UNIVERSITY OF NAPLES – FEDERICO II

DEPARTMENT OF PHARMACY

**Ph.D. THESIS IN
"PHARMACEUTICAL SCIENCE"**

Formulation of novel Functional Foods and Nutraceutical Products from Annurca apple with healthy human effects

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Functional Food formulation

Annurca apple puree enriched with different strains of acid lactic bacteria as a potential functional food: In vitro and in vivo studies

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Abbreviations

AAE : Annurca apple extract

AFA : Apple fruit cv Annurca

AMD : Annurca nutraceutical formulation

AMS : AppleMets

AMSbz : AppleMetS hair

ANN : Annurca

APC : Adenomatous polyposis coli gene

BMI : Body Mass Index

CFU : Colony forming units

CLP : Capsule

CVD : Cardiovascular Disease

DAD : PhotoDiode Array Detector

DigAAE : Polyphenolic content of: *in vitro* digested Annurca Apple Extract

DigLAE : Polyphenolic content of: *in vitro* digested Limoncella Apple Extract

DPPH : 2,2-diphenyl-1-picrylhydrazyl

EGCG : Epigallocatechin gallate

FAO : Food and Agricultural Organization of the United Nations

FAP: Familial Adenomatus Polyposis

FAS : Full Analysis Set

FDA : Food and drug administration

FMO : Flavin-containing monooxygenase

FZD : Frizzled

GAE : Gallic acid equivalents sulfate-reducing bacteria

GD : Golden Delicious

GI : Gatrointestinal

HDL : High density lipoprotein cholesterol

IndAAE : Industrail Annurca extract

IndLAE : Industrail Limoncella extract

KAP : Keratin associted proteins

LADME : Liberation, Absorption, Distribution, Metabolism and Excretion

LAE : Limonella apple extract

LDL : Low density lipoprotein cholesterol

LP : Lactofermented puree

ND : Not Detected

NLP : Not Lactofermented puree

NMR : Nuclear magnetic resonance

NOE : Overhouser effect

PAL : Phenylalanine ammonia lipase

PC : Phosphatydilcholine

PCW : Polysaccharides cell wall

PEF(AAE) : Polyphenolic purified fraction of AAE

PEF(LAE) : Polyphenolic purified fraction of LAE
PGI : Protected Geographical Indication
PL : Pink Lady
SD : Standard Deviation
SRB : Sulfate-reducing bacteria
STD : Nuclear magnetic resonance saturation transfer difference
TGF : Trasforming growth factor
TMA: Trimhetylamine
TMAO : trimhetylamine-N-oxide
TPC : Total Polyphenol Content
WNT : Wingless-related integration site

Abstract

The present thesis work aimed to the *in vitro* and *in vivo* evaluation of the nutraceutical potential of whole fruit or only of its polyphenolic fraction mainly obtained from a specific apple *Malus pumila* Miller cv Annurca. This apple fruit is used in various manners, the whole fruit is enabled to produce a functional food, while the polyphenolic extract is used to design and project different nutraceutical formulations and vegetal extract innovative using as potential medical device. Various healthy human bioactivities of Annurca apple formulations have been investigated, from the cholesterol lowering-effect, atherosclerosis prevention to anticancer activity. Particularly, the whole fruit Annurca apple is used to the preparation of probiotic apple puree, a probiotic juice, with various purposes: no dairy probiotic product, with higher bioaccessibility (percentage increases of 40.12% of TC, and of 11.89%of antioxidant activity) and consequently higher bioavailability of phenolic compounds than in simple puree. Additionally preliminary *in vivo* study, has shown a reduction of TMAO blood levels (63,12%), due to the combination effects of probiotic and Annurca apple polyphenols daily intake. Industrial scale-up of row lab scale polyphenolic Annurca apple extract, resulting in two products AMS (polyphenolic extract microencapsulated with maltodextrins (400mg)) and AMSbz (polyphenolic extract microencapsulated with maltodextrins (400 mg), biotin (0.20 mg), selenomethionine (80.0 µg), and zinc acetate (21.0 mg)). From randomized clinical trial, was obtained on average, after two months of treatment a % increase of hairs growth, weight and keratin content by 118.3, 37.3, and 35.7%. After, *in vitro* treatment of HaCaT cells (human keratinocyte cell line) increased the keratin expression of three fold against the control. These effects were ascribable a dimeric procyanidin. The same compound is responsible of interaction at duodenal level, with intestinal micelle, particulary interacting with phosphatidylcholine determine a breakage micellar system, causing the cholesterol precipitation, limiting its intestinal absorption. Finally, lab and industrial scale of polyphenolic Annurca apple extracts were tested on *in vitro* cultures of cells carrying FAP (Familial Adenomatous Polyposis) mutations and on *ex vivo* biopsies of FAP patients, the WNT inhibitory activity of Annurca was studied. Duodenal digested apple extract has showed no inhibitory activity on WNT pathway, the signaling via responsible of FAP, but stronger inhibitory activity has been shown by industrial scale product, based on another apple cultivar named "Limoncella" *Malus Domestica* cv Limoncella. This effect was ascribable to the quercetin content of Limoncella's industrial product higher than the equivalent Annurca version.

Research Objectives

The evidence that the regular consumption of apples leads to beneficial health effects, ranging from a reduction of CVD (cardiovascular disease) risk to anticancer prevention, encouraged me to explore and deepen the nutraceutical potential of the "Annurca" apple whole fruit or only its polyphenolic extract, by both *in vitro* and *in vivo* studies.

Annurca apple polyphenolic extract's bioactivities explored were :

- ❖ promoting hair growth, both *in vitro* and in humans;

- ❖ lowering the cholesterol solubility in an *in vitro* model of duodenal bile micellar solution;

- ❖ WNT inhibitory activity *in vitro* and on *ex-vivo* biopsies (inhibitors of WNT/β catenin pathway have been shown to efficiently act as chemopreventive agents for patients affected by Familial Adenomatous Polyposis (FAP)).

Functional food based on Annurca apple whole fruit shows the following bioactivites :

- ❖ Increase polyphenolic bioaccessibility *in vitro*;

- ❖ Decrease of TMAO (cardiovascular risk marcker) blood levels *in vivo*.

Date and proven the previous biological activities, aim of the present thesis work is to formulate nutraceuticals or functional foods that will find application in disease prevention. Today, the nutraceutical product, whitch its formulation and activity, were described in this thesis work is available in Italian commerce with the name of : AppleMets hair[TM].

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Introduction

Hippocrates said, "Let food be thy medicine, and medicine be thy food". It was the 377 a.c., when Hippocrates with this sentence underlined the ancient concept of the importance of food for health. The human evolution goes with the food evolution, and the food products and manufacturing changes relating to the human needs. "An apple a day keeps the doctor away", these is a folk saying most famous. Today it is known that all the beneficial healthy effects associated to the apple consumption are ascribable, not an "unknown" and "mysterious" compounds, but largely to their characteristic polyphenolic complex (Knekt et al., 1996; 2000; 2002; Aprikian et al., 2001). Particularly, in the present thesis work, my attention has been addressed on an apple cultivar typical of south Italy: *Malus pumila* Miller named "Annurca", which is listed as a PGI product and is known as "Mela Annurca Campana IGP" product from the European Council [Commission Regulation (EC) No. 417/2006)]. This apple has been already shown to possess nutraceutical potential in virtue of its ability to reduce cellular glucose levels and lipid uptake (Tenore et al. 2013,2014,2016 and Sommella et al. 2015). The whole fruit or only the polyphenolic content of this apple cultivars are formulated and elaborated in various ways to evaluate and test new different bioactive activities both in *vitro* and in *vivo* systems. The formulations and applications prepared were the following :

- ❖ Nutraceuticals

- ❖ Functional foods

- ❖ Medical device

 In 1989 Stephen De Felice, coined the term "nutraceutical" the union of the terms "nutrition" and "pharmaceutical", he was the founder and chairman of the Foundation for Innovation in Medicine (FIM), Cranford, NJ in 1989. 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 et al., 2003). Nutraceutical formulations have a potential to deliver in concentrated form, the presumed food bioactive agents , presented in a non-food matrix and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods (Zeisel, 1999), they look like a drug and are generally sold in form of : pills, capsules, tablets etc. (Gul et al.,2016).

Functional foods are food and food derivatives that provide beneficial health effects in addition to their basic nutritional properties (Menrad 2003). It should be a food similar in appearance to a conventional food (beverage, food matrix), consumed as part of a usual diet, contains biologically active components with demonstrated physiological benefits, and offers the potential of reducing the risk of chronic diseases beyond basic nutritional functions (Food and Agricultural Organization of the United Nations (FAO), 2007). Functional foods are often enriched or fortified with bioactive substances through a process called nutrification, examples should be food products that contain specific minerals, vitamins, fatty acids, or dietary fiber, foods with addition of biologically active substances such as phytochemicals or other antioxidants and probiotics. The possible categories of functional foods products are shown in the following table (Gul et al., 2016).

Category	*Definition*	*Examples*
Basic Food	Food or food product that naturally contains bioactive compounds	• Carrots naturally contains Beta-carotene
Processed Foods with added bioactives	Bioactive agent is added to the food during the processing	• Orange juice added with calcium • Yogurt with increased level of probiotic • Fermented Juice
Foods enhanced to have more of bioactive	Normally Bioactive's level is modified or concentrated	• Tomatoes with increased lycopene content • Eggs with increased levels of omega-3 fatty acid

Table 1 : Categories of functional foods (Gul et al., 2016).

Directive 2007/47/EC defines a medical device as : any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, together with any accessories, including the software intended by its manufacturer to be used specifically for

diagnostic and/or therapeutic purposes and necessary for its proper application, intended by the manufacturer to be used for human beings for the purpose of:

- Diagnosis, prevention, monitoring, treatment, or alleviation of disease;

- Diagnosis, monitoring, treatment, alleviation of, or compensation for an injury or handicap;

- Investigation, replacement, or modification of the anatomy or of a physiological process;

- Control of conception.
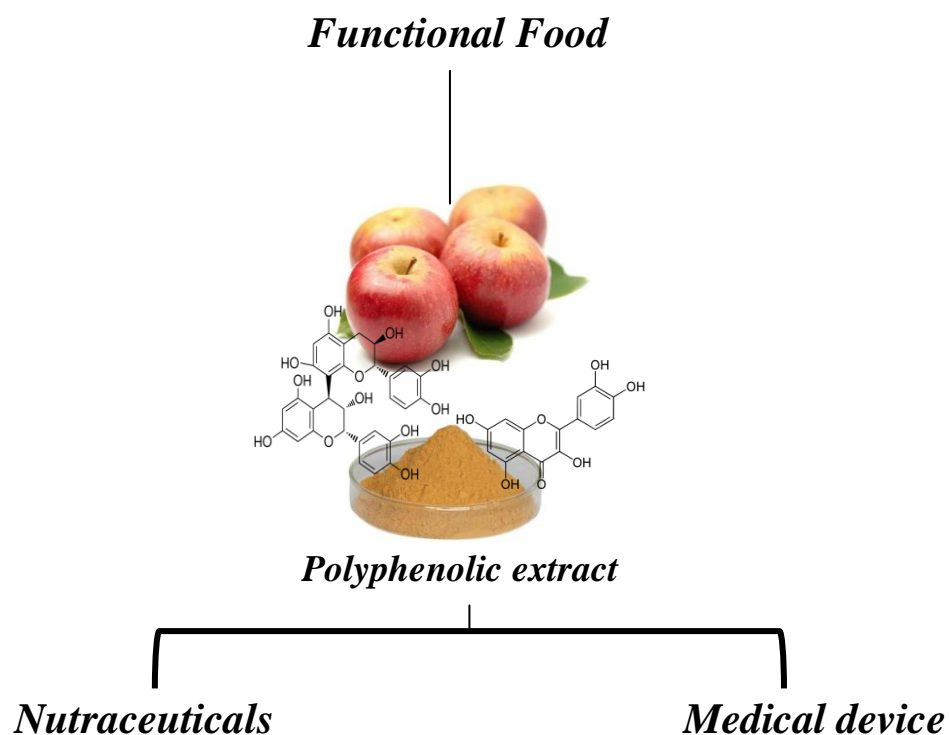
Fig 1 : Graphical illustration of different formulations of Annurca Apple whole fruit or of its polyphenolic extract

Annurca apple, the queen of the apples. An overview of history, origin and cultivation

"Mela Annurca Campana IGP" is a precious apple cultivar (*Malus pumila* Miller), typical of Campania region. It is defined the 'queen of the apples' thanks to its marked organoleptic qualities: flavor, taste and scent. This apple is famous for its white compact and crisp flesh, agreeably slightly sour and perfume.

Its description in the paintings found in the excavations of Ercolano and its documentation in "*Naturalis Historia*" by Pliny the Elder testifies its very ancient relationship with the Campania Felix. It is present in this geographical area for at least two millennia, particularly the precise origin area seems to be the area near Pozzuoli. This information, is confirmed by the most ancient name attributed to this apple cultivar by Pilinys the Elder, " Mala Oracula", because of its production in proximity to Averno Lake (situated in the Pozzuoli region), which, according to the ancients, was the location of the underworld, so it was produced near the Orcus. In the time this denomination changed in anorcola, annorcola, until the 1987, when Giuseppe Antonio Pasquale in his opera *" Manuale de Abrocoltura"* named this cultivar "Annurca". Today, this product is listed as a PGI product and is known as "Mela Annurca Campana IGP" product from the European Council [Commission Regulation (EC) No. 417/2006)].

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, thus its production is possible only in 137 municipalities of Campania region. The

strong disciplinary, explain the particular and unique cultivation, harvasted and ripening methods. According to these guidelines, 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. 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.

Fig 2 : Melannurca Campana IGP

Polyphenols

Eating five servings of fruits and vegetables per day! This is what is highly recommended and heavily advertised nowadays to the general public to stay fit and healthy! Drinking green tea on a regular basis, eating chocolate from time to time, as well as savoring a couple of glasses of red wine per day have been claimed to increase life expectancy even further! Why? The answer is in fact still under scientific scrutiny, but a particular class of compounds naturally occurring in fruits and vegetables is considered to be crucial for the expression of such human health benefits: the polyphenols! Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets. Ranging from simple phenolic molecules to highly polymerized compounds with molecular weights greater than 30,000 Da (Quideau et al., 2011). In the following paragraphs will be illustrated the chemical classification, and several polyphenols bio-features such as healthy biological activities, bioaccessibility and bioavailability.

Classification

From chemical structure point of view the polyphenols can be divided in two classes, related with the number of cycle rings and the type of functional group. Practically, the most used classification is the distinction in flavonoids and not flavonoids compounds (Cheynier, 2005).

Flavonoids

The term flavonoids is a collective noun for plant pigments, mostly derived from benzo-γ-pyrone. 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. In base on the saturation level, C-ring substitution pattern and opening of the central pyran ring they are classified in (Middleton et al., 2000):

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

Flavonols present a double bond between C2 and C3, a ketonic function in C4 position and a hydroxyl group at the C3 position (De la Rosa et al., 2010). They are present in food in free or glycosylate form and the sugar portion could be : glucose, rhamnose, galactose, arabinose, xylose and glucuronic acid. The most diffuse aglycone compounds are the Quercetin, Kampherol and Myricetin. These compounds are typically present in the red wine and are particularly interesting in terms of their effects on human health, predominantly because of their role as antioxidants, although the precise mechanism of the oxidation process is not entirely understood. However, it is known that their activity depends from their structure and in particular the substitution with a 4-keto and 3- or 5-hydroxy group is considered essential for the metal ion chelation function. This ability to complex ion contributes to their antioxidant properties, to prevent the formation of free radicals. The presence of these compounds in the wine differs from that present in the grapes must due to the presence of aglycone forms, which are the result of hydrolysis of the glycosylated form during vinification, maturation and aging of wine. Other vegetable sources are: onions, curly kale, leeks, broccoli, blueberries. (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 present largely in the mandarin skin and its content is up to 6.5 g/L of essential oil of mandarin (D'Archivio et al., 2007).

Flavan-3-ols or flavanols are found in the solid part of grapes (peels, seeds and stems) in monomeric or polymeric forms. The basic units of these compounds are four monomers: catechin, epicatechin, gallate and epigallocatechin. The first two are ortho-hydroxylated at position 3 'and 4' of ring B while the remaining ones have a further 5 'substitution; then it is possible to replace with gallic acid leading to esterification. The asymmetricity of positions 2 and 3 causes the flavanols's split into two classes of diastereoisomers. These base units are generally organized to form a polymers, the main representatives are proanthocyanidins, named also condensed tannins, when the constituent units ranging from 2 to 11 base units (D'Archivio et al., 2007). These compounds are very abundant in tea and fruit, grapes and chocolate and are responsible of astringent character of fruit (grapes, apples, berries, etc.) and beverages (wine, cider, tea, beer etc) and for the bitterness of chocolate (Rasmusen et al., 2005).

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)

Anthocyanidins are colored molecules responsible of red/ purple color of fruit, flower and vegetables. In vegetables they are present in aglycone form and in glycosylate called antocyanidins (D'Archivio et al., 2007).The glycosidation most frequent is characterized by the sugar moiety mainly attached at the 3-position on the C-ring or at the 5, 7-position on the A-ring, while the substitution in 3'-, 4'-, 5'-position on the B-ring is very rare (Mazza et al., 1993). Food contents are generally proportional to itself color intensity, they are present largely in: peels apple, blackcurrants, blackberries, cabbage, beans, onions, radishes (D'Archivio et al., 2007).
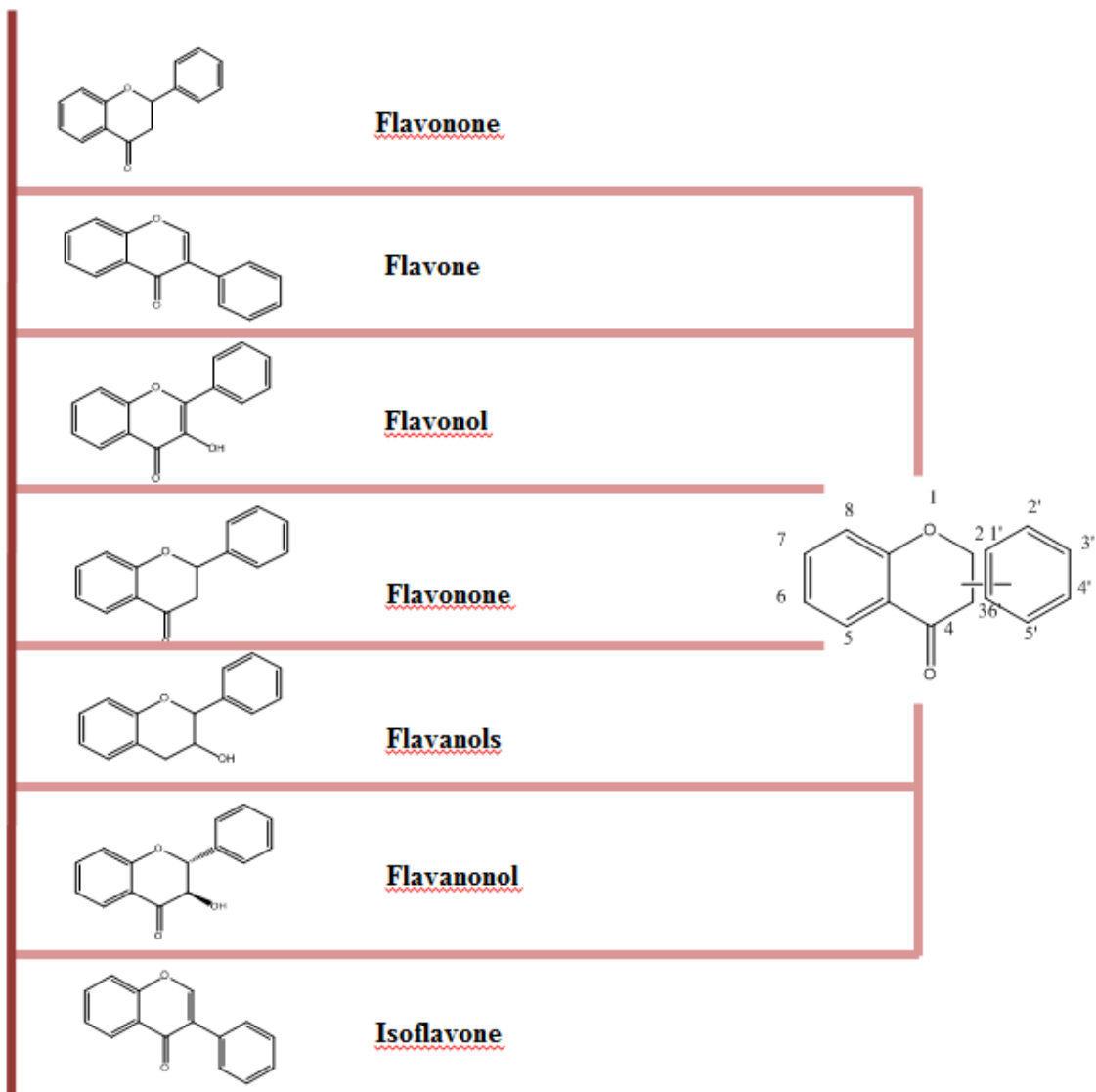
Fig. 3: General chemical structure of flavonoids and their different classes (Ravishankar et. al 2013)

Not flavonoid compounds

Not-flavonoid compounds are characterised by heterogeneous chemical structures and are classified into three groups: phenolic acids, lignans and stilbenes (Cheynier, 2005).

Phenolic acids can be distinguished in two classes: derivatives of benzoic acid and derivatives of cinnamic acid (D'Archivio et al., 2007). The major sources of benzoic acid are red fruits, black radish, onions and tea. However their concentrations in edible plants

and fruit are very low, several mg/Kg of fresh weight (FW), exception for the gallic acid which is present in the concentration of 4g/ kg (FW) of tea (Barberan et al., 2000). These compounds are often arranged with other molecules, to form very complex structures such as hydrolysable tannins (gallotannins in mangoes and ellagitannins in red fruit such as strawberries, raspberries, and blackberries) (Clifford et al. 2000). Hydroxybenzoic acids, both free and esterified, are found in only a few plants eaten by humans, thus they have not been extensively studied and are not currently considered to be of great nutritional interest. The most important are protocatechuic acid and gallic acid.
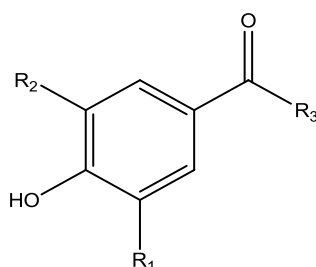
	R1	R2	R3
p-Hydroxybenzoic acid	H	H	OH
Protocatechuic acid	OH	H	OH
Gallic acid	OH	OH	OH
Vanillic acid	OCH3	H	OH
Syringic acid	OCH3	OCH3	OH
Methyl p-hydroxybenzoate	H	H	OCH3
Mhetyl gallate	OH	OH	OCH3
Ethyl protocatechuate	OH	H	OCH2CH3

Fig. 4: Hydroxybenzoic acid structure and its possible derivatives.

Hydroxycinnamic acids are more common than hydroxybenzoic acids and consist mainly of p-coumaric acid, caffeic acid, ferulic acid and sinapic acid. Their content in edible plant is very low excepted for blackberries which contain up to 270 mg/kg fresh (Shaidi et al., 1995).It's very difficult to find these compounds in free form in the edible plant and fruit , because they are present mostly in the glycosylated and esters derivatives of quinic acid, shikimic acid and tartaric acid (Manach et al., 2004). The most abundant in the fruits is the

caffeic acid, which represents the 75% of the total hydroxycinnamic acid in the fruits, both in the free and glycosylated form. The most studied for nutraceutical purpose is the chlorogenic acid, a combination of quinic and caffeic acid. Chlorogenic acid is largely studied for his ipoglycemic activity, it is very abundant in the coffe. A single cup of coffe may contain a 70-350 mg of chlorogenic acid for cup, it is established the maximum intake of chlorogenic acid by drinking coffe is 0.5-1.0 g daily (Jhonston et al., 2003; Clifford 2000 and Olthof et al., 2000).
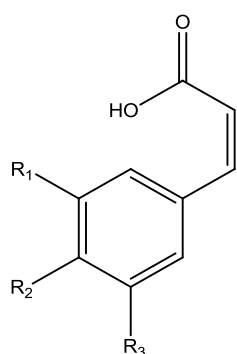
R_2 = OH : p-Coumaric Acid
R_2 = R_3 = OH Caffeic Acid
R_3 = OCH_3, R_2 = OH : Ferulic Acid
R_1 = OCH_3, R_2 = OH, R_3 = OCH_3 : Sinapic Acid

Fig. 5: Hydroxycinnamic acid and his major derivates.

Lignans are formed of 2 phenylpropane units (Figure 6). They are present in very low concentration in grains, fruit, cereal and especially in the linseed (up to 3.7 g/kg dry wt) where they have a concentration of 1000 times more high than in other food sources.
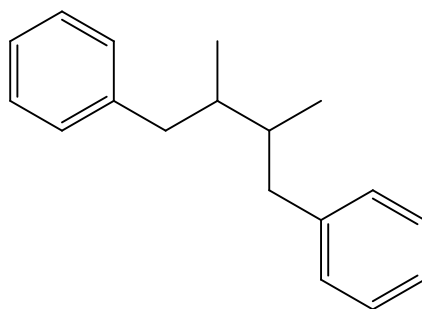
Fig. 6: Lignan structure.

