THE TISSUE BIOAVAILABILITY, BIOMARKERS, AND EFFECTS OF ANTHOCYANINS ON HUMAN HEALTH. STUDIED THROUGH SYSTEMATIC REVIEWS ON ANTHOCYANIN-RICH FOODS AND A NUTRITIONAL PRE-CLINICAL STUDY WITH ANTHOCYANIN-RICH RED FLESHED-APPLES. THE APPLECOR PROJECT.

Berner Andrée Sandoval Ramírez

UNIVERSITAT
ROVIRA i VIRGILI

The tissue bioavailability, biomarkers, and effects of anthocyanins on human health. Studied through systematic reviews on anthocyanin-rich foods and a nutritional pre-clinical study with anthocyanin-rich red fleshed-apples. The AppleCOR Project.

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FAIG CONSTAR que aquest treball, titulat "**La biodisponibilitat en teixits, biomarcadors i efectes de les antocianines en la salut humana: Estudi a través de revisions sistemàtiques sobre aliments rics en antocianines i un estudi pre-clínic amb pomes de polpa vermella rica en antocianines. El projecte AppleCOR.",** que presenta **Berner Andrée Sandoval-Ramírez** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al **Departament de Medicina i Cirurgia** d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "**La biodisponibilidad en tejidos, biomarcadores y efectos de las antocianinas en la salud humana: Estudio a través de revisiones sistemáticas sobre alimentos ricos en antocianinas y un estudio preclínico con manzanas de pulpa roja rica en antocianinas. El proyecto AppleCOR.**", que presenta **Berner Andrée Sandoval-Ramírez** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el **Departamento de Medicina y Cirugía** de esta universidad.

I STATE that the present study, entitled "**The tissue bioavailability, biomarkers, and effects of anthocyanins on human health. Studied through systematic reviews on anthocyanin-rich foods and a nutritional pre-clinical study with anthocyanin-rich red fleshed-apples. The AppleCOR Project.**", presented by **Berner Andrée Sandoval Ramírez** for the award of the degree of Doctor, has been carried out under my supervision at the **Department of Medicine and Surgery** of this university.

Tarragona, 17 de noviembre de 2020.

El/s director/s de la tesi doctoral

El/los director/es de la tesis doctoral

Doctoral Thesis Supervisor/s

[signatura] / [firma] / [signature]

Rosa Maria Solà Alberich

[signatura] / [firma] / [signature]

Úrsula Catalán Santos

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Studied through systematic reviews on anthocyanin-rich foods and a nutritional pre-clinical study with anthocyanin-rich red fleshed-apples. The AppleCOR Project.

International doctoral thesis

Supervised by:

MD, PhD Rosa SOLÀ ALBERICH

PhD, Úrsula CATALÁN SANTOS

Department of Medicine and Surgery

Universitat Rovira i Virgili

UNIVERSITAT ROVIRA i VIRGILI

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UNIVERSITAT ROVIRA i VIRGILI

Faculty of Medicine and Health Sciences
Department of Medicine and Surgery

Carrer Sant Llorenç, 21
43201, Reus, Spain
Tel: +34 977 759 306
Fax: +34 977 759 352

HERE I STATE that the present work entitled: "**The tissue bioavailability, biomarkers, and effects of anthocyanins on human health. Studied through systematic reviews on anthocyanin-rich foods and a nutritional pre-clinical study with anthocyanin-rich red fleshed-apples. The AppleCOR Project**", presented by **Berner Andrée Sandoval-Ramírez** to obtain the title of Doctor, has been performed under my direction in the department of Medicine and Surgery of this University, and that it fulfills the requirements to be eligible for the acquisition of a doctoral degree with international mention.

Reus, January, 23rd, 2021.

The doctoral thesis directors

MD, PhD Rosa SOLÀ ALBERICH

PhD, Úrsula CATALÁN SANTOS

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Dedicated to the people who with their support, patience,
understanding, and love, have made all this possible.
I will be forever grateful to YOU.

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Berner Andrée Sandoval-Ramírez
ORCID: 0000-0002-6242-922X.
Researcher ID: Y-3114-2018.
Scopus Author ID: 57195537775.

ABBREVIATIONS

Anthocyanins (ACNs)

Phenolic compounds (PCs)

Cardiovascular disease (CVD)

Total cholesterol (TC)

Low-density lipoprotein cholesterol (LDLc)

High-density lipoprotein cholesterol (HDLc)

Proprotein convertase subtilisin/kexin type 9 (PCSK9)

C-reactive protein (CRP)

Growth-differentiation factor (GDF)

LDL receptor (LDLr)

Hydroxymethylglutrayl (HMG)-CoA

Scavenger receptors (SR)

Adhesion molecule-1 (VCAM-1)

Nuclear factor kappa beta (NF-kB)

Activator protein (AP)

Interleukin (IL)

American College of Cardiologist (ACC)

American Heart Association (AHA)

Sodium-glucose cotransporter 2 (SGLT2)

Glucagon-like peptide (GLP)

Acetylsalicylic acid (ASA)

Angiotensin-converting enzyme inhibitors (ACEIs)

Angiotensin-II receptor (ARA-II)

Dietary Approaches to Stop Hypertension (DASH)

Observational studies (OS')

Randomized controlled trials (RCTs)

Preferred Reporting Items for Systematic Reviews (PRISMA)

International Prospective Register of Systematic Reviews (PROSPERO)

Food and Drug Administration (FDA)

Food intake biomarkers (FIBs)

Organic cation transporter 1 (OCT1)

Organic anion transporter 2 (OAT2)

Sodium/monocarboxylate transporters (SMCT)

Cyanidin-3-glucoside (C3G)

Systematic review and meta-analysis (SRM)

Type 2 diabetes mellitus (T2DM)

High-fat diet (HFD)

Nitric oxide (NO)

Glutathione S-transferase Mu 2 (GTSM2)

Protein kinase cAMP-activated catalytic subunit alpha (PRKACA)

IQ motif containing GTPase activating protein 1 (IQGAP1)

Fibromodulin (FMOD)

Transgrelin (TAGLN)

Adenylyl cyclase-associated protein 1 (CAP1)

Alpha-1-antitrypsin (SERPINA1)

Enoyl-CoA hydratase 1 (ECH1)

Glutathione peroxidase 1 (GPX1)

Myoglobin (MB)

Four and a half LIM domains protein 1 (FHL1)

Nitric oxide synthase (iNOS)

Cyclooxygenase (COX)

Heme oxygenase (HO)

Acute lung injury (ALI)

Lipopolysaccharide (LPS)

Partial pressure of oxygen in arterial blood (PaO2)

Fraction of inspired oxygen (FiO2)

Bronchoalveolar fluid (BALF)

Myeloperoxidase (MPO)

Mitogen activated protein kinase (MAPK)

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BACKGROUND: Anthocyanins (ACNs) are phenolic compounds present in foods. However, various ACN properties considered relevant to human health are still unknown. In consequence, the present work aims to increase the scientific knowledge on ACNs, providing answers to various questions such as the ACN tissue bioavailability, consumption biomarker, and the effects of the ACN oral intake on human health.

METHODS: 1 animal experiment on hypercholesterolemic rats and 6 systematic reviews from the scientific literature were conducted following the most adequate methodological proceedings between February 2018 and February 2021.

RESULTS: From various systematic reviews: 1st, ACNs can be detected in multiple target tissues with medical relevance, suggesting some health effects. 2nd, C3G is the only ACN fulfilling the criteria to be considered an adequate food-intake biomarker for berry consumption in human plasma and urine. 3rd, Consuming 100-150 g/day of whole-apples improves multiple CVD risk factors and reduces the risk of CVD and CVD mortality. 4th, From the animal intervention, ACN-rich apples have an anti-inflammatory effect, while white-fleshed apples reduce complement system-related proteins in hearts and aortas of hypercholesterolemic rats, suggesting a beneficial apple matrix effect, regardless of their ACN content. 5th, The oral ACN intake, regardless of its source, is associated with a reduced risk of T2DM and hypertension in humans. Moreover, oral ACNs also improve the plasmatic lipid profile, glucose metabolism, and endothelial function, reasonably explaining the T2DM risk reduction associated with the dietary ACN intake. 6th, The oral PC administration in animals improves the intestinal barrier integrity and function from three main mechanisms: a) The reduction of pro-inflammatory molecules, b) the improvement in tight-junction protein expression, and c) the improvement of the antioxidant intracellular activity. 7th, Resveratrol, and other phenolic compounds (PCs) significantly reduce the acute lung injury secondary to sepsis in diverse animal models.

CONCLUSION: Therefore, as a result of the present work, our hypothesis is vertified, and the anthocyanins provided by fruits, extracts or other products help improve cardiovascular risk factors and other diseases. It can be concluded that, regardless of their source, the whole-apple, and ACN oral intakes should be considered effective for the prevention and treatment of cardiometabolic disease in humans. Moreover, in animal models, resveratrol or other PCs showed an improvement of the intestinal barrier integrity loss and in the management of the acute lung injury associated with the systemic inflammation in critical illnesses, such as sepsis, opening new promising application in humans.

JUSTIFICATION

In 2017, around the world approximately 17.8 million people died from cardiovascular disease (CVD), making CVD the leading cause of death [1]. Accordingly, over the last decades, nutrition has acquired an important role in healthcare systems primarily due to the increase of the aged population and the prevalence of non-communicable diseases such as CVD, obesity, type 2 diabetes, metabolic syndrome, and hypertension [2,3].

Traditionally, most of the health benefits associated with frequent fruit and vegetable consumption have been attributed to their content of fiber, vitamins, minerals, and more recently, to naturally occurring bioactive molecules present in plants called phenolic compounds (PCs) [4–8]. PCs are a complex and extensive family of biomolecules, evidence suggests that although some PCs share effects [5,7,9], most of their specific mechanisms of action and effects remain unclear. Nevertheless, flavonoids, particularly anthocyanins (ACNs), have demonstrated the potential to prevent diverse chronic [10–13], and neurodegenerative diseases [14].

ACNs are natural plant pigments responsible for the red-blue colors present in plants, especially in the skin and flesh of berries, red-fleshed apples, and other fruits [15–17]. Current research has been focused on the study of the ACN effects using extracts [9]. However, studying fruits and vegetables as whole-foods remains essential, as whole-foods are the most common way in which people consume any given nutrient [18,19].

Despite the efforts made to discover the properties of various ACN-rich fruits, essential aspects such as the ACN tissue bioavailability, the health-related consequences of the ACN presence on different target tissues, or the existence of an ACN-rich food intake biomarker, have not been determined. The reasons range from the absence of methods allowing the detection of ACNs in living human tissues, forcing us to rely on animal experimentation, or the economic and technical limitations making currently impossible the determination of an intake biomarker for large food groups (e.g. ACN-rich fruits) from one single randomized controlled trial. Nonetheless, the tissue bioavailability of ACNs would fairly explain the positive health effects and associations for which apples and other ACN-rich fruits are healthy and amongst the most consumed fruits in the world [20].

It is important to remind that the phenolic content and composition of apples, and probably from other ACN-rich fruits, can fluctuate significantly influenced by aspects such as the soil quality, growth period, harvest moment, storage conditions, maturity status altering their matrix composition, and natural differences between varieties [21]. In consequence, more research is needed to determine the health effects of ACN-rich fruits like berries, but also from novel fruit varieties, such as Redlove apples.

In accordance, a multicentric research project called "The AppleCOR Project" was designed by investigators from the Rovira i Virgili and Lleida universities with the main goal of advancing the knowledge of the effects of ACNs on human health, more specifically of ACN-rich fruits such as *Redlove* apple variety on different CVD risk factors. The AppleCOR project is a large two-part project including first, a randomized controlled trial in humans, and second, an *in vivo* animal experiment, both designed to assess the cardiovascular effects and to determine the mechanisms of action of ACN-rich *Redlove* apple variety, an ACN-free white-fleshed apple and an ACN-rich infusion obtained from *Aronia melanocarpa*.

Current evidence suggests that ACN intake may be beneficial for the prevention of disease and seem to have significant potential for the treatment of different human illnesses and conditions. Therefore, the present work aims to increase the scientific knowledge on ACNs, providing answers to various ACN-related questions such as the ACN tissue bioavailability, consumption biomarkers, and the effects of ACN and ACN-rich fruit intake on human health.

Finally, two novel applications for ACNs on human health are discussed and proposed as a part of this work, exploring the possible health benefits of the ACN supplementation on different pathological alterations suffered by critically ill patients such as the intestinal barrier integrity and the acute lung injury associated to the condition. A summarize of the previous justification of the present doctoral Thesis has been represented in **FIGURE 1**.

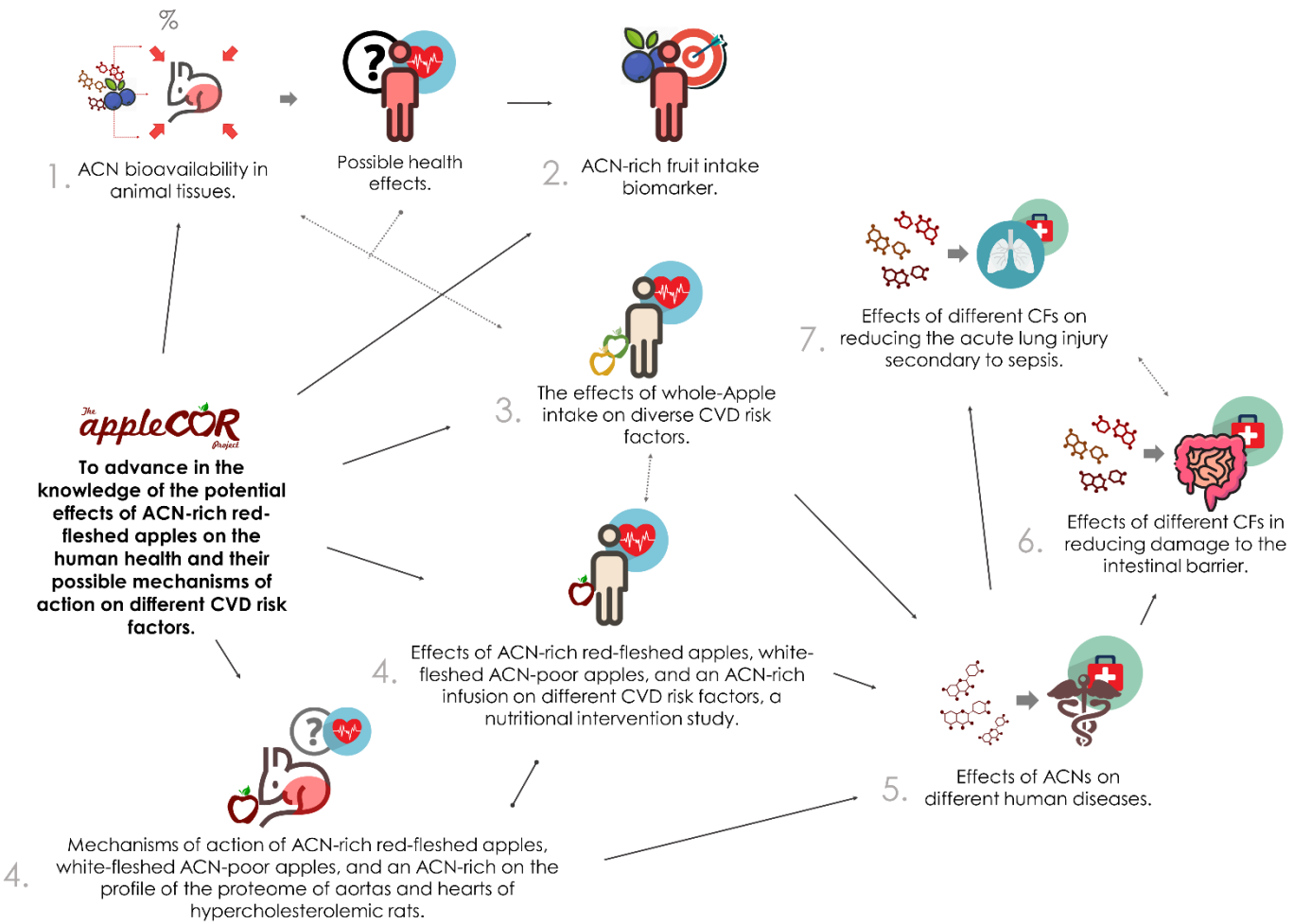

Figure 1. Concept map for the thesis entitled: "The tissue bioavailability, biomarkers, and effects of anthocyanins on human health. Studied through systematic reviews on anthocyanin-rich foods and a nutritional pre-clinical study with anthocyanin-rich red fleshed-apples. The AppleCOR Project". The solid lines show the relationship between two concepts; the pointed lines show the indirect relationship between two concepts. The sense on which the arrow points indicates the causality between concepts. Abbreviations: ACN, anthocyanin; CVD, cardiovascular disease; CF, phenolic compound.

1. INTRODUCTION

1.1. CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the leading cause of death worldwide. CVD-related deaths are most frequently caused by ischemic heart disease [22–24]. However frequent, CVD has a heterogeneous distribution among populations [22,23].

It has been estimated that in developed countries like the United States, more than16.5 million individuals over 20 years old already have some degree of coronary heart disease, whereas the prevalence of myocardial infarction in the same population is around 3% [25]. Consequently, only in the United States, at least one person dies every 40 seconds from CVD [25].

CVD is preventable [26]. Estimates say that almost 90% of all cardiovascular events could be prevented from improving various risk factors such as consuming a healthy diet [27–30], increasing physical activity, or avoiding tobacco and alcohol [31–35]. Also, the treatment of risk factors such as the plasmatic lipids, blood pressure, and plasmatic glucose values is considered beneficial for CVD prevention [36–38].

However, despite the efforts to reduce CVD, the latest reports confirm that CVD is still the leading cause of death [1,22,39,40]. Although, the CVD mortality rates have declined between 1979 and 2015 when CVD accounted for more than 2.7 million (74.2%) of all deaths [41].

Among CVD, coronary heart disease is the most frequent pathology accounting for 43.8% of deaths, followed by stroke (16.8%), elevated blood pressure (9.4%), heart failure (9.0%), arterial diseases (3.1%), and other CVDs (17.9%) [41]. As a result, economic projections reveal that by the year 2035 over 130 million US adults will suffer some form of CVD, with expected associated costs reaching 1.1 trillion US dollars in 2035 [41]. The global trends in CVD mortality reveal that it is constantly rising at a relatively low rate in a rise-and-fall pattern [41].

Since CVD is comprehended by a heterogeneous group of diseases, there is no single underlying mechanism, for instance, coronary heart disease and stroke are mainly caused by atherosclerosis [23,42–45], while aortic aneurisms can be congenital or caused by an altered blood flow [46]. However, stroke, coronary artery disease, and peripheral artery disease, all share atherosclerosis as an important pathophysiological element [23,42–45]. Therefore, for the present thesis, only pathologies secondary to atherosclerosis will be considered CVDs.

1.1.1. **CVD Risk factors.**

In epidemiology, a risk factor is the behavior, circumstance, or condition, increasing the risk of developing CVD [47]. The cardiovascular-related risk factors can be classified as modifiable (i.e. plasmatic lipids and blood pressure values) or as non-modifiable (i.e. sex, ethnicity, age, and others) [48]. Since the novel cardiovascular risk factors have not been fully established, only the traditional risk factors will be described in the following sections, while full information on the known CVD risk factors is contained in TABLE 1.

TABLE 1: Update on the reported cardiovascular disease risk factors.

Area	Risk factors	
	Group	Sub-group
Socioeconomic and environmental		
	• Sanitation and living conditions. • Natural and physical environment.	• Increased air pollution. • Increased noise. • Reduced green spaces. • Extreme temperatures. • Low socioeconomic status.
	• Social environment.	• Low socioeconomic status. • Low education • Poverty. • Increased inequality.
Behavioral factors and infections		
	• Physical inactivity. • Smoking. • Alcohol consumption. • Infections. • Poor diet.	

- High intake of processed meat.
- High intake of salt.
- Low intake of fruits and vegetables.
- Low intake of fiber.
- Low intake of whole grains.
- High intake of trans fats.

Increased adiposity

Biological risk factors

- Hypertension.
- Dyslipidemia.
  - High TC.
  - High LDLc.
  - Low HDLc.
- Diabetes and high blood glucose.
- Thrombosis and inflammation.
- Altered metabolome, epigenome, proteome, transcriptome.
- Altered intestinal microbiome.
- Obesity.

Subclinical atherosclerosis

Early markers due to genetic polymorphism

- Positive family history.
- Increased lipoprotein (a).
- High homocysteine levels.

Markers of existing disease

- Increased coronary artery calcium score.
- Increased C-reactive protein.
- Increased apolipoprotein A1 and B.
- Increased lipoprotein (a).
- Increased carotid intima-media thickness.
- High myeloperoxidase
- F2-isoprostanes.
- Increased ankle-brachial index
- Increased leukocyte count.
- increased fasting blood glucose levels.
- Periodontal disease.
- High homocysteine levels.
- Low vitamin D.

- Increased lipoprotein phospholipase A2.
- Increased myeloperoxidase.
- Increased F2 isoprostanes.
- Brain and pro-brain natriuretic peptide
- Renal function markers

- Increased creatinine
- Increased urea.

Gene modification targets	• D3774Y gene • LDLr • LYSM-Cre

Table elaborated from [24,49–51].

1.1.2. Established CVD risk factors

1.1.2.1. Lipids

The plasmatic lipids are strongly associated with CVD in humans [52,53]. This association was confirmed from the strong relationship between high serum total cholesterol (TC) and the risk of CVD [52,53]. Moreover, increased low-density lipoprotein cholesterol (LDLc) levels in plasma are also associated with an increased CVD risk in a concentration-dependent manner [54,55]. Interestingly, from the information on proprotein convertase subtilisin/kexin type 9 (PSCK9) inhibitor drugs, it has been suggested that targeting for extremely low LDLc concentrations might be beneficial for human health [56]. On the other hand, high-density lipoprotein (HDL) has demonstrated an inverse relationship with cardiovascular risk while other functional properties of HDL particles, might beneficially influence inflammation, oxidation, angiogenesis, and glucose homeostasis [5,57–59]. As a consequence, the reduction of TC (≤200 mg/dL), and LDLc (≤120 mg/dL), as well as the increase of HDLc (≥60 mg/dL) are considered therapeutic targets in current guidelines to prevent CVD [60,61].

1.1.2.2. Hypertension

As a result of the Framingham study, in 1980 it was demonstrated that blood pressure and isolated systolic hypertension were associated with CVD [62,63]. Currently, the normal blood pressure values are still under debate, as a consequence, there are different criteria for the classification of normal blood pressure and hypertension [64,65]. The ranges

and classifications for the normal blood pressure values and hypertension diagnosis in humans are presented in TABLE 2.

Consequently, current American guidelines suggest that systolic pressure values ≤130/80 mmHg should be considered the therapeutic target for hypertension. However, the optimum blood pressure target is still under strong debate, as meta-analysis suggest that achieving blood pressure levels under 135/85 mmHg does not provide further benefit for CVD prevention [66], thus, supporting the European target of ≤135/85 mmHg for hypertensive patients.

TABLE 2. Blood pressure classification and hypertension diagnosis.

Variable	ACC/AHA		ESC/ESH	
	Classification	Value (mmHg)	Classification	Value (mmHg)
Normal blood pressure	Normal	≤120/≤80	Optimal	≤120/≤80
	Elevated	120-129/≤80	Normal	120-129/≤80-84
			High normal	130-139/85-89
Hypertension	Stage I	130-139/80-89	Grade I	140-159/90-99
	Stage II	≥140/≥90	Grade II	160-179/100-109
			Grade III	≥180/≥110
Hypertension diagnosis value	≥130/≥80		≥140/≥90	

ACC, American College of Cardiology; AHA, American Heart Association; ESC, European Society of Cardiology; ESH, European Society of Hypertension; mmHg, millimeters of mercury. Table elaborated from [64,65].

1.1.2.3. Smoking

In 1962 smoking tobacco was first associated with an increased risk of CVD also as a result of the Framingham study [49], and confirmed in other epidemiological studies not only for active but also for passive smoking

[49,67,68]. As a consequence, smoking cessation is considered one of the most important and effective measures for the reduction of CVD in humans [69]. The life expectancy of smoking individuals is reduced between 2.5 years (past smokers) and 12 years (25+ cigarettes/day) [70].

1.1.2.4. Diabetes

Type 2 diabetes mellitus is significantly associated with a 3-fold increase in the risk of developing CVD [71], in particular for women [72,73]. Furthermore, glucose intolerance represents a 1.5-fold increase in the CVD risk [74]. As a result, achieving a target HbA1c <7% significantly reduces microvascular complications [75]. However, HbA1c targets should range between 6.0–6.5% in young patients with no evidence of CVD, while an HbA1c <8% should be the goal for elderly patients [75].

1.1.2.5. Physical Inactivity

Physical inactivity is considered an established CVD risk factor [76–78]. Compared to sedentary individuals, the people that achieve the recommended 2.5 h/week of moderate-intensity aerobic activity are associated with a lower CVD mortality risk (-23%), and incidence (-17%) [78]. Furthermore, individuals performing physical activity for ≥5 h/week can expect to live between 2-3 years longer than their inactive counterparts (0.5-2 h/week) [70].

1.1.2.6. Obesity

Obesity is considered a chronic metabolic disease that has been linked to CVD [79–81] and reduces life expectancy up to 5.5 years [70]. Additionally, childhood obesity has been positively associated with CVD [78]. Currently, the concept of the "metabolically healthy obese" has been introduced to name the paradox that suggests that obesity is not always related to metabolic abnormalities and increased risk of CVD [82]. However, the information from the systematic review and meta-analysis of observational [83], and mendelian randomization studies [84], demonstrate

that obesity is independently associated with CVD in humans [84]. Thus, achieving ideal body weight is the ultimate goal for CVD prevention [85]. However, bodyweight reduction should be encouraged for most patients with overweight/obesity, and weight maintenance should be promoted when weight loss cannot be achieved [85].

1.1.3. **Emerging CVD risk factors**

Apart from traditional CVD risk factors, some molecules appear to be emerging as biomarkers of cardiovascular risk [86]. For instance, the increase of the high-sensitivity C-reactive protein (CRP) as a biomarker of inflammation, is significantly and independently associated with CVD[86], however, with an undetermined causal association [86]. Other biomarkers such as the growth-differentiation factor (GDF)-15 which is considered a strong predictor of cardiovascular events and a potential tool for the stratification of CVD risk [86], and other markers such as fibrinogen or uric acid since both have been positively associated with an elevated risk of CVD [86]. Thus, the field on novel CVD biomarkers is under frequent updates.

Furthermore, genetic modification seems to be the future for CVD risk reduction. An interesting target might be the D377Y gene in mice (equivalent to D374Y in humans) as the CRISPR genetic modification of this gene increases its activity and reduces the atheroma plaque formation [51]. Thus, suggesting the importance of D374Y in humans [51]. Additionally, the LDL receptor (LDLr) seems a promising target for CVD risk, in particular, the LYSM-Cre modification in macrophages seems to lead to reduced atherosclerosis and reduced activity of hydroxymethylglutrayl (HMG)-CoA finally leading to a reduced atheroma plaque formation even in hypercholesterolemic conditions [51].

1.2. THE PATHOPHYSIOLOGY OF CARDIOVASCULAR DISEASE

The atheroma plaque is the key element of many CVDs including coronary heart disease, stroke, and peripheral artery disease [23,42–45]. The atheroma plaque formation is complex and multiple systems like the coagulation and immune systems are involved (FIGURE 2) [23,42–45]. It is considered that hypercholesterolemia is one of the main drivers of atherosclerosis [87,88], however, the same process is also favored by abnormal arterial blood flow and a pro-/anti-coagulant imbalance (FIGURE 2-A) [42,43,45].

The increased plasmatic cholesterol enhances the vascular endothelial permeability, mainly from increasing the expression of scavenger receptors (SR) such as CD36 and LOX-1 [45,87], allowing LDLc particles into the arterial wall [42–45,87] (FIGURE 2-A). Additionally, several circulating monocytes are recruited by the monocyte chemoattractant released by the endothelial cells [23,43], recruiting monocytes and causing their attachment to the vascular wall via selectins or adhesion molecule-1 (VCAM-1) expressed by endothelial cells (FIGURE 2-A) [23,87]. These changes allow the monocyte migration into the sub-endothelial space via diapedesis (FIGURE 2-B) [43,45].

Once in the sub-endothelial space, monocytes migrate either into a pro- or anti-inflammatory phenotype depending on unknown local stimuli (FIGURE 2-C) [23,44]. After expressing a pro-inflammatory phenotype, the macrophages located in the sub-endothelial space increase their production and release of nuclear factor kappa B (NF-kB) and activator protein (AP)-1 (FIGURE 2-D) [42,43]. NF-kB and AP-1 are responsible for increased necrosis of the endothelial cells, which in turn release interleukin (IL)-6, IL-1β, and IL-8, further increasing the monocyte recruitment (FIGURE 2-F) [42]. On the other hand, the pro-inflammatory macrophages located in the intima layer, start turning into foam cells (FIGURE 2-E) from the increased

expression of multiple SRs (AI, AII, AIII, BI, BII, CD36, LOX-1), causing an increase in the macrophage's cholesterol uptake capacity, and from the decrease in the macrophage's cholesterol efflux capacity [43,45] **(FIGURE 2-D)**.

Foam cells are macrophages located in the fatty deposits of blood vessel walls that became laden with lipids after saturation with LDLc [89]. It has been reported foam cells enable the angiogenesis and the necrotic pool creation that increases atherosclerosis and plaque instability [89,90]. Foam cells are considered fundamental in the atheroma plaque formation [43,91,92] due to their release of tumor necrosis factor (TNF), reactive oxygen species, and metalloproteases that increase inflammation **(FIGURE 2-G)** [23,43,45]. Additionally, foam cells release myeloperoxidase, 12/15 lipoxygenase, and other cytokines causing further monocyte chemo-attraction **(FIGURE 1-H)** and further enhancing the oxidation of other LDLc particles in the intima layer or the arterial wall **(FIGURE 2-I)** increasing the plaque instability causing it to finally break and form a clot that will obstruct blood flow and cause the clinical manifestations of CVD [89–91].

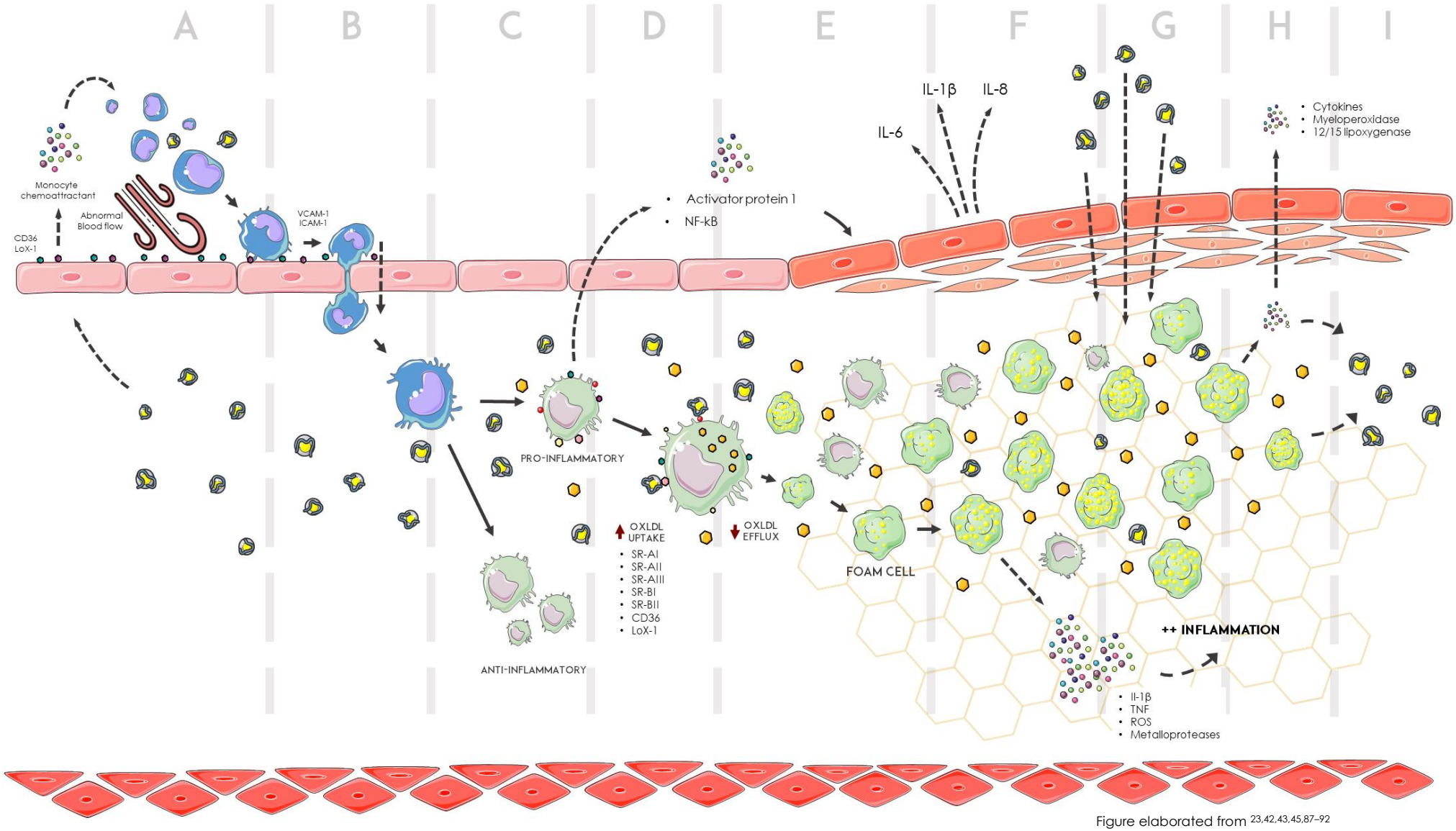

Figure elaborated from [23,42,43,45,87–92]

FIGURE 2. **THE PATHOPHYSIOLOGY OF THE ATHEROMA PLAQUE FORMATION. 2-A)** Hypercholesterolemia, an abnormal blow flow, and the expression of scavenger receptors such as CD36 and LOX-1 favor the atheroma plaque formation. **2-B)** The sub-endothelial migration of monocytes via diapedesis occurs. **2-C)** The monocyte differentiation into a pro- or anti-inflammatory phenotype occurs from unknown stimuli. **2-D)** The macrophages located in the sub-endothelial space increase their production and release of nuclear factor kappa beta (NF-kB) and activator protein (AP)-1 causing inflammation and further monocyte recruitment occurs. Additionally, the macrophage's cholesterol uptake capacity is increased. **2-E)** The cytokine release from the NF-kB and AP-1 activation causes further inflammation. **2-F)** Foam cell formation starts. **2-G)** NF-kB and AP-1 increase the necrosis of the endothelial cells releasing interleukin (IL)-6, IL-1β, and IL-8. **2-H)** Cytokines cause further monocyte recruitment. **2-I)** The intima layer LDL particle oxidation is increased, and the plaque becomes unstable.

1.3. THE CVD RISK TREATMENT AND PREVENTION

1.3.1. CVD primary prevention

The CVD primary prevention is the reduction in CVD risk that is conducted before a CVD event takes place for the first time, and is achieved from the modification of diverse CVD risk factors [85]. Consequently, from the 2019 American College of Cardiologist (ACC)/American Heart Association (AHA) guidelines for the primary prevention of CVD [85]:

1st. The most effective preventive measure for the prevention of CVD is obtained through a healthy lifestyle [85].

2nd. Whenever possible a team-based multidisciplinary strategy assessing not only the biological but also the social determinants of CVD should be followed [85].

3rd. Individuals aged between 40 – 75 years old who are under current evaluation for CVD prevention should undergo CVD risk estimation and discuss their pathology with their clinicians before starting pharmacological therapy [85].

4th. Adults should consume a diet rich in fruits, vegetables, nuts, lean protein (animal or vegetal), and fish while decreasing the intake of red and processed meats, and refined sugars, for adults with overweight or obesity weight loss should be recommended [85].

5th. A 150 minute/week target of moderate-intensity or 75 minutes/week of intense physical activity is recommended for adults who are looking for CVD prevention [85].

6th. The improvement of dietary habits and exercise are crucial for CVD prevention in T2DM patients, if medication is needed, metformin should be considered the first-line drug, followed by either a sodium-glucose cotransporter 2 (SGLT2) inhibitor or a glucagon-like peptide (GLP)-1 receptor agonist [85].

7th. Smoking cessation is strongly advised [85].

8th. Acetylsalicylic acid (aspirin) should not be used frequently to prevent CVD due to a lack of effect [85].

9th. Statins are considered the first-line drugs for CVD primary prevention in patients with increased LDLc levels, patients with T2DM in the ages between 40 – 75, and those with an increased CVD risk [85].

10th. In individuals with high blood pressure or hypertension, pharmacological and non-pharmacological interventions are recommended to achieve a blood pressure target of

≤130/80 mmHg [85], however as mentioned before the last recommendation is still under strong debate [64,65].

1.3.2. CVD secondary prevention

The secondary prevention of CVD consists of the prevention in the progression or recurrence of CVD after a first event has occurred [93,94]. Secondary prevention consists in further enforcing the measures recommended for primary prevention such as smoking cessation or achieving ideal body weight [93,94], however, the pharmacological treatment with lipid-lowering agents [94–96], or the use of anti-diabetic [93,97–99], anti-platelet [100], or anti-hypertensive drugs [101], and the implementation of a healthy diet are also strongly recommended.

Regarding the plasmatic LDLc cholesterol values, LDLc targets of <70 mg/dL (<1.8 mmol/L), or a 50% reduction in plasmatic LDLc if the baseline is between 70 – 135 mg/dL (1.8 – 3.5 mmol/L) is recommended [95,96]. Statins like atorvastatin (40 - 80 mg/day), or rosuvastatin (20 - 40 mg/day) are considered the first-line drugs for the secondary prevention of CVD showing some therapeutic equivalence [95,96]. Additionally, the proprotein convertase subtilisin-Kexin type 9 (PCSK-9) inhibitors, such as evolocumab or alirocumab, are also recommended as PCSK-9 inhibitors significantly reduce up to 60% of plasmatic LDLc if associated with statins [94–96].

For glucose management, the recommendation for a target HbA1c level of <7% (< 53 mmol/mol) is recommended for most adults with T1DM or T2DM but not for pregnant women [85]. Metformin has demonstrated to reduce the risk of CVD in all-cause and cardiovascular mortality in patients with coronary artery diseases [102]. However, metformin's cardio-protective effects are also being demonstrated beyond its glucose-lowering effect from an unknown mechanism of action [103]. To reduce cardiovascular and all-cause mortality, SGLT2 inhibitors, such as empagliflozin or canagliflozin, should be considered early in the course of CVD [93,97–99].

Regarding the anti-platelet drug management, the use of acetylsalicylic acid (ASA) is recommended in all patients in secondary prevention to all individuals that suffered an acute coronary syndrome and are without contraindications at an initial loading ASA dose of 150 -300 mg posteriorly reducing it to 81 mg/day (acceptable: 75 -100 mg/day) [100]. Moreover, a P2Y12 inhibitor such as clopidogrel (75 mg/day), or prasugrel (10 mg/day) should be added as soon as possible and maintained for at least 12 months [100]. It should be noted that no differences in clinical efficacy and safety have been noted between P2Y12 inhibitors [100].

To reduce CVD risk, achieving blood pressure values <140/90 mmHg is recommended [93]. The use of angiotensin-converting enzyme inhibitors (ACEIs) such as enalapril or captopril, angiotensin-II receptor (ARA-II) blockers like valsartan or losartan, and the use of diuretics significantly reduced the risk of cardiovascular events [101]. Moreover, ACEIs significantly reduce all secondary outcomes, while the calcium channel blockers and diuretics significantly reduce stroke [101].

Finally, the Dietary Approaches to Stop Hypertension (DASH) pattern demonstrated an improvement in the cardiac function, blood pressure values, functional capacity, oxidative stress, and mortality in the secondary CVD prevention [104]. Additionally, other dietary patterns, like the Mediterranean diet, significantly improved inflammation, cardiac function, and quality of life in cross-sectional studies [104]. While patterns such as the hyper-proteic or low-carbohydrate diets suggest some improvement in the functional capacity of patients with heart failure [104].

1.4. EPIDEMIOLOGICAL STUDIES.

In medical sciences, the epidemiological studies on human populations are an attempt to correlate diverse health effects in humans, to its underlying cause [105]. For these purposes, the epidemiological study designs can be classified as observational or experimental [105]. In observational studies (OS'), scientists limit themselves to observing nature's course consequently describing what happens to provide with associations between an exposure and an outcome, while in experimental study designs, one or all factors from a certain case are controlled, allowing the identification of causal relationships between exposures and outcomes [105].

1.4.1. Observational studies

OS' are usually formed by two components, one descriptive and one analytical. The descriptive side of OS' provides answers for the questions "who, what, where, and when" of a health event, while the analytical component tries to answer "how" a health event occurs [105–107]. OS' can be classified as:

- **Ecological studies:** are usually a retrospective designed basic type of OS that is usually used to compare clusters of people that have been grouped based on their location or temporal association and providing prevalence ratios [105–107].

- **Report of case-series:** reports the clinician's experience usually providing basic common characteristics, on one single patient, a small group of patients sharing the diagnosis, or a factor potentially causing illness [105–107].

- **Case-control studies:** are retrospective studies where individuals suffering from a disease (cases), are compared against a group of individuals that do not suffer from the disease (controls), to look back for potential exposures causing the disease in the cases but not in the controls [105–107]. It provides the odds ratio, prevalence odds ratio, prevalence ratio, and prevalence difference [105–107].

- **Cross-over studies:** cross-sectionals are also known as "prevalence studies" because of their utility for assessing the population's prevalence of a disease at a single point in time [105–107]. Individuals are selected based on their exposure and not their outcome which is obtained after the participant's enrolment [105–107]. Cross-over studies are particularly useful to determine the odds ratio, prevalence odds ratio, prevalence ratio, and prevalence difference [105–107].

- **Cohort studies:** in cohorts, subjects are included and followed for a long time based on their status on exposures, meaning that cohorts are followed through time, starting disease-free, to posteriorly assess the outcome results and look back for exposures in sick individuals [105–107]. Cohort studies are especially useful to determine the odds ratio, prevalence ratio, prevalence odds ratio, prevalence difference, attributable risk, incidence ratio, relative risk, and risk ratio hazard ratio [105–107].

1.4.2. Experimental studies

For experimental or intervention studies, an experimental model is employed to confirm a causal relationship previously evidenced from OS', or to test the causality of new hypothesis [105,106]. There are different types of experimental designs:

- **Pre-post trial:** This type of study assesses the effects after the implementation of a particular intervention not controlled by the investigator (i.e. banning smoking in closed spaces) [105,106].
- **Non-randomized trials:** Are interventional study designs that compare a group where an intervention was performed with a group where there was no intervention. These

studies are considered particularly predisposed to distinct types of bias for which are not considered a preferred study design [105,106].

- **Randomized controlled trials (RCTs):** are often medical experiments aiming to reduce bias when testing the effectiveness of a new treatment by randomly assigning subjects into two or more groups with different treatments, to posteriorly compare them against an untreated control group [105,106].

- **Cross-over RCTs:** are interventional studies where participants are intentionally switched to the other treatment arm [105,106]. Cross-over RCTs, begin as a traditional RCT, however, at the end of each treatment phase, each participant is re-allocated to another group with a different treatment usually after a wash-out period [105,106]. Cross-over studies demonstrate the reversibility of intervention and compensate for bad randomization [105,106].

- **Field trials:** like randomized controlled trials, field trials are interventions where subjects are randomly assigned to a treated or control group to determine causal relationships [108]. Field trials are distinguished from RCTs because they are usually conducted discretely in the "real world" relying on an external force (i.e. government disposition or climatic event) to act as an exposure [108].

- **Community-based trials:** are clinical trials commonly designed to be executed by primary care physicians, community health centers, or local health facilities directly at a community level rather than inside of research facilities [109].

1.5. SYSTEMATIC REVIEWS OF THE SCIENTIFIC LITERATURE

The systematic reviews and meta-analyses are considered the pinnacle of evidence [110]. Systematic reviews improve the complex interaction that exists between the information from scientific studies and the clinical experience from day-to-day practice to stay up to date with the ever-increasing amounts of information [111]. It is for those reasons that systematic reviews and meta-analyses are often used as the starting point to develop new guidelines and solve other challenging questions that cannot be solved in other ways due to methodological complications [112,113].

The rationale behind the position of systematic reviews and meta-analyses at the top of the pyramid focuses on the methodological design of studies to determine the value of their contributions [110]. However, the study design does not ensure the certainty of the information or account for bias. Thus, challenging the traditional pyramidal design [110]. In consequence, it is currently considered that systematic reviews should be seen as tools to assess the information from all the other levels of evidence (e.g. RCTs and OS') since the quality of the designs themselves challenge the limits on the quality of information and the traditional pyramid design [110]. In other words, systematic reviews should be considered not only the highest level of the pyramid but also the lens through which large amounts of information should be analyzed to find the most accurate answers and provide new conclusions [110,113].

FIGURE 3.

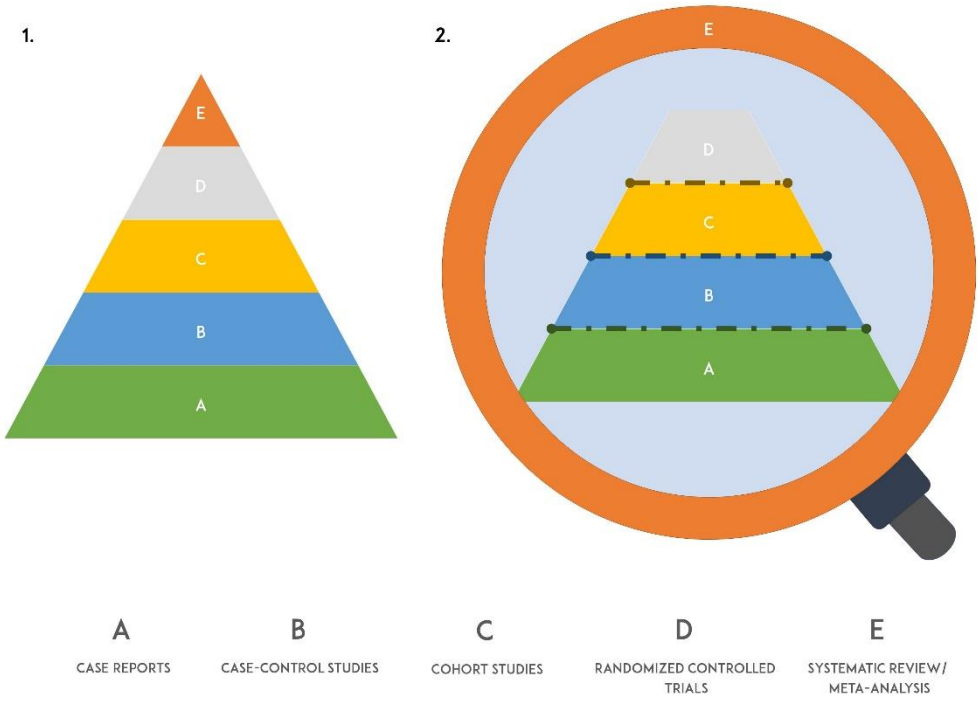

FIGURE 3. THE NEW PYRAMID OF EVIDENCE. 1) classical evidence pyramid: the classical representation with increasing validity from base to top. 2) the new pyramid of evidence: The lines dividing each level become blurry as the quality of information determines the value of each level, the top of the pyramid is separated and surrounds the other studies as systematic reviews and meta-analysis should be seen as a lens providing a better understanding of all other levels of information. Figure adapted from [110].

1.5.1. Types of reviews

Review articles are one of the most cited types of articles suggesting that the summary of large amounts of information from a critical point of view is highly valued amongst scientists [113]. Accordingly, at least 14 types of reviews have been identified, although many review types lack explicit methodologies and are not mutually exclusive [113]. More information on the characteristics of the known types of reviews is presented in TABLE 3.

TABLE 3. The characteristics of the reported types of reviews.

Type	Description	Goals	Search	Quality assessment	Analysis of content
Critical review	Demonstrates the writer's extensive search and critical assessment on a topic beyond the level of a simple description usually resulting in a hypothesis.	To identify the most relevant information in the field.	Not required for the report.	Not mandatory (recommended PRISMA statement).	A significant analytical component usually in a narrative form.
Literature narrative review	It is the generic term for the materials that examine current literature. Usually cover at different levels of complexity.	Undetermined (specific goal-oriented).	Might be reported (not required).	Not mandatory (recommended PRISMA statement).	Undetermined (specific goal-oriented).
Mapping review	Maps and categorizes the existing literature to determine future reviews or primary research	To identify gaps in the literature to provide new research topics.	A complete search must be reported and determined using time or scope constraints.	Not required.	Has an emphasis on the characteristics: the quantity and quality of literature, not the content nor the results. Usually presented in a graphical or tabular form.
Meta-analysis	It is a statistical set of techniques that combine the results of quantitative or qualitative studies.	To provide a precise measurement of the effect of the results of the analyzed studies.	Aims for a complete and detailed search that must be reported.	Exhaustive and comprehensive. Might use other statistical techniques like the funnel plot to assess its completeness. Following the PRISMA statement.	It is the numerical analysis of the measurement of the effect, in the absence of heterogeneity. Presented in graphical, tabular, and commented in a narrative form.
Mixed methods review	It is the combination of methods where the central component is a scientific literature review, usually systematic.	To provide a combined approach to solve a complex question.	A broad, and separate search must be performed to retrieve all studies relevant for the variables included.	This method requires either a generic appraisal tool or the use of separate adequate tools to assess bias independently. Following the PRISMA statement.	Analyzes both results and look for correlations, characteristics, discrepancies, and gaps used to provide new conclusions.

Overview	It is the generic term for the summary of the literature usually in the medical field.	To survey the (medical) literature and describe its characteristics.	Might include a systematic search, depending on the nature of the overview (systematic or not).	Might include quality assessment, depending on the nature of the overview (systematic or not).	The analysis is presented following the nature of the overview. It might be conceptual, thematic, chronological, etc.
Qualitative systematic review	It is the method to integrate and compare the results from qualitative studies.	To identify common themes or constructs between different individual qualitative studies.	Might be selective or purpose-depending.	Usually assess to justify the union of messages or concepts, not to include or exclude publications.	It provides a thematic analysis that might include conceptual models in a narrative form.
Rapid review	It is the assessment of what is already known about a specific practice or policy issue, using systematic review methods for the search and assessment of the included publications.	To provide with a scope on the direction of effects in the literature.	Must include a complete search determined by time and scope limitations. The complete search term, process, inclusion, and exclusion criteria should be published.	Thorough quality assessment using the most adequate tool depending on the study design and purpose oriented. Following the PRISMA statement.	Focused on the quantity, quality, and direction of effect found from literature, usually presented in a narrative form with tabular elements.
Scoping review	It is the preliminary assessment of the size and scope of literature.	To identify the nature and extent of research evidence, usually including the ongoing projects.	Includes a complete search determined by time and scope limitations. It usually includes ongoing research.	No formal quality assessment required.	Gives insights on the quantity and quality of literature focused on a specific key feature in an attempt to identify a possible viable review.
State-of-the-art review	Addresses current matters in contrast to other approaches, current or previous.	To offer new perspectives on an issue or to point out an area or research.	Aims for a complete and comprehensive search, ideally reported (not required).	No formal quality assessment required.	Focuses on the current state of knowledge to provide new insights and ideas for future investigation or research.
Systematic review	It is the systematic search of all literature on a specific topic, often adhering to guidelines on the conduction of the review.	Aims to appraise and synthesize all research evidence on a predetermined specific subject to provide answers to complex questions and drive new conclusions not apparent from individual studies.	Must include a complete search determined by time and scope limitations. The complete search term, process, inclusion, and exclusion criteria should be published.	Thorough quality assessment using the most adequate tool depending on the study design and purpose oriented. Following the PRISMA statement.	Focuses on what is known, gives recommendations for practice. Identifies what is still unknown and suggests future research.

Systematic search and review	It is a review method that combines the strengths of the critical review with a comprehensive search process.	To produce the "best evidence synthesis"	Must include a complete search that might be determined by time and scope limitations.	Might include quality assessment (not required).	Focuses on what is known, giving recommendations for practice in a minimal narrative, and presenting a tabular summary of the studies. Highlights limitations.
Systematized review	It is an attempt to include the elements of a systematic review while not fully complying with all formal requirements. Usually conducted as postgraduate assignments.	To appraise and summarize the information on a specific topic. It does not need to be thorough.	Aims for a comprehensive search, ideally reported (not required).	No formal quality assessment required.	Focuses on what is known and the limitations of the methodology, usually presented in a narrative manner with a tabular component.
Umbrella review	It refers to the compilation of multiple reviews into one accessible and usable document. It focuses on ample conditions or problems for which there are multiple reviews, interventions, or results.	To broadly explain what is known and provide answers to broad and complex questions.	Aims for a comprehensive search, focusing only on the identification of the reviews to be included in the analysis, does not search for primary components. The search must be reported as if in a systematic review.	Thorough quality assessment for the included reviews using the most adequate depending on the study design and purpose oriented. The quality assessment determines inclusion or exclusion.	Focuses on what is known on complex questions, providing answers and recommendations for practice. Identifies what is still unknown and suggests future research.

PRISMA, Preferred Reporting Items for Systematic Reviews, and Meta-Analyses. Table elaborated from [112–117]

1.5.2. Scientific review's quality assessment

To ensure the quality of systematic reviews, protocols must be elaborated usually using the Preferred Reporting Items for Systematic Reviews (PRISMA) protocols [118], or the Methodological Expectations for Cochrane Intervention Reviews (MECIR) [119], and should be registered in an international registry such as the "International Prospective Register of Systematic Reviews" (PROSPERO), established by the University of York or the registries established by the Campbell or Cochrane collaborations. Additionally, as a general recommendation, a web-based search must be performed in at least two scientific libraries using the most fitting set of words depending on the specific goals [114,117,120–122]. Moreover, the reporting of the results should be done in a structured manner, using methodologies such as the PRISMA methodology [123]. A detailed list of the quality assessment tools for different types of studies is presented in TABLE 4.

TABLE 4. Tools for study quality and risk of bias assessment.

Name	Acronym	Uses	Authors	Country
Randomized controlled trials				
Risk of Bias Tool	ROB 2.0	Individually and cluster-randomized parallel-group trials, and individually randomized cross-over trials.	Cochrane collaboration.	United Kingdom
Critical Appraisal Skills Program	CASP	Systematic Reviews, Randomized Controlled Trials, Cohort Studies, Case-Control Studies, Economic Evaluations, Diagnostic Studies, Qualitative studies, and Clinical Prediction Rule.	Public Health Resource Unit, NHS, England.	United Kingdom
Joanna Briggs Institute assessment tools	JBI	Analytical cross-sectional studies, case-control studies, case reports, case series, cohorts, diagnostic test accuracy studies, economic evaluations, prevalence studies, qualitative research studies, quasi-experimental research, randomized controlled trials, systematic reviews.	Joanna Briggs Institute	Australia
Non-randomized controlled trials and observational studies				

Risk of Bias for non-randomized (observational) studies or cohorts of Interventions	ROBINS-I	Non-randomized studies or cohorts of interventions.	Cochrane Bias Methods Group and the Cochrane Non-Randomized Studies of Interventions Methods Group.	The United Kingdom.
Risk of Bias for non-randomized (observational) studies or cohorts of Exposures	ROBINS-E	Non-randomized studies or cohorts of exposures.	Cochrane Bias Methods Group and the Cochrane Non-Randomized Studies of Interventions Methods Group.	The United Kingdom.
Newcastle-Ottawa scale	NOS	Case-control or cohort studies.	Department of Epidemiology and Community Medicine, University of Ottawa.	Canada.
Institute of Health Economics Case Series Studies Quality Appraisal Tool	IHE	Case series studies (modified Delphi technique).	Institute of Health Economics.	Canada.
Joanna Briggs Institute Assessment Tools	JBI	Analytical cross-sectional studies, case-control studies, case reports, case series, cohorts, diagnostic test accuracy studies, economic evaluations, prevalence studies, qualitative research studies, quasi-experimental research, randomized controlled trials, systematic reviews.	Joanna Briggs Institute.	Australia.
Critical Appraisal Skills Program	CASP	Systematic Reviews, Randomized Controlled Trials, Cohort Studies, Case-Control Studies, Economic Evaluations, Diagnostic Studies, Qualitative studies, and Clinical Prediction Rule.	Public Health Resource Unit, NHS, England.	The United Kingdom.
The Methodological Index for Non-Randomized Studies	MINORS	Non-randomized intervention studies.	Slim, K. et al.	France.
Agency for Healthcare Research and Quality	AHRQ	Provides with multiple documents on the applications of systematic reviews and systematic review quality assessment.	Agency for Healthcare Research Quality.	The United States.

critical appraisal tool to assess the quality of cross-sectional studies	AXIS	Cross-sectional studies.	Downes, M. et al.	Australia/United Kingdom.
Transparent Reporting of Evaluations with Nonrandomized Designs	TREND	Non-randomized controlled trials.	Centers for Disease Control and Prevention (CDC).	The United States.
Strengthening the reporting of observational studies	STROBE	Cohort, case-control, cross-sectional.	STROBE Group.	Multiple countries.
McMaster Critical Review Forms and Guidelines for Qualitative Studies		Qualitative studies	The evidence-based practice research group, McMaster University.	Canada.
Mixed Methods Appraisal Tool	MMAT	Complex systematic literature reviews that include qualitative, quantitative and mixed methods studies (mixed studies reviews)	Mc Gill University	Canada
Systematic Reviews				
Assessing the Methodological Quality of Systematic Reviews	AMSTAR 2	Systematic reviews of controlled or non-controlled interventions.	Bruyère Research Institute	Canada
Critical Appraisal Skills Program	CASP	Systematic Reviews, Randomized Controlled Trials, Cohort Studies, Case-Control Studies, Economic Evaluations, Diagnostic Studies, Qualitative studies, and Clinical Prediction Rule.	Public Health Resource Unit, NHS, England.	United Kingdom
Agency for Healthcare Research and Quality	AHRQ	Provides with multiple documents on the applications of systematic reviews and systematic review quality assessment.	Agency for Healthcare Research Quality	United States
Joanna Briggs Institute assessment tools	JBI	Analytical cross-sectional studies, case-control studies, case reports, case series, cohorts, diagnostic test accuracy studies, economic evaluations, prevalence studies, qualitative research studies, quasi-experimental research, randomized controlled trials, systematic reviews.	Joanna Briggs Institute	Australia
Animal Studies				

Systematic Review Centre for Laboratory Animal Experimentation	SYRCLE	Risk of bias in animal studies.	Hooijmans, C. et al.	Netherlands
Animal Research: Reporting of In Vivo Experiments	ARRIVE	Animal studies	National Centre for the Replacement, Refinement, and Reduction of Animals in Research	United Kingdom

1.6. PHARMACOLOGY CONCEPTS IN NUTRITION

In pharmacology, bioavailability is defined by the Food and Drug Administration (FDA) as ''the rate and extent to which the active ingredient or moiety is absorbed from a drug product and becomes available at the site of action'' [124]. However, the putative benefits coming from the ingestion of nutraceuticals (natural compounds claiming to have beneficial effects in the treatment or prevention of a disease or condition [125]), such as flavonoids or ACNs, are not often adequately evidenced due to their low and variable oral bioavailability [126].

It is considered that the low and variable bioavailability of nutraceuticals might be secondary to several physicochemical and physiological aspects such as the food matrix, their solubility on gastrointestinal fluids, the formation of insoluble complexes with other compounds, their low permeability across the intestinal barrier, and several molecular modifications suffered from the intestinal microbiota [126]. Additionally evidencing the need for biomarkers that can help assess the adequate consumption and effects of various nutrients with unrelated bioavailability [127].

Consequently, the concepts of nutritional bioavailability and nutrition biomarkers will be briefly introduced in the following sections.

1.6.1. **Definitions**

- Bioavailability: "The extent to which a xenobiotic can be used by the body" [128].
- Systemic availability: "The proportion of the dose of a xenobiotic that reaches the systemic circulation intact after oral administration" [128].
- Apparent bioavailability: For PCs that suffer the first-pass metabolism is the proportion of the "original" PC that reaches the systemic circulation after oral administration [128].
- Total bioavailability: "The absorbed proportion of a xenobiotic that is absorbed through the gastrointestinal wall and enters the systemic circulation both in its original form and as a metabolite(s) produced by the first-pass metabolism" [128].

1.6.2. **Bioavailability in nutrition**

To characterize the most important factors that restrict the oral bioavailability of drugs, a scheme was developed where compounds were classified from class I – IV depending on their high or low permeability and solubility [126]. However, as noted before, the bioavailability of nutraceuticals (i.e. ACNs) is highly dependent on multiple factors [126].

Accordingly, a new scheme was developed to better understand the factors that limit the bioavailability of nutritional compounds [126]. In this new model, the nutraceutical's bioavailability can be classified accordingly within 3 major classes and 10 subclasses that will be presented in FIGURE 4, and briefly summarized and explained in the following sections.

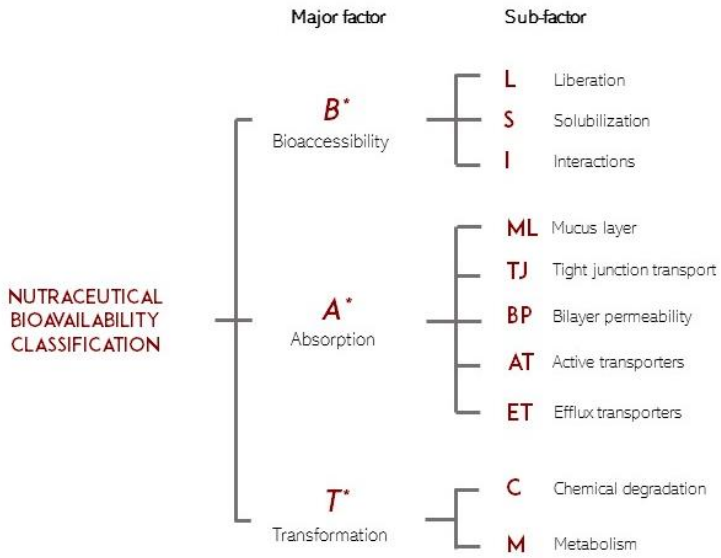

FIGURE 4. THE NUTRACEUTICAL BIOAVAILABILITY CLASSIFICATION SCHEME

Figure adapted from [126]

1.6.2.1. Bioaccessibility (*B**)

The Bioaccessibility class refers to the group of limiting factors that are related to the Bioaccessibility of the nutraceutical within the intestinal fluids of the gastrointestinal tract. In other words, the nutraceutical must be present in a physical form suitable for absorption [126]. Nutraceuticals with high Bioaccessibility (>75%) are designated as B*(+), while those with low Bioaccessibility (<75%) are classified as B*(-) [126]. Additionally, the bioaccessibility can be further classified into three sub-classes of limitations:

- Liberation limited (*L*): when the bioavailability of a molecule is limited by the nutraceutical's ability to be released from the food matrix [126].

- Solubility limited (*S*): when the bioavailability of a molecule is limited by the nutraceutical's ability to be solubilized within the intestinal fluids [126].

- Interaction limited (*I*): when the bioavailability of a molecule is limited by its interaction with other components present in the intestinal fluids [126].

1.6.2.2. Absorption (*A**)

The absorption describes the factors that are related to the trespassing of the nutraceutical from the gastrointestinal fluids, across the epithelium and into the systemic circulation [126]. Additionally, the absorption can be further classified into five sub-classes of limitations:

- Mucin layer transport limited (*ML*): when the bioavailability of a molecule is limited by the pass of the molecule through the mucus layer that protects the gastrointestinal tract due to its size, electrostatic or hydrophobic interactions of the molecule with the mucus layer [126].

- Bilayer permeability limited (*BL*): when the bioavailability of a molecule is limited by the ability of the molecule to travel across the nonpolar phospholipid bilayer of the cell membrane [126]. The BL characterizes the absorption of molecules from their oil-water partition coefficient, where a high coefficient favors the molecule's absorption, and low oil-water partition coefficient values are associated with the low absorption of a molecule [126].

- Tight junction transport limited (*TJ*): when the bioavailability of a molecule is limited by the molecule's ability to be transported across the epithelium through tight junctions under physiological conditions (pore radios >0.7 nm) [126], a type of cell-cell junction in the intestinal epithelia, primarily composed by occludin and claudin proteins, and forming a barrier impermeable most soluble molecules [129].

- Active transport limited (*AT*): when the bioavailability of a molecule is limited by active transport mechanisms, and defined as the absence of an active transport mechanism for a given molecule or by the saturation of said action mechanism [126].

- Efflux transported limited (*ET*): when the bioavailability of a molecule is limited by efflux transporters located within the epithelium cell membranes that actively carry the molecule back into the intestinal lumen [126].

1.6.2.3. Transformation (*T**)

The bioavailability of a molecule could be limited by its transformation into an inactive form in the gastrointestinal tract [126]. Additionally, the transformation can be further classified into two sub-classes of limitations:

- Chemical degradation limited (*C*): when the bioavailability of a molecule is limited by its chemical transformation (i.e. oxidation, reduction, or hydrolysis) [126].

- Metabolism limited (*M*): when the bioavailability of a molecule is limited by their metabolization from specific enzyme systems in the gastrointestinal tract [126]. Such is the case for many phenolic compounds like ACNs, resveratrol, quercetin, and epicatechin that are susceptible to phase I and II metabolism reactions within the gastrointestinal tract [126].

1.6.2.4. Interpretation and use of the nutraceutical bioavailability classification scheme

To be of use, the scheme classifies molecules by a *B** *A** *T** label following the major factors that limit its bioavailability [126]. A plus sign (+) is added next to each major physicochemical factor that is considered nonlimiting, however, if it is considered a limiting factor a minus sign (-) will be placed instead [126]. Additionally, to specify which single or multiple specific sub-factors are the ones that might be influencing the

bioavailability for a specific molecule, their abbreviations should be placed in subscript next to their appropriate major factors [126]. For instance, resveratrol would be classified as $B^*(-)_S$; $A^*(-)_{ET}$; $T^*(-)_{M,C}$, since resveratrol has low solubility [126], it is efflux-transported back into the intestinal lumen by the multidrug resistance-associated protein 2 (MRP2)transporter protein [126] and suffers from methylation, sulfonation, auto-oxidation and degradation processes within the intestinal lumen [126].

1.6.3. **Biomarkers in nutrition**

The suitability of the nutritional status of an individual is determined by the extent to which the physiological nutrient requirements were achieved at a particular stage in life [130]. In medicine, the development of biomarkers has been largely driven by their uses for the identification and quantification of a disease or its progression, while in nutrition the purpose of biomarkers is focused on the prevention of disease and the promotion of health [130]. As a consequence, a different approach suggesting the consideration of biomarkers to be for health instead of disease [130].

Consequently, there is a need to assess the adequate intake of any given nutrient at a stage of life in an objective and precise manner [130]. However, the dietary assessment and nutritional status of an individual is usually performed using dietary intake recalls, food records, and food frequency questionnaires with inherent limitations such as the subjective nature of their data, the insufficient information in food composition tables, and other factors that influence the bioavailability of the nutrients [130]. Therefore, there is a need for molecules that can be detected through biochemical analysis indicating their consumption, further lowering methodological errors, and detecting deficiency states in a more precise manner than a dietary assessment [130]. Such biomarkers are clinically useful, in particular, to detect deficiencies and suggest treatment [130].

However, there is a lack of well-accepted biomarkers of food intake for commonly eaten food groups such as alcoholic and non-alcoholic beverages, dairy products, meat, fatty fish, eggs, and others, consequently resulting in an urgent need to discover and validate new biomarkers [131].

Currently, a considerable number of candidate food intake biomarkers (FIBs) are being noted from the metabolomic studies, systematic reviews, and intervention studies, however, the FIB candidates must be validated [132]. The validation of an FBI does not depend only on the analytical validity of the biomarker, the nutritional or biological validity should also be judged [132]. Therefore, to consider the validation and application of a biomarker the following characteristics should be taken into consideration:

1.6.3.1. Plausibility:

Takes into consideration various confounding factors, such as the molecule's presence in other foods, its industrial uses, or its endogenous production from other compounds demonstrating the causal relationship between the presence of the molecule in the analyzed fluids or tissues, and the ingestion of specific food or group [132].

1.6.3.2. Dose-response:

It refers to the existing relationship between the dose of an ingested molecule and its presence in organs or tissues, determined by its bioavailability, and taking into consideration the molecule's kinetics [132].

1.6.3.3. Time-response:

Assesses the time at which the candidate molecule's maximum concentration is reached in various body fluids and the range of time for its measurement taking into account its absorption and elimination rates under the specific purpose of the biomarker [132].

1.6.3.4. Stability:

Evaluates the best practices that are needed to collect and store the samples to avoid their degradation and thus the ability to determine their concentration [132].

1.6.3.5. Analytical performance:

Denotes the reliability of a molecule's chemical analysis taking into consideration its accuracy, precision, and measurement variability. This criterion is minimally fulfilled by a molecule's capability of being measured at different concentrations [132].

1.6.3.6. Reproducibility:

Evaluates the detection of a molecule performed in at least two different laboratories, and assessed through inter-laboratory comparison tests [132].

1.6.3.7. Robustness:

The robustness refers to the candidate knowledge on the biomarker's behavior in complex diets from intervention studies [132]. In other words, it evaluates the interaction between the candidate biomarker and other food components that might affect its use as a biomarker [132].

1.6.3.8. Reliability:

It evaluates the candidate for a biomarker and compared it against the current gold-standard methodology to give a good measure of exposure [132]. Additionally, a comparison between the candidate biomarker and a dietary assessment tool should be performed [132].

1.7. PHENOLIC COMPOUNDS

The PCs are a group of molecules characterized by the presence of at least one phenol unit as a part of their chemical structures usually with a hydroxy group attached directly to the phenyl or another aryl group [133]. From their chemical structure, PCs can then be subdivided into phenolic acids, tannins, quinones, curcuminoids, stilbenes, lignans, quinones, coumarins, and flavonoids [133].

1.7.1. **Flavonoids**

Flavonoids are PCs characterized by a *flavan* nucleus and represent one of the most common classes of PCs present in fruits and vegetables [134–136]. There are more than 8000 flavonoid derivates identified in plants where they are usually responsible for their red or blue pigmentation also protecting from pathogens and ultraviolet radiation [134,136].

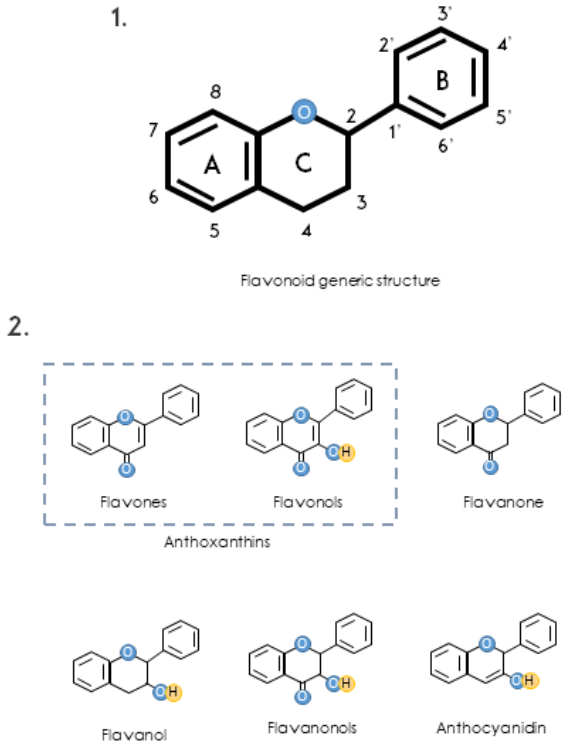

FIGURE 5. THE CHEMICAL STRUCTURE OF FLAVONOIDS. The chemical structure of a flavonoid consists of a skeleton made from 15 carbons, and 3 rings, two phenyl rings (A and B), and one heterocyclic (C) [137,138]. Additionally, from their basic structure, flavonoids can be classified as anthoxanthins, flavanones, flavanols, flavanonols, and flavans [137,138]

Figure elaborated from [143,144]

Flavonoid consumption is considered beneficial for human health [135]. Some epidemiological studies suggest that flavonoids have anti-inflammatory [139], and anticancer properties [140–142]. Additionally, various studies demonstrate that oral flavonoid intake significantly reduces the risk of CVD mortality [143,144]. However, due to the extent of the information on flavonoids, the following sections will be limited to anthocyanins, the glycoside version of anthocyanidins [145,146].

1.8. ANTHOCYANINS

ACNs are phenolic compounds part of the flavonol subclass and one of the most studied and well-known group of bioactive molecules [147]. ACNs are water-soluble plant pigments considered responsible for the red, pink, purple, or blue coloration in the leaves, flowers, fruits, and seeds of many plants [9].

1.8.1. Chemical structure

ACNs molecules soluble and unstable in aqueous solution, existing in six core aglycone versions (cyanidin, delphinidin, pelargonidin, malvidin, peonidin, and petunidin) (FIGURE 6). however, the addition of either a sugar such as glucose, galactose, arabinose, or others in different positions of their C- or A-rings yields over 700 derivative glycoside structures [128]. ACNs are characterized by their positively charged oxygen atom making them potent antioxidants [148], as a result, ACNs are highly reactive to changes in pH, shifting from its flavylium form when diluted in acid solutions (pH 1-3) to their carbinol or chalcone forms at higher pH values (>4) [128]. (FIGURE 6).

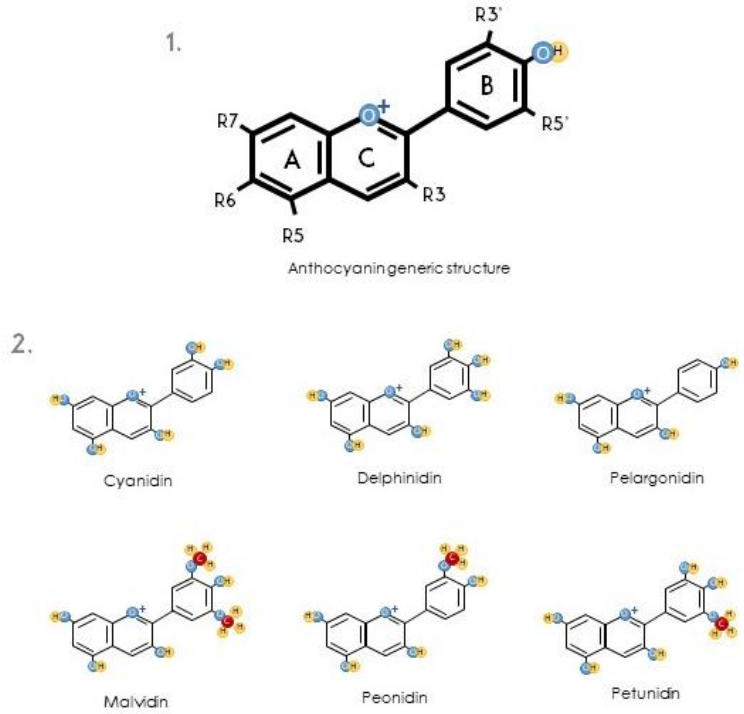

FIGURE 6. THE CHEMICAL STRUCTURE OF ACNS.

Figure adapted from [128]

1.8.2. **ACN natural occurrence**

ACNs are commonly found in the skin of various red and blue colored fruits, as well as in the flesh of some berries or apples at a concentration that ranges between 0.1% and 1.0% of their dry weight [149,150]. The known natural sources of ACNs are summarized in (TABLE 5).

TABLE 5. ACN content in fruits and vegetables.

Food	Scientific name	ACN mg/100 g of fresh fruit or vegetable weight	Average content (mg/100 g)
Apple	*Malus Domestica*	0-60.00	30
Golden delicious		24.52	24.52
Red love		6.87	6.87
Gala		0.86	0.86
Fuji		3.68	3.68
Granny Smith		1.36	1.36
Bilberry	*Vaccinium myrtillus*	300–698	499
Black bean	*Phaseolus vulgaris*	24.1–44.5	32.8
Black currant	*Ribes nigrum*	130–476	303
Black olives	*Olea europaea*	42–228	135
Black rice	*Oryza sativa*	10–493	251.5
Blackberry	*Rubus sp.*	82.5–325.9	204.2
Blueberry	*Vaccinium corymbosum*	61.8–299.6	180.7
Bog whortleberry	*Vaccinium uliginosum*	154	154
Cherry	*Prunus sp.*	2–450	226
Chokeberry	*Aronia melanocarpa*	410–1480	945
Cranberry	*Vaccinium Oxycoccus*	67–140	103.5
Crowberry	*Empetrum nigrum*	360	360
Eggplant	*Solanum melongena*	8–85	46.5
Elderberry	*Sambucus*	664–1816	1240
Goji	*Lycium barbarum*	49.4	49.4
Gooseberry	*Ribes uva-crispa*	2.0–43.3	22.65
Grapefruit	*Citrus × paradisi*	5.9	5.9
Lettuce	*Lactuca sativa*	2.5–5.2	3.85
Nectarine	*Prunus persica var. nucipersica*	2.4	2.4
Peach	*Prunus persica*	4.2	4.2
Pear	*Pyrus sp.*	5–10	7.5
Plum	*Prunus subg. Prunus*	2–25	15
Purple corn	*Zea mays L.*	1642	1642
Raspberry	*Rubus idaeus*	20–687	353.5
Red cabbage	*Brassica oleracea var. capitata f. rubra*	322	322
Red currant	*Ribes rubrum*	22	22
Red grape	*Vitis vinifera*	30–750	390
Red onion	*Allium cepa*	23.3–48.5	35.9
Red radish	*Raphanus sativus*	100–154	127

Rhubarb	*Rheum rhabarbarum*	4–200	102
Rowanberry	*Sorbus aucuparia*	14	14
Saskatoon berry	*Amelanchier alnifolia*	234	234
Strawberry	*Fragaria × ananassa*	19–55	37
		ACN mg/100 mL of liquid	
Pomegranate, unprocessed juice	*Punica granatum*	0.15–2.52	1.33
Red wine		16.4–35	25.7

Table elaborated from [16,151].

1.8.3. **ACN pharmacokinetics**

ACNs can be absorbed in different rates and extents depending on their reactive groups and chemical complexity [128]. Additionally, ACNs suffer multiple structural changes during their pass through the human body [152–154]. Due to the complexity and importance of the ACN's pharmacokinetics, the absorption and metabolism of ACNs will be described in the following sections and presented in FIGURE 7.

1.8.3.1. The absorption and metabolism of ACNs

ACNs can be absorbed intact despite their chemical structure and the pH conditions along the gastrointestinal tract [128,152–154]. Additionally, the co-ingestion of ACNs with other foods or beverages has an impact on its bioavailability [128,155], for instance, the co-ingestion of ACNs with milk significantly reduces their bioavailability [128,155], on the other hand, the ACN ingestion along with dairy cream (48% fat) does not impact the ACNs' C_{max} but it significantly increases the T_{max} [155].

I. Oral cavity

There is not much information on the biotransformation or absorption of ACNs from the oral cavity, however, it has been demonstrated that ACNs can be degraded in the oral cavity by the interaction with undetermined salivary proteins as has been demonstrated with malvidin-3-glucoside [155].

II. Stomach

After the initial degradation that occurred in the oral cavity, the ACNs travel into the gastric lumen, where 19 – 37% of total ACNs are absorbed from the gastric fluids, usually in an intact form [128,154].

Traditionally, the gastric absorption has been attributed mainly to bilitranslocase [128,154,156], an organic anion trans-membrane carrier with a high affinity for bilirubin [157]. However, it has been demonstrated that ACNs can also be absorbed from the gastric lumen and into the systemic circulation via GLUT1 [154], GLUT3 [154], organic cation transporter 1 (OCT1) [154], organic anion transporter 2 (OAT2) [154], and the sodium/monocarboxylate transporters (SMCT) 1 and 2 [154].

Finally, it is considered that ACNs do not suffer any structural changes in the gastric lumen since they remain in their glycosidic form at the gastric pH (1-4) [128,151,154,155].

III. Small intestines

After gastric absorption, ACNs continue their travel through the gastrointestinal tract into the duodenum where 10.4 ± 7.6 % of ACNs are absorbed [128], the remaining ACNs continue to the jejunum [128,154,155,158,159].

In the small intestine, ACNs can be actively absorbed from the intestinal lumen through SGLT1, GLUT2, and bilitranslocase transporters located in the intestinal brush border [158,160,161]. On the other hand, ACNs can also be absorbed through passive diffusion after the hydrolyzation of ACNs, and thus, their conversion into anthocyanidins by the β-glucosidase or lactase-phlorizin hydrolase enzymes located in the brush border of the intestinal epithelium [154,158].

It has been demonstrated that around 7% of ingested ACNs can be detected in the intestinal walls of animals after 2 h after the oral ACN ingestion [128,162], and concentrations appear to be time-accumulative [128,159]. However, only trace amounts of ACNs are absorbed in the small intestine, while the majority of ACNs are absorbed in the large intestine [155].

Some of the ACNs that are absorbed into the intestinal epithelial cells suffer the sulfation, methylation, or glucuronidation of their chemical structures yielding multiple ACN metabolites inside the intestinal epithelial cell that will be then transported along with the unaltered ACNs into the inferior vena cava circulation [158].

