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Effects of grape polyphenols on cardiometabolic risk factors

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1. General introduction and outline of the thesis

Polyphenols are a heterogeneous group of compounds contained in plant foods. The main sources of polyphenols are whole grains, fruit, especially red fruits, soy, cocoa, tea, coffee and wine. The scientific interest for polyphenols is increasing thanks to epidemiological studies that suggest an association between the consumption of polyphenol-rich foods and prevention of some chronic-degenerative diseases, such as type 2 diabetes (DMT2), cardiovascular diseases (CVD) and several types of malignancies [1–4].

Polyphenols are reported to improve some biological functions, including endothelial function, platelet aggregation, lipid and glucose metabolism and to reduce oxidative stress [5,6]. It is important to consider that the bioavailability of polyphenols, which is quite low (<10% of polyphenols or their metabolites are found in urine and plasma) influence their activities while their chemical structure influence their intestinal absorption [7,8]. For these reasons, great attention is currently given to biovailability and structure of polyphenols.

Evidence on the beneficial effects of polyphenols mainly derive from *in vitro* and animal studies [9–13]. These studies focused on the beneficial effects of the individual classes of polyphenols, in particular flavonoids. Moreover, these studies used pharmacological doses of these compounds that are difficult to obtain with the usual diet.

Red grape is a great source of polyphenols, especially anthocyanin and resveratrol. Acute studies demonstrated that grape polyphenols are able to reduce plasma glucose concentration in both animals and humans [14,15]. Moreover, it has been observed an improvement in glucose metabolism after chronic consumption of grape polyphenols in subjects with the metabolic

syndrome or type 2 diabetes [16–18]. Grape supplementation seems to have also a favorable effect on others cardio-metabolic risk factors, such as plasma lipid levels, LDL oxidation and blood pressure [19].

In order to highlight the importance of grape polyphenols in the prevention of cardiovascular diseases, the present project was designed to assess: (1) the bioavailability of grape polyphenols and their plasma pharmacokinetic profile after assumption of phenolic-rich drink made from grape pomace; (2) the impact of grape polyphenols on some cardio-metabolic risk factors.

2. Overview on polyphenols

Chemical compounds extracted from plants (phytochemical extracts) are classified into primary and secondary metabolites.

Primary metabolites include sugars, amino acids, fatty acids, nucleic acids and other compounds. They have been found in all plants as regulators of growth and as components of cell wall.

In planta secondary metabolites have various functions, including protecting plants from herbivores and microbial infection, as attractants for pollinators and seed-dispersing animals, as allopathic agents, UV protectants, and signal molecules in the formation of nitrogen-fixing root nodules [20,21].

Polyphenols are a large and heterogeneous group of phytochemical compounds, ubiquitous in the plant world mainly contained in fruit, vegetables, whole grains, olives, legumes, chocolate and in some beverages such as tea, coffee and wine [22–24]. They have an important role in the physiology of the plants; in fact, they contribute to the pigmentation and to the organoleptic characteristics of the plants [25]. It has been estimated that

western population have a daily intake of polyphenols of about 1 g [8,25].

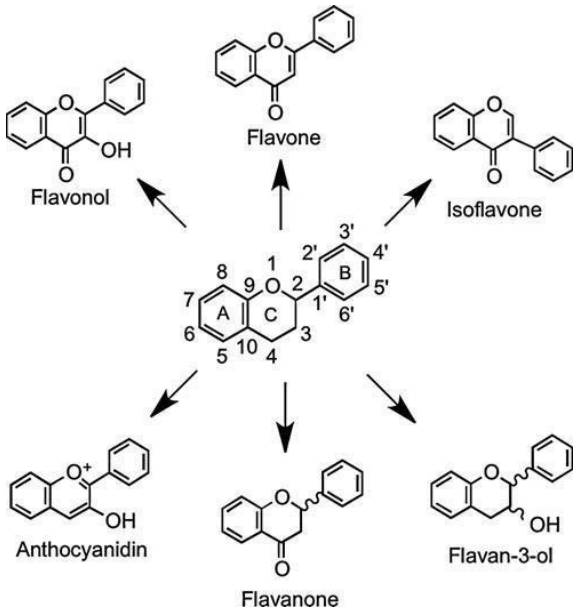
2.1 Structure and Classification

"Polyphenols" definition includes many classes of compounds that have a common chemical structure: an aromatic ring with one or more hydroxyl groups attached [8]. Over 8,000 chemical structures of polyphenols have been reported [26]. On the basis of their structure they can be classified as flavonoids and non- flavonoids.

2.1.1 Flavonoids

Flavonoids are polyphenolic compounds comprising 15 carbons with two aromatic rings connected by a three-carbon bridge (fig.1). The main subclasses of these C6–C3–C6 compounds are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins. The basic flavonoid skeleton can have numerous substituents. The majority of flavonoids occur naturally as glycosides rather than aglycones.

Figure 1. Structure of the flavonoid skeleton.



2.1.1.1 Flavonols

Flavonols occur widely throughout the plant kingdom with the exception of fungi and algae. The most common flavonols, kaempferol, quercetin, isorhamnetin, and myricetin are typically found as glycosides with conjugation occurring at the 5, 7, 3', 4', and 5' positions. Although the number of aglycones is limited, there are more than 200 sugar conjugates of kaempferol [26]. There is information on the flavonol content of commonly consumed fruits, vegetables, and beverages with sizable differences in the amounts found in seemingly similar produce, possibly due to local growing conditions, seasonal changes, and varietal differences [27–30]. Yellow and red onions *(Allium cepa)* are a rich source of flavonols, containing high concentrations of quercetin-4'-*O*-glucoside and quercetin- 3,4'-*O*-diglucoside. The disaccharide quercetin-3-*O*-rutinoside is a common dietary component.

2.1.1.2 Flavones

Flavones, such as apigenin, luteolin, wogonin, and baicalein, are similar structurally to flavonols, except for the lack of oxygenation at C-3. A wide range of substitutions is possible with flavones, including hydroxylation, methylation, *O*- and *C*-glycosylation, and alkylation. In general, flavones are not distributed widely, although substantial amounts have been detected in celery *(Apium graveolens)*, parsley *(Petroselinum hortense)*, and some herbs. Many flavones occur as 7-*O*-glycosides, although rooibos tea, a caffeine-free beverage prepared from leaves of the South African shrub *Aspalathus linearis*, contains small amounts of apigenin-8-*C*- glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin), luteolin-8-*C*- glucoside (orientin), and luteolin-6-*C*-glucoside (iso-orientin) [20,31]. Polymethoxylated flavones, such as nobiletin and tangeretin, occur in citrus species.

2.1.1.3 Isoflavones

Isoflavones have the B-ring attached at C-3 rather than at the C-2 position (fig.1). They are present almost exclusively in leguminous plants with substantial quantities of daidzein and genistein occurring in soybean *(Glycine max)* principally as 7-*O*-(6"-*O*-malonyl) glucosides with lower amounts of the corresponding 7-*O*-(6"-*O*-acetyl) glucosides, 7-*O*- glucosides, and the aglycones. Fermented soy products can be rich in the aglycones as a result of hydrolysis of the glycosides, whereas products whose manufacture involves heating, such as soy milk and tofu, contain reduced amounts of isoflavones, mainly in the form of the daidzein and genistein glucosides, which form as a result of degradation of malonyl- and acetylglucosides [32]. Because of their

structural similarity to estrogen, isoflavones are classified as phytoestrogens, as are the non-flavonoid lignans, which are a diverse group of compounds that occur in high concentrations principally in cereal grains.

2.1.1.4 Flavanones

Flavanones such as naringenin and hesperetin are characterized by the absence of $\Delta^{2,3}$ double bond and the presence of a chiral center at C-2 (fig.1). *In planta,* flavanones occur predominantly as the S- or (-)- enantiomer with the C-ring attached to the B-ring at C-2 in the α- configuration [3]. Flavanones occur as hydroxyl, glycosylated, and *O*- methylated derivatives. They are present in especially high amounts in flavedo of citrus fruits. The most common flavanone glycoside is hesperetin-7-*O*-rutinoside (hesperidin). Flavanone rutinosides are tasteless, in contrast to flavanone neohesperidoside conjugates, such as hesperetin-7-*O*-neohesperidoside (neohesperidin) from bitter oranges (*Citrus aurantium*) and naringenin-7-*O*-neohesperidoside (naringin) from grapefruit (*Citrus paradise*), which have an intense bitter taste.

2.1.1.5 Anthocyanidins

The most common anthocyanidin aglycones are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, which form conjugates with sugars and organic acids to generate a multitude of anthocyanins of differing colors, ranging from orange and red to blue and purple, and as a consequence, they are readily visible in fruits and flowers [33,34].

2.1.1.6 Flavan-3-ols

Flavan-3-ols are the most complex subclass of flavonoids, ranging from the simple monomers to the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins. The two chiral centers at C2 and C3 of the monomeric flavan-3-ol (fig. 1) produce four isomers for each level of B-ring hydroxylation, two of which, (+)-catechin and (-)- epicatechin, are widespread in nature, while others such as (-)- epiafzelechin have a more limited distribution [3,35]. Pairs of enantiomers can be resolved by chiral chromatography, but not with the more commonly used reversed-phase high-performance liquid chromatography (HPLC), and as a consequence, they are easily overlooked. Oligomeric and polymeric proanthocyanidins have an additional chiral center at C4 in the upper and lower units. Type B proanthocyanidins are formed from (+)- catechin and (-)- epicatechin by oxidative coupling between the C-4 of the upper monomer and the C-6 or C-8 of the adjacent lower or extension unit to create oligomers or polymers. Type A proanthocyanidins have an additional ether bond between C-2 in the B-ring of one monomer and C-7 in the A-ring of the other monomer. Proanthocyanidins can occur as polymers of up to 50 units. Proanthocyanidins that consist exclusively of (epi)catechin units are called procyanidins, and are the most abundant type of proanthocyanidins in plants. The less-common proanthocyanidins containing (epi)afzelechin or (epi)gallocatechin subunits are propelargonidins and prodelphinidins, respectively [20]. Green tea (*Camellia sinensis*) contains very high levels of flavan-3-ol monomers with the main components being (-)-epigallocatechin, (-)-epigallocatechin-3-*O*-gallate, and (-)-epicatechin-3-*O*-gallate. The levels of these flavan-3-ols decline during

fermentation of the green leaves to produce black tea, principally as a result of the action of polyphenol oxidase, and there is a concomitant accumulation of theaflavins and thearubigins [36]. Theaflavin, theaflavin-3- *O*-gallate, theaflavin-3'-*O*-gallate, and theaflavin-3,3'-*O*-digallate are dimer-like structures that contribute to the quality of the black tea beverage. The brownish, water-soluble, high-molecular-weight thearubigins are the major phenolic fraction in black tea. Recent pioneering studies have established that black teas contain on average 5000 thearubigin components in the mass range of 1000–2100 *amu* [37,38]. A typical cup of black tea contains ~ 100mg of thearubigins.

2.1.2 Non-flavonoids

Among the non-flavonoids of dietary significance there are the C6–C1 phenolic acids. Gallic acid is the commonest phenolic acid, and occurs widely as complex sugar esters in gallotannins, such as 2-*O*-digalloyl- tetra-*O*-galloyl-glucose, which are minor dietary components. The related ellagic acid-based ellagitannins, such as sanguiin H-6 and punicalagin, are found in a diversity of food, including raspberries (*Rubus idaeus*), strawberries (*Fragaria ananassa*), blackberries (*Rubus spp.*), and many other fruits, including pomegranate (*Punica granatum*) and persimmon (*Diospyros kaki*), as well as walnuts (*Juglans regia*), hazelnuts (*Corylus avellana*), and oak-aged wines where they are leached from the oak during maturation of the wines [39]. The ellagitannin content of some food products can be high with a glass of pomegranate juice and a 100 g serving of raspberries providing ~300 mg, and four walnuts ~400mg [40]. The C6–C3 hydroxycinnamates occur mainly as

conjugates, for example, with tartaric acid or quinic acid, and collectively are referred to as chlorogenic acids. Chlorogenic acids, principally 3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids, form ~10% of green robusta coffee beans (*Coffea canephora*). Regular consumers of coffee may have a daily intake in excess of 1 g of chlorogenic acids, and this will be the major dietary phenolics for many people. Accumulating in the flesh of grapes, caftaric acid is the main hydroxycinnamate in both red and white wines produced from *Vitis vinifera* and well as Concord grape juice, which is a product of grapes of *Vitis lambrusca* [41]. Stilbenes have a C6–C2–C6 structure and are phytoalexins produced by plants in response to disease, injury, and stress[42]. The main stilbene is resveratrol (3,5,4'-trihdroxystilbene), which occurs as cis and trans isomers as well as conjugated derivatives, including *trans*-resveratrol-3-*O*-glucoside (*trans*-piceid). The woody root of the noxious weed *Polygonum cuspidatum* (Japanese knotweed or Mexican bamboo) contains unusually high levels of *trans*-resveratrol and its glucoside with concentrations of up to 377 mg/100 g dry weight [43]. Red wines contain a diversity of stilbene derivatives, but invariably in very low concentrations compared to the levels of other (poly)phenolic components

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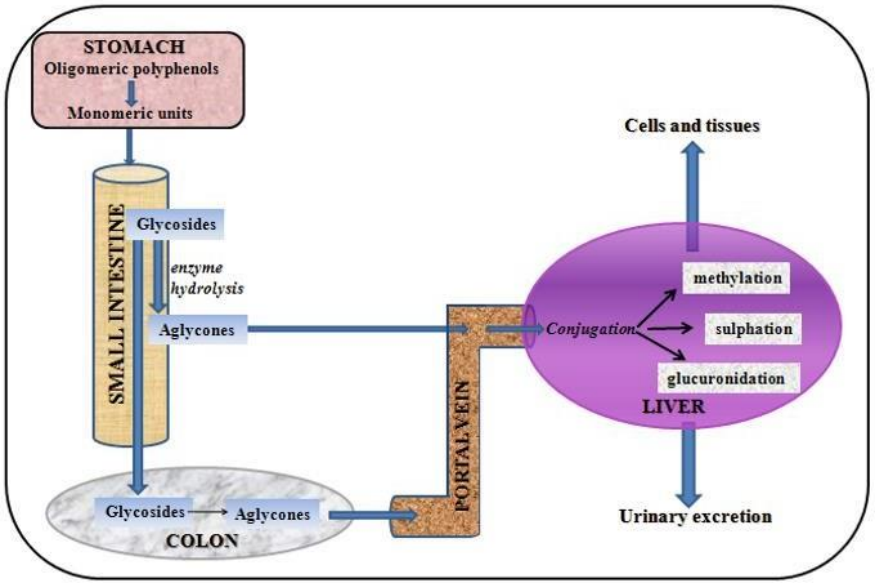
2.2 Bioavailability

The commonly accepted definition of bioavailability is: “the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways”. Consequently, it is important not only to know how much of a nutrient is present in specific food or dietary supplement, but even more important is to know how much of that is bioavailable [24]. The chemical structure of polyphenols, more than their concentration, determines the rate and extent of absorption and the nature of the metabolites circulating in the plasma[45]. Generally, polyphenols are little absorbed, largely metabolized and rapidly eliminated; their metabolism has a common pathway as shown in figure 2 [24].

