (88.3)	44 (83.0)	0.510
Body mass index, Kg/m^2	32.7 ± 3.50	32.0 ± 2.99	32.7 ± 3.64	32.4 ± 3.76	33.6 ± 3.53	0.100
Current smoker, *n* (%)	37 (16.1)	8 (13.6)	10 (17.2)	6 (10.0)	13 (24.5)	0.189
Leisure-time physical activity, MET·min/week	2525 ± 2458	1780 ± 1855	2276 ± 1890	3064 ± 3248	3019 ± 2368	0.011

F&V, fruit and vegetables; MET, metabolic task equivalents. Values are percentages for categorical variables and means ± SD for continuous variables. *P*-values were calculated by analysis of variance–one factor for continuous variables and the χ^2-test for categorical variables, $p < 0.05$.

Table 3. Main dietary nutrient intake and food consumption by group.

	Low F&V		High F&V		
	Low-to-Moderate fat	Very high fat	Low-to-Moderate fat	Very high fat	*p*-value*
Mediterranean diet adherence score (range 0-17)	7.29 ± 2.33	6.67 ± 2.56	10.0 ± 2.49	9.83 ± 2.79	< 0.001
Total energy, Kcal/day	1701 ± 511	2803 ± 420	2026 ± 373	3161 ± 356	< 0.001
Nutrient intake					
Carbohydrates, g/day	180 ± 77.7	256 ± 74.8	249 ± 66.7	324 ± 67.9	< 0.001
Fiber, g/day	15.5 ± 6.15	19.2 ± 4.79	35.3 ± 6.39	42.5 ± 8.87	< 0.001
Protein, g/day	70.8 ± 18.2	99.1 ± 19.6	89.8 ± 19.5	127 ± 21.7	< 0.001
Total fat, g/day	64.8 ± 15.3	140 ± 14.6	69.7 ± 12.6	142 ± 16.9	< 0.001
SFA, g/day	17.7 ± 4.44	36.9 ± 7.19	17.8 ± 4.66	34.5 ± 7.14	< 0.001
MUFA, g/day	32.4 ± 8.69	72.8 ± 11.0	33.7 ± 7.81	75.5 ± 12.4	< 0.001
PUFA, g/day	9.87 ± 3.56	22.5 ± 6.10	12.2 ± 3.98	25.3 ± 7.75	< 0.001
Cholesterol, mg/day	292 ± 105	437 ± 130	317 ± 97.1	459 ± 134	< 0.001
Alcohol, g/day	16.3 ± 20.9	17.3 ± 18.1	6.08 ± 11.1	11.6 ± 13.4	< 0.001
Food consumption, g/day					
F&V	278 ± 68.7	300 ± 52.7	1265 ± 299	1328 ± 270	< 0.001
Legumes	14.2 ± 7.95	18.3 ± 7.94	24.3 ± 16.4	30.4 ± 17.2	< 0.001
Cereals	140 ± 88.1	170 ± 85.3	129 ± 78.7	177 ± 82.4	0.005
Dairy	244 ± 113	360 ± 226	297 ± 213	380 ± 216	0.001
Meat	117 ± 44.9	151 ± 58.4	120 ± 48.0	171 ± 67.9	< 0.001
Fish	64.6 ± 38.5	92.6 ± 53.8	100 ± 48.0	136 ± 56.5	< 0.001
Nuts	5.11 ± 6.53	20.6 ± 21.4	10.5 ± 10.5	39.1 ± 30.7	< 0.001
Olive oil	23.2 ± 11.1	53.4 ± 15.5	24.0 ± 10.6	48.1 ± 16.4	< 0.001
Sunflower oil	1.40 ± 3.76	2.04 ± 5.72	0.91 ± 3.53	2.34 ± 7.22	0.455
Butter	0.75 ± 2.22	1.87 ± 4.77	0.56 ± 2.18	0.87 ± 2.08	0.092
Margarine	0.68 ± 1.65	1.38 ± 3.44	0.35 ± 0.98	1.34 ± 3.08	0.065
Pastries	18.4 ± 20.2	40.9 ± 43.6	14.6 ± 18.8	31.8 ± 32.1	< 0.001

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; F&V, fruit and vegetables. Values are percentages for categorical variables and means ± SD for continuous variables. * *P*-values were calculated by analysis of variance–one factor for continuous variables and the χ^2-test for categorical variables, $p < 0.05$.

The predominant carotenoids in plasma were: astaxanthin, lutein, canthaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene. Other carotenoids were all below the LoQ. Plasma concentrations of retinol were comparable in the four groups.

Individuals with high F&V consumption showed greater plasma total carotenoid concentrations (+3.04 μmol/L (95 % CI: 0.90, 5.17), *p*-value = 0.005) than participants with low F&V consumption. The differences were statistically significant when the intake of fat was low-to-moderate (+3.83 μmol/L (0.97, 6.7), *p* -value = 0.009, P for interaction = 0.161), but not when the intake of fat was very high. In the same direction, plasma concentration of carotenes, xanthophylls and the provitamin A fraction significantly increased particularly when fat intake was low-to-moderate, but not when it was very high (**Table 4**).

Participants with very high fat consumption tended to present with a lower concentration of carotenoids (-2.69 μmol/L (-5.54; 0.16), *p*-value = 0.064) in comparison with individuals with low-to-moderate fat intake. Similarly, although the difference did not achieve statistical significance, when the consumption of F&V was high the results were on the same direction (-2.52 μmol/L (-6.10; 1.05), *p*-value = 0.166). Low consumption of F&V elicited no differences between the low-to-moderate fat intake and the very high fat intake groups. Plasma concentrations of xanthophylls and the provitamin A fraction were significantly lower in high fat consumers, particularly, when F&V intake was high, which was significant for xanthophylls and showed a trend in that direction for the provitamin A fraction (**Table 5**).

The comparison between the groups with extreme conditions is compiled in **Table 6**. High F&V and low-to-moderate fat consumers had significantly higher carotenoid concentrations than low F&V and very high fat intake (+3.86 μmol/L (0.86; 6.85), *p*-value = 0.012). Xanthophyll differences were less dispersed but smaller in magnitude relative to carotenes. The provitamin A fraction showcased the same results. No significant differences were found between the group high in both F&V and fat and the group low in F&V and low-to-moderate in fat.

Table 4. Differences in total carotenoid, carotenes, xanthophylls, provitamin A and non-provitamin A plasma concentrations (µmol/L) between F&V consumption groups.

		High F&V vs. Low F&V	*p*-value	High F&V vs. Low F&V (Low-to-Moderate Fat)	*p*-value	High F&V vs. Low F&V (Very high Fat)	*p*-value
Total carotenoids	Median	5.31 vs. 2.08		6.75 vs. 2.48		4.23 vs. 1.71	
	ß [CI]-model 1	3.64 [1.85; 5.44]	**<0.001**	5.01 [2.54; 7.48]	**<0.001**	2.12 [-0.43; 4.67]	0.104
	ß [CI]-model 2	2.97 [1.18; 4.76]	**0.001**	4.13 [1.67; 6.60]	**0.001**	1.67 [-0.84; 4.18]	0.192
	ß [CI]-model 3	3.04 [0.90; 5.17]	**0.005**	3.83 [0.97; 6.70]	**0.009**	1.33 [-1.64; 4.30]	0.379
Carotenes	Median	3.00 vs. 0.25		4.26 vs. 0.95		1.36 vs. 0.19	
	ß [CI]-model 1	3.47 [1.50; 5.44]	**< 0.001**	4.70 [1.99; 7.42]	**< 0.001**	2.06 [-0.75; 4.88]	0.150
	ß [CI]-model 2	2.77 [0.79; 4.74]	**0.006**	3.79 [1.07; 6.51]	**0.006**	1.60 [-1.17; 4.37]	0.257
	ß [CI]-model 3	2.80 [0.46; 5.14]	**0.019**	3.53 [0.38; 6.68]	**0.028**	1.35 [-1.92; 4.62]	0.419
Xanthophylls	Median	2.04 vs. 1.09		2.44 vs. 1.04		2.03 vs. 1.09	
	ß [CI]-model 1	1.00 [0.67; 1.33]	**< 0.00001**	1.27 [0.81; 1.73]	**< 0.00001**	0.69 [0.22; 1.17]	**0.004**
	ß [CI]-model 2	0.89 [0.55; 1.23]	**< 0.00001**	1.13 [0.67; 1.59]	**< 0.00001**	0.62 [0.15; 1.09]	**0.009**
	ß [CI]-model 3	0.88 [0.48; 1.27]	**< 0.001**	1.00 [0.48; 1.53]	**< 0.001**	0.42 [-0.13; 0.96]	0.136
Provitamin A	Median	2.88 vs. 0.52		3.69 vs. 0.43		1.86 vs. 0.56	
	ß [CI]-model 1	2.50 [1.44; 3.55]	**< 0.00001**	3.37 [1.92; 4.82]	**< 0.00001**	1.50 [0.003; 3.00]	0.050
	ß [CI]-model 2	2.13 [1.07; 3.19]	**< 0.001**	2.90 [1.45; 4.35]	**< 0.001**	1.26 [-0.22; 2.73]	0.095
	ß [CI]-model 3	2.41 [1.15; 3.67]	**< 0.001**	2.88 [1.19; 4.56]	**< 0.001**	1.21 [-0.54; 2.96]	0.177
Non-provitamin A	Median	1.89 vs. 1.06		2.27 vs. 1.23		1.33 vs. 0.99	
	ß [CI]-model 1	1.15 [0.21; 2.09]	**0.017**	1.63 [0.33; 2.94]	**0.014**	0.62 [-0.73; 1.97]	0.369
	ß [CI]-model 2	0.84 [-0.10; 1.79]	0.081	1.24 [-0.07; 2.55]	0.064	0.41 [-0.92; 1.75]	0.544
	ß [CI]-model 3	0.63 [-0.50; 1.76]	0.275	0.96 [-0.56; 2.48]	0.215	0.13 [-1.45; 1.70]	0.874

ß, difference between groups; CI, confidence interval. Model 1—adjusted for age and sex. Model 2—adjusted for age, sex and physical activity. Model 3—adjusted for the variables used in model 2 plus energy intake, the modified Mediterranean diet adherence score (subtracting the questions regarding F&V, fat and wine) and alcohol consumption (g/day). *P*-values < 0.05 were considered significant.

Table 5. Differences in total carotenoid, carotenes, xanthophylls, provitamin A and non-provitamin A plasma concentrations (μmol/L) between fat intake groups.

		Very high fat vs. Low-to-Moderate Fat	*p*-value	Very high fat vs. Low-to-Moderate Fat (Low F&V)	*p*-value	Very high fat vs. Low-to-Moderate Fat (High F&V)	*p*-value
Total carotenoids	Median	2.35 vs. 5.02		1.71 vs. 2.48		4.23 vs. 6.75	
	ß [CI]-model 1	-1.46 [-3.31; 0.39]	0.122	0.04 [-2.45; 2.53]	0.976	-2.85 [-5.39; -0.30]	**0.028**
	ß [CI]-model 2	-1.59 [-3.37; 0.19]	0.081	-0.29 [-2.72; 2.15]	0.816	-2.75 [-5.24; -0.27]	**0.030**
	ß [CI]-model 3	-2.69 [-5.54; 0.16]	0.064	-0.02 [-3.54; 3.50]	0.991	-2.52 [-6.10; 1.05]	0.166
Carotenes	Median	0.37 vs. 2.48		0.19 vs. 0.95		1.36 vs. 4.26	
	ß [CI]-model 1	-1.19 [-3.21; 0.82]	0.245	0.23 [-2.55; 3.00]	0.873	-2.41 [-5.18; 0.35]	0.087
	ß [CI]-model 2	-1.32 [-3.27; 0.63]	0.185	-0.12 [-2.85; 2.60]	0.928	-2.31 [-5.02; 0.39]	0.094
	ß [CI]-model 3	-2.36 [-5.51; 0.79]	0.142	0.13 [-3.81; 4.07]	0.948	-2.05 [-5.97; 1.87]	0.305
Xanthophylls	Median	1.25 vs. 1.54		1.09 vs. 1.04		2.03 vs. 2.44	
	ß [CI]-model 1	-0.35 [-0.71; 0.002]	0.051	-0.05 [-0.51; 0.42]	0.847	-0.62 [-1.09; -15.00]	**0.010**
	ß [CI]-model 2	-0.38 [-0.72; -0.03]	**0.031**	-0.10 [-0.55; 0.35]	0.668	-0.61 [-1.07; -0.14]	**0.010**
	ß [CI]-model 3	-0.88 [-1.41; -0.35]	**0.001**	-0.19 [-0.84; 0.45]	0.555	-0.78 [-1.44; -0.13]	**0.019**
Provitamin A	Median	0.81 vs. 2.06		0.56 vs. 0.43		1.86 vs. 3.69	
	ß [CI]-model 1	-1.16 [-2.26; -0.06]	**0.039**	-0.19 [-1.64; 1.27]	0.803	-2.06 [-3.55; -0.56]	**0.007**
	ß [CI]-model 2	-1.23 [-2.30; -0.17]	**0.023**	-0.36 [-1.80; 1.07]	0.618	-2.01 [-3.47; -0.54]	**0.007**
	ß [CI]-model 3	-2.18 [-3.87; -0.48]	**0.012**	-0.22 [-2.30; 1.85]	0.834	-1.89 [-4.00; 0.21]	0.078
Non-provitamin A	Median	1.24 vs. 1.78		0.99 vs. 1.23		1.33 vs. 2.27	
	ß [CI]-model 1	-0.30 [-1.25; 0.65]	0.537	0.22 [-1.09; 1.54]	0.738	-0.79 [-2.14; 0.55]	0.248
	ß [CI]-model 2	-0.36 [-1.29; 0.58]	0.455	0.08 [-1.22; 1.37]	0.910	-0.75 [-2.07; 0.57]	0.267
	ß [CI]-model 3	-0.51 [-2.00; 0.98]	0.502	0.20 [-1.66; 2.07]	0.832	-0.63 [-2.53; 1.26]	0.514

ß, difference between groups; CI, confidence interval. Model 1—adjusted for age and sex. Model 2—adjusted for age, sex and physical activity. Model 3—adjusted for the variables used in model 2 plus energy intake, the modified Mediterranean diet adherence score (subtracting the questions regarding F&V, fat and wine) and alcohol consumption (g/day). *P*-values < 0.05 were considered significant.

Table 6. Differences in total carotenoid, carotenes, xanthophylls, provitamin A and non-provitamin A plasma concentrations (µmol/L) between extreme groups.