Alternatively, the unabsorbed ACNs continue their passing through the intestinal tract into the colon, where they can be degraded into phenolic acids or aldehydes within the colonic lumen by the 300 - 500 bacterial species that make the colonic microbiota [154,158].

IV. Large intestine and the influence of the intestinal microbiota

After the majority of ingested ACNs arrive at the colon, the 3-O-glycosidic sugar moiety of ACNs is hydrolyzed by bacteria from the Clostridium, Bifidobacterium, Bacteroides, and Eubacteria species, thus converting some of the remaining ACNs into anthocyanidins, and another phase I and II conjugates [128,154,155,158].

It has been described that the release of deglycosylation enzymes from the intestinal bacteria and ACN bacterial metabolization results in the production of several metabolites including the protocatechuic, vanillic, benzoic, caffeic, and *p*-coumaric acids amongst many other PCs [128,154,158,163].

The importance of the ACN colonic metabolites should not be underestimated, as it has been demonstrated that at least 30% of ingested ACNs could remain stable in their original form after 8 h of ingestion and their intestinal passage [164]. Moreover, only 20% of the metabolites are absorbed from the small intestine suggesting that the colon is a major absorption site for ACNs and their metabolites [164]. While the remaining ACNs are excreted through feces [128,154,155,158,159,163].

1.8.3.2. The classification of ACNs according to the nutraceutical bioavailability classification scheme

ACNs are considered highly bioaccessible molecules (>75%), soluble, and easily liberated from the food matrix, however, due to their size and physicochemical properties its absorption is limited by the intestinal bilayer permeability which is mediated through active transport, finally, the bioavailability of ACNs might be limited by the pre-absorptive metabolization of their structures [126]. Thus, ACNs can be classified from their absorptive properties as:

$$B^*(+);\ A^*(-)_{BP};\ T^*(-)_C$$ [126].

1.8.4. **ACNs in the systemic circulation**

If ACN metabolites and catabolites are taken into consideration, ACNs are more bioavailable than previously thought [155]. Parent ACNs can be rapidly detected in the systemic circulation reaching their maximum peak (C_{max}: 141 nM) and disappearance from the bloodstream at 1.8 and 6 h post-consumption respectively [158]. Additionally, the ACN metabolite maximum peak (C_{max}: 0.2−2 μM) has been detected in the first 10 h after the parent ACN oral ingestion and remains detectable in circulation for up to 48 h [158].

This evidences a biphasic ACN profile in the blood with a first peak encountered between 0 and 5 h, and a second and more intense peak around 6 and 48 h, corresponding to the gastric/intestinal and colonic absorption peaks, respectively [155,158].

Once in the circulation ACNs undergo massive biotransformation processes mainly in the liver where phase I and II metabolization reactions occur, however, the same reactions have been demonstrated in the lungs and kidneys of humans however to a lesser extent where they are also excreted [128,154,155,158,159,163].

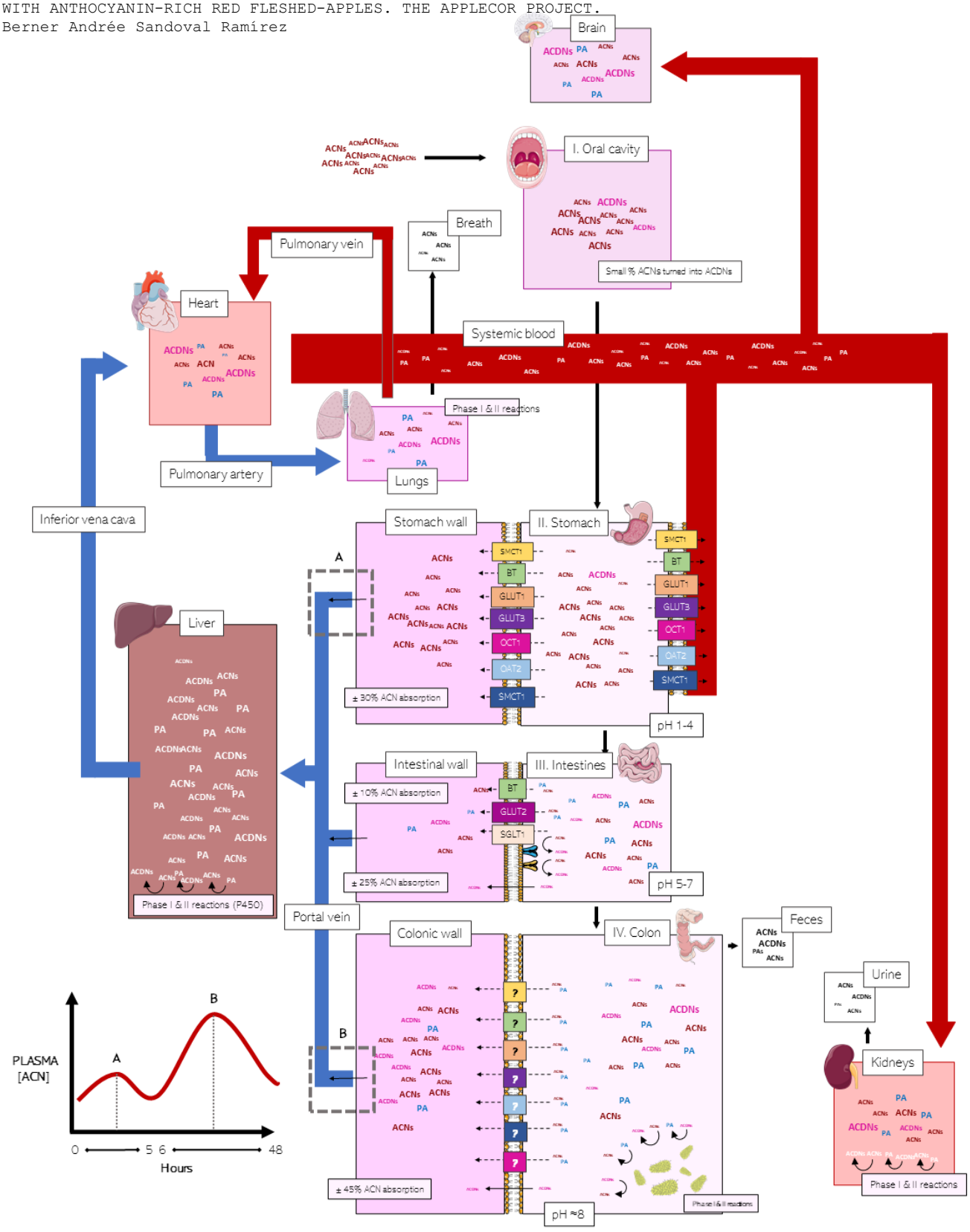

FIGURE 7. THE PHARMACOKINETICS OF ACNS.

Figure elaborated from [128,151,163,151,154–159,162]

1.8.5. **The health benefits of ACNs**

From observational studies, it has been determined that the dietary ACN intake is inversely associated with the risk of CVD in both European and US populations [143]. Additionally, consuming around 20-320 mg of ACNs significantly decreases serum LDLc between 16-25% when compared against placebo [165]. On the other hand, ACNs seem to be able to beneficially modify the low-grade systemic inflammation [166], and ACNs have a potent antioxidant activity, thus reducing the damage caused by the reactive oxygen species [9]. Finally, the dietary ACN intake seems to be beneficial for the prevention of type 2 diabetes mellitus, some types of cancer, and hypertension [17,167,168]. A more detailed description of the ACNs health properties is enclosed within the present thesis.

Therefore, the main objective of the present thesis is to determine the effect of anthocyanin consumption on different cardiovascular risk factors and other diseases.

2. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS:

The anthocyanins provided by fruits, extracts, or other products help to improve cardiovascular risk factors and other diseases.

2.1. MAIN OBJECTIVE:

To determine the effect of anthocyanin consumption on different cardiovascular risk factors and other diseases.

2.2. SPECIFIC OBJECTIVES:

2.2.1. **OBJECTIVE 1.** To determine the presence of anthocyanins in different animal tissues and their possible effects on health. (Systematic review 1: Anthocyanin tissue bioavailability in animals: possible implications for human health. A systematic review) (published IF: 6.500; Q1;D1 (8/89).

2.2.2. **OBJECTIVE 2.** To determine the existence of a biomarker of consumption of red fruits rich in anthocyanins. (Systematic review 2: "Possible biomarkers for anthocyanin-rich berry intake in human body fluids a systematic review and qualitative metaanalysis") (published).

2.2.3. **OBJECTIVE 3.** To determine the known effects of apple consumption on different cardiovascular risk factors. (Narrative review 3: "The effects and associations of whole-apple intake on diverse cardiovascular risk factors. A narrative review") (published IF: 3.571; Q1;D1 (28/135).

2.2.4. **OBJECTIVE 4.** To determine the effects of the sustained consumption of red-fleshed apples and other anthocyanin-rich fruits on diverse cardiovascular disease risk factors in humans, and the protein expression of the tissues in the hearts and aortas of rats. (Article 5: "Red-and white-fleshed apples modulate aorta and heart proteome in hypercholesterolemic rats. The AppleCOR project") (under peer review).

2.2.5. **OBJECTIVE 5.** To determine the known effects and associations of anthocyanin consumption on different diseases and risk factors. (Umbrella review 4: "The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and clinical controlled trials") (under peer review).

2.2.6. **OBJECTIVE 6.** To determine the effects of different phenolic compounds in the reduction of the damage to the structure and barrier function of the intestinal epithelium. (Systematic review 6: "Exploring the effects of phenolic compounds to reduce intestinal damage and improve the intestinal barrier integrity: A systematic review of *in vivo* and *in vitro* studies") (published IF: 6.360; Q1;D2 (9/89).

2.2.7. **OBJECTIVE 7.** To determine the effects of different phenolic compounds in the reduction of the damage suffered by the lungs during the acute lung injury induced by sepsis. (Systematic review 7: "Exploring the Effects of the Phenolic Compound Administration to Reduce the Acute Lung Injury Secondary to Lipopolysaccharide Administration in Animals: A Systematic Review of *in vivo* Animal Studies") (Submitted).

3. METHODS AND RESULTS

3.1. Chapter 1

Cyanidin-3-glucoside as a possible biomarker of anthocyanin-rich berry intake in body fluids of healthy humans: a systematic review of clinical trials.

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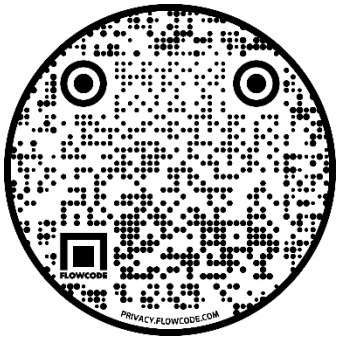

Reference: *Sandoval-Ramírez BA, Catalán Ú, Fernández-Castillejo S, Pedret A, Llauradó E, Solà R. Cyanidin-3-glucoside as a possible biomarker of anthocyanin-rich berry intake in body fluids of healthy humans: a systematic review of clinical trials. Nutr Rev. 2020 Jul 1;78(7):597-610. doi: 10.1093/nutrit/nuz083. PMID: 31858139; PMCID: PMC7279666.*

Special Article

Cyanidin-3-glucoside as a possible biomarker of anthocyanin-rich berry intake in body fluids of healthy humans: a systematic review of clinical trials

Berner Andrée Sandoval-Ramírez, Úrsula Catalán, Sara Fernández-Castillejo, Anna Pedret, Elisabet Llauradó, and Rosa Solà

Context: *Anthocyanins are phenolic compounds found in berries. They exhibit promising health benefits in humans, but no accurate biomarkers of berry intake have been identified thus far.* ***Objective:*** *The aim of this systematic review is to propose a biomarker of anthocyanin-rich berry intake in human plasma and urine.* ***Data Sources:*** *PubMed and Cochrane databases were searched from January 2008 to January 2019.* ***Study Selection:*** *Databases were searched for human intervention studies that assessed the presence of anthocyanins in human body fluids using high-throughput techniques. Non-English articles and studies publishing targeted analyses were excluded.* ***Data Extraction:*** *Ten clinical trials, in which 203 phenolic compounds were identified, were included and assessed qualitatively. The following criteria were used to identify biomarkers of berry intake: frequency, plausibility, dose-response, time response, robustness, reliability, stability, analytical performance, and reproducibility. Sensitivity and specificity of potential biomarkers were determined by the receiver operating characteristic curve.* ***Results:*** *Of the 203 phenolic compounds identified in human samples, the anthocyanin cyanidin-3-glucoside was the molecule found most frequently in urine (58.06%) and plasma (69.49%). Cyanidin-3-glucoside fulfills the essential criterion of plausibility as well as the dose-response, time response, stability, and analytical performance criteria. Its positive predictive value is 74% ($P = 0.210$) in plasma, which is acceptable, and 61.7% ($P = 0.402$) in urine.* ***Conclusions:*** *Current evidence suggests that cyanidin-3-glucoside is a potential biomarker of anthocyanin-rich berry intake in plasma and urine of healthy humans.*
PROSPERO registration number: *CRD42018096796.*

Affiliation: *B.A. Sandoval-Ramírez, Ú. Catalán, S. Fernández-Castillejo, A. Pedret, E. Llauradó,* and *R. Solà* are with the Department of Medicine and Surgery, the Functional Nutrition, Oxidation, and Cardiovascular Diseases Research Group, Universitat Rovira i Virgili, Reus, Spain. *Ú. Catalán* is with the Institut d'Investigació Sanitària Pere Virgili, Reus, Spain. *S. Fernández-Castillejo* and *A. Pedret* are with the Fundació EURECAT—Centre Technològic de Nutrició Salut, Reus, Spain. *R. Solà* is with the Hospital Universitari Sant Joan de Reus, Reus, Spain.

Correspondence: *Ú. Catalán*, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Facultat de Medicinia i Ciències de la Salut, Functional Nutrition, Oxidation, and Cardiovascular Disease Research Group (NFOC-Salut), C/Sant Llorenç, 21, 43201 Reus, Spain. Email: ursula.catalan@urv.cat.

Key words: anthocyanins, berry, biomarker, cyanidin-3-glucoside, plasma, urine.

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INTRODUCTION

In recent years, interest in the implementation of healthy diets high in fruits and vegetables,[1–6] shown to have positive effects on disease prevention[7–9] and life expectancy, has been increasing.[10] Some of the health benefits attributed to the frequent consumption of fruits and vegetables can be attributed, at least in part, to phenolic compounds, which are naturally produced biomolecules contained in a variety of plants.[11,12] Phenolic compounds, however, are classified into widely varying families of biomolecules, and not all families have the same effects on maintenance of health and prevention of disease.[13,14] One of the most important classes of phenolic compounds are the flavonoids, particularly the anthocyanins, which have shown promising potential in the prevention of diseases and conditions such as obesity,[15–17] type 2 diabetes,[17–19] hypertension,[20] prostate cancer,[21–23] lung cancer,[24–26] heart failure,[27] renal failure,[28–30] Alzheimer disease, and Parkinson disease.[31–35] Anthocyanins are water-soluble pigments responsible for the red or blue coloration of certain flowers, seeds, fruits, and plants.[36] They are most commonly found in great concentrations in both the flesh and the skin of red-fleshed apples[37] and in most berries.[38] They can be further divided into parent anthocyanins or anthocyanin metabolites, depending on their chemical structure or their metabolization.[39] Parent anthocyanins are cyanidin-3-glucoside (C3G), peonidin-3-glucoside, and malvidin-3-glucoside, while anthocyanin metabolites are cyanidin-3-glucuronide, peonidin-3-*O*-arabinoside, and malvidin-3-galactoside.[39]

Despite the important role of fruits and vegetables in human health, only a few biochemical markers that can assess individual intake of specific food items have been described.[40,41] The identification of biomarkers of anthocyanin-rich foods such as berries[42] could help further elucidate the specific health benefits of anthocyanins.

It is possible, however, that a biomarker of anthocyanin intake could have low specificity. One possible explanation for the lack of specific biomarkers described thus far might be the heterogeneous composition of foods, which hinders the identification of viable biomarker candidates. Hence, there is a need to identify specific biomarkers to assess the consumption of berries. Recently, guidelines for addressing the complex assessment and validation of biomarkers of food intake have been proposed.[42,43] Dragsted et al[43] were the first to propose a comprehensive guideline for the validation of biomarkers of food intake after conducting a systematic review of the literature. They proposed a novel process, based on the analysis of different criteria, for the identification of possible biomarkers of food intake. Therefore, the aim of the present systematic review of clinical trials, conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, is to propose a possible biomarker of phenolic compounds present in human body fluids after the ingestion of anthocyanin-rich berries.

METHODS

Search strategy and selection criteria

The present systematic review of human clinical trials of berry consumption was conducted in accordance with the PRISMA methodology[44] and was previously registered in University of York's PROSPERO (International Prospective Register of Systematic Reviews) with the registration number CRD42018096796.

A Web-based search of the PubMed and Cochrane Library databases was performed using with the following terms, which included all berries with high anthocyanin content: (blackcurrant OR "blackcurrant extract" OR blackberry OR "blackberry extract" OR raspberry OR "raspberry extract" OR blueberry OR "blueberry extract" OR cherry OR "cherry extract" OR redcurrant OR "redcurrant extract" OR grape) AND (biomarker* OR marker* OR metabolite* OR pharmacokinetics OR biokinetics) AND (intake OR ingestion OR consumption OR eating OR diet) AND (human OR men OR women OR patient) AND (urine OR blood OR plasma OR serum OR faeces OR feces) NOT (animal OR mouse OR mice OR rat OR pig OR juice OR drink OR cell OR in vitro OR questionnaire OR self-reported OR systematic review).

Published articles were screened on the basis of their titles, abstracts, and full texts, with the following inclusion criteria applied: (1) intervention studies performed in humans; (2) healthy male or female participants; (3) participant age between 18 and 90 years; (4) parallel studies; (5) untargeted analytical approach; (6) diet free of phenolic compounds for $\geq$ 24 hours before the start of the study; (7) analysis of body fluids (blood, plasma or urine, feces, or saliva) performed; (8) body fluids analyzed at baseline; (9) dose characterization performed; (10) use of a controlled, polyphenol-free diet during the intervention; and (11) studies published from January 1, 2008, to January 10, 2019. The following exclusion criteria were applied: (1) articles not published in English; (2) crossover washout period of less than 48 hours; (3) administration of phenolic compound–rich fruits in conjunction with other nutritional elements; (4) presence of gastrointestinal

pathologies such as inflammatory bowel disease or chronic malabsorption syndrome; (5) participants under any kind of medication or treatment; (6) targeted (ie, nongeneral) analytical approach; (7) nonhealthy individuals; and (8) any inclusion criterion not fulfilled. Further information about the PICOS (Population, Intervention, Comparison, Outcomes, and Study design) criteria is detailed in Table 1.

Process for selecting a biomarker candidate

In the present systematic review, the following process is proposed for selecting a biomarker candidate. First, a systematic review was performed to search for all studies of possible phenolic compounds present or absent in human body fluids. Second, the risk of bias of all included clinical trials was assessed to determine study quality. Third, of the phenolic compounds found in human body fluids, the one described most frequently was identified. Fourth, the 8 criteria proposed by Dragsted et al[43] for validating biomarkers of food consumption were applied to the biomarker candidates found most frequently. This was done to identify the most viable candidates. Special emphasis was placed on the plausibility criterion, which is an essential criterion for any biomarker of food intake.[43] Fifth, to determine which phenolic compounds identified would be most viable as biomarker candidates, further statistical analysis was conducted to determine the specificity and sensitivity of each compound by calculating the area under the curve (AUC) receiver operating characteristic (ROC) curve. Finally, results were discussed in relation to the bibliographic references available, and conclusions were drawn.

Data extraction and analysis

Using the PRISMA criteria,[44] 2 authors (Ú.C. and B.A.S-R.) independently extracted published data from the main text and tables. Any differences were resolved by a third author (R.S.). The following information was extracted from all included articles: (1) study characteristics, including type (postprandial, parallel, or crossover) and length of study, type of samples analyzed, sources of anthocyanins, routes of administration used, and number and size of doses used; (2) number and characteristics of participants, including age, sex, and health status; and (3) total or specific amounts of phenolic compounds reported. The PRISMA checklist is provided as Table S1 in the Supporting Information online.

Risk-of-bias assessment

Two authors (B.A.S-R. and Ú.C.) independently assessed the potential risk of bias of the studies that met the inclusion criteria, using the methodological index for nonrandomized studies (MINORS).[45] Items were scored with 0 if no data were reported; with 1 when data were reported but were inadequate; and with 2 when data were both reported and adequate. If, after applying the MINORS criteria, a study received a score of 13 or above, with 24 being the maximum score, risk of bias was considered low.[45] Additionally, in accordance with the PRISMA guidelines,[44] 2 authors (B.A.S-R. and Ú.C.) reached a consensus, and a third author (R.S.) independently resolved any discrepancies between the other 2 authors. The potential risk of bias of the present systematic review was assessed by AMSTAR 2, a tool for the critical appraisal of systematic reviews that include randomized or nonrandomized studies of healthcare interventions.[46] AMSTAR 2 is based on the evaluation of 16 items by means of simple response categories. The results of the systematic review can then be rated as being of high, moderate, low, or critically low quality.

Criteria for assessment of biomarkers of berry intake

The assessment and validation of biomarkers of food intake was performed according to Praticò et al[42] and Dragsted et al[43] and included the 8 criteria proposed by Dragsted et al[43]: (1) plausibility or specificity, to determine if the biomarker was specific or could be attributed to a food or food group. If the phenolic compound composition of the berries was not reported in the included studies, it was retrieved from the Phenol-Explorer database[47]; (2) dose-response, to determine if the concentration of the biomarker in the sample(s) analyzed increased or decreased in agreement with the dose of molecule used; (3) time response, to determine if the description of the kinetics of the molecule is adequate to enable prudent choices about sample type and time window; (4) robustness, to determine if the biomarker remains robust even after the ingestion of different types of complex meals; (5) reliability, to determine if the biomarker compares well with other biomarkers or other data following intake of the same food or food group; (6) stability, to determine if the marker is stable during collection, processing, and storage; (7) analytical performance, to determine if the specificity and sensitivity of the molecule are adequate, and if the molecule can be measured with accuracy; and (8) reproducibility, to determine if analysis of the molecule has been successfully reproduced in another laboratory.[43] To evaluate each of the 8 criteria, the researcher could answer as follows: (a) yes, which indicates the phenolic compound was investigated and meets the criterion completely; (b) no, which indicates the phenolic compound was investigated but did not

Table 1. PICOS criteria for inclusion and exclusion of studies

Criteria	Inclusion criteria	Exclusion criteria
Population	Healthy men and women	Participants receiving any kind of medication or treatment; nonhealthy individuals; individuals with gastrointestinal pathologies such as inflammatory bowel disease or chronic malabsorption syndrome
Intervention	Oral or intravenous administration of an anthocyanin-rich berry; analysis of body fluid (blood, plasma, urine, feces, or saliva) performed	Administration of fruits rich in phenolic compounds in conjunction with other nutritional elements
Comparison	None	None
Outcome	Bioavailability of phenolic compounds in plasma and urine	None
Study design	Human intervention studies, randomized controlled trials, and randomized controlled crossover trials	Observational studies, studies without a washout period, studies that used a targeted analytical approach

meet the minimum requirements for the criterion to be met; or (c) undetermined, which indicates the phenolic compound has not yet been researched or the results obtained were uncertain. However, a molecule could be considered a specific biomarker of food intake even if not all these criteria were answered with "yes" responses. If a phenolic compound candidate fulfills all criteria except the plausibility criterion, it must be discarded as a potential biomarker. Moreover, each phenolic compound should be analyzed in accordance with the objectives of its specific use. For instance, an unstable biomarker might be useful for short-term, but not long-term, assessment of exposure.

Statistical analyses

The frequency with which each phenolic compound appeared in human body fluids was calculated and expressed as a percentage. The dose of anthocyanin-rich berries consumed was expressed as the mean and standard deviation (SD). Since quantitative information about the concentration of each phenolic compound in each sample could not be obtained from the studies included in the review, the concentrations that were reported have been translated into a qualitative dichotomic variable expressed as the presence or absence of any given phenolic compound molecule in plasma and urine. A cutoff point of phenolic compound metabolites that appeared in more than 40% of the plasma or urine samples of all individuals analyzed was established. The χ^2 test was used to compare the presence of the most frequent phenolic compound metabolites in the 2 matrices. Sensitivity (true positive) and specificity (false positive) values were calculated using the AUC ROC, which was obtained from the calculation of the logistic regression model that was constructed for phenolic compounds deemed most promising on the basis of the trapezoidal rule. Thus, AUC values close to 100% indicate better discriminatory power. Results from the qualitative variables were expressed as percentages. It is possible that biomarker candidates could be composed of 2 or more less-specific phenolic compounds, and therefore the bivariate Pearson correlation was used to calculate correlations between phenolic compounds found in urine or plasma. All statistical analyses were performed using IBM SPSS software, version 25.0. All P values less than 0.05 were considered statistically significant.

RESULTS

Literature search and study selection

The initial screening identified 103 trials published between January 1, 2008, and January 10, 2019. After the further analysis, 79 studies were excluded for not meeting the inclusion criteria, and 6 were excluded as duplicate publications. As a result, 18 publications met the inclusion criteria and were examined further. Eight of these 18 publications were excluded for other reasons, shown in Figure 1; thus, 10 articles were included in the review.[48–57] One of the 10 articles performed both short- and long-term exposure experiments.[49] Therefore, these experiments were included separately in the review, resulting in a total of 11 studies. No study had a control group. The complete selection process is shown in Figure 1.

Risk-of-bias assessment

The MINORS methodology was used to determine the risk of bias in all 10 articles included. Six studies had no conflict of interest,[48,49,51,53,54,57] 3 did not report any information about possible conflicts of interest,[50,55,56] which led to a lower score, and 1 study reported conflicts of interest for receiving funding from the alcoholic beverage industry, though the funding did not influence the study results.[52] All 10 articles received a score above 13 points (mean ± SD, 18.10 ± 1.45 points), which was considered a low risk of bias. The MINORS scores for

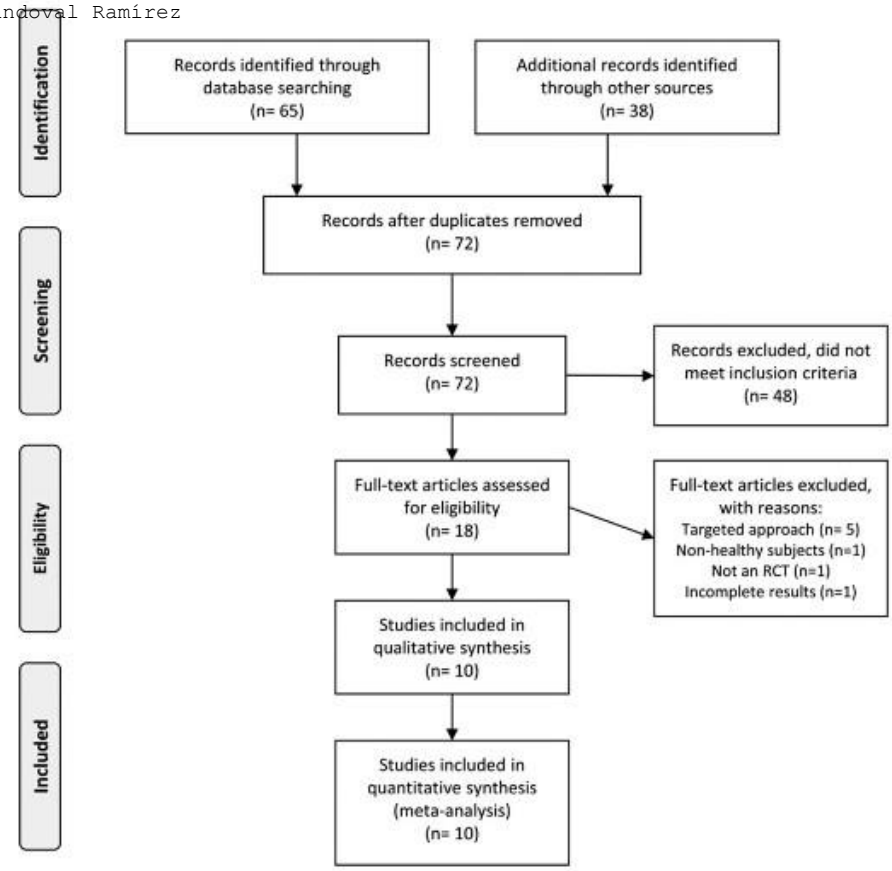

Figure 1 **Flow diagram of the literature search process.** *Abbreviation*: RCT, randomized controlled trial.

all studies that met the inclusion criteria, along with the results of further analysis, are provided in Table S2 in the Supporting Information online.

The AMSTAR 2 tool was used to assess the non-randomized clinical trials included in the present review. The results show this review to be of high quality for its low risk of bias (see Appendix S1 in the Supporting Information online).

Overview of findings

Consumption of the following berries was reported in the 10 articles included: red raspberry,[48] raspberry,[51] wild blueberry,[49,53] black raspberry,[50] grape pomace,[52] mixed berry puree,[54] blackberry,[55,57] elderberry, and lowbush blackberry.[56]

Since none of the included studies reported the composition of the different berries administered, the Phenol-Explorer database[47] was used to determine the polyphenol content of berries. The daily oral dose of anthocyanins ranged between 12 g of elderberry extract[56] and 500 mL of mixed berry puree, which was described as being equivalent to 500 g.[54] The mean dose of anthocyanins administered was 181 ± 130 g/d. A total of 105 participants were included in the analysis (39 men, 36 women, and 30 individuals whose sex was not reported).

The following methods were used for the detection of phenolic compounds: high-performance liquid chromatography (HPLC) in 5 studies[50–52,56,57] HPLC coupled with quadrupole high-resolution time of flight mass spectrometry (HPLC-QTOF-MS) in 2 studies,[49,53] HPLC coupled with a diode-array detector (HPLC-DAD) in 1 study,[54] HPLC–electrospray ionization tandem mass spectroscopy (HPLC-ESI-MS/MS) in 1 study,[55] and ultra HPLC-QTOF-MS (UHPLC-QTOF-MS) in 1 study.[48]

Of the 10 studies included in this review, 1 evaluated phenolic compounds in plasma only,[53] 5 evaluated phenolic compounds in urine only,[50,52,54–56] and

Table 2 Characteristics of included studies in which parent anthocyanin or anthocyanin metabolites were assessed in human body fluids after ingestion of anthocyanin-rich berries

Type of study	Reference	Source of anthocyanins	No. of doses	Dose given	Duration of intervention	Study participants				Sample	
						Sex		Age (y)	No.		
						M (no.)	F (no.)			Plasma	Urine
Long-term											
	Zhang et al (2018)[48]	Red raspberry	32	125 g	32 d	NR	NR	NR	2	X	X
	Feliciano et al (2016)[49]	Wild blueberry	60	100 g	30 d	18	–	18–70	18	X	X
	Tian et al (2006)[50]	Black raspberry	7	40 g	7 d	NR	NR	NR	10	NP	X
Short-term											
	Ludwig et al (2015)[51]	Raspberry	1	300 g	48 h	4	5	22–44	9	X	X
	Feliciano et al (2016)[49]	Wild blueberry	1	100 g	24 h	19	–	18–71	18	X	X
	Sasot et al (2017)[52]	Grape pomace extract	1	200 mL	24 h	6	6	24–40	12	NP	X
	Zhong et al (2017)[53]	Wild blueberry beverage	1	150 g	24 h	6	6	20–45	12	X	NP
	Pimpão et al (2014)[54]	Mixed berry puree	1	500 mL	24 h	3	6	23–54	9	NP	X
	Felgines et al (2005)[55]	Blackberry	1	200 g	24 h	02	03	NR	5	NP	X
	Wu et al (2002)[56]	Elderberry extract	1	12 g	24 h	–	04	60–70	4	NR	X
		Lowbush blueberry	1	189 g	24 h	–	06	60–70	6	NR	X
	Marques et al (2016)[57]	Blackberry	1	250 g	2 h	NR	NR	NR	18	X	X

Abbreviations and symbols: NR, not reported; NP, not performed; X, analysis performed.

4 evaluated phenolic compounds in both urine and plasma[48,49,51,57]; of the last 4 studies, 1 included short-term and long-term experiments in a single study.[49] None of the included studies assessed the presence of parent anthocyanins or anthocyanin metabolites in matrices other than plasma or urine, such as in saliva, blood, or feces.

With regard to the period of berry consumption, 7 studies were conducted as short-term trials, which included periods ranging between 2 hours and 48 hours,[49,51–57] 2 studies were performed as long-term trials, which ranged between 7 days and 32 days,[48,50] and 1 study performed both long-term and short-term experiments.[49] The mean (± SD) length of the long-term interventions was 23 ± 11.34 days. Complete information about the included studies is reported in Table 2.[48–57]

Phenolic compounds detected in plasma and urine

Phenolic compounds detected most frequently. A total of 203 different phenolic compounds were detected in urine and plasma after consumption of anthocyanin-rich berries in the human studies included in the present review (see Table S3 in the Supporting Information online). Ninety-seven phenolic compounds were found in both plasma and urine, 21 were found only in plasma, and 85 were found only in urine. Of these, those that appeared in more than 40% of samples (the cutoff value established to designate the most frequently detected phenolic compounds, since a higher cutoff significantly limited the number of candidate phenolic compounds) of human plasma, urine, or both were selected, resulting in 19 different phenolic compounds (Table 3).

The following 10 phenolic compounds, in decreasing order, were detected most frequently in urine: C3G (58.06%); 3,4-dihydroxyphenylacetic acid (44.09%); 4-hydroxybenzoic acid (44.09%); caffeic acid (44.09%); gallic acid (44.09%); hippuric acid (44.09%); 2-methylpyrogallol-*O*-sulfate (41.94%); 4-hydroxyhippuric acid (41.94%); dihydrocaffeic acid (41.94%); and protocatechuic acid (41.94%).

In plasma, the following 15 phenolic compounds, in decreasing order, were detected in more than 40% of samples: C3G (69.49%); cyanidin-3-glucuronide (69.49%); 4-hydroxyphenylacetic acid (54.24%); ferulic acid (54.24%); hippuric acid (54.24%); *p*-coumaric acid (54.24%); 4-hydroxybenzoic acid (50.85%); caffeic acid (50.85%); syringic acid (50.85%); vanillic acid (50.85%); 3,4-dihydroxyphenylacetic acid (49.15%); 4-hydroxyhippuric acid (45.76%); ferulic acid 4-*O*-glucuronide (45.76%), ferulic acid 4-sulfate (45.76%); and isoferulic acid 3-*O*-β-D-glucuronide (45.76%).

The phenolic compounds C3G, 3,4-dihydroxyphenylacetic acid, 4-hydroxybenzoic acid, caffeic acid, hippuric acid, and 4-hydroxyhippuric acid were present in high frequencies in either plasma or urine samples. In particular, C3G was the phenolic compound most frequently detected in both urine (58.3%, n = 54 of 93 samples) and plasma (69.49%, n = 41 of 59 samples). Cyanidin-3-glucuronide was frequent in plasma (69.49%, n = 41 of 59 samples), but not in urine (26.88%, n = 25 of 93 samples). Complete information on the 19 most frequently detected phenolic compounds is shown in Table 3.

Table 3. Phenolic compounds found in more than 40% of urine or plasma samples, as indicated by gray shading.

Metabolite	Urine ($N = 93$) No. (%)	Plasma ($N = 59$) No. (%)
Cyanidin-3-glucoside	54 (58.06)	41 (69.49)
3,4-Dihydroxyphenylacetic acid	41 (44.09)	29 (49.15)
4-Hydroxybenzoic acid	41 (44.09)	30 (50.85)
Caffeic acid	41 (44.09)	30 (50.85)
Hippuric acid	41 (44.09)	32 (54.24)
4-Hydroxyhippuric acid	39 (41.94)	27 (45.76)
Gallic acid	41 (44.09)	2 (3.39)
2-Methylpyrogallol-*O*-sulfate	39 (41.94)	18 (30.51)
Dihydrocaffeic acid	39 (41.94)	18 (30.51)
Protocatechuic acid	39 (41.94)	12 (20.34)
Cyanidin-3-glucuronide	25 (26.88)	41 (69.49)
4-Hydroxyphenylacetic acid	32 (34.41)	32 (54.24)
Ferulic acid	29 (31.18)	32 (54.24)
p-Coumaric acid	14 (15.05)	32 (54.24)
Syringic acid	30 (32.26)	30 (50.85)
Vanillic acid	29 (31.18)	30 (50.85)
Ferulic acid 4-*O*-glucuronide	9 (9.68)	27 (45.76)
Ferulic acid 4-sulfate	30 (32.26)	27 (45.76)
Isoferulic acid 3-*O*- β-D-glucuronide	18 (19.35)	27 (45.76)

Correlation between phenolic compounds found most frequently. The 19 phenolic compounds found most frequently in this systematic review (Table 3) were used to create a correlation matrix to determine whether any correlations exist between the phenolic compounds. In plasma, 15 phenolic compounds were found to be significantly correlated ($P < 0.05$), whereas in urine, 10 phenolic compounds were correlated ($P < 0.05$). Cyanidin-3-glucoside was correlated with cyanidin-3-glucuronide in plasma ($r = 1.00$; $P < 0.001$), but not in urine. The correlation matrix of the most frequent metabolites found in plasma or in urine is presented in Figure 2.

Assessment of a candidate biomarker of anthocyanin-rich berry intake. The 19 phenolic compounds detected most frequently in this systematic review (Table 3) were assessed as potential biomarkers of anthocyanin-rich berry intake, using the 8 criteria proposed by Dragsted et al[43] (Figure 3). Cyanidin-3-glucoside fulfills the plausibility criterion, which is an essential criterion for any biomarker of food intake, as well as the dose-response, time response, stability, and analytical performance criteria. Cyanidin-3-glucuronide, a metabolite of C3G, also fulfills the plausibility criterion, but its presence in plasma and urine has scarcely been studied. The other 17 phenolic compounds do not fulfill the plausibility criteria and thus were excluded as candidate biomarkers of berry intake.

Sensitivity and specificity analyses of C3G as a biomarker of anthocyanin-rich berry intake. To test the sensitivity and specificity of C3G as a biomarker of berry intake, the AUC ROC of C3G in plasma and urine was calculated. The AUC was 74% ($P = 0.210$) in plasma and 61.7% ($P = 0.402$) in urine (Figure 4).

DISCUSSION

Of the 203 phenolic compounds identified in the present review, 2 anthocyanins are detected most frequently after consumption of anthocyanin-rich berries. Cyanidin-3-glucoside is the phenolic compound and anthocyanin detected most frequently in both urine and plasma, while cyanidin-3-glucuronide is the second most frequent anthocyanin in plasma, but not in urine.

As noted in the section "Assessment of a Candidate Biomarker of Anthocyanin-Rich Berry Intake" C3G is one of two phenolic compounds that fulfill the criterion of plausibility. In order for a molecule to be considered a biomarker of intake, it must meet the plausibility criterion.[43] Since the other phenolic compounds did not fulfill the plausibility criterion, they were not considered viable candidates as biomarkers of anthocyanin-rich berry intake. Cyanidin-3-glucoside also met several other criteria. Consequently, each of the 8 Dragsted et al[43] criteria is analyzed and discussed below, but only for C3G.

Criteria for validation of C3G as a biomarker of anthocyanin-rich berry intake

Plausibility. The plausibility of a specific molecule within a certain food or food group to become a biomarker requires consideration of different confounding factors, such as the molecule's presence in other foods as well as whether the molecule is used as an additive or is generated endogenously from the metabolization of other compounds.[43] As a result, plausibility demonstrates a causal relationship between the ingestion of an anthocyanin-rich berry and the presence of the anthocyanin in biological samples. According to Phenol-Explorer, C3G is found in the anthocyanin-rich berries consumed in the studies included in this review.[47] The results show that C3G was present in 69.49% of the plasma samples and 58.06% of the urine samples obtained from participants after intake of anthocyanin-rich berries in clinical trials.

Cyanidin-3-glucoside is a relatively infrequent molecule in nature, present mostly in red- or blue-pigmented fruits such as berries but also in certain vegetables such as red cabbage and olives.[47] Additionally, C3G has been found in several organs, such as the brain, liver, vascular endothelium, kidney, and lungs, in different animal models following ingestion of diverse anthocyanin-rich foods.[58–60]

Cyanidin-3-glucoside is not a derivative of other chemical substances commonly present in mammalian

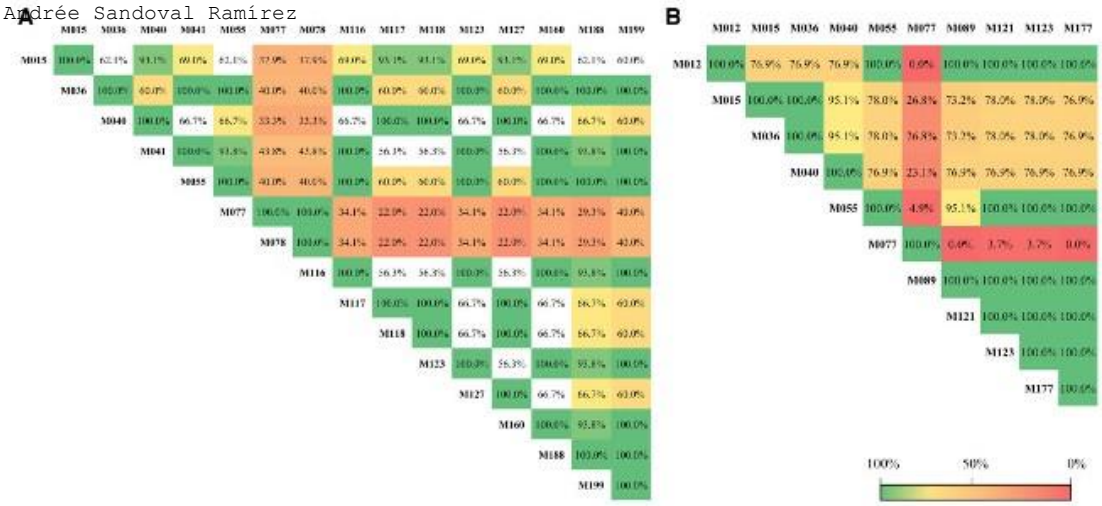

Figure 2 **Correlation matrix of the most frequent metabolites found in plasma or urine.** (A) Correlation between the significant metabolites found in plasma. (B) Correlation between the significant metabolites found in urine. All percentages of correlation values are statistically significant ($P < 0.05$) unless otherwise indicated; asterisks (*) indicate nonsignificant values. *Metabolites*: M012, 2-methylpyrogallol-*O*-sulfate; M015, 3,4-dihydroxyphenylacetic acid; M036, 4-hydroxybenzoic acid; M040, 4-hydroxyhippuric acid; M041, 4-hydroxyphenylacetic acid; M055, caffeic acid; M077, cyanidin-3-glucoside; M078, cyanidin-3-glucuronide; M089, dihydrocaffeic acid; M116, ferulic acid; M117, ferulic acid 4-*O*-glucuronide; M118, ferulic acid 4-sulfate; M121, gallic acid; M123, hippuric acid; M127, isoferulic acid 3-*O*-*β*-D-glucuronide; M160, *p*-coumaric acid; M177, protocatechuic acid; M188, syringic acid; M199, vanillic acid.

organs, such as 3,4-dihydroxyphenylacetic acid, a derivative of dopamine that can be found in animal brains,[61–63] or ferulic acid, which can be obtained from the metabolization of caffeic acid and is also present in diverse foods such as some fermented alcoholic beverages,[64] fruit,[65] cereals,[66] vegetables,[67,68] vegetable oils,[69] and seeds.[67,68] Moreover, C3G is not used as an additive for the preservation of processed foods.[70] Cyanidin-3-glucuronide, a metabolite of C3G, also fulfills the plausibility criterion, but information about its metabolization and presence in food is scarce.

Dose-response. The dose-response criterion refers to the relationship between the dose of anthocyanins ingested and the presence of anthocyanins in organs or tissues. The dose response depends on the bioavailability of C3G, taking into account the limits/levels of saturation and the saturation kinetics of C3G.[43] After a single oral ingestion of 150 g of wild blueberry puree, the mean C_{max} of C3G was 2.4 ± 0.2 nmol/L.[53] After oral ingestion of 300 g of raspberries, the mean C_{max} was 0.2 ± 0.1 nmol/L.[51] After a single oral ingestion of 250 g of blackberries, the mean C_{max} was 47 ± 8 ng/mL.[57] An oral dose of 500 mg of ^{13}C-labeled C3G resulted in a maximal C3G concentration of 141nM ± 70nM in human serum.[71] Several aspects determine the bioavailability of C3G. First, C3G is chemically composed of a benzopyran core, which, in turn, is integrated by a phenolic ring, a pyran ring, and a benzoyl ring.[72] Second, it has a molecular weight of 449.4 g/mol, a polar surface area of log $P = 0.39$, a partition coefficient of Å = 191, and known hydrophobic/hydrophilic characteristics.[72] Third, it can undergo glycosylation and methylation, both of which affect its pharmacokinetics.[73] For instance, a second glycosylation in the fifth carbon is known to increase the hydrophilic properties of C3G, thereby increasing its bioavailability, while an extra malonyl group makes it more hydrophobic, thereby decreasing its bioavailability.[72,74,75] Fourth, C3G's absorption saturation is observed around an ingested dose of 200nM, primarily because C3G binds and interacts with other molecules (eg, cellulose) while being absorbed.[72] On the basis of these findings about the bioavailability of C3G, it seems likely that C3G meets the dose-response criterion to become a suitable biomarker of berry intake.

Time response. The time response criterion refers to the time required for the maximum C3G concentration to be reached in plasma and urine as well as to the range of time that C3G concentrations are measurable,[43] which in turn is influenced by the absorption and elimination of C3G. When evaluating whether the time response criterion is met, the specific purpose of the biomarker should be considered. In the case of C3G, time-response kinetic parameters have been determined

Figure 3 **Criteria for validating biomarkers of food intake, applied to molecules that appeared in more than 40% of samples.** Dark green: yes; the criterion is fulfilled for at least some use of the biomarker; light green: partial yes; the criterion is fulfilled, but more information is needed for complete validation; yellow: undetermined; information is insufficient to validate the criterion; red: no; the criterion has been investigated but is not fulfilled.

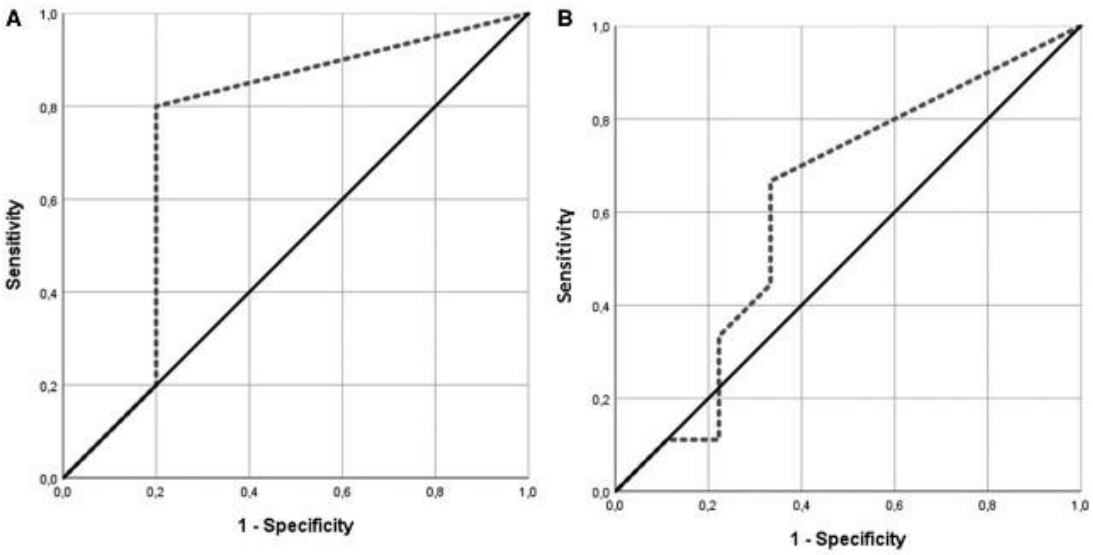

Figure 4 **Results of the receiver operating characteristic (ROC) analysis, performed to determine the sensitivity and specificity of C3G in (A) plasma (AUC 74%; $P = 0.210$) or (B) urine (AUC 61.7%; $P = 0.402$).**

through the present systematic review. In human plasma, the mean (± SD) T_{max} of C3G was 1.8 ± 0.3 hours after a single oral dose of 150 g of wild blueberry puree, and the AUC was 62.2 ± 10.3 nmol/h/L.[53] The rapid absorption of C3G has also been confirmed by the following values in human plasma: T_{max} of 66 ±15 minutes after ingestion of 250 g of blackberries,[57] and T_{max} of 1 hour after ingestion of 300 g of raspberries.[51] In human serum, the T_{max} of C3G was 1.8 ± 0.2 hours after a single oral dose of 500 mg of ^{13}C-labeled C3G.[71] In another study, C3G was found to have a minimum mean bioavailability of 12.38% ± 1.38% in humans.[73]

Several animal studies have reported different peak C3G levels, depending on the animal, the specimen, or the organs investigated.[39] In mice plasma, the oral administration of C3G extract at 500 mg/kg resulted in an elimination rate constant of 0.43 h^{-1}, and the intravenous administration of 1 mg/kg resulted in an elimination rate constant of 1.84 h^{-1},[60] with the half-life of C3G being 1.6 hours after oral ingestion and 0.4 hour after intravenous administration.[60] In addition, Marczylo et al[60] reported AUC values of 25.1 nmol/h/mL after C3G oral intake and 3.01 nmol/h/mL after intravenous administration in mice. Moreover, the maximum peak concentration of C3G in the heart tissue of mice, 1.40 × 10^4 pmol/g, was reached 10 minutes after oral administration of C3G extract at a dosage of 500 mg/kg.[39,60]

In brain tissue of mice, the maximum peak concentration of 44.98 pmol/g was reached 15 seconds after intravenous administration of 668 nmol of C3G extract.[39,59] In brain tissue of pigs, the concentration of C3G was 3.17 pmol/g after long-term (3 weeks) oral administration of bilberry extract at 82.5 mg/kg/d.[39,76]

Stability. The stability criterion refers to the best practices needed for collection and storage of biological specimens to avoid degradation of C3G, thereby permitting C3G concentrations to be measured in such specimens.[43] Like other anthocyanins, C3G is unstable at high temperatures.[77] For example, microwave heating with 300 W at high temperatures reduces the content of C3G in pomegranate juice.[72] Similarly, longer exposures to lower baking temperatures (30 minutes at 140°C and 15 minutes at 240°C) also reduce the amount of C3G found in muffins enriched with raspberry and cranberry pomace powder.[78] Storage temperature can significantly affect the stability of C3G in foods kept at 5°C, 20°C, and 35°C, even if phenolic acids, procyanidins, and flavone glycosides are added to protect the structure of C3G from degradation.[79] The stability of C3G is also affected by pH.[78] At low pH values (pH ≤ 4.0), C3G exists as a flavylium cation and, as pH rises, transforms into either its carbinol (pH = 5.2) or its quinoidal (pH = 5.5–6.0) form, whereas at higher pH values (pH ≥ 6.0), C3G reaches equilibrium and acquires a *cis*-chalcone conformation.[80]

Analytical performance. The analytical performance criterion for C3G refers to the reliability of its chemical analysis, ie, whether analytical variability, accuracy, sensitivity, and specificity are adequate.[43] The minimum requirement for this criterion to be fulfilled by C3G is the ability to measure C3G at different concentrations, a so-called semiquantitative analysis.[43] In the case of C3G, high-throughput analysis techniques such as HPLC allow identification and quantification of C3G in a wide range of different concentrations in human body fluids[52,81,82] and in diverse animal organs and tissues.[39,59,76,83]

For instance, Czank et al[73] demonstrated that ^{13}C-marked C3G molecules could be identified and measured in human blood, urine, breath, feces, and plasma, with maximum concentrations in plasma ranging between 1.4 and 592 nmol/L.[84] Moreover, the analytical performance of C3G has also been evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS), which has a precision that ranges from 1.2% to 14.5% and an intraday and interday accuracy of 5.1% to 13.6% and 11.5% to 10.9%, respectively. As a consequence, LC-MS/MS was found to be a reproducible, reliable, and accurate method for measurement of C3G.[85]

In order for C3G to meet the criterion of analytical performance and be fully validated as a suitable biomarker, a stable isotope-labeled standard would need to be present in every sample.[43] The gold standard of this isotope-labeled molecule for C3G has not been described to date.

Reproducibility, robustness, and reliability. Reproducibility is achieved only when the analysis of C3G detection has been performed identically in at least 2 different laboratories and evaluated by interlaboratory comparison tests. Such standardized analysis is not commonly performed and represents an area of uncertainty.

Another criterion not fulfilled by C3G is robustness, which evaluates the behavior of C3G in complex diets. Like most putative biomarkers, C3G has been identified in a limited number of well-designed, highly controlled intervention studies.[60] To date, however, it has not been evaluated when ingested along with other nutrients as a part of a complex meal.

Lastly, the purpose of the reliability criterion is to compare C3G as a new biomarker against the current gold-standard methodology, HPLC, in a controlled setting with supervised food intake and then validate the results via direct comparison using methods such as

Passing-Bablok regression.[88] Since this has not yet been done, the reliability criterion has not yet been met.

C3G as a useful biomarker of anthocyanin-rich berry consumption. Of all the possible phenolic compounds that were found to be viable biomarkers of berry intake, C3G is the molecule found most frequently in plasma and urine. It therefore fulfills the key criterion of plausibility because its presence has been demonstrated in anthocyanin-rich berries,[47] supporting C3G as a biomarker of anthocyanin-rich berry intake. Moreover, it also fulfills 4 additional criteria for use as a biomarker, ie, dose-response, time response, stability, and analytical performance.

Since C3G is a relatively rare anthocyanin in plants, and because it is not produced by the metabolization of other compounds, the presence of C3G in human body fluids after the consumption of food is caused directly by C3G ingestion. The positive predictive value of C3G as a biomarker of anthocyanin-rich berry intake is 74% in plasma and 61.7% in urine, although these values are not significant. Predictive values between 70% and 80% are considered acceptable,[87] and thus a 74% predictive value of C3G in plasma is acceptable, suggesting that, in plasma, C3G could be a suitable biomarker of anthocyanin-rich berry intake, although further confirmatory studies are warranted.

C3G and human health. The ability to detect C3G might play an important role in human health, particularly since epidemiological studies have shown that the consumption of more than 2 cups of blueberries per week is associated with a significantly slower rate of lung function loss.[88]

Cyanidin-3-glucoside has also shown anticancer properties in cell and animal experiments in which it was isolated and administered intravenously at relatively high doses of 250nM and 500nM.[60,89,90] Among the acknowledged activities of C3G, its roles in DNA protection,[91] in reduction of body weight and amelioration of insulin resistance in mice with diet-induced obesity, in reduction of hepatic steatosis,[92] and in activation of fatty acid metabolism[93] are worth highlighting, along with its anti-inflammatory[89] antimicrobial, and protective epigenetic effects in cancer and neurological diseases.[72,93–95]

Limitations

One of the main limitations of this systematic review is the design of the studies originally screened, which were nonrandomized clinical trials. Such studies often use untargeted analysis to assess the presence of a specific biomarker in a single anthocyanin-rich food, usually berries such as strawberries or blueberries, or other fruits like apples. Thus, authors commonly place special emphasis on the specific phenolic compound found in a single anthocyanin-rich food when reporting their results, while complete reports of the phenolic compounds found in all samples analyzed are usually not described.[48–57] On the other hand, studies that used a targeted analytical approach were not included in the present review, and only a few studies met the inclusion criteria for use of an untargeted analytical approach in plasma and urine. The systematic review methodology for identifying biomarkers of food intake by untargeted analysis might not have detected or measured all compounds in a specimen, and therefore it is possible that another biomarker of anthocyanin-rich berry intake might exist. However, at this time, and with the information available, C3G remains the best candidate as a biomarker of anthocyanin-rich berry intake. Moreover, although C3G is a good biomarker of berry intake, it might also be suitable as a biomarker for intake of other foods that contain C3G, but this requires further study.

Another important limitation of the present review is the variation in the sample size of the clinical trials included. To account for the small sample size of some studies, a qualitative meta-analysis of the data was performed. As a result, all studies of different sizes contributed equally to a pool of information, which was further analyzed to reach conclusions.

Another objective of the present review was to identify an anthocyanin biomarker in different human body fluids. Thus far, however, there is insufficient information about the presence of anthocyanins in body fluids other than plasma and urine, such as saliva, which can be collected easily and noninvasively to obtain biological information.

The methodological heterogeneity of anthocyanin identification and quantification in human plasma and urine also represents a critical limitation of this qualitative review. As result, quantitative data could not be analyzed because of differences in the doses used for experimentation, the methods used for quantification, and the sources of anthocyanins administered.

The dose of anthocyanin-rich berries used in most of the included studies varied widely, from 12 g/d to 300 g/d orally, but the complete chemical composition of the ingested berries was not described. Furthermore, the time between administration of the dose and collection of body fluid samples also presented an important variable.

CONCLUSION

Up to 203 different phenolic compounds have been reported in plasma and urine samples from healthy

humans. The phenolic compound identified most frequently after consumption of anthocyanin-rich berries is C3G, found in 69.49% of plasma samples and in 58.06% of urine samples. When the process proposed here for the selection of a biomarker candidate is applied, C3G meets the critically important plausibility criterion, as well as the dose-response, time response, stability, and analytical performance criteria. In addition, it has an acceptable positive predictive value of 74% in plasma. Thus, C3G is a promising biomarker of anthocyanin-rich berry consumption in plasma and urine samples of healthy humans.

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Declaration of interest. The authors have no relevant interests to declare.

Supporting Information

The following Supporting Information is available through the online version of this article at the publisher's website.

Appendix S1 **AMSTAR 2 results**

Table S1 **PRISMA checklist**

Table S2 **Risk-of-bias assessment of studies screened by means of the MINORS methodology**

Table S3 **Phenolic compounds detected in urine and plasma in the studies included for analysis**

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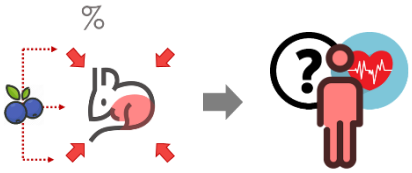

3.2. Chapter 2

Anthocyanin Tissue Bioavailability in Animals: Possible Implications for Human Health.

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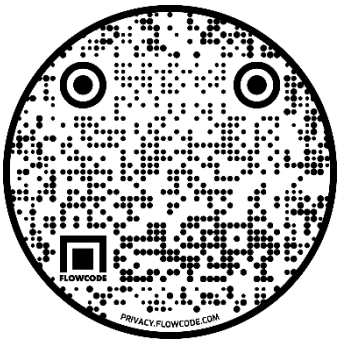

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Review

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Anthocyanin Tissue Bioavailability in Animals: Possible Implications for Human Health. A Systematic Review

Berner Andrée Sandoval-Ramírez,[†] Úrsula Catalán,[*,†,§] Sara Fernández-Castillejo,[†] Laura Rubió,[∥] Alba Macià,[∥] and Rosa Solà[†,‡]

[†]Faculty of Medicine and Health Sciences, Medicine and Surgery Department, Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut), Universitat Rovira i Virgili, 43201 Reus, Spain

[‡]Hospital Universitari Sant Joan de Reus (HUSJR), 43204 Reus, Spain

[§]Institut d'Investigació Sanitària Pere Virgili (IISPV), 43204 Reus, Spain

[∥]Food Technology Department, Agrotecnio Research Center, University of Lleida, Av/78 Alcalde Rovira Roure 191, 25198 Lleida, Spain

ABSTRACT: Anthocyanins (ACNs) are promising health-enhancing phenolic compounds. We focus on ACN animal tissue bioavailability to provide an evidentiary link between tissue ACNs and their associated health properties. We performed a systematic review of electronic libraries; 279 results were retrieved, and 13 publications met inclusion criteria. Extracted information included animal model employed, administration route, doses, analysis method, and ACN concentration values in tissues. Total ACN concentrations were detected in mice kidney (2.17×10^5 pmol/g), liver (1.73×10^5 pmol/g), heart (3.6×10^3 pmol/g), and lung (1.16×10^5 pmol/g); and in pig brain (6.08×10^3 pmol/g). ACNs showed a predominance of parent ACNs in long-term experiments versus an ACN metabolite predominance in short-term experiments. ACNs detected in animal tissues, such as cyanidin-3-glucoside, suggest it may have an important role in human health. This information could be useful to determine proper ACN-intake biomarkers in biological samples in futures studies.

KEYWORDS: *anthocyanins, tissue bioavailability, health, animal studies, mechanism-of-action, phenolic compounds*

INTRODUCTION

In recent years, preventive medicine has acquired a significantly more important role in healthcare systems primarily due to the increase in aged population and the prevalence of obesity, diabetes, metabolic syndrome, and hypertension[1,2] in which a healthy diet and lifestyle play a significant role.[3] Dietary changes and healthy eating patterns such as the Mediterranean diet are characterized by a high intake of fruit, vegetables, fish, nuts, and olive oil, that can reduce the risk for chronic noncommunicable diseases.[4,5] However, most bioactive molecules present in fruits and vegetables and the corresponding biochemical pathways that grant them their associated health benefits are still unclear.

Among the most well-known bioactive molecule groups found in fruits and vegetables are the phenolic compounds such as anthocyanins (ACNs). ACNs are water-soluble plant pigments responsible of giving red and blue coloration in fruits, flowers, seeds, and plants.[6] They are frequently found in the skin of many fruits and in the flesh of some berries,[7] with concentrations ranging from 0.1% up to 1.0% of the fruit's or vegetable's dry weight.[8] ACNs consist of a wide range of molecule classes and subclasses,[9] each one with its own properties and most of them capable of modulating or regulating wide and diverse biochemical pathways.[10,11]

It is well-known that the ACN's absorption site[12] and the chemical structure of anthocyanins[13] have an impact on the phenolic profiles that can be detected in human body fluids. These differences can be attributed to changes in pH between the gastric and colonic lumens,[14,15] but also because of an important ACN's metabolization by colonic microbiota which can occur within the first 2 h after the ingested ACNs reach the human colon.[16] Thus, colonic metabolization often results in new metabolites that were not present in the original ACN food source.[17−19] These colonic ACN metabolites have shown interesting potential for human health and could even have greater biological activity than their parent molecules, since colonic ACN metabolites are more abundant and are quite often better absorbed than their ACN parents.[20,21] Consequently, ACNs colonic metabolization has a direct impact in the amount and half-life of different metabolites of ACNs.[22] The mentioned differences in ACN absorption and metabolization render different plasmatic phenolic profiles that in turn may be of paramount importance when determining tissue profiles and the benefits of long-term versus short-term ACN ingestion; however, this remains an area of uncertainty. Thus, absorption and metabolization probably contributes to the ACN tissue bioavailability, and this might be a key relevant aspect for determining biomarkers to assess the intake of ACN containing foods. Moreover, bioavailability of ACNs in tissues and cells show great potential for the treatment and prevention of different pathological entities.[23−27]

In the current Review, we hypothesize on how the ACNs, either parent ACNs, such as cyanidin-3-glucoside (C3G),

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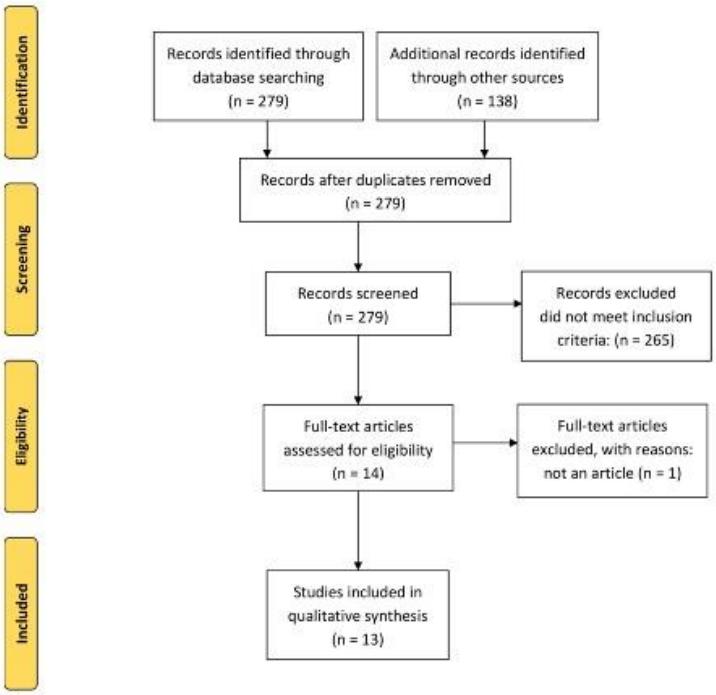

Figure 1. PRISMA statement flowchart for anthocyanin tissue availability adapted to animal studies.

delphindin-3-glucoside, malvidin-3-glucoside, peonidin-3-glucoside (P3G), and petunidin-3-glucoside, or their metabolites could be detected in target tissues might exert an effect in the cells in which they are present. In consequence, providing a link between ACNs present in tissues after their consumption and the described intracellular mechanisms of action are an explanation of ACN reported beneficial effects on human health. We adapted the concept published by de Ferrars et al.[15] and defined "parent" ACNs as an ACN structure from which other molecules, namely, metabolites, are obtained by substituting or adding radicals through the methylation, conjugation, sulfation, and glucuronidation. Thus, our primary goal is to perform a systematic review of the current knowledge reported on the tissue bioavailability of ACNs in different animal tissues after the administration of diverse ACN sources and consequently identifying possible bioactive molecules that could be clinically relevant. Moreover, through an up-to-date descriptive review of in vitro experiments, we aspire to provide an explanation for the reported in vivo ACN health effects.

MATERIALS AND METHODS

Search Strategy and Selection Criteria. For the present Review, our group adapted the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (http://www.prisma-statement.org/), designed for clinical trials,[28] to the systematic review of animal studies due to the lack of a better and more standardized screening method. An electronic-based search in the scientific libraries PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Scopus (http://www.scopus.com) was performed. A broad search term of "anthocyanin AND bioavailability" was used for our search, since more specific options did not render all studies that could meet the inclusion criteria used for the present Review. Results were screened based on their titles, abstracts, and full-text availability according to our inclusion criteria: (1) animal interventions using ACNs extracts or ACN supplementation and (2) ACNs tissue bioavailability evaluation. All non-English publications, and studies that did not report specific metabolite concentration values in tissues were excluded from the discussion.

Data Extraction, Standardization, and Analysis. Two independent authors (B.A.S.-R. and Ú.C.) extracted published data from text and tables; for data published as graphics, approximates were estimated through scaling; all differences were resolved by a third reviewer (R.S.). The following information was extracted from all reviewed studies: (1) study characteristics including the animal model used, ACN administration routes, doses, and sample analysis method; and (2) total ACN, parent ACN, or ACN metabolite concentration assessed in animal tissues.

All concentration units were converted and homogenized into the international system of units (molar) using metabolite molecular weight published in the Phenol-Explorer (http://phenol-explorer.eu/).[29] After homogenization, data was analyzed and text, Tables, and Figures were elaborated, presented, and discussed.

RESULTS

Literature Search, Studies Selection, and Characteristics. A total of 279 results published from inception up to February 2018 were found in our initial screening. All entries titles and abstracts were assessed for relevance according to inclusion criteria; 265 studies did not meet inclusion criteria and 1 study was excluded because it was not a paper. Thirteen publications[10,11,30−40] were identified and further examined as full texts. The flow diagram of the selection process used is shown in Figure 1.

Of all publications evaluated, 7 short-term experiments[11,33−37,40] assessed ACN tissue bioavailability after a single dose of ACN

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administered either orally or intravenously (IV). The remaining 6 experiments[10,30−32,38,39] assessed ACN tissue bioavailability after a long-term oral-controlled ACN supplementation. Out of the 13 studies, 5 were performed on Wistar rats,[30,33,35−37] 3 on mice,[11,32,34] 2 on Sprague−Dawley rats,[10,40] 2 on pigs,[31,38] and 1 on Zucker rats.[39]

High-performance liquid chromatography and mass spectrometry were the most used analysis techniques to determine ACN concentration in tissues. Kidney, liver, and brain were the most frequently analyzed organs with highest total ACN concentrations: 2.17×10^5 pmol/g in mice kidneys,[11] 1.73×10^5 pmol/g in mice liver,[32] and 6.08×10^3 pmol/g in young swine brain.[38] Complete information on general characteristics of studies that assessed ACN tissue bioavailability in animals as well as the total ACN concentrations detected in analyzed tissues are presented in Table 1.

Short versus Long-Term Tissue ACNs Bioavailability. One of the differences that was noted through our findings was that the ACN bioavailability profiles in animal tissues after a short versus long-term administration of ACNs showed different patterns. We observed a clear predominance of parent ACNs in long-term experiments, as shown in Table 3, versus ACNs metabolites predominance in short-term experiments reflected in Table 2. These differences will be further addressed and explained in more detail in the following sections.

Tissue Bioavailability in Short-Term Experiments. In short-term experiments, high methodology variability was observed. These experiments were performed after a single dose of an ACN concentrate either orally or IV. Oral doses presented high variability between 8 mg/kg grape extract[37] and 500 mg/kg C3G extract,[11] while IV doses ranged between 1 mg/kg C3G extract[11] and 5 mg/kg bilberry extract.[33] Animals were sacrificed between 0 min and 24 h after administration, although sacrifice was most commonly performed 15 min after ACN either oral or IV administration.[11,33,35,36]

4′-*O*-methylcyanidin-3-*O*-β-glucopyranoside highest concentration (19.18 pmol/g) in liver was found 15 min after a 5 mg/kg IV ACN dose after bilberry extract administration,[33] while in kidneys petunidin-3-glucoside was the highest ACN (2317.00 pmol/g) found at 15 min after a 670 nmol ACN IV dose.[36] C3G was the parent ACN found at highest concentrations in brain at 0.25 min (40.46 pmol/g) after the IV administration of bilberry extract.[35] Moreover, C3G was also found in gastrointestinal tract (8×10^5 pmol/g), lung (5.19×10^4 pmol/g), and prostate (4×10^4 pmol/g) with peak concentrations at different times after an oral single dose of C3G extract.[11] Complete information regarding peak concentrations of parent ACNs and ACN metabolites at different doses and times from short-term animal interventions are reported in Table 2.

Tissue Bioavailability in Long-Term Experiments. High heterogeneity in oral ACN administrated doses was also noted for long-term interventions: doses ranged from 27.5 mg/kg/day[38] up to 617.6 mg/kg/day.[32] Animal sacrifices were performed at different periods comprehended between 10 days and 8 weeks. In long-term experiments, animal brains were the most common organs analyzed.[10,30−32,35,38,41] In the analyzed brains, the highest concentration for an ACN was found for malvidin-3-glucoside (4.43 pmol/g) after an 82.5 mg/kg/day oral supplementation with bilberry extract for 3 weeks in young swine pigs.[38]

Cyanidin-3-rutinoside-5-β-D-glucoside was found in the heart (1.50×10^{-4} pmol/g), brain (1.85×10^{-4} pmol/g), liver (3.34×10^{-5} pmol/g), kidney (9.24×10^{-4} pmol/g), and bladder (2.16×10^{-4} pmol/g) after 2 weeks of 200 mg/kg/day oral supplementation with bilberry in Wistar rats.[30] Complete information regarding doses, maximum ACN peak or metabolite concentration, and animal organ tissues analyzed for long-term animal interventions are reported in Table 3.

ACNs Bioavailability in Animal Tissues. *Cardiac Tissue Bioavailability.* Out of the 13 studies assessed, 3 studies evaluated the presence of ACNs in animal hearts using different ACN sources.[11,30,32] For mice hearts obtained after a 2 week ACN 617.6 mg/kg/day oral supplementation with C3G extract, tissue ACN concentrations were not detected,[32] whereas ACN concentrations of 1.11 pmol/g were reported in heart tissue for oral ACN doses as low as 200 mg/kg/day in a 3 week intervention using bilberry extract in Wistar rats.[30] These differences in heart ACN detection were probably due to differences in sample processing proceedings, ACN source, or animal model used since analysis methods used for ACN detection did not differ between both studies. Interestingly, higher supplementation oral doses of 2000 mg/kg/day using bilberries in Wistar rats reported a 54% (0.51 pmol/g) reduction for heart tissue ACN bioavailability compared with lower doses (200 mg/kg/day) of bilberry extract,[30] suggesting the possible increase or upregulation in ACN's degradation and elimination mechanisms available inside cardiac cells.

In mice hearts, ACNs were also found in short-term experiments performed when a single C3G extract oral dose of 500 mg/kg was administered to mice, the study reported peak of C3G (1.4×10^4 pmol/g) 10 min post administration.[11] Cyanidin metabolites were the most commonly in animal hearts analyzed, with the highest concentration reported for long-term studies was 4.95×10^{-4} pmol/g after administrating a 200 mg/kg/day oral dose of bilberry for 3 weeks in Wistar rats,[30] and 1.40×10^4 pmol/g on heart for short-term studies after a single 500 mg/kg oral dose of C3G extract in mice.[11]

There is no consensus on ACN daily doses and administration times that are needed to detect ACNs in target tissues. However, as stated before, the minimum ACN time and oral dose supplementation to achieve heart tissue detection has been demonstrated in rats after 3 weeks of bilberry extract supplementation with 200 mg/kg/day.[30] This oral dose of 200 mg/kg/day in rats represents a human oral dose of 32.4 mg/kg/day, representing around 2 g of bilberry extract for a standard 70 kg human.[42]

Brain Tissue Bioavailability. ACN brain bioavailability was evaluated in 7 out of the 13 studies that met inclusion criteria for the present review.[10,30−32,35,38,43] ACNs were not detected in brain tissue after a 48 mg/kg/day oral ACN extract supplementation for an 8 week period with wild blueberry in Sprague−Dawley rats,[10] after 509 mg/kg/day oral dose of blueberry powder after 10 days in Zucker rats, or after a 617.6 mg/kg/day oral grape seed extract supplementation for 2 weeks in mice.[39]

However, a maximum ACN concentration of 6.08×10^3 pmol/g was found in analyzed brains after a 3 week experiment performed on young swine pigs in which an 82.5 mg/kg/day oral dose of tart cherry was administered, whereas an ACN distribution pattern was determined for every brain region.[38] In same study, also a direct correlation between dose consumed and tissue bioavailability was observed.[38]

In the brain, after an 82.5 mg/kg/day ACN oral dose of tart cherry, petunidin-3-glucoside was the ACN found in highest concentration (6.66 pmol/g), followed by malvidin-3-glucoside (4.43 pmol/g) and P3G (4.40 pmol/g).[38] Moreover, in

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Table 1. General Characteristics of Studies Performing Anthocyanin Bioavailability in Animals and Total Anthocyanin Concentrations Detected in Analyzed Tissues[a]

			administration routes and dosage						total anthocyanin mean concentration (pmol/g) in different tissues										
			oral		IV														
author and ref	animal model	anthocyanin source	single dose	long-term	single dose	human equivalent	experiment duration	analysis method	heart	brain	liver	kidney	bladder	R. Fat	prostate	lung	testes	spleen	thymus	muscle	eyes
Del Bò et al.[10]	SD rat	wild blueberry powder		48 mg/kg/day		7.8 mg/kg/day	4 and 8 wk	HPLC		ND	ND										
Ichiyanagi et al.[33]	Wistar rat	bilberry extract	400 mg/kg		5 mg/kg		15 min	HPLC			206.14	673.26									
Aqil et al.[34]	athymic nude mice	blueberry powder	10 mg/mouse	5% diet sup			120 min/10 days	UPLC/LC-MS								≤0.25 ng[b]					
Forna7saro et al.[35]	Wistar rat	C3G extract			668 nmol		0, 25, 5, 10, 15, 20 min	UPLC-MS/MS		44.98[c]	599.05[c]	3.68×10^{3c}									
Vanzo et al.[36]	Wistar rat	C3G extract			670 nmol		0.25, 1, 5, 15 min	HPLC-DAD-MS			1011.00	138.00									
Chen et al.[59]	Zucker rat	grape seed extract		509 mg/kg/day		82.5 mg/kg/day	10 days	LC-MS OR LC-MS/MS		ND											
Marczylo et al.[11]	mice	C3G extract	500 mg/kg			40.5 mg/kg	5, 10, 20, 30, 60, 90, 120 min	HPLC	3.6×10^3		3.53×10^4	2.17×10^5			4.96×10^4	2.83×10^4					
					1 mg/kg		5, 10, 15, 20, 30, 60, 90, 120 min	HPLC	720.00		80.00	970.00			ND	430.00					
Chen et al.[58]	Pig	bilberry extract		27.5 mg/kg/day		20 mg/kg/day	3 wk	LC-MS/MS		1.76×10^{3cf}											
				82.5 mg/kg/day		60 mg/kg/day	3 wk	LC-MS/MS		6.08×10^{3cf}											
Borges et al.[30]	SD rat	raspberry juice	2.77 mL juice				0, 1, 2, 3, 4, 6, 12, 24 h	HPLC-PDA-MS/MS			ND	ND	ND								
Kirakosyan et al.[10]	Wistar rat	tart cherry powder		200 mg/kg/day		32.4 mg/kg/day	3 wk	LC-MS/MS	1.11	0.27	0.43	2.07	1.33	ND							
				2000 mg/kg/day		324.3 mg/kg/day	3 wk	LC-MS/MS	0.51	0.43	1.52	1.65	3.66	ND							
Vanzo et al.[37]	wistar rat	grape extract	8 mg/kg			1.3 mg/kg	10 min	HPLC			2.65×10^3	3.03×10^3									
							30 min	HPLC			3.02×10^3	2.11×10^3									
Milbury et al.[31]	pig	blueberry powder		2% blueberry powder			8 wk	HPLC-MS/MS		0.1683[cf]											
Hiroyuki et al.[32]	mice	bilberry extract		617.6 mg/kg		50.1 mg/kg	2 wk	HPLC-DAD & LC-MS/MS	ND	ND	1.73×10^5	1.14×10^5		ND		1.16×10^5	1.49×10^5	ND	ND	ND	ND

[a]IV, intravenous; HPLC, high-performance liquid chromatography; UPLC, ultrahigh performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; ND, not detected; G.I., gastrointestinal; R. Fat, retroperitoneal fat; SD, Sprague−Dawley; wk, week. [b]Exact values not reported. [c]Maximum detected value. [d]Data extracted from figures.

