

UNIVERSITY OF NAPLES – FEDERICO II

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**Ph.D. THESIS IN
“PHARMACEUTICAL SCIENCE”**

**Nutraceutical potential of vegetal food products
typical of Campania region: Mela Annurca
Campana IGP**

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ABSTRACT

The nutraceutical potential of a specific apple cultivar, “Annurca”, was evaluated by both *in vitro* and *in vivo* studies. Particularly, the effects of Annurca polyphenolic extract on lipid uptake and metabolism were evaluated and compared with those obtained from polyphenolic extracts coming from other conventional cultivars.

In vitro experiments performed on HepG2 (human hepatocellular liver carcinoma) cells indicated Annurca flesh polyphenolic extract as the most active in reducing cholesterol and triglyceride uptake (about, 15 and 20 times than control cells, respectively), in inhibiting lipase activity (-55% than control cells), in enhancing low-density lipoprotein (LDL) receptor binding activity (+46% than control cells), in increasing Apolipoprotein A1 (ApoA1) cell expression (+71.4% above control levels) and in protecting from oxidative stress (cell proliferation: +47.5%; cell radical levels: -33.3% than control cells). All the amazing *in vitro* properties of Annurca flesh polyphenolic extract were attributed to its high content in procyanidins, and good linear correlations between procyanidin content and cellular effects were registered.

In vitro experiments of gastrointestinal digestion showed a good bioaccessibility of apple procyanidins during the three digestive steps. After salivary digestion, their bioaccessibility was 35% of undigested samples, suggesting a potential good absorption through the oral mucosa, and it increased during gastric (+5.1% compared to salivary digestion) and intestinal (+16% in respect to gastric digestion) phases. However, their bioavailability was very low (6.7% of their native pattern and 12.0% of the intestinal digestion), suggesting they would act mainly in the gut. The small portion absorbed was strongly bound to plasma proteins (93.4%), especially to high-density lipoproteins (HDLs, 58.7%), suggesting a role in cholesterol metabolism.

The results of the first clinical trial showed that Annurca apple was the most effective, since it decreased total cholesterol (TC, -8.4%) and low-density lipoprotein cholesterol (LDL-C, -14.5%) levels, and raised high-density lipoprotein cholesterol levels (HDL-C, +14.0%).

The results of the second clinical trial showed that the treatment with AppleMetSTM (AMS) resulted in a significant decrease in plasma LDL-C levels (-37.5%), an effect comparable to that obtained after treatment with 40 mg of simvastatin or 10 mg of atorvastatin. Moreover, it increased HDL-C levels of about 49.2%, an effect never observed in any pharmaceutical or nutraceutical substance. These amazing results suggested that Annurca polyphenols represent an important tool for the treatment of mild hypercholesterolemia, especially in elderly people with higher values of baseline total cholesterol.

RESEARCH OBJECTIVES

The evidence that the regular consumption of apples leads to beneficial health effects, especially to a reduction of CVD (cardiovascular disease) risk, encouraged to deepen the nutraceutical potential of the polyphenolic fraction of a specific apple cultivar, “Annurca”, by both *in vitro* and *in vivo* studies, and to compare its effects on lipid uptake and metabolism with those obtained from polyphenolic extracts of other conventional cultivars. In fact, dyslipidemia is a pathological imbalance of circulating lipoproteins, such as LDLs (low-density lipoproteins), VLDLs (very low-density lipoproteins) and HDLs (high-density lipoproteins) and it is considered as a major risk factor for the development of cardiovascular diseases (Rosenson et al., 2013).

The research project was structured as follows:

- qualitative and quantitative profiling of phenolic compounds contained in the various apple cultivars by HPLC-DAD/ESI-MS analysis;
- determination of the effects on metabolic parameters (glucose and lipid uptake, cell physiological oxidative stress and hepatic lipid metabolism) by experiments performed on HepG2 (human hepatocellular liver carcinoma) cells;
- *in vitro* definition of some pharmacokinetic parameters (bioaccessibility, bioavailability and plasma protein interaction);
- determination of plasma lipid profile after the regular consumption of different apple cultivars (functional foods) or of a nutraceutical consisting of Annurca polyphenols (AppleMetSTM) by healthy subjects afflicted by mild hypercholesterolemia.

INTRODUCTION

The word “nutraceutical” was coined in 1989 by Stephen DeFelice, founder and chairman of the Foundation of Innovation in Medicine (FIM), and it derived from the crasis of the terms “nutrition” and “pharmaceutical”. A nutraceutical can be defined as “a food or a part of a food that provides medical or health benefits, including the prevention and/or the treatment of a disease” (Kalra, 2003).

Functional foods are food and food derivatives that provide beneficial health effects in addition to their basic nutritional properties (Menrad, 2003). Functional foods are often enriched or fortified with bioactive substances through a process called nutrification. Some important examples of functional foods are represented by enriched cereals, breads, sport drinks, fortified snack foods, baby foods, prepared meals and more. The specific bioactive compounds deriving from foods are known as dietary supplements or nutraceuticals and they can be taken in the form of pills, powders or other medicinal liquid forms (Wildman, 2001).

It is well known that some ancient civilizations, such as Indians, Egyptians, Chinese and Sumerians, used food as medicine. Hippocrates said, “Let food be thy medicine, and medicine be thy food”, suggesting that the ancients were well aware of the importance of food for health. The concept of food as medicine is found also in folk sayings, such as “An apple a day keeps the doctor away” or “Good wine makes good blood”, which suggest that a certain type of food or beverage could prevent some kinds of disease.

Currently, there are many data that allow stating the importance of the combination between good nutrition and good health. For example, epidemiological and clinical trials have demonstrated that a nutrition rich in fish is associated with a minor risk of emergence of cardiovascular diseases, due to the effects of omega-3 fatty acids, such as EPA (eicosapentaenoic acid, C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3) (Kris-Etherton et al., 2002). Other epidemiological trials have reported the existence of anticancer properties in green tea, due to its content in phenolic compounds, such as (-)-epigallocatechin gallate (EGCG) (Jankun et al., 1997).

Thus, this is the context of my Ph.D. research project in Pharmaceutical Science.

My research activity has been focused on the evaluation of the nutraceutical potential of vegetal food products typical of Campania region, an area situated in the southern part of the Italian peninsula. The ancient Romans named it “*Campania Felix*” because of its prosperity, which is due to the conspicuous presence of volcanic soils that ensure high land fertility and are responsible for a particularly developed agricultural sector. Many typical products of this region are considered as products of excellence and have qualitative characteristics that depend essentially or exclusively on the territory where they grow. Some of these products obtained recognitions by European Union, such as PGI (Protected Geographical Indication) and PDO (Protected Designation of Origin).

Particularly, my attention has been addressed to a typical apple cultivar (*Malus pumila* Miller) named “Annurca”, which is listed as a PGI product and is known as “Mela Annurca Campana IGP”. The choice of studying this fruit arose from the evidence that the regular consumption of apples is associated with beneficial effects on health. It has been reported that the regular consumption of apples results in a reduction of cardiovascular risk (Knekt et al., 1996) and cerebrovascular diseases (Knekt et al., 2000). The protective effect of apples against the occurrence of cardiovascular events could be due to their potential cholesterol-lowering ability. Aprikian et al. reported that rats fed with cholesterol and lyophilised apples show lower levels of plasma and liver cholesterol, higher concentration of high-density lipoproteins and increased cholesterol excretion in the feces (Aprikian et al., 2001). Apple consumption is also associated with a reduced risk of developing type II diabetes (Knekt et al., 2002). Moreover, eating apples on a regular basis may reduce the emergence of certain types of cancer, such as liver, colon, lung and prostate cancers (Riboli et al., 2003).

The magic ingredients contained in apples and considered as the responsible for their healthful properties are polyphenols, which include mainly flavonoid derivatives, such as flavonols, flavanols, anthocyanins, dihydrochalcones and phenolic acids (Alonso-Salces et al., 2004).

CHAPTER 1

Mela Annurca Campana IGP

1.1 Origin and history

“Mela Annurca Campana IGP” is a precious apple cultivar (*Malus pumila* Miller), typical of Campania region. It is present in this geographical area for at least two millennia, since some depictions of this fruit were found in some paintings in the ruins of Herculaneum. Specifically, its place of origin would be the area near Pozzuoli, as it can be deduced from the “*Naturalis Historia*” by Pliny the Elder. He named it “Mala Orcula” because of the proximity of Pozzuoli to Averno Lake, which, according to the ancients, was the location of the underworld, so it was produced near the Orcus. From “Mala Orcula” the names “anorcola” and “annorcola” were derived and they were used until 1876, when the name “Annurca” appeared for the first time in the “*Manuale di Arboricoltura*” written by the botanist Giuseppe Antonio Pasquale. In the past, this cultivar was traditionally grown in Phlegraean and Vesuvian areas, often in small companies and along with vegetables and other fruits, but, because of the regression of the agricultural areas in Naples, it is currently cultivated in other areas of Campania region, especially in the areas of Caserta and Benevento.

1.2 Product description

“Mela Annurca Campana IGP” is considered as the queen of apples (Figure 1) and it has specific characteristics, described in its disciplinary of production.



Figure 1: Mela Annurca Campana IGP.

The fruit is medium to small and slightly asymmetrical. The minimum dimensions required are 60 mm in diameter and 100 g in weight. The shape is flattened and roundish and there is a short and weak stalk. When the fruit is harvested, the peel appears yellowish-green, with red stripes on 50-80% of the surface, but they can reach 90-100% of the surface after the reddening on the ground. The rind is smooth, waxy, moderately rusty, especially in the stem cavity, and has numerous small lenticels. The flesh is white, juicy, firm and crispy and has a very high acid/sugar ratio, which is responsible for a pleasantly acidulous and fragrant flavour.

Two different ecotypes are possible: “*Annurca*” and “*Annurca Rossa del Sud*”, which is a natural mutant and is characterised by fruits with red skin before the reddening on the ground.

1.3 Production

The disciplinary of production of Mela Annurca Campana IGP indicates that its production is possible only in 137 municipalities of Campania region.

The cultivation takes place in apple orchards, where it is possible to find other apple cultivars up to a maximum of 10% of the plants. Several cultivation methods are allowed; the number of plants per hectare is variable, but it cannot be greater than 1200 plants/Ha.

The harvesting takes place in mid-September and it is made by hand. After the collection, apples are subjected to the reddening on the ground in the so-called “melai”, which are small plots of land, not larger than 1.50 meters. “Melai” are covered by soft material layers, such as hemp, pine needles, wood shavings or other plant materials and they are arranged in order to avoid water stagnation and to be protected from the sun. During their permanence there, apple fruits are arranged in rows; they are sprayed daily with water and cannot receive any plant health treatment. When a part of the surface of the fruit becomes red, the apples are turned to allow the reddening on the opposite side (Figure 2).



Figure 2: A typical “melaio”.

Finally, apples are accurately inspected in order to discard fruits that appear damaged or rotten. Moreover, fruits must not have parasites or pesticide residues. This peculiar treatment lasts for about one month and allows the final product to reach its typical taste and quality characteristics (Insero, 2004). The harvesting and reddenning operations have to be concluded by December, 15th.

1.4 PGI Recognition

PGI is the acronym of Protected Geographical Indication. According to the Regulation (EC) No. 510/2006, “it is the name of an area, a specific place or, in exceptional cases, the name of a country, used as a description of an agricultural product or a foodstuff,

- which comes from such an area, place or country;
- which has a specific quality, goodwill or other characteristic property, attributable to its geographical origin;
- at least one of the stages of production, processing or preparation takes place in the area.”

Thus, in order to receive the PGI recognition, the entire product must be traditionally and at least partially manufactured (prepared, processed or produced) within the specific region and consequentially acquire unique properties.

Annurca apple cultivar received PGI recognition from the European Council [Commission Regulation (EC) No. 417/2006] according to the conditions and requirements established by Regulation (EC) No. 2081/1992 (Figure 3).



Figure 3: “Mela Annurca Campana IGP” logo.

The Italian Ministry of Agriculture, Food and Forestry Policies authorised Is.Me.Cert (Istituto Mediterraneo per la Certificazione dei prodotti e dei processi nel settore agroalimentare) to perform conformity checks in order to give the authorization to use the registered name “Mela Annurca Campana IGP” (Decree of 26 February 2003). Checks are performed both in the office and in the companies and include document controls, inspections and analytical test on batches. The aim of these controls is to verify the compliance to the established requirements, such as origin, conformity of cultivation methods and of product, traceability of products, respect of labeling rules and so on. Moreover, the Italian Ministry of Agriculture, Food and Forestry Policies recognised the existence of “Consorzio di tutela Mela Annurca Campana IGP”, that aims to protect, to monitor and to promote the product (Decree of 18 April 2007).

1.5 Economical and production data

The annual production of Annurca apple cultivar is about 60000 tonnes and it represents 60% of apple production of Campania region and 5% of Italian apple production (D’Abrosca et al., 2006). Currently, about 2/3 of the harvest are sold in Campania and Lazio regions, while the remaining 20% arrive in Liguria, Lombardia, Piemonte and Toscana regions.

Data published in 2005 reported that the surface enrolled in the PGI certification system is 288 hectares and the farms entered in the register are 86, so the total certified production is about 25000 tonnes.

1.6 Nutritional and health value

Table 1 shows the nutrient composition of Annurca apple, according to the data reported in the database of C.R.E.A. (Centro di Ricerca per gli Alimenti e la Nutrizione). Although the quantity of micronutrients is not shown in this table, several types of vitamins (such as thiamine, riboflavin, niacin and ascorbic acid), and different kinds of minerals (such as potassium, iron, phosphorus and manganese) are contained.

Table 1: Nutrient composition of Annurca apple.	
Nutrient component	Quantity in 100 g
Water (g)	84.3
Proteins (g)	0.3
Lipids (g)	0.1
Cholesterol (g)	0
Available carbohydrates (g)	10.2
Soluble sugars (g)	10.2
Total fiber (g)	2.2
Energy (kcal)	40
Energy (kJ)	169

In addition to the above-mentioned nutritional properties, Annurca apple has shown several beneficial health effects. As reported in the introduction, all apple cultivars are rich in secondary metabolites, whose positive effects on health have already been reported in previous studies. Thus, several authors have studied Annurca apple cultivar in order to analyse its contribution to health.

Annurca secondary metabolites have been reported to have an important antioxidant activity. Cefarelli et al. reported that they have inhibiting activity against the autoxidation of methyl linoleate, peroxide scavenging activity and DPPH scavenging activity (Cefarelli et al., 2006). The strong antioxidant compounds contained in Annurca apple are able to prevent endogenous damage both *in vitro* (human gastric epithelial cells) and *in vivo* (rat gastric mucosa). Particularly, Annurca polyphenolic extract may counteract the damaging effect deriving from ROS (Reactive Oxygen Species) and such a protective effect is

mainly attributable to its major components, catechin and chlorogenic acid (Graziani et al., 2005).

Another work reported that Annurca polyphenolic extract, which is rich in chlorogenic acid, catechin and epicatechin, has potent demethylating activity due to the capacity of inhibiting DNA methyltransferase and of regulating apoptosis and cell viability; as a result, it could be considered an excellent chemopreventive of colorectal cancer, since it is known that hypermethylation of the promoter of hMLH1 and subsequent microsatellite instability occurs in 12% of sporadic colorectal cancers (Fini et al., 2007).

Mari et al. compared three different apple cultivars and reported that Annurca polyphenolic extract is richer than those obtained from Red Delicious and Golden Delicious cultivars. Furthermore, it was the most effective in decreasing cell viability and in increasing the activity of the enzyme caspase-3, which has a key role in apoptosis cascade. Both the cytotoxic and the apoptotic effects depend on the qualitative composition of polyphenols and not only on the total content (Mari et al., 2010).

Annurca polyphenolic extract has also shown a good antimicrobial activity, since it determines inhibition halos against some pathogen bacteria such as *Bacillus cereus* and *Escherichia coli*. Moreover, the same work reported that Annurca polyphenolic extract does not induce any evident gene mutations, suggesting that it also exhibits antimutagenic properties (Fратиanni et al., 2007).

To sum up, all the papers reported herein agree on the recognition of polyphenols as the compounds responsible for these important and positive effects both *in vitro* and *in vivo*.

CHAPTER 2

Polyphenols

Polyphenols represent a large and heterogeneous family of secondary metabolites, widely spread in plant kingdom and commonly found both in edible and non edible plants (El Gharras, 2009). These compounds are chemicals produced by plants in order to confer some properties, such as resistance against ultraviolet radiations and aggressions by pathogens (Beckman, 2000). Moreover, polyphenols are considered as the responsible for some characteristics of foods and beverages deriving from plants, since they contribute to the taste, colour and nutritional properties (Cheynier, 2005).

The main sources of polyphenols are fruit and beverages, such as red wine, coffee and tea; other important sources are vegetables, cereals and leguminous plants (El Gharras, 2009). Some of these compounds are ubiquitous (e.g. quercetin is present in all plant products); others are specific to particular plants (e.g. isoflavones are found only in soy products; flavanones are typical of citrus fruit and phloridzin is contained only in apples). In general, foods contain complex mixtures of polyphenols, but, unfortunately, they are too often scarcely characterised (Manach et al., 2004).

2.1 Classification

Polyphenolic compounds are divided in different classes according to their number of phenolic rings and the structural elements that allow the binding between the rings (El Gharras, 2009). Nevertheless, they are usually distinguished in flavonoids and nonflavonoids (Cheynier, 2005).

2.1.1 Flavonoid compounds

Among polyphenolic compounds, flavonoids represent the largest group: more than 4000 molecules have been identified and the list is constantly growing (Harborne et al., 2000). Such a complexity is due to the occurrence of numerous substitution patterns in which primary substituents can be replaced by other chemical groups;

for example, the molecules can be glycosylated or acylated, forming highly complex structures.

Flavonoids have a skeleton of diphenylpropanes. They consist of two phenolic rings, named A and B, that are linked together by three carbon atoms, forming an oxygenated heterocyclic ring, named C. According to the oxidation state of the heterocyclic ring, flavonoids are divided in six different subclasses (Figure 4) (Manach et al., 2004):

- flavonols;
- flavones;
- flavanones;
- isoflavones;
- anthocyanidins;
- flavanols (or flavan-3-ols).

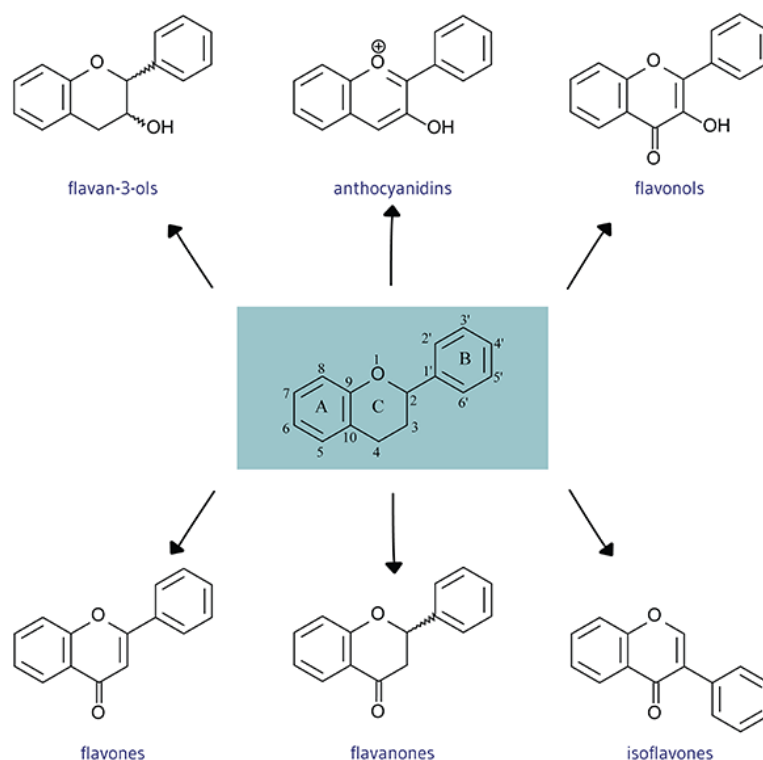


Figure 4: Different subclasses of flavonoids.

The most ubiquitous flavonoids in foods are flavonols, which are generally present at relatively low concentrations (about 15-30 mg/kg FW). Flavonols present a

double bond between C2 and C3, an oxygen atom at the C4 position and a hydroxyl group at the C3 position (de la Rosa et al., 2010). The main representatives of this class are quercetin and kaempferol. Generally, these molecules are in glycosylated forms and the sugar portion is represented by glucose, rhamnose, galactose, arabinose, xylose and glucuronic acid. The richest foods and beverages are onions, curly kale, leeks, broccoli, blueberries, red wine and tea (Manach et al., 2004).

Flavones have a double bond between C2 and C3 and a ketone group at the C4 position (de la Rosa et al., 2010). They are much less spread in fruit and vegetables than flavonols, and they are found principally in parsley and celery. This class includes mainly glycosides of luteolin and apigenin (Manach et al., 2004).

Flavanones are also named dihydroflavones. In these molecules, the ring C is saturated and presents an oxygen atom at the C4 position (de la Rosa et al., 2010). The main sources in human foods are tomatoes and some aromatic plants, such as mint, but the richest are citrus fruits. These compounds appear generally glycosylated by a disaccharide at position 7: the main aglycones are naringenin (present in grapefruit), hesperetin (found in orange) and eriodictyol (found in lemon) (Manach et al., 2004).

Isoflavones have B and C rings linked through C3 position, instead of C2 position, peculiar of the other kinds of flavonoids (de la Rosa et al., 2010). They are found almost exclusively in leguminous plants, both as glycosides and aglycones. The most important sources in human diet are represented by soy and soybean-derived products. Genistein, daidzein and glycitein are the most important compounds that belong to this class (Manach et al., 2004).

The ring C of flavanols (or flavan-3-ols) is saturated and has a hydroxyl group at the C3 position (de la Rosa et al., 2010). These molecules can be found both in monomeric and in polymeric forms. Among monomers, the most important are catechins, such as (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (+)-gallocatechin and their gallate derivatives; among polymers, the main representatives are proanthocyanidins, named also condensed tannins, constituted by 4-11 units. The monomers catechin and epicatechin are the main flavanols contained in fruit, while gallocatechin, epigallocatechin and epigallocatechin gallate

are present mainly in teas. The polymers proanthocyanidins are found mainly in grapes (Manach et al., 2004).

The last class of flavonoids is represented by anthocyanidins, which exist mainly in the glycosylated and esterified forms (anthocyanins), because of the high instability of the aglycone form (Manach et al., 2004). Anthocyanins are based on the flavylium salt structure (de la Rosa et al., 2010); the glycosylation occurs mainly with glucose, galactose, rhamnose, xylose, arabinose, and fructose, whereas the esterification occurs with various organic acids (such as citric acid or malic acid) and phenolic acids. Fruit, red wine, some varieties of cereals and some kinds of vegetables (aubergines, cabbage, beans, onions and radishes) are the richest foods and beverages in human diet. The most common anthocyanidin in food is represented by cyanidin (Manach et al., 2004).

2.1.2 Nonflavonoid compounds

Nonflavonoid compounds are characterised by heterogeneous chemical structures and are classified into three groups: phenolic acids, lignans and stilbenes (Cheynier, 2005).

Phenolic acids are distinguished in derivatives of benzoic acid and derivatives of cinnamic acid. Hydroxybenzoic acids are found in small quantities only in a few plants used by humans, both in free and in esterified forms: the most important are protocatechuic acid and gallic acid. Hydroxybenzoic acids are the components of more complex structures, such as gallotannins and ellagitannins. Hydroxycinnamic acids are more common than hydroxybenzoic acids and consist mainly of *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid. These compounds are found in the form of glycosylated derivatives and esters of quinic acid, shikimic acid and tartaric acid (Figure 5) (Manach et al., 2004).

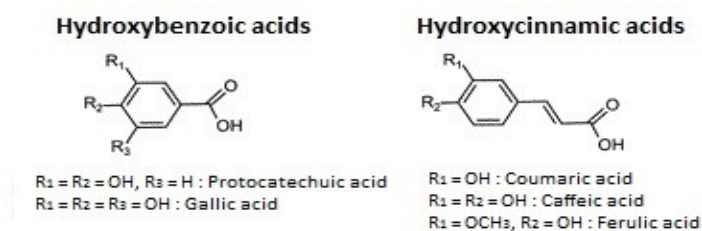


Figure 5: Phenolic acids.

Lignans are diphenolic compounds containing a 2,3-dibenzylbutane structure. They can be found in traces in cereals, grains, fruit and some vegetables, but the richest source is linseed, which contains chiefly secoisolariciresinol and small quantities of matairesinol (Manach et al., 2004) (Figure 6).

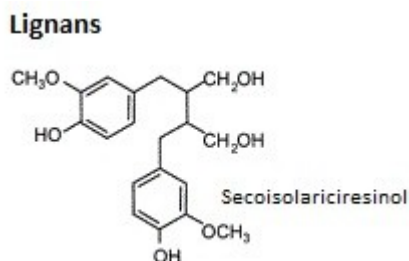


Figure 6: Lignans.

Finally, stilbenes are hydrocarbons consisting of ethene substituted with a phenyl group on both carbon atoms involved in the double bond. The most important exponent is resveratrol, which is contained chiefly in red wine (Manach et al., 2004) (Figure 7).

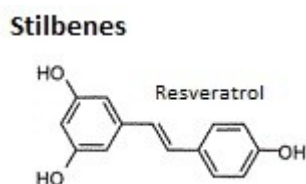


Figure 7: Stilbenes.

2.2 Biosynthesis

The biosynthesis of these molecules is the result of several chemical reactions catalysed by various enzymes, which are regulated by both exogenous (such as light, temperature, water availability and pathogens) and endogenous (such as hormones) factors. In higher plants, the majority of phenolic compounds derive from the amino acid phenylalanine, a product of the shikimic acid pathway, which converts products deriving from the metabolism of carbohydrates, such as phosphoenolpyruvate and erythrose-4-phosphate, in essential aromatic amino acids, such as phenylalanine, tyrosine and tryptophan.

The biosynthesis of flavonoids needs as initial substrates one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA. *P*-coumaroyl-CoA is formed through a synthetic pathway, involving three steps and enzymes. Firstly, the amino acid phenylalanine is deaminated and transformed in *trans*-cinnamic acid through an important enzyme, named phenylalanine ammonia lyase (PAL). Subsequently, *trans*-cinnamic acid is converted in *p*-coumaric acid thanks to the action of the enzyme cinnamate 4-hydroxylase (CA4H). Finally, the action of the enzyme 4-coumaroyl-CoA ligase (4CL) turns *p*-coumaric acid in the corresponding CoA thioester. Malonyl-CoA is formed in the reaction catalysed by the enzyme acetyl-CoA carboxylase (ACC) (Vogt, 2010).

Thus, three molecules of malonyl-CoA and one molecule of *p*-coumaroyl-CoA are substrates for the enzyme chalcone synthase (CHS), which catalyses a series of sequential decarboxylations and condensations, leading to the formation of naringenin chalcone. Naringenin chalcone is turned into the flavanone naringenin, by the action of chalcone isomerase (CHI). Naringenin represents the progenitor of all successive flavonoid structures (de la Rosa et al., 2010).

Malonyl-CoA and *p*-coumaroyl-CoA are also the substrates of stilbene synthase (STS), which is an important enzyme for the formation of stilbene skeleton (de la Rosa et al., 2010).

As regards phenolic acids and lignans, they have a common precursor that is represented by *p*-coumaric acid (de la Rosa et al., 2010).

2.3 Biological properties

As reported above, phenolic compounds are secondary metabolites that are useful for the plants which produce them. Firstly, these molecules can give a certain resistance against some agents, such as ultraviolet rays and pathogens (Beckman, 2000). Since they are coloured pigments, these compounds are the responsible for the colour of flowers and fruits in which they are contained: flavonols and flavones are yellow (Cheynier, 2005), while anthocyanins exhibit pink, red, purple or blue colours according to the chemical structure and the pH (Manach et al., 2004). Polyphenols are also considered as the responsible for the taste of foods and

beverages in which they are contained, and they give an astringent and bitter taste (Cheynier, 2005).

During the last decades, a continuous and growing interest for polyphenols has been occurred and numerous studies have been devoted to these molecules, their occurrence in plants and food coming from plants, and their effects on food quality. Furthermore, a lot of epidemiological studies and associated meta-analysis have reported that polyphenols or polyphenol-rich diet may play a significant role in health, since they can protect against the development and progression of some pathologies that are associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases, diabetes and osteoporosis (Graf et al., 2005; Arts et al., 2005). Polyphenols, in fact, are strong antioxidant and they can act both as chain breakers and as radical scavengers (Rice-Evans, 2001), thanks to the fact that the phenolic groups can accept an electron and form stable phenoxyl radicals. Thus, by virtue of their antioxidant properties, they may protect cell constituents from oxidative damage and reduce the risk of onset of various degenerative diseases associated with oxidative stress (Pandey et al., 2009).

Polyphenols may reduce the number of tumors and their growth. Such an effect has been observed for various types of cancer, such as mouth, stomach, duodenum, colon, liver, lung, mammary gland and skin tumors. The chemopreventive effects of polyphenols are due to several mechanisms of action, such as antioxidant and anti-inflammatory activities, inhibition of proliferation, induction of cell cycle arrest, induction of apoptosis, induction of cytochrome P450 enzymes and changes in cellular signalling (Yang et al., 2001; García-Lafuente et al., 2009).

As regards the cardio-protective effects, polyphenols are considered as potent inhibitors of LDL oxidation, which is associated with the development of atherosclerosis and the resulting coronary heart diseases and myocardial infarctions (Aviram et al., 2000). In addition, polyphenols may exert protective effects against cardiovascular diseases by performing antioxidant, antiplatelet and anti-inflammatory actions, by increasing HDL levels and by improving endothelial functions (García-Lafuente et al., 2009).

Polyphenols have also shown neuroprotective action. Since they have antioxidant activity, they may protect against the development of pathologies characterised by

oxidative damage, such as Alzheimer's and Parkinson's diseases (Dai et al., 2006; Aquilano et al., 2008).

Finally, polyphenols have been found to have other several health beneficial effects (Pandey et al., 2009). Dietary polyphenols are efficient in the treatment of asthma; in fact, epidemiological studies have shown the occurrence of a decreased prevalence and incidence of asthma and an enhanced pulmonary function after apple intake. Isoflavones are considered beneficial for the treatment of osteoporosis. Different kinds of polyphenols have shown antidiabetic effects, through different mechanisms that include the inhibition of glucose absorption into the intestine or of glucose uptake by peripheral tissues.

Specifically, flavonoids have been reported as the main responsible for antioxidant, anticarcinogenic and antiatherosclerotic actions (Wang et al., 2000).

2.4 Bioaccessibility and bioavailability

Although polyphenols are widely distributed in the plant kingdom, their total content in plant products is often difficult to estimate, because of the occurrence of several factors, such as ripeness at the time of harvest, environmental factors (e.g. sun exposure, rainfall, type of culture, fruit yield per tree), storage and methods of culinary preparation (e.g. cooking and peeling) (D'Archivio et al., 2007). Moreover, there are not unequivocal data about the daily intake of polyphenols, because it depends chiefly on dietary habits and preferences of the individuals and may vary considerably in relation to the type, quality and quantity of the plant product consumed. For example, heavy coffee drinkers will likely consume more phenolic acids than flavonoids, whereas people that consume fruits or beverages such as red wine, tea, chocolate or beer, will likely take flavanols, such as catechins, proanthocyanidins, anthocyanins and their oxidation products (Scalbert et al., 2000).