Stilbenes, finally, 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) and particularly in fresh skin of red grapes reach a concentration of : 50-100 g/kg net weight (Baliga et al., 2005). (Figure 7).
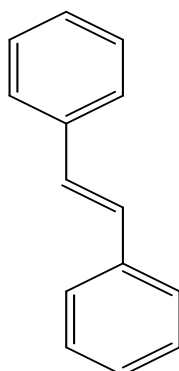
Fig. 7: Stilbenes structure.

Human biological activity

Several epidemiological studies and associated meta-analysis reported that polyphenols or polyphenols-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 to oxidative stress such as Alzheimer's and Parkinson's diseases (Dai et al., 2006; Aquilano et al., 2008; Pandey et al., 2009). 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 signaling

(Yang et al., 2000; García-Lafuente et al., 2009). Additionally, they play a strong role in the prevention of metabolic syndrome .They are able to inhibit the production of LDL oxide, the major responsible of the develop of atherosclerosis process (Aviram et al., 2000), determine the increase of HDL and improving endothelial functions (García-Lafuente et al., 2009). Polyphenols, in particular ,found application also in the diabetic pathologies, they inhibit the α–amylase, α-glucosidase and glucose intestinal absorption (Iwai et al., 2004 and Tadera et al., 2006). One of the most important property of polyphenols recently identified is their preventive effect against long-term diabetes complications including retinopathy, nephropathy and neuropathy. For example, anthocyanins facilitate blood flow and prevent diabetes-induced microangiopathy, increase microvascular permeability, decrease leucocytes aggregation in vascular cell wall and improve capillary filtration of albumin (Ghosh et al., 2007; Rhee et al., 2002). Polyphenolic compounds, also, have wonderful modulatory effects on many aspects of metabolic, endocrine and cellular signaling transduction of adipose tissue. Some polyphenols such as catechins increase β-oxidation in adipocytes, down-regulate the enzymes and genes involved in lipogenesis and fatty and up-regulate lipolysis pathways via induction of hormone sensitive lipase (Nakazato et al.,2006; Osada et al., 2006). Some polyphenols, including green tea catechins and epicatechins, chlorogenic acid, ferulic acids, caffeic and tannic acids, quercetin and naringenin, could interact with absorption of glucose from intestine via inhibition of Na+-dependent glucose transporters, SGLT1 and SGLT2 (Kobayashi et al., 2000; Jhonston et al., 2005). Some investigations have shown that polyphenolic compounds are also able to regulate postprandial glycemia and inhibit the development of glucose intolerance by a facilitated insulin response and attenuated secretion of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like polypeptide-1 (GLP-1) (Jhonston et al., 2003; Dao et al., 2011). Epidemiological studies have shown the occurrence of a decreased prevalence and incidence of asthma and an enhanced pulmonary function after apple intake. Considering flavan-3-ols, the antibacterial activity of catechins is known from the 1990s, when it was demonstrated that these compounds, largely present in oolong tea and above all green tea (*Camellia sinensis*), inhibited the *in vitro* growth of several bacterial species, such as *Vibrio cholerae, Streptococcus mutans, Campilobacter jejuni, Clostridium perfringes, and Escherichia coli* (Sakanake et al 1992; Diker et al 1991; Ahn et al 1991; Isogoi et al 1998 and Daglia 2012). In order to achieve beneficial effects the maximum polyphenols daily intake is 1g/day (Chun et al., 2007).

Bioaccessibility and bioavaiability

Although the biological activity of polyphenols is largely studied, as reported in the last paragraph, it is important consider that not all the amounts present in polyphenolic rich food or beverage, are available to the intestinal absorption. In order to obtain many effects 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. As shown in this scheme the liberation of the bioactive compounds from the food matrix, is a key passage, fundamental to the consequently availability. So it is important underlines, that the polyphenols availability is not only limited from the enzymatic, mechanical or chemical transformation that happen during the gastrointestinal digestion, following by their potential inactivation, but also by all processes of splitting the bioactive compounds from the food matrix. So it is correct to consider before the bioaccessibility and after the bioavailability. The concept of bioaccessibility can be defined as the quantity or fraction of bioactive compounds which is released from the food matrix in the GI tract and becomes available for absorption. This includes digestive transformations of food into material ready for assimilation, the absorption/assimilation into intestinal epithelium cells, and lastly, the presystemic metabolism (Fernández-García et al., 2009). After the liberation from food matrix, the rate of absorption depending from the chemical structure of the compounds, their hydrophobicity, the presence of charge etc. Most of polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form (low hydrophobicity) (Manach et al., 2004) and related to their chemical structures changes the absorption site : at gastric level are absorbed flavonols like quercetin, but not its glycosidate form, and anthocyanins (Pandey et al., 2009). Most of glycoside resist to gastric digestion and arrive untransformed in the intestine. At this level the glycosides could be absorbed by enterocyte membrane sodium dependent glucose transporter SGLT1 and in the enterocyte they are directly hydrolyzed by β-glucosidase in aglycone form (Passamonti et al., 2005 and D'Archivio et al., 2007). Fourthmore the substances, that reach the lower tract of intestine, must be hydrolyzed by intestinal enzymes or by the colonic microflora before they may be absorbed. When the intestinal flora is involved, the efficiency of absorption is often reduced because the flora also could degrades the aglycones producing various simple aromatic acids (Manach et al., 2004; Manach et al., 1995 and Hollman et al., 1997). The mechanism involved for polyphenols intestinal

absorption is the passive passage thought the gut wall, for the hydrophobic molecules, but the membrane carriers that could be involved in polyphenolic free form absorption have not been identified. To date, the unique active transport mechanism that has been described is a Na-dependent saturable transport mechanism involved in cinnamic and ferulic acid absorption in the rat (Ader et al., 1996). 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 (Day et al., 2001), increasing their hydrophilicity and promoting their excretion through bile and urine: thus, circulating polyphenols are conjugated derivatives, strongly bound to plasmatic proteins, especially albumin and the binding force depending by the structural characteristics of the phenolic compounds (Manach et al., 2004; Pandey et al., 2009). The digestion would change the structure of polyphenolic compounds, and release molecules without biological activity, but sometimes, could release molecules that demostrated a new strong biological activity. For example, the soy isoflavone daidzein is microbially biotransformed to equol, which has a more potent estrogenic action than the precursor itself (Bowey et al., 2003). Finally, the sum of all these steps, determines a reduction of polyphenol amount available for the intestinal absorption. Bioaccessibility studies have been conducted on grapes, they have found 62.4% of total polyphenols were bioaccessible in the simulated pancreatic juice while the remaining were in part degraded and in part not extracted from the solid matrix. Regarding flavonoids, 56.1% of total flavonoids were bio-accessible in the simulated pancreatic juice while only 7.6% of anthocyanins were found in the pancreatic digested samples. These different bioaccessibility values between flavonoid and anthocyanins are related to their chemical structures. (Tagliazucchi et al., 2010) Flavonoids like cathecin or quercetin are stable at alkaline intestinal pH (loss of catechin of less than 8% in simulated intestinal fluid (Neilson et al., 2007; Laurent et al., 2007)), while the low quantity of anthocyanins in the simulated pancreatic juice confirms their low chemical stability at gastric pH (Pérez-Vicente et al., 2002).
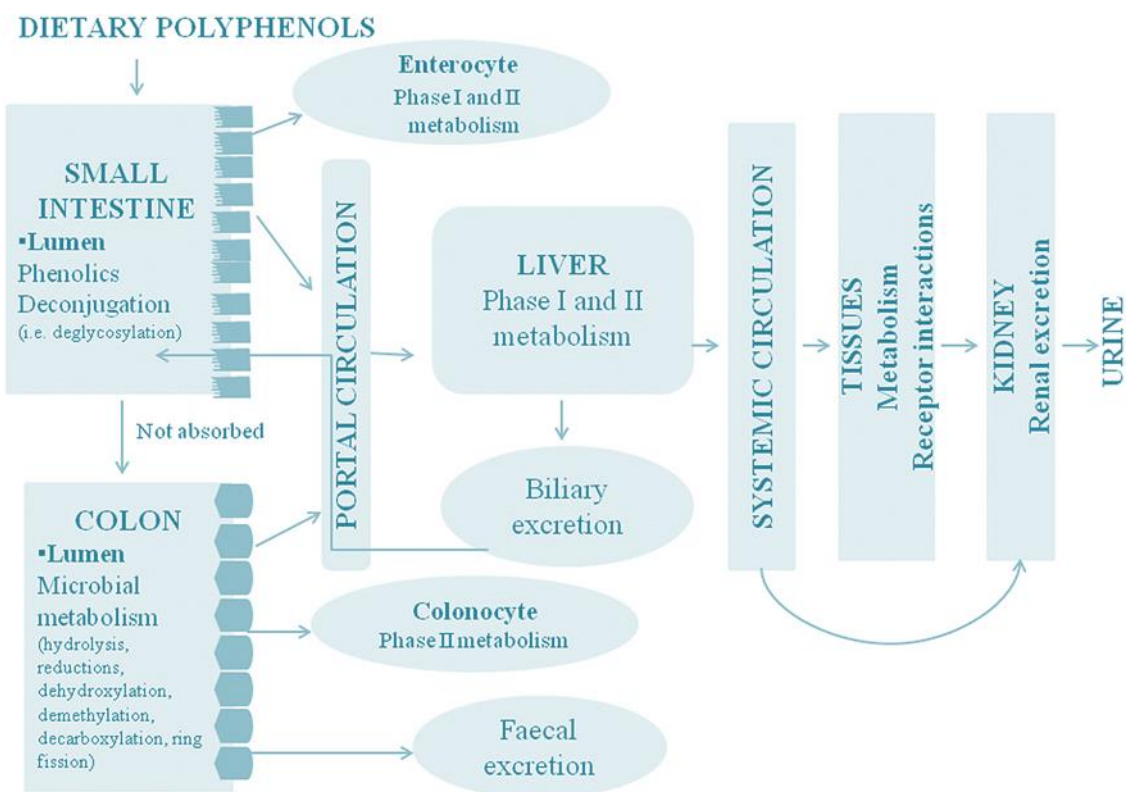
Fig. 8: Routes for dietary polyphenols and their metabolites in humans. Within the host, dietary polyphenols and their microbial metabolites successively undergo intestinal and liver Phase I and II metabolism, biliary secretion, absorption in the systemic circulation, interaction with organs and excretion in the urine (Cardona et al., 2013).

1.2.5 Polyphenols contained in apples

The apple is an important source of polyphenolic compounds. A lot of studies were produced to identity and characterize the apple's polyphenolic composition, particularly flavonoids (the major phenolics in apples) and related total antioxidant activities based on chemical extraction, typically using methanol or water/methanol mixtures (Lamperi et al., 2008; Lee et al., 2003; Neveu et al., 2010 and Wojdylo et al., 2008). It is difficult to performs a unique characterization of apple polyphenols, fix a unique profile. The qualitative profile depended from the different cultivars studied, and the amount of single molecules analyzed is distributed in different way between the pulp and the peel (Tsao et al., 2003 and Lamperi et al., 2008). In general, the concentration of polyphenols is major in

the peel than in the palp. It is normal, because the peel is the defense organ of the fruit, and a part of polyphenols have a protection activity against the external attacks. In general the apple polyphenolic profile is characterized by the presence of compounds belonging to the following classes: flavanols, flavonols, anthocyanins, hydroxycinnamic acids and dihydrochalcones (Alonso-Salces et al., 2004).

Flavonols are mostly represented by quercetin derivatives : 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). These compounds have a maximum UV absorbance at 360 nm .
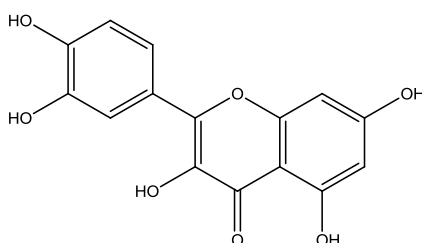
Fig. 9: Quercetin structure

The Chlogenic acid (4-p-coumaroylquinic acid) is the most representative Hyroxycinnamic acid, it is the 4 % to 18% of apple polyphenols, and has two UV Absorbance maximum : 280 and 360 nm.
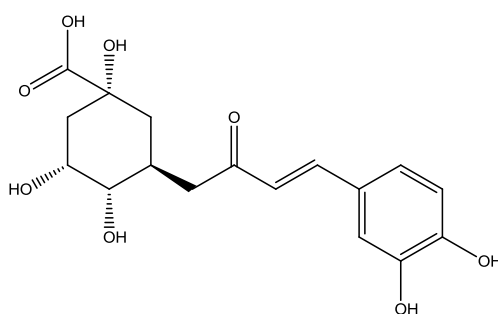
Fig. 10: Chlogenic Acid structure

The anthocyanins are characterized by strong red color, in fact they are present mostly in the apple with brilliant red or dark peels. According to these feature they present a maximum UV absorbance at 520 nm. The anthocyanins present in higher concentration in apple, are glycoside derivative of cyaniding (cyanidin-3-O-galactoside).
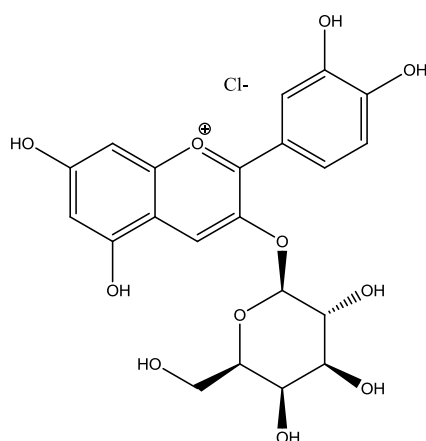
Fig. 11: Cyanidin-3-O-galactoside structure

Among flavanols the most representative are (71-90% of total polypheolic content) : (-)-epicatechin and (+)-catechin. According to their structures, they have a maximum absorbance at 280 nm.
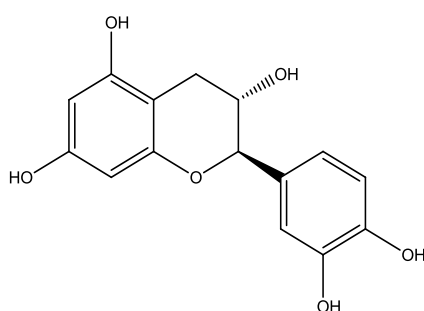
Fig. 12: (+)Catechin structure

These compounds are present in polymerized form : the procyanidins (polimeralization of catechin and epicatechin). The polymeric procyanidins are constituted by a repetition of the same base units, and according to the number of repetition the procyanidyns can be divided in 5 class: dimeric (n=2) Procyanidin B2-B8 and A1-A2. In B-type procyanidins, monomeric units are connected through a single C4-C8 or C4- C6 linkage, while A-type procyanidins have an additional C2-O-C7 or C2-O-C5 linkage (Fig. 13) . The monomeric units could reach higher polymerization grade to form a trimeric (n=3 C1 and C2) (Fig.12), tetrameric, pentameric, and high molecular weight procyanidins when its structure is formed by more base unit (6< n<11). (Appeldoon et al., 2009)
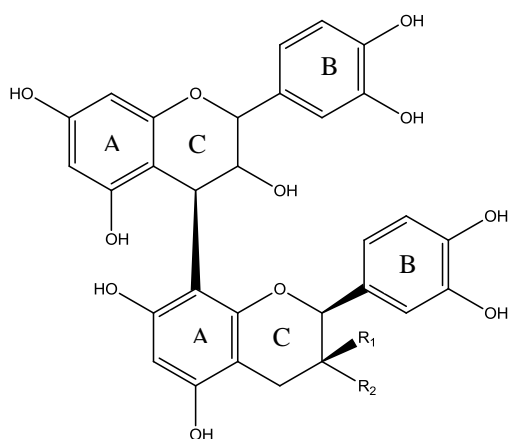
Procyanidin B1 : R₁ = OH, R₂ = H
Procyanidin B2 : R₁ = H, R₂ = OH
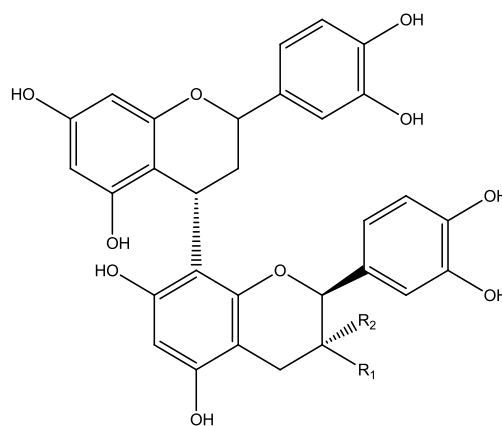
Procyanidin B3 : R₁ = OH, R₂ = H
Procyanidin B4 : R₁ = H, R₂ = OH
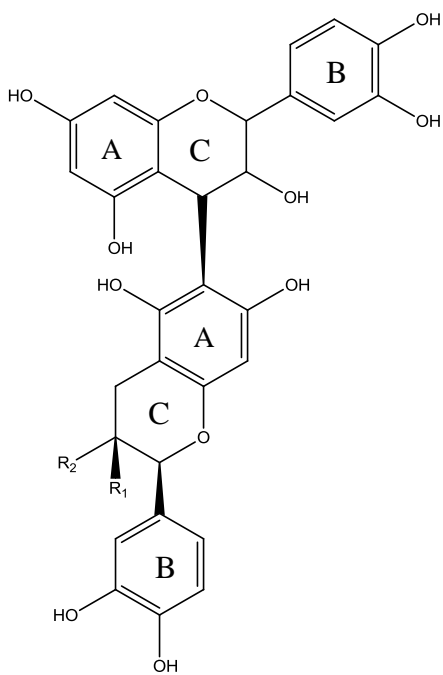
Procyanidyn B5: R₁ = H, R₂ = OH
Procyanidyn B7: R₁ = OH, R₂ = OH
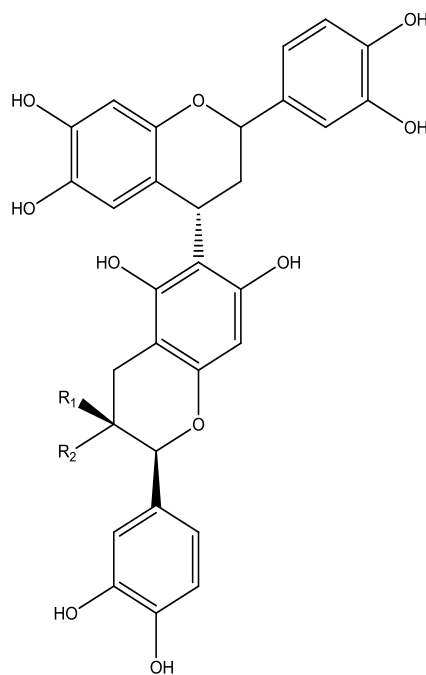
Procyanydin B6 : R₁ = OH, R₂= H
Procyanidin B8 : R₁ = OH, R₂ OH

Fig. 13: Different structures of dimeric Procyanidins (Bombardelli and Marazzoni, 1995).

Procyanidin C2   Procyanidin C1
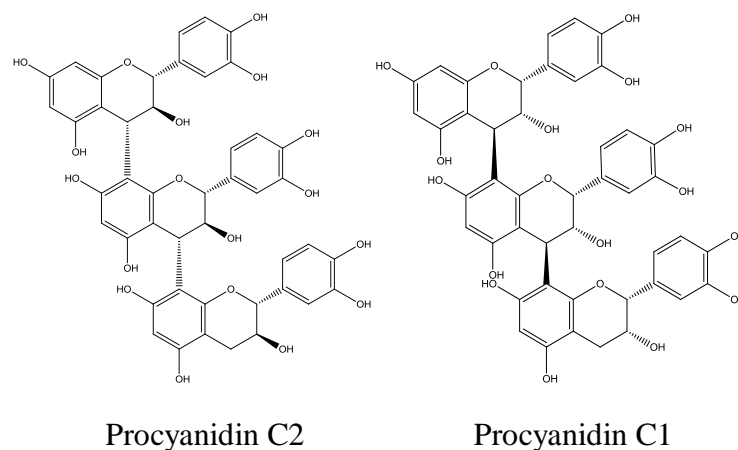
Fig. 14: Trimeric procyanidin structures.

A comparative study about the phenolic composition in different apple cultivars has been performed by Tenore et al.(2013a), which showed that, the Annurca apple has a higher total phenol content than other cultivar studied : Pink Lady, Red delicious, Golden Delicious, Fuji and Granny Smith (Annurca's total phenol content is 135.32 mg/100g FW and 101.57, 95.87, 45.27, 32.13 mg/100g FW are the concentrations present respectively in Red Delicious, Granny Smith, Fuji and Golden Delicious cultivar). Particularly, the data that attract my attention is the extraordinary amount of procyanidin B2 in this apple cultivar 62.91 mg/100g FW against a lower concentration in the other studied cultivar. These data are linked to the particular protocol of ripening. According to PGI guide line, the Annurca apples are harvested when they present a green- yellow peel, and the repining are completed on the ground for 15 days. During this period the peel color change until to be dark red and the concentration of phenolic compounds tends to increase. This effect could be due to the ethylene action, a hormone able to stimulate the activity of phenylalanine ammonia lypase (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).In the last years on Procianydin B2 was addressed particular interest for its peculiar chemopreventive actions and strong effects on the metabolic syndrome (Tenore et al., 2013a,b;2014). Thus, in this study, I have used largely the annurca apple as source of phenolic extract, particularly rich in procyandin B2, to evaluate and explore new potential nutraceutical applications. In the following chapters will be shown different applications and formulations of this precious fruit and its valuable polyphenolic content.

Chapter 1

Functional food formulation

Annurca apple puree enriched with different strains of acid lactic bacteria as a potential functional food: In vitro and in vivo studies

1.1 Introduction

Probiotics are defined as live microbial food supplements, which upon ingestion in sufficient quantities exert health benefits including the improvement of the intestinal microbial balance (Menrad, 2003). The consumption of foods and beverages containing functional probiotic microorganisms is a growing, global consumer trend (Verbeke, 2005). Encouraged from this new global tendency, I projected a new potential functional food : LactoFermented Annurca apple puree .This product was a symbiotic system, composed of two different actors : the prebiotic matrix (Annurca apple puree) and the probiotic strains. This project could be considered as an unusual study of bioaccessibility, which is normally evaluated by *in vitro* digestion procedures. This parameter was examined from another point of view : how the fermentation by acid lactic bacteria would improve the amounts of free phenolic compounds released from the fiber, mainly represent by pectin in Annurca apple puree. This study was aimed to increase the polyphenolic bioaccessibility, that is normally limited from several factors. It's commonly known that the association of polyphenols with dietary fiber rich food, caused a decrease of bioaccessibility of ingested polyphenols in gastric and small intestinal digestion because they form a specific and unspecific bond with the fiber (Hagl et al., 2011; Manach et al., 2004; Saura-Calixto et al., 2007). The Polyphenolic bioaccessibility is not only limited by different diet associations, therefore, in vegetable and fruit it is limited by the interaction with the food matrix and mainly with polysaccharides cell wall (PCW). The interactions between polyphenols and plant cell walls can occur spontaneously and extensively, as soon as polyphenols come into contact with either plant cell wall analogues (e.g. cellulose, cellulose/pectin) (Padayachee et al., 2012a,b) or PCW materials (Bindon et al., 2010; Renardet al., 2001). Several studies have investigated the interactions between procyanidins and model polysaccharides, that represent cell wall components (Bourvellec et al., 2005; Ruiz et al., 2014). In agreement with these studies were possible various type of interactions between polyphenols and

PCW, and they derive from the structure of the phenolic compounds and the polysaccharides constituents of the PCW. In particular, in the apple puree, the pectin was the most abundant component of apple fiber. As some studies showed, a gel-like pectin model preferentially interacts with procyanidins with a greater apparent binding affinity than xyloglucan and cellulose (Le Bourvellec et al., 2005). In addition, Padayachee et al. (2012a,b) contributed to the current understanding of the mechanism for the binding selectivity of polyphenols to different PCW components through an investigation of the interactions of anthocyanins and phenolic acids in purple carrot juice with either cellulose or cellulose-pectin composites. Padayachee's study found that polyphenols selectively and extensively bind to different types of cell wall components, normally present in the primary PCW, with the saturated binding levels predicted to be in the range of 0.3–1.5 g adsorbed polyphenols/g dry weight of PCW. It can be inferred that a high proportion of ingested polyphenols might be bound to cell walls (dietary fiber) in plant-based food products, during food processing, preparation, and consumption, whenever polyphenols have the opportunity to come into contact with the PCW. In fact, the interaction between polyphenols and PCWs may occur quickly in real food systems, since the binding phenomenon happens spontaneously under the practical conditions of the food processing of several common food products such as wine (Bindon et al., 2013), fruit juice (Le Bourvellec et al., 2007), or after consumption inside the human digestive tract (Hagl et al., 2011; Manach et al., 2004; Saura-Calixto et al.,2007). Importantly, if the binding is relatively stable, with limited bioaccessibility during gastric and small intestinal digestion (Hagl et al., 2011; Mandalari et al., 2011; Padayachee et al., 2012), many polyphenols might be carried by PCW materials to the large intestine, where they would be expected to be released/metabolized by the resident microbiota (Fogliano et al., 2011; Scalbert et al., 2002). Taking inspiration from this last concept, I formulated the Lactofermentated Annurca apple puree, it is a prototype of functional food, where the probiotic activity is an arm to increase the theoretical polyphenols bioaccessibility, that conjugated with probiotic activity, could reduce the blood concentration of TMAO (trymethilammine-N-oxide). This molecule is considered a new risk factor of cardiovascular disease with a linear correlation between the TMAO blood concentration and cardiovascular events (Mente et al., 2015). Per blood concentration < 2.43 μM, is associated a low risk of cardiovascular diseases, moderate risk for concentration between 2.43 and 3.66 μM, high risk between 3.66 and 6.18 μM and very high risk for TMAO concentration > 6.18 μM (Wilson et al., 2013).