		Low-to-Moderate Fat & High F&V vs. Very high Fat & Low F&V	*p*-value	Very high Fat & High F&V vs. Low-to-Moderate Fat & Low F&V	*p*-value
Total carotenoids	Median	6.75 vs. 1.71		4.23 vs. 2.48	
	ß [CI]-model 1	4.97 [2.49; 7.45]	**<0.001**	2.16 [-0.39; 4.70]	0.096
	ß [CI]-model 2	4.42 [1.98; 6.86]	**<0.001**	1.38 [-1.15; 3.91]	0.284
	ß [CI]-model 3	3.86 [0.86; 6.85]	**0.012**	1.31 [-3.27; 5.89]	0.575
Carotenes	Median	4.26 vs. 0.19		1.36 vs. 0.95	
	ß [CI]-model 1	4.48 [1.75; 7.21]	**0.001**	2.29 [-0.52; 5.10]	0.110
	ß [CI]-model 2	3.92 [1.22; 6.61]	**0.004**	1.48 [-1.32; 4.27]	0.300
	ß [CI]-model 3	3.40 [0.08; 6.72]	**0.045**	1.48 [-3.58; 6.54]	0.567
Xanthophylls	Median	2.44 vs. 1.09		2.03 vs. 1.04	
	ß [CI]-model 1	1.32 [0.86; 1.77]	**<0.00001**	0.65 [0.18; 1.12]	**0.007**
	ß [CI]-model 2	1.23 [0.77; 1.68]	**<0.00001**	0.52 [0.05; 0.99]	**0.031**
	ß [CI]-model 3	1.20 [0.65; 1.75]	**<0.001**	0.22 [-0.62; 1.06]	0.608
Provitamin A	Median	3.69 vs. 0.56		1.86 vs. 0.43	
	ß [CI]-model 1	3.56 [2.10; 5.02]	**< 0.00001**	1.32 [-0.18; 2.81]	0.084
	ß [CI]-model 2	3.26 [1.82; 4.70]	**< 0.00001**	0.89 [-0.60; 2.38]	0.240
	ß [CI]-model 3	3.10 [1.33; 4.87]	**< 0.001**	0.98 [-1.72; 3.68]	0.475
Non-provitamin A	Median	2.27 vs. 0.99		1.33 vs. 1.23	
	ß [CI]-model 1	1.41 [0.10; 2.72]	**0.035**	0.84 [-0.50; 2.19]	0.220
	ß [CI]-model 2	1.16 [-0.14; 2.46]	0.080	0.49 [-0.86; 1.83]	0.477
	ß [CI]-model 3	0.76 [-0.83; 2.35]	0.350	0.33 [-2.10; 2.76]	0.791

ß, difference between groups; CI, confidence interval. Model 1— adjusted for age and sex. Model 2—adjusted for age, sex and physical activity. Model 3—adjusted for the variables used in model 2 plus energy intake, the modified Mediterranean diet adherence score (subtracting the questions regarding F&V, fat and wine) and alcohol consumption (g/day). *P*-values < 0.05 were considered significant.

Health effects of the bioactive compounds of the Mediterranean diet

The Mediterranean diet is rich in carotenoids and polyphenols, whose ingestion has been associated with a healthier status. To test whether the actual circulating or excreted concentrations of these compounds or their metabolites, which more accurately reflect tissue exposure, we performed two studies.

The samples analyzed for the first objective were used for the assessment of the cross-sectional relationship between circulating carotenoids and early markers of cardiovascular disease.

A manuscript entitled "**Circulating carotenoids are associated with favorable lipid and fatty acid profiles in an older population at high cardiovascular risk.**" that comprises these results has been submitted to be considered for publication and is currently under review.

Table 7 depicts the general characteristics of the population, as explained in the previous section, separated in women and men.

Table 7. General characteristics of the population.

Characteristics	All	Women	Men
No. of subjects	230	106	124
Age, years	66.1 ± 4.40	66.8 (4.43)	65.5 (4.24)
Type 2 diabetes, n (%)	55 (23.6)	21 (19.8)	34 (27.4)
Hypercholesterolemia, n (%)	155 (67.4)	78 (73.6)	77 (62.1)
Hypertension, n (%)	200 (87.0)	90 (84.9)	110 (88.7)
Medications, n (%)			
Insulin	8 (3.5)	1 (0.9)	7 (5.7)
Metformin	44 (19.1)	16 (15.1)	28 (22.6)
Other hypoglycemic drugs	37 (16.1)	13 (12.3)	24 (19.4)
Cholesterol-lowering agents	115 (50.0)	54 (50.9)	61 (49.2)
Anti-hypertensive agents	191 (83.0)	86 (81.1)	105 (84.7)
Leisure time physical activity, MET·min/week	2525 ± 2458	1995 (1829)	2979 (2819)
Body mass index, Kg/m^2	32.7 ± 3.50	33.0 (3.77)	32.3 (3.27)
Current smoker, n (%)	37 (16.1)	12 (11.3)	25 (20.2)
Dietary intake			
Total energy intake, kcal/day	2400 ± 716	2268 ± 705	2513 ± 708

Table 7. (*cont'd*)

Characteristics	All	Women	Men
Carbohydrate, % kcal/day	41.8 ± 8.19	42.0 ± 8.16	41.6 ± 8.25
Protein, % kcal/day	16.4 ± 3.03	17.2 ± 2.90	15.7 ± 2.97
Fat, % kcal/day	38.1 ± 8.06	39.1 ± 8.16	37.4 ± 7.92
Alcohol, g/day	12.8 ± 16.92	5.42 ± 9.33	19.2 ± 19.3
Mediterranean diet adherence score (range 0-17)	8.4 ± 2.9	8.7 ± 2.8	8.2 ± 3.0

MET, metabolic task equivalents. Values are number and percentages for categorical variables and means ± SD for continuous variables.

When associating the circulating carotenoids with cardiovascular risk factors, regarding anthropometric variables, only higher concentrations of plasma xanthophylls were significantly associated with lower body mass index in women (**Figure 10**).

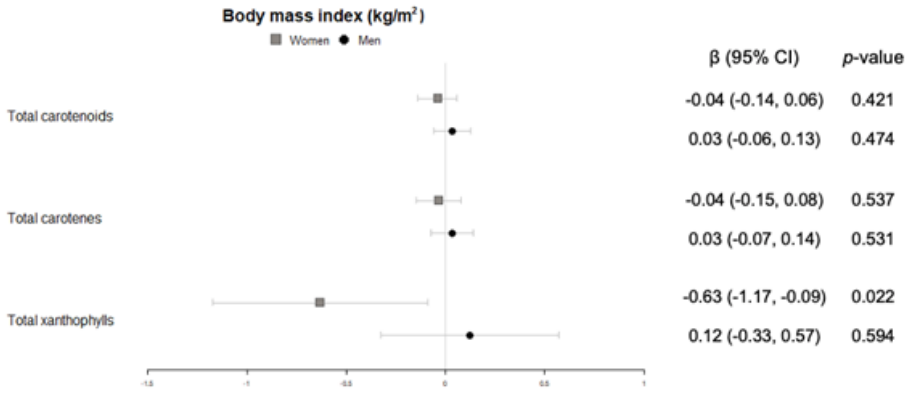


Figure 10. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with body mass index (Kg/m2) (95 %-CI), and their respective *p*-values.

In men, significant inverse associations were found for xanthophylls with waist circumference (**Figure 11**), total carotenes with heart rate (**Figure 12**) and total carotenoids, as well as xanthophylls, with systolic blood pressure (**Figure 13**).

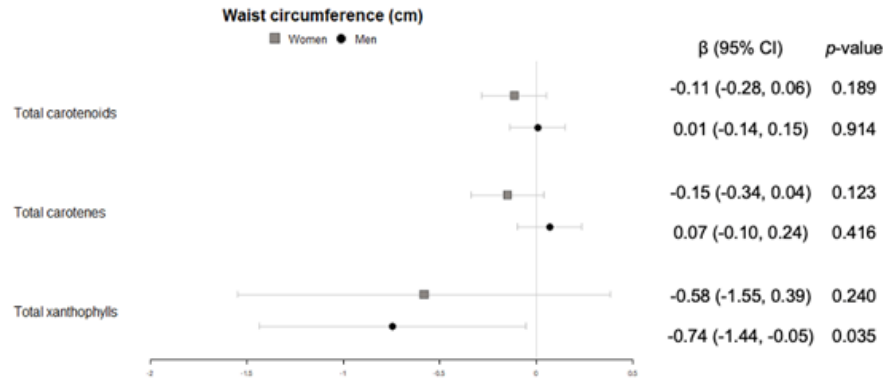


Figure 11. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with waist circumference (cm) (95 %-CI), and their respective *p*-values.

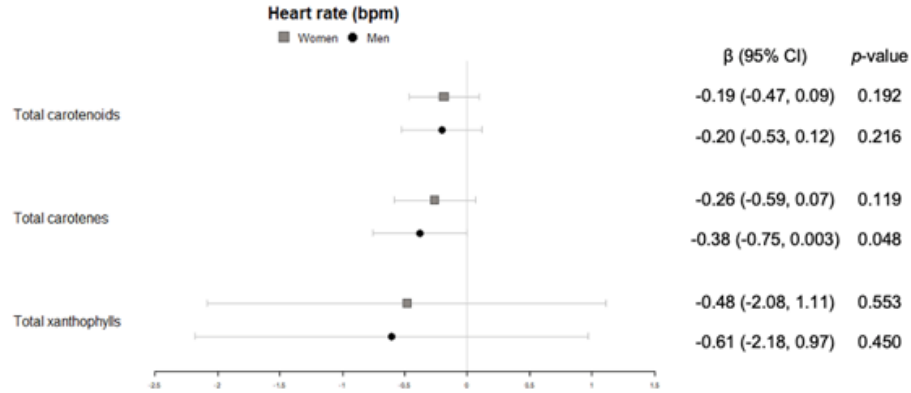


Figure 12. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with heart rate (bpm) (95 %-CI), and their respective *p*-values.

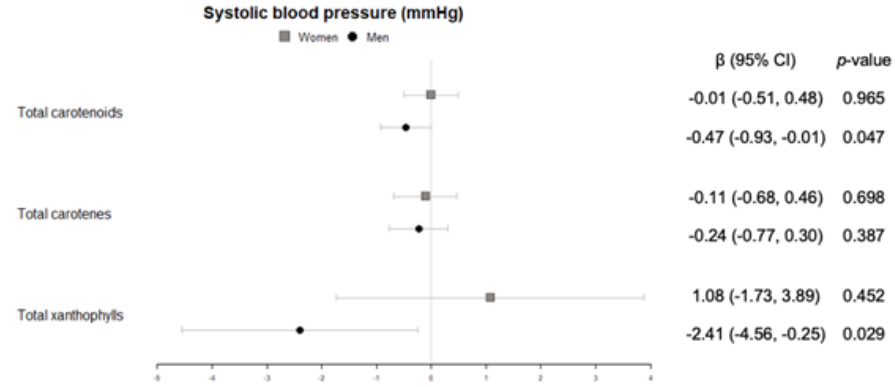


Figure 13. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with systolic blood pressure (mmHg) (95 %-CI), and their respective *p*-values.

Significantly lower levels of plasmatic glucose were observed in men with higher carotenoid concentrations, both carotenes and xanthophylls (**Figure 14**). A trend in the same direction (lower levels) of glycated hemoglobin A1c was observed for higher carotenoid and xanthophyll concentrations (**Figure 14**). These associations were not found in women.

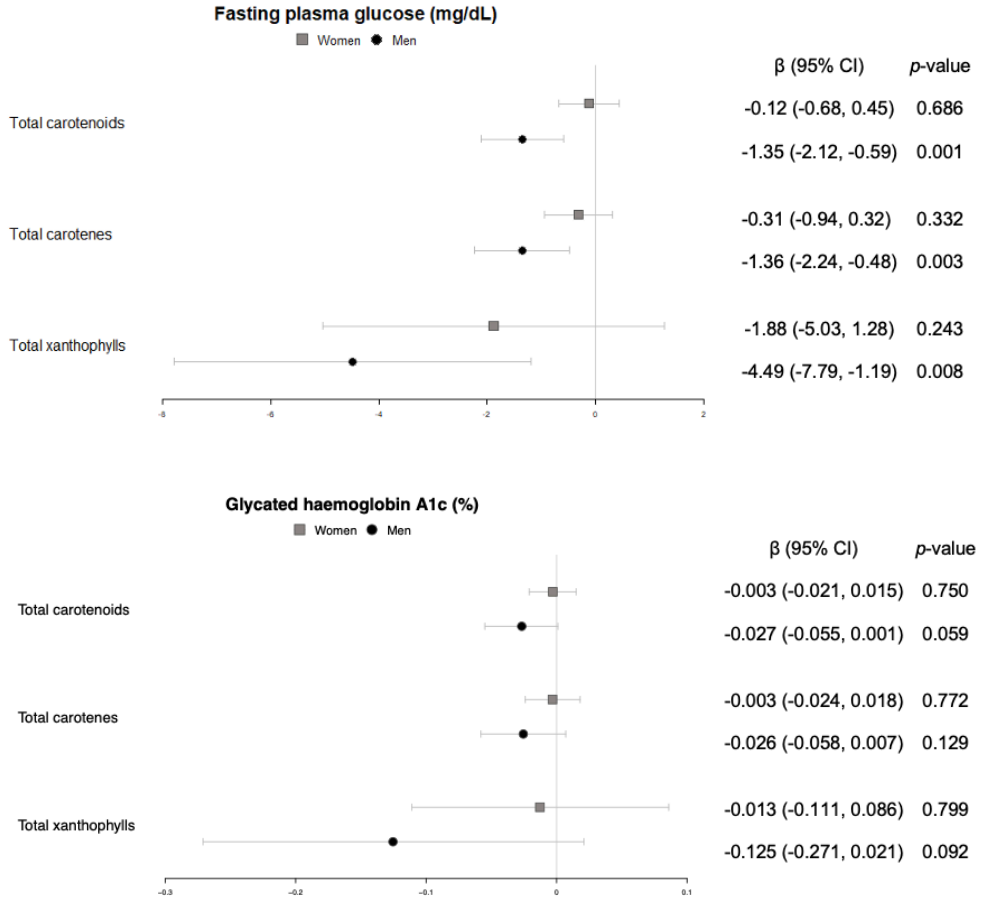


Figure 14. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with fasting plasma glucose (mg/dL) and glycated hemoglobin A1c (%) (95 %-CI), and their respective *p*-values.

Total carotenoids and carotenes were significantly and inversely associated with triglycerides in plasma, in both women and men. A trend in the same direction for the sum of xanthophylls was found only in women (**Figure 15**).

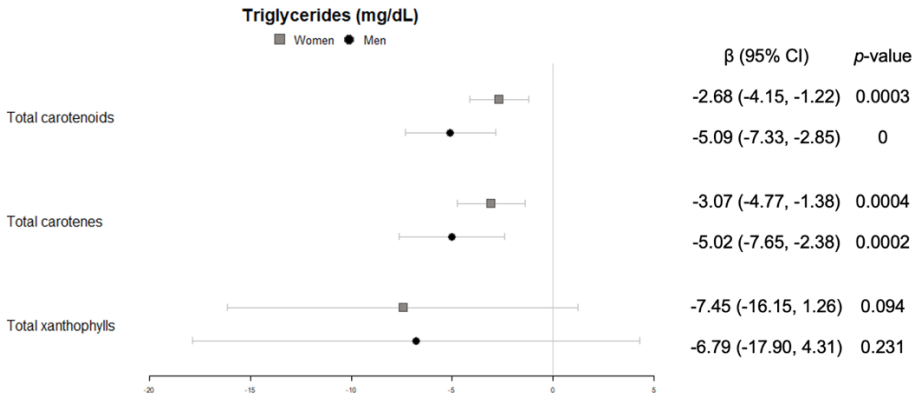


Figure 15. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with blood triglycerides (mg/dL) (95 %-CI), and their respective *p*-values.

Total carotenoids, together with both carotenes and xanthophylls, showed a significantly positive correlation with HDL-cholesterol only in women (**Figure 16**).

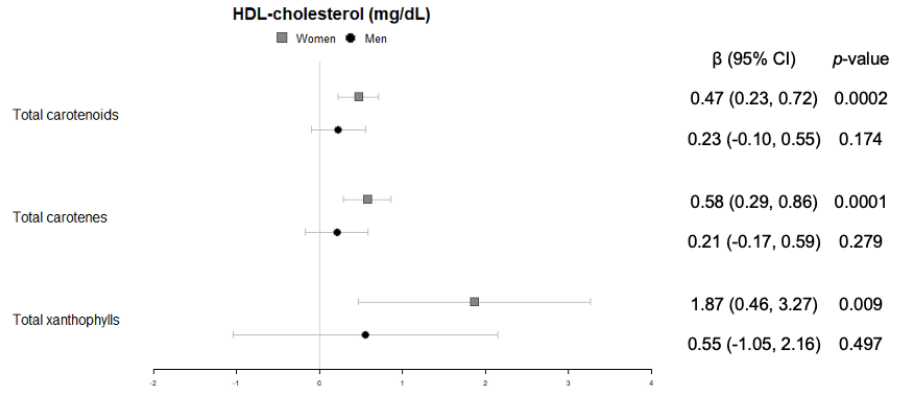


Figure 16. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with blood HDL-cholesterol (mg/dL) (95 %-CI), and their respective *p*-values.