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Table 2. Peak Parent and Anthocyanin Metabolite Concentration at Different Doses and Times From Short-Term Animal Interventions[41]

					parent or metabolite maximum concentration (pmol/g) at different time points (min) in analyzed tissues																					
					heart		brain			liver					kidney					prostate		lung				
parent or metabolite ACN	ref	dose	ACN source	administration route	10	0.25	2	15	0.25	1	10	15	20	30	0.25	2	10	15	30	5	10	15	30	5	10
4′-*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside	33	5 mg/kg	bilberry	IV								19.18						46.12							
4′-*O*-methyl delphinidin 3-*O*-β-D-galactopyranoside	33	5 mg/kg	bilberry	IV								15.73						55.09							
4′-*O*-methyl petunidin 3-*O*-β-D-galactopyranoside	33	5 mg/kg	bilberry	IV								11.11						21.60							
cyanidin 3-*O*-β-D-galactopyranoside	33	5 mg/kg	bilberry	IV								5.41						14.19							
cyanidin-3-glycoside	35	668 nmol	C3G extract	IV			40.46		56.61						1.69									4×10^4	5.19×10^4
	11	500 mg/kg	C3G extract	oral	1.40×10^4									3.23×10^4			3.86×10^5								
cyanidin-3-*O*-β-D-glycoside-glucuronide	33	5 mg/kg	bilberry	IV								2.13						18.36							
delphinidin-3-glucoside	36	670 nmol	C3G extract	IV					641.00																
	37	8 mg/kg	grape extract	oral										50.00					110.00						
malvidin 3-*O*-β-D-galactopyranoside	33	5 mg/kg	bilberry	IV								13.50						21.84							
malvidin 3-*O*-β-D-glucopyranoside	33	5 mg/kg	bilberry	IV								2.59						2.40							
malvidin-3-6-*O*-acetylglucoside	37	8 mg/kg	grape extract	oral								140.00		160.00			220.00		930.00						
malvidin-3-6-*O*-*p*-coumaryl-glucoside	37	8 mg/kg	grape extract	oral								290.00		580.00			40.00		120.00						
malvidin-3-glucoside	36	670 nmol	C3G extract	IV					370.00									546.00							
	37	8 mg/kg	grape extract	oral								1260.00		1830.00			1170.00		1450.00						
peonidin 3-glucoside	35	668 nmol	C3G extract	IV					539.38						1.99										
	37	8 mg/kg	grape extract	oral			2.07					290.00		310.00			230.00		270.00						
peonidin 3-*O*-β-D-galactopyranoside	33	5 mg/kg	bilberry	IV								7.24						54.07							
petunidin 3-*O*-β-D-galactopyranoside	33	5 mg/kg	bilberry	IV								3.46						9.53							
petunidin-3-glucoside	35	668 nmol	C3G extract	IV				2.45					3.06			2.82									
	36	670 nmol	C3G extract	IV														2317.00							

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Table 2. continued

parent or metabolite ACN	ref	dose	ACN source	administration route	parent or metabolite anthocyanin maximum concentration (pmol/g) at different time points (min) in analyzed tissues																					
					heart		brain			liver						kidney					prostate	lung				
					10	0.25	2	15	0.25	1	10	15	20	30	0.25	2	10	15	30		5	10				
	37	8 mg/kg	grape extract	oral							660.00			880.00			1366.00		700.00							

[a]ND, not detected; IV, intravenous; G.I., gastrointestinal; C3G, cyanidin-3-glucoside. Homogenized values obtained from data collection. Parent anthocyanins are presented in bold.

brain, a P3G peak concentration of 2.07 pmol/g was found after 2 min for a single 8 mg/kg oral raspberry dose.[37]

Liver Tissue Bioavailability. Total ACN concentrations were reported in liver, ranging from not detected in Sprague–Dawley rats after an 8 week period of oral intervention of 48 mg/kg/day with wild blueberry extract,[10] to a detected concentration of 1.73×10^5 pmol/g in mice livers after a 2 week oral intervention with 617.6 mg/kg/day of bilberry extract.[32] In short-term studies, 4′-*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside was the ACN metabolite found in highest concentration (19.18 pmol/g), 15 min after a 5 mg/kg IV dose of bilberry extract in Wistar rats.[33] In long-term experiments, cyanidin-3-rutinoside-5-β-D-glucoside was the ACN metabolite found in highest concentrations (1.16×10^{-3} pmol/g), after a 2000 mg/kg/day oral dose for 3 weeks of tart cherry extract in Wistar rats.[30]

Kidney Tissue Bioavailability. ACN concentrations detected in kidney ranged from 1.65 pmol/g after an oral supplementation of 2000 mg/kg/day with bilberry for 3 weeks,[30] up to 2.17×10^5 pmol/g for a C3G extract unique oral dose of 500 mg/kg presenting a maximal C3G concentration at 10 min after oral administration in rats.[11] In kidney a maximal C3G concentration was found at 10 min after a unique oral C3G extract administration,[11] while after a single oral dose of 500 mg/kg of raspberry other ACN metabolites such as malvidin-3-glucoside, P3G, and petunidin-3-glucoside maximum peaks were detected at 10, 10, and 15 min, respectively.[37]

Lung Tissue Bioavailability. In lung tissue, C3G was found at a peak concentration of 3.86×10^5 pmol/g after a single 5 mg/kg C3G extract IV administrated dose in mice;[11] and at a concentration of 1.15×10^4 pmol/g after an oral supplementation with 617.6 mg/kg of a C3G extract for 2 weeks in mice.[32] Beyond C3G, other parent ACNs such as delphinidin-3-glucoside (3.40×10^4 pmol/g) and P3G (8.80×10^4 pmol/g) were also found in lungs after oral doses of 617.6 mg/kg of supplementation with a C3G extract for 2 weeks in mice.[32]

ACN Bioavailability in Other Animal Tissues. ACNs were also detected in prostatic tissue of mice (total concentrations of 4.96×10^4 pmol/g), after a single oral 500 mg/kg dose of C3G extract,[11] and on testes of mice (1.16×10^5 pmol/g) after a 2 week intervention using a dose of 617.6 mg/kg/day of bilberry oral supplementation.[32] However, to date not enough bioavailability assays have been published regarding ACN presence in these tissues to properly determine the importance of specific phenolic compounds in relation of the health of these organs.

DISCUSSION

The present work aims to summarize the knowledge reported on the ACN's tissue bioavailability after the administration of different ACN sources in animals. Thus, identifying possible bioactive molecules in animal tissues could suggest clinical relevance for humans. Surprisingly, there is a remarkable lack of studies that describe both ACN tissue bioavailability and their pharmacodynamics explaining ACN effects. In order to solve this issue, we describe the relationship between the ACNs detected concentrations in animal tissues and their possible health effects, by providing a link between the findings of ACNs in in vivo animal assays and in vitro experiments, leading to determine specific mechanisms of action in cell or/and tissues. This allowed us to provide a potential explanation of ACN's health effects in humans.

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Table 3. Maximum Peak Parent or Metabolite Anthocyanin Concentration Detected in Animal Tissues Obtained After Long-Term Animal Intervention Studies[a]

				parent or metabolite anthocyanin maximum concentration (pmol/g) at different time points (weeks) found in analyzed tissues													
				heart	brain		liver		kidney		lung	testes		bladder			
parent or metabolite ACN	ref	ACN source	dose	3	3	8	2	3	2	3	2	2	2	3			
cyanidin-3-rutinoside-5-β-D-glucoside	30	tart cherry	200 mg/kg/day	1.50×10^{-4}	1.85×10^{-4}			3.34×10^{-5}		9.24×10^{-4}				2.16×10^{-4}			
cyanidin-3-arabinoside	30	tart cherry	2000 mg/kg/day	2.28×10^{-4}	4.00×10^{-4}			1.16×10^{-3}		6.10×10^{-4}				4.32×10^{-4}			
	31	blueberry powder	2% sup			1.48×10^{-3}											
	38	bilberry extract	27.5 mg/kg/day		1.50												
	38	bilberry extract	82.5 mg/kg/day		3.18												
	31	blueberry powder	2% sup			5.92×10^{-4}											
cyanidin-3-glucoside	38	bilberry extract	27.5 mg/kg/day		0.85												
	38	bilberry extract	82.5 mg/kg/day		3.17												
	31	blueberry powder	2% sup			8.14×10^{-3}											
cyanidin-3-glucosyl rutinoside	32	bilberry	617.6 mg/kg/day				2.30×10^{4}		2.50×10^{4}		1.15×10^{4}	ND					
	30	tart cherry	200 mg/kg/day	6.26×10^{-5}	ND			ND		ND				5.69×10^{-4}			
cyanidin-3-rutinoside	30	tart cherry	2000 mg/kg/day	1.03×10^{-4}	ND			ND		ND				3.09×10^{-3}			
	30	tart cherry	200 mg/kg/day	3.98×10^{-4}	ND			2.46×10^{-4}		1.90×10^{-4}				4.64×10^{-4}			
delphinidin-3-galactoside	30	tart cherry	2000 mg/kg/day	1.67×10^{-4}	ND			ND		4.63×10^{-4}				ND			
	38	bilberry extract	27.5 mg/kg/day		0.21												
	38	bilberry extract	82.5 mg/kg/day		3.32												
	31	blueberry powder	2% sup			5.18×10^{-3}											
delphinidin-3-glucoside	38	bilberry extract	27.5 mg/kg/day		0.21												
	38	bilberry extract	82.5 mg/kg/day		2.90												
malvidin-3-galactoside	32	bilberry	617.6 mg/kg/day				3.40×10^{4}		1.14×10^{5}		3.40×10^{4}	ND					
	38	bilberry extract	27.5 mg/kg/day		0.93												
	38	bilberry extract	82.5 mg/kg/day		3.97												
	31	blueberry powder	2% sup			1.85×10^{-2}											
malvidin-3-glucoside	38	bilberry extract	27.5 mg/kg/day		1.43												

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Table 3. continued

					parent or metabolite anthocyanin maximum concentration (pmol/g) at different time points (weeks) found in analyzed tissues											
					heart	brain		liver		kidney		lung	testes	bladder		
parent or metabolite ACN	ref	ACN source	dose		3	3	8	2	3	2	3	2	2	3		
	38	bilberry extract	82.5 mg/kg/day			4.43										
	31	blueberry powder	2% sup				5.33×10^{-2}									
malvidin-3-glucoside/peonidin-3-glucoside	32	bilberry	617.6 mg/kg/day					1.08×10^{5}		8.80×10^{4}		7.05×10^{4}	1.49×10^{5}			
peonidin 3-glucoside	38	bilberry extract	27.5 mg/kg/day			0.51										
	38	bilberry extract	82.5 mg/kg/day			4.40										
	31	blueberry powder	2% sup				1.55×10^{-1}									
peonidin-3-arabinoside	31	blueberry powder	2% sup				2.22×10^{-3}									
peonidin-3-galactoside	38	bilberry extract	27.5 mg/kg/day			NR										
	38	bilberry extract	82.5 mg/kg/day			3.80										
	31	blueberry powder	2% sup				3.70×10^{-3}									
peonidin-3-rutinoside	30	tart cherry	200 mg/kg/day		4.95×10^{-4}	8.37×10^{-5}			3.35×10^{-4}		9.52×10^{-4}			7.71×10^{-5}		
	30	tart cherry	2000 mg/kg/day		1.54×10^{-5}	3.33×10^{-5}			1.17×10^{-4}		5.74×10^{-4}			1.43×10^{-4}		
petunidin-3-galactoside	38	bilberry extract	27.5 mg/kg/day			ND										
	38	bilberry extract	82.5 mg/kg/day			3.84										
	31	blueberry powder	2% sup				2.52×10^{-2}									
petunidin-3-glucoside	38	bilberry extract	27.5 mg/kg/day			0.23										
	38	bilberry extract	82.5 mg/kg/day			6.66										
	32	bilberry	617.6 mg/kg/day					8.00×10^{5}		ND		ND	ND			

[a]ND, not detected; sup, supplementation; NR, Not reported. Homogenized values obtained from data collection. Parent anthocyanin are presented in bold.

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Tissue ACN Profile Differences in Short versus Long-Term in Animal Oral Interventions. From current data, we suggest that the difference in tissue ACN bioavailability profiles obtained after short and long-term oral assays performed in animal interventions could be explained by the saturation of the absorption mechanisms, mainly at gastric level via bilitranslocase, and by the further colonic metabolization of non-absorbed ACNs after an oral consumption.[10] Gastric absorption is then followed by hepatic metabolization in which ACN metabolites are created by glucosidation, methylation, and glucuronidation, and then liberated into the plasma, causing a rise of ACN metabolite forms that are further delivered to the different tissues.[17]

Furthermore, from our results, in oral short-term experiments, a predominance of parent ACNs in tissues is detected, whereas the long-term oral administration of ACNs can cause the saturation of the ACN gastric absorption mechanisms, mainly bilitranslocase located in the mucosecretory and parietal cells of the stomach.[44] This saturation leads to the hydrolysis of parent ACNs into their metabolites. As a result, total ACN metabolite concentration increases in the stomach, following later stages of the digestion process when ACNs are susceptible to be metabolized by colonic microbiota.[12,17] Therefore, in long-term ACN administration, colonic microbiota reconvert ACNs into their metabolized versions, that are then absorbed through the colonic epithelia, transferred to the bloodstream, and from there distributed to the rest of tissues in animals.[17] This colonic process is also probably the same for humans. Whereas in short-term exposure, the main absorption is performed through the gastric epithelia.[22,44]

A good example of how ACN gut microbiota metabolization and colonic absorption are relevant to their bioavailability is the generation of the two major molecules product of C3G's degradation: protocatechuic acid (PCA) and pholoroglucinaldehyde (PGA). After C3G's ingestion and gastric absorption, C3G's remnant concentrations arrive at the colon, where gut microbiota start its degradation yielding PCA out of C3G's B-ring and PGA out of its A-ring.[11,22] Afterward, these molecules are absorbed, giving rise to their respective plasmatic concentration peaks and therefore rending maximum concentration times (T-max; 3.3 ± 0.7 h for PGA and 2.8 ± 1.1 h for PCA), much later than their parent C3G (1.8 ± 0.2 h).[13] This information suggests that the observed time/dose response over tissue ACN profiles might be of relevance for tissue-specific health effects derived from ACN administration. As consequence, the result is that short-term oral exposure to ACNs leads to a higher presence of parent ACNs, while the long-term administration renders a more diverse pattern in which ACN metabolites are more commonly found.

ACN's Mechanisms of Action and Possible Health Repercussions. Since ACN mechanisms of action in humans cannot be determined due to the intrinsic methodologic limitations and ethical concerns to detect their presence in human tissues, this subject has not yet been properly studied. Because of that, complementary to animal studies, in vitro studies performed on cellular models become relevant when determining important bioactive ACN molecules and their effects on biochemical pathways involved in the treatment or prevention of a series of pathologies.[45−48] ACNs have shown great potential in cellular models of experimentation as bioactive molecules capable of not just reducing oxidative stress,[49−51] but also possibly being capable of modifying pathways from different types of cancer (hepatocarcinoma,[52] colorectal cancer,[53,54] and breast cancer[55,56]) or modulating obesity and its associated low-grade inflammation state.[57]

ACNs and Cardiac Health. Cardioprotective effects of some ACNs such as C3G have been demonstrated in mice, both in cellular and in animal model experiments,[58] since it has been shown to be able to reduce doxorrubicin's, an anticancer drug, cardiotoxicity in myocytes; where after high doses of pure C3G extract, cellular death was reduced after a 3 week oral supplementation.[58] Another study evaluated on an isoproterenol-induced myocardial infarction mice model, after 28 days of ACN oral administration. In this study, mice showed reduced plasmatic protein levels of creatine kinase muscle/brain (CK-MB), a cardiac necrosis biomarker, increased levels of intracellular enzymatic antioxidants, and decreased levels of apoptotic markers probably achieving C3G's effects through the activation of β_1 adrenergic receptors in cardiac cells,[23] which in turn could reduce cytochrome *c* intracellular concentrations, therefore leading to fewer cardiac cellular damage and apoptosis.[59]

Moreover, after 4 weeks of 10 mg/kg/day C3G oral supplementation in rats with myocardial infarction, left ventricle dilatation and body mass loss were prevented while not showing any improvements in cardiac structure and function.[58] These cardioprotective effects of C3G were not observed after 8 weeks of ACN administration of the same doses.[60] The discrepancy the results obtained after 4 and 8 weeks of daily C3G consumption need to be addressed.

However, ACN metabolization in the heart might be generated to counteract possible adverse effects of ACN activity, since high concentrations of phenolic compounds can cause rather negative intracellular effects, the opposite of what has been demonstrated for lower ACN concentrations as reported before.[61]

Up to the date of the present Review, few studies have evaluated the effect of oral ACNs in humans and showed a lower systolic blood pressure, lower plasmatic triglyceride (TG) levels, and healthier TG/HDL cholesterol ratio.[6,62−64] No experimental studies assessing protective effects of ACN supplementation after myocardial infarction or heart failure in humans have been performed.

ACNs and Brain Health. In brain, it has been demonstrated that ACNs are able to cross the blood-brain barrier;[65] however, to the best of our knowledge, the exact mechanism by which it happens is still unclear.

In animal models, ACN natural dietary supplementation not only reduced oxidative stress but also neurodegeneration and memory impairment for a mice model of Alzheimer's disease, which could be explained by the regulation of the phosphorylated-phosphatidylinositol 3-kinase-Akt-glycogen synthase kinase 3 beta (p-Pl3k/Akt/GSK3β) pathway.[27] The p-Pl3k/Akt/GSK3β reduced reactive oxygen species elevations, further preventing apoptosis, neurodegeneration,[27] and glial cell death induced by H_2O_2, as a consequence delaying the age-related degenerative changes in brain cells.[66] Some ACN's possible mechanisms of action have been described in in vitro experiments were the improvement of free radical scavenging, reactive carbonyl trapping, antiglycation, anti-amyloid β (Aβ) fibrillation, and microglial neuroprotective effects in murine cell cultures,[50] and in the human neuroblastoma cell line SK-N-SH.[24]

The effects of ACNs on brain tissue were observed at oral doses as low as 15 mg/kg/day in mice,[67] which represents a human equivalent of 1.3 mg/kg/day (91 mg/day for a 70 kg average person).[42] The ACN brain effects could be enhanced

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by the use of nanovehicles by loading polyethylene glycol-gold nanoparticles with ACNs to increase brain bioavailability.[67] As result, ACN exerts an enhanced cellular protection against Aβ induced oxidative stress involved in Alzheimer's diseases.[68] The nanoparticle delivery system could be a good strategy to increase intracellular concentrations of ACNs in other tissues. Moreover, ACN antioxidant protective effects could provide beneficial effects not only in Alzheimer's disease, but also in other neurodegenerative diseases such as Huntington's demonstrated in mice where supplementation with ACNs for 3 weeks showed an improvement in motor functions,[69,70] and Parkinson's disease where ACNs acted mainly by their free radical scavenging properties.[71] Thus, the demonstrated ACN effects support their potential for the treatment and prevention of neurodegenerative diseases.

ACNs hold various pharmacokinetic and pharmacodynamics profiles due to their structural differences, as demonstrated with models that used C-labeled ACN molecules that evidenced their specific properties. In humans, after a 500 mg bolus dose of isotopically labeled C3G, maximum plasmatic concentrations ranged between 10 and 2000 nM and T-max values comprehended between 2 and 20 h.[13] In accordance with these findings, we suggest that sustained consumption and ACN source could be of importance when analyzing their profiles present in the tissues.

ACNs and Hepatic Health. There is a considerable amount of published evidence about the presence of ACNs on hepatic tissue, surely because its paramount role in ACN metabolization,[11,13,22] and its central role in the degradation and excretion of many molecules. Evidence indicates that ACNs could help reduce liver inflammation demonstrated by the lower activity of alanine aminotransferase and aspartate aminotransferase, two key hepatic inflammation biomarkers, reported in a murine model of hepatic damage induced by IV injected lipopolysaccharide and *Propionibacterium acnes,* in which a positive reduction of inflammation was observed after the intake of 50−150 mg/kg/day bilberry extract for 7 days.[72] However, there is a remarkable lack of studies that assess the impact of specific ACN presence in the liver as to prevent hepatic diseases.

ACNs and Renal Health. The evidence that suggests that the presence of malvidin-3-glucoside, P3G, and petunidin-3-glucoside in renal tissue prevented and delayed the progression not only of renal disease against cisplatin-induced acute kidney injury that is produced after its use as an anticancer treatment,[73] but also of ischemia-reperfusion injury as demonstrated in mice.[74] In consequence, these results suggest a potential aid in preventing and delaying the progression of acute kidney injury in humans. On the other hand, ACNs have shown nonspecifically inhibition properties against the connective tissue growth factor's expression, also known as CCN2. This growth factor has been identified as an important molecule in the development of kidney failure in diabetic nephropathy, through the retardation of tumor growth factor-β signaling pathway.[75] ACN supplementation with doses as low as 10 mg/kg every 2 weeks for 4 doses in humans retarded glomerular angiogenesis and inhibited endothelial tube formation promoted by high glucose-exposed mesangial conditioned media.[75]

We suggest that ACN source is of importance when analyzing different polyphenol profiles in different animal tissues; therefore, not all ACN sources could provide the same health benefits for humans. Despite the promising effects of ACN on kidney protection, up to date no human studies regarding the applications of ACN therapy in kidney disease or glomerular injuries have been described.

Health Implications of ACNs in Lung and Other Tissues. ACNs have shown anti-lung-cancer properties as supported by the results from ACNs' successful inhibition of lung cancer cell migration and invasion by suppressing matrix metalloproteinase (MMP)-2 and MMP-9, as well as different proteins related to in cancer proliferation, adhesion, and angiogenesis involved in lung cancer development.[76] These anticancer benefits might be specifically provided by P3G, an ACN which has demonstrated to inhibit the invasion, motility, and secretion of MMPs such as MMP-2, MMP-9, and urokinase-type plasminogen activator in lung cancer cells, by inhibiting extracellular signal-regulated kinase (ERK)-1/2, a mitogen-activated protein kinase (MAPK) family member involved in the regulation of MMP molecules as demonstrated in a lung cancer cell in vitro model.[77] Furthermore, so-called suboptimal in vitro concentrations of a combination of ACNs have demonstrated to act synergistically inhibiting the growth of aggressive non-small-cell lung cancer cells, possibly by their inhibitory effects on molecules like β-catenin, cyclin B1, and MMP-9 as well as the inhibition of TNFα-induced nuclear factor-kappa B (NF-κB) activation.[78]

Delphinidin is another ACN that has shown anticancer properties. It is capable of inducing cell apoptosis in lung cancer cells by inhibiting the epidermal growth factor receptor (EGFR)/vascular endothelial growth factor receptor 2 (VEGFR2) pathway,[79] and through the suppression of hypoxia-inducible factor 1-alpha (HIF-1α).[80] However, up to date, studies performed on humans have not shown lung cancer preventive benefits, as is the case of the Kuopio Ischemic Heart Disease Risk Factor Study, an ongoing prospective study performed in 2682 middle-aged men from Finland, in which a nonsignificant lung cancer risk reduction of 20% for men was found when comparing the highest and lowest ACN consumption quartiles.[81]

One other relevant area of interest that has not been addressed in this Review is the importance of ACNs in the gastrointestinal tract for the prevention of colorectal cancer,[53,54] mainly due to the shortage of studies that provide evidence of a complete ACN profile in gastrointestinal tissues. Nonetheless, reports of total ACN concentration values of 8.1 μg/g[82] or 4.48 × 105 pmol/g[11] of intestinal mucosa were described in mice. Moreover, evidence exists regarding the ability of ACNs to inhibit intestinal tumor development in ApcMin mice and growth of human colon cancer cell lines,[83,84] and to induce cytotoxicity and decrease viability of Caco-2 cells, facts that could hinder the growth of tumoral cells in vivo.[85] These studies demonstrate the presence of ACNs in colorectal tissues and ACN capability for colon cancer prevention. Though, the lack of more studies in animals and human populations make difficult the correlation of ACN consumption and colorectal cancer prevention.

Limitations of the Review. One of the greatest limitations on performing our review was the heterogeneity between the study methodologies, regarding the animal models employed, doses used, experiment duration, ACN sources and profiles, which could hinder the direct comparisons between studies. Another limitation observed was that ACN source composition was rarely described in published articles, making difficult to determine the molecular origin of the ACN profiles demonstrated, which is of paramount importance to differentiate the possible best ACN sources for specific tissues or health effects or pathologies. Published studies were found to be either focused on the metabolism, bioavailability, or

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health effects, but there are no studies that integrate this information altogether. As a consequence, under these conditions, it is not easy to determine which parent or ACN metabolite is actually responsible for observed health effects in in vivo studies.

Final Remarks. The presence of parent or ACN metabolites in animal tissues could explain the myriad of health benefits attributed to oral or IV administration of ACNs. C3G and its metabolites are one of the most frequently found metabolites in tissues. ACN source, dose, and consumption time are of paramount importance when analyzing ACN profiles in target tissues.

From the analyzed information obtained through this Review, we suggest that C3G, present in target tissues, could have an interesting potential for the reduction of myocardial infarction negative tissue effects and neurodegenerative diseases such as Alzheimer's and Parkinson's, and could also help delay or even reverse acute renal failure. Therefore, the published evidence indicates that the ACNs detected in animal tissues, such as C3G, may have an important role and could be one of the most promising bioactive molecules for human health. Moreover, this information could be useful to determine proper ACN-intake biomarkers in biological samples in futures studies.

AUTHOR INFORMATION

Corresponding Author

*Tel: (+34) 977 75 93 75. E-mail: ursula.catalan@urv.cat.

ORCID

Úrsula Catalán: 0000-0001-8884-9823

Author Contributions

Study conception and design: B.A.S-R., Ú.C., and R.S. Acquisition of data: B.A.S-R. and Ú.C. Analysis and interpretation of data: B.A.S-R., Ú.C., R.S., and S.F.-C. Drafting of the manuscript: B.A.S-R. Critical revision: Ú.C., R.S., S.F.-C., L.R., and A.M.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACN, anthocyanin; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; IV, intravenous; P3G, peonidin-3-glucoside; C3G, cyanidin-3-glucoside; CK-MB, creatine kinase; Aβ, amyloid β; Pl3k/Akt/GSK3β, phosphorylated-phosphatidylinositol 3-kinase-Akt-glycogen synthase kinase 3 beta; CCN2, connective tissue growth factor; TNF-α, tumor necrosis factor α; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; EGFR, epidermal growth factor receptor; VEFGR2, vascular endothelial growth factor receptor 2

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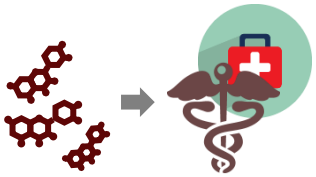

3.3. Chapter 3

The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and clinical controlled trials.

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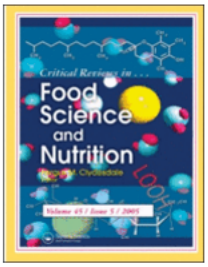

The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and clinical controlled trials.

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Keywords:	Blood pressure, Cancer, Cholesterol, Endothelial function, Diabetes, Health

Fergus M. Clydesdale, Ph.D.

Editor-in-Chief, *Critical Reviews in Food Science and Nutrition*

13/03/2020

Dear Prof. Clydesdale,

Please, find enclosed our manuscript entitled **"The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and clinical controlled trials."** which we would like to be considered for its publication on the *Critical Reviews in Food Science and Nutrition* Journal.

Anthocyanins (ACNs) are phenolic compounds part of the flavonol subclass present in most red-colored fruits, such as berries, with promising health properties. However, the health-related benefits of ACN consumption remain unclear. The present umbrella review aims to bring information concerning the effects (from the systematic review and meta-analysis (SRM) of randomized controlled trials; RCTs) and associations (from the SRM of observational studies; OS') for ACNs and human health. Thus, providing information regarding ACN's health properties. To the best of our knowledge, **no other study has summarized and analyzed all the information from SRMs regarding the impact of ACNs on multiple health outcomes**.

The highest level of evidence regarding ACNs is obtained from the systematic reviews and meta-analyses of randomized controlled trials and observational studies; accordingly, the methodology proposed to determine the currently known properties of ACNs is an umbrella review. We believe that is the Interpretation of the results obtained from both RCTs and OS' that a better understanding of the efficacy/effectiveness and safety of a food or bioactive compound can be attained. Meta-analyses using both RCTs and OS' should be used to highlight some questions that neither an RCT, nor an OS would not solve by themselves.

Accordingly, from OS', the main results of the present work demonstrated that ACNs are significantly associated with a reduction in the risk of type 2 diabetes mellitus, and hypertension. Moreover, from RCTs, ACNs also significantly improved the plasmatic lipid profile, glucose metabolism, and vascular health in different populations without effects

on the blood pressure values. Moreover, the ACN dietary intake did not show significant associations with the risk of breast cancer, nor of gastric cancer. Moreover, due to the conjunct analysis of both OS- and RCT-SRMs, considered the highest level of scientific information, **For the first time, the possible mechanism of action for the T2DM risk reduction associated with the chronic ACN dietary intake has been provided from the analysis of both OS'- and RCT-SRMs.**

On behalf of the co-authors, I affirm that the data has not been previously published, the manuscript is not under consideration for publication by any other journal, and all of the authors comply with the criteria needed for authorship. Any more data could be sent upon request if during the review process is needed.

We believe that the findings of our umbrella review make a valuable contribution to the current body of knowledge regarding the scientifically proven properties of ACNs coming from fruits or extracts, and would be of interest to the general as well as the specialized reader of the *Critical Reviews in Food Science and Nutrition* journal.

We look forward to your opinion as to the suitability of our manuscript for the inclusion in *Critical Reviews in Food Science and Nutrition*.

Yours sincerely,

Úrsula CATALÁN, PhD

Universitat Rovira i Virgili (URV)

Faculty of Medicine and Health Sciences

Functional Nutrition, Oxidation, and Cardiovascular Disease Research Group (NFOC-Salut)

Fundació EURECAT-Technological Center of Nutrition and Health (CTNS), Reus, Spain.

Avda/ Universitat, 1. CP/ 43204 Reus, Spain

Tel: (+34) 977 75 93 77

E-mail: ursula.catalan@eurecat.org / ursula.catalan@urv.cat

Type of the Paper: Umbrella review

The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and clinical controlled trials.

Berner-Andrée Sandoval-Ramírez [1], Úrsula Catalán [1, 2, 3 *], Elisabet Llauradó [1], Rosa-María Valls[1], Patricia Salamanca [1, 5], Laura Rubió [6], Silvia Yuste [6], and Rosa Solà [1, 3, 4].

[1] Universitat Rovira i Virgili, Faculty of Medicine and Health Sciences, Medicine and Surgery Department, Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut), Reus, Spain.

[2] Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain.

[3] Fundació EURECAT-Technological Center of Nutrition and Health (CTNS), Reus, Spain.

[4] Hospital Universitari Sant Joan de Reus (HUSJR), Reus, Spain.

[5] Grup de recerca CENIT (Grup Col·laboratiu en Estils de Vida, Nutrició i Tabaquisme), Institut Universitari d'Investigació en Atenció Primària - IDIAP Jordi Gol, Barcelona, España.

[6] Food Technology Department, Agrotecnio Research Center, University of Lleida, Av/ 78 Alcalde Rovira Roure 191, 25198-Lleida, Spain.

***Corresponding author**

Úrsula CATALÁN, PhD

Universitat Rovira i Virgili (URV)

Faculty of Medicine and Health Sciences

Functional Nutrition, Oxidation, and Cardiovascular Disease Research Group (NFOC-Salut)

Fundació EURECAT-Technological Center of Nutrition and Health (CTNS), Reus, Spain.

Avda/ Universitat, 1. CP/ 43204 Reus, Spain

Tel: (+34) 977 75 93 77

E-mail: ursula.catalan@eurecat.org / ursula.catalan@urv.cat

The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and clinical controlled trials.

Abstract:

Background: Anthocyanins (ACNs) are phenolic compounds, called flavonols, present in foods whereas ACN's health benefits remain undefined. The present umbrella review aims to bring information concerning the effects (from systematic reviews and meta-analyses (SRM) of randomized controlled trials; RCTs) and associations (from SRM of observational studies; OS') between ACNs and human health. **Methods:** Following the PRISMA methodology, the PubMed, SCOPUS, and Cochrane databases were searched up to December 1st, 2019 for OS-SRMs and RCT-SRMs regarding ACNs and different health outcomes. The RCT-SRM's risk of bias was evaluated using the AMSTAR 2, while for OS-SRMs the Joanna Briggs Institute methodology was employed. **Results:** From four OS-SRMs (including 38 studies and 1,532,282 participants), ACNs of different sources were significantly associated with the reduction of the risk of hypertension, and type 2 diabetes mellitus. From six RCT-SRMs (including 103 interventions and >2,668 participants), ACNs of different sources improved the plasmatic lipid profile, glucose metabolism, and endothelial function without significant effects on blood pressure. In contrast, no associations between ACNs and the breast, nor gastric cancer risks were found. **Conclusion:** ACNs open new pathways in the management of the glucose metabolism, plasmatic lipid profile, and the improvement of the endothelial function in humans.

Word count: 200/200

Keywords: cancer, blood pressure, cholesterol, endothelial function, diabetes, health.

Total word count: 12,224

ORCID No:

Berner Andrée Sandoval-Ramírez: 0000-0002-6242-922X

Úrsula Catalán: 0000-0001-8884-9823

Elisabet Llauradó: 0000-0002-7439-9531

Laura Rubió: 0000-0001-8973-2942

Patricia Salamanca: 0000-0002-9606-6762

Silvia Yuste: 0000-0003-0775-2179

Rosa Solà: 0000-0002-8359-235X

1. Background:

Anthocyanins (ACNs) are phenolic compounds part of the flavonol subclass (Marczylo et al. 2009). ACNs are natural plant pigments responsible for the red, pink, blue and purple colors present in the seeds, flowers, fruits, and leaves of different plants (Khoo et al. 2017). Despite ACNs are soluble and unstable molecules when in aqueous solution, ACNs exist in at least six main aglycone versions (cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin), however, the addition of either glucose, galactose, arabinose, rutinose, rhamnose or xylose in different positions of their C- or A-rings generate more than 700 different derivative glycoside structures (Fang 2014).

ACNs possess a positively charged oxygen atom as a part of their molecular structure, making them potent hydrogen-donating antioxidants (Kong et al. 2003). As a result, when in aqueous solution, ACNs are highly responsive to pH changes, shifting into a flavylium cation conformation in acid solutions (pH= 1-3), while at higher pH values (>4), ACNs change their structural conformation into a carbinol and/or chalcone form (Lila et al. 2016; Fang 2014). The structural conversions endured by ACNs modify their metabolization throughout the gastrointestinal tract due to different pH values in the gastric and intestinal lumens, as a result, changing the ACN's absorption site, and determining the ACN bioavailability and ulterior presence in the diverse body fluids and tissues (Wu, Yang, and Chiang 2018; Keppler and Humpf 2005; Fang 2014; Sandoval-Ramírez et al. 2018).

Thus, in intestinal lumen, ACNs undergo an important metabolization process by the colonic microbiota after the first 2 hours of ingestion (Keppler and Humpf 2005; Marczylo et al. 2009) often yielding metabolites not present in the original food source (Hribar and Ulrih 2014; Kamiloglu et al. 2015), increasing the diversity, concentration, tissue presence and half-life profiles of ACNs (Sandoval-Ramírez et al. 2018).

From the aforementioned characteristics of ACNs emerges their potential use for the prevention and treatment of different diseases. Hence, the benefits of ACN consumption and its impact on human health such as the prevention of cardiovascular, neurological, renal disease and other human conditions including the low-grade systemic inflammation should be compiled (Sandoval-Ramírez et al. 2018; Lila et al. 2016; Lee et al. 2017; Skrovankova et al. 2015; Marczylo et al. 2009; Lin et al. 2017).

The interpretation of the results obtained from both randomized clinical trials (RCTs) and observational studies (OS') can help determine the effectiveness, safety and health properties of whole-foods and bioactive

compounds (Faraoni and Schaefer 2016). Moreover, the combination of both, RCTs and OS', may help understand and solve questions that could not be answered by the analysis of either one type of study alone (Faraoni and Schaefer 2016). In accordance, to provide with a full summary on the available data regarding the health properties of ACNs, our work integrates the information on both the associations between ACNs and diverse health outcomes (from OS'), as well as the ACN effects on diverse cardiovascular and metabolic disease risk factors (from RCTs). Moreover, the available information on ACNs from multiple systematic and meta-analysis (SRMs) can be compiled into an umbrella review (Aromataris et al. 2015) to provide the reader with one accessible and usable document on the proven health benefits of ACNs. Therefore, the present umbrella review aims to appraise and summarize the current knowledge regarding the effects (RCT-SRMs) and associations (OS-SRMs) of ACNs on diverse aspects of human health.

2. Methods

2.1. Search Strategy and Selection Criteria

As an umbrella review, a summary of the existing SRMs on a specific subject, such as ACN health benefits from the ACN consumption in humans. Thus, an umbrella review is a way to provide decision-makers with the all known information obtained from systematically performed research, therefore organizing and assessing the evidence from various health outcomes associated with the ACN consumption (Aromataris et al. 2015).

In this sense, for the present umbrella review, our group followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methodology (http://www.prisma-statement.org/) (Moher et al. 2009). The protocol of the present umbrella review was previously registered in the University of York's International Prospective Register of Systematic Reviews (PROSPERO; https://www.crd.york.ac.uk/prospero/) with the registration number: pending.

A web-based search in three of the most important reference scientific libraries: PubMed (http://www.ncbi.nlm.nih.gov/pubmed), the SCOPUS library (www.scopus.com), and the Cochrane Library (http://www.cochranelibrary.com/) was performed. The following search term was used to retrieve all possible included articles: "(Anthocyanin OR ACN OR anthocyanins) AND ("systematic review" OR meta-analysis OR

metaanalysis OR meta analysis) AND (health OR cardiovascular OR cancer OR disease OR metabolic OR chronic OR diabetes OR illness OR mortality OR risk)".

The results were screened based on their titles, abstracts, and full texts according to the previously established inclusion as follows: 1) SRMs of RCTs regarding the effects of the consumption or supplementation of ACNs on any health outcome; 2) SRMs of OS' regarding the epidemiological associations between the ACN consumption/supplementation and any health outcome; and 3) studies published from inception up to December 1st, 2019. All non-English articles, articles presenting the results from non-clinical outcomes, articles not fulfilling the inclusion criteria, articles reporting incomplete methodology, as well as low-quality SRMs were excluded from our analysis.

2.2. Data extraction and analysis

Two independent authors extracted the published data from the main text and tables (Ú.C. and BA. S-R.), and all differences were resolved by a third author (R.S.) as recommended by the PRISMA criteria (Moher et al. 2009). The following information was extracted from the finally included articles: 1) general information including title, author, year published, and health outcome assessed; 2) type of review performed; 3) assessed population; 4) databases searched; 5) date range of database search; 6) intervention type of the reviewed articles; 7) publication date range of the included articles; 8) quality appraisal tool used for bias assessment; and 9) main conclusions. However, the extraction of the findings and results for an umbrella review ought to be limited to those results obtained directly from the results of SRMs; thus, primary study level data should not be reported in an umbrella review (Aromataris et al. 2015). Finally, for heterogeneity (I^2) assessment, the cut-off values for a low, moderate and high heterogeneity were 25%, 50%, and 75% respectively (Higgins et al. 2003).

2.3. SRM Risk of bias assessment

The potential risk of bias was independently assessed by two authors (BA.S-R. and Ú.C.) for the SRMs that met our inclusion criteria, using the critical appraisal tool for systematic reviews including randomized or non-randomized studies (AMSTAR 2) (Shea et al. 2017). The AMSTAR 2 was based on the evaluation of 16 items for which simple response categories must be fulfilled, the results rate the assessed SRMs as of high, moderate, low or critically low quality (Shea et al. 2017). Moreover, the risk of bias of the meta-analysis of

OS' was performed using the Joanna Briggs Institute risk of bias assessment (Aromataris et al. 2015). Following the PRISMA criteria, two authors (BA.S-R. and Ú.C.) reached a consensus for the risk of bias evaluation scores, all discrepancies were resolved by a third independent author (R.S.).

3. Results

3.1. Literature search, studies selection, and characteristics

From our initial screening, 57 SRMs published from inception up to December 1st, 2019 were retrieved. After assessing all titles and abstracts, 45 SRMs were excluded for not meeting the inclusion criteria, while no studies were excluded as repeated entries.

The resultant 12 SRMs were further examined, and 2 out of the 12 eligible publications were excluded due to low-quality risk of bias assessment (n=1), and incomplete results (n=1) (**Figure 1**), leading to the final inclusion of 10 SRMs (Guo et al. 2016; Godos et al. 2019; Liu et al. 2016; Ellwood et al. 2019; L. Yang et al. 2017; Hui et al. 2013; D. Y. Yang et al. 2019; Yongjian Zhu et al. 2016; Fairlie-Jones et al. 2017; Wallace, Slavin, and Frankenfeld 2016).

From the 10 SRMs finally included in the present umbrella review, 4 OS-SRMs comprising the information from 38 OS and 1,532,282 participants (1 prospective study; 1 cross-sectional and prospective studies; and 2 prospective with case-control studies) (Guo et al. 2016; Godos et al. 2019; Hui et al. 2013; D. Y. Yang et al. 2019). Moreover, as a part of the present umbrella review, 6 RCT-SRMs were included, summarizing the information from 103 RCTs and >2668 volunteers (Fairlie-Jones et al. 2017; Wallace, Slavin, and Frankenfeld 2016; Yongjian Zhu et al. 2016; Liu et al. 2016; Ellwood et al. 2019; L. Yang et al. 2017) were included. Regarding the ACN-related outcomes assessed on the included SRMs:

a) The type 2 diabetes mellitus (T2DM) risk associations with ACNs and their effects over diverse glycemic control biomarkers were assessed in 2 SRMs. The first OS-SRM including 3 prospective cohort studies (Guo et al. 2016) assessing the risk of T2DM, and 1 RCT-SRM including 32 RCTs to assess the effects of ACNs on the diverse glycemic control biomarkers (L. Yang et al. 2017).

b) The effects of ACNs on hypertension and the blood pressure levels were assessed in 3 SRMs: 1 OS-SRM including 20 OS' (15 cross-sectional trials and 7 prospective OS') (Godos et al. 2019); 1 RCT-

SRM including 6 RCTs (1 of them a cross-over) (Yongjian Zhu et al. 2016), and finally 1 RCT-SRM where 6 RCTs were included (Ellwood et al. 2019).

c) The effects of ACNs on diverse cardiovascular disease (CVD) biomarkers, such as lipids, were evaluated from 2 RCT-SRMs: 1 including 12 RCTs (Wallace, Slavin, and Frankenfeld 2016), and 1 RCT-SRM including 6 RCTs (Liu et al. 2016).

d) The effects of ACNs on the vascular function were assessed from 1 RCT-SRM including 29 RCTs (8 acute and 21 chronic interventions) (Fairlie-Jones et al. 2017).

e) Finally, the associations between the ACN dietary intake and cancer were assessed:

1- The breast cancer risk and its associations with the ACN dietary intake were evaluated from 1 OS-SRM from 12 OS' (6 prospective cohorts, 1 nested case-control, 2 population-based case-control, and 3 hospital-based case-controls) (Hui et al. 2013), while the associations between

2- The gastric cancer risk and the dietary ACN consumption were assessed from 1 OS-SRM including 6 articles (2 cohorts and 4 case-control studies) (D. Y. Yang et al. 2019).

Complete information on the general characteristics of the included OS-SRM and RCT-SRM is presented in **Table 1**.

In relation with the ACN sources, due to the diverse ACN sources (purified ACNs, n=37; fruit extracts, n=42; freeze-dried fruits, n=11; fruit juices, n=13) reported in the original RCTs assessed in the SRMs included as a part of our umbrella review, the specific source for attaining ACN's benefits could not be identified. Accordingly, the ACN results should be interpreted as the effects or associations of a given ACN dose/dietary intake regardless of its source (i.e. extracts or fruits).

3.2. Methodological quality and funding from the included studies

The AMSTAR 2 was used to assess the methodological quality of the included RCT-SRMs, all of which were selected from inception (Shea et al. 2017). As a result, 6 of the RCT-SRM included were scored as high quality (Wallace, Slavin, and Frankenfeld 2016; Fairlie-Jones et al. 2017; Yongjian Zhu et al. 2016; Liu et al. 2016; Ellwood et al. 2019; L. Yang et al. 2017), and 1 systematic review was excluded from this analysis due to a critically low score (Igwe et al. 2019).

None of the RCT-SRM included reported any relevant conflict of interest concerning their funding. Complete analysis of the AMSTAR 2 risk of bias assessment from the included RCT-SRMs is shown in **Table 2**. Moreover, the risk of bias of the compiled OS-SRM (Guo et al. 2016; Godos et al. 2019; Hui et al. 2013; D. Y. Yang et al. 2019) was assessed with the Joanna Briggs Institute risk of bias assessment tool (Aromataris et al. 2015) (**Table 3**).

3.3. The effects and associations of ACNs on human health

3.3.1. Glucose metabolism and type 2 diabetes mellitus

The associations between dietary ACN consumption and the risk of T2DM was assessed in 1 high-quality OS-SRMs of 3 prospective cohort studies published in 2012, from a total sample of 200,894 participants (12,611 diagnosed as T2DM patients) (Guo et al. 2016). As a result, the individuals consuming the highest dietary ACN intake (≈22 mg/day), estimated through food frequency questionnaires, showed a significant reduction of 15% in the risk of T2DM [relative risk (RR) = 0.85; 95% CI: 0.80, 0.91; I^2: 14.5%] (Guo et al. 2016). Moreover, it was noted that the risk of T2DM was decreased in 5% per each 7.5 mg/day increment of dietary ACN intake (RR = 0.95; 95% CI: 0.93, 0.98; I^2: 0.00%) (Guo et al. 2016). Also, significant curvilinear associations were described for the dietary intake of ACNs (P for nonlinearity = 0.006) (Guo et al. 2016).

The effects of ACNs on different glucose metabolism biomarkers were appraised in the high-quality RCT-SRM, including 1,491 volunteers (732 cardiometabolic patients / 759 controls) from 32 RCTs published between 2004 - 2016 (L. Yang et al. 2017). As a result, the supplementation with 200-400 mg/day of ACNs significantly reduced the fasting glucose levels in 0.31 mmol/L equal to 5.58 mg/dL (SMD: -0.31; 95% CI: -0.59, -0.04; I^2 = 80.7%), and also decreased the glycated hemoglobin (HbA1c) values in 0.65% (SMD: -0.65; 95% CI: -1.00, -0.29; I^2 = 72.7%) of cardiometabolic patients when compared against control subjects (L. Yang et al. 2017).

Furthermore, the ACN consumption in overweight-obese subjects significantly reduced the HOMA-IR in 0.65 units (SMD: -0.65; 95% CI: -1.23, -0.06; I^2 = 45.2%) (L. Yang et al. 2017), and the 2-h postprandial glucose values in 0.82 mg/dL (SMD: -0.82; 95% CI: -1.49, -0.15; I^2 = 77.7%). However, due to scarce data

described in RCT-SRM (L. Yang et al. 2017), the precise ACN dose needed for reduction of the 2-h postprandial glucose response was not defined (L. Yang et al. 2017).

In short, from OS', the ACN chronic dietary intake (mainly from fruits) was associated with a reduced risk of T2D, this association can be explained by the effects of ACNs from RCTs (extracts or purified ACNs), which cause the significant decrease of the fasting glucose levels, HbA1c, and HOMA-IR, as T2DM biomarkers.

3.3.2. Endothelial Function Assessment by Arterial Flow-mediated Dilation (FMD).

The effects of ACNs on the endothelial function were assessed in one high-quality RCT-SRM of 29 RCTs [Acute (n = 8), chronic (n = 21)] (Fairlie-Jones et al. 2017). The included 29 RCTs were published between 2006-2016 and conducted in adult male, female and post-menopausal female populations, aged over 18 years old, from the United Kingdom, North America, China, Korea, Italy, Australia, Greece and Israel (Fairlie-Jones et al. 2017). As a result, the acute ACN intake with doses between 1 - 724 mg/day from different sources caused a significant 3.92% improvement in the arterial FMD [standard mean deviation (SMD): 3.92%, 95% CI: 1.47-6.38, P= 0.002, I^2= 91.8%]. In the same way, the chronic ACN consumption from diverse sources significantly improved the arterial FMD (SMD: 0.84%, 95% CI: 0.55-1.12, P < 0.001; I^2= 62.5%) (Fairlie-Jones et al. 2017).

Finally, the acute ACN supplementation significantly enhanced the pulse wave velocity in -1.27 m/s (SMD: -1.27, 95% CI: -1.96, -0.58, P < 0.001; I^2 = 17.8%), and increased the vascular reactivity in 141% (SMD: 2.41, 95% CI: -0.91, -3.91, P= 0.002; I^2 = 92.6%) (Fairlie-Jones et al. 2017). These vascular benefits were noted equally for healthy and non-healthy populations including obese, overweight and hypertensive subjects (Fairlie-Jones et al. 2017). No significant differences were noted for the arterial augmentation index, a measure of systemic arterial stiffness (Fairlie-Jones et al. 2017). Hence, ACNs (mostly as extracts or purified ACNs) significantly improve the endothelial function in both healthy and non-healthy subjects such as obese, overweight and hypertensive subjects.

3.3.3. Blood pressure and hypertension

In the OS-SRM of 20 OS' [cross sectional (n = 15), prospective cohort (n = 7)], published in 2002 – 2018, the association between the dietary ACN intake and the occurrence of hypertension was assessed from 200,256

participants (45,732 cases of hypertension). As a result, the dietary ACN intake was significantly associated with an 8% reduction in the risk of hypertension, when comparing the highest against the lowest dietary exposure to ACNs (RR: 0.92, 95% CI: 0.88, 0.97; $I^2 = 74\%$) (Godos et al. 2019).

However, the effects of ACNs on blood pressure and other CVD biomarkers were assessed in the RCT-SRM from 12 RCTs published between 2005 – 2014 (Wallace, Slavin, and Frankenfeld 2016), while no significant effects of the ACN interventions and the participants' blood pressure values were noted for doses between 19.2 - 640.0 mg/day (Wallace, Slavin, and Frankenfeld 2016). Interestingly, no adverse effects of anthocyanins were reported across studies at levels up to 640 mg/day (Wallace, Slavin, and Frankenfeld 2016). Nonetheless, one of the RCTs included in the RCT-SRM an 85 mg/day dose of a flavonoid-rich chokeberry extract (*Aronia melanocarpa*; 25% ACNs) or a placebo were added to the statin therapy of post-myocardial infarction patients for 6 weeks resulting in significant decreases of both the systolic and diastolic blood pressure values (Naruszewicz et al. 2007; Wallace, Slavin, and Frankenfeld 2016).

Accordingly, in an RCT-SRM from 6 clinical studies [RCTs (n = 5), crossover (n = 1)], including 472 post-menopausal, light smokers or healthy participants (China, England, Norway and Italy), ACN doses between 162-640 mg/day had no effects over the systolic blood pressure (Weighted Mean Difference (WMD): 1.15 mmHg, 95% CI: -3.17, 5.47; $I^2 = 56\%$), nor the diastolic blood pressure were identified (WMD: 1.06 mmHg, 95% CI: -0.71, 2.83; $I^2 = 0.00\%$) (Yongjian Zhu et al. 2016).

Additionally, in another RCT-SRM the effects of the flavonoid-rich fruit intake, compared the administration of any type of flavonoid-rich fruit or equivalent supplement, including ACNs, against a placebo or other interventions in 119 adult subjects with hypertension from 3 RCTs published between 2007 - 2016 (Ellwood et al. 2019). The subgroup analysis showed no significant effects between the ACN intake and the systolic or diastolic blood pressure values (MD: 0.96, 0.96, 95% CI: 3.22, 1.30; P = 0.41, $I^2 = 0.00\%$), when compared against a placebo (Ellwood et al. 2019). In accordance, no significant effects were observed on the systolic or diastolic blood pressure ($p>0.05$) (L. Yang et al. 2017).

Therefore, despite the positive association between the chronic intake of ACNs and a decreased risk of hypertension found in OS', no effects of the ACNs (mostly as extracts or purified ACNs) on hypertension were determined from the RCTs. This discrepancy between the effects and associations of ACNs and hypertension

might be explained by undetermined confounding factors reducing the risk of hypertension; moreover, other food ingredients could determine the effects of ACNs on the blood pressure.

3.3.4. Plasmatic lipid profile

The effects of ACNs on the lipid profile of humans were assessed in 3 RCT-SRMs (Wallace, Slavin, and Frankenfeld 2016; L. Yang et al. 2017; Liu et al. 2016).

In the systematic review of 12 RCT articles, representing 10 studies describing the effects of ACNs on diverse CVD biomarkers were assessed in European (n = 5), Chinese (n = 2), Iranian (n = 2), and Mexican (n = 1) populations (Wallace, Slavin, and Frankenfeld 2016). As a result, the consumption of ACN doses between 19.2 - 640.0 mg/day, demonstrated significant decreases of ≈ 14.08% in the values of plasmatic low-density lipoprotein cholesterol (LDLc), notably all 4 RCTs reporting positive results were conducted on hyperlipidemic (n=3) and dyslipidemic populations (n=1), while healthy populations did not report significant changes in LDLc (Wallace, Slavin, and Frankenfeld 2016). The different responses of LDLc blood concentration in hypercholesterolemic or healthy populations could be explained by the higher baseline LDLc levels in hypercholesterolemic patients.

Moreover, from another RCT-SRM, the effects of ACN supplementation with the same doses also showed significant increases of ≈11.79% in the high-density cholesterol (HDLc) plasmatic levels in hyperlipidemic subjects (n = 2), dyslipidemic (n =1), healthy individuals (n = 2), metabolic syndrome patients (n = 1), and in pre-hypertensive patients (n = 1) (Wallace, Slavin, and Frankenfeld 2016). Finally, ACNs significantly reduced the TC values between 5.86 - 25.53% ($p<0.01$), in patients with metabolic syndrome (n=1) or hyperlipidemia (n=2) (Wallace, Slavin, and Frankenfeld 2016).

In addition, the cholesterol lowering properties of ACNs were assessed in the RCT-SRM of 586 dyslipidemic subjects coming from 6 RCTs (Liu et al. 2016). As a result, when compared against the placebo group, the consumption of ACN doses of 90 - 320 mg/day for 4 - 24 weeks significantly reduced the plasmatic levels of total cholesterol (TC) in 24 mg/dL (Mean difference (MD): -24.06, 95% CI: -45.58, -2.64 mg/dL; I^2 = 93%), of triglycerides in 26.14 mg/dL (TG; MD = -26.14, 95% CI: -40.20, -3.08 mg/dL; I^2 = 66%), and of LDLc in 22.10 mg/dL (MD: -22.10, 95% CI: -34.36, -9.85; mg/dL; I^2 = 61%), while increasing the HDLc in 5.58 mg/dL (MD: 5.58, 95% CI: 1.02, 10.14 mg/dL; I^2 = 90%) (Liu et al. 2016).

These ACN effects were particularly true for Iranian [MD: -50.58, 95% CI: -86.52, -14.64 mg/dL, I^2 = 89%) and Chinese (MD: -6.59, 95% CI: -12.44, -0.73 mg/dL, I^2 = 1%) populations (Liu et al. 2016).

Moreover, in a high quality RCT-SRM of 27 trials, the effects of ACNs over the glycemic control and plasmatic lipids were assessed from 1491 volunteers (732 cardiometabolic patients) (L. Yang et al. 2017). As a result, the intake of 200 - 400 mg/day of ACNs, from diverse sources, was associated with the decrease of TC in 0.33 mmol/L [(12.76 mg/dL) SMD: -0.33; 95% CI: -0.62, -0.03; I^2 = 86.9%], and of LDLc in 0.35 mmol/L [(13.53 mg/dL) SMD: -0.35; 95% CI: -0.66, -0.05; I^2 = 85.2%], while marginally increasing HDLc values in 0.24 mmol/L [(9.28 mg/dL) (SMD: 0.24; 95% CI: 0.00, 0.49; I^2 = 81.1%)] (L. Yang et al. 2017). Moreover, the TG, ApoA1 and ApoB plasmatic values were not significantly modified (L. Yang et al. 2017).

The information available on the effects of ACN intake (mostly as extracts or purified ACNs) on the plasmatic lipids showed a reduction in the TG, TC, and LDLc while increasing the HDLc plasmatic values.

3.3.5. Cancer

The associations between the dietary ACN intake and cancer were assessed in 1 moderate- (Hui et al. 2013), and 1 high-quality OS-SRM (D. Y. Yang et al. 2019). In the first OS-SRM, the associations between the ACN dietary intake and the breast cancer risk were assessed from 9,513 breast cancer patients and 181,906 controls involved in 12 OS' [prospective cohort (n = 6), nested case-control (n=1), population-based case-control (n = 2), hospital-based case-control (n = 3)], published between 1997 - 2010 (Hui et al. 2013). As a result, there were no significant associations between the ACN dietary intake and the breast cancer risk (Relative Risk (RR) = 0.97, 95% CI: 0.87, 1.08) (Hui et al. 2013).

Additionally, the associations between the ACN dietary intake and the gastric cancer risk were assessed in 949,226 patients and controls from 6 OS' [cohort (n = 2), case-control (n = 4)], published between 2004 - 2017 (D. Y. Yang et al. 2019). As a result, no significant associations between the dose-response relationship was not found in this OS-SRM (RR = 0.92, 95% CI: 0.81, 1.04) (D. Y. Yang et al. 2019). Additionally, no significant associations were found for the linear or the non-linear dose-response of ACNs in the subgroup analysis by gender (men: RR = 1.02, 95% CI: 0.73, 1.40; women: RR = 0.80, 95% CI: 0.52, 1.23), nor for tumor location (cardia: RR = 0.90, 95% CI: 0.62, 1.31; non-cardia: RR = 0.86, 95% CI: 0.69, 1.07) (D. Y. Yang et al. 2019).

Thus, the ACN dietary intake showed no significant associations with breast cancer nor with the gastric cancer risks.

4. Discussion

In the present umbrella review of 10 SRMs about ACNs, from four OS-SRMs (38 studies and 1,532,282 participants), ACNs of different sources were significantly associated with the reduction of the risk of hypertension, and type 2 diabetes mellitus. From 6 RCT-SRMs (including 103 interventions and >2,668 participants), ACNs mainly from extracts improved the plasmatic lipid profile, glucose metabolism, and endothelial function without significant effects on blood pressure.

In consequence, the information obtained from OS-SRMs and RCT-SRMs provides new perspectives in the management of cardiometabolic diseases in humans.

4.1. ACNs, glucose metabolism and T2DM

T2DM is characterized by insulin resistance and high blood sugar with diverse long-term complications including coronary heart disease, stroke, kidney failure and reduced blood flow to the extremities (Brunton 2016). In recent years, T2DM has considerably increased its prevalence among the general population, particularly in younger individuals, contributing to longer disease exposure and an increased risk of complications with numerous adverse effects (Lascar et al. 2018). As a result, the prevention and treatment of T2DM acquired significant interest from the scientific and medical communities. In accordance, an umbrella analysis on the effects of ACNs on glycemic control and the associations between ACNs over the risk of T2DM was conducted. As a result, the OS-SRM (200,894 participants), suggests that individuals consuming the highest dietary intake of ≈ 22 mg/day of ACNs from different sources have a 15% reduction in the risk of T2DM (Guo et al. 2016). Moreover, an additional 5% reduction in the risk of T2DM has been noted per each increment of 7.5 mg/day on the dietary ACN intake in the prospective OS' (Guo et al. 2016).

The reduction of the T2DM risk from the ACN intake is in accordance with the information retrieved from the RCT-SRM regarding the effects of ACNs (mostly as extracts or purified ACNs) on diverse glucose metabolism biomarkers, where the ACN supplementation with doses between 200 - 400 mg/day significantly reduced the fasting glucose levels in 5.58 mg/dL and of Hb1Ac in 0.65% (L. Yang et al. 2017).

Furthermore, ACN consumption significantly decreased the HOMA-IR, an index used to express the level of insulin resistance (Shashaj et al. 2016), by 0.65 HOMA units in overweight-obese subjects (L. Yang et al. 2017). The ACN glucose metabolism regulation assessed by *in vitro* studies performed on HepG2 cells have demonstrated that ACNs promoted the glycogen synthesis and the reduction of gluconeogenesis (Yan et al. 2016). The reduction of gluconeogenesis, can be explained by a decreased activity of the phosphoenolpyruvate carboxykinase and the glucose-6-phosphatase enzymes, secondary to the activation of PPARγ in HepG2 cells from ACNs (Yan et al. 2016; Yan, Dai, and Zheng 2016) and adipocytes (Choi et al. 2017; Luna-Vital, Weiss, and Gonzalez de Mejia 2017). Consequently, ACNs seem to be able to increase the cellular glucose intake and reduce insulin resistance by adipocytes and hepatic cells. Moreover, in a human intestinal Caco-2 cells model, ACNs downregulated the expression of the GLUT2 transporter, possibly explaining the diminished intestinal glucose absorption and the postprandial glycemia (Alzaid et al. 2013; Luna-Vital, Weiss, and Gonzalez de Mejia 2017).

Finally, ACNs significantly reduce the risk and aid in the management of T2DM in humans. Moreover, since SRMs are considered the highest level of scientific evidence (Murad et al. 2016), the analysis of the information from both OS'-SRMs and RCT-SRMs evidenced, for the first time, a summary of the effects from ACNs which act as the possible mechanism of action for the T2DM risk reduction that is associated with the chronic ACN dietary intake.

4.2. ACNs and the Arterial Flow-mediated Dilation Functional Assessment.

So far, the effects of ACNs on the endothelial function have been assessed in one high-quality RCT-SRM. As a result, the acute ACN supplementation with doses between 1 - 724 mg/day significantly improved the arterial FMD in 3.92%, and reduced the pulse wave velocity in 1.27 m/s, while also improving the vascular reactivity (Fairlie-Jones et al. 2017).

On the other hand, the chronic ACN intake revealed a smaller, however significant, improvement of the arterial FMD of 0.84%, in both healthy and non-healthy populations (Fairlie-Jones et al. 2017). It has been suggested that the effects observed after the acute intake of ACNs (mostly as extracts or purified ACNs) might be related to the plasmatic presence of vanillic and benzoic acids on the first 2 hours after ACN consumption,

while the effects after 6 hours might be related to the added presence of hippuric and homovanillic acids (Rodriguez-Mateos et al. 2013).

Moreover, the effects on the vascular function improvement by the ACN intake appears related to the regulation in the expression of diverse proteins involved in the nitrous oxide (NO) metabolism, in consequence enhancing the endothelial function (Rodriguez-Mateos et al. 2013; Speciale et al. 2014). The NO-related regulation by ACNs is also linked to the prevention of the peroxynitrite-mediated endothelial dysfunction by cyanidin-3-glucoside, an ACN metabolite, as demonstrated in a human umbilical vein endothelial cell model (Speciale et al. 2014). Additionally, ACNs reduced the expression of diverse adhesion molecules in endothelial cells, leading to lesser monocyte presence, monocyte activity and inflammation in mice (Rodriguez-Mateos et al. 2013). As a result, the ACN's mechanisms described explain the observed effects of ACNs in the significant improvement of the endothelial function in humans.

4.3. ACNs and blood pressure

Only the OS-SRMs have demonstrated associations between the chronic ACN intake and a reduced risk of hypertension (Godos et al. 2019). As a result of the present umbrella review, the ACN supplementation with doses between 162 - 640 mg/day showed no significant effects on the reduction of either the systolic nor of the diastolic blood pressure values neither in post-menopausal women, light smokers nor in healthy subjects (Yongjian Zhu et al. 2016). However, a noteworthy exception exists for patients after an acute myocardial infarction as reported in an RCT which was analyzed in one of the RCT-SRMs (Yongjian Zhu et al. 2016) which is included in present umbrella reviews (Naruszewicz et al. 2007). Where the combined therapy of statins supplemented with a flavonoid-rich extract (25% ACNs) in a group of post-myocardial infarction patients resulted in significantly lower (-11 mmHg; $p<0.03$) systolic pressure values when compared against a statin only treated group (-7.2 mmHg; $p<0.03$) (Naruszewicz et al. 2007). Nonetheless, the same effects for ACNS were not noted in any other of the assessed populations.

From current evidence, the ACN effects (from RCTs) on the blood pressure reduction do not coincide with the beneficial association of the ACN dietary intake and a lower hypertension risk. The discrepancies between the observational and experimental results might be due to multiple causes. First, the inherent limitations and biases related to the data recollection and design in food frequency questionnaires used in OS'.

Second, there might exist inadvertent and unanalyzed confounding variables that could explain a lesser risk of hypertension in individuals who are also consuming larger amounts of ACN-rich fruits (Rodrigo et al. 2015; Béjar and Vázquez-Limón 2017). Third, the observed effects of ACNs in the RCT-SRMs might come from a higher ACN extract intake for relatively short periods, rather than lower ACN doses consumed as a part of the dietary intake from participants in the OS-SRMs. And finally, the observed ACN associations with blood pressure might be related to the chronic intake of other bioactive compounds also linked to the ACN-rich fruits, such as fiber, influencing the associations between ACNs and the risk of hypertension (Streppel et al. 2005). The above-mentioned inconsistencies between OS' and RCTs also represent a void in the current body of knowledge as well as a research opportunity regarding the possible effects of ACNs on blood pressure and hypertension.

4.4. ACNs and the plasmatic lipid profile

The ACN effects from doses between 200 - 400 mg/day (mostly from extracts or purified ACNs) have demonstrated to significantly decrease the plasmatic concentrations of TC in 12.76 mg/dL, of LDLc in 13.53 mg/dL mg/dL, and of TG in 26.14 mg/dL, as well as the increase of HDLc plasmatic values in 9.28 mg/dL, observed not only for hyperlipidemic subjects, in individuals suffering from metabolic syndrome, and in pre-hypertensive patients but also, in a lesser magnitude in healthy volunteers.

The possible ACN mechanisms of action on the lipid metabolism can be related to: The improvement in the reverse cholesterol transport and the HDL particle functionality beyond the simple increase in the HDLc concentrations by ACNs, as previously reviewed (Millar, Duclos, and Blesso 2017). Additionally, ACNs enhance the cholesterol efflux in HDL particles, inhibiting the cholesteryl ester transfer protein (CETP) in dyslipidemic subjects (Liu et al. 2016). Also, ACNs improve the paraoxonase-1 activity in HDL particles leading to increased HDL functionality in hypercholesterolemic subjects (Yanna Zhu et al. 2014) and reduce the aortic cholesterol levels as proven in a hyperlipidemia mice model (Farrell et al. 2015). In *in vitro* assays, diverse ACN metabolites, predominantly cyanidin-3-glucoside and peonidin-3-glucoside, demonstrated the significant suppression of the cholesterol uptake in Caco-2 cells *via* competitive inhibition, therefore increasing the luminal precipitation of cholesterol (Yao et al. 2013), and explaining the higher fecal ACN excretion and lower plasmatic cholesterol values observed in hamsters (Liang et al. 2013).

Finally, in mice, cyanidin-3-glucoside, significantly inhibited the aortic sinus plaque formation, reduced hypercholesterolemia, promoted the fecal bile acid excretion and upregulated hepatic cholesterol 7a-hydroxylase expression (CYP7A1) in mice (Wang et al. 2012).

Thus, ACN intake produces a more beneficial lipid profile and improves the HDL functionality in humans.

4.5. ACNs and Cancer

The present umbrella review analyzed the associations between the ACN dietary intake and the cancer risk in humans from two high-quality OS-SRMs of cohort studies. As a result, the ACN dietary intake was not associated with a decreased risk of breast cancer in a sample of 191,419 participants from 12 OS' (Hui et al. 2013), nor of gastric cancer in a sample of 949,226 participants from 6 OS' (D. Y. Yang et al. 2019). Thus, so far, ACNs are not related to a reduced risk of developing gastric nor breast types of cancer.

On the other hand, despite the lack of positive results in the SRM from OS', ACNs have shown promising results for the prevention of breast cancer in previous animal and cellular models discussed below.

However, *in vitro* models, ACNs have been related to the inhibition of the HER-2-positive breast cancer epithelial-mesenchymal transition-mediated metastasis by suppressing FAK signaling (Zhou et al. 2017), and have also the ability to promote the apoptosis of triple-negative breast cancer cells (Wang et al. 2016). Additionally, the oral administration of 150 mg/kg/day of ACNs to nude mice reduced the tumor growth, inhibited the pulmonary metastasis and decreased the occurrence of tumoral nodules (Luo et al. 2014). Besides, ACNs might have a protective role in the onset of other cancer types such as prostate cancer, the *in vitro* evidence demonstrates that ACNs induce the apoptosis of DU-145 cells while inhibiting the xenograft growth of prostate cancer (Ha et al. 2015), causing the apoptosis and cell differentiation of the prostate cancer cells (Sorrenti et al. 2015).

Additionally, ACNs have also shown potential in the prevention of colon cancer, where ACNs significantly increase the activation of the apoptotic pathway through the activation of caspase-3 and the inactivation of diverse transcription factors (Forester et al. 2014). Accordingly, in animal experiments, mice consuming 10% of ACNs showed a significantly lower number of colon tumors when compared against the control and 1% ACN-supplemented mice (Lippert et al. 2017).

Furthermore, the ACNs and ACN-metabolites present in the plasma of healthy volunteers have shown to inhibit the pancreatic cancer cell migration (Kuntz, Kunz, and Rudloff 2017), therefore deserving further attention as a potential bioactive phytochemicals in cancer prevention.

On the other hand, ACNs have demonstrated the potential to reduce the cancer progression by inducing apoptosis and autophagy of thyroid cancer affected cells *in vitro* (Long et al. 2018).

Despite the discouraging findings arising from the results of our umbrella review, the potential beneficial associations of ACNs on the risk of other types of cancer, such as the prostate or colon cancer, have not been addressed in high-quality SRMs so far, thus representing an ongoing opportunity for future research in the field.

5. Conclusions

The information obtained from SRMs including 38 OS', gathering more than 1.5 million participants, revealed that ACNs provided by fruits are associated with the reduction of the T2DM and hypertension risks while showing no association with the breast nor gastric cancer risk.

Additionally, the umbrella review of the SRM of 103 RCTs regarding the effects of the ACN supplementation (mostly as extracts or purified ACNs) to >2668 volunteers demonstrated the ACN consumption significantly reduces the fasting glucose levels, the Hb1Ac, the HOMA-IR, and the plasmatic values of TC, triglycerides, and LDLc, while increasing the HDLc values. Moreover, ACNs showed a significant effect on increasing the arterial FMD, the pulse wave velocity, and vascular reactivity.

For the first time, the possible mechanism of action for the T2DM risk reduction associated with the chronic ACN dietary intake has been provided from the analysis of both OS'- and RCT-SRMs.

The current evidence demonstrated that ACNs, regardless of their source, have no effects on the systolic blood, nor the diastolic blood pressure.

The precise dose needed to attain most of the benefits related to the ACNs consumption could not be acquired from the current evidence due to scarce information; however, doses between 200 - 400 mg/day seem to provide significant health benefits, particularly for cardiometabolic health. The determination of the precise sources of ACNs (i.e. fruits, extracts, juices, etc.) to attain their related benefits could not be determined therefore, the positive effects and associations of ACNs are related to their total intake amount regardless of their source.

Finally, the chronic ACN dietary intake, regardless of its source, aids in the prevention of T2D and hypertension, while the ACN-rich extract or purified ACN supplementation should be considered in the management of glucose metabolism, hypercholesterolemia, and the improvement of the endothelial function in humans.

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7. Declaration of interests

The authors declare no conflicts of interest.

8. Contributions

B.A.S-R., Ú.C., S.Y., P.S., and R.S. were responsible for study conception and design. B.A.S-R. and Ú.C. acquired the data. B.A.S-R., Ú.C., and E.L. analyzed and interpreted the data. B.A.S-R., Ú.C., and R.S. drafted the manuscript. Ú.C., R.S., L.R., and E.L. performed a critical revision of the manuscript.

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Zhu, Yongjian, Yacong Bo, Xi Wang, Wenjie Lu, Xule Wang, Zhanying Han, and Chunguang Qiu. 2016. "The Effect of Anthocyanins on Blood Pressure." *Medicine (United States)* 95 (15): e3380. https://doi.org/10.1097/MD.0000000000003380.

Table 1. General characteristics of the included systematic reviews and meta-analysis.

Title	Author and year	Type of review	Outcome/s	Population	Databases	Data range	N =	Intervention type	Participants involved in meta-analysis [Total (participants/cases)]	The publication date range of the included studies	Quality appraisal tool used	ACN Dose/Intake needed for outcome	Reported outcomes
Type 2 diabetes mellitus and glucose metabolism													
Associations of dietary intakes of anthocyanins and berry fruits with risk of type 2 diabetes mellitus: a systematic review and meta-analysis of prospective cohort studies.	(Guo et al. 2016)	Systematic review and meta-analysis	Type 2 Diabetes Mellitus risk	General population / Diabetic subjects	Cochrane Library, Embase, PubMed.	Inception - January 2016	3	Cohort (n=3)	200,894 / 12,611	2012	Newcastle-Ottawa Scale	≈22 mg/day	Dietary anthocyanin consumption (≈22 mg/day) is associated with a 15% reduction of T2DM risk (summary RR = 0.85; 95% CI: 0.80, 0.91). Significant curvilinear associations were found for the dietary intake of anthocyanins (P for nonlinearity =

												0.006). The risk of T2DM was decreased by 5%, per each 7.5 mg/day increment of dietary anthocyanin intake (RR = 0.95; 95% CI: 0.93, 0.98). Anthocyanins significantly reduced fasting glucose (SMD: -0.31; 95% CI: -0.59, -0.04; I2 = 80.7%), 2-h postprandial glucose (SMD: -0.82; 95% CI: -1.49, -0.15; I2 = 77.7), glycated hemoglobin
Effects of anthocyanins on cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials.	(L. Yang et al. 2017)	Systematic review and meta-analysis	Glycemic regulation and lipid profile	Healthy population / cardiometabolic patients	MEDLINE, Embase, Cochrane database, OVID EBM Reviews, clinicaltrials.gov.	Inception - February 2017	32	RCTs (n=32)	1,491 (759 / 732)	2004 - 2016	Cochrane risk of bias tool	N/A

												(SMD: -0.65; 95% CI: -1.00, -0.29; I2 = 72.7%).
Endothelial function												
The effect of anthocyanin-rich foods or extracts on vascular function in adults: a systematic review and Meta-analysis of randomized controlled trials.	(Fairlie-Jones et al. 2017)	Systematic review and meta-analysis	Vascular function	United kingdom, North America, China, Korea, Italy, Australia Greece and Israel Adults over 18 years old	EMBASE, MedLine, Cochrane, CINAHL, Scopus.	Inception - June 2017	29	Acute RCT (n=8), chronic RCT (n=21)	NR	2006 - 2016	Jadad scoring system	1 - 724 mg/day	Anthocyanin consumption was significantly associated to an improvement in the flow-mediated dilation after the acute (SMD: 3.92%, 95% CI: 1.47-6.38, *p*= 0,002; I2= 91.8%) and the chronic supplementation (SMD: 0.84%, 95%

													CI: 0.55-1.12, P=0.000; I2=62.5%). Moreover, the acute anthocyanin supplementation is significantly associated to a pulse wave velocity improvement (SMD=-1.27 m/s, 95% CI: -1.96, -0.58, P=0,000; I2=17.8%)
Blood pressure and hypertension													
Dietary polyphenol intake, blood pressure, and hypertension: a systematic review and meta-analysis of observational studies.	(Godos et al. 2019)	Systematic review and meta-analysis	Blood pressure and hypertension	General population/hypertensive subjects ranging 40 - 70 years-old	PubMed, Embase.	Inception - April 2019	20	Cross sectional (n=15), Prospective Cohort (n=7)	200,256 / 45,732	2002 - 2018	Funnel plot analysis	"Highest dietary intake"	When compared the highest vs. Lowest exposures, the dietary anthocyanin intake was associated with an 8% reductio

Systematic review of anthocyanins and markers of cardiovascular disease.	(Wallace, Slavin, and Frankenfeld 2016)		Cardiovascular disease markers	European, Mexican, Chinese, and Iranian population	PubMed, Web of Science, BIOSIS	Inception - July 2014	12	RCT (n=12)	NA	2005 - 2014	Scottish Intercollegiate Guidelines Network (SIGN) grading system	19.2 - 640.0 mg/day	n in the risk of hypertension, (RR: 0.92, 95% CI: 0.88, 0.97). No significant effects of the ACN interventions and the participants' blood pressure values were noted (p>0.05). There was no significant association between the anthocyanin supplementation (162 mg/d - 640 mg/d) and systolic blood pressure (WMD: 1.15 mmHg, 95%
The effect of anthocyanins on blood pressure: A PRISMA-Compliant Meta-Analysis of Randomized Clinical Trials.	(Yongjian Zhu et al. 2016)	Systematic review and meta-analysis	Blood Pressure	Chinese, English, Norwegian and Italian populations	PubMed, Web of Science, Wanfang Database, China National Knowledge Infrastructure.	Inception - 2015	6	RCTs (n=5), cross-over (n=1)	472	2008 - 2015	Cochrane risk of bias tool	162 - 640 mg/day	

Effects of flavonoid-rich fruits on hypertension in adults: a systematic review.	(Elwood et al. 2019)	Systematic review and meta-analysis	Hypertension	Hypertensive subjects	MEDLINE, Embase, Cochrane Trials (CENTRAL), CINAHL.	Inception - September 2018	3	RCTs (n=3)	119 / 119	2007 - 2016	Critical appraisal tools from the Joanna Briggs Institute	N/A	CI: -3.17, 5.47; I2=56%) nor in diastolic blood pressure (WMD: 1.06 mmHg, 95% CI: -0.71, 2.83; I2=0%). Subgroup analysis by type of flavonoids demonstrated no significant difference in the systolic blood pressure among those who received anthocyanins versus those who received placebo (MD=0.96, 0.96,

													95% CI: 3.22, 1.30; P=0.41, I2=0%).
Effects of anthocyanins on cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials.	(L. Yang et al. 2017)	Systematic review and meta-analysis	Glycemic regulation and lipid profile	Healthy population / cardiometabolic patients	MEDLINE, Embase, Cochrane database, OVID EBM Reviews, clinicaltrials.gov.	Inception - February 2017	32	RCTs (n=32)	1,491 (759 / 732)	2005 - 2016	Cochrane risk of bias tool	N/A	No significant effects were observed on the systolic or diastolic blood pressure (p>0.05).
Plasmatic lipid profile													
Systematic review of anthocyanins and markers of cardiovascular disease.	(Wallace, Slavin, and Frankenfeld 2016)	Systematic review and meta-analysis	Cardiovascular disease markers	European, Mexican, Chinese, and Iranian population	PubMed, Web of Science, BIOSIS	Inception - July 2014	12	RCT (n=12)	NA	2005 - 2014	Scottish Intercollegiate Guidelines Network (SIGN) grading system	19.2 - 640.0 mg/day	After the systematic review, the anthocyanin interventions suggest significant decreases of LDLc in hyperlipidemic populations, however not in healthy individ

uals or other cardiovascular-related diseases. Moreover, HDLc showed statistically significant increases in hyperlipidemic subjects, healthy individuals, individuals with metabolic syndrome and prehypertensive subjects. Finally, ACNs significantly reduced the TC values between 5.86 - 25.53% in patients with metabolic syndrome, and in

													patients with dyslipidemia.
	Effects of anthocyanin on serum lipids in dyslipidemia patients: a systematic review and meta-analysis.	(Liu et al. 2016)	Systematic review and meta-analysis	Serum lipids	Dyslipidemic subjects	PubMed, Web of Science, MEDLINE, Cochrane Library, China National Knowledge Infrastructure, Wanfang Database.	Inception - July 2015	6	RCTs (n=6)	586 / 586	2008 - 2014	Cochrane risk of bias tool	90 - 320 mg/day	In dyslipidemic patients, the anthocyanin supplementation significantly reduced the levels of TC (MD = -24.06, 95% CI: -45.58, -2.64 mg/dL; I2 = 93%) of TG (MD = -26.14, 95% CI: -

40.20, -3.08 mg/dL; I2 = 66%), of LDLc (MD= -22.10, 95% CI: -34.36, -9.85; mg/dL; I2 = 61%), and significantly increased the levels of the HDLc (MD = 5.58, 95% CI: 1.02, 10.14 mg/dL; I2 = 90%) when compared against the placebo group.

Effects of anthocyanins on cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials.	(L. Yang et al. 2017)	Systematic review and meta-analysis	Glycemic regulation and lipid profile	Healthy population / cardiometabolic patients	MEDLINE, Embase, Cochrane database, OVID EBM Reviews, clinicaltrials.gov.	Inception - February 2017	32	RCTs (n=32)	1,491 (759 / 732)	2005 - 2016	Cochrane risk of bias tool	N/A	As a result, the intake of 200 - 400 mg/day of ACNs, from diverse sources, was associated with the decrease of TC in 0.33 mmol/L [(12.76 mg/dL) SMD: -0.33; 95% CI: -0.62, -0.03; I^2 = 86.9%], and of LDLc in 0.35 mmol/L [(13.53 mg/dL) SMD: -0.35; 95% CI: -0.66, -0.05; I^2 = 85.2%], while marginally increasing HDLc values in 0.24 mmol/L

												[(9.28 mg/dL) (SMD: 0.24; 95% CI: 0.00, 0.49; I^2 = 81.1%)]. Moreover, the TG, ApoA1 and ApoB plasmatic values were not significantly modified.
Cancer												
Flavonoids, flavonoid subclasses and breast cancer risk A meta-analysis of epidemiologic studies.	(Hui et al. 2013)	Meta-analysis	Breast cancer risk	NR	Cochrane Library, MEDLINE, Embase, PubMed.	Inception - July 2012	12	Prospective cohort (n=6), nested case-control (n=1), population-based case-control (n=2), hospital-based case-control (n=3)	9,513 / 181,906	1997-2010	Funnel plot analysis	N/A	No significant associations for anthocyanins and breast cancer risk were observed (RR= 0.97, 95% CI: 0.87, 1.08).

Intake of Anthocyanins and Gastric Cancer Risk: A Comprehensive Meta-Analysis on Cohort and Case-Control Studies.	(D. Y. Yang et al. 2019)	Systematic review and meta-analysis	Gastric cancer risk	NR	PubMed, EMBASE, Web of Science, Cochrane Library.	Inception - June 2018	6	Cohort (n=2), case-control (n=4)	949,226	2004 - 2017	GRADE system	1 mg/day	There was no significant association between the anthocyanin intake and the gastric cancer risk (RR=0.92, 95%CI: 0.81, 1.04). Moreover, there is no significant evidence of an association by subgroup analysis for gender (men: RR=1.02, 95% CI: 0.73, 1.40; women: RR=0.80, 95% CI: 0.52, 1.23) and tumor location (cardia: RR=0.90, 95% CI:

											0.62, 1.31; non-cardia: RR= 0.86, 95% CI: 0.69, 1.07) was observed nor for the linear and nonlinear dose-responses.	

Note: N/A, not available; RCT, randomized controlled trial; TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; MD, mean difference; SMD, standard mean deviation; RR, relative risk; WMD, weighted mean difference; CINAHL, cumulative index to nursing and allied health.

Table 1. General characteristics of the included systematic reviews and/or meta-analysis.