TMAO is a secondary metabolite produced in two-steps process. Firstly, its precursor TMA (trimethylamine) is derived *in vivo* from the gut-microbial cleavage of trimethylamine spices such as choline and carnitine or phosphatidylcholine (Ufnal et al., 2015). Choline is absorbed by the small intestine through a carrier saturated at a concentration of 200-300 micromolar (Sheard et al., 1989), when the carrier was saturated, choline excess reaches crass intestine and it was metabolized by intestinal bacteria to form TMA (trimethylamine)(Wang et al., 2011) and the major part of TMA produced, passed into the bloodstream and then rapidly oxidized to TMAO by FMO3 a liver isoform of flavin monooxygenase (FMO, Flavin-containing monooxygenase) (Warrier et al., 2015). The formation of this molecule was a multi-factorial event, is related to the diet habits (high consumption of red meat, eggs, fish) and gut microbiota composition (Wang et al., 2011). The effects of association between apple polyphenolic content and acid lactic bacteria were studied both in *vitro* (increase of free phenolic compounds and antioxidant activity) and in *vivo* (valuation of blood concentration of TMAO).

1.2 Material and mhetods

Lactobacillus plantarum and *Lactobacillus rhamnosus ATCC 11982* (Farmalabor, Canosa, Italy) were reactivated by culturing twice in MRS broth (meat peptone 10.0 gL−1; dextrose 20.0 gL−1; yeast extract 5.0 gL−1; beef extract 10.0 gL−1; disodium phosphate 2.0 gL−1; sodium acetate 5.0 gL−1; ammonium citrate 2.0 gL−1; magnesium sulfate 0.1 gL−1; manganese sulphate 0.05 gL−1; Tween 80 1.0 gL−1) (Thermo scientific, Whalthan, Massachusetts, USA) at 37 ◦C for 18 h. Pellets were washed in sterile physiological solution (NaCl 8.5 gL−1) and suspended in 5mL the same solution.

1.2.1 Annurca 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 used for the preparation of apple puree.

1.2.2 Annurca apple puree preparation

The whole (pulp and peel) Annurca apples were cut, homogenized with boiling water in ratio (1ml :1 g) until to achieve a good consistence and a smooth puree. It was sterilized for 15 min at 121°C.

1.2.3 Fermentation of Annurca apple puree

Fermentation experiments were conducted in test tubes, each of them containing 30 mL of apple puree and in sterile conditions. All samples were inoculated with two 18 h cultures (final concentration 2.5 and 5×10^6 CFU /mL) and incubated at 37 ∘C. The control puree was also incubated and stored under the same conditions where the volume of inoculum is replaced by the same volume of sterile physiological solution (NaCl 8.5 gL−1). The samples were left in incubation for 24 and 48 h at 37°C.

1.2.4 pH measurement

After the incubation time 24 and 48 h, the tubes were opened and the pH was measured by pH meter (Crison, Barcellona, Spain).

1.2.5 Biochemical analysis

Apple puree samples were centrifuged at $11 \times g$ for 10min (Biofuge, Beckman, Los Angeles, CA, USA). The supernatant was filtered through 0.45 μm nylon syringe filter (Phenomenex ,Torrance, CA, USA,) and used for biochemical analyses.

1.2.6 Antioxidant activity

The antioxidant activity of annurca apple puree was measured with respect to the radical scavenging ability of the antioxidants present in the sample using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich St. Louis, MO, USA). The analysis was performed by adding 50 μL of the apple puree supernatant to 950 μL of a methanol solution of DPPH (153 mmol L−1). The decrease in absorbance was determined with a UV-visible spectrophotometer (Beckman, Los Angeles, CA, USA). The absorbance of DPPH radical without antioxidant, i.e., the control, was measured as basis. All determinations were in triplicate. Inhibition was calculated according to the formula [(1− Af /Ab)] × 100, where Af is the absorbance after 6min, and Ac is the absorbance of the blank at time zero. (Brand-Williams et al., 1995).

1.2.7 Total Polyphenol Content

Total polyphenol content (TPC) was determined through Folin-Ciocalteau's method, using gallic acid as standard (Sigma-Aldrich St. Louis, MO, USA). In brief, the 0.1 mL of samples (properly diluted with water in order to obtain an absorbance value within the linear range of the spectrophotometer) underwent an addition of: 0.5 mL of Folin-Ciocalteau's (Sigma-Aldrich St. Louis, MO, USA) reagent and 0.2 mL of an aqueous solution of Na_2CO_3 (20%; w/v %), bringing the final volume to 10 mL with water. After mixing, the samples were kept in the dark for 90 min. After the reaction period, the absorbance was measured at 760 nm. Each sample was analyzed in triplicate and the concentration of total polyphenols was calculated in terms of gallic acid equivalents (GAE) (Di Lorenzo et al., 2015).

1.2.8 Study population and protocol

The study was a multicentered, placebo-controlled trial, conducted at the Samnium Medical Cooperative and the UCCP center (Benevento, Italy). Participants in the study were recruited by the Cooperative Medica Samnium and the UCCP Center (Primary Care Complex Unit) (Benevento, Italy). Patients were recruited in April 2017. Patients between the ages of 18 and 70 had baseline values of cholesterol values outside the optimal values: TC 200-260mg / dL; HDL-C 30-45mg / dL; and LDL-C 189-206mg / dL. Subjects were asked to keep their eating habits unchanged throughout the study and to compile a Food Frequency Questionnaire (FFQ) to provide information on their eating habits. Exclusion criteria were as follows: pregnant women, women suspected of being pregnant, women who tried to undergo a pregnancy, women in the breast-feeding period. Subjects received oral and written information about the study before giving their written consent. All patients underwent a standardized Physical Examination based on body weight measurement and waist circumference and BMI evaluation, an assessment of medical history (up to 5 years before enrollment), and blood analysis. Volunteer's protocol and letter, and synoptic documents on the study were presented to the scientific committee of scientific ethics at AO Rummo Hospital (Benevento, Italy). The study was approved by the committee and carried out in accordance with the Helsinki Declaration of 1964 (as revised in 2000).

The duration of the study was 16 weeks: all subjects were undergoing 4 weeks of placebo treatment, followed by 8 weeks of treatment and 4 weeks of follow-up. Clinical visits and blood collection samples were performed after 12 hours of fasting at weeks 0, 4, 12, 16.

All blood samples were taken in the morning. Subjects were advised not to drink alcohol or to perform intense physical activity within 48 h before blood sampling. The blood samples was collected in 10 mL EDTA 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 levels of TMAO are analyzed by LC-MS system.

1.2.9 Randomization, concealment, and blinding

A total of 56 eligible patients were randomly assigned to three groups to receive simple apple puree (control), fermented apple puree and a *Lactobacilli* capsules, these three different treatments were coded with different colors and given in random order. The code was not broken until all analyses were completed and the results were analyzed statistically. If a patient dropped out before receiving functional food, he or she was replaced by the next eligible patient enrolled at the same center. The concealed allocation was performed by an Internet-based randomization 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.

1.2.10 Study treatments

The group of 53 patients (30–83 years of age) was randomly divided into three subgroups (14,16 and 23 subjects). The three subgroups were instructed to drink 120 mL of fermented apple puree, 120 mL of simple apple puree or a not-gastroprotect capsules of *Lactobacillus* mix per day. Noteworthy, the daily dose of apple puree, adopted for the clinical trial, was in full accordance with the maximum polyphenolic 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.

A group of 14 subjects, who have been given the indication of taking 125mL of lacto-fermented pure. Puree was supplied weekly in 1000g packs and stored at 4°C during the

week treatment, these samples were prepared by addition of *Lactobacilli* in concentration of 2.5×10^6CFU/mL, 24h before theirs delivery.

A group of 23 subjects took a not-gastroprotected capsules of *Lactobacilli* (*Lb. rhamnosus* and *Lb. plantarum* 50:50) formulated to contain the same amount of bacteria used for the preparation of daily apple puree dose. The capsules were given to subjects weekly (7 capsules), and during the treatment were stored at 4°C.

The third group of 16 subjects took 125mL simple apple daily. Puree was supplied weekly in 1000g packs and stored at 4°C during the week treatment.

At the end of the 8-week treatment, a month of follow-up was performed, during subjects stopped taking the capsules and puree.
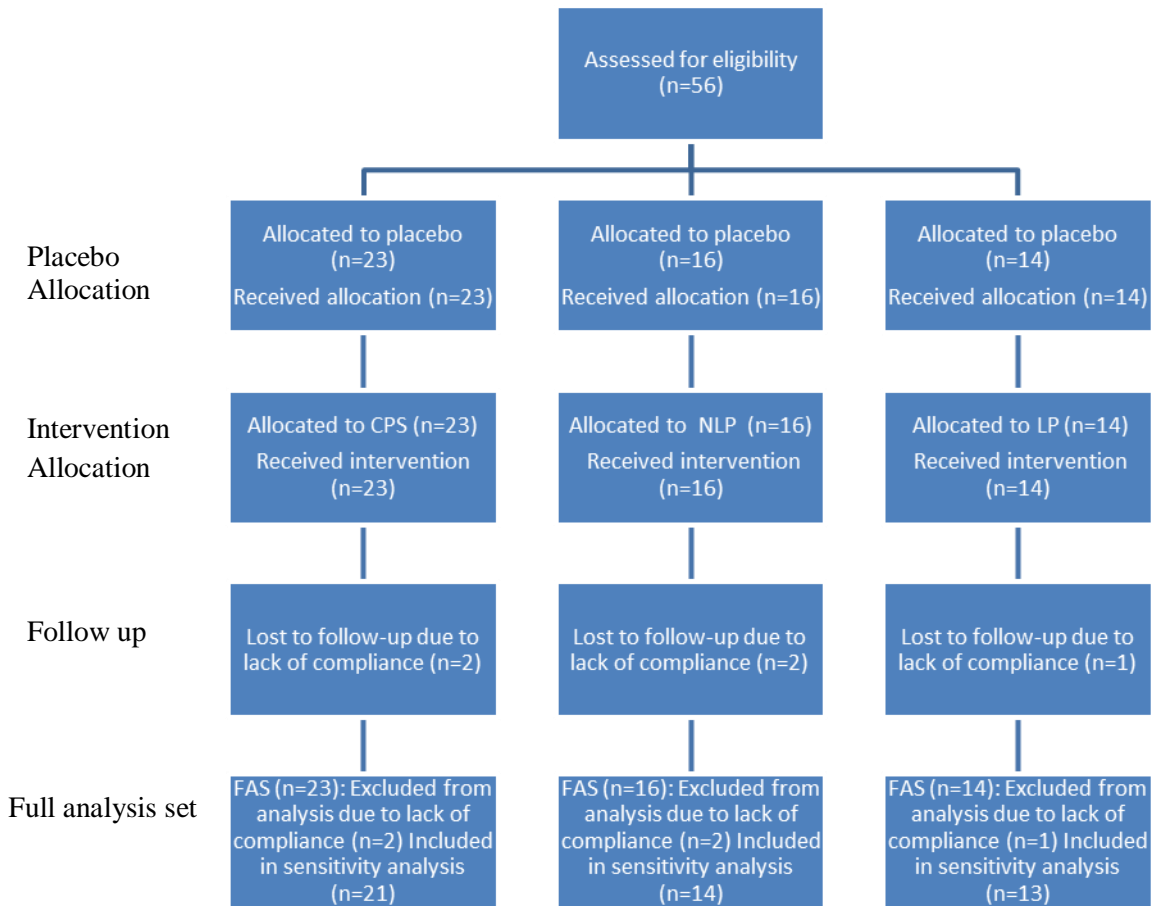
Fig. 15: Study flowchart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from pre-screening to data collection, and the extent of exclusions, loss to follow-up and completeness of diary documentation available across the entire trial period. CPL, capsule of *Lactobacillus*; NLP, Not Lactofermented Puree; LP, Lactofermented Puree. FAS, full analysis set.

1.2.11 Study outcomes and data collection

The unique endpoint measured was the TMAO blood levels.

1.2.12 Safety

The product is made from apple puree, each weekly dose contains approximately 1,2g of polyphenolic extract (daily dose of 170 mg). For clinical trials, puree's polyphenolic content is in full compliance with the maximum daily intake of polyphenolic extract (1000 mg), via dietary supplements and novel foods, as indicated by the updated version (January 2015) of Regulation (EC) No. 258/1997 of the Commission, as a safe and healthy state of polyphenolic quantity.

1.2.13 TMAO LC-MS Analysis

To 80 μL of plasma were added 160 μL of methanol. The mixture are vortex for 2 min, and after centrifuged at 12.000 rpm. The supernatant was collected, and used for the LC-MS analysis.

The quantitative analysis was performed by LC-MS. The HPLC system Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) has a DAD detector coupled with Advion Expression mass spectrometer (Advion Inc., Ithaca, NY). For the identification (ESI) source has to operate in positive ion mode. For separation of the analytes a Luna Hilic (5μ particle size, 150x3mm) and security guard colon both supplied by Phenomenex (Torrance, CA, USA,) were used. The column temperature was maintained at 60 °C during analysis. Mobile phase composition comprised (A) 0.15% formic acid in water containing a final concentration of 10 mM ammonium acetate and (B) 100% methanol (LC/MS grade) in the ratio 80:20 (A:B), run isocratically at a flow rate of 0.35 mL min1 for 6 min, with a 5 μl injection volume. After use, the column was stored in 100% acetonitrile and was routinely cleaned according to the manufacturer's instructions. TMAO is identified and quantified by analytical standard (Sigma-Aldrich St. Louis, MO, USA) calibration curve (Beal et al., 2016)

1.2.14 Statistical analysis

All of the experimental data were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was carried out 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

1.3 Resultes

1.3.1 Ph reductions

Two strains of acid lactic bacteria were used in my study : *L.rhamnosus* and *L.plantarum*. No general agreement has been reached regarding the concentration of probiotics necessary to achieve beneficial effects; usually counts from 10^6 to 10^8 CFUmL−1 are recommended (Shah 2001). In agreement with this guidelines were projected two possible formulations with different probiotic concentrations. The first with a starting probiotic concentration of 2.5 CFU $x10^6$/mL and a second of 5 CFU $x10^6$/mL. The pH values were measured at T0 (time of puree preparation) and after 24 and 48 h of incubation for both the strains tested. A linear decrease of the pH was observed in both the products, with a reduction proportional to the time of incubation and the number of acid lactic bacteria added to the apple puree. This event let me hypothesize about a forceful fermentative action of the two strains, capability was more evident for *L. rhamnosus*, which caused a higher reduction of pH compared to the effect of *L.plantarum* calculated as % pH decrease against the control (the same volume of puree incubated in the same condition of the inoculated products). The data were shown in the following figure 16, where were reported the values of the pH of 120 mL of apple puree, measured after 24 and 48 h of the incubation, in relation to the number and the strain of *Lacatobacillus* used. For the *L.rhamnosus*, were obtained a decreases of 10.24% ($2.5x10^6$CFU/mL) and 15.18 % ($5x10^6$ CFU/mL) after 24 h of incubation at 37°C and respectively 11.00% and 17.45% after 48h of incubation. For the *L.plantarum* the data obtained were the following: reductions of 5.40% and 9.64% after 24 h pass to 9.01% and 12.94% after 48 h, respectively for the incubated products $2.5x10^6$CFU/mL and $5.0x10^6$CFU/mL. My results are in agreement with other study which suggested a pH decrease of prebiotic matrix after probiotic fermentation, caused by the formation of short chain fatty acid (Nazzaro et al., 2009 and 2008) and that vegetable and fruit juices could be a good media for the probiotic growing (Babu et al., 1992, Yoon et al., 2006 and 2015).
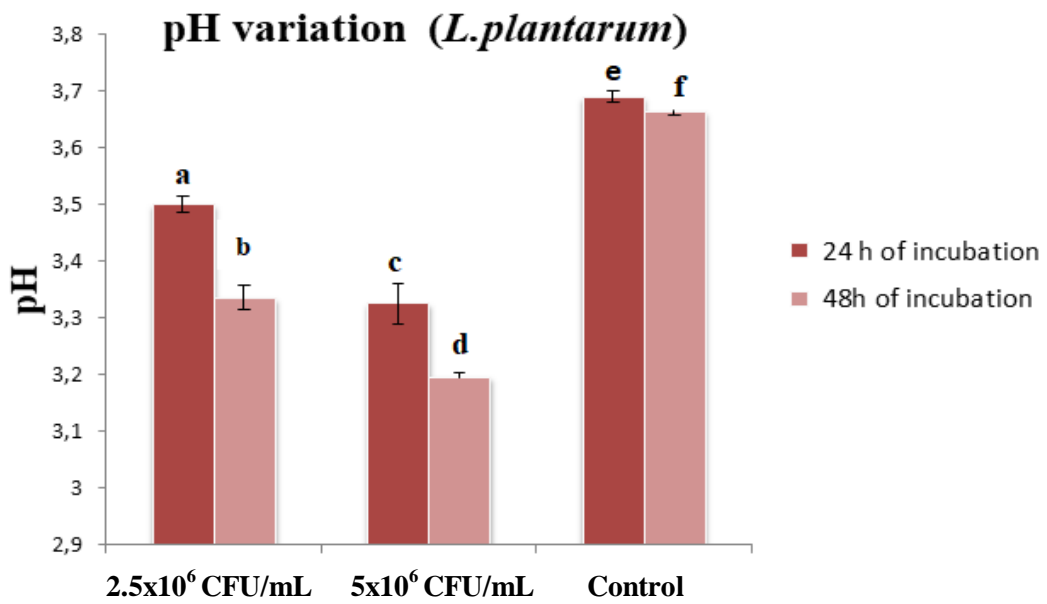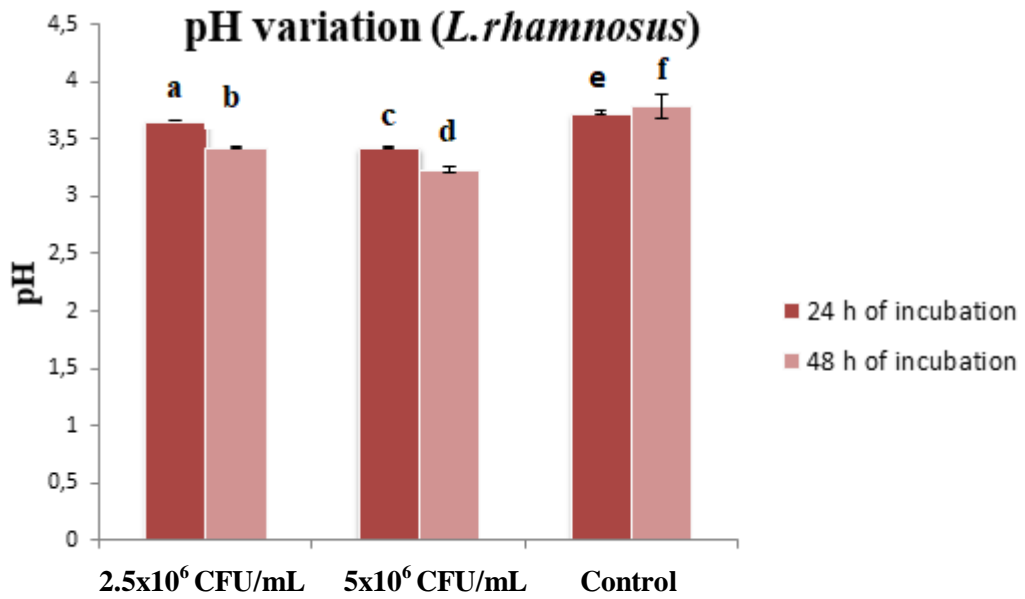
Fig. 16: pH values of two different probiotic starting concentration of Annurca apple puree fermented products (2.5 UFC x10^6 /mL and 5 UFC x10^6 /mL in 120 mL of apple puree) and the control. Data are the means ± SD (n = 3). Results were significantly different at a level of P = 0.001. [abcd]Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test

1.3.2 Antioxidant activity and total phenol content of fermented products

The capacity of two tested strains to increase the bioaccessibility of phenolic compounds were evaluated by two different types of test. The antioxidant activity of annurca apple puree was measured with respect to the radical scavenging ability of the antioxidants present in the sample using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the total polyphenol content (TPC) was determined through Folin-Ciocalteau's method. These tests were performed on the apple puree supernatant, within could be a possible enrichment of free phenolic compounds, released from the matrix after the probiotic fermentation. The data regard the antioxidant activity and the total polyphenolic content were shown in the following table.

Starting Concentration (CFU/mL)	Incubation time (h)	% increase of antioxidant activity *	% increase of total polyphenols content**
L.rhamnosus 2.5x10^6 CFU/mL	24	11,89 ± 3,96***	40,12± 5.6
L.rhamnosus 2.5x10^6 CFU/mL	48	13,30 ± 3,26	18,67 ± 3,96
L.rhamnosus 5x10^{6}CFU/mL	24	nd	1,34 ± 5,7
L.rhamnosus 5x10^{6}CFU/mL	48	nd	2,93 ± 7,9
L.plantarum 2.5x10^{6}CFU/mL	24	nd	7,59 ± 6,94
L.plantarum 2.5x10^{6}CFU/mL	48	nd	8,88 ± 8,16
L.plantarum 5x10^{6}CFU/mL	24	nd	6,64 ± 8,40
L.plantarum 5x10^{6}CFU/mL	48	nd	3,79 ± 2,70

Table 2:*% increase of antioxidant activity was calculated against the control and expressed in μM TE (trolox equivalent).**% increase of total polyphenols was calculated against the control and expressed in mgEGA (Gallic acid mg equivalent/120 mL of puree).*** Values are means ± SD (n = 3).

According to these results, the best solution was obtained by the incubation, of *L.rhamnosus* than *L.plantarum*, with an important increase of the free phenolic compounds (40.12%) against the 7.59% of the *L.plantarum* fermented products. The same seems for the antioxidant activity, where the higher increase was registered in *L.rhamnosus* fermented products (11.89% after 24h and 13.30 after 48 h). It is very important to

evaluate these results are not only linked to the strains tested, but also to the starting concentration using for the fermentation (2.5 and 5×10^6 CFU/mL). These concentrations were chosen according to the recommendation for probiotic foods: minimal counts of 7. Log CFU/mL for better efficacy in regulating beneficial effects (Vinderola et al., 2000). The probiotic concentration tends to increase during the incubation time, to reach a concentration of 8/8.5 Log CFU/mL (Perera et al., 2011). For the products with an initial concentration higher (5×10^6 CFU/mL), were shown a not important increase of free phenolic compounds and a decrease of the antioxidant activity. These events were linked to the degradation of polyphenols by too high concentration of acid lactic bacteria. (Lee et al., 2006 and Tzounis et al., 2011). According to the data reported in the table n°2, there were several formulations characterized by an increase of total phenols content against an absence of antioxidant activity increase .This event could be explained by the antioxidant activity test used: The DPPH test measure. This assay is based on the chromophoric changes measurable at 515 nm that accompany reaction of the DPPH radical with a hydrogen or an electron donor. But not all the different classes of polyphenols have the same hydrogen donator attitude. In same studies a good correlation was found between the percentage of reduced DPPH and the concentration of total flavan-3-ols ($R = 0.77$). On the contrary, hydrogen donor ability was not apparently related to the content of dihydrochalcones ($R = 0.46$) or hydroxycinnamates ($R = 0.17$) (Panzella et al., 2013). Others authors, therefore, indicate a greater DPPH scavenging activity of procyanidin B2 and epicatechin respect to chlorogenic acid. (Carbone et al., 2011). I could hypothesize according to the last affirmation, that cinnamic acid derivatives or dihydrochalcones were released from the matrix and not degraded by the lactic fermentation. Thus it was possible to observe an increase of total phenolic content, against a not increase of antioxidant activity.

1.3.3 Blood TMAO concentration

The TMAO hematic concentration after treatment (60 days) of involved patients are present in the following table :

Table.3 Effect of functional product on blood levels of TMAO						
	LP	**Δ%**	**NLP**	**Δ%**	**Capsule**	**Δ%**
	t0	2.1± 0.2		0.02± 0.006		1.7 ± 0.7	
TMAO (microM)	t60	0.8 ± 0.008	-63.12	0.01 ± 0.003	-42.3	1.2 ± 0.6	-25.8
Subjects consumed 120mL of puree or 1 capsule of Lactobacillus for 2 months per day						
Value are means ± SD (n = 5).						
Results are significantly different at a level of P = 0.001.						
LP, Lactofermented Puree; NLP, Not Lactofermented Puree; CPL, Capsules.						