Lastly, both total carotenoids and the sum of carotenes were correlated with lower saturated fatty acids in all individuals, and a trend towards the same direction was found for the sum of xanthophylls in women (**Figure 17**). No association was observed in women for monounsaturated fatty acids, but a significant inverse association for total carotenoids, and a trend in the same direction for carotenes, were found in men (**Figure 17**). Polyunsaturated fatty acids tended to be higher with higher total carotenoids in women. This association was significant for men and, when looking at carotenes, in all individuals (**Figure 17**).

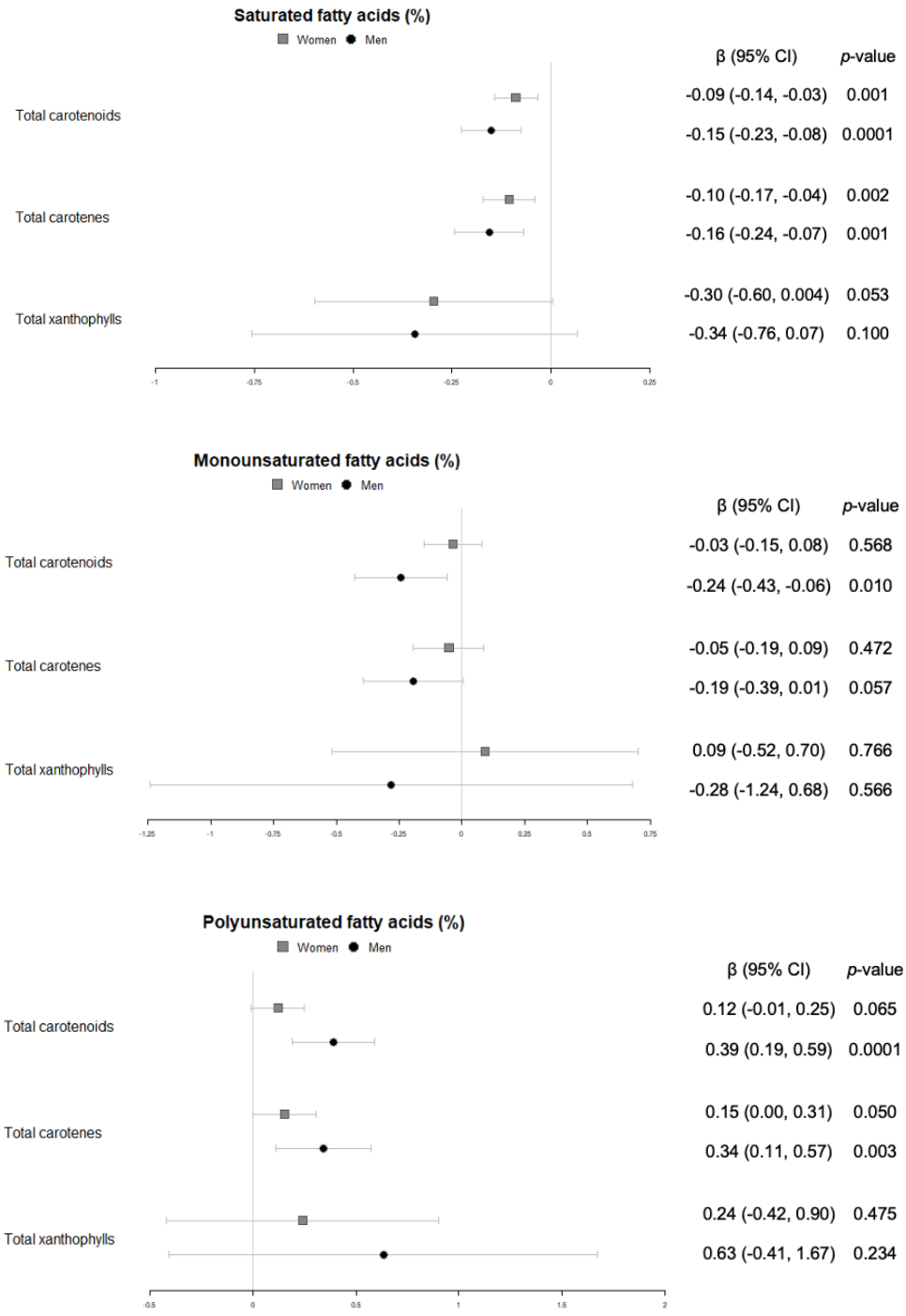


Figure 17. Cross-sectional association of plasma carotenoids and subclasses (per unit increase) with plasma saturated, monounsaturated, and polyunsaturated fatty acids (%) (95 %-CI), and their respective *p*-values.

Then, to study the association between microbial phenolic metabolites concentration in urine and type 2 diabetes development we designed a case-control study within the PREDIMED study, as explained in the previous section. The results of this work have been submitted to be considered for publication with the title: "**One-year changes in urinary microbial phenolic metabolites and the risk of type 2 diabetes. A case-control study.**"

The general characteristics of the population at baseline are shown in **Table 8** according to whether they developed type 2 diabetes during a mean of 3.6 years of follow-up. None of the participants were under hypoglycemic agent treatment even though their fasting plasma glucose was significantly higher at baseline in cases.

Table 8. General characteristics of the population at baseline.

	Case	**Control**	***p*-value**
No. of subjects	46	126	
Women, n (%)	26 (56.5)	84 (66.8)	0.220
Age (years)	65.9 ± 6.0	67.9 ± 5.7	**0.039**
Intervention group, n (%)			0.334
Mediterranean Diet + EVOO	18 (39.1)	54 (42.9)	
Mediterranean Diet + nuts	12 (26.1)	42 (33.3)	
Control	16 (34.8)	30 (23.8)	
Dyslipidemia, n (%)	38 (82.6)	98 (77.8)	0.491
Hypertension, n (%)	45 (97.8)	113 (89.7)	0.084
Body mass index (Kg/m^2)	30.9 ± 3.0	30.6 ± 3.9	0.721
Energy intake (Kcal/day)	2381 ± 544	2276 ± 488	0.226
Smoking habit, n (%)			0.682
Current smoker	8 (17.4)	20 (15.9)	
Past smoker	12 (26.1)	26 (20.6)	
Never smoker	26 (56.5)	80 (63.5)	
Physical activity (METs-min/day)	258.2 ± 176.3	218.4 ± 208.6	0.250
Level of education, n (%)			
High and medium studies	12 (26.1)	25 (15.8)	0.378
Fasting plasma glucose (mg/dL)	118.8 ± 18.1	96.5 ± 12.8	**<0.001**

EVOO, extra virgin olive oil; MET, metabolic task equivalents. Values are percentages for categorical variables and means ± SD for continuous variables. *p*-values were calculated by t-test for continuous variables and the χ^2-test for categorical variables.

The associations between individual urinary MPM with the odds of developing type 2 diabetes are presented in **Table 9**. Compared to participants from the lowest tertile of the 1-year to baseline difference of hydroxybenzoic acid glucuronide, those in the highest tertile had significantly decreased odds of developing type 2 diabetes (OR [95 % CI], 0.39 [0.23-0.64]; $p < 0.001$ for trend). A 1-SD increment in the 1-year difference of this same MPM concentration was also associated with reduced odds of development of type 2 diabetes. Although the trend was not maintained for the highest tertile, participants from the middle tertile of 4-hydroxybenzoic acid, hydroxytyrosol sulphate, and vanillic acid sulphate concentration change had significantly diminished odds of developing type 2 diabetes compared to those in the lowest tertile. All these associations remained significant after adjusting for multiple comparisons (**Figure 18**).

Because of the differences in baseline plasma glucose between the cases and controls we created an extra model further adjusted for fasting plasma glucose (Multivariable model 2). After this adjustment, most of the differences were not maintained. Only participants from the middle tertile of 4-hydroxybenzoic acid concentration change stayed at significantly decreased odds of developing type 2 diabetes compared to those in the lowest tertile. And participants from the middle tertile of *m*-coumaric acid concentration change showed up to be at significantly increased odds of developing type 2 diabetes compared to the participants in the lowest tertile after adjusting for multiple comparisons. The third tertile of vanillic acid sulphate, also showed a significantly higher likelihood of developing the disease than the first tertile when adjusting for glucose but not after the correction for multiple comparisons.

Table 9. Likelihood (OR [95 % CI]) of incident type 2 diabetes by tertiles of 1-year changes in urinary concentrations of MPM in the PREDIMED Study.

	T1	T2	T3	P_{Trend}	1-SD Increment
No. of cases	58	57	57		
4-Hydroxybenzoic acid					
Basic model	1.00 (ref)	**0.31 [0.18-0.54]**	0.81 [0.38-1.71]	0.579	0.91 [0.70-1.19]
Multivariable model 1	1.00 (ref)	**0.31 [0.19-0.53]**	0.88 [0.31-2.49]	0.813	0.94 [0.70-1.26]
Multivariable model 2	1.00 (ref)	**0.22 [0.14-0.36]**	0.67 [0.30-1.48]	0.284	0.78 [0.60-1.01]
Hydroxybenzoic acid glucuronide					
Basic model	1.00 (ref)	**0.54 [0.34-0.86]**	**0.41 [0.26-0.65]**	**<0.001**	**0.63 [0.51-0.79]**
Multivariable model 1	1.00 (ref)	**0.52 [0.32-0.86]**	**0.39 [0.24-0.64]**	**<0.001**	**0.61 [0.49-0.77]**
Multivariable model 2	1.00 (ref)	0.49 [0.13-1.84]	0.61 [0.19-1.97]	0.341	0.69 [0.44-1.09]

Table 9. (*cont'd*)

	T1	T2	T3	P_{Trend}	1-SD Increment
Enterolactone glucuronide					
Basic model	1.00 (ref)	0.97 [0.40-2.35]	0.79 [0.54-1.14]	0.208	1.08 [0.93-1.25]
Multivariable model 1	1.00 (ref)	1.05 [0.51-2.13]	0.73 [0.45-1.19]	0.209	1.10 [0.89-1.36]
Multivariable model 2	1.00 (ref)	1.17 [0.79-1.73]	0.79 [0.29-2.13]	0.789	1.06 [0.73-1.54]
m-coumaric acid					
Basic model	1.00 (ref)	1.35 [0.75-2.44]	1.78 [0.82-3.87]	0.147	1.26 [0.97-1.63]
Multivariable model 1	1.00 (ref)	1.48 [0.75-2.91]	1.71 [0.89-3.32]	0.110	1.24 [0.99-1.55]
Multivariable model 2	1.00 (ref)	**1.46 [1.13-1.89]**	1.58 [0.91-2.73]	0.110	1.11 [0.96-1.30]
Hydroxytyrosol sulphate					
Basic model	1.00 (ref)	**0.54 [0.41-0.72]**	0.57 [0.22-1.50]	0.258	0.93 [0.58-1.49]
Multivariable model 1	1.00 (ref)	**0.59 [0.51-0.68]**	0.57 [0.30-1.09]	0.090	0.94 [0.63-1.39]
Multivariable model 2	1.00 (ref)	0.38 [0.17-0.86]	0.34 [0.07-1.72]	0.265	0.71 [0.31-1.65]
Protocatechuic acid					
Basic model	1.00 (ref)	1.51 [0.40-5.67]	1.75 [0.73-4.20]	0.208	1.26 [0.87-1.83]
Multivariable model 1	1.00 (ref)	1.83 [0.54-6.15]	1.91 [0.83-4.40]	0.129	1.30 [0.91-1.87]
Multivariable model 2	1.00 (ref)	3.00 [0.75-12.02]	2.08 [0.89-4.88]	0.068	1.35 [0.85-2.13]
Vanillic acid glucuronide					
Basic model	1.00 (ref)	0.99 [0.48-2.04]	0.76 [0.31-1.84]	0.546	0.94 [0.60-1.48]
Multivariable model 1	1.00 (ref)	0.95 [0.39-2.35]	0.89 [0.48-1.63]	0.702	1.04 [0.71-1.50]
Multivariable model 2	1.00 (ref)	1.17 [0.44-3.10]	1.53 [0.61-3.81]	0.399	1.31 [0.82-2.08]
Vanillic acid sulphate					
Basic model	1.00 (ref)	**0.83 [0.74-0.93]**	1.13 [0.71-1.81]	0.608	1.02 [0.75-1.38]
Multivariable model 1	1.00 (ref)	**0.87 [0.77-0.97]**	1.11 [0.79-1.55]	0.554	1.01 [0.79-1.31]
Multivariable model 2	1.00 (ref)	0.76 [0.29-2.01]	1.38 [0.97-1.97]	0.046	1.06 [0.70-1.61]

Inverse normal transformation was applied to raw values of metabolites. The basic model was adjusted for sex, age, and intervention group. To the covariables in the basic model we added body mass index, physical activity, smoking status, education level, hypertension, dyslipidemia, and energy intake to build model 1. Model 2 was further adjusted for baseline fasting plasma glucose. Robust variance estimators were used to account for recruitment center. In bold, tertiles that were significantly different from the reference after adjusting the *p*-values to account for multiple testing using the Simes procedure. CI, confidence interval; MPM, microbial phenolic metabolite; SD, standard deviation; OR, odds ratio; Ref., reference.

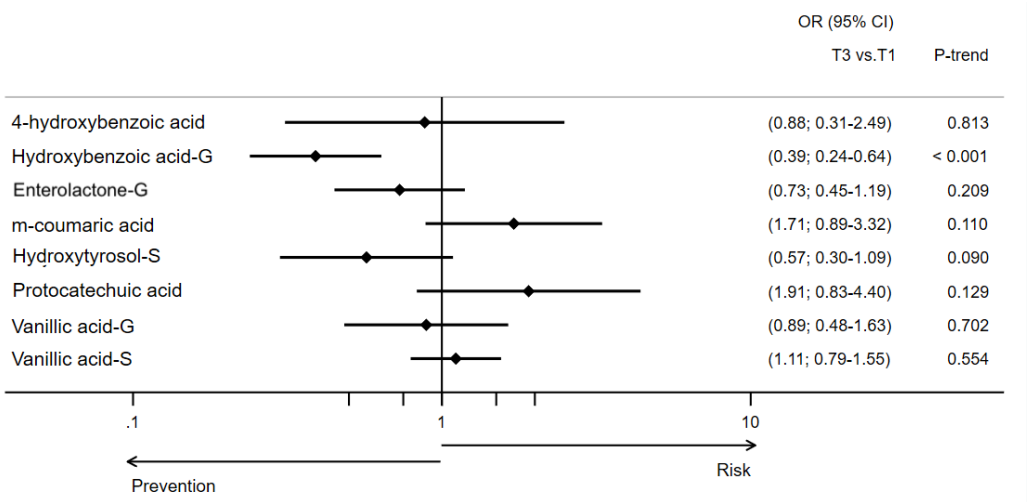


Figure 18. Likelihood (OR [95 % CI]) of development of type 2 diabetes by 1-year changes in urinary concentrations of MPM in the PREDIMED Study.

This model was adjusted for sex, age, intervention group, body mass index, physical activity, smoking status, education level, hypertension, dyslipidemia, and energy intake.