Title	Author	Year	Type of review	Outcome/s	Population	Databases	Data range	N=	Intervention type	Participants involved in meta-analysis [Total (participants/cases)]	The publication date range of the included studies	Quality appraisal tool used
Type 2 diabetes mellitus and glucose metabolism												

1.Associations of dietary intakes of anthocyanins and berry fruits with risk of type 2 diabetes mellitus: a systematic review and met a-analysis of prospective cohort studies.	Guo, X. *et al.*	2016	Systematic review and meta-analysis	Type 2 Diabetes Mellitus risk	General population / Diabetic subjects	Cochrane Library, Embase, PubMed.	Inception - January 2016	4	Cohort (n=4)	200,894 / 12,611	2012 - 2013	Newcastle-Ottawa Scale	Dietary anthocyanin associated with a 15 risk (summary RR = 0.91). Significant cu were found for the di anthocyanins (P for t The risk of T2DM w each 7.5 mg/day incr anthocyanin intake (1 0.93, 0.98).
2. Effects of anthocyanins on cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials.	Yang, L. *et al.*	2017	Systematic review and meta-analysis	Glycemic regulation and lipid profile	Healthy population / cardiometabolic patients	MEDLINE, Embase, Cochrane database, OVID EBM Reviews, clinicaltrials.gov.	Inception - February 2017	32	RCTs (n=32)	1,491 (759 / 732)	2005 - 2016	Cochrane risk of bias tool	Anthocyanins signifi glucose (SMD: -0.31 I2 = 80.7%), 2-h pos (SMD: -0.82; 95% C 77.7), glycated heme 95% CI: -1.00, -0.29 (SMD: -0.33; 95% C 86.9%), and LDLc (S 0.66, -0.05; I2 = 85.2 were observed on bl inflammation marker
Blod pressure and hypertension												
3. The effect of anthocyanins on blood pressure: A PRISMA-Compliant Meta-Analysis of Randomized Clinical Trials.	Zhu, Y. *et al.*	2016	Systematic review and meta-analysis	Blood Pressure	Chinese, English, Norwegian and Italian populations	PubMed, Web of Science, Wanfang Database, China National Knowledge Infrastructure.	Inception - 2015	6	RCTs (n=5), cross-over (n=1)	472	2008 - 2015	Cochrane risk of bias tool	There was no signifi the anthocyanin supp - 640 mg/d) and syst (WMD: 1.15 mmHg, I2=56%) nor in diast (WMD: 1.06 mmHg, I2= 0%).

4. Effects of flavonoid-rich fruits on hypertension in adults: a systematic review.	Ellwood, L. *et al.*	2019	Systematic review and meta-analysis	Hypertension	Hypertensive subjects	MEDLINE, Embase, Cochrane Trials (CENTRAL), CINAHL.	Inception - September 2018	6	RCTs (n=6)	119 / 119	2007 - 2016	Critical appraisal tools from the Joanna Briggs Institute	Subgroup analysis by demonstrated no sign systolic blood pressu received anthocyanir received placebo (M 3.22, 1.30; P=0.41, I
5. Dietary polyphenol intake, blood pressure, and hypertension: a systematic review and meta-analysis of observational studies.	Godos, J. *et al.*	2019	Systematic review and meta-analysis	Blood pressure and hypertension	General population/hypertensive subjects ranging 40 - 70 years-old	PubMed, Embase.	Inception - April 2019	20	Cross sectional (n=15), Prospective Cohort (n=7)	200,256 / 45,732	2002 - 2018	Funnel plot analysis	When compared the exposures, the dietar was associated with a risk of hypertension, 0.88, 0.97).
Cardiovascular disease markers													
6. Systematic review of anthocyanins and markers of cardiovascular disease.	Wallace, T. *et al.*	2016	Systematic review and meta-analysis	Cardiovascular disease markers	European, Mexican, Chinese, and Iranian population	PubMed, Web of Science, BIOSIS	Inception - July 2014	12	RCT (n=12)	NA	2005 - 2014	Scottish Intercollegiate Guidelines Network (SIGN) grading system	After the systematic interventions suggest LDLc in hyperlipide however not in healt cardiovascular-relate HDLc showed statist increases in hyperlip individuals, individu syndrome and prehyp

7. Effects of anthocyanin on serum lipids in dyslipidemia patients: a systematic review and meta-analysis.	Liu, C. *et al.*	2016	Systematic review and meta-analysis	Serum lipids	Dyslipidemic subjects	PubMed, Web of Science, MEDLINE, Cochrane Library, China National Knowledge Infrastructure, Wanfang Database.	Inception - July 2015	6	RCTs (n=6)	586 / 586	2008 - 2014	Cochrane risk of bias tool	In dyslipidemic patie supplementation sign levels of TC (MD = - -2.64 mg/dL; I2 = 93 26.14, 95% CI: -49.2 66%), of LDLc (MD 34.36, -9.85; mg/dL; significantly increas HDLc (MD = 5.58, 9 mg/dL; I2 = 90%) wi the placebo group.
Cancer												
8. Flavonoids, flavonoid subclasses and breast cancer risk: A meta-analysis of epidemiologic studies.	Hui, C. et *al.*	2013	Meta-analysis	Breast cancer risk	NR	Cochrane Library, MEDLINE, Embase, PubMed.	Inception - July 2012	12	Prospective cohort (n=6), nested case-control (n=1), population-based case-control (n=2), hospital-based case-control (n=3)	181,906 / 9,513	1997- 2010	Funnel plot analysis	No significant associ and breast cancer risk 0.97, 95% CI: 0.87,
9. Intake of Anthocyanins and Gastric Cancer Risk: A Comprehensive Meta-Analysis on Cohort and Case-Control Studies.	Yang, D. *et al.*	2019	Systematic review and meta-analysis	Gastric cancer risk	NR	PubMed, EMBASE, Web of Science, Cochrane Library.	Inception - June 2018	6	Cohort (n=2), case-control (n=4)	949,226	2004 - 2017	GRADE system	There was no signifi the anthocyanin intal risk (RR=0.92, 95%(Moreover, there is n an association by sub gender (men: RR=1.(women: RR=0.80, 9 tumor location (cardi 0.62, 1.31; non-cardi 0.69, 1.07) was obse and nonlinear dose-r
Vascular function												

10.The effect of anthocyanin-rich foods or extracts on vascular function in adults: a systematic review and Meta-analysis of randomized controlled trials.	Fairlie-Jones, L. *et al.*	2017	Systematic review and meta-analysis	Vascular function	United kingdom, North America, China, Korea, Italy, Australia Greece and Israel Adults over 18 years old	EMBASE, MedLine, Cochrane, CINAHL, Scopus.	Inception - June 2017	29	Acute RCT (n=8), chronic RCT (n=21)	NR	2006 - 2016	Jadad scoring system	Anthocyanin consum associated to an impr mediated dilation aft 3.92%, 95% CI: 1.47 91.8%) and the chro (SMD: 0.84%, 95% I2= 62.5%). Moreov anthocyanin supplem associated to a pulse improvement (SMD- 1.96, -0.58, P=0,000

Note: RCT, randomized controlled trial; TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; MD, mean difference; SMD, standard mean deviation; RR, relative risk; WMD, wei CINAHL, cumulative index to nursing and allied health; NOI,

Table 2. AMSTAR 2 Quality assessment results for the intervention studies.

Title	Reference	Year	1. PICO components	2. Review methods	3. Study selection	4. Literature search	5. Study selection	6. Data extraction	7. Exclusion justification	8. Study description	9. Included studies risk of bias	10. Included studies funding	11. Statistical methods	12. Risk of bias impact	13. Risk of bias accountability	14. Heterogeneity	15. Publication bias	16. Conflict of interest	Results
A systematic literature review of the effect of anthocyanins on gut microbiota populations.	(Igwe et al. 2019)	2019	Y	Y	Y	PY	Y	Y	N	Y	N	N	N/A	N/A	N	Y	N/A	Y	CRITICALLY LOW
Effects of anthocyanin on serum lipids in dyslipidemia patients: a systematic review and meta-analysis.	(Liu et al. 2016)	2016	Y	Y	Y	Y	Y	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	HIGH
Effects of flavonoid-rich fruits on hypertension in adults: a systematic review.	(Ellwood et al. 2019)	2019	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	HIGH
Effects of anthocyanins on cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials.	(L. Yang et al. 2017)	2017	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	HIGH
The effect of anthocyanins on blood pressure: A PRISMA-Compliant Meta-Analysis of Randomized Clinical Trials.	(Yongjian Zhu et al. 2016)	2016	Y	Y	Y	PY	Y	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	HIGH

The effect of anthocyanin-rich foods or extracts on vascular function in adults: a systematic review and meta-analysis of randomized controlled trials.	(Fairlie-Jones et al. 2017)	2017	Y	Y	Y	PY	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	**HIGH**
Systematic review of anthocyanins and markers of cardiovascular disease.	(Wallace, Slavin, and Frankenfeld 2016)	2016	Y	Y	Y	PY	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	**HIGH**

Note: Y, yes; N, No; PY, partial yes; N/A, not applicable.

Table 3. Joanna Briggs Institute risk of bias assessment from the meta-analysis of observational studies.

Item No.	Criteria	Godos, J. *et al* (Godos et al. 2019)	Hui, C. *et al* (Hui et al. 2013)	Yang, D. *et al* (Guo et al. 2016)	Guo, X. *et al* (Guo et al. 2016)

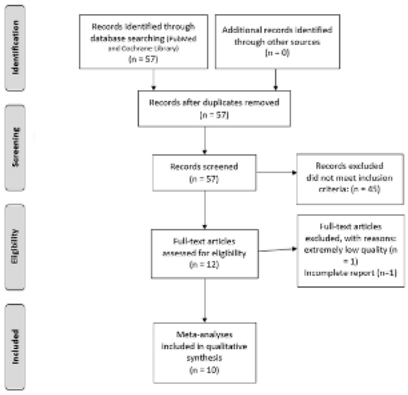
1	Is the review question clearly and explicitly stated?	Yes	Yes	Yes	Yes
2	Were the inclusion criteria appropriate for the review question?	Yes	Yes	Yes	Yes
3	Was the search strategy appropriate?	Yes	Yes	Yes	Yes
4	Were the sources and resources used to search for studies adequate?	Yes	Yes	Yes	Yes
5	Were the criteria for appraising studies appropriate?	Yes	Yes	Yes	Yes
6	Was critical appraisal conducted by two or more reviewers independently?	Yes	Yes	Yes	Yes
7	Were there methods to minimize errors in data extraction?	Yes	Yes	Yes	Yes
8	Were the methods used to combine studies appropriate?	Yes	Yes	Yes	Yes
9	Was the likelihood of publication bias assessed?	Yes	Yes	Yes	Yes
10	Were recommendations for policy and/or practice supported by the reported data?	Yes	Yes	Yes	Yes
11	Were the specific directives for new research appropriate?	Yes	Yes	Yes	Yes
	Overall appraisal	Included	Included	Included	Included

Figure Legends

Figure 1. PRISMA flowchart for the included studies.

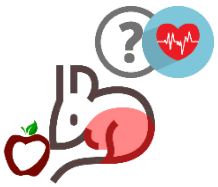
URL: http://mc.manuscriptcentral.com/bfsn Email: fergc@foodsci.umass.edu

Figure 1. PRISMA Flow Diagram



PRISMA flowchart for the included studies.

254x190mm (96 x 96 DPI)



3.4. Chapter 4

Red and white-fleshed apples modulate aorta and heart proteome in hypercholesterolemic rats. The AppleCOR project.

Please feel free to download the full article here:

Reference: *Pending*

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Manuscript Draft

Manuscript Number:

Title: Red-fleshed apples rich in anthocyanins and white-fleshed apples modulate aorta and heart proteome in hypercholesterolemic rats. The AppleCOR study

Article Type: Research Article (max 7,500 words)

Keywords: anthocyanins; phenolic compounds; proteomics; aorta and heart rat tissues; apple

Corresponding Author: Dr. Anna Pedret,

Corresponding Author's Institution:

First Author: Úrsula Catalán

Order of Authors: Úrsula Catalán; Anna Pedret; Sílvia Yuste; Laura Rubió; Carme Piñol; Berner A Sandoval-Ramírez; Judit Companys; Elisabet Foguet; Pol Herrero; Núria Canela; MªJosé Motilva; Rosa Solà

Abstract: New mechanisms-of-action of anthocyanins (ACNs) provided by a red-fleshed apple compared with a white-fleshed apple, and with an ACN-rich extract on the proteome profile of aorta and heart as cardiovascular key tissues were determined. Hypercholesterolemic Wistar rats were separated into the corresponding groups to analyze the proteomic profile of the aorta and heart tissues using nano-liquid chromatography coupled to mass-spectrometry. Red-fleshed apple downregulated CRP, C1QB and CFP related-inflammation. White-fleshed apple reduced C1QB, CFB, CFD, C3, and C9 related to the complement system, reduced MB and CP related to iron metabolism, and increased ME1, PKM, and PC related to energy homeostasis. ACN-rich extract increased FMOD, TAGLN, and CAP1 related to cellular structure and decreased PRKACA, IQGAP1, and HSP90AB1 related to cellular signaling. Red-fleshed apple rich in ACNs suggested an anti-inflammatory effect while white-fleshed apple reduced the complement system protein-related. An apple matrix effect reduced inflammatory proteins regardless their ACN content.

Cover Letter

Paul Finglas, Ph.D.

Editor-in-Chief, *Food Chemistry*

08-05-2020

Dear Dr. Finglas,

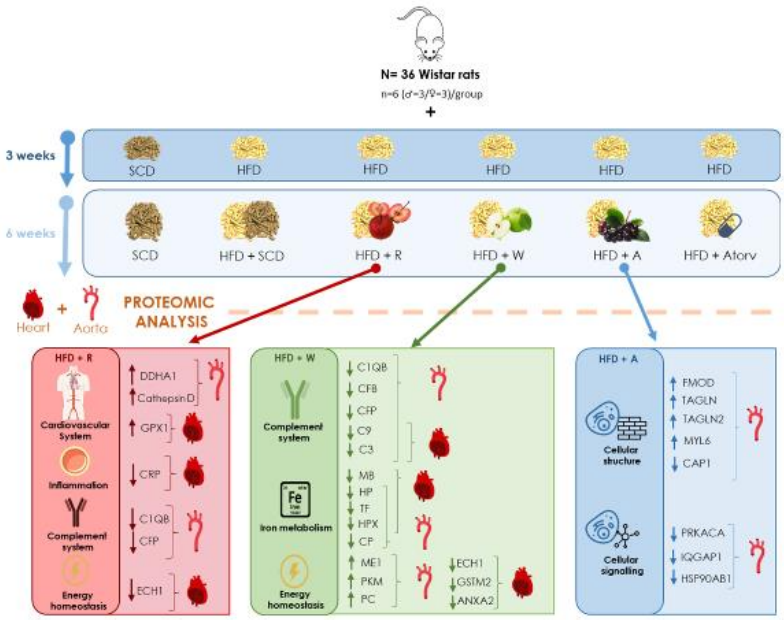
Please, find enclosed our manuscript entitled **"Red-fleshed apples rich in anthocyanins and white-fleshed apples modulate aorta and heart proteome in hypercholesterolemic rats. The AppleCOR study"** which we would like to be considered for its publication on the *Food Chemistry*.

It is known that apples are amid the most popular fruits consumed around the world, however, despite the growing efforts to study the mechanisms by which apple consumption can improve cardiovascular disease, the effects of different apple varieties that differ between each other in anthocyanin composition is still unknown. The aim of the current paper is **to describe for the first time the effects of a high-fat diet supplemented with a red-fleshed apple rich in anthocyanins, a white-fleshed apple without anthocyanins used as matrix apple control, and anthocyanin-rich extract from *Aronia melanocarpa* used as a control of anthocyanin intake on the proteome profile of aorta and heart as cardiovascular key tissues in hypercholesterolemic Wistar rats.**

The main findings of the study revealed that red-fleshed apple rich in anthocyanins consumption suggested an anti-inflammatory effect on the heart in hypercholesterolemic rats. White-fleshed apple reduced mainly the complement system protein-related in heart and aorta while the anthocyanin-

rich extract intake modified the expression of different structural and signaling proteins related to cardiovascular disease in the aorta. Moreover, an apple matrix effect was observed by reducing inflammatory proteins on the aorta and/or heart regardless of their anthocyanin content.

Here we show you a graphical abstract that summarizes the main findings of the study.



Moreover, on behalf of the co-authors, I affirm that the data has not been previously published nor the manuscript is under consideration for publication by any other journal in the same or a substantially similar form. As requested, all of the authors comply with the criteria needed for authorship and originality, and no copyright to any other work was breached in the manuscript's creation.

We believe that the findings of the present scientific article make a valuable contribution to the current body of knowledge, regarding the scientifically proven properties of whole-fruits, particularly apples, and would be of interest to the general as well as the specialized reader of the *Food Chemistry*.

Without any other concern, we look forward to your opinion as to the suitability of our manuscript for the inclusion in *Food Chemistry*.

Yours sincerely,

Anna PEDRET, PhD

Universitat Rovira i Virgili (URV)

Faculty of Medicine and Health Sciences

Medicine and Surgery Department

Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut)

Fundació EURECAT-Technological Center of Nutrition and Health (CTNS), Reus, Spain.

Avda/ Universitat, 1. CP/ 43204 Reus, Spain

Tel: (+34) 977 75 93 75 / Fax: (+34) 977 75 93 22

E-mail: anna.pedret@eurecat.org

***Highlights (for review)**

HIGHLIGHTS

- Red-fleshed apple rich in ACNs exerted an anti-inflammatory effect in rat heart
- White-fleshed apple reduced the complement system proteins in heart and aorta
- ACN-rich extract up-or downregulated CVD structural and signaling proteins in aorta
- An apple matrix effect regardless of the ACN content reduced inflammatory proteins

Red-fleshed apples rich in anthocyanins and white-fleshed apples modulate aorta and heart proteome in hypercholesterolemic rats. The AppleCOR study

Úrsula Catalán[1, 2, 3], Anna Pedret[1, 3, *], Silvia Yuste[4], Laura Rubió[4], Carme Piñol[5, 6], Berner Andrée Sandoval-Ramírez[1], Judit Companys[1, 3], Elisabet Foguet[7], Pol Herrrero[7], Núria Canela[7], MªJosé Motilva[8], and Rosa Solà[1, 3, 9]

[1] Universitat Rovira i Virgili, Faculty of Medicine and Health Sciences, Medicine and Surgery Department, Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut), Reus, Spain.

[2] Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain.

[3] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Catalonia, Spain.

[4] Food Technology Department, Universitat de Lleida-AGROTECNIO Center, Lleida, Spain

[5] Department of Medicine, Universitat de Lleida, Lleida, Spain.

[6] Institut de Recerca Biomèdica de Lleida. Fundació Dr. Pifarré-IRBLleida, Lleida, Spain.

[7] Eurecat, Centre Tecnològic de Catalunya. Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT. Reus, Spain

[8] Instituto de Ciencias de la Vid y del Vino-ICVV (CSIC, Gobierno de La Rioja, Universidad de La Rioja), Logroño, La Rioja, Spain.

[9] Hospital Universitari Sant Joan de Reus (HUSJR), Reus, Spain.

*Corresponding author:

Anna PEDRET, PhD

Universitat Rovira i Virgili (URV)

Faculty of Medicine and Health Sciences

Medicine and Surgery Department

Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut)

Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Catalonia, Spain.

Avda/ Universitat, 1. CP/ 43204 Reus, Spain

Tel: (+34) 977 75 93 75 / Fax: (+34) 977 75 93 22

E-mail: anna.pedret@eurecat.org

Abbreviations:

ABCA1: phospholipid-transporting ATPase 1

ACNs: Anthocyanins

C1QB: C1q subcomponent B

C4BPA: C4 binding protein alpha chain

CAP1: adenylyl cyclase-associated protein 1

CFB: complement factor B

CFD: complement factor D

CRP: C-reactive protein

CVD: cardiovascular disease

ECH1: enoyl-CoA hydratase 1

FC: fold-change

FMOD: fibromodulin

GLRX3: glutaredoxin-3

GPX1: glutathione peroxidase 1

HDLc: high-density lipoprotein cholesterol

HFD: high-fat diet

HFD+A: high-fat diet plus Aronia infusion

HFD+Atorv: high-fat diet plus atorvastatin

HFD+R: high-fat diet plus red-fleshed apple

HFD+W: high-fat diet plus white-fleshed apple

HSP90AB1: heat shock protein 90-beta

IQGAP1: IQ motif containing GTPase activating protein 1

LDLc: low-density lipoprotein cholesterol

LDLR: low-density lipoprotein receptor

MYL6: myosin light polypeptide 6

NO: nitric oxide

PCs: phenolic compounds

SCD: Standard chow diet

TAGLN: transgelin

Abstract

New mechanisms-of-action of anthocyanins (ACNs) provided by a red-fleshed apple compared with a white-fleshed apple, and with an ACN-rich extract on the proteome profile of aorta and heart as cardiovascular key tissues were determined. Hypercholesterolemic Wistar rats were separated into the corresponding groups to analyze the proteomic profile of the aorta and heart tissues using nano-liquid chromatography coupled to mass-spectrometry. Red-fleshed apple downregulated CRP, C1QB and CFP related-inflammation. White-fleshed apple reduced C1QB, CFB, CFD, C3, and C9 related to the complement system, reduced MB and CP related to iron metabolism, and increased ME1, PKM, and PC related to energy homeostasis. ACN-rich extract increased FMOD, TAGLN, and CAP1 related to cellular structure and decreased PRKACA, IQGAP1, and HSP90AB1 related to cellular signaling. Red-fleshed apple rich in ACNs suggested an anti-inflammatory effect while white-fleshed apple reduced the complement system protein-related. An apple matrix effect reduced inflammatory proteins regardless their ACN content.

Data are available via ProteomeXchange with identifier PXD018885.

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1. INTRODUCTION

In 2025, the mortality target a 25% reduction in premature mortality from noncommunicable diseases, including cardiovascular disease (CVD), the leading cause of death worldwide (Roth et al., 2017).

Lifestyles performing frequent physical activity and consuming high amounts of vegetables and fruits (Warburton & Bredin, 2017), including apples, demonstrated protection on CVD (Medina-Remón, Kirwan, Lamuela-Raventós, & Estruch, 2018).

In particular, apples are amid the most popular fruits consumed around the world due to their geographical distribution, organoleptic properties, and seasonal availability (N. Wang et al., 2018). Moreover and throughout history, apples have enjoyed a good reputation in terms of their beneficial health properties, traditionally attributed to their high amounts of fiber, vitamins, minerals (Veronese et al., 2018), and high content of phenolic compounds (PCs) (Bondonno et al., 2018). The apple PC content ranges between 5,230 to 27,240 mg/kg of their dry weight, out of which the most common PC types are the hydroxycinnamic acids (50-3,000 mg/kg), flavanols (4,622-25,480 mg/kg), anthocyanins (ACNs; 10-551 mg/kg), and dihydrochalcones (49-434 mg/kg) (Hyson, 2011). Notwithstanding, the phenolic pattern of the apple fruit changes amongst their different varieties, and also by the weather, season, geographical distribution, and maturity of the fruit at the time of the harvest between other factors (Stirpe et al., 2017).

ACNs are phenolic compounds part of the flavonol subclass. ACNs exist naturally as plant pigments responsible for the red, pink, blue and purple colors present in the seeds, flowers, fruits, and leaves of different plants. The health benefits related to ACNs consumption and their impact on humans are the prevention of CVD, neurological, and renal disease (Sandoval-Ramírez et al., 2018). In the last few years, there has been an

increasing interest in potential crops for coloring food naturally without transgenic programs in order to obtain added healthy properties, such as red-fleshed apples containing a high amount of ACNs in their flesh. Due to the enhanced content of ACNs reported in these specific apple varieties, different studies have shown that the total phenolic content and antioxidant capacity were significantly higher compared to common white-fleshed apples, which indicates that these red-fleshed apples could have presumably added healthy properties (Bars-Cortina, Macià, Iglesias, Romero, & Motilva, 2017).

In humans, whole-apple intake, regardless of its PC, demonstrated the capability of improving CVD risk factors (Sandoval-Ramírez et al., 2020).

Apple's particular mechanisms of action on CVD have been studied for example, of up-regulating the expression of different antioxidant proteins such as the glutathione peroxidase, catalase dismutase and superoxide dismutase in the liver, as well as up-regulating the hepatic genes associated to PPARα, therefore providing a healthier metabolic profile in obese Zucker rats (Manzano et al., 2016).

Therefore, despite the growing efforts to study the mechanisms by which regular apple consumption could protect from CVD, the interest of the new red-fleshed apple varieties that differ between each other in ACN composition are still unknown. On the other hand, to explore the underlining mechanisms, the tissues proteomic analysis offers new perspectives to investigate how apple intake could regulate protein expression to potentially protect from CVD. Thus, present study aims to determine new mechanisms-of-action of ACNs provided by a red-fleshed apple variety compared with a white-fleshed apple without ACNs, and with an extract rich in ACNs from *Aronia*

melanocarpa on the proteome profile of aorta and heart as cardiovascular key tissues in hypercholesterolemic rats.

2. MATERIAL AND METHODS

2.1. Preparation of the supplemented diets in Wistar rats

To compare the ACNs effect provided by apples, two different apple varieties were selected: i) the red-fleshed "Redlove" apple variety, a new genotype naturally biofortified in ACN, and ii) the white-fleshed Granny Smith apple variety. Additionally and to study the effect of ACNs without the possible interaction of the apple matrix, an ACN-rich extract was selected.

Both apple varieties were provided by NUFRI SAT (Mollerussa, Lleida, Spain), and had a similar phytochemical profile except for ACNs. To prepare feed, freeze-dried flesh apple was used to preserve the ACNs and the rest of phenolic compounds. First, the apple cores were removed and the whole-apples (with peel) were cut into 1 cm-sized cube. Then, the apple cubes were frozen in liquid nitrogen and then lyophilized on a Lyobeta 15 TELSTAR Lyophilizer (Terrassa, Spain) and were immediately transferred to airtight plastic containers and refrigerated (2 ºC) until their use in the preparation of the supplemented diets. -To obtain the ACN-rich extract, a cold water infusion of an *Aronia melanocarpa* fruit powder (Aronia Pulver, BIOJOY, Nuremberg, Germany) was prepared, which was equivalent in dose and type of ACNs with red-fleshed apple. The Aronia powder was mixed with distilled water (1:1 proportion) and the mixture was homogenized (Kinematica Polytron, Polytron Corporation, Montreal, Canada) for 60 seconds. The resulting infusion was centrifuged and the supernatant was analyzed and added to the drinking water of the rats. The phenol characterization of the freeze-dried flesh apples and the ACN-extract is shown in **Supplementary Table S1**.

158 **2.2. Animals and experimental procedure**

159 Thirty-six Wistar rats weighing between 300 and 350 g were purchased from Charles
160 River Laboratories (Barcelona, Spain). The rats were divided into 6 groups of 6
161 animals each (3 males and 3 females) as follows, Group 1: standard chow diet (SCD)
162 (2014, rodent maintenance diet, Envigo, Huntingdon, Cambridgeshire, United
163 Kingdom); Group 2: high-fat diet (HFD) (Atherogenic Rodent Diet TD. 02028, Envigo,
164 Huntingdon, Cambridgeshire, United Kingdom) to induce hypercholesterolemia; Group
165 3: HFD + red-fleshed apple (HFD+R); Group 4: HFD + white-fleshed apple (HFD+W),
166 Group 5: HFD + ACN-rich extract (HFD+A); and Group 6: HFD + Atorvastatin
167 (HFD+Atorv) as hypolipemiant drug.

168 Group 1 was fed with chow diet for 9 weeks (**Supplementary Figure 1**). The other
169 groups were fed during 3 weeks with a HFD and the following 6 weeks with the HFD
170 supplemented with the different products. For HFD+R and HFD+W groups (Groups 3
171 and 4), HFD pellets were crushed in a mill along with the freeze-dried flesh apples. For
172 HFD+A (Group 5), the Aronia extract was dissolved daily in the drinking water. Rats
173 from group HFD+Atorv (Group 6) were given the drug Atorvastatin (Pfizer-Egypt
174 Company) at a dosage of 4 mg/kg/day dissolved daily in the drinking water. Moreover,
175 the HFD (Group 2) and the Groups 5 and 6 diets were modified by adding 25% of chow
176 diet in the same proportion as apples, so that all groups except Group 1, would take the
177 same proportion of HFD during the supplementation period. (**Supplementary Figure**
178 **1**). HFD+R and HFD+A were supplemented with the same dose and type of ACNs, 1.8
179 and 1.9 mg/day/rat, respectively. The ACNs administered dose results in a human
180 equivalent dose of 70 mg/day, which was calculated according to(Reagan-Shaw, Nihal,

181 & Ahmad, 2007). The nutritional composition regarding macronutrients and energy of
182 each diet used in the study is shown in **Supplementary Table S2.**

183 During the study, rats were housed in cages on a 12 h light-12 h dark schedule at a
184 controlled temperature (20 ± 2 ºC) and humidity (55 ± 10%). Food and water were
185 available *ad libitum*. The body weight, food, and water intake were recorded every 3
186 days.

187 At the end of the study, the rats were anesthetized with isoflurane (IsoFlo, Veterinarian
188 Esteve, Bologna, Italy) and sacrificed by cardiac puncture. The rats were perfused with
189 an isotonic (0.9%) sodium chloride solution, to remove the remaining blood in the
190 tissues. The hearts and the descending portion of the aortas were excised and
191 immediately snap-frozen in liquid nitrogen. All rat procedures were conducted
192 following the European Communities Directive 2010/63/EU regulating animal research
193 guidelines. All protocols were approved by the Animal Ethical Committee of the
194 University of Lleida (CEEA 01-10/17) and performed under a Generalitat de Catalunya
195 Project License (10038). The study complies with the ARRIVE guidelines developed by
196 the NC3Rs (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

197 **2.3. Proteomic analysis**

198 **2.3.1. Protein extraction and quantification**

199 To determine total protein content, the aortas and hearts were weighed (25-30 mg),
200 lysed following the radio-immunoprecipitation assay buffer protocol (Thermo Fisher
201 Scientific, Madrid. Spain), and processed as explained in more detail in the
202 **Supplementary Information**.

203 **2.3.2. Protein digestion and peptide 10-plex Tandem Mass Tag (TMT) labeling**

A total of 30 µg of proteins obtained from the aorta or heart tissues were reduced with 4mM 1.4-Dithiothreitol for 1h at 37°C and alkylated with 8 mM iodoacetamide for 30 min at 25ºC in the dark. Afterward, the samples were digested overnight (pH 8.0, 37ºC) with sequencing-grade trypsin (Promega Biotech Iberica SL, Alcobendas, Madrid) at an enzyme: protein ratio of 1:50. Digestion was quenched by acidification with 1% (v/v) formic acid, and peptides were desalted on the Oasis HLB SPE column (Waters, Cerdanyola del Vallès, Barcelona, Spain) before Tandem Mass Tag (TMT 10-plex labeling (Thermo Fisher Scientific, Madrid, Spain) following manufacturer instructions. Samples were normalized along with the different TMT-multiplexed batches using, a TMT-126 tag labeled pool containing all the samples was included in each TMT batch.

The different TMT 10-plex batches were desalted on Oasis HLB SPE columns before the nano-liquid chromatography coupled to mass spectrometry (LC-MS) analysis.

2.3.3. Off-gel nano LC-(Orbitrap) MS/MS analysis

The multiplexed and labeled aortas or hearts were fractionated by Off-gel (Agilent, Madrid, Spain) as instructed in the manufacturer's protocol. Samples were fractioned in 12 non-linear pH 3-10 fractions (**Supplementary information**). The chromatographic separation was performed with a 90 min gradient using Milli-Q water (0.1% formic acid) and acetonitrile (0.1% formic acid) as a mobile phase at a flow rate of 300 nL/min.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro from Thermo Fisher Scientific by an enhanced Fourier transform-resolution MS spectrum (R=30,000 FHMW) followed by a data-dependent the Fourier Transform coupled to double Mass Spectrometer (FT-MS/MS) acquisition (R=15,000 FHMW, 40% HCD) from the most intense ten parent ions with a charge state rejection of one and dynamic exclusion of 0.5 min.

228 **2.3.4. Protein identification/quantification**

229 The aorta or heart protein identification and quantification were performed on the
230 Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific, Madrid, Spain) by
231 Multidimensional Protein Identification Technology combining the 6 raw data files
232 obtained after the Strong cation-exchange chromatography fractionation. For protein
233 identification, all MS and MS/MS spectra were analyzed using the Mascot search
234 engine (v.2.5). The Mascot was set up to search SwissProt_2018_03.fasta database
235 (557012 entries), restricting for Human taxonomy (20317 sequences) and assuming
236 trypsin digestion. Two missed cleavages were allowed, and an error of 0.02 Da for FT-
237 MS/MS fragmentation mass and 10.0 ppm for an FT-MS parent ion mass were allowed.
238 TMT-10plex was set as the quantification, modification, and oxidation of methionine,
239 and the acetylation of N-termini was set as a dynamic modification, whereas
240 carbamidomethylation of cysteine was set as a static modification. The false discovery
241 rate and protein probabilities were calculated by Perclorator.
242 For protein quantification, the ratios between each TMT-label against each 126-TMT
243 label were used, and the results were normalized based on protein median values. No
244 protein western blot confirmation was conducted supported by the argument of
245 Aebersold R. *et al*., who confirmed that the results obtained by MS-based proteomics
246 (also recognized by the journal Nature Methods as the method of the year 2012
247 ("Method of the Year 2012.," 2013) are vastly superior to the obtained by western blot
248 for several reasons (Aebersold, Burlingame, & Bradshaw, 2013). Moreover, analyzing
249 individual biological aorta or heart replicates instead of pooling samples, as is the case
250 of our study, gives more statistical power to the differentially expressed proteins and
251 makes the use of additional methods for validating the findings unnecessary. The MS
252 proteomics data have been deposited to the ProteomeXchange Consortium via the

PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD018885.

2.4. Statistical analysis

2.4.1. Data pre-processing

For statistical analyses, only those proteins present in ≥ 67% of the samples in all groups were considered. In addition, log base 2 (log2) transformations were applied to the data including the variance stabilization, data range compression, and normalizing the data distribution.

Another important advantage of using log2 transformation is the ratio comparisons, such as the fold-change (FC), when comparing, for example, the HFD vs the SCD (HFD/SCD ratio).

Finally, the protein data set was mean-centered and Pareto scaled by being divided by the square root of the standard deviation (SD) of each variable to reduce the influence of intense peaks while emphasizing weaker peaks that may have more biological relevance, although not giving to much relevance to noise signals.

2.4.2. Multivariate statistical analysis

A multivariate statistical approach was initially performed in protein identified using the Metaboanalyst 4.0 (http://www.metaboanalyst.ca/) software. The modeling made use of hierarchical clustering and other supervised methods including the partial least squares discriminant analysis and an orthogonal projection to latent structures discriminant analysis (**Supplementary information**). All these methods were applied using a Pareto scaling.

Multivariate analysis was based on the Eigen-decomposition of a cross-product matrix (e.g., covariance matrix) and thus require complete datasets. To estimate missing values,

277 we used a Bayesian principal component analysis for values missing at random. The
278 protein component analysis is calculated using Bayes theorem while the Bayesian
279 estimation is used to calculate the likelihood of an estimated value.

280 **2.4.3. Univariate statistical analysis**

281 A univariate test was performed for each protein. For the univariate case, data were not
282 Pareto scaled. A Kolmogorov-Smirnov test was carried out to check for distribution
283 normality. For pairwise comparisons, either a Student's t-test or a Wilcoxon test was
284 performed depending on each protein's distribution. In the first case, a test for equality
285 of variances was performed before the t-test analysis. *P* values were adjusted using the
286 Benjamini-Hochberg method for multiple testing considerating a 5% false discovery rate.
287 The reported results included the fold change and the *p* values for each group. For
288 comparisons involving more than two groups, an analysis of variance (ANOVA) or a
289 Kruskal-Wallis test was performed. The Tukey HSD or the Nemenyi post-hoc tests
290 were carried out accordingly. In any case, the *p* values of the post-hoc pairwise
291 comparisons were further corrected for multiple testing for all the variables using a
292 Benjamini-Hochberg false discovery rate correction. A *P*-value <0.02 was considered to
293 be statistically significant.

294 **2.5. Clustering and pathway analysis**

295 An initial functional evaluation was performed using the UniProt (www.uniprot.org)
296 database, with a focus on the protein function and relevant biological processes.

297 The Ingenuity Pathway Analysis (IPA software; Ingenuity System Inc., Redwood, CA,
298 USA; www.ingenuity.com) was employed to examine the functional correlations within
299 groups. Datasets containing protein identifiers (UniProt-KB) and their corresponding
300 expression values (FC) of each two comparative groups were uploaded. Each protein

identifier was mapped to its corresponding protein object in the Ingenuity Pathways Knowledge Base. All mapped proteins were differentially expressed with $p < 0.05$ and overlaid onto global molecular networks developed from information contained in the knowledge base. The networks were then algorithmically generated based on their connectivity. Networks were "named" in the most prevalent functional group(s) present. Networks were ranked by a score that defines the probability of a collection of nodes being equal to or greater than the number in a network achieved by chance alone. Canonical pathways, Diseases, and Bio Functions, Ingenuity Tox List, and Molecule Activity Predictor tools were overlaid on the networks.

3. RESULTS AND DISCUSSION

3.1. Proteomic analysis in aorta and heart rat tissues.

After proteomic analysis, a total of 1163 in the aorta and 1149 proteins in heart tissues from the Wistar rats were identified.

The complete information regarding the protein's relative quantification and identification, the protein coverage as well as the peptides identified from the proteomic analysis in the aortas and hearts of rats are shown in **Supplementary Tables S3 and S4** respectively.

After the 70% frequency filter was applied, 750 proteins were considered for further statistical analysis in the aorta samples and a total of 761 proteins in the heart samples.

3.2. Tissue proteome modulation by the different diets in the aorta and the heart tissues

322 When we compared between groups split by sex, no differences were found in the
323 proteomic analyses. Therefore, the results comparing the different diet groups are not
324 gender split (n=6/group).

325 **3.2.1. HFD *versus* SCD**

326 After HFD treatment we observed a significant increase or decrease of certain proteins
327 compared to the SCD in both tissues, aorta, and heart Wistar rat tissue, which are described in
328 **Supplementary Table S5**

329 **3.2.2. The red-fleshed apple effects**

330 **Table 1** shows the results of the significantly expressed up- or downregulated proteins in the
331 aorta or the heart tissues after the HFD+R diet compared to the HFD ($p<0.02$).

332 **3.2.2.1. The red-fleshed apple effects on cardiovascular-related proteins.**

333 In the aorta tissues, the HFD+R diet significantly ($p<0.02$) upregulated the expression of N(G),
334 N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1) compared to the HFD, an enzyme
335 that catalyzes the hydrolyzation of two endogenous inhibitors of the NO synthases inhibiting
336 their protecting activity from cardiovascular morbidity. The significantly upregulated
337 expression of both DDAH1 and DDAH2 was observed by ACNs isolated from the cornelian
338 cherry fruit in atherosclerotic New Zealand rabbits induced by diet (Sozański et al., 2019).

339 Additionally, in the present study, the HFD+R treatment significantly upregulated the
340 expression of glutathione peroxidase 1 (GPX1) in heart tissue. GPX1 is an antioxidant enzyme
341 able to restore the endothelial phenotype in some high oxidative stress pathologies such as
342 hyperhomocysteinemia, and the activity of GPX1 has been inversely correlated to CVD in
343 patients with coronary artery disease (Blankenberg et al., 2003).

344 In the heart of Wistar rats, our findings demonstrate for the first time that the ingestion of an
345 HFD supplemented with an ACN-rich apple can significantly upregulate the GPX1 expression
346 despite the detrimental effects of an HFD.

347 In rat aorta's tissues, the HFD+R supplementation significantly upregulated the expression of
348 cathepsin D (**Table 1**), a cholesterol efflux inducing molecule that increases the expression of
349 the phospholipid-transporting ATPase 1 (ABCA1) and the apolipoprotein A-I, mediated lipid
350 efflux.

351 Therefore, the HFD+R-mediated the upregulation of DDAH1 in aorta and GPX1 in heart tissue,
352 consistent with a healthier pattern of CVD biomarkers in rats. These findings support the
353 beneficial role of red-fleshed apples rich in ACNs for the prevention of CVD.

354 **3.2.2.2. The red-fleshed apple effects on the CRP, complement system proteins, and energy**
355 **homeostasis**

356 In the heart tissue, the HFD+R significantly reduced the expression of CRP compared to HFD,
357 suggesting an anti-inflammatory effect (**Table 1**). CRP is a pro-inflammatory molecule
358 involved in diverse reactions that are related to the activation of the inflammatory process
359 associated with the development of atherosclerosis and other cardiovascular events.

360 In addition, in aorta tissue, the HFD+R treatment significantly downregulated the expression of
361 C1QB and CFP involved in the complement system (**Table 1**), and also downregulated ECH1
362 involved in energy homeostasis in heart tissue. As discussed below, similar changes were
363 observed in HFD plus white-fleshed apple (HFD+W) group.

364 **3.2.3. The white-fleshed apple effects.**

365 The differentially expressed proteins modified after the HFD+W diet and classified by tissues
366 are shown in **Table 2**.

367 Regarding the HFD+W treatment compared to HFD, a decrease of 7 differentially expressed
368 proteins in both tissues, in the aorta and also in the heart tissue: C3, CP, TF, SERPINA3N, C9,
369 HP, and HPX were observed (**Supplementary Figure S2**; $p<0.02$).

370 **3.2.3.1. The white-fleshed apple effects on the complement system and anti-inflammatory**
371 **proteins.**

372 The HFD+W treatment downregulated the expression of proteins involved in the activation of
373 both the classical and alternative complement pathways, such as the complement factor 3 (C3)
374 and C9 in the hearts and aortas of rats, while complement factor B (CFB), properdin (CFP),
375 C4BPA, and C1QB were reduced only in the rat aortic tissue.

376 A decrease of the C3 concentration produces the reduction of the spontaneous conversion of C3
377 into hydrolyzed C3 [C3(H2O)] (McGrath et al., 2006). In turn, C3(H2O) should functionally
378 bind to the CFB, which was also downregulated by the HFD+W diet, and to complement factor
379 D (CFD) to generate the metastable molecule C3b, a key opsonizing molecule part of the innate
380 immune system (Hertle, Stehouwer, & van Greevenbroek, 2014), protecting against infections
381 in mammals.

382 The HFD+W treatment also significantly downregulated the expression of the C4 binding
383 protein alpha chain (C4BPA), and complement C1q subcomponent subunit B (C1QB) in the rat
384 aorta, while reducing the complement factor 9 (C9) in both aorta and heart tissues (**Table 2**).
385 Reduction of C9 expression could reflect a reduction of the atherosclerotic plaque formation
386 process; since it has been demonstrated that high concentrations of C9 are present as deposits in
387 the intima layer of grade II atherosclerotic lesions in the human aorta (R. Vlaicu, Rus,
388 Niculescu, & Cristea, 1985). Hence, the novel results of the present study, regarding the effects
389 of an HFD+W supplementation in rats, showed significant downregulation in the expression of
390 proteins involved in the complement system such as CFP, CFB, C3, C4BPA, C1QB, and C9.
391 Such downregulations might be involved in the reduction of CVD risk as the formation of the
392 atherosclerotic plaque is a complex process performed between the modified lipid particles and
393 diverse innate immune system molecules (S. I. Vlaicu et al., 2016). Additionally, the HFD+W
394 supplementation in rats reduced other pro-inflammatory molecules such as the SERPINA 1 (α1-
395 antitrypsin) in heart tissue, and SERPINA3N (α1-antichymotrypsin) in both heart and aorta,
396 suggesting a positive effect of the HFD+W on the cardiovascular risk through the regulation of
397 the inflammatory process.

398 **3.2.3.2. The white-fleshed apple effects on the iron homeostasis proteins.**

399 As a result of our experiment, HFD+W treatment significantly reduced the expression of
400 proteins involved in the iron homeostasis such as myoglobin (MB) in the heart tissue, while
401 reducing the expression of haptoglobin (HP), serotransferrin (TF), hemopexin (HPX), and
402 ceruloplasmin (CP) in both heart and aorta tissues, when compared to the HFD group (**Table 2**).

403 The iron-binding molecule, myoglobin (MB), serves as a dioxygen reservoir in the muscles of
404 mammals. MB can act as a potent nitric oxide (NO) scavenger, thus representing a control
405 system for the preservation of mitochondrial respiration. These findings suggest that a reduction
406 in the expression of myoglobin might be beneficial for hypertensive states where there is a
407 lesser bioavailability of vascular NO (Hermann, Flammer, & Lüscher, 2006). Moreover, the
408 HFD+W supplementation downregulated the expression of CP, a copper-binding glycoprotein
409 with ferroxidase activity and antioxidant properties, linked to the promotion of deleterious
410 vascular effects being considered a risk factor for CVD (Grammer et al., 2014). In addition to
411 the already described effects, the HFD+W treatment significantly reduced the expression of
412 transferrin, an iron-binding protein able to control ferric iron concentrations in human body
413 fluids (W. Wang, Knovich, Coffman, Torti, & Torti, 2010).

414 High transferrin concentrations (>160 mg/dL) are associated with an increased CVD mortality
415 risk in individuals where both transferrin and LDLc levels are elevated (Shipra et al., 2014).
416 Thus, the HFD+W supplementation resulted in the decrease of myoglobin, transferrin, and
417 ceruloplasmin proteins involved in the iron homeostasis which participates in essential
418 reduction-oxidation reactions for several fundamental biological processes.

419 Finally, the HFD+W treatment significantly reduced the expression of HP, an acute-phase
420 protein, in heart, and aorta tissues. This reduction is considered positive, and it has also been
421 observed in a study with olive oil phenolic compounds (Pedret et al., 2015), where the reduction
422 in the expression of haptoglobin was related to an improvement in the cholesterol efflux

capacity of the HDL particles in humans. Therefore, HFD+W supplementation can exert a positive effect over the CVD through the regulation of iron homeostasis related proteins.

3.2.3.3. The white-fleshed apple effects on the energy homeostasis proteins.

The HFD+W treatment downregulated the expression of enoyl-CoA hydratase 1 (ECH1) in the heart tissue (**Table 2**), an enzyme in charge of catalyzing the second step in the fatty-acid β-oxidation and the metabolization of branched-chain amino acids. The downregulation of ECH1 has been linked to enhanced resistance to ischemia-reperfusion injury in the hearts of Brown Norway rats (Du et al., 2013).

Moreover, the HFD+W supplementation also significantly downregulated the expression of Glutathione S-transferase Mu 2 (GSTM2) in heart, a molecule that reduces the activity of the ryanodine receptors in the sarcoplasmic reticulum, causing a reduction in spontaneous contraction frequency and the myocyte shortening (Hewawasam, Liu, Casarotto, Board, & Dulhunty, 2016), therefore improving the heart's contractility.

One interesting finding is that the HFD+W treatment downregulated the expression of annexin A2 in the hypercholesterolemic heart rat tissue, a calcium-regulated binding protein that reduces the expression of the proprotein convertase subtilisin/Kexin Type 9 (PCSK9) enzyme (Seidah et al., 2012). The PCSK9 receptor is an enzyme known for its capability to bind the LDL receptors (LDLR) on the liver promoting their degradation, hence a reduction in the degradation of LDLRs, increases the clearance of the cholesterol inside of LDL molecules, consequently reducing the LDLc plasmatic levels.

Moreover, the HFD+W treatment upregulated the expression of the pyruvate kinase levels in the aorta, and the pyruvate carboxylase and the NADP dependent malic enzyme (ME1) in hearts tissue. The upregulation of these enzymes, increases the intracellular concentrations of oxaloacetate and malate, substrates needed to start the tricarboxylic acid cycle, therefore, suggesting a possible increase in the intracellular energy levels (Jitrapakdee & Wallace, 1999).

448 **3.2.4. The ACN-rich extract effects**

449 After evaluating the impact of ACNs diet supplementation through the apple, we evaluated the
450 impact of ACNs minimizing the apple matrix effect. Regarding the up- or downregulation of the
451 differentially expressed proteins modified by the HFD+A compared to HFD, we observed that
452 the diet only modified 3 significant proteins in heart tissue (PHYH, GLRX3, and MRPL38;
453 $p<0.02$)). However, many more proteins were modulated in aorta tissue by the HFD+A diet
454 compared to the HFD, as shown in **Table 3** ($p<0.02$).

455 **3.2.4.1. The ACN-rich extract effects on cellular signaling proteins.**

456 In the aorta, the ACN-rich extract significantly modified the expression of different proteins
457 such as downregulation the expression of the protein kinase, cAMP-dependent, catalytic alpha
458 (PRKACA). The decrease of PRKACA expression observed after the HFD+A supplementation
459 would favor the inhibition of the spontaneous and pathological blood clot formation in blood
460 vessels (Gambaryan et al., 2010) as result, potentially reducing the risk of cardiovascular events.

461 Additionally, the HFD+A downregulated the expression of the IQ motif containing GTPase
462 activating protein 1 (IQGAP1) in aorta tissue (**Table 3**), a protein with a crucial role in
463 regulating the assembly and dynamics of the actin cytoskeleton. On the other hand, IQGAP1's
464 overexpression has been associated with the cell proliferation, migration, and rearrangement of
465 the vascular smooth muscle cells in varicose veins (Huang et al., 2014).

466 The HFD+A also downregulated the expression of the heat shock protein HSP 90-beta
467 (HSP90AB1) in the aorta, a molecule necessary for a large number of cellular processes, acting
468 as a chaperone promoting the maturation and structural maintenances of different specific
469 proteins involved in the cell cycle control and signal transduction (Haase & Fitze, 2016).

470 **3.2.4.2. The ACN-rich extract effects on cellular structure related proteins.**

471 In the aorta, the HFD+A upregulated fibromodulin (FMOD) in the rat. FMOD is a protein
472 participating in the assembly of the collagen fibers in the extracellular matrix and known for

473 triggering the platelet aggregation through the activation of a collagen-specific receptor. This
474 upregulation supports the interest of ACNs, as a positive moderator of the intravascular
475 coagulation process.

476 Additionally in the aorta, the HFD+A treatment raised the aortic expression of transgelin
477 (TAGLN) and TAGLN2, proteins that are involved in the calcium-related contractile properties
478 of the cell. Moreover, the HFD+A diet upregulated the aortic protein expression of the myosin
479 light polypeptide 6 (MYL6), a structural protein acting as a non-calcium binding regulatory
480 protein of myosin.

481 Furthermore, in the aorta, the HFD+A diet significantly downregulated the expression of the
482 adenylyl cyclase-associated protein 1 (CAP1), a human resistin-receptor that increases the
483 expression of CD36 mRNA, associated with the coronary artery disease. Moreover, CAP1 has
484 also been identified as one important regulator of the PCSK9, a modulator of LDL receptor
485 degradation in the liver (Jang et al., 2019).

486 **3.2.5. HFD+Atorv *versus* HFD**

487 The results regarding the HFD+Atorv diet are shown in Supplementary **Table S6**. In aorta
488 tissue, the treatment of the HFD+Atorv only increased of MYPOP and a decrease of SEPT9,
489 MAP4, and FHL1 (p<0.02) compared to HFD. However, in heart tissue, HFD+Atorv, increased
490 TNS1, PCBP2, DPYSL2, LMNA, GPX1, and ES1 protein homolog, mitochondrial and a
491 decrease of ECH1, MB, GSTM2, PHB, CBR1, NDUFB7, PGAM1, NME2, AKR1C15, and
492 CKMT2 (p<0.02) compared to HFD. After the hearta and aorta proteome analysis has been
493 demonstrated the pleiotropic effects of Atorvastatin, which can affect the cardiovascular system
494 beyond their effect on the lipid profile (Blum & Shamburek, 2009).

495 **3.2.6. The apple matrix effect**

496 In the aorta of the hypercholesterolemic rats which consumed the HFD+R or the HFD+W diets,
497 regardless of their ACN content, common significant downregulation of CFP and C1QB was

observed. C1QB is a protein-related to the activation of the complement classical pathway due to its important role as an important fragment of C1, which is the first component and main activator of the classical pathway of the complement system (Son, Diamond, & Santiago-Schwarz, 2015). The downregulated effects of the apple's matrix on C1QB was accompanied by the reduction of the complement system regulator, CFP (Harboe et al., 2017), thus, leading to a stimulus for the reduction in the complement system activation. The HFD+W significantly decreased the expression of CFP and C1QB largely than the HFD+R supplemented group.

In addition, in aorta tissue, the HFD+R treatment significantly downregulated the expression of SERPINA1 and ECH1 in heart tissue, thus HFD+R showed similar effects than the observed in HFD+W. Consequently, the apple consumption, independently of the ACN content present in red-fleshed apples, induced a comparable effect on aorta proteome involved in the complement system.

Furthermore, the HFD+R and the HFD+W treatments downregulated the expression of ECH1 in rat hearts, showing the same effects as observed in HFD+Atorv.

3.2.7. Red and white-fleshed apples and ACN-rich extract comparisons with atorvastatin

The Atorvastatin diet was used as a control due to its hypolipemiant and antioxidant properties but it could have also other attributed effects.

In addition to the apple matrix effect, it was noted that the rats consuming an HFD+W, HFD+R, or HFD+A diets, promoted a change in proteins also modified by the HFD+Atorv intervention (**Figure 1**), which was the positive control. The HFD+Atorv decreased the protein expression of ECH1, this was also observed in our rats after the HFD+W and the HFD+R interventions, an effect that has not been reported up to date by other authors.

In addition, GPX1 was downregulated in the HFD+R group, while GSTM2 and MB were both downregulated in the HFD+Atorv and the HFD+W groups. Finally, both the HFD+A and the HFD+Atorv evidenced the same downregulation in the Four and a half LIM domains protein 1

(FHL1), a protein whose specified functions remain unknown. However, FHL1 is significantly increased in cardiac failure, cardiac hypertrophy, pulmonary hypertension, and arrhythmias (Chu & Chen, 2011). Therefore, we suggest that apples and atorvastatin share common mechanisms of action which impact positively on diverse CVD risk factors.

The summary of the main findings after the HFD+R, HFD+W, and HFD+A supplementation diets are represented in **Supplementary Figure S3**.

3.3. Common proteins modified after the different diets in the aorta and heart tissue

3.3.1. Aorta tissue

The common differentially expressed proteins after the different treatments in aorta tissue are represented in **Figure 1A.**

FHL1 was reduced in aorta tissue after HFD+Atorv (-1.465 FC, p=0.0108) and after HFD+A (-1.669 FC, p=0.0022) diets compared to the HFD.

PRKACA (-1.122 FC, p=0.0043), CCT3 (-1.634 FC, p=0.0152), and GLUD1 (-1.256 FC, p=0.0162) were reduced, and MYL6 (1.653 FC, p=0.0119) was increased after HFD+A diet compared to HFD diet. However, the inverse effect was observed after HFD being PRKACA, CCT3, and GLUD1 increased (1.240 FC; p=0.0064, 1.622 FC; p=0.0087, and 1.297 FC; p=0.0118, respectively) and MYL6 reduced (-1.631 FC, p=0.0167) compared to the SCD.

DDAH1 (1.554 FC, p=0.0090) and DDT (1.427 FC, p=0.0164) increased after HFD+R diet compared to the HFD, and the same proteins were decreased after HFD (-1.365 FC, p=0.0190 and -1.466 FC, p=0.0120, respectively) compared to the SCD.

C3 (-2.158 FC, p<0.0001) was decreased after HFD+W compared to the HFD whereas increased after HFD (1.489 FC, p=0.0019) compared to SCD.

545 C1QB was decreased after HFD+W (-2.554 FC, p=0.0004) and after HFD+R (-1.674 FC,
546 p=0.0087) diets compared to HFD and increased after HFD (2.001 FC, p=0.0111) compared to
547 SCD.

548 CFP was decreased after HFD+W (-1.701 FC, p=0.0087) and after HFD+R (-1.454 FC,
549 p=0.0152) compared to HFD.

550 Thus, in rat aorta tissue, CFP and C1QB decreased after HFD+W and HFD+R suggesting an
551 apple matrix effect. DDAH1 and DDT were increased after HFD+R suggesting an ACN effect
552 provided by red-fleshed apple. In the same tissue, PRKACA, CCT3, FHL1, and GLUD1 were
553 reduced while MYL6 was increased after HFD+A suggesting an ACN-rich extract effect.

554 **3.3.2. Heart tissue**

555 The common differentially expressed proteins that were commonly modified after the different
556 treatments in heart tissue are represented in **Figure 1B.**

557 HFD+A diet did not modify any common protein modified by other different treatments in heart
558 tissue.

559 PCBP2 was increased after HFD+Atorv diet (1.245 FC, p=0.0022) compared to the HFD, and
560 PCBP2 was reduced after HFD (-1.273 FC, p=0.0022) compared to the STD.

561 GSTM2 (-1.354 FC, p=0.0041), CKMT2 (-1.499 FC, p=0.0172), and MB (-1.727 FC,
562 p=0.0027) were reduced after HFD+Atorv diet and also after HFD+W diet (-1.326 FC;
563 p=0.0047, -1.418 FC; p=0.0116, and -1.512 FC; p=0.0199, respectively) compared to HFD but,
564 in contrast, GSTM2 (1.386 FC, p=0.0034), CKMT2 (1.532 FC, p=0.0015), and MB (1.609 FC,
565 p=0.0100) were increased after HFD compared to SCD.

566 However, TNS1 was increased after HFD+Atorv diet (1.297 FC, p=0.0008) and after HFD+W
567 (1.273 FC, p=0.0083) compared to HFD but decreased after HFD (-1.280 FC, p=0.0176)
568 compared to SCD.

569 LMNA and GPX1 were increased after HFD+Atorv diet (1.267 FC, p= 0.0023 and 1.177 FC,
570 p= 0.0065, respectively) and after HFD+R diet (1.153 FC, p=0.0187 and 1.179 FC, p=0.0110,
571 respectively) compared to HFD.

572 SERPINA1 was decreased after HFD+W diet (-1.273 FC, p=0.0188) and after HFD+R diet (-
573 1.263 FC, p=0.0054) compared to HFD. In contrast, SERPINA1 was found to increase after
574 HFD (1.234 FC, p=0.0177) compared to SCD.ECH1 was decreased after HFD+Atorv (-1.298
575 FC, p=0.0012), HFD+W (-1.305 FC, p=0.0152), and HFD+R (-1.144 FC, p=0.0133) compared
576 to HFD. But, ECH1 was found increased after HFD (1.175 FC, p=0.0083) compared to SCD.

577 In rat heart tissue, GSTM2, CKMT2, and MB were reduced and TNS1 was increased after
578 HFD+W suggesting a differential effect of white-fleshed apple. SERPINA1 and ECHI were
579 decreased after HFD+W and HFD+R suggesting and apple matrix effect. LMNA and GPX1
580 were increased after HFD+Atorv and HFD+R suggesting an ACN effect provided by red-
581 fleshed apple similar to the hypolipemiant drug effect.

582 **3.4. Pathway analysis of the differentially expressed proteins in heart and aorta tissue**
583 **modulated by HFD+W or HFD+A**

584 To evaluate the interest of the presence of anthocyanins in apple flesh, we conducted the
585 clustering and pathway analysis through the IPA software with the differentially expressed
586 proteins (p<0.02) in the aorta and heart Wistar rat tissues after HFD+W as a standard apple
587 intake source or HFD+A as an anthocyanin-apple source. Due to the origin of the differentially
588 express proteins modified by the HFD+R treatment, the pathway analysis could not be predicted
589 by IPA software to construct a predicted network.

590 After the HFD+W diet, the top network found by IPA was "Neurological Disease,
591 Hematological Disease, and Cardiovascular Disease" (score=25). 15/46 differentially expressed
592 proteins formed part of this network (**Figure 2**).

593 Furthermore, after the HFD+A diet, the top network found by IPA was "Energy Production,
594 Cellular Function and Maintenance, and Post-translational Modifications" (score=17). 10/28
595 differentially expressed proteins formed part of this network (**Figure 3**).

596 The graphical representation of the main Networks modified by HFD+W or HFD+A is shown
597 in **Figure 2** and **Figure 3,** respectively, in which the modulated proteins or others predicted
598 proteins involved in the Network are located in the cell compartments. The differentially
599 expressed proteins modified by HFD+W or HFD+A have been highlighted in color and
600 indicated when the proteins were up- or downregulated (red means upregulated and green
601 means downregulated) compared to HFD.

602 The top canonical pathways modulated after the proteome analysis of the HFD+W diet were
603 Acute Phase Response Signaling (C3, C9, C4BPA, CFB, CP, HP, HPX, ORM1, SERPINA1,
604 SERPINA3, and TF), Complement System (C3, C9, C1QB, C4BPA, and CFB), LXR/RXR
605 Activation (C3, C9, HPX, ORM1, SERPINA1, and TF), FXR/RXR Activation (C3, C9, HPX,
606 ORM1, SERPINA1, and TF) and Coagulation System (F9, F12, and SERPINA1) Iron
607 homeostasis signaling pathway (CP, HP, HPX, and TF). The top canonical pathways modulated
608 after the proteome analysis of the HFD+A diet were CDK5 Signaling (LAMC1, PRKACA, and
609 RRAS2), Epithelial Adherens Junction Signaling (IQGAP1, MYL6, and RRAS2), Glutamate
610 Biosynthesis II (GLUD1), Glutamate Degradation X (GLUD1), PPARα/RXRα Activation
611 (HSP90AB1, PRKACA, and RRAS2), Actin Cytoskeleton Signaling (IQGAP1, MYL6, and
612 RRAS2).

613 **3.5. Upstream regulators of the protein dataset modified after HFD+W or HFD+A of the**
614 **heart and aorta rat tissue**

615 The IPA upstream regulators identify the cascade of upstream transcriptional regulators that can
616 explain the observed gene expression changes in the proteins dataset. After the HFD+W diet,
617 the top five upstream regulators of the protein dataset modified in aorta and heart rat tissue are
618 TNF, AGTR2, NFE2L2, EGR1, and PRL. Regarding the HFD+A diet, the top five upstream

regulators of the protein dataset modified in the aorta and heart Wistar rat tissue, are FLNA, FRS2, MYOCD, PIAS1, and *Yap1*. Those regulators help to illuminate the biological activities occurring in the tissues or cells.

3.6. Top relevant diseases and biological functions after HFD+W or HFD+A

The top relevant diseases and biological functions after HFD+W diet in aorta and heart tissues and the main proteins involved in Blood coagulation (C3, C9, F12, and F9), Homeostasis of iron ion (CP and TF), Complement activation (C3 and CFB), Transport of transition metal ion (CP and TF), Complement-mediated lysis of red blood cells (CFB), Classical complement pathway (C3), Transport of iron ion (TF), Transport of Cu^{2+} (CP), Myocardial infarction (C3), and Contraction of aortic ring tissue (HPX).

Regarding the top relevant diseases and biological functions after HFD+A diet were Nervous system development (PRKACA, CLTC, CAP1, SEPT2, and IQGAP1), Cell death and survival (PRKACA, RRAS2, HSP90AB1, PPIA, and NNT) Small molecule biochemistry (AK2, ATP5PB, GLUD1, and NNT).

4. CONCLUSION

Red-fleshed apple, white-fleshed apple, and ACN-rich extract, all three interventions were able to modify the expression of multiple proteins on aorta and heart tissues in hypercholesterolemic rats, in different pathways, which are positively related to the CVD benefits.

Concretely, the red-fleshed apple was involved in the downregulation of C1QB and CFP on aorta tissue and CRP on heart tissue related to the complement system and inflammation.

The white-fleshed apple consumption was involved in the downregulation of proteins related to the complement system (C1QB, CFB, CFP, C9, and C3 on aorta tissue and also C9 and C3 in heart tissue), the iron homeostasis system (CP, HP, TF, and HPX on aorta tissue, and also HP, TF, HPX, and MB on heart tissue), while regulated proteins positively involved in the cellular

643 energetic homeostasis (upregulation of ME1, PKM, and PC in aorta tissue and downregulation
644 of ECH1, GSTM2, and ANXA2 in heart tissue).

645 Moreover, red-fleshed and white-fleshed apple consumption, independently of their ACN
646 content, reduced proteins related to the complement system, suggesting an anti-inflammatory
647 effect of the apple matrix, which could be related to other phenolic compounds different from
648 ACN, or with other apple components as soluble fiber.

649 On the other hand, ACN-rich extract intervention significantly regulated proteins related to the
650 cellular structure (upregulation of FMOD, TAGLN, TAGLN2, and MYL6, and downregulation
651 of CAP1), while downregulating proteins related to the cellular signaling pathways (PRKACA,
652 IQGAP1, and HSP90AB1) on aorta rat tissue.

653 The proteomic data obtained revealed more information about the metabolic pathways
654 modulated by ACNs from apple and its matrix, thereby increasing our understanding of the
655 underlying mechanism by which apple regulates protein expression to potentially protect the
656 heart and aorta tissues from CVD.

657 From our results, we conclude that red-fleshed apple rich in ACNs consumption suggested an
658 anti-inflammatory effect based on the heart proteome modulation in hypercholesterolemic rats.
659 White-fleshed apple reduced mainly the complement system protein-related in heart and aorta
660 while the ACN-rich extract intake modified the expression of different structural and signaling
661 proteins related to CVD in the aorta. Moreover, the apple matrix could have an effect on
662 reducing inflammatory proteins on the aorta and/or heart regardless of their ACN content.
663 Therefore, these findings provide a more complete picture of the biological roles of apple intake
664 in both inflammation and other aspects of cellular biology.

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683 **Úrsula Catalán and Anna Pedret:** Conceptualization, Data curation, Formal analysis, Funding
684 acquisition, Investigation, Methodology, Validation, Visualization, Roles/Writing - original
685 draft, and Writing - review & editing. **Sílvia Yuste:** Conceptualization and Methodology.
686 **Laura Rubió:** Conceptualization, Funding acquisition, Investigation, Methodology,
687 Supervision, and Writing - review & editing. **Carme Piñol:** Methodology. **Berner Andrée**
688 **Sandoval-Ramírez:** Data curation, Investigation, Visualization, and Roles/Writing - original
689 draft. **Judit Companys:** Investigation. Elisabet Foguet: Data curation, Validation. **Pol Herrero:**
690 Data curation, Formal analysis, Software, and Validation. Núria Canela: Validation, Software,
691 and Validation. **MªJosé Motilva and Rosa Solà:** Conceptualization, Funding acquisition,
692 Investigation, Project administration, Resources, Supervision, and Writing - review & editing.

693 7. DECLARATION OF COMPETING INTEREST

694 The authors declare that they have no known competing financial interests or personal
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9. FIGURE CAPTIONS

Figure 1. Venn diagram of the common differentially expressed proteins compared to the different treatments. A) Aorta, and B) heart tissue. SCD, standard chow diet; HFD, high-fat diet;

844 HFD+R, HFD+red-fleshed Apple; HFD+W, HFD+white-fleshed Apple; HFD+A, HFD+Aronia
845 (anthocyanin-rich extract); HFD+Atorv, HFD+Atorvastatin. The common proteins up- (↑) or
846 down-regulated (↓) by the different treatments are shown in the diagram.

847 **Figure 2.** Network of Neurological, hematological and cardiovascular disease system showing
848 the differentially expressed proteins in heart and aorta Wistar rat tissues after HFD+white-
849 fleshed apple treatment. Proteins are represented in red or green color if the protein is up- or
850 downregulated, respectively.

851 **Figure 3.** The network of Energy production, cellular function and maintenance, and post-
852 translational modifications showing the differentially expressed proteins in heart and aorta
853 Wistar rat tissues after HFD+Aronia (anthocyanin-rich extract) treatment. Proteins are
854 represented in red or green color if the protein is up- or downregulated, respectively.

855 **Supplementary Figure S1**. 36 Wistar rats were divided into 6 groups of 6 animals each (3
856 males and 3 females). Group 1: standard chow-diet (SCD), Group 2: high-fat diet (HFD), Group
857 3: high-fat diet + red-fleshed apple (HFD+R), Group 4: high-fat diet + white-fleshed apple
858 (HFD+W), Group 5: high-fat diet + Aronia (anthocyanin-rich extract; HFD+A); and Group 6:
859 high-fat diet + Atorvastatin (HFD+Atorv).

860 **Supplementary Figure S2.** Venn diagram showing the significant protein changes after
861 HFD+W versus HFD in the aorta, and B) heart tissue. Proteins colored in green means decrease
862 and proteins colored in red means increase compared to HFD.

863 **Supplementary Figure S3.** Summary of the main findings in the aorta and heart proteome after
864 the supplementation with red-fleshed apple, white-fleshed apple, and anthocyanin-rich extrat.

865

Table 1

Table 1. Proteome changes on aorta or heart tissue of high-fat diet + red fleshed apple (HFD+R) *versus* high-fat diet (HFD).

Tissue	UniProt ID	Gene	Protein name	FC	*p*-value*
AORTA	P24268	*Ctsd*	Cathepsin D	1.284	0.0083
	O08557	*Ddah1*	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	1.554	0.0090
	P80254	*Ddt*	D-dopachrome decarboxylase	1.427	0.0164
	Q6IRK8	*Sptan1*	Spectrin alpha chain, non-erythrocytic 1	-1.580	0.0081
	G3V7N9	*C1qb*	Complement C1q subcomponent subunit B / Adiponectin A	-1.674	0.0087
	F1LST1	*Fn1*	Fibronectin	-1.600	0.0136
	B0BNN4	*Cfp*	Complement factor properdin	-1.454	0.0152
	P69897	*Tubb5*	Tubulin beta-5 chain	-1.202	0.0161
HEART	M0RAM5	*Gpx1*	Glutathione peroxidase	1.179	0.0110
	P41499	*Ptpn11*	Tyrosine-protein phosphatase non-receptor type 11	1.340	0.0165
	P49242	*Rps3a*	40S ribosomal protein S3a	1.153	0.0180
	P24368	*Ppib*	Peptidyl-prolyl cis-trans isomerase B	1.342	0.0185
	G3V8L3	*Lmna*	Lamin A, isoform CRA_b	1.153	0.0187
	P48199	*Crp*	C-reactive protein	-1.653	0.0031
	Q5M9G3	*Caprin1*	Caprin-1	-1.507	0.0036
	A0A0G2JY31	*Serpina1*	Alpha-1-antiproteinase	-1.263	0.0054
	Q6P7Q4	*Glo1*	Lactoylglutathione lyase	-1.338	0.0121
	Q62651	*Ech1*	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	-1.144	0.0133
	P11960	*Bckdha*	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial (Fragment)	-1.212	0.0152

FC, fold change.
T-test or Wilcoxon test of pairwise comparisons was performed depending on each protein's distribution.
* $p<0.02$ was considered statistically significant

Table 2

Table 2. Proteome changes on aorta or heart tissue of high-fat diet + white fleshed apple (HFD+W) *versus* high-fat diet (HFD).

Tissue	UniProt ID	Gene	Protein name	FC	*p*-value*
AORTA	A0A0G2K1S6	*Me1*	Malic enzyme	1.670	0.0022
	P43278	*H1f0*	Histone H1.0	1.259	0.0110
	P11980	*Pkm*	Pyruvate kinase PKM	1.268	0.0128
	P52873	*Pc*	Pyruvate carboxylase, mitochondrial	1.538	0.0140
	Q9EQS0	*Taldo1*	Transaldolase	1.293	0.0187
	M0RBF1	*C3*	Complement C3	-2.158	< 0.0001
	G3V7K3	*Cp*	Ceruloplasmin	-2.244	< 0.0001
	G3V7N9	*C1qb*	Complement C1q subcomponent subunit B	-2.554	0.0004
	P12346	*Tf*	Serotransferrin	-2.387	0.0007
	A0A0H2UHI5	*Serpina3n*	Ab1-233	-2.793	0.0013
	Q63514	*C4bpa*	C4b-binding protein alpha chain	-2.706	0.0017
	Q62930	*C9*	Complement component C9	-3.006	0.0022
	A0A0G2K896	*RGD1310507*	Similar to RIKEN cDNA 1300017J02	-1.780	0.0038
	B0BNN4	*Cfp*	Complement factor properdin	-1.701	0.0087
	G3V615	*Cfb*	Complement factor B	-1.526	0.0094
	A0A0H2UHM3	*Hp*	Haptoglobin	-2.912	0.0152
	D3ZTE0	*F12*	Coagulation factor XII	-1.323	0.0158
	P20059	*Hpx*	Hemopexin	-1.925	0.0167
HEART	F1LN42	*Tns1*	Tensin 1	1.273	0.0083
	Q3V5X8	*Endog*	Endonuclease G	1.376	0.0120
	P24268	*Ctsd*	Cathepsin D O	1.131	0.0168
	A0A0G2K2T1	*Txlnb*	Taxilin beta	1.239	0.0186
	Q62930	*C9*	Complement component C9	-2.267	<0,0001
	Q63798	*Psme2*	Proteasome activator complex subunit 2	-1.997	<0,0001
	Q6MG34	*RT1-CE10*	RT1 class I, CE10	-2.250	<0,0001
	A0A0H2UHM3	*Hp*	Haptoglobin	-2.728	0.0006

P02764	*Orm1*	Alpha-1-acid glycoprotein	-3.506	0.0007
G3V7K3	*Cp*	Ceruloplasmin	-2.038	0.0016
Q6P9V7	*Psme1*	Proteasome (Prosome, macropain) activator subunit 1	-2.216	0.0043
P12346	*Tf*	Serotransferrin	-1.928	0.0043
P97532	*Mpst*	3-mercaptopyruvate sulfurtransferase	1.205	0.0047
P08010	*Gstm2*	Glutathione S-transferase Mu 2	-1.326	0.0047
P20059	*Hpx*	Hemopexin	-1.891	0.0064
P01048	*Map1*	T-kininogen 1	-2.852	0.0092
P09605	*Ckmt2*	Creatine kinase S-type, mitochondrial	-1.418	0.0116
D3ZKU6	*Gbp1*	Uncharacterized protein	-1.603	0.0123
P16296	*F9*	Coagulation factor IX	-1.183	0.0129
P07943	*Akr1b1*	Aldose reductase	-1.244	0.0134
P27605	*Hprt1*	Hypoxanthine-guanine phosphoribosyltransferase	-1.446	0.0137
Q68FS4	*Lap3*	Cytosol aminopeptidase	-1.443	0.0151
Q62651	*Ech1*	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	-1.305	0.0152
M0RBJ7	*C3*	Complement C3	-1.563	0.0153
A0A0H2UHI5	*Serpina3n*	Ab1-233	-1.809	0.0161
Q07936	*Anxa2*	Annexin A2	-1.182	0.0176
A0A0G2JY31	*Serpina1*	Alpha-1-antiproteinase	-1.273	0.0188
Q9QZ76	*Mb*	Myoglobin	-1.512	0.0199

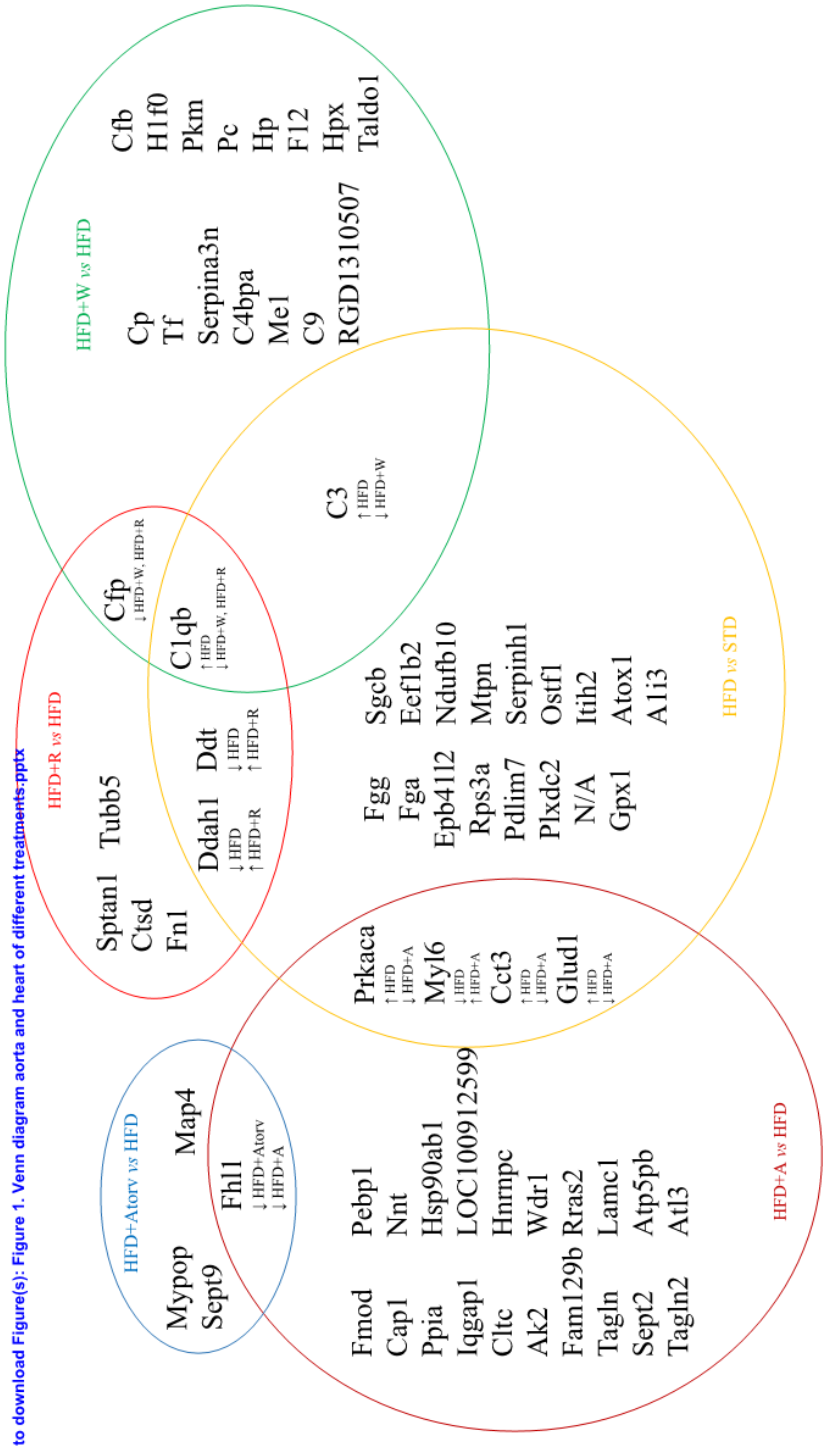
FC, fold change.
T-test or Wilcoxon test of pairwise comparisons was performed depending on each protein's distribution.
* $p<0.02$ was considered statistically significant

Table 3. Proteome changes on aorta or heart tissue of high-fat diet + Aronia (HFD+A) *versus* high-fat diet (HFD).