Figure 2. Metabolic cycle of dietary polyphenols.



Many polyphenols are present in food in form of esters, glycosides, or polymers that cannot be absorbed in native form. Before absorption, these compounds must be hydrolyzed by intestinal enzymes or by the colonic

microflora.

In recent years, growing interest has been focused on the study of the absorption, distribution, metabolism and excretion of flavonoids and their metabolites.

2.2.1 Bioavailability of flavonoids

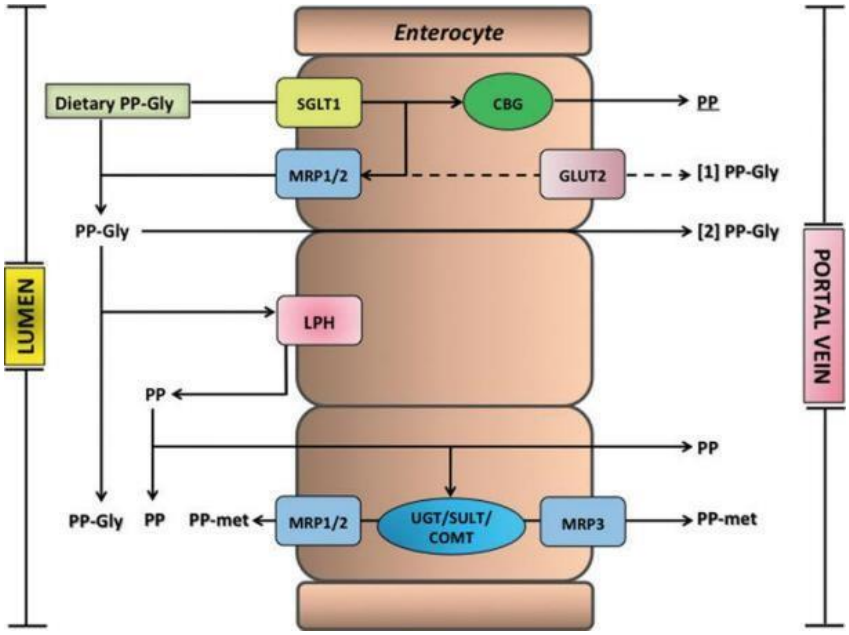
After ingestion, the absorption of most flavonoids occurs in the small intestine. Typically, the absorption of flavonoid glycosides, as illustrated in figure 3, is associated with cleavage and release of the aglycone as a result of the action of lactase phloridzin hydrolase (LPH) in the brush border of the small intestine epithelial cells. LPH exhibits broad substrate specificity for flavonoid-O-β-D- glucosides, a compound related to flavonoids, and the released aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane [46]. An alternative hydrolytic step is mediated by a cytosolic b-glucosidase (CBG) within the epithelial cells. The CBG-catalyzed hydrolysis involves the active sodium- dependent glucose transporter 1 (SGLT1) that is albe to transport the polar glucosides into the epithelial cells [47].

Thus, there are two possible routes by which the glycoside conjugates are hydrolyzed, and the resultant aglycones appear in the epithelial cells, namely LPH/ diffusion and transport/CBG (fig.3).

Before passing into the bloodstream, aglycones are further metabolized (during the phase II Metabolism) to form sulphate, glucuronide, and/or methylated metabolites through the respective action of sulfotransferases

(SULTs), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-*O*- methyltransferases (COMTs). There is also efflux of some of the metabolites back into the lumen of the small intestine, and this is thought to involve members of the adenosine triphosphate-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein (Fig. 3). MRP-3 and the glucose transporter GLUT2 have also been implicated in the efflux of metabolites from the basolateral membrane of the enterocytes [48,49]. Once in the portal bloodstream, metabolites rapidly reach the liver, where they can be subjected to further phase II metabolism, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion [50]. Polyphenol conjugates with sugar moieties that are resistant to the action of LPH/CBG [51] are not absorbed in the small intestine to any degree and pass to the colon.

Figure 3. Proposed mechanisms for the absorption and metabolism of polyphenolic compounds in the small intestine.



As a result of more recent studies on the bioavailability of dietary polyphenolic compounds [52], there is a growing evidence that polyphenolic glucuronide, methyl, and sulfate conjugates are treated by the body as xenobiotics, and instead of accumulating in the circulatory system, they are rapidly turned over and removed by excretion via the kidneys. As a consequence, although plasma pharmacokinetics of these metabolites provides useful information, estimates such as of area-under-the curve values do not necessarily yield accurate quantitative data on absorption. In the circumstances, urinary excretion provides a more realistic assessment.

3. Effects of grape polyphenols on cardio-metabolic risk factors: literature data

The beneficial effect of grape, a very rich source of flavonoids, on human health has been described in some studies [53,54]. In particular, grape is rich in flavan-3-ols, anthocyanins, and resveratrol, which are the most investigated polyphenols due to their putative cardio-protective and chemo-preventive effects [55–57].

The benefits of grape polyphenols against cardiovascular diseases seem to be mediated by their favorable effects on several cardiometabolic risk factors, such as plasma glucose and lipid levels, LDL oxidation, and inhibition of platelet aggregation, inflammation and blood pressure [19].

3.1 Blood glucose and insulin sensitivity

Most of the data available on the effects of grape polyphenols on glucose and insulin metabolism have been obtained on animal models [9–13].

Humans studies evaluating the effects of grape polyphenols intake on glucose and insulin levels have been mainly performed in subjects with type 2 diabetes (T2D) or metabolic syndrome (MetS) [16,58–64].

Some years ago, in a double-blinded, randomized, crossover trial performed in 32 subjects with T2D, Kar and colleagues showed that the intake of 600 mg/day of grape seed extract for four weeks resulted in a significant reduction of fructosamine ($p = 0.0004$), whole blood glutathione (GSH; $p < 0.01$), high sensitivity C reactive protein (hsCRP) ($p = 0.0006$) and total cholesterol concentration ($p = 0.05$), whereas no significant changes in HOMA–IR[17]

(tab.1) were seen.

Moreover, in a randomized, controlled study conducted in 38 males with at least one component of MetS, the daily consumption of 20g of wine grape pomace flour containing 822 mg of polyphenols for 16 weeks was significanltly associated with a reduction in postprandial insulin and fasting glucose levels compared with the baseline ($p < 0.05$). However, no significant differences in fasting insulin, postprandial glucose and insulin, glycosylated hemoglobin and HOMA–IR between the grape pomace group and the control group were seen[16] (tab. 1).

Previously to these findings, Banini et al. studied the effect of the consumption of dealcoholized muscadine grape wine on plasma insulin levels in type 2 diabetic subjects[18] (tab. 1). After 28-day intervention, subjects receiving the dealcoholized wine showed lower fasting insulin levels and an increased fasting glucose: insulin ratio increased from 8.5 to 13.1 during the 28-day intervention, indicating an improvement of insulin sensitivity.

In addition, a recent meta-analysis of 15 trials on the effect of grape seed extract (GSE) intake on several cardiovascular risk factors showed a significant decrease in fasting plasma glucose concentrations (WMD: −2.01; 95% CI: −3.14, −0.87) in high risk subjects [65].

As grape skins and red wine are important sources of resveratrol, several studies focused on its effect on glucose metabolism.

A study conducted in nineteen subjects with T2D who received 5 mg of resveratrol for four weeks showed an improvement in insulin sensitivity assessed with HOMA–IR, a reduction in glucose levels and a delay in glucose peak following a standard meal. Unfortunately, there was no control group or

placebo used [66] (tab. 1).

The favorable effect of resveratrol supplementation on glucose and insulin metabolism has been also highlighted in a meta-analysis of 11 RCTs. The results showed a significant reduction in fasting glucose concentrations (-35.22 mg/dL), fasting insulin concentrations (-4.55 μIU/mL), Hb A1c (-0.79%) and HOMA-IR (-2.25) in subjects with diabetes compared with control subjects [67].

However, a combination of other polyphenolic compounds present in red wine or grape are likely to contribute to these beneficial effects, since resveratrol is known to have a low bioavailability.

In summary, the available evidence on the beneficial effect of grape polyphenols on glucose metabolism at fasting and in people with T2D is clear. However, few data from clinicl trials are available about the effects of grape polyphenols intake on glucose metabolism in healthy subjects and on post-prandial glucose and insulin response.

Table 1. Clinical trials on the impact of grape polyphenols on glucose metabolism.

Study Ref	**Subjects (n)**	**Study Design**	**Source and Dose**	**Duration**	**Principal Outcomes**
Kar et al. 2009[17]	T2D (32)	Crossover RC	Grape seed extract (600mg/d)	4wk	↓fructosamine (p=0.0004)
Urquiaga et al. 2015[16]	MetS (38)	Crossover RC	Red grape pomace (20g/d)	16 wk	↓Postprandial insulin (p<0.05)

Banini et al. 2006[18]	T2D 29	Crossover RC	Dealcoholized muscadine grape wine (150mL/d)	28days	↓blood glucose, insulin and glycated hemoglobin
Brasnyo et al. 2011[66]	T2D 19	Crossover RC	trans-resveratrol (10mg/d)	4 wk	↓$HOMA_{IR}$

MetS= metabolic syndrome; T2D= type 2 diabetes C= controlled; R=randomized; wk= week.

3.2 Blood lipids

Polyphenols from red wine play a role in the hepatic cholesterol and lipoprotein metabolism. Indeed, polyphenols can reduce cholesterol absorption and decrease the delivery of cholesterol to the liver, which, in turn, reduces plasma cholesterol level. Additionally, polyphenols affect apolipoproteins (apo) A and B, which are emerging as risk factors for CVD [68], modify Very Low Density Lipoproteins (VLDL) particles and reduce plasma triglyceride (TG) levels due to possible increased activity of lipoprotein lipase (LPL) [69].

Previous studies by Castilla et al. [70,71], Khadem-Ansari et al. [72] and Albers et al. [73] indicate that grape juice can exert an effect on blood lipids. Studies conducted by Castilla et al. using 100 mL/day of grape juice for 14 days showed a decrease in total cholesterol (TC), LDL-C and apo B-100 ($p < 0.001$) in 38 [70] and 32 [71] healthy and hemodialysis patients, respectively; whereas the apo A-1 value increased ($p < 0.01$ [71]). Two other studies with grape juice [72,73], resulted in increased HLD- C, while an increase of apo B was also shown in the study of Khadem-Ansari and colleagues [72] (tab. 2). Two additional studies with Concord grape juice found an increase in TG ($p < 0.001$ [74] and $p < 0.05$

[75]) and no change in TC, HDL-C or LDL-C levels (tab. 2). Other studies described different results depending on grape extract administration and the patients' disease. In this context, no changes in LDL-C or TG were found in 24 overweight pre- hypertensive and/or pre-diabetic individuals, although an increase in HDL-C and a decrease in TC were achieved (p = 0.001 and p = 0.037, respectively), when the individuals were supplemented with 350 mg/day of whole grape extract for 6 weeks[76], whereas in a study conducted in 52 mild hyperlipidemic individuals supplemented with 200 mg/day of GSE, no change was observed in HDL-C or TG (tab. 2). Indeed, decreases in TC and LDL-C were found[77] (tab. 2). Another human supplementation study with 2 x 300 mg/day of MegaNatural (R) Gold, a high-quality GSE, for 3 weeks, found that TC, LDL-C and HDL-C concentrations were significantly decreased in 8 hypercholesterolemic subjects (TC and LDL-C, $p< 0.01$; HDL-C, $p< 0.05$), while no effect was found in 9 healthy individuals in another sudy [78] (tab. 2).