Cell biology of vitamin A in the lung and when subject to acute lung injury

To gain better understanding of how retinoid metabolism within the lung, upstream of the actions of all-*trans*-retinoic acid and the retinoic acid receptors, influences the development of acute lung disease, we employed different mouse knockout models and single cell RNA-sequencing coupled with bioinformatic analyses.

A manuscript entitled "**Retinoids stored locally in the lung are required to attenuate the severity of acute lung injury.**" that describes these results has been submitted to be considered for publication and is currently under review.

HPLC analysis of wild type lung homogenates, from three-month-old chow-fed mice, corroborated the presence of both retinyl esters (555.6 ± 93.6 nmol/g) and all-*trans*-retinol (9.6 ± 3.3 nmol/g) at about 40 % and 10 % of hepatic levels (1485.1 ± 197.1 nmol/g and 91.7 ± 21.8 nmol/g, respectively) (**Figures 19A** and **19B**). Interestingly, while retinyl palmitate accounts for about 75 % of retinyl esters in the liver, lung retinyl ester acyl composition was almost in equimolar concentrations palmitate and stearate (**Figure 20**).

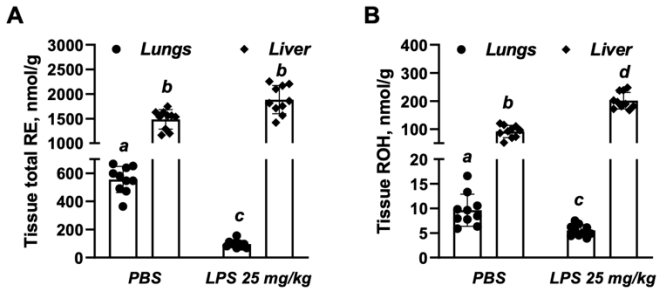


Figure 19. Tissue retinoid concentrations of mice during LPS-induced acute lung injury.

Total retinyl ester (RE) (***panel A***) and retinol (ROH) (***panel B***) concentrations (nmol/g) in lungs and liver. Values marked with different letters (a, b, c or d) are statistically different, $p < 0.05$. All values are given as the mean ± 1 S.D.

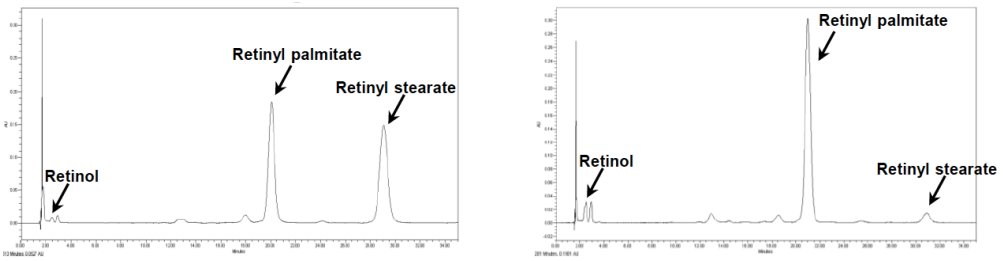


Figure 20. HPLC profiles for retinoid species extracted from lung (**left**) and liver (**right**).

After acute lung injury elicited by LPS (25 mg/Kg) intranasal instillation, lung retinyl ester concentrations declined more than 80 % in 7 days, and retinol more than 40 %, a decline that was not associated with significant changes in hepatic retinyl esters (**Figures 19A** and **19B**). Plasma retinol and RBP4, which is a known negative acute phase response protein, were also lower in injured animals (**Figure 21A** and **21B**). This decline was induced by the acute phase response, evidenced by elevated plasma C-reactive protein concentrations (**Figure 21C** and **21D**).

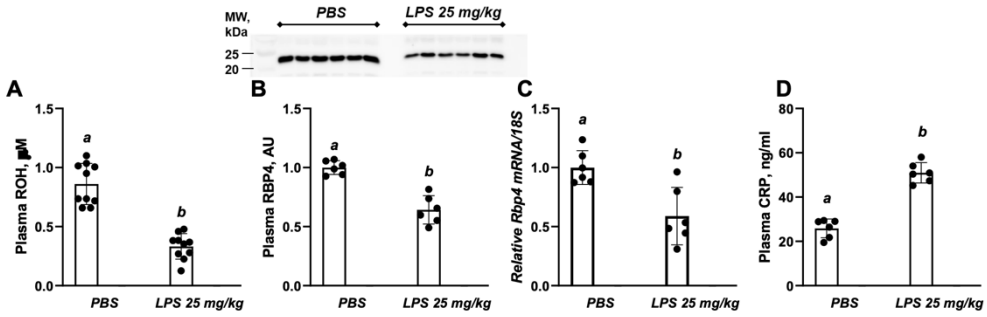


Figure 21. Plasma retinol (ROH), RBP4 and C-reactive protein (CRP) concentrations and hepatic RBP4 expression of mice during LPS-induced acute lung injury.

Panel A – Plasma retinol concentrations (μM) determined by HPLC. ***Panel B*** – quantitative representation of retinol-binding protein 4 (RBP4) relative concentrations determined by immunoblot (upper insert) in plasma of mice. Each lane represents an extract prepared from an individual animal, n = 10 for each group. ***Panel C*** – hepatic *Rbp4* mRNA expression determined by qRT-PCR in mice. ***Panel D*** – C-reactive protein concentration determined by ELISA in plasma of mice, n = 6 for each group. All seven days after intranasal instillation of LPS (25 mg/Kg of body weight) or vehicle PBS alone Values marked with different letters (a, b, c or d) are statistically different, $p < 0.05$. All values are given as the mean ± 1 S.D.

Two models of retinoid-insufficiency were used to understand the abovementioned results: one lacking the ability to store retinoids due to the deletion of the gene encoding LRAT (*Lrat*-deficient or *Lrat*$^{-/-}$ mice) and one that cannot mobilize hepatic retinol due to the lack of RBP4 (*Rbp4*-deficient or *Rbp4*$^{-/-}$ mice). When maintained on a retinoid-sufficient diet and under normal physiological conditions, pulmonary and plasma concentrations of retinol in *Lrat*$^{-/-}$ mice are about 10 and 50 % of their wild type counterparts (**Figure 22A-22C**). These animals synthetize enough all-*trans*-retinol to be phenotypically normal. In the same conditions, extrahepatic tissues of *Rbp4*-deficient mice depend on postprandially delivered retinoids for maintenance of vitamin A signaling. Lungs of *Rbp4*$^{-/-}$ mice have, nevertheless, the same retinyl ester and retinol concentrations than their wild type counterparts (**Figure 22D-22F**).

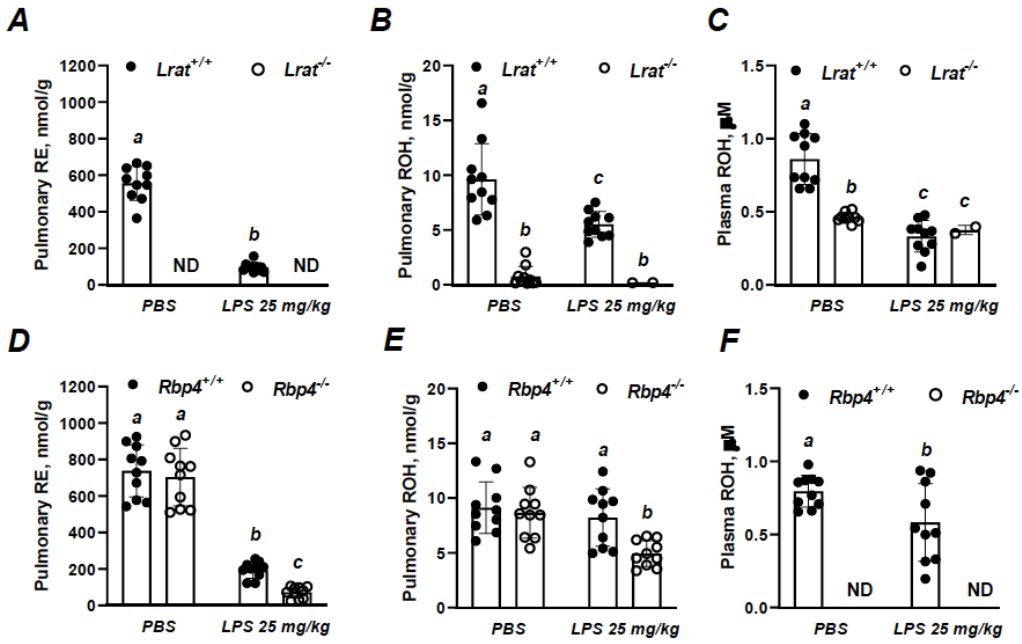


Figure 22. Retinoid concentrations in mouse lungs and plasma during LPS-induced ALI.

Lung total retinyl ester (RE) (***Panels A and D***) and retinol (ROH) (***Panels B and E***) concentrations (nmol/g) and plasma retinol (***Panels C and F***) concentrations (μM) determined by HPLC in mice 7 days after intranasal instillation of LPS (25 mg/Kg of body weight in PBS) or PBS alone.

After a lethal dose of intranasally instilled LPS, *Lrat*-deficient mice survival rapidly declined to 15 % while survival rate of *Rbp4*-deficient mice did not differ significantly from their controls (**Figure 23**).

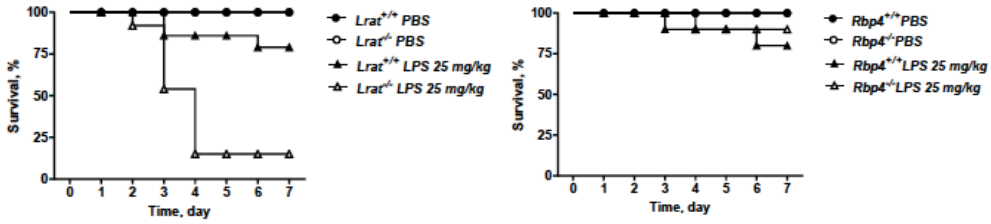


Figure 23. Survival curves of mice subject to acute lung injury.

Kaplan-Meier survival curves for *Lrat*$^{-/-}$ (**left**) and *Rbp4*$^{-/-}$ (**right**) mice as well as for their respective littermate controls (*Lrat*$^{+/+}$ and *Rbp4*$^{+/+}$) over the 7-day period after intranasal instillation of a LPS (25 mg/Kg of body weight in PBS) or PBS alone. n = 14 for each group.

About $5 \cdot 10^5$ pulmonary retinoid-containing cells were isolated by FACS using the autofluorescence of retinoids (emission at 455 nm upon excitation at 350 nm) to gain deeper insight into cellular aspects of retinoid storage in the lungs. Most of the freshly isolated cells had an elongated quiescent fibroblast morphology in culture, with intracellular lipid droplets that declined after 3 days, in parallel to the acquisition of spindle-shaped morphology. These changes were explained by a decrease in *Lrat* expression and increase in *Acta2*, a marker of fibrosis (**Figure 24**).

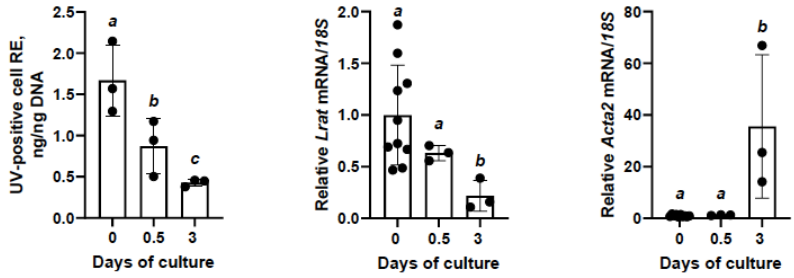


Figure 24. Characterization of FACS isolated cells.

Total retinyl ester concentrations (ng/ng DNA) determined by HPLC and *Lrat* and *Acta2* mRNA expression determined by qRT-PCR in retinoid-containing cells in culture.

The isolated cells were subject to single-cell RNA sequencing and postprocessing analyses allowed for the identification of 10 clusters (clusters 0-9) based on principal component analysis of the gene expression. Highly discriminative tissue-specific representative markers were used to distinguish four major cell groups. Unexpectedly, pulmonary fibroblasts were not the only lung cell type accumulating retinoids, although it was the most abundant, representing 83 % of total collected cells. This group consisted of 3 distinct but related clusters that were enriched with general fibroblast/stromal marker transcripts, including collagen type I alpha 1 chain (*Col1a1)* (**Figure 25A** and **25B**), *Pdgfra*, and *Ptgis* (**Figure 25C**). In addition, endothelial cells (cluster 3), enriched in platelet and endothelial cell adhesion molecule 1 (*Pecam1*) (**Figure 25B**) as well as transcripts for other endothelial marker genes, including cadherin 5 (*Cdh5*) and plasmalemma vesicle-associated protein (*Plvap*) (**Figure 25C**) accounted for 7 % of total cells. The remaining 10 % was equally divided in a homogenous group of epithelial cells (cluster 4), distinguished by its enrichment in epithelial cell adhesion molecule (*Epcam*) (**Figure 25A** and **25B**) that consisted exclusively of alveolar type 2 cells expressing surfactant protein genes like *Sfpta1* and *Sfptb* (**Figure 25C**); and a set of myeloid cells, that showed high levels of protein tyrosine phosphatase receptor type C (*Ptprc*) (**Figure 25A** and **25B**) and were represented by 5 clusters (clusters 5-9) differentially expressing immune cell marker genes, including interleukin-1β (*Il-1β*) and lymphocyte-specific protein 1 (*Lsp1*) (**Figure 25C**).

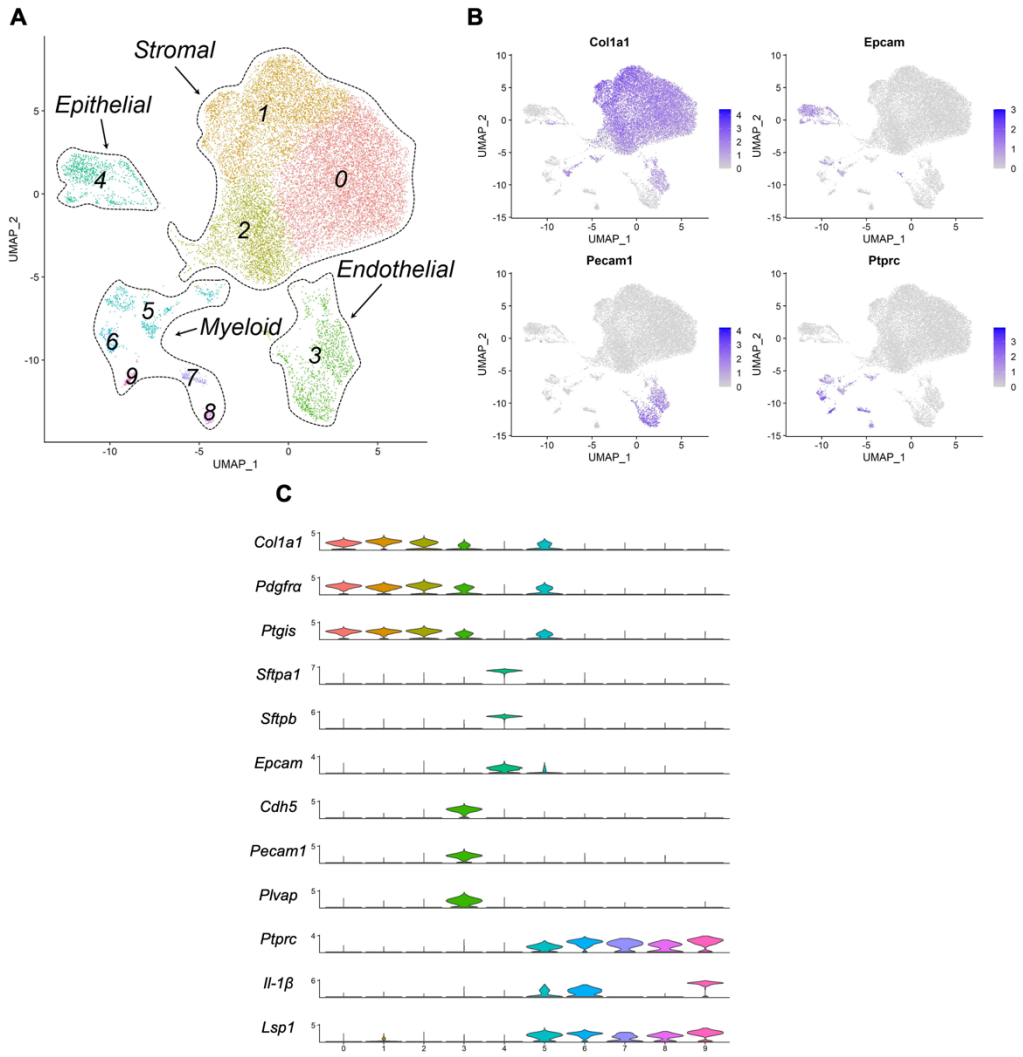


Figure 25. Single cell RNA sequencing of mouse lung retinoid-containing cells.