Tissue	UniProt ID	Gene	Protein name	FC	*p*-value*
AORTA	G3V6E7	*Fmod*	Fibromodulin	1.495	0.0002
	P10111	*Ppia*	Peptidyl-prolyl cis-trans isomerase A	1.290	0.0049
	A0A0G2JSG6	*Ak2*	Adenylate kinase 2, mitochondrial	1.296	0.007
	P31232	*Tagln*	Transgelin	1.379	0.0099
	A0A0G2K6J5	*Myl6*	Myosin light polypeptide 6	1.653	0.0119
	Q5XFX0	*Tagln2*	Transgelin-2	1.360	0.0123
	P31044	*Pebp1*	Phosphatidylethanolamine-binding protein 1	1.569	0.0129
	D3ZCZ9	*LOC100912599*	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	1.492	0.0139
	Q9WUH4	*Fhl1*	Four and a half LIM domains protein 1	-1.669	0.0022
	Q08163	*Cap1*	Adenylyl cyclase-associated protein 1	-1.472	0.0037
	A1L1M0	*Prkaca*	Protein kinase, cAMP-dependent, catalytic, alpha	-1.122	0.0043
	G3V7Q7	*Iqgap1*	IQ motif containing GTPase activating protein 1 (Predicted), isoform CRA_b	-1.507	0.006
	F1M779	*Cltc*	Clathrin heavy chain	-1.582	0.0064
	B4F7E8	*Fam129b*	Niban-like protein 1	-1.204	0.0091
	Q91Y81	*Sept2*	Septin-2	-1.414	0.0109
	Q5BJZ3	*Nnt*	Nicotinamide nucleotide transhydrogenase OS	-1.422	0.013
	P34058	*Hsp90ab1*	Heat shock protein HSP 90-beta	-1.254	0.0137
	A0A140TAI3	*Hnrnpc*	Heterogeneous nuclear ribonucleoprotein C, isoform CRA_b	-1.504	0.0144
	Q5RKI0	*Wdr1*	WD repeat-containing protein 1	-1.349	0.0146
	Q5BJU0	*Rras2*	RAS-related 2	-1.229	0.0147
	F1MAA7	*Lamc1*	Laminin subunit gamma 1	-1.413	0.0152
	P19511	*Atp5pb*	ATP synthase F(0) complex subunit B1, mitochondrial	-1.308	0.0152
	Q6P502	*Cct3*	T-complex protein 1 subunit gamma	-1.634	0.0152
	P10860	*Glud1*	Glutamate dehydrogenase 1, mitochondrial	-1.256	0.0162
	A0A0G2JSS9	*Atl3*	Atlastin-3	-1.198	0.0196
HEART	P57093	*Phyh*	Phytanoyl-CoA dioxygenase, peroxisomal	1.152	0.026
	A0A0G2K5P8	*Glrx3*	Glutaredoxin-3	-1.106	0.0263
	Q5PQN9	*Mrpl38*	39S ribosomal protein L38, mitochondrial	-1.256	0.0281

FC, fold change.
T-test or Wilcoxon test of pairwise comparisons was performed depending on each protein's distribution.
* $p<0.02$ was considered statistically significant

Figure 1
Click here to download Figure(s): Figure 1. Venn diagram aorta and heart of different treatments.pptx




B)
HFD+R vs HFD
Crp
Caprin1
Glo1
Bckdha
Ptpn11
Rps3a
Ppib
Lmna
↑ HFD+Atorv, HFD+R
Gpx1
↑ HFD+Atorv, HFD+R
HFD+W vs HFD
C9
Psme2
RT1-CE10
Hp
Orm1
Cp
Psme1
Tf
Mpst
Hpx
Map1
Endog
Gbp1
F9
Akr1b1
Hprt1
Lap3
C3
Serpina3n
Ctsd
Anxa2
Txlnb
Serpina1
↑ HFD
↓ HFD+W, HFD+R
Ech1
↑ HFD
↓ HFD+Atorv, HFD+W, HFD+R
Gstm2
↑ HFD
↓ HFD+Atorv, HFD+W
Tkt
↑ HFD
↓ HFD+Atorv, HFD+W
Ckmt2
↑ HFD
↓ HFD+Atorv, HFD+W
Mb
↑ HFD
↓ HFD+Atorv, HFD+W
Pcbp2
↓ HFD
↑ HFD+Atorv
HFD+A vs HFD
Phyh
Glrx3
Mrpl38
HFD vs STD
Pgam2
Apobec2
Ndufs8
Cyb5a
Fhl3
Vps26c
Fabp4
Gpc1
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Phb
Cbr1
Ndufb7
Pgam1
Nme2
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Dpysl2

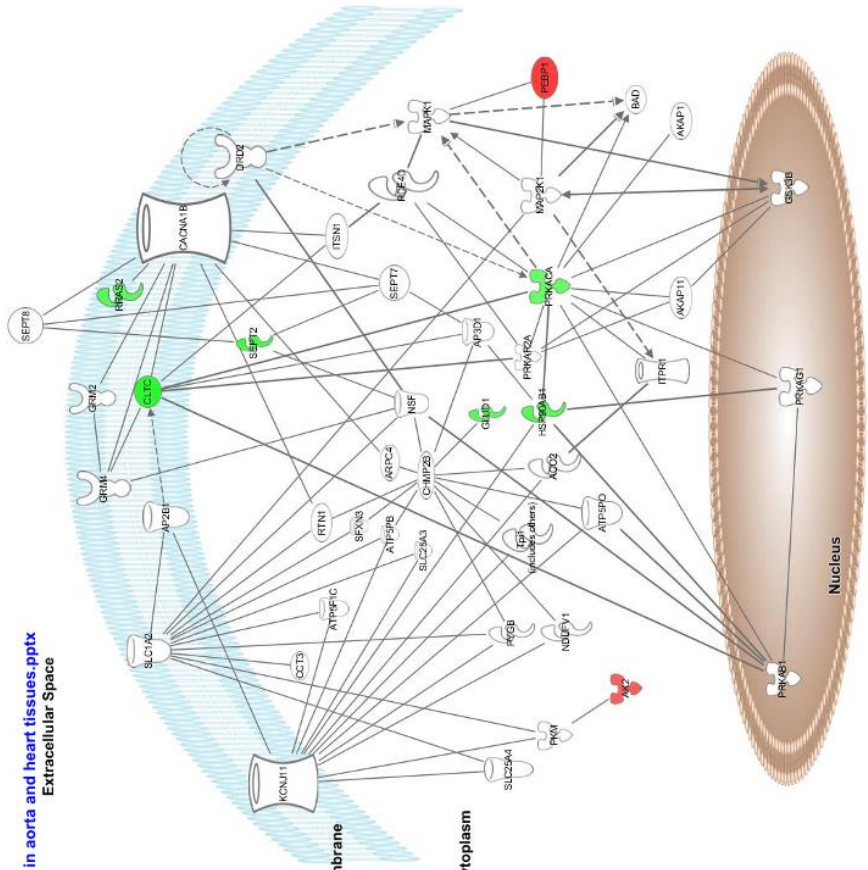

Figure 3
Click here to download Figure(s): Figure 3. IPA HFD+A in aorta and heart tissues.pptx

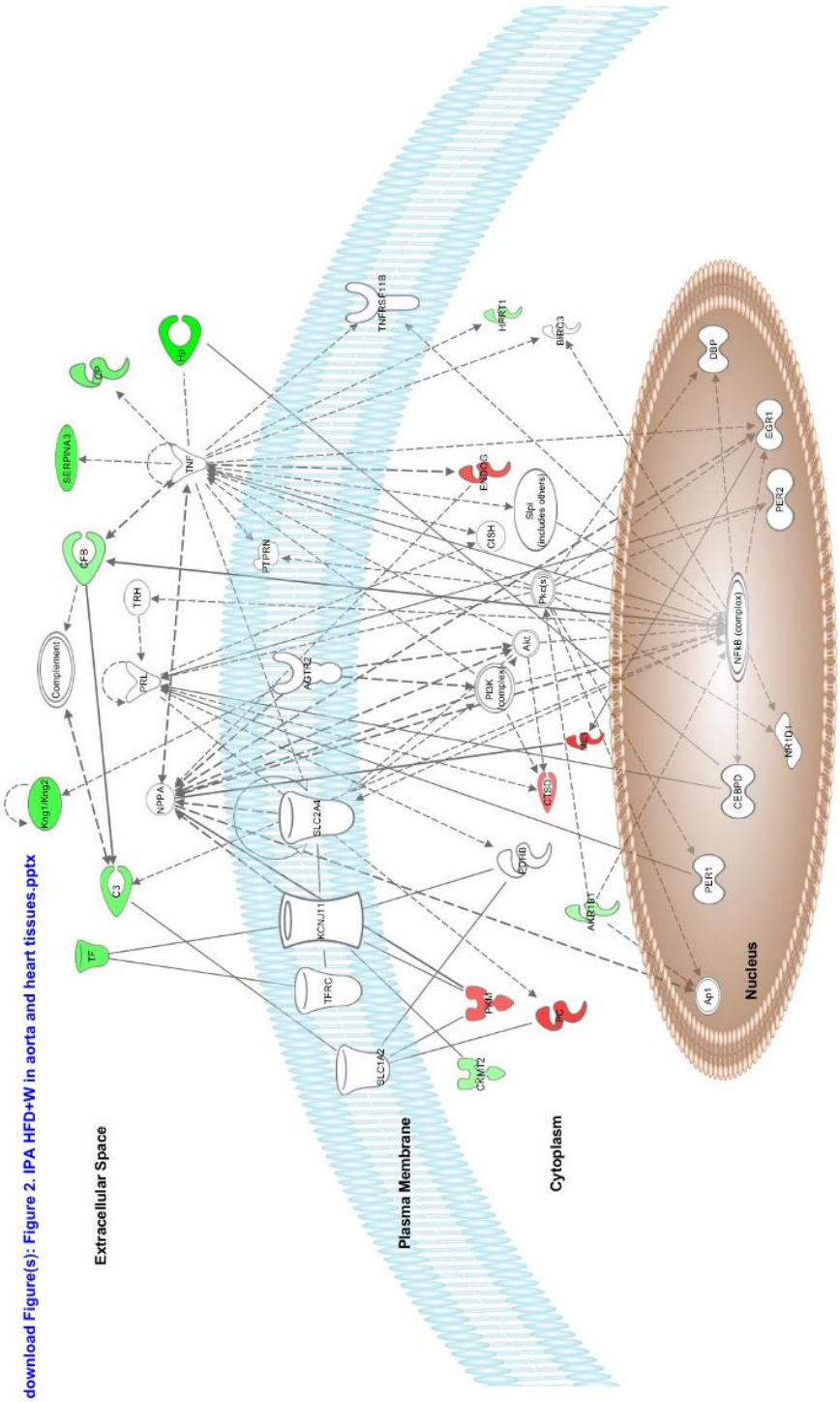

Figure 2
Click here to download Figure(s): Figure 2. IPA HFD+W in aorta and heart tissues.pptx

Supplementary Information
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SUPPORTING INFORMATION

2. Material and Methods

2.3. Proteomic analysis

2.3.1. Protein extraction and quantification

First, samples were frozen with liquid nitrogen. Second, the samples were mixed with 1 mL of a radio-immunoprecipitation buffer and homogenized completely with a BlueBender using frozen/drawn cycles. Third, the samples were agitated for 1 hour at 4 ºC and centrifuged (13,000 x *g*). Fourth, after centrifugation, the samples were sonicated with a 30s pulse at 50% amplitude. Fifth, the samples were then centrifuged at 13,000 x *g* for 15 min at 4ºC and supernatants were collected for protein precipitation with the addition of 10% trichloroacetic acid (TCA)/acetone. And Sixth, the protein pellets were re-suspended in 6M urea/50 mM ammonium bicarbonate solution and quantified by Bradford's method.

2.3.3. Offgel-nano LC-(Orbitrap) MS/MS analysis

Thus, fraction 1 (F1) was mixed with F7, F2 was mixed with F8, and so on until all fractions were mixed accordingly. In total 6 fractions were obtained and separated onto a C-18 reversed-phase nano-column (75µm I.D.; 15cm length; 3µm particle diameter, Nikkyo Technos Co. LTD, Japan) on an EASY-II nanoLC from Thermo Fisher Scientific.

2.4. Statistical analysis

2.4.1. Data pre-processing

Ratios have the disadvantage of showing a non-intuitive scale. Thus, those proteins with a two-fold increase in rat aorta or heart tissue samples will have an HFD/SCD ratio of 2, whereas those with a two-fold decrease will have an HFD/SCD ratio of 0.5. The use of a log2 scale has the advantage of producing a continuous spectrum of values and treating a two-fold change in a similar fashion. Hence, a two-fold increase in the

patients will be given by log2(2) = +1, whereas a two-fold decrease will be given by log2(1/2)=-1.

2.4.2. Multivariate statistical analysis

2.4.2.1. Unsupervised methods

Unsupervised methods were initially applied to identify trends, groupings, and outliers. These methods work on unlabeled data, that is, they do not incorporate information such as a sample class (Y: treatments/controls). PCA is a projection method that summarizes the multivariate data (X) in a small number of principal components (which are linear combinations of the original variables X) based on the largest variation in the dataset. In the PCA scores plot, each dot represents the complete proteomic profile of one sample.

On the other hand, HCA clusters observations based on the similarity of their proteomic profiles and results are usually visualized as dendrograms and heat maps. For a comparative analysis across different proteins, data were standardized as *z*-scores across samples for each protein before clustering so that the mean was 0 and the standard deviation was 1. The standardized matrix was used in unsupervised HCA for samples and proteins using Euclidean-based distances from which hierarchical clusters were generated using a Ward-linkage.

2.4.2.2. Supervised methods

Supervised methods incorporate additional information about the samples into the models to identify variation in the data that is correlated with the phenotypic response variables. Similar to PCA, PLS is a projection method that captures in its components the maximum covariance between the data (X) and the variable of interest (Y: response/class/phenotype). It is a multivariate regression technique to predict the response variable (Y) from linear combinations of the original variables (components). To evaluate the performance of each model, the goodness of fit (R2X) and the predictive performance (Q2Y), which relate to the explained and predicted variance, respectively, were calculated. The R2X always increases with the number of components, from 0, indicating that no variation in the data is modeled, to 1, where all the variation is modeled. On the other hand, Q2Y varies from -∞, which means that your model is

not all predictive or is overfitted, to 1, which reflects a perfect predictive precision. Unlike R2X, it increases with the number of components but at some point it falls, indicating the no more components should be added. The difference between Q2Y and R2X is a rough measure of overfitting.

The importance of each individual X-variable on the model was estimated the Variable Importance for the Projection (VIP), which is a weighted sum of squares (SS) of the PLS weights, $w_{a,k}$, with the weights calculated from the amount of Y-variance of each PLS component.

OPLS is similar to PLS but incorporates and orthogonal signal correction filter to improve interpretation, although it has the same predictive performance as PLS. It works by decomposing the data (X) in the so-called predictive component, related to the response variable Y, and the orthogonal components, containing the non-related information to the response. In order to assess the significance of class discrimination, a permutation test was performed.
In order to avoid overfitting, model validity was established by permutation testing (1000 permutations). It consists of comparing the Q2Y obtained for the original dataset with the distribution of Q2Y values calculated when the original Y values are randomly assigned to the individuals. Then, the position of the Q2Y for the original model in the distribution of Q2Y values obtained from the permutations is used to calculate a *p*-value to estimate the significance of the OPLS model.

In both PLS and OPLS methods, the response variable can be continuous or categorical. In the latter case, the term discriminant analysis (DA) is used and the response variable refers to the class membership. In this case, the objective is to discriminate/classify two or more classes and investigate the causes for class separation (in our case proteins that are in higher/lower concentration in patients compared to controls).
A tool for visualization and interpretation of multivariate classification OPLS-DA models is the S-plot. It visualizes both the covariance (*p[1]*), also called model loadings, and correlation (*p(corr)[1]*):

The p[1] axis describes the magnitude of each variable in X, whereas the p(corr)[1] axis represents the reliability of each variable in X. Biomarkers should have high reliability. However, peaks with high reliability but low magnitude/intensity are close to the noise level and there is a high risk for spurious correlations. Therefore, ideal biomarkers have high magnitude and high reliability and can be easily identified by both extremes of the S-plot.

Supplementary Table S1

Click here to download Supplementary Material: Supplementary Table S1_Phenolic composition in the snacks and aronia RATS

Supplementary Table S1. Phenolic composition of the main phenolics (µg phenolic/day/ rat) in the white-fleshed apple snack, red-fleshed apple snack, and anthocyanin-rich extract from *Aronia melanocarpa.*

Phenolic compounds	White-fleshed apple snack (mean ± SD)	Red-fleshed apple snack (mean ± SD)	Anthocyanin-rich extract (mean ± SD)
Cyanidin arabinoside	6.88 ± 6.12	167 ± 6.00	480 ± 18.0
Cyanidin galactoside	21.8 ± 18.1	1690 ± 36.0	1426 ± 48.0
Total Anthocyanins	***28.6 ± 24.2***	***1857 ± 42.0***	***1906 ± 67.0***
Protocatechuic acid	n.d.	108 ± 67.3	462 ± 65.0
Coumaric acid hexoside	48.7 ± 7.03	48.7 ± 7.03	2.00 ± 0.00
Ferulic acid hexoside	67.3 ± 7.44	134 ± 16.4	5.00 ± 0.00
Vanillic acid	n.d.	n.d.	14.0 ± 1.00
Vanillic acid hexoside	58.2 ± 4.05	271 ± 7.24	138 ± 22.0
5-*O*-caffeoylquinic acid	1386 ± 28.08	5004 ± 174	814 ± 434
3-*O*-caffeoylquinic acid	n.d.	n.d.	362 ± 97.0
Gallic acid	n.d.	n.d.	36.0 ± 5.00
Gallic acid hexoside	n.d.	n.d.	25.0 ± 6.00
Caffeic acid	n.d.	n.d.	32.0 ± 1.00
Homogentisic acid	n.d.	n.d.	21.0 ± 7.00
Total Phenolic acids	***1837 ± 56.2***	***5566 ± 211***	***1911 ± 638***
Catechin	377 ± 25.3	n.d.	n.d.
Epicatechin	1867 ± 27.1	353 ± 49.3	12.0 ± 1.00
Dimer	3663 ± 121	438± 18.1	32.0 ± 7.00
Trimer	350 ± 51.6	82.0± 7.34	8.00 ± 1.00
Total Flavan-3-ols	***6259 ± 226***	***875 ± 74.9***	***52.0 ± 9.00***
Quercetin arabinoside	166 ± 24.3	232 ± 28.7	5.00 ± 1.00
Quercetin rhamnoside	230 ± 26.6	587 ± 59.7	n.d.
Quercetin glucoside	541 ± 81.0	279± 35.9	133 ± 11.0
Quercetin rutinoside	n.d.	n.d	87.0 ± 12.0
Total Flavonols	***938 ± 132***	***1098 ± 124.5***	***225 ± 24.0***
Eriodictyol hexoside	n.d.	26.4 ± 1.42	7.00 ± 2.00
Naringenin	n.d.	n.d.	n.d.
Total Flavanones	***n.d.***	***26.4 ± 1.42***	***7.00 ± 2.00***
Phloretin glucoside	247 ± 34.5	1371 ± 160	n.d.
Phloretin xylosyl glucoside	276 ± 17.6	739 ± 32.7	n.d.
Hydroxyphloretin xylosyl glucoside	14.2 ± 1.01	20.5 ± 1.52	n.d.
Total Dihydrochalcones	***536 ± 53.1***	***2130 ± 195***	***n.d.***
TOTAL PHENOLICS	***9598 ± 487***	***11552 ± 1420***	***4101 ± 740***

n.d., non-detected; SD, standard deviation.

Supplementary Table S2

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Supplementary Table S2. Nutrient composition of each diet used in the study.

Nutrient information	Diet of different groups											
	SCD		**HFD**		**HFD+R[a]**		**HFD+W[a]**		**HFD+A[b, c]**		**HFD+Atorv[b, d]**	
	Per 100 g	Per 20 g daily dose	Per 100 g	Per 20 g daily dose	Per 100 g	Per 20 g daily dose	Per 100 g	Per 20 g daily dose	Per 100 g	Per 20 g daily dose	Per 100 g	Per 20 g daily dose
Protein	14.3	2.9	17.3	3.5	13.7	2.7	13.6	2.7	16.6	3.3	16.6	3.3
Carbohydrate	48.0	9.6	46.9	9.4	58.6	11.7	58.3	11.7	51.7	10.3	47.2	9.4
Fat	4.0	0.8	21.2	4.2	16.1	3.2	16.1	3.2	16.9	3.4	16.9	3.4
Kcal	290.0	58.0	450.0	90.0	396.3	79.3	406.6	81.3	428.0	85.6	410.0	82.0

SCD, standard chow diet; HFD, high-fat diet; HFD+R, HFD + red-fleshed apple; HFD+W, HFD + white-fleshed apple; HFD+A, HFD + Aronia; HFD+Atorv, HFD + Atorvastatin

[a] Composed by 75% HFD + 25% apple snack (white or red-fleshed)

[b] Composed by 75% HFD + 25% SCD

[c] 100 g feed + 1 L of aronia infusion (daily dose: 20 g feed + 20 mL of aronia infusion)

[d] 4 mg Atorvastatin/day in the water

Supplementary Table S3

Click here to download Supplementary Material: Supplementary Table S3_BBDD aorta.xlsx

Accession	Description	Σ# Proteins
P11517	Hemoglobin subunit beta-2 OS=Rattus norvegicus OX=10116 PE=1 SV=2 - [HBB2_RAT]	4
P01946	Hemoglobin subunit alpha-1/2 OS=Rattus norvegicus OX=10116 GN=Hba1 PE=1 SV=3 - [	2
A0A0G2JSV6	Globin c2 OS=Rattus norvegicus OX=10116 GN=Hba-a2 PE=1 SV=1 - [A0A0G2JSV6_RAT	2
A0A0G2JSW3	Globin a4 OS=Rattus norvegicus OX=10116 GN=Hbb PE=1 SV=1 - [A0A0G2JSW3_RAT]	4
Q5XFX0	Transgelin-2 OS=Rattus norvegicus OX=10116 GN=Tagln2 PE=1 SV=1 - [TAGL2_RAT]	1
Q62669	Beta-globin OS=Rattus norvegicus OX=10116 GN=LOC103694855 PE=1 SV=1 - [Q62669	1
P05964	Protein S100-A6 OS=Rattus norvegicus OX=10116 GN=S100a6 PE=1 SV=3 - [S10A6_RAT	1
P08010	Glutathione S-transferase Mu 2 OS=Rattus norvegicus OX=10116 GN=Gstm2 PE=1 SV=2	7
P80254	D-dopachrome decarboxylase OS=Rattus norvegicus OX=10116 GN=Ddt PE=1 SV=3 - [D	1
P62329	Thymosin beta-4 OS=Rattus norvegicus OX=10116 GN=Tmsb4x PE=1 SV=2 - [TYB4_RAT	1
P62738	Actin, aortic smooth muscle OS=Rattus norvegicus OX=10116 GN=Acta2 PE=2 SV=1 - [A	6
G3V8C3	Vimentin OS=Rattus norvegicus OX=10116 GN=Vim PE=1 SV=1 - [G3V8C3_RAT]	41
P31232	Transgelin OS=Rattus norvegicus OX=10116 GN=Tagln PE=1 SV=2 - [TAGL_RAT]	3
P02770	Serum albumin OS=Rattus norvegicus OX=10116 GN=Alb PE=1 SV=2 - [ALBU_RAT]	1
A0A0G2JSH5	Serum albumin OS=Rattus norvegicus OX=10116 GN=Alb PE=1 SV=1 - [A0A0G2JSH5_RA	1
Q08290	Calponin-1 OS=Rattus norvegicus OX=10116 GN=Cnn1 PE=1 SV=1 - [CNN1_RAT]	1
P36972	Adenine phosphoribosyltransferase OS=Rattus norvegicus OX=10116 GN=Aprt PE=1 SV=	1
Q7M0E3	Destrin OS=Rattus norvegicus OX=10116 GN=Dstn PE=1 SV=3 - [DEST_RAT]	2
P63102	14-3-3 protein zeta/delta OS=Rattus norvegicus OX=10116 GN=Ywhaz PE=1 SV=1 - [143	2
P10111	Peptidyl-prolyl cis-trans isomerase A OS=Rattus norvegicus OX=10116 GN=Ppia PE=1 SV=	4
Q9QZ76	Myoglobin OS=Rattus norvegicus OX=10116 GN=Mb PE=1 SV=3 - [MYG_RAT]	1
P62963	Profilin-1 OS=Rattus norvegicus OX=10116 GN=Pfn1 PE=1 SV=2 - [PROF1_RAT]	1
P07150	Annexin A1 OS=Rattus norvegicus OX=10116 GN=Anxa1 PE=1 SV=2 - [ANXA1_RAT]	1
P70623	Fatty acid-binding protein, adipocyte OS=Rattus norvegicus OX=10116 GN=Fabp4 PE=1 S	5
P45592	Cofilin-1 OS=Rattus norvegicus OX=10116 GN=Cfl1 PE=1 SV=3 - [COF1_RAT]	2
P26772	10 kDa heat shock protein, mitochondrial OS=Rattus norvegicus OX=10116 GN=Hspe1 PE	2
P47875	Cysteine and glycine-rich protein 1 OS=Rattus norvegicus OX=10116 GN=Csrp1 PE=1 SV=	1
P48500	Triosephosphate isomerase OS=Rattus norvegicus OX=10116 GN=Tpi1 PE=1 SV=2 - [TPI	3
P85972	Vinculin OS=Rattus norvegicus OX=10116 GN=Vcl PE=1 SV=1 - [VINC_RAT]	3
Q63610	Tropomyosin alpha-3 chain OS=Rattus norvegicus OX=10116 GN=Tpm3 PE=1 SV=2 - [TP	1
Q9WUH4	Four and a half LIM domains protein 1 OS=Rattus norvegicus OX=10116 GN=Fhl1 PE=2 S	5
Q6P725	Desmin OS=Rattus norvegicus OX=10116 GN=Des PE=1 SV=1 - [Q6P725_RAT]	9
Q923Z2	Tropomyosin 1, alpha, isoform CRA_a OS=Rattus norvegicus OX=10116 GN=Tpm1 PE=1	1
P05065	Fructose-bisphosphate aldolase A OS=Rattus norvegicus OX=10116 GN=Aldoa PE=1 SV=	4
P11030	Acyl-CoA-binding protein OS=Rattus norvegicus OX=10116 GN=Dbi PE=1 SV=3 - [ACBP_I	2
A0A0G2JVG3	Pyruvate kinase OS=Rattus norvegicus OX=10116 GN=Pkm PE=1 SV=1 - [A0A0G2JVG3_I	2
P61983	14-3-3 protein gamma OS=Rattus norvegicus OX=10116 GN=Ywhag PE=1 SV=2 - [1433(	3
P11980	Pyruvate kinase PKM OS=Rattus norvegicus OX=10116 GN=Pkm PE=1 SV=3 - [KPYM_RA	3
M0R5J4	Uncharacterized protein OS=Rattus norvegicus OX=10116 PE=3 SV=1 - [M0R5J4_RAT]	3
B5DFD8	SH3 domain-binding glutamic acid-rich-like protein OS=Rattus norvegicus OX=10116 GN=	1
P39069	Adenylate kinase isoenzyme 1 OS=Rattus norvegicus OX=10116 GN=Ak1 PE=1 SV=3 - [K	3
A0A0G2JSQ4	Tropomyosin 1, alpha, isoform CRA_p OS=Rattus norvegicus OX=10116 GN=Tpm1 PE=1	1
A0A0G2JSZ2	RCG32102, isoform CRA_a OS=Rattus norvegicus OX=10116 GN=Svs4 PE=4 SV=1 - [A0A	2
G3V6D3	ATP synthase subunit beta OS=Rattus norvegicus OX=10116 GN=Atp5f1b PE=1 SV=1 - [(	2
P04636	Malate dehydrogenase, mitochondrial OS=Rattus norvegicus OX=10116 GN=Mdh2 PE=1 S	1
P11762	Galectin-1 OS=Rattus norvegicus OX=10116 GN=Lgals1 PE=1 SV=2 - [LEG1_RAT]	1
Q99MC0	Protein phosphatase 1 regulatory subunit 14A OS=Rattus norvegicus OX=10116 GN=Ppp1	1
G3V913	Heat shock 27kDa protein 1 OS=Rattus norvegicus OX=10116 GN=Hspb1 PE=1 SV=1 - [(	2
Q63607	Alpha-tropomyosin 3 OS=Rattus norvegicus OX=10116 GN=Tpm1 PE=1 SV=1 - [Q63607_	1
A0A0G2K6J5	Myosin light polypeptide 6 OS=Rattus norvegicus OX=10116 GN=Myl6 PE=1 SV=1 - [A0A	6
P07943	Aldose reductase OS=Rattus norvegicus OX=10116 GN=Akr1b1 PE=1 SV=3 - [ALDR_RAT	1
Q07936	Annexin A2 OS=Rattus norvegicus OX=10116 GN=Anxa2 PE=1 SV=2 - [ANXA2_RAT]	1
P09527	Ras-related protein Rab-7a OS=Rattus norvegicus OX=10116 GN=Rab7a PE=1 SV=2 - [R	2
P04276	Vitamin D-binding protein OS=Rattus norvegicus OX=10116 GN=Gc PE=1 SV=3 - [VTDB_	2

Supplementary Table S4

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Accession	Description	Σ# Proteins	Σ# Unique Peptides
Q9QZ76	Myoglobin OS=Rattus norvegicus OX=10116 GN=Mb PE=1 SV=3	1	21
P08733	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	3	20
A0A0G2JSW3	Globin a4 OS=Rattus norvegicus OX=10116 GN=Hbb PE=1 SV=1	4	6
P01946	Hemoglobin subunit alpha-1/2 OS=Rattus norvegicus OX=10116 G	2	2
P11517	Hemoglobin subunit beta-2 OS=Rattus norvegicus OX=10116 PE=	4	5
A0A0G2JSV6	Globin c2 OS=Rattus norvegicus OX=10116 GN=Hba-a2 PE=1 SV	2	2
P07483	Fatty acid-binding protein, heart OS=Rattus norvegicus OX=1011	1	9
P23928	Alpha-crystallin B chain OS=Rattus norvegicus OX=10116 GN=Cry	1	8
Q5XFX0	Transgelin-2 OS=Rattus norvegicus OX=10116 GN=Tagln2 PE=1	2	7
O88767	Protein/nucleic acid deglycase DJ-1 OS=Rattus norvegicus OX=10	2	10
P26772	10 kDa heat shock protein, mitochondrial OS=Rattus norvegicus O	2	9
P39069	Adenylate kinase isoenzyme 1 OS=Rattus norvegicus OX=10116 G	3	10
Q63362	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	1	7
P48500	Triosephosphate isomerase OS=Rattus norvegicus OX=10116 GN=	3	18
P80254	D-dopachrome decarboxylase OS=Rattus norvegicus OX=10116 G	1	5
P16409	Myosin light chain 3 OS=Rattus norvegicus OX=10116 GN=Myl3 P	1	13
P31399	ATP synthase subunit d, mitochondrial OS=Rattus norvegicus OX=	2	10
P05065	Fructose-bisphosphate aldolase A OS=Rattus norvegicus OX=1011	6	21
P10111	Peptidyl-prolyl cis-trans isomerase A OS=Rattus norvegicus OX=10	4	7
P04692	Tropomyosin alpha-1 chain OS=Rattus norvegicus OX=10116 GN=	9	23
P70623	Fatty acid-binding protein, adipocyte OS=Rattus norvegicus OX=1	5	7
P02770	Serum albumin OS=Rattus norvegicus OX=10116 GN=Alb PE=1 S	1	3
A0A0G2JSH5	Serum albumin OS=Rattus norvegicus OX=10116 GN=Alb PE=1 S	1	2
P31044	Phosphatidylethanolamine-binding protein 1 OS=Rattus norvegicus	1	12
G3V9U2	3-ketoacyl-CoA thiolase, mitochondrial OS=Rattus norvegicus OX=	3	24
Q9Z2L0	Voltage-dependent anion-selective channel protein 1 OS=Rattus n	1	17
P63039	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus O	1	35
P04642	L-lactate dehydrogenase A chain OS=Rattus norvegicus OX=1011	4	14
D4A4D5	Similar to 60S acidic ribosomal protein P2 OS=Rattus norvegicus O	3	4
Q05962	ADP/ATP translocase 1 OS=Rattus norvegicus OX=10116 GN=Slc2	4	13
P11030	Acyl-CoA-binding protein OS=Rattus norvegicus OX=10116 GN=D	2	3
D4A5L9	Uncharacterized protein OS=Rattus norvegicus OX=10116 PE=3 S	3	10
P29419	ATP synthase subunit e, mitochondrial OS=Rattus norvegicus OX=	1	6
Q5M9I5	Cytochrome b-c1 complex subunit 6, mitochondrial OS=Rattus nor	1	2
G3V6D3	ATP synthase subunit beta OS=Rattus norvegicus OX=10116 GN=	2	30
P13803	Electron transfer flavoprotein subunit alpha, mitochondrial OS=Rat	1	20
P11980	Pyruvate kinase PKM OS=Rattus norvegicus OX=10116 GN=Pkm	5	30
D4A0T0	NADH:ubiquinone oxidoreductase subunit B10 OS=Rattus norvegic	1	11
Q06647	ATP synthase subunit O, mitochondrial OS=Rattus norvegicus OX=	1	10
D3ZD09	Cytochrome c oxidase subunit OS=Rattus norvegicus OX=10116 G	2	7
A0A0G2K3Z9	Uncharacterized protein OS=Rattus norvegicus OX=10116 PE=4 S	3	8
P45592	Cofilin-1 OS=Rattus norvegicus OX=10116 GN=Cfl1 PE=1 SV=3 -	2	2
F1LPG5	NADH:ubiquinone oxidoreductase subunit B4 OS=Rattus norvegicu	3	4
M0R5J4	Uncharacterized protein OS=Rattus norvegicus OX=10116 PE=3 S	2	16
B5DEL8	NADH dehydrogenase (Ubiquinone) Fe-S protein 5 OS=Rattus nor	2	4
O35115	Four and a half LIM domains protein 2 OS=Rattus norvegicus OX=	1	13
P67779	Prohibitin OS=Rattus norvegicus OX=10116 GN=Phb PE=1 SV=1	2	17
P19804	Nucleoside diphosphate kinase B OS=Rattus norvegicus OX=1011	2	5
P04636	Malate dehydrogenase, mitochondrial OS=Rattus norvegicus OX=	1	24
G3V8C3	Vimentin OS=Rattus norvegicus OX=10116 GN=Vim PE=1 SV=1 -	12	17
Q62669	Beta-globin OS=Rattus norvegicus OX=10116 GN=LOC103694855	1	8
G3V7U0	Cysteine and glycine-rich protein 3 OS=Rattus norvegicus OX=101	3	8
D3ZE15	NADH:ubiquinone oxidoreductase subunit A13 OS=Rattus norvegic	2	4
O35244	Peroxiredoxin-6 OS=Rattus norvegicus OX=10116 GN=Prdx6 PE=	1	13

Supplementary Table S5
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Supplementary Table S5. Proteome changes on aorta and heart tissue of high-fat diet *versus* standard chow died.

Tissue	UniProt ID	Gene	Protein name	FC	*p*-value*
AORTA	P02680	*Fgg*	Fibrinogen gamma chain	2.228	< 0.001
	Q7TQ70	*Fga*	Ac1873	1.989	0.0010
	M0RBF1	*C3*	Complement C3	1.489	0.0019
	A0A0G2K162	*Epb41l2*	Erythrocyte membrane protein band 4.1-like 2	1.197	0.0022
	P49242	*Rps3a*	40S ribosomal protein S3a	1.302	0.0027
	Q9Z1Z9	*Pdlim7*	PDZ and LIM domain protein 7	1.239	0.0043
	A1L1M0	*Prkaca*	Protein kinase, cAMP-dependent, catalytic, alpha	1.240	0.0064
	D4A5E5	*Sgcb*	Sarcoglycan, beta	1.331	0.0085
	Q6P502	*Cct3*	T-complex protein 1 subunit gamma	1.622	0.0087
	G3V7N9	*C1qb*	Complement C1q subcomponent subunit B	2.001	0.0111
	P10860	*Glud1*	Glutamate dehydrogenase 1, mitochondrial	1.297	0.0118
	Q5RJR9	*Serpinh1*	Serine (Or cysteine) proteinase inhibitor, clade H, member 1, isoform CRA_b	1.093	0.0126
	D3ZFH5	*Itih2*	Inter-alpha-trypsin inhibitor heavy chain 2	1.698	0.0154
	P14046	*A1i3*	Alpha-1-inhibitor 3	1.494	0.0166
	B5DEZ8	*Plxdc2*	Plexin domain containing 2 (Predicted)	-1.122	0.0043
	Q5U2Q3	*N/A*	Ester hydrolase C11orf54 homolog	-1.426	0.0049
	M0RAM5	*Gpx1*	Glutathione peroxidase	-1.144	0.0071
	B5DEN5	*Eef1b2*	Eukaryotic translation elongation factor 1 beta 2	-1.260	0.0111
	D4A0T0	*Ndufb10*	NADH:ubiquinone oxidoreductase subunit B10	-1.397	0.0116
	P80254	*Ddt*	D-dopachrome decarboxylase	-1.466	0.0120
	P62775	*Mtpn*	Myotrophin	-1.416	0.0122
	Q6P686	*Ostf1*	Osteoclast-stimulating factor 1	-1.225	0.0152
	Q9WUC4	*Atox1*	Copper transport protein ATOX1	-1.819	0.0165
	A0A0G2K6J5	*Myl6*	Myosin light polypeptide 6	-1.631	0.0167
	O08557	*Ddah1*	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	-1.365	0.0190
HEART	P09605	*Ckmt2*	Creatine kinase S-type, mitochondrial	1.532	0.0015

P08010	*Gstm2*	Glutathione S-transferase Mu 2	1.386	0.0034
P16290	*Pgam2*	Phosphoglycerate mutase 2	1.260	0.0053
B4F789	*Apobec2*	Apolipoprotein B editing complex 2 (Predicted), isoform CRA_a	1.403	0.0055
Q62651	*Ech1*	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.175	0.0083
B0BNE6	*Ndufs8*	NADH dehydrogenase (Ubiquinone) Fe-S protein 8 (Predicted), isoform CRA_a	1.427	0.0096
P00173	*Cyb5a*	Cytochrome b5	1.303	0.0100
Q9QZ76	*Mb*	Myoglobin	1.609	0.0100
D3ZPF0	*Fhl3*	Four and a half LIM domains 3	1.150	0.0139
E9PU42	*Vps26c*	Down syndrome critical region gene 3 (Predicted), isoform CRA_c	1.679	0.0152
A0A0G2JY31	*Serpina1*	Alpha-1-antiproteinase	1.234	0.0177
P35053	*Gpc1*	Glypican-1	1.291	0.0182
Q4V8F6	*Pcbp2*	Pcbp2 protein	-1.273	0.0022
P70623	*Fabp4*	Fatty acid-binding protein, adipocyte	-1.346	0.0157
F1LN42	*Tns1*	Tensin 1	-1.280	0.0176
P13086	*Suclg1*	Succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	-1.288	0.0196
M0R7S5	*Plin4*	Perilipin 4	-1.497	0.0197

FC, fold change.
T-test or Wilcoxon test of pairwise comparisons was performed depending on each protein's distribution.
* $p<0.02$ was considered statistically significant

Supplementary Table S6
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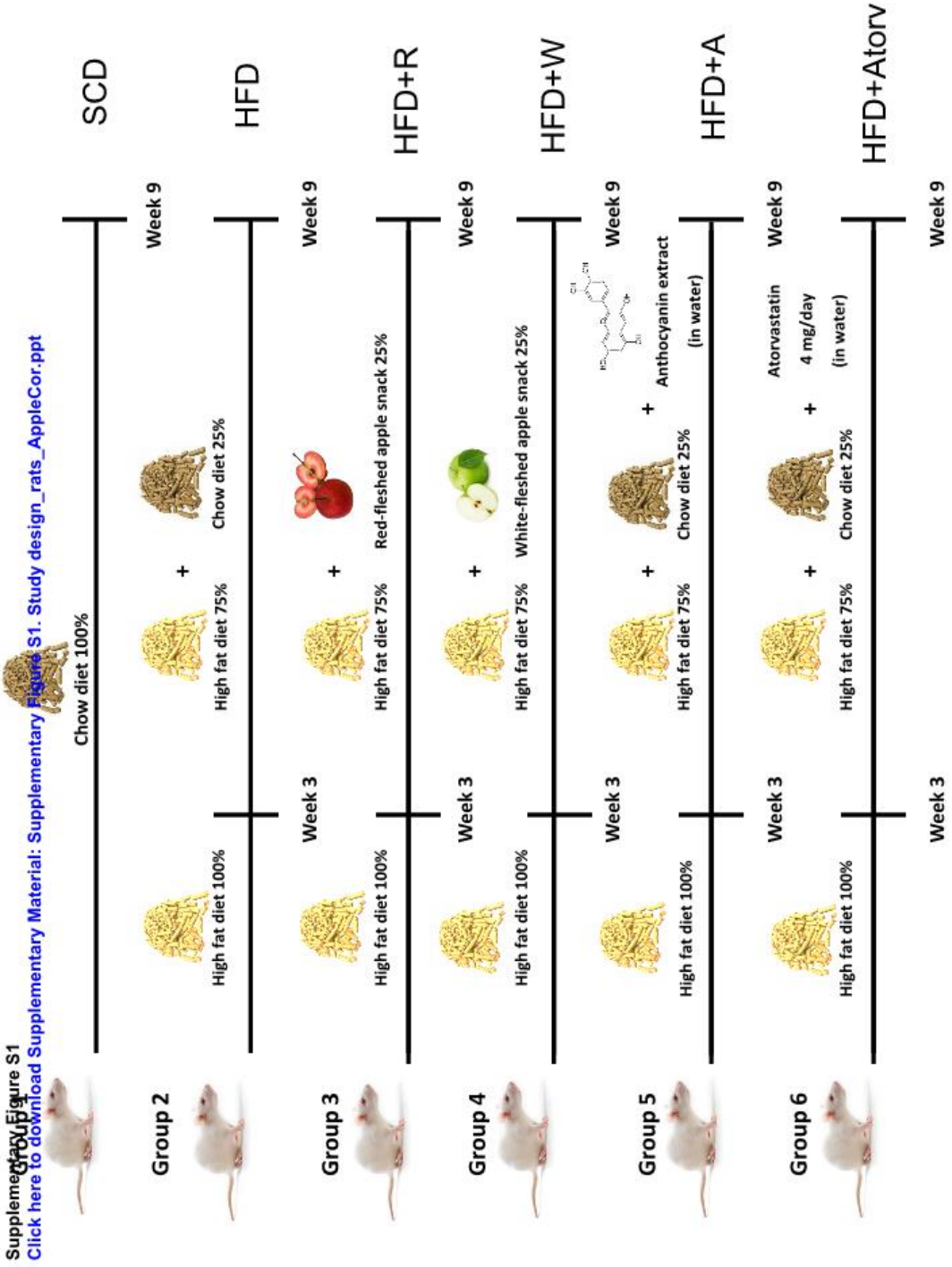
Supplementary Table S6. Proteome changes on aorta or heart tissue of high-fat diet + Atorvastatina *versus* high-fat diet.

Tissue	UniProt ID	Gene	Protein name	FC	*p*-value*
AORTA	A0A0G2JUZ3	*Mypop*	Myb-related transcription factor, partner of profilin	1.331	0.0047
	Q9QZR6	*Sept9*	Septin-9	-1.238	0.0086
	A0A0G2JW88	*Map4*	Microtubule-associated protein	-1.582	0.0087
	Q9WUH4	*Fhl1*	Four and a half LIM domains protein 1	-1.465	0.0108
HEART	F1LN42	*Tns1*	Tensin 1	1.297	0.0008
	Q4V8F6	*Pcbp2*	Pcbp2 protein	1.245	0.0022
	P47942	*Dpysl2*	Dihydropyrimidinase-related protein 2	1.188	0.0022
	G3V8L3	*Lmna*	Lamin A, isoform CRA_b	1.267	0.0023
	M0RAM5	*Gpx1*	Glutathione peroxidase	1.177	0.0065
	P56571	*N/A*	ES1 protein homolog, mitochondrial	1.159	0.0148
	Q62651	*Ech1*	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	-1.298	0.0012
	Q9QZ76	*Mb*	Myoglobin	-1.727	0.0027
	P08010	*Gstm2*	Glutathione S-transferase Mu 2	-1.354	0.0041
	P67779	*Phb*	Prohibitin	-1.142	0.0085
	P47727	*Cbr1*	Carbonyl reductase [NADPH] 1	-1.300	0.0130
	D3ZLT1	*Ndufb7*	NADH dehydrogenase (Ubiquinone) 1 beta subcomplex, 7 (Predicted)	-1.613	0.0131
	P25113	*Pgam1*	Phosphoglycerate mutase 1	-1.237	0.0148
	P19804	*Nme2*	Nucleoside diphosphate kinase B	-1.356	0.0152
	D3ZF77	*Akr1c15*	Aldo-keto reductase family 1 member C15	-1.235	0.0163
	P09605	*Ckmt2*	Creatine kinase S-type, mitochondrial	-1.499	0.0172

FC, fold change.
T-test or Wilcoxon test of pairwise comparisons was performed depending on each protein's distribution.
* $p<0.02$ was considered statistically significant


Supplementary Figure S1
Click here to download Supplementary Material: Supplementary Figure S1. Study design_rats_AppleCor.ppt
SCD
HFD
HFD+R
HFD+W
HFD+A
HFD+Atorv
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Chow diet 100%
Week 9
Week 3
High fat diet 100%
High fat diet 75%
Chow diet 25%
Red-fleshed apple snack 25%
White-fleshed apple snack 25%
Anthocyanin extract
(in water)
Atorvastatin
4 mg/day
(in water)

Supplementary Figure S2
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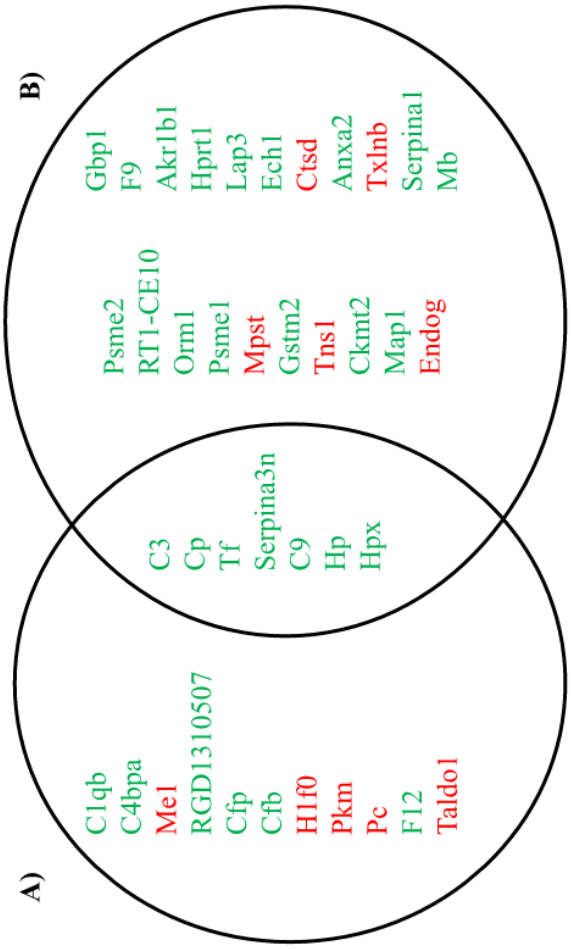
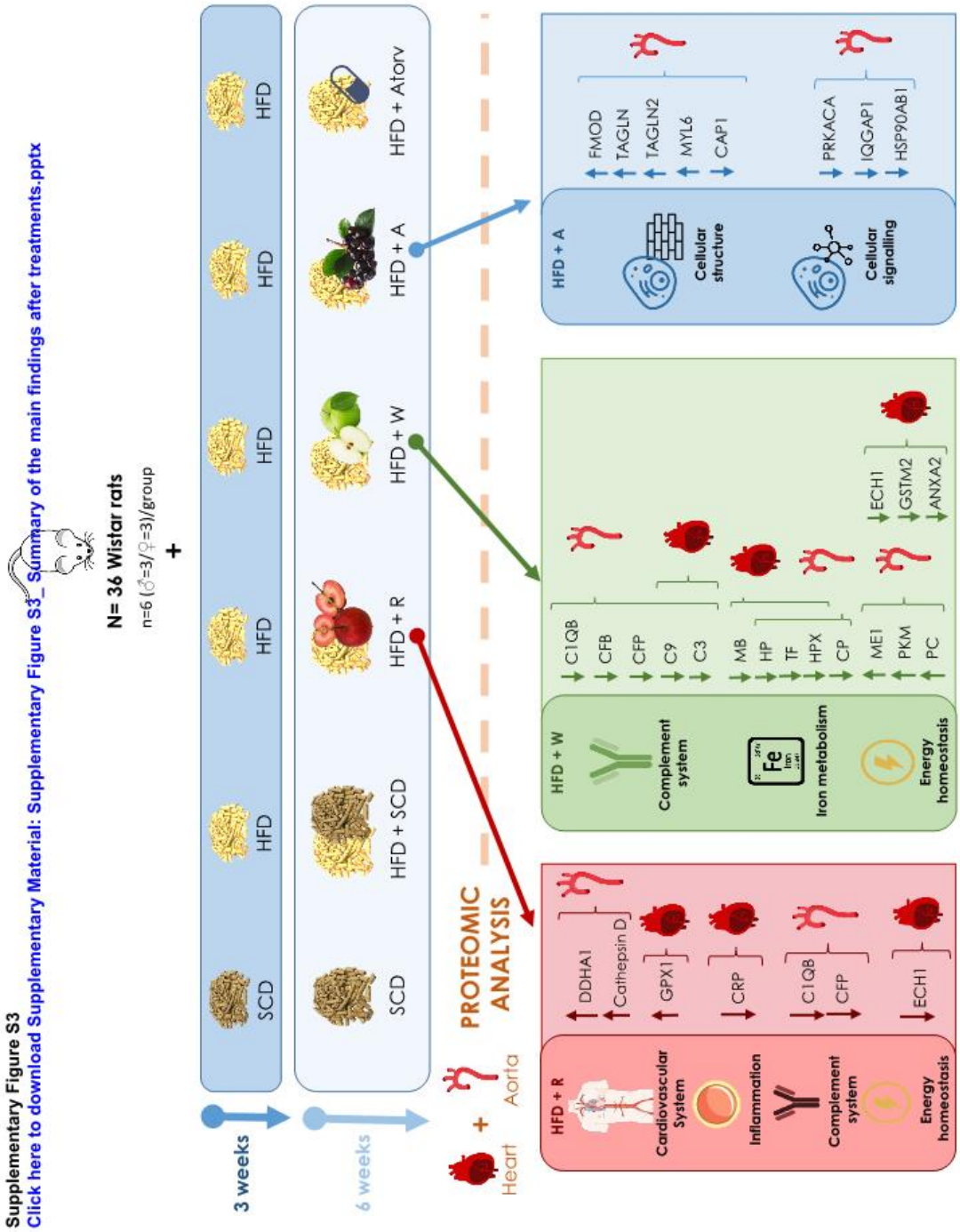


Supplementary Figure S3
Click here to download Supplementary Material: Supplementary Figure S3_ Summary of the main findings after treatments.pptx



***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

3.5. Chapter 5

The effects and associations of whole-apple intake on diverse cardiovascular risk factors. A narrative review.

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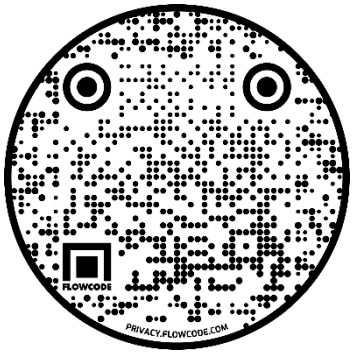

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REVIEW

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The effects and associations of whole-apple intake on diverse cardiovascular risk factors. A narrative review

Berner Andrée Sandoval-Ramírez[a], Úrsula Catalán[a,b], Lorena Calderón-Pérez[a,c], Judit Companys[c,a], Laura Pla-Pagà[c,a], Iziar A. Ludwig[a], Ma Paz Romero[d], and Rosa Solà[a,e]

[a]Faculty of Medicine and Health Sciences, Medicine and Surgery Department, Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut), Universitat Rovira i Virgili, Reus, Catalonia, Spain; [b]Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Catalonia, Spain; [c]Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Catalonia, Spain; [d]Food Technology Department, XaRTA-TPV, Agrotecnio Center, Escola Tecnica Superior d'Enginyeria Agraria, University of Lleida, Lleida, Catalonia, Spain; [e]Hospital Universitari Sant Joan de Reus (HUSJR), Reus, Catalonia, Spain

ABSTRACT

Apples are among the world's most consumed fruits. However, while the impact of whole-apple intake on cardiovascular disease (CVD) remains unknown. This narrative review summarizes a novel integrated view of whole-apple intake, CVD risk association (through observational studies; OSs), and the effects on CVD risk factors (randomized trials; RTs). In 8 OSs, whole-apple intake was associated with a reduced risk of CVD mortality, ischemic heart disease mortality, stroke mortality, all-cause mortality, and severe abdominal aortic calcification, as well as with lower C-reactive protein (CRP) concentrations. In 8 RTs, whole-apple consumption reduced total cholesterol, low-density lipoprotein cholesterol, systolic blood pressure, pulse pressure, and plasma inflammatory cytokines, and noticeably reduced CRP, whereas it increased high-density lipoprotein cholesterol (HDLc) and improved endothelial function. Thus, consuming between 100 and 150 g/day of whole apples is associated with a lower CVD risk and decreases in blood pressure, pulse pressure, total cholesterol, low-density lipoprotein cholesterol, and inflammation status as well as with increases in HDLc and endothelial function. These results, support the regular consumption of whole apples as an aid in the prevention of CVD.

KEYWORDS

Apple; blood pressure; cardiovascular; cholesterol; health

Introduction

Cardiovascular disease (CVD) is the current leading cause of death worldwide, despite the important efforts toward the prevention of cardiovascular events through the implementation of healthy lifestyles (Onor et al. 2017; Warburton and Bredin 2017; Joseph et al. 2017), which include dietary management and physical activity (Martínez-González et al. 2015). Consequently, more strategies are needed to address the management and prevention of CVD. Additionally, consuming high amounts of fruits and vegetables is associated with diverse cardiovascular health benefits, such as the reduction in various cardiovascular risk factors and the associated risk of cardiovascular events and CVD mortality (Slavin and Lloyd 2012; Miller, Thangthaeng, et al. 2017; Alissa and Ferns 2017; Collese et al. 2017). Apples are one of the most popular fruits worldwide due to their seasonal availability, geographical distribution and organoleptic properties (Wang et al. 2018). Currently, apples' health effects are attributed mostly to their high content of phenolic compounds (PCs) (GutiErrez-Grijalva et al. 2016; Guasch-Ferré et al. 2017; Dias et al. 2017) and fiber (Veronese et al. 2018). However, the total phenolic composition, pattern, and content of apples differ significantly between varieties (Kalinowska et al. 2014). Furthermore, apple diversity is influenced by a set of environmental factors including the soil, growing season, and harvest season, as well as their storage conditions and maturity status (Kalinowska et al. 2014; Stirpe et al. 2017). Despite these differences, the most frequently found PCs in apples are hydroxycinnamic acids (0.05–3 g/kg), flavanols (4.6–25.48 g/kg), dihydrochalcones (0.049–0.434 g/kg), and anthocyanins, which are mainly in the red peel (0.010–0.551 g/kg), which contribute to a total phenolic content that ranges between 5.23 and 27.24 g/kg of their dry weight (Hyson 2011; Gerhauser 2008). In addition, apples contain by a complex set of components such as vitamins A, B1, B2, C, and K, and minerals including iron, phosphorus, and potassium, as well as natural sugars (Stirpe et al. 2017). Therefore, to reveal the impact of whole-apple consumption on CVD risk factors, information from observational studies (OSs) and randomized trials (RTs) was

CONTACT Úrsula CATALÁN ursula.catalan@urv.cat Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili (URV), Facultat de Medicinia i Ciències de la Salut, Functional Nutrition, Oxidation, and Cardiovascular Disease Research Group (NFOC-Salut), C/Sant Llorenç, 21. 43201 Reus, Catalonia, Spain.

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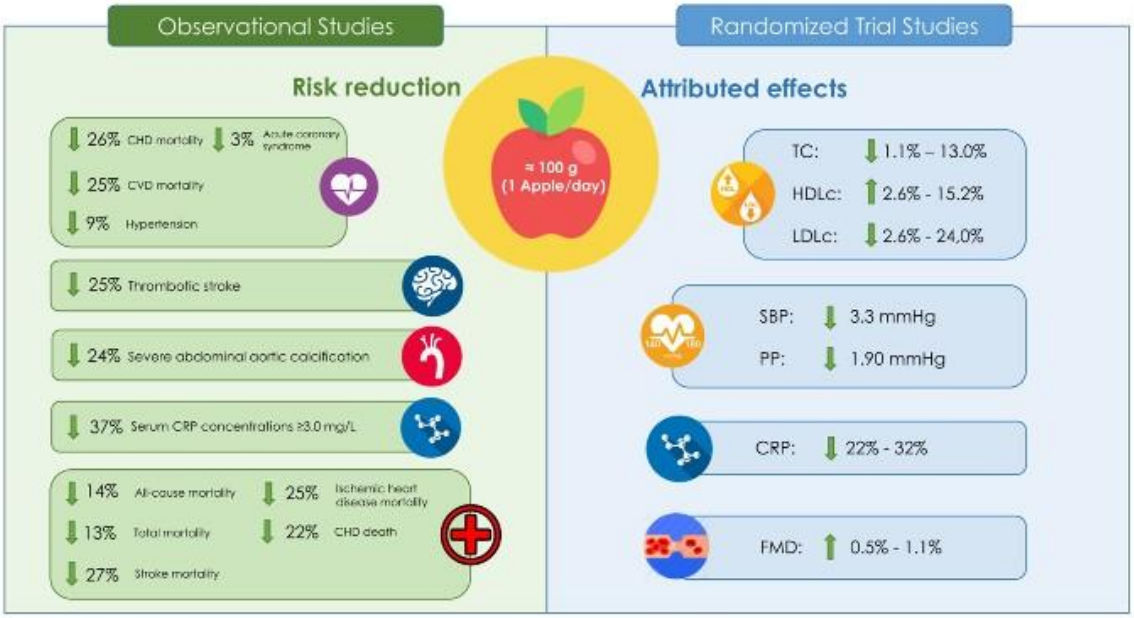

Figure 1. Beneficial effects of whole-apple consumption from observational studies (associated risk reductions) and randomized trial studies (attributed effects). The figure showed the health benefits of the consumption of approximately 100 g of whole-apple, corresponding to 1 apple a day, on diverse cardiovascular risk factors. Low-density lipoprotein cholesterol (LDLc), total cholesterol (TC), high-density lipoprotein cholesterol (HDLc), C-reactive protein (CRP), millimeters of mercury (mmHg), systolic blood pressure (SBP), pulse pressure (PP), coronary heart disease (CHD), cardiovascular disease (CVD), flow-mediated dilatation (FMD). All values presented, are statistically significant ($p < 0.05$) with the exception of the CRP values in randomized clinical trials.

analyzed. First, the associations between whole-apple consumption and CVD risk protection were assessed based on the analysis of OS. Second, the causal effects of whole-apple consumption on CVD risk factors were assessed through RTs. As a food, the causal effects of whole-apple consumption on CVD risk factors in humans should be considered with attention to particular methodological considerations regarding the compliance of different key quality characteristics of randomized controlled trials (RCTs) such as the impossibility for a double-blinded trial and the lack of adequate controls and/or placebos (Kalinowska et al. 2014; Hébert et al. 2016).

Such is the case of the seminal study by Gormley et al. conducted in 1977, which involved noncontrolled and non-randomized parallel intervention trial design, on 76 free-living male volunteers aged between 30 and 50 years old (Gormley et al. 1977). The participants were divided into two groups based on similar cholesterol levels: Group 1 consumed between 2 and 3 Irish Golden Delicious (GD) apples/day whereas Group 2 consumed only 3 Irish GD apples/week. Both groups were evaluated before and after a period of 4 months (Gormley et al. 1977). Despite the positive results reported by the authors regarding a serum cholesterol reduction of 8.1% when comparing both groups at the end of the intervention (Gormley et al. 1977), the study design does not comply with the present-day quality standards of an RCT (Schulz, Altman, and Moher 2010). Moreover, since there is a current interest in assessing the effects of whole-food intake, we focused on the effects of the intake of whole apples instead of their individual components, extracts or juices. In that context, the objective of this narrative review is to render a novel and integrated view of whole-apple consumption by summarizing its associations with different CVD risk factors through OSs as well as the effects of whole-apple intake on CVD risk factors such as blood pressure, endothelial function, lipids, and inflammation in RTs.

Literature search

This narrative review is structured based on the criteria proposed by different authors (Green, Johnson, and Adams 2006; Ferrari 2015) and utilizes a methodological systematic approach to the literature search based on the general principles published in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al. 2009). The PRISMA flowchart (Supplementary material, Figure 1) and checklist (Supplementary material, Table 1) for the present narrative review are provided. The Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies from the National Heart, Lung and Blood Institute (NHLBI) was used to assess the included observational studies (National Institutes of Health 2014) for the OSs included in this narrative review (Supplementary material, Table 2). For the included RTs, the Revised Tool to Assess Risk of Bias in Randomized Trials (RoB 2) (Higgins et al. 2011) was employed (Supplementary material, Table 3 and Supplementary Data).

Information sources and search strategy

The scientific web libraries SCOPUS (https://www.scopus.com), Cochrane Library (https://www.cochranelibrary.com/), and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) were explored using the following search terms for this narrative review: "(Apples OR malus) AND (health OR health outcome) AND (RCT OR randomized clinical trial OR epidemiological OR observational OR randomized control* trial OR cohort OR cohort studies OR case-control studies) NOT (review)". Only studies published in the last 20 years, from January 1999 to April 2019 were finally selected.

Article's selection criteria employed

Our group identified 802 articles from the database search after duplicates were removed, and no additional articles were found by hand-searching. Only peer-reviewed articles written in English were included. Two independent authors (B.A.S.-R. and Ú.C.) extracted the published data and a third reviewer (R.S.) resolved all differences. After the analysis of the titles and abstracts, a total of 16 articles (Arts et al. 2001; Mink et al. 2007; Hodgson et al. 2016; Bondonno et al. 2012; Bondonno et al. 2018; 2016; Knekt et al. 2002; Chun et al. 2008; Chai et al. 2012; Hansen et al. 2010; Borgi et al. 2016; Tenore et al. 2017; Vafa et al. 2011; Ravn-Haren et al. 2013; Auclair et al. 2010; Liddle et al. 2019) were included according to our search strategy for this narrative review.

The effects and associations of whole-apple intake on cardiovascular risk factors

The information regarding the associations between whole-apple consumption and cardiovascular risk factors provided by the OSs and the beneficial effects attributed to whole-apple intake on CVD risk factors, acquired from the RTs, are shown in Table 1.

Observational studies

Our research strategy identified 8 OSs that reported associations between whole-apple intake and variouscardiovascular risk factors (Mink et al. 2007; Hodgson et al. 2016; Bondonno et al. 2016; Knekt et al. 2002; Arts et al. 2001; Chun et al. 2008; Hansen et al. 2010; Borgi et al. 2016). Of these articles, 7 were cohort studies (Mink et al. 2007; Hodgson et al. 2016; Bondonno et al. 2016; Knekt et al. 2002; Arts et al. 2001; Hansen et al. 2010; Borgi et al. 2016), and 1 was a cross-sectional study, which was included in our analysis due to scarce data (Chun et al. 2008).

Observed cardiovascular effects of whole-apple intake

Many beneficial associations have been consistently demonstrated in OSs between higher intakes of PCs, obtained through fruit consumption, and various CVD risk factors (Aune et al. 2017; Miller, Thangthaeng, et al. 2017). However, to the best of our knowledge, a comprehensive analysis on the associations between whole-apple intake and different CVD risk factors has not been performed to date. In that context, the Iowa Women's Health Study, a prospective cohort study performed on a total sample of 34,489 postmenopausal women aged between 55 and 69 years old, between 1986 and 2002, sought to unveil the possible health-related associations between the fruit and vegetable intake, determined through food-frequency questionnaires (FFQ), and various cardiovascular outcomes (Mink et al. 2007). The authors reported that the postmenopausal women who consumed >1 whole-apple/day reported a significant 25% reduction in CVD mortality [hazard rate ratio (HRR) (95% confidence interval; CI)] [0.75 (0.68, 0.82; $p < 0.001$)] (Mink et al. 2007). Additionally, it was demonstrated that consuming >1 whole-apple/day is also associated with a 26% reduction in coronary heart disease (CHD) mortality [0.74 (0.65, 0.84; $p < 0.001$)] and with a 27% reduction in stroke mortality [0.73 (0.59, 0.90; $p = 0.018$)] compared with postmenopausal women who consumed <1 apple/week (Mink et al. 2007). Additionally, in another cohort study in which the total fruit intake of 1,456 elderly women (>70 years old) was assessed through FFQs for a period of 15 years (Hodgson et al. 2016), the results of the study reveal that, after multivariable adjustments, for each 53 g/day increase in whole-apple intake there is a significant 14% risk reduction in the all-cause mortality among the nonsmoking population [HR = 0.86 (0.75, 0.97; $p < 0.05$)]. However, the same effects were not observed for the smoking population (Hodgson et al. 2016).

Furthermore, in the "Calcium Intake Fracture Outcome Study" (CAIFOS), a five-year-long (1998–2003) prospective cohort study, in which the whole-apple consumption was assessed through FFQs on a sample of 1052 elderly women (over 70 years old), and abdominal aortic calcification (AAC) was assessed as a biomarker for subclinical vascular disease (N. P. Bondonno et al. 2016). After multivariable adjustments, the results showedthat for each extra 50 g/day of whole-apple intake there was a 24% lower odds of having severe abdominal aortic calcification [odds ratio (OR): 0.76 (0.62, 0.93), $p = 0.009$)] (N. P. Bondonno et al. 2016).

Similar results have also been published in a 6 year (1966–1972) cohort study in which the flavonoid intake of 10,054 Finnish men and women between 52 and 65 years old was determined through dietary history (Knekt et al. 2002). The results showed that in comparisons between the highest and lowest quartiles of consumption, the ingestion of quercetin, an apple-attributed flavanol, reduced the total mortality relative risk (RR) by 13% [0.87 (0.77, 0.99; $p = 0.003$)], the RR for ischemic heart disease mortality by 25% [0.75 (0.60, 0.94; $p = 0.007$)], and the RR of thrombotic stroke by 25% [0.75 (0.57, 0.99; $p = 0.009$)] (Knekt et al. 2002) following multivariable adjustment. Moreover, the association between total mortality and the whole-apple consumption was further described in the Iowa Women's Health Study population (Arts et al. 2001). In this cohort study, the whole-apple intakes of 34,492 postmenopausal women (55–69 years old) were assessed for a 12-year period (1986–1998) through FFQs (Arts et al. 2001). The results

Table 1. General information on studies that assessed the effect of whole-apple intake on cardiovascular risk factors.

Title	Author	*N*	Population	Duration of the intervention/follow-up period	Study type	Observational evaluation method/Clinical intervention	Associations or effects between whole-apple intake and cardiovascular risk factors
Observational studies							
Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women	Mink et al. (2007)	34,489	55–69 year-old post-menopausal women	16 years	Prospective cohort	Food-frequency questionnaire	HRR demonstrates that consuming >1 apples/week has a significant inverse association (HR (95% CI)) of stroke mortality 0.73 (0.59, 0.90) $p = 0.018$, CHD mortality 0.74 (0.65, 0.84) $p < 0.001$, and CVD mortality 0.75 (0.68, 0.82) $p < 0.001$, when adjusted for age and energy.
Apple intake is inversely associated with all-cause and disease-specific mortality in elderly women.	Hodgson et al. (2016)	1,456	> 70-year-old elderly women	15 years	Cohort	Food-frequency questionnaire	HR demonstrates that for each standard deviation increase of 53 g/day of apple intake is associated (HR (95% CI)) with a lower risk of all-cause mortality in nonsmokers 0.86 (0.75, 0.979) $p < 0.05$.
Fruit intake and abdominal aortic calcification in elderly women: A prospective cohort study	Bondonno et al. (2016)	1,052	> 70-year-old elderly women	5 years	Prospective cohort	Food-frequency questionnaire	Each standard deviation (SD; 50 g/day) increase in apple intake was associated with a 24% lower odds of having severe AAC (AAC score >5) OR (95% CI): 0.76 (0.62, 0.93), $p = 0.009$ after a multivariable-adjusted logistic regression.
Flavonoid intake and risk of chronic diseases.	Knekt et al. (2002)	10,054	52–65 year-old men and women	6 years	Cohort	Dietary history	The RR (95% CI) between highest and lowest quartiles of quercetin consumption, attributed to apple intake after a multivariable adjustment was of 0.87 (0.77, 0.99) $p = 0.003$, for total mortality; 0.75 (0.60, 0.94) $p = 0.007$; for mortality from ischemic heart disease; and 0.75 (0.57, 0.99) $p = 0.009$, for thrombotic stroke.
Dietary catechins in relation to coronary heart disease death among postmenopausal women	Arts et al. (2001)	34,492	55–69-year-old post-menopausal women	12 years	Prospective cohort	Food-frequency questionnaire	Apples are inversely associated with CHD death (Risk ratio (95% CI)) 0.78 (0.62, 0.98) $p < 0.05$, after multivariable-adjusted.
Serum C-Reactive Protein Concentrations Are Inversely Associated with Dietary Flavonoid Intake in U.S	Chun et al. (2008)	8,335	≥19 year-old adults	3 years	Cross-sectional	24 hour dietary recall	Individuals who consumed more than 138 g/apple/day had a significant decrease in serum C-reactive protein concentration of 1.49 (0.10) mg/L with and an OR for CRP ≥ 3 mg/L (OR (95% CI)) 0.63 (0.39, 1.04) $p < 0.05$.
Fruit and vegetable intake and risk of the acute coronary syndrome.	Hansen et al. (2010)	53,383	50–64-year-old men and women	7.7 years	Prospective cohort	Food-frequency questionnaire	An inverse association for apple intake and the risk of the acute coronary syndrome was noted for men with a multivariable-adjusted IRR (95% CI) of 0.97 (0.94, 0.99) $p < 0.05$, per each 25 g/day of apple consumed. The same trend was observed in women, although not significant.
Fruit and Vegetable Consumption and the Incidence of Hypertension in Three Prospective Cohort Studies	Borgi et al. (2016)	123,059	25–55-year-old women 40–75-year-old men	8 years	Cohort	Semi-quantitative food-frequency questionnaire	Consumption levels of ≥4 apples/week were associated with a decreased risk of hypertension after a multivariable pooled HR (95% CI) analysis of 0.91 (0.88–0.95) $p < 0.001$.

Randomized trial studies							
Lipid profile							
Annurca (*Malus pumila* Miller cv. Annurca) apple as a functional food for the contribution to a healthy balance of plasma cholesterol levels: results of a randomized clinical trial	Tenore et al. (2016)	250	31–56-year-old mildly hypercholesterolemic healthy subjects	2 months	Chronic RT	**Group 1:** 1 RD apple/day **Group 2:** 1 GS apple/day **Group 3:** 1 F apple/day **Group 4:** 1 GD apple/day **Group 5:** 2 ANN apples/day	The significant difference ($p < 0.05$) between the end of the intervention study ($t = 2$ months) and the baseline ($t = 0$) of each group on plasma biochemical parameters: **TC reduction:** ANN (−8.4%)> GS (−4.5%)>RD (−2.9%)>F (−2.3%)>GD (-1.1%) **LDLc reduction:** ANN (−14.5%)> GS (-8.3%)>RD (−5.8%)>F (−4.7%)>GD (-2.6%) **HDLc increase:** ANN (+15.2%)> GS (+4.5%)>RD (+4.2%)>F (+4.1%)>GD (+2.6%) **TG increase:** GS (+12.7%)>F (+10.8%)>GD (+9.3%)>RD (+8.3%) >ANN (+6.1%)
Effects of apple consumption on lipid profile of hyperlipidemic and overweight men	Vafa et al. (2011)	46	30–50-year-old hyperlipidemic and overweighed men	2 months	Chronic RCT	**Group 1:** control group **Group 2:** 300 g GD apple/day	No significant differences were observed regarding TC, LDLc, HDLc, LDL/HDL ratio, Lp(a), and ApoB serum levels. There was an observed increase of TG and VLDL at the end of the study when compared to the baseline in Group 2, although not significant. Moreover, there was an observed increase in TG and VLDL when comparing Group 2 against Group 1 at the end of the study ($p < 0.05$).
Daily apple versus dried plum: impact on cardiovascular disease risk factors in postmenopausal women	Chai et al. (2012)	160	50–61-year-old healthy post-menopausal women	12 months	Chronic RCT	**Group 1:** Dried apple 75 g/day **Group 2:** Dried plum 100 g/day (comparative control)	In Group 1, after 3 months, there was a 9% reduction in serum TC and a 16% reduction of LDLc when compared to baseline, these reductions were further increased to 13% and 24% respectively at the end of the study ($p < 0.05$). Moreover, there was a reduction in TC values but not in LDLc, when comparing Group 1 versus Group 2 at 6 months ($p < 0.05$). There were no significant differences between HDLc and TG, and HDLc/LDLc ratio between and within the groups. Moreover, after dried apple consumption, HDLc increased by 3%, TG decreased by 9%, and the ratio HDL/LDL increased after 12 months when compared to the baseline, although non-significant.
Intake of whole apples or clear apple juice has contrasting effects on plasma lipids in healthy volunteers	Ravn-Haren et al. (2013)	23	18–69-year-old healthy volunteers	4 week each intervention + 1-week wash-out	Crossover chronic RCT	**Group 1**: Control period **Group 2**: 550 g/day of Shampion whole fresh apple **Group 3:** 22 g/day of Shampion apple pomace **Group 4:** 500 ml/day of Shampion cloudy apple juice **Group 5:** 500 ml/day of Shampion clear apple juice.	After 4 weeks, Group 2 showed a reduction in plasma TC and in LDLc of 5.6% and 6.7% respectively when compared to Group 1, although non-significant. Group 2 did not show any differences in TG, HDLc, and TC/HDLc ratio when compared to Group 1.

(*continued*)

Table 1. Continued.

Title	Author	N	Population	Duration of the intervention/follow-up period	Study type	Observational evaluation method/Clinical intervention	Associations or effects between whole-apple intake and cardiovascular risk factors
The regular consumption of a polyphenol-rich apple does not influence endothelial function: a randomized double-blind trial in hypercholesterolemic adults.	Auclair et al. (2010)	30	47–60-year-old hypercholesterolemic men	4 week	Crossover chronic RT	**Group 1:** 40 g of lyophilized MD apple (polyphenol-poor; = 2 MD fresh apples (270 g)) **Group 2:** 40 g of lyophilized MD apple (polyphenol-rich; = 2 MD fresh apples (270 g))	After 4 weeks of intervention, Group 2 showed no significant differences in TC, TG, LDLc, HDLc, Apo A1, Apo B, and Apo B/Apo A1 ratio when compared to Group 1 and when compared the baseline *versus* the end of the study
Inflammation							
Daily apple versus dried plum: impact on cardiovascular disease risk factors in postmenopausal women	Chai et al. (2012)	160	50–61-year-old healthy post-menopausal women	12 months	Chronic RCT	**Group 1:** Dried apple 75 g/day **Group 2:** Dried plum 100 g/day (comparative control)	Serum CRP levels noticeably decreased by 22% and 32% in Group 1 after 6 months and 12 months of intervention respectively when compared to baseline.
Intake of whole apples or clear apple juice has contrasting effects on plasma lipids in healthy volunteers	Ravn-Haren et al. (2013)	23	18–69-year-old healthy volunteers	5 × 4 week	Crossover RCT	**Group 1**: Control period **Group 2**: 550 g/day of Shampion whole fresh apple **Group 3:** 22 g/day of Shampion apple pomace **Group 4:** 500 ml/day of Shampion cloudy apple juice **Group 5:** 500 ml/day of Shampion clear apple juice.	Group 2 showed no significant reduction in serum hs-CRP after 4 weeks when compared to Group 1.
Assessing the Effects of Acute and Chronic Whole Apple Consumption on Biomarkers of Inflammation in Overweight and Obese Adults (P21-011-19)	Liddle et al. (2019)	46	44–48-year-old overweight and obese adults	6 weeks	Chronic RCT	**Group 1:** Control **Group 2:** 3 Gala apples (200 g) /day	Group 2, consisting of chronic apple consumption, decreased fasting unstimulated (IL-6) and LPS-stimulated (IL-6, INF-γ, TNF-α) PBMC-secreted inflammatory cytokines ($P < 0.05$), as well as plasma IL-6 ($P < 0.05$).
Assessing the Effects of Acute and Chronic Whole Apple Consumption on Biomarkers of Inflammation in Overweight and Obese Adults (P21-011-19)	Liddle et al. (2019)	26	42–48-year-old overweight and obese adults	1 dose (4 h and 6 h)	Crossover acute RCT	**Group 1:** 3 Gala apples (200 g)	Acute apple consumption decreased 4 h postprandial unstimulated (IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1β, TNF-α) and LPS-stimulated (IL-6, TNF-α) PBMC-secreted inflammatory cytokines ($P < 0.05$).
The regular consumption of a polyphenol-rich apple does not influence endothelial function: a randomized double-blind trial in hypercholesterolemic adults.	Auclair et al. (2010)	30	47–60-year-old hypercholesterolemic men	4 week	Crossover chronic RT	**Group 1:** 40 g of lyophilized MD apple (polyphenol-poor; = 2 MD fresh apples (270 g)) **Group 2:** 40 g of lyophilized MD apple (polyphenol-rich; = 2 MD fresh apples (270 g))	Apple intake showed no significant differences in CRP after 4 weeks of intervention between both groups, nor when comparing the end of the intervention with their respective baselines.
Blood pressure and endothelial function							
Flavonoid-rich apples and nitrate-rich spinach augment nitric	Bondonno et al. (2012)	30	33–70-year-old healthy men and women	1 dose	Crossover acute RCT	**Group 1:** Control (CP apple flesh)	Group 2 significantly reduced SBP in 3.3 mmHg (95% CI), 4.6, 1.8 ($p < 0.001$) and

oxide status and improve endothelial function in healthy men and women: A randomized controlled trial.						**Group 2:** Apple (120g CP apple flesh + 80g CP apple skin) **Group 3:** Spinach (200g) **Group 4:** Apple + Spinach (120g CP apple flesh + 80g CP apple skin + 200g spinach)	showed a non-significant tendency to lower DBPs when compared to Group 1. Moreover, Group 2 also decreased PP in 1.9 mmHg (95% CI), 3.2, 0.3 ($p = 0.02$) and showed a significant increase in brachial artery FMD in 1.1% (95% CI), 0.7, 1.5 ($p < 0.0001$) when compared to Group 1.
Flavonoid-Rich Apple Improves Endothelial Function in Individuals at Risk for Cardiovascular Disease: A Randomized Controlled Clinical Trial.	Bondonno et al. (2018)	30	47–70-year-old men and women	12 h	Acute RCT	**Group 1:** Control (2 CP apple flesh) **Group 2:** Apple (2 CP whole-apple + CP apple skin)	Group 2 improved brachial FMD at 1 h in 1% (95% CI), 0.8, 1.3 ($p < 0.001$), and at 2 h in 0.8% (95% CI), 0.6, 0.9 ($p < 0.001$) post intervention. Moreover, there were no significant differences in SBD or DBP between both groups after the acute intervention.
Flavonoid-Rich Apple Improves Endothelial Function in Individuals at Risk for Cardiovascular Disease: A Randomized Controlled Clinical Trial.	Bondonno et al. (2018)	30	47–70-year-old men and women	4 week each intervention + 2-week wash-out	Crossover Chronic RCT	**Group 1:** Control (2 CP apple flesh/day) **Group 2:** Apple (2 CP whole-apple + CP apple skin/day)	Group 2 significantly improved brachial artery FMD in 0.5% (95% CI), 0.4, 0.7 ($p < 0.001$) after 4 weeks of intervention. There were no significant differences in SBP and DBP between groups at the end of the chronic intervention.
The regular consumption of a polyphenol-rich apple does not influence endothelial function: a randomized double-blind trial in hypercholesterolemic adults.	Auclair et al. (2010)	30	47–60-year-old hypercholesterolemic men	4 week	Crossover chronic RT	**Group 1:** 40 g of lyophilized MD apple (polyphenol-poor; = 2 MD fresh apples (270 g)) **Group 2:** 40 g of lyophilized MD apple (polyphenol-rich; = 2 MD fresh apples (270 g))	Apple intake showed no significant differences in brachial artery FMD after 4 weeks of intervention between both groups, nor when comparing the end of the intervention with their respective baselines. Moreover, apple intake showed no differences in SBP, DBP, and PP.
Intake of whole apples or clear apple juice has contrasting effects on plasma lipids in healthy volunteers	Ravn-Haren et al. (2013)	23	18–69-year-old healthy volunteers	5x4 week	Crossover chronic RCT	**Group 1**: Control period **Group 2**: 550 g/day of Shampion whole fresh apple **Group 3:** 22 g/day of Shampion apple pomace **Group 4:** 500 ml/day of Shampion cloudy apple juice **Group 5:** 500 ml/day of Shampion clear apple juice.	Group 2 showed no differences in SBP and DBP when compared to Group 1 at the end of the intervention.

RT, randomized trial; RCT, randomized controlled trial; HRR, hazard rate ratio; HR, hazard ratio; RR, relative risk; OR, odds ratio; IRR, incidence rate ratio; CHD, Coronary heart disease; CVD, cardiovascular disease; TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; TG, triglycerides; VLDLc, very-low-density lipoprotein cholesterol; CRP, C-reactive protein; BP, blood pressure; RCT, Randomized Clinical Trial; IL-6, Interleukin-6; TNF-α, Tumoral nuclear factor-alpha; INF-γ, Interferon-gamma; LPS, lipopolysaccharide; AAC, abdominal aortic calcification; RD, red delicious; GS, granny smith; ANN, Annurca; F, Fuji; GD, golden delicious; hs-CRP, high sensitivity C-reactive protein; CP, Cripps Pink; DBP, diastolic blood pressure; SBP, systolic blood pressure; FMD, flow-mediated dilatation; PP, pulse pressure; MD, Malus Domestica.

following multivariable adjustments showed that the whole-apple consumption was associated with a 12% lower risk ratio of CHD mortality [0.78 (0.62, 0.98; $p < 0.05$)] (Arts et al. 2001).

Furthermore, the cardiovascular effects of the whole-apple intake were also assessed through FFQs, for a mean of 7.7 years (1993–2000), in a sample of 53,383 men and women that were a part of the "Diet, Cancer, and Health Prospective Cohort Study" (Hansen et al. 2010). The volunteers were aged between 50 and 64 years old and had no previous diagnosis of cancer in the Danish Cancer Registry (Hansen et al. 2010). The researchers found that for each 25 g/day of whole-apple that was consumed there was an associated 3% reduction in the incidence rate ratio (IRR) of the acute coronary syndrome in men [0.97 (0.94, 0.99; $p < 0.05$)] (Hansen et al. 2010). The same trend was observed in women, although the results were borderline significant (Hansen et al. 2010).

Similarly, other health benefits of whole-apple intake on the risk of hypertension were noted in the OSs.For instance, a prospective study included the volunteers who were recruited in the following three large longitudinal cohorts: the Nurses' Health Study (NHS; $n = 62{,}175$ women), the Nurses' Health Study II (NHS II; $n = 88{,}475$ women), and the Health Professionals Follow-up Study (HPFS; $n = 36{,}803$ men) (Borgi et al. 2016). The resulting sample of 123,059 participants was followed for 8 years, and the results following multivariable pooled HRR demonstrated that consuming $\geq$4 apples/week is associated with a 9% reduction in the risk of hypertension [0.91 (0.88, 0.95; $p < 0.001$)] (Borgi et al. 2016).