Overall, the evidence on the effect of grape products on lipid metabolism are still scant and controversial. Several factors such as the type of supplementation, polyphenol content, study duration, study design and participant characteristics could explain the heterogeneity of the observed results.

Table 2. Clinical trials on the impact of grape polyphenols on plasma lipids.

Study Ref	Subjects (n)	Study Design	Source and Dose	Duration	Principal Outcomes
Castilla et al. 2006[70]	Healthy and hemodialysis (38)	Crossover R PC	Red grape juice (100mL/d)	14 days	↓TC, ↓LDL-C, ↓apo B-100 (p<0.001); ↑HPL-C and Apo A-1 (p<0.001)
Castilla et al. 2008[71]	Hemodialysis (32)	Crossover R PC	Red grape juice (100mL/d)	14 days	↓TC, ↓LDL-C, ↓ApoB-100 (p<0.001); ↑ApoA-1 (p<0.01)
Albers et al. 2004[73]	CAD patients (40)	Crossover R PC DB	Purple grape juice (7mL/kg/d)	14 days	↑TG (p<0.05) and no change in TC, HDL-C, LDL-C
O' Byrne et al. 2002[75]	Healthy (36)	Parallel R	Concord grape juice (10mL/kg/d)	2 wk	↑TG (p<0.05) and no change in TC, HDL-C, LDL-C
Evans et al. 2014[76]	Pre-hypertensive/ pre-diabetic (24)	Crossover R PC DB	Grape extract (350mg/d)	6 wk	↑HDL-C (p=0.001); ↓TC (p=0.037) and no change in LDL-C or TG
Razavi et al. 2013[77]	Mild hyperlipidemic (52)	Crossover R PC DB	Grape seed extract (200mg/d)	8 wk	↓TC (p=0.015); ↓LDL-C (p=0.014)

Vinson et al. 2001[78]	Healthy and hypercolestorelomic (17)	Parallel R	Grape seed extract (600mg/d)	3 wk	↓TC and ↓LDL-C (p<0.01); ↓HDL-C (p<0.05)

CAD= coronary artery disease; DB=double blind; PC= placebo controlled; R=randomized; wk= week.

3.3 Blood pressure

Several studies have evaluated the effects of grapes on blood pressure (BP) in cohorts whose average baseline BP was in the pre-hypertensive range [46,79–87]. Studies by Sivaprakasapillai et al. [46], in which 27 adults with the metabolic syndrome were randomized into three groups (placebo, 300 mg per day of GSE and 150 mg per day of GSE) and those carried out by Clifton [82], a double-blind randomized crossover control trial with a 12 weeks long period in 36 men and women with above-average vascular risk, reported a decrease in SBP of -11 mmHg and -3 mmHg, respectively (tab. 3). Moreover, Sivaprakasapillai et al. [46] also observed the same tendency for diastolic blood pressure (DBP), with a decrease from -7 to -11 mmHg with the two different dosages. A meta-analysis of nine RCTs showed that GSE significantly lowered SBP by -1.54 mm Hg (p = 0.02), but no significant effect was observed on DBP [88]. In contrast, a double blind RCT 8-week intervention study carried out by Ras et al. [81], with seventy pre- and stage 1 hypertensive subjects who consumed either 300 mg/day of GSE or a placebo, evidenced a change in SBP values in both groups (-5.2 mmHg and -2.2 mmHg, p = 0.01, for GSE and placebo groups, respectively). The DBP also changed by -2.5 mmHg (p = 0.01) in the GSE group and by -1.1 (p = 0.01) mm Hg in the placebo group (tab. 3). Another study by Draijer et al. [89] demonstrated that the

consumption of a polyphenol-rich grape extract containing 800 mg of polyphenols lowered SBP by -3 mmHg and DBP by -2 mmHg in 60 untreated mildly hypertensive subjects and also confirmed that 24-h ambulatory BPs were significantly lower in the grape-wine extract intervention (135.9±1.3 mmHg), compared to placebo (138.9±1.3 mmHg).

In contrast, three studies found no changes in BP values [83–85]; however, these studies were performed in healthy people or individuals with pre- and stage 1 hypertension (tab. 3). A RCT study published in 2015 by Vaisman et al. [90] which investigated the effect of daily dietary consumption of red grape cell powder (RGC) on blood pressure in 50 subjects with prehypertension and mild hypertension who consumed 200, 400 mg RGC or placebo daily for 12 weeks, found a significant decrease in DBP in the 200 mg RGC group compared to the placebo group ($p = 0.032$) (tab. 3).

Therefore, more trials in patients with pre- and stage 1 hypertension are needed to confirm the beneficial effect of grape on BP.

Table 3. Clinical trials on the impact of grape polyphenols on blood pressure.

Study Ref	Subjects (n)	Study Design	Source and Dose	Duration	Principal Outcomes
Ras et al. 2013[81]	Healthy (70)	Parallel R PC	Grape seed extract (300mg/d)	8 wk	↓SBP ($p = 0.01$) ↓DBP ($p = 0.01$)
Sivaprakasapillai et al. 2009[46]	Metabolic Syndrome (27)	Crossover R PC	Grape seed extract (300mg/d) (500mg/d)	4 wk	↓SBP ($p = 0.05$) ↓DBP ($p = 0.05$)
Clifton et al. 2004[82]	Above average vascular risk (36)	Crossover R PC DB	Grape seed extract (2g/d)	4 wk	↓SBP ($p = 0.05$) ↑FMD ($p < 0.05$)

Sano et al. 2007[83]	Healthy (61)	Crossover R PC DB	Grape seed extract (200 mg/d) (400 mg/d)	12 wk	No significant change in BP
van Mierlo et al. 2010[84]	Healthy (35)	Crossover R PC DB	Grape seed extract (800mg/d)	2 wk	No change in BP or FMD
Ward et al. 2005[85]	Hypertensive (69)	Parallel PC DB	Grape seed extract (1000mg/d)	6 wk	No change in BP or FMD
Vaisman et al. 2015[90]	Pre- and mild-hypertension (50)	Parallel R PC DB	Red grape cell powder (200mg/d) (400mg/d)	12 wk	↓DBP (p =0.032) in 200 mg group

DB=double blind; PC= placebo controlled; R=randomized; wk= week; FMD= flow-mediated dilatation measured in brachial artery.

3.4 LDL oxidation and oxidative stress

Polyphenols, especially those from red wine and grape juice, inhibit LDL oxidation and thus attenuate the development of atherosclerosis [73,91–96]. Studies in hemodialysis patients showed that a consumption of 100 mL/d of concentrated Bobal grape juice for 14 days [70,71] was associated with a 35% and a 65% decrease in oxidized LDL (*ox*-LDL), respectively. Studies conducted with Concord grape juice found discrepant results; the first one in 1999 found a 35% increase in LDL lag time ($p = 0.015$) in patients with stable atherosclerotic coronary artery disease after consuming 7.7 mL/kg per day for 14 days [74]. On the other hand, the study by O'Byrne et al. in 2002 observed a 10% increase in LDL lag time ($p < 0.001$) and a 9% decrease in LDL oxidation rate ($p < 0.01$) in healthy individuals after consuming 10 mL/kg per day for 14 days [75]. A recent randomized, controlled, crossover study demonstrated that the acute consumption of 400 mL of grape juice significantly decreased thiobarbituric acid reactive substances (TBARS) levels compared to the

control intervention ($p < 0.05$), in 24 healthy subjects [97].

Some studies have been conducted with grape seed extract to evaluate LDL oxidation. The first one, published in 2003, was carried out by Vigna et al. [98] in 24 healthy male heavy smokers during 4 weeks of treatment with two capsules daily of 75 mg of a grape procyanidin extract. Among oxidative indexes, TBARS concentration was significantly reduced in subjects taking the standardized formulation of a polyphenolic grapes extract versus placebo treatment (-14.7% ± 21.1% vs. +5.0% ± 18.1%, $p < 0.01$). Similarly, the lag phase of LDL oxidation was prolonged with respect to baseline (+15.4% ± 24.4% after procyanidins and +0.1% ± 16.0% after placebo, $p < 0.05$). On the other hand, a study carried out by Ward et al. in 2005 [85], found no change in ox-LDL levels in a randomized, double- blind, placebo controlled, factorial trial in which 18 hypertensive individuals were treated with 1000 mg/day grape seed extract for 6 weeks. The research published in 2007 by Sano et al. [83] examined the effect in healthy individuals of 200 or 400 mg/day administration of proanthocyanidin equivalent for 12 weeks. Both dosages decreased the concentration of *ox*-LDL by 12%–14% ($p < 0.05$), measured as plasma maloaldehyde (MDA). The last study published in 2013 by Razavi et al.[77] found a decrease in *ox*-LDL ($p = 0.008$) after 8 weeks treatment with 200 mg/day of red grape seed extracts in individuals with mild hyperlipidemia. In 2012 a study was carried out by Tomé-Carneiro et al. [99], in which 75 patients undergoing primary prevention of cardiovascular disease participated in a triple- blinded, randomized, placebo-controlled trial with resveratrol-enriched grape extract (8 mg of resveratrol) for 6 months. At the end of the treatment, a decrease in LDL-C ($p = 0.04$), Apo-B ($p = 0.014$) and

ox-LDL (p = 0.001) was observed, thus reducing the atherogenic potential. The presence of resveratrol in the GE was necessary to achieve these effects.

Oxidative stress reflects an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. The generation of free radicals as superoxide ($O2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), peroxynitrite (ONO_2^-), and nitric oxide (NO) is necessary, since they play a role in growth, repair, and immune functions that are essential for human cells. However, these molecules also have the ability to oxidize signaling molecules, DNA, macromolecules, and cell structures such as lipid membranes, thus damaging healthy cells.

Long-term oxidative stress has been linked to several cardiovascular diseases. A number of human studies have documented a decrease in markers of oxidative stress, as superoxide and F2-isoprostanes, following supplementation with grapes. In 2001, Freedman et al. [100]found a significant decrease in platelet-dependent superoxide (from 29.5 ± 5.0 to 19.2 ± 3.1, p < 0.05) among 20 healthy subjects who consumed 7 mL/kg per day of purple grape juice for 14 days. Similarly, in 2004 Albers et al. [73] found a similar result (50 au after placebo vs. 34.5 au after Concord grape juice, p = 0.02) after 14 days of supplementation with 7 mL/kg per day of Concord grape juice in 20 subjects with coronary artery disease. In 2008, Castilla et al. [71] described a decrease in NADPH oxidase-dependent production of superoxide by neutrophils (p < 0.01) in stable hemodialysis patients after 14 days of 100 mL concentrated Bobal grape juice. With regard to to F2-isoprostanes as an indicator of oxidative stress [101], controversial results have been found. In 2001,

Caccetta et al. [102] reported a decrease in plasma and urine F-isoprostane ($p < 0.05$) following the consumption of dealcoholized red wine in a RCT of 18 smokers. One year later, O'Byrne et al. [75] and did not find any change in urinary F2- isoprostanes in healthy young adults consuming 10 mL/kg per day of grape juice for 2 weeks. In 2005, Zern et al. [103] reported a decrease in urinary F2-isoprostanes ($p < 0.05$) after 4 weeks of treatment with 36 g of lyophilized grape powder (~200 g fresh) in pre- and post-menopausal women. Ward et al. [85] found no change in plasma or urinary F2-isoprostanes in hypertensive individuals who received a supplementation of 1000 g/day of grape seed extract for 6 weeks. In 2013, Hokayem et al. [104] found an increase in systemic oxidative stress expressed as higher levels of urinary F2-isoprostanes after 8 weeks of 2 g/day grape polyphenol supplementation in 38 overweight/obese, first degree relatives of type 2 diabetic patients in a randomized double-blind controlled trial.

Two studies have examined DNA damage as a biomarker of oxidative stress. The first one, performed in hypertensive men with a supplementation of 5.5 mL/kg per day of Concord grape juice [105], reported a decrease in lymphocyte DNA damage ($p < 0.01$) after 8 weeks of treatment. Recently, Corredor et al. [106] observed a significant decrease in *ox*-DNA damage when dialysed patients were supplemented with 100 mL of unfermented grape juice for 6 months. All supplement trial informations are collected in table 4.

Table 4. Clinical trials on the impact of grape polyphenols on LDL oxidation and oxidative stress.

Study Ref	Subjects (n)	Study Design	Source and Dose	Duration	Principal Outcomes
Toaldo et al. 2015[97]	Healthy (24)	Crossover R C	Grape juice (400mL/d)	2 wk	↓TBARS (p<0.05)
Vigna et al. 2003[98]	Heavy smokers (24)	Crossover R PC DB	Grape extract (75mg/d)	4 wk	↓TBARS (p<0.01) and ↑*ox*-LDL (p<0.05)
Zern et al. 2005[103]	Pre- and post-menopausal women (44)	Crossover R PC	Grape powder (36g/d)	4 wk	↓ F2-isopreastones and TBARS (p<0.05)
Hokayem et al. 2013[104]	Healthy overweight/obese T2D (38)	Crossover R PC DB	Grape polyphenols (2g/d)	8 wk	↓ F2-isopreastones and TBAS (p<0.05)
Corredor et al. 2016[106]	Chronic kidney disease (39)	Crossover R C	Unfermented grape juice (100mL/d)	6 months	↓ ox-DNA damage
Park et al. 2009[105]	Hypertensive men (40)	Crossover R PC DB	Grape juice (5.5mL/kg/d)	8 wk	↓ DNA damage (p<0.01)

C= controlled; DB=double blind; PC= placebo controlled; R=randomized; wk= week.