In Patients treated with simple puree was registered a decrease in TMAO levels of 42.3%, and this effect is amplified by the addition of *Lactobacillus* in lacto-fermented puree, 63.12% of decrease was registered in subjects treated with this product, while *Lactobacillus* alone contributed to a less pronounced lowering of 25.8%. These results could suggest a possible relation between the increased polyphenols bioaccessibility and TMAO metabolism.

1.4 Discussion

The idea which inspired this project is aimed to realize a probiotic juice, where acid lactic bacteria, were inoculated and growth in Annurca apple puree. This apple cultivar, as reported above, is largely studied for its important beneficial effects linked to its particular polyphenolic composition. These product could be considered like a "reinforce" apple puree, thanks to the more abundant free phenolic compounds. Therefore this potential functional food was a no diary source of acid lactic bacteria, the strains used are *L. rhamnosus and L.plantarum*. These bacteria were added to apple puree in two different start concentrations $2.5x10^6$CFU/mL and $5x10^6$CFU/mL. The *in vitro* studies, showed that, there was a decrease of pH values, excepted according to saccharolytic activity of *Lactobacillus*. These was related to their ability to degrade the polysaccharides fiber and release short chain fatty acid, like : butirryc, lactic, acetic acids (to these compounds are ascribed a several bioactivites) (Nazzaro et al. 2009), responsible of the pH decrease.

These events were considered, also, as a confirm of the growth of *Lactobacillus* in this food matrix. The best incubation conditions, according to the data obtained, regards to the % increase of total phenol content and antioxidant activity was 2.5×10^6 CFU/mL of *L.rhamnosus* after only 24 h of incubation. In respect of *in vitro* results, these conditions were used to prepare the products for *in vivo* tests . The clinical trial performed is, only a preliminary screening, aimed only to understood the real influence or interference of this product on a new parameter, the level of TMAO, causes of atherosclerosis via macrophages activation (Wang et al., 2011). Although the patients enrolled didn't have TMAO levels considered dangerous (<2.5 mM), was registered an important decrease of blood levels of this molecule. The most significative decrease was achieved, after treatment with lactofermented apple puree (-63.12%). This result reflects the data already present in the literature, a diet rich in polyphenols favors a change in the intestinal bacterial composition, in favor of saccharolytic bacteria. Administration of extraction juices from apples increased fecal counts of *Lactobacillus* and *Bifidobacterium* (Sembries al., 2006), against other gut microbiota strains such as *Clostidium* strain that has a strong implication in the TMA formation. In fact, Romano et al., 2015 said that, the bacteria able to process the choline in TMA were bacteria strains such as : *Anaerococcus hydrogenalis, Clostridium asparagiforme, Clostridium hathewayi, Clostridium sporogenes, Escherichia fergusonii, Proteus penneri, Providencia rettgeri, and Edwardsiella tarda*. In patients treated with fermented puree was achieved a decrease 1.5 times greater than the results obtained in patients treated with simple puree. This data was related to % increase of free phenolic compounds released from the pectin, that conducted a bacteriostatic or bactericide activity was responsible of lower grade growth of *Clostidium* at intestinal level (lowering TMA formation) (Semla et al., 2009). Therefore these data are associable also, to a potential increase of bioavailability confirmed from Pereira-Caro et al.(2015), they reported that, chronic administration of probiotic enhances the bioavailability of orange juice flavanones in humans of 70%, related to lower growth of sulfate-reducing bacteria (SRB) are those bacteria that can obtain energy by oxidizing organic compounds or molecular hydrogen (H_2) while reducing sulfate (SO_2^{-4}) to hydrogen sulfide (H_2S), it means a minor polyphenolic (oxidizing organic compounds) degradation and follow to major phenolic bioavailability. It means potential higher polyphenolic blood concentration in patients treated with fermented puree, that could exerct their antioxidant activity and cause a potential conversion or reduction of TMAO levels.

1.5 Conclusions

The bioaccessibility, and consequential bioavailability of polyphenols are increased respectively, thanks to the release of polyphenols from the fiber (bioaccessibility) due to fermentation proved by *in vitro* increase of TC and antioxidant activity, and to the inhibition of SRB growth induced by chronic treatment with probiotic (bioavailability). The TMAO levels, is influenced by changing and modelling microbiota composition related to major intake of polyphenols and probiotic, both present in the same product, and from potential major blood polyphenols rate.

Chapter 2

Nutraceutical formulations

2.1 Annurca Apple Nutraceutical Formulation Enhances Keratin Expression in a Human Model of Skin and Promotes Hair Growth and Tropism in a Randomized Clinical Trial

2.1.1 Introduction

Several pharmaceutical products have been formulated over the past decades for the treatment of male and female alopecia and pattern baldness (Kelly et al., 2016). These most common forms of hair loss in men and women, affecting approximately 50% of the adult population, are non-stop processes which flow toward definite pattern of alopecia in genetically predisposed individuals. Although clinically different, the pathogenic pathways leading to this type of hair loss are thought to be similar in both sexes (Hoffman., 2002). Minoxidil, which was originally synthesized as a potassium channel opener (Buhl et al., 1993) and initially prescribed for hypertension, is an FDA approved topical medication for curing male pattern baldness (Kulik., 1988 and Olsen., 1989). Recently, finasteride, which is a type II 5α-reductase inhibitor and was initially used for has been approved by the FDA as an oral drug for male hair loss therapy (Kaufman et al., 1998). Nevertheless, side effects have been reported for both agents ,thereby, considerably limiting their clinical use (Wilson et al., 1991; Boek et al., 1996; Nikel et al. 1996;Wilton et al., 1996). In this perspective, the pharmaceutical and medical attention has recently focused on the discovery of new and safer remedies. Particularly, in search of biological products which possess hair growing activity, great interest has been attracted by oligomeric procyanidins, extracted from grape seeds, able to promote hair epithelial cell growth as well as to induce anagen phase, at almost the same intensity of minoxidil (Takaheshi et al., 1999). Specifically, procyanidin B2 has demonstrated to be one of the most effective and safest natural compound in promoting hair growth, both *in vitro* (Kamimura et al., 2002) and in humans by topical applications (Kamimura et al., 2000 and Takahashi et al. 2001). During hair growth and keratinisation process along the hair fiber, several keratins arrange into filaments to contribute to the production of the hair shaft (Makar., 2007). As ascribed

above, apple fruits of cv Annurca (AFA), native of Southern Italy, has the highest contents of oligomeric procyanidins, and, specifically, of procyanidin B2, with respect to more common apple samples, such as Red Delicious, Granny Smith, Pink Lady, Fuji, and Golden Delicious (Tenore et al., 2013). Encouraged by these results, the second aim of the present work was to explore the bioactivity of AFA polyphenolic extract, highlighting the effects of its supplementation on cellular keratin expression by targeted bioscreen *in vitro*. For this reason, we focused on a well-characterised human experimental model of adult skin based on normal human keratinocyte (HaCaT cells)(Miniaci et al. 2016). This cell line retains a capacity for differentiation similar to normal human epidermal keratinocytes, and hence offers a suitable model for the study of keratinisations as well as of epidermal homeostasis (Breitkreutz et al. 1998). Then, the second goal was to evaluate the effects of AFA extract supplementation as a nutraceutical, on the hair growth and tropism in healthy subjects. To this regard, according to our previous knowledge about the effects of salivary and gastric digestion on the bioaccessibility of active components, was used a specific formulation consisting of gastro-protected capsules. (Tenore et al. 2013b)

The present part of my thesis work shows, for the first time, the impact of procyanidin B2, contained in apple natural extract, on keratins biosynthesis *in vitro*, and represents the first attempt of its oral administration to humans as potential hair growing nutraceutical agent.

2.1.2 Materials and method

2.1.2.1 Apple collection

Annurca (*M. pumila* Miller cv Annurca) apple fruits (each, 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 for about 30 days (Lo Scalzo et al., 2001) and then analyzed.

2.1.2.2 Preparation of AFA supplements (AMS and AMSbzs)

AFA (whole fruits, peel and pulp) have been repeatedly extracted with water, the obtained solution has been filtered, centrifuged, and concentrated. Then, the solution has been spry-dried in combination with maltodextrins, obtaining a fine powder named AppleMetS (AMS). As regards the clinical trial, the AFA supplements used in this study consisted in two different types of gastric-resistant capsules, containing: 1) AFA polyphenolic extract microencapsulated with maltodextrins (400 mg) (AMS); 2) AFA polyphenolic extract

microencapsulated with maltodextrins (400 mg), biotin (0.20 mg), selenomethionine (80.0 μg), and zinc acetate (21.0 mg), named AppleMetS Hair (AMSbzs). The choice of using gastric-resistant capsules was due to our previous knowledge about the effects of salivary and gastric digestion on the bioaccessibility of bioactive components (Tenore et al., 2013). Both supplements were formulated by the Department of Pharmacy, University of Naples "Federico II" (Naples, Italy). Large-scale production of both supplements was accomplished by MB-Med Company (Turin, Italy).

2.1.2.3 Cell cultures and treatments

HaCaT cells, an immortalized, non-tumourigenic human keratinocyte cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) containing high glucose (4.5 g/L), supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/mL, Sigma) and streptomycin (100 μg/mL, Sigma Milan, Italy) at 37 °C in a humidified 5% CO_2 atmosphere.

For *in vitro* experiments, subconfluent cultures of HaCaT cells were incubated in presence or not of FBS with a range of AMS concentrations from 0.009 to 2.3 mg/ml for established times.

2.1.2.4 In vitro bioscreens

Before evaluating the biological effects of AFA polyphenolic extract (AMS) on the regulation of keratin expression, preliminary experiments were allowed to finalize the experimental model and to define specific protocols for suitable *in vitro* treatments. Biochemical and toxicological investigations were thereby performed in order to explore AMS bioactivity in human keratinocytes (HaCaT cell line) *in vitro*, focusing on the evaluation of cell viability as well as on cell growth and proliferation following the exposure up to 48 h to various AMS concentrations. As follows, optimal experimental conditions of cell growth and protein biosynthesis, relative to both extract concentration and incubation times, were set up by means of targeted bioscreens. In detail, the experimental procedure involved the estimation of a "cell survival index", arising from the combination of cell viability evaluation with cell counting (Vitiello et al., 2015). HaCaT cells were inoculated in 96-microwell culture plates at a density of 10^4 cells/well, and allowed to grow for 24 h. The medium was then replaced with fresh medium and cells were treated for further 48 h with a range of concentrations (0.009, 0.09, 0.23, 0.46, 0.92,

1.68 and 2.3 mg/ml) of extract. Using the same experimental procedure, cell cultures were also incubated with cisplatin (cDDP), as a positive control for cytotoxic effects. Cell viability was evaluated using the MTT assay procedure, which measures the level of mitochondrial dehydrogenase activity using the yellow 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as substrate. The assay is based on the redox ability of living mitochondria to convert dissolved MTT into insoluble purple formazan. Briefly, after the treatments, the medium was removed and the cells were incubated with 20 μl/well of a MTT solution (5 mg/mL) for 1 h in a humidified 5% CO_2 incubator at 37 °C. The incubation was stopped by removing the MTT solution and by adding 100 μl/well of DMSO to solubilize the obtained formazan. Finally, the absorbance was monitored at 550 nm using a microplate reader (iMark microplate reader, Bio-Rad, Milan, Italy). Cell number was determined by TC10 automated cell counter (Bio-Rad, Milan, Italy), providing an accurate and reproducible total count of cells and a live/dead ratio in one step by a specific dye (trypan blue) exclusion assay. Bio-Rad's TC10 automated cell counter uses disposable slides, TC10 trypan blue dye (0.4% trypan blue dye w/v in 0.81% sodium chloride and 0.06% potassium phosphate dibasic solution) and a CCD camera to count cells based on the analyses of captured images. Once the loaded slide is inserted into the slide port, the TC10 automatically focuses on the cells, detects the presence of trypan blue dye and provides the count. When cells are damaged or dead, trypan blue can enter the cell allowing living cells to be counted. Operationally, after treatments in 96-microwell culture plates, the medium was removed and the cells were collected. Ten microliters of cell suspension, mixed with 0.4% trypan blue solution at 1:1 ratio, were loaded into the chambers of disposable slides. The results are displayed as total cell count (number of cells per ml). If trypan blue is detected, the instrument also accounts for the dilution and shows live cell count and percent viability. Total counts and live/dead ratio from random samples for each cell line were subjected to comparisons with manual hemocytometers in control experiments. The calculation of the concentration required to inhibit the net increase in the cell number and viability by 50% (IC_{50}) is based on plots of data ($n = 6$ for each experiment) and repeated five times (total $n = 30$). IC_{50} values were obtained by means of a dose response curve by nonlinear regression using a curve fitting program, GraphPad Prism 5.0, and are expressed as mean values ± SEM ($n = 30$) of five independent experiments.

The evaluation of the relative number of live and dead cells was performed by a MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, WI). Briefly, cells were plated

into 96-well plates (5×10^3/well) and, after the treatments with AMS as before described, the solution containing the fluorogenic substrates for the measurements of live-cell and dead-cell protease activities was added to the cells. Following 30 min at 37 °C, the fluorescence was determined in a Perkin Elmer microplate reader (Perkin Elmer LS 55 Luminescence Spectrometer, Beaconsfield, UK) using a filter combination with an excitation wavelength of 400/485 nm and an emission wavelength of 505/520 nm (slits 5 nm). Data are expressed as percentage of viable cell versus control cultures.

2.1.2.5 Cell morphology analysis

Human keratinocyte cell line was grown on 60 mm culture dishes by plating 5×10^5 cells. After reaching the subconfluence, cells were incubated for 24 and 48 h with a range of concentrations of AMS (0.09, 0.23, 0.46, 0.92 and 2.3 mg/ml) under the same *in vitro* experimental conditions described above, and were then morphologically examined by a phase-contrast microscope (Labovert microscope, Leitz). Microphotographs at a 200 × total magnification (20 × objective and 10 × eyepiece) were taken with a standard VCR camera (Nikon).

2.1.2.6 Preparation of cellular extracts

HaCaT cells were grown for 48 h in the presence or absence of AMS at different concentrations (0.23 and 0.46 mg/ml), as established after preliminary bioscreens. Following treatments, protein extracts were prepared according to previously described procedure (Achtstaetter et al.,1986). Briefly, cell pellets were lysed at 4 °C for 10 min with buffer containing 1% Triton X-100, 5 mM EDTA, in PBS (pH 7.4) containing protease inhibitors. After centrifugation at 14,000 × g for 10 min at 4 °C, the supernatant was collected as the soluble fraction and stored at -80 °C. The pellet was homogenized at 4 °C for 30 min in 1 ml of 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% Triton X-100, and protease inhibitors. The supernatant fraction was obtained by centrifugation at 15.000 × g for 10 min at 4 °C and then stored at − 80 °C. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Milan, Italy) (Miniaci et al., 2016).

2.1.2.7 Western blot analysis

Defined amounts (70 μg) of proteins from soluble cellular extracts were loaded and separated on 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (GE

Healthcare, UK) using a Bio-Rad Transblot (Bio-Rad). Proteins were visualized by reversible staining with Ponceau-S solution and destained in PBS. Membranes were blocked at room temperature in milk buffer [1×PBS, 5–10% (w/v) non-fat dry milk, 0.2% (v/v) Tween-20] and then incubated at 4 °C overnight with 1:250 monoclonal anti-pan cytokeratin antibodies (mixture) (Sigma-Aldrich, Milan, Italy) which recognize the following human cytokeratins, according to their molecular weight: K1 (68 kDa), K4 (59 kDa), K5 (58 kDa), K6/K10 (56 kDa), K13 (54 kDa), K8 (52 kDa), K18 (45 kDa) and K19 (40 kDa). As secondary antibody a goat anti-mouse IgG + IgM (1:5000, Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA) was used. The immune complexes were visualized by the ECL chemoluminescence method (ECL, Amersham Biosciences, UK) and analysed by an imaging system (ImageQuant^{TM} 400, GE Healthcare Life Sciences). Densitometric analyses were conducted using the GS-800 imaging densitometer (Bio-Rad). GAPDH antibody (Sigma-Aldrich, Milan, Italy) was used to normalize the results.

2.1.2.8 Study population and protocol

Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in November 2015. Patients aged 30-83 years were eligible for enrolment if they showed evidence of pattern baldness. The subjects were asked to keep their dietary habits unchanged throughout the entire study. Exclusion criteria were: smoking, obesity (BMI >30 kg/m^2), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, drug therapy or supplement intake for hair growth, drug therapy or supplement intake containing apple polyphenols, heavy physical exercise (>10 h/week), pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding, 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, 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, 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 hair sampling were performed at weeks 0, 4, 8, 12, and 16. Hair counts were carried out per area (cm^2) of a pre-determined site of bald head skin, by three independent investigators three times each in double-blind mode. A number of 100 hairs (1 cm long) were sampled in the neck. Hair were weighed by using a ABJ 320-4NM analytical balance (Zetalab Ltd, Padova, Italy). The hair keratin content was evaluated by first defatting hair samples with *n*-hexane and petroleum ether (4:1) for 4 h in a Soxhlet apparatus, and then, estimating the protein content according to the Kjieldahl method 920.87, by using a PBI International model Mineral SIX digester (PBI International, Milan, Italy) and a Buchi model B-324 distillation unit (Buchi, Flawil, Switzerland)(Prosky et al. 1988). Clinic visits, and blood sampling to test hepatic and renal toxicity, 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, 2.200 g, 4 °C). All samples were stored at -80 °C until analysis. Plasma AST, ALT, γ-GTP, ALP, LDH, albumin, total bilirubin, and creatinine levels, were determined on a Diacron International Free Carpe Diem, using commercially available kits from Diacron International (Grosseto, Italy). In addition to these five meetings, five standardised telephone interviews were performed starting from the first meeting, to verify compliance and increase protocol adherence. In particular, 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 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.

2.1.2.9 Randomisation, concealment, and blinding

A total of 250 eligible patients were randomly assigned to two groups in order to receive AMS or AMSbzs supplements. Supplements 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 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.

2.1.2.10 Study treatments

The group of 250 patients (116 men and 134 women, 30-83 years of age) was randomly divided into two subgroups (each one of 125 subjects, 58 men and 67 women). The two subgroups were instructed to take two capsules of AMS or AMSbzs per day (one capsule in the morning, and one capsule in the evening). Noteworthy, the daily dose of polyphenolic extract, 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. Moreover, the quantities of biotin, selenium, and zinc, have been calculated on the basis of what established by the Italian Ministry of Health (DM Revision June 2016) as regards the maximum levels of vitamins and minerals allowed in food supplements, in accordance with what reported by the current European regulation about the minimum daily requirement (Nutrient Reference Values, NRVs) of vitamins and minerals (Reg. UE 1169/2011).

2.1.2.11 Study outcomes and data collection

Primary and secondary efficacy outcomes. The primary endpoint measured was the increase in the hair number/cm^2 area of bald head skin (> 5% after 30 days, > 10% after 60 days), while key secondary outcomes were the increase in the weight and keratin content of hair samples collected (in both cases: > 5% after 30 days, > 10% after 60 days). 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 (unpublished standard operating procedure) before unblinding in order to define conclusive primary and secondary efficacy data from a clinical perspective.

2.1.2.12 Safety

Although no specific toxicity studies have been performed herein, mutagenicity tests, acute/subacute toxicity studies have long since demonstrated the safety of polyphenol content of apples both in mice and human beings. Specifically, the Commission Regulation (EC) No. 258/1997 established 1000 mg as maximum polyphenolic extract daily intake in humans. Accordingly, the polyphenolic extract dose adopted for the trial was an amount reasonably lower than that regarded as safe in humans. Moreover, the daily administration of biotin, selenium, and zinc, respected what disposed by the Italian Ministry of Health (DM Revision June 2016) as regards the maximum levels of vitamins and minerals allowed in food supplements, in accordance with what established by the current European regulation about the minimum daily requirement (Nutrient Reference Values, NRVs) of vitamins and minerals (Reg. UE 1169/2011). 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 body mass index), 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. During the test, all subjects underwent a clinical diagnosis by a dermatologist, focusing on any adverse dermatological reactions such as inflammation, erythema or eczema.

2.1.2.13 Statistics

Methodology. All the *in vitro* experiments have been repeated four times. Data were presented as mean ± SEM and the statistical analysis was performed using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA), and ANOVA test for multiple comparisons followed by Bonferroni's test. 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 G 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. All of the trial experimental results were expressed as mean ± standard deviation (SD) of four replications for each patient. 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 polyphenols dose, and Jadad score. Treatment effects were analysed using PROC MIXED with treatment (placebo, apple supplements) 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 parameters and blood pressure values. Data that could not meet the criteria of variance

homogeneity (Levenes test) and normal distribution (determined by residual plot examination and Shapiro–Wilks test) even after log transformation were analysed by a nonparametric test (Friedman). The level of significance (α-value) was 95% in all cases ($P < 0.05$).

2.1.2.14 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 (for example, poor compliance, errors in treatment assignment). Secondly, they had to have been on treatment for at least 50 days counting from day of first intake (completion of a certain prespecified 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.

2.1.2.15 Patient involvement

No patients were involved in setting the research question or the outcome measures, nor were they 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.

2.1.3 Results

2.1.3.1 In vitro bioscreen

The results are presented in Figure 17 by concentration-effect curves, and clearly prove that AMS does not show any significant interference with the cell survival index, even at high in vitro concentrations (>0.5 mg/ml). As well, we even report a slight increase of cell viability in the presence of low micromolar concentrations of the extract. In line with this outcome, inversely correlated fluorescent measurements for the estimation of the live/dead cells ratio showed that AMS, for all the screened concentrations and times, does not

interfere with the HaCaT keratinocytes viability (see Fig.17-B). In addition and to support, we monitored the cellular morphology throughout the in vitro trials. End-points imaging by light microscopy were taken from cells to assess potential cellular monolayers modifications induced by incubation with different concentrations of the polyphenolic extract. The occurrence of changes in cell morphology as well as in monolayers appearance are generally suggestive of distinctive hallmarks of cell death pathways activation. Accordingly with the cell survival index analysis, AMS proves to be very biocompatible, also at high final concentrations and after 48 h of incubation, so that no significant modifications of keratinocyte subconfluent monolayers were evidenced (Fig. 18). In conclusion, in vitro bioscreens suggest the theoretical safety of AMS administration to human keratinocytes under a wide range of experimental conditions, thus allowing to design further in vitro experiments on viable cells.
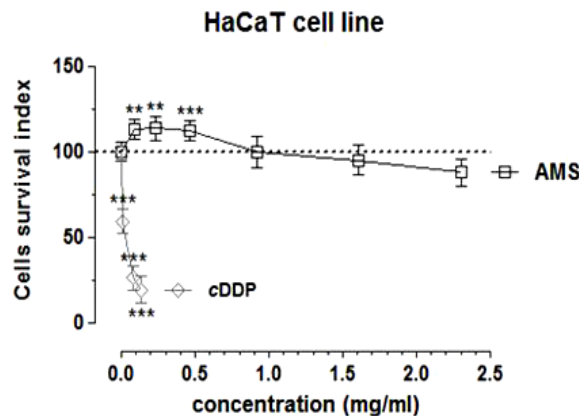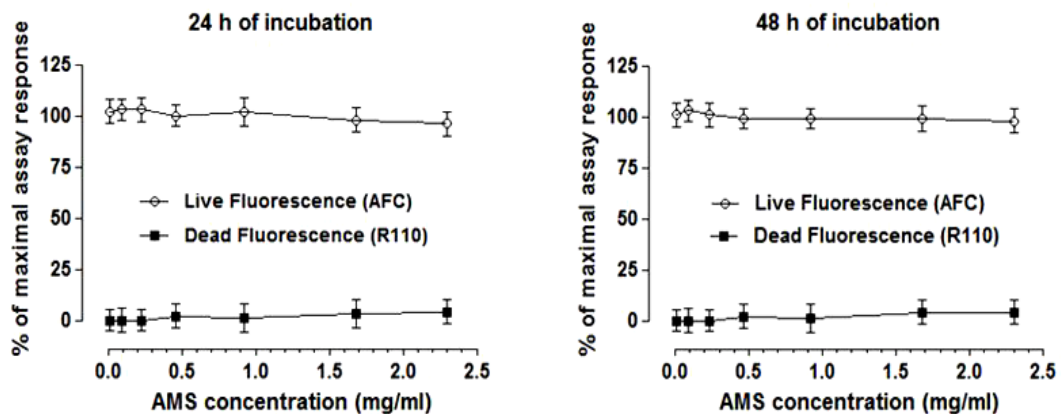
Fig. 17: AMS bioactivity in vitro. (A) Cell survival index, evaluated by the MTT assay and the determination of live/dead cell ratio. Human keratinocytes (HaCaT cells) were treated for 48 h with

a range of concentrations (from 0.09 to 2.3 mg/mL) of AMS (Annurca apple polyphenolic extract microencapsulated with maltodextrins, AppleMetS). Results are expressed in line graph as the percentage of cell survival index to untreated control cultures and are reported as mean – SEM (n = 5) of three independent experiments. Cisplatin is the positive control for cytotoxicity (cDDP). The amounts of cisplatin used to estimate its concentration/effect curve are reported as mg/mL ($3 \cdot 10^{-3}$, $7.5 \cdot 10^{-3}$, $1.5 \cdot 10^{-2}$), corresponding to 10, 25, and 50 micromolar concentrations, respectively. ***$P < .001$ versus control (untreated cells); **$P < .01$ versus control (untreated cells). (B) Inversely correlated fluorescent measures for live and dead cell counting after AMS treatments. HaCaT cells were incubated with AMS for the indicated times (24 and 48 h) and concentrations (from 0.009 to 2.3 mg/mL), and then the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega) was performed according to the manufacturer's instructions. Results are reported in line graphs as mean percentage – SEM (n = 5) of live and dead cells of four independent experiments. cDDP, cis-diamminedichloroplatinum; SEM, standard error of the mean
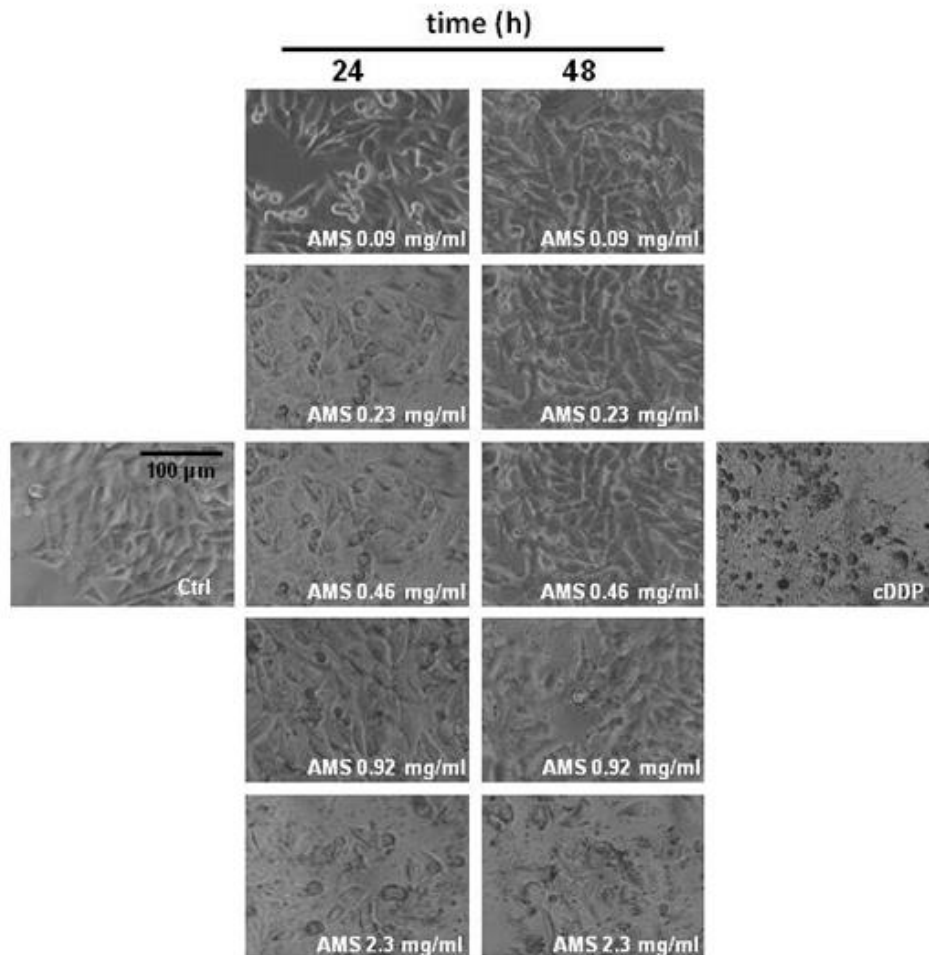
Fig. 18: **AMS effects on cell monolayers**. Representative microphotographs at a 200 · magnification (20 · objective and a 10 · eyepiece) by phase-contrast light microscopy of human keratinocytes treated for the indicated times (24 and 48 h) with a range of concentrations (from