Additional benefits were noted for the inflammatory status, as was demonstrated in a cross-sectional study in which the serum C-reactive protein (CRP) concentrations were determined for 8,335 adults over 19 years old who were enrolled and followed in the National Health and Nutrition Examination Survey (NHANES) between 1999 and 2002 (3 years). Whole-apple intake was estimated with the USDA flavonoid databases matched with a 24-h dietary recall (Chun et al. 2008). As a part of the methodology, the individuals were divided into tertiles according to their whole-apple intake as the following: nonconsumers, individuals who consumed between 0 g and 106 g of whole-apple/day (low consumers), and individuals consuming between 106 g and 138 g of whole-apple/day (medium consumers). The reported CRP concentrations (means ± standard error of the mean; SEM) were 1.90 ± 0.02 mg/L for nonconsumers, 1.84 ± 0.12 mg/L for low consumers, and 1.49 ± 0.08 g/L for medium consumers (Chun et al. 2008). In addition, individuals who consumed more than 138 g of whole-apple/day (high consumers) showed significantly lower values of serum CRP concentrations of 1.49 ± 0.10 mg/L ($p < 0.05$) (Chun et al. 2008), similar to the concentrations for the medium consumers, and reported a clinically relevant OR of 0.63 (0.39, 1.04; $p < 0.05$) for CRP $\geq$3.0 mg/L (Chun et al. 2008). The authors observed the presence of a linear trend in a decrease of the serum CRP concentrations for whole-apple consumption in the range of 0–138 g ($p < 0.05$).

Randomized trials

Our screening identified 8 articles that described RTs (Tenore et al. 2017; Vafa et al. 2011; Chai et al. 2012; Ravn-Haren et al. 2013; Auclair et al. 2010; Liddle et al. 2019; Bondonno et al. 2012; Bondonno et al. 2018). Of these, 2 were not controlled trials (Auclair et al. 2010; Tenore et al. 2017), whereas 6 were RCTs (Vafa et al. 2011; Chai et al. 2012; Ravn-Haren et al. 2013; Liddle et al. 2019; Bondonno et al. 2012; Bondonno et al. 2018) according to the information that the authors declared in the content of the article. Moreover, out of the 8 included RTs, 5 articles reported the effects of whole-apple intake on serum or plasma lipid profiles (Tenore et al. 2017; Vafa et al. 2011; Chai et al. 2012; Ravn-Haren et al. 2013; Auclair et al. 2010), 4 described the effects of whole-apples on inflammation (Chai et al. 2012; Ravn-Haren et al. 2013; Liddle et al. 2019; Auclair et al. 2010), and 4 articles assessed the effects of whole-apple intake on, blood pressure and vascular health effects (Bondonno et al. 2012; Bondonno et al. 2018; Auclair et al. 2010; Ravn-Haren et al. 2013). Additionally, out of the 8 RTs included, 5 involved only a chronic intervention (Tenore et al. 2017; Vafa et al. 2011; Chai et al. 2012; Ravn-Haren et al. 2013; Auclair et al. 2010), 1 RT involved only an acute intervention (Bondonno et al. 2012); and 2 RTs reported results on both acute and a chronic interventions (Liddle et al. 2019; Bondonno et al. 2018). Additionally, out of these 8 studies, 5 RTs used a crossover design intervention (Ravn-Haren et al. 2013; Auclair et al. 2010; Liddle et al. 2019; Bondonno et al. 2012; Bondonno et al. 2018).

Lipid profile

The impact of serum or plasma cholesterol levels on CVD is well-established and is recognized as an independent risk factor for CVD (Goldstein and Brown 2015). According to the American Heart Association (AHA) and the American College of Cardiologists (ACC), total cholesterol (TC) levels $\leq$150 mg/dL, and/or low-density lipoprotein cholesterol levels (LDLc) $\leq$100 mg/dL, are considered to be optimal for humans (Grundy et al. 2019). Accordingly, the AHA and the ACC recommended in their latest guidelines, published in 2018, that individuals with an established atherosclerotic CVD are considered to be at high risk of cardiac complications, and should reach an LDLc target of $\leq$70 mg/dL (Grundy et al. 2019). Moreover, it has been demonstrated that each 38.67 mg/dL (1 mmol/L) decrease in the plasma LDLc concentrations is associated with a 30% reduction in CVD risk (Joseph et al. 2017). In this context, 5 RTs that assessed the effects of whole-apple consumption on the lipid profile in humans were identified through our screening (Tenore et al. 2017; Vafa et al. 2011; Chai et al. 2012; Ravn-Haren et al. 2013; Auclair et al. 2010).

In a recent single-blinded and placebo-controlled RCT, a group of 250 volunteers between 31 and 56 years old were randomly divided into 5 groups of 50 subjects each (22 women and 28 men per group) to determine the effects of the whole-apple consumption on the following CVD risk factors: TC, LDLc, high-density cholesterol (HDLc), and

triglycerides (TG) (Tenore et al. 2017). For a period of 60 days, four of the five groups consumed one apple a day of one of the following apple varieties, (1) red delicious (RD), (2) granny smith (GS), (3) GD and (4) Fuji (F), while the fifth group was assigned to consume 2 Annurca (ANN) apples/day (Tenore et al. 2017). The authors justify the use of two ANN apples instead of oneas an attempt to match the different apple's overall macronutrient composition. At the end of 2 months, ANN apples demonstrated the most beneficial lipid profile changes when compared to the RD, GS, GD, and F apple varieties. The summary of the results are presented in decreasing order as follows, for TC: ANN (−8.4%), GS (−4.5%), RD (−2.9%), F (−2.3%), and GD (−1.1%), $p < 0.05$ (Tenore et al. 2017). For the LDLc reduction: ANN (-14.5%), GS (−8.3%), RD (−5.8%), F (−4.7%), and GD (−2.6%), $p < 0.05$ (Tenore et al. 2017). Furthermore, all apples showed a significant increase in HDLc as follows: ANN (+15.2%), GS (+4.5%), RD (+4.2%), F (+4.1%), and GD (+2.6%), $p < 0.05$ (Tenore et al. 2017). In addition, all apples showed an increase in the TG values at different rates: GS (+12.7%), F (+10.8%), GD (+9.3%), RD (+8.3%), and ANN (+6.1%), $p < 0.05$ (Tenore et al. 2017). In summary, this intervention demonstrated that the overall effectiveness of apples, as modulators of plasma lipids differs depending on the apple variety consumed. Thus, the assessed whole apples were able to reduce TC values between 1.1% (GD) and 8.4% (ANN) and LDLc between 2.6% (GD) and 14.5% (ANN), while raising HDLc values between 2.6% (GD) and 15.2% (ANN), and TG values between 6.1% (ANN) and 12.7% (GS) (Tenore et al. 2017). The ANN apple effects on the lipid profile can be explained, at least in part, by their higher concentration of PCs in comparison with the rest of the compared apples (Stirpe et al. 2017). ANN apples are known to have approximately 146 ± 2.64 mg/100 g of total polyphenols, as well as high concentrations of procyanidins, quercetin, and phloretin which are known for their positive health properties (Mari et al. 2010).

In another chronic RCT study, the effects of consuming 75 g/day of a nondescribed dried apple variety on the plasma lipid profile were compared against the effects of consuming 100 g/day of dried plums (Chai et al. 2012). The intervention was performed on 160 postmenopausal women aged between 50 and 61 years old for a total of 12 months (Chai et al. 2012). As stated by the authors, the dry plum dosage was chosen based on a comparable amount of energy, fiber, carbohydrates and fat, while providing a different phenolic profile than that of the dried apple (Chai et al. 2012). The results at 3 months demonstrated a significant 9% reduction in TC and 16% in LDLc in the dried apple group compared to baseline ($p < 0.05$) (Chai et al. 2012). Moreover, at the end of the study (12 months), these reductions were further increased to 13% for TC and 24% for LDLc ($p < 0.05$) (Chai et al. 2012). However, the researchers observed no significant differences in HDLc, TG, or the HDLc/LDLc ratio, neither between nor within groups (Chai et al. 2012).

Furthermore, the effects of whole-apple consumption on the lipid profile in humans were also assessed in a crossover RCT performed on 23 healthy volunteers between 18 and 69 years old (Ravn-Haren et al. 2013). For the study, the volunteers were randomly assigned to one of five different intervention arms for a period of 4 weeks with a one-week wash-out after completing each intervention. This process was repeated until all the volunteers completed all the interventions (Ravn-Haren et al. 2013). The interventions were as follows: Intervention 1 was used as a control period; for intervention 2, the volunteers ingested 550 g/day of whole Shampion apples; for intervention 3, the volunteers consumed 22 g/day of Shampion apple pomace; for intervention 4, the volunteers drank 500 mL/day of cloudy Shampion juice; and for intervention 5, the volunteers drank 500 mL/day of clear Shampion juice (Ravn-Haren et al. 2013). The result showed that eating 550 g/day of Shampion whole-apple caused a borderline statistically significant reduction in plasma TC of 5.6% ($p = 0.066$) whereas LDLc showed a nonsignificant reduction of 6.7% ($p = 0.12$) when compared against the control intervention after four weeks (Ravn-Haren et al. 2013).

However, two of the included studies reported no significant effects of whole apples on diverse cardiovascular risk factors. In the first study, 46 overweight and hyperlipidemic men between 30 and 50 years old were divided into two groups. The first group was asked to consume 300 g/day of fresh whole Golden Delicious (GD) apples for a period of 2 months and the second group acted as a control (Vafa et al. 2011). The results showed no significant differences with respect to the TC, LDLc, HDLc, LDL/HDL ratio, Lp (a), or ApoB serum levels. However, the GD apple intake was significantly associated with an increase in the plasma TG and VLDL values when compared against the control group at the end of the study ($p < 0.05$) (Vafa et al. 2011).

Furthermore, in a 4-week crossover chronic RCT on 30 hypercholesterolemic men between 47 and 60 years old, the participants were randomly assigned into one of two groups: Group 1 was assigned to consume 40 g/day of lyophilized "polyphenol-rich" Malus Domestica (MD) apples (≈ 2 whole-apples), while Group 2 consumed 40 g of lyophilized "polyphenol-poor" MD apples (Auclair et al. 2010). After 4 weeks of intervention, Group 1 did not show any significant differences in TC, TG, LDLc, HDLc, apolipoprotein (Apo) A1, Apo B, and Apo A1/Apo B ratio when compared against Group 2or when compared to baseline values (Auclair et al. 2010).

Inflammation

There is a current focus on so-called low-grade inflammation, based on the concept that the steady production of pro-inflammatory cytokines can alter the correct and normal metabolic functions of the human body (Minihane et al. 2015). This low-grade inflammation is known to play a central role in the pathophysiology of a vast number of human diseases including CVD, obesity, osteoarthritis, cancer and other illnesses (Grandl and Wolfrum 2018; Guzik and Touyz 2017; Corte et al. 2016; Asghar and Sheikh 2017; Guarner and Rubio-Ruiz 2015). The effect of whole-apple intake on low-grade inflammation has been examined in 4

RCTs (Chai et al. 2012; Ravn-Haren et al. 2013; Liddle et al. 2019; Auclair et al. 2010) to date. Among the included articles, 1 RCT reported both an acute and a chronic intervention (Liddle et al. 2019), while the remaining 3 RCTs described only chronic interventions (Chai et al. 2012; Ravn-Haren et al. 2013; Auclair et al. 2010).

In a combined study that included both an acute and a chronic intervention, the effects of whole-apple intake on diverse low-grade inflammation biomarkers other than CRP were assessed (Liddle et al. 2019). For the acute intervention, 200 g of Gala apples were given in a postprandial study to 26 overweight or obese adults (Liddle et al. 2019). The results showed that acute apple consumption significantly decreased fasting unstimulated interleukin (IL)-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1β, and tumor necrosis factor α (TNF-α; $p < 0.05$) (Liddle et al. 2019). Furthermore, the acute whole-apple intake produced significant reductions in the IL-6 and TNF-α inflammatory cytokines that were secreted from postprandial peripheral blood mononuclear cells (PBMC) isolated from whole blood and stimulated with 10 ng/mL lipopolysaccharide (LPS) for 24 h ($p < 0.05$) (Liddle et al. 2019). In the chronic intervention, 46 overweight or obese adults were randomly assigned to either Group 1 (the control group) or to Group 2, in which the volunteers consumed 3 whole Gala apples per day (~200 g) for a 6-week intervention period (Liddle et al. 2019). The results demonstrated, a significant decrease in the fasting unstimulated IL-6 and the LPS-stimulated IL-6, interferon-γ, and TNF-α, as well as plasma IL-6 in the intervention group, Group 2 (Liddle et al. 2019).

CRP, one of the traditional inflammation biomarkers, was assessed in one RCT in which 160 postmenopausal women (50–61 years old) who were recruited in the United States from 2007 to 2009 were randomly assigned into one of two groups. Group 1 was assigned to consume 75 g/day of a nondescribed variety of whole dried apples whereas in Group 2, the volunteers consumed 100 g/day of dried plums (Chai et al. 2012). To assess the effects of the whole-apple intake on various cardiovascular risk factors, blood samples were analyzed at 0, 3, 6, and 12 months. Compared to the baseline, Group 1 showed noticeable decreases in the serum CRP concentration levels of 22% and 32%, after 6 and 12 months of intervention respectively ($p < 0.05$) (Chai et al. 2012).

However, CRP was also evaluated in the study conducted by Ravn-Haren et al. that has already been described in this article's section "Lipid profile". Lipid Profile section (Ravn-Haren et al. 2013). The results showed that consuming 550 g/day of fresh whole Shampion apples caused no significant reductions in serum high-sensitive CRP when compared to the control group after 4 weeks of intervention (Ravn-Haren et al. 2013). Similarly, another study reported negative results for the effects of whole-apple intake on CRP. In the crossover RCT conducted by Auclair et al., also described in this review's section "Lipid profile". Lipid Profile section (Auclair et al. 2010), the intake of 40 g of lyophilized MD apple, equivalent to two fresh whole apples, had no significant effect on CRP after 4 weeks of intervention, neither when compared with the control group nor when compared with the baseline values (Auclair et al. 2010).

Blood pressure and endothelial function

High blood pressure and endothelial function are considered to be modifiable risk factors for CVD (James 2017; Ettehad et al. 2016). High blood pressure is one of the most studied parameters implicated in the development of CVD. For every reduction of 10 mmHg in systolic blood pressure, there is an associated 20% (RR 0.80; CI 95% 0.77–0.83) reduction in CVD events (Ettehad et al. 2016). Moreover, endothelial function also plays a key role in all stages of atherosclerosis (Gimbrone and García-Cardeña 2016; Matsuzawa et al. 2015). Hence, endothelial function is now considered to be one of the earliest predictors of CVD and has been proposed as an independent CVD risk factor due to its profound association with cardiovascular pathologies (Gimbrone and García-Cardeña 2016; Matsuzawa et al. 2015; Daiber et al. 2017). Furthermore, high values of pulse pressure (PP), defined as the difference between the systolic and the diastolic blood pressures (Homan and Cichowski 2019) are associated with a significant risk for the development of CVD (Homan and Cichowski 2019).

In the present narrative review, 4 RTs were included that addressed the effects of whole-apple intake on the blood pressure or PP and endothelial function (Bondonno et al. 2012; Auclair et al. 2010; Bondonno et al. 2018; Ravn-Haren et al. 2013). Of the 4 included RTs, 1 RCT showed a significant reduction in blood pressure and PP (Bondonno et al. 2012) whereas 2 RCTs reported a significant increase in endothelial function (Bondonno et al. 2012; Bondonno et al. 2018).

In a crossover acute RCT performed on 30 healthy men and women aged between 33 and 70 years old, the researchers assessed the acute effects of whole apple's flavonoid intake on blood pressure and flow-mediated dilatation (FMD) of the brachial artery as primary outcomes and assessed the nitrosylated species (RXNO) and nitric oxide (NO) concentrations as secondary outcomes (Bondonno et al. 2012). For these purposes, the participants were randomly assigned into one of the following intervention groups: Group 1 consumed 120 g of Cripps Pink (CP) apple flesh as a control; Group 2 consumed 120 g of CP apple flesh + 80 g of CP apple skin; Group 3 was asked to consume 200 g of spinach; and Group 4 consumed 120 g of CP apple flesh + 80 g of CP apple skin + 200 g of spinach (Bondonno et al. 2012). The results showed that the acute intake of Group 2 significantly reduced the systolic blood pressure (SBP) mean values by 3.3 mmHg [95% CI, 4.6, 1.8 ($p < 0.001$)] and showed a nonsignificant tendency to lower diastolic blood pressure (DBP) values when compared to Group 1, the control group (C. P. Bondonno et al. 2012). Moreover, in Group 2, the PP values also decreased by 1.9 mmHg [95% CI, 3.2, 0.3 ($p = 0.02$)], and there was a significant increase in brachial artery FMD of 1.1% [95% CI,

0.7, 1.5 ($p < 0.0001$)] compared to the control Group 1 (Bondonno et al. 2012).

Additionally, another study consisted of both an acute 12 h intervention and a chronic crossover study comprising two four-week intervention periods plus a two-week washout period (Bondonno et al. 2018). To assess the effects of whole-apple intake on endothelial function, 30 men and women aged between 47 and 70 years old were randomly assigned to one of two groups. Group 1, was assigned to eat only the flesh of 2 CP apples/day (as a control), while Group 2 was assigned to eat 2 whole CP apples/day plus the skin of a second CP apple blended with water (Bondonno et al. 2018). As a result, for the acute intervention, Group 2 improved the brachial artery FMD by 1% [95% CI; 0.8, 1.3 ($p < 0.001$)] 1 h after the intervention and by 0.8% [95% CI; 0.6, 0.9 ($p < 0.001$)] 2 h after the intervention (N. P. Bondonno et al. 2018). Furthermore, as a result of the chronic intervention, Group 2 significantly improved the brachial artery FMD by 0.5% [95% CI; 0.4, 0.7 ($p < 0.001$)] after 4 weeks of intervention compared to the baseline values (Bondonno et al. 2018). However, despite the positive effects of the acute or chronic intake of whole-apples on brachial artery FMD (Group 2), there were no significant differences in the SBP nor in DBP between groups at the end of either the acute and the chronic interventions (Bondonno et al. 2018).

However, two trials did not report positive effects regarding whole-apple intake and vascular health or endothelial function (Ravn-Haren et al. 2013; Auclair et al. 2010). In the first study, a double-blinded crossover chronic RT performed by Auclair et al. and already described in section "Lipid profile". Lipid Profile section (Auclair et al. 2010), it was reported that for hypercholesterolemic males, neither consuming 40 g/day of a polyphenol-rich apple nor consuming 40 g/day of a polyphenol-poor apple caused any significant differences between the two groups in brachial artery FMD, SBP, DBP or PP or in comparisons against their respective baselines (Auclair et al. 2010). Moreover, in the final study, another crossover chronic RCT that has also been described in this paper's section "Lipid profile". Lipid Section (Ravn-Haren et al. 2013), it was reported that consuming 550 g/day of whole-fresh Shampion apple showed no significant differences in either SBP and DBP compared to those of the control group at the end of the intervention (Ravn-Haren et al. 2013).

Final remarks

The analysis of the observational evidence provided by these studies demonstrated that the intake of at least 1 whole-apple/day (≈100 g/day) is significantly associated with a reduction in the all-cause mortality risk by 14% (Hodgson et al. 2016), the stroke mortality risk by 27% (Mink et al. 2007), the ischemic heart disease mortality risk by 25% (Knekt et al. 2002), the CHD mortality risk by 26% (Mink et al. 2007), the CVD mortality risk by 25% (Mink et al. 2007), the hypertension risk by 9% (Borgi et al. 2016), the thrombotic stroke event risk by 25% (Knekt et al. 2002), and the risk of severe abdominal aortic calcification by 24% (Bondonno et al. 2016). Moreover, whole-apple consumption is associated with a 37% reduction in the risk of high serum CRP concentrations (≥3.0 mg/L) (Chun et al. 2008). Furthermore, the whole-apple intake effects tested in RTs showed an overall hypocholesterolemic effect. In particular, intake of the whole ANN variety apples reduced TC by 8.4% and LDLc by 14.7%, while increasing HDLc by 15.2%, whereas the GS, F, RD, and GD apple varieties (listed from higher to lower efficacy) were able to reduce TC and LDLc to various degrees (Tenore et al. 2017). These results suggest that at least some of the noted beneficial effects of whole apples are not primarily driven by a matrix effect as much as they are by its phenolic content. Moreover, eating 200 g/day of Gala apples for at least 6 weeks significantly decreases the production of various pro-inflammatory cytokines such as the fasting unstimulated IL-6, as well as the LPS-stimulated IL-6, INF-γ, and TNF-α in PMBCs. Additionally, the intake of 75 g/day of whole apples for 12 months noticeably reduced the inflammatory status by decreasing the CRP serum levels by 32% (Chai et al. 2012). Likewise, whole-apple intake caused a reduction of 3.3 mmHg in SBP and a reduction of 1.9 mmHg in PP (Bondonno et al. 2012), while improving the endothelial function in 1.1% as assessed using brachial artery FMD (Bondonno et al. 2012; Bondonno et al. 2018). A summary of the beneficial risk associations and effects of whole-apple consumption as shown by the OSs and RTs are displayed in Figure 1.

Additionally, most of the articles included in the present work described neither the geographical origin nor the nutritional composition of the apples used. In particular, the geographical origin of most of the apple varieties included in the present review was diverse (ANN, Italy; RD, Peru/Iowa; GS, Australia; GD, United States; F, Japan; Shampion, Czech Republic; Gala, New Zealand; CP, Australia) and was not available in all cases from the original authors. The geographical origin of apples seems to be of paramount importance when analyzing their health properties, since conditions such as the weather, the harvest season, and the type of soil significantly change the apple's nutritional composition (i.e., phenolic content or their mineral, fiber and vitamin content) (Wang et al. 2018; Kalinowska et al. 2014; Stirpe et al. 2017), which affect the various health effects on CVD. Furthermore, quality labels such as the "Protected Geographical Indication" product, given to ANN apples, ensures a specific nutritional composition linked to their geographical origin, which guarantees a standardized apple and the positive cardiovascular effects associated with their consumption.

Therefore, the common English-language proverb of Welsh origin "one apple a day keeps the doctor away," is currently being scientifically demonstrated in the context of the prevention of CVD.

Future considerations

The authors of the present narrative review consider that performing RCTs to explore the properties of foods

consumed as a whole and not only their extracts is of paramount importance for both the scientific community and general society to increase the applicability and quality of the evidence-based clinical recommendations for a healthy lifestyle, not only for disease prevention but also for individuals suffering from diverse metabolic pathologies. Moreover, we argue that the generalization of the conclusions acquired from clinical trials that use apple extracts to infer part of the properties of whole-foods can only provide researchers with partial information of their effects, due to the frequently unaccounted properties of the food matrix, which is known for its capacity to modify the bioavailability of phenols and other compounds among other possible variables (Gleize et al. 2016; Cusack, Fernandez, and Volek 2013). Additionally, our group contends that the combination of the causal evidence acquired from RCTs along with the associations obtained from OSs is the most appropriate way to determine the properties of chronic intake of whole apples and other whole foods.

Conclusion

The regular consumption of 100–150 g/day of whole apples, corresponding to one regular fresh apple, can be considered a positive action for the prevention of CVD and CVD mortality. Additionally, whole-apple intake improves different CVD risk factors through the reduction of blood pressure, PP, TC, LDLc, and inflammation status, while increasing HDLc and improving the endothelial function. This information supports regular whole-apple consumption as an effective tool in the management of CVD.

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Disclosure statement

The authors have declared no conflicts of interest. Complete Declaration of Interest forms for each author has been uploaded at the time of manuscript submission.

Author's Contributions

Study conception and design: B.A.S-R., Ú.C., L.C., J.C., L.P., and R.S.
Acquisition of data: B.A.S-R., and Ú.C.
Analysis and interpretation of data: B.A.S-R., Ú.C., and R.S.
Drafting of the manuscript: B.A.S-R, and Ú.C.
Critical revision: Ú.C., M-P.R., I.L., and R.S.

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ORCID

Berner Andrée Sandoval-Ramírez http://orcid.org/0000-0002-6242-922X
Úrsula Catalán http://orcid.org/0000-0001-8884-9823
Lorena Calderón-Pérez http://orcid.org/0000-0003-0766-0733
Judit Companys http://orcid.org/0000-0003-1485-0818
Laura Pla-Pagà http://orcid.org/0000-0003-3033-6691
Iziar A. Ludwig http://orcid.org/0000-0001-5506-3293
Ma Paz Romero http://orcid.org/0000-0001-9892-4874
Rosa Solà http://orcid.org/0000-0002-8359-235X

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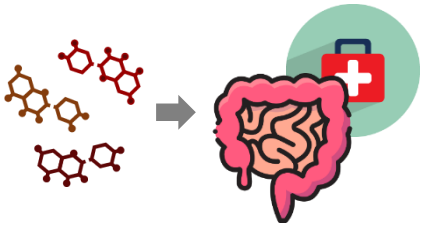

3.6. Chapter 6

Exploring the effects of phenolic compounds to reduce intestinal damage and improve the intestinal barrier integrity: A systematic review of in vivo animal studies.

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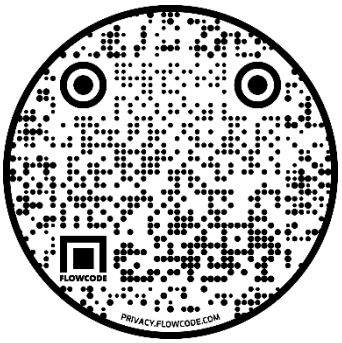

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Meta-analyses

Exploring the effects of phenolic compounds to reduce intestinal damage and improve the intestinal barrier integrity: A systematic review of *in vivo* animal studies

Berner Andrée Sandoval-Ramírez [a], Úrsula Catalán [a, b, *], Anna Pedret [a, b], Rosa M. Valls [a], Mª José Motilva [c], Laura Rubió [d], Rosa Solà [a, b, e]

[a] Universitat Rovira i Virgili, Faculty of Medicine and Health Sciences, Medicine and Surgery Department, Functional Nutrition, Oxidation, CVD Research Group (NFOC-Salut), Reus, Catalonia, Spain
[b] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Catalonia, Spain
[c] Instituto de Ciencias de la Vid y del Vino-ICVV (CSIC), Gobierno de La Rioja, Universidad de La Rioja, Logroño, La Rioja, Spain
[d] Food Technology Department, XaRTA-TPV, Agrotecnio Center, Escola Tecnica Superior d'Enginyeria Agraria, University of Lleida, Lleida, Catalonia, Spain
[e] Hospital Universitari Sant Joan de Reus (HUSJR), Reus, Catalonia, Spain

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SUMMARY

Background & aims: The integrity of the intestinal barrier in the diseased is key to prevent further complications and disease such as sepsis and death, whereas, the role of food bioactive molecules (i. e. phenolic compounds (PCs) on the intestinal barrier, is still unknown. The current aim was to explore the benefits of the oral PC administration on the intestinal barrier integrity in animals.
Methods: The effects of PCs on the intestinal barrier integrity in *in vivo* animal models of intestinal inflammation were assessed up-to August 2020 from the PubMed, SCOPUS, and Cochrane Library databases under the PRISMA methodology. The risk of bias was assessed from ARRAY and SCYRCLE tools.
Results: From 1241 articles, 14 studies were included. In animals, oral resveratrol (n = 6) improves the intestinal barrier integrity and reduces intestinal damage. Additionally, grape seed extract (n = 2), curcumin (n = 1), genistein (n = 1), chlorogenic acid (n = 1), grape pomace (n = 1), olive leaf (n = 1) or cranberry extract (n = 1) improve the intestinal barrier integrity downregulating various inflammatory molecules (TNF-α, and other interleukins), and increasing the antioxidant enzymes in animals. Furthermore, resveratrol, quercetin, epigallocatechin, and other PCs improve the epithelial barrier integrity and pro-inflammatory molecule expression in the intestinal epithelia.
Conclusions: The oral PC administration in animals improves the intestinal barrier integrity and function from three main mechanisms: 1) The reduction of pro-inflammatory molecules, 2) the improvement in tight-junction protein expression, and 3) the improvement of the antioxidant intracellular activity suggesting the potential use of PCs in the management of intestinal injury in humans, particularly for resveratrol, the most studied PC.

1. Introduction

The gastrointestinal tract is a key player in the pathogenesis of the multiorgan dysfunction found in critically ill patients which is secondary to the breakdown of the intestinal barrier, consequently impairing its protective role. The loss of the intestinal barrier integrity is common amongst critically ill patients causing higher mortality rates when compared against individuals with a preserved intestinal barrier integrity [1].

On the other hand, during a life-threatening intestinal disease such as necrotizing enterocolitis (NEC) in preterm infants [2], the gastrointestinal tract epithelia is characterized for because of increased permeability and inflammation secondary to an augmented rate of apoptosis [3,4]. NEC is characterized by the patchy necrosis of the small intestine with variable effects on the colon that might progress to systemic sepsis, multisystem organ failure, and finally death [2]. The increase in the intestinal permeability, evidenced as an increased plasmatic lipopolysaccharide

* Corresponding author. Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut., Av. de la Universitat, 1, 43204 Reus, Spain.
E-mail address: ursula.catalan@eurecat.org (Ú. Catalán).

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(LPS) levels, is caused by the overexpression of different proteins such as the toll-like receptor 4 (TLR4) [2].

Moreover, in humans, the intestinal gut integrity is disrupted during critical illness [5,6]. The disruption is caused by an increased intestinal permeability during critical illness from the down-regulation of the B-cell lymphoma 2 (Bcl-2) protein expression [7,8]. As has been demonstrated, the downregulation of Bcl-2 is significantly associated with the necrosis and loss of function in the intestines of septic transgenic mice, while the Bcl-2 overexpression was associated with the inhibition of intestinal epithelial apoptosis and the improvement of survival in septic mice [7,8]. Finally, in animal models of critical illness the expression tight junction (TJ) proteins such as claudin-2, claudin-5, and zonula occludens (ZO)-1 all decrease, further evidencing the loss of the intestinal barrier integrity [9–11], thus demonstrating the importance of the TJ proteins in the development of sepsis [12,13].

Consequently, new therapeutic approaches for improving the intestinal integrity and barrier function are currently needed [14].

In that sense, the phenolic compounds (PCs) are a large group of natural bioactive molecules or phytochemicals present in plants, PCs including different sub-classes such as flavonoids, stilbenes, phenolic acids, and lignans [15], with promising potential for the treatment and prevention of diverse chronic diseases with an inflammatory component such as type 2 diabetes mellitus (T2DM), cancer and cardiovascular disease (CVD) [16–18].

PCs are considered to be safe for human consumption due to their low rates of adverse effects, even when consumed at high doses [15,19–21], and their tissue bioavailability has been confirmed after oral intake in diverse animals, suggesting that their presence in diverse target tissues leads to their associated health benefits [18].

From previous reviews, it has been demonstrated that in *in vivo* animal studies, PCs and PC extracts can lower the severity of colitis by modifying various intracellular signaling cascades in the intestinal epithelium and showing anti-inflammatory effects [22]. Additionally, a clinical trial using pomegranate extract as a PC source demonstrated to significantly reduce the plasmatic concentrations of the LPS-binding protein in humans, decreasing endotoxemia in overweight-obese individuals who were suffering from intestinal inflammation [23]. Furthermore, the supplementation with curcumin, a PC, has demonstrated to improve the intestinal disease activity in patients suffering from ulcerative colitis partially from the reduction of oxidative stress in a randomized, double-blinded, placebo-controlled pilot study [24]. In other studies where healthy humans consumed wine with 1758 mg/L of total polyphenols improved the expression of diverse pro-inflammatory cytokines improving the intestinal barrier integrity was demonstrated [25].

Due to the lack of a model able to precisely recreate the changes occurring in human intestines during critical illness [26], diverse animal models of induced intestinal damage and intestinal inflammation were used to assess the potential benefits of PCs on the intestinal barrier integrity during critical illness [3,27,28].

The present systematic review aims to explore the potential effects of the oral PC administration on the intestinal barrier integrity from *in vivo* animal models of induced intestinal damage.

2. Materials and methods

2.1. Literature search

This systematic review is structured following the general principles published in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [29]. The PRISMA flowchart (Fig. 1) and PRISMA checklist (Supplementary Table 1) for the present systematic review are presented.

2.1.1. Information sources and search strategy

The scientific web libraries Scopus (https://www.scopus.com), Cochrane Library (https://www.cochranelibrary.com/), and PubMed (https://pubmed.ncbi.nlm.nih.gov/Error! Hyperlink reference not valid.) were explored. For the present systematic review articles regarding the experimental use of PCs on human or *in vivo* animal research was searched through the following terms: "(phenolic compound OR polyphenol) AND (intestinal OR intestine OR bowel OR jejunum OR duodenum OR colon) AND (barrier OR disease OR inflammation OR inflammatory OR sepsis OR permeability) AND (human OR *in vivo*) NOT (review)".

2.1.2. Article's selection criteria

Our group identified the articles from the database search, duplicates were removed, and 3 additional articles were found by hand-search. Published articles were screened based on their titles, abstracts, and full texts according to the following inclusion criteria:

1) *in vivo* animal studies regarding the effects of at least one PC on the intestinal barrier integrity.
2) Studies where the effects of PCs were compared against both positive and negative controls in *in vivo* animal models.
3) Studies published in the last 20 years, from January 1st 2000 up to August 1st 2020.

The exclusion criteria were the following: 1) Non-English articles; 2) Low-quality after the risk of bias assessment for *in vivo* animal studies; 2) incomplete data publication; 3) Review articles; 4) studies assessing the effects from the metabolization of phenolic compounds by the intestinal microbiome, and 5) not fulfilling the inclusion criteria. After full-text analysis, the following information was extracted from the included articles: title, author information, type of study performed, assessed outcome/s, intervention target (animal used), administered dose, length of study, administration route, doses administered, and main conclusions. Whenever possible, the dose administered to animals was converted into its human equivalent dose (HED) defined as the conversion of the animal dose into its HED and expressed as the 24 h dose for an average human being weighting 70.0 kg [30].

Under the PRISMA methodology, two independent authors (B.A.S.-R. and U.C.) analyzed the titles, abstracts, and full-text articles for inclusion while a third reviewer (R.S.) resolved all differences if present.

2.1.3. Quality assessment

The reporting quality of the included animal study articles was assessed and interpreted following the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines [31], while the risk of bias for the included animal studies was assessed using the SCYRCLE's tool for assessing the risk of bias in animal studies [32].

3. Results

3.1. Literature search, study selection, and characteristics

From our initial database screening, 1241 articles published from January 1st, 2000 up to August 1st, 2020 were retrieved from all databases. All titles and abstracts were assessed; as a result, 1183 articles were excluded after the initial screening, 7 articles were excluded as duplicates, 16 articles were excluded as *in vitro* studies, 5 publications were excluded for being reviews, 13 publications

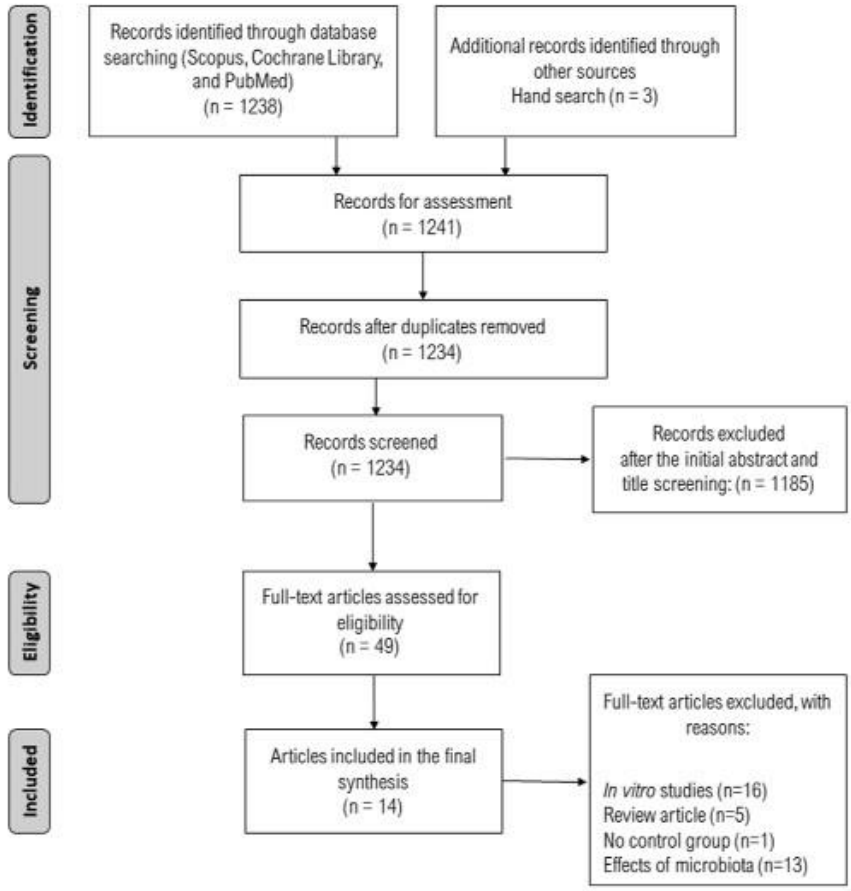

Fig. 1. PRISMA flow diagram for the included studies.

were excluded for assessing the effects of the intestinal metabolization of PCs, and 1 experiment was excluded for not having a control group leaving a total of 14 articles for final inclusion [33–46].

The complete PRISMA statement flow diagram for the included studies is presented in Fig. 1. No studies performed on humans were found regarding the effects of the PC supplementation on the intestinal health and barrier integrity of critically ill humans.

The following information was retrieved from the included studies:

Regarding the animal models used in the 14 included *in vivo* animal studies: Wistar rats (n = 4) [31,34,36,40], C57BL/10 SnSn mice (n = 1) [35], C57BL/6J mice (n = 1) [37], both C57BL/6 and IL-deficient mice (n = 1) [38], both C57BL/6 and CD1 mice (n = 1) [41], C57BL/6 mice alone (n = 1) [43], weaned pigs (n = 2) [33,44], Sprague–Dawley rats (n = 1) [32], IL-10 deficient mice (n = 1) [39], and BALB/C mice (n = 1) [45]. The current animal models used to assess intestinal inflammation are summarized in Table 1.

Most of the included studies assessed different PCs individually, with the exception of two studies where resveratrol and curcumin were evaluated separately in the same experiment [35,46]. In *in vivo* animal studies, resveratrol as an extract was the most commonly used source of PCs (n = 6) [31,32,34,35,44,45]; after its oral (n = 5) [31,34,35,44,45], or intravenous administration (n = 1) [32]. Other PC sources used in *in vivo* animal studies were: oral grape seed extract (GSE) (n = 2) [38,39], oral grape pomace extract (n = 1) [40], oral cranberry extract (n = 1) [37], oral curcumin (n = 1) [35], oral hesperidin (n = 1) [43], oral Olive (*Olea europaea*) leaves extract (n = 1) [41], genistein by intestinal injection (n = 1) [36], and oral chlorogenic acid (n = 1) [33]. Complete information on the included animal studies is reported in Table 2.

3.1.1. Quality assessment

The reporting quality of the 14 included animal studies was assessed through the ARRIVE guidelines. As a result it was determined that the included animal articles adequately reported their results. Moreover, the risk of bias of the 14 included animal studies was assessed using the SCYRCLE's tool; as a result, all the included articles were of moderate quality. Nonetheless, after a thorough assessment of the included animal articles, it was determined that despite their moderate score, there is a low risk of bias in the included publications. The complete results on the ARRIVE guidelines and SCYRCLE's tool for risk of bias assessment are reported on Supplementary Tables 2 and 3, respectively.

Table 1
Summary of the current models used to assess intestinal inflammation.

Model type	Model sub-type	Model used	Description
Animal model			
	Knockout mice		
		IL-10	Involved in anti-inflammatory pathways
		IL-23R	Involved in T-cell differentiation
		CD4+ CD25+	Involved in the T-cell glycoprotein adaptation
		NOD2/CARD15	Peptidoglycan involved in apoptosis
		TGF-β1	Involved in T-cell development and immune response regulation
		RAG	Involved in lymphocyte maturation
		ATG16L1	Involved in pathogen regulation
		$APC^{MIN/+}$	Used for intestinal tumorogenesis
		IL-2	Involved in the inflammatory process
		TNF-α	Involved in the activation of apoptosis
		STAT3	Involved in intestinal mucosa regeneration
		NFkβ	Involved in inflammation and cell survival
		MUC2	Involved in the production of mucin
		IFN-γ	Involved in the inflammatory process
		MYD88	Involved in the signaling process for toll-like receptors and NFkβ
		TLR	Involved in the microbial surface identification
	Rats		Frequently employed along with the use of chemical agents to induce inflammation. Furthermore, HLA-B27 models spontaneously develop intestinal inflammation
	Nematodes and insects		
		Caenorhabditis elegans	*Caenorhabditis elegans* has been used to examine host–microbiome interactions in apical surface of intestinal epithelia cells.
		Drosophila melanogaster	Models have been used to explore the alterations in the innate immune response related to chronic inflammation and cancer development
	Fish		
		Zebrafish (*Danio rerio*)	Zebrafish have been used to test adaptative and immune responses due to intestinal cell similarities with mammalians such as the enterocytes, goblet cells and microvilli. Especially useful for motility and peristaltic studies.
	Pigs		Pigs have been used because of their anatomical similarities with the human intestines, particularly for the stomach and small intestine. However, several differences in the expression of IFN-γ, IL-12 and IL-10 production must be taken into consideration when compared against humans.
	Non-human primates		
		Macaques	Non-human primate animal models are considered the gold standard for the study of the mechanisms involved in chronic and acute inflammation due to the similarities in physiology, immunology, anatomy, and the intestinal microbiome.
Surgical procedures			
		Xenograft	The procedure involves the transplantation of fetal intestinal segments from one species into another.
		Cannulation	Commonly applied to obtain gastrointestinal samples and to examine nutritional metrics.
		Intestinal loops	Are complex procedures useful to study host–pathogen interactions with an added advantage of replicating the normal intestinal characteristics by creating intestinal segments partitioned into "loops"
Chemical agents		Dextran sulphate sodium	Causes basal crypt and epithelial cell damage after long term administration with an increase in the production of pro-inflammatory cytokines
		Trinitrobenzene sulfonic acid	Causes a Th1 mediated immune response with an increase in the production of pro-inflammatory cytokines
		Oxazolone	Causes a Th2 mediated immune response with an increase of interleukins 4, 5, and 13.
		Azoxymethane	Commonly used in conjunction with dextran sulphate sodium to induce an increased production of IL-21, IL-17α, and IL-6.
Biological agents			
	Bacteria	*Citrobacter rodentium*	Used to cause acute inflammation in the colon, producing ulcerative and proliferative intestinal lesions
		Helicobacter pylori	Has been used for its ability to occupy the gastric and intestinal epithelia causing damage after cytotoxin release in the presence of urease
		Salmonella enterica	Used to induce chronic models of intestinal inflammation in mice causing deep injury in the intestinal layers
		Mycobacterium avium	Used to cause intestinal changes like the ones found in humans with Crohn's disease
	Helminths	*Trichuris muris*	The most used nematode for intestinal inflammation in murine models, characterized for the loss in barrier function of the colon
	Protozoa	*Toxoplasma gondii*	Commonly used to cause a robust Th1 immune response in the small intestine resulting in an increased expression of IL-12 and IFN-γ

Note: IL, interleukin; IFN-γ, interferon gamma; HLA, human leucocitary antigen; NFkβ, nuclear factor kappa beta; NOD2, nucleotide-binding oligomerization domain-containing protein 2; CARD15, caspase recruitment domain-containing protein 15; TGF-β1, tumor growth factor beta 1; RAG, recombinant activation gene; ATG16L1, autophagy related 16 like 1; TNF-α, tumor necrosis factor alpha; STAT3, signal transducer and activator of transcription 3; MUC2, mucin 2; MYD88, myeloid differentiation primary response 88; TLR, toll like receptor.

3.2. In vivo animal studies

3.2.1. Resveratrol

The effects of resveratrol on the intestinal barrier were assessed on seven different animal studies [33–35,41,45,53,55]. The animal models used were BALB/C mice (n = 1) [32], Wistar rats (n = 2) [33,41], C57BL/6/10ScSn mice (n = 1) [35], piglets (n = 2) [45,55], and Sprague–Dawley rats (n = 1) [34].

In a dextran sodium sulfate (DSS) colitis model induced for 14 days to 21 male BALB/C mice, the oral dietary supplementation

Table 2
General information of the included studies assessing the effects of polyphenols on the intestinal barrier integrity and intestinal health in diverse animal (*in vivo*) studies.

Title	Author	Year	Outcome	Intervention target	N (m/f)	Phenolic compound used	Animal dose	Human equivalent dose (70 kg human)	Length of study	Administration route	PC treatment	Doses administered (N)	Main conclusions
Enteral resveratrol supplementation attenuates intestinal epithelial inducible nitric oxide synthase activity and mucosal damage in experimental necrotizing enterocolitis.	Ergün, O. et al.	2007	Nitric oxide synthase activity and musocal wall integrity	Newborn Wistar rats	27 (NR)	Resveratrol	15 mg/kg/BID	85 mg/day	4 days	Oral	Oral PC treatment started on day 0 and continued for 4 days until animal sacrifice. Necrotizing enterocolitis was provoked on day 0 using a chemical agent and from hypoxia with a 5% oxygen room air until sacrifice in day 4.	8	The resveratrol treated group showed no changes in the macroscopic intestinal appearance (intestinal edema, pneumatosis intestinalis and ileal necrosis). The Western blot analysis revealed that resveratrol caused a marked decrease in the elevation of the NO synthase protein expression (0.6 ± 5.1; $p < 0.01$), when compared against the NEC group (3.7 ± 2.9). When compared against the NEC group (191.4 ± 4.1 μmol/L·g), resveratrol significantly reduced the ileal nitrate/nitrite levels (181 ± 3.6 μmol/L·g; $p < 0.01$) in a significant manner.
The protective effect of resveratrol on the intestinal mucosal barrier in rats with severe acute pancreatitis.	Jha, R.K. et al.	2008	Intestinal mucosal barrier	Sprague −Dawley rats	54 (m)	Resveratrol	10 mg/kg	1.61 mg/day	12 h	Intravenous	One single PC dose was administered on minute 0 after a bile duct clipping surgery to induce pancreatitis and intestinal inflammation.	1	The resveratrol treated animals showed lower endotoxin levels at 3, 6, and 12 h when compared against the pancreatitis group ($p < 0.05$). At 12 h, resveratrol significantly reduced the endotoxin levels in ≈51% ($p < 0.05$), as well as the pancreatic and intestinal mucosal congestion, edema, inflammatory cell infiltration, when compared against the pancreatitis group ($p < 0.05$). The intravenous administration of resveratrol significantly lowered the apoptotic cell index of the mucosal cells ($p < 0.05$), decreased the expression of the Bax protein ($p < 0.05$), and increased the expression of the Bcl-2 protein.
Anti-inflammatory effects of resveratrol, curcumin and simvastatin in acute small intestinal inflammation.	Bereswill, S. et al.	2010	Intestinal inflammation	C57BL/10ScSn mice	NR	Resveratrol Curcumin	20 mg/day 100 mg/day	1.62 mg/day 8.13 mg/day	10 days	Oral	Oral PCs were administered on day 0 until animal sacrifice in day 10. The intestinal inflammation (ileitis) was induced in day 2 from a Toxoplasma *gondii* inoculation.	10	After 19 days, the resveratrol group showed higher survival rates (40% survival; $p < 0.005$), when compared against the control group (0% survival; $p < 0.005$). Resveratrol significantly decreased the animal weight loss in 9% (11%; $p < 0.005$) when compared against the control group (20%; $p < 0.005$). The animals treated either with resveratrol or curcumin presented only mild signs of inflammation (edema and cell-free exudate) in the visual exploration of the ileal mucosa, while maintaining an intact epithelium ($p < 0.0001$). Both treatments reported a lower increase in T lymphocytes ($p < 0.05$), a 20−30% increase in the FOXP3+ cell numbers ($p < 0.05$), and 25−50% fewer MPO-7+ cells ($p < 0.05$), while significantly reducing the total bacterial load in 1−2 orders of magnitude when compared against the placebo control ($p < 0.05$).
Protective effect of soy isoflavone genistein on ischemia-reperfusion in the rat small intestine.	Sato, Y. et al.	2011	Ischemmia-reperfusion intestinal injury	Male Wistar rats	NR (m)	Genistenin	500 μl.	NA	NA	Intestinal injection	One single PC dose was directly administered into the intestinal lumen 30 min after the induction of intestinal ischemia from mesenteric artery clamping.	1	Genistein significantly reduced the damage and loss of villi to the intestinal crypts ($p < 0.05$), mainly inhibiting the intestinal xanthine oxidase activity, and enhancing the reactive oxygen species scavenging activity.

(*continued on next page*)

Table 2 (continued)

Title	Author	Year	Outcome	Intervention Target	N (m/f)	Phenolic compound used	Animal dose	Human equivalent dose (70 kg human)	Length of study	Administration route	PC treatment	Doses administered (N)	Main conclusions
A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice	Anhe, F. et al.	2014	diet-induced intestinal inflammation and metabolic endotoxemia	C57BL/6J mice	36 (m)	Cranberry extract (phenolic acids, flavonols, anthocyanins, proanthocyanidins)	200 mg/kg	1120 mg/day	8 weeks	Oral	Oral PC administration started after a 2-week acclimation period along with a high fat and high sucrose diet to induce intestinal inflammation	56	The oral administration of cranberry extract completely prevented a twofold increase in circulating levels LPS levels after 8 weeks of a high fat/high sucrose diet in
Grape seed extract improves epithelial structure and suppresses inflammation in ileum of IL-10-deficient mice.	Yang, G. et al.	2014	Ileal inflammation	C57BL/6 mice and IL-deficient mice	NR (f)	GSE	1% w/w supplemented diet	NA	16 weeks	Oral	Oral PC administration started on day 0 until week 16. Intestinal inflammation was provoqued by the genetic IL-10 deficiency.	112	The GSE decreased the intestinal crypt depth ($p < 0.05$) and increased the ratio of villus vs. crypt length in the terminal ileum ($p < 0.05$). GSE significantly decreased the proliferation and enhanced the differentiation of the intestinal epithelial cells ($p < 0.05$). suppressing the NF-kβ in activated B-cells ($p < 0.05$). Finally, GSE significantly reduced the intestinal cell autophagy by decreasing the expression of beclin-1 ($p < 0.05$).
Favorable effects of grape seed extract on intestinal epithelial differentiation and barrier function in IL10-deficient mice.	Yang, G. et al.	2015	Intestinal epithelial differentiation and barrier function	IL10-deficient female mice	NR (f)	GSE	140 mg/kg/day 160 mg/kg/day	798 mg/day 910 mg/day	12 weeks	Oral	Oral PC administration started on day 0 until the end of week 12 when animals were sacrificed. Intestinal inflammation was provoqued by IL-10 deficiency	84	After 12 weeks of oral GSE supplementation with 140 or 160 mg/kg/day in mice, the *in vivo* intestinal permeability significantly decreased with additional reductions in the fecal total antioxidant capacity, and serum TNF-α levels ($p < 0.01$) In addition, the GSE significantly reduced the number of proliferating nuclear antigen-positive cells per crypt ($p < 0.01$), and downregulated the MAP kinase's growth signaling in the colon ($p < 0.01$).
Dietary Supplementation with a Low Dose of Polyphenol-Rich Grape Pomace Extract Prevents Dextran Sulfate Sodium-Induced Colitis in Rats	Boussenna, A. et al.	2016	dextran sulfate sodium (DSS)-induced colitis in rats	Wistar rats	40	Grape pomace extract (GPE)	0.1%, 0.5% and 1%	NA	21	Oral	Oral PCs were administered from day 0 until day 21. colitis was induced on day 14 from DSS oral administration.	21	The GPE 0.1% diet significantly delayed the onset of colitis symptoms, prevented the decrease in food intake, weight loss, the colon shortening, and the polimorphonuclear infiltration of the intestinal wall when compared against the positive control group. 0.5% and 1% GPE doses did not show significant effects.
Resveratrol Protects Oxidative Stress-Induced Intestinal Epithelial Barrier Dysfunction by Upregulating Heme Oxygenase-1 Expression.	Wang, N. et al.	2016	Epithelial barrier disfunction and oxidative stress	Male Wistar rats	60 (m)	Resveratrol	10 mg/kg 20 mg/kg	112 mg/day 225 mg/day	7 days	Oral	One single PC dose was administered orally after bile duct ligation to cause intestinal inflammation	1	When compared against the control group (3.19 ± 0.90), both resveratrol treatments significantly decreased the intestinal permeability as evidenced in the decrease of the lactulose/mannitol ratio in 70.5% by the 10 mg/kg dose (0.94 ± 0.43; $p < 0.05$), and in 86.8% by the 20 mg/kg dose (0.42 ± 0.25; $p < 0.05$). Resveratrol upregulated the hemeoxygenase-1 protein expression ameliorating the TJ protein disruption ($p < 0.05$).
Immunomodulatory properties of Olea europaea leaf extract in intestinal inflammation	Vezza, T. et al.	2017	dextran sulfate sodium-induced intestinal inflammation	C57BL/6J	NR (m)	Olive (Olea europaea) leaves extract	0.5, 1, and 10 mg/kg	2.8, 5.6, and 56 mg/day	11	Oral	PC doses started at day 0 and were administered until animal sacrifice on day 11. Intestinal inflammation was induced on day 0 until sacrifice on day 11 using DSS.	11	The olive leaf extract at 1 and 10 mg/kg doses significantly improved the evolution of the colitis severity, enhanced the intestinal functionality from the improvement of intestinal permeability assessed by FITC-dextran permeability. Additionally, the olive leaf extract improved epithelial regeneration, reduced the inflammatory cell infiltration and edema in the intestinal mucosa.

		dinitrobenzenesulfonic acid-induced intestinal inflammation	CD1 mice	NR (m)	Olive (Olea europaea) leaves extract	1, 10, and 25 mg/kg	5.6, 56, and 140 mg/day	6	Oral	PC doses started on day 0 and were administered until animal sacrifice on day 6. Intestinal inflammation was induced using dinitrobenzene sulfonic acid on day 2 and continued until sacrifice on day 6.	6	The olive leaf extract 1 mg/kg dose significantly improved inflammation in the colon of treated mice, reduced the expression of IL-1β, TNF-α, IL-6, IL-17, and MIP-2. Additionally, the olive leaf extract significantly upregulated the ICAM-1, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the intestines of treated animals.
Resveratrol Ameliorates Intestinal Barrier Defects and Inflammation in Colitic Mice and Intestinal Cells.	Mayangsari, Y. et al.	2018	Intestinal barrier defects and inflammation	BALB/C mice	21 (m)	Resveratrol	0.1% w/w supplemented diet	NA	14 days	Oral	Oral PC administration started at day 0 and continued until day 14 when animals were sacrificed	14	When compared against the control, resveratrol significantly prevented the body weight loss in 28% (100 ± 1%; $p < 0.05$), the shortening of the colon length in 0.8 cm (4.6 ± 0.1 cm; $p < 0.05$). Additionally, resveratrol reduced the plasmatic levels of IL-1β in 89.9% (5.9 ± 1.8 AU; $p < 0.05$) and of IL-6 in 96% (10 ± 3 AU; $p < 0.05$). Resveratrol reduced the expression of the CXCL-2 (14 ± 7 AU; $p < 0.05$), increased the expression of occludin (0.76 ± 0.06 AU; $p < 0.05$), and reduced the neutrophil infiltration (9.3 ± 0.7 cells; $p < 0.05$).
Chlorogenic acid improves intestinal barrier functions by suppressing mucosa inflammation and improving antioxidant capacity in weaned pigs.	Chen, J. et al.	2018	Intestinal barrier function	Weaned pig	24 (NR)	Chlorogenic acid	1000 mg/kg/day	38.85 g/day	2 weeks	Oral	Oral PC administration started at 24 days of age when pigs were weaned and continued for 2 weeks until animal sacrifice.	14	Chlorogenic acid significantly decreased the serum D-lactic acid content and diaminooxidase activity ($p < 0.05$). Chlorogenic acid showed a tendency for lower endotoxin levels ($p < 0.10$), however, no significant differences in the levels of cortisol and corticotrophin-releasing hormone were observed between the two groups. Chlorogenic acid reduced the histamine contents in the jejunum and ileum ($p < 0.05$), while reducing the tryptase levels only in the jejunum ($p < 0.05$) Significant decreases in the counts of tryptase-positive mast cells in the duodenum and jejunum of pigs were noted after the chlorogenic acid supplementation ($p < 0.05$). Moreover, chlorogenic acid also downregulated the expression of IL-1β, and TNF-α in the small intestine of pigs ($p < 0.05$), of the IL-6 and TNF-α levels in the ileum ($p < 0.05$), and up-regulated the jejunal and ileal expression of claudin-1. Finally chlorogenic acid downregulated the expression of diverse inflammatory proteins such as IL-23p19 ($p < 0.001$), and TNF-α ($p < 0.01$).
Resveratrol improves intestinal barrier function, alleviates mitochondrial dysfunction and induces mitophagy in diquat challenged piglets	Cao, S. et al.	2019	Intestinal injury induced by diquat (herbicide)	Piglets	24	Resveratrol	100 mg/kg/day	4.97 g/day	14 days	Oral	Oral PC administration started on day 0 until day 13 along with diquat to induce intestinal inflammation and compared against a diquat-free control group.	13	Resveratrol significantly increased the total intestinal antioxidant capacity in treated piglets. Additionally, oral resveratrol reversed the increase in the mucosal concentration of hydrogen peroxide and malondialdehyde in the jejunum. The oral resveratrol administration also improved the intestinal barrier function evidenced as an improvement in the TEER reduction provoqued by the herbicide. Moreover, resveratrol prevented reduction of occludin, claudin-1 and ZO-1 levels in the jejunal mucosa.

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Table 2 (continued)

Title	Author	Year	Outcome	Intervention target	N (m/f)	Phenolic compound used	Animal dose	Human equivalent dose (70 kg human)	Length of study	Administration route	PC treatment	Doses administered (N)	Main conclusions
Hesperidin Protects Against Intestinal Inflammation by Restoring Intestinal Barrier Function and Up-Regulating Treg Cells	Gao, K. et al.	2019	dextran sulfate sodium (DSS)-induced colitis in mice	C57BL/6 mice	30	Hesperidin	10 mg/kg 20 mg/kg 40 mg/kg	56 mg/day 112 mg/day 224 mg/day	13 days	Oral	PC treatment started on day 0 and continued until animal sacrifice on day 13. Inflammation was induced from day 0 until day 7 using DSS	13	Hesperidin significantly reduced the disease activity score, and pathological changes in the intestines such as edema, hyperemia, inflammatory infiltration, and necrosis. Additionally, hesperidin significantly decreased TNF-α, IL-6 and increased IL-10 and IFN-γ in the intestines of treated animals.

Note: GSE, grape seed extract; NF-kβ, nuclear factor kappa-light-chain-enhancer; BID, twice a day; MAP, mitogen-activated protein; AU, arbitrary units; CXCL2, chemokine motif ligand 2; TJ, tight junction; NO, nitric oxide; NEC, necrotizing enterocolitis; MP, myeloperoxidase; AhR, aryl hydrocarbon receptor; Nrf2, nuclear factor erythroid 2–related factor 2; IL, interleukin; NR, not reported; m, male; f, female; DSS, dextran sulfate sodium; PC, phenolic compound.

with resveratrol 0.1% (w/w) resulted in a 28% reduction of the animal's weight loss (100 ± 1%; $p < 0.05$), and prevented the colon shortening by 0.8 cm (4.6 ± 0.1 cm; $p < 0.05$) when compared against the untreated control group [53]. Additionally, oral resveratrol reduced the plasmatic interleukin (IL)-1β concentrations in 89.9% (5.9 ± 1.8 arbitrary units (AU); $p < 0.05$), and the plasmatic IL-6 concentrations in 96% (10 ± 3 AU; $p < 0.05$) [53].

Additionally, a single oral resveratrol dose of either 10 or 20 mg/kg (HED: 112 or 125 mg/day) of resveratrol on the intestinal barrier integrity was appraised 1 week after its administration to 60 male Wistar rats with severe intestinal damage induced by the bile duct ligation (BDL) [41]. As a result, the treatment with resveratrol 10 mg/kg significantly decreased the intestinal permeability in 6% (0.94 ± 0.43; $p < 0.05$) while the 20 mg/kg dose decreased the intestinal permeability of Wistar rats in 58% (0.42 ± 0.25; $p < 0.05$) when compared against the BDL control group (3.19 ± 0.90) [41]. Finally, a single oral resveratrol dose of 20 mg/kg dose, significantly upregulated the heme oxygenase (HO)-1 protein, improving the intestinal antioxidant capacity ($p < 0.05$) when compared against the BDL controls [41].

The effects of an oral 15 mg/kg resveratrol dose (HED: 85 mg/day) twice for 4 days were assessed in a necrotizing enterocolitis (NEC) model developed on 27 newborn Wistar rats [33]. As a result, the NEC Wistar rats treated with resveratrol showed no changes in their macroscopic intestinal appearance, whereas the rats on the non-treated NEC group presented different degrees of intestinal edema, pneumatosis intestinalis and ileal necrosis [33]. Additionally, the Western blot analysis revealed that resveratrol in NEC Wistar rats caused a marked decrease in the elevation of the nitric oxide (NO) synthase protein expression (0.6 ± 5.1; $p < 0.01$) when compared against the NEC control group (3.7 ± 2.9) [33]. Finally, when compared against the NEC Wistar rat group [191.4 ± 4.1 μmol/(L·g)], 15 mg oral resveratrol dose twice daily for 4 days significantly reduced the ileal nitrate/nitrite levels [181 ± 3.6 μmol/(L·g); $p < 0.01$] [33].

The intestinal effects of the oral supplementation with either resveratrol 20 mg/day (HED: 1.62 mg/day) or curcumin 100 mg/day (HED: 8.13 mg/day), and simvastatin for 10 days were assessed from the intestines of C57BL/10ScSn mice, an animal model commonly used to study inflammation [35]. As a result, on the 8th day after the induction of an acute ileitis with *Toxoplasma gondii*; the resveratrol, curcumin, and simvastatin treated C57BL/10ScSn mice showed lesser hyper-acute inflammation in the small intestine [35]. At 10 days, the end of the intervention, the resveratrol treated group showed higher survival rates (40% survival; $p < 0.005$), when compared against the control group (0% survival; $p < 0.005$) [35]. Additionally, in C57BL/10ScSn mice, resveratrol significantly decreased the weight loss by 9% (11%; $p < 0.005$) when compared against the control group (20%; $p < 0.005$) [35]. The C57BL/10ScSn mice treated either with resveratrol or curcumin presented only mild signs of inflammation (edema and cell-free exudate) in the visual exploration of the ileal mucosa while maintaining an intact epithelium ($p < 0.0001$) [35]. Both treatments, curcumin or resveratrol, reported a lower increase in T lymphocytes ($p < 0.05$), a 20–30% increase in the numbers of a T regulatory lymphocyte cell sub-type important for the immune system's tolerance and homeostasis called FOXP3+ cells ($p < 0.05$), and 25–50% fewer myeloperoxidase (MPO)-7+ cells ($p < 0.05$), while significantly reducing the total serum bacterial load in 1–2 orders of magnitude when compared against the placebo control C57BL/10ScSn mice ($p = 0.05$) [35].

The effects of a single 10 mg/kg (HED: 1.61 mg/kg) intravenous resveratrol dose on the intestinal barrier integrity were assessed in 54 male Sprague–Dawley rats after the induction of severe acute pancreatitis induced by the clipping of the bile and biliopancreatic

ducts [34]. As a result, the intravenous treatment of severe pancreatitis using resveratrol on Sprague–Dawley rats showed lower endotoxin levels at 3, 6, and 12 h when compared against the pancreatitis control group ($p < 0.05$) [34]. At 12 h of intravenous administration, resveratrol significantly reduced the endotoxin levels in ≈51% ($p < 0.05$) [34], as well as the pancreatic and intestinal mucosal congestion, edema, and the inflammatory cell infiltration, when compared against the pancreatitis control group ($p < 0.05$) [34]. The intravenous administration of resveratrol significantly lowered the apoptotic cell index of the mucosal cells ($p < 0.05$) [34], decreased the expression of the Bax protein ($p < 0.05$) [34], and increased the expression of the Bcl-2 protein compared with Sprague–Dawley rats with severe pancreatitis as a control [34].

The effects of a resveratrol 100 mg/kg/day (HED: 4.97 g/day) on the intestinal inflammation induced by diquat, a herbicide, were assessed on 24 piglets [45]. For the experiment, the oral resveratrol administration started on day 0 until day 13 along with diquat to induce intestinal inflammation, the effects were compared against a diquat-free control group [45]. As a result, resveratrol significantly increased the intestinal antioxidant capacity, in addition, resveratrol reversed the increased concentrations of hydrogen peroxide and malondialdehyde caused by diquat in the jejunum [45]. Moreover, resveratrol improved the intestinal barrier function, evidenced as an improvement in the TEER reduction secondary to diquat administration [45]. Finally, resveratrol prevented the reduction of various TJ proteins in the jejunal mucosa (occludin, claudin-1, ZO-1) [45].

3.2.2. Grape seed extract (GSE) and grape pomace extract (GPE)

The effects of GSE on the intestinal health were assessed from 2 animal interventions [38,39]. In mice, after 12 weeks of oral GSE supplementation with 140 or 160 mg/kg/day (HED: 798 or 910 mg/day respectively), the *in vivo* intestinal permeability significantly decreased while additionally increasing the fecal total antioxidant capacity, and serum TNF-α levels ($p < 0.01$) compared with the untreated group as a control [39]. Also, the GSE significantly reduced the number of proliferating nuclear antigen-positive cells per crypt ($p < 0.01$) and downregulated the mitogen-activated protein (MAP) kinase's growth signaling evidenced the reduced phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) in the colon ($p < 0.01$) compared with the control mice [39].

In another mice experiment, interleukin (IL)-10-deficient and C57BL/6 wild type female mice as a multi-hit model where a colitogenic trigger initiates the inflammatory process [56], were used to test the effects of the oral water supplementation with GSE at 0.1% w/w on the ileal inflammation [38]. As a result in IL-10-deficient mice, GSE significantly decreased the intestinal crypt depth ($p < 0.05$) and increased the villus vs. crypt length ratio in the terminal ileum of animals ($p < 0.05$) when compared against wild type mice used as controls [38]. Furthermore, in separate IL10-deficient mice, the oral GSE administration significantly decreased the proliferation and enhanced the differentiation of the intestinal epithelial cells ($p < 0.05$), suppressing the nuclear factor kappa-light-chain-enhancer (NF-kβ) in activated B-cells ($p < 0.05$) compared against the GSE free control group [38]. Finally, oral GSE significantly reduced the intestinal cell autophagy by decreasing the expression of beclin-1 ($p < 0.05$) when compared against C57BL/6 wild type mice as a control [38]. Thus, oral GSE exerts protective effects on the ileal epithelial structure in IL-10-deficient mice, possibly through the suppression of the intestinal inflammatory response.

The effects from the oral administration of various GPE doses (0.1%, 0.5%, and 1%) on the intestinal inflammation secondary to the DSS oral administration were assessed on 40 Wistar rats, for the experiment GPE was administered from day 0 until day 21, on day 14 colitis was induced from the DSS oral administration [40]. As a result, GPE 0.1% significantly delayed the onset of colitis symptoms, prevented the decrease in food intake, animal weight loss, colon shortening, and the polymorphonuclear infiltration of the intestinal wall when compared against the positive control group [40]. The other GPE doses (0.5% and 1%) did not show the same effects [40].

3.2.3. Other phenolic compounds

The effects of oral chlorogenic acid on the intestinal barrier integrity were assessed in an experiment where a dose of 1000 mg/kg/day (HED: 38.85 g/day) of chlorogenic acid, a natural polyphenol present in human diet and plants, was orally administered to 24 weaned pigs for 14 days, as weaning is considered a cause of intestinal inflammation [44]. As a result, oral chlorogenic acid decreased the serum D-lactic acid content and diamine oxidase activity ($p < 0.05$), while showing a tendency for lower endotoxin levels ($p < 0.10$) when compared with the control group without oral chlorogenic acid [44]. No significant differences in the levels of cortisol and corticotrophin-releasing hormone were observed between the two weaned pig groups [44]. Moreover, oral chlorogenic acid reduced the histamine contents in the jejunum and ileum of weaned pigs ($p < 0.05$), while reducing the tryptase levels only in the jejunum of the treated animals ($p < 0.05$) when compared against the control group [44]. After chlorogenic the acid intake, significant decreases in the counts of tryptase-positive mast cells in the duodenum and jejunum of the weaned pigs were noted when compared against the control group ($p < 0.05$) [44]. Moreover, the chlorogenic acid consumption also downregulated the expression of IL-1β and TNF-α in the small intestine of the treated weaned pigs ($p < 0.05$), the IL-6 and TNF-α levels in the ileum of treated pigs ($p < 0.05$) [44], and upregulated the jejunal and ileal expression of claudin-1 in the supplemented animals [44]. However, the expression of inflammation repressors (suppressor of cytokine signaling 1 and toll-interacting protein) was up-regulated by the chlorogenic acid administration whereas chlorogenic acid downregulated the expression of diverse inflammatory proteins such as IL-23p19 ($p < 0.001$), and TNF-α ($p < 0.01$) compared against the weaned pig control [44]. The results suggest that oral chlorogenic acid ameliorates the intestinal barrier disruption in weaned pigs mediated by the suppression of the toll-like receptor (TLR)4/NF-κβ and the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/HO-1 signaling pathways [44].

Urolithin A, a major gut microbial PC metabolite derived from the transformation of the ellagitannins in berries and pomegranate fruits showing anti-inflammatory, anti-oxidative, and anti-aging activities in *Caenorhabditis elegans* [57] was also studied. The effects of urolithin A on the intestinal barrier function were assessed in one experiment where five urolithin A doses of 20 mg/kg (HEA: 1.62 mg/kg) were administered at 0 h, 6 h, 12 h, 18 h, and 24 h while LPS was administered intraperitoneally to induce inflammation at hour 24 in C57BL/6 wild type, Nrf2−/−, and AhR−/− mice [54]. As a result, urolithin A significantly modified the expression of 437 different genes ($p < 0.05$), mainly in the eukaryotic initiation factor 2 (eIF2), mammalian target of rapamycin (mTOR), and mitochondrial dysfunction pathways in C57BL/6 mice [54]. Moreover, urolithin A significantly upregulated the expression of claudin-4 ($p < 0.05$), Cyp1A1 ($p < 0.05$), and HO-1 ($p < 0.05$) in all mice when compared against baseline values. Finally, urolithin A significantly improved the intestinal TJ health through the activation of the aryl hydrocarbon receptor (AhR) - nuclear factor erythroid 2–related factor 2 (Nrf2)-dependent pathways [54], and attenuated colitis in wild type, Nrf2−/−, and AhR−/− mice by

remedying the barrier dysfunction in addition to different anti-inflammatory activities [54].

The effects of genistein, a phytoestrogen part of the isoflavonone family, on the intestinal barrier integrity were assessed in an intestinal injury model on Wistar rats [36]. For this experiment, the Wistar rats were divided into two groups; one of them was pre-treated with a single intestinal injection of 500 μL of genistein, while the second group received a placebo treatment with saline solution [36]. After the single intestinal injection, an ischemia-reperfusion injury was induced in both groups, then the animals were euthanized and the oxidative status and epithelial integrity were assessed [36]. As a result, the single intestinal genistein injection significantly reduced the damage and loss of villi to the intestinal crypts ($p < 0.05$) [36], mainly inhibiting the intestinal xanthine oxidase activity, and enhancing the reactive oxygen species scavenging activity [36].

The effects of an olive leaf extract (OLE) were assessed on two models of intestinal inflammation [42]. For the first model an OLE dose of 0.5, 1 or 10 mg/kg (HED: 0.04, 0.08 or 0.8 mg/kg) was administered on a colitis model induced by DSS in C57BL/6J mice starting at day 0 until animal sacrifice in day 11 [42]. As a result, OLE at 1 and 10 mg/kg doses significantly improved the colitis severity, and enhanced the intestinal functionality from an improvement in the intestinal permeability [42]. Additionally, OLE improved epithelial regeneration, reduced inflammatory cell infiltration, and edema in the intestinal mucosa of the treated animals [42]. In the second model, OLE doses of 1, 10 or 25 mg/kg (HED: 0.08, 0.8, or 2 mg/kg) starting at day 0 until animal sacrifice were used. OLE was administered daily to CD1 mice with induced colitis from the oral administration of dinitrobenzene sulfonic acid at day 2 and was continued until animal sacrifice on day 6 [42]. As a result, 1 mg/kg dose significantly improved the intestinal inflammation of treated mice, reduced the intestinal expression of IL-1β, TNF-α, IL-6, IL-17, and the macrophage inflammatory protein (MIP)-2. Additionally, the OLE significantly upregulated intracellular adhesion molecule (ICAM)-1 expression, the inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX) 2 in the intestines of the treated animals [42].

The effects of the oral administration of a daily 10, 20, or 40 mg/kg hesperidin doses (HED: 0.8, 1.6, or 3.2 mg/kg) on a DSS-induced intestinal inflammation were assessed on 30 C57BL/6 mice [46]. For the treatment, the hesperidin doses were administered from day 0 until day 13, while the intestinal inflammation was induced from day 0 until day 7 from the oral DSS administration [46]. As a result, hesperidin was able to significantly reduce the disease activity score and the pathological changes provoked by DSS in the intestines of untreated animals such as edema, hyperemia, inflammatory cell infiltration, and necrosis [46]. In addition, hesperidin significantly decreased the TNF-α, IL-6, and increased IL-10 and IFN-α concentrations in the intestines of treated animals [46].

Finally, the effects of the oral administration of a 200 mg/kg (HED: 16 mg/kg/day) dose of a cranberry extract on the diet-induced intestinal inflammation and metabolic endotoxemia provoked by a high-fat high sucrose diet in 36 C57BL/6J mice were assessed [37]. For the experiment, the cranberry extract was administered along with a high fat and high sucrose diet for 8 weeks [37]. As a result, the cranberry completely prevented the two-fold increase in the circulating LPS levels after 8 weeks of the high fat/high sucrose diet in the treated animals when compared against the control group [37].

Notably, no side effects were observed for any of the PCs administered in all the included articles even when the PCs were given at doses only achievable from the PC extract administration [33–40,42–46,54,55].

The effects of the oral administration of resveratrol and other PCs on the intestinal barrier integrity are summarized in Fig. 2.

4. Discussion

The nutrition of patients is a vital necessity that should be treated as such [58]. In that sense, early enteral nutrition (EN) is

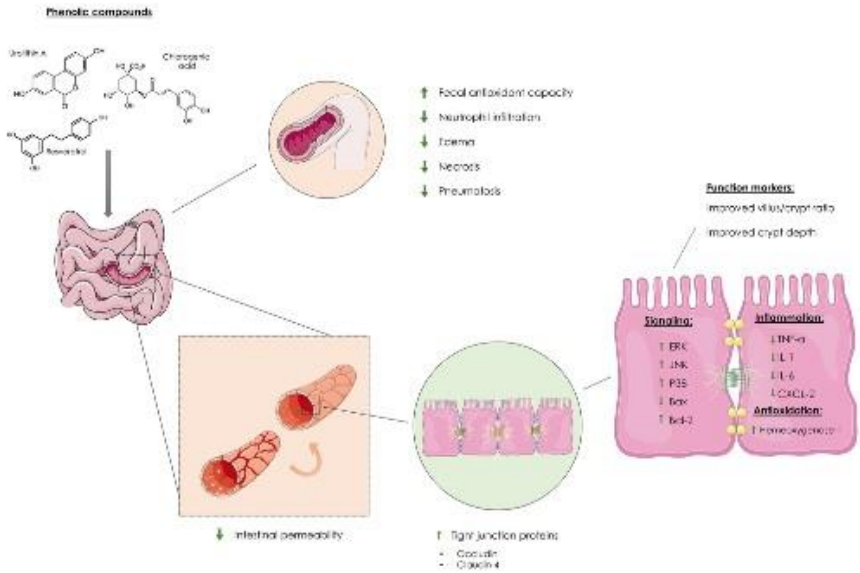

Fig. 2. The main effects of phenolic compounds (PCs) on the intestinal health and barrier function. The ingestion of various different PCs has demonstrated to significantly reduce the intestinal permeability, increasing the expression of different tight junction proteins and modulating the expression of various pro-inflammatory or antioxidant proteins resulting in the reduction of intestinal tissue damage, healthier cytological characteristics, lesser neutrophil infiltration, and an improved antioxidant capacity. *Abbreviations*: ERK, extracellular signal-regulated kinase; JNK, Janus kinase; Bax; Bcl-2, B-cell lymphoma 2; TNF-α, tumor necrosis factor alpha; IL; CXCL-2, Macrophage Inflammatory Protein 2.

recommended in current guidelines as it has demonstrated to reduce the mortality rates in intensive care units, helping to maintain the integrity of the intestinal barrier, leading to fewer gastrointestinal hemorrhages, infectious complications, the subsequent organ failure, and finally death in critically ill patients [59,60]; suggesting that composition of EN formulae is one area of medical interest.

As evidenced by the present systematic review, various PCs seem to have promising beneficial effects on the intestinal inflammation. For instance, it has been demonstrated that in animal models, the oral administration of various PCs at different doses significantly decreases various macroscopic signs of intestinal inflammation [33–36,38,39,53]. For instance, oral resveratrol significantly improves the intestinal edema, *pneumatosis intestinalis*, and ileal necrosis in newborn rats with necrotizing enterocolitis [33], the mucosal congestion, edema, and inflammatory cell infiltration in adult rats with intestinal inflammation secondary to acute pancreatitis [34], and also in C57BL/10ScSn mice with acute intestinal inflammation [35].

Additionally, resveratrol, genistein, and the oral GSE demonstrated to improve various characteristics of the intestinal epithelium such as the villus/crypt ratio, and crypt depth, in IL-10 deficient mice, Wistar rats and BALB/C mice [36,38,39,53] suggesting the improvement of the intestinal functionality and structure in diverse animal models. Thus, PCs might promote various beneficial effects to treat the decrease of the intestinal crypt proliferation and crypt/callus axis that occurs in critical patients secondary to systemic inflammation that leads to the reduction of the intestinal villus length which, in turn, causes nutrient malabsorption [2,12,13].

From all comments, the improvements in the intestinal function and structure after the oral PC administration seem to be caused by three main mechanisms in accordance with the information obtained from this review:

First, PCs improve the expression of various pro-inflammatory molecules as has been evidenced for resveratrol which improves the expression of pro-inflammatory proteins such as TNF-α, IL-1β, IL-6, and CXCL-2 in a dose-dependent manner [53]. Similar improvements on the intestinal cell expression of pro-inflammatory molecules have been observed for other PCs, for instance, GSE demonstrated to therapeutically improve the intestinal concentrations of TNF-α after 12 weeks of supplementation to IL-10 deficient mice [39]. Additionally, the oral administration of an olive leaf extract improved the expression of IL-1β, TNF-a, IL-6, and IL-17 in mice when administered in conjunction with the induction of colitis for 6 days [42]. Finally chlorogenic acid significantly improved the expression of endotoxin, histamine, tryptase, IL-1β, TNF-α, and IL-6 concentrations in the duodenum, jejunum, or ileum in various segments of the intestines of weaned pigs [44].

The reduced secretion of pro-inflammatory molecules in the intestinal epithelium of animals treated with PCs is evidenced as a smaller neutrophil infiltration rate in Wistar rats with intestinal inflammation secondary to bile duct ligation [41], lower T lymphocytes and MPO-7+ cells counts, as well as additional increases in the FOXP3+ cell numbers in the intestinal epithelium of C57BL/10ScSn mice treated with curcumin or resveratrol [35].

It must be noted that further benefits from oral PCs on the intestinal inflammatory profile might come from the improvement in the activity of proteins related to the inflammatory process as demonstrated after the olive leaf extract administration which significantly upregulated the expression of ICAM-1, iNOS, and COX-2 in the intestines of C57BL/6J and CD1 mice [42]. Such anti-inflammatory properties of PCs might constitute one of the most important mechanism of action for the improvement of intestinal health observed in IL-10 deficient mice [39], weaned pigs [44], Wistar rats [41], and C57BL/6J and CD1 mice [42].

Second, PCs improve the expression or prevent the reduction of different TJ proteins in the intestinal epithelium as demonstrated for resveratrol which significantly improved the protein expression of occludin in BALB/c mice [53], ameliorated the TJ protein disruption by upregulating the expression of antioxidant enzymes such as HO-1 in rats with intestinal inflammation secondary to bile duct ligation [41], and prevented the reduction of occludin, claudin-1 and zonula occludens (ZO)-1 levels in the jejunal mucosa of piglets with herbicide-induced intestinal inflammation [45]. Additionally, the prevention of the reduction of occludin, claudin-1 and ZO-1 levels in the jejunal mucosa is the mechanism that counteracts the alterations in the expression of various TJ proteins causes by inflammation such as the increase in the expression of claudin-2, and the decrease of claudin-5 and the ZO-1 proteins, possibly preventing the loss in the intestinal barrier function that characterizes critically ill patients [9–11] and in consequence possibly improving the intestinal permeability.

Third, PCs decrease the concentration of reactive oxygen species by increasing the activity of antioxidant enzymes such as HO-1 and xanthine oxidase as demonstrated in Wistar rats with intestinal inflammation and treated with resveratrol [41] or genistein [36] respectively, suggesting that the antioxidant benefits observed from the oral PC administration do not come only from their chemical structure but also from their metabolic regulatory activities.

Noticeably, the intestinal beneficial effects of PCs can also be attained from the intravenous administration of resveratrol which significantly lowered the endotoxin levels in plasma, as well as the intestinal edema, the inflammatory cell infiltration, and the apoptotic index of intestinal cells by improving the expression of pro-apoptotic proteins such as Bax and Bcl-2 in intestinal damage secondary to severe pancreatitis in Sprague–Dawley rats [34]. This might be of human benefit for the treatment of the increase in the intestinal apoptosis in critically ill patients where the intestinal disfunction has demonstrated to be regulated by a decreased expression of the Bcl-2 protein, causing intestinal necrosis and loss of function [7,8].