4. Personal research areas

4.1 Bioavailability and pharmacokinetic profile of a phenolic-rich drink made from grape pomace

Polyphenol bioavailability in humans is quite variable, and the inter-individual variability in the production of phenolic metabolites has not been comprehensively assessed to date. So, we performed a study to investigate the pharmacokinetic and excretive profiles of phenolic metabolites after the acute administration of a drink prepared with a red grape pomace extract in 10

healthy volunteers.

Specifically, we focused on the analysis, of human polyphenol metabolites in plasma and urine samples of the participants after the administration of a grape pomace drink, by using the UHPLC-ESI-MS/MS techniques.

The main clinical characteristics of participants are reported in table 5.

Table 5. Characteristics of participants in the study (n=12).

Age (years)	26 ± 3[a]
BMI (kg/m^2)	26 ± 2
Systolic blood pressure (mmHg)	125 ± 11
Diastolic blood pressure (mmHg)	77 ± 6
Fasting plasma glucose (mg/dl)	96 ± 3
Fasting plasma insulin (μU/ml)	12 ± 6
Fasting plasma cholesterol (mg/dl)	152 ± 29
Fasting plasma triglycerides (mg/dl)	86 ± 34
Fasting plasma HDL-cholesterol (mg/dl)	39 ± 5
Homa Index	2.8 ± 1.1

[a] = Means ± SD (all such values)

Participants were asked to consume a low-polyphenol diet during the experimental period (at least 3 days before and 2 days after the test days). On the day of the test, participants were admitted at the Clinical Research Center, after 12 h overnight fast for the baseline blood drawing; thereafter, they consumed 250 mL of an aqueous extract drink of red grape pomace (RGPD;

9.8 g/100 mL of soluble carbohydrates and 625 mg/100 mL of total polyphenols). Three hours after RGPD consumption, participants consumed a standard low-polyphenol meal, consisting in white bread (150 g), fatless ham (70 g), spreadable cheese (80 g) and plumcake (33 g) (903 kcal, 18% protein, 30% fat, 52% carbohydrates). Plasma samples were collected every hour over 8h after drink intake. The next day, a fasting plasma sample was also collected, 24 h after the test drink. In addition, urine samples were collected at fasting and after the RGPD consumption at: 0–3, 3–6, 6–10, 10–24, 24–36, and 36–48 hours.

Firstly, we analyzed phenolic composition of our grape pomace drink and we identified and quantified a total of 25 phenolic compounds in the RGPD (tab. 6).

Table 6. Phenolic composition of Red Grape Pomace Drink (RGPD).

Compounds	**RT**	**$[M-H]^-$ (*m/z*)**	**µmol/250 mL RGPD**
Phenolic acids			
Gallic acid	1.48	169	22.49
Flavan-3-ols			
Catechin	3.35	289	462.75
Epicatechin	3.65	289	387.58
Gallotannins			
Galloyl glucose	2.22	331	24.57
Flavonols			
Quercetin rhamnoside	4.41	447	5.78
Quercetin-3-*O*-glucoside	4.17	463	15.97

Quercetin-3-*O*-glucuronide	4.13	477	25.04
Myricetin hexoside	3.84	479	14.29
Syringetin hexoside	4.45	507	6.90
Quercetin rutinoside	4.00	609	4.03
Procyanidins			
Procyanidin dimer B-type	3.04	577	41.76
Procyanidin dimer B-type	3.20	577	19.64
Procyanidin B2	3.42	577	28.28
Procyanidin dimer gallate B-type	3.73	729	11.50
Procyanidin trimer B-type	3.62	865	10.37
Procyanidin trimer B-type	3.26	865	13.79
Procyanidin trimer B-type	2.07	865	7.80
Anthocyanins		**$[M]^+$(*m/z*)**	
Cyanidin-3-*O*-glucoside	4.5	449	1.70
Delphinidin-3-*O*-glucoside	3.24	465	41.73
Petunidin-3-*O*-glucoside	3.46	479	378.95
Malvidin-3-*O*-glucoside	3.67	493	2117.33
Malvidin-3-*O*-acetylglucoside	4.2	535	27.21
Petunidin-3-*p*-coumaroylglucoside	4.44	625	3.75
Malvidin-3-*p*-coumaroylglucoside	4.68	639	30.08
Malvidin-diglucoside	4.39	655	5.89
Total sum (poly)phenols in drink			**3709.2**

The 250 mL of RGPD used contained 3.7 mmol of total phenolic compounds. Anthocyanins were the most abundant class of phenolic compounds (70%),

followed by flavan-3-ol monomers (23%) and procyanidins (4%). Small amounts of flavonols, galloyl glucose, and gallic acid were also present in the drink.

To assess the bioavailability of polyphenols contained in the RGPD, we analyzed the excretion of phenolic metabolites in urine and we identified 35 phenolic compounds over 48 h after ingestion of the RGPD (tab. 7).

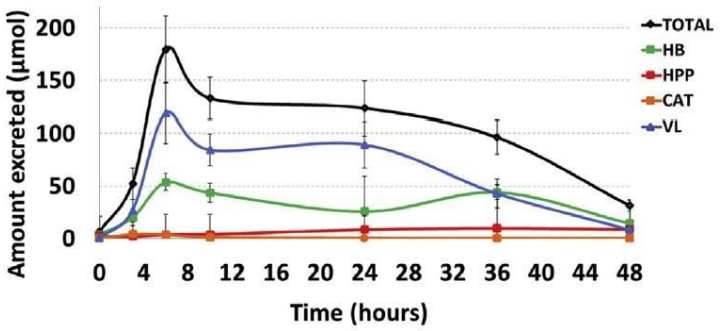
Table 7. Phenolic metabolites detected in urine.
Data on total excretion (0-48h) are expressed as means ± SEM.

Phenolic compounds	**Total (0–48 h) N=9**	**CV (%) N=9**
Gallic acid	0.93 ± 0.06	19.9
Vanillic acid-4-glucuronide	19.17 ± 4.17	68.7
Protocatechuic acid	0.55 ± 0.06	32.8
Protocatechuic acid-3-sulphate	13.68 ± 1.11	25.6
Benzoic acid-4-sulphate	26.02 ± 4.27	51.8
Vanillic acid-4-sulphate	42.12 ± 5.12	38.4
Catechol-sulphate	1.25 ± 0.66	166.8
Methylpyrogallol-sulphate	93.61 ± 10.83	36.6
Methylcatechol-sulphate	4.72 ± 0.37	24.5
Total simple phenols and hydroxybenzoic acids	**202.05 ± 13.59**	**21.3**
Ferulic acid-4-glucuronide	5.09 ± 0.73	45.3
Feruloylglycine	12.43 ± 1.12	28.5
Dihydrocaffeic acid-sulphate	1.39 ± 0.30	67.3
Dihydroferulic acid-sulphate	1.69 ± 0.22	42.0
Ferulic acid-4-sulphate	3.69 ± 0.53	45.7
Sinapic acid-sulphate	1.50 ± 0.22	45.7
Hydroxyphenylpropionic acid-sulphate	12.94 ± 1.75	42.7
Total hydroxyphenylpropionic and hydroxycinnamic acids	**38.73 ± 1.77**	**14.5**
(Epi)catechin glucuronide-sulphate	0.37 ± 0.08	66.4
(Epi)catechin-glucuronide	1.74 ± 0.15	28.0
(Epi)catechin-sulphate, isomer 1	2.63 ± 0.73	88.0
(Epi)catechin-sulphate, isomer 2	2.44 ± 0.49	62.9
Methyl(epi)catechin-sulphate, isomer 1	0.38 ± 0.07	59.1
Methyl(epi)catechin-sulphate, isomer 2	1.98 ± 0.32	51.2
Total (epi)catechin derivatives	**9.55 ± 1.67**	**55.4**
5-(phenyl)-γ-valerolactone-sulphate-glucuronide	7.52 ± 1.13	47.5
5-Phenyl-valeric acid-sulphate-glucuronide	0.39 ± 0.05	43.4
5-(phenyl)-γ-valerolactone-sulphate-methoxy	0.24 ± 0.03	44.1
5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-glucuronide	22.91 ± 3.51	48.4
5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	99.47 ± 15.35	48.8
5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-sulphate	3.62 ± 1.03	90.2
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone	0.29 ± 0.06	70.0
5-(Hydroxyphenyl)-γ-valerolactone-sulphate isomers	205.05 ± 35.34	54.5
5-Phenyl-γ-valerolactone-3′-glucuronide	14.28 ± 3.52	77.9
5-Phenyl-γ-valerolactone-3′-sulphate	10.37 ± 2.20	67.1
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	0.66 ± 0.09	41.5
Total phenyl-γ-valerolactones and phenyl-valeric acids	**364.80 ± 48.93**	**42.4**
Total phenolic metabolites	**615.13 ± 51.87**	**26.7**
Hippuric acid	953.65 ± 143.22	47.5
4-Hydroxyhippuric acid	88.38 ± 5.49	19.7

Several classes of phenolic compounds were detected, including simple phenols (catechol derivatives), hydroxybenzoic acids, hydroxyphenylpropionic acids, hydroxycinnamic acids, phenyl-valeric acids, phenyl-γ-valerolactones, and epicatechin conjugates (tab. 7). Phenyl-γ-valerolactones were the main compounds excreted in urine, followed by simple phenols and hydroxybenzoic acids, hydroxyphenylpropionic and hydroxycinnamic acids and epicatechins (fig. 4). The maximum excretion of phenyl-γ-valerolactones was found in the 3–6 h urine samples (32.5% of the total excretion).

The maximum excretion of the 9 simple phenols and hydroxybenzoic acids was reached after 6 h from the ingestion of RGPD; the same pattern was observed for phenyl-γ-valerolactones; these metabolites also showed an additional peak after 36 h following RGPD consumption (fig. 4). In addition, a total of 7 hydroxyphenylpropionic acids and hydroxycinnamic acids were identified in urine (tab. 7). The total 0–48 h excretion of these metabolites was 38.7 ± 1.8 μmol, much lower than for phenyl-γ-valerolactones and simple phenols and hydroxybenzoic acids. Finally, a total of 6 phase II epicatechin metabolites were detected in urine (tab. 7) with a total excretion equal to 9.6 ± 1.7 μmol. In contrast to the other phenolic classes, these flavan-3-ol metabolites were excreted more rapidly.

Figure 4. Total 0–48 h excretion of phenolic metabolites after RGPD consumption.



HB = simple phenols and hydroxybenzoic acids; HPP = hydroxyphenylpropionic acids and hydroxycinnamic acids; CAT = epicatechins; VL = phenyl-γ-valerolactones

As regards the pharmacokinetic characteristics, we identified 28 compounds in plasma samples. For each compound, we calculated maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}), area under the plasma concentration-time curve (AUC_{0-24}) and half-life of elimination ($t_{1/2}$) (tab. 8).

Table 8. Pharmacokinetic parameters of phenolic metabolites detected in plasma.