In order to obtain any effect in specific tissues or organs, polyphenols must be available and their bioavailability is influenced by several processes that occur in the LADME (Liberation, Absorption, Distribution, Metabolism and Excretion) scheme. Therefore, the step of liberation of the bioactive compounds from the food matrix in the gastrointestinal tract is of fundamental importance to ensure that they

enter into the systemic circulation and become bioavailable. As regards the intestinal absorption, aglycones can be directly absorbed from the small intestine, whereas glycosides have to be hydrolysed by the intestinal enzymes or by the colonic microflora. Later, polyphenols undergo both in the intestine and in the liver to highly efficient metabolic conjugation processes (such as methylation, sulfation and glucuronidation) that aim to detoxify these xenobiotics, increasing their hydrophilicity and promoting their excretion through bile and urine: thus, circulating polyphenols are conjugated derivatives, strongly bound to plasmatic proteins, especially albumin (Manach et al., 2004; Pandey et al., 2009).

Considering the above, despite the consumption of polyphenols is usual, not necessarily these compounds are able to exert biological activity within the organism. In fact, polyphenol bioavailability is not the same for the various molecules and there is no relationship between their concentration in food and their bioavailability in human body because only their chemical structure is responsible for the absorbed quantity and for the type of circulating metabolites (Pandey et al., 2009).

Ingested phenolic compounds could have a lower intrinsic activity or be scarcely absorbed from the intestine, highly metabolised or quickly eliminated (Manach et al., 2004). In fact, gastrointestinal digestion conditions may lead to drastic structural changes that could alter the stability, bioavailability and bioactivity of food constituents. Cilla et al. reported that the digestion process may lead to a reduction in phenolic content by at least 47% in digested fruit beverages compared to those not subjected to digestion (Cilla et al., 2009). Another work reported that only 62% of originally present polyphenols in grapes are bioaccessible after gastrointestinal digestion (Tagliazucchi et al., 2010).

The evidence of the absorption of polyphenols through the gut is given by both direct and indirect methods. Direct methods consider the measuring of the concentration of phenolic compounds in plasma and urine after the ingestion of polyphenol-rich foods (Young et al., 1999; Tian et al., 2006). Indirect methods consider the increase in plasma antioxidant capacity after the consumption of polyphenol-rich foods (Duthie et al., 1998; Young et al., 1999).

Finally, it is important to understand that there is a great inter-individual variability in producing metabolites and that they may have lost or preserved their biological activity, so their identification and quantification represent an important field of research (D'Archivio et al., 2007).

2.5 Polyphenols contained in apples

As mentioned above, foods and beverages contain complex mixtures of polyphenols, but unfortunately they are too often poorly characterised. However, certain studies have focused on the characterisation of the phenolic fraction that is contained in apples. Therefore, data on the polyphenolic profile of apples exist, but as regards the quantity of polyphenols, there are no univocal data because it depends on a lot of factors. In addition, the quantitative profile is different when considering the flesh or the peels. In general, these compounds are more abundant in the peel than in the flesh, maybe because the skin is subjected to injuries more than the pulp, so it needs more protection from pathogen attacks (Escarpa et al., 1998; Lamperi et al., 2008). Since apple peels contain more polyphenols than apple pulp, the antioxidant activity and the bioactivity for the peel may be higher than for the flesh (Boyer et al., 2004).

The polyphenolic compounds present in apples belong to different classes: flavanols, flavonols, anthocyanins, hydroxycinnamic acids and dihydrochalcones (Alonso-Salces et al., 2004; Vrhovsek et al., 2004).

Flavan-3-ols are the most abundant polyphenols contained in apples. In fact, they constitute between 71% and 90% of total phenolic amount and consist mainly of (-)-epicatechin and (+)-catechin, present in the form of both monomers (Figure 8) and oligomers, named procyanidins (Figure 9).

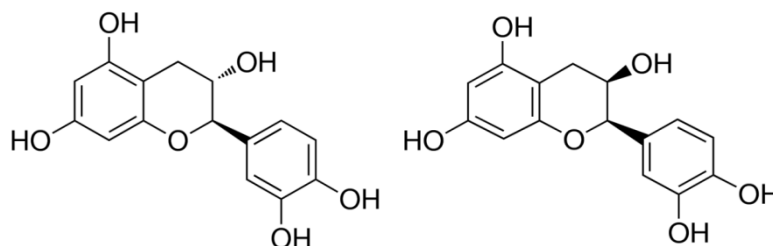


Figure 8: (+)-catechin (on the left) and (-)-epicatechin (on the right).

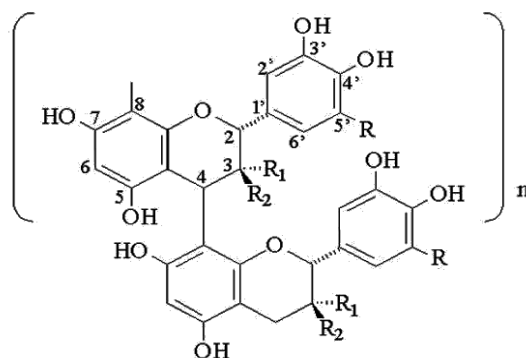


Figure 9: Basis structure of procyanidins.

Flavonols are the strongest antioxidant compounds. They range from 1% to 11% of total phenolic amount and are represented chiefly by quercetin (Figure 10) and some of its glycosides, such as hyperin (quercetin-3-*O*-galactoside), isoquercitrin (quercetin-3-*O*-glucoside), rutin (quercetin-3-*O*-rutinoside), avicularin (quercetin-3-*O*- α -arabinofuranoside) and quercitrin (quercetin-3-*O*-rhamnoside).

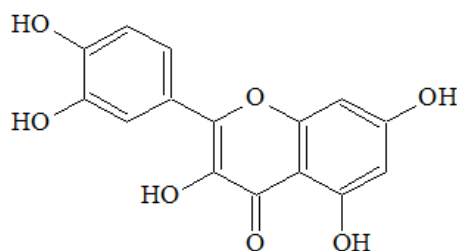


Figure 10: Quercetin.

The main anthocyanin present in apples is a glycoside of cyanidin, also named ideain (cyanidin-3-*O*-galactoside) (Figure 11). Its concentration goes from 1% to 3% of total phenolic content in red apples.

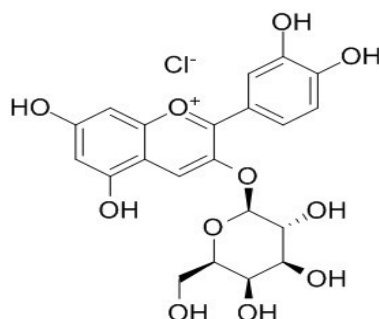


Figure 11: Cyanidin-3-*O*-galactoside or ideain.

Apples also contain hydroxycinnamic acids in a percentage ranging from 4% to 18% of apple polyphenols: the most representative is chlorogenic acid (Figure 12), followed by 4-*p*-coumaroylquinic acid.

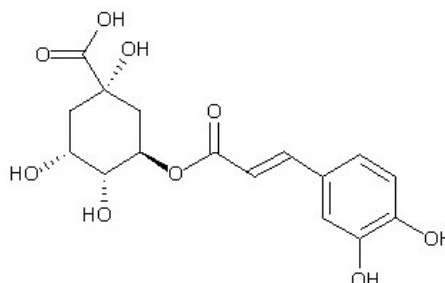


Figure 12: Chlorogenic acid.

Finally, apples contain dihydrochalcones, that are bicyclic flavonoids, ranging from 2% to 6% of total polyphenols: the most important are phloretin (Figure 13) and its glycosides, phloretin-2'-*O*-glucoside (also known as phloridzin) and phloretin-2'-*O*-xyloglucoside.

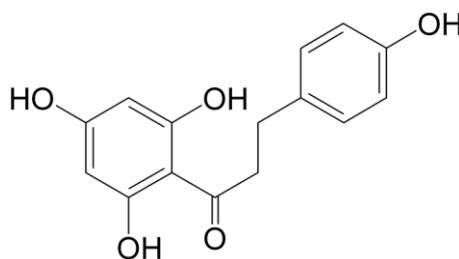


Figure 13: Phloretin.

The above-mentioned differences in concentration ranges are due to the fact that there are large varietal differences and, in the context of a same apple variety, there may be differences due to environmental factors, such as sun exposure, use of certain fertilization methods and stage of fruit development. Apple storage in controlled conditions does not affect the concentration of phytochemicals, whereas processing results in a very significant decrease in phenolic compounds and antioxidant activity (Boyer et al., 2004).

In relation to post-harvest operations, it is very important not to forget that the apple cultivar considered in this thesis work is subjected to a peculiar treatment on the basis of its disciplinary of production. Does the reddenning on the ground

treatment affect the phenolic composition of Annurca apple? According to a paper previously published, the concentration of phenolic compounds tends to increase during the reddening-ripening process in the peels, but not in the flesh. This effect could be due to the ethylene action, a hormone able to stimulate the activity of phenylalanine ammonia lyase (PAL), which is the most important enzyme in polyphenol biosynthetic pathway. Consequently, the antioxidant activity of peels increases during the reddening process, so polyphenols coming from Annurca are considered relatively stable and maintain health benefits also after storage (D'Angelo et al., 2007).

CHAPTER 3

Characterisation of apple polyphenolic extracts and in vitro metabolic effects

3.1 Materials and methods

3.1.1 Reagents and standards

All chemicals and reagents used were either analytical-reagent or HPLC grade. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA) before use. The standards used for the identification and quantification of phenolic acids and flavonoids were: chlorogenic acid, (+)-catechin, (-)-epicatechin, isorhamnetin, myricetin, phloretin, phloridzin (phloretin-2-*O*-glucoside), procyanidin B2, quercetin, quercitrin (quercetin-3-*O*-rhamnoside), rutin (quercetin-3-*O*-rutinoside), isoquercitrin (quercetin-3-*O*-glucoside), hyperin (quercetin-3-*O*-galactoside) and cyanidin-3-*O*-galactoside chloride (Sigma Chemical Co., St. Louis, MO). Acetonitrile and methanol were of HPLC grade (Carlo Erba, Milano, Italy).

3.1.2 Fruit collection and sample preparation

Annurca (*Malus pumila* Miller cv Annurca) apple fruits were collected in Valle di Maddaloni (Caserta, Italy) in October when fruits had just been harvested (green peel). Fruits were reddened, following the typical treatment (Lo Scalzo et al., 2001) for about 30 days, and then analysed. Other four apple varieties analysed in this study, Red Delicious (RD) (*Malus pumila* Miller cv Red Delicious), Pink Lady (PL) (*Malus pumila* Miller cv Pink Lady), Fuji (F) (*Malus pumila* Miller cv Fuji) and Golden Delicious (GD) (*Malus pumila* Miller cv Golden Delicious), were purchased in a local supermarket. Lyophilised apple peels and flesh (10 g) were treated with 100 mL of a methanol/water mixture (80:20, v/v) acidified with 0.5% formic acid for 24 h at 4 °C in order to extract phenolic compounds. After centrifugation, the supernatant was slowly filtered and purified through an Amberlite XAD-2 column packed as follows: 10 g of resin (pore size 9 nm; particle size 0.3-1.2 mm; Supelco, Bellefonte, PA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 x 2 cm). The column was washed with

100 mL of acidified water (pH 2) and 50 mL of deionised water in order to remove sugar and other polar compounds. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen. The polyphenolic extracts were stored at -80 °C until analysis.

3.1.3 HPLC-DAD/ESI-MSⁿ analysis

HPLC separation and quantification of phenolic compounds in apple peel and flesh extracts were performed according to previous studies with some modifications (Mari et al., 2010). The identification of phenolic compounds was possible by recording chromatograms at different wavelengths, 280 nm for flavonols, procyanidins, dihydrochalcones and hydroxycinnamic acids, and 500 nm for anthocyanins and by comparing spectra and retention times with those of commercial standards and with those reported in previous works (Mari et al., 2010). Elution conditions consisted in 2% acetic acid (solvent A) and 0.5% acetic acid in acetonitrile and water (50:50, v/v) (solvent B) gradient at a flow rate of 1.0 mL/min. The column selected was a Hypersil BDS C18 column (250 mm x 4.6 mm, 5 µm) (Thermo, Bellefonte, PA). Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA) provided with photodiode array detector (DAD). The gradient conditions were: 0-5 min, 10% B; 55 min, 55% B; 65 min, 95% B, followed by 5 min of maintenance. The identity of phenolic acids and flavonoids was confirmed with LC-ESI/MS/MS experiments and data were compared with those of commercial standards and with those reported in previous works (Mari et al., 2010). The same chromatographic conditions were applied to a HP1100 HPLC system (Agilent, Palo Alto, CA) coupled to a PE-Sciex API-2000 triple-quadrupole mass spectrometer (AB Sciex, Warrington, UK) equipped with a Turbospray (TSI) source. Individual phenols were separated on a Hypersil BDS C18 column (250 mm x 2.1 mm, 5 µm) (Thermo Electron, Bellefonte, PA) at a flow rate of 200 µL/min. Mass spectra were recorded from m/z = 50 to 1500, in both negative and positive ionisation mode. For negative ion mode, the capillary voltage was set at -13 V, the spray voltage was at 4.5 kV and the tube lens offset was at -15 V, while in positive ion mode, the capillary voltage was set at

13 V, the spray voltage was at 5 kV and the tube lens offset was at -35 V. The capillary temperature was 275 °C. Data were acquired in MS, MS/MS and MSⁿ scanning modes.

3.1.4 Cell culture

Human hepatocellular liver carcinoma cells (HepG2, HB-8065) were obtained from American Type Culture Collection. Monolayers of cells were grown in T75 flasks and maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% antibiotic/antimycotic solution (Gibco-Invitrogen, Mount Waverley, Australia), 0.375% NaHCO₃ and 20 mmol/L HEPES, pH 7.4, and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. The cells were dispersed with trypsin-EDTA (0.05% trypsin and 0.02% EDTA) and plated on the bottom of a transparent black 96-well plate in a 200 µL growth medium at a density of 1.0×10^5 cells/mL. Assays were performed by incubating the culture with 1 µg/mL of apple polyphenolic extract for 72 h.

3.1.5 Glucose uptake

Cells were washed twice with 2 mL of HEPES buffer solution A (HEPES, 20 mM; NaCl, 137 mM; KCl, 4.7 mM; MgSO₄, 1.2 mM; CaCl₂, 1.8 mM) in both upper and lower compartments (pH 7.4). After washing, 1 mL of HEPES buffer containing D-[U-¹⁴C] glucose 0.5 mCi/mL and 1 mM total glucose (solution B) was added to the upper compartment. Cells were then incubated for 30 min at 37 °C, the electrical resistance measured, and buffer from the upper and lower compartments removed. Glucose uptake was stopped by washing each membrane twice with ice-cold PBS. After that, 1 mL of NaOH solution (0.1 mol/L) was added to lyse the cells, and aliquots were removed for scintillation counting and protein measurement. Scintillation solution (5 mL, Ecoscint XR scintillation solution, National Diagnostics) and 0.5 mL of the different test solutions were mixed and analysed by scintillation counting using a Packard Liquid Scintillation Analyzer 1600TR.

3.1.6 [³H] cholesterol uptake

To determine inhibition of cholesterol uptake, cells were incubated in an F-12 medium containing 10 nmol/L [1,2-³H] cholesterol (PerkinElmer Pty Ltd., Melbourne, Australia). The cells were washed twice in F-12 to remove unbound [³H] cholesterol, and 0.5 mL of 0.1% sodium dodecyl sulphate (SDS) was added to each well to solubilise the cells. Three milliliters of Ultima Gold scintillant was added, and the radioactivity of the solubilised cells was determined using Wallac 1410 liquid scintillation counter (Pharmacia, Turku, Finland). For cholesterol efflux experiments, cells were washed in F-12 and incubated for 45 min in F-12 containing 20 nmol/L [³H] cholesterol to “load” the cells with cholesterol. Cells were then washed twice in F-12 and then incubated (for up to 100 min) containing the test samples as shown in the figure legend to determine cholesterol efflux. As a positive control, 1% methyl- β -cyclodextrin (M β CD) was used to acutely deplete cellular cholesterol. Cells were washed twice in F-12, supernatant was removed, and then 0.5 mL of 0.1% SDS was added to solubilise the cells for β counting as above.

3.1.7 Cell proliferation

Cell proliferation was determined using Cell-Titer Blue (Promega, Alexandria, Australia) as per manufacturer’s instructions. Briefly, cells were plated and test samples were prepared in as described above. After 48 h, 20 μ L of Cell-Titer Blue was added, and plates were mixed gently for 5 s and incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO₂ in air. Fluorescence was then determined with excitation and emission wavelengths λ_{ex} = 550 nm and λ_{em} = 615 nm, respectively, on a Victor3 multilabel plate counter (PerkinElmer, Waltham, MA).

3.1.8 Oxidative stress

2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, 5 μ M) was used to detect intracellular Reactive Oxygen Species (ROS) levels. Then, the cells were washed once with PBS and lysed in 3 mL ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.2% sodium dodecyl sulphate (SDS). The cell lysates were collected and centrifuged at 2000 g for 5 min at 4 °C. The fluorescence of the supernatants

was measured using a LS50B PerkinElmer luminescence spectrometer (PerkinElmer, Waltham, MA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. For medium NO quantification, cells were treated with or without positive control N(G)-nitro-L-arginine methyl ester (L-NAME) and incubated at 37 °C, 5% CO₂ for 24 h. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cells were then stimulated with 100 µg/mL lipopolysaccharide (LPS). After 24 h, cell-free supernatants were collected and stored at -80 °C until NO determination using the Griess reaction. Absorbance at 540 nm was then measured using an automated 96-well Varioskan Ascent plate reader (Thermo Electron, Bellefonte, PA).

3.1.9 Lipase assay

Cells were grown in 6-well plates and 2 mL medium/well. After 48 h incubation, the media was removed and the cells were incubated for an additional 12 h in 1 mL/well of fresh medium containing 25 IU heparin (Leo Pharmaceuticals, Breda, The Netherlands). Lipase activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl with a gum acacia-stabilised glycerol [¹⁴C] trioleate emulsion as substrate. Assays were performed for 2 h at 30 °C. Activities are expressed as m-units (nmol of free fatty acids released per min). In a total assay volume of 125 µL, the release of free fatty acids was linear with time and sample volume up to 50 µL for the cell-free medium and 20 µL for the cell lysates. In immunoinhibition assays, 40 µL of the cell-free medium or 10 µL of the cell lysates was preincubated for 3 h on ice in a total volume of 50 µL with 50 µg of goat anti-(human HL) IgG. This antibody was raised against human HL partly purified from post-heparin plasma. After centrifugation (10 min, 10000 g, 4 °C), 75 µL of substrate mixture was added to the supernatant and the residual immunoresistant triacylglycerol hydrolase activity was determined.

3.1.10 LDL receptor binding activity

The effect of apple peel and flesh polyphenolic extracts on HepG2 cell low-density lipoprotein (LDL) receptor binding activity was indirectly evaluated by measuring the LDL concentration in the cell medium. HepG2 cells were grown in 12-well

culture plates, followed by washing PBS and incubation for 24 h in DMEM serum-free media. The cells were then washed in PBS and incubated for a further 6 h in DMEM serum-free media containing fluorescent-labeled Dil-LDL (10 mg/L, Biomedical Technologies, Stoughton, MA). The fluorescence of the cell medium was measured at 514 nm (excitation) and 550 nm (emission) wavelengths by using a LS50B luminescence spectrophotometer (PerkinElmer, Waltham, MA).

3.1.11 Quantification of Apolipoprotein A1 in culture media

The concentration of Apolipoprotein A1 (ApoA1) in the cell culture media was quantified in 96-well plates using an enzyme-linked immunosorbent assay (E90519Hu 96 test kit; Gentaur Molecular Products Kampenhout, Belgium), according to the manufacturer's instructions.

3.1.12 Statistics

Unless otherwise stated, all the experimental results were expressed as mean \pm standard deviation (SD) of five determinations. Statistical analysis of data was performed by a one-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. *P* values less than 0.05 were regarded as significant. The degree of linear relationship between two variables was measured using the Pearson product moment correlation coefficient (*R*). Correlation coefficients (*R*) were calculated by using Microsoft Office Excel application.

3.2 Results and discussion

3.2.1 Polyphenolic profiling of apple peels and flesh

The results obtained through HPLC-DAD/ESI-MSⁿ analysis about the polyphenolic content in peel and flesh extracts of various apple cultivars are reported in Table 2. The qualitative and quantitative composition of apple phytochemicals is complex enough, so the attention was focused on some specific compounds, procyanidins, rutin and phloridzin, because they are considered as the responsible for some important metabolic effects (Tenore et al., 2013a; Tenore et al., 2014).

Table 2: Polyphenolic contents in apple peel and flesh extracts determined by HPLC-DAD-MS analysis.					
	ANN	RD	PL	F	GD
PEELS					
TP	173.23 ± 2.1	281.73 ± 1.3	152.17 ± 1.1	92.26 ± 2.2	70.14 ± 0.2
Procyanidins	13.35 ± 1.95	44.21 ± 3.21	13.50 ± 1.98	8.21 ± 1.12	6.03 ± 1.01
Rutin	27.81 ± 2.10	36.84 ± 3.23	43.84 ± 3.52	17.09 ± 1.78	10.04 ± 1.36
Phloridzin	42.77 ± 3.23	68.54 ± 3.75	18.23 ± 1.65	16.34 ± 1.44	5.20 ± 1.10
FLESH					
TP	114.99 ± 1.9	73.40 ± 2.2	68.25 ± 0.8	36.04 ± 1.0	25.12 ± 1.0
Procyanidins	78.76 ± 3.52	37.70 ± 3.01	52.77 ± 3.24	25.44 ± 2.96	16.01 ± 1.65
Rutin	10.64 ± 1.21	1.50 ± 0.86	2.49 ± 0.98	1.94 ± 1.78	1.04 ± 0.98
Phloridzin	2.54 ± 1.85	10.21 ± 2.1	1.60 ± 1.65	1.46 ± 1.31	1.10 ± 0.99
Values are expressed as mg/100 g FW ± SD (<i>n</i> = 5). Results were significantly different at a level of <i>P</i> = 0.001. ANN: Annurca; RD: Red Delicious; PL: Pink Lady; F: Fuji; GD: Golden Delicious; TP: Total Polyphenols.					

The obtained data confirmed that the level of total phenolic compounds was higher in the peels than in the pulp, as reported in earlier works (Tsao et al., 2003; Lamperi et al., 2008). Such differences are attributable to the fact that polyphenols are produced by plants in order to protect themselves from the attack of pathogens, which act mainly on the skin, so their defensive role has significance mainly for the peels. Obviously, the values may considerably change if considering different apple varieties.

The richest peel extract was represented by Red Delicious (281.73 mg/100 g FW), whereas the poorest one was Golden Delicious (70.14 mg/100 g FW); the other apple varieties had a polyphenolic content that was intermediate between these values. Red Delicious extract was the richest in phloridzin (68.54 mg/100 g FW) and procyanidins (44.21 mg/100 g FW), whereas Pink Lady extract was the richest in rutin (43.84 mg/100 g FW).

Among flesh extracts, the richest one was represented by Annurca (114.99 mg/100 g FW), whereas the poorest one was Golden Delicious (25.12 mg/100 g FW). The

main compounds contained in all the pulp coming from different apple cultivars were procyanidins, which ranged from 51.4% to 77.3%. Particularly, Annurca flesh extract was the richest in procyanidins (78.76 mg/100 g FW) and possessed the highest levels of rutin (10.64 mg/100 g FW), too. Red Delicious extract, instead, was the richest in phloridzin (10.21 mg/100 g FW).

3.2.2 Glucose uptake

The capacity of apple peel and flesh polyphenolic extracts to inhibit glucose uptake and transport was tested by using HepG2 cells (human hepatocellular carcinoma). The choice of using this type of cells arose from the evidence that malignant cells express higher levels of facilitated glucose transporters (GLUTs), through which the energy-independent transport of glucose across the plasma membrane occurs. The major expression of these transporters is due to an enhanced rate of glycolysis by cancer cells. Several glucose transporters have been identified, and they are classified into three groups on the basis of sequence similarities and characteristics. Glucose transporters are tissue-specific because they reflect the different glucose needs of different tissues: liver cells, kidney proximal renal tubule cells, pancreatic islet β -cells and intestinal epithelial cells express mainly GLUT2 (Wu et al., 2009). The results regarding glucose uptake are shown in Figure 14. The extracts of Red Delicious and Annurca peels showed the best glucose uptake inhibition capacity, whereas the other samples had less significant activity (Figure 14A). As regards flesh extracts, no sample was reported to have significant capacity (Figure 14B) (Tenore et al., 2013a).

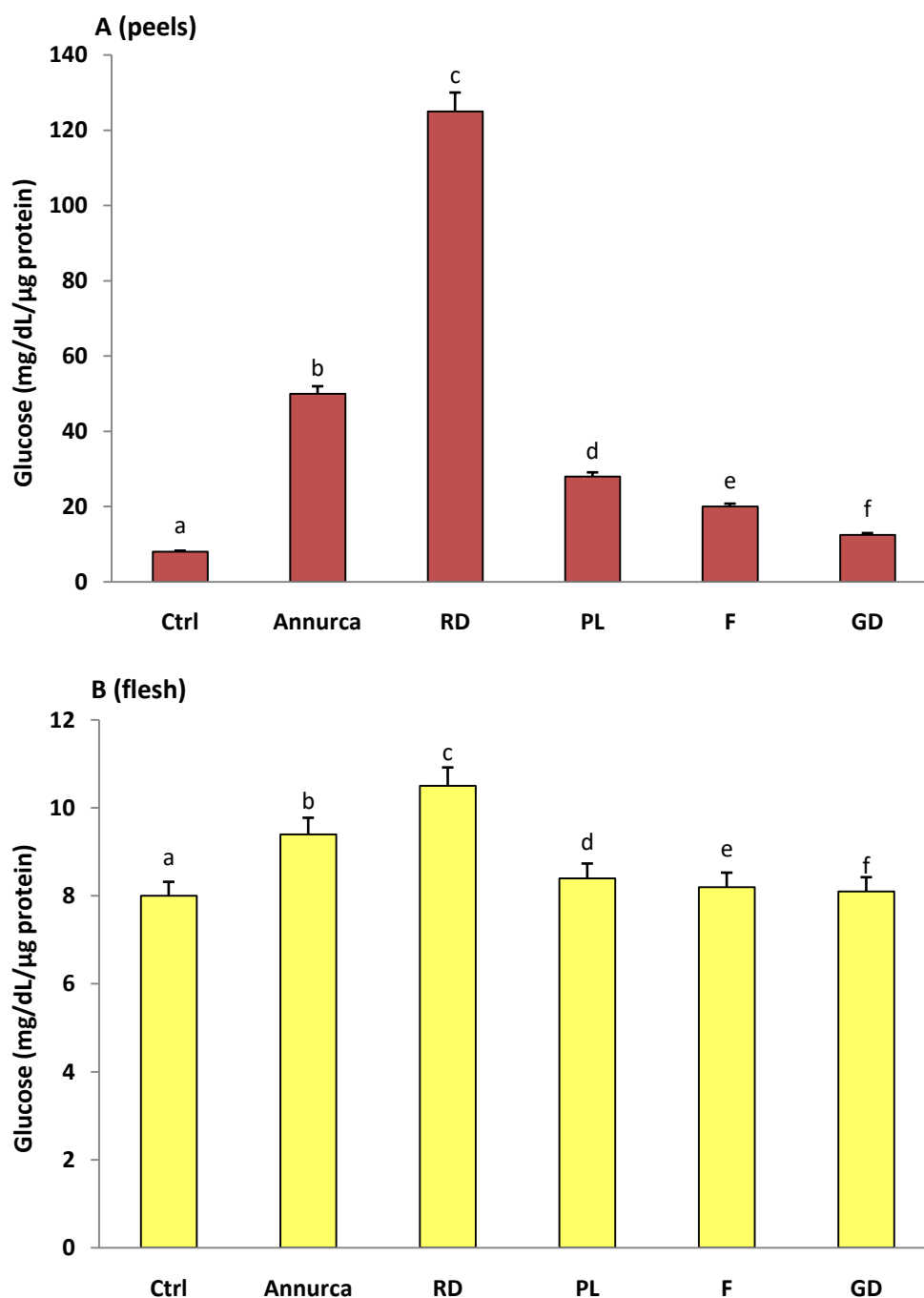


Figure 14: Effect of polyphenolic extracts from apple peels and flesh on glucose uptake by HepG2 cells. Data are referred to glucose concentration in the cell medium and are the means \pm SD ($n = 3$).

^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

Such an effect has been ascribed to phloridzin, a dihydrochalcone typical of *Rosaceae* plants. In fact, phloridzin is able to reduce glucose uptake by human intestinal (Caco-2) cells, thanks to the inhibition of GLUT2, which represents the

major apical intestinal sugar transporter when the concentration of glucose is high (Manzano et al., 2010). Another work reported that it produces renal glycosuria and it blocks intestinal glucose absorption thanks to the inhibition of the Na^+ -glucose symporters that are located in the kidney proximal renal tubule and in the mucosa of the small intestine (Ehrenkranz et al., 2005).

The results regarding glucose uptake by HepG2 cells agreed with those regarding the content of phloridzin in apple peel and flesh extracts found by HPLC analysis. In fact, a statistically significant correlation between phloridzin content and glucose uptake inhibition was found both for peels ($R = 0.9650$) and for flesh ($R = 0.9234$).

3.2.3 Cholesterol uptake

The determination of cholesterol uptake was also performed by using HepG2 cell line, since it is well known that intestine and liver cells are involved in the absorption and the metabolism of lipids.

The data regarding cholesterol uptake are reported in Figure 15. The results revealed that flesh polyphenolic extracts were more active in inhibiting cell cholesterol uptake than those ones deriving from peels, and, particularly, the most active was Annurca apple flesh extract (Tenore et al., 2013a).