0.09 to 2.3 mg/mL) of AMS (Annurca apple polyphenolic extract microencapsulated with maltodextrins, AppleMetS). The shown images are representative of three independent experiments. IC50 concentrations of cisplatin (cDDP) are used as positive control for cytotoxicity.

2.1.3.2 In vitro AMS supplementation strongly enhances keratin content in human skin cells

To explore AMS effect on the regulation of keratin expression, HaCaT cells were cultured in standard growth medium in presence of tested concentrations of the extract (0.23 and 0.46 mg/ml) for 24 and 48 h, as determined by preliminary bioscreens. After incubations, the total cellular levels of keratins were evaluated by Western blot analysis using a broad spectrum anti pan-keratin antibodies, as described in the experimental section. Cytokeratin bands identification was achieved by molecular weight markers position (MW). As clearly shown, in vitro treatments for 48 h resulted in a remarkable increase of keratins expression, and this also occurs with a similar extent using a serum-free culture medium to exclude unspecific stimuli (see Fig. 19). The cellular content of cytokeratins was clearly enhanced in all the examined conditions, reaching a notable maximum increase of about 3-fold with respect to untreated control cells following 48 h of exposure to extracts at 0.46 mg/ml. This effect was concentration- and time-dependent (see Fig. 20), definitely due to the presence of AMS, as further suggested by the concurrent analysis of GAPDH protein, herein used as housekeeping gene. Noteworthy, the high weight molecular cytokeratin isoforms - i.e. K1, K4, K5 and K6/K10 - visibly reached a very high expression level throughout treatments - an effect probably due to induction of protein synthesis coupled to protein accumulation. Accordingly, we can assume that, without affecting cell growth and proliferation, the AFA extract supplementation is able to induce a remarkable increase in keratins levels in human skin cells, under many experimental conditions and even at both relatively low concentration (<0.5 mg/ml) and short exposure times (24 h).
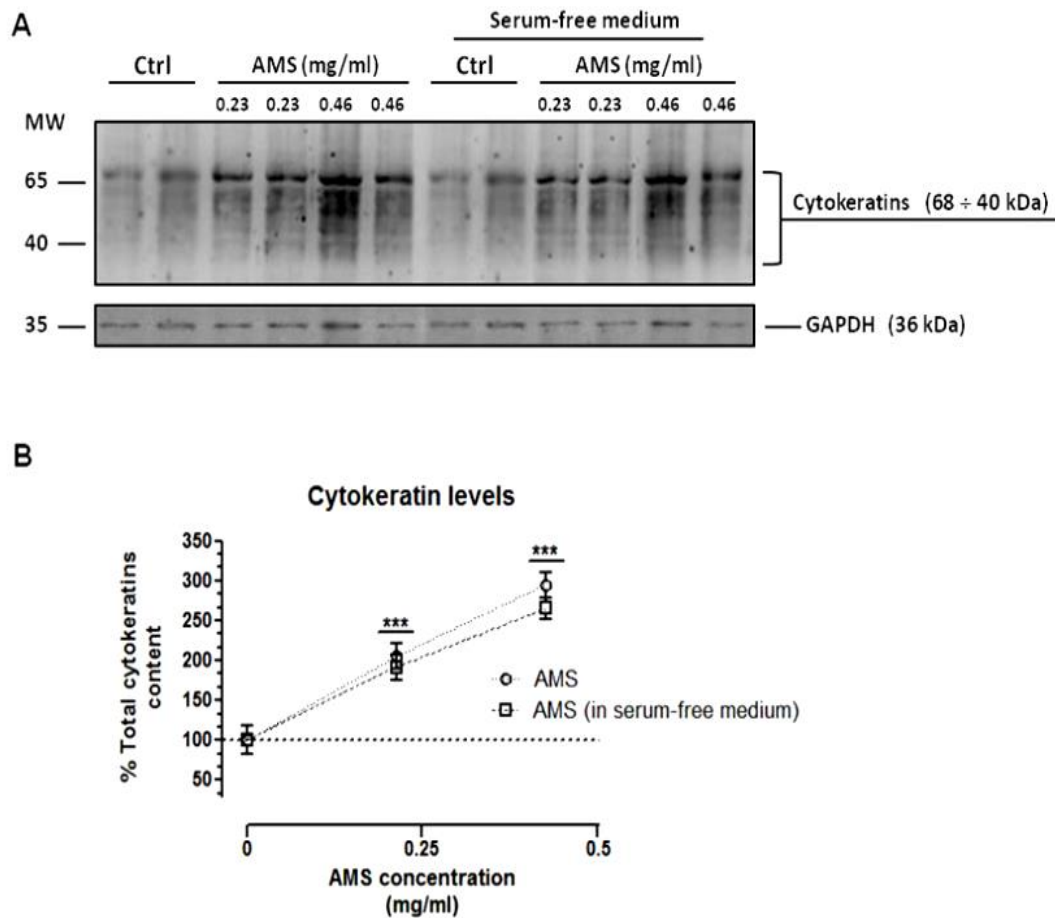
Fig. 19: Concentration-dependent effects of AMS on keratin expression in HaCaT cells. (A) Western blot analysis showing the keratin levels in HaCaT cells exposed to AMS (Annurca apple polyphenolic extract microencapsulated with maltodextrins, AppleMetS) (0.23 and 0.46 mg/mL) for 48 h, in the presence (left lanes) or absence of serum (right lanes), as indicated in figure. Equal amounts of proteins (70 lg) were separated on a 10% SDS–polyacrylamide gel and subjected to Western blot analysis using monoclonal anti-pan-cytokeratin antibodies that recognize human AU16 c cytokeratins ranging from 68 to 40 kDa, as reported in the Experimental section. Shown are blots representative of three independent experiments. (B) After chemiluminescence, the keratin bands within each lane were quantified by densitometric analysis and plotted in line graph as percentage of control. The anti-GAPDH antibody was used to standardize the amounts of proteins in each lane. Shown are the averages – SEM values of three independent experiments. ***$P < .001$ versus control (untreated cells).
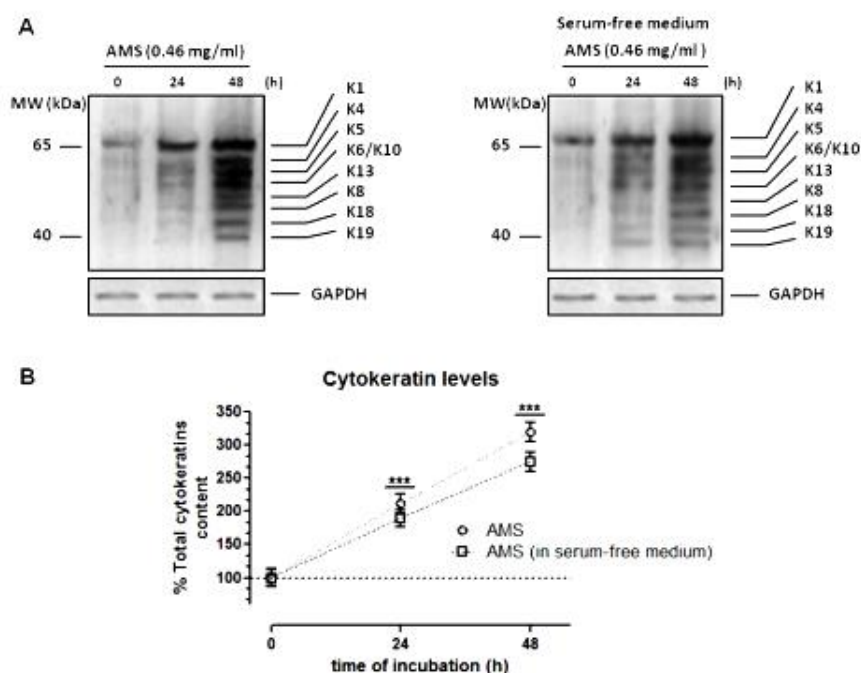
Fig. 20: Time course effects of AMS on keratin expression in HaCaT cells. (A) Western blot analysis for the evaluation of the keratin total content in HaCaT cells exposed for 24 and 48 h to 0.46 mg/mL of AMS (Annurca apple polyphenolic extract microencapsulated with maltodextrins, AppleMetS), in the presence (left blot) or absence (right blot) of serum. Equal amounts of proteins (70 lg) were separated on a 10% SDS–polyacrylamide gel and subjected to Western blot analysis using monoclonal anti-pan-cytokeratin antibodies that recognize the following human cytokeratins according to their molecular weight: K1 (68 kDa), K4 (59 kDa), K5 (58 kDa), K6/K10 (56 kDa), K13 (54 kDa), K8 (52 kDa), K18 (45 kDa), and K19 (40 kDa). Shown are blots representative of three independent experiments. (B) The cytokeratin bands within each lane were quantified by densitometric analysis and plotted in line graph as percentage of control. The anti-GAPDH antibody was used to standardize the amounts of proteins in each lane. Shown are the averages – SEM values of three independent experiments. ***$P < .001$ versus control (untreated cells).

2.1.3.3 Enrolment and subject attrition

Patients were enrolled in November 2015. A total of 340 patients were screened for eligibility; 90 patients (26.5%) did not pass the screening stage; 250 patients were randomised. The most common reason was that patients did not meet the inclusion criteria at baseline (n=38), followed by general refusal to participate for no specific reasons (n=14), and concerns about the protocol, especially fear of placebo (n=4). Some fulfilled exclusion criteria (n=34).

Overall, 250 patients were assigned to the group assuming the two supplements: they were divided into two subgroups (each one made of 125 patients), according to the two different supplements chosen for this study. Patients of both subgroups underwent a placebo period

during the first 4 weeks before the treatment period of 8 weeks. Follow-up period lasted other 4 weeks. Figure 21 shows the flow of participants through the trials together with the completeness of diary information over the entire treatment period.

No patient prematurely terminated study participation before allocation to treatment. Figure 21 follows the CONSORT PRO reporting guideline34 and reveals that within the assessment period, the following percentage of patients for each subgroup provided data for the primary endpoint: subgroup AMS, 74.6% (85 of 114 patients); subgroup AMSbzs, 73.4% (83 of 113 patients). In each subgroup, a few patients did not submit any diaries, giving no specific reason for this. Completeness of the patient diaries did not differ between the treatment groups.
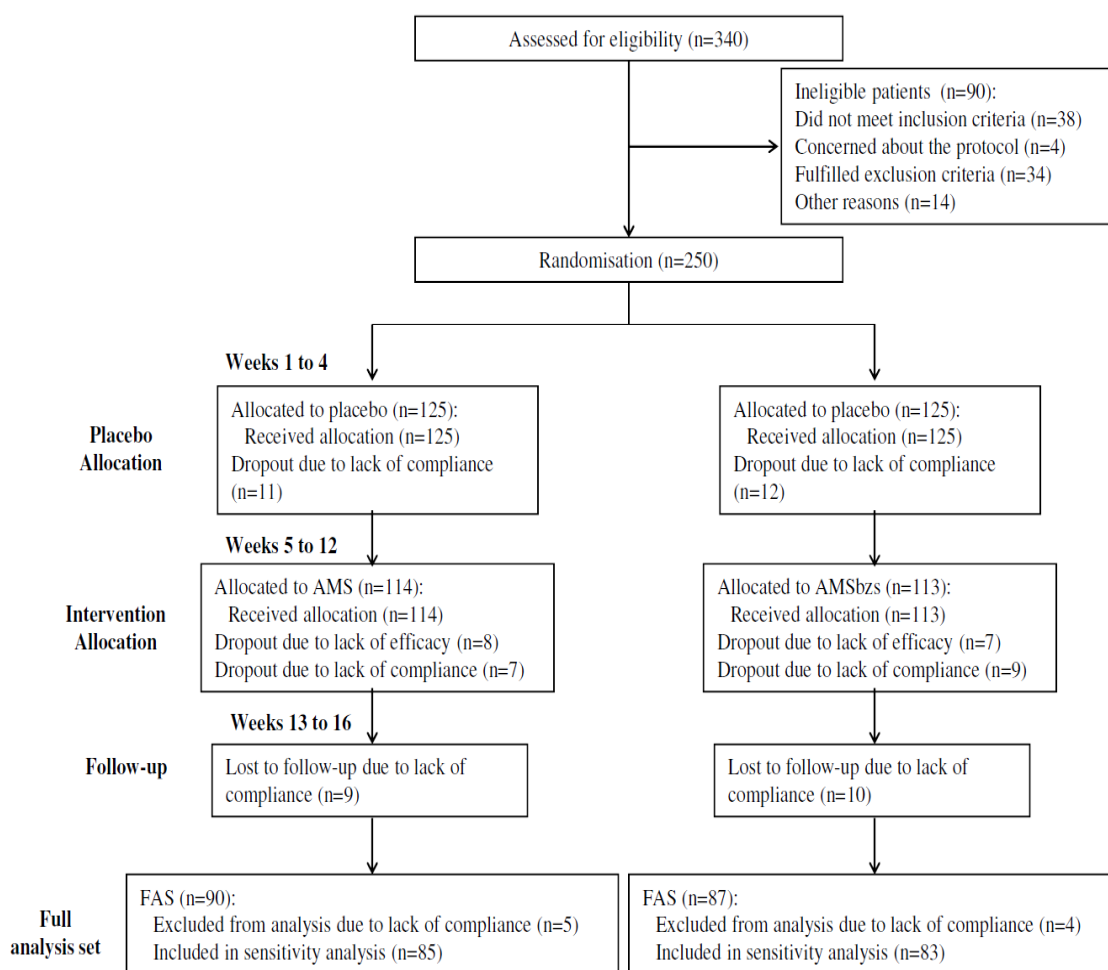
Fig. 21: Study flowchart according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrollment and primary efficacy endpoints based on patient diaries, from prescreening to data collection; and the extent of exclusions, loss to follow-up, and completeness of diary documentation available across the entire trial period. AMS, Annurca apple polyphenolic

extract microencapsulated with maltodextrins (AppleMetS); AMSbzs, formulation including Annurca apple polyphenolic extract microencapsulated with maltodextrins, biotin, selenium, and zinc (AppleMetS Hair); FAS, full analysis set

2.1.3.4 Participants' baseline characteristics

Table 4 shows the demographic and clinical characteristics assessed at the baseline visit of all 250 patients randomised. Interestingly, more than half of the randomised patients were female; the total age range was 30-83 years. The groups were well balanced.

Table 4. Baseline characteristics of intention to treat sample according to study treatment.		
Placebo		
Characteristics	**AMS (n=125)**	**AMSbzs (n=125)**
Demographics		
Age (years)	42.4 ± 10.6	41.8 ± 10.8
Male sex (No (%))	58 (46.4%)	58 (46.4%)
White ethnicity (No (%))	125 (100%)	125 (100%)
Clinical parameters		
Hair number/cm^2	16.4 ± 4.6	15.7 ± 4.8
Hair weight (mg)*	30.1 ± 8.1	29.6 ± 7.9
Keratin content (mg)**	27.4 ± 6.4	26.9 ± 5.9
Treatment		
Characteristics	**AMS (n=114)**	**AMSbzs (n=113)**
Demographics		
Age (years)	43.5 ± 10.2	42.1 ± 11.1
Male sex (No (%))	51 (44.7%)	50 (44.2%)
White ethnicity (No (%))	114 (100%)	113 (100%)
Hair number/cm^2	15.9 ± 4.8	15.3 ± 4.5
Hair weight (mg)*	29.9 ± 7.8	30.1 ± 8.0
Keratin content (mg)**	27.2 ± 5.5	27.4 ± 6.1
Value are means ± SD. AMS: Annurca apple polyphenolic extract microencapsulated with maltodextrins (AppleMetS); AMSbzs: formulation including Annurca apple polyphenolic extract microencapsulated with maltodextrins, biotin, selenium, and zinc (AppleMetS Hair). * The weight is referred to an aliquot of 100 hairs (1 cm long). ** The keratin content is referred to an aliquot of 100 hairs (1 cm long).		

2.1.3.5 Primary and secondary efficacy outcome measures

No significant variation of hair number, weight, and keratin content with respect to the baseline values was registered at the end of placebo period in subjects belonging to both supplement-supplied groups (Table 5). Analyzing results, we can assert that the administration of both supplements led to a statistically significant variation of all of the clinical parameters. Specifically, AMSbzs supplement exerted the most important effects, increasing the hair number/cm2 by 125.2% (95% CI: -2.54, P = 0.0095), the hair weight by 42.1% (95% CI: -3.41, P = 0.0019), and the keratin content by 40.1% (95% CI: -1.22, P = 0.0042), at the end of the trial period (Table 5) (Fig. 22). Noteworthy, very significant results were achieved already after one month of intervention study.

Table 5. Effects of Annurca apple supplements on clinical parameters.					
		AMS	Δ (%)	AMSbzs	Δ (%)
Hair number/cm^2	*t 0*	16.4 ± 4.6		15.7 ± 4.8	
	t 30	25.3 ± 4.8	*+54.3*	25.1 ± 4.4	*+60.1*
	t 60	35.8 ± 5.1	*+118.3*	35.3 ± 5.7	*+125.2*
Hair weight (mg)*	*t 0*	30.1 ± 8.1		29.6 ± 7.9	
	t 30	36.1 ± 7.6	*+20.1*	37.2 ± 6.8	*+25.7*
	t 60	41.3 ± 8.5	*+37.3*	42.1 ± 7.9	*+42.1*
Keratin content (mg)**	*t 0*	27.4 ± 6.4		26.9 ± 5.9	
	t 30	32.4 ± 7.0	*+18.4*	33.2 ± 7.1	*+23.5*
	t 60	37.2 ± 6.9	*+35.7*	37.7 ± 6.7	*+40.1*
Value are means ± SD (*n* = 5). AMS: Annurca apple polyphenolic extract microencapsulated with maltodextrins (AppleMetS); AMSbzs: formulation including Annurca apple polyphenolic extract microencapsulated with maltodextrins, biotin, selenium, and zinc (AppleMetS Hair). Subjects were administered with 2 AMS or AMSbzs capsules/day for 2 months. Results were significantly different at a level of *P* = 0.001. * The weight is referred to an aliquot of 100 hairs (1 cm long). ** The keratin content is referred to an aliquot of 100 hairs (1 cm long).					

2.1.3.6 Safety issues

All the laboratory analysis concerning the hepatic and renal function indicated no alteration of values after two months of AMS treatment (Table 6). Other safety assessments, such as vital signs, blood pressure, or electrocardiographic findings, were all periodically monitored and baseline values did not change substantially during and at the end of the trial.

Table 6. Effects of Annurca apple supplements on plasma indicators of hepatic and renal function in subgroups.

AMS

	AST (GOT) (U/L)			ALT (GPT) (U/L)			γ-GTP (U/L)			ALP (U/L)		
	t 0	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*
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 (%)		*-0.5*	*-0.5*		*-1.4*	*-1.1*		*-2.7*	*-7.5*		*-0.98*	*-3.0*
	LDH (U/L)			Albumin (g/dL)			Total bilirubin (mg/dL)			Creatinine (mg/dL)		
	t 0	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*
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*

AMSbzs

	AST (GOT) (U/L)			ALT (GPT) (U/L)			γ-GTP (U/L)			ALP (U/L)		
	t 0	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*
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)		
	t 0	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*	*t 0*	*t 30*	*t 60*
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 (%)		*-0.28*	*-3.84*		*+0.23*	*-3.48*		*-*	*-5.26*		*-2.38*	*-4,76*

Value are means ± SD (*n* = 5).

AMS: Annurca apple polyphenolic extract microencapsulated with maltodextrins (AppleMetS); AMSbzs: formulation including Annurca apple polyphenolic extract microencapsulated with maltodextrins, biotin, selenium, and zinc (AppleMetS Hair).

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

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

2.1.3.7 Study strength and limitations

The major strengths of the clinical trial herein presented reside in the originality of the study and in the evaluation of the treatment effects in real-world settings. The positive results, herein reported, can inform physicians about a novel treatment/intervention, which can represent a valuable alternative in the clinical practice. It has also been reported that the anagen ratio undergoes seasonal changes: it rises to a maximum in March and falls to a minimum in September (Randall et al., 1991).

Our clinical test was performed from January to July, so the effects of seasonal changes are unlikely to have influenced the overall results. Conversely, the main limitations of our study include the short-term assessment for the treatment of a chronic condition and the choice of exclusively white race.

2.1.4 Discussion

Polyphenols have always been important antioxidants of the human diet (Ferreira et al., 2002; Cos et al., 2004 and Fu et al., 2015). Among them, oligomeric procyanidins have also shown remarkable hair growth stimulant effects *in vitro* and *in vivo*, being able to promote hair epithelial cell growth and anagen induction of the hair cycle (Kamimura et al., 2006). In particular, procyanidins B2 and B3 show evidence of protective actions versus apoptosis in hair epithelial cell cultures, thereby restricting catagen induction in the hair cycle (Kamimura et al., 2006). Analysing results (Table 5), we found that, on average, after two months, hair growth, weight, and keratin content, increased by 118.3, 37.3 and 35.7%, respectively. Surprisingly, the co-formulation named as AppleMetS Hair (AMSbzs), obtained by adding to the AMS supplement the vitamin biotin, and the minerals zinc and selenium, three elements well known for their positive general effects on hair and skin (Reg. EU n. 1924/2006), led the second subgroup of subjects to only slightly higher results in terms of hair growth, weight, and keratin content (Table 5), clearly indicating for AMS a major role in promoting hair growth and tropism. In spite of the small number of subjects and the short period over which the trial was carried out, we observed a clear trend and significant results for both supplements in each subgroup, which were revealed by some statistical analyses towards increased hair growth and density (Table 5).