Id.#	Phenolic compounds	Cmax (nM)	Tmax (h)	$t_{1/2}$ (h)	$AUC_{0\text{-}24}$ (nmol h L^{-1})
Simple phenols and hydroxybenzoic acids					
1	Gallic acid	124.3 ± 31.9	3.8 ± 0.7	20.5 ± 9.4	607.2 ± 211.3
2	Vanillic acid-4-glucuronide	61.3 ± 9.0	5.7 ± 0.4	6.2 ± 1.4	410.9 ± 82.2
4	Protocatechuic acid	5.9 ± 1.2	4.1 ± 0.8	40.4 ± 13.7	40.9 ± 7.5
3	Protocatechuic acid-3-glucuronide	3.1 ± 0.6	3.0 ± 0.8	6.7 ± 2.3	20.5 ± 3.8
9	Protocatechuic acid-3-sulphate	408.5 ± 68.7	2.1 ± 0.3	3.8 ± 0.4	1088.6 ± 126.3
10	Benzoic acid-4-sulphate	56.7 ± 9.4	3.0 ± 0.7	37.2 ± 14.6	521.3 ± 76.3
13	Vanillic acid-4-sulphate	117.0 ± 30.2	4.0 ± 0.5	4.8 ± 0.6	381.9 ± 121.7
6	Catechol-sulphate	2.9 ± 1.0	11.2 ± 2.9	6.3 ± 1.2	20.8 ± 7.5
7	Methylpyrogallol-sulphate	512.4 ± 117.5	5.9 ± 0.5	3.6 ± 0.5	2724.2 ± 584.2
8	Methylcatechol-sulphate	47.1 ± 7.6	2.4 ± 0.5	3.3 ± 0.6	184.6 ± 23.6
5	4-Hydroxyhippuric acid	98.7 ± 15.1	4.6 ± 0.5	14.8 ± 4.2	874.0 ± 173.4
Hydroxyphenylpropionic and hydroxycinnamic acids					
12	Ferulic acid 4-glucuronide	72.8 ± 19.9	7.0 ± 2.0	5.7 ± 1.4	567.5 ± 208.1
24	Feruloylglycine	26.0 ± 3.9	9.3 ± 3.3	26.3 ± 4.9	175.1 ± 28.0
23	Dihydrocaffeic acid-sulphate	8.3 ± 1.5	7.0 ± 2.0	40.1 ± 17.0	50.8 ± 19.9
26	Dihydroferulic acid-sulphate	7.7 ± 1.3	8.5 ± 2.7	17.5 ± 6.8	56.4 ± 16.9
28	Ferulic acid-4-sulphate	10.0 ± 1.8	5.2 ± 0.6	17.9 ± 7.0	63.4 ± 16.2
(Epi)catechin derivatives					
11	(Epi)catechin-glucuronide-sulphate	6.8 ± 1.5	4.8 ± 0.6	0.9 ± 0.3	20.0 ± 4.5
20	(Epi)catechin-glucuronide	135.5 ± 14.2	1.7 ± 0.3	2.3 ± 0.4	459.9 ± 44.5
27	(Epi)catechin-sulphate, isomer 1	87.0 ± 22.8	1.6 ± 0.2	1.9 ± 0.4	166.2 ± 31.1
29	(Epi)catechin-sulphate, isomer 2	94.9 ± 22.8	2.5 ± 0.5	2.9 ± 0.5	290.2 ± 50.1
32	Methyl(epi)catechin-sulphate, isomer 1	12.6 ± 1.9	2.7 ± 0.8	8.1 ± 1.3	53.1 ± 10.2
Phenyl-γ-valerolactones and phenyl-valeric acids					
31	5-Phenyl-valeric acid-sulphate-glucuronide	11.0 ± 1.1	7.9 ± 2.0	36.0 ± 14.1	106.2 ± 29.1
17	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-glucuronide	268.4 ± 68.2	5.3 ± 0.6	2.1 ± 0.5	1098.2 ± 219.2
19	5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	1171.2 ± 242.7	5.2 ± 0.6	12.4 ± 8.3	6224.8 ± 1175.4
30	5-(Hydroxyphenyl)-γ-valerolactone-sulphate isomers	893.7 ± 201.3	6.3 ± 2.0	4.8 ± 1.1	5196.3 ± 1457.9
25	5-Phenyl-γ-valerolactone-3′-glucuronide	88.4 ± 44.7	9.1 ± 2.5	11.0 ± 2.7	617.0 ± 178.2
35	5-Phenyl-γ-valerolactone-3′-sulphate	69.2 ± 25.2	11.0 ± 2.9	8.1 ± 1.9	441.0 ± 135.7
21	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	14.3 ± 3.2	7.0 ± 2.0	39.5 ± 26.4	66.1 ± 16.5

(All values are reported as Mean ± SEM, N=10).

Phenyl-γ-valerolactones were the most abundant class of phenolic metabolites in circulation. In particular, the glucuronide- and sulphate-conjugated isomers of 5- (3′,4′-dihydroxyphenyl)-γ-valerolactone (**17**, **19**, and **30**) represented the most abundant compounds, with mean C_{max} ranging from 268 to 1171 nM.

Phenyl-γ-valerolactones peak started after 4 h from the drink ingestion (fig. 5). Their amount decreased progressively 8 h after RGPD consumption, reaching concentration values < 100 nM for all the phenyl-γ-valerolactones after 24 h from the drink intake.

A total of 10 hydroxybenzoic acids and simple phenols were detected in

plasma, being methylpyrogallol-sulphate (**7**) and protocatechuic acid-3-sulphate (**9**) the most representative compounds. The maximum C_{max} of hydroxybenzoic acids and

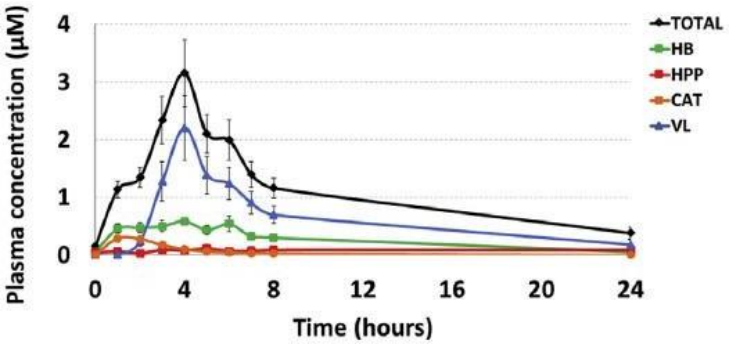
simple phenols varied significantly among metabolites (tab. 8). Some compounds

peaking during the first few hours (**1**, **3**, **4**, **9**, and **10**) showed a second concentration peak around 6–8 h (data not shown).

Regarding hydroxyphenylpropionic acids and hydroxycinnamic acids, 5 compounds were identified in plasma (tab. 8). Ferulic acid-4-glucuronide (**12**) was the most abundant compound, followed by another phase II conjugate of ferulic acid, feruloylglycine (**24**). Most of these compounds had a t_{max} at 5–9 h (tab. 8), although both ferulic acid-4-glucuronide (**12**) and ferulic acid-4-sulphate (**29**) presented a first peak at 1 h. A total of 5 epicatechin derivatives were detected in plasma. The C_{max} of epicatechin conjugates in plasma were around 100 nM, peaking during the first hours after RGPD intake (tab. 8).

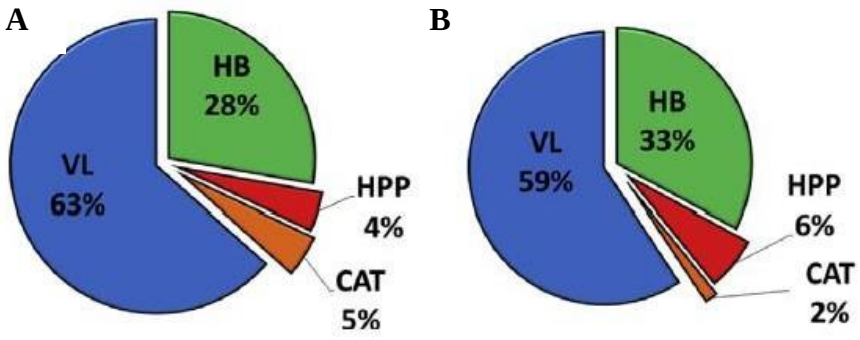
Although some subjects showed a clear pharmacokinetic profile, others did not show a trend associated with the intake and metabolism of phenolic compounds. The relative contribution of the individual classes of phenolic metabolites detected in plasma to the pool of circulating metabolites is shown in figure 6A and it was quite similar to that registered for urine (fig. 6B).

Figure 5. Metabolites detected in plasma.



HB = simple phenols and hydroxybenzoic acids; HPP = hydroxyphenylpropionic acids and hydroxycinnamic acids; CAT = epicatechins; VL = phenyl-γ-valerolactones.

Figure 6. A) Relative plasma AUC_{0-24} calculated for phenolic metabolites by class after RGPD consumption. **B)** 48 h-excretion in urine of phenolic metabolites.

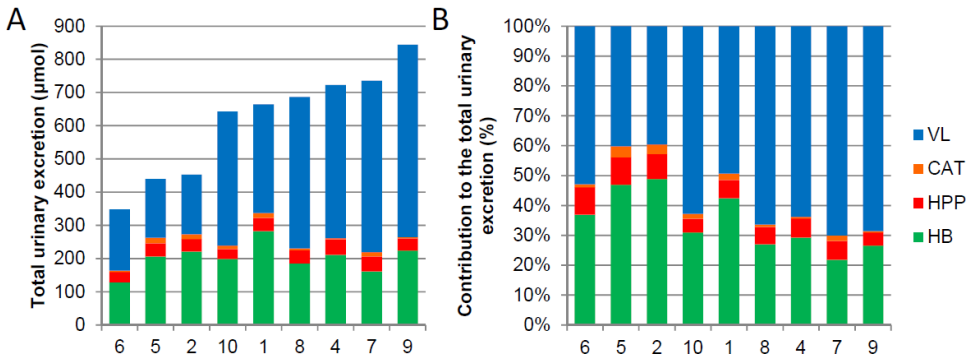


HB = simple phenols and hydroxybenzoic acids; HPP = hydroxyphenylpropionic acids and hydroxycinnamic acids; CAT = epicatechins; VL = phenyl-γ-valerolactones.

In conclusion, we focused on the inter-individual variability in the production and excretion of phenolic metabolites. As reported in table 7, we registered a

high coefficients of variation (CV) in the urinary excretion of phenolic metabolites. The CV% for each metabolite ranged from 20 to 167%, with a mean CV of 52%. Total phenolic compounds urinary excretion varied between 348 and 844 μmol (fig. 7A), the CV being 27% (tab. 7). This inter-individual variability in the urinary excretion was also observed among classes of phenolic compounds (fig. 7A), and entailed notable differences in the qualitative urinary profile of each volunteer, mainly linked to variations in phenyl-γ-valerolactones, hydroxybenzoic acids and simple phenols. (fig. 7B).

Figure 7. A) Cumulative urinary excretion of phenolic metabolites at 48 h. **B)** Relative contribution of each class of phenolic compounds to the total urinary excretion at 48 h.



HB = simple phenols and hydroxybenzoic acids; HPP = hydroxyphenylpropionic acids and hydroxycinnamic acids; CAT = epicatechins; VL = phenyl-γ-valerolactones.

The high inter-variability in the absorption and excretion of phenolic derivatives observed in plasma and urine samples is a key point point to be considered.

Future investigations should focus both on the causes behind the observed

inter- individual variability and how these differences may impact the putative health properties of this drink.

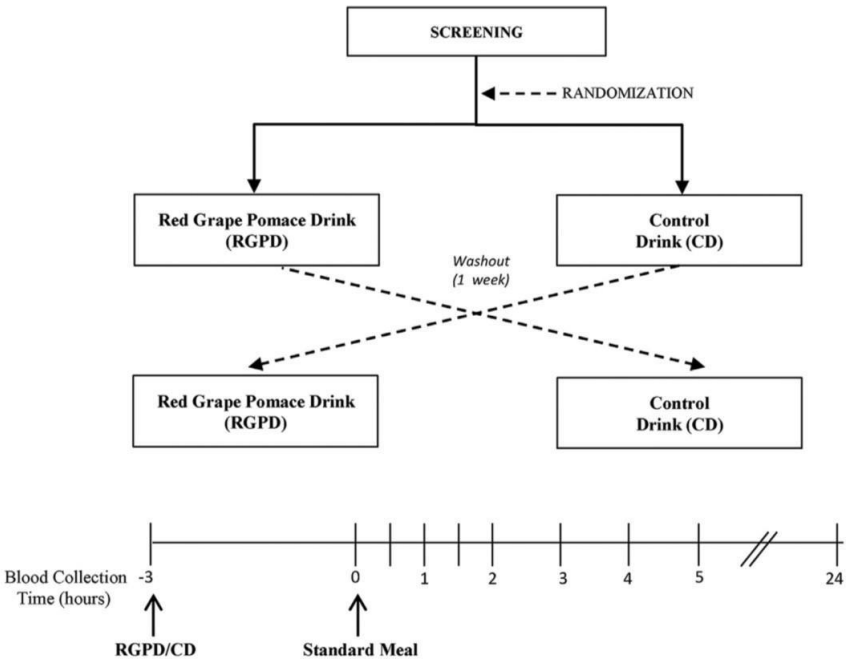
4.2 Acute metabolic effects of the consumption of a phenolic-rich drink made from red grape pomace in healthy individuals

Studies in both animals and humans focusing on the effects of grape polyphenols on glucose and insulin metabolism demonstrated that the supplementation of grape polyphenols was able to reduce plasma glucose concentration [14,15]. An improvement in glucose metabolism was also observed after a prolonged supplementation of grape polyphenols in people with the metabolic syndrome and type 2 diabetes [16–18]. However, so far, a detailed analysis of the metabolic pathways of the polyphenols involved in the modulation of glucose metabolism and insulin action is not available yet. In fact, since most of the studies investigating the effects of grape polyphenols on clinical outcomes in humans have not measured their metabolites, the relation between the polyphenol metabolic pathways and their clinical effects remains unclear. Thus, we performed a pilot study to evaluate the acute effects of the consumption of a RGPD on glucose/insulin and lipid responses to a standard meal in healthy individuals. In addition, we assessed the relationship between circulating phenolic metabolites, reported in the previous section, and the main processes regulating glucose metabolism, i.e., insulin secretion and insulin sensitivity.

The characteristics of participants are reported in the section above. Participants were asked to consume a low-polyphenol diet during the experimental period. The study was conducted with a randomized, controlled, cross-over design (fig. 8). On the two experimental days, at one-week interval,

participants were admitted to the research center after a 12 h overnight fast, for baseline blood drawing; thereafter, they consumed in random order 250 mL of a red grape pomace drink [RGPD; 24 g of soluble carbohydrates, 92 kcal and 1562 g of total polyphenols, as gallic acid equivalents (GAE)] or 250 mL of a control drink without polyphenols (CD; 24 g of soluble carbohydrates, 92 kcal). Randomization was made by a web-based program. Three hours after RGPD or CD consumption, participants consumed a standard low-polyphenol meal, consisting of white bread (150 g), fatless ham (70 g), spreadable cheese (80 g) and plumcake (33 g) (903 kcal, 18% protein, 30% fat, 52% carbohydrates). At the end of the test day, participants received another standard meal (pasta omelet) (905 kcal, 14% protein, 46% fat, 40% carbohydrates) to be consumed at 9.00 p.m. for dinner.