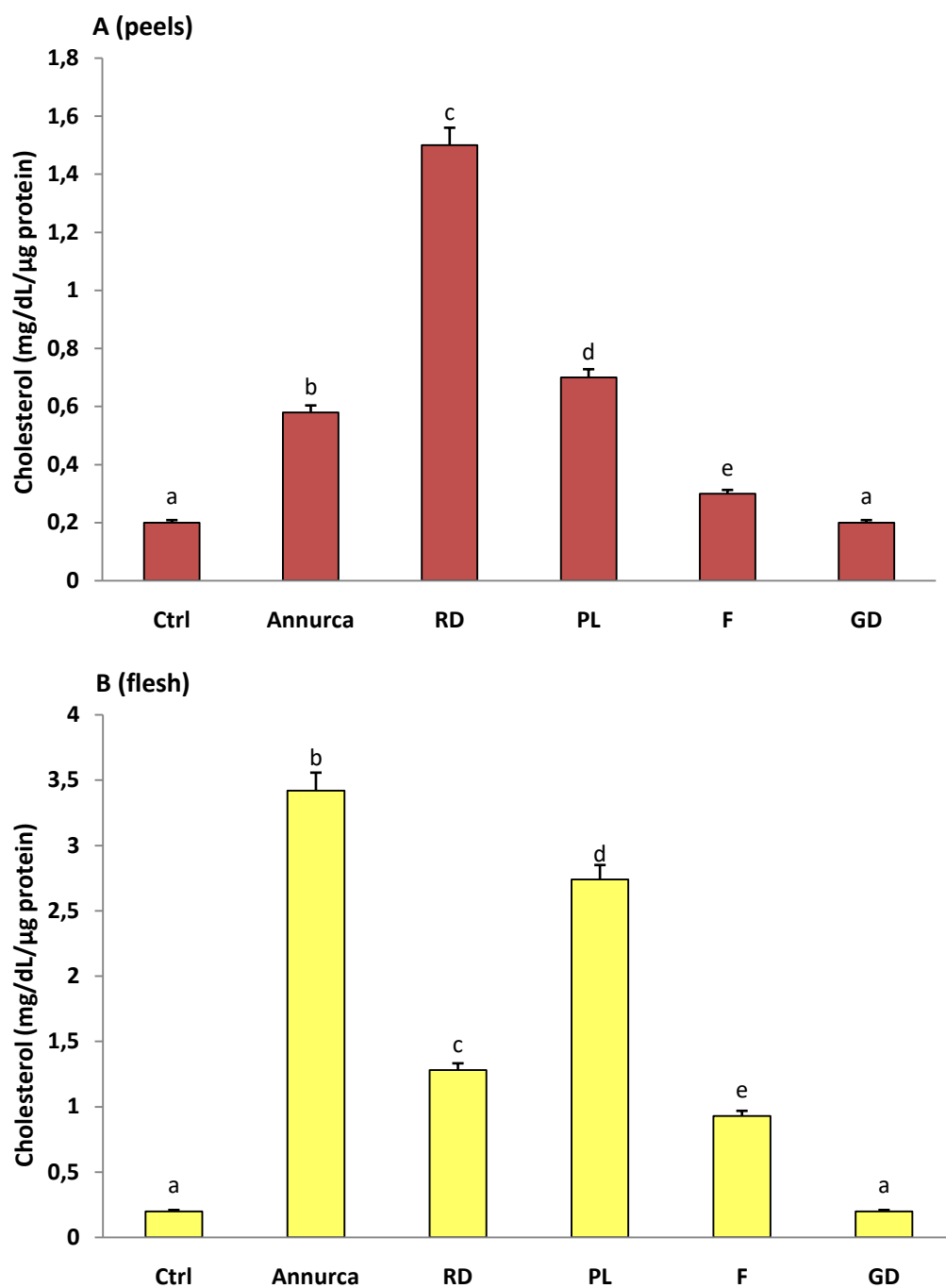


Figure 15: Effect of polyphenolic extracts from apple peels and flesh on cholesterol uptake by HepG2 cells. Data are referred to cholesterol concentration in the cell medium and are the means \pm SD ($n = 3$). ^{abcde}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

Oligomeric procyanidins have been considered as the responsible for this effect. In fact, in a previous work, Leifert et al. evaluated the effects on cholesterol uptake by polyphenolic extracts obtained from grape seed and red wine and concluded that

they would be able to act as β -cyclodextrins, molecules known to bind cholesterol (Yu et al., 2006). Procyanidin oligomers would promote the formation of complexes similar to β -cyclodextrins, which incorporate cholesterol in a selectively and intensively manner. Thus, they would prevent cholesterol uptake and would give a false impression of inhibited cholesterol membrane transport in these cells (Leifert et al., 2008).

The results regarding cellular cholesterol uptake agreed with those concerning the content in procyanidins of extracts analysed by HPLC-DAD/ESI-MSⁿ. In fact, procyanidins represented the most abundant polyphenolic compounds contained in apple flesh, and Annurca flesh possessed the highest quantity. It was found a good linear correlation between the levels of procyanidins contained in the samples and the inhibition of cholesterol uptake both for peels ($R = 0.9838$) and for flesh ($R = 0.9919$).

3.2.4 Cell proliferation and oxidative stress

HepG2 cells were also used in order to evaluate the effects of apple peel and flesh polyphenolic extracts on cell proliferation and oxidative stress.

The results concerning the effects of polyphenolic extracts coming from different apple peels are shown in Figure 16. Peel polyphenols revealed to be cytotoxic. In particular, Annurca, Red Delicious and Pink Lady peel extracts decreased cell proliferation by 48.0%, 62.5% and 37.5%, respectively, by comparison with control cells, while Fuji and Golden Delicious peel extracts did not show any significant difference compared to control cells (Figure 16A). The lower cell proliferation of peel polyphenols coming from Annurca, Red Delicious and Pink Lady may be attributed to their capacity to induce the production of ROS and the resulting oxidative stress, as corroborated by higher TBARS levels (especially for Annurca and Red Delicious) and lower NO levels in the cell medium (Figure 16B and 16C) (Tenore et al., 2013a).

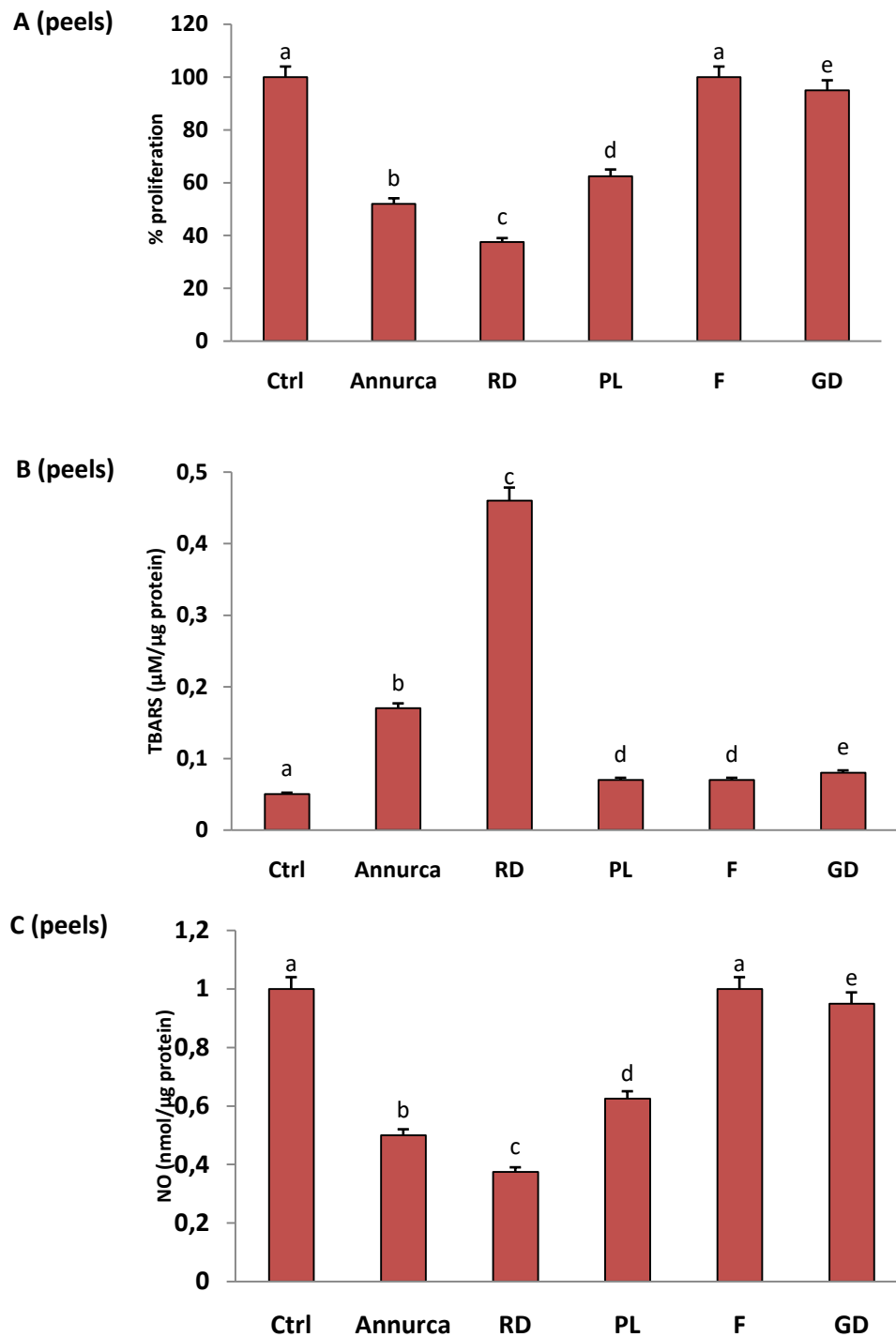


Figure 16: Effect of polyphenolic extracts from apple peels on HepG2 proliferation (A), oxidative stress (B) and on nitric oxide levels in the cell medium (C). Data are the means \pm SD ($n = 3$).

^{abcde}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

It may appear strange that the strong antioxidant polyphenolic compounds could exert pro-oxidant effects. Actually, *in vitro* evidence suggests that these

phytochemicals may have different effects depending on the cell line used or on their structure and concentration, so they can either scavenge ROS or generate more oxidative stress (Choueiri et al., 2012). The occurrence of pro-oxidant effects would be directly correlated to the total amount of hydroxyl groups. Specifically, quercetin and catechin derivatives, which are characterised by multiple hydroxyl groups, significantly increase the production of hydroxyl radicals in a Fenton system, resulting to be cytotoxic and pro-apoptotic (Babich et al., 2011; Forester et al., 2011).

Nitric oxide (NO) is a diffusible, transcellular and multifunctional messenger involved in the regulation of numerous both physiological and pathological conditions. Nitric oxide results from the transformation of L-arginine to L-citrulline by the action of the enzyme nitric oxide synthase (NOS) in the presence of O₂ and NADPH. There are three isoforms of NOS: eNOS, expressed in the vascular endothelium; nNOS, present in central and peripheral neurons; iNOS, found in several types of cells, such as macrophages and hepatocytes. eNOS and nNOS isoforms are constitutively active and their continuous release of NO ensures the maintenance of the endothelium in an activated state and the correct functioning of the peripheral nervous system, respectively. iNOS isoform produces NO after stimulation of immunological or inflammatory pathways and acts as second messenger in modulating signalling pathways (Akifusa et al., 2008). When a condition of oxidative stress occurs, nitric oxide can combine with superoxide radicals (O₂^{•-}), determining the formation of peroxynitrite (ONOO⁻), which is involved in damaging cell constituents and in inducing apoptotic cell death (Chung et al., 2001). Thus, lower concentration of NO in cell medium is associated with a major consumption of this compound by cells and it is considered as an indicative marker of oxidative stress.

The results concerning the effects of polyphenolic extracts coming from different apple flesh are shown in Figure 17. On average, flesh samples revealed to be more protective than peels; particularly, Annurca and Red Delicious flesh extracts increased cell proliferation by 47.5% and 12.5%, respectively, compared to control cells (Figure 17A). This effect was corroborated by lower levels of TBARS and

higher levels of NO in the cell medium (Figure 17B and 17C) (Tenore et al., 2013a).

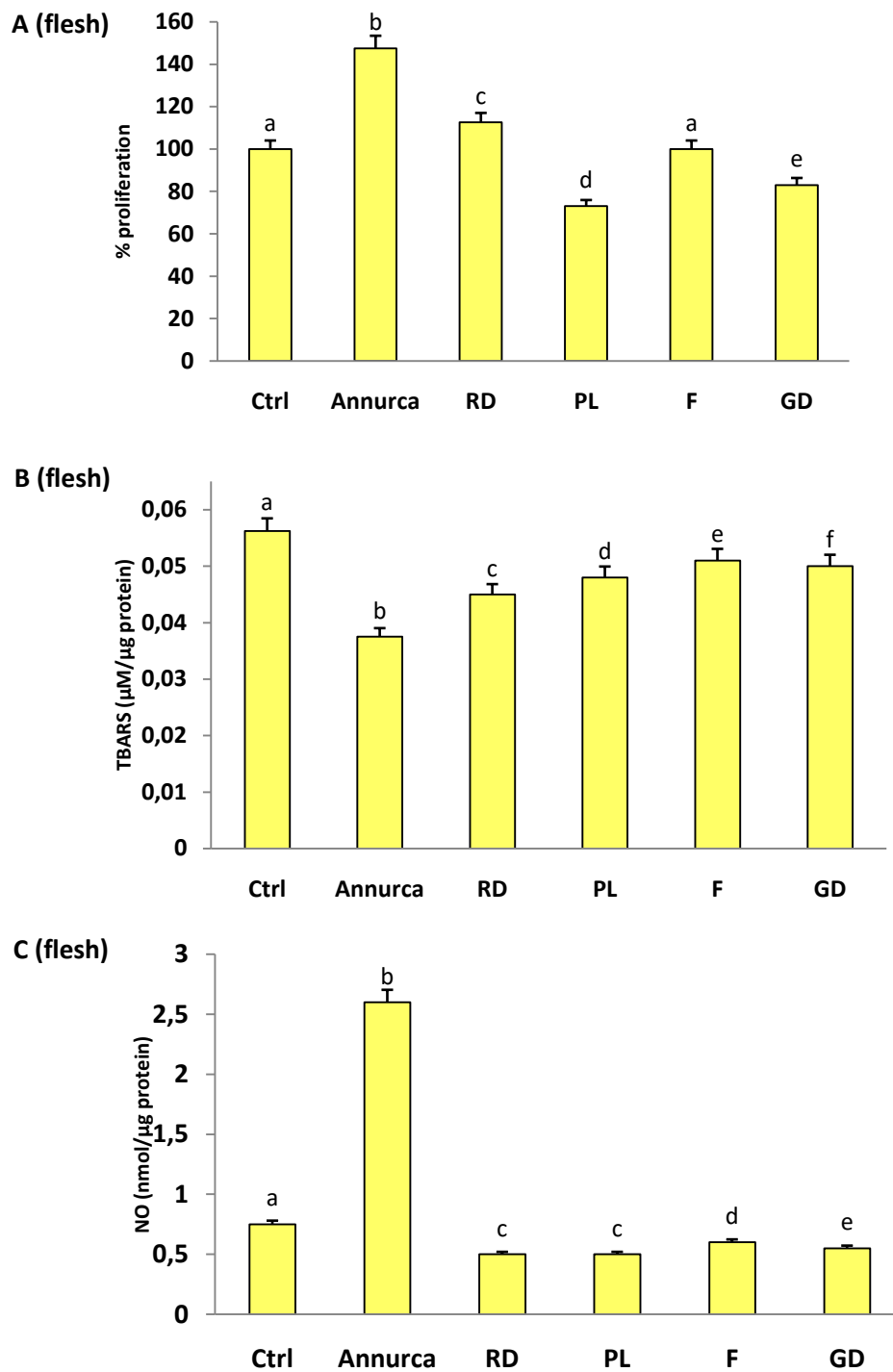


Figure 17: Effect of polyphenolic extracts from apple flesh on HepG2 proliferation (A), oxidative stress (B) and on nitric oxide levels in the cell medium (C). Data are the means \pm SD ($n = 3$).

^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

The best values were obtained for Annurca flesh. Annurca polyphenols decreased the physiological intracellular ROS by 33.3%, while the polyphenols obtained from the other analysed cultivars had minor effects and ranged from 9.25% to 19.9%. Annurca flesh extracts also increased NO release by cells by about five times by comparison with the other flesh extracts. Particularly, the latter result is very interesting, if considering the important both physiological (vasorelaxation and neuronal signal transduction) and pathological (cell apoptosis) actions that this compound exerts in different tissues.

The reasons of the differences found in the behaviour of apple peel and flesh polyphenolic extracts of various cultivars relative to cell proliferation and oxidative stress have to be sought in the different polyphenolic composition of peels and pulp. The most abundant polyphenols in flesh are procyanidins, and oligomeric procyanidins have been reported to adsorb to membranes thanks to associations with the polar portion of phospholipids and to generate a “flavonoid coat”, which would be able to protect against oxidants and other external aggressors. Such a coat could limit the access of oxidants to the phospholipid bilayer thanks to the formation of a compact structure and it could control the propagation of free radical chain reactions that occur in the hydrophobic core membrane (Ugartondo et al., 2007).

3.2.5 Triglyceride uptake

The inhibition capacity on triglyceride uptake of apple peel and flesh polyphenolic extracts coming from different apple cultivars was evaluated by using HepG2 cell line, because of the knowledge of the role of intestine and liver cells in the metabolism of lipids.

The results concerning the effects on triglyceride uptake are shown in Figure 18. As regards peels, Red Delicious extract determined an increase in the triglyceride cell medium concentration of approximately 854% above control cells, while the other polyphenolic extracts led to less relevant effects (Figure 18A). Instead, as regards flesh samples, all the extracts determined a reduction in triglyceride deposition, by increasing their concentration in the cell medium. The best results were obtained with Annurca extract (+2237% than control cells), followed by Pink Lady (+1467%

than control cells), Red Delicious (+1012% than control cells), Fuji (+655% than control cells) and Golden Delicious (+375% than control cells) extracts (Figure 18B) (Tenore et al., 2014).

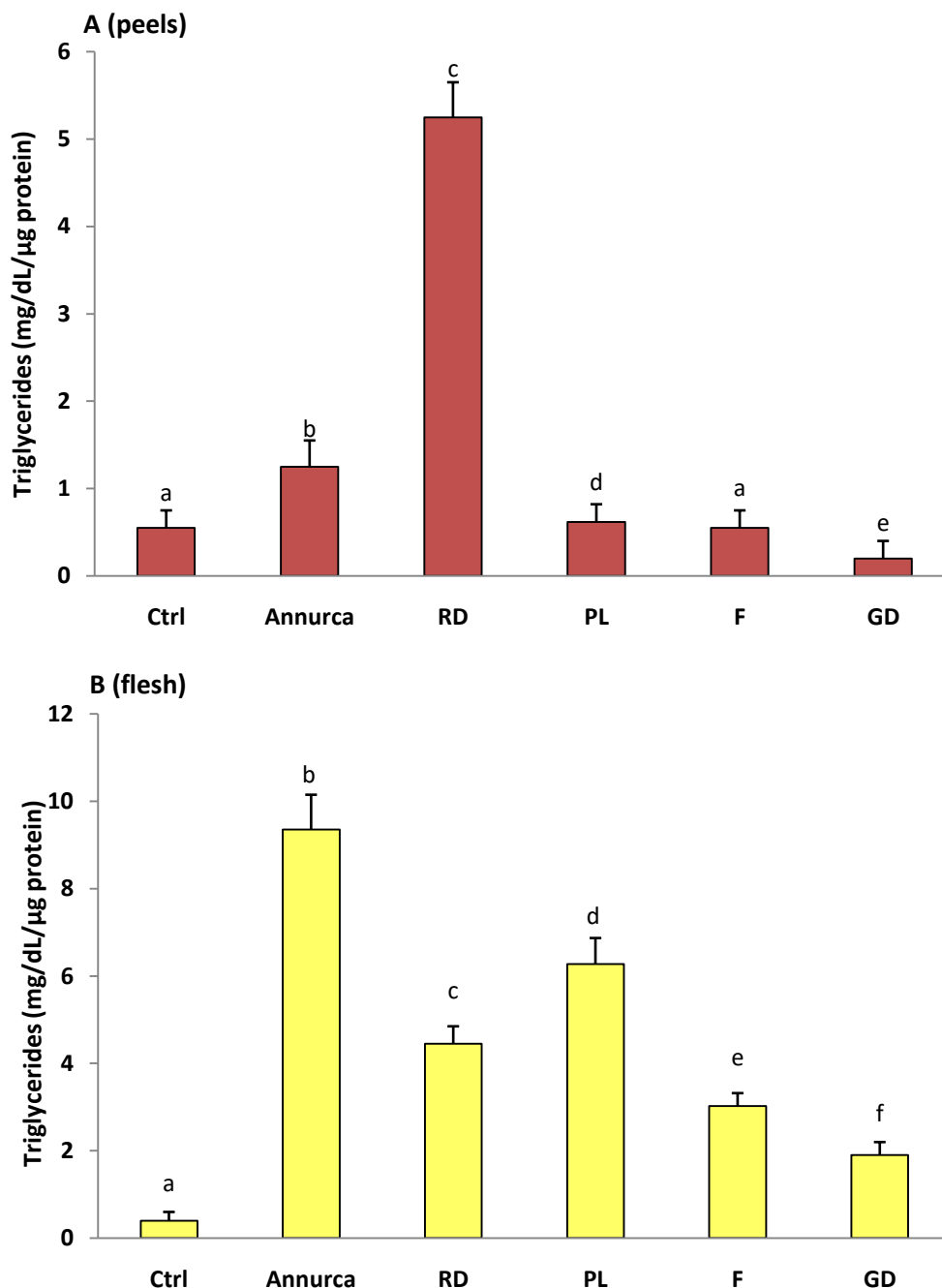


Figure 18: Inhibition of HepG2 cell triglyceride uptake by apple peel (A) and flesh (B) polyphenolic extracts expressed as remaining triacylglycerols in the medium. Data are the means \pm SD ($n = 5$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($P < 0.05$).

Procyanidins are considered as the responsible for this effect, because they would be able to inhibit *in vivo* triglyceride uptake (Sugiyama et al., 2007). Moreover, oligomeric procyanidins would promote the formation of complexes similar to β -cyclodextrins, which incorporate triglycerides in a selectively and intensively manner. Thus, they would prevent triglyceride uptake and would give a false impression of inhibited triglyceride membrane transport in these cells (Leifert et al., 2008).

The results regarding cellular triglyceride uptake agreed with the polyphenolic profiling obtained by HPLC-DAD/ESI-MSⁿ. In fact, procyanidins represented the most abundant polyphenolic compounds contained in apple flesh, and Annurca flesh had the highest quantity. A good linear correlation between the levels of procyanidins contained in the samples and the inhibition of triglyceride uptake both for peels ($R = 0.9915$) and for flesh ($R = 0.9395$) was found.

3.2.6 Lipase activity

The incubation of HepG2 cells with apple peel and flesh polyphenolic extracts led to the inhibition of triacylglycerol hydrolase, an important enzyme that intervenes in lipid digestion.

The results are shown in Figure 19. Among peels, Red Delicious extract showed the highest percentage of inhibition of the enzyme (-30% than control cells), followed by Annurca (-8% than control cells), Pink Lady (-7% than control cells), Fuji (-4% than control cells) and Golden Delicious (not detectable) extracts (Figure 19A). As regards flesh extracts, the percentage of inhibition for Annurca was higher than those found in other apple peel and flesh extracts (-55% than control cells). The other flesh extracts showed the following percentages of inhibition compared to control cells: Pink Lady, -30%; Red Delicious, -21%; Fuji, -14% and Golden Delicious, -9% (Figure 19B) (Tenore et al., 2014).

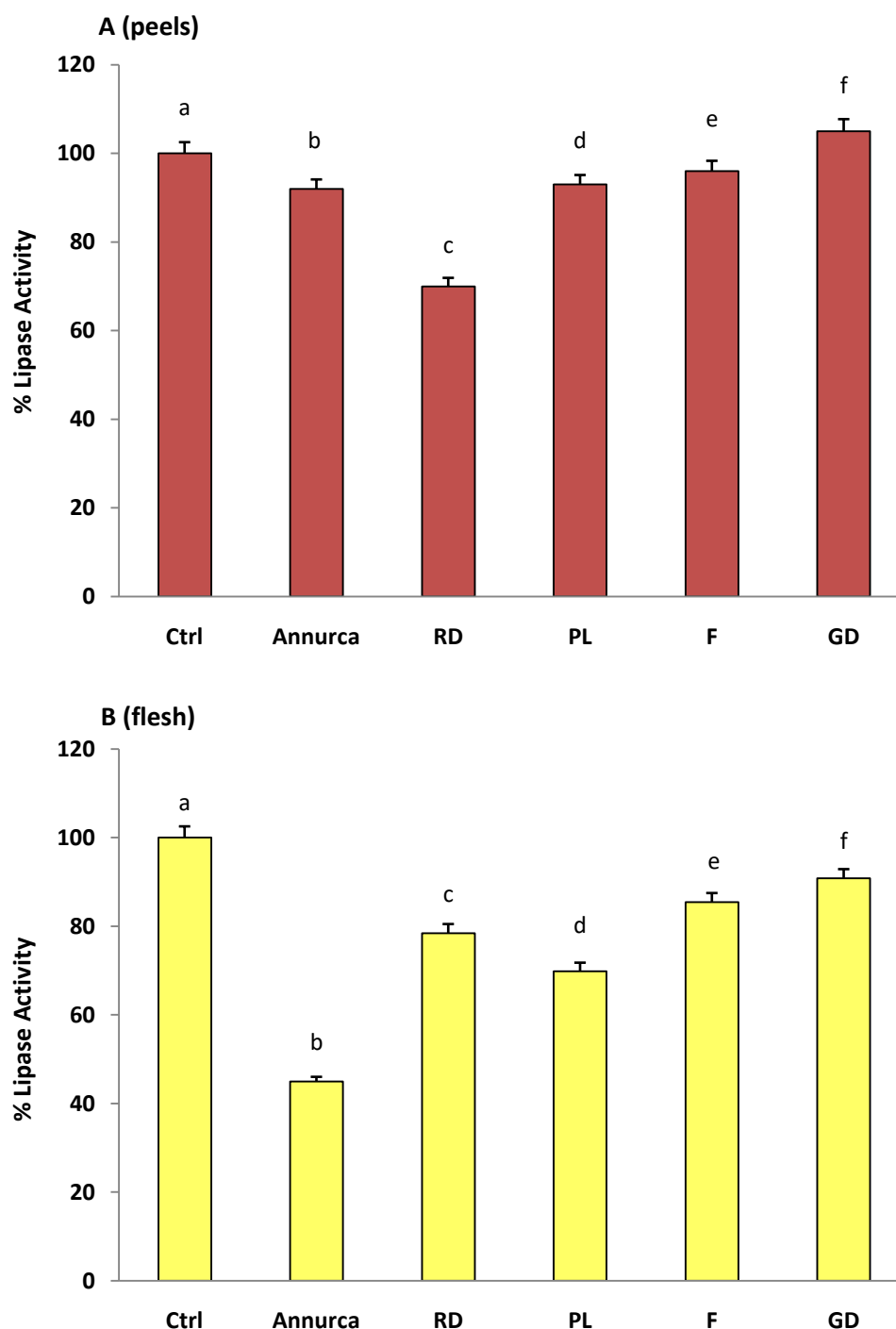


Figure 19: Inhibition of lipase activity in HepG2 cells by apple peel (A) and flesh (B) polyphenolic extracts. Data are the means \pm SD ($n = 5$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

An earlier work reported that apple polyphenolic extracts are able to inhibit *in vitro* pancreatic lipase activity in a dose-dependent manner and to reduce *in vivo* triglyceride absorption (Sugiyama et al., 2007). In particular, oligomeric procyanidins are considered as the apple polyphenols responsible for the inhibition

of this enzyme, because they have been reported to interact with proteins thanks to hydrophobic effects, hydrogen bonds and proline residues (Haslam, 1996). Specifically, procyanidins characterised by a higher degree of polymerization would be associated with an increase in the inhibition of pancreatic lipase, which reached its maximal level with pentameric or greater procyanidins (Sugiyama et al., 2007).

The results regarding the inhibition of pancreatic lipase agreed with the procyanidin content found in the extracts through HPLC-DAD/ESI-MSⁿ analysis. In fact, procyanidins represented the most abundant polyphenolic compounds contained in apple flesh, and Annurca flesh expressed the highest quantity. A good linear correlation between the levels of procyanidins contained in the samples and the inhibition of enzyme activity both for peels ($R = 0.9727$) and for flesh ($R = 0.9903$) was found.

The inhibition of pancreatic lipase would be associated with a reduced hydrolysis of dietary lipid esters and the resulting absorption of fatty acids (Sbarra et al., 2005), even if no data on hepatic cells are now available.

The obtained data showed a good linear correlation between the inhibition of triacylglycerol hydrolase activity and triglyceride uptake both for peels ($R = 0.9689$) and for flesh ($R = 0.9843$).

3.2.7 Low-density lipoprotein (LDL) receptor binding activity

The incubation of HepG2 cells with apple peel and flesh polyphenolic extracts obtained from different cultivars led to an enhancement in binding activity of low-density lipoprotein (LDL) receptor, that was evaluated indirectly by measuring the LDL concentration.

The results are shown in Figure 20. Among peels, Red Delicious extract was the most effective in increasing the binding activity of LDL receptor (-26% than control cells) (Figure 20A). As regards flesh, instead, Annurca extract was the most effective and showed the highest results (-46% than control cells), followed by Pink Lady (-30% than control cells), Red Delicious (-20% than control cells), Fuji (-6% than control cells) and Golden Delicious (not detectable) (Figure 20B) (Tenore et al., 2014).

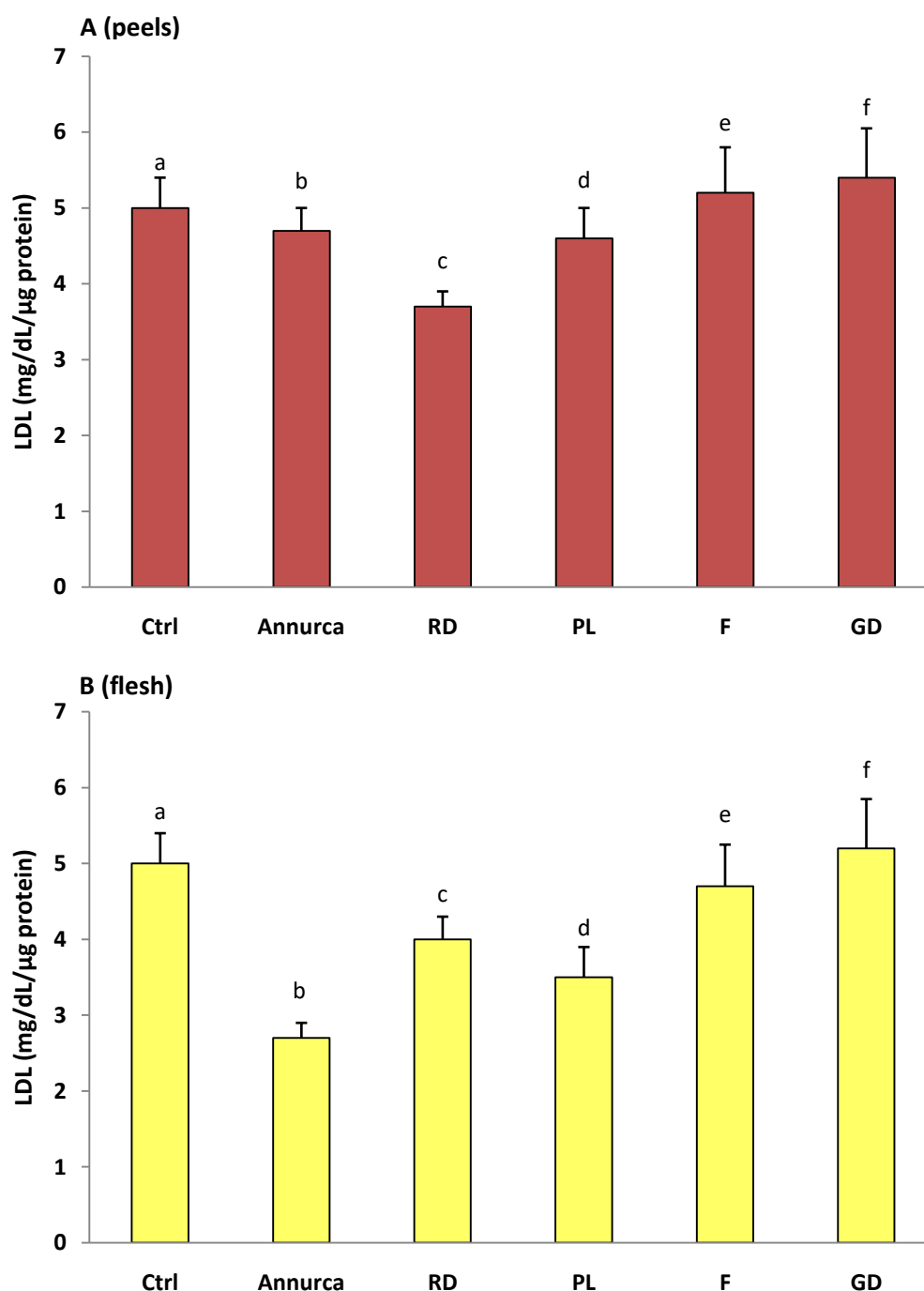


Figure 20: Enhancement of HepG2 cell low-density lipoprotein (LDL) receptor binding activity by apple peel (A) and flesh (B) polyphenolic extracts expressed as remaining LDL in the medium. Data are the means \pm SD ($n = 5$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

Procyanidins are the compounds that may be responsible for such an effect. In fact, these molecules are constituted by catechin and epicatechin monomers, which have already been reported to enhance LDL receptor binding activity of HepG2 cells in a

dose-dependent manner, by facilitating the active SREBP-1 (Sterol Regulatory Element Binding Protein-1) transcription factor form. Thus, procyanidins would act in a manner similar to that of green tea catechins (Bursill et al., 2006).