Panel A – UMAP visualization of cell type clustering inferred from mouse lung retinoid-containing cell scRNA-seq data; Ten retinoid-containing cell clusters were identified. ***Panel B*** – UMAP visualization of relative expression of canonical cell type specific markers to validate stromal (*Col1a1*), epithelial (*Epcam*), endothelial (*Pecam1*), and myeloid (*Ptprc*) cell clusters. The intensity of expression is indicated by blue coloring. ***Panel C*** – Violin plot representation showing known stromal (*Col1a1*, *Pdgfra*, and *Ptgis*), epithelial (*Sfpta1, Sfptb*, and *Epcam*), endothelial (*Pecam1, Cdh5,* and *Plvap*), and myeloid (*Ptprc*, *Il-1β,* and *Lsp1*) marker gene expression across all clusters. The y-axis indicates normalized Log2 expression value, the x-axis indicates cell cluster number.

Regarding retinoid uptake and storage related genes, strong mRNA expression of *Lrat* and retinol-binding protein 1 (*Rbp1*), a gene encoding a protein needed for intracellular transport of the hydrophobic retinol, was detected predominantly in the stromal and endothelial cell clusters (**Figure 26A**). *Rbp4,* which is secreted predominantly by hepatocytes, was found in one of the pulmonary fibroblast clusters (**Figure 26A**). Consistent with the hypothesis that lung cells do not rely substantially on retinol delivered to the tissue by RBP4, none of the cell clusters expressed the gene encoding for the protein STRA6. In fact, different cell clusters expressed genes encoding proteins involved in lipoprotein derived retinoid uptake like *Lpl* (fibroblast cluster 1, endothelial cells, alveolar epithelial cells type 2 and myeloid cell cluster 5); glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (*Gpihbp1*) which mediates the transport and anchoring of LPL in capillaries (endothelial cells) or *Cd36* (endothelial cells, alveolar epithelial cells type 2, and myeloid cell clusters 5 and 6) (**Figure 26A and 26B**). All cell clusters, except for endothelial cluster 3 and myeloid cluster 6, had low levels of transcripts for scavenger receptor class B type 1 (*Scarb1*), the gene encoding for SR-B1. The very low-density lipoprotein receptor (*Vldlr*) gene was highly expressed in most of the fibroblast clusters, endothelial, epithelial, and myeloid cluster 5, while transcripts for low-density lipoprotein receptor (*Ldlr*) were detected only in alveolar epithelial cells type 2 and one immune cell cluster (cluster 6). Low-density lipoprotein receptor-related protein 1 (*Lrp1*), an endocytic cell surface receptor proposed to mediate retinoid uptake in the placenta and fetal lungs, was highly expressed in all fibroblast clusters but less strongly by endothelial and selected immune cell clusters (clusters 5 and 6). Lastly, apolipoprotein E (*ApoE*) was another highly expressed gene encoding a protein critically involved in lipid metabolism was expressed in the fibroblast clusters. Only one myeloid cell cluster (cluster 9) expressed mRNA for diacylglycerol O-acyltransferase 1 (*Dgat1*) (**Figure 26B**) which can account for some RE synthesis when very high levels of retinoids are present in the gut.

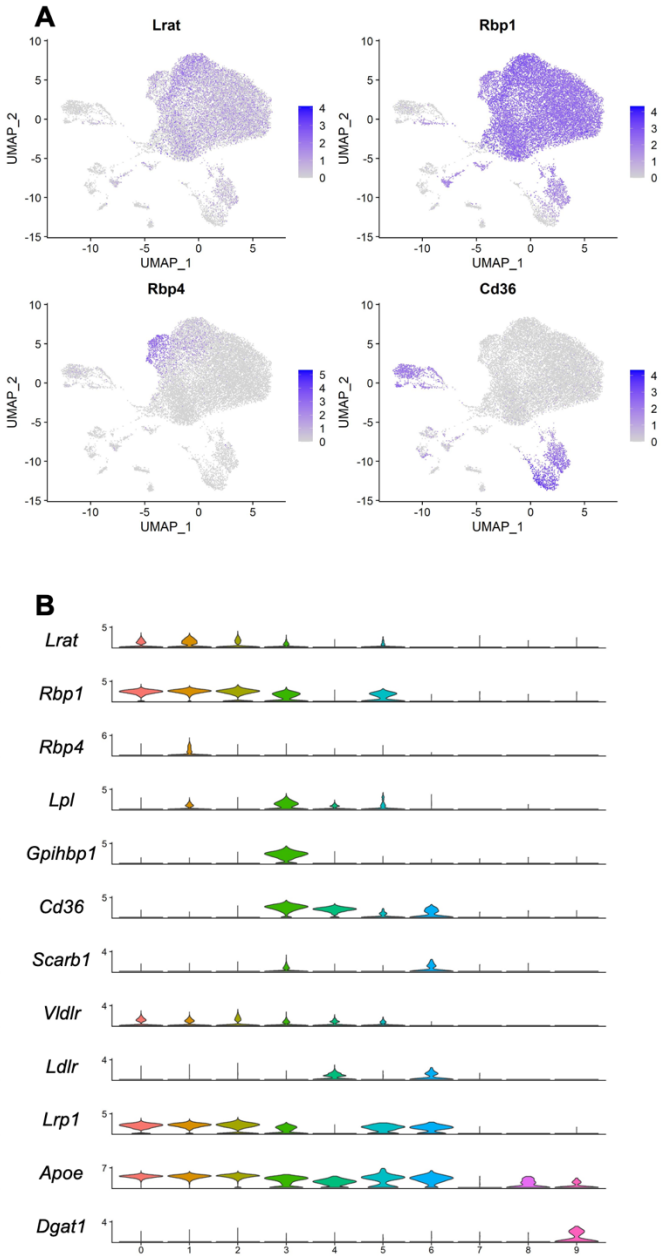


Figure 26. Post-processing analysis of scRNA-seq data.

Panel A – UMAP visualization of relative expression of *Lrat*, *Rbp1*, *Rbp4*, and *Cd36* in cell clusters. The intensity of expression is indicated by blue coloring. ***Panel B*** – Violin plot representation showing expression of genes involved in retinoid (*Lrat*, *Rbp1*, and *Rbp4*) and lipid (*Lpl*, *Gpihbp1*, *Cd36*, *Scarb1*, *Vldlr*, *Ldlr*, *Lrp1*, *Apoe*, and *Dgat1*) metabolism across all clusters. The y-axis indicates normalized Log2 expression value, the x-axis indicates cell cluster number.

Fibroblast clusters were then subject to differential gene expression analysis to understand their heterogeneity. The comparison with previously reported gene patterns for pulmonary fibroblasts (**Supplementary Figure 1**) (61-65) allowed for the categorization of fibroblasts into two groups. Clusters 0 and 2 shared high expression of marker genes that have previously been proposed to characterize alveolar fibroblasts, comprising *Col13a1*$^{+}$ matrix fibroblasts (*Slc7a10*, *Tagln*, *Fgfr4*, *Limch1*, *Col13a1*, *Pdgfra*, *Npnt*, *Bsg*, *Itga8*, *Vegfd*, *Tbx2*, *Ptgis*, *Mfap4*, *Spon1*, and *Cdh11*) and lipofibroblasts (*Lrat, ApoE*, *Rbp1*, *Lpl*, *Ces1d*, *Lrp1*) (**Figure 27A**). Because these traits were not discriminating the clusters, the group was further differentiated by the expression of several genes (**Figure 27B**). Genes encoding proteins involved primarily in cell proliferation in response to growth factor stimulation (transcription factors, activators, corepressors, and enhancer proteins like *Jun*, *Junb*, *Jund*, *Fos*, *Fosb*, *Ier2*, *Egr1*, *Atf3, Cebpd, Btg2, Klf6* as well as proteins regulating transduction of growth factor signaling (*Ccn1*, *Ccn2, Rhob, Ppp1r15a*, *Socs3*, *Dusp1*, *Sat1*) were characteristic of cluster 0, which was annotated as "proliferative" lipofibroblasts. At the same time, cluster 2 was enriched in genes encoding for cell adhesion (*Pdzd2*), growth (*Igfbp3*, *Cdkn2c*), differentiation (*Tagln*, *Hopx*), amino acid uptake (*Slc7a10*), and lipid hydrolysis (*Ces1d*), lipofibroblast-specific transcripts that led to the annotation of this cluster as "conventional" lipofibroblasts.

On the other hand, cluster 1 cells shared high expression levels of marker genes attributed to adventitial fibroblasts like *Col1a1*, *Col1a2*, *Igfbp7*, *Bgn*, *Gpc3*, *Eln*, *C3*, *Mfap5*, *Fstl1*, *Dcn*, *Dkk3*, *Cygb*, *Mmp3*, *Heyl*, and *Col14a1* (**Figure 27A**). It also comprised cells expressing genes that some investigators associate with myofibroblasts and/or pericytes, including *Crip1*, *Htra1*, *Pdgfrβ*, *Acta2*, *Tgfβi*, *Hhip*, and *Aspn* but their low expression did not provide sufficient confidence to classify them as one or the other. Their expression of genes required for lipid metabolism such as *Lpl*, *Lrp1*, *Lrat* and *Rbp1* was seen as these cells being lipofibroblasts committed to the lipogenic-to-myogenic switch and was annotated as "myofibroblast-committed" lipofibroblasts. It included a significant number of upregulated genes encoding proteins aimed at blocking proliferation (*Pmepa1*, *Cav1, Nr4a1*), inducing differentiation (*Klf2*, *Klf4*), promoting growth (*Igfbp4*, *Igfbp6*) and enabling growth arrest (*Sfrp1*, *Nbl1*, *Gas1*, *Gas6*, and *Cdkn1a*). In addition, proteins aimed at acquiring functions attributed to extracellular matrix homeostasis, both structure (*Col1a1*, *Col3a1*, *Col14a1*, *Eln*, *Fbln1*, *Dpt*, *Dcn*, *Mfap5*) and remodeling (*Mmp2*, *Mmp3*, *Timp1*, *Cpxm1*, *Man2a1*, *Cygb*, *Mt1*, *Mt2*, *Fxyd5, Fxyd6*); and lipid metabolism (*Rbp4*, *Pltp*, *C3*, *C4b*) (**Figure 27B**) were also upregulated.

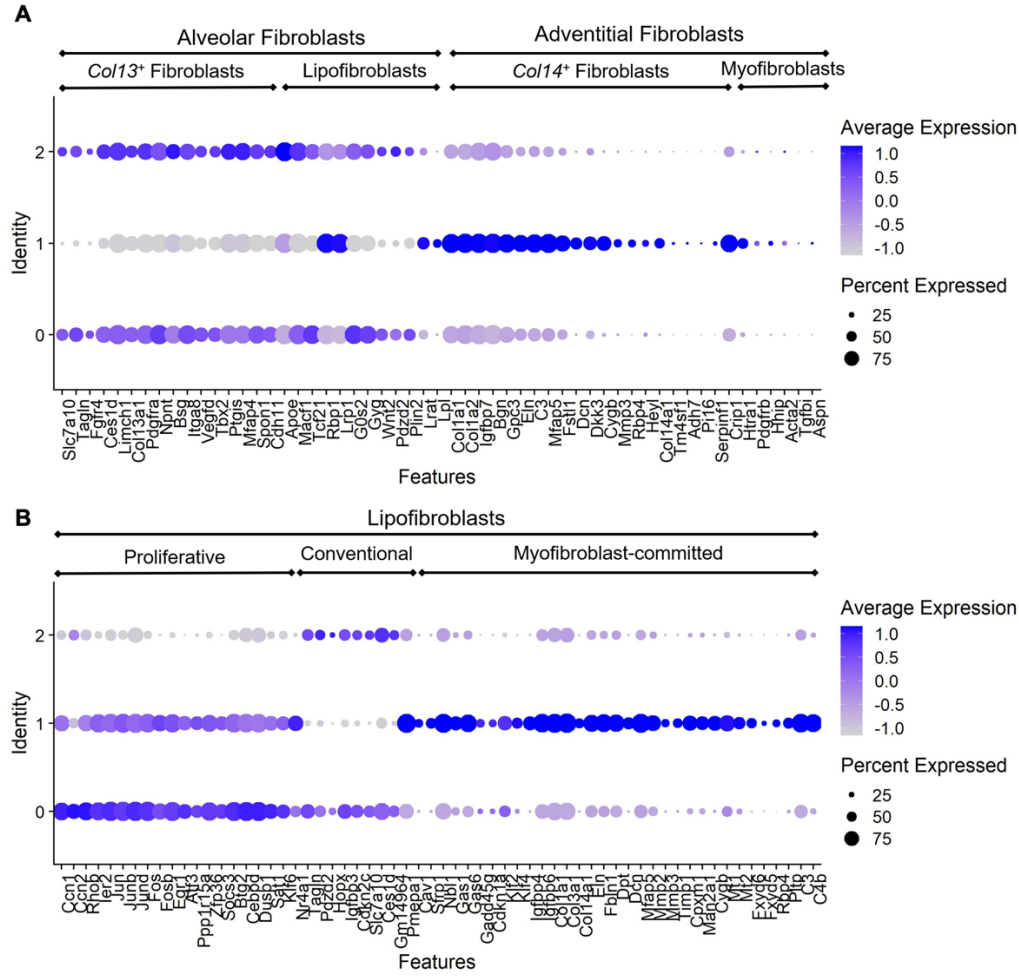
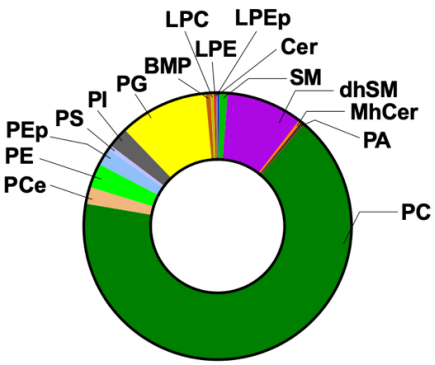


Figure 27. Pulmonary fibroblast characterization.

Panel A – Dot plot visualization of marker gene expression of lung fibroblasts (alveolar and adventitial, *Col13*$^{+}$ and *Col14*$^{+}$, lipofibroblasts, and myofibroblasts) in stromal clusters from retinoid-containing cell scRNA-seq datasets. ***Panel B*** – Dot plot visualization of differentially expressed genes (DEGs) in stromal clusters (proliferative, conventional, and myofibroblast-committed lipofibroblasts) from retinoid-containing cell scRNA-seq datasets. In both cases, the x-axis (features) gives gene names and the y-axis (identity) indicates cluster numbers. The level of expression is specified by the color legend. Size of the cell population expressing the gene of interest is indicated by the size of the circle as specified by the legend.