Some clinical trials have extensively demonstrated the positive hair-growing effects of procyanidin B2, a major dimeric procyanidin occurring in apples (Kamimura et al., 2000; 2002 and Takahashi et al., 2001). Actually, these studies have exclusively investigated the

potential benefits of topical applications of procyanidin B2 under the form of alcoholic solutions in mixture with other bioactive components. Kamimura et al. (2000) have reported the effects of 1% procyanidin B2 tonic on human hair growth after sequential use for 6 months. A double-blind clinical test involving a total of 29 male subjects was performed. It was observed an increase of 6.7 total hairs/0.25 cm2 after 6 months of procyanidin therapy. The same effects have been reported with minoxidil (Kreindler et al., 1987, Roberts et al., 1987 and Savin et al., 1987) and finasteride (an inhibitor of type II 5α-reductase, Merck)(Kaufman et al., 1998) therapy for androgenetic alopecia. In 2% minoxidil treatment, an increase of 250 total hairs/5.1 cm^2 (calculated as 12.3 total hairs/0.25 cm2) after 12 month therapy was reported (Kreindler et al. 1987). In finasteride treatment (1 mg/day, oral administration), an increase of 86 total hairs/5.1 cm2 (calculated as 4.22 total hairs/0.25 cm2) after 12 month therapy was reported (Kaufman et al., 1998). The level of efficacy of 1% procyanidin B2 is concluded to compare favourably with minoxidil and finasteride therapy (Kamimura et al., 2000). As far as the procyanidins are concerned, it has been demonstrated that in murine epithelial cells both procyanidins B2 and B3 are capable of increase the anagen growth phase within hair cycle by means of their protective action on cellular apoptosis induced by the androgen-inducible cytokine Transforming Growth Factor (TGF) β1 and β2, which normally cause a cell cycle block in favor of differentiation processes and are thereby supposed to trigger catagen induction in hair follicle (Hibino et al., 2004). In the same way, procyanidins exhibited intensive biological effects in promoting hair epithelial cell growth in murine models *in vivo*, leading to increased hair density and terminal hair formation (Kamimura et al., 2006). Indeed, the addition of TGF-β1 or TGF-β2 to cultured murine hair epithelial cells dose-dependently decreased cell growth by increasing apoptosis via intrinsic caspase network, whereas the concurrently addition of procyanidin B2 to the culture medium counteracted both TGF-β1 and TGF-β2 effects, protecting cells from apoptotic pathways activation. Interestingly, Kamimura et al. (2006) also showed that procyanidin B2 upregulates the expression of MAPK/ERK Kinase (MEK-1/2) in the hair epithelial cells, as well as their activation (Kamimura et al., 2006). MEK is a member of the MAPK signaling cascade that is generally activated by mitogens, so that when MEK is inhibited, cell proliferation is blocked and apoptosis is induced (Wellbrock et al., 2016). Not surprisingly, components of the MAPK/ERK pathway were mainly discovered in cancer cells (Santarpia et al., 2012) in which drugs able to reverse the "on" or "off" switch are currently being investigated as cancer chemotherapeutics (Asati et al., 2016, and Ruscono et al., 2012). However, it is still

unclear whether these two suggestions are correlated, and especially if procyanidins are able to interact with distinct cell membrane receptors involved in MAPK/ERK signalling pathways and/or to directly interfere with this cascade of intracellular proteins.

During hair growth and proliferation, several cytokeratins expressed by epithelial cells orchestrate the assembly of typical intermediate filaments, which contribute in common with keratin-associated proteins (KAPs) in the production of the hair shaft within the keratinisation process of the hair fiber (Makar et al., 2007). All the well-known 54 human keratins belonging to the IF proteins share common structural and functional features. In addition, evidently bundled as tonofilaments in epidermal tissue, keratin filaments are endowed with key functional role in the integrity and mechanical stability of both the single epithelial and hair cells and, via cell contacts, of the whole epithelial tissue (Schweizer et al., 2006). As well, the central function of epidermis-type keratins is demonstrated by knock-out mouse models but also throughout different human hereditary keratin diseases affecting skin and hair (Moll et al., 2008). Therefore, since keratins are fundamental in epidermal and hair development and homeostasis, the regulation of keratins expression appears to be a central event for rapid proliferating epidermal cells, as well as in hair follicle growth and maturation. To date, despite the use of dietary nutraceutics and functional food supplements has greatly increased, studies concerning their biological effects on hair loss have produced conflicting results (Miniaci et al., 2016). Conversely, by targeted bioscreen we have herein demonstrated that AMS supplementation *in vitro* is able to promote a remarkable up-regulation of keratin expression in human keratinocytes, resulting in keratins accumulation within cells. These data are fully consistent with those arising from our clinical trial - which indicates an important increase of the keratin hair content, of about 35% more than in untreated group - thereby validating the biological effects of AMS on hair growing and proliferation. To the best of our knowledge, no correlation between the administration of nutraceuticals and actual increased expression of keratin in human skin cells had been reported so far. As well, this is the first study demonstrating the effectiveness of a natural procyanidin B2-containing gastro-protected apple extract as hair growth-stimulant via oral administration. For these reasons, we believe that the reported findings concerning the use of AMS as oral nutraceutical supplement are of great interest, as they reveal an efficacy in promoting hair growth fully comparable with or even higher than those obtained from trials performed with pharmaceutical therapies nowadays regarded as the leading treatments for the pattern baldness. In addition, our proposed therapy, apart not giving any sign of hirsutism in all of

the enrolled subjects, would allow to avoid the possible inconvenience deriving from the topical application of a generic alcoholic solution (skin burning, reddening, and cracking), or, more importantly, specific side effects, which are typical of finasteride, such as sexual dysfunctions (Venkataram et al., 2014) or of minoxidil, such as cardiovascular (Pettinger et al., 1980; Reichgott et al., 1981; Krehlik et al., 1985) immunologic (Tunkel et al., 1987; Mitas et al.,1981) and endocrine disorders (Nguyen et al., 2009).In fact, no evidences of cytotoxic effect nor morphological changes on cultured human keratinocytes were hitherto observed after exposure to AMS under different experimental conditions. Similarly, with the exception of antiproliferative effects on cancer cells and antibacterial activities, any type of cytotoxicity induced by apple bioactive components has never been reported in scientific literature (Schiavano et al., 2015; Sudan et al., 2014; Hyson et al., 2009 and Luo et al., 2016)
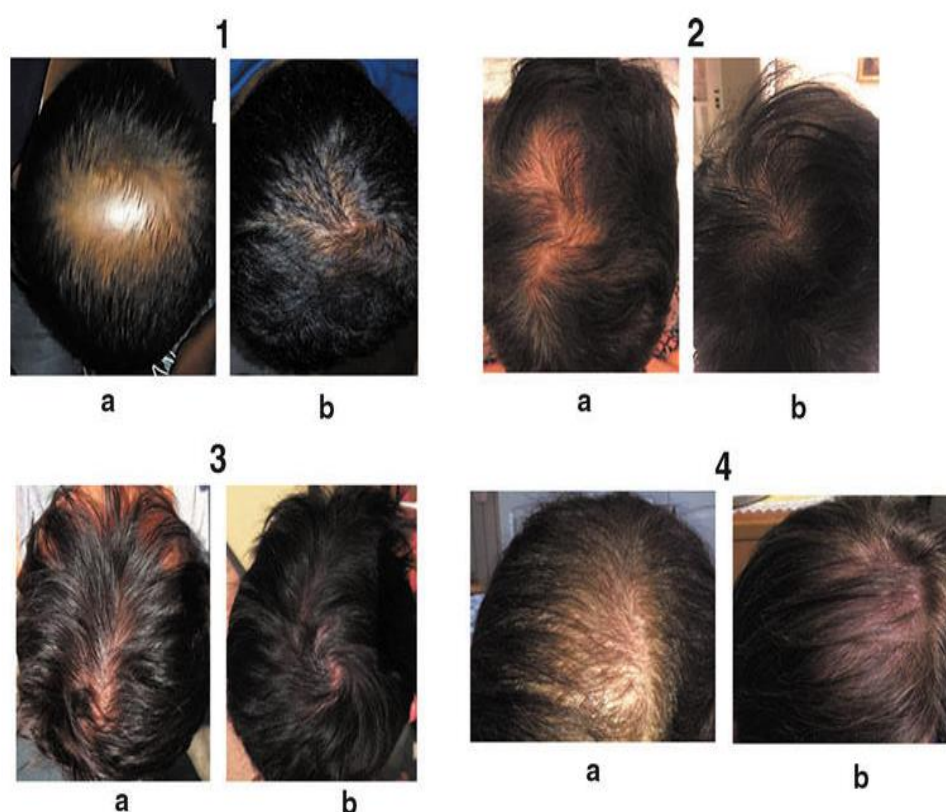
Fig. 22: Some examples of subjects (1,2,3 male and 4 female),who have consumed 2capsules/day of AMSbz supplement for 60 days (T0 and T60)

2.1.5 Conclusion

Male and female pattern baldness is a highly common condition affecting approximately 50% of the adult population, and potentially causing a significant negative impact on the quality of life. The results of this clinical trial, in combination with in vitro evidence of increased cellular keratin content, provide strong support that a dietary supplement acting as nutraceutical and based on a procyanidin B2-containing AFA natural extract can effectively promote hair growth and improve skin quality, at once increasing hair density, weight, and keratin content. Further studies are ongoing to better clarify the molecular basis of the procyanidins-induced growth promotional effects at the skin level, focusing both on the regulation of cells homeostasis and on the functional role played by keratins selectively induced via nutraceutical supplementation.

Chapter 2

Nutraceutical Formulations

2.2 Protection from gastrointestinal digestion is required to preserve the WNT inhibitory activity exerted by apple extracts on colon-rectal cells carrying FAP mutations

2.2.1 Introduction

Familial adenomatous polyposis (FAP) is an inherited colorectal cancer syndrome characterized by the formation of a large number of adenomas throughout the large bowel. The birth frequency of FAP in European populations is estimated at roughly 1 in 20,000 live births and progression to colorectal cancer is very high probably in the age of 35–40 yr (Galiastatos et al., 2006). While colectomy remains the optimal prophylactic treatment, the identification of chemopreventive agents represents one of the major challenges for the future (Galiastatos et al., 2006).

FAP is caused by a germline mutation in the adenomatous polyposis coli (APC) gene on chromosome 5q21. This locus contains a tumor suppressor gene encoding for the protein APC, that functions intracellularly as scaffold in the large protein complex known as " β-catenin destruction complex". This includes the serine/threonine kinase Glycogen synthase kinase-3 β, Axin, and Casein Kinase I. The complex represents an important intracellular check point since it reduces the intracellular levels of β-catenin by targeting it to proteasomal degradation. Its activity avoids translocation of β-catenin into the nucleus and its binding to the transcription factors TCF and LEF, both promoting the expression of mitogenic genes c-myc and cyclinD1(Clevers et al., 2012; Dijksterhvis et al., 2014; Miki et al., 2011 and Krousova et al., 2014).

Wild Type APC counterbalances the activity of the WNT (Wingless-related integration site) pathway, a signaling cascade regulating development in embryos and tissue homeostasis in adult organs (Clevers et al., 2012). In the gastrointestinal (GI) tract, WNT supports the self-renewal capacity of epithelial stem cells allowing GI organs to be the most intensively self replenishing in mammals. (Burges et al. 2011). The Frizzled (FZD) GPCRs' family members act as WNT receptors and, upon activation, recruit and disassemble the β-catenin destruction complex inhibiting its function and causing β-catenin intracellular accumulation and nuclear translocation. This is the reason for why FAP

mutations, by abolishing APC function, lead to constitutively active WNT signaling and in turn to uncontrolled proliferation of colon cells, formation of polyps and adenocarcinomas. Apple extracts have been shown to mediate several biological cellular effects that might be of interest with respect to chemoprevention of colorectal diseases. Such activity mostly relies on the high amount of polyphenols they contain (Kernet al., 2006). Polyphenol-rich apple extracts have been shown to suppress human colon cancer cell growth in several in vitro culture models. Moreover, in a murine model of FAP, the APC$^{Min/+}$ mice, the assumption by beverage of apple polyphenol extracts affected number and growth of colon polyps, reduced colorectal bleeding and high-grade dysplasia (Fini et al., 2011). The action of the apple polyphenols has been so far mainly ascribed to their antioxidant potential. However, new researches have recently shown several polyphenols being endowed of inhibitory activity toward specific protein targets, among the others, many of the components WNT signaling pathway (Ishibeshi et al., 2015).

The aim of this part of my thesis work was to test the WNT inhibitory activity of two apple cultivars native to Southern Italy, namely "Annurca" and "Limoncella", identify the polyphenols mainly responsible for their inhibitory activity and their mechanism of action.

Malus pumila Miller cv. Annurca is a widespread apple and accounts for the 5% of national apple production. It is listed as Protected Geographical Indication (PGI) product from the European Council [Commission Regulation (EC) No. 417/2006)]. This apple has been already shown to posses nutraceutical potential in virtue of its ability to reduce cellular glucose levels and lipid uptake (Tenore et al., 2013;2014;2016 and Sommella et al. 2015). Malus domestica cv 'Limoncella' is a juicy and aromatic variety of apple known since ancient roman times (D'Ambrosca et al., 2007). It resists to long time storage and survive to cold winters. Differently from Annurca, Limoncella's nutraceutical potential has not yet been fully documented.

I tested on in vitro cultures of cells carrying FAP mutations and on ex vivo biopsies of FAP patients, the WNT inhibitory activity of "Annurca" and "Limoncella" apple extracts, identified the mechanism underpinning their activity and evaluate their potency upon *in vitro* simulated gastrointestinal digestion.

2.2.2 Materials and methods

2.2.2.1 Reagents

Salt and organic solvents were from Applichem (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA). Polyethylenimina (PEI, #408727, Sigma-Aldrich); Lipofectamine 2000 (#11660-019, Invitrogen, Thermo Fisher Scientific, MA, USA).

Cell Cultures - HEK293, CaCo-2, and U87MG cells were grown in DMEM (#41965-039, GIBCO, Thermo Fisher Scientific) supplemented with 10% FBS (#10270, GIBCO), Glutamax (#35050-061, GIBCO) and Pen/Strep (#15070-063, GIBCO). HEK293 transfection was performed using PEI (Generoso et al. 2015). CaCo-2 transfection was performed using Lipofectamine (Invitrogen) following manufacturer instruction. Cell viability was measured with the Trypan Blue and Propridium Iodide. Percentage of cells in apoptosis was measured with mitotracker red (Life Technologies) following manufacturer instructions.

2.2.2.2 Excision and culturing of human biopsies from FAP patients

Biopsies from Familial Adenomatosis Poliposis patients were kindly provided by Prof. G. B. Rossi. The study was approved by the Ethic Committee of the Istituto Nazionale Tumori "Fondazione G. Pascale", IRCCS, Napoli, Italy and of the University Federico II of Naples. Immediately after excision, biopsies were rinsed in physiological saline. Samples were then digested with Trypsin for 10 min at RT and centrifuged at 400 rpm for 10 min at RT. Isolated cells present in the supernatant were then counted and cultured at the confluency of 100,000 cell per ml in DMEM supplemented with 10 % FBS, Glutamax and Pen/Strep. When indicated Limoncella extract was added at the concentration of 400mg/l. After 24 and 48 hours of incubation, cell viability was measured with the Trypan Blue assay and Propridium Iodide.

2.2.2.3 DNA

All DNA constructs were synthesized at GeneScript (USA). The cDNA coding for N terminally HA tagged FZD4wt (HA-FZD4-wt), was cloned in the expression vector pCDNA3.1 (Invitrogen). For the reporter construct WNT reporter GFP construct: 8 repeats of the optimized TCF/LEF binding sequence [5'-AGATCAAAGGGG-3'] (interspaced by the triplet 5'-GTA-3') were positioned upstream to a minimal TATA box promoter [5'-tagagggtatataatggaagctcgaattccag-3'] and a KOZAC region [5'-

cttggcattccggtactgttggtaaaaagcttggcattccggtactgttggtaaagccacc-3']. The sequence was cloned in the vector pCDNA 3.1 (+) GFP between the restriction sites for NruI and HindIII. This replacement substitutes the CMV promoter of the original vector with the TCF/LEF responsive sequences. Correctness of the sequences were verified by DNA sequencing.

2.2.2.4 WNT pathway activity measurement using the TCF-GFP constructs

HEK293 cells were seeded (5 x 10^3 per well) in 96-well black Optyplates (Perkin Elmer). After 24 hours, cells were co-transfected with both WNT reporter GFP construct and HA-FZD4-wt. Transfection mixtures were prepared as follow: for each well 0,25μg of PEI (pH 7.0) was supplemented with 0,08 μg of DNA(both diluted in 4 μl of DMEM). The mixture was incubated at room temperature for 30 min to be then diluted in culture medium and added to the cells. 24 hours after transfection, medium was replaced and cells incubated with WNT5A conditioned medium. Human glyoblastoma U87MG cells were used as source of WNT5A conditioned medium. 16 Confluent 6 cm plates of U87MG cells were incubated for 3 days in DMEM, 10% FBS and Glutamax in the absence of antibiotics. Conditioned medium was thus collected and stored at -20°C. Cells were then rinsed with fresh medium and cultivated for a further period of 3 days. The conditioned medium obtained after the second incubation was pooled with the first one. The pooled conditioned medium was used to stimulated TCF/LEF activity in HEK293 cells. It was used undiluted (EC100). When indicate conditioned medium was supplemented with the indicated compounds or apple extracts at the indicate concentration. After 48 hours cells were fixed in 3.7% formaldehyde in PBS pH 7.4 for 30 min. Formaldehyde was quenched by incubating the cells for 30 min in 0.1 M Glycine in PBS 1X. The activity of the compounds was evaluated by measuring GFP expression.

2.2.2.5 WNT Pathway Activity Measurement in CaCo-2 Cells Using the TCF-GFP Constructs

CaCo-2 cells, growing on glass coverslips, were transfected by Lipofectamine , according to the to the manufacturer's procedures. Twenty-four hours after transfection, the medium was replaced and cells were incubated with the indicated extracts for 24–48 h. The cells were then fixed in a solution of 3.7% formaldehyde, in PBS with a pH 7.4, for 30 min. Formaldehyde was quenched by incubating the cells for 30 min in 0.1 M glycine in PBS.

2.2.2.6 Immunofluorescence

CaCo-2 cells were grown on glass coverslips. Cells were fixed in 3.7% formaldehyde/PBS (pH 7.4) for 30 min. Formaldehyde was quenched by incubating the cells for 30 min in 0.1 M glycine in PBS 1x. Then, cells were permeabilized in 0.1% Triton/PBS, pH 7.4, for 10 min at 25 °C and then incubated with a rabbit polyclonal anti-β-catenin antibody (H-102, sc-7199, Santa Cruz, Dallas, TX, USA) diluted 1:200 in PBS and a goat anti-rabbit IgG (H&L), DyLight 594 conjugate, (ImmunoReagents, Raleigh, NC, USA) (diluted 1:500 in PBS), for 1 h and 45 min, respectively.

2.2.2.7 Fruit Collection and Sample Preparation

Annurca (*Malus Pumila* Miller cv Annurca) apple fruits and Limoncella (*Malus Domestica* cv Limoncella) were collected from Valle di Maddaloni (Caserta, Italy), in October 2016, when fruits had just been harvested. Annurca fruits were reddened for about 30 days, and then analyzed (Lo Scalzo et al.,2001). Lyophilised peels and flesh (10 g) of Limoncella and Annurca apple samples were treated with 60 mL of 80% methanol (0.5% formic acid), homogenized for 5 min by ultra-turrax (T25-digital, IKA, Staufen im Breisgau, Berlin, Germany) and shaken on an orbital shaker (Sko-DXL, Argolab, Carpy, Italy) at 300 rpm for 15 min. Then, the samples were placed in an ultrasonic bath for another 10 min, before being centrifuged at 6000 rpm for 10 min. The supernatants were collected and stored in darkness, at 4 °C. The pellets obtained were re-extracted, as described above and with another 40 mL of the same mixture of solvents. Finally, the extracts obtained were filtered under vacuum, the methanol fraction was eliminated by evaporation, and the water fraction was lyophilized. To obtain polyphenol-enriched fractions of Annurca apple extract (AAE) and Limoncella apple extract (LAE) (in the text referred to as PEF(AAE) and PEF(LAE), respectively) the dry extracts were dissolved in distilled water and slowly filtered through an Amberlite XAD-2 column, packed as follows: Resin (10 g; pore size 9 nm with particle sizes of 0.3–1.2 mm; Supelco, Bellefonte, PA, USA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 × 2 cm). The column was washed with 100 mL of acidified water (pH 2) and 50 mL of deionized water for removal of sugars 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 extracts were stored at −80 °C until HPLC analysis. Before performing the biological tests, each extract was dissolved in DMSO at a final concentration of 300 mg/mL. Food grade Limoncella apple extracts (IndLAE) were produced at MB-Med (Turin, Italy) starting from

fresh Limoncella Apples. Upon lyophilization of peels and flesh of Limoncella apples, samples were treated with ethanol/water (70:30 v/v) for 24 h at 40 °C to extract phenolic compounds and generate food grade Limoncella Apple Extracts (in the text referred to as IndLAE).

2.2.2.8 HPLC-DAD analysis

Extracts from the three different apple varieties and of industrial products were solubilized with 1% formic acid. Analyses were run on a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) provided with photodiode array detector (DAD). The column selected was a Kinetex® C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA). The analyses were performed at a flow rate of 1 mL/min, with solvent A (2% acetic acid) and solvent B (0.5% acetic acid in acetonitrile and water 50:50, v/v). After a 5 min hold at 10% solvent B, elution was performed according to the following conditions: from 10% (B) to 55% (B) in 50 min and to 95% (B) in 10 min, followed by 5 min of maintenance. Flavonols, procyanidins, dihydrochalcones, flavanols and hydroxycinnamic acids were monitored at 280 nm and anthocyanins at 520 nm. For quantitative analysis, standard curves for each polyphenol standard were prepared over a concentration range of 0.1-1.0 μg/μL with six different concentration levels and duplicate injections at each level. The identity of polyphenols was confirmed by comparison to those of commercial standards.

2.2.2.9 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$_2$PO$_4$, 88.8 g/L), sodium sulphate (Na$_2$SO$_4$, 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.

2.2.2.10 Statistical Analysis

EC50 were calculated from dose-response data using nonlinear regression analysis of Prism software 6.0 (GraphPad, GraphPad Software, San Diego California USA, www.graphpad.com). All data were analyzed using the two-tailed Student's t-test. Two way ANOVA test was used to compare columns. $P < 0.05$ were considered statistically significant.

2.2.3 Results

2.2.3.1 HPLC-DAD analysis

Total (flesh and peel) polyphenolic contents in Limoncella and Annurca apple extracts were determined by HPLC-DAD analysis and are reported in the following table :

	AAE*	LAE*	PEF(AAE)**	PEF(LAE) **
Chlorogenic Acid	3.9 ±0.2	4.7 ±0.1	10.2 ±0.6	9.7 ±0.9
[+] Catechin	0.8 ±0.1	1.3 ±0.1	3.0 ±0.2	5.5 ±0.4
[-] Epicatechin	0.9 ±0.2	1.3 ±0.1	2.8 ±0.1	4.0 ±0.2
Isoquercetin	1.4 ±0.1	2.9 ±0.2	3.4 ±0.3	2.7 ±0.3
Rutin	11.2 ±0.2	0.4 ±0.3	34.7 ±0.1	1.7 ±0.2
Phloridzin	2.1 ±0.3	1.2 ±0.5	7.5 ±0.2	3.0 ±0.1
Procianidine B2	1.3 ±0.1	2.8 ±0.1	4.1 ±0.1	10.8 ±0.1
Phloretin	n.d.	n.d.	n.d.	n.d.
Quercetin	n.d.	n.d.	n.d.	n.d.

Table 7: Polyphenolic content present in: Annurca Apple Extract (AAE), Limoncella Apple Extract (LAE), a polyphenolic purified fraction of AAE (PEF(AAE)), a polyphenolic purified fraction of LAE ((PEF(LAE)). * mg/100g of DW; ** mg/100mg DW; n.d., non detected; Values are reported as mean ± s.d.(n=3);

AAE (Annurca apple extract) and LAE (Limoncella apple extract) present qualitatively similar polyphenolic repertoires. They contain similar amount of Chlorogenic acid, the most abundant hydroxycinnamic acid in apples as well as of the flavan-3-ols [+] Catechin and [-] Epicatechin. The Quercetin-glycosides, Rutin and the phloretin–glycoside, phloridzin are more abundant in AAE than in LAE, that on the contrary contains, as already reported, higher amounts of Procyanidine B2 and isoquercetin (D'Ambrosca et al., 2007 and Sommella et al., 2015). The aglycones Quercetin and Phloretin are absent in both the extracts (Sommella et al., 2015). PEF(AAE) and PEF(LAE), were respectively, the purified extracts of Annurca and Limoncella, more rich , as shown in the table, in polyphenlic compounds.

HPLC-DAD analysis of the flavonoid content of the IndLAE (Industrial Limoncella extract) did not reveal major change in the amount of polyphenols compared to AAE and LAE. However, I realized the presence of a discrete amount of Quercetin , which was absent in the correlative non industry extract. Probably this quantity of quercetin derives from the conversion of quercetin glycosides into their aglycone form, during the industrial production. In IndAAE is not reached this Quercetin concentration increase so high. (data not showed).