The results regarding the enhancement of LDL receptor binding activity agreed with those regarding the procyanidin content in apple peel and flesh samples. In fact, procyanidins represented the major component contained in apple flesh, and, particularly, Annurca flesh had the highest quantity. A good linear correlation between the levels of procyanidins contained in the samples and the LDL receptor binding up-regulation was registered both for peels ($R = 0.9488$) and for flesh ($R = 0.9887$).

3.2.8 Apolipoprotein A1 (ApoA1) expression

The incubation of HepG2 cells with apple peel and flesh polyphenolic extracts led to an increased expression of Apolipoprotein A1 (ApoA1), which is the major protein component of HDL particles. HDL particles are involved in the cholesterol transport from extrahepatic tissues to the liver, in order to process or excrete cholesterol in bile acids. Moreover, ApoA1 activates the enzyme LCAT (lecithin:cholesterol acyltransferase), so it is essential for the reverse cholesterol pathway (Jonas, 1998).

The results are shown in Figure 21. Among peels, Red Delicious extract was the most effective in increasing the expression of Apolipoprotein A1 (+35.7% than control cells) (Figure 21A). As regards flesh, instead, Annurca extract was more effective than other apple peel and flesh extracts (+71.4% than control cells), followed by Pink Lady (+54.3% than control cells), Red Delicious (+21.4% than control cells), Fuji (not detectable) and Golden Delicious (not detectable) extracts (Figure 21B) (Tenore et al., 2014).

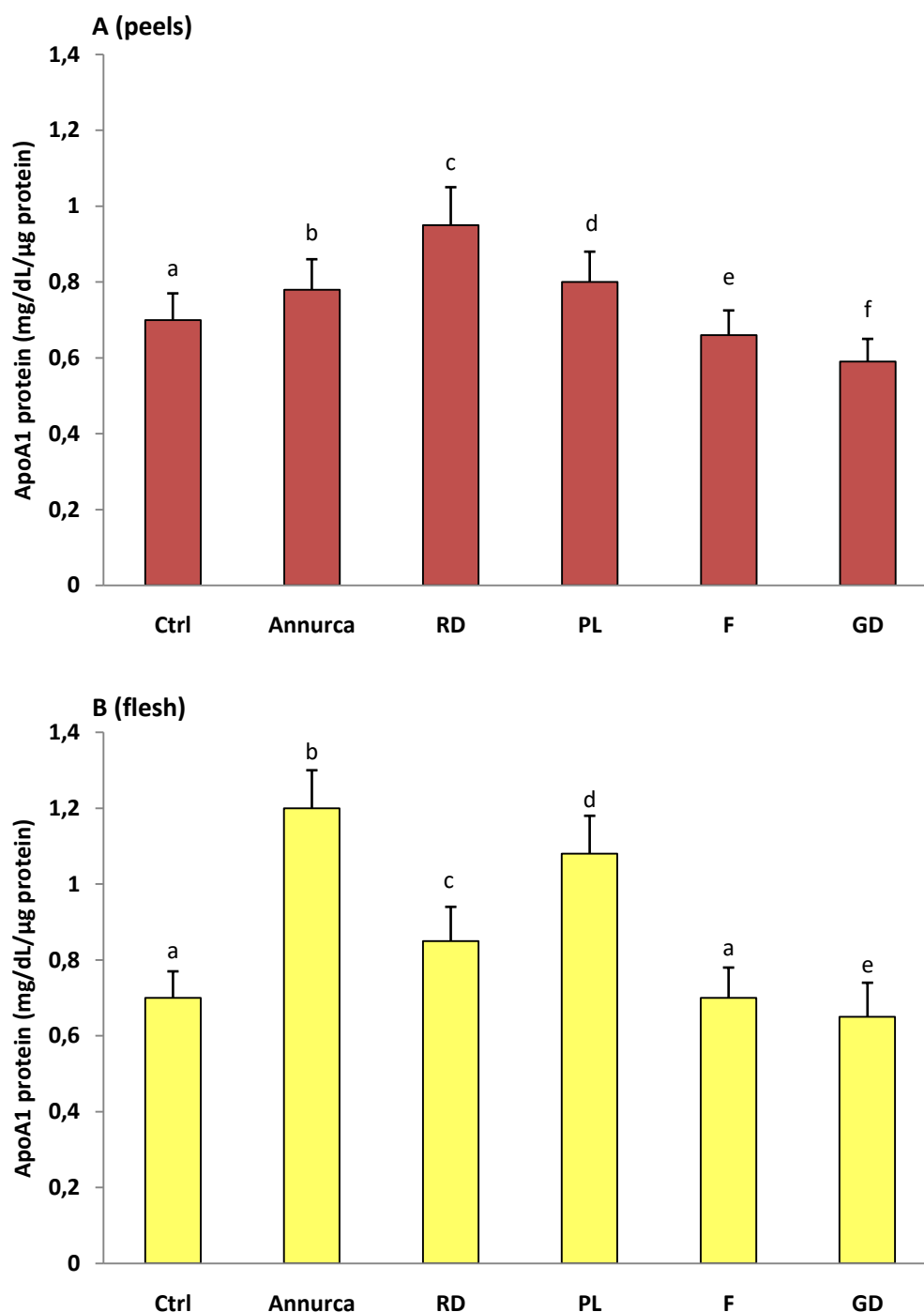


Figure 21: Enhancement of HepG2 cell ApoA1 protein expression by apple peel and flesh polyphenolic extracts expressed as ApoA1 protein concentrations in the medium. Data are the means \pm SD ($n = 5$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

It has been reported that several polyphenolic compounds may increase ApoA1 levels in HepG2 cells (Yasuda et al., 2011) and tea catechins are able to enhance HDL plasma levels both in animals (hyperlipidemic rats) and humans (patients with

obesity and metabolic syndrome) (Gong et al., 2010; Basu et al., 2010). Moreover, the enzyme LCAT would be more active after the interaction of (+)-catechin and procyanidins with ApoA1 and the resulting change of its conformation (Jonas, 1998).

A good linear correlation between the levels of procyanidins contained in the samples and the increase in ApoA1 cell expression was registered both for peels ($R = 0.8946$) and for flesh ($R = 0.9772$).

CHAPTER 4

In vitro evaluation of bioaccessibility, bioavailability and plasma protein interaction of digested apple peel and flesh polyphenols

4.1 Materials and methods

4.1.1 Reagents and standards

All chemicals and reagents used were either analytical-reagent or HPLC grade. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA) before use. The standards used for the identification and quantification of phenolic acids and flavonoids were: chlorogenic acid, (+)-catechin, (-)-epicatechin, isorhamnetin, myricetin, phloretin, phloridzin (phloretin-2-*O*-glucoside), procyanidin B2, quercetin, quercitrin (quercetin-3-*O*-rhamnoside), rutin (quercetin-3-*O*-rutinoside), isoquercitrin (quercetin-3-*O*-glucoside), hyperin (quercetin-3-*O*-galactoside) and cyanidin-3-*O*-galactoside chloride (Sigma Chemical Co., St. Louis, MO). Chemicals and reagents used to simulate the gastrointestinal digestion and interaction with plasma proteins were: potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH₂PO₄), sodium sulphate (Na₂SO₄), sodium chloride (NaCl), sodium bicarbonate (NaHCO₃), urea, α -amylase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts, human serum albumin (HSA, $\geq 97.0\%$), high-density lipoprotein (HDL, $\geq 95.0\%$), low-density lipoprotein (LDL, $\geq 95.0\%$) and very low-density lipoprotein (VLDL, $\geq 95.0\%$) (Sigma Chemical Co., St. Louis, MO). Acetonitrile and methanol were of HPLC grade (Carlo Erba, Milano, Italy).

4.1.2 Fruit collection and sample preparation

Annurca (*Malus pumila* Miller cv Annurca) apple fruits were collected in Valle di Maddaloni (Caserta, Italy) in October when fruits had just been harvested (green peel). Fruits were reddened, following the typical treatment (Lo Scalzo et al., 2001) for about 30 days, and then analysed. Other four apple varieties analysed in this study, Red Delicious (RD) (*Malus pumila* Miller cv Red Delicious), Pink Lady (PL) (*Malus pumila* Miller cv Pink Lady), Fuji (F) (*Malus pumila* Miller cv Fuji) and Golden Delicious (GD) (*Malus pumila* Miller cv Golden Delicious), were

purchased in a local supermarket. Lyophilised apple peels and flesh (10 g) were treated with 100 mL of a methanol/water mixture (80:20, v/v) acidified with 0.5% formic acid for 24 h at 4 °C in order to extract phenolic compounds. After centrifugation, the supernatant was slowly filtered and purified through an Amberlite XAD-2 column packed as follows: 10 g of resin (pore size 9 nm; particle size 0.3-1.2 mm; Supelco, Bellefonte, PA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 x 2 cm). The column was washed with 100 mL of acidified water (pH 2) and 50 mL of deionised water in order to remove sugar and other polar compounds. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen. The polyphenolic extracts were stored at -80 °C until analysis.

4.1.3 In vitro gastrointestinal digestion

In vitro gastrointestinal digestion was performed according to a procedure described previously (Raiola et al., 2012), with slight modification. *In vitro* gastrointestinal digestion was distinguished into different steps: salivary, gastric and duodenal digestive steps. For the salivary digestion, apple samples (20 g) were mixed with 6 mL of artificial saliva consisting of: potassium chloride (KCl, 89.6 g/L), potassium thiocyanate (KSCN, 20 g/L), monosodium phosphate (NaH_2PO_4 , 88.8 g/L), sodium sulphate (Na_2SO_4 , 57.0 g/L), sodium chloride (NaCl, 175.3 g/L), sodium bicarbonate (NaHCO_3 , 84.7 g/L), urea (25.0 g/L) and α -amylase (290 mg). The pH of the solution was adjusted to 6.8 with HCl 0.1 N. Then, the mixture was introduced in a plastic bag containing 40 mL of water and homogenised in a Stomacher 80 Microbiomaster (Seward, Worthing, UK) for 3 min. Immediately, 0.5 g of pepsin (14800 U) dissolved in HCl 0.1 N was added, the pH was adjusted to 2.0 with HCl 6 N, and then incubated at 37 °C in a Polymax 1040 orbital shaker (250 rpm) (Heidolph, Schwabach, Germany) for 2 h. After the gastric digestion, the duodenal step was simulated as follows: the pH was increased to 6.5 with NaHCO_3 0.5 N and then 5 mL of a mixture constituted by pancreatin (8.0 mg/mL) and bile salts (50.0 mg/mL) (1:1, v/v), dissolved in 20 mL of water, was added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h. After each step of

digestion, 10 mL of the obtained extract were centrifuged at 4000 rpm and 4 °C for 1 h: before each following step, the digestion procedure was started over again. To determine the polyphenolic profile, the supernatants were extracted with a mixture of acetonitrile-water (84:16; v/v) and then analysed by HPLC.

4.1.4 Cell culture and in vitro study of polyphenolic transepithelial transport

Human colon carcinoma cells Caco-2 (HTB-37) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells were cultured routinely in HEPES buffered Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose and supplemented with 12.5% heat-decomplemented fetal calf serum (FCS), 1% nonessential amino acids, 5 mM L-glutamine, 40 U/mL penicillin, 100 µg/mL gentamycin and 40 µg/mL streptomycin (DMEMc). Cells were maintained at 37 °C in a humidified atmosphere of CO₂/air (5:95) and passaged every 7 days by trypsinisation. They were seeded in transwells at 6×10^4 cells/cm². The medium (15 mL DMEM containing 10% FCS) was changed every 2 days until cells reached confluence (7-8 days). The integrity of the monolayers was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell-ERS device (Millipore, Zug, Switzerland) before and after the treatments. To evaluate transepithelial permeability, medium was removed from the apical and basal sides of the cultures and replaced by 2 mL of the transport solution consisting of Hanks' balanced salt solution (HBSS) and intestinal apple polyphenols, and pH was adjusted to 6 or 7.4. After 4 h of incubation at 37 °C, apical and basal solutions were collected and in order to determine the polyphenolic profile, aliquots (5 mL) were immediately mixed with 1 mL of methanol and filtered on 0.45 µm Millex-HV filter units. Samples were stored at -20 °C until HPLC analysis.

4.1.5 In vitro study of plasma protein interaction with polyphenols

The *in vitro* transepithelial permeated polyphenols were added to plasma protein solutions simulating, each one, the average fasting plasma protein concentrations. The basic composition of plasma protein solutions consisted in 20 mM HEPES (pH

7.5), 0.9% NaCl, 0.5 M NaHCO₃. Each plasma protein solution was constituted by: human serum albumin (HSA, 42 mg/mL), high-density lipoprotein (HDL, 1450 µg/mL), low-density lipoprotein (LDL, 780 µg/mL) and very low-density lipoprotein (VLDL, 120 µg/mL). Polyphenols were incubated with plasma proteins for 30 min at 37 °C to allow equilibration. Then, the samples were centrifuged at 25 °C for 30 min at 14000 g. The supernatant containing the free polyphenolic fraction was collected and subjected to HPLC analysis.

4.1.6 HPLC-DAD/ESI-MSⁿ analysis

HPLC separation and quantification of phenolic compounds in apple peel and flesh extracts and samples obtained from *in vitro* digestion, transepithelial permeation, and plasma protein binding experiments were performed according to previous studies with some modifications (Mari et al., 2010). The identification of phenolic compounds was possible by recording chromatograms at different wavelengths, 280 nm for flavonols, procyanidins, dihydrochalcones and hydroxycinnamic acids, and 500 nm for anthocyanins and by comparing spectra and retention times with those of commercial standards and with those reported in previous works (Mari et al., 2010). Elution conditions consisted in 2% acetic acid (solvent A) and 0.5% acetic acid in acetonitrile and water (50:50, v/v) (solvent B) gradient at a flow rate of 1.0 mL/min. The column selected was a Hypersil BDS C18 column (250 mm x 4.6 mm, 5 µm) (Thermo, Bellefonte, PA). Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA) provided with photodiode array detector (DAD). The gradient conditions were: 0-5 min, 10% B; 55 min, 55% B; 65 min, 95% B, followed by 5 min of maintenance. The identity of phenolic acids and flavonoids was confirmed with LC-ESI/MS/MS experiments and data were compared with those of commercial standards and with those reported in previous works (Mari et al., 2010). The same chromatographic conditions were applied to a HP1100 HPLC system (Agilent, Palo Alto, CA) coupled to a PE-Sciex API-2000 triple-quadrupole mass spectrometer (AB Sciex, Warrington, UK) equipped with a Turbospray (TSI) source. Individual phenols were separated on a Hypersil BDS C18 column (250 mm x 2.1 mm, 5 µm) (Thermo Electron, Bellefonte, PA) at a flow rate of 200 µL/min. Mass spectra were recorded from *m/z*

= 50 to 1500, in both negative and positive ionisation mode. For negative ion mode, the capillary voltage was set at -13 V, the spray voltage was at 4.5 kV and the tube lens offset was at -15 V, while in positive ion mode, the capillary voltage was set at 13 V, the spray voltage was at 5 kV and the tube lens offset was at -35 V. The capillary temperature was 275 °C. Data were acquired in MS, MS/MS and MSⁿ scanning modes.

4.1.7 Statistics

All the experimental results were expressed as mean \pm standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student's *t* test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The level of significance (α -value) was 95% in all cases ($P < 0.05$). The degree of linear relationship between two variables was measured using the Pearson product moment correlation coefficient (*R*). Correlation coefficients (*R*) were calculated by using Microsoft Office Excel application.

4.2 Results and discussion

As already reported, the parameters studied were bioaccessibility, bioavailability and interactions with plasma proteins (Tenore et al., 2013b). Bioaccessibility is considered as the nutrient fraction that is extracted from the food matrix in the gastrointestinal tract and that becomes available for the absorption (Tagliazucchi et al., 2010), while bioavailability is the fraction that reaches the systemic circulation and that is available for the action (Manach et al., 2004). The amount of the considered polyphenols before digestion of apple peel and flesh is reported in Figure 22.

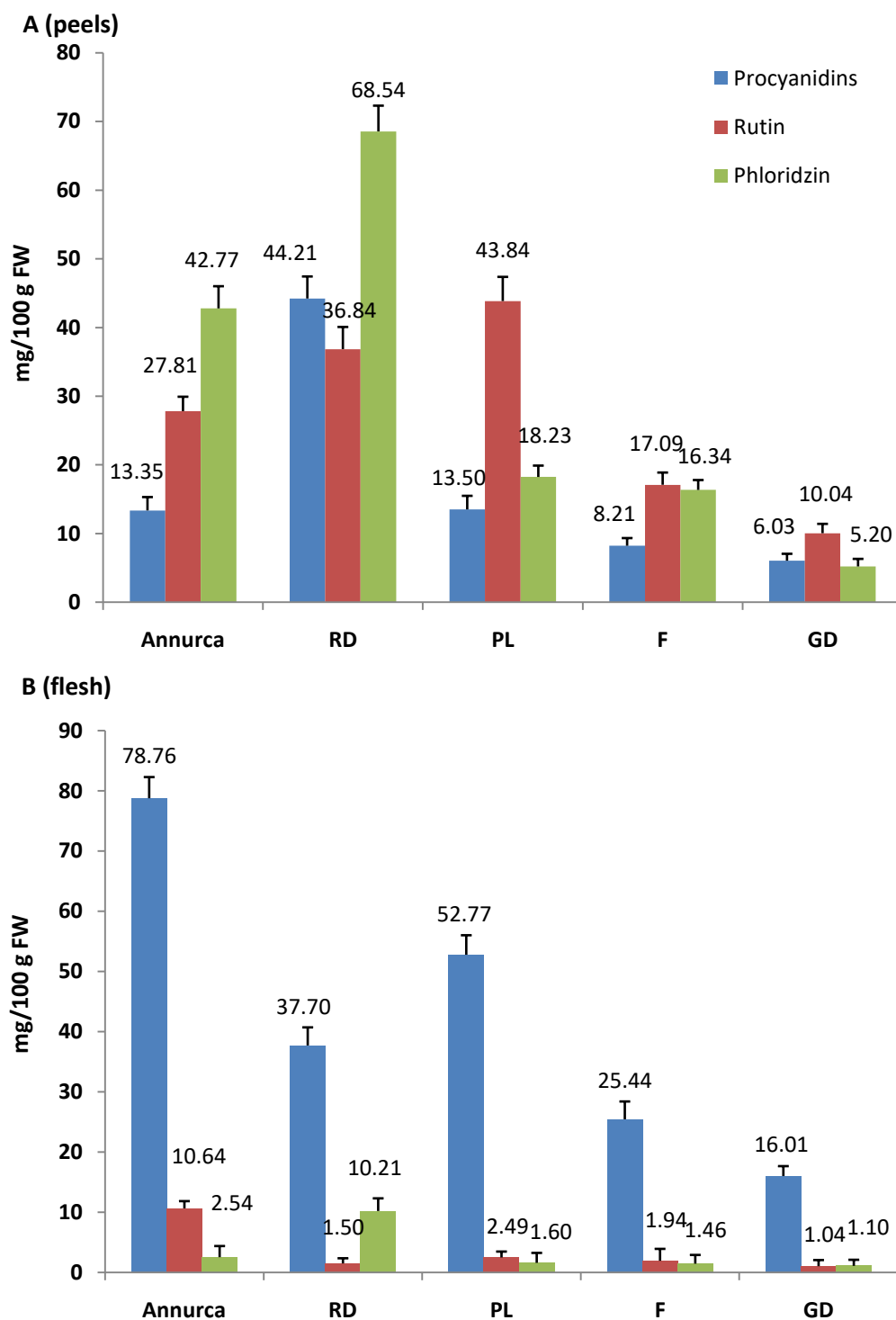


Figure 22: Polyphenolic profile of apple peel and flesh extracts. Polyphenolic contents are expressed as mg/100 g FW \pm SD ($n = 5$). Means are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

4.2.1 Bioaccessibility after salivary digestion

The results regarding the bioaccessibility after salivary digestion are shown in Figure 23. The first digestive step revealed to be very important for the simulated digestive extraction process of apple polyphenols. Procyanidins, rutin and phloridzin coming from apple peels and flesh were detected in this step at levels of about 35.0%, 35.1% and 27.3%, respectively, of the undigested samples. As expected on the basis of the values obtained before the *in vitro* digestion process, experimental results showed for Red Delicious and Pink Lady peels the greatest bioaccessibility in phloridzin (18.71 mg/100 g FW) and rutin (15.39 mg/100 g FW), respectively, and for Annurca pulp the best bioaccessibility in procyanidins (27.57 mg/100 g FW).

Bioaccessibility of apple peel and flesh polyphenols after salivary digestion

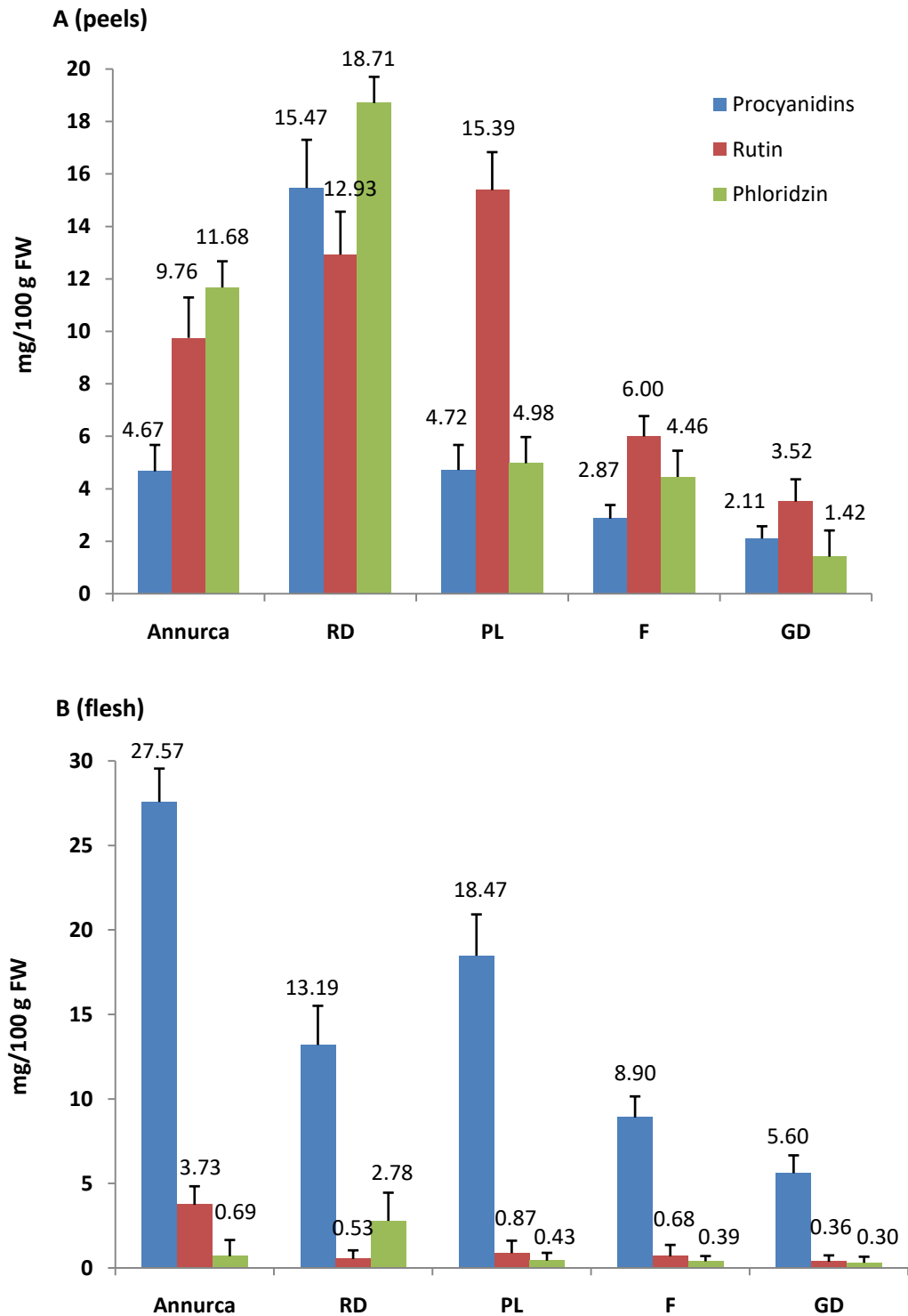


Figure 23: Salivary bioaccessibility of polyphenols from apple peels and flesh. Polyphenolic contents are expressed as mg/100 g FW \pm SD ($n = 5$). Means are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

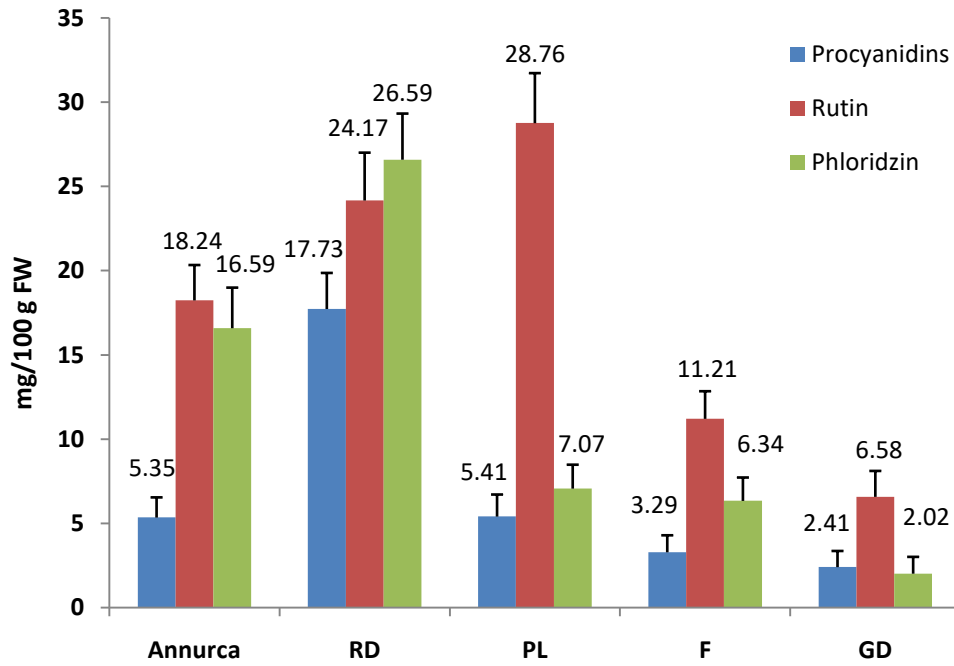
Salivary digestion represents a key step in a simulated *in vitro* gastrointestinal digestion because it is well known that the epithelium of oral mucosa is highly vascularised and allows both polar and hydrophobic molecules to be absorbed and to reach rapidly the systemic circulation, without the passage through the gastrointestinal tract. Thus, since a lot of molecules, both nutrients and non-nutrients, are sensitive to pH variations and/or are poorly absorbed by the epithelium of the intestinal mucosa, good extraction processes at this level may be preliminary to a better bioavailability of such compounds, because they cannot be degraded in the stomach and/or destroyed in the intestine and/or subjected to metabolism by the liver (Oulianova et al., 2007). Moreover, it has been reported that procyanidins and condensed tannins may inhibit some digestive enzymes, such as α -amylase, which degrades starch to dextrins. Thus, these molecules may act as antinutritional compounds and for procyanidin-rich foods mastication is more important than salivary enzymes in defining their bioaccessibility (Gonçalves et al., 2011).

4.2.2 Bioaccessibility after gastric digestion

The data regarding the bioaccessibility after the gastric phase are reported in Figure 24. The gastric phase revealed an increase in the bioaccessibility of procyanidins, rutin and phloridzin compared to salivary step (about, +5.1%, +30.5% and +11.5%, respectively), both for peels and for flesh. As expected on the basis of the values obtained before the *in vitro* digestion process, experimental results showed for Red Delicious and Pink Lady peels the greatest bioaccessibility in phloridzin (26.59 mg/100 g FW) and rutin (28.76 mg/100 g FW), respectively, and for Annurca flesh the best bioaccessibility in procyanidins (31.58 mg/100 g FW).

Bioaccessibility of apple peel and flesh polyphenols after gastric digestion

A (peels)



B (flesh)

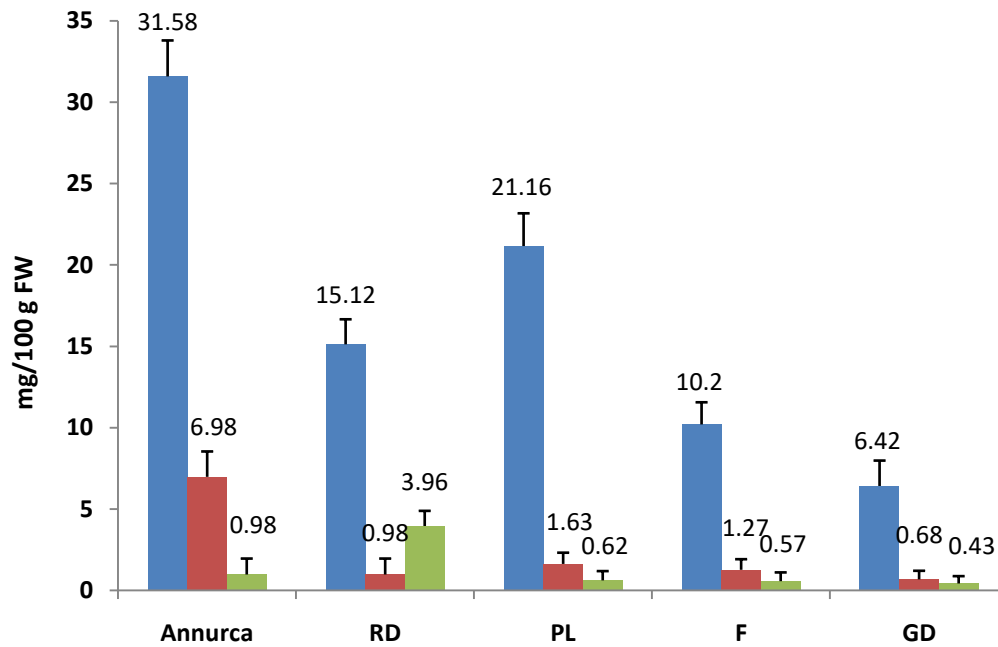


Figure 24: Gastric bioaccessibility of polyphenols from apple peels and flesh. Polyphenolic contents are expressed as mg/100 g FW \pm SD ($n = 5$). Means are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

Greater values of accessible polyphenols in the gastric phase than in the salivary phase highlight two important aspects: firstly, there is a continuation of extractive processes of polyphenolic compounds from food matrix; secondly, polyphenols are stable at highly acid pH in the stomach.