With the previous results, only *Lrat* mice were used for experiments from then on, analyzing the initial events preceding the lethality observed for *Lrat*$^{-/-}$ mice at an early time (24 hours after instillation) and employing a nonlethal LPS concentration (5 mg/Kg) that is capable of triggering an acute inflammatory response in the lung. Higher BAL cell numbers (**Figure 29A**), and higher BAL and plasma TNFα concentration (**Figure 29B** and **29C**) in *Lrat*$^{-/-}$ mice evidenced significantly greater inflammatory lung injury. A small but statistically significant increase in EVLW was also observed (**Figure 29D**). Other parameters that define lung functionality, like surfactant proteins B and C were downregulated in *Lrat*$^{-/-}$ and wild type mice. *Sftpb* downregulation was significantly greater in *Lrat*-deficient mice (**Figure 29E**), while *Sftpc* did not show a significant downregulation in these mice (**Figure 29F**). This dysregulation in surfactant protein gene expression was associated with significant alteration in surfactant lipid composition (**Figure 29G**). Phosphatiylcholines (PC) and phosphatidylglycerols (PG), the major surfactant associated lipid species (**Figure 28**), were in significantly lower concentrations in LPS-instilled *Lrat*$^{-/-}$ mice (**Figure 29H** and **29I**). Lastly, consistent with the significantly elevated EVLW (**Figure 29D**), mRNA expression of genes encoding junction proteins needed for maintaining alveolar barrier integrity, including *Cdh5*, *Ocln*, and *Tjp1* was significantly downregulated in *Lrat*$^{-/-}$ lungs 24 hours after LPS instillation compared to littermate controls (**Figure 29J-29M**).



Lipid species	Mol %
Phosphatylcholine (PC)	66.87835
Phosphatidylglycerol (PG)	11.32748
Dihydrosphingomyelin (dhSM)	8.153958
Phosphatidylethanolamine (PE)	2.886557
Phosphatidylinositol (PI)	2.797658
Plasmalogen phosphatidylethanolamine (Pep)	2.154491
Ether phosphatidylcholine (PCe)	2.058167
Sphingomyelin (SM)	1.015562
Bis(monoacylglycero)phosphate (BMP)	0.591869
Phosphatidylserine (PS)	0.503343
Monohexosylceramide (MhCer)	0.364123
Lysophosphatidylcholine (LPC)	0.326848
Lysophosphatidylethanolamine (LPE)	0.176749
Sulfatide (Sulf)	0.161722
Ceramide (Cer)	0.140697
Phosphatidic acid (PA)	0.140075
Plasmogen Lysophosphatidylethanolamine (LPEp)	0.096730
Lysophosphatidylinositol (LPI)	0.077462
Lysophosphatidylserine (LPS)	0.061962
Acyl Phosphatidylglycerol (AcylPG)	0.022806
Lactosylceramide (LacCer)	0.022361
Monosialodihexosylganglioside (GM3)	0.019581
Ether lysophosphatidylcholine (LPCe)	0.005497
Dihydroceramide (dhCer)	0.004984
N-Acyl Phosphatidylethanolamine (NAPE)	0.004202
N-Acyl Serine (NSer)	0.003848
Globotriaosylceramide (GB3)	0.002660
N-Acyl Phosphatidylserine (NAPS)	0.000251

Figure 28. Mouse BAL fluid lipid composition.

Lipid species detected by UPLC-MS/MS and their mol % in mouse BAL fluid.

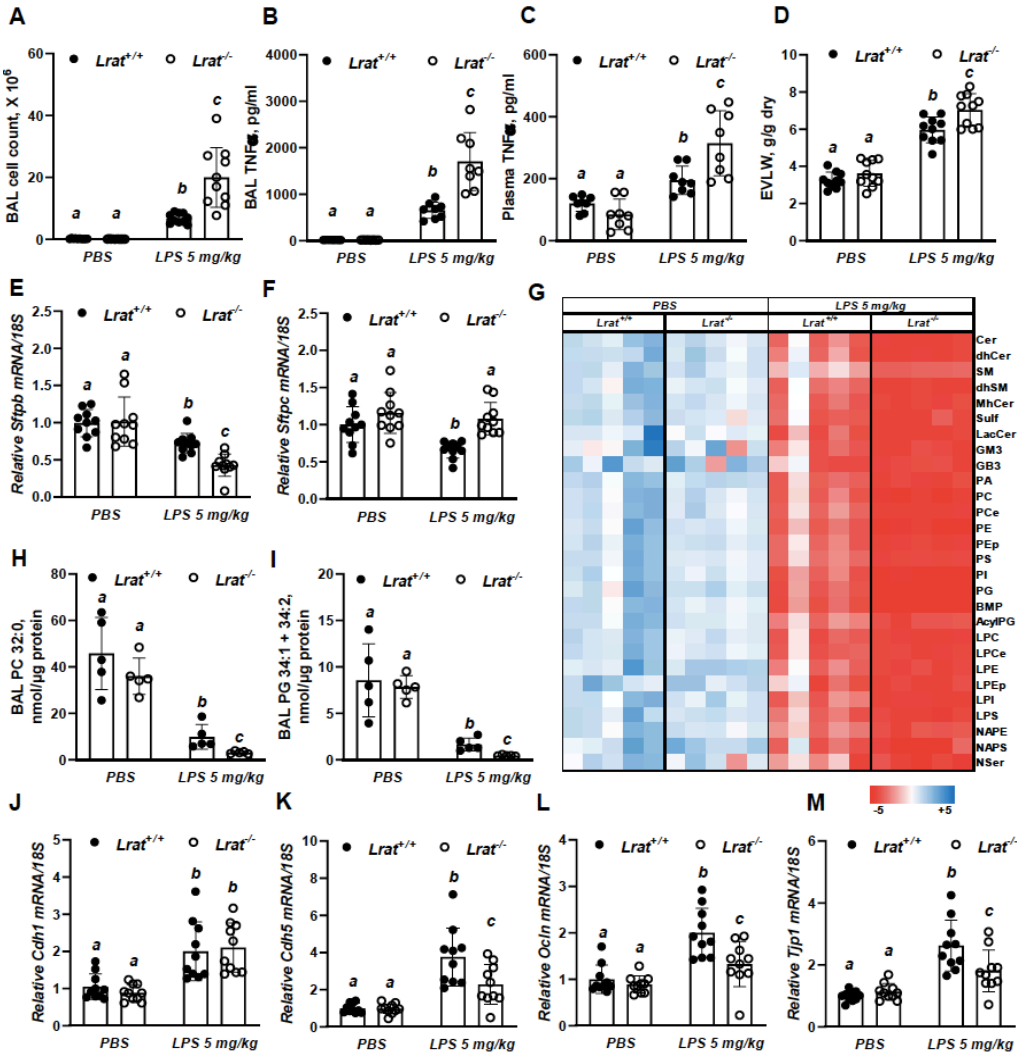


Figure 29. Lung functional parameters in mice during LPS-induced inflammatory stress.

Panel A – Bronchoalveolar lavage (BAL) fluid cell numbers. TNFα concentrations determined by ELISA in cell-free BAL fluid (***panel B***) and plasma (***panel C***). ***Panel D*** – Extravascular lung water (EVLW). Whole lung *Sftpb* (***panel E***) and *Sftpc* (***panel F***) mRNA expression determined by qRT-PCR. Heatmap (***panel G***) indicating relative concentrations of all BAL lipids and BAL concentrations of dipalmitoylphosphatidylcholine (PC 32:0) (***panel H***) and phosphatidylglycerol (PG 34:1 + PG 34:2) (***panel I***) determined by UPLC-MS. ***Panels J-M*** – whole lung *Cdh1*, *Cdh5*, *Ocln*, and *Tjp1* mRNA expression determined by qRT-PCR Values marked with different letters (a, b, c) are statistically different, $p < 0.05$. All values are given as the mean ± 1 S.D., n = 10 for each group. All in mice 24 hours after intranasal instillation of LPS (5 mg/Kg of body weight in PBS) or PBS alone.

In line with the observations of the lethal dose study, the 5 mg/Kg non-lethal dose resulted in 50 % decline in pulmonary retinyl esters of wild type mice (**Figure 30A**), associated with a significant increase in retinol (**Figure 30B**) and a more than 5-fold elevation in all-*trans*-retinoic acid (**Figure 30C**). On the contrary, *Lrat*-deficient mice could not mobilize any retinoids and so, their all-*trans*-retinoic acid remained low (**Figures 30A-30C**).

qRT-PCR analyses indicated that the ones involved in retinoid metabolism and signaling were highly responsive to inflammatory insult within the first 24 hours (**Figure 30D-30H**). Among the highly upregulated genes were those needed to facilitate the uptake of retinol from lipoproteins (*Lpl*, *Cd36*, *Scarb1*) (**Figure 30D**) and, surprisingly, *Stra6*, which was nearly undetectable in the normal lung and was upregulated more than 10-fold, presumably as an additional route for acquiring needed retinoid (**Figure 30E**). Furthermore, about a 5-fold upregulation was seen for *Rdh10*, the first enzyme needed for all-*trans*-retinoic acid synthesis from retinol (**Figure 30F**). *Rara* and *Rarγ*, genes encoding nuclear proteins that mediate all-*trans*-retinoic acid-dependent transcriptional regulation, were elevated in the mouse lungs 24 hours after LPS instillation (**Figure 30G**). Although transcription of *Rarβ*, the least highly expressed RAR isoform in the lung, was downregulated.

Concurrently with these data, expression of *Lrat*, which could negatively affect retinol use for all-*trans*-retinoic acid production by converting it into retinyl esters, as well as *Rbp4*, which could contribute to retinol efflux from the lungs, were significantly downregulated (**Figure 30E** and **30H**). Other genes mediating retinoid metabolism in the lung remained unchanged, although showing greater variability in LPS instilled mice. These included a gene for the intracellular retinol-binding protein *Rbp1* as well as *Raldh1*, which encodes the second and final enzyme needed for all-*trans*-retinoic acid synthesis (**Figure 30E** and **30F**). Importantly, mRNA expression of *Cyp26a1* and *Cyp26b1*, were upregulated reflecting high intracellular all-*trans*-retinoic acid concentrations. Their upregulation is aimed at buffering all-*trans*-retinoic acid excess, suggesting that there is dysregulation of pulmonary retinoid homeostasis during the early stages of acute lung injury.

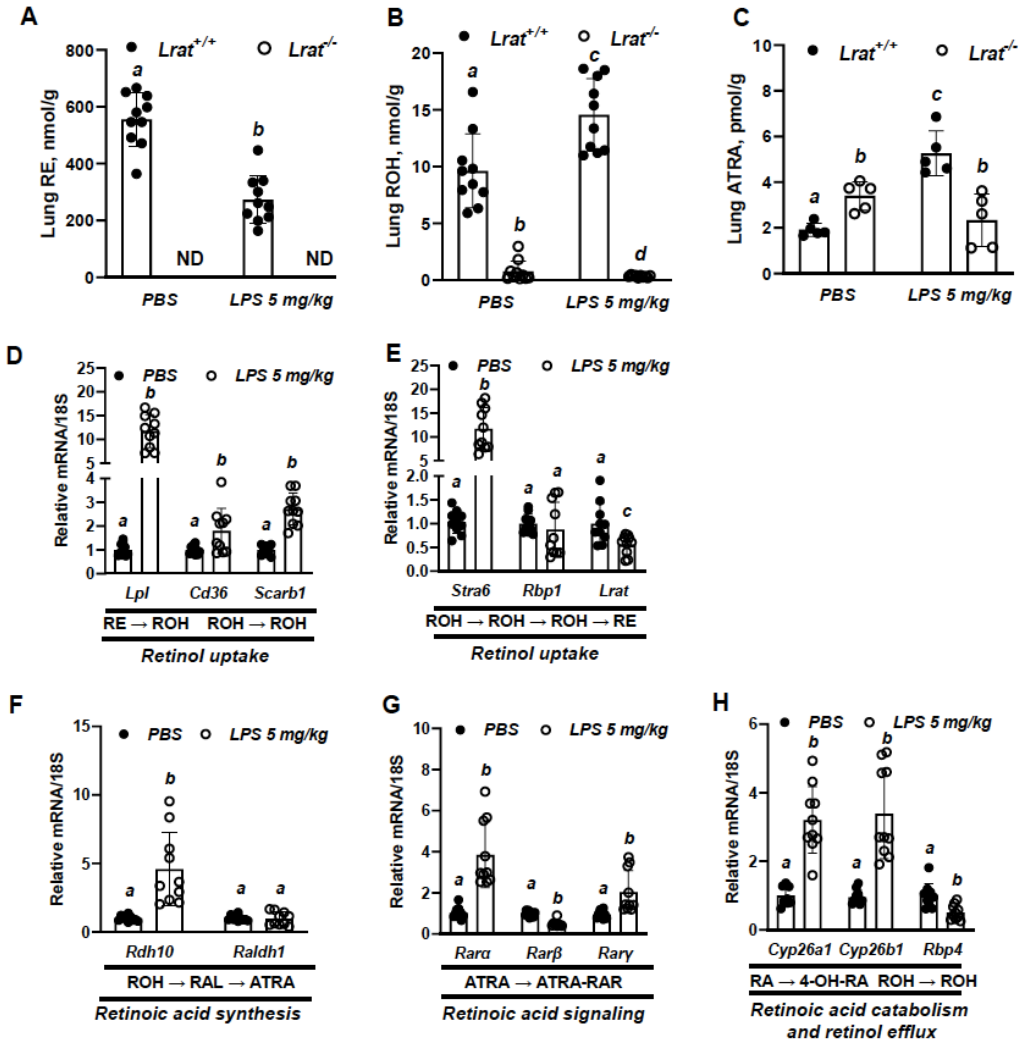


Figure 30. Retinoid concentrations and retinoid-related transcript levels of genes mediating retinoid metabolism in mouse lungs during early periods of ALI.

Lung total retinyl ester (RE) (***panel A***) and retinol (ROH) (***panel B***) concentrations (nmol/g) determined by HPLC and all-*trans*-retinoic acid (ATRA) concentrations (pmol/g) (***panel C***) determined by UPLC-MS/MS in mice 24 hours after intranasal instillation of LPS (5 mg/Kg of body weight in PBS) or PBS alone. ***Panels D-H*** – whole lung mRNA expression of genes mediating retinol uptake (*Lpl*, *Cd36*, *Scarb1*, *Stra6*, *Rbp1*, *Lrat*), all-*trans*-retinoic acid synthesis (*Rdh10*, *Raldh1*), signaling (*Rara, Rarβ*, *Rarγ*), catabolism (*Cyp26a1*, *Cyp26b1*) and efflux (*Rbp4*) in mice 24 hours after intranasal instillation of LPS (5 mg/Kg of body weight in PBS) or PBS alone. Values marked with different letters (a, b, c) are statistically different, $p < 0.05$. All values are given as the mean ± 1 S.D., n = 10 for each group.

Lastly, a gain-of-function study was carried out to establish the extent to which lipoprotein-derived retinoids are important for maintaining or improving lung health. LPS (25 mg/Kg) instilled *Lrat*$^{-/-}$ mice were given by gavage a daily dose of 300 IU of retinol over a 7-day study period. In *Lrat*$^{-/-}$ mice, supplementation resulted in reduced lethality by 30 %, bringing the survival rate to almost 50 % (as compared to 15 % in LPS instilled *Lrat*$^{-/-}$ mice not receiving the oral retinoid) (**Figure 31**).