In accordance with the animal results, the *in vitro* evidence for resveratrol demonstrates that their oral administration significantly reduces the trans epithelial electrical resistance (TEER) provoked by different noxious agents in induced pluripotent cells (IPC)-J2 and caco-2 cells [51], counteracting their deleterious effects on diverse signaling proteins such as ERK, JNK, and p38 [51]. Similar cell effects were observed for other PCs [48,52].

Moreover, several *in vitro* studies provide other evidence suggesting that PCs improve the intestinal barrier integrity. For instance, a 20 μg/mL concentration of a proanthocyanin-rich purple potato extract significantly increased the expression of occludin, claudin-1, and the ZO-1 TJ proteins in caco-2 colon cancer cells [47]. Moreover, the same proanthocyanin-rich purple potato extract significantly increased the production of different intestinal transcription factors such as Elf3, and Hes1, demonstrating that different PCs can enhance the epithelial barrier integrity by increasing epithelial cell differentiation in caco-2 cells [47]. Finally, the extracts from three different passion fruits [48], routin extract [49], quercetin extract [49], epigallocatechin extract [49], and resveratrol extract [50,51], all demonstrated to be capable of increasing the TEER values, reflecting a better intestinal barrier integrity in *in vitro* models of intestinal damage [48–51].

The reduction of apoptosis, the increase of the villus length, and an increased expression of different TJ proteins coming from the early oral administration of PCs might counteract the increase in gut permeability that starts within the 1st hour of critical illness

and lasts for at least 48 h in humans [3,61]. Preventing the increase of the intestinal permeability might lead to lesser bacterial translocation, which in turn would reduce the subsequent systemic infection and distant organ damage [62], thus, the animal results suggest that the PC administration, through an oral or intravenous pathway might improve the intestinal integrity and barrier function of critically ill patients and possibly other patients suffering from intestinal inflammation [14].

From the above mentioned comments, the oral PC administration seems to exert beneficial effects directly from their interaction with diverse intracellular proteins in the intestinal epithelium [33–46]. However, it must be noted that the intestinal metabolization of PCs might also be of paramount importance for the intestinal barrier integrity since many of the PC's beneficial effects on the intestines might be caused by the first- or second-pass metabolization by the intestinal microbiota resulting in a more varied PC profile in the intestinal lumen of animals [63]. For instance, urolithin A, a PC resulting from the ellagitannin transformation by gut microbiota [64], significantly improved the intestinal barrier integrity, upregulating the expression of the TJ protein, claudin-4, and also the antioxidant enzyme HO-1, from the activation of the AhR-Nrf2 dependent pathway in mice with intestinal inflammation secondary to LPS injection [54].

Additionally, similar results on the improvement of TJ proteins were reported for curcumin and resveratrol when tested in combination with an antibiotic to assess the role of the intestinal microbiota in the alleviation of intestinal inflammation in 180 hybrids weaned piglets [55]. As a result of the intervention, curcumin and resveratrol at 300 mg/kg doses were able to significantly regulate the gut microbiota and reduced the intestinal inflammation from the decrease in the expression of the TLR4 signaling pathway in the jejunum and ileum of weaned piglets [55].

In same study, curcumin and resveratrol at all doses significantly reduced the IL-1β, TNF-α, while improving the IL-10, IgG, and IgA ($p < 0.05$) concentrations in the intestines of weaned pigs, thus, demonstrating not only that PCs might play a beneficial role for intestinal health but also the important role of the intestinal microbiota on the intestinal barrier health and function [55].

Therefore, as a result of the present systematic review of animal studies, it has become ostensible that the oral PC administration has the potential for improving the intestinal barrier integrity during severe inflammation, from an improvement in the inflammatory profile in intestinal epithelial cells, increasing the TJ protein expression thus reducing the intestinal permeability, and improving antioxidation in the intestinal epithelia. In consequence, the translation of PC animal results into human health problems suggests a possible improvement in the intestinal barrier function, possibly reducing further complications such as sepsis and death in some patients, such as critically ill patients and other patients suffering from intestinal inflammation from the improvement of the intestinal integrity.

5. Limitations

The current systematic review summarizes the results from various animal interventions to determine the possible beneficial effects of the PC supplementation on human nutrition to improve the intestinal inflammation, however, the animal results must be validated in a randomized control trial on humans to determine the effects of PCs on human intestinal health. A major limitation of the present review is that because of the complexity and extent of the information on the effects from the intestinal PC metabolization by the intestinal microbiota, and their possible effects over the intestinal health have only been briefly mentioned as a part of our work. However, the authors consider this as a major topic of increasing interest amongst the scientific community and it is worthy of study in future reviews focusing both on the microbiota and intestinal barrier.

6. Conclusions

From the present systematic review, in animals, the oral administration of various PCs improves the intestinal barrier integrity and function from three main mechanisms: 1) The reduction of several pro-inflammatory molecules, 2) the improvement in the expression of TJ proteins, and 3) the improvement of the antioxidant intracellular activity from chemical interactions and the increased expression of antioxidant enzymes. Furthermore, resveratrol, the most studied PC in different animal models of intestinal damage, improves the intestinal barrier integrity through an increase in the expression of various anti-inflammatory and antioxidant proteins in animals. Thus, suggesting the possible use of resveratrol or other PCs in the management of the intestinal injury associated with various intestinal and systemic pathologies in humans. However, the precise dose and time for the oral administration of resveratrol or other PCs in humans are still undetermined.

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Contributions

Study conception and design: B.A.S-R., ÚC, RS.
Acquisition of data: B.A.S-R., and Ú.C.
Analysis and interpretation of data: B.A.S-R., Ú.C., AP, RMV, LR, and R.S.
Drafting of the manuscript: B.A.S-R, and Ú.C.
Critical revision: Ú.C., MJM, and R.S.

Conflict of interest

The authors have declared no conflicts of interest. Complete Declaration of Interest forms for each author has been uploaded at the time of manuscript submission.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2020.09.027.

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3.7. Chapter 7

Exploring the effects of phenolic compounds to reduce the acute lung injury secondary to sepsis: A systematic review of in vivo studies.

Exploring the Effects of the Phenolic Compound Administration to Reduce the Acute Lung Injury Secondary to Lipopolysaccharide Administration in Animals: A Systematic Review of *in vivo* Animal Studies.

Authors: Berner Andrée Sandoval-Ramírez [1], M.D. M.Sc.; Úrsula Catalán [1, 2, 3] *, Ph.D.; and Rosa Solà [1, 3, 4], M.D., Ph.D.

Affiliations:

[1] Universitat Rovira i Virgili, Faculty of Medicine and Health Sciences, Medicine and Surgery Department, Functional Nutrition, Oxidation, and CVD Research Group (NFOC-Salut), Reus, Catalonia, Spain.

[2] Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Catalonia, Spain.

[3] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Catalonia, Spain.

[4] Hospital Universitari Sant Joan de Reus (HUSJR), Reus, Catalonia, Spain.

***Corresponding author**

Úrsula CATALÁN, PhD

Universitat Rovira i Virgili (URV)

Faculty of Medicine and Health Sciences

Functional Nutrition, Oxidation, and Cardiovascular Disease Research Group (NFOC-Salut)

39 **Abstract**

40 **Background:** The acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)
41 are a continuum of disease without specific treatment. In that sense, the role of various
42 phenolic compounds (PCs) on ALI/ARDS is still unknown. **Aim:** To explore the potential
43 benefits of PC supplementation from various models of LPS-induced ALI/ARDS in
44 animals. **Methods:** *in vivo* animal experiments assessing the effects of a PC on the LPS-
45 induced ALI and published over the last decade were retrieved from PubMed, SCOPUS,
46 and the Cochrane Library databases under the PRISMA methodology. The risk of bias was
47 assessed from ARRAY and SCYRCLE tools. **Results:** from 151 articles, 20 studies were
48 finally included. In animals, resveratrol, curcumin, zingerone, phloretin, chlorogenic acid,
49 thymol, and protocatechuic acid significantly improved the alveolar wall thickness,
50 pulmonary edema, and inflammatory cell infiltration at different rates. The benefits appear
51 to be caused by the downregulation of the NF-ƙβ and MAPK's activity causing a drop in
52 the pro-inflammatory molecule's production and cell infiltration, the reduction of ROS in
53 the lung tissue, and the improvement of the iNOS activity and NO concentrations.
54 **Conclusions:** After the analysis of all animal results, PCs appear to be promising agents for
55 the treatment of ALI/ARDS in humans.

56 **Word count: 200/200**

57 **Keywords:** sepsis, nutrition, supplementation, polyphenols, critical illness, pneumology **6/6**

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61 **Conflict of interest:** The authors declare no conflict of interest.

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Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Catalonia, Spain.

Avda/ Universitat, 1. CP/ 43204 Reus, Spain.

Tel: (+34) 977 75 93 77.

E-mail: ursula.catalan@eurecat.org / ursula.catalan@urv.cat

Word count:

ORCID No:

Berner Andrée Sandoval-Ramírez: 0000-0002-6242-922X

Úrsula Catalán: 0000-0001-8884-9823

Rosa Solà: 0000-0002-8359-235X

63 **1. Introduction**

64 The acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are a
65 continuum of disease, currently without specific treatment, that is characterized for the
66 onset of severe hypoxemia (PaO2/FiO2 ≤100 - 300 mmHg) with bilateral infiltrates that
67 cannot be explained by the left atrial hypertension (capillary wedge pressure <18
68 mmHg) within the first 7 days of a known insult (Parekh, Dancer, and Thickett 2011;
69 Riviello et al. 2016).

70 ARDS is diagnosed in 2.3% - 3% of all pediatric intensive care unit admissions
71 (Matthay et al. 2018; Khemani et al. 2019), with mortality rates between 17% - 33% in
72 children under 5 years old (Matthay et al. 2018; Khemani et al. 2019). In adults, ALI's
73 crude incidence is estimated at around 78.9 per 100,000 person-years, while ALI's in-
74 hospital mortality is estimated at around 38.5% and responsible for at least 74,500
75 deaths each year (Rubenfeld et al. 2005).

76 During ALI/ARDS, a disruption in the integrity of the alveolar-capillary membrane
77 increases its permeability and causes by excessive neutrophil migration that releases
78 considerable amounts of oxidant agents, proteases, leukotrienes, and other pro-
79 inflammatory signals (Butt, Kurdowska, and Allen 2016; Johnson and Matthay 2010).
80 In addition to neutrophil migration, significant amounts of cytotoxic and pro-
81 inflammatory mediators such as interleukin (IL)-1, IL-6, IL-8, and IL-10 are released
82 into the alveolar space, enhancing the lung endothelium disruption and further
83 decreasing the epithelial barrier's function (Butt, Kurdowska, and Allen 2016; Johnson
84 and Matthay 2010).

The resulting exudate progressively collapses and consolidates the distal airspaces from surfactant inactivation causing the loss of the lung's gas exchange surface area (Griffiths et al. 2019). If the lung's vascular tone is not yet paralyzed, the hypoxic vasoconstriction of the lung circulatory system occurs allowing deoxygenated blood to be oxygenated in newly recruited lung units (Griffiths et al. 2019). The combination of both the loss of gas exchange space and hypoxic vasoconstriction causes profound hypoxemia and respiratory failure (Griffiths et al. 2019). The pathophysiology of ALI has been summarized in **Figure 1**.

Most of the information available on the pathophysiology of ALI was acquired from experiments developed in various animal models that have been comprehensively reviewed elsewhere (Matute-Bello, Frevert, and Martin 2008). However, most animal models were designed to recreate at least one of the known risk factors for ALI/ARDS development like sepsis, the use of mechanical ventilation, pulmonary embolisms, acid aspiration, bacterial infections, or the damage secondary to ischemia-reperfusion (Matute-Bello, Frevert, and Martin 2008).

Despite all efforts, currently, there is no approved standard treatment for patients suffering from ALI/ARDS (Griffiths et al. 2019; Papazian et al. 2019). Existing guidelines and research focus mostly on ensuring and improving the patient's ventilatory support taking measures the use of high-frequency oscillation ventilation, lung-protective ventilation, or the use of extracorporeal membrane oxygenation amongst other techniques (Griffiths et al. 2019; Papazian et al. 2019).

However, most of the available therapies have shown insufficient efficacy over the unacceptably high ALI/ARDS mortality rates (Griffiths et al. 2019; Papazian et al. 2019), consequently, new treatment options are needed.

In that sense, several naturally occurring phytochemicals present in fruits and vegetables called phenolic compounds (PCs) have proved to prevent diseases where inflammation plays an important role like cancer, diabetes, and cardiovascular disease (CVD) (Minatel et al. 2017). Moreover, PCs are considered safe for humans even when consumed at high doses (Ross and Kasum 2002; Kay 2006; Manach et al. 2005; Mennen et al. 2005), while the reports on the PC tissue bioavailability in animals suggest that the presence of various PCs in target tissues after their oral intake might lead to health several benefits (Sandoval-Ramírez et al. 2018).

Additionally, from a recent systematic review of *in vivo* animal studies regarding the effects of the oral administration of PCs on the intestinal inflammation and barrier function has been suggested that, in animals, the oral administration of PCs significantly improves the intestinal health and barrier function from three main mechanisms of action: First, PCs reduce pro-inflammatory molecule production; Second, PCs improve the intestinal expression of various tight-junction proteins; and Third, PCs enhance the intracellular antioxidant activity of the intestinal epithelia (Sandoval-Ramírez et al. 2020).

All and all, current research suggests that PC's might prove useful in the management of various complications secondary to systemic inflammation in critically ill patients (Meng, Klingensmith, and Coopersmith 2017; Sandoval-Ramírez et al. 2020).

As a consequence, the present systematic review aims to summarize the effects from the oral PC administration on the lipopolysaccharide (LPS)-induced ALI in animals, to explore their potential use as a part of the management of critically ill patients.

2. Materials and methods

132 **2.1. Literature Search**

133 This systematic review was structured following the general principles published in
134 the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)
135 guidelines (Moher et al. 2009). The PRISMA flowchart (**Figure 1)** and the PRISMA
136 checklist (**Supplementary Table 1)** are reported as a part of the present article.

137 *2.1.1. Information Sources and Search Strategy.*

138 Three scientific web libraries: PubMed (https://pubmed.ncbi.nlm.nih.gov/), The
139 Cochrane Library (https://www.cochranelibrary.com/), and SCOPUS
140 (https://www.scopus.com) were explored. All articles regarding the effects of a PC in an
141 LPS-induced animal model of ALI and published over the last decade (2010-2020) were
142 retrieved using the following term:

143 "(polyphenol OR phenol OR phenolic compound) AND (sepsis OR septic OR LPS
144 OR lipopolysaccharide OR LPS-induced OR endotoxin) AND (acute) AND (lung* OR
145 pulmonary OR alveolar) AND (damage OR injury) AND (human OR animal OR RCT
146 OR randomized controlled trial OR rat OR mice OR pig) NOT (review)"

147 *2.1.2. Article's Selection Criteria*

148 Following the PRISMA methodology, two independent authors (B.A.S.-R. and
149 Ú.C.) analyzed the titles and abstracts for possible inclusion, while a third reviewer
150 (R.S.) resolved all differences. The article's full texts were screened for the following
151 criteria:

A) *In vivo* studies assessing the effects coming from the oral, intragastric intravascular, or intraperitoneal administration of at least one phenolic compound on the sepsis- or LPS-induced ALI in animal models.

B) *In vivo* Animal studies where the results of PCs were compared against both positive and negative controls.

C) Studies published from January 1st, 2000 up to November 1st, 2020.

All non-English articles, low-quality publications, publications with a high risk of bias, articles reporting incomplete data, review articles, experiments that administered >1 PC simultaneously, and articles not fulfilling the inclusion criteria were excluded from the present systematic review.

2.1.3. Quality assessment

The reporting quality of the included animal studies was assessed using the "Animal Research Reporting of In Vivo Experiments" (ARRIVE) methodology (Kilkenny et al. 2010), while the risk of bias was evaluated using the "SCYRCLE's tool for assessing the risk of bias in animal studies" (Hooijmans et al. 2014).

3. Results

3.1. Literature search, study selection, and characteristics.

From our database search, 151 articles published between January 1st, 2000 up to November 1st, 2020 were retrieved. After the careful assessment of all titles and abstracts, 128 articles were excluded for not falling under the scope of the present systematic review, 1 article was excluded for intranasal PC administration, 1 article was excluded for assessing the effects of PCs on organs other than the lungs, and 1 article

was excluded for insufficient quality in reporting results, finally leaving 20 articles for analysis (Yao et al. 2018; Xie et al. 2014; Wan et al. 2018; Jingyan et al. 2017; Huang et al. 2016; Xiuli Zhang et al. 2015; Xu Zhang et al. 2010; Xiao et al. 2012; Cheng et al. 2018; Kumari, Dash, and Singh 2017; Y. F. Liu et al. 2017; Kim et al. 2016; Xu et al. 2013; Wang et al. 2018; Hu et al. 2019; Jiang et al. 2016; Z. Zhang et al. 2014; H. X. Zhang et al. 2014; Cao et al. 2011; Xing et al. 2019). The selection process has been summarized in **Figure 2**.

Resveratrol was the PC most commonly used (n=6) (Cao et al. 2011; H. X. Zhang et al. 2014; Z. Zhang et al. 2014; Jiang et al. 2016; Hu et al. 2019; Wang et al. 2018), along with curcumin (n=6) (Xu et al. 2013; Kim et al. 2016; Y. F. Liu et al. 2017; Kumari, Dash, and Singh 2017; Cheng et al. 2018; Xiao et al. 2012), then followed by thymol (n=2) (Wan et al. 2018; Yao et al. 2018), chlorogenic acid (n=1) (Xu Zhang et al. 2010), zingerone (n=1) (Xie et al. 2014), protocatechuic acid (n=1) (Xiuli Zhang et al. 2015), phloretin (n=1) (Huang et al. 2016), salidroside (n=1) (Jingyan et al. 2017), and epicatechin (n=1) (Xing et al. 2019).

Regarding the animal models most preferred, BALB/c mice (n=6) were the animals most commonly used for ALI induction (Huang et al. 2016; Xie et al. 2014; Wan et al. 2018; Z. Zhang et al. 2014; Kim et al. 2016; Yao et al. 2018), followed by Sprague-Dawley rats (n=4) (Jingyan et al. 2017; Cheng et al. 2018; Xiao et al. 2012; Xu et al. 2013), C57BL6 mice (n=3) (Xing et al. 2019; Hu et al. 2019; Jiang et al. 2016), ICR mice (n=3) (Cao et al. 2011; H. X. Zhang et al. 2014; Xu Zhang et al. 2010), Wistar rats (n=1) (Wang et al. 2018), albino rats (n=1) (Y. F. Liu et al. 2017), swiss albino mice (n=1) (Y. F. Liu et al. 2017), and Kunming mice (n=1) (Xiuli Zhang et al. 2015). More information on the included studies can be found in **Table 1**.

*(please insert **Table 1** near this position)*

3.1.1. Quality assessment

The reporting quality 21 animal studies were assessed for inclusion using the ARRIVE guidelines. As a result it was determined that 1 of the possibly included animal did not report adequately its results. Moreover, the risk of bias for the remaining 20 included animal studies was assessed using the SCYRCLE's tool; as a result, it was determined that all the articles were of moderate quality. However, after analysis a low risk of bias was determined that despite the moderate score in SCYRCLE's tool. Complete results on the ARRIVE and SCYRCLE's tools are disclosed in the **Supplementary Table 2** and **Supplementary Table 3**, respectively.

3.1.2. The effects of resveratrol on the LPS-induced ALI in animal models.

The effects of resveratrol on the ALI were assessed in 6 animal experiments (Cao et al. 2011; H. X. Zhang et al. 2014; Z. Zhang et al. 2014; Jiang et al. 2016; Hu et al. 2019; Wang et al. 2018).

1st, in ICR mice that were given resveratrol doses of 1 mg/kg for 3 days as a pretreatment before ALI was induced from an intraperitoneal LPS injection, resveratrol 1 mg/kg showed to prevent the increases in alveolar wall thickness and edema observed in the lungs of control animals (Cao et al. 2011). Besides, the resveratrol pretreatment also reduced the lung wet-to-dry (W/D) weight ratio by 2% when the treated animals were compared against the control group ($p<0.01$) (Cao et al. 2011). Furthermore, resveratrol suppressed the increases in the ARN expression of IL-1β ($p<0.01$), MIP-1α ($p<0.01$), and nitric oxide synthase (iNOS) in the lungs of treated animals ($p<0.01$) (Cao

et al. 2011). Finally, resveratrol significantly downregulated the NF-ƙβ signaling pathway ($p<0.01$) (Cao et al. 2011).

2nd, in BALB/c mice where resveratrol 45.5 mg/kg was orally administered once a day for 3 days before ALI induction from intrabronchial LPS administration, resveratrol demonstrated to reduce the pulmonary congestion, alveolar wall thickness, edema, hemorrhage, and alveolar cavity exudation when compared against the control group (Z. Zhang et al. 2014). Additionally, resveratrol 45.5 mg/kg significantly reduced the lung W/D weight ratio ($p<0.05$), the leukocyte and neutrophil infiltration ($p<0.05$), and inhibited the expression of the toll-like receptor 4 (TLR4) ($p<0.05$), myd88 ($p<0.05$), cyclooxygenase (COX)-2 ($p<0.05$), and the nuclear factor kappa beta (NF-κβ) ($p<0.05$) (Z. Zhang et al. 2014).

3rd, in ICR mice where one resveratrol 0.3 mg/kg dose was administered intraperitoneally immediately before the induction of ALI from LPS injection, resveratrol 0.3 mg/kg proved to reduce the alveolar edema, wall thickening, and air space decreased in the lungs of treated animals when compared against the control group (H. X. Zhang et al. 2014). Also, resveratrol 0.3 mg/kg significantly reduced the histopathologic lung injury score in treated animals (1.60 ± 0.31) when compared against the control group (3.17 ± 0.21; $p<0.01$) (H. X. Zhang et al. 2014).

4th, in C57BL/6 mice where one resveratrol 30 mg/kg dose was administered intraperitoneally immediately before ALI from bronchial LPS administration, resveratrol 30 mg/kg reduced the alveolar congestion, inflammatory cell infiltration, lung injury score ($p<0.05$), and lung W/D weight ratio ($p<0.05$) when compared against the control group (Jiang et al. 2016). Besides, resveratrol 30 mg/kg significantly reduced the total cell, and neutrophil infiltration ($p<0.05$) (Jiang et al. 2016).

Furthermore, resveratrol 30 mg/kg significantly reduced both the myeloperoxidase (MPO) expression and activity ($p<0.05$), as well as the IL-1β and IL-18 expression in the lungs of treated animals when compared against the control group (Jiang et al. 2016). Finally, resveratrol 30 mg/kg also inhibited the mRNA expression of the NLR family pyrin domain-containing (Nlrp3; $p<0.05$), Asc ($p<0.05$), and caspase-1 ($p<0.05$), inhibited the NLRP3 inflammasome activation and depressed the NF-κβ translocation and DNA binding-activity (Jiang et al. 2016).

5th, in Wistar rats where one resveratrol 30 mg/kg dose was administered intraperitoneally one hour before the induction of ALI from the cecal ligation and puncture, resveratrol 30 mg/kg reduced the bronchoalveolar lavage fluid (BALF) concentrations of macrophage inflammatory protein (MIP)-2 (15.3 ± 2.8 pg/mL; $p<0.05$) and IL-18 (4.2 ± 0.5 pg/mL; $p<0.05$) when compared against the control group (MIP-2, 28.6 ± 3.5 pg/mL; IL-18, 10.6 ± 0.9 pg/mL), and significantly increased the pulmonary IL-10 concentrations (783.4 ± 45.3 pg/mL) when compared against the control group (425.6 ± 26.4 pg/mL) (Wang et al. 2018).

Furthermore, the rats treated with resveratrol 30 mg/kg significantly also reduced the lung concentrations of malondialdehyde ($p<0.05$), and 8-oxo-2'-deoxyguanosine ($p<0.05$). Also, resveratrol increased both the expression and activity of the heme oxygenase-1 and nuclear factor erythroid 2-related factor 2 (nrf2) proteins (Wang et al. 2018).

Finally, resveratrol 30 mg/kg significantly reduced the lung W/D ratio, inflammatory cell infiltration, interstitial hyperemia, and edema in the lungs of treated rats ($p<0.05$), consequently lowering the lung tissue damage score in a significant manner ($p<0.05$) (Wang et al. 2018).

6th, in C57BL/6 mice where 2 intraperitoneal injections of resveratrol 30 mg/kg were administered 24 and 3 hours before the induction of ALI from LPS administration, resveratrol 30 mg/kg significantly reversed the LPS-induced inflammatory infiltrates ($p<0.01$), epithelial cell hyperplasia ($p<0.01$), and lung fibrosis ($p<0.01$) in the lungs of treated animals when compared against the control group (Hu et al. 2019). The animals treated with resveratrol 30 mg/kg reduced the bronchial deposition of alpha-1 type I collagen ($p<0.01$), and alpha-smooth muscle actin ($p<0.01$) (Hu et al. 2019). Additionally, resveratrol 30 mg/kg decreased the CD45+ Sigle F− macrophage percentage ($p<0.01$) and reduced the STAT3 protein activation ($p<0.01$) (Hu et al. 2019). Finally, resveratrol 30 mg/kg decreased the expression of IL-1β ($p<0.01$), and CXCL5 in the lungs of treated mice ($p<0.01$) (Hu et al. 2019).

3.1.3. The effects of curcumin on the LPS-induced ALI in animal models.

The effects of curcumin on the ALI were assessed in 6 animal experiments (Xu et al. 2013; Kim et al. 2016; Y. F. Liu et al. 2017; Kumari, Dash, and Singh 2017; Cheng et al. 2018; Xiao et al. 2012).

1st, in a Sprague-Dawley rat model where 2 intraperitoneal doses of curcumin (50 mg/kg or 100 mg/kg) were administered 2 and 12 hours after the induction of ALI from the ligation and puncture of the cecum, both curcumin doses (50 mg/kg or 100 mg/kg) significantly improved the PaO2/FiO2 ratio at 6, 12, and 24 h ($p<0.01$) (Xiao et al. 2012). Furthermore, both curcumin doses (50 mg/kg or 100 mg/kg) significantly improved the lung W/D weight ratio in the lungs of treated animals ($p<0.01$) (Xiao et al. 2012).

The microscopic examination of the BALF in treated rats revealed that both curcumin doses (50 mg/kg or 100 mg/kg) significantly reduced the lymphocyte ($p<0.01$), neutrophil ($p<0.01$), and total cell count ($p<0.01$) (Xiao et al. 2012).

Likewise, both curcumin doses (50 mg/kg or 100 mg/kg) significantly reduced the malonaldehyde and superoxide dismutase activity in treated animals ($p<0.01$), as well as the expression of tumor necrosis factor (TNF)-α ($p<0.01$), IL-8 ($p<0.01$), and MIF ($p<0.01$) in the lungs of the treated rats (Xiao et al. 2012).

Lastly, the 72 h survival rate was increased from 40% in the sepsis group to 80% in the curcumin 50 mg/kg group ($p<0.05$), and to 90% in the curcumin 100 mg/kg group ($p<0.05$) (Xiao et al. 2012).

2nd, in a Sprague-Dawley rat model of sepsis-induced ALI where one curcumin 200 mg/kg dose was administered from intraperitoneal injection at the same time of ALI induction, curcumin 200 mg/kg significantly reduced the lung W/D weight ratio at 24 and 48 hours ($p<0.05$) (Xu et al. 2013). Additionally, curcumin 200 mg/kg reduced the alveolar space fibrin accumulation, cellular necrosis, and erythrocyte count in the lungs of treated rats when compared against the control group (Xu et al. 2013). Finally, curcumin 200 mg/kg significantly reduced the transforming growth factor (TGF)-β1's gene expression and plasma concentrations at 24 and 28 h ($p<0.05$) (Xu et al. 2013).

3rd, in a BALB/C mice model of LPS-induced lung injury where one intraperitoneal injection of curcumin 200 mg/kg was administrated immediately before ALI induction from intrabronchial LPS, curcumin 200 mg/kg significantly increased the AMP-activated protein kinase (AMPK) phosphorylation in the lung tissue of the treated mice ($p<0.05$) (Kim et al. 2016). Moreover, curcumin 200 mg/kg significantly decreased the

313 TNF-α ($p<0.05$), MIP-2 ($p<0.05$), and IL-6 production ($p<0.05$) (Kim et al. 2016).
314 Curcumin 200 mg/kg also prevented the phosphorylation of IκBα in the lungs ($p<0.05$),
315 and significantly reduced the white blood cell and neutrophil counts in the BALF of
316 treated mice (Kim et al. 2016). Finally, curcumin 200 mg/kg significantly reduced the
317 lung W/D weight ratio and MPO activity in the lungs of the treated mice ($p<0.05$) (Kim
318 et al. 2016).

319 4th, in an albino rat model of sepsis-induced chronic lung injury where a daily dose of
320 curcumin 50 mg/kg or 100 mg/kg was orally administered for 45 days after ALI
321 induction from the ligation and puncture of the cecum, curcumin 50 mg/kg significantly
322 reduced the lung W/D weight ratio in 36.36% ($p<0.05$) (Y. F. Liu et al. 2017).
323 Curcumin 50 mg/kg also reduced the lung's neutrophil count in 28.57% ($p<0.05$), the
324 lymphocyte count in 30% ($p<0.05$), and the total cell count 32% ($p<0.05$) (Y. F. Liu et
325 al. 2017). Furthermore, curcumin 50 mg/kg significantly reduced the MPO enzyme
326 activity in 26.32% ($p<0.05$), and the lipid peroxidation in 25% ($p<0.05$) (Y. F. Liu et al.
327 2017).

328 On the other hand, curcumin 100 mg/kg caused a 41.84% reduction in the total protein
329 content of the treated rat's lungs ($p<0.05$), significantly reduced the lung W/D weight
330 ratio by 45.45% ($p<0.05$) (Y. F. Liu et al. 2017). Curcumin 100 mg/kg also reduced the
331 lung's neutrophil count in 61.9% ($p<0.05$), the lymphocyte count in 65% ($p<0.05$), and
332 the total cell count 64% ($p<0.05$) (Y. F. Liu et al. 2017). Moreover, curcumin 100
333 mg/kg significantly reduced the MPO enzyme activity in 63.16% ($p<0.05$), the lipid
334 peroxidation in 39.28% ($p<0.05$) (Y. F. Liu et al. 2017).

335 Finally, both curcumin doses (50 mg/kg and 100 mg/kg) significantly increased the
336 superoxide dismutase (SOD; $p<0.05$), and catalase activities ($p<0.05$) and reduced the

IL-8 ($p<0.05$), TNF-a ($p<0.05$), and macrophage migration inhibitory factor (MIF) content in treated rats when compared against controls ($p<0.05$) (Y. F. Liu et al. 2017).

5th, in a swiss albino mice model of LPS-induced airway inflammation where a single intraperitoneal dose of curcumin 20 mg/kg was administered 1 hour before ALI induction from an intraperitoneal LPS injection, curcumin 20 mg/kg significantly reduced lethal endotoxemia from 50% in controls to 30% mortality in the treated mice ($p<0.05$) (Kumari, Dash, and Singh 2017). Besides, curcumin 20 mg/kg significantly reduced the concentrations of TNF-α ($p<0.05$), reactive oxygen species (ROS; $p<0.01$), and nitrite levels ($p<0.05$) in the plasma of treated mice (Kumari, Dash, and Singh 2017). Furthermore, the lungs of the treated albino mice evidenced significant reductions in the MPO activity ($p<0.01$), had lower cell counts ($p<0.01$), and lower protein leakage in the BALF ($p<0.05$) (Kumari, Dash, and Singh 2017). Finally, curcumin 20 mg/kg significantly reduced hydroxyproline levels ($p<0.05$), the expression of the LPS-induced TGF-β1 ($p<0.05$), iNOS ($p<0.05$), and TLR-4 ($p<0.05$) in the lungs of the treated albino mice ($p<0.05$) (Kumari, Dash, and Singh 2017).

6th, in a Sprague-Dawley rat model of LPS-induced neonatal lung injury where one dose of curcumin 1.5 mg/kg, 3 mg/kg, or 6 mg/kg was administered once daily for seven days through intraperitoneal injection (Cheng et al. 2018). Curcumin doses started immediately after ALI induction from LPS-administration (Cheng et al. 2018). As a result, curcumin at all doses (1.5 mg/kg, 3 mg/kg, or 6 mg/kg) significantly reduced the lung's W/D weight ratio and increased the PaO2 in a dose-dependent manner ($p<0.05$). Additionally, curcumin at all doses (1.5 mg/kg, 3 mg/kg, or 6 mg/kg) decreased the concentration of high mobility group box 1 (HMGB1) protein, TNF-α, IL-6, and TGF-β1 in the BALF of the treated rats in a dose-dependent manner ($p<0.05$).

361 *3.1.4. The effects of various PCs on the LPS-induced ALI in animal models.*

362 Regarding the less frequently used PCs to assess the possible beneficial effect from the
363 PC supplementation to treat ALI in animal models:

364 One intraperitoneal dose of thymol 20 mg/kg, 40 mg/kg, or 80 mg/kg in a BALB/c mice
365 model of LPS-induced lung injury demonstrated to significantly reduce the treated
366 animal's alveolar wall thickening, lung W/D weight ratio ($p<0.01$), edema, and cellular
367 infiltration in a dose-dependent manner (Yao et al. 2018). Additionally, thymol 20
368 mg/kg, 40 mg/kg and 80 mg/kg also reduced, in a dose-dependent manner, the BALF
369 concentrations of TNF-α ($p<0.01$), IL-6 ($p<0.01$), and IL-1β ($p<0.01$), as well as the
370 MPO ($p<0.01$), and malondialdehyde ($p<0.01$) activities in the lungs of treated mice
371 (Yao et al. 2018).

372 Similar results were observed in another experiment for intraperitoneal thymol 100
373 mg/kg administered 0.5 hours before ALI induction in BALB/c mice (Wan et al. 2018).
374 As a result, thymol 100 mg/kg also reduced the concentrations of TNF-α ($p<0.01$), IL-6
375 ($p<0.01$), and proteins ($p<0.01$) in the BALF of treated animals (Wan et al. 2018).
376 Additionally, thymol 100 mg/kg attenuated the alveolar hemorrhage, wall thickening,
377 and neutrophil infiltration in the lungs of the treated mice (Wan et al. 2018). Thymol
378 100 mg/kg also attenuated the NF-ƙβ ($p<0.05$) and increased the SOD activity in the
379 lungs of treated mice ($p<0.05$) (Wan et al. 2018). Finally, thymol 100 mg/kg
380 significantly reduced the total cell count ($p<0.01$), and MPO activity ($p<0.01$) in the
381 BALF of treated mice (Wan et al. 2018).

382 Furthermore, a single intraperitoneal administration of chlorogenic acid 50 mg/kg in an
383 ICR mice model of ALI significantly reduces the pulmonary wall thickening, interstitial

384 edema, neutrophil infiltration, MPO activity ($p<0.01$), and cell count in the lungs of
385 treated mice ($p<0.05$) (Xu Zhang et al. 2010). Other reductions in polymorphonuclear
386 and leukocyte infiltration ($p<0.01$), as well as the NO concentration ($p<0.01$) were
387 noted in the BALF of ICR mice that were treated with chlorogenic acid 50 mg/kg (Xu
388 Zhang et al. 2010). The reduction in NO concentration was caused by a reduction in the
389 iNOS activity in the lungs of treated mice ($p<0.001$) (Xu Zhang et al. 2010).

390 Zingerone 20 mg/kg and 40 mg/kg significantly reduced, in a dose-dependent manner,
391 the lung W/D weight ratio, NF-ƙβ, MAPK, and MPO activities in the lung tissue of
392 BALB/c mice that were orally treated one hour before the induction of ALI from LPS
393 administration (Xie et al. 2014). Additionally, zingerone significantly reduced the
394 neutrophil ($p<0.01$), macrophage ($p<0.01$), and total cell number count ($p<0.01$) as well
395 as the TNF-α ($p<0.01$), and IL1β ($p<0.01$) concentrations in the BALF of treated mice
396 (Xie et al. 2014).

397 Seven intraperitoneal doses of phloretin 5 mg/kg or 20 mg/kg to a BALB/c mice model
398 on which ALI was provoked from the tracheal administration of LPS revealed that only
399 phloretin 20 mg/kg was able to significantly reduce the lung's W/D weight ratio
400 ($p<0.05$), neutrophil infiltration ($p<0.05$), and MPO activity in treated mice ($p<0.05$)
401 (Huang et al. 2016). Additionally, phloretin 5 mg/kg and 20 mg/kg significantly reduced
402 the neutrophil count ($p<0.05$), and the concentrations of IL-6 ($p<0.05$), TNF-α ($p<0.05$),
403 IL-1β ($p<0.05$), and MCP-1 ($p<0.05$) in the BALF of treated animals, in a dose-
404 dependent manner (Huang et al. 2016). Moreover, both phloretin doses (5 mg/kg or 20
405 mg/kg) caused significant decreases in the NF-ƙβ ($p<0.05$), and MAPK's ($p<0.05$)
406 pathway activity in the lungs of treated animals, whereas in plasma, both doses of

407 phloretin significantly reduced the TNF-α ($p<0.05$), and IL-6 concentrations ($p<0.05$)
408 (Huang et al. 2016).

409 Protocatechuic acid, when administered intraperitoneally in a single 10 mg/kg dose to
410 Kunming mice 1 h before ALI induction from LPS-administration, can significantly
411 reduce the inflammatory cell infiltration, alveolar hemorrhage, and wall thickening in
412 treated mice (Xiuli Zhang et al. 2015). Additionally, protocatechuic acid 10 mg/kg
413 significantly reduced BALF amount ($p<0.05$) as well as the TNF-α ($p<0.05$), and IL-1β
414 ($p<0.05$) concentrations in the blood and the lungs of treated Kunming mice (Xiuli
415 Zhang et al. 2015). Finally, protocatechuic 10 mg/kg acid significantly reduced the
416 TLR4 ($p<0.05$), and NF-ƙβ expression in the lungs of treated mice ($p<0.05$) (Xiuli
417 Zhang et al. 2015).

418 Salidroside can reduce in a dose-dependent manner the lung W/D weight ratio ($p<0.01$),
419 and the MPO lung levels ($p<0.01$) of septic rats as demonstrated in a Sprague-Dawley
420 rat model of LPS-induced ALI (Jingyan et al. 2017). For the experiment, salidroside 20
421 mg/kg or 50 mg/kg was administered directly into the animal's stomach for 3 days, on
422 the third day, ALI was induced from LPS-intraperitoneal injection (Jingyan et al. 2017).
423 Additionally, both salidroside doses demonstrated their ability to restore in a dose-
424 dependent manner the intracellular concentrations of SOD ($p<0.01$), catalase ($p<0.01$),
425 glutathione ($p<0.01$), and glutathione peroxidase ($p<0.01$) (Jingyan et al. 2017).
426 Furthermore, salidroside at both doses (20 mg/kg or 50 mg/kg) significantly reduced the
427 IL-6 ($p<0.01$), IL-1β ($p<0.01$), and TNF-α ($p<0.01$) serum levels in treated animals
428 from a reduced TLR/NF-ƙβ expression in the lungs of the treated rats ($p<0.01$) (Jingyan
429 et al. 2017).

Finally, 3 intragastric doses of epicatechin 15 mg/kg administrated to C57BL6/N mice immediately after, 30 min, and 12 h after ALI induction from intraperitoneal LPS injection demonstrated that epicatechin 15 mg/kg can significantly reduce the lung injury score ($p<0.05$) and alveolar wall thickness ($p<0.05$) in treated mice (Xing et al. 2019). Also, epicatechin 15 mg/kg significantly reduced the protein concentration and neutrophil infiltration in the BALF of treated mice ($p<0.05$), reduced the MAPK activity in the lungs of treated mice ($p<0.05$), and decreased the TNF-α ($p<0.05$), the IL-6 ($p<0.05$) concentrations in both the BALF and lungs of the treated mice (Xing et al. 2019).

4. Discussion

The ALI and subsequent ARDS suffered by critically ill patients are considered to be responsible for at least 74,500 deaths each year only in the United States (Rubenfeld et al. 2005). Current estimations determine that ARDS mortality is situated around an unacceptably high 48% (Griffiths et al. 2019).

As previously mentioned, the treatment for ALI and ARDS currently focuses on various measures designed to improve or maintain the ventilatory function of the lungs such as adjusting the tidal volumes, monitoring the plateau pressure, avoiding high-frequency oscillatory ventilation, and changing the patient to a prone position (Griffiths et al. 2019; Papazian et al. 2019) all with little efficacy. Besides, several drugs have been tested for ALI/ARDS treatment with limited or minimal success in improving survival (Raghavendran et al. 2008).

For instance, the inhalation prostacyclin has been tried to increase alveolar perfusion with few beneficial results (Dembinski et al. 2001), the beta-2 agonist therapy only demonstrated to minimally improve the alveolar fluid clearance and the reduce of edema (Manocha et al. 2006; Matthay and Calfee 2008), some platelet aggregation and neutrophil elastase inhibitors have been used to reduce the vascular occlusion with promising, yet insufficient results (Sahebnasagh et al. 2020; Chen et al. 2020), while the inhaled NO therapy showed only transient improvements in oxygenation without a reduction in mortality secondary to NO-induced renal damage (Gebistorf et al. 2016). Hence, new therapies are still needed for improving ALI/ARDS treatment and reducing mortality.

On that matter, as suggested from the systematic review of animal models, supplementing the nutrition of critically ill patients with PCs during ALI/ARDS could result in the patient's mortality reduction for the following reasons:

First, resveratrol (Cao et al. 2011; Z. Zhang et al. 2014; H. X. Zhang et al. 2014), chlorogenic acid (Xu Zhang et al. 2010), protocatechuic acid (Xiuli Zhang et al. 2015), and epicatechin (Xing et al. 2019) proved to significantly reduce the alveolar wall thickness caused LPS administration in various animal models, possibly from the reduction of lung cell hyperplasia, and collagen deposition (Hu et al. 2019). Also, curcumin demonstrated to improve the PaO2/FiO2 ratio in a dose-dependent manner when administered at doses as low as 1.5 mg/kg in Sprague-Dawley rats (Xiao et al. 2012; Cheng et al. 2018). Consequently, suggesting that the administration of various PCs, particularly curcumin, would most likely cause the reduction of the alveolar wall thickness and improve the gas exchange in animals suffering from ALI.

476 **Second,** resveratrol (Cao et al. 2011; Z. Zhang et al. 2014; Jiang et al. 2016; Wang et al.
477 2018), curcumin (Xiao et al. 2012; Xu et al. 2013; Y. F. Liu et al. 2017; Cheng et al.
478 2018), chlorogenic acid (Xu Zhang et al. 2010), zingerone (Xie et al. 2014), phloretin
479 (Huang et al. 2016), thymol (Yao et al. 2018; Wan et al. 2018), and salidroside (Jingyan
480 et al. 2017) were all able to reduce the lung's W/D weight ratio in LPS-induced animal
481 models of ALI, thus, suggesting from all the included animal studies that the
482 administration of a PC to septic animals will most likely cause the pulmonary edema
483 reduction pulmonary during ALI.

484 **Third,** resveratrol (Cao et al. 2011; H. X. Zhang et al. 2014; Z. Zhang et al. 2014; Jiang
485 et al. 2016; Hu et al. 2019; Wang et al. 2018), curcumin (Xiao et al. 2012; Kim et al.
486 2016; Y. F. Liu et al. 2017; Kumari, Dash, and Singh 2017), thymol (Yao et al. 2018;
487 Wan et al. 2018), chlorogenic acid (Xu Zhang et al. 2010), zingerone (Xie et al. 2014),
488 protocatechuic acid (Xiuli Zhang et al. 2015), and epicatechin (Xing et al. 2019) proved
489 to significantly reduce the neutrophil and leukocyte infiltration in the lungs and BALF
490 in animals, suggesting that the PC administration lessens the immune response in
491 animals suffering from LPS-induced ALI.

492 Finally, some signs of a possible reduction in ALI's mortality from PC administration
493 can be found in two of the included animal studies (Kumari, Dash, and Singh 2017;
494 Xiao et al. 2012) where curcumin was able to significantly reduce the animal's mortality
495 in ≈ 50% when doses between 20 mg/kg and 100 mg/kg were administered. However,
496 since the mortality was not the primary outcome of any of the included articles, no
497 conclusions on the effects of the PC administration on ALI's mortality can be drawn
498 from this systematic review of *in vivo* animal studies.

What is more, from the available information regarding the effects of resveratrol, curcumin, zingerone, phloretin, chlorogenic acid, thymol, and protocatechuic acid on various LPS-induced ALI animal models, proposes that PCs can significantly improve various key characteristics of ALI such as the alveolar wall thickness, pulmonary edema, and inflammatory cell infiltration from three main mechanisms:

First, PCs reduce the signaling activity in two key pathways: a) the NF-ƙβ activity, downregulated from the resveratrol, zingerone, protocatechuic acid, phloretin, or salidroside administration (Z. Zhang et al. 2014; Jiang et al. 2016; Cao et al. 2011; Xie et al. 2014; Xiuli Zhang et al. 2015; Huang et al. 2016); and b) the MAPK activity, which was downregulated after curcumin, zingerone, phloretin, or epicatechin administration in the assessed animal studies of LPS-induced ALI (Kim et al. 2016; Xie et al. 2014; Huang et al. 2016; Xing et al. 2019).

Since regulating the activity of signaling pathways like the NF-ƙβ or MAPK is considered crucial for the regulation of various aspects of the immune response such as the cytokine and interleukin production (T. Liu et al. 2017), the downregulation of the NF-ƙβ and MAPK pathways from various PCs, fairly explains the reductions in the inflammatory cell infiltration and expression of several pro-inflammatory proteins including IL-1β, IL-6, IL-8, IL-18, and TNF-α that was observed after PC administration in diverse animal models of LPS-induced ALI (Yao et al. 2018; Xie et al. 2014; Wan et al. 2018; Jingyan et al. 2017; Huang et al. 2016; Xiuli Zhang et al. 2015; Xu Zhang et al. 2010; Xiao et al. 2012; Cheng et al. 2018; Kumari, Dash, and Singh 2017; Y. F. Liu et al. 2017; Kim et al. 2016; Xu et al. 2013; Wang et al. 2018; Hu et al. 2019; Jiang et al. 2016; Z. Zhang et al. 2014; H. X. Zhang et al. 2014; Cao et al. 2011; Xing et al. 2019).

Additionally, the reduction in the lung's pro-inflammatory protein production and alveolar inflammatory cell infiltration also explain the improvement in the alveolar wall thickness, and edema observed in all the included animal studies (Yao et al. 2018; Xie et al. 2014; Wan et al. 2018; Jingyan et al. 2017; Huang et al. 2016; Xiuli Zhang et al. 2015; Xu Zhang et al. 2010; Xiao et al. 2012; Cheng et al. 2018; Kumari, Dash, and Singh 2017; Y. F. Liu et al. 2017; Kim et al. 2016; Xu et al. 2013; Wang et al. 2018; Hu et al. 2019; Jiang et al. 2016; Z. Zhang et al. 2014; H. X. Zhang et al. 2014; Cao et al. 2011; Xing et al. 2019)

Second, PCs reduce the tissue oxidation and ROS concentration in the lungs of animals suffering from ALI after LPS administration. The most commonly observed benefit appears to be caused by a drop in MPO activity as demonstrated from curcumin (Kim et al. 2016; Y. F. Liu et al. 2017; Kumari, Dash, and Singh 2017), resveratrol (Jiang et al. 2016), zingerone (Xie et al. 2014), phloretin (Huang et al. 2016), salidroside (Jingyan et al. 2017), and chlorogenic acid (Xu Zhang et al. 2010) after their administration in different animal models.

MPO is an enzyme with reported antibacterial activities that involve the production of ROS (H_2O_2) and reactive nitrogen species which are released into the extracellular fluid in response to the oxidative stress and the inflammatory cell response (Khan, Alsahli, and Rahmani 2018). It is considered that the controlled liberation of MPO is crucial for its efficient activity, while its unrestrained release and degranulation increase inflammation and causes damage in surrounding tissues (Khan, Alsahli, and Rahmani 2018). Consequently, the reduction in the MPO activity from the administration of various PCs partly explains the reduction in the damage caused by the release of ROS into the lung tissue of treated animals (Kim et al. 2016; Y. F. Liu et al. 2017; Kumari,

547 Dash, and Singh 2017; Jingyan et al. 2017; Xu Zhang et al. 2010; Jiang et al. 2016;
548 Huang et al. 2016; Xie et al. 2014).

549 Furthermore, additional benefits appear to come from the increased expression of other
550 anti-oxidant enzymes like HO-1 induced from the resveratrol or salidroside
551 administration (Jingyan et al. 2017; Wang et al. 2018), and the improvement of SOD's
552 activity, enhanced after phloretin or salidroside administration in animal models of ALI
553 (Huang et al. 2016; Jingyan et al. 2017).

554 **Third**, PCs appear to trigger an improvement in the pulmonary vasodilatation that is
555 caused by hypoxia from a reduction in the iNOS activity and NO concentrations in the
556 lungs of animals treated with resveratrol, curcumin, or chlorogenic acid, and suffering
557 from and LPS-induced ALI (Cao et al. 2011; Kumari, Dash, and Singh 2017). Thus,
558 PCs could reduce the hypoxic vasoconstriction of the lung's circulatory system that
559 occurs during ALI (Griffiths et al. 2019), this allows the deoxygenated blood to be
560 oxygenated in newly recruited lung units consequently improving some ventilatory
561 parameters.

562 Thus, suggesting from the comprehensive analysis of the animal results, that PCs could
563 effectively counteract various of the mechanisms of action that cause ALI/ARDS, and
564 potentially improve the unacceptable mortality rates in critically ill patients.

565 **5. Future Considerations**

566 The current systematic review summarizes the results from various animal interventions
567 suggesting some possible beneficial effects of PCs in the ALI caused by sepsis, these
568 results, along with previous information on the beneficial effects of PCs on intestinal
569 health (Sandoval-Ramírez et al. 2020), suggesting the PC supplementation might prove

570 to be beneficial in critically ill patients, however, before any conclusions are drawn, all
571 animal results must be validated in a randomized control trial on humans to determine
572 the effects of PCs on human health.

573 **1. Conclusions**

574 From the present systematic review, in animals, the oral administration of various PCs
575 improves the intestinal barrier integrity and function from three main mechanisms: 1)
576 The reduction of several pro-inflammatory molecules, 2) the improvement in the
577 expression of TJ proteins, and 3) the improvement of the antioxidant intracellular
578 activity from chemical interactions and the increased expression of antioxidant
579 enzymes. Furthermore, resveratrol, the most studied PC in different animal models of
580 intestinal damage, improves the intestinal barrier integrity through an increase in the
581 expression of various anti-inflammatory and antioxidant proteins in animals. Thus,
582 suggesting the possible use of resveratrol or other PCs in the management of the
583 intestinal injury associated with various intestinal and systemic pathologies in humans.
584 However, the precise dose and time for the oral administration of resveratrol or other
585 PCs in humans are still undetermined.

586 **6. Conclusions**

587 From the results of the present systematic review of animal studies can be concluded
588 that in animal models of LPS-induced ALI, PCs significantly improve various key
589 characteristics such as the alveolar wall thickness, pulmonary edema, and inflammatory
590 cell infiltration secondary 1) to the reduction in the signaling activity the NF-ƙβ and the
591 MAPK activity which caused smaller concentrations of pro-inflammatory molecules
592 and reduced inflammatory cell infiltration, 2) the reduction in the concentration of ROS

593 in the lung tissue, and 3) the improvement of the iNOS activity and NO concentrations
594 consequently reducing the lung's hypoxic vasoconstriction.

595 Therefore, from all animal results, PCs should be considered promising agents for the
596 treatment of ALI/ARDS in humans.

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611 ***Declaration of Interests***

612 The authors have declared no conflicts of interest. Complete Declaration of Interest
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614 **Contributions**

615 Study conception and design: B.A.S-R., Ú.C., and R.S.

616 Acquisition of data: B.A.S-R., and Ú.C.

617 Analysis and interpretation of data: B.A.S-R., Ú.C., and R.S.

618 Drafting of the manuscript: B.A.S-R, and Ú.C.

619 Critical revision: Ú.C., and R.S.

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830 **Figure legends**

831 **Tables**

Table 1. General information of the included studies assessing the effects of polyphenols on the lung and heart damage secondary to LPS administration in animal models.

Title	Author	Year	Outcome	Intervention target	N (m/f)	Phenolic compound used	Animal dose	Human equivalent dose (70 kg human)	Length of study	PC treatment methodology	Dose number (administration route)	Main results
LPS-induced lung damage												
Resveratrol												
Protective effect of resveratrol on acute lung injury induced by lipopolysaccharide in mice	Cao, Q. *et al.*	2011	LPS-induced lung injury	ICR mice	NR(m)	Resveratrol	1 mg/kg		3 days	Resveratrol was given 3 days before the induction of sepsis from an intraperitoneal LPS injection. To some animals, a 25 mg/kg resveratrol dose was given with an intraperitoneal injection to compare against intraperitoneal dexamethasone as positive treatment control. The animals were sacrificed 8 hours after LPS injection, and the lungs were analyzed.	3 (oral) or 1 (intraperitoneal)	Resveratrol 3-day pretreatment significantly reversed the alveolar wall thickness, edema, and inflammatory cell infiltration caused by LPS ($p<0.05$). Resveratrol reduced the pulmonary lung W/D ratio (-2%; $p<0.01$). Resveratrol effectively suppressed the increase in the ARN expression of IL-1β and MIP-1α caused by LPS ($p<0.05$). Resveratrol reduced iNOS expression ($p<0.01$). Finally, resveratrol downregulated the NF-kβ signaling pathway ($p<0.01$).
Protective effect of resveratrol against acute lung injury induced by lipopolysaccharide via inhibiting the myd88-dependent Toll-like receptor 4 signaling pathway	Zhang, Z. *et al.*	2014	LPS-induced acute lung injury	BALB/c mice	144 (NR)	Resveratrol	45.5 mg/kg		3 days	Resveratrol was administered 3 days before the ALI from LPS intrabronchial administration. Animals were sacrificed 3 days after LPS injection.	3 (oral)	When compared against the control group: Resveratrol reduced the pulmonary congestion, leukocyte infiltration, alveolar wall thickness, edema, hemorrhage, and alveolar cavity exudation. Additionally, reduced the lung W/D ratio ($p<0.05$) in the lungs of treated animals. Resveratrol reduced the leukocyte and neutrophil pulmonary infiltration ($p<0.05$) when compared against the control group. Resveratrol inhibited the expression of TLR4 ($p<0.05$), myd88 ($p<0.05$), COX-2 ($p<0.05$), and NF-κB ($p<0.05$) in the lungs of treated animals.

The protective effect of resveratrol against endotoxemia-induced lung injury involves the reduction of oxidative/nitrative stress	Zhang, H-X. *et al.*	2014	LPS-induced acute lung injury	ICR mice	50 (m)	Resveratrol	0.3 mg/kg	24 hours	Resveratrol was administered immediately before ALI induction from LPS injection. All mice were sacrificed 24 h after LPS injection.	1 (intraperitoneal)	When compared against the control group: Resveratrol reduced the diffuse alveolar edema, alveolar wall thickening, interstitial leukocyte infiltration, and the alveolar air space decrease. Resveratrol significantly reduced the histopathologic lung injury score from 3.17 ± 0.21 in the control group to 1.60 ± 0.31 in the treated group ($p<0.01$). When compared against the control group: Resveratrol reduced the alveolar congestion and inflammatory cell infiltration in the lungs of treated animals. Resveratrol significantly reduced the lung injury score ($p<0.05$), and the lung wet-to-dry ratio ($p<0.05$) in the lungs of treated animals. Resveratrol significantly reduced total cell and neutrophil lung tissue infiltration ($p<0.05$) in treated animals. Resveratrol reduced MPO expression and activity ($p<0.05$). Resveratrol reduced the IL-1β ($p<0.05$), and IL-18 expression in the lung tissue of treated animals ($p<0.05$). Resveratrol inhibited the mRNA expression of the Nrp3 ($p<0.05$), Asc ($p<0.05$), and caspase-1 proteins ($p<0.05$) in the lungs of treated animals. Resveratrol inhibited the NLRP3 inflammasome activation ($p<0.05$) and depressed the NF-κβ translocation and DNA binding activity in the lungs of treated mice ($p<0.05$).
Resveratrol ameliorates LPS-induced acute lung injury via NLRP3 inflammasome modulation	Jiang, L. *et al.*	2016	LPS-induced lung injury	C57BL6 mice	30 (m)	Resveratrol	30 mg/kg	6 hours	Resveratrol was administered by intraperitoneal injection immediately before LPS administration. Animals were euthanized 6 hours after ALI induction from LPS administration.	1 (intraperitoneal)	

Alleviation of Acute Lung Injury in Rats with Sepsis by Resveratrol via the Phosphatidylinositol 3-Kinase/Nuclear Factor-Erythroid 2 Related Factor 2/Heme Oxygenase-1 (PI3K/Nrf2/HO-1) Pathway	Wang, Y. *et al.*	2018	sepsis-induced acute lung injury	Wistar rat	36 (m)	Resveratrol	30 mg/kg	24 hours	Resveratrol was administered by intraperitoneal injection 1 hour after the induction of ALI from a cecal ligation and puncture procedure. All animals were euthanized 24 hours after ALI induction.	1 (intraperitoneal)	Resveratrol reduced the bronchoalveolar lavage fluid (BALF) concentrations of macrophage inflammatory protein (MIP)-2 (15.3 ± 2.8 pg/mL; $p<0.05$) and IL-18 (4.2 ± 0.5 pg/mL; $p<0.05$) when compared against the control group (MIP-2, 28.6 ± 3.5 pg/mL; IL-18, 10.6 ± 0.9 pg/mL), and significantly increased the pulmonary IL-10 concentrations (783.4 ± 45.3 pg/mL) when compared against the control group (425.6 ± 26.4 pg/mL). The rats treated with resveratrol significantly also reduced the lung concentrations of malondialdehyde ($p<0.05$), and 8-oxo-2'-deoxyguanosine ($p<0.05$). Besides, resveratrol increased both the expression and activity of the heme oxygenase-1 and nuclear factor erythroid 2-related factor 2 (*nrf2*) proteins. Resveratrol significantly reduced the lung W/D ratio, inflammatory cell infiltration, interstitial hyperemia, and edema in the lungs of treated rats ($p<0.05$), consequently lowering the lung tissue damage score in a significant manner ($p<0.05$). When compared against the control group: resveratrol significantly reversed the LPS-induced inflammatory infiltrates ($p<0.01$), epithelial cell hyperplasia ($p<0.01$), and lung fibrosis ($p<0.01$) in the lungs of treated animals when compared against the control group. Resveratrol reduced the bronchial deposition of alpha-1 type I collagen ($p<0.01$), and alpha-smooth muscle actin ($p<0.01$) in the lungs of treated animals. resveratrol decreased the CD45+ Sigle F– macrophage percentage ($p<0.01$) and reduced the STAT3 protein activation ($p<0.01$). Resveratrol decreased the expression of IL-1β ($p<0.01$), and CXCL5 in the lungs of treated animals ($p<0.01$).
Resveratrol decreases CD45 + CD206 - subtype macrophages in LPS-induced murine acute lung injury by a SOCS3 signaling pathway	Hu, L. *et al.*	2019	LPS- induced acute lung injury/acute respiratory distress syndrome	C57BL/6 mice	NR (m)	Resveratrol	30 mg/kg	24 hours	Resveratrol was administered by intraperitoneal injection 3 and 24 hours after ALI induction from LPS treatment.	2 (intraperitoneal)	

Curcumin													
Curcumin protects against sepsis-induced acute lung injury in rats	Xiao, X. *et al.*	2012	Sepsis-induced acute lung injury	Sprague-Dawley rats	100 (m)	Curcumin	50 mg/kg or 100 mg/kg	24 hours	Curcumin was administrated by intraperitoneal injection 2 and 12 hours after ALI induction from a cecal ligature and puncture procedure. Animals were euthanized 6, 12, and 24 h after ALI induction.	2 (intraperitoneal)	When compared against the control group; both curcumin doses (50 mg/kg or 100 mg/kg) significantly improved the PaO2/FiO2 ratio at 6, 12, and 24 h ($p<0.01$). Both curcumin doses (50 mg/kg or 100 mg/kg) significantly improved the lung W/D weight ratio in the lungs of treated animals ($p<0.01$). Both curcumin doses (50 mg/kg or 100 mg/kg) significantly reduced the lymphocyte ($p<0.01$), neutrophil ($p<0.01$), and total cell count ($p<0.01$) in the BALF of treated rats. Both curcumin doses (50 mg/kg or 100 mg/kg) significantly reduced the malonaldehyde and superoxide dismutase activity in treated animals ($p<0.01$), as well as the expression of TNF-α ($p<0.01$), IL-8 ($p<0.01$), and MIF ($p<0.01$) in the lungs of the treated rats. The 72 h survival rate was increased from 40% in the sepsis group to 80% in the curcumin 50 mg/kg group ($p<0.05$), and to 90% in the curcumin 100 mg/kg group ($p<0.05$). When compared against the control group; curcumin significantly reduced the wet-to-dry ratio in treated rat's lungs at 24 and 48 hours ($p<0.05$). Curcumin significantly reduced the alveolar space fibrin exudation and cell necrosis, while impeding the erythrocytes leakage exhibiting normal lung tissue in disperse areas and preventing damage in the histopathologic examination. Curcumin significantly reduced the TGF-β1 gene expression and plasma concentrations at 24 and 48 h ($p<0.05$).
The effect of curcumin on sepsis-induced acute lung injury in a rat model through the inhibition of the TGF-β1/SMAD3 pathway	Xu, F. *et al.*	2013	sepsis-induced acute lung injury	Sprague-Dawley rats	125 (m)	Curcumin	200 mg/kg	48 hours	Curcumin was administrated by intraperitoneal injection at the time of ALI induction. Animals were euthanized at 0, 6, 12, 24, and 48 h after ALI induction.	1 (intraperitoneal)	

Effect of curcumin (Curcuma longa extract) on LPS-induced acute lung injury is mediated by the activation of AMPK	Kim, J. *et al.*	2016	LPS-induced acute lung injury	BALB/c mice	NR (m)	Curcumin	200 mg/kg	24 hours	Curcumin was administrated by intraperitoneal injection before the induction of ALI from bronchial LPS administration. Animals were euthanized 24 h after the ALI induction.	1 (intraperitoneal)	When compared against the control group: curcumin significantly increased the AMP-activated protein kinase (AMPK) phosphorylation in the lung tissue of the treated mice ($p<0.05$). Curcumin significantly decreased the TNF-α ($p<0.05$), MIP-2 ($p<0.05$), and IL-6 production ($p<0.05$). Curcumin also prevented the phosphorylation of IκBα in the lungs ($p<0.05$), and significantly reduced the white blood cell and neutrophil counts in the BALF of treated mice. Curcumin significantly reduced the lung W/D weight ratio and MPO activity in the lungs of the treated mice ($p<0.05$). When compared against the control group: curcumin 50 mg/kg significantly reduced the lung's W/D weight ratio by 36.36% ($p<0.05$). Curcumin 50 mg/kg also reduced the lung's neutrophil count in 28.57% ($p<0.05$), the lymphocyte count in 30% ($p<0.05$), and the total cell count 32% ($p<0.05$). Furthermore, curcumin 50 mg/kg significantly reduced the MPO activity by 26.32% ($p<0.05$), and the lipid peroxidation by 25% ($p<0.05$). On the other hand, curcumin 100 mg/kg caused a 41.84% reduction in the total protein content of the treated rat's lungs ($p<0.05$). significantly reduced the lung W/D weight ratio by 45.45% ($p<0.05$). Curcumin 100 mg/kg also reduced the lung's neutrophil count in 61.9% ($p<0.05$), the lymphocyte count in 65% ($p<0.05$), and the total cell count 64% ($p<0.05$). Curcumin 100 mg/kg significantly reduced the myeloperoxidase enzyme activity in 63.16% ($p<0.05$), the lipid peroxidation in 39.28% ($p<0.05$). Finally, both curcumin doses (50 mg/kg and 100 mg/kg) significantly increased the SOD ($p<0.05$) and catalase activities ($p<0.05$) and reduced the IL-8 ($p<0.05$), TNF-α
Efficacy and Therapeutic Potential of Curcumin Against Sepsis-Induced Chronic Lung Injury in Male Albino Rats	Liu, Y.-F. *et al.*	2017	sepsis-induced chronic lung injury	Albino rats	24 (hr)	Curcumin	50 mg/kg or 100 mg/kg	45 days	Curcumin was orally administered after ALI induction from a cecal ligation and puncture procedure. Animals were euthanized 45 days after ALI induction.	45 (oral)	

											(p<0.05), and macrophage migration inhibitory factor (MIF) content in treated rats when compared against controls (p<0.05).
Curcumin inhibits lipopolysaccharide (LPS)-induced endotoxemia and airway inflammation through modulation of sequential release of inflammatory mediators (TNF-α and TGF-β1) in a murine model	Kumari, A. *et al.*	2017	LPS-induced release of inflammatory mediators in the lung	Swiss albino mice	60 (nr)	Curcumin	20 mg/kg	3 days	Curcumin was administrated by intraperitoneal injection 1 h before the induction of ALI from intraperitoneal LPS administration.	1 (intraperitoneal)	When compared against controls: curcumin significantly reduced lethal endotoxemia from 50% in controls to 30% mortality in the treated mice (p<0.05). Curcumin significantly reduced the concentrations of TNF-α (p<0.05), ROS (p<0.01), and nitrite levels (p<0.05) in the plasma of treated mice. Furthermore, the lungs of the treated albino mice evidenced significant reductions in the MPO activity (p<0.01), had lower cell counts (p<0.01), and lower protein leakage in the BALF (p<0.05). Finally, curcumin 20 mg/kg significantly reduced hydroxyproline levels (p<0.05), the expression of the LPS-induced TGF-β1 (p<0.05), iNOS (p<0.05), and TLR-4 (p<0.05) in the lungs of the treated albino mice (p<0.05).

Curcumin Attenuates Pulmonary Inflammation in Lipopolysaccharide-Induced Acute Lung Injury in Neonatal Rat Model by Activating Peroxisome Proliferator-Activated Receptor γ (PPARγ) Pathway	Cheng, K. *et al.*	2018	LPS-induced neonatal acute lung injury	Sprague-Dawley rats	NR	Curcumin	1.5 mg/kg, 3 mg/kg or 6 mg/kg	7 days	ALI was induced from LPS intraperitoneal injection at day 0. Curcumin was administrated by intraperitoneal injection immediately after ALI induction, and once daily for 7 days more.	7 (intraperitoneal)	When compared against the control group: curcumin at all doses significantly reduced the lung's W/D weight ratio and increased the PaO2 in a dose-dependent manner ($p<0.05$). Additionally, curcumin at all doses significantly decreased the concentration of the high mobility group box 1 (HMGB1) protein, TNF-α, IL-6, and TGF-β1 in the BALF of the treated rats in a dose-dependent manner ($p<0.05$).
Other PCs												
Chlorogenic acid protects mice against lipopolysaccharide-induced acute lung injury	Zhang, X.*et al.*	2010	LPS-induced lung injury	ICR mice	NR (m)	Chlorogenic acid	50 mg/kg	24 hours	chlorogenic acid was administered by intraperitoneal injection either 30 min or 3 h after ALI induction from LPS intratracheal administration.	1 (intraperitoneal)	When compared against the control group: chlorogenic acid significantly reduces the pulmonary wall thickening, interstitial edema, neutrophil infiltration, MPO activity ($p<0.01$), and cell count in the lungs of treated mice ($p<0.05$). Chlorogenic acid significantly reduces the polymorphonuclear and leukocyte infiltration ($p<0.01$), as well as the NO concentration ($p<0.01$) in the BALF of treated mice. Chlorogenic acid significantly reduces iNOS activity in the lung tissue of treated mice. When compared against controls: both zingerone doses significantly reduced, in a dose-dependent manner, the lung W/D weight ratio ($p<0.01$), NF-kβ ($p<0.01$), MAPK ($p<0.01$), and MPO ($p<0.01$) activities in the lung tissue of BALB/c mice. Zingerone significantly reduced the neutrophil ($p<0.01$), macrophage ($p<0.01$), and total cell number count ($p<0.01$) as well as the TNF-α ($p<0.01$), and IL1β ($p<0.01$) concentrations in the BALF of treated mice.
Zingerone attenuates lipopolysaccharide-induced acute lung injury in mice	Xie, X. *et al.*	2014	LPS-induced acute lung injury	BALB/c mice	NR	Zingerone	20 mg/kg or 40 mg/kg	24 hours	Zingerone was administered 1 hour before the induction of ALI from intranasal LPS administration. Animals were sacrificed 24 h after ALI induction.	1 (intragastric)	

Protective effects of protocatechuic acid on acute lung injury induced by lipopolysaccharide in mice via p38/MAPK and NF-κB signal pathways	Zhang, X.et al.	2015	LPS-induced acute lung injury	Kunming mice	NR	Protocatechuic acid	10 mg/kg	6 hours	Protocatechuic acid was administered by intraperitoneal injection 1 h before ALI induction from LPS administration. Animals were sacrificed 6 h after ALI induction.	1 (intraperitoneal)	When compared against the control group: protocatechuic acid can significantly reduce the inflammatory cell infiltration, alveolar hemorrhage, and wall thickening in treated mice. Protocatechuic acid significantly reduced BALF amount ($p<0.05$), TNF-α ($p<0.05$), and IL-1β ($p<0.05$) concentrations in the blood and the lungs of treated Kunming mice. Finally, protocatechuic acid significantly reduced the TLR4 ($p<0.05$), and NF-kβ expression in the lungs of treated mice ($p<0.05$). When compared against the control group: phloretin 20 mg/kg significantly reduced the lung's W/D weight ratio ($p<0.05$), neutrophil infiltration ($p<0.05$), and MPO activity in treated mice ($p<0.05$). Additionally, phloretin 5 mg/kg and 20 mg/kg significantly reduced the neutrophil count ($p<0.05$), and the IL-6 ($p<0.05$), TNF-α ($p<0.05$), IL-1β ($p<0.05$), and MCP-1 ($p<0.05$) concentrations in the BALF of treated animals, in a dose-dependent manner. Moreover, both phloretin doses (5 mg/kg or 20 mg/kg) significantly decreased the NF-kβ ($p<0.05$), and MAPK's ($p<0.05$) pathway activity in the lungs of treated animals, whereas in plasma, both doses of phloretin significantly reduced the TNF-α ($p<0.05$), and IL-6 concentrations ($p<0.05$). When compared against the control group: Salidroside can reduce the lung W/D weight ratio ($p<0.01$), and the MPO lung levels ($p<0.01$) of treated rats in a dose-dependent manner. Additionally, salidroside can restore the intracellular concentrations of SOD ($p<0.01$), catalase ($p<0.01$), glutathione ($p<0.01$), and glutathione peroxidase ($p<0.01$) in a dose-dependent manner. Salidroside at both doses (20 mg/kg or 50 mg/kg)
Phloretin attenuates LPS-induced acute lung injury in mice via modulation of the NF-κB and MAPK pathways	Huang, W-C. *et al.*	2016	LPS-induced acute lung injury	BALB/c mice	32 (f)	Phloretin	5 mg/kg or 20 mg/kg	7 days	Phloretin was administered in combination with a small LPS dose by intraperitoneal injection once a day for seven days. On day 7, ALI was caused in animals from tracheal LPS administration. 4 hours after ALI induction, all animals were euthanized.	7 (intraperitoneal)	
Salidroside Attenuates LPS-Induced Acute Lung Injury in Rats	Jingyan, L. *et al.*	2017	LPS-induced lung injury	Sprague-Dawley rats	50 (mr)	Salidroside	20 mg/kg or 50 mg/kg	3 days	Salidroside was administered intragastrical once a day for three days. On the third day, ALI was induced with LPS by intraperitoneal injection. All animals were euthanized 6 hours after ALI induction.	3 (intragastric)	

												significantly reduced the IL-6 ($p<0.01$), IL-1β ($p<0.01$), and TNF-α ($p<0.01$) serum levels in treated animals. Finally, salidroside also reduced the TLR/NF-kβ expression in the lungs of the treated rats ($p<0.01$).
Protective effects of thymol on LPS-induced acute lung injury in mice	Yao, L. *et al.*	2018	LPS-induced acute lung injury	BALB/c mice	60 (m)	Thymol	20 mg/kg, 40 mg/kg or 80 mg/kg	12 hours	Thymol was administered by intraperitoneal injection 1 hour after ALI induction from the tracheal LPS administration. All animals were sacrificed 12 h after ALI induction.	1 (intraperitoneal)	When compared against controls: thymol significantly reduced the alveolar wall thickening, lung W/D weight ratio ($p<0.01$), edema, and cellular infiltration in a dose-dependent manner. Additionally, thymol at all doses also reduced in a dose-dependent manner the BALF concentrations of TNF-α ($p<0.01$), IL-6 ($p<0.01$), and IL-1β ($p<0.01$), as well as the MPO ($p<0.01$), and malondialdehyde ($p<0.01$) activities in the lungs of treated mice. When compared against the control group thymol 100 mg/kg significantly attenuated the alveolar hemorrhage, alveolar wall thickening, and neutrogoil infiltration into the alveolar space when administered both as a preventive and treatment. Thymol 100 mg/kg significantly reduced the total cell count ($p<0.01$), and MPO activity ($p<0.01$) in the BALF of treated mice when administered previously and after ALI induction. Thymol 100 mg/kg significantly reduced the TNF-α ($p<0.01$), and IL-6 ($p<0.01$), and protein concentrations ($p<0.01$) in the BALF of treated animals when administered previously and after ALI induction. Finally, thymol 100 mg/kg significantly attenuated the NF-kβ ($p<0.05$) and increased the SOD activity in the lungs of treated animals ($p<0.05$) when administered previously and after ALI induction.
Preventive and Therapeutic Effects of Thymol in a Lipopolysaccharide-Induced Acute Lung Injury Mice Model	Wan, L. *et al.*	2018	LPS-induced acute lung injury	BALB/c mice	NR (m)	Thymol	30 mg/kg or 100 mg/kg	6 hours	Thymol was administered by intraperitoneal injection 0.5 h before or after ALI induction from LPS intraperitoneal injection. Animals were euthanized 6 h after ALI induction.	1 (intraperitoneal)	

Epicatechin alleviates inflammation in lipopolysaccharide-induced acute lung injury in mice by inhibiting the p38 MAPK signaling pathway	Xing, J. *et al.*	2019	LPS-induced acute lung injury	C57BL6/N mice	32 (m)	Epicatechin	15 mg/kg	24 hours	Epicatechin was administered intragastrical at 0, 0.5, and 12 h after ALI induction from LPS intraperitoneal administration. The animals were sacrificed 24 h after ALI induction.	3 (intragastric)	When compared against the control group: epicatechin significantly reduced the lung injury score ($p<0.05$) and alveolar wall thickness ($p<0.05$) in treated mice. Epicatechin significantly reduced the protein concentration and neutrophil infiltration in the BALF of treated mice ($p<0.05$), reduced the MAPK activity in the lungs of treated mice ($p<0.05$), and decreased the TNF-α ($p<0.05$), the IL-6 ($p<0.05$) concentrations in both the BALF and lungs of the treated mice.

Note: ALI, acute lung injury; LPS, lipopolysaccharide; NF-κß, nuclear factor kappa beta; IL, interleukin; MIP-1α, macrophage inflammatory protein-1 alpha; iNOS, inducible nitric oxide synthase; Sirt1, sirtuin 1; MYD88, Myeloid differentiation primary response 88; COX, cyclooxygenase; NLRP, NLR family pyrin domain-containing; ASC, Apoptosis-associated speck-like protein; BALF, bronchoalveolar lavage fluid; MIF, macrophage migration inhibitory factor; ROS, reactive oxygen species; HMBG1, High-mobility group box 1; TGF-β1, transforming growth factor β1; HO-1, heme oxygenase-1; W/D; wet-to-dry

833 **Figures.**

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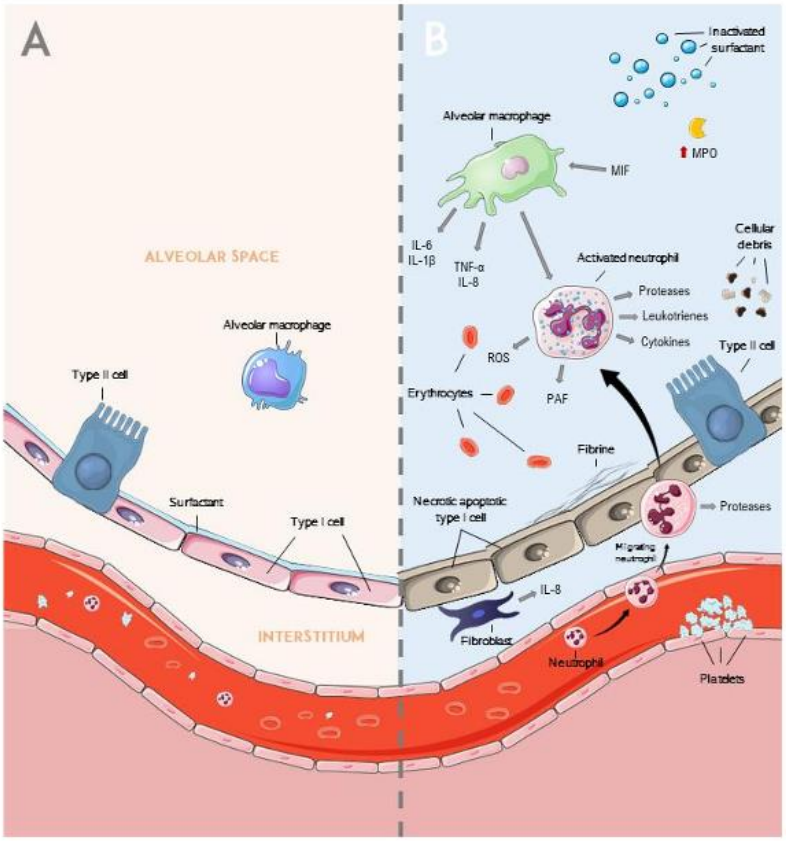

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836 **Figure 1.** A) the normal alveolar space; B) the pathophysiological changes occurring
837 during the acute lung injury. *Abbreviations:* IL, interleukin; ROS, reactive oxygen
838 species; MPO, myeloperoxidase; TNF-α, tumor necrosis factor; MIF, macrophage
839 migration inhibitory factor; PAF, platelet-activating factor.

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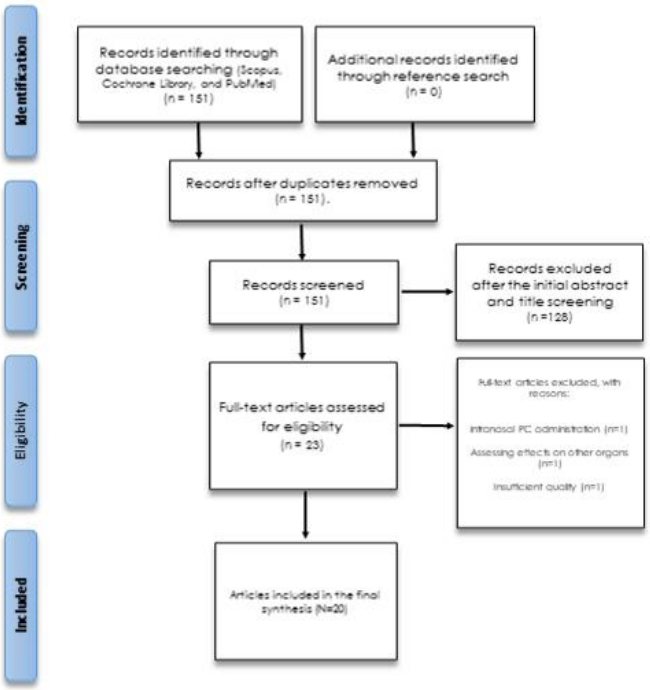
Figure 1. PRISMA flow diagram



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842 **Figure 2.** PRISMA flowchart diagram.