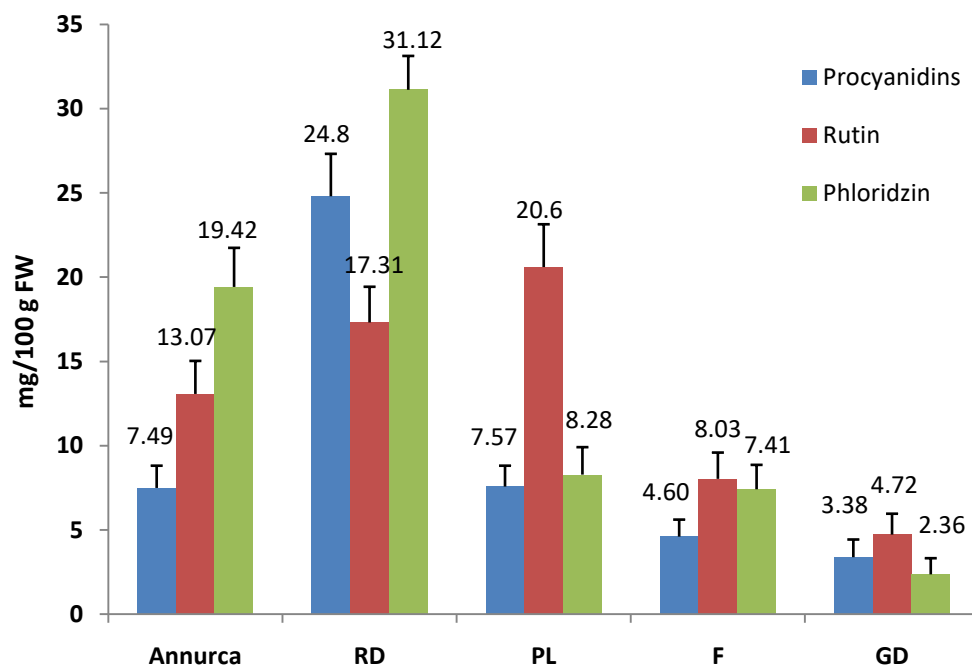
However, our results were partially in contrast with some previously reported. As regards rutin and phloridzin, they have already been reported to be resistant enough to acid hydrolysis and to reach the intestine in their native form, without any transformation to corresponding aglycones (Manach et al., 2004; Bouyaed et al., 2012). As regards procyanidins, previous studies reported that they are sensitive to gastric acid pH and are degraded to their monomers within few hours (Zhu et al., 2002). Nevertheless, these data refer mainly to procyanidin B1, B2 and B5, which are dimeric compounds, but apple extracts are rich in complex mixtures of procyanidins characterised by a higher degree of polymerization (such as trimers, tetramers and pentamers) (Mari et al., 2010). Earlier *in vivo* studies reported that cocoa procyanidins, which exhibit a higher degree of polymerization respect to dimeric compounds, would be more resistant to the pH of the stomach and would not be degraded into the corresponding monomers in such a little time (Rios et al., 2002).

4.2.3 Bioaccessibility after intestinal digestion

The results concerning the intestinal bioaccessibility are shown in Figure 25. After intestinal digestion, the levels of procyanidins and phloridzin were higher (about, +16.0% and +6.6%, respectively), whereas rutin levels were lower (approximately, -18.6%) than those found in gastric step. As expected on the basis of the values obtained before the *in vitro* digestion process, experimental results showed for Red Delicious and Pink Lady peels the greatest bioaccessibility in phloridzin (31.12 mg/100 g FW) and rutin (20.6 mg/100 g FW), respectively, and for Annurca flesh the best bioaccessibility in procyanidins (44.18 mg/100 g FW).

Bioaccessibility of apple peel and flesh polyphenols after intestinal digestion

A (peels)



B (flesh)

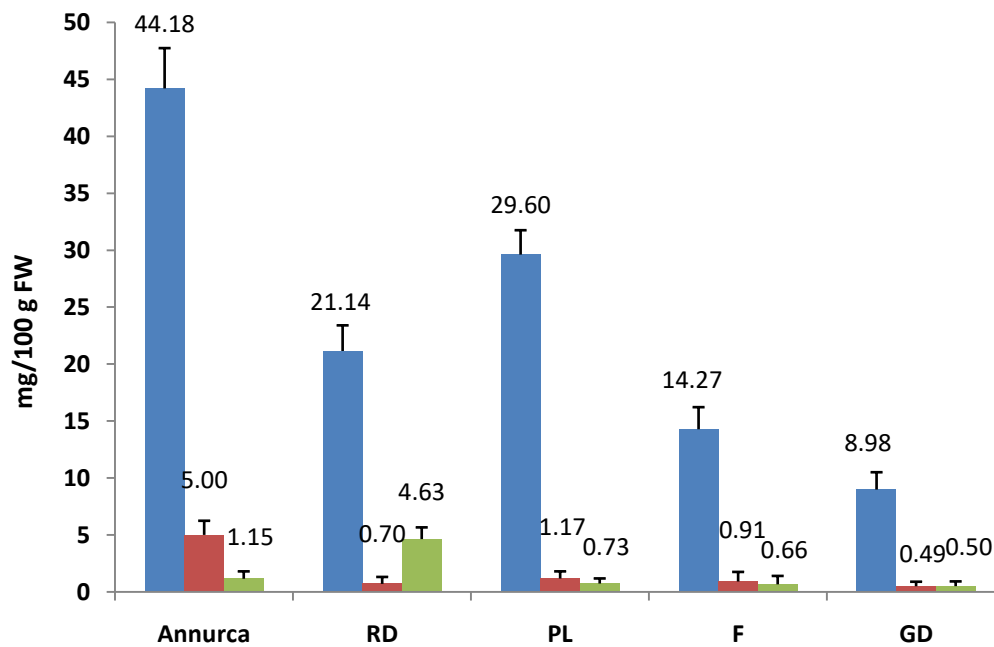


Figure 25: Intestinal bioaccessibility of polyphenols from apple peels and flesh. Polyphenolic contents are expressed as mg/100 g FW \pm SD ($n = 5$). Means are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

Our results were in agreement with previous studies as regards intestinal bioaccessibility of rutin and phloridzin (Bouayed et al., 2012). Instead, the results regarding the intestinal bioaccessibility of procyanidins were in contrast with some previously reported. In fact, Zhu et al. highlighted the complete degradation of procyanidin B2, a dimeric compound, in the intestinal medium into unknown products (Zhu et al., 2002). However, similarly to what stated for the gastric phase, procyanidins with a higher degree of polymerization (for example, trimers, tetramers and pentamers) would be more stable than dimeric compounds.

4.2.4 Bioavailability of permeated polyphenols

In order to study the bioavailability of polyphenols coming from apple peels and flesh, single layers of Caco-2 cells were used. They were treated with apple peel and flesh polyphenols coming from intestinal digestion only after the evaluation of their biocompatibility through MTT assay. The choice of using this type of cell line, which is considered as a model of absorption in the small intestine, arose from the evidence that experiments performed by using a semipermeable cellulose membrane do not consider other mechanisms involved in the absorption of polyphenols. In fact, dialysis bags simulate only the intestinal passive diffusion, but polyphenols can be absorbed by enterocyte bilayer also through facilitated diffusion and active transport. As reported in Chapter 2, most part of glycosides are hydrolysed by specific enzymes and only the aglycone moiety can be absorbed in the small intestine; only specific glucosides can be absorbed without undergoing hydrolysis process, through the Na^+ -dependent glucose transporter (SLGT1) (Manach et al., 2004).

Rutin is a quercetin glycoside, constituted by the flavonol quercetin and the disaccharide rutinose. It has been reported that polyphenols linked to a rhamnose moiety reach the colon and are hydrolysed by rhamnosidases before absorption (Graefe et al., 2001). Phloridzin is a phloretin glucoside, constituted by phloretin and glucose. It has been reported that there is a first step of extracellular hydrolysis by the enzyme phloridzin hydrolase present in the brush-border membrane; then, there is a second step of passive diffusion of aglycone moiety across the brush border (Day et al., 2000). As regards procyanidins, it has been reported they are

poorly absorbed in their native form because of their high molecular weight (Déprez et al., 2001).

Our experimental results showed that all the considered polyphenols are scarcely absorbed, in contrast with a previous study (Bouyaed et al., 2012), whose results may appear overestimated. The aliquots of permeated rutin, phloridzin and procyanidins were about 5.21%, 5.16% and 6.7%, respectively, of their native patterns and about 11.1%, 11.4% and 12.0%, respectively, of their intestinal levels. In spite of such scarce values, these molecules may accumulate in the intestine and exert important local activity. First of all, their strong antioxidant capacity could protect the gut from oxidising agents, to which it is highly exposed, and so they could prevent the onset of various diseases, such as inflammation and cancer (Halliwell et al., 2000). Furthermore, the high molecular weight procyanidins may lead to a decrease in cholesterol uptake, by acting as β -cyclodextrins and forming micelle-like complexes in which cholesterol is selectively and intensively incorporated (Leifert et al., 2008).

All the data are shown in Table 3 and in Figure 26.

Table 3: Percentages of bioavailability of polyphenols from apple peels and flesh.			
	Rutin	Phloridzin	Procyanidins
Bioavailability (%) vs native patterns	5.21	5.16	6.7
Bioavailability (%) vs intestinal levels	11.1	11.4	12.0

Bioavailability of apple peel and flesh polyphenols after intestinal digestion

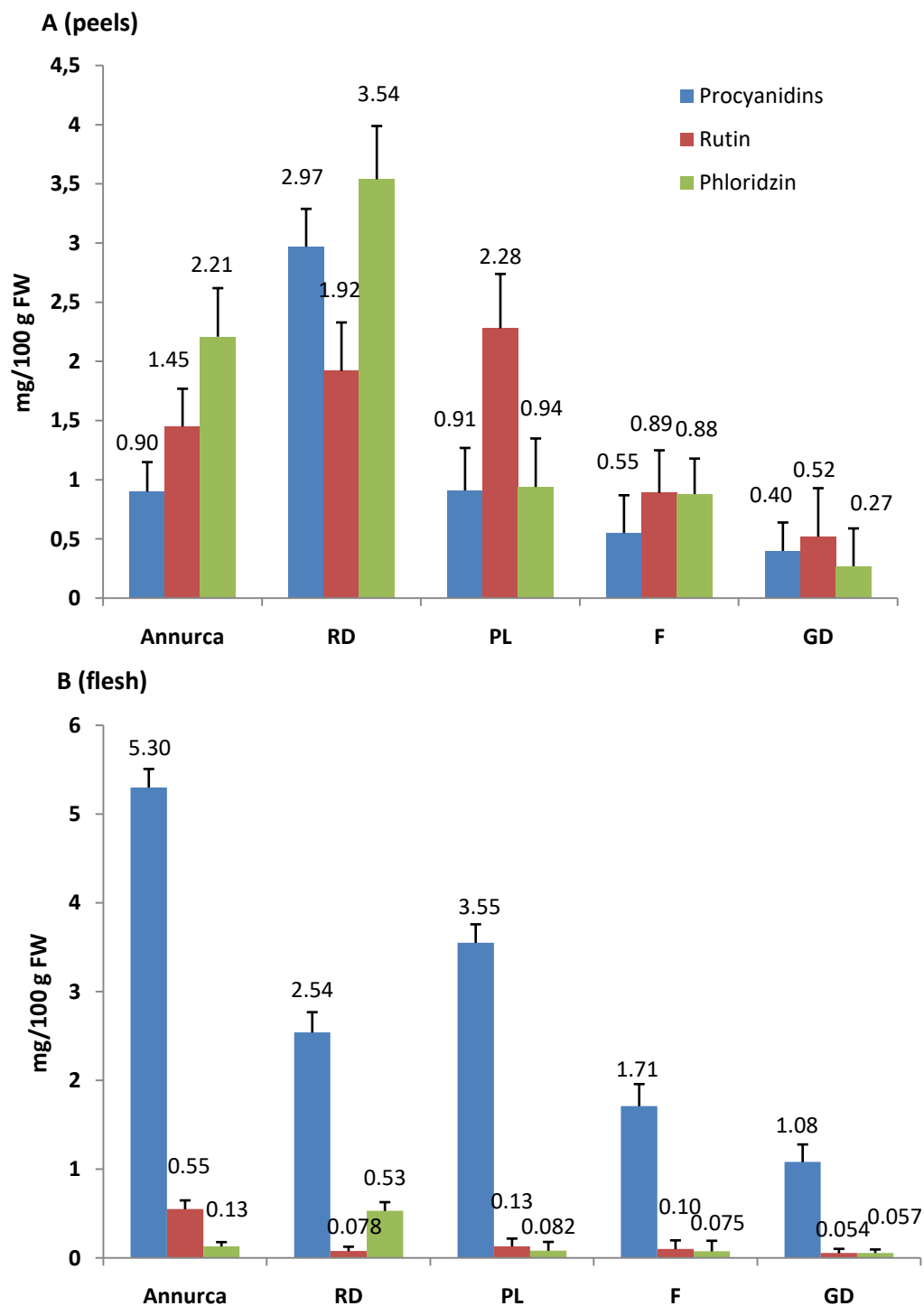


Figure 26: Bioavailability of polyphenols from apple peels and flesh evaluated by using single layers of Caco-2 cells as a model of absorption in the small intestine. Polyphenolic levels are expressed as mg/100 g FW \pm SD ($n = 5$). Means are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

4.2.5 Interaction with plasma proteins

Finally, the interactions of permeated polyphenols coming from apple peel and flesh with plasma proteins were evaluated. All the considered polyphenols showed a generally very high percentage of binding to plasma proteins, on average 93%. Rutin and phloridzin interacted chiefly with albumin (about 56.5% and 55.9%, respectively), as already reported in previous studies (Manach et al., 2004; Xiao et al., 2012), whereas procyanidins preferentially bound to HDLs (about 58.7%). Polyphenolic compounds also bound significantly to LDLs (on average, 16.2-19.6%), but they interacted scarcely with VLDLs (on average, 0.4-0.5%).

The experimental results are shown in Table 4.

Table 4: Percentages of bioavailable apple polyphenolic compounds bound to plasma proteins.				
	HSA	HDL	LDL	VLDL
Procyanidins	18.1 ± 0.9	58.7 ± 1.6	16.2 ± 1.1	0.4 ± 0.3
Rutin	56.5 ± 1.5	17.1 ± 1.0	19.6 ± 0.3	0.5 ± 0.1
Phloridzin	55.9 ± 2.0	16.8 ± 1.2	18.9 ± 0.8	0.4 ± 0.5
Data are mean values ± SD (<i>n</i> = 5) of polyphenols from peels and flesh of apple samples. Means are significantly different by the Tukey-Kramer multiple comparison test (<i>P</i> < 0.05). HSA: Human Serum Albumin; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; VLDL: Very Low-Density Lipoprotein.				

Protein-polyphenol interactions may be either reversible or irreversible; the type of binding depends on several factors, such as pH, temperature and concentration of both proteins and ligands (Xiao et al., 2012). However, the consequences of these interactions are not clear, but they would generally affect biological activities, leading to different effects (Manach et al., 2004; Xiao et al., 2012).

It has been demonstrated that procyanidins form a stable complex with protein ApoA1, the main protein component of HDL particles (Brunet et al., 2002). Protein ApoA1 is involved in the reverse cholesterol pathway because it activates the enzyme LCAT, that would be more active after the interaction of protein ApoA1 with procyanidins or their monomers and the resulting change of its conformation (Jonas, 1998). Thus, the interaction between procyanidins and HDLs suggests a possible role of procyanidins in the reverse transport and metabolism of cholesterol.

Moreover, the binding between polyphenols and LDLs may have an important role in the protection of cardiovascular system from oxidising agents and atherosclerosis (Manach et al., 2004).

CHAPTER 5

In vivo effects on plasma levels of TC, LDL-C and HDL-C after regular consumption of various apple cultivars

5.1 Materials and methods

5.1.1 Apple samples

Annurca (*Malus pumila* Miller cv Annurca) apple fruits (each weighing about 100 g) were collected in Valle di Maddaloni (Caserta, Italy) in October, when fruits had just been harvested (green peel). Fruits were reddened following the typical treatment (Lo Scalzo et al., 2001) for about 30 days and then analysed. Four other apple varieties (each weighing about 200 g) analysed in this study, Red Delicious (RD) (*Malus pumila* Miller cv Red Delicious), Granny Smith (GS) (*Malus pumila* Miller cv Granny Smith), Fuji (F) (*Malus pumila* Miller cv Fuji) and Golden Delicious (GD) (*Malus pumila* Miller cv Golden Delicious), were purchased from a local supermarket.

5.1.2 Study population and protocol

The study is listed on the ISRCTN registry with ID ISRCTN15653394. Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in November 2015. Patients aged 18-83 years were eligible for enrolment if they had the following values of serum cholesterol parameters at baseline: total cholesterol (TC), 200-260 mg/dL; high-density lipoprotein cholesterol (HDL-C), 30-45 mg/dL; low-density lipoprotein cholesterol (LDL-C), 189-206 mg/dL. The subjects were asked to keep their dietary habits unchanged throughout the entire study.

Exclusion criteria were: smoking, obesity (body mass index - BMI - $> 30 \text{ kg/m}^2$), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, drug therapy or supplement intake for hypercholesterolemia, drug therapy or supplement intake containing apple polyphenols, heavy physical exercise ($> 10 \text{ h}$ per week), pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding women, birch pollen allergy, use of

vitamin/mineral supplements 2 weeks prior to entry into the study and donation of blood less than 3 months before the study.

The subjects received oral and written information concerning the study before they gave their written consent. Protocol, letter of intent of volunteers and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy). The study was approved by the committee (protocol 14307) and carried out in accordance with the Helsinki Declaration of 1964 (as revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports. The study was a monocentric, randomised, single-blind, placebo-controlled trial, conducted at the Samnium Medical Cooperative (Benevento, Italy).

The study duration was 16 weeks: the same group underwent 4 weeks of placebo treatment, consisting of the habitual diet without apples, followed by 8 weeks of intervention study and 4 weeks of follow-up. Both the examinations and the intervention study were performed in an outpatient setting. Clinic visits and blood sampling were performed after 12 h of fasting at weeks 0, 4, 8, 12 and 16. Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected in 10-mL EDTA-coated tubes (Becton-Dickinson, Plymouth, UK) and plasma was isolated by centrifugation (20 min, 2200 g, 4 °C). All samples were stored at -80 °C until analysis. Plasma TC, HDL-C and LDL-C levels were determined using commercially available kits from Diacron International (Grosseto, Italy). Analyses were performed on a Diacron International Free Carpe Diem, and intra- and inter-day variations were 1.4% and 1.6% for TC, 1.6% and 2.2% for LDL-C, and 2.0% and 2.3% for HDL-C, respectively. In addition to these five meetings, six standardised telephone interviews were performed every 14 days starting from the first meeting, to verify compliance and increase protocol adherence. These interviews reminded patients to complete their intake-checking table for the intervention study and to record any discontinuation or adverse events they might have experienced in the meantime (which were also documented regularly on the case report forms during each telephone and clinic visit).

All patients underwent a standardised physical examination, assessment of medical history (for up to 5 years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. At each clinic visit, patients had to complete three self-administered questionnaires on quality-of-life aspects, and their diaries were checked for data completeness and quality of documentation to ensure patient comprehension of the diary items.

5.1.3 Randomisation, concealment and blinding

A total of 250 eligible patients were randomly assigned to five groups in order to receive apple samples. On the first day of week 5 and once a week during the following 8 weeks, subjects were supplied with apples to be consumed during the following week. Apples were coded with different colours and given in random order. The code was not broken until all analyses were completed and the results were analysed statistically. If a patient dropped out before receiving apples, he or she was replaced by the next eligible patient enrolled at the same centre. The concealed allocation was performed by an Internet-based randomisation schedule, stratified by study site. The random number list was generated by an investigator with no clinical involvement in the trial. Patients, clinicians, core laboratories and trial staff (data analysts, statisticians) were blind to treatment allocation.

5.1.4 Study treatments

A group of 250 patients (168 men and 132 women, 18-69 years of age) was randomly divided into five subgroups (each one of 50 subjects: 28 men and 22 women). The volunteers enrolled in this study had the following values of serum cholesterol parameters at baseline: TC, 200-260 mg/dL; HDL-C, 31-45 mg/dL; LDL-C, 179-206 mg/dL. Four groups were assigned to consume one apple per day among the following: Red Delicious (RD), Granny Smith (GS), Fuji (F), Golden Delicious (GD). The fifth group was asked to consume two Annurca apples per day, since the weight of this cultivar is on average half that of the commercial varieties in this study.

5.1.5 Study outcomes and data collection

Primary and secondary efficacy outcomes

Primary endpoints measured were the variations in TC, HDL-C and LDL-C levels, while key secondary outcomes collected during clinic visits were measurements of blood pressure and heart rate, and evaluation of BMI.

All raw patient ratings were evaluated in a blinded manner at the site of the principal investigator. The decision process was performed according to a consensus document before unblinding in order to define conclusive primary and secondary efficacy data from a clinical perspective.

Safety

Although no specific toxicity studies have been performed herein, mutagenicity tests and acute/subacute toxicity studies have long since demonstrated the safety of polyphenol content of apples, both in mice and humans. Nevertheless, we assessed safety from reports of adverse events as well as laboratory parameters concerning hepatic and renal function, vital signs (blood pressure, pulse, height, weight and BMI), and physical or neurological examinations. Safety was assessed over the entire treatment period at weeks 0, 4, 8, 12 and 16, including adverse events occurring in the first 3 weeks after cessation of treatments.

5.1.6 Statistics

Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalised linear mixed-effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR) (Little et al., 2002), but also provides reasonably stable results even when the assumption of MAR is violated (Molenberghs et al., 2004; O’Kelly et al., 2014). Patients who did not provide any diary data (leading to zero evaluable days) were excluded from the MAR-based primary efficacy analysis, according to an “all observed data approach” as proposed by White and colleagues (White et al., 2012).

This approach is statistically efficient without using multiple imputation techniques (Carpenter et al., 2007). Data retrieved after withdrawal of randomised study treatment were also included in the analysis.

Unless otherwise stated, all the experimental results were expressed as mean \pm standard deviation (SD) of at least five replications. Statistical analysis of data was performed using Student's *t*-test or two-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed using Cochran's test ($P < 0.1$). The I^2 statistic was also calculated and $I^2 > 50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. Percentage change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% CIs, *P*-values, or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration and Jadad score. Treatment effects were analysed using PROC MIXED with treatment (placebo, fresh apples) and period as fixed factors, subject as random factors and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in serum cholesterol and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levene's test) and normal distribution (determined by residual plot examination and Shapiro-Wilk test) even after log transformation were analysed using a nonparametric test (Friedman). The level of significance (α -value) was 95% in all cases ($P < 0.05$).

Analysis sets

The full analysis set population included all patients randomised and who did not fail to satisfy a major entry criterion. We excluded patients who provided neither primary nor secondary efficacy data from efficacy analyses. The per protocol set consisted of all patients who did not substantially deviate from the protocol; they had two characteristics. Firstly, this group included patients for whom no major protocol violations were detected (e.g. poor compliance, errors in treatment

assignment). Secondly, they were on treatment for at least 50 days, counting from day of first intake (completion of a certain pre-specified minimal exposure to the treatment regimen). Hence patients who prematurely discontinued the study or treatment before day 44 were excluded from the per protocol sample.

Determination of sample size

Pilot data from an interventional study supported the assumption that the probability to achieve a better result on apple extract than placebo is 0.71 (Nagasako-Akazome et al., 2005). Hence a sample size of 21 patients in each group would have 77% power to detect the difference between two groups using a two-sided Mann-Whitney *U* test at a 5% significance level. Assuming a dropout rate of about 37%, a minimum of 34 patients in each treatment group had to be enrolled.

Patient involvement

No patients were involved in setting the research question or the outcome measures, nor they were involved in developing plans for participant recruitment, or the design and implementation of the study. There are no plans to explicitly involve patients in dissemination. Final results will be sent to all participating sites.

5.2 Results and discussion

In order to verify that the *in vitro* properties of Annurca apple were also maintained *in vivo*, a 16-week clinical trial was performed. Specifically, it aimed to understand if the cholesterol-lowering effect after Annurca daily consumption was really superior to that of other cultivars regularly consumed (Tenore et al., 2016). Differently from *in vitro* studies, it was decided to use in *in vivo* studies Granny Smith cultivar instead of Pink Lady apple, for various reasons. Firstly, Granny Smith and Pink Lady apples possess similar polyphenolic profiles, so equivalent *in vivo* effects could be reasonably supposed. Secondly, Granny Smith apple is the only cultivar currently employed for the formulation of a nutraceutical product (Applephenon[®]) of which clinical study results, regarding the effects on plasma cholesterol levels, are available (Nagasako-Akazome et al., 2005). Last, but not least, Granny Smith apple is more widespread and commercially interesting than Pink Lady.

5.2.1 Enrolment and subject attrition

Patient enrolment occurred in November 2015. A total of 340 patients were screened for eligibility; 90 patients (26.5%) did not pass the screening stage, while the remaining 250 patients were randomised. The exclusion of 90 patients was due to various factors. The most common cause was that patients did not have the inclusion criteria regarding values of serum cholesterol parameters at baseline ($n = 38$), followed by general refusal to participate for no specific reason ($n = 14$) or for concerns about the protocol, especially fear of placebo ($n = 4$). The other patients were excluded because they had one or more exclusion criteria ($n = 34$).

Successively, the 250 patients were assigned to the group consuming apples. They were divided into five subgroups, each one formed by 50 patients, on the basis of the different apple cultivars chosen for the study. All the patients underwent a placebo period for the first 4 weeks, during which they did not consume any kind of apple in the habitual diet; then, they started the treatment period of 8 weeks, during which they were asked to consume a different apple cultivar one per day (Red Delicious, Granny Smith, Fuji or Golden Delicious) or two per day (Annurca); finally, there was a follow-up period during the last 4 weeks, and patients were asked to stop the consumption of apple fruits. No patient dropped out from study participation before allocation to treatment.

The flow of participants through the trials and the completeness of diary information over the entire treatment period are shown in Figure 27. It follows the CONSORT PRO reporting guideline (Calvert et al., 2013) and reveals that, during the assessment period, the following percentages of patients for each group and subgroup provided data for the primary endpoint: RD, 88.6% (39 of 44 patients); GS, 88.9% (40 of 45 patients); F, 89.4% (42 of 47 patients); GD, 86.7% (39 of 45 patients); Annurca, 86.7% (39 of 45 patients). In each group, a few patients did not hand in any diaries, not giving any specific reason. However, completeness of the patient diaries did not differ between the treatment groups.

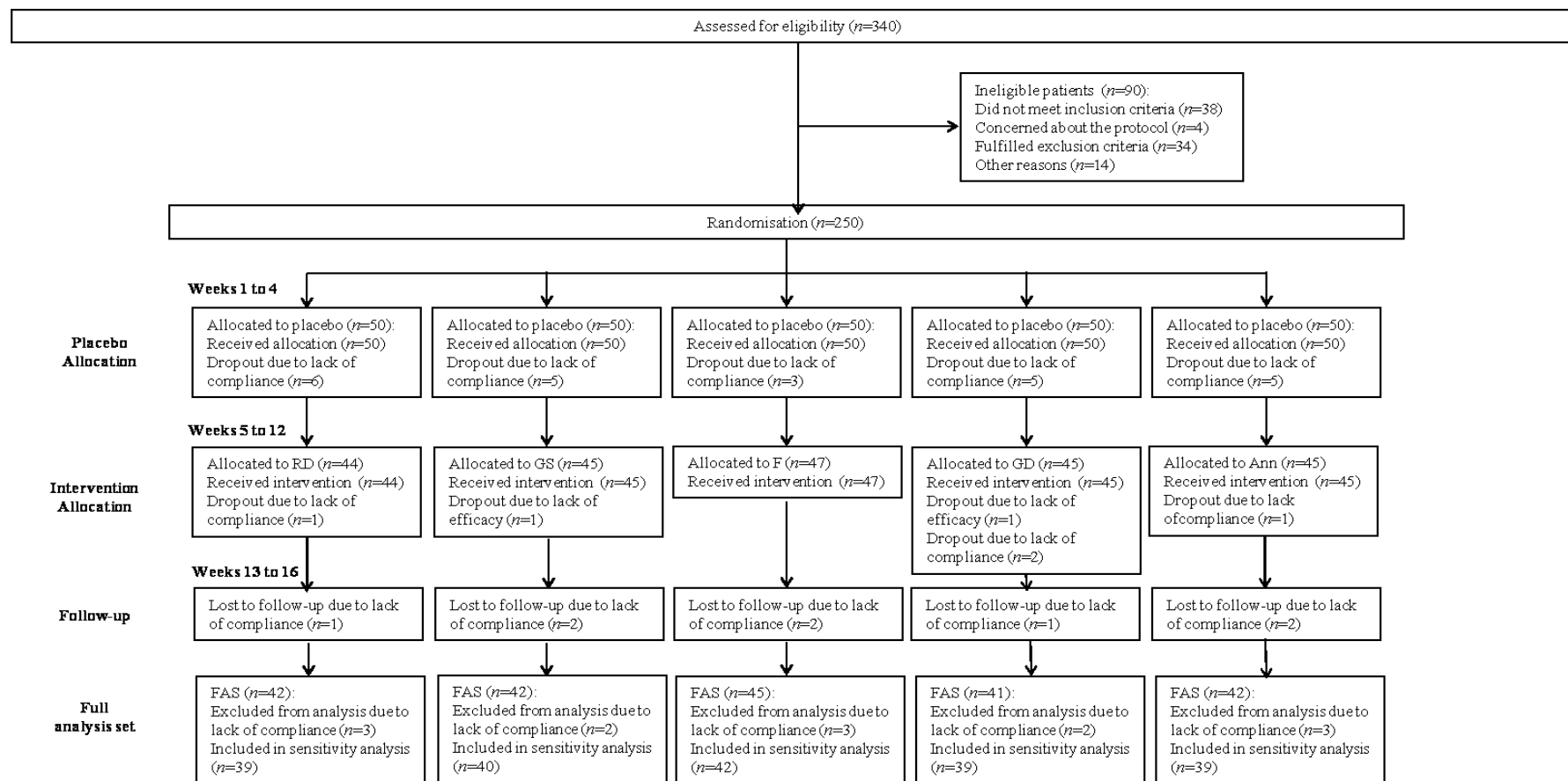


Figure 27: Study flow chart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from pre-screening to data collection, and the extent of exclusions, loss to follow-up and completeness of diary documentation available across the entire trial period. RD, Red Delicious apple; GS, Granny Smith apple; F, Fuji apple; GD, Golden Delicious apple; Ann, Annurca apple; FAS, full analysis set.

5.2.2 Participants' baseline characteristics

The demographic and clinical characteristics assessed at the baseline visit of all 250 patients randomised are reported in Table 5. The subjects involved were white and ranged from 18 to 83 years old; about half of the randomised subjects were female. The five groups were well balanced for demographics and clinical parameters.

Table 5: Baseline characteristics of intention-to-treat sample according to study treatment.