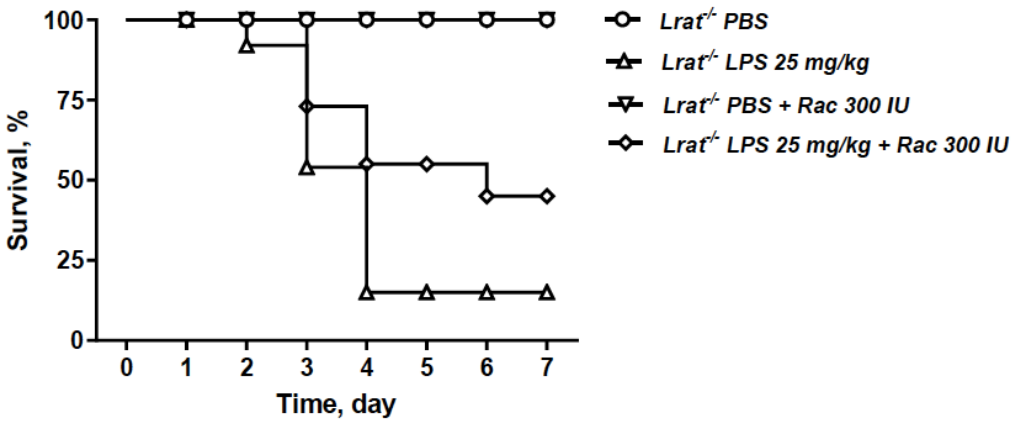


Figure 31. Survival of mice subject to acute lung injury and supplemented with retinol.

Kaplan-Meier survival curves for *Lrat*$^{-/-}$ mice supplemented with 300 IU of retinol over the 7-day period after intranasal instillation of a LPS (25 mg/Kg of body weight in PBS) or PBS alone. n = 14 for each group.

Global discussion

In the field of preventive nutrition, the benefits of a balanced diet have been and continue to be proven by large cohorts and interventional trials. In addition to a correct macronutrient intake for eliciting proper energetic and metabolic responses, maintaining a good micronutrient status is critical for the correct functioning of the body both in health and when subject to injuries. In the last decades, the study of bioactive compounds has searched for the potential role of non-essential components in enhancing the health status of the general population and people with chronic diseases that pose a burden on their lives.

In the present work, we have tried to broaden current knowledge about the relevance of a micronutrient like vitamin A and bioactive compounds like carotenoids and polyphenols in health and disease. Because of the geographical location, this investigation has largely been performed from a Mediterranean point of view. The traditional dietary habits in this area are characterized by an elevated consumption of fruits and vegetables, together with healthy fats, like extra virgin olive oil and nuts. All these plant foodstuffs are rich in carotenoids and polyphenols. In addition, vitamin A can be obtained from this diet from these sources as its precursor, provitamin A carotenoids, or from animal foodstuffs also present in the Mediterranean diet like goat cheese, eggs, or offal.

Reported intake inferred from food frequency questionnaires has been traditionally used when assessing risk factors in large cohorts and more recently compounds found in biological samples are being used as biomarkers of food intake. The results of this dissertation regarding risk factors are an example of the latter. Understanding the relationship between both is valuable because, even though the biological samples give accurate and objective information, sometimes these samples cannot be retrieved, the population is so large that the analysis is too time consuming, or the compounds are difficult to detect or quantify because the levels are too low in biological samples or because they have a short half-life in such samples.

In this regard, carotenoids have long been considered biomarkers of fruits and vegetable intake (185). We observed this as well in the PREDIMED-Plus trial, in which the participants at baseline had a mean fruit and vegetable consumption around the 600 g/day recommended by the World Health Organization, the first decile not meeting the requirement and the tenth decile surpassing it greatly (186). However, the circulating levels of carotenoids are not only influenced by the consumption of these foods but also by their bioaccesibility and bioavailability. One of the main players in this case is dietary fat, which, because carotenoids are liposoluble, was thought to be

an adjuvant in their appearance in plasma after ingestion. This hypothesis was supported by different studies performed mostly *in vitro* and with animal models, and using moderate amounts of fat (2.5 to 10 %) (120–122).

The mean fat intake at baseline of the PREDIMED-Plus trial was 30 grams higher than the 70 g/day that has been considered a normal amount (187). Because fat is a central nutrient in the Mediterranean diet, fat intake of participants in the first quartile was barely below the regular 70 g/day amount: around 30 % of the daily energy intake. For this, we defined the first quartile as low-to-moderate fat consumers, while the fourth quartile doubled that amount, being considered a very high fat intake. Contrary to what we were expecting, there were no significant differences in carotenoid plasma concentrations between fat consumers. In fact, there was a trend towards being higher in low-to-moderate fat consumers. Additionally, when separating the groups in both F&V and fat intake the results were similar, the only group showing significantly higher plasma concentrations of carotenoids was the one comprised by high F&V and low-to-moderate fat consumers. The associations were particularly significant for xanthophylls and the provitamin A fraction but of greater magnitude for carotenes. No differences were found in the non-provitamin A fraction. Djuric et al. (2006) also proposed that there is an interaction between F&V and fat in carotenoid absorption, a combination of high F&V consumption and low-fat intake recommendations increased plasma carotenoid levels (188). In addition, an in vitro study in which different F&V were digested *in vitro* and the digested fraction was given to cells together with different fats, carotenoid micellarization did not increase when fat was added in high amounts (120).

The explanation for these findings might be helped by the absorption mechanisms of these molecules. Fat, in very high concentrations, might saturate the mixed micelles, hindering the integration of carotenoids and reducing their bioaccessibility (120,189). The fat transporters might also be a place of competition between carotenoids and fats, in which differential affinity might favor fat absorption when in very high concentrations. Because fat intake influences the secretion of bile acids, this might also be altering the final concentrations of carotenoids in plasma (190,191). Finally, genetic differences in the transporters and the enzymes that cleave carotenoids might be leading to these differences (192). All in all, the results suggest that, when using carotenoids as F&V consumption biomarkers, dietary fat should be considered as a confounder.

After this first approach, the investigation was focused on assessing the association of carotenoids, but also polyphenols, with the risk of developing diseases that are

highly influenced by dietary habits. The risk of developing these diseases starts accumulating much earlier than the moment where they represent a burden, which is especially from middle-to-advanced stages of life. When investigating the role of dietary bioactive compounds on risk factors or on the risk of developing diseases, large interventional trials are compelling because they consider free-living populations where there is variability in both dietary intake and risk or risk factors. For all these reasons, the population of studies like the PREDIMED and PREDIMED-Plus was a good match for our investigation.

Selecting a portion of participants from these interventional trials has allowed for the precise analysis of bioactive compounds in minimally invasive samples and associate them with cardiovascular risk factors and risk of developing type 2 diabetes. Others have addressed these questions from the assessment of intake of these compounds (77,133,193–199) or in different populations (200–203).

When assessing the relationship between carotenoids and cardiovascular risk factors, both anthropometric and biochemical, the quantified samples from the previous study were used. That is because even though the food intake was clearly compartmentalized, the plasma concentrations of carotenoids were not. Carotenoid concentrations were dispersed and ranged from almost non-detectable to more than 40 μmol/L.

Regarding anthropometric variables, waist circumference and body mass index were related to circulating xanthophylls in men and total carotenoids in women, respectively. Findings that are somewhat in line with others, who have not investigated actual plasma concentrations (197,198) nor separated between sex (204). Total carotenoids were found to be beneficially associated with heart rate and systolic blood pressure only in men. In a similar contingency as before, others had described this for a non-stratified population (205,206). These effects might be explained by both the antioxidant and provitamin A activities of carotenoids described *in vitro* and in animal studies (207) although the difference between women and men needs to be addressed.

Concerning the biochemical parameters of cardiovascular health, total plasma carotenoids were found to be associated with lower fasting plasma glucose and with a trend towards lower glycated hemoglobin only in men, something that is in agreement with other studies, that have even found a direct relationship in women (208), or no association in obese and overweight women (209), which is the case of the population studied here. On the contrary, only in women were total carotenoids associated with higher levels of HDL-cholesterol. The association with lower triglycerides was found

for both females and males. These two outcomes have been previously described for a non-separated population (210). In addition, no correlation was found with total cholesterol. These results suggest that carotenoids might have a role in maintaining a salutary plasma lipid profile in older populations at risk of developing cardiovascular disease. Carotenoids have been described to enhance long-chain fatty acids hydrolysis and β-oxidation related enzymes (211,212), a trait that might explain our results.

Going one step further, we investigated the relationship of carotenoids with circulating fatty acids, something that, to the best of our knowledge, has not been examined *in vivo*. Carotenoids were associated with a better fatty acid profile in both women and men: lower saturated and higher poly-unsaturated fatty acids. Whether this relationship is caused by carotenoids, fatty acids, or it is a spurious correlation derived from the diet remains to be clarified. It is clear that there is need for understanding not only the relationship of fatty acids and carotenoids after ingestion but also after absorption of both lipidic families.

Later, we continued the investigations of bioactive compounds and health by addressing polyphenols, molecules that are famed for being anti-inflammatory compounds and whose health promoting properties are nowadays subject to study. After developing a method for detecting and quantifying urinary MPMs (146), we examined their association with the development of type 2 diabetes. Out of the polyphenols and metabolites that we could quantify, one-year changes in urinary hydroxybenzoic acid glucuronide, a final microbial derivative from dietary polyphenols (213), were found to be strongly associated with a lower likelihood of developing the disease. The drawback of these results is that, when fasting plasma glucose was considered as a covariable, the significance was lost. This is probably caused by the baseline difference in plasma glucose, a common situation in baseline analyses of this study (214–217), which was already very significant and indicating insulin resistance in the future cases of type 2 diabetes.

Even though there is need for studies in even earlier stages of the disease to assess if hydroxybenzoic acid glucuronide is indeed a marker of type 2 diabetes prevention, there is already evidence that it might be. One of the isomers of this metabolite, 4-hydroxybenzoic acid, is a precursor of ubiquinone or Coenzyme Q, an electron transporter in the mitochondria (218) whose deficiency is being said to be connected with glucose tolerance impairment (219,220). Some other studies agree in the potential beneficial role of this molecule (221–225), and even suggested that it might modulate the insulin receptor and glucose transporter 4 (GLUT4) (226).

An increase in 4-hydroxibenzoic acid was observed in urine after an intervention with organic products carried out by our group (227), as well as an increase of constituents from the family of hydroxybenzoic acids after different types of foodstuffs like oranges, cocoa and almonds, or beverages like green tea, coffee, red wine and gin (228–234). In addition, another study performed in our group showed that intake of hydroxybenzoic acids, among other polyphenols, was correlated with lower incidence of type 2 diabetes (235). These results lead to the hypothesis that not only this family might be related to type 2 diabetes onset, but its concentrations are related to the dietary pattern and the gut microbial population, which might, in turn, be modified by dietary components (236).

Some other one-year changes in urinary metabolites were also seen to be somewhat associated with type 2 diabetes development, between the first and second tertiles, the second was less likely to develop the disease. One of these metabolites was hydroxytyrosol, one of the main polyphenols found in olive oil (237) that can be found in human samples as well, and one of whose degradation products is hydroxybenzoic acid (137). The use of this metabolite for reduction of oxidative damage risk was approved by the European Food Safety Authority in 2011 and *in vitro* and animal studies have demonstrated its protective activity (238,239). Vanillic acid, the other metabolite found associated with type 2 diabetes, similar to hydroxytyrosol, has been associated with the rescuing of glucose and insulin levels in plasma and other potential favorable mechanisms (240–242).

With these two studies we have tried to broaden current knowledge on cardiovascular risk factors and risk of development of type 2 diabetes by assessing bioactive compounds' concentrations in easily obtainable and minimally invasive human samples.

Lastly, due to the COVID-19 outbreak and pandemic, research all around the globe started focusing on the lung in attempts of finding potential treatments for this disease. In this context, the research stay that was initially planned in the understanding of the implication of vitamin A in obesity, was shifted and centered on its presence in the lung and role in lung injury.

Up until now, the lungs were thought to take up vitamin A from the circulation coming from liver stores rather than postprandially (243). Aside from the regulatory role of this micronutrient in lung development (244,245), there were no other known roles that would require the lungs to have permanent stores available for use. Nevertheless, the first approaches involving two of the major animal models used for studying retinoid biology in animal models showed that animals that did not have retinoid stores within the lung, albeit ones having available circulating vitamin A, were less likely to

survive after acute lung injury. The greater mortality of these mice suggests that local stores and uncompromised retinoid metabolism in the lung restrict the severity of acute lung injury. Both retinyl ester and retinol decline in the lung after inflammation resulted from their direct use locally, rather than from hepatic stores, to buffer against the experimentally induced insult. In addition, the observed decline in plasma retinol was due to the lessened retinol-RBP4 secretion from the liver and not because of increased utilization by the inflamed lung. These unexpected results prompted an investigation aimed at deciphering the different cell types that contain and store retinoids within the lung.

Single cell transcriptomic analyses allowed for the identification of ten clusters of retinoid-containing cells. More than 80 % of these cells were fibroblasts including "conventional", "proliferative", and "myofibroblast-committed" lipofibroblasts, but there also included microvascular endothelial cells, type 2 epithelial cells, and different types of immune cells. These findings are paradigm shifting, as not only one, but more than 5 types of cells within the lung contain sufficient retinoids to be selected by their autofluorescence and emission upon excitation. The transcriptional signatures of these cells underlying retinoid uptake, accumulation, and metabolism suggest that subpopulations of these cell types have actions in pulmonary retinoid metabolism.

The data from our studies using both lethal and non-lethal doses of LPS lead us to propose that the early period of acute lung injury is associated with extensive local pulmonary retinoid mobilization and changes in retinoid- and lipid-related gene expression aimed at providing local substrate needed for ensuring all-*trans*-retinoic acid synthesis, which is required for maintaining RAR-dependent transcription, as described to a smaller extent in supplementation studies investigating other pulmonary injuries (246,247). Another interesting result was that a dietary retinoid intervention after the lung injury was able to reduce lethality. This supports the idea that postprandial or lipoprotein-derived retinoids supply the lungs for maintenance or improvement of their health. It also opens way for new experiments on the possible benefits of vitamin A in the face of lung injury.

A main limitation of the studies that comprise this dissertation has been the scarce literature on both sex-related differences when assessing carotenoid effect on cardiovascular health outcomes and MPM effect on type 2 diabetes, which makes a comparison with previous results difficult. In addition, the cross-sectional design in the case of carotenoid studies, together with the impossibility to determine food processing, microbial metabotypes or other confounders in dietary bioactive compound uptake and effect represent weaknesses. Lastly, the large difference in baseline fasting

plasma glucose, which is the main predictor of type 2 diabetes, prevents from establishing strong conclusions regarding MPMs effects on the development of this disease.

The precise extraction and detection methodologies used for the assessment of bioactive compounds present in plasma and urine are the main strengths of these studies, together with the statistical methodologies used in the carotenoid studies that allow for the interpretation of interval-censored variables, both as response and explanatory variables. The longitudinal analysis in the MPM association with type 2 diabetes risk is also a good feature of this study. As is the use of a novel technology like single cell transcriptomics, which enables the proper annotation of different cells coming from the same tissue or separation technique.

This dissertation has been developed both through human and animal studies, giving a broad overview in the role of bioactive compounds and vitamin A in nutrition and metabolism in health and disease. To the best of our knowledge, this is the first time that the relevance of both the source (F&V) and the vehicle (fat) involved in carotenoid bioavailability have been assessed in a large cohort study using high-throughput metabolomic techniques. The description of the association of bioactive compounds to risk factors or disease risk is not ground-breaking, but both the analytical and statistical techniques used are precise and novel. The same can be said about the animal model experiments, the combination of traditional with innovative techniques adds great value to the investigation.