Figure 8. Study design.



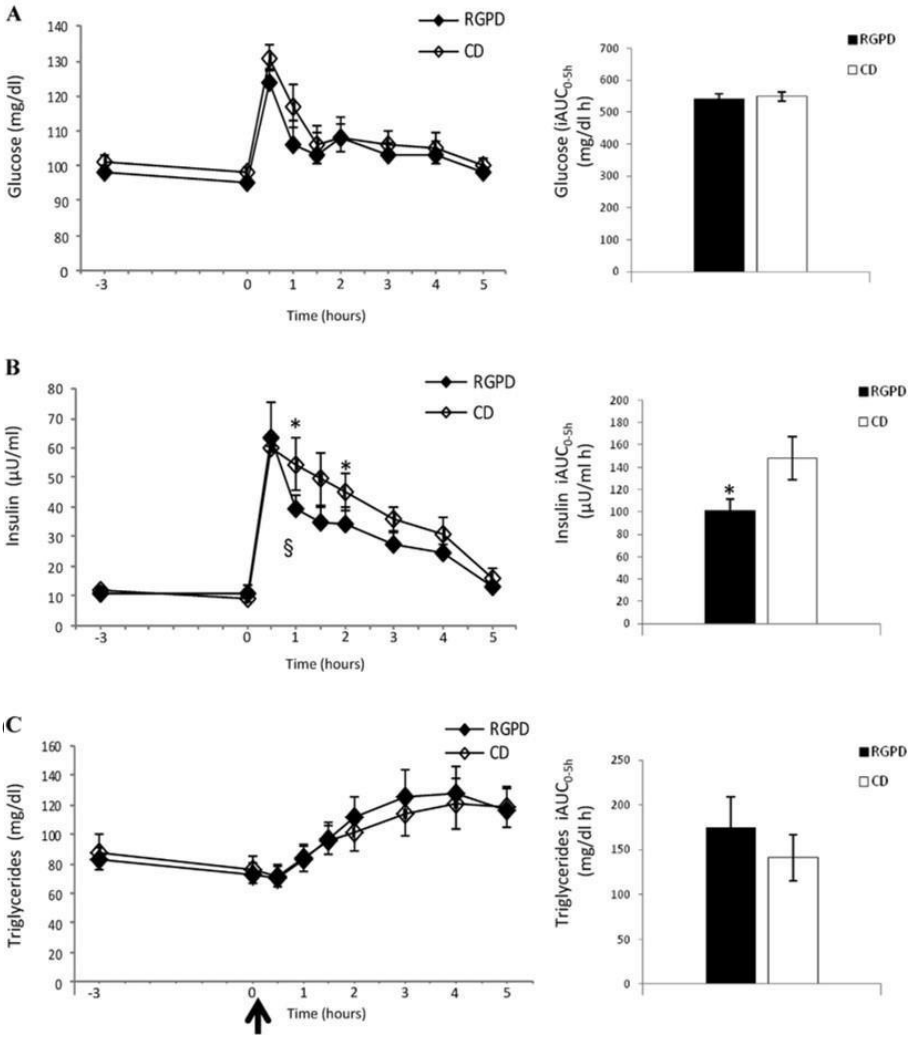
The polyphenol composition of the RGPD has been reported in table 6.

Glucose, insulin and triglyceride levels at fasting and in response to the standard meal and the corresponding iAUC were analized (fig. 9).

On the two experimental days, there were no differences in the fasting levels of glucose, insulin and triglycerides. After RGPD or CD consumption, the post-meal glucose response was not different (p = 0.299, drink effect; repeated measures ANOVA), evaluated either at any time point of the curve or as iAUCs [540 ± 16 *vs* 549 ± 15 mg/dl x 5 h, respectively; p = 0.903] (fig. 9A). Conversely, post-meal insulin levels were lower after RGPD than after CD consumption (p = 0.025), drink effect; repeated measures ANOVA), reaching a statistically significant difference at 1 and 2 h after the meal (p < 0.05); consistently, the corresponding iAUCs was significantly lower after RGPD than CD [102 ± 10 *vs* 148 ± 19 µU/ml x 5 h (M±SEM), respectively; p = 0.036] (fig. 9B).

Post-meal plasma triglyceride levels were not different after consuming RGPD or CD (p = 0.675, meal effect; repeated measures ANOVA), either at any timepoint of the curve or as iAUCs [173 ± 35 *vs* 141 ± 26 mg/dl x 5 h, (M±SEM), respectively; p = 0.176] (fig. 9C).

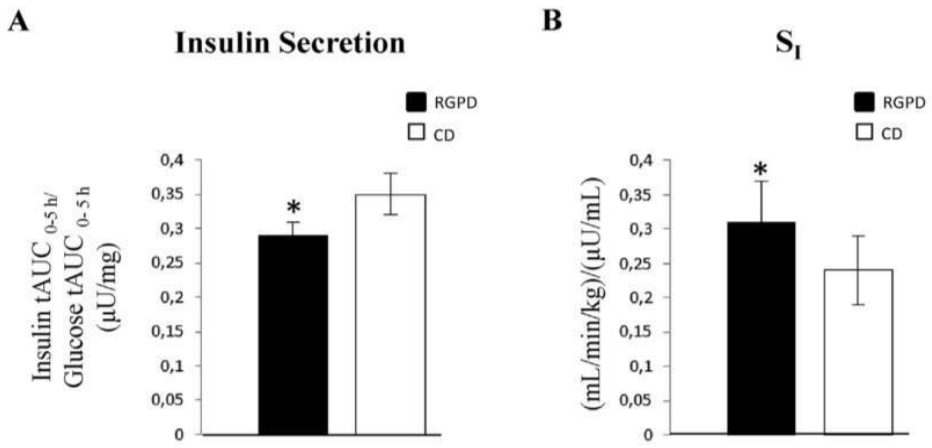
Figure 9. Postprandial plasma glucose **(A)** insulin **(B)** and triglycerides **(C)** concentrations and corresponding incremental area under the curve (iAUC) (means ± SEM) after the intake of the Red Grape Pomace Drink (RGPD) and Control Drink (CD) in young volunteers.



Insulin sensitivity index (S_I), over 2 h after the meal, was significantly higher (+36%) after RGPD as compared to CD intake [0.34 ± 0.06 *vs.* 0.25 ± 0.06 mL/min/kg/µU/mL, respectively; $p = 0.037$] (fig.10). Moreover, the insulin secretion index calculated over 5 h after the standard meal was significantly lower (-18%) after RGPD compared to CD consumption [0.29 ± 0.03 *vs.* 0.35 ± 0.04 µU/mg, respectively; $p = 0.016$] (fig. 10). Twenty-four hours after

the test day, fasting glucose, insulin and triglyceride concentrations were similar after consumption of RGPD or CD (glucose: 97 ± 2 *vs* 98 ± 2 mg/dl; insulin: 11 ± 2 *vs* 12 ± 1 µU/ml; triglyceride: 81 ± 8 *vs* 79 ± 12 mg/dl; (M±SEM), respectively).

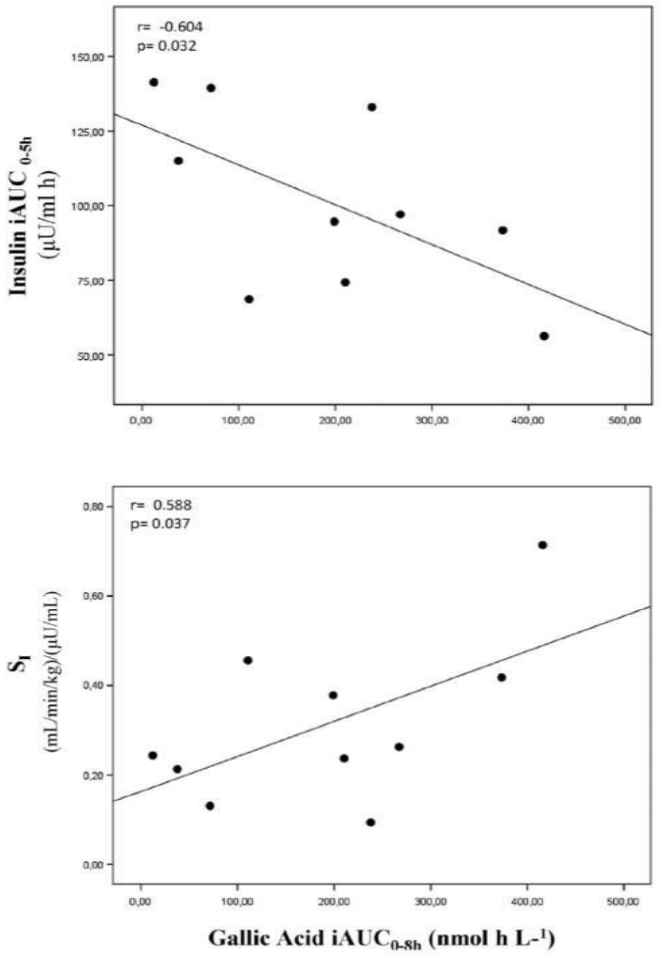
Figure 10. Insulin Secretion **(A)** and Insulin Sensitivity **(B)** Indices (means±SEM) after the intake of the Red Grape Pomace Drink (RGPD) and Control Drink(CD) in young volunteers.



Moreover, we analyzed the correlation between the plasma concentration of phenolic metabolites (tab. 8) and the metabolic parameters. Interestingly, we found that plasma gallic acid concentration (Gallic Acid $iAUC_{0\text{-}8h}$) was the only phenolic metabolite being inversely correlated with post-meal plasma insulin response (Insulin $iAUC_{0\text{-}5h}$) ($r = -0.604$, $p = 0.032$) after RGPD consumption, and positively correlated with the Insulin Sensitivity Index (S_I) ($r = 0.588$, $p = 0.037$) (fig. 11). No other statistically significant correlation was found between any of the phenolic metabolites and insulin secretion and insulin sensitivity indices. Stepwise linear regression, using the S_I index as dependent variable and plasma phenolic metabolites as independent variables,

showed that gallic acid was the best predictor of S_I index (β = 0,707, p = 0.033), followed by epicatechin- glucuronide (β = 0,274, p = 0.001) and dihydrocaffeic acid-sulphate (β = 0,089; p = 0.015).

Figure 11. Correlation between plasma gallic acid concentration ($iAUC_{0e8h}$) and Postprandial Insulin Response ($iAUC_{0e5h}$) and Insulin Sensitivity Index (S_I).



These findings indicate that the polyphenols contained in the RGPD, consumed away from meal, are able to improve insulin sensitivity and reduce insulin secretion; this effect is likely mediated by the increase in plasma levels

of gallic acid.

4.3 Effects of grape polyphenols consumption on lipid metabolism

To date, the effects of grape polyphenols on lipid metabolism are less clear [98,107], with some studies demonstrating a significant lipid-lowering effect [70,71,77]and others failing to find any change in lipid profile [75,76,108]. Therefore, we conducted a meta-analysis of intervention cohort studies to assess the effect of grape products on lipid profile, i.e. TC, HDL-C, LDL-C, TG and main Apo concentrations taking into account some possible confounders.

A total of 24 articles (25 datasets, 618 subjects assigned to grape juice/extracts/products administration and 587 subjects assigned to placebo) were included in the analysis [16,17,107,109–117,18,118–120,46,80,90,98,99,103,105]. In detail, 21 studies (22 data-sets) reported data on TC, 22 studies (23 data-sets) on HDL-C, 20 studies (21 data-sets) on TG, 20 studies (21 data-sets) on LDL-C, 4 studies (4 data-sets) on oxLDL-C, 2 studies (2 data-sets) on apo A, 3 studies (3 data-sets) on apo B.

All included studies were RCTs; the major characteristics of the studies analyzed are shown in table 9.

Table 9. Characteristics of the studies included.