Placebo					
Characteristics	Ann (n=50)	RD (n=50)	GS (n=50)	F (n=50)	GD (n=50)
Demographics					
Age (years)	42.4 ± 10.6	41.8 ± 10.8	43.8 ± 10.5	44.8 ± 11.2	42.7 ± 11.2
Male sex ((No (%))	28 (56%)	26 (52%)	27 (54%)	25 (50%)	26 (52%)
White ethnicity ((No (%))	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)
Clinical parameters					
TC (mg/dL)	240.2 ± 11.5	236.3 ± 12.4	239.1 ± 13.4	233.5 ± 12.6	236.1 ± 13.8
LDL-C (mg/dL)	187.9 ± 12.1	185.2 ± 11.9	191.0 ± 11.3	184.1 ± 12.3	187.8 ± 13.1
HDL-C (mg/dL)	39.9 ± 6.8	36.2 ± 7.7	36.8 ± 6.1	36.3 ± 7.2	32.9 ± 7.3
Glucose (mg/dL)	111.2 ± 9.2	88.7 ± 2.2	99.5 ± 8.1	91.4 ± 9.4	100.1 ± 7.3
Triglycerides (mg/dL)	87.1 ± 9.2	102.8 ± 12.1	93.6 ± 11.8	92.4 ± 10.2	102.3 ± 12.1
Treatment					
Characteristics	Ann (n=45)	RD (n=44)	GS (n=45)	F (n=47)	GD (n=45)
Demographics					
Age (years)	43.5 ± 10.2	42.1 ± 11.1	44.2 ± 10.6	45.5 ± 10.7	43.8 ± 12.2
Male sex ((No (%))	25 (55.5%)	23 (52.3%)	24 (53.3%)	23 (48.9%)	23 (51.1%)
White ethnicity ((No (%))	45 (100%)	44 (100%)	45 (100%)	47 (100%)	45 (100%)
Clinical parameters					
TC (mg/dL)	239.1 ± 11.9	235.9 ± 13.7	237.6 ± 14.3	235.5 ± 13.3	238.4 ± 15.3
LDL-C (mg/dL)	189.6 ± 11.7	183.7 ± 12.3	189.1 ± 11.6	185.7 ± 11.1	189.1 ± 11.6
HDL-C (mg/dL)	38.5 ± 7.2	37.9 ± 8.1	35.4 ± 6.7	36.7 ± 7.5	34.2 ± 6.9
Glucose (mg/dL)	109.3 ± 8.9	89.5 ± 2.7	98.6 ± 8.5	90.5 ± 10.0	101.2 ± 9.3
Triglycerides (mg/dL)	85.4 ± 8.3	101.5 ± 16.8	95.5 ± 9.0	90.3 ± 10.4	100.5 ± 11.8

Values are means ± SD.

Ann: Annurca apple; RD: Red Delicious apple; GS: Granny Smith apple; F: Fuji apple; GD: Golden Delicious apple.

5.2.3 Primary efficacy outcome measures

Firstly, patients enrolled underwent 4 weeks of placebo treatment, consisting in the habitual diet without apples. At the end of placebo period no significant variation of plasma TC, LDL-C and HDL-C levels compared to the baseline values was registered, as shown in Table 5.

Then, the four groups of patients were asked to consume for 8 weeks one apple per day among the following: Red Delicious, Granny Smith, Fuji and Golden Delicious. The fifth group had to consume two Annurca apples per day, since the weight of this cultivar is on average half that of the other commercial cultivars. The administration of apples led, in all groups, to a statistically significant variation of plasma TC, LDL-C and HDL-C levels. All the results are reported in Table 6.

Table 6: Effects of apple samples on plasma cholesterol, glucose and triglyceride metabolism.											
		Ann	Δ (%)	RD	Δ (%)	GS	Δ (%)	F	Δ (%)	GD	Δ (%)
TC (mg/dL)	<i>t</i> 0	239.1 \pm 11.9		235.9 \pm 3.7		237.6 \pm 4.3		235.5 \pm 3.3		238.4 \pm 5.3	
	<i>t</i> 30	219.2 \pm 12.8	-8.3	228.5 \pm 4.2	-3.1	227.2 \pm 3.5	-4.4	230.8 \pm 4.1	-2.0	235.3 \pm 1.9	-1.3
	<i>t</i> 60	218.9 \pm 12.1	-8.4	229.0 \pm 3.6	-2.9	226.9 \pm 3.8	-4.5	230.1 \pm 3.4	-2.3	235.8 \pm 2.0	-1.1
LDL-C (mg/dL)	<i>t</i> 0	189.6 \pm 11.7		183.7 \pm 2.3		189.1 \pm 1.6		185.7 \pm 1.1		189.1 \pm 1.6	
	<i>t</i> 30	161.6 \pm 10.9	-14.7	171.7 \pm 1.3	-6.5	172.4 \pm 0.8	-8.8	177.6 \pm 1.7	-4.4	184.1 \pm 2.0	-2.6
	<i>t</i> 60	161.9 \pm 11.2	-14.5	173.1 \pm 1.6	-5.8	173.4 \pm 1.2	-8.3	176.9 \pm 2.1	-4.7	184.1 \pm 2.3	-2.6
HDL-C (mg/dL)	<i>t</i> 0	38.5 \pm 7.2		37.9 \pm 8.1		35.4 \pm 6.7		36.7 \pm 7.5		34.2 \pm 6.9	
	<i>t</i> 30	44.3 \pm 7.9	+15.2	39.5 \pm 7.5	+4.2	37.6 \pm 8.0	+6.2	37.7 \pm 6.8	+2.7	34.7 \pm 6.4	+1.5
	<i>t</i> 60	43.9 \pm 8.1	+14.0	39.9 \pm 7.0	+5.3	37.0 \pm 8.3	+4.5	38.2 \pm 6.0	+4.1	35.1 \pm 5.6	+2.6
Glucose (mg/dL)	<i>t</i> 0	109.3 \pm 8.9		89.5 \pm 2.7		98.6 \pm 8.5		90.5 \pm 10.0		101.2 \pm 9.3	
	<i>t</i> 30	118.3 \pm 12.0	+8.2	103.4 \pm 10.8	+15.5	100.9 \pm 12.4	+2.3	99.4 \pm 8.3	+9.8	111.3 \pm 10.1	+10.1
	<i>t</i> 60	120.3 \pm 13.1	+10.1	101.2 \pm 11.6	+13.1	105.4 \pm 10.3	+6.9	104.5 \pm 10.1	+15.5	109.3 \pm 9.8	+8.0
Triglycerides (mg/dL)	<i>t</i> 0	85.4 \pm 15.2		101.5 \pm 16.8		95.5 \pm 13.0		90.3 \pm 18.4		100.5 \pm 11.8	
	<i>t</i> 30	91.5 \pm 16.4	+7.2	111.3 \pm 19.3	+9.7	111.2 \pm 21.1	+16.5	102.7 \pm 16.7	+13.7	107.7 \pm 13.4	+7.2
	<i>t</i> 60	90.6 \pm 17.3	+6.1	109.9 \pm 14.7	+8.3	107.6 \pm 15.2	+12.7	100.0 \pm 14.2	+10.8	109.8 \pm 16.5	+9.3
<p>Values are means \pm SD ($n = 5$).</p> <p>Subjects consumed either one apple/day or two Annurca apples/day for 2 months.</p> <p>Results were significantly different at a level of $P = 0.001$.</p> <p>Ann: Annurca; RD: Red Delicious; GS: Granny Smith; F: Fuji; GD: Golden Delicious.</p>											

All the discussed results refer to the second month of intervention study, but actually noteworthy results were obtained already after one month.

The highest hypocholesterolemic effect was exerted by Annurca apple, which decreased TC levels by 8.4% (95% CI: -2.54, $P = 0.0095$). It was followed by Granny Smith and Red Delicious apples, which led to a decrease by 4.5% and 2.9%, respectively. The lowest efficacy was observed for Fuji and Golden Delicious apples, which reduced TC levels by 2.3% and 1.1%, respectively.

Currently, several human studies have been carried out in order to investigate the hypocholesterolemic effects of apples or apple products. These studies concluded that dietary intervention with whole fresh apples leads to the most appreciable results, whereas no significant effects were registered with other apple products, such as juice and whole dried apples. Gormley et al. conducted a study involving 76 men afflicted by mild hypercholesterolemia. The design considered two groups: the intervention group had to eat an average of three apples (Irish Golden Delicious) a day and the control group had to eat a maximum of three apples per week, both over 4 months. The plasma cholesterol levels of the intervention group decreased by 8.1% (Gormley et al., 1977). Other two studies used a design consisting of a single intervention with supplementation of two to three apples (Red Delicious or Golden Delicious) a day, without using a control group (Sablé-Amplis et al., 1983; Girault et al., 1988). The study performed by Sablé-Amplis and colleagues involved 30 hypercholesterolemic men and women, who had to maintain their habitual diet and to add apples as prescribed. In both groups a significant decrease in TC levels was observed (by 9 to 12%) after 1 month of intervention (Sablé-Amplis et al., 1983). The study performed by Girault et al. did not have any inclusion or exclusion criteria and lasted 2 months. This study involved 235 men and women, and 109 of these subjects were overweight or hypercholesterolemic. Only among this latter group significant results were found, since TC levels decreased by 5% (Girault et al., 1988). Ravn-Haren and colleagues performed a single-blind, randomised, cross-over human study in order to evaluate the effect on plasma cholesterol in 34 healthy men and women, who were assigned to consume 550 g of whole apples (Shampion cultivar) per day over a period of 4 weeks. A slight decrease (5.6%) in TC levels was found (Ravn-

Haren et al., 2013). The other available human studies performed using whole apples did not show any significant variation of plasma TC levels (Canella et al., 1963; Mayne et al., 1982; Mahalko et al., 1984).

The same trend found for TC (Annurca > GS > RD > F > GD) was observed as regards LDL-C lowering effects. In fact, on average apple consumption led to a significant decrease in plasma LDL-C levels and Annurca apple was about 1.7-fold more active in reducing the LDL-C (-14.5%, 95% CI: -3.41, $P = 0.0019$) than Granny Smith apple (-8.3%).

Published human studies reported that dietary intervention with whole fresh apples leads to a slight reduction in plasma LDL-C levels, while no significant effects were registered with other apple products, such as juice and whole dried apples. The above-mentioned human study performed by Girault et al. reported that plasma LDL-C levels decreased by 5.5% (Girault et al., 1988). Hyson and colleagues evaluated the effect on plasma total cholesterol in 25 healthy men and women, who were asked to eat 340 g of whole apples a day, through an unblind, randomised, cross-over human study, and they observed a small, but not significant, reduction (5%) in plasma LDL-C levels (Hyson et al., 2000). The above-mentioned human study performed by Ravn-Haren and colleagues showed a decrease in plasma LDL-C levels by 6.7% (Ravn-Haren et al., 2013).

A similar trend was observed for HDL-C values, too. In fact, Annurca apple showed the greatest effect (+14.0%, 95% CI: -1.22, $P = 0.0042$) compared to Granny Smith (+4.5%), Red Delicious (+5.3%), Fuji (+4.1%) and Golden Delicious (+2.6%) cultivars.

Previous human dietary intervention studies with both whole fresh apples and apple products reported contrasting results as regards plasma HDL-C levels. The above-mentioned studies performed by Gormley et al. and Girault et al., both using apples, registered an increase by 12.5% and a decrease by 5.8%, respectively, in HDL-C levels (Gormley et al., 1983; Girault et al., 1988). A cross-over study considered the administration for 6 weeks of 568 mL of filtered apple juice per day to 25 hypercholesterolemic men and showed a significant decrease (-9%) in HDL-C plasma levels (Mee et al., 1997). Other studies,

involving the administration of either fresh apples or apple products, did not report any significant variation of plasma HDL-C levels (Canella et al., 1963; Mayne et al., 1982; Sablé-Amplis et al., 1983; Mahalko et al., 1984; Davidson et al., 1998; Hyson et al., 2000; Ravn-Haren et al., 2013).

The molecular mechanism of action that underlies these effects is still to be clarified, but, according to the *in vitro* studies, it can be hypothesised that apple procyanidins would play a major role in regulating plasma cholesterol levels. In fact, an impressive correlation was found when comparing the *in vivo* hypolipidemic effect of each single apple cultivar with their procyanidin content. Annurca had the highest content in procyanidins and showed to be the most effective, whereas Fuji and Golden Delicious were the poorest in procyanidin amount and were also the least active in affecting cholesterol serum levels.

However, the daily intake of apples increased significantly plasma glucose and triglyceride levels, as reported in Table 6. The major rise in glucose levels occurred in patients that consumed Fuji apples (+15.5%, 95% CI: -5.68, $P = 0.0042$), whereas the least effective on raising plasma glucose levels was Granny Smith apple (+6.9%, 95% CI: -4.21, $P = 0.0032$). As regards triglyceride levels, Granny Smith apple increased their levels by about 12.7% (95% CI: -2.21, $P = 0.0032$). All the other apple cultivars affected the triglyceride levels to a lesser extent (Fuji, +10.8%, 95% CI: -3.54, $P = 0.0036$; Golden Delicious, +9.3%, 95% CI: -5.13, $P = 0.0029$; Red Delicious, +8.3%, 95% CI: -5.06, $P = 0.0012$; Annurca, +6.1%, 95% CI: -2.46, $P = 0.0027$).

These effects are not surprising, because about 90% of the energy from an apple derives from its carbohydrates, mainly sugars, such as fructose and glucose. Fructose is lipogenic, since its breakdown products can either enter the Krebs cycle or serve as substrates for the formation of mevalonate and triglycerides (Dekker et al., 2010).

5.2.4 Safety issue, study strength and limitations

The safety of polyphenolic content of apples was assessed through mutagenicity tests and acute/subacute toxicity studies both in mice and humans, although no

specific toxicity studies were performed during this study. Nevertheless, patients were periodically monitored in order to assess vital signs, blood pressure and electrocardiographic findings and baseline values did not change substantially during and at the end of the trial (data not reported).

The major strengths of this clinical trial reside in the originality of the study and in the evaluation of the treatment effects in real-world setting, while the main limitations of this study are short-term assessment for the treatment of a chronic condition and the choice of treating exclusively white race.

CHAPTER 6

In vivo effects on plasma levels of TC, LDL-C and HDL-C after administration of the polyphenolic fraction coming from Annurca apple cultivar

6.1 Materials and methods

6.1.1 Apple collection

Annurca (*Malus pumila* cv Annurca) apple fruits (each weighing about 100 g) were collected in Valle di Maddaloni (Caserta, Italy) in October when fruits had just been harvested (green peel). The fruits were reddened, following the typical treatment (Lo Scalzo et al., 2001) for about 30 days, and then analysed. Four other apple varieties (each weighing about 200 g) analysed in this study, Red Delicious (RD) (*Malus pumila* Miller cv Red Delicious), Granny Smith (GS) (*Malus pumila* Miller cv Granny Smith), Fuji (F) (*Malus pumila* Miller cv Fuji) and Golden Delicious (GD) (*Malus pumila* Miller cv Golden Delicious), were purchased from a local supermarket.

6.1.2 Preparation of apple extracts

Lyophilised apples (10 g) were treated with 100 mL of a methanol/water mixture (80:20, v/v) acidified with 0.5% formic acid for 24 h at 4 °C in order to extract phenolic compounds. After centrifugation, the supernatant was slowly filtered and purified through an Amberlite XAD-2 column packed as follows: 10 g of resin (pore size 9 nm; particle size 0.3-1.2 mm; Supelco, Bellefonte, PA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 x 2 cm). The column was washed with 100 mL of acidified water (pH 2) and 50 mL of deionised water in order to remove sugar and other polar compounds. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen. The polyphenolic extracts were stored at -80 °C until analysis.

6.1.3 HPLC-DAD/ESI-MSⁿ analysis

HPLC separation and quantification of phenolic compounds in apple extracts were performed according to previous studies with some modifications (Mari et al., 2010). The identification of phenolic compounds was possible by recording chromatograms at different wavelengths, 280 nm for flavonols, procyanidins, dihydrochalcones and hydroxycinnamic acids, and 500 nm for anthocyanins and by comparing spectra and retention times with those of commercial standards and with those reported in previous works (Mari et al., 2010). Elution conditions consisted in 2% acetic acid (solvent A) and 0.5% acetic acid in acetonitrile and water (50:50, v/v) (solvent B) gradient at a flow rate of 1.0 mL/min. The column selected was a Hypersil BDS C18 column (250 mm x 4.6 mm, 5 µm) (Thermo, Bellefonte, PA). Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA) provided with photodiode array detector (DAD). The gradient conditions were: 0-5 min, 10% B; 55 min, 55% B; 65 min, 95% B, followed by 5 min of maintenance. The identity of phenolic acids and flavonoids was confirmed with LC-ESI/MS/MS experiments and data were compared with those of commercial standards and with those reported in previous works (Mari et al., 2010). The same chromatographic conditions were applied to a HP1100 HPLC system (Agilent, Palo Alto, CA) coupled to a PE-Sciex API-2000 triple-quadrupole mass spectrometer (AB Sciex, Warrington, UK) equipped with a Turbospray (TSI) source. Individual phenols were separated on a Hypersil BDS C18 column (250 mm x 2.1 mm, 5 µm) (Thermo Electron, Bellefonte, PA) at a flow rate of 200 µL/min. Mass spectra were recorded from $m/z = 50$ to 1500, in both negative and positive ionisation mode. For negative ion mode, the capillary voltage was set at -13 V, the spray voltage was at 4.5 kV and the tube lens offset was at -15 V, while in positive ion mode, the capillary voltage was set at 13 V, the spray voltage was at 5 kV and the tube lens offset was at -35 V. The capillary temperature was 275 °C. Data were acquired in MS, MS/MS and MSⁿ scanning modes.

6.1.4 Cell cultures and in vitro experiments

Human hepatocellular liver carcinoma cells (HepG2, HB-8065) were obtained from American Type Culture Collection. Monolayers of cells were grown in T75 flasks and maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% antibiotic/antimycotic solution (Gibco-Invitrogen, Mount Waverley, Australia), 0.375% NaHCO₃ and 20 mmol/L HEPES, pH 7.4, and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. The cells were dispersed with trypsin-EDTA (0.05% trypsin and 0.02% EDTA) and plated on the bottom of a transparent black 96-well plate in a 200 µL growth medium at a density of 1.0×10^5 cells/mL. Assays were performed by incubating the culture with 1 µg/mL of apple polyphenolic extract for 72 h.

To determine inhibition of cholesterol uptake, cells were incubated in an F-12 medium containing 10 nmol/L [1,2-³H] cholesterol (PerkinElmer Pty Ltd., Melbourne, Australia). The cells were washed twice in F-12 to remove unbound [³H] cholesterol, and 0.5 mL of 0.1% sodium dodecyl sulphate (SDS) was added to each well to solubilise the cells. Three milliliters of Ultima Gold scintillant was added, and the radioactivity of the solubilised cells was determined using Wallac 1410 liquid scintillation counter (Pharmacia, Turku, Finland). For cholesterol efflux experiments, cells were washed in F-12 and incubated for 45 min in F-12 containing 20 nmol/L [³H] cholesterol to "load" the cells with cholesterol. Cells were then washed twice in F-12 and then incubated (for up to 100 min) containing the test samples as shown in the figure legend to determine cholesterol efflux. As a positive control, 1% methyl-β-cyclodextrin (MβCD) was used to acutely deplete cellular cholesterol. Cells were washed twice in F-12, supernatant was removed, and then 0.5 mL of 0.1% SDS was added to solubilise the cells for β counting as above.

The effect of apple polyphenolic extracts on HepG2 LDL receptor binding activity was indirectly evaluated by measuring the LDL concentration in the cell medium. HepG2 cells were grown in 12-well culture plates, followed by washing in PBS and incubation for 24 h in DMEM serum-free media. The cells were then washed in PBS and incubated for a further 6 h in DMEM serum-free media

containing fluorescent-labeled Dil-LDL (10 mg/L, Biomedical Technologies, Stoughton, MA). The fluorescence of the cell medium was measured at 514 nm (excitation) and 550 nm (emission) wavelengths by using a LS50B luminescence spectrophotometer (PerkinElmer, Waltham, MA).

The concentration of Apolipoprotein A1 (ApoA1), the main constituting protein of nascent discoidal HDL particles, was quantified in the cell culture media in 96-well plates using an enzyme-linked immunosorbent assay (E90519Hu 96 test kit; Gentaur Molecular Products Kampenhout, Belgium), according to the manufacturer's instructions.

6.1.5 Annurca supplement (AMS) preparation

The Annurca apple supplement used in this study consisted of gastric-resistant capsules containing Annurca apple polyphenolic extract (400 mg/cps) and maltodextrin (100 mg). The extract was encapsulated in gastric-resistant capsules due to the previous knowledge of salivary and gastric digestion of some polyphenolic components. The product was formulated by the Department of Pharmacy, University of Naples "Federico II" (Naples, Italy), and registered with the name of AppleMetSTM (AMS). Large-scale production of AMS has been accomplished by MB-Med Company (Turin, Italy). Apples have been extracted with pure ethanol, and the obtained extract solution has been kept at -20 °C for 24 h in order to allow sugar elimination. After centrifugation, the extract has been spray-dried, obtaining a fine powder, which has been used to formulate gastric-resistant capsules.

6.1.6 Study population and protocol

The study is listed on the ISRCTN registry with ID ISRCTN15653394. Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in November 2015. Patients aged 18-83 years were eligible for enrolment if they had the following values of serum cholesterol parameters at baseline: total cholesterol (TC), 200-260 mg/dL; high-density lipoprotein cholesterol (HDL-C), 30-45 mg/dL; low-density lipoprotein

cholesterol (LDL-C), 189-206 mg/dL. The subjects were asked to keep their dietary habits unchanged throughout the entire study.

Exclusion criteria were: smoking, obesity (body mass index - BMI - $> 30 \text{ kg/m}^2$), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, drug therapy or supplement intake for hypercholesterolemia, drug therapy or supplement intake containing apple polyphenols, heavy physical exercise ($> 10 \text{ h/week}$), pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding women, birch pollen allergy, use of vitamin/mineral supplements 2 weeks prior to entry into the study and donation of blood less than 3 months before the study.

The subjects received oral and written information concerning the study before they gave their written consent. Protocol, letter of intent of volunteers and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy). The study was approved by the committee (protocol 14303), and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports. The study was a monocentric, double-blind, placebo-controlled trial conducted at the Samnium Medical Cooperative (Benevento, Italy).

The study duration was 16 weeks: the group underwent 4 weeks of placebo treatment, consisting in the administration of identically appearing capsules containing only maltodextrin, followed by 8 weeks of nutraceutical treatment, and 4 weeks of follow-up. Both the examinations and the study treatment were performed in an outpatient setting. Clinic visits and blood sampling were performed after 12 h of fasting at weeks 0, 4, 8, 12 and 16. Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected in 10-mL EDTA-coated tubes (Becton-Dickinson, Plymouth, UK) and the plasma was isolated by centrifugation (20 min, 2200 g, 4 °C). All samples were stored at -80 °C until analysis. Plasma TC, HDL-C and LDL-C levels were determined

using commercially available kits from Diacron International (Grosseto, Italy). Analyses were performed on a Diacron International Free Carpe Diem, and intra- and inter-day variations were 1.4% and 1.6% for TC, 1.6% and 2.2% for LDL-C and 2.0% and 2.3% for HDL-C, respectively. In addition to these five meetings, six standardised telephone interviews were performed every 14 days starting from the first meeting, to verify compliance and increase protocol adherence. These interviews reminded patients to complete their intake-checking table for the intervention study and to record any treatment discontinuation, or adverse events they might have experienced in the meantime (which were also documented regularly on the case report forms during each telephone and clinic visit).

All patients underwent a standardised physical examination, assessment of medical history (for up to five years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. At each clinic visit, patients had to complete three self-administered questionnaires on quality of life aspects, and their diaries were checked for data completeness and quality of documentation to ensure patient comprehension of the diary items.

6.1.7 Randomisation, concealment and blinding

A total of 250 eligible patients were assigned to the group in order to receive AMS supplement. AMS supplement and placebo were coded with different colours and given in random order. The code was not broken until all analyses were completed and the results were analysed statistically. If a patient dropped out before receiving AMS supplement, he or she was replaced by the next eligible patient enrolled at the same centre. The concealed allocation was performed by an Internet-based randomisation schedule, stratified by study site. The random number list was generated by an investigator with no clinical involvement in the trial. Patients, clinicians, core laboratories, and trial staff (data analysts, statisticians) were blind to treatment allocation.

6.1.8 Study treatments

The group of 250 patients (116 men and 134 women, 30-83 years of age) was instructed to take two capsules of AMS per day (one capsule at lunch, and one at

dinner). The volunteers enrolled in this study had the following values of serum cholesterol parameters at baseline: TC, 214-254 mg/dL; HDL-C, 30-43 mg/dL; LDL-C, 150-205 mg/dL. AMS product consisted in gastric-resistant capsules, each containing 400 mg of Annurca apple polyphenolic extract, and registered with the name of AppleMetSTM (AMS). Large-scale production of AMS had been accomplished by MB-Med Company (Turin, Italy) with full respect of all the good manufacturing practices. Noteworthy, the dose of AMS supplement (800 mg/day) adopted for the clinical trial was in full accordance with the maximum polyphenolic extract daily intake (1000 mg), through food supplements and novel foods, indicated by the revised form (January 2015) of the Commission Regulation (EC) No. 258/1997, as the safe polyphenolic daily amount compatible with a good health state.

6.1.9 Study outcomes and data collection

Primary and secondary efficacy outcomes

Primary endpoints measured were the variations of TC, HDL-C and LDL-C levels, while key secondary outcomes collected during clinic visits were measurements of blood pressure and heart rate, and evaluation of BMI. All raw patient ratings were evaluated in a blinded manner at the site of the principal investigator. The decision process was performed according to a consensus document before unblinding in order to define conclusive primary and secondary efficacy data from a clinical perspective.

Safety

Although no specific toxicity studies have been performed herein, mutagenicity tests and acute/subacute toxicity studies have long since demonstrated the safety of polyphenol content of apples both in mice and humans. Specifically, the Commission Regulation (EC) No. 258/1997 established 1000 mg as the maximum polyphenolic extract daily intake in humans. Accordingly, the AMS dose adopted for the trial was of 800 mg/day, an amount reasonably lower than that regarded as safe in humans. Nevertheless, we assessed safety from reports of adverse events as well as laboratory parameters concerning the hepatic and renal function, vital signs (blood pressure, pulse, height, weight and BMI), and physical or

neurological examinations. Safety was assessed over the entire treatment period at weeks 0, 4, 8, 12 and 16, including adverse events occurring in the first three weeks after cessation of treatments.

6.1.10 Statistics

Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalised linear mixed effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR) (Little et al., 2002), but also provides reasonably stable results even when the assumption of MAR is violated (Molenberghs et al., 2004; O’Kelly et al., 2014). Patients who did not provide any diary data (leading to zero evaluable days) were excluded from the MAR based primary efficacy analysis, according to “an all observed data approach” as proposed by White and colleagues (White et al., 2012). This approach is statistically efficient without using multiple imputation techniques (Carpenter et al., 2007). Data retrieved after withdrawal of randomised study treatment were also included in the analysis.

Unless otherwise stated, all the experimental results were expressed as mean \pm standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student’s *t* test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed by using Cochran’s test ($P < 0.1$). The I^2 statistic was also calculated and $I^2 > 50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. Percent change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% CIs, *p*-values or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status,

study design, type of intervention, duration, total polyphenol dose and Jadad score. Treatment effects were analysed using PROC MIXED with treatment (placebo, fresh apples, apple supplement) and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in serum cholesterol and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levene's test) and normal distribution (determined by residual plot examination and Shapiro-Wilk test) even after log transformation were analysed by a nonparametric test (Friedman). The level of significance (α -value) was 95% in all cases ($P < 0.05$).

Analysis sets

The full analysis set population included all randomised patients and patients who did not fail to satisfy a major entry criterion. We excluded patients who provided neither primary nor secondary efficacy data from efficacy analyses. The per protocol set consisted of all patients who did not substantially deviate from the protocol; they had two characteristics. Firstly, this group included patients for whom no major protocol violations were detected (e.g. poor compliance, errors in treatment assignment). Secondly, they were on treatment for at least 50 days counting from day of first intake (completion of a certain pre-specified minimal exposure to the treatment regimen). Hence, patients who prematurely discontinued the study or treatment before day 44 were excluded from the per protocol sample.

Determination of sample size

Pilot data from an interventional study supported the assumption that the probability to achieve a better result on apple extract than placebo is 0.71 (Nagasako-Akazome et al., 2005). Hence, a sample size of 21 patients in each group would have 77% power to detect the difference between two groups using a two sided Mann-Whitney U test on a 5% significance level. Assuming a dropout rate of about 37%, 34 patients in each treatment group had to be enrolled.

Patient involvement

No patients were involved in setting the research question or the outcome measures, nor they were involved in developing plans for participant recruitment, or the design and implementation of the study. There are no plans to explicitly involve patients in dissemination. Final results will be sent to all participating sites.

6.2 Results and discussion

6.2.1 Polyphenolic extracts profiling and in vitro effects on cholesterol metabolism

The incubation of HepG2 cells with polyphenolic extracts coming from different apple cultivars (Annurca, Red Delicious, Granny Smith, Fuji and Golden Delicious) indicated that Annurca polyphenolic extract was more effective on cholesterol metabolism than the other apple polyphenolic extracts, as it increased TC levels by 5.5 times (Figure 28A), decreased LDL-C concentrations by 48% (Figure 28B) and enhanced ApoA1 levels by 2 times (Figure 28C) in the cell medium with respect to control cells.