	Ind(LAE)
Chlorogenic Acid	8.6±0.1
Catechin	1.4±0.2
Epicatechin	0.7±0.1
Isoquercetin	0.5±0.1
Rutin	2.6±0.3
Phoridzin	7.0±0.4
Procianidine B2	2.5±0.1
Quercetin	1.2±0.1
Phloretin	n.d.

Table 8: Polyphenolic content of: Industrial Limoncella Extract (IndLAE) mg/100g DW;Values are reported as mean ± s.d.

2.2.3.2 In vitro digestion

Results of HPLC-DAD analysis of the polyphenolic contents in digested AAE and LAE are reported in Table 9. As expected, *in vitro* digestion decreased the overall polyphenolic content of around 40-50%. For both cultivars, digestion converted procyanidins into monomeric catechins, and the amount of chlorogenic acid, rutin, epicatechin and isoquercetin was drastically reduced. Interestingly, GI digestion reduced the content of phloridzin in AAE but, not in LAE where the molecule almost completely resisted to GI.

	digAAE*	digLAE*
Chlorogenic Acid	0.9 ±0.1	0.7 ±0.1
[+]Catechin	2.5 ±0.1	2.8 ±0.1
[-] Epicatechin	0.1 ±0.1	0.3 ±0.1
Isoquercetin	0.1±0.1	0.1 ±0.1
Rutin	0.3 ±0.2	n.d.
Phloridzin	n.d.	0.3 ±0.1
Procianidine B2	n.d.	0.2 ±0.1
Phloretin	n.d.	n.d.
Quercetin	n.d.	n.d.

Table 9: Polyphenolic content of: *in vitro* digested Annurca Apple Extract (digAAE), *in vitro* digested Limoncella Apple Extract (digLAE); * mg/100g of DW.
Values are reported as mean ± s.d.

2.2.3.3 WNT pathway activity measurement using the TCF-GFP constructs

The activity of the apple extracts on WNT/β-catenin signaling was assayed in a reconstituted recombinant system. I used, as a biological platform, human embryonic HEK293 cells, transiently expressing both the WNT receptor Frizzled 4 (FZD4) and a WNT pathway reporter DNA construct. Three different WNT reporter constructs were used. The first, hereinafter referred to as TCF-wt GFP, presents the coding sequence of GFP under the control of an optimized WNT pathway responsive promoter. In the second, hereinafter referred to as TCF-mut GFP, the WNT responsive promoter was mutagenized to become unresponsive to WNT. Finally, a third reporter construct (cmv GFP) presents the coding sequence of GFP under the control of a constitutive cmv promoter. The TCF-mut GFP and cmv GFP constructs were here considered as negative controls, and used to monitor WNT unrelated change in GFP expression. Activation of canonical WNT pathway

upon treatment with the FZD4 agonist WNT-5A induces GFP expression and increases the fluorescence of the cells (Figure 23A).
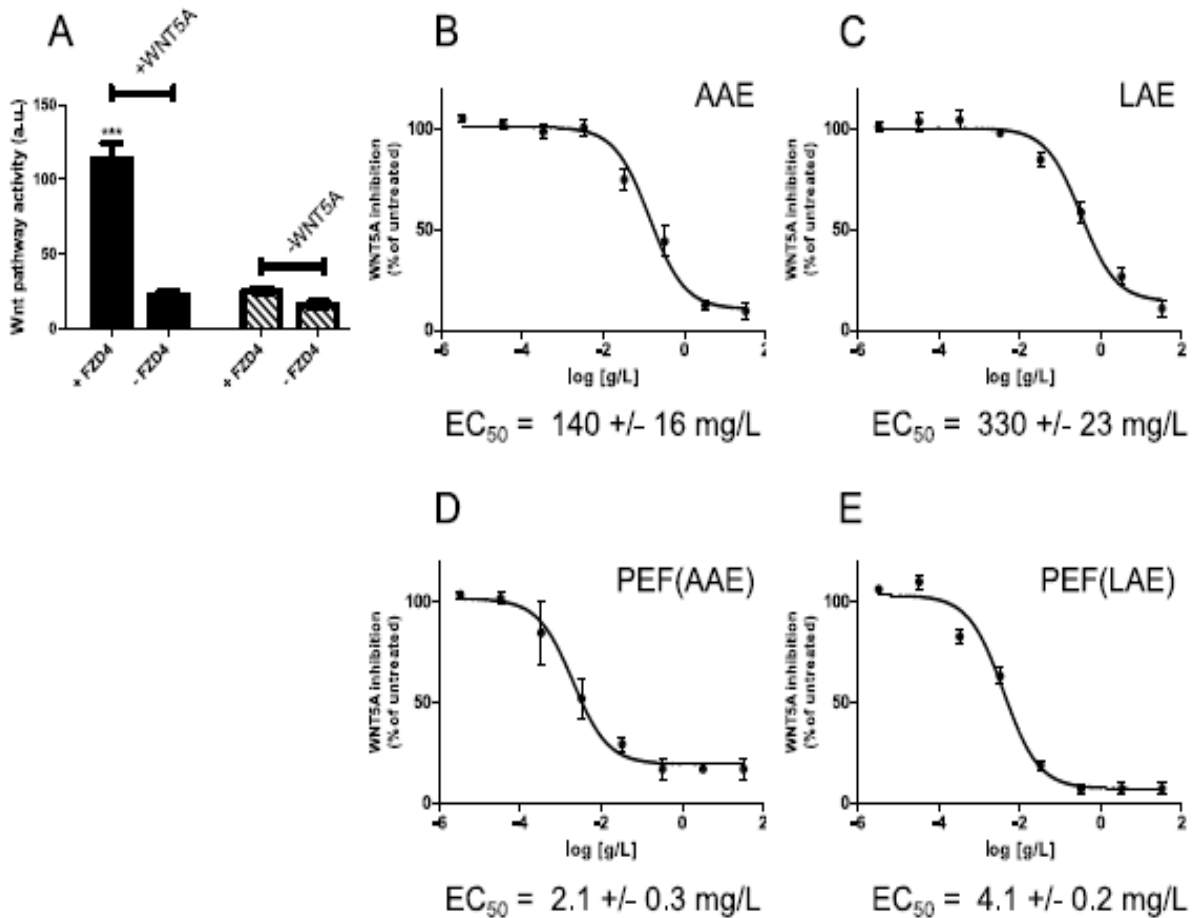

Fig. 23: AAE and LAE act as WNT pathway inhibitors mainly due to their polyphenolic content. (A). HEK293 cells were transfected with cDNA coding for the WNT reporter GFP construct and the WNT receptor FZD4. Activation of the pathway by WNT5A (a FZD4 agonist) induces expression of GFP. The histogram shows the increase in WNT pathway activity after treatment with WNT5A in the presence or in the absence of the WNT receptor FZD4 (B-C) Dose-response curves for AAE and LAE modulation of WNT/β-catenin pathway in HEK293 cells co-expressing FZD4 and the WNT reporter construct. Values indicate changes in GFP expression (expressed as percentage of change over untreated samples).(D-E) Dose-response curves for PEF(AAE) and PEF(LAE) modulation of WNT/β-catenin pathway. In A values are reported as mean +/- s.e.m (n=5). ANOVA testing *** $P<0.05$. IN B-E values are reported as mean +/- s.d. (n=3). EC_{50} values for each sample are shown under the corresponding graph and are reported as mean +/- s.e.m (n=5).

AAE and LAE both worked efficiently as WNT inhibitors and reduced WNT activity elicited by WNT5A. EC_{50} of WNT pathway inhibition were of 140 +/- 16 mg/L and 330 +/- 23 mg/L for AAE and LAE, respectively (Figure 23B-C). The WNT inhibitory activity

of both the extracts mainly depends on their flavonoid content. AAE and LAE fractions enriched in polyphenols, PEF(AAE) and PEF(LAE)) (Table7) presented WNT inhibitory potency increased compared to total fresh fruit extracts (2.1 +/- 0.3 mg/L and 4.1 +/- 0.2 mg/L for PEF(AAE) and PEF(LAE), respectively) (Figure 23D-E). The WNT inhibitory activity of both full extracts and polyphenolic fractions cannot be ascribed to their toxicity on cells, that occurs at 10g/L (data not shown), a concentration higher than their EC_{50} of inhibition of the WNT pathway activity.

I thus moved to identify which polyphenols were mostly contributing to WNT inhibitory activity of the apple extracts (Figure 24 and Table10). Pure Epicatechin and Catechin showed very weak inhibition of the WNT5A elicited TCF/LEF activity. Chlorogenic acid presented a WNT inhibithory activity of 3.4 ± 0.2 μM. Rutin and Isoquercetin presented EC_{50} of inhibition at 1.8 ± 0.1 μM and 3.1 ± 0.4 μM, respectively The dihydrochalcone Phloridzin presented EC_{50} at 12.9 ± 0.5 μM while Procianidine B2 of 1.4 ± 0.3 μM . Considering their low abundance in both the apple extract, none of the abovementioned molecules would reach WNT inhibitory effective concentrations in AAE and LAE solutions diluted at their EC_{50} (Table10). Thus, while polyphenols surely contribute to the WNT inhibitory activity of the apple extracts, none of the polyphenol by itself can account for the full activity of AAE and LAE, that seem to require, on the contrary, the presence of all the components of the polyphenolic fraction.

	[a] EC_{50}(M)	[b] M
Chlorogenic Acid	3.4 ± 0.2 x 10^{-6}	≈ 5.3 x 10^{-8}
Catechin	2.5 ± 0.3 x 10^{-4}	≈ 1.8 x 10^{-8}
Epicatechin	$>> 10^{-4}$	≈ 2.6 x 10^{-8}
Isoquercetin	1.8 ± 0.1 x 10^{-6}	≈ 7.8 x 10^{-9}
Rutin	3.1 ± 0.4 x 10^{-6}	≈ 2.6 x 10^{-9}
Phoridzin	1.2 ± 0.2 x 10^{-5}	≈ 1.1 x 10^{-8}
Procianidine B2	1.4 ± 0.3 x 10^{-6}	≈ 1.9 x 10^{-8}

Table 10: [b] EC_{50} of WNT pathway inhibition of the indicated compounds. Values are reported as mean ± s.e.m (n=3)[a] Molarity of the indicated compounds in a solution of LAE at its EC_{50} concentration.
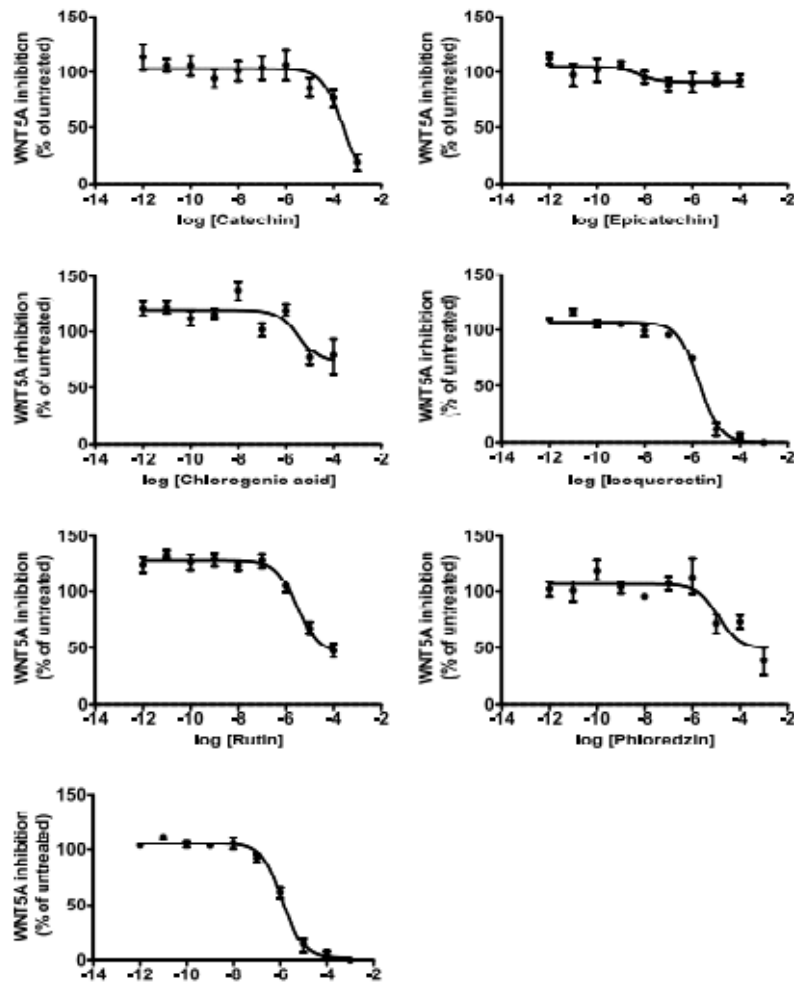
Fig. 24: **WNT inhibitory activity of apple polyphenols.** Dose-response curves for the indicated compounds modulation of WNT pathway in HEK293 cells. Values as reported as mean +/- s.d. (n=3). EC_{50} values for each compound are reported in Table10.

I thus moved to identify the WNT pathway branches inhibited by AAE and LAE. This is very important especially when searching for therapeutic agents to use for FAP patients. Since APC is a midstream component of the WNT pathway, its mutations make manipulation of upstream components of the WNT pathway therapeutically ineffective. To be active in FAP patients, WNT inhibitors should act either downstream to APC or on "non canonical" WNT pathway branches. One of the most active non-canonical branches positively contributing to WNT signaling is the one involving the EGF Receptor (EGFR). Once activated, the EGFR pathway bypasses APC and leads, via AKT, to β-catenin activation. I thus challenged AAE and LAE to compete with LiCl and EGF, two inducers of the WNT pathway, both acting downstream to APC. LiCl binds directly to GSK-3β and inhibits the β-catenin destruction complex. In contrast, EGF activates the EGFR pathway, that, via AKT, promotes β-catenin detachment from the Plasma Membrane and its nuclear translocation. In this biological system, both LiCl and EGF induced, in a dose-response

manner, GFP expression in HEK293 transfected with TCF-wt GFP (Figure 24A–C). On the contrary, they both did not have an effect on cells transfected either with TCF-mut GFP or with cmv GFP (Figure 24A–C). AAE and LAE failed to inhibit activation of the WNT pathway induced by 15 and 30 mM LiCl. However, they both reduced WNT pathway activity induced by 5 and 10 mM LiCl (the results for LAE are depicted in Figure 3B). Moreover, at all the tested concentrations, the extract abolished the WNT stimulatory activity of EGF [34] (Figure 24D). These results prove that the apple extracts inhibit WNT pathway activation induced by LiCl and EGF and are thus suitable WNT inhibitors for FAP cells carrying APC mutations.
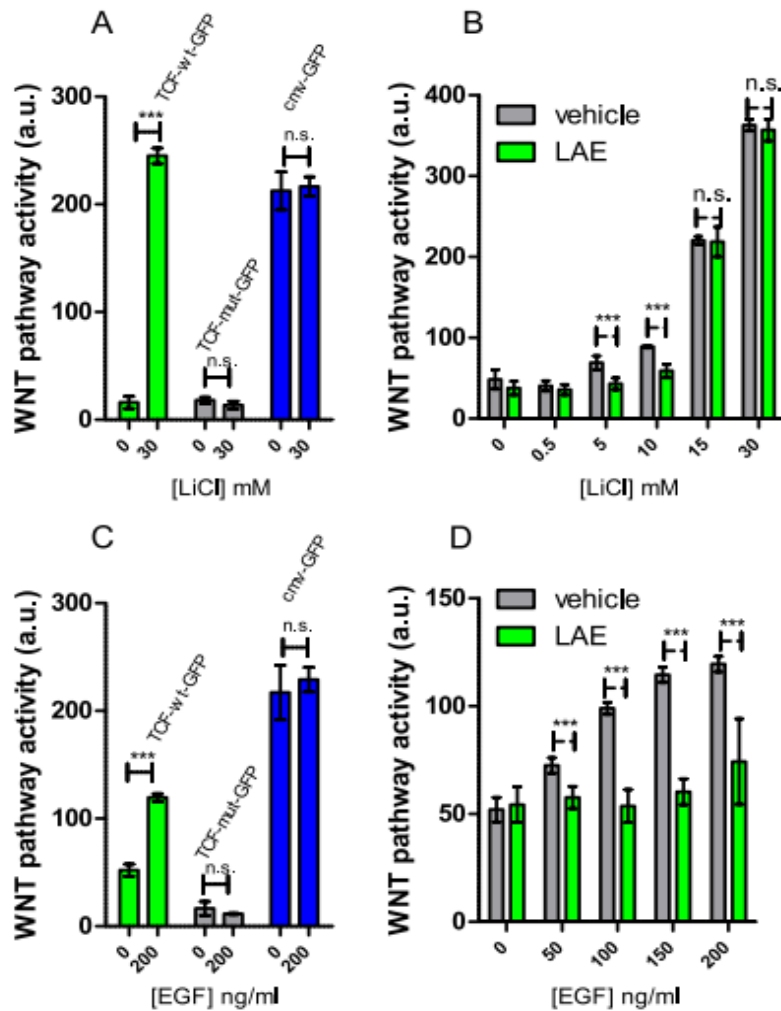
Fig 24: .LAE inhibits the WNT pathway, acting downstream to APC. (A) The histogram shows the WNT pathway activity induced by LiCl (30 mM) in HEK293 cells transfected with TCF-wt GFP (green bars), TCF-mut GFP (grey bars) and cmv GFP (blue bars); (B) WNT pathway activity of cells treated with the indicated concentration of LiCl in the presence (green bars) or in the absence (grey bars) of LAE (400 mg/L); (C) WNT pathway activity induced by EGF (200 ng/mL) in HEK293 cells transfected with TCF-wt GFP (green bars), TCF-mut GFP (grey bars) and cmv GFP (blue bars); (D) WNT pathway activity of cells treated with the indicated concentration of EGF in the presence (green bars) or in the absence (grey bars) of LAE (400 mg/L). Values are reported as mean $\pm$ SEM (n = 5). *** $p < 0.05$, n.s. indicates non-statistical.

This was further proved by testing the WNT inhibitory activity that AAE and LAE exerted on CaCo-2 cells, a colon cancer cell line presenting mutation in the APC gene and commonly used as *in vitro* cell culture prototype for FAP cells (Figure 25B). CaCo-2 cells were transiently transfected with the WNT reporter GFP construct. Due to the APC mutation, these cells have a constitutively active WNT pathway as shown by the number of GFP positive cells. Upon treatment with AAE or LAE, the number of GFP expression decreased, indicating inhibition of the WNT pathway.

Finally, AAE and LAE were tested in an *ex vivo* system of FAP cells. Human colon biopsies were cultured *in vitro* soon after their resection from FAP patients (Figure 25C). In unsupplemented medium, these *ex-vivo* samples duplicate for up to two days until their growth rapidly arrest. Treatment with AAE or LAE resulted in a decreased survival of the *ex-vivo* cultures.
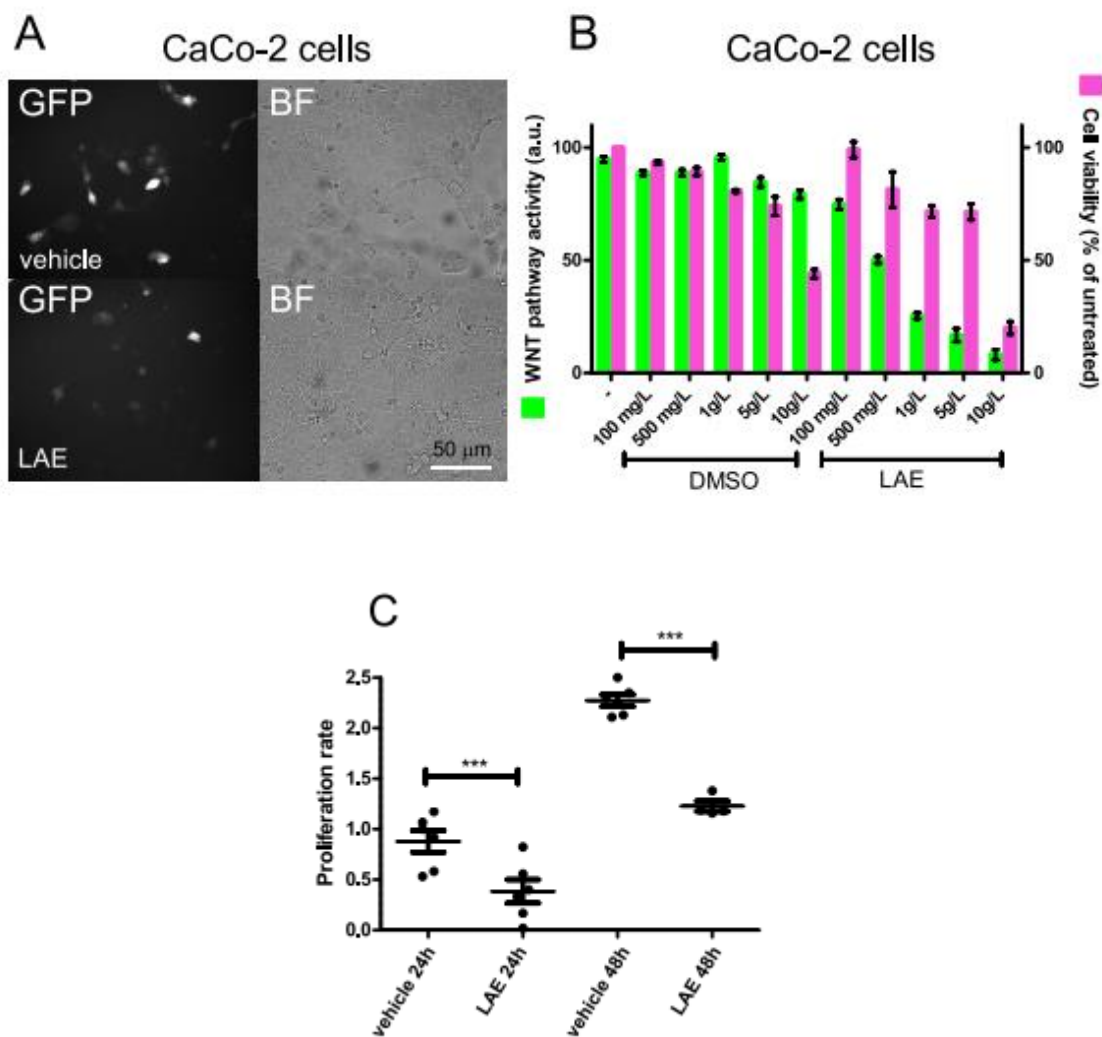
Fig. 25: AAE and LAE act as WNT inhibitors in CaCo-2 cells. (A) Activity of the WNT reporter construct TCF-wt GFP in CaCo-2 cells cultivated for 48 h in the presence or in the absence of LAE (400 mg/L) (representative of five experiments) (BF = Bright Field; scale bar is shown); (B) WNT pathway activity (green bars) and cell viability (magenta bars) of CaCo-2 cells transfected with TCF-wt GFP and cultivated in the absence (−) or in the presence of the indicated concentration of LAE (or of the corresponding dilution of DMSO). Values on the left axes indicate changes in GFP expression (a.u.). Values on the right axes indicate changes in cell viability expressed as percentage of untreated cells. (C) Proliferation rate of human colonic biopsies in culture medium supplemented with LAE or with vehicle. Values are expressed as mean +/- s.e.m (n=9). Anova testing. *** P<0.05.

In an attempt to formulate a nutraceutical product, the industrial versions, IndLAE and IndAAE were tested. IndLAE presents a WNT inhibitory activity increased compared to the one got at laboratory low-scale (EC$_{50}$ of WNT pathway inhibition were of 47.4 +/- 0.9 mg/L) (Figure 26A).
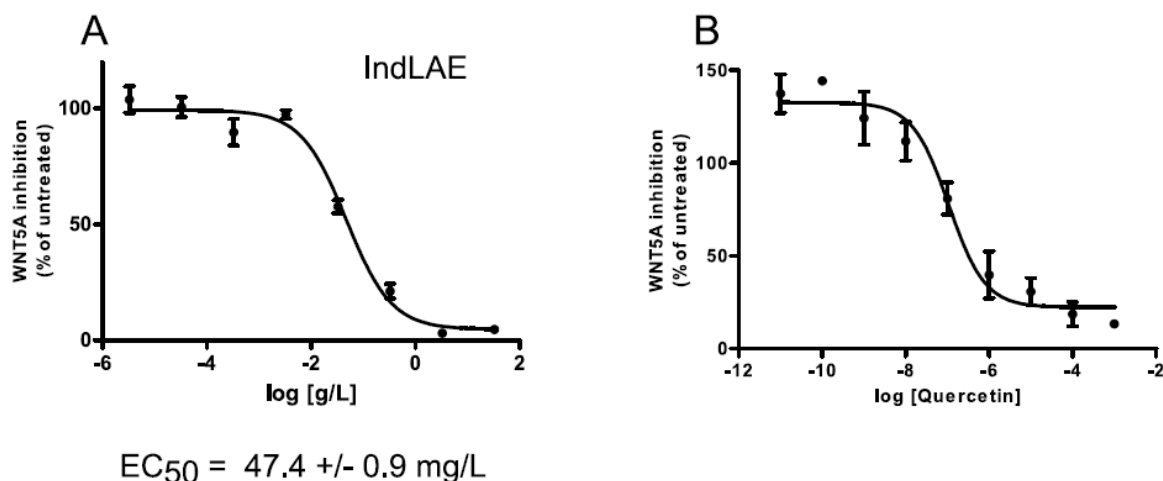
Fig. 26. WNT inhibitory activity of IndLAE and Quercetin. A) Dose-response curves for Ind(LAE) modulation of WNT/β-catenin pathway. Values are reported as mean +/- s.d. (n=3). EC$_{50}$ value for Ind(LAE) is shown under graph and is reported as mean +/- s.e.m (n=5). **B)** Dose-response curves for Quercetin modulation of WNT pathway in HEK293 cells. Values as reported as mean +/- s.d. (n=3). EC$_{50}$ values for Quercetin is reported in Table2.