Author	Study design	Population (n)	Follow-up (weeks)	Type of grape product	Type of control	Reported outcomes	Age (years)	Male gender (%)
Argani 2016	RCT-parallel double-blind	70 mild to moderate hyperlipidemia	8	SE	placebo	TC, HDL-C, TG, LDL-C, apo A	47,0	32,7
Banini 2006	RCT-parallel open	23 T2DM	4	J	no supplement	TC, HDL-C, TG, LDL-C	53,9	47,6
Dohadwala 2010	RCT-crossover double-blind	64 preHT/stage 1 HT	8	J	placebo	TC, HDL-C, TG, LDL-C	42,6	68,8
Han 2016	RCT-parallel double-blind	50 healthy subjects	10	WG	placebo	apo B	NA	47,9
Hansen 2005	RCT-parallel double-blind	35 healthy subjects	4	WG	placebo	TC, HDL-C, TG, LDL-C	52,0	45,6
Hollis 2009	RCT-parallel open	50 healthy subjects	12	J	no supplement	TC, HDL-C, TG, LDL-C	25,0	NA
Jiménez 2008	RCT-parallel open	43 non-smokers	16	WG	no supplement	TC, HDL-C, TG, LDL-C	35,3	37,2
Kar 2009	RCT-crossover double-blind	32 T2DM	4	SE	placebo	TC, HDL-C, TG	62,0	50,0
Martínez-Maqueda 2018	RCT-crossover open	98 subjects with MetS**	6	WG	no supplement	TC, HDL-C, TG, LDL-C	42,6	55,1
Mellen 2010	RCT-crossover double-blind	50 subjects with CAD or ≥1 CV risk factor	4	SE	placebo	TC, HDL-C, TG, LDL-C	52,1	50,0
Millar 2018	RCT-crossover double-blind	40 subjects with MetS	4	WG	placebo	TC, HDL-C, TG	53,5	60

Park 2009	RCT-parralel double-blind	40 healthy subjects	8	J	placebo	TC, HDL-C, TG, LDL-C	44,4	100,0
Preuss 2000	RCT-parallel double-blind	19 subjects with hyperlipidemia	8	SE	placebo	TC, HDL-C, LDL-C	NA	NA
Sano 2007	RCT-parallel single-blind	35 subjects with hyperlipidemia	12	SE	placebo	TC, HDL-C, TG, LDL-C, apo A, apo B	53	47,5
Siasos 2013	RCT-crossover double-blind	26 healthy smokers	2	J	placebo	TC, LDL-C	26,3	38,5
Sivaprakasapillai 2009	RCT-parallel double-blind	18 subjects with MetS	4	SE	placebo	TC, HDL-C, TG, LDL-C, oxLDL-C	46,5	38,5
Taghizadeh 2016	RCT-parallel double-blind	40 healthy females	8	SE	placebo	TC, HDL-C, TG, LDL-C	20,7	0,0
Tomé-Carneiro 2012	RCT-parallel triple-blind	50 T2DM or hyperlipidemia under statins	24	WG	placebo	TC, HDL-C, TG, LDL-C, oxLDL-C, apo B	59,5	54,0
Urquiaga 2015	RCT-parallel open	38 male with MetS*	16	WG	no supplement	HDL-C, TG	44,0	100,0
Vaisman 2015	RCT-parallel double blind	32 heterogeneous§	12	WG	placebo	TC, HDL-C, TG, LDL-C	57,0	74,4
Vigna 2003	RCT-crossover double-blind	24 healthy males heavy smokers	4	WG	placebo	TC, HDL-C, TG, LDL-C	54,0	100,0
Yubero 2013	RCT-parallel double-blind	60 healthy subjects	8	SK	placebo	TC, HDL-C, LDL-C, oxLDL-C	51,0	NA
Zern 2005	RCT-crossover single-blind	44 pre-/post-menopausal women	4	WG	placebo	TC, HDL-C, TG, LDL-C	39,7 (premenopausal) 58,5 (postmenopausal)	0,0

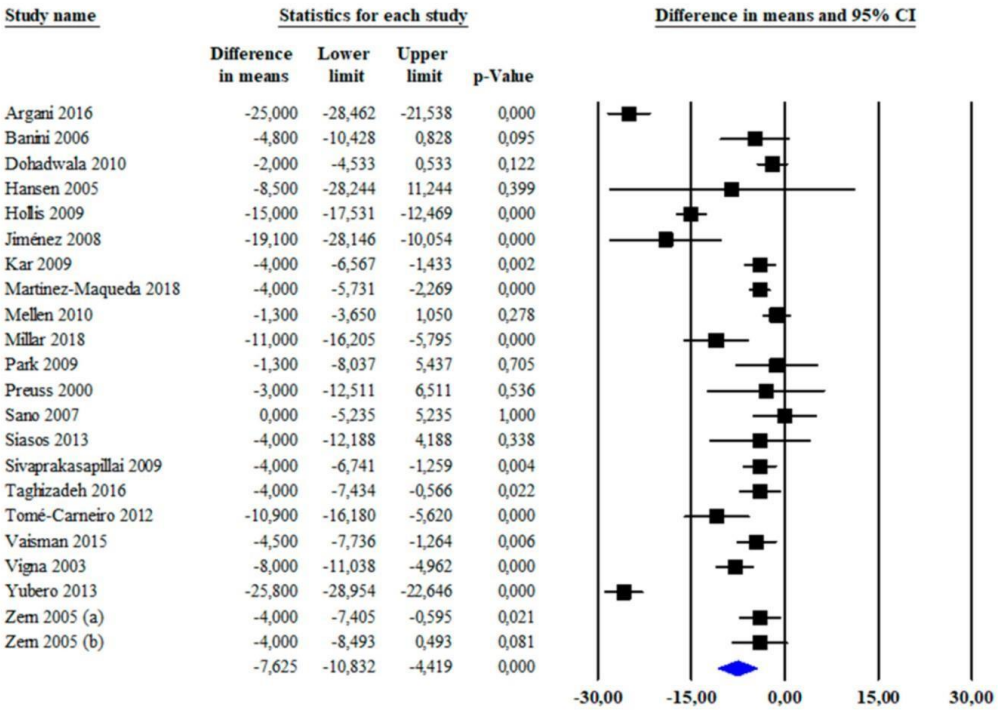
Zunino 2014	RCT-crossover double-blind	24 obese	3	WG	placebo	HDL-C, TG, LDL-C, oxLDL-C	36,0	33,3

SE: grape seed extract; J: grape juice; WG: whole grape product; SK: grape skin extract; TC: total cholesterol; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; TG: triglycerides; oxLDL-C: oxidized low-density lipoprotein cholesterol; apo A: apolipoprotein A; apo B: apolipoprotein B; NA: not available; T2DM: type 2 diabetes mellitus; HT: hypertension; MetS: metabolic syndrome.* at least one MetS criteria; **at least two MetS criteria; §35–70 years, BMI <40 kg/m^2, systolic blood pressure <154 mmHg and diastolic blood pressure <93 mmHg.

Eleven studies evaluated the effect of whole grape products (grape powder or pomace) [16,90,120,98,99,103,109,111,112,114,116], 5 studies the grape juice [18,80,105,113,117], 7 studies grape seed extracts [17,46,83,107,110,115,118] and 1 study the effect of grape skin extracts [119].

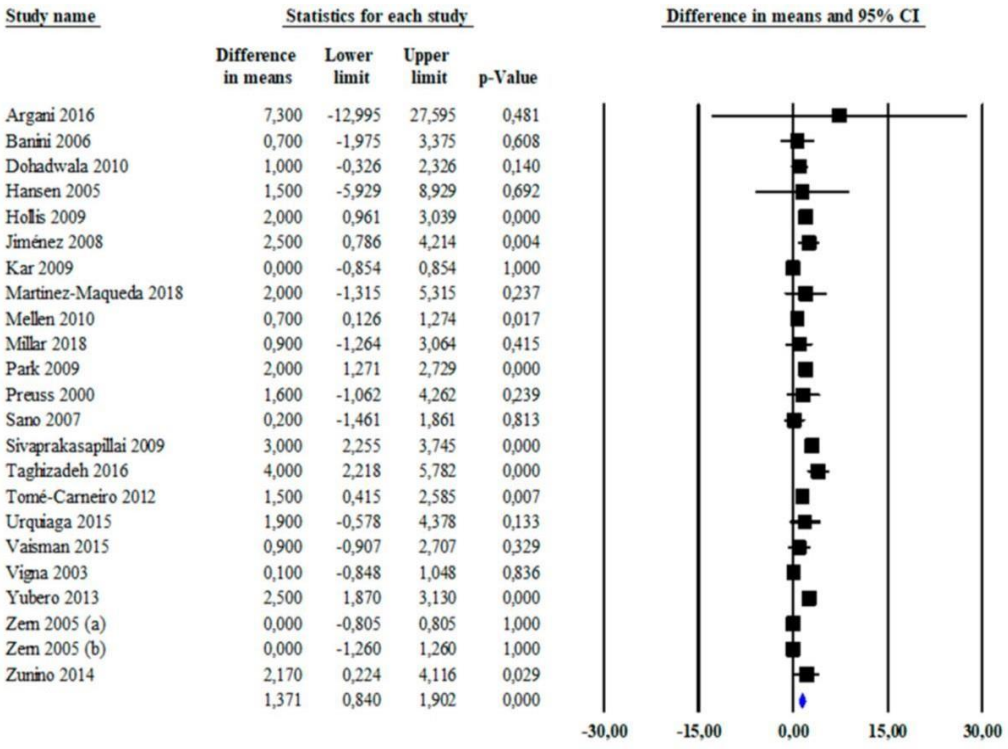
We found a greater reduction in TC levels after administration of grape products as compared with placebo (MD: -7.6 mg/dl [-0.2 mmol/l]; 95%CI: -10.8, -4.4; p<0.001) (fig. 12). Heterogeneity among studies was significant (I^2=94.3%, p<0.001) and no reduction in the overall heterogeneity was found after excluding one study at the time. In the intervention group, we found a trend toward a significant decrease in TC levels after supplementation with grape products as compared with pre-intervention TC levels (MD: -5.0 mg/dl [-0.13 mmol/l]; 95%CI: -10.2, 0.1; p=0.057, I^2=41.9%, p=0.021).

Figure 12. Change in TC levels after administration of grape products as compared with controls.



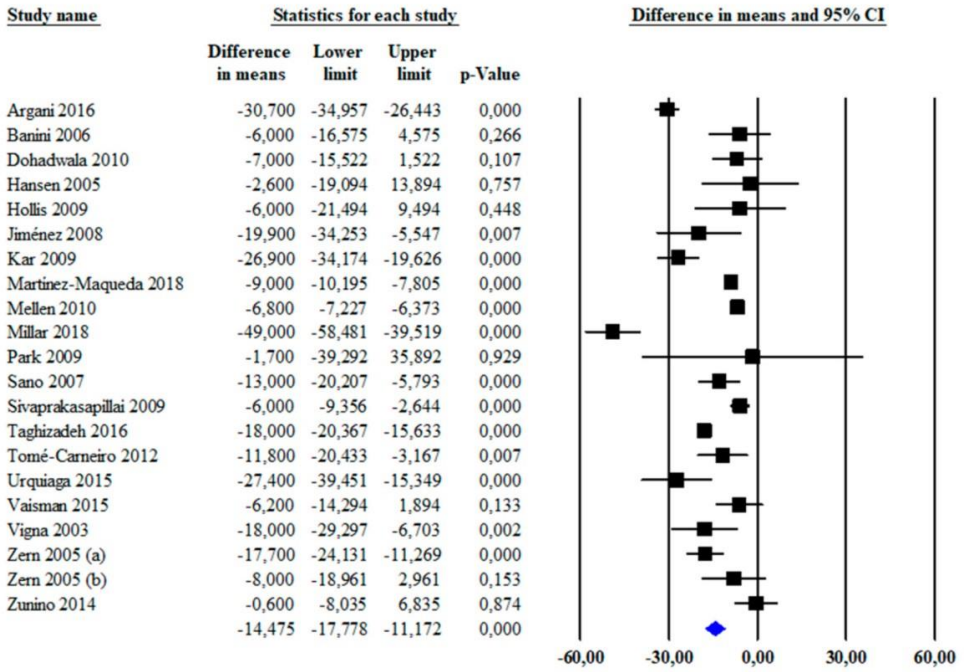
Although administration of grape products resulted in a more significant increase in HDL-C levels as compared with placebo (MD: 1.4 mg/dl [0.04 mmol/l]; 95%CI: 0.8, 1.9; $p < 0.001$, I^2=74.7%, $p < 0.001$) (fig. 13), no significant change in HDL-C was found in the intervention group between pre- and post- supplementation levels (MD: 0.9 mg/dl [0.02 mmol/l]; 95%CI: -0.3, 2.1; p=0.122, I^2=0%, p=1.000).

Figure 13. Change in HDL-C levels after administration of grape products as compared with controls.



We observed a greater decrease in TG levels after supplementation with grape products as compared with placebo (MD: -14.5 mg/dl [-0.16 mmol/l]; 95%CI: - 17.7, -11.2; p<0.001) (fig. 14). Heterogeneity among these studies was significant (I^2=94.1%, p<0.001) and no reduction in the overall heterogeneity was found after excluding one study at the time. However, we found no significant changes in TG levels between pre- and post-supplementation in the intervention group (MD: 0.60 mg/dl [0.01 mmol/l]; 95%CI: -2.0, 3.2; p=0.654, I^2=0%, p=0.881).

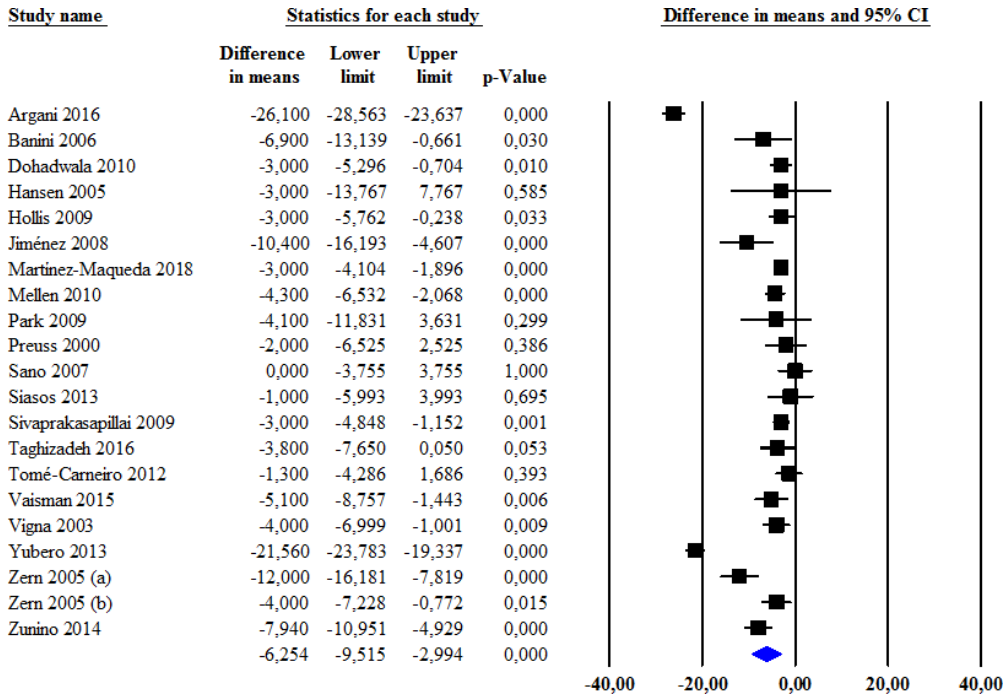
Figure 14. Change in TG levels after administration of grape products as compared with controls.