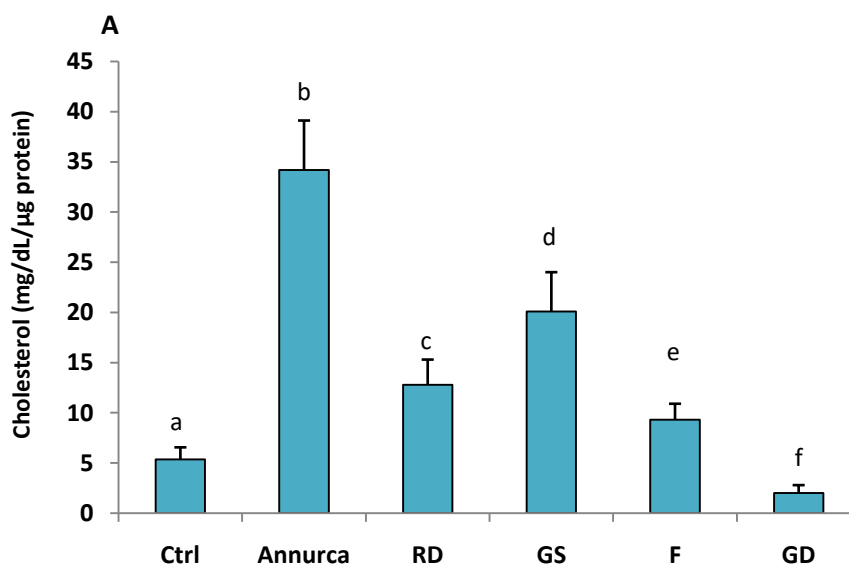


Figure 28A: Effects of apple polyphenolic extracts on HepG2 cell line cholesterol uptake. Data are referred to concentrations in the cell medium and are the means \pm SD ($n = 3$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

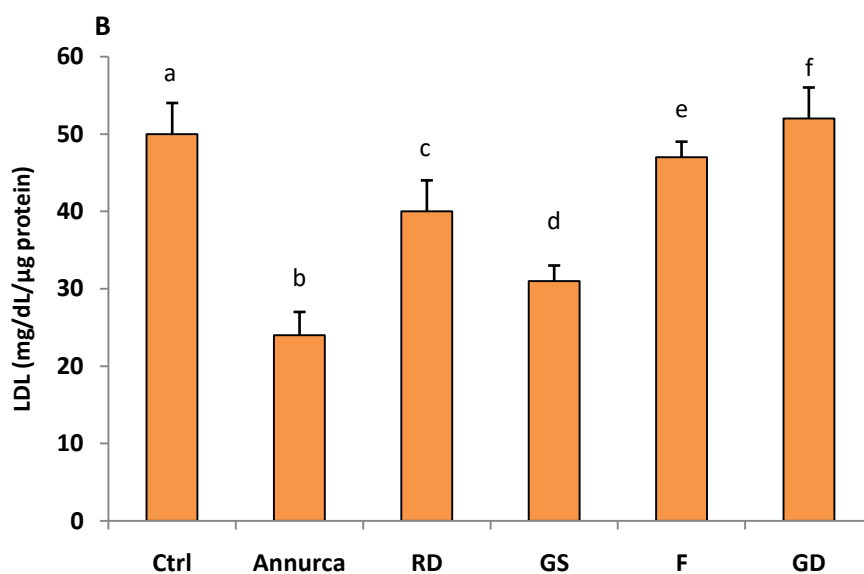


Figure 28B: Effects of apple polyphenolic extracts on HepG2 cell line low-density lipoprotein (LDL) receptor binding activity. Data are referred to concentrations in the cell medium and are the means \pm SD ($n = 3$). ^{abcde}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

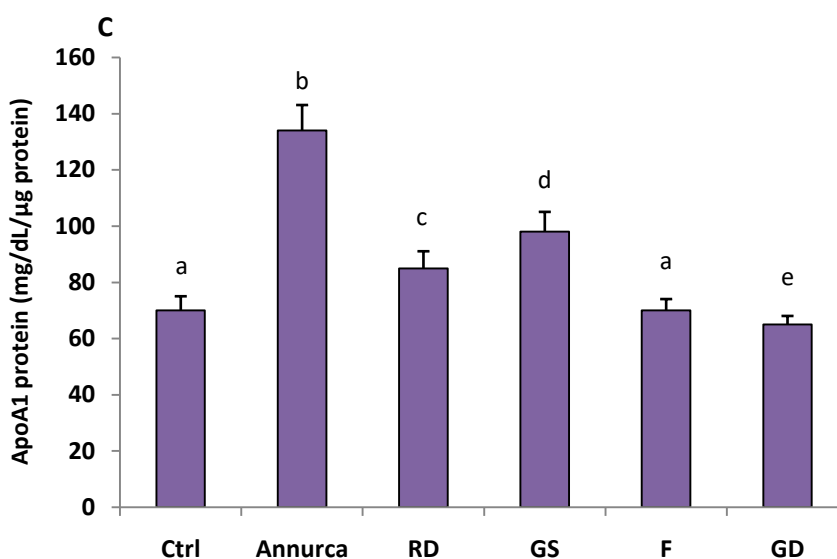


Figure 28C: Effects of apple polyphenolic extracts on HepG2 cell line ApoA1 protein expression. Data are referred to concentrations in the cell medium and are the means \pm SD ($n = 3$). ^{abcde}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

To understand the reasons underlying these effects, a combination of High Performance Liquid Chromatography (HPLC) with Diode Array Detection (DAD)

and Mass Spectrometry (MS) was used, so the qualitative and quantitative profile of whole apple samples was determined. The results are reported in Table 7 and 8.

Table 7: Antioxidant composition (% \pm SD; $n = 5$) of apple polyphenolic extracts and Annurca supplement (AMS) determined by HPLC-DAD-MS analysis.

	ANN	RD	GS	F	GD	AMS
Procyanidins	62.91 \pm 2.1	41.47 \pm 1.8	62.40 \pm 2.2	58.01 \pm 1.0	51.70 \pm 0.9	64.07 \pm 2.5
Dihydrochalcones	5.88 \pm 0.4	16.80 \pm 0.9	3.98 \pm 0.5	6.82 \pm 0.08	5.04 \pm 0.4	4.06 \pm 0.8
Flavonols	29.86 \pm 0.7	37.72 \pm 1.4	30.64 \pm 0.6	31.17 \pm 1.0	41.51 \pm 1.1	29.77 \pm 1.7
Anthocyanins	1.50 \pm 0.08	2.93 \pm 0.2	1.90 \pm 0.04	1.19 \pm 0.03	0.09 \pm 0.0	1.42 \pm 0.1

Data are the means \pm SD ($n = 3$).

Results were significantly different at a level of $P = 0.001$.

ANN: Annurca; RD: Red Delicious; GS: Granny Smith; F: Fuji; GD: Golden Delicious.

The analyses showed that the polyphenolic profile of Annurca was similar to that found in other apple cultivars and consisted of dihydrochalcones (5.88%), flavonols (29.86%), anthocyanins (1.5%), but it was particularly rich in procyanidins (62.91%). Particularly, Annurca and Granny Smith polyphenolic extracts were very similar, with an almost identical content in procyanidins (62.91% and 62.40%, respectively) (Table 7).

Table 8: Polyphenolic composition of apple samples determined by HPLC-DAD-MS analysis.

	Annurca	RD	GS	F	GD
Total polyphenols	135.31 \pm 2.1	101.57 \pm 1.7	95.87 \pm 2.3	45.27 \pm 1.1	32.13 \pm 1.2
Procyanidins	85.09 \pm 2.2	42.12 \pm 1.5	59.82 \pm 0.6	26.26 \pm 0.2	16.61 \pm 0.8
Dihydrochalcones	7.82 \pm 0.5	17.06 \pm 0.9	3.82 \pm 0.2	3.09 \pm 0.06	1.62 \pm 0.08
Flavonols	40.41 \pm 0.6	38.31 \pm 1.0	29.38 \pm 0.4	14.11 \pm 0.8	13.34 \pm 0.5
Anthocyanins	2.03 \pm 0.09	2.98 \pm 0.1	1.82 \pm 0.03	0.54 \pm 0.01	0.03 \pm 0.00

Data are expressed as mg/100 g FW and are the means \pm SD ($n = 3$).

Results were significantly different at a level of $P = 0.001$.

RD: Red Delicious; GS: Granny Smith; F: Fuji; GD: Golden Delicious.

However, Annurca apple cultivar showed the highest total amount of polyphenols (135.31 mg/100 g FW), and, in particular, procyanidin levels were greater (from 1.4 to 5.1 times) than those found in the other apple cultivars (Table 8).

Table 9 indicates that procyanidins occurred in apples as a mix of dimers (mainly, procyanidin B2), trimers (e.g. procyanidin C1), tetramers, pentamers or higher molecular weight oligomers. All apple cultivars exhibited a comparable oligomeric procyanidin profile, with the exception of Annurca, which resulted richer in dimers than the other cultivars tested herein.

Table 9: Composition (% \pm SD; $n = 5$) of apple and Annurca supplement (AMS) procyanidin fractions determined by HPLC-DAD-MS analysis.

	ANN	RD	GS	F	GD	AMS
Dimers ($n = 2$)	26.91 \pm 1.1	7.24 \pm 0.7	11.13 \pm 0.6	10.06 \pm 0.7	8.70 \pm 0.7	28.50 \pm 0.9
Trimers ($n = 3$)	9.06 \pm 0.8	8.02 \pm 0.9	12.34 \pm 1.2	11.14 \pm 1.0	8.64 \pm 0.9	8.95 \pm 1.2
Tetramers ($n = 4$)	3.84 \pm 0.5	5.68 \pm 0.4	8.75 \pm 0.5	7.88 \pm 0.6	6.12 \pm 0.5	3.77 \pm 0.8
Pentamers ($n = 5$)	5.25 \pm 0.2	3.84 \pm 0.2	5.87 \pm 0.6	5.34 \pm 0.4	4.62 \pm 0.2	5.18 \pm 0.5
Hexamers ($n = 6$)	6.62 \pm 0.4	3.29 \pm 0.3	4.92 \pm 0.4	4.44 \pm 0.2	3.84 \pm 0.3	6.54 \pm 0.4
Oligomers ($7 \leq n \leq 10$)	11.22 \pm 1.8	13.6 \pm 0.9	19.38 \pm 1.5	18.71 \pm 1.4	16.38 \pm 1.2	11.13 \pm 1.5
Total procyanidins	62.91 \pm 2.1	41.47 \pm 1.8	62.40 \pm 1.8	58.01 \pm 1.0	51.70 \pm 0.9	64.07 \pm 2.5
Data are the means \pm SD ($n = 3$). Results were significantly different at a level of $P = 0.001$. ANN: Annurca; RD: Red Delicious; GS: Granny Smith; F: Fuji; GD: Golden Delicious.						

Thus, to understand if there was a correlation between the different procyanidin profile and the *in vitro* effects on cholesterol uptake, LDL receptor binding activity and ApoA1 protein expression, HepG2 cells were incubated with each procyanidin oligomeric form ($n = 2, 3, 4, 5$ and the mix of $7 \leq n \leq 10$) at the same molar concentrations. The results indicated that dimers (mostly procyanidin B2) were the most effective in decreasing LDL-C levels (-60% than control cells) (Figure 29A) and in increasing ApoA1 levels (+85.7% than control cells) (Figure 29B), whereas the mixture of oligomers ($7 \leq n \leq 10$) was the most effective in increasing total cholesterol levels (almost 9 times than control cells) (Figure 29C) in the cell medium.

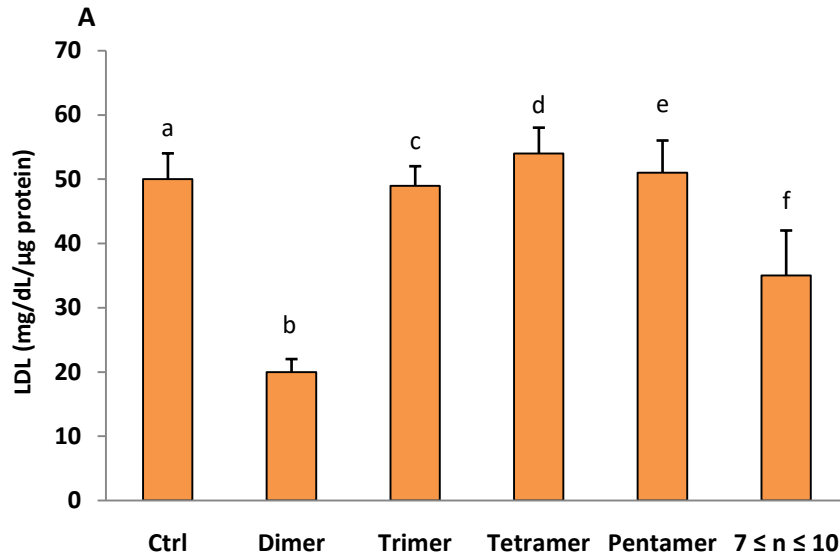


Figure 29A: HepG2 cell line low-density lipoprotein (LDL) receptor binding activity. Data are referred to concentrations in the cell medium and are the means \pm SD ($n = 3$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

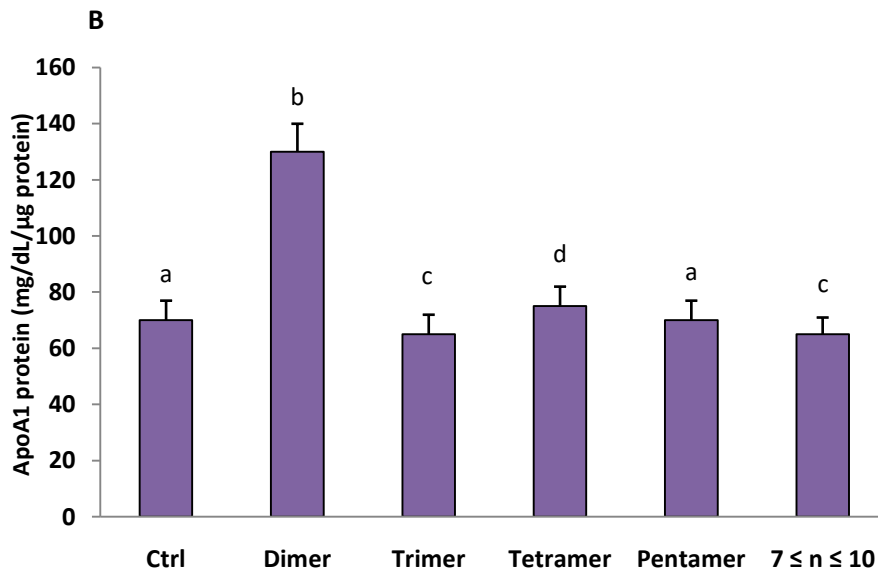


Figure 29B: HepG2 cell line ApoA1 protein expression. Data are referred to concentrations in the cell medium and are the means \pm SD ($n = 3$). ^{abcd}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

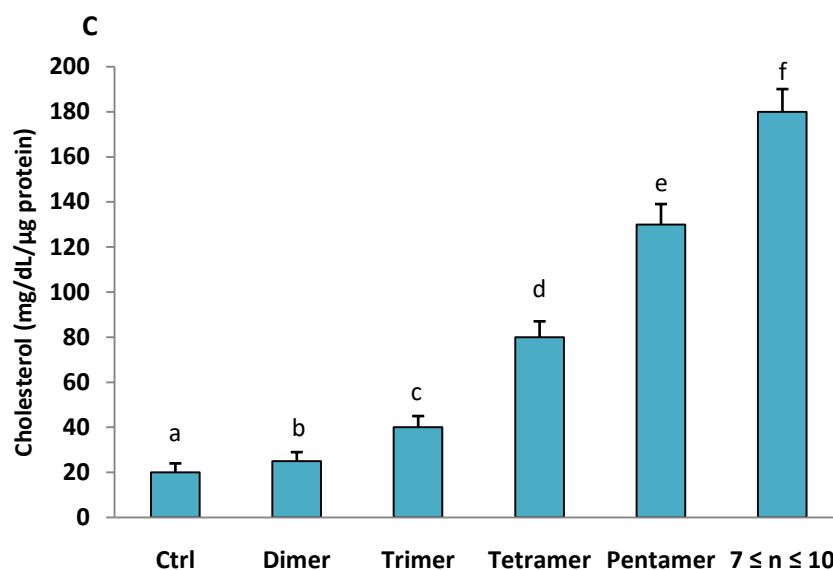


Figure 29C: HepG2 cell line cholesterol uptake. Data are referred to concentrations in the cell medium and are the means \pm SD ($n = 3$). ^{abcdef}Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test. Results were significantly different at a level of $P = 0.001$.

Procyanidin B2 is the most abundant component of dimeric fraction in Annurca apple. It is a dimer of (-)-epicatechin, a molecule commonly found in green tea and cocoa. In a previous work, the treatment of HepG2 cells with green tea catechins led to a decrease in cholesterol content in the cell medium (-30%) and to the activation of SREBP-1 transcription factor, which determined an up-regulation of LDL receptors (Bursill et al., 2006). Another *in vitro* experiment reported that HepG2 cells treated with procyanidin B2 (a dimer) showed an up-regulation of SREBP-1 greater than those treated with procyanidin C1 (a trimer), while an increase in the expression of SREBP-2 was registered by both B2 treated cells and C1 treated cells (Yasuda et al., 2011).

Thus, the activation of SREBP pathway leads to an induction of LDL receptor over-expression, an effect also exploited by the most used hypolipidemic drugs in the world, statins. In fact, statins inhibit liver cholesterol biosynthesis, a block sensed by the SREBP pathway, which enhances its active form and induces LDL receptor over-expression (DeBose-Boyd et al., 2001). Therefore, dimeric procyanidins may be considered able to exert a statin-like LDL-lowering effect. However, differently from statins, which are not able to rise HDL-C plasma levels ($< 10\%$), dimeric procyanidins affect *in vitro* ApoA1 concentration (+85.7%),

which is an important factor for the formation of the nascent discoidal HDL and for the activation of LCAT, an enzyme essential for cholesterol esterification and HDL maturation (Mei et al., 2015).

Thus, in order to verify if the *in vitro* properties of Annurca were maintained *in vivo*, too, capsules containing polyphenolic extract deriving from Annurca apple were administered for two months to 250 mildly hypercholesterolemic healthy subjects. Specifically, Annurca polyphenolic extract was encapsulated in gastric-resistant capsules (400 mg/capsule) and registered as AppleMetSTM (AMS). Patients enrolled were asked to take two capsules per day, accounting 800 mg/day, corresponding to six Annurca apples, an amount per day which is unlikely to be reached only through diet (Tenore et al., 2017).

6.2.2 Enrolment and subject attrition

Patient enrolment occurred in November 2015. A total of 352 patients were screened for eligibility; 102 patients (29.0%) did not pass the screening stage, while the remaining 250 patients were randomised. The exclusion of 102 patients was due to various factors. The most common cause was that patients did not have the inclusion criteria regarding values of serum cholesterol parameters at baseline ($n = 41$), followed by general refusal to participate for no specific reason ($n = 17$) or for concerns about the protocol, especially fear of placebo ($n = 7$). The other patients were excluded because they had one or more exclusion criteria ($n = 37$). Successively, the 250 patients were assigned to the group assuming the AMS product. Firstly, they underwent a placebo period for the first 4 weeks, during which they were given identically appearing capsules containing only maltodextrin; then, they started the treatment period of 8 weeks; finally, there was a follow-up period during the last 4 weeks, and patients were asked to stop the administration of AMS. No patient dropped out from study participation before allocation to treatment.

The flow of participants through the trials and the completeness of diary information over the entire treatment period are shown in Figure 30. It follows the CONSORT PRO reporting guideline (Calvert et al., 2013) and reveals that, during the assessment period, the following percentage of patients provided data for the

primary endpoint: AMS, 83.4% (191 of 229 patients). A few patients did not hand in any diaries, not giving any specific reason.

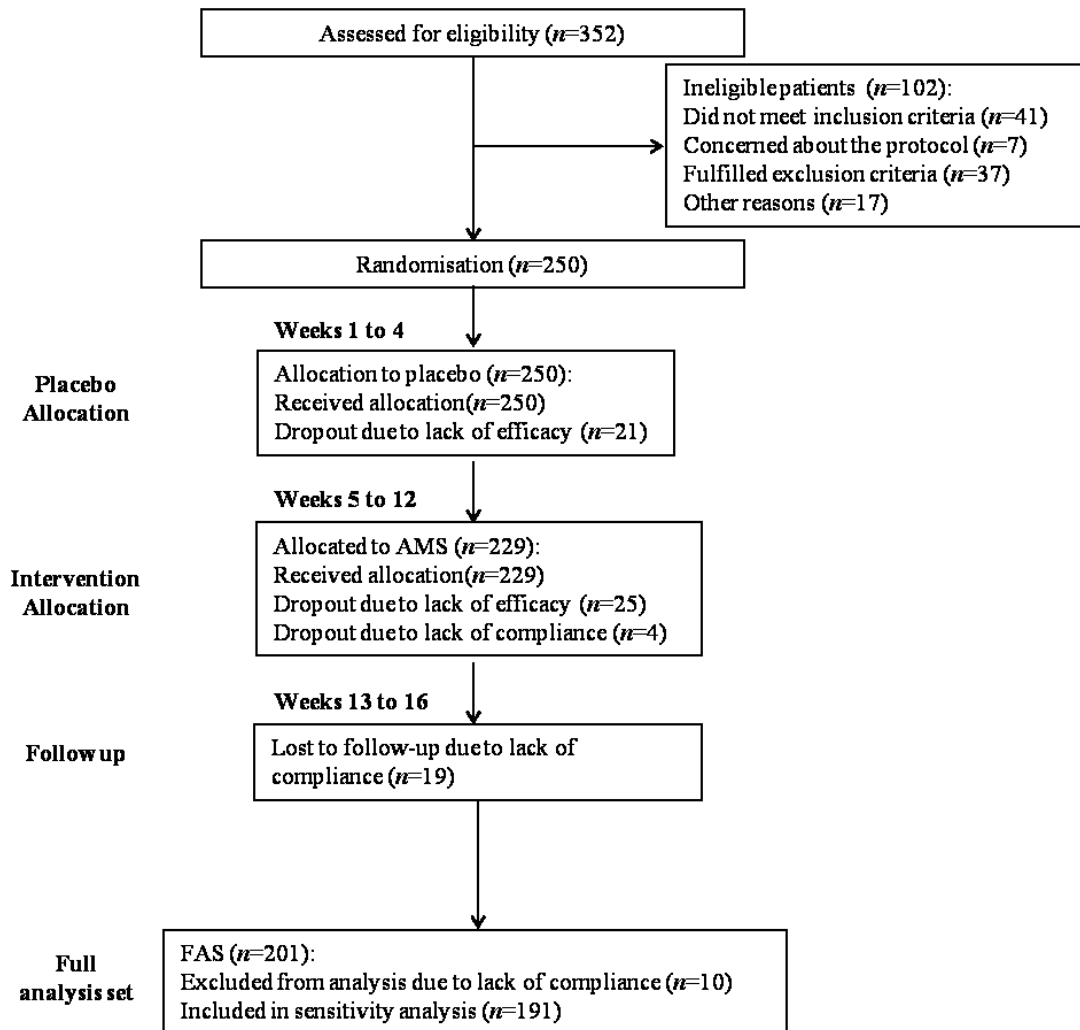


Figure 30: Study flowchart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from pre-screening to data collection, and the extent of exclusions, loss to follow-up, and completeness of diary documentation available across the entire trial period. AMS, AppleMetSTM; FAS, full analysis set.

6.2.3 Participants' baseline characteristics

The demographic and clinical characteristics assessed at the baseline visit of all 250 patients randomised are reported in Table 10. The subjects involved were

white and they ranged from 18 to 83 years old; about half of the randomised subjects were female.

Table 10: Baseline characteristics of intention-to-treat sample according to study treatment.

Placebo	
Characteristics	AMS (<i>n</i>=250)
Demographics	
Age (years)	34.7 ± 12.4
Male sex ((No (%))	116 (46%)
White ethnicity ((No (%))	250 (100%)
Clinical parameters	
TC (mg/dL)	234.9 ± 13.1
LDL-C (mg/dL)	183.3 ± 11.4
HDL-C (mg/dL)	36.2 ± 8.5
Glucose (mg/dL)	108.2 ± 8.4
Triglycerides (mg/dL)	97.0 ± 9.3
Treatment	
Characteristics	AMS (<i>n</i>=229)
Demographics	
Age (years)	36.1 ± 13.8
Male sex ((No (%))	105 (45.8%)
White ethnicity ((No (%))	229 (100%)
Clinical parameters	
TC (mg/dL)	233.6 ± 13.7
LDL-C (mg/dL)	181.3 ± 12.3
HDL-C (mg/dL)	38.0 ± 8.1
Glucose (mg/dL)	110.3 ± 9.9
Triglycerides (mg/dL)	95.1 ± 8.2
Values are means ± SD. AMS: AppleMetS™.	

6.2.4 Primary efficacy outcome measures

At the end of placebo period no significant variation of plasma TC, LDL-C and HDL-C levels compared to the baseline values was registered, as shown in Table 10.

All the discussed results refer to the second month of intervention study, but actually noteworthy results were obtained already after one month. After treatment with AppleMetSTM supplement, the patients showed a reduction in TC and LDL-C levels by 24.9% (95% CI: -5.36, $P = 0.0011$) and 37.5% (95% CI: -4.07, $P = 0.0021$), respectively, and an increase in HDL-C levels by 49.2% (95% CI: -2.14, $P = 0.0030$). Patients who assumed AMS did not show any variation of glucose (CI: -0.64, $P = 0.0022$) or triglyceride (CI: -0.93, $P = 0.0048$) levels. All the results are reported in Table 11.

Table 11: Effects of Annurca supplement (AMS) on plasma cholesterol, glucose and triglyceride metabolism.

		AMS	Δ (%)
TC (mg/dL)	<i>t</i> 0	233.6 ± 13.7	
	<i>t</i> 30	176.1 ± 14.2	-24.6
	<i>t</i> 60	175.4 ± 13.6	-24.9
LDL-C (mg/dL)	<i>t</i> 0	181.3 ± 12.3	
	<i>t</i> 30	112.7 ± 11.3	-37.8
	<i>t</i> 60	113.4 ± 11.6	-37.5
HDL-C (mg/dL)	<i>t</i> 0	38.0 ± 8.1	
	<i>t</i> 30	56.8 ± 7.5	+49.4
	<i>t</i> 60	56.8 ± 7.0	+49.2
Glucose (mg/dL)	<i>t</i> 0	110.3 ± 9.9	
	<i>t</i> 30	109.3 ± 10.2	-0.9
	<i>t</i> 60	110.8 ± 11.1	+0.4
Triglycerides (mg/dL)	<i>t</i> 0	95.1 ± 14.2	
	<i>t</i> 30	96.2 ± 14.4	+1.1
	<i>t</i> 60	95.9 ± 13.3	+0.8
Subjects were administered with 2 AMS capsules/day for 2 months. Values are means ± SD ($n = 5$). Results were significantly different at a level of $P = 0.001$.			

Of outmost importance is the impact on HDL-C, since very few natural or pharmaceutical substances are able to enhance HDL-C levels. Currently, a number of HDL-targeted therapies are emerging and consist of ApoA1 mimetics, reconstituted HDL, and CETP (cholesteryl ester transfer protein) inhibitors. However, ApoA1 mimetics and reconstituted HDL are able to increase only temporarily HDL-C levels, whereas the most potent CETP inhibitor, torcetrapib, increases HDL-C levels by up to 106%, but is associated with high mortality (Linsel-Nitschke et al., 2005; Kingwell et al., 2014).

Differently from an apple-rich diet, which could increase blood glucose and triglyceride levels, since the fructose contained is lipogenic (Dekker et al., 2010), AMS did not have any effect on these parameters.

A nutraceutical product or a natural remedy may be regarded as significantly effective if it is capable of determining a 20% variation of each plasma value of TC, LDL-C and HDL-C. Particularly, AMS supplement made possible to 50%, 75.6% and 85.6%, respectively, of the enrolled subjects to reach these endpoints, as shown in Figure 31, 32 and 33, respectively.

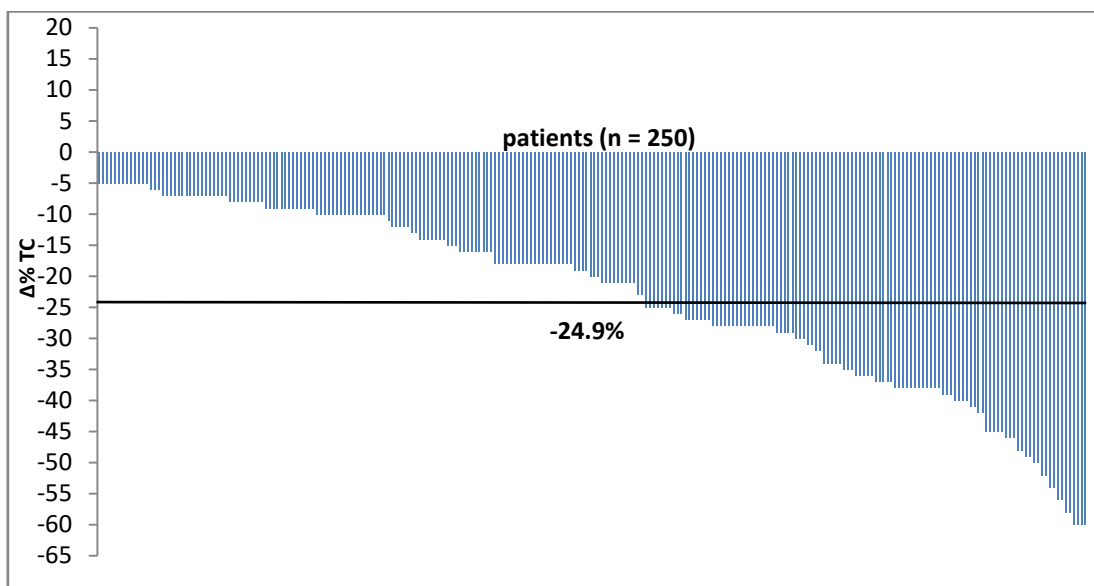


Figure 31: Effects of Annurca supplement (AMS) on plasma total cholesterol (TC). Subjects were administered with 2 AMS capsules/day for 2 months. Values are means \pm SD ($n = 5$). Results were significantly different at a level of $P = 0.001$.

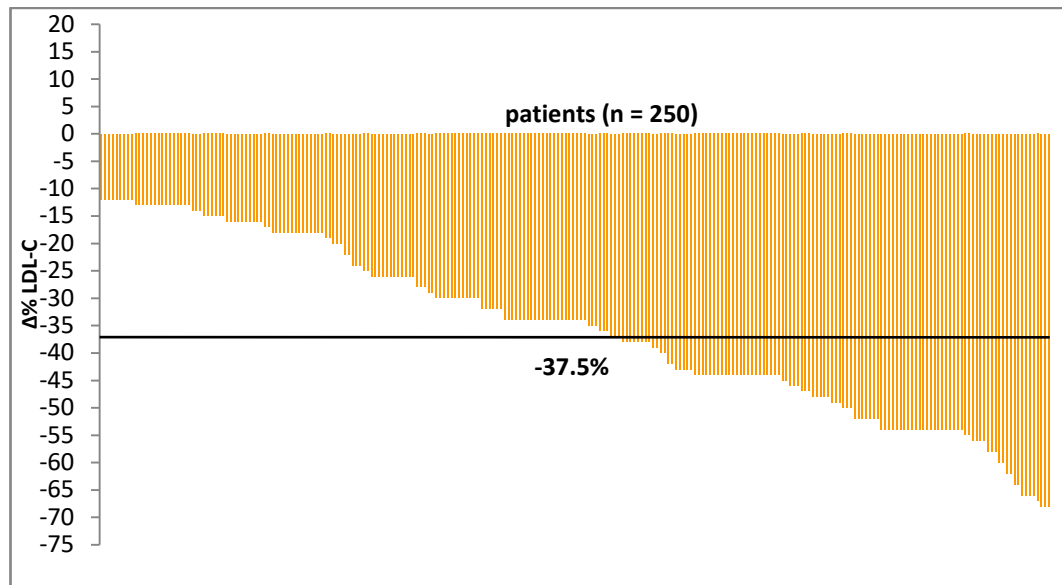


Figure 32: Effects of Annurca supplement (AMS) on plasma LDL-C. Subjects were administered with 2 AMS capsules/day for 2 months. Values are means \pm SD ($n = 5$). Results were significantly different at a level of $P = 0.001$.

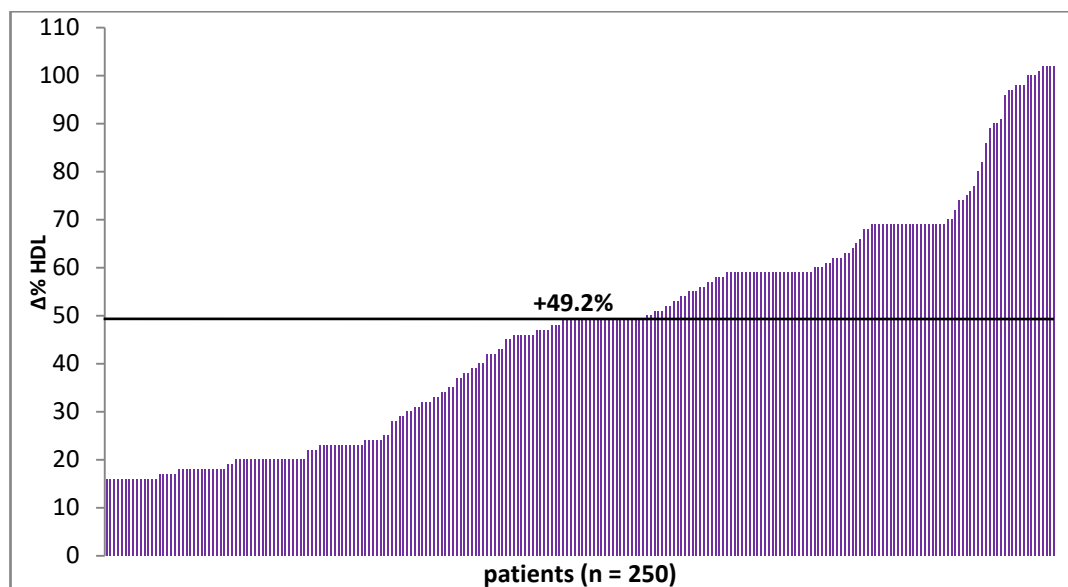


Figure 33: Effects of Annurca supplement (AMS) on plasma HDL-C. Subjects were administered with 2 AMS capsules/day for 2 months. Values are means \pm SD ($n = 5$). Results were significantly different at a level of $P = 0.001$.