If the results of this dissertation and the hypotheses that arise from it are confirmed, nutrition could be optimized for a proper bioactive compound absorption and health effect both in prevention and prognosis of highly prevalent diseases.

Conclusions

The results of this dissertation lead to the postulation of the following conclusions:

In a senior population at high risk of developing cardiovascular disease:

1. High fruit and vegetable consumption together with a low-to-moderate fat intake is associated with higher plasma carotenoids compared to a low fruit and vegetable consumption regardless of the fat intake, or to a high fruit and vegetable consumption together with a very high fat intake.
2. Plasma carotenoid concentrations correlate with lower plasma triglyceride concentrations and fitter fatty acid profile. In women they also correlate with higher plasma HDL-cholesterol levels and in men with fasting plasma glucose.
3. One-year changes in urinary hydroxybenzoic acid glucuronide concentration are associated with a lower likelihood of developing type 2 diabetes when baseline plasma glucose is not considered.

In mice models:

4. Local retinoid stores in the lung, not systemic retinoid transport and metabolism, are critical in the metabolic maintenance of this tissue during acute lung injury ensuring the maintenance alveolar integrity, surfactant production, and inflammatory response.
5. Ten distinct cell-types store retinoids in the lung and have been described and annotated.
6. Dietary retinol supplementation during acute lung injury reduces lethality compared to no intervention.

Future perspectives

There are new compelling hypotheses and questions that arise from the results of this dissertation, and these will require further research.

Regarding carotenoid intake and plasma concentrations:

- Is there a limit up to which carotenoid concentrations in plasma rise with an increase in fat intake?
- Does dietary fat have a dual role in carotenoid appearance in plasma after ingestion promoting carotenoid absorption when the amounts of fat are low but competing with them when in high concentrations?
- Are different types of fats distinct in their association with carotenoid concentration in plasma?
- Does vitamin A have an active role in the appearance of provitamin A carotenoids in plasma?

Regarding bioactive compounds in disease risk prediction:

- What is the exact role of carotenoids in relationship with cardiovascular risk factors?
- Would MPMs, or a particular MPM, be useful as a predictor of type 2 diabetes?

Regarding vitamin A in acute lung injury:

- Do human lungs store vitamin A in the same way as mice?
- How does vitamin A stored in the lung help in the prognosis of acute lung injury?

In all cases, further investigation that ends in properly designed clinical trials are needed to contrast and prove the results of this dissertation and the abovementioned new hypotheses. The development and improvement of appropriate statistical techniques to accurately assess the role of bioactive compounds and other molecules measured in biological samples will also be very profitable.

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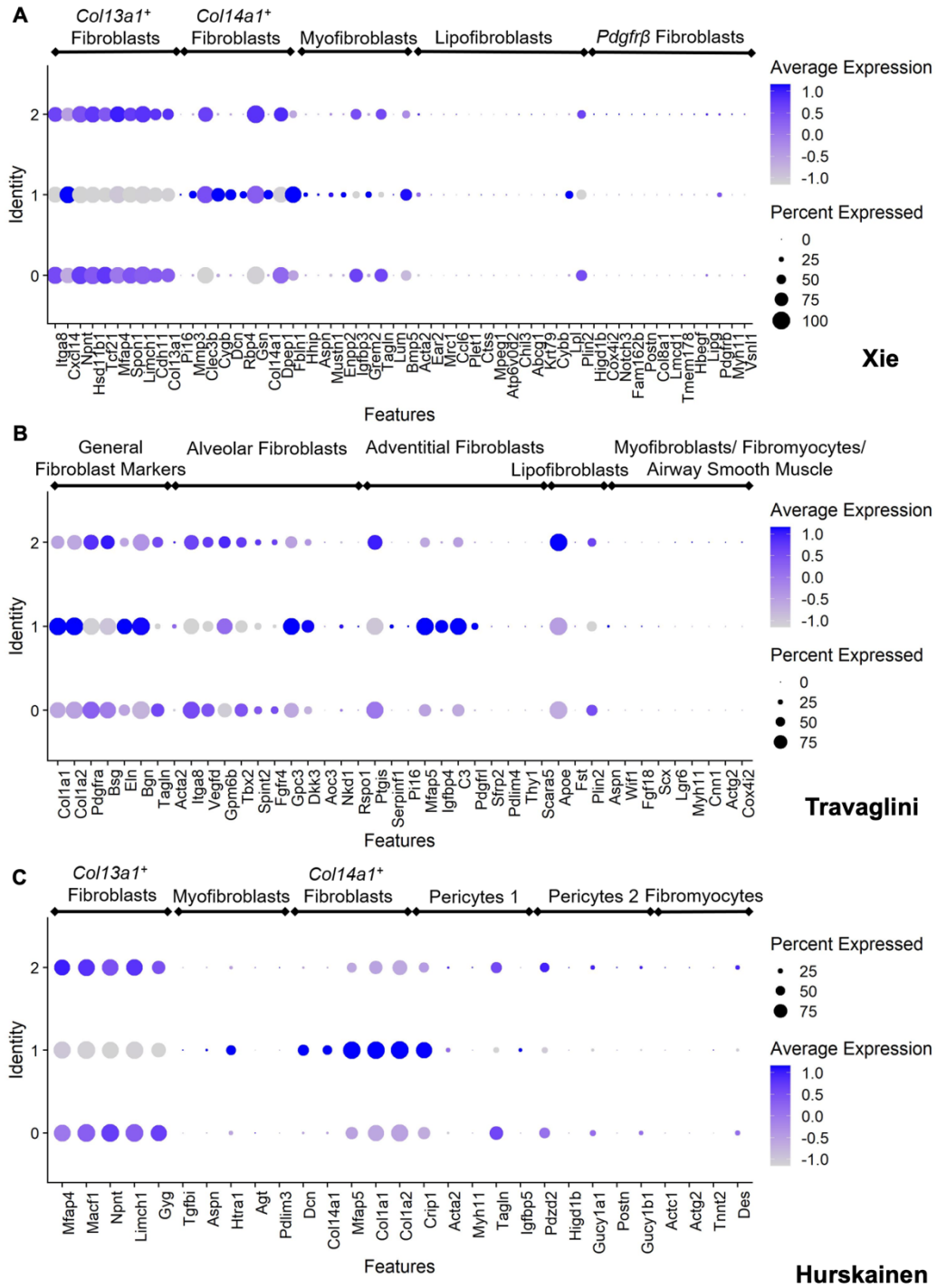
Annex

Supplementary material

Supplementary Table 1. Primer sequences used for qRT-PCR

Gene name	Accession number	Primers (forward/reverse)
18S		5'-CCATCCAATCGGTAGTAGCG
		5'-GTAACCCGTTGAACCCCATT
Lrat	NM_023624	5'-AGTTCAAGACTAGCCTGCTCA
		5'-TACAAGCTGGCCTTCGAC
Rbp1	NM_011254	5'-AGAAGACAGAACCACACGA
		5'-GGTGGTCAGAGACCCAAG
Aldh1a1	NM_001361503	5'-TGTTGAGGAGTCAGTTTATGATG
		5'-GGCTCCTTCTTTCTTCCC
Rdh10	NM_133832	5'-TCTACCGGGACCTGGAGGC
		5'-TCCAGAAACCACACCAGCA
Cyp26a1	NM_007811	5'-AGAGCAATCAAGACAACAAGTTAG
		5'-ATCGCAGGGTCTCCTTAAT
Cyp26b1	NM_175475	5'-TCTCTGCCAGGTGTACTTAG
		5'-CCTTCAGAAGAACCCGTATC
Rara	NM_009024	5'-AATCTGCACGCGGTACA
		5'-TCAGCATCGTCCATCTCC
Rarβ	NM_011243	5'-CAAGTTCAAGTGGGAATATAGCAGA
		5'-ACTGACTGACTCCACTGTT
Rarγ	NM_011244	5'-CAGAGGGTCGCCACCATT
		5'-ATCTCCTCCGAGCTGGTGC
Rbp4	NM_001159487	5'-GAGTCCGTCTTCTGAGCAAC
		5'-CTTGAACTTGGCAGGATCT
Stra6	NM_001162476	5'-CCTGGGCCTTCTCCCATCAT
		5'-CCTGGTAAGTGGCTGTTCCTGTCA
Lpl	NM_008509	5'-GCTGGTGGGAAATGATGT
		5'-ACCAGTAATTCTATTGACCTTCTTAT
Cd36	NM_001159558	5'-GCTGTGTTTGGAGGCATTCT
		5'-CCTTGATTTTGCTGCTGTTC
Scarb1	NM_016741	5'-CCCAAACGAGGTCCTCA
		5'-ACTTGTCAGGCTGGAAAT
Sftpb	NM_147779	5'-CCCAGCTCTAACTACAGAC
		5'-CCCTTCTGAAGGCTTCCA
Sftpc	NM_011359	5'-TGATGGAGAGTCCACCGGAT
		5'-CCACCACAACCACGATGAGA
Cdh1	NM_009864	5'-GGTCTTTCAGCTCCTTCC
		5'-AGGCACAGTTTATATCTCAGCA
Cdh5	NM_009868	5'-TTATTATAAGACTCTAGTTCTCACAGACA
		5'-CATTATCCTTACAGCAATGACTAC
Ocln	NM_001360536	5'-CTGACCTTGAGTGTGGA
		5'-CACCTGTCGTGTAGTCTG
Tjp1	NM_009386	5'-GGTCAAATGAAGACAATTACTTATTGTAT
		5'-CCCACTAGGGTAAGGCA


A
Col13a1+ Fibroblasts
Col14a1+ Fibroblasts
Myofibroblasts
Lipofibroblasts
Pdgfrβ Fibroblasts
Identity
Features
Average Expression
Percent Expressed
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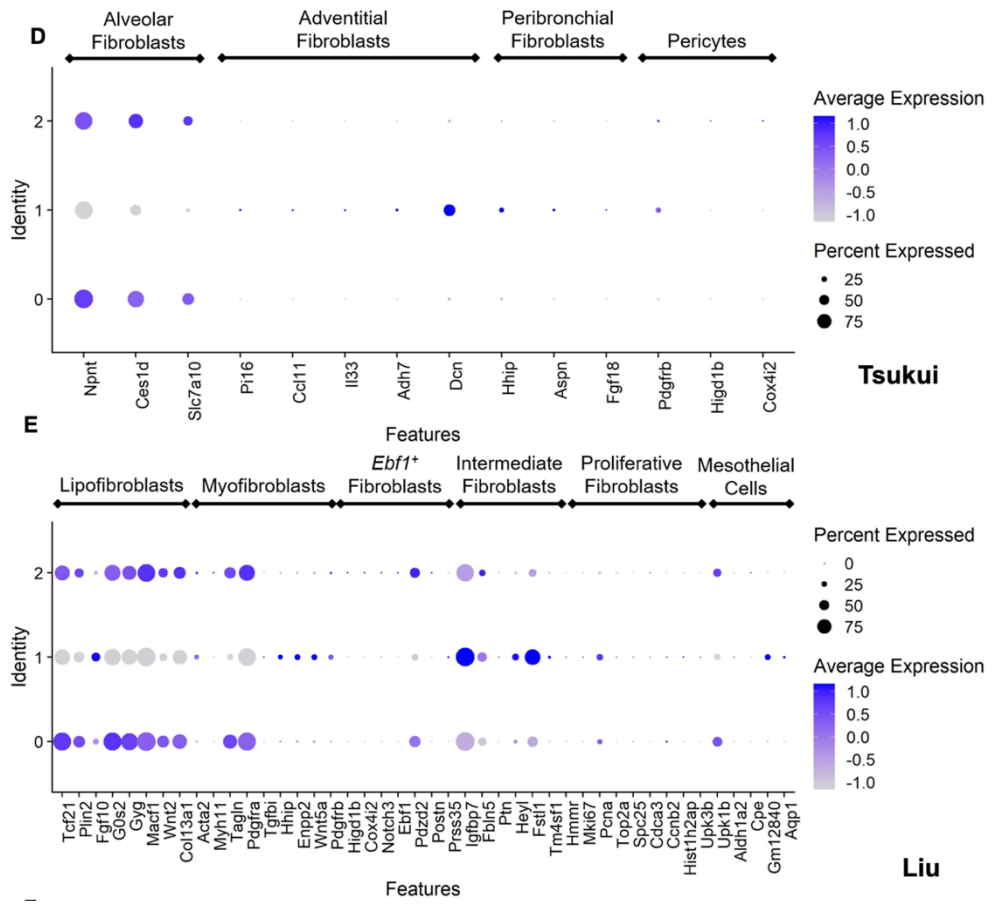
B
General Fibroblast Markers
Alveolar Fibroblasts
Adventitial Fibroblasts
Lipofibroblasts
Myofibroblasts/ Fibromyocytes/ Airway Smooth Muscle
Identity
Features
Average Expression
Percent Expressed
Travaglini
C
Col13a1+ Fibroblasts
Myofibroblasts
Col14a1+ Fibroblasts
Pericytes 1
Pericytes 2
Fibromyocytes
Identity
Features
Percent Expressed
Average Expression
Hurskainen



Supplementary Figure 1. Post-processing analysis of scRNA-seq data.

Panel A – Dot plot visualization of marker gene expression reported by Xie et al. (180) to annotate *Col13a1*$^{+}$ fibroblast, *Col14a1*$^{+}$ fibroblast, myofibroblast, lipofibroblast, and *Pdgfrβ* fibroblast clusters. ***Panel B*** – Dot plot visualization of marker gene expression reported by Travaglini et al. (40) to annotate general fibroblast, alveolar fibroblast, adventitial fibroblast, lipofibroblast, myofibroblast, fibromyocyte, and airway smooth muscle cell clusters. ***Panel C*** – Dot plot visualization of marker gene expression reported by Hurskainen et al. (182) to annotate *Col13a1*$^{+}$ fibroblast, *Col14a1*$^{+}$ fibroblast, myofibroblast, pericyte, and fibromyocyte clusters. ***Panel D*** – Dot plot visualization of marker gene expression reported by Tsukui et al. (181) to annotate alveolar fibroblast, adventitial fibroblast, peribronchial fibroblast, and pericyte clusters. ***Panel E*** – Dot plot visualization of marker gene expression reported by Liu et al. (183) to annotate alveolar lipofibroblast, myofibroblast, *Ebf1*$^{+}$ fibroblast, intermediate fibroblast, proliferative fibroblast, and mesothelial cell clusters. The x-axis (features) gives gene names, the y-axis (identity) indicates cluster numbers. The level of expression is specified by the color legend. Size of the cell population expressing the gene of interest is indicated by the size of the circle as specified by the legend. All visualizations were applied to stromal clusters from retinoid-containing cell scRNA-seq datasets generated in our study.

Other publications

Original articles

Emily P. Laveriano-Santos*, **María Marhuenda-Muñoz***, Anna Vallverdú-Queralt, Miriam Martínez-Huélamo, Anna Tresserra-Rimbau, Elefterios Miliarakis, Camila Arancibia-Riveros, Olga Jáuregui, Ana María Ruiz-León, Sara Castro-Baquero, Ramón Estruch, Patricia Bodega, Mercedes de Miguel, Amaya de Cos-Gandoy, Jesús Martínez-Gómez, Gloria Santos-Beneit, Juan M. Fernández-Alvira, Rodrigo Fernández-Jiménez and Rosa M. Lamuela-Raventós. "Identification and quantification of urinary microbial phenolic metabolites by HPLC-ESI-LTQ-Orbitrap-HRMS and their relationship with dietary polyphenols in adolescents." *Antioxidants,* 2022, 11(6), 1167; DOI: 10.3390/antiox11061167

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*Equally contributing authors

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