Meta-regression models showed that an increasing age was associated with a less significant improvement in HDL-C after grape products supplementation as compared with placebo (Z-value: -2.58, p = 0.001). None of the other clinical and demographic variables influenced differences in changes in TC, HDL-C, TG.

A significantly greater reduction in LDL-C levels was observed after administration of grape products as compared with placebo (MD: -6.3 mg/dl [-0.16 mmol/l]; 95%CI: -9.5, -3.0; p<0.001) (fig. 15). Heterogeneity among studies was significant (I^2=96.3%, p<0.001) and no reduction in the overall heterogeneity was found after excluding one study at the time.

Figure 15. Changes in LDL-C levels after administration of grape products as compared with controls.



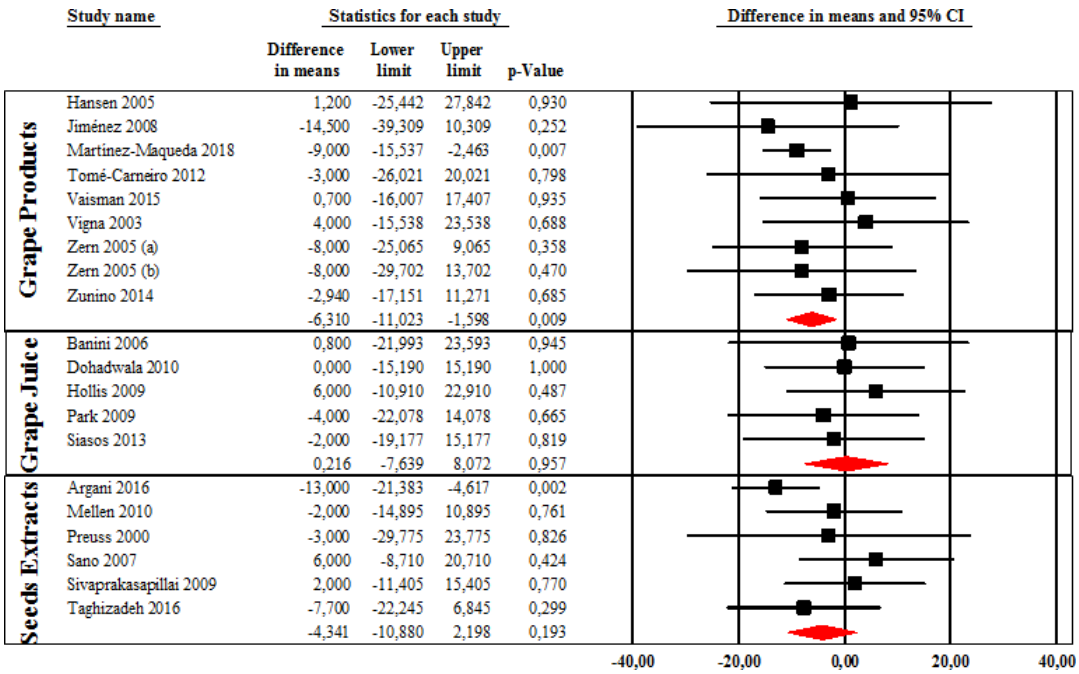
Meta-regression analysis showed that none of the evaluated clinical and demographic variables influenced differences in changes in LDL-C after grape products supplementation as compared with placebo.

In the intervention group, we found a significant decrease in LDL-C after supplementation with grape products as compared with pre-intervention LDL-C levels (MD: -5.6 mg/dl [-0.14 mmol/l]; 95%CI: -9.5, -1.7; p=0.005) with a non- significant heterogeneity (I^2=29.1%, p=0.105). Stratifying the population according to grape product types, we found a significant reduction in LDL-C levels in subjects taking whole grape products (MD: -6.3 mg/dl [-0.16 mmol/l]; 95%CI: -11.0, -1.6; p=0.009, I^2=0%, p=0.901) while no significant difference in LDL-C was found after the supplementation with grape seeds extracts (MD: -4.3 mg/dl [-0.11 mmol/l]; 95%CI: -10.9, 2.2;

p=0.193, I^2=28.9%, *p*=0.218) and with grape juice (MD: 0.21 mg/dl [0.01 mmol/l]; 95%CI: -7.6, 8.1; *p*=0.957, I^2=0%, *p*=0.948) (fig. 14). Only 1 study [119] reported a significant reduction in LDL-C after consumption of grape skin extracts. Moreover, analyzing data according to polyphenol content of the supplements used in each study, we found a significant reduction in LDL-C levels in subject taking supplements with a polyphenol content >400 mg/die (MD: -5.8 mg/dl [-0.15 mmol/l]; 95%CI: -10.7, -0.8; *p*=0.022, I^2=0%, *p*=0.662), but not in those receiving supplements with a lower polyphenol content (MD: -4.3 mg/dl [-0.11 mmol/l]; 95%CI: -11.9, 3.2; *p*=0.260, I^2=57.2%, *p*=0.013).

A significantly greater reduction in *ox*LDL-C levels was observed after administration of grape products as compared with placebo (MD: -4.5 U/l; 95%CI: -7.5, -1.5; *p*=0.003, I^2=90.6%, *p*<0.001), as well as after supplementation as compared to baseline values (MD: -5.0 U/l; 95%CI: -8.8, -1.2; *p*=0.010, I^2=0%, *p*=0.470).

Figure 13. Changes in LDL-C levels in subjects taking whole grape products, grape juice and grape seeds extracts.



Finally, we found no significant change in apo A levels both considering administration of grape products vs placebo (MD: 7.7 mg/dl [2.8 µmol/l]; 95%CI: -7.5, 22.9; p=0.320 - I^2=97.7%, p<0.001) and considering pre- vs post-supplementation levels (MD: 7.1 mg/dl [2.5 µmol/l]; 95% CI: -0.2, 14.4; p=0.055 - I^2=0%, p=0.361).

A significant reduction in apo B levels was found after administration of grape products as compared with placebo (MD: -2.4 mg/dl [-0.05 µmol/l]; 95% CI: -4.5, -0.3; p=0.026) with a non-significant heterogeneity among studies (I^2=62.8, p=0,068). However, in the intervention group, no significant change in apo B was found after supplementation with grape products as compared with pre- supplementation levels (MD: -3.2 mg/dl [-0.06 µmol/l]; 95%CI: -8.2, 1.9; p=0.218) without heterogeneity among studies (I^2=0%, p=0.993).

Overall, the results of this meta-analysis show that the administration of grape

products is associated with a significant improvement of lipid profile, as evidenced by changes in TC, HDL-C, LDL-C, oxLDL-C, apo B and TG levels in subjects receiving grape products compared to placebo.

5. Discussion and Conclusion

The available evidence supports the efficacy of grape polyphenols intake on several cardiometabolic risk factors. However, polyphenol bioavailability in humans is not well understood, the inter-individual variability in the production of phenolic metabolites has not been comprehensively assessed and the specific role of phenolic metabolites in the protection against several human chronic diseases is still little known.

- In our first study investigating the pharmacokinetic and excretive profiles of phenolic metabolites after the acute administration of a drink made from red grape pomace, a total of 35 and 28 phenolic metabolites were quantified in urine and plasma, respectively. Our results showed that the main circulating metabolites included phenyl-γ-valerolactones, hydroxybenzoic acids, simple phenols, hydroxyphenylpropionic acids, hydroxycinnamates, and (epi)catechin phase II conjugates. Moreover, a high inter-individual variability was shown both in urine and plasma samples, and different patterns of circulating metabolites were observed by applying a multivariate analysis. Besides the huge variability in the production of metabolites of colonic origin, an important variability was observed due to phase II conjugates. These results are of interest to further understand the potential health benefits of phenolic metabolites on individual basis.

- In the other one study, our aim was to evaluate the metabolic effects related to the consumption in acute conditions of the experimental RGPD, rich in polyphenols. Our resuts showed a significative reduction of post-meal insulin response and an improvement of insulin sensitivity after the RGPD intake, compared to control drink (CD) intake. Indeed, $iAUC_{0\text{-}5h}$ was 31% lower and the SI index was 36% higher after the RGPD consumption compared to CD. Similarly, insulin secretion index, calculated over 5 h after the standard meal, was 18% lower. Insulin sensitivity and insulin secretion have long been acknowledged as the main processes regulating glucose homeostasis. They are linked by a close and inverse relationship and, therefore, changes in one process produce adaptation of the other one. Based on this notion, the lower post-meal insulin secretion observed in our study after the consumption of the RGPD drink should be interpreted as a compensatory reciprocal change induced by the improvement in insulin sensitivity following RGPD. The fact that post-meal glucose response was similar after both RGDP and the control drink despite a different insulin response, further supports an improvement of insulin action. The finding that RGPD is associated with a lower post-meal insulin response has important clinical implications in the light of previous studies showing that postprandial hyperinsulinemia is a risk factor for the development of T2D and cardiovascular diseases. An interesting observation of our research is that post-meal levels of gallic acid were inversely correlated with post-meal plasma insulin response and positively with SI index, suggesting that the improvement of insulin sensitivity and the reduction of insulin secretion could be mediated by the

increase in plasma levels of gallic acid.

- The latest study aimed to evaluate the relationship between the intake of grape products and lipid levels. We did a meta-analysis of current evidence on this topic. Our results showed that the administration of grape products is associated with a significant improvement of lipid profile, as evidenced by changes in TC, HDL-C, LDL-C, oxLDL-C, apo B, and TG levels in subjects receiving grape products compared to placebo. With regard to the extent of the lipid-lowering effect, the reduction was −5.6 mg/dL (−0.14 mmol/L) for LDL-C and −5.0 U/L for *ox*LDL-C.

Although the magnitude of the effect is not impressive in absolute terms, it may still be noteworthy for the prevention of CV diseases on a population basis. Indeed, for each 1% decrease in LDL-C, there is a 1% decrease in cardiovascular event rate. Of particular clinical relevance is also the reduction in *ox*LDL-C, an important player in the atherosclerotic process.

To the best of our knowledge, this is to date the largest meta-analysis evaluating the relationship between grape products intake and lipid profile.

Meta-regression analysis showed that the presence of metabolic diseases, such as diabetes, obesity, and dyslipidemia, did not affect the differences in the changes of the lipid fractions, suggesting that the benefits of grape supplementation take place in individuals with or without metabolic diseases.

A critical factor to be considered in examining the currently available literature on grape polyphenols and cardiovascular benefits relates to the dose of grape product supplementations. In fact, the dose was quite variable among the studies analyzed, ranging from 22.4 to 2370 mg/day; thus, it cannot be excluded that in some studies, the dose of grape supplementation was too low

to exert measurable effects, which could erroneously lead to the conclusion of negative results. The importance of the dose clearly emerges from our study; in fact, the subgroup analyses showed that the reduction in LDL-C reached statistical significance when the daily grape polyphenols supplementation was >400 mg/day. This finding should be taken into account in supporting clinical recommendations as well as in designing future intervention studies.

We evaluated also the individual impact of various grape products on lipid profile and we found a favorable effect of whole grape products, while no significant change in plasma lipid levels was observed after supplementation with grape seed extracts and grape juice. This finding could be explained by the fact that whole grape products include skin, pomace, and seeds, which supply a mixture of polyphenol compounds with a potential synergistic effect on lipid metabolism. From a clinical point of view, the results of this study support the nutraceutical- based approach as a useful complement to nutritional and pharmacological therapies to improve lipid profile, as stated in recently published guidelines.

In conclusion, the intake of a drink prepared from red grape pomace can deliver significant amounts of different phenolic metabolites to the human system.

In addition, polyphenols from a RGPD, consumed away from meal, improve insulin sensitivity and reduce insulin secretion. Furthermore, these effects likely mediated by the increase in plasma levels of gallic acid.

For these reasons, our experimental drink could be included in the usual diet and could contribute to increase the daily intake of polyphenols with potential

health benefits. Notably, the sugar content of our RGPD is very low compared with other fruit juices, especially those based on grapes; this characteristic represents a further health advantage, particularly for individuals with abnormal glucose metabolism. However, further intervention studies are needed to better clarify the impact of grape polyphenols and their metabolites on the metabolic parameters related to type 2 diabetes risk.

Finally, grape polyphenols exert a favorable effect on lipid profile in humans by significantly reducing plasma levels of LDL-C and oxLDL-C. Additional trials specifically in patients with dyslipidemia or diabetes mellitus are required to confirm this finding.

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