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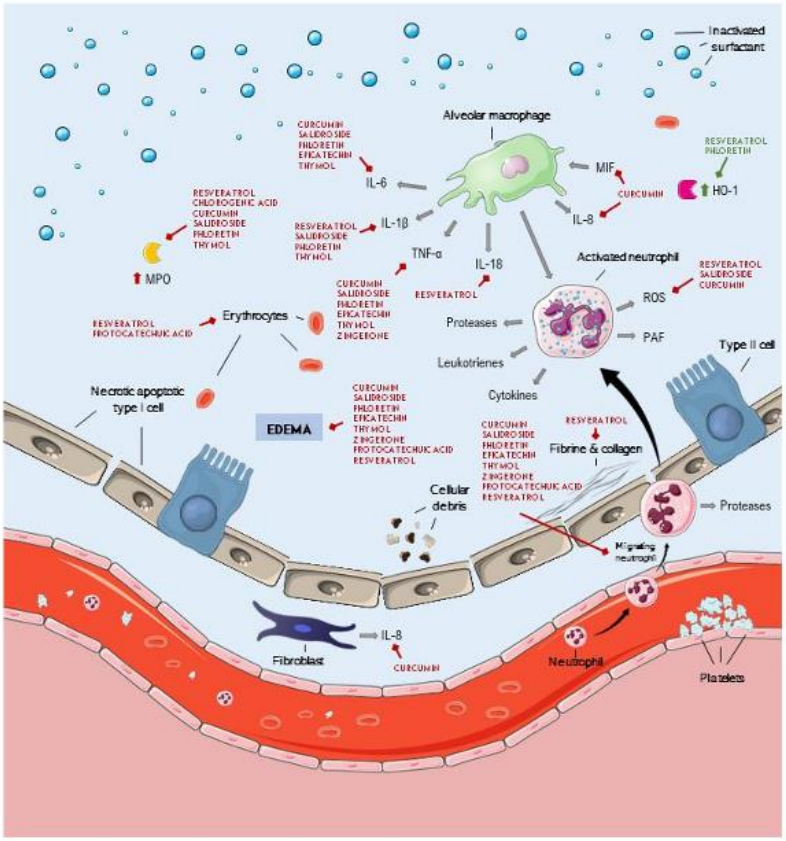

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850 **Figure 3.** The mechanisms of action of various phenolic compounds in LPS-induced
851 animal models of acute lung injury. Red arrows indicate inhibition or reduction; green
852 arrows indicate enhamcement or increase. *Abbreviations:* IL, interleukin; ROS, reactive
853 oxygen species; MPO, myeloperoxidase; TNF-α, tumor necrosis factor; MIF,
854 macrophage migration inhibitory factor; PAF, platelet-activating factor; HO-1, heme
855 oxygenase 1.

4. DISCUSSION

The hypothesis proposed on the effects of anthocyanins in the present doctoral Thesis has been confirmed, demonstrating that regardless of their source, ACNs improve various risk factors associated with cardiovascular and other diseases. Moreover, from the ACN effects, additional properties such as their beneficial effects on the intestinal barrier integrity and other organs emerged for other PCs. As a result, opening new opportunities for the prevention, treatment, or management of various diseases.

So far, research on ACNs has been focused on the study of their effects from extract administration [9], however, ACNs are pigments naturally present in plants [15–17], thus, to properly understand the benefits from fruit intake, it is essential to study the effects of "whole-foods" instead of their individual components since whole-foods are the most common presentation for nutrient intake [18,19].

To determine the effects of ACN consumption on health, multiple systematic reviews, and a randomized animal trial were employed. Their results will be discussed in the following sections, divided in accordance with each separate study.

4.1. Regarding the ACN-rich fruit biomarker of intake:

Despite the important role of fruits and vegetables on human health, to properly assess the connection between fruit intake and their effects or associations with human health, an exposure biomarker is needed [131]. Currently, dietary exposure is estimated from tools such as food frequency questionnaires assessing regular food consumption or 24-h dietary recalls for a more detailed assessment of short-term food intake, however, such methods are usually associated with systematic inherent errors secondary to their subjective nature [131]. Therefore, the information of a food-intake biomarker should be obtained directly from biological

samples, however, the determination of an adequate biomarker of consumption for commonly consumed berries and other ACN-rich fruits has been elusive due to methodological complications for a single RCT [131].

Nonetheless, from the systematic review of multiple human studies, it has been demonstrated that up to 203 PCs can be detected in human urine and plasma from which a 40% detection cut-off was considered to reduce the number of candidates. Out of the 97 remaining PCs, C3G was the most frequently found PC in 69.49% of the plasma samples and in 58.06% of the urine samples of healthy humans [169]. Thus, C3G is the most frequently found molecule in the plasma or urine of healthy humans [169].

C3G seems to be a highly specific molecule, presence is relatively rare in plants [170], and it is not used as a food additive nor secondary to the human metabolization of other compounds [132]. As a result, the evidence plausibility demonstrates the causal relationship between ACN's intake and bioavailability, demonstrating that C3G's in human plasma or urine is directly caused by the ingestion of berries [169], thus fulfilling the crucial food biomarker criterion of plausibility [131,132,169]. Similarly, C3G fulfills the analytical performance, stability, dose-, and time-response standards, therefore complying with most of the criteria needed to be considered a biomarker [131,132,169].

Finally, C3G's has a positive predictive value of 74% in plasma and of 61.7% in urine, although not significantly, such values are considered acceptable [169,171]. As a consequence, it has been determined that C3G is the most adequate biomarker to assess the adequate intake of ACN-rich berry in human plasma or urine [169].

4.2. Regarding the ACN tissue bioavailability in animals:

To explain the ACN effects beyond their intestinal bioavailability, the ACNs that are absorbed at different degrees and rates into living systems must be available at the tissue site to exert any physiological activity [172]. Thus, a key point to explain the ACN effects is the ACN tissue bioavailability, however, since it is currently impossible to attain such information directly from humans, it was obtained from animals [172].

Accordingly, from short-term animal experiments using a single ACN dose either oral or intravenous (IV), and the information from the long-term oral ACN administration to animals, a difference in the short- *versus* the long-term ACN tissue profiles were noted [127]. Parent ACNs were predominant in long-term animal experiments, while the ACN metabolites were more frequent in the short-term interventions [127].

The difference in ACN profiles could be explained by the saturation of the absorption mechanisms (mainly at gastric level via bilitranslocase) and by the further colonic metabolization of the non-absorbed ACNs [127,173]. Suggesting that the time and dose effects over ACN tissue profile might be relevant to attain tissue-specific health effects derived from ACN administration [127].

Moreover, the parent or ACN metabolite tissue bioavailability could explain the health benefits attributed to ACNs, orally, or intravenously administrated [127]. In that sense, cyanidin-3-glucoside (C3G) and its metabolites are the most frequently found ACNs in the hearts, kidneys, lungs, and brains of animals, also demonstrating multiple health benefits in separate animal experiments [127]. consequently suggesting that C3G, a bioavailable ACN in target tissues, could have an interesting potential for the reduction of cardiac, neurodegenerative, or renal diseases [127].

Thus, it has been evidenced that ACNs that are bioavailable in animal tissues, like C3G, might have an important role in human health [127]. However, it should be kept in mind that the ACN source, dose, and time of administration cause different ACN profiles in target tissues [127].

4.3. Regarding the effects and associations of ACN intake on human health, from the umbrella review of the information from the systematic review and meta-analysis (SRM) of RCTs and OS'

The results from both RCTs and OS' could aid in the determination of the effectiveness, safety, and health properties of whole-foods and bioactive compounds [174]. Moreover, the conjunct analysis of RCTs and OS' might assist the understanding and aid solving complex questions, such as the significance of the relationship between the ACN's effects and associations, that could not be answered from either one type of study alone [174]. Accordingly, to provide accessible and practical information on the proven health benefits of ACNs, our umbrella review [117] integrates the information on the ACN effects and associations from SRMs of RCTs and OS' respectively.

Accordingly, to analyze the increasing number of information from systematic reviews, the accomplishment of "umbrella" reviews, allowing the relevant findings from other reviews to be compared and contrasted, is the logical "next step" to provide healthcare workers with the evidence required to make adequate decisions [117].

As a result, from OS'-SRMs, the dietary intake of ≈ 22 mg/day of ACNs, regardless of their source, is significantly associated to a 15% reduction in the risk of type 2 diabetes mellitus (T2DM) [175], what is more, an additional 5% reduction in the risk of T2DM has been noted per each increment of 7.5 mg/day of ACN intake [175]. Similarly, the dietary ACN intake is significantly associated with the reduction of the hypertension

risk [176], while no associations were noted for gastric nor breast cancers [177,178].

In addition, the information from RCT-SRMs reveals that the oral administration of ACNs extracts to humans, causes a significant improvement in the TC, LDLc, and HDLc plasmatic concentrations increase the cellular glucose uptake, reduces the insulin resistance, and improves the endothelial function without showing significant effects on the blood pressure values [165,179–181]. The effects of oral ACNs in human RCTs could explain the reduction in the risk of T2DM associated with the chronic ACN intake observed in humans.

In consequence, the chronic ACN dietary intake, regardless of its source, aids in the prevention of T2DM and hypertension, while oral ACN supplementation should be considered in the management of glucose metabolism, hypercholesterolemia, and in the improvement of the endothelial function in humans.

4.4. Regarding the effects of red-fleshed apples, white-fleshed apples, and an ACN-rich extract on the heart and aortic tissue proteome concerning their effects on CVD in animals.

When compared against common white-fleshed apples, different studies demonstrate that the total phenolic content and antioxidant capacity reported is higher in red-fleshed apples from their enhanced ACN content, indicating that red-fleshed apples presumably have additional health properties [16]. As a consequence, determining the possible mechanisms-of-action of ACNs from red-fleshed apples and compared against white-fleshed apples (ACN-free control), and an ACN-rich extract from *Aronia melanocarpa* (apple matrix control) on the proteomic profile of the aortas and hearts of hypercholesterolemic rats.

On that matter, the results from the ACN's mechanisms of action obtained after the sustained administration of red-fleshed apples, white-fleshed apples, or an ACN-rich extract to hypercholesterolemic rats reveal that multiple changes in the expression of various proteins can be identified in the aortas and hearts of Wistar rats. These results are further discussed in the following sections; associating the effects on the proteome of the hearts and aortas of rats after each nutritional intervention, with a cardiovascular-related outcome to understand their possible implications of said changes on human health.

4.4.1. *The red-fleshed apple effects* on the heart and aortic tissue proteome.

The red-fleshed-apple-mediated modification in the proteome of the hearts and aortas of hypercholesterolemic rats is consistent with a healthier pattern of CVD biomarkers.

When compared to the high-fat diet (HFD) group in the aortas of hypercholesterolemic rats, red-fleshed apples upregulate DDAH1, an enzyme that prevents some protein degradation products from causing CVD in humans [182,183], and of cathepsin D, a cholesterol efflux inducing molecule [184]. Similarly, in the rat's hearts, red-fleshed apples significantly increased the expression of GPX1, an antioxidant enzyme that can restore the endothelial phenotype of some high oxidative stress pathologies and suggesting positive effects on CVD prevention [185,186].

Additionally, red-fleshed-apples significantly downregulated the expression of CRP, C1QB, and CFP in the heart tissues of rats, all three proteins involved in the complement system activation [187,188], thus, demonstrating a beneficial effect on various anti-inflammatory proteins related to CVD [189] from the consumption of red-fleshed apples.

As a consequence, these findings demonstrate for the first time that despite the detrimental effects of a HFD, the oral supplementation with ACN-rich red-fleshed apples to rats beneficially modifies the expression of proteins related to CVD in humans, thus supporting the beneficial role of red-fleshed apples for the prevention of CVD.

4.4.2. *The white-fleshed apple effects* on the heart and aortic tissue proteome.

White-fleshed apples downregulated the expression of complement 3 (C3) and C9 in the hearts and aortas of rats, while other molecules such as the complement factor B, properdin, C4BPA, and C1QB were reduced only in the rat's aortas. These proteins are involved in the activation or are a part of both the classical and alternative complement pathways [187,188], and could most likely influence CVD [189].

The reduction in the expression of proteins such as C9 could reflect a reduction of the atherosclerotic plaque formation process. High concentrations of C9 are deposited as a part of the intima layer in grade II aortic atherosclerotic lesions in humans [190]. Moreover, the downregulation of the expression of proteins involved in the complement system caused by white-fleshed apples demonstrates a particular anti-inflammatory effect from the white-fleshed apple consumption and suggests a possible effect in the reduction of the atheroma plaque formation with a consequent reduction in the risk of CVD.

On the other hand, the white-fleshed apples significantly reduced the expression of iron homeostasis proteins such as myoglobin in the hearts, and haptoglobin, hemopexin, and ceruloplasmin in the aortas of rats. These changes could benefit cardiovascular health through the reduction in the expression of myoglobin, a potent nitric oxide (NO) scavenger [191], might be beneficial for hypertensive states where there is

a lesser bioavailability of vascular NO, or from the downregulation of ceruloplasmin, a promoter of deleterious vascular effects and a known CVD risk factor [192–194].

Additionally, in the hearts of rats, the oral intake of white-fleshed apples downregulates the expression of ECH1, a reduction that has been linked to enhanced resistance to ischemia-reperfusion injury in the hearts of Brown Norway rats [195], and of glutathione s-transferase mu 2 (GTSM2), a molecule capable of improving the heart's contractility [196]. Interestingly, white-fleshed apples downregulate the expression of annexin A2, a calcium-regulated binding protein that reduces the expression of the PCSK9 enzyme [197], hence increasing the cholesterol clearance from LDL particles, supporting the beneficial role of white-fleshed apples in CVD prevention.

However, it was demonstrated that white-fleshed apples particularly influence proteins in the "Neurological Disease, Hematological Disease, and Cardiovascular Disease" with a score of 25 points and where 15/46 of the differentially expressed proteins were a part of this network. The evidence demonstrates that the effects of the white-fleshed apple intake could influence various relevant diseases and biological functions such as the blood coagulation (from the modification of C3, C9, F12, and F9), the iron homeostasis and transport (CP and TF), the complement activation (C3 and CFB), and from various other protein modifications.

4.4.3. *The ACN-rich extract effects* on the heart and aortic tissue proteome.

To assess the ACN properties, and reduce the apple matrix effect, an ACN-rich extract was used. As a result, it was demonstrated that in the aortas of hypercholesterolemic rats, the ACN-rich extract significantly reduces the expression or the protein kinase cAMP-activated catalytic

subunit alpha (PRKACA), favoring the inhibition of the spontaneous and pathological blood clot formation in blood vessels [198], and of IQ motif containing GTPase activating protein 1 (IQGAP1), a protein associated with the cell proliferation, migration, and rearrangement of the vascular smooth muscle cells in varicose veins [199]. Thus, potentially reducing the risk of cardiovascular events.

Additionally, the ACN-rich extract upregulates fibromodulin (FMOD), a protein known for triggering the platelet aggregation through the activation of a collagen-specific receptor [200], and other molecules with cardiovascular interest such as transgrelin (TAGLN) and adenylyl cyclase-associated protein 1 (CAP1) involved in the heart's contractility [201], and a modulator of LDL receptor degradation in the liver respectively [202], suggesting that ACNs positively improve the cardiovascular risk.

Furthermore, the ACN-rich extract particularly influenced the "Energy Production, Cellular Function, and Maintenance, and Post-Translational Modifications" pathway with a score of 17 points, and where 10/28 of the differentially expressed proteins were a part of this network. While the ACN-rich extract modifies proteins that could have an impact on the nervous system development (PRKACA, CLTC, CAP1, SEPT2, and IQGAP1), cell death and survival (PRKACA, RRAS2, HSP90AB1, PPIA, and NNT) and other biochemical functions.

4.4.4. The apple matrix effect on the heart and aortic tissue proteome.

After the oral intake of both the red- and white-fleshed apples, in the aorta of the hypercholesterolemic rats, common downregulations in the expression of CFP, a complement system regulator [187], and C1QB, part of the first component and main activator of the complement system [188], were observed regardless of the apple's ACN content, thus, leading to a stimulus for the reduction in the complement system activation.

Additionally, also in the aortas of hypercholesterolemic rats, both apple varieties significantly downregulated the expression of alpha-1-antitrypsin (SERPINA1), and enoyl-CoA hydratase 1 (ECH1), both considered potent inflammatory molecules [203,204], suggesting an anti-inflammatory effect from the apple matrix.

Apple consumption induced a comparable effect on the complement system's proteome in the aortas of hypercholesterolemic rats, despite their different ACN content. Consequently, it can be stated that apples share common effects on the complement system that could be beneficial for health in humans, probably from their shared matrix composition (i.e. oral fiber or other PCs).

4.4.5. ***Red-fleshed apples, white-fleshed apples, and ACN-rich extract comparisons with atorvastatin***

The rats consuming red-fleshed apples, white-fleshed apples, or the ACN-rich extract, promoted a decreased expression of ECH1 that was also observed after the administration of atorvastatin, an effect not previously reported. Additionally, the red-fleshed apples and atorvastatin reduced the expression of glutathione peroxidase 1 (GPX1), while GSTM2 and myoglobin (MB) were both downregulated by the white-fleshed apples and atorvastatin. Finally, the ACN-rich extract and atorvastatin downregulated the four and a half LIM domains protein 1 (FHL1), a protein with unknown functions but found significantly increased in cardiac failure, cardiac hypertrophy, pulmonary hypertension, and arrhythmias [205]. Therefore, apples and atorvastatin, one of the most used lipid-lowering drugs, share common mechanisms of action that might impact positively on diverse CVD risk factors.

Thus, the evidence demonstrates that apples and ACNs influence the expression of multiple proteins related to CVD in a beneficial manner in

animals, suggesting that apples and ACNs could be considered for the prevention of CVD in humans.

4.5. Regarding the effects of whole-apple intake on diverse cardiovascular disease (CVD) risk factors.

The previous results demonstrate that apples have a beneficial role CVD prevention, that all apples share effects, probably from their matrix composition and that the intensity of the various apple's effects depends on their PC composition. In that sense, to reveal the influence that whole-apple intake has over CVD, the associations between whole-apple consumption and CVD risk protection were assessed from OS', while the effects from the whole-apple intake on multiple CVD risk factors were evaluated from various randomized controlled and non-controlled trials (RTs).

Accordingly, the OS evidence demonstrates that consuming at least 1 whole-apple/day (≈100 g/day) is significantly associated with a reduction in the mortality risks for CVD, stroke, and ischemic heart disease, and all-cause death separately [206–209]. Additionally, from OS', 1 whole-apple a day reduces de risk of suffering from severe abdominal aortic calcification [210], hypertension, or a thrombotic stroke, and reduces the risk of serum CRP concentrations ≥3.0 mg/L [211].

On the other hand, the whole-apple intake effects tested in RTs evidence an overall beneficial effect over the plasmatic lipids. As it was demonstrated, the intake of the different whole-apple varieties reduced TC and LDLc while increasing HDLc to various degrees [212–216], suggesting that at least some of the noted beneficial effects of the whole-apple consumption are not driven by a matrix effect and modified by each apple variety's phenolic content.

Moreover, apples also demonstrated to significantly decrease various pro-inflammatory cytokines such as IL-6, INF-γ, and TNF-a in PMBC *in vitro* models and noticeably decreasing the CRP serum levels in humans [214–217]. Similarly, the whole-apple intake reduces the systolic blood pressure and pulse pressure values, while also improving the endothelial function [215,216,218].

The results also evidence that the geographical origin of apples is important when analyzing their health properties, since conditions such as the weather, harvest season, and type of soil significantly change the apple's phenolic content, mineral, fiber and vitamin content consequently influencing their effects on CVD and health. Therefore, demonstrating that apples, should be considered in the management and prevention of CVD, regardless of their variety.

4.6. Regarding the effects of the oral phenolic compound (PC) administration on the intestinal barrier integrity.

Because of the previous results, it has been determined that ACNs, regardless of their source, should be considered in the acute management of cardiometabolic disease in humans. However, the ACN effects suggest further possible uses in the management of other conditions, where like in CVD, inflammation plays an important role such as critical illness like necrotizing enterocolitis in preterm infants, or sepsis in adults.

Accordingly, the information from intestinal inflammation animal and cellular models that share common pathophysiological characteristics with the intestines of critically ill patients reveals that PCs might be useful as an aid in the reduction of intestinal damage and improve the intestinal barrier integrity.

From animal models it has been determined that the oral administration of various PCs at different doses significantly decreases various macroscopic signs of intestinal inflammation such as the intestinal edema, pneumatosis intestinalis, mucosal congestion, inflammatory cell infiltration and ileal necrosis [219–225].

From all comments, the improvement in the intestinal function and structure observed after the oral PC administration seems to be caused by three main mechanisms:

1st) the oral PC administration causes the improvement in the expression of various pro-inflammatory molecules such as TNF-α, IL-1β, IL-6, and CXCL-2 in a dose-dependent manner [225–228], and the reduction in the activity of various pro-inflammatory proteins such as the ICAM-1, nitric oxide synthase (iNOS)and cyclooxygenase (COX)-2 [225,227–229].

2nd) The oral PC administration improves the expression or prevent the reduction of various tight junction proteins such as occludin, claudin-1 and ZO-1 in the intestinal epithelia [226,228,230].

3rd) The oral PC administration decreases the concentration of reactive oxygen species by increasing the activity enzymes such as heme oxygenase (HO)-1 [224,228].

Therefore, the results suggest that the oral PC administration has the potential for improving the intestinal barrier integrity during severe inflammation, from various improvements in the intestinal epithelia. Thus, suggesting the possibility of reducing complications like sepsis and death in critically ill patients and other patients suffering from intestinal inflammation.

4.7. Regarding the effects of the PC administration on the sepsis-induced acute lung injury (ALI).

In addition to the results on the intestinal inflammation, from various animal models it has been determined that the PC administration could effectively counteract various of the mechanisms of action that cause ALI or acute respiratory distress syndrome for the following reasons:

First, resveratrol [231–233], chlorogenic acid [234], protocatechuic acid [235], and epicatechin [236] significantly reduced the lipopolysaccharide (LPS)-induced increase in the alveolar wall thickness in various animal models, possibly reducing lung cell hyperplasia, and collagen deposition. Also, curcumin 1.5 mg/kg improved the partial pressure of oxygen in arterial blood (PaO2)/fraction of inspired oxygen (FiO2) ratio in a dose-dependent in Sprague-Dawley rats [237,238]. Thus, the administration of various PCs, particularly curcumin, could reduce the alveolar wall thickness and improve the gas exchange in animals suffering from ALI.

Second, all analyzed PCs: resveratrol [231,232,239,240], curcumin [237,238,241,242], chlorogenic acid [234], zingerone [243], phloretin [244], thymol [245,246], and salidroside [247] significantly reduced the lung's W/D weight ratio in LPS-induced ALI in animals. Thus, PCs most likely cause the pulmonary edema reduction pulmonary during ALI.

Third, resveratrol [231–233,239,240,248], curcumin [237,242,249,250], thymol [245,246], chlorogenic acid [234], zingerone [243], protocatechuic acid [235], and epicatechin [236] significantly decreased the inflammatory cell infiltration in the lungs and bronchoalveolar fluid (BALF) in animals. Thus, PCs lessen the immune response during LPS-induced ALI in animals. While some signs of a possible reduction in ALI's mortality were described from curcumin administration [237,250].

Furthermore, resveratrol, curcumin, zingerone, phloretin, chlorogenic acid, thymol, and protocatechuic acid seem to improve ALI's pathophysiology from three main mechanisms:

First, PCs reduce the NF-ƙβ [231,232,235,239,243,244]; and MAPK activity [236,243,244,249].

Downregulating the NF-ƙβ and the mitogen-activated protein kinase (MAPK) explains the reductions in the inflammatory cell infiltration and several pro-inflammatory proteins observed after PC administration in diverse animal models of LPS-induced ALI [231,232,241–250,233–240], also explaining the improvements in the edema and alveolar wall thickness observed in animal models of LPS-induced ALI [231,232,241–250,233–240].

Second, the improvement in tissue oxidation and ROS concentration in the lungs of animals mostly from a drop in the myeloperoxidase (MPO) activity as demonstrated from curcumin [242,249,250], resveratrol [239], zingerone [243], phloretin [244], salidroside [247], and chlorogenic acid [234] since MPO's controlled liberation is crucial an efficient activity, while its unrestrained activity increases inflammation and damage in tissues [251].

Third, an improvement the lung's vasodilatation secondary hypoxia from a reduction in the iNOS activity causing the reduction in NO concentrations in the lungs of animals treated with various PCs [231,250]. Thus, counteracting one of the main pathological mechanisms of ALI (Griffiths et al. 2019), and allowing the oxygenation of deoxygenated blood in newly recruited lung units.

Therefore, suggesting that PCs could potentially various aspects of the ALI in humans.

5. OVERALL DISCUSSION

ACNs are naturally occurring molecules present in berries and other red-colored fruits, with various positive effects and associations on human health [15–17]. Accordingly, increasing the knowledge of ACNs might provide new perspectives for the treatment and prevention of numerous diseases in humans.

Despite the efforts made to discover the effects of the oral ACN intake in humans, the absence of adequate detection methods, and the economic and technical limitations for the determination of some ACN-rich fruit properties, such as an intake biomarker from a single RCT, have caused that aspects like the ACN tissue bioavailability, and the health-related consequences from the ACN presence on different target tissues, remain poorly understood.

However, tools like systematic reviews and meta-analyses, considered the pinnacle of the evidence pyramid [252], are capable of providing reliable answers to complex questions, consequently improving the intricate relations between evidence-based medicine and clinical experience [111]. Thus, systematic reviews can be considered as a starting point when facing questions related to complex scientific challenges [112]. However, the quality of a systematic review, and its answers, is highly dependent on its methodology [117].

Accordingly, to provide the most accurate information, various processes must be followed to ensure the quality of any systematic reviews produced: First, whenever possible, the research protocols should be registered in international databases such as the "International Prospective Register of Systematic Reviews" (PROSPERO) established by York University (https://www.crd.york.ac.uk/prospero/) to provide a comprehensive listing of systematic reviews, to avoid duplication and reduce bias. Second, use complex keyword terms should be used in more than one electronic search engine (i.e. PubMed,

Scopus, Cochrane library, etc.) to retrieve the most relevant scientific publications to be included. Third, the most adequate tools (AMSTAR, RoB2, etc.) designed to assess the risk of bias both within the included studies and to the study itself, should be applied to warrant the quality of the included information. And fourth, the most adequate standardized statements (STROBE, PRISMA, CONSORT, etc.) designed to assess the quality of the report should be applied to warrant the quality of the publications (**FIGURE 8**).

As a result, from the systematic review of various animal studies, it was determined that in human randomized controlled trials, after the oral intake of different ACN-rich berry intake, up to 203 ACN metabolites can be detected in human urine and plasma. However, out of all PCS, C3G is the most frequently identified ACN in both the plasma and urine of healthy humans and shows a tendency for positive predictive value in both fluids considered acceptable after the oral intake of different berries [169]. What is more, C3G complies with the paramount plausibility criterion and meets the requirements to comply with other criteria such as the dose-response, time-response, stability, and analytical performance. Thus, C3G is the most promising biomarker for the oral ACN-rich fruit intake in the plasma or urine of healthy humans [169].

Additionally, C3G and other ACN metabolites are bioavailable in the kidneys, livers, hearts, and lungs of mice as well as in the brains of pigs [127]. Whereas, the same ACNs have demonstrated positive health effects in the same tissues on separate animal experiments [127].

Nevertheless, ACNs are not equally distributed in animal tissues and their profiles vary according to the time for their measure, evidencing a parent ACN predominance in long-term experiments while in short-term experiments ACN metabolites become more frequent. The difference in ACN profiles can be explained by the saturation of bilitranslocase, yielding multiple metabolites which are posteriorly re-converted into

parent ACNs by the colonic microbiota and absorbed into plasma by the intestinal epithelia [127].

Tissue bioavailable ACNs like C3G, show potential for reducing the impact of oxidative stress, modifying cancer-related pathways, or the reduction of the adverse effects of obesity and other chronic diseases in humans [127]. Therefore, suggesting that ACNs, and particularly C3G, have an important role in the preservation of human health [127].

The ACN's tissue bioavailability and possible effects determined from animal tissues suggest that the ACN oral intake from natural sources, such as berries or apples, might be beneficial for human health, particularly for CVD, the current most prevalent cause of death.

With that in mind, and in accordance with previous results, ACN-rich apple varieties, such as *red-fleshed* apples, beneficially modify the expression of proteins related to the complement system in humans, on the other hand, white-fleshed apples regulate the expression of various inflammatory proteins, and the ACN-rich extract improved proteins related to the cellular signaling. Thus, supporting the beneficial role of apples for the prevention of CVD. These results evidence first, that **apples share beneficial effects** probably from their matrix composition; second, that although all apple varieties are beneficial, their effects depend on their PC composition, and third, that ACNs have positive effects on cardiovascular health and the prevention of CVD in particular.

In that sense, consuming at least one whole-apple/day (**≈100 g/day**) beneficially modifies the plasmatic concentrations of TC, LDLc, HDLc and various pro-inflammatory cytokines such as CRP in various degrees also improving the systolic blood pressure, endothelial function, and pulse pressure in humans [253]. Similarly, the observational evidence demonstrates that consuming at least **one whole-apple/day** is significantly associated with a clinically relevant reduction in the risk of cardiovascular mortality and chronic low-grade inflammation [253].

The formerly described effects and associations were evidenced for apples, regardless of their variety and phenolic content. However, it should be noted that environmental conditions such as the weather, harvest season, and type of soil, influence the apple's composition and subsequent health effects [253].

What is more, in humans, the systematic review of observational studies gathering more than 1.5 million participants demonstrates that the dietary intake of ACN doses between **200 - 400 mg/day** is associated with a significant reduction in the risk of T2DM and hypertension.

On the other hand, from the acute oral supplementation with ACN doses between 200 - 400 mg/day to human subjects, it was determined that oral ACNs significantly reduce the fasting glucose levels, the Hb1Ac, and the HOMA-IR. In addition, oral ACNs also improve the TC, triglyceride, LDLc, and HDLc plasmatic concentrations, as well as the vascular function in humans.

Therefore, the evidence demonstrates that ACNs have positive effects on multiple CVD risk factors, explaining the relationship between the positive ACN effects from *in vitro* cellular studies, *in vivo* animal research, and the benefits associated with the dietary ACN intake in humans.

In consequence, **ACNs**, **regardless of their source**, should be considered in the acute management of glucose metabolism, endothelial dysfunction, and hypercholesterolemia as well as in the prevention of T2DM and hypertension in humans. Nevertheless, the ACN effects suggest that ACNs are could be used in the management of other conditions, where like in CVD, inflammation plays an important role.

Such is the case of a life-threatening critical illness like necrotizing enterocolitis in preterm infants, or sepsis in adults, where an increase in apoptosis and the intestinal epithelial permeability secondary to inflammation could lead to systemic sepsis, multisystem organ failure,

and finally death. Thus, demonstrating that the gastrointestinal tract is a target in the management in critically ill patients.

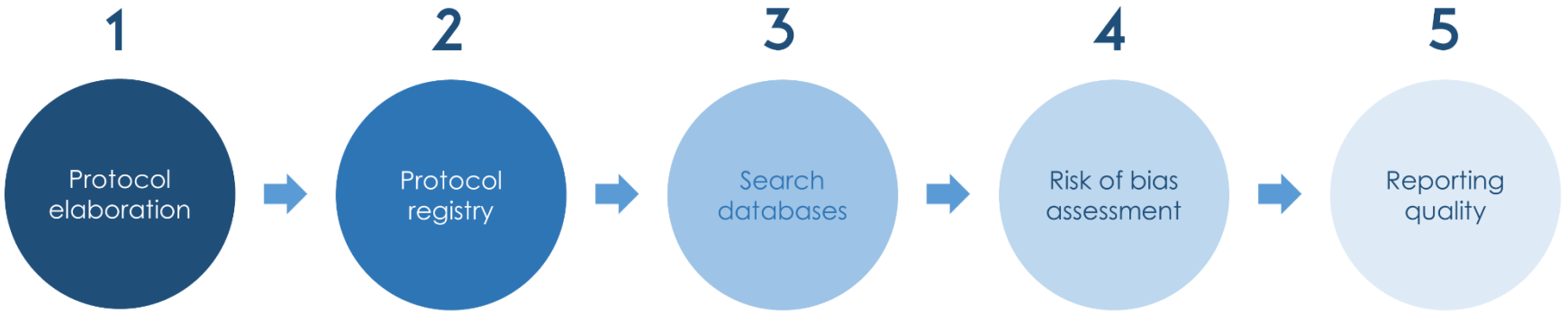
In that sense, the evidence from *in vivo* animal models of intestinal damage shows that the oral administration of resveratrol, the most studied PC, improves the intestinal barrier integrity, reduces intestinal damage, and decreases neutrophil infiltration, as a result improving the intestinal barrier integrity in animals [254].

In animals, the beneficial effects of resveratrol come from three main mechanisms of action: 1) the reduction of pro-inflammatory molecules, 2) the increase of antioxidant enzymes, and 3) the upregulation in the expression of TJ proteins in the intestinal epithelia [254]. While the *in vitro* results corroborate that PCs significantly reduce the TEER increase secondary to diverse noxious-agent action in cells, therefore counteracting their deleterious effects on signaling pathway proteins [254].

Consequently, the evidence fairly explains the improvement in multiple clinical parameters of intestinal damage observed after the oral resveratrol or other PC administration in animals. Suggesting that in humans, the oral administration of resveratrol or other PCs should be considered in the management of the intestinal and acute lung injuries associated with various local and systemic pathologies.

As a result, it has become apparent that PCs might improve the intestinal barrier integrity, increase the TJ protein expression, reduce the intestinal permeability, and to possibly decrease further complications during severe inflammation as a result of the improvement of the intestinal health in some patients including critically ill patients. Nonetheless, due to scarce information, the precise dose and time for the oral administration of resveratrol or other PCs in humans, remain undetermined.

Figure 8. Five critical steps for the elaboration of good quality systematic reviews



- **MECIR** (Methodological Expectations for Cochrane Intervention Reviews).
- **PRISMA** (Preferred Reporting Items for Systematic Reviews protocols).

- **Campbell** Collaboration. (no charge).
- **Cochrane** Collaboration. (no charge).
- **PROSPERO** international prospective register of systematic reviews(no charge).

- **PubMed.** (https://pubmed.ncbi.nlm.nih.gov/)
- **Embase.** (www.embase.com)
- **MEDLINE.** (https://www.medline.com/)
- **Web of Science** (www.webofknowledge.com)
- **Google Scholar.** (www.scholar.google.com)
- **SCOPUS.** (www.scopus.com)

- **AHRQ** (Agency for Healthcare Research and Quality).
- **Downs-Black tool.**
- **Cochrane's RoB 2.**
- **SYRCLE** (Systematic Review Centre for Laboratory animal Experimentation).
- **RoBANS** (Risk of Bias Assessment tool for Non-randomized Studies).
- **Reid tool.**
- **ROBINS** (Risk Of Bias In Non-randomized Studies - of Interventions).
- **MINORS I & II**
- **Joanna Briggs institute** assessment tool

- **STROBE** (Strengthening the Reporting of Observational studies in Epidemiology).
- **CONSORT** (Consolidated Standards of Reporting Trials).
- **PRISMA** (Preferred Reporting Items for Systematic Reviews protocols).

OTHER USEFUL TOOLS

- **RefMan** (EndNote reference manager).
- **The Covidence** online software product improves healthcare evidence synthesis.
- **Mendeley** reference manager.

6. FUTURE PERSPECTIVES.

As a result of this work, a methodological framework for the determination of an intake biomarker for berries has been provided. However, to confirm C3G as the most adequate berry-intake biomarker, an RCT must be performed. One option to confirm C3G as a berry intake biomarker is to divide a group of healthy humans fit for study (N= 180), into 7 randomly assigned smaller groups of 25 volunteers each (G1, G2, G3, G4, G5, G6, G7) (FIGURE 9).

After 48 hours of a PC-free diet and an overnight fast, basal blood and urine samples would be collected from all volunteers (0 min). Once the samples are collected, each group would consume 300 g of a puree, with an already known PC profile, made from one of seven different commonly consumed berry varieties (blueberry, red cranberry, strawberry, cherry, chokeberry or blackberry), while one group would consume a 300 g banana puree as an ACN-free control (FIGURE 9).

After the puree consumption, an IV catheter would be placed on the volunteers and blood samples extracted at 15, 30, 45 min, and 1, 3, 6 h. On the other hand, the urine samples would be collected 3 and 6 h after intake, in accordance with the already existing information on the ACNs bioavailability (FIGURE 9).

After sample collection, processing, and analysis with a high-throughput technique, the results should render both, the PC composition profiles of the 6 berry varieties, and the PC profile in the urine and plasma samples of the volunteers. These results can be compared between groups (G1 vs G2 vs G3, etc.) to determine the "common denominator" which would be the intake biomarker for berry intake. Furthermore, the comparison between the fluid and fruit PC profiles would help understand the metabolites arising from specific fruit consumption and would provide traceability for all metabolites (FIGURE 10).

Furthermore, the methodology used to determine C3G as the most probable intake biomarker for berries can be used to determine other biomarkers for popular fruit groups such as pomes or citrics, and to other highly consumed foods like the artificial sweeteners, dairy products, coffee or tea.

Due to the fact that ACN's are red/blue pigments with positive health effects regardless of their source, ACNs could be used to give color or supplement different foods and beverages in order to increase their beneficial properties and prevent cardiometabolic diseases. Moreover, ACN extracts could be used as supplements to aid in the treatment of T2DM & hypertension and to prevent CVD in humans.

From the specific ACN tissue bioavailability, a set of RCTs could be designed to test the beneficial effects of the ACNs that are bioavailable in various specific organs on a matching disease, e.g. the effects of the brain bioavailable delphinidin-3-galactoside to reduce Alzheimer disease or the effects of the kidney bioavailable malvidin-3-glucoside to reduce acute kidney disease.

Finally, to test the possible effects of the oral PC administration on the improvement of the intestinal barrier integrity and the reduction of the acute organ damage associated to severe inflammation, 90 patients diagnosed with sepsis is proposed. Volunteer should be recruited from intensive care units during a three-month period.

After enrollment, the included patients will be randomly divided into two groups. For the intervention, patients in G1 will be supplemented with an oral resveratrol dose of 5 mg/kg/day during their entire stay on the ICU, using their enteral nutrition (EN) formula as a vehicle for administration, while the EN formula for patients in G2 will be treated with a placebo.

All standard clinical parameters (blood pressure, respiratory frequency, heart rate, urine output, etc.) will be measured and recorded at least every 2 hours. Blood samples will be collected as required, however, LPS (intestinal permeability biomarker), troponins or BNP (heart health biomarker), and creatinine (renal health biomarker) will be sampled from all patients at least once a day. Other parameters such as body weight, consciousness status, ventilator parameters, and amine doses will be recorded for further analysis. At the end of 3 months, all data will be analyzed using the IBM SPSS software and the results will be presented accordingly (FIGURE 11).

Figure 9. Future perspectives

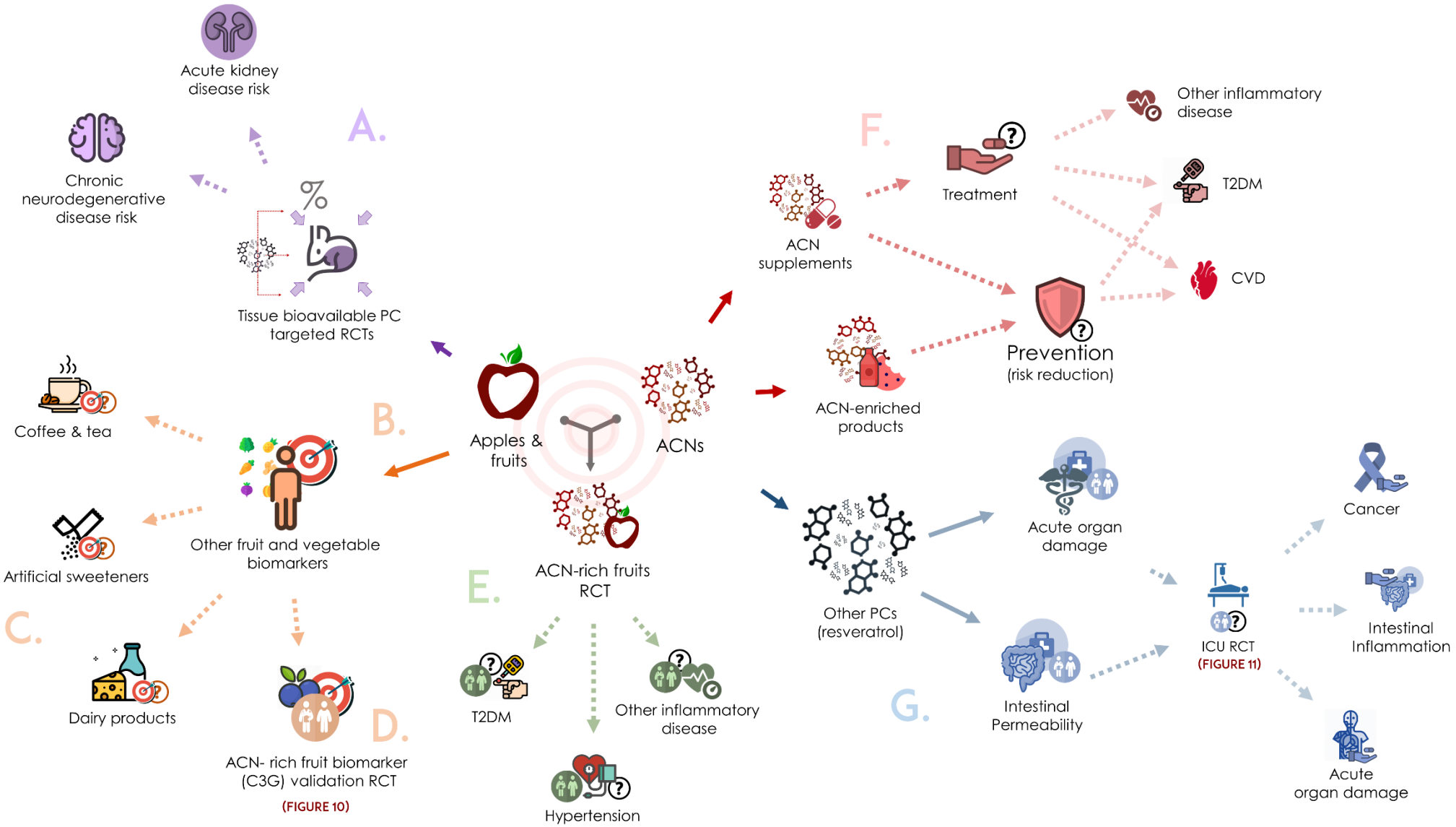


Figure 10. Berry intake biomarker RCT concept.

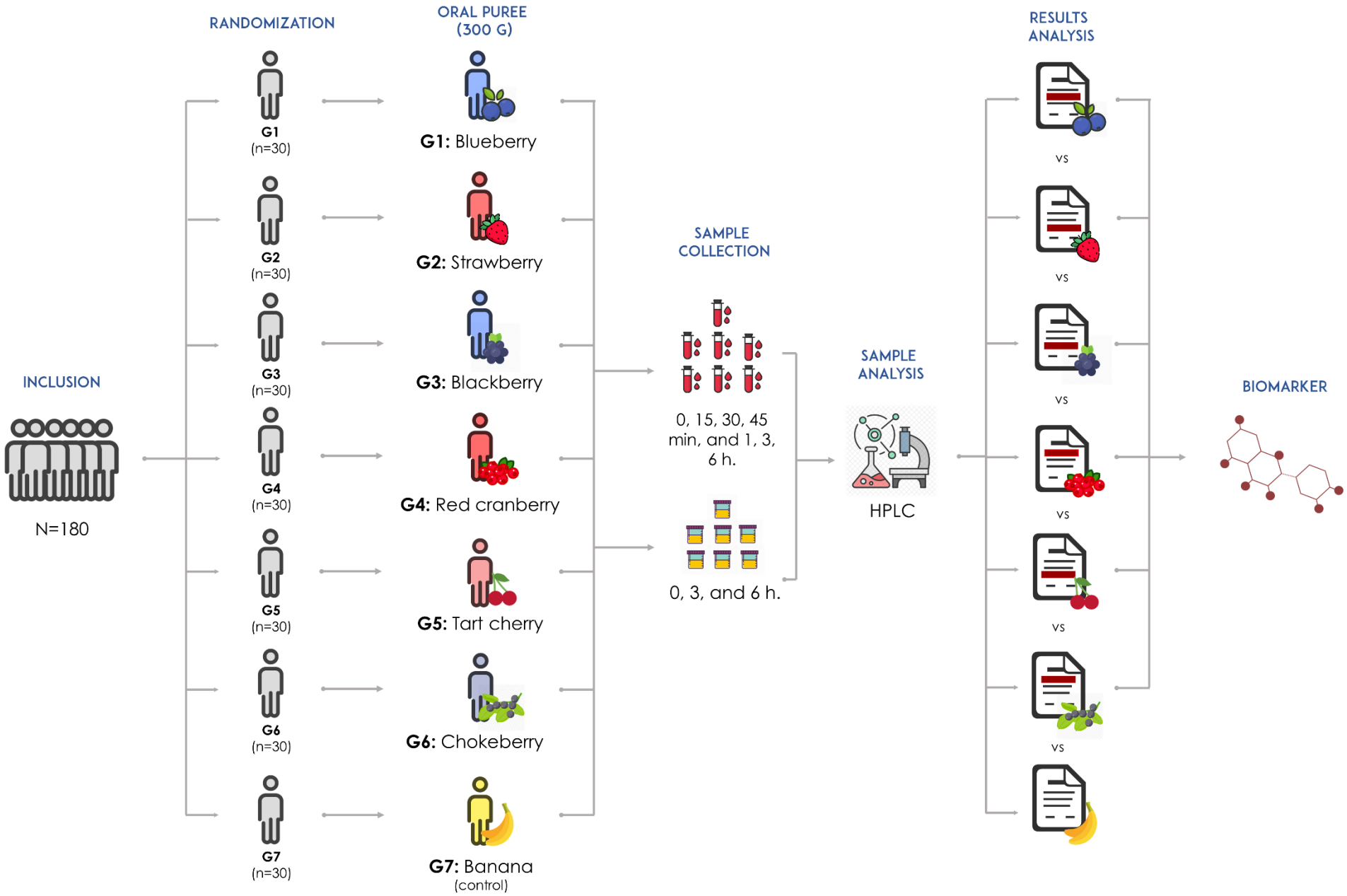
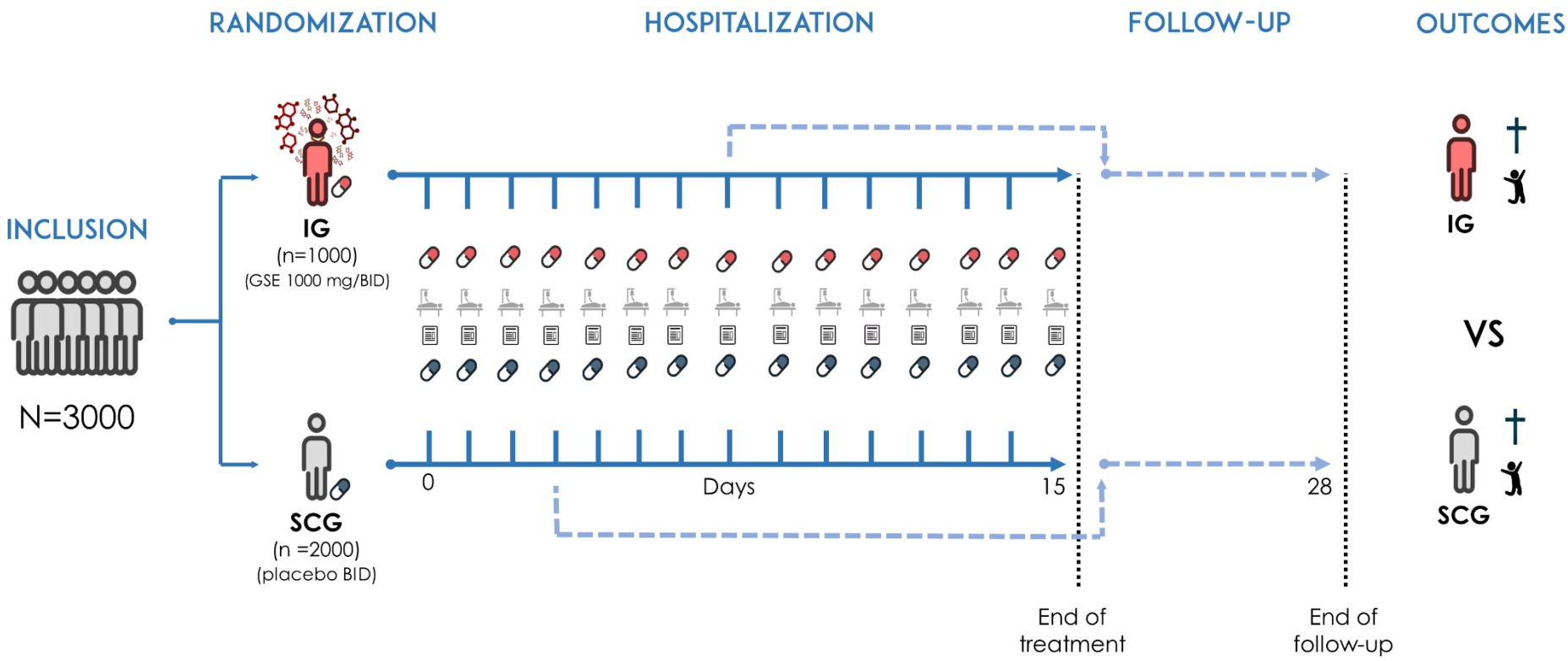


Figure 11. RCT to assess the possible effects of PCs on the intestinal barrier integrity and organ damage.



7. CONCLUSIONS

7.1. Regarding the ACN-rich fruit biomarker of intake:

7.1.1. After the oral intake of different berries (ACN-rich fruits) in human RCTs, C3G is the most frequently identified ACN in the plasma (69.49%) and urine (56.06%) of healthy humans.

7.1.2. C3G showed a tendency for an acceptable positive predictive value in plasma (74%; $p = 0.210$), and urine (61.7%; $p = 0.402$) after the oral intake of different berries (ACN-rich fruits).

7.1.3. C3G fulfills the plausibility, and other important criteria to be considered an adequate biomarker of berry intake such as the dose-response, time response, stability, and analytical performance.

7.1.4. C3G is the most promising biomarker for oral ACN-rich fruit intake in the plasma or urine of healthy humans.

7.2. Regarding the ACN tissue bioavailability in animals:

7.2.1. After ACN-rich berry intake, C3G and other ACNs are identified in the kidneys, livers, hearts, and lungs of mice as well as in the brains of pigs.

7.2.2. ACNs showed a predominance of parent ACNs in long-term experiments versus an ACN metabolite predominance in short-term experiments.

7.2.3. ACNs such as C3G may have an important role in human health.

7.3. Regarding the effects and associations of ACN intake on human health, from the umbrella review of the information by SRMs of RCTs and OS'.

7.3.1. From humans RCTs, the ACN oral intake significantly improves the plasmatic lipid profile, glucose metabolism, and endothelial function.

7.3.2. From human OS', the oral ACNs consumption is significantly associated with the reduction of the risk of hypertension and T2DM.

7.3.3. The effects of oral ACNs in human RCTs could explain the reduction in the risk of T2DM associated with the chronic ACN intake observed in humans.

7.3.4. ACNs should be considered in the management of glucose metabolism, endothelial dysfunction, and hypercholesterolemia, as well as in the prevention of T2DM and hypertension in humans.

7.4. Regarding the effects of red-fleshed apples, white-fleshed apples, and other ACN-rich extracts on the proteome of the aorta and heart tissues of hypercholesterolemic rats.

7.4.1. The red-fleshed apple consumption suggests an anti-inflammatory effect based on the downregulation of the aortic expression of complement system proteins such as C1QB and CFP and the expression of CRP in the cardiac tissue of hypercholesterolemic rats.

7.4.2. White-fleshed apples downregulate the expression of the complement system-related proteins C1QB, CFB, CFP, C9, and C3 in the aorta while the ACN-rich extract intake modified the expression and C9 and of C9 and C3 in heart tissue.

7.4.3. White-fleshed apples downregulate the expression of the iron homeostasis related proteins CP, HP, TF, and HPX in aortas, and of HP, TF, HPX, and MB in the hearts of hypercholesterolemic rats.

7.4.4. The ACN-rich extract significantly regulates FMOD, TAGLN, TAGLN2, and MYL6, proteins related to the cellular structure and downregulated proteins related to the cellular signaling pathways such as PRKACA, IQGAP1, and HSP90AB1 in the aortas of rats.

7.4.5. Both the red-fleshed and white-fleshed apples reduce proteins related to the complement system, suggesting an anti-inflammatory effect of the apple matrix independently of their ACN content, possibly related with other apple components as soluble fiber.

7.5. Regarding the effects of whole-apple intake on diverse cardiovascular disease (CVD) risk factors.

7.5.1. Consuming between 100 and 150 g/day of whole-apples in RCTs improves multiple CVD risk factors such as the plasmatic lipids and blood pressure values in humans.

7.5.2. Consuming between 100 and 150 g/day of whole-apples reduces the risk of CVD and CVD mortality in prospective observational studies.

7.5.3. The whole-apple consumption should be considered as an aid in the prevention of CVD.

7.6. Regarding the effects of the oral phenolic compound (PC) administration on the intestinal barrier integrity.

7.6.1. Oral resveratrol, the most studied PC in different animal models of intestinal damage, improves the intestinal barrier integrity increasing the expression of various anti-inflammatory and antioxidant proteins.

7.6.2. The *in vitro* evidence fairly explains the improvement in multiple clinical parameters of intestinal damage observed after the oral resveratrol administration in animals.

7.6.3. The oral PC administration in animals improves the intestinal barrier integrity and function from three main mechanisms: 1) The reduction of pro-inflammatory molecules, 2) the improvement in tight-junction protein expression, and 3) the improvement of the antioxidant intracellular activity.

7.6.4. The oral intake of resveratrol or other PC's (grape seed extract, genistein, urolithin A) should be considered in the management of the intestinal injury associated with local and systemic pathologies. However, the precise dose and time for the oral administration of resveratrol or other PCs in humans are still undetermined.

7.7. Regarding the effects of the PC administration on acute lung injury (ALI) induced by LPS-administration.

7.7.1. PCs improve the alveolar wall thickness in animal models of ALI, possibly reducing lung cell hyperplasia, and collagen deposition.

7.7.2. PCs significantly reduce the lung's W/D weight ratio in LPS-induced ALI in animal models of ALI.

7.7.3. PCs significantly decrease the inflammatory cell infiltration and improves the immune response in the lungs and BALF in animal models of ALI.

7.7.4. PCs significantly downregulate the NF-ƙβ and MAPK's activity in the lungs of animal models with ALI.

7.7.5. PCs improve tissue oxidation and ROS concentration in the lungs of animals mostly from a decrease in MPO activity in animal models of ALI.

7.7.6. PCs reduce the iNOS activity, and NO concentrations the lung's improving the vasodilatation secondary hypoxia in animal models of ALI.

8. OVERALL CONCLUSION

Therefore, as a result of the present work, our hypothesis is vertified, and the anthocyanins provided by fruits, extracts or other products help improve cardiovascular risk factors and other diseases. It can be concluded that, regardless of their source, the whole-apple, and ACN oral intakes should be considered effective for the prevention and treatment of cardiometabolic disease in humans. Moreover, in animal models, resveratrol or other PCs showed an improvement of the intestinal barrier integrity loss and in the management of the acute lung injury associated with the systemic inflammation in critical illnesses, such as sepsis, opening new promising application in humans.

9. Other contributions

Sandoval-Ramírez, Berner Andrée, Rosa M. Lamuela-Raventós, Ramon Estruch, Gemma Sasot, Monica Doménech, and Anna Tresserra-Rimbau. 2017. "Beer Polyphenols and Menopause: Effects and Mechanisms - A Review of Current Knowledge." Oxid Med Cell Longev. 2017;2017:4749131. doi: 10.1155/2017/4749131.

Murchland, Audrey R., Anna Gottschlich, Kristin Bevilacqua, Andres Pineda, **Berner Andrée Sandoval-Ramírez**, Christian S Alvarez, Gina S Ogilvie, et al. 2019. "HPV Self-Sampling Acceptability in Rural and Indigenous Communities in Guatemala: A Cross-Sectional Study." BMJ Open 9 (10): e029158. doi: 10.1136/bmjopen-2019-029158.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 4749131, 9 pages
https://doi.org/10.1155/2017/4749131

Review Article

Beer Polyphenols and Menopause: Effects and Mechanisms—A Review of Current Knowledge

Berner Andrée Sandoval-Ramírez,[1] Rosa M. Lamuela-Raventós,[1,2] Ramon Estruch,[2,3] Gemma Sasot,[1,2] Monica Doménech,[2,3] and Anna Tresserra-Rimbau[1,2]

[1]*Department of Nutrition, Food Science and Gastronomy, XaRTA, INSA, School of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain*
[2]*CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain*
[3]*Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain*

Correspondence should be addressed to Anna Tresserra-Rimbau; annatresserra@ub.edu

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Academic Editor: Giuseppe Cirillo

Beer is one of the most frequently consumed fermented beverages in the world, and it has been part of the human diet for thousands of years. Scientific evidence obtained from the development of new techniques of food analysis over the last two decades suggests that polyphenol intake derived from moderate beer consumption may play a positive role in different health outcomes including osteoporosis and cardiovascular risk and the relief of vasomotor symptoms, which are commonly experienced during menopause and are an important reason why women seek medical care during this period; here, we review the current knowledge regarding moderate beer consumption and its possible effects on menopausal symptoms. The effect of polyphenol intake on vasomotor symptoms in menopause may be driven by the direct interaction of the phenolic compounds present in beer, such as 8-prenylnaringenin, 6-prenylnaringenin, and isoxanthohumol, with intracellular estrogen receptors that leads to the modulation of gene expression, increase in sex hormone plasma concentrations, and thus modulation of physiological hormone imbalance in menopausal women. Since traditional hormone replacement therapies increase health risks, alternative, safer treatment options are needed to alleviate menopausal symptoms in women. The present work aims to review the current data on this subject.

1. Introduction

Beer is one of the most frequently consumed alcoholic beverages in the world. Beer consumption ranks first in Europe, slightly above wine consumption, according to the World Health Organization [1] and third amongst alcoholic beverage preferences in North America [2]. Archaeological findings show that Chinese villagers brewed fermented alcoholic drinks as far back as 7000 BC on a small individual scale, with a production process and methods similar to those of ancient Egypt and Mesopotamia [3]. Throughout human history, products, ingredients, procedures, and techniques have evolved due to technological advances and the implementation of industrialized processes [4] further enhancing the long history of beer as a part of the human diet.

During the last two decades, scientific evidence has suggested that moderate consumption of alcoholic beverages has positive outcomes on different aspects of cardiovascular risk, as evidenced by Nogueira et al. who correlated regular daily intake of 330 ml of beer with positive changes in insulin sensitivity and lipid profiles [5]. Fermented beverages have also shown positive associations with different cardiovascular disease endpoints such as coronary heart disease, peripheral arterial disease, chronic heart failure, and stroke in which regular moderate consumption of alcohol reduced the prevalence of adverse events [6], and fermented beverages have shown anti-inflammatory properties [7]; these findings may explain the benefits of regular and moderate alcohol intake on cardiovascular disease risk [8–11]. In the last decade, the development of new techniques for food analysis has allowed

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HPV self-sampling acceptability in rural and indigenous communities in Guatemala: a cross-sectional study

Audrey R. Murchland,[1] Anna Gottschlich,[1] Kristin Bevilacqua,[1] Andres Pineda,[2] Berner Andrée Sandoval-Ramírez,[3] Christian S Alvarez,[1] Gina S Ogilvie,[4] Thomas E Carey,[5] Mark Prince,[6] Michael Dean,[7] Carlos Mendoza Montano,[2] Alvaro Rivera-Andrade,[2] Rafael Meza[1,8]

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► Prepublication history and additional material for this paper are available online. To view these files, please visit the journal online (http://dx.doi.org/10.1136/bmjopen-2019-029158).

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For numbered affiliations see end of article.

Correspondence to
Dr Rafael Meza;
rmeza@umich.edu

ABSTRACT

Introduction Cervical cancer disproportionately burdens low-income and middle-income countries (LMICs) such as Guatemala. Self-collection testing for human papillomavirus (HPV) has been suggested as a form of cervical cancer screening to facilitate access in LMICs. This study assessed and compared the acceptability of self-collection HPV testing in two rural, indigenous and ethnically distinct communities in Guatemala: Santiago Atitlán, Sololá and Livingston, Izabal.

Methods All participants, women between the ages of 18 and 60, completed a questionnaire. Eligible participants were also asked to self-collect a vaginal sample and complete a questionnaire regarding comfort and acceptability. Self-collected samples were tested for high-risk HPV using the real-time PCR Hybribio kit.

Results In the indigenous community of Santiago Atitlán, of 438 age-eligible participants, 94% completed self-collection. Of those, 81% found it comfortable and 98% were willing to use it as a form of screening. In the multiethnic (Afro-Caribbean, indigenous) community of Livingston, of 322 age-eligible participants, 53% chose to self-collect. Among those who took the test, 83% found it comfortable and 95% were willing to use it as a form of screening. In Livingston, literacy (can read and/or write vs cannot read or write) was higher in women who chose to self-collect (prevalence ratio 2.25; 95% CI 1.38 to 3.68). Ethnicity, history of screening and reproductive history were not associated with willingness to self-collect in Livingston. Women in Santiago reported less prior use of healthcare than women in Livingston. Overall, 19% (106/549) of samples tested positive for high-risk HPV.

Conclusion Among women willing to self-collect in rural and indigenous communities in Guatemala, self-collection for HPV testing is highly acceptable. However, willingness to try self-collection might vary across communities and settings. Women from a community that used less healthcare were more likely to choose self-collection. Further research is necessary to determine what factors influence a woman's choice to self-collect.

Strengths and limitations of this study

- ► To our knowledge, little is known about the acceptability of self-collection human papillomavirus testing across the diverse communities within Guatemala and Latin America, and in particular among indigenous populations.
- ► Our study provided not only a larger sample size compared with previous studies but was also conducted in two differing communities.
- ► Due to the sensitive nature of the questions related to sexual history, it is possible that a social desirability bias may have resulted in over-reporting of perceived 'good behaviours', such as screening or use of protection, in addition to under-reporting of perceived 'bad behaviours', such as number of lifetime sexual partners and other sexual behaviour measures.
- ► Sampling methods differed between the two communities due to the lack of reliable census counts in one community, but our sample in this community is reflective of the overall population structure in terms of ethnic, age and other metrics, suggesting that influential selection bias into the study might be limited.

INTRODUCTION

Cervical cancer, primarily caused by human papillomavirus (HPV) infection, has a very good prognosis when detected in premalignant or early malignant stages.[1] However, it disproportionately burdens low-income and middle-income countries (LMICs), such as Guatemala, compared with high-income countries (HICs).[2–4] HICs currently use Pap smears to detect abnormal cervical lesions that can be removed, greatly reducing the risk of cervical cancer.[3 5] However, there are many barriers to implementing successful Pap smear (cytology-based) screening programmes in LMICs, including difficulties establishing sustainable laboratory infrastructure, training and retaining adequate numbers of trained pathologists or cytologists, overburdened primary care clinics, and time and travel limitations for women in reaching screening

10. Oral presentations, conferences, posters, and dissemination impact.

NuGO week 2018: Mitochondria, nutrition, and health.

Congress attendance.
Nutrigenomics Organization (NuGO).
Newcastle, England.
Date: 09/03/2018 - 09/06/2018.

The power of nutrition: improving cardiovascular risk with functional foods.

Oral Presentation. (Awarded)
LX National Congress of Medicine and IV Congress of Health Sciences.
College of Physicians and Surgeons of Guatemala.
Guatemala, Guatemala.
Date: 11/28/2019.

Bioavailability of anthocyanins in animal tissues, red fruits and their effects on health.

Poster presentation.
XVIII Latin American Nutrition Congress.
Latin American Nutrition Society (SLAN).
Guadalajara, Mexico.
Date: 11/11/2019 - 11/15/2019.

Eat your fruits and vegetables, science and our grandmas are right.

Scientific dissemination
2019 European Researchers Night.
Marie-Sklodowska Curie Actions.
Tarragona, Spain.

BIODISPONIBILIDAD DE ANTOCIANINAS EN TEJIDOS ANIMALES:

LOS FRUTOS ROJOS Y SUS EFECTOS SOBRE LA SALUD.

Autores: Berner Andrée Sandoval-Ramírez [1], Úrsula Catalán [1,3], Sara Fernández-Castillejo [1], Laura Rubió [4], Alba Macià [4] y Rosa Solà [1,2]

[1] Universitat Rovira i Virgili, facultad de Medicina y Ciencias de la Salud, Departamento de Medicina y Cirugía. Reus, Spain.
[2] Hospital Universitari Sant Joan de Reus (HUSJR), Reus, Spain.
[3] Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain.
[4] Departamento de Tecnología de los Alimentos, Agrotecnio Research Center, University of Lleida, Lleida, Spain.

Si deseas leer más escanea el código QR.
bernerandree.sandoval@urv.cat

ANTECEDENTES:

Las Antocianinas (ACNs) son pigmentos naturales, solubles en agua, provenientes de las plantas y se encuentran con mayor frecuencia en la piel y la pulpa de algunas bayas. Las ACNs son responsables de dar las coloraciones rojo/azul en algunas frutas, flores, semillas y plantas. Entre los efectos de las ACNs, se ha descrito que su consumo se ha asociado positivamente a la prevención de la enfermedad cardiovascular, algunos tipos de cáncer, Alzheimer y otras enfermedades. No obstante, la absorción y metabolización de las ACNs determinan su biodisponibilidad y los diferentes perfiles fenólicos en plasma y en los tejidos. En consecuencia, la relación entre la biodisponibilidad en tejidos y los beneficios de la ingesta de ACNs a largo plazo frente a corto plazo continúa siendo un área de incertidumbre.

OBJETIVO:

Evaluar la biodisponibilidad de ACNs en diferentes tejidos de animales. Además establecer un vínculo entre la biodisponibilidad tisular de las ACNs, los beneficios para la salud asociados a su consumo y los mecanismos de acción mediante los cuales podrían ejercer estos beneficios.

MÉTODOS:

- Se adaptó el "*Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA) *statement*" utilizado para la realización de revisiones sistemáticas de ensayos clínicos, debido a la falta de una metodología más especializada aplicado para la revisión de estudios animales.
- Se realizó una búsqueda en las bibliotecas científicas electrónicas PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Scopus (http://www.scopus.com) con las siguientes características: 1) administración de ACNs en animales, y 2) análisis de biodisponibilidad tisular de ACNs.

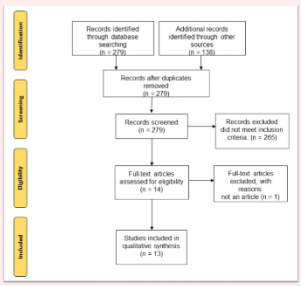
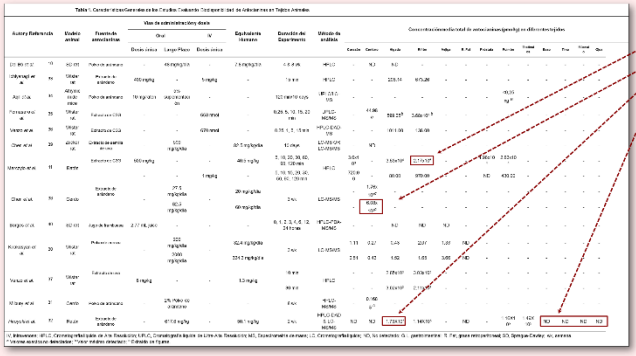


Figura 1: Prisma statement flowchart

RESULTADOS Y DISCUSIÓN:

Tabla 1:



De las 13 publicaciones que cumplieron con los criterios de inclusión, se observó que tanto las ACNs *Parent*, como sus metabolitos han sido detectadas en distintos tejidos animales dentro de los cuales se encuentran el corazón, cerebro, hígado, riñones, vejiga, y pulmones. Los órganos más frecuentemente analizados fueron los riñones, el hígado y el cerebro. Las concentraciones máximas presentadas fueron:

- 2.17×10^3 pmol/g en riñón de ratón.
- 6.08×10^3 pmol/g en cerebro de cerdo.
- 1.73×10^3 pmol/g en hígado de ratón.
- Por otro lado no se detectaron ACNs en tejidos como: Bazo, timo, ojos, músculo y grasa retroperitoneal.

Definimos ACN *parent* (ej. cianidina-3-glucosido, peonidina-3-glucosido, malvindina-3-glucosido, etc.) como una estructura de ACN a partir de la cual se derivan otra moléculas que son denominadas metabolitos. Estos son obtenidos mediante la sustitución o adición de radicales a través de metilación, conjugación, sulfatación y glucuronidación.

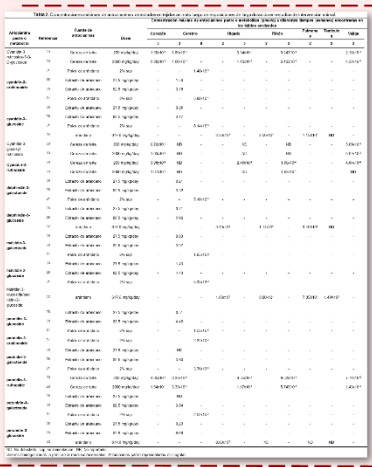


Tabla 2:

Se encontró una gran variedad de especies de ACNs en los experimentos evaluados, que evidencian un predominio de especies *parent* en experimentos de larga duración (semanas) *versus* el predominio de metabolitos de ACNs en experimentos de corta duración (horas) después de administrar ACNs por vía oral.

Esta diferencia entre la administración oral de ACNs a largo y corto plazo, podría ser explicado por la saturación de los mecanismos de absorción de antocianinas en la pared gástrica que sucede en experimentos de larga duración. Principalmente a través de la biltranslocasa ubicada en las células mucosecretoras y parietales:

1. La administración oral constante de ACNs causaría la saturación de sus mecanismos de absorción gástricos de ACNs (biltranslocasa).
2. Esta saturación lleva a la hidrolisis de las ACNs *parent* en sus metabolitos.
3. Como resultado la concentración de ACNs aumenta en la luz gástrica que en procesos posteriores de la digestión, llegarán al colon
4. Allí serán susceptibles metabolización por la microbiota intestinal, quienes reconvertirán los metabolitos de vuelta en sus ACNs *parent*.
5. Estos serán absorbidas a través de la pared intestinal hacia el plasma y posteriormente a los tejidos diana en animales.

CONCLUSIONES

- Las ACNs encontradas en tejidos animales, en particular la cianidina-3-glucosido y la peonidina-3-glucosido han demostrado actividad biológica para la prevención de distintas enfermedades.
- El consumo adecuado de frutos ricos en ACNs son capaces de reducir el daño celular cardiaco, el progreso de algunas enfermedades neurodegenerativas como el Alzheimer, reducir el daño renal agudo y también frenar la proliferación celular en el cáncer de pulmón.

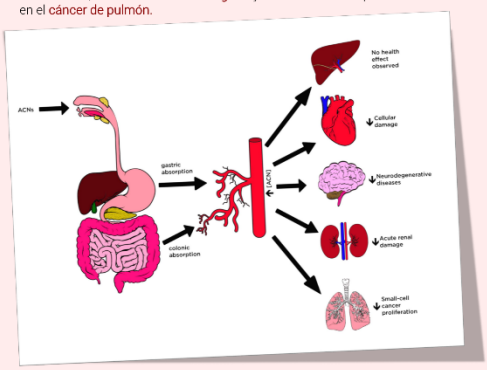


Figura 2: Distribución de ACNs, luego de su administración a largo plazo (semanas) en diferentes tejidos animales y sus posibles efectos beneficiosos sobre la salud.

Agradecimientos:
The AppleCOR Project (Subproject AGL2019-76943-C2-2-R and Suproject AGL2019-76943-C2-1-R) has been possible with the support of Ministerio de Economía, Industria y Competitividad, the Agencia Estatal de Investigación (AEI) and the European Regional Development Fund (ERDF). Sandoval-Ramírez, Berner Andrée enjoys a 2017MDT-COFUND-30 pre-doctoral fellowship contract. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 713679 and from the Universitat Rovira i Virgili (URV). UC has a PFIs estratégic de recerca i innovació en salut (PERIS) post doctoral grant (SLT002/16/00229; Catalunya, Spain) from Generalitat de Catalunya. NFOC-Salut group is a consolidated research group of Generalitat de Catalunya, Spain (2017 SGR 522).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Jueves, 27 de Febrero de 2020

Salud

Constatan los beneficios de la ingesta de manzana para la prevención de enfermedades cardiovasculares

Se dice en múltiples ocasiones que comer una manzana al día es un hábito saludable. Sin embargo, a pesar de su popularidad, aún resulta difícil determinar con precisión ¿hasta qué punto son buenas las manzanas para la salud? Para conocer la información de forma más precisa, investigadores del grupo NFOC-Salut de la URV (Cataluña, España) han analizado de manera integrada cuáles son los efectos del consumo de una manzana entera y de qué manera su consumo se encuentra asociado con el riesgo de padecer enfermedades

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Comer manzanas reduce el riesgo de padecer enfermedades cardiovasculares
Investigadores de la URV analizan los beneficios de esta fruta para la salud
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Regió7
Menjar una poma al dia redueix el risc de patir malalties cardiovasculars
Investigadors d'un grup de recerca de la URV analitzen els estudis fets fins ara sobre els beneficis d'aquesta fruita


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Menjar una poma al dia redueix en un 25% el risc de patir un infart cardiac o un ictus
Investigadors de la Universitat Rovira i Virgili analitzen els estudis fets fins ara sobre els beneficis d'aquesta fruita


Menjar una poma al dia redueix les malalties cardiovasculars, segons la URV


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Menjar una poma al dia redueix el risc de patir malalties cardiovasculars
Investigadors de la URV analitzen els estudis fets fins ara sobre els beneficis d'aquesta fruita


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Menjar una poma al dia prevé malalties cardiovasculars
Investigadors del grup NFOC-Salut de la URV determinen que qui ingereix una poma sencera al dia durant un any, redueix en un 14% el risc de morir per qualsevol causa, en un 27% el risc de morir per ictus i en un 25% el risc de morir per un infart cardiac i de patir un ictus
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Investigadors del grup NFOC-Salut de la URV han analitzat de manera integrada els efectes de menjar una poma sencera al dia


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INVITACIÓ:
ÀREA METROPOLITANA
Una recerca de la URV revela el gran benefici que té per la salut menjar una poma al dia
Investigadors d'un grup de recerca de la URV analitzen els estudis fets fins ara sobre els beneficis d'aquesta fruita

RECERCA

Prendre una poma al dia limita el risc de malalties del cor

Investigadors del Grup de Recerca en Nutrició Funcional, Oxidació i Malaltia Cardiovascular (NFOC-Salut) de la Universitat Rovira i Virgili (URV) han constatat els beneficis de la ingesta de pomes per a la prevenció de malalties cardiovasculars. El grup ha analitzat els estudis fets fins ara sobre l'efecte del consum de pomes de polpa vermella riques en antocianines per reduir el colesterol, en el marc del projecte *AppleCor*. La prova científica demostra que els individus que ingereixen almenys una poma sencera al dia -uns 100 grams-, com a mínim durant un any, redueixen en un 14% el risc de morir per qualsevol causa, en un 27% el risc de morir per ictus i en un 25% el risc de morir per un infart cardiac i de patir un ictus. A més, es redueix en un 9% el risc d'hipertensió i en un 24% el risc que l'aorta es calcifiqui de manera greu.

DIARI T21 TARRAGONA TARRAGONÈS BAIX CAMP

TARRAGONA POLÍTICA SOCIETAT ESPORTS CULTURA AGENDA PAR

Andrée Sandoval, l'investigador guatemalenc sorprès per com des de la URV 'juguem a les grans lligues'

24/11/2019

Andreé Sandoval s'ha integrar en un equip d'investigadors destinat a completar un estudi sobre l'efecte del consum de pomes de polpa vermella en la reducció del colesterol d'LDL

Bernard Andrée Sandoval és un guatemalenc de 29 anys seduït per l'oferta de màster de la URV. «Hi vaig arribar des del meu país gairebé per accident. Jo m'acabava de graduar com a metge i després de quatre o cinc mesos d'estudiant de cirurgià vaig decidir donar un tomb a la meva vida», explicava. «Buscant per internet diverses opcions per ampliar estudis em vaig trobar amb el màster de Nutrició i Metabolisme de la URV. I en el transcurs del màster em va picar el cuquet de la investigació i l'interès per cursar un doctorat».

Ara bé, davant «del problema existent a Espanya de la manca de fons», Sandoval admet que «em vaig rendir i vaig deixar d'intentar obrir-me pas en el camp de la investigació. Però tot just quan faltaven dos mesos per acabar el meu màster van llençar una convocatòria per una beca Martí Franquès». Cofinançada per la universitat i per la UE (a través de la Fundació Marie Curie), l'oferta era una via de sortida que en inici «no vaig valorar. Ho havia intentat altres vegades i no me n'havia sortit. Però davant la insistència de les meves amigues, literalment el darrer dia vaig decidir fer el pas i em vaig presentar a la convocatòria».

Investigadors prometedors d'arreu del món

Dels gairebé cinquanta aspirants, ell va ser qui finalment es va fer amb la plaça. «Quan em van dir que havia guanyat gairebé no m'ho creia», assenyalava Sandoval. La beca, amb caràcter general, està convocada per a «investigadors prometedors d'arreu del món. La idea és formar-te no només en l'àrea de la teva elecció sinó també en capacitats transversals, és a dir, que et permetin anar més enllà de la vida acadèmica i poder dur a terme des de divulgació cientifica fins a treballar a la indústria o en recerca».

La beca concedida a aquest jove amant de la lectura, el dibuix i del gimnàs li ha permès integrar-se dins d'un equip d'investigadors destinat a completar un estudi sobre l'efecte del consum de pomes de polpa vermella en la reducció del colesterol d'LDL, conegut com a colesterol dolent. La recerca la impulsen des del Centre Tecnològic de Nutrició i Salut de Reus (integrat dins de la xarxa de l'Eurecat) i comparen l'impacte del consum de pomes de polpa vermella amb les de polpa blanca i les infusions d'un fruit vermell anomenat aròmia.

Com reduir el colesterol

L'objectiu és analitzar l'efecte de les antocianines, components d'origen natural presents en algunes varietats d'aquestes fruites, en la reducció d'aquest tipus de colesterol. «El que nosaltres fem són assajos clínics controlats. Ara que ja sabem que menjar els fruits rics en antocianines redueixen el risc de malalties cardiovasculars, volem esbrinar com ho fan. I per això agafem un grup de persones aleatòries, les dividim en dos o més grups tractats tant amb pomes vermelles com amb placebos i al final comparem les diferències. D'aquesta manera –relata- podem determinar-ne efectes específics. Com els diferents grups són iguals, nosaltres som els causants de les diferències, per la qual cosa els podem atribuir una relació causa-efecte». La funció de Sandoval, en tant que metge, «és determinar si les persones són aptes per entrar i continuar en l'estudi, i intentar veure si tenen efectes secundaris. I posteriorment fer l'anàlisi dels resultats i el processament de les dades».

El grup de recerca està encapçalat per la doctora Rosa Solà, «cinc investigadores postdoctorals i entre sis i set predoctorals». Un equip que s'està forjant en una àrea que interessa també al mercat, perquè pretén «determinar de quina manera diferents components de diferents plantes afecten el risc cardiovascular, la tensió arterial o el flux de sang a les venes. I particularment – puntualitza Sandoval- al nostre grup el contracten les empreses per intentar determinar propietats dels seus productes». Entre ells el govern de l'estat de Florida, que «volia invertir per conèixer si un determinat component que tenen les taronges ajudava a baixar la tensió. El nostre grup va presentar un pla d'estudi i va guanyar-ne finalment la licitació. I amb això els fons per poder-lo executar».

Tot aquest cúmul d'oportunitats han de permetre Sandoval, que es defineix com a «metge de base», ampliar horitzons professionals. «Em feia molta falta adquirir coneixements en l'àrea de la investigació. I particularment en un camp com el de la nutrició, encara molt novedós, molt complex i amb molts interrogants per desvetllar. Gràcies a aquesta experiència puc compaginar el meu vessant més clínic amb el d'investigador».

De fet, un cop acabi el doctorat el febrer del 2021 el propòsit de Sandoval «és tornar a casa. És el Tercer Món i hi ha moltes necessitats de moltes coses, i metges especialitzats en nutrició n'hi ha molt pocs». Les estadístiques parlen per si soles. «A Guatemala es detecta desnutrició severa en sis de cada deu nens menors de 5 anys. Aquest és un problema real, i tinc molt d'interès a aprendre per poder traslladar aquest coneixement al meu país», afirmava mentre feia valdre la qualitat de l'ensenyament rebut a la URV. «M'ho va dir l'altre dia la nostra investigadora en cap i tenia tota la raó. Ens hem d'adonar que des d'aquí juguem a les grans lligues», malgrat reconèixer els hàndicaps la manca de fons en el contractament de recursos humans o en la necessitat de compartir l'ús dels aparells. «Amb les habilitats adquirides aquí sé que puc competir a tot arreu. I la meva intenció és ser algú reconegut en un futur a escala mundial. Dit això, i si em permets que et sigui honest, no m'esperava el gran nivell de qualitat de la URV, particularment en nutrició», es deixa anar.

«Des d'una ciutat de poc més de cent mil habitants es coordinen estudis internacionals. Un dia li vaig preguntar a la meva cap com podia ser que una zona tan petita fos tan potent, i la seva resposta em va semblar molt encertada: al final l'aposta per la qualitat i l'exigència dels investigadors principals dels diferents grups de recerca ha actuat com a pool d'atracció, més enllà de la dimensió de la població».

Jordi Siré

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THE TISSUE BIOAVAILABILITY, BIOMARKERS, AND EFFECTS OF ANTHOCYANINS ON HUMAN HEALTH. STUDIED THROUGH SYSTEMATIC REVIEWS ON ANTHOCYANIN-RICH FOODS AND A NUTRITIONAL PRE-CLINICAL STUDY WITH ANTHOCYANIN-RICH RED FLESHED-APPLES. THE APPLECOR PROJECT.

Berner Andrée Sandoval Ramírez

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