Moreover, considering that plasma levels of TC, LDL-C and HDL-C good for health are 200, 140 and 50 mg/dL, respectively, AMS supplement made possible to 87%, 70.6% and 68.4% of the enrolled subjects to reach the respective endpoints.

Thus, if the only dietary intervention is not enough for an effective primary prevention of cardiovascular diseases, the daily administration of a nutraceutical formulation, which provides for the polyphenolic content of six Annurca apples a day, may be regarded as a significant therapeutic tool. In particular, the ratio LDL-C/HDL-C in the plasma samples of subjects treated with AMS decreased from 6.26 to 2.30, and it was correlated to a substantial reduction of the cardiovascular risk. The ratio between LDL-C and HDL-C levels is established to maximum levels of 3.0-3.5 (women-men) and 2.5-3.0 (women-men) for the primary and secondary prevention, respectively (Millán et al., 2009).

Finally, gender, total cholesterol baseline and age were evaluated in order to understand if these variables could affect the efficacy of AMS supplement. The results are reported in Table 12.

The analysis of men and women subgroups revealed that there were not major differences as regards TC (men = 95% CI: -0.85, $P = 0.0016$; women = 95% CI: -0.63, $P = 0.0013$), LDL-C (men = 95% CI: -0.74, $P = 0.0024$; women = 95% CI: -0.84, $P = 0.0036$) and HDL-C (men = 95% CI: -0.91, $P = 0.0012$; women = 95% CI: -0.74, $P = 0.0022$) variations (Table 12).

Table 12: Effects of Annurca supplement (AMS) on plasma cholesterol, glucose and triglyceride metabolism in subgroups men and women.					
		Men	Δ (%)	Women	Δ (%)
TC (mg/dL)	<i>t</i> 0	234.2 ± 12.1		233.0 ± 12.3	
	<i>t</i> 30	175.4 ± 14.3	-25.2	177.4 ± 13.1	-23.7
	<i>t</i> 60	175.6 ± 12.5	-25.1	176.8 ± 11.9	-24.1
LDL-C (mg/dL)	<i>t</i> 0	180.5 ± 11.3		181.7 ± 12.9	
	<i>t</i> 30	110.5 ± 14.0	-38.7	114.5 ± 10.2	-37.0
	<i>t</i> 60	112.7 ± 10.8	-37.6	113.9 ± 9.8	-37.4
HDL-C (mg/dL)	<i>t</i> 0	38.6 ± 5.6		37.5 ± 6.4	
	<i>t</i> 30	57.9 ± 7.1	+50.0	55.9 ± 3.9	+49.1
	<i>t</i> 60	58.0 ± 4.5	+50.4	55.6 ± 7.2	+48.3
Glucose (mg/dL)	<i>t</i> 0	111.4 ± 10.7		108.9 ± 11.3	
	<i>t</i> 30	110.4 ± 11.3	-1.0	108.1 ± 10.3	-0.7
	<i>t</i> 60	111.7 ± 12.3	+0.3	109.7 ± 11.5	+0.6
Triglycerides (mg/dL)	<i>t</i> 0	96.3 ± 11.1		94.6 ± 10.4	
	<i>t</i> 30	97.4 ± 10.3	+1.2	95.4 ± 12.1	+0.8
	<i>t</i> 60	97.1 ± 11.2	+0.8	94.8 ± 11.0	+0.2
Subjects were administered with 2 AMS capsules/day for 2 months. Value are means ± SD (<i>n</i> = 5). Results were significantly different at a level of <i>P</i> = 0.001.					

More interesting data emerged when the effectiveness analyses were performed on patients divided in subgroups based either on baseline TC values or on age. In fact, AMS supplement revealed to be more effective when administered to subjects characterised by the highest TC values range (Table 12 *TC*) (men 214-229 mg/dL, TC = 95% CI: -2.07, *P* = 0.0024; LDL-C = 95% CI: -2.94, *P* = 0.0040; HDL-C = 95% CI: -4.67, *P* = 0.0042; men 230-245 mg/dL, TC = 95% CI: -5.77, *P* = 0.0048; LDL-C = 95% CI: -5.22, *P* = 0.0026; HDL-C = 95% CI: -4.83, *P* = 0.0018; men 246-254 mg/dL, TC = 95% CI: -5.67, *P* = 0.0038; LDL-C = 95% CI: -5.30, *P* = 0.0034; HDL-C = 95% CI: -3.23, *P* = 0.0019; women 214-229 mg/dL, TC = 95% CI: -5.17, *P* = 0.0014; LDL-C = 95% CI: -5.73, *P* = 0.0010; HDL-C = 95% CI: -4.60, *P* = 0.0043; women 230-245 mg/dL, TC = 95% CI: -4.17, *P* = 0.0032; LDL-C = 95% CI: -5.43, *P* = 0.0040; HDL-C = 95% CI: -4.23,

$P = 0.0015$; women 246-254 mg/dL, TC = 95% CI: -2.98, $P = 0.0028$; LDL-C = 95% CI: -3.43, $P = 0.0048$; HDL-C = 95% CI: -4.63, $P = 0.0014$) or to elder subjects (Table 12 *Age*) (men 30-45 years old, TC = 95% CI: -3.07, $P = 0.0034$; LDL-C = 95% CI: -3.49, $P = 0.0040$; HDL-C = 95% CI: -5.57, $P = 0.0036$; men 46-60 years old, TC = 95% CI: -4.38, $P = 0.0025$; LDL-C = 95% CI: -2.44, $P = 0.0043$; HDL-C = 95% CI: -3.44, $P = 0.0039$; men 61-83 years old, TC = 95% CI: -2.77, $P = 0.0028$; LDL-C = 95% CI: -5.12, $P = 0.0026$; HDL-C = 95% CI: -4.89, $P = 0.0030$; women 30-45 years old, TC = 95% CI: -4.72, $P = 0.0025$; LDL-C = 95% CI: -6.73, $P = 0.0011$; HDL-C = 95% CI: -3.42, $P = 0.0023$; women 46-60 years old, TC = 95% CI: -5.61, $P = 0.0037$; LDL-C = 95% CI: -5.28, $P = 0.0047$; HDL-C = 95% CI: -4.13, $P = 0.0015$; women 61-83 years old, TC = 95% CI: -3.84, $P = 0.0024$; LDL-C = 95% CI: -3.87, $P = 0.0041$; HDL-C = 95% CI: -5.63, $P = 0.0026$).

These data seem to be of outmost importance as the recipients of any hypolipidemic treatment are represented mainly by elderly people with an unbalanced lipid profile.

Table 12 Men TC: Effects of Annurca supplement (AMS) on plasma cholesterol, glucose and triglyceride metabolism in men subgroup according to total cholesterol (TC) at baseline.

		Men (TC:214-229)	Δ (%)	Men (TC: 230-245)	Δ (%)	Men (TC: 246-254)	Δ (%)
TC (mg/dL)	<i>t</i> 0	222.1 \pm 10.2		233.1 \pm 12.3		248.0 \pm 12.5	
	<i>t</i> 30	169.2 \pm 12.5	-23.2	176.3 \pm 10.4	-25.3	181.2 \pm 14.3	-27.1
	<i>t</i> 60	169.7 \pm 13.0	-23.0	176.6 \pm 11.1	-25.2	181.6 \pm 13.1	-27.0
LDL-C (mg/dL)	<i>t</i> 0	165.3 \pm 12.7		180.6 \pm 13.1		195.1 \pm 12.7	
	<i>t</i> 30	104.4 \pm 13.2	-36.9	110.6 \pm 12.8	-38.9	116.3 \pm 13.3	-40.5
	<i>t</i> 60	104.8 \pm 12.6	-36.3	112.3 \pm 12.8	-37.5	117.6 \pm 11.7	-39.3
HDL-C (mg/dL)	<i>t</i> 0	41.1 \pm 3.8		38.9 \pm 5.3		35.6 \pm 4.2	
	<i>t</i> 30	59.6 \pm 4.2	+45.0	58.7 \pm 6.1	+50.3	55.2 \pm 4.4	+55.1
	<i>t</i> 60	60.0 \pm 3.7	+46.1	58.7 \pm 4.8	+50.5	55.5 \pm 6.1	+55.0
Glucose (mg/dL)	<i>t</i> 0	109.7 \pm 11.1		113.4 \pm 11.8		111.2 \pm 14.1	
	<i>t</i> 30	108.9 \pm 12.9	-0.9	112.2 \pm 12.5	-1.2	110.5 \pm 11.8	-0.8
	<i>t</i> 60	109.8 \pm 13.6	-0.1	113.7 \pm 10.3	+0.3	111.6 \pm 12.7	+0.3
Triglycerides (mg/dL)	<i>t</i> 0	100.2 \pm 11.9		90.9 \pm 9.3		97.6 \pm 11.1	
	<i>t</i> 30	101.0 \pm 12.4	+0.8	92.6 \pm 11.2	+1.7	98.7 \pm 11.4	+0.8
	<i>t</i> 60	101.5 \pm 11.7	+1.3	91.3 \pm 10.6	+0.4	98.2 \pm 10.2	+0.6

Subjects were administered with 2 AMS capsules/day for 2 months.

Values are means \pm SD (*n* = 5).

Results were significantly different at a level of *P* = 0.001.

Table 12 Men Age: Effects of Annurca supplement (AMS) on plasma cholesterol, glucose and triglyceride metabolism in men subgroup according to age.

		Men (Age: 30-45)	Δ (%)	Men (Age: 46-60)	Δ (%)	Men (Age: 61-83)	Δ (%)
TC (mg/dL)	<i>t</i> 0	228.6 \pm 11.5		233.6 \pm 12.8		240.1 \pm 13.1	
	<i>t</i> 30	174.4 \pm 12.5	-23.9	175.7 \pm 13.8	-24.8	176.2 \pm 14.1	-26.9
	<i>t</i> 60	174.6 \pm 13.0	-23.6	175.3 \pm 12.3	-25.1	175.7 \pm 12.5	-26.8
LDL-C (mg/dL)	<i>t</i> 0	168.9 \pm 13.0		181.1 \pm 14.5		192.4 \pm 13.8	
	<i>t</i> 30	109.1 \pm 12.7	-35.4	110.8 \pm 12.0	-38.8	112.5 \pm 14.4	-41.5
	<i>t</i> 60	108.3 \pm 12.9	-35.9	111.7 \pm 13.8	-38.3	112.0 \pm 12.9	-41.8
HDL-C (mg/dL)	<i>t</i> 0	42.8 \pm 3.7		39.1 \pm 6.2		34.2 \pm 5.3	
	<i>t</i> 30	61.4 \pm 4.4	+43.2	58.6 \pm 5.2	+49.1	54.1 \pm 4.8	+58.1
	<i>t</i> 60	62.1 \pm 3.8	+45.3	59.0 \pm 5.1	+50.7	53.3 \pm 6.2	+56.2
Glucose (mg/dL)	<i>t</i> 0	107.2 \pm 13.8		112.9 \pm 13.6		113.4 \pm 11.9	
	<i>t</i> 30	106.2 \pm 12.5	-0.9	111.2 \pm 12.4	-1.2	112.2 \pm 13.1	-1.5
	<i>t</i> 60	106.7 \pm 14.6	-0.5	114.4 \pm 10.9	+1.3	114.4 \pm 10.3	+0.9
Triglycerides (mg/dL)	<i>t</i> 0	98.2 \pm 10.5		91.1 \pm 10.3		99.4 \pm 10.3	
	<i>t</i> 30	98.5 \pm 11.2	+0.3	91.7 \pm 12.8	+0.7	100.2 \pm 13.0	+0.8
	<i>t</i> 60	99.5 \pm 11.8	+1.3	91.2 \pm 11.6	+0.1	99.6 \pm 11.5	+0.2

Subjects were administered with 2 AMS capsules/day for 2 months.

Values are means \pm SD ($n = 5$).

Results were significantly different at a level of $P = 0.001$.

Table 12 Women TC: Effects of Annurca supplement (AMS) on plasma cholesterol, glucose and triglyceride metabolism in women subgroup according to total cholesterol (TC) at baseline.

		Women (TC:214-229)	Δ (%)	Women (TC: 230-245)	Δ (%)	Women (TC: 246-254)	Δ (%)
TC (mg/dL)	<i>t</i> 0	220.0 \pm 11.7		232.0 \pm 12.5		249.0 \pm 12.1	
	<i>t</i> 30	172.5 \pm 13.5	-21.6	177.1 \pm 13.1	-23.7	189.0 \pm 13.5	-25.1
	<i>t</i> 60	172.0 \pm 11.8	-21.8	177.7 \pm 10.9	-23.4	188.2 \pm 11.5	-25.4
LDL-C (mg/dL)	<i>t</i> 0	167.4 \pm 13.4		182.3 \pm 12.6		198.1 \pm 12.7	
	<i>t</i> 30	110.3 \pm 12.5	-34.1	114.7 \pm 14.2	-37.1	119.6 \pm 11.2	-39.6
	<i>t</i> 60	109.0 \pm 11.9	-34.9	115.0 \pm 12.9	-36.9	120.6 \pm 12.7	-39.1
HDL-C (mg/dL)	<i>t</i> 0	41.2 \pm 4.5		37.9 \pm 5.5		34.5 \pm 4.3	
	<i>t</i> 30	59.4 \pm 5.2	+44.3	56.7 \pm 4.6	+49.7	53.2 \pm 5.7	+54.1
	<i>t</i> 60	60.0 \pm 5.8	+44.8	56.5 \pm 5.7	+49.1	53.1 \pm 3.8	+54.2
Glucose (mg/dL)	<i>t</i> 0	114.5 \pm 14.1		108.2 \pm 12.7		102.7 \pm 13.9	
	<i>t</i> 30	113.9 \pm 12.1	-0.5	107.8 \pm 13.9	-0.4	101.3 \pm 12.4	-1.4
	<i>t</i> 60	115.5 \pm 10.4	+1.3	109.2 \pm 11.8	+0.9	103.6 \pm 14.8	0.9
Triglycerides (mg/dL)	<i>t</i> 0	95.2 \pm 12.5		98.7 \pm 13.9		88.7 \pm 10.1	
	<i>t</i> 30	96.0 \pm 11.3	+0.8	98.1 \pm 10.5	-0.6	89.3 \pm 10.3	+0.7
	<i>t</i> 60	95.8 \pm 14.7	+0.2	99.4 \pm 13.8	+1.3	89.0 \pm 10.2	+0.1

Subjects were administered with 2 AMS capsules/day for 2 months.

Values are means \pm SD (*n* = 5).

Results were significantly different at a level of *P* = 0.001.

Table 12 Women Age: Effects of Annurca supplement (AMS) on plasma cholesterol, glucose and triglyceride metabolism in women subgroup according to age.

		Women (Age: 30-45)	Δ (%)	Women (Age: 46-60)	Δ (%)	Women (Age: 61-83)	Δ (%)
TC (mg/dL)	<i>t</i> 0	226.1 \pm 13.6		234.5 \pm 11.8		238.4 \pm 13.2	
	<i>t</i> 30	177.3 \pm 10.8	-21.6	177.6 \pm 12.5	-24.1	178.5 \pm 12.1	-25.0
	<i>t</i> 60	176.6 \pm 12.0	-21.9	177.3 \pm 11.9	-24.4	178.0 \pm 13.5	-25.2
LDL-C (mg/dL)	<i>t</i> 0	170.3 \pm 11.5		182.6 \pm 13.2		193.5 \pm 12.1	
	<i>t</i> 30	112.2 \pm 13.1	-34.1	113.9 \pm 14.1	-37.6	116.1 \pm 13.8	-40.0
	<i>t</i> 60	111.9 \pm 12.4	-34.3	113.5 \pm 13.5	-37.8	114.9 \pm 11.8	-40.6
HDL-C (mg/dL)	<i>t</i> 0	40.2 \pm 3.2		38.2 \pm 5.2		33.8 \pm 4.3	
	<i>t</i> 30	64.3 \pm 5.9	+42.1	56.7 \pm 6.4	+48.5	53.1 \pm 5.7	+57.2
	<i>t</i> 60	56.9 \pm 6.1	+41.5	57.5 \pm 5.3	+50.6	51.9 \pm 3.8	+53.6
Glucose (mg/dL)	<i>t</i> 0	111.4 \pm 12.3		109.2 \pm 13.3		105.2 \pm 10.2	
	<i>t</i> 30	112.2 \pm 10.6	+1.0	107.8 \pm 14.0	-1.3	104.3 \pm 12.3	-0.8
	<i>t</i> 60	109.1 \pm 10.1	-1.1	110.4 \pm 10.0	+1.1	104.8 \pm 13.1	-0.4
Triglycerides (mg/dL)	<i>t</i> 0	88.2 \pm 11.1		93.3 \pm 12.4		102.4 \pm 12.5	
	<i>t</i> 30	89.3 \pm 12.7	+1.3	91.9 \pm 10.7	+0.7	102.7 \pm 13.0	+0.3
	<i>t</i> 60	88.4 \pm 10.1	+0.2	94.3 \pm 11.8	+1.1	103.2 \pm 11.2	+0.8

Subjects were administered with 2 AMS capsules/day for 2 months.

Value are means \pm SD (*n* = 5).

Results were significantly different at a level of *P* = 0.001.

Good linear correlations between TC ranges and the entity of variation of plasma TC, LDL-C and HDL-C levels were registered (Table 12). The values of these correlations were: men with TC 214-229 mg/dL *vs* Δ TC, $R = 0.95$; men with TC 230-245 mg/dL *vs* Δ HDL-C, $R = 0.98$; men with TC 246-254 mg/dL *vs* Δ LDL-C, $R = 0.96$; women with TC 214-229 mg/dL *vs* Δ TC, $R = 0.99$; women with TC 230-245 mg/dL *vs* Δ HDL-C, $R = 0.95$; women with TC 246-254 mg/dL *vs* Δ LDL-C, $R = 0.96$.

Table 12 also highlights the existence of good linear correlations between age ranges and the entity of variation of plasma TC, LDL-C and HDL-C levels. The values of these correlations were: men aged 30-45 years old *vs* Δ TC, $R = 0.98$; men aged 46-60 years old *vs* Δ HDL-C, $R = 0.98$; men aged 61-83 years old *vs* Δ LDL-C, $R = 0.96$; women aged 30-45 years old *vs* Δ TC, $R = 0.97$; women aged 46-60 years old *vs* Δ HDL-C, $R = 0.94$; women aged 61-83 years old *vs* Δ LDL-C, $R = 0.98$. Moreover, the entity of variation of plasma TC, LDL-C and HDL-C parameters is rather different according to the age subgroup. Specifically, the TC variation between the men 30-45 and 61-83 age subgroups is about -3%, the LDL-C variation between the same groups is about -6%, whereas the change of HDL-C between the same groups is approximately +11%.

On the basis of the obtained results, it can be asserted that two capsules of AMS exhibited a LDL-C lowering effect equivalent to 80 mg of fluvastatin, or 40 mg of simvastatin, or 10 mg of atorvastatin (Law et al., 2003), which belong to the pharmaceutical class of statins, the first-line lipid-lowering drug available on the market. Additionally, AMS may increase HDL-C levels by 49.2%, an effect far above to that obtained after administration of statins (+5-16%) and niacin (+30%). Finally, AMS is safer than statins, which have been related to several side effects, such as myopathy, type 2 diabetes onset and, recently, cancer risk incidence. The increase in cancer risk emerged in a trial that aimed to study the effect of the association simvastatin/ezetimibe (at 40 and 10 mg, respectively) in patients characterised by mild-to-moderate asymptomatic aortic stenosis. Specifically, it was observed a greater incidence of cancer in the group treated with simvastatin/ezetimibe (105 subjects) than in the group administered with placebo (70 subjects) (Rossebø et al., 2008).

Thus, to sum up the results obtained thanks to this clinical trial, AMS formulation:

- strongly impacts on plasma HDL-C levels (+49.2%);
- decreases LDL-C levels (-37.5%) and TC levels (-24.9%);
- does not affect plasma glucose or triglyceride levels;
- is more effective on elder people with higher values of baseline TC.

The mechanism of action through which procyanidins exert lipid-lowering action has not been elucidated, but, on the basis of *in vitro* studies, it can be asserted that they may act in two different ways. Dimeric compounds (such as procyanidin B2) would have a statin-like mechanism (DeBose-Boyd et al., 2001; Bursill et al., 2006; Yasuda et al., 2011), while polymeric compounds would not be absorbed by the intestine and may accumulate in the gut, where they may act with a β -cyclodextrin-like mechanism (Leifert et al., 2008).

6.2.5 Pharmacokinetics

It is almost impossible to determine the pharmacokinetic profile of AMS in terms of absorption, distribution, metabolism and excretion because it does not contain a pure pharmaceutical substance, but it is constituted by a phytocomplex. Maybe, in order to obtain pharmacokinetic data, volunteers could be provided with AMS pure components (procyanidins, flavonols, dihydrochalcones and anthocyanins), but from literature data it is known that the pharmacokinetic profile within the same chemical group can be extremely variable (Manach et al., 2005). Nevertheless, data regarding the human pharmacokinetic profile of many constituents of AMS are already available from previous studies. In 2005 Manach and colleagues published a review that took into the account 97 studies, which investigated the kinetics and extent of polyphenolic compounds absorption in humans and showed that the flavanol epicatechin had mean values of T_{\max} (which is the time necessary to reach the C_{\max}) of 1.8 hours, the C_{\max} (which is the maximal plasma concentration) was at maximum 1.10 $\mu\text{mol/L}$ and the half-life was of 2.5 hours. With regard to metabolism, several types of circulating metabolites were found: epicatechin-3'-*O*-glucuronide, 4'-*O*-methyl-epicatechin-3'-*O*-glucuronide, 4'-*O*-methylepicatechin-5-*O*-glucuronide, 4'-*O*-methylepicatechin-7-*O*-glucuronide and the aglycones epicatechin and 4'-*O*-

methyl-epicatechin (Manach et al., 2005). As regards procyanidins, dimeric compounds were detected in human plasma 30 minutes after their consumption and reached the maximal plasma concentration of 41.4 nmol/L by 2 h (Holt et al., 2002), whereas high molecular weight procyanidins were not absorbed by the intestine and could act locally (Ottaviani et al., 2012).

6.2.6 Safety issue, study strength and limitations

The safety of polyphenolic content of apples was assessed through mutagenicity tests and acute/subacute toxicity studies both in mice and humans, although no specific studies were conducted during this study. Particularly, the Commission Regulation (EC) No. 258/1997 established 1000 mg as the maximum polyphenolic extract daily intake in humans, because below this dose no significant hematological, clinical, chemical, histopathological or urinary effects were observed. For this trial, the dose adopted was of 800 mg/day, an amount close to but lower than that considered as safe in humans. All the laboratory analyses indicated that there were no alterations regarding the hepatic and renal function after two months of AMS treatment (Table 13). Moreover, patients were periodically monitored in order to assess vital signs, blood pressure and electrocardiographic findings and baseline values did not change substantially during and at the end of the trial (data not reported).

The major strengths of this clinical trial reside in the originality of the study and in the evaluation of the treatment effects in real-world setting, while the main limitations of this study are short-term assessment for the treatment of a chronic condition and the choice of treating exclusively white race.

Table 13: Effects of Annurca supplement (AMS) on plasma indicators of hepatic and renal function in subgroups men and women.												
Men												
	AST (GOT) (U/L)			ALT (GPT) (U/L)			γ-GTP (U/L)			ALP (U/L)		
	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>
Mean value	21.6	21.5	21.5	27.9	27.5	27.6	37.2	36.2	34.4	222.6	220.4	215.9
Δ (%)		-0.46	-0.46		-1.43	-1.07		-2.68	-7.53		-0.99	-3.00
	LDH (U/L)			Albumin (g/dL)			Total bilirubin (mg/dL)			Creatinine (mg/dL)		
	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>
Mean value	177.0	176.5	170.2	4.31	4.32	4.16	0.57	0.57	0.54	0.84	0.82	0.80
Δ (%)		-0.28	-3.84		+0.23	-3.48		-	-5.26		-2.38	-4.76
Women												
	AST (GOT) (U/L)			ALT (GPT) (U/L)			γ-GTP (U/L)			ALP (U/L)		
	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>
Mean value	21.8	21.8	21.7	27.3	26.9	27.0	37.4	35.7	34.8	222.8	220.6	214.7
Δ (%)		-	-0.46		-1.46	-1.10		-4.54	-6.95		-0.99	-3.63
	LDH (U/L)			Albumin (g/dL)			Total bilirubin (mg/dL)			Creatinine (mg/dL)		
	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>	<i>t 0</i>	<i>t 30</i>	<i>t 60</i>
Mean value	179.9	178.8	172.2	4.41	4.32	4.23	0.59	0.57	0.54	0.83	0.80	0.79
Δ (%)		-0.61	-4.28		-2.04	-4.08		-3.39	-8.47		-3.61	-4.82
Subjects were administered with 2 AMS capsules/day for 2 months.												
Value are means ± SD (<i>n</i> = 5).												
Results were significantly different at a level of <i>P</i> = 0.001.												

The positive results obtained suggest physicians a novel treatment/intervention, which may be a valuable alternative in the clinical practice. In fact, the Food and Drug Administration (FDA) has recently approved in the United States a nutraceutical product, constituted of Granny Smith apple polyphenolic extract and named Applephenon[®], with the indication of reducing serum cholesterol levels and preventing obesity. A randomised human placebo-controlled intervention study with Applephenon[®] at different dosages showed that the maximum dose (1500 mg/day) decreased plasma TC and LDL-C levels by 5% and 8%, respectively, and increased plasma HDL-C levels by 1% (Nagasako-Akazome et al., 2005). Instead, AppleMetS[™] was able to reduce TC and LDL-C levels by 24.9% and 37.5%, respectively, and to raise HDL-C levels by 49.2%. These great discrepancies are due to the different polyphenolic composition (especially in the procyanidin fraction) of the different apple cultivars.

CONCLUSIONS

The present thesis work aimed to the *in vitro* and *in vivo* evaluation of the nutraceutical potential of the polyphenolic fraction obtained from a specific apple cultivar, Annurca. Particularly, the effects of Annurca polyphenolic extract on lipid uptake and metabolism were evaluated and put in comparison with those obtained from other commercial cultivars. In *in vitro* studies the analyses were performed by using the following apple cultivars: Red Delicious, Pink Lady, Fuji and Golden Delicious; however, in *in vivo* experiments the cultivar Pink Lady was replaced by the cultivar Granny Smith, because of the reasons above-listed.

In vitro experiments performed on HepG2 cells showed that, among peels, Red Delicious peel extract was the most active in reducing cholesterol and glucose uptake and in affecting lipid metabolism, since it reduced triglyceride uptake, inhibited lipase activity and increased LDL receptor binding activity and ApoA1 expression. However, it revealed to be cytotoxic and able to induce oxidative stress, as shown by the decrease in cell proliferation, the increase in TBARS levels and the reduction in NO concentration. Thus, on the one hand these results confirmed that food supplements formulated with polyphenolic extracts obtained from this apple cultivar are effective, but on the other hand they address to the possible risks resulting from an incorrect use. Instead, the experiments conducted with Annurca flesh extract indicated that Annurca flesh polyphenols may be regarded as a good potential nutraceutical. In fact, they were able to decrease cholesterol uptake and to affect positively lipid metabolism also more than Red Delicious polyphenolic peel extract, but they did not show any kind of cytotoxicity.

These amazing effects were attributed to procyanidins, which represent the most abundant polyphenols contained in apple flesh, and good linear correlations between procyanidin content and cellular effects were found.

However, in order to achieve any effect in a specific tissue or organ, polyphenols must be available, so the successive step was the *in vitro* evaluation of some important parameters, such as bioaccessibility, bioavailability and plasma protein interaction. For this sort of analysis, *in vitro* experiments of gastrointestinal

digestion were performed, reproducing as closely as possible all the reactions that occur in human body.

Polyphenols revealed to have a good bioaccessibility but a scarce bioavailability; moreover, the poor absorbed part was strongly bound to plasma proteins. Nevertheless their scarce absorption, procyanidins may accumulate in the gut, protecting the intestine from the attack of numerous oxidising agents and inhibiting dietary cholesterol uptake. Furthermore, bioavailable procyanidins interact significantly with plasma proteins, especially HDLs, suggesting they may play a role in the reverse transport and metabolism of cholesterol.

In order to verify if the amazing *in vitro* properties of the polyphenolic fraction of Annurca apple were also maintained *in vivo*, two different clinical trials were performed.

The first one aimed to evaluate *in vivo* effects on plasma TC, LDL-C, and HDL-C levels after the regular consumption of different apple cultivars (Annurca, Red Delicious, Granny Smith, Fuji and Golden Delicious), which were considered as functional foods. The data obtained after the first clinical trial showed that the effect of daily apple consumption on plasma cholesterol levels depends on the kind of apple cultivar and its polyphenolic composition. Particularly, the consumption of two Annurca apples per day had good effects for serum cholesterol levels in healthy subjects, as it decreased TC and LDL-C levels by 8.4% and 14.5%, respectively, and increased HDL-C levels by 14.0%. This last effect seems to be the most important because very few natural or pharmaceutical substances have been reported to exert a positive influence on the so-called “good cholesterol”. Thus, these data indicate Annurca apple as a functional food for the maintenance of the healthy balance of plasma cholesterol levels, and it may be considered as a useful tool for the prevention of CVD risk through diet.

The second *in vivo* study was based on the evaluation of the effects on plasma TC, LDL-C and HDL-C after the administration of the only polyphenolic fraction coming from Annurca apple cultivar. The administration of two capsules per day of AppleMetSTM (AMS) formulation (equivalent to the consumption of six Annurca apples per day) led to really excellent results on plasma values of TC,

LDL-C and HDL-C (-24.9%, -37.5% and +49.2%, respectively). Particularly, the effect on LDL-C is comparable to 10 mg of atorvastatin, which is the most potent statin available to date, whereas the important effect on HDL-C is not found in any pharmaceutical or nutraceutical substance. In particular, this nutraceutical product was more effective on elder people with higher values of baseline TC, who represent the major recipients of any hypolipidemic treatment.

The first clinical trial demonstrates that a severe dietary intervention is necessary for cardiovascular disease prevention, but it is unrealistic to think that nutrition habits will change easily in industrialised countries. Instead, the second clinical trial leads to really excellent results and offers a simplified remedy with respect to diet transformation. Thus, the nutraceutical product may be considered as a complementary and/or alternative safe substance, suitable for the treatment of mildly hypercholesterolemic subjects who do not present occurrence of atheromatous plaques yet.

The mechanism that underlies the cholesterol-lowering effects after the administration of whole fresh Annurca apple or AMS product would involve the action of procyanidins, which would act in different ways. Dimeric compounds (such as procyanidin B2) would have a statin-like LDL-lowering effect, by activating the SREBP-1 pathway and enhancing the expression of LDL receptor. Moreover, they would raise ApoA1 concentration and activate LCAT, exerting good effects also on plasma values of HDL. Polymeric compounds, instead, would not be absorbed by the intestine and may accumulate in the gut, where they may act with a β -cyclodextrin-like mechanism.

To sum up, on the basis of the positive results obtained by *in vitro* experiments and *in vivo* clinical trials, Annurca polyphenols may represent a novel treatment/intervention thanks to their beneficial effects on cholesterol uptake and metabolism.

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