HPLC-DAD analysis of the flavonoid content of the IndLAE did not reveal major change in the amount of poliphenols compared to AAE and LAE. However, I realized the presence of a discrete amount of Quercetin a molecule that was absent in the apple extracts and that it probably arose from the conversion of quercetin glycosides into their aglycone form. Tested in WNT inhibitory assay, Quercetin presented a strong inhibitory activity on the WNT pathway (EC$_{50}$ at 110 ± 5 nM) compared to the other polyphenols alone suggesting

that the molecule is definitely contributing to the WNT inhibitory activity of the industrial extract.

To achieve WNT inhibitory activity in the colon, apple polyphenolic must be bioaccessible, i.e. they should be extracted from the food matrix, reisist to gastrointestinal (GI) digestion and reach the colon-rectal section of the intestine. However, GI digestion may affect substantially native apple polyphenolic patterns and polyphenol concentrations as well as induce drastic structural changes of the food constituents. During GI digestion polyphenols may be further degraded (such as Anthocyanins in the small intestine), or hydrolyzed, deglycosylated or cleaved by esterases. To measure the bio-accessibility of the WNT inhibitory pool of apple polyphenol we subjected apple extracts to *in vitro* simulated digestion. Upon digestion, AAE and LAE drastically lost their WNT inhibitory activity (Figure 27A-B). As expected, *in vitro* digestion decreased the overall polyphenolic content of around 40-50%. This results indicate that very likely the polyphenols ingested by assumption of fresh fruit or fresh fruit extract will not reach the colon-rectal segment of the intestinal tract and strongly points towards the encapsulation of the extract in gastro-protect tablets as essential requirement to preserve apple extract WNT inhibitory activity.

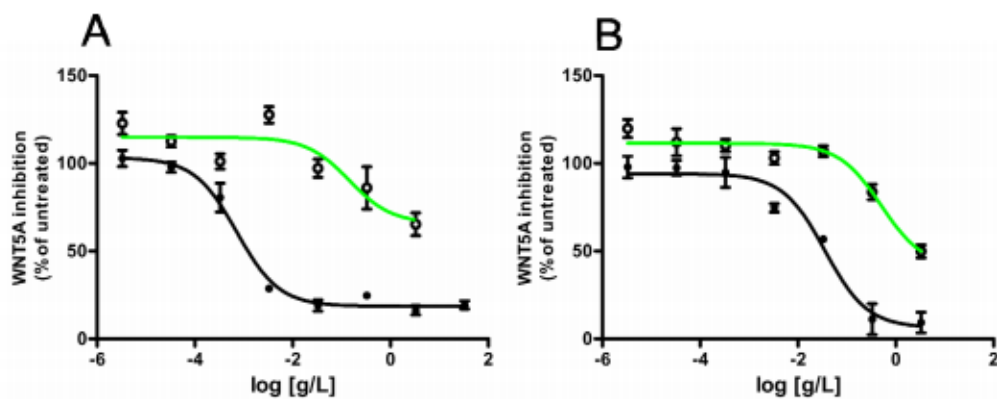**Fig 27:** *In vitro* **simulated gastrointestinal digestion reduced WNT inhibithory activity of AAE and LAE.** Dose-response curves for AAE **(A)** and LAE **(B)** modulation of WNT/β-catenin pathway before (continuous lines) or after *in vitro* simulated digestion (dotted lines). Values are reported as mean +/- s.d. (n=3).

2.2.4 Discussion

Polyphenol consumption has been related to several health beneficial effects, such as reduced incidence of cancer and cardiovascular diseases. The diet is the principal human source of polyphenolic compounds. Polyphenol-rich foods include fruits, vegetables, and whole grains. Here was shown that Apple Extracts from both Annurca (AAE) and Limoncella (LAE) possess WNT inhibitory activity. Was proven that both AAE and LAE inhibit the WNT pathway affecting a non-canonical branch involving PI3K and thus, bypassing APC for their activity, they are potentially suitable for the treatment of FAP patients.

AAE and LAE WNT inhibitory activity can be partially ascribed to the polyphenolic fraction these apples are particularly rich in. However, none of the constituent of the polyphenolic fraction, tested as pure molecule, exerted strong WNT inhibitory activity, suggesting the importance of the entire pool of flavonoids for the mixture to exert its action. This confirms what is nowadays an accepted concept of nutraceutical studies i.e. that phyto-complexes are always more active than pure compounds. This could be in part ascribed to the protection that the anti-oxidant environment of the polyphenolic mixture is able to exert on each of its component. This is an important aspect to underline considering that clinical trials are mainly focused on supplementation of single compounds, whereas the epidemiologic evidence supports a scenario where mixture of bioactive compounds, as they naturally occur in the diet, may act synergistically to be effective.

We here show that alimentary grade large-scale manufacturing alters the flavonoid composition of LAE drastically increasing the WNT inhibitory activity of the extract. This could be ascribed to the presence of Quercetin, a potent β-catenin inhibitor absent in the original extract and probably originated by the conversion of Quercetin-glycosides into their aglycone form. Probably, it process hasn't happened during indAAE industrial preparation, resulting in not liberation of Quercetin free form, so the Annurca version of industrial extract shows a less activity on WNT inhibitory pathway (data not shown). Anyway , this result should encourage researchers in testing the activity of the extract after large scale manufacturing because the latter drastically alters composition of the extract even if this event, as we have here shown, does not always have to be detrimental for the properties of the extract.

Finally was shown, that both LAE and AAE loose activity upon in vitro simulated gastrointestinal digestion as result of a severe loss of polyphenols. This strongly points towards encapsulation in gastro-resistant tablets for example enteric coatings as essential requirement to preserve apple extract WNT inhibitory activity in the colon-rectal section of the intestinal tract.

2.2.5 Conclusion

Apple extracts inhibit WNT pathway in colon cells carrying FAP mutations and represent a valid nutraceutical alternative for the treatment of this pathology. Enteric coating is advisable to preserve the activity of the extracts in the colon-rectal section of the digestive tract.

Capther 3

Potential medical device

A potential medical device application from Annurca apple polyphenolic extracts : A strong inhibition of in vitro micellar cholesterol solubility by a novel Annurca apple based nutraceutical formulation: a NMR study.

3.1 Introduction

Previous studies have extensively demonstrated that Annurca polyphenolic extract is able to positively influence cholesterol metabolism. Specifically, *in vitro* experiments have proved its capacity to enhance Apolipoprotein A1 expression, the main protein constituent of nascent discoidal high density lipoprotein cholesterol (HDL-C), and favor low density lipoprotein cholesterol (LDL-C) receptor binding activity, in HepG2 cell lines (Tenore et al., 2013; 2014). Later, Annurca polyphenolic extract has been administered to mildly hypercholesterolemic healthy subjects under the form of a nutraceutical formulation, according to a randomised trial which has confirmed the previous *in vitro* results in terms of increase in plasma HDL-C and decrease in LDL-C levels (Tenore et al., 2017). These studies would indicate oligomeric procyanidins, mainly the dimeric procyanidin B2, as the major responsible for such effects on both *in vitro* and clinical HDL-C and LDL-C parameters. Nevertheless, the molecular mechanisms underlying these effects are still scarcely known.

It has been recently reported that green tea catechins are the most effective polyphenolic compounds in inhibiting the micellar cholesterol solubility in the small intestine. This effect may be the cause of the increased fecal excretion of cholesterol observed in experimental animals and hypocholesterolemic activity in experimental animals and humans (Kobayashi et al.,2005; Kajimoto et al., 2003; Kajimoto et al 2006). Ogawa et al. 2016 have tried to clarify this mechanism, by performing a nuclear magnetic resonance (NMR) study to investigate the interaction between tea catechins and cholesterol micelles (Ogawa et al., 2016). The data indicated the ability of epigallocatechin gallate (EGCG) to lower the solubility of phosphatidylcholine (PC) and cholesterol in micellar solutions due to their elimination from the micelles by interaction between taurocholic acids and EGCG.

Considering that oligomeric procyanidins occurring in apples consist of catechin monomeric units, it can be hypothesized that the lower molecular weight compounds (dimers, trimers, …) may have a similar mechanism of action to that of monomeric catechins as regards their effects on micellar cholesterol solubility. To clarify this aspect, the aim of the present work will be to evaluate the *in vitro* effect of Annurca apple polyphenolic extract on micellar cholesterol solubility in a model reproducing the duodenal environment. Specifically, a main goal will be to elucidate the molecular mechanism of action by performing a NMR study for the interaction between Annurca polyphenols and bile acids. The attention will be focused on the effects of an industrial nutraceutical product formulated by using Annurca water extract microencapsulated with maltodextrins (AMD).

3.2 Materials and methods

3.2.1 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 B$_2$, 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 methyl alcohol were of HPLC grade (Carlo Erba, Milano, Italy). Sodium taurocholate, phosphatidylcholine, cholesterol, NaCl, and sodium phosphate, were purchased from Sigma Chemical Co.

3.2.2 Fruit collection and sample preparation

Annurca (*M. pumila* Miller cv Annurca) apple fruits were collected in Valle di Maddaloni (Caserta, Italy) in October 2016 when fruits had just be harvested (green peel). Fruits were reddened, following the typical treatment (Lo Scalzo et al., 2001) for about 30 days, and then analysed. Other two apple varieties analysed in this study, Pink Lady (PL) (*M. pumila*

Miller cv Pink Lady), and Golden Delicious (GD) (*M. pumila Miller* cv Golden Delicious), were acquired in a local supermarket. Lyophilised apples (10 g) were treated with 100 mL of 80% methanol (0.5% formic acid) for 24 h at 4 °C to extract phenolic compounds. After centrifugation, the supernatant was slowly filtered through an Amberlite XAD-2 column packed as follows: resin (10 g; pore size 9 nm; particle size 0.3-1.2 mm; Supelco, Bellefonte, PA, USA) 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 for sugar and other polar compound removal. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen.

3.2.3 Industrial preparation of Annurca nutraceutical product (AMD)

AMD consisted of Annurca apple extract microencapsulated with maltodextrins. Large-scale production of AMS was accomplished by MB-Med Company (Turin, Italy). Apples were extracted with water at 35 °C. After centrifugation, the extract was spray-dried in combination with maltodextrins, obtaining a fine powder with a maltodextrins/extract ratio 4:1.

3.2.4 HPLC-DAD/ESI-MS analysis

Extracts from the three different apple varieties and AMD were solubilized with 1% formic acid. Analyses were run on a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) provided with photodiode array detector (DAD). The column selected was a Kinetex® C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA). The analyses were performed at a flow rate of 1 mL/min, with solvent A (2% acetic acid) and solvent B (0.5% acetic acid in acetonitrile and water 50:50, v/v). After a 5 min hold at 10% solvent B, elution was performed according to the following conditions: from 10% (B) to 55% (B) in 50 min and to 95% (B) in 10 min, followed by 5 min of maintenance. Flavonols, procyanidins, dihydrochalcones, flavanols and hydroxycinnamic acids were monitored at 280 nm and anthocyanins at 520 nm. For quantitative analysis, standard curves for each polyphenol standard were prepared over a concentration range of 0.1-1.0 μg/μL with six different concentration levels and duplicate injections at each level. The identity of

polyphenols was confirmed by LC-ESI/MS experiments and data were compared to those of commercial standards. The same chromatographic apparatus and conditions (HPLC system, gradient elution, column, temperature) was coupled to an Advion Expression mass spectrometer (Advion Inc., Ithaca, NY) equipped with an Electrospray (ESI) source. Mass spectra were recorded from m/z = 50 to 1200, both in negative and in positive ionization mode. The capillary voltage was set at − 28 V, the spray voltage was at 3 kV and the tube lens offset was at − 10 V in negative ion mode, while the capillary voltage was set at 34 V, the spray voltage was at 3.5 kV and the tube lens offset was at 55 V in positive ion mode. The capillary temperature was 275 °C. Data were acquired in full scan and SIM modes.

3.2.5 *In vitro effects of apple polyphenolic extracts on cholesterol micellar solubility*

The effects of purified apple polyphenolic extracts and AMD on the micellar solubility of cholesterol were examined as described by previous authors (Ikeda et al; 2003). A bile salt micellar solution containing 6.6 mmol/L sodium taurocholate, 0.6 mmol/L PC, 0.5 mmol/L cholesterol, 132 mmol/L NaCl, and 15 mmol/L sodium phosphate (pH 6.8) was prepared by sonication and stored at 37 °C for at least 24 h. Aliquots (100 μL) of apple polyphenolic extract solutions and AMS (100 μg/mL) in deionized water stored at 37 °C were added to the 3 mL micellar solutions. The mixture was incubated for 1 h at 37 °C. The solution reveals an evident opalescence due to cholesterol precipitation (Fig. 28). The supernatant was passed through a 0.2 μm syringe filter (25 mm; GDD/X; Whatman Inc., Piscataway, NJ, USA), and the cholesterol content originating from the phospholipids was analysed by gas chromatography using an SPB-1 column (Supelco, Bellefonte, PA, USA) and a DB-WAX column (Agilent Technologies, Santa Clara, CA, USA), respectively.
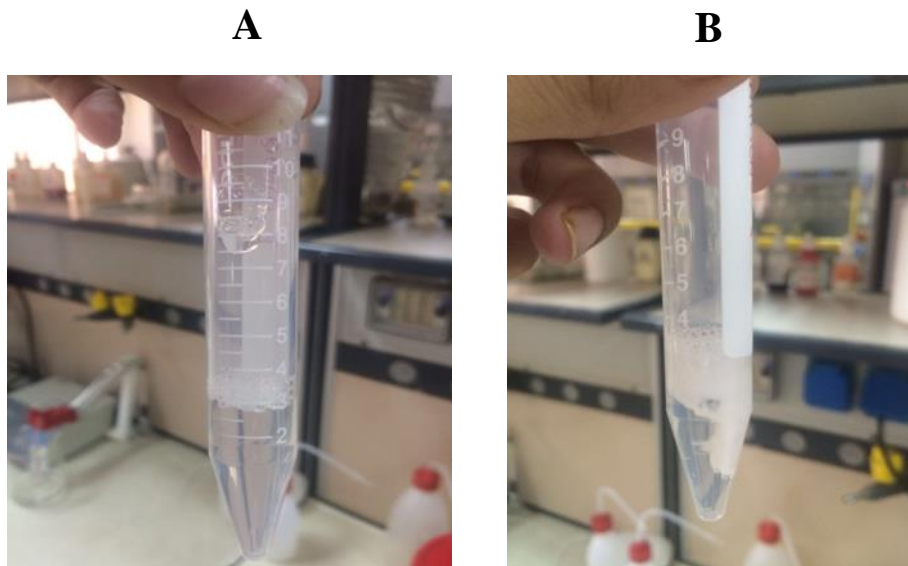
Fig. 28: (A) Micellar solution of cholesterol; (B) Precipitation of cholesterol after addition of apple polyphenolic extract solutions.

3.2.6 Statistics

All of the experimental data were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was carried out 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

3.3 Results and Discussion

3.3.1 Effects of apple extracts and AMD on in vitro cholesterol micellar solubility

The effects of the three apple extracts and AMD on *in vitro* micellar solubility of cholesterol was tested. As shown in Figure 29, Annurca was able of the highest influence on cholesterol solubility among the apple samples.
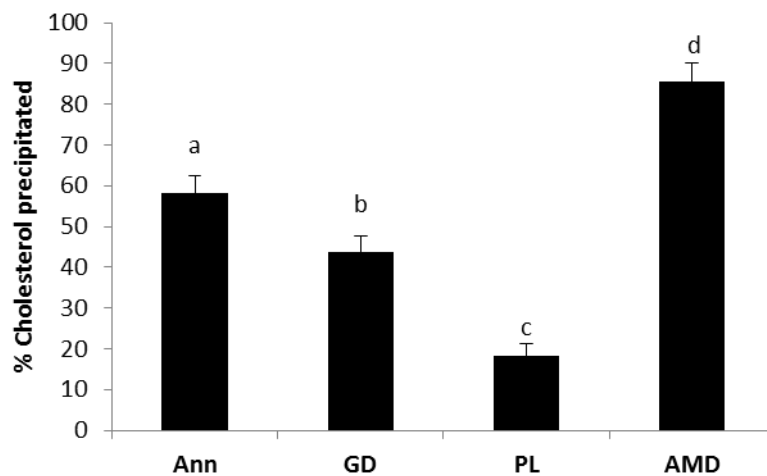
Fig. 29: Effect of apple sample extracts and Annurca nutraceutical formulation (AMD) on micellar solubility of cholesterol *in vitro*. Ann: Annurca; GD: Golden Delicious; PL: Pink Lady.Data are means ± SE of triplicate experiments. Results were significantly different at a level of P = 0.001. [abcd]Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test.

Previous authors have demonstrated that the addition of green tea catechins with a galloyl moiety to a bile salt micellar solution precipitated cholesterol and decreased the micellar solubility of cholesterol in a dose-dependent manner (Ikeda et al; 1992). In contrast, green tea catechins without a galloyl moiety did not precipitated cholesterol. When purified EGCG was added to the bile salt micellar solution, the amount of EGCG precipitated from the micellar solution was highly positively correlated with the amount of cholesterol precipitated. These results strongly suggested that EGCG eliminated the cholesterol from the bile salt micelles and co-precipitated with cholesterol. Later, Kobayashi et al. (2014) showed for the first time that green tea catechins with a galloyl moiety, but not those without a galloyl moiety, eliminated not only cholesterol but also PC from bile salt micelles (Kobayashi et al., 2014). When bile salt micelles contained a phospholipid other than PC, neither cholesterol nor the phospholipid was eliminated by the addition of EGCG. These observations suggest that green tea catechins with a galloyl moiety interact with PC and the binding of EGCG to PC decreases PC solubility. The same authors have confirmed these results by performing NMR studies on the same *in vitro* models of cholesterol vesicles and green tea catechins. An intermolecular nuclear Overhauser effect (NOE) was observed between PC and EGCG in bile salt micelles with EGCG added, but not between

cholesterol and EGCG. These observations strongly suggested that EGCG decreases the micellar solubility of cholesterol via specific interaction with PC. Apple procyanidins are oligomeric compounds consisting of catechin monomeric units. Specifically, dimeric procyanidins are the most structurally similar to the green tea catechins with a galloyl moiety (Figure 30).

	R_1	R_2
Procyanidin B1	OH	H
Procyanidin B2	H	OH

	R_1	R_2
Procyanidin B3	OH	H
Procyanidin B4	H	OH

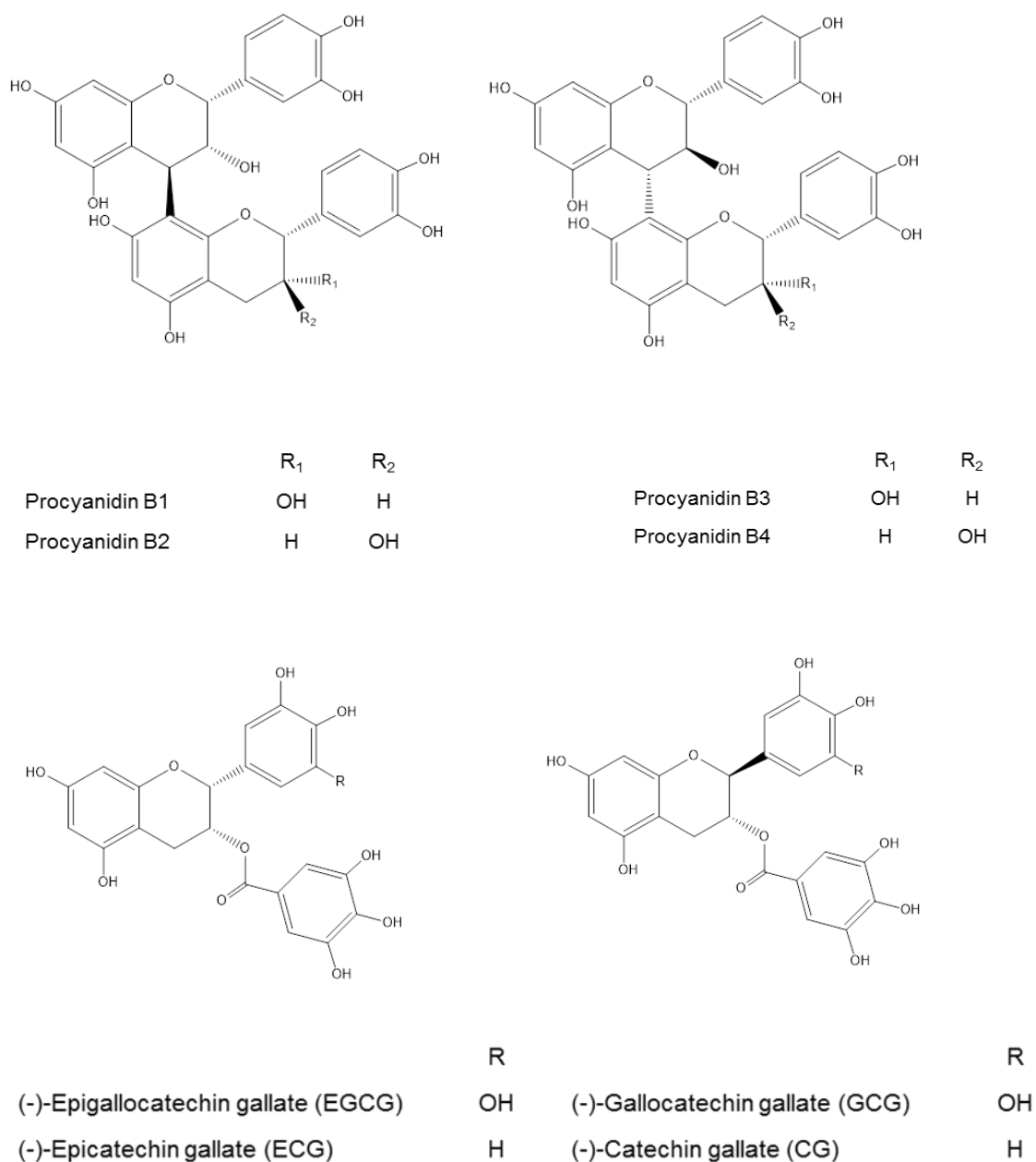|	R		R
(-)-Epigallocatechin gallate (EGCG)	OH	(-)-Gallocatechin gallate (GCG)	OH
(-)-Epicatechin gallate (ECG)	H	(-)-Catechin gallate (CG)	H

Fig. 30: Chemical structures of apple dimeric procyanidins and green tea galloylated catechins.

Therefore, it could be hypothesized that these dimers may play a major role as regards our observed effects of apple extract samples on *in vitro* cholesterol micellar solubility (Figure 28). This effect was related to different quantity of dimeric procyanidins in the extracts tested. The results of LCMS analysis are reported in the following table

Table 11. Polyphenolic composition of apple extracts and nutraceutical formulation (AMS) determined by HPLC-MS

	Annurca	Pink Lady	Golden Delicious	AMS
Chlorogenic acid	106.2 ± 10.2	100.4 ± 8.7	104.2 ± 7.2	0.04 ± 0.001
p-Coumaroylquinic acid	ND	ND	ND	ND
[+]-Catechin	62.4 ± 4.7	59.7 ± 4.2	57.2 ± 3.7	0.3 ± 0.02
[−]-Epicatechin	68.1 ± 5.2	60.3 ± 4.6	59.4 ± 4.0	0.3 ± 0.02
Procyanidin B_1	6.52 ± 0.5	4.62 ± 0.7	5.71 ± 0.9	0.2 ± 0.01
Procyanidin trimer	12.6 ± 1.9	53.8 ± 3.9	10.1 ± 1.7	0.3 ± 0.01
Procyanidin B_2	16.0 ± 1.8	6.01 ± 0.8	10.2 ± 1.7	0.04 ± 0.001
Procyanidin trimer (isomer)	21.3 ± 2.1	63.7 ± 5.1	16.1 ± 1.8	0.2 ± 0.02
Cyanidin-3-*O*-galactoside	0.41 ± 0.04	ND	ND	ND
Rutin (Quercetin-3-*O*-rutinoside)	42.7 ± 3.5	38.4 ± 2.8	36.1 ± 2.2	0.06 ± 0.002
Hyperin (Quercetin-3-*O*-galactoside)	109.1 ± 11.3	100.4 ± 9.2	98.1 ± 8.1	ND
Isoquercitrin (Quercetin-3-*O*-glucoside)	72.6 ± 4.3	69.5 ± 4.4	67.1 ± 3.5	ND
Reynoutrin (Quercetin-3-*O*-xyloside)	76.8 ± 3.8	70.0 ± 4.5	68.4 ± 3.4	ND
Guajaverin (Quercetin 3-*O*-arabinopyranoside)	72.5 ± 3.7	67.2 ± 3.8	64.2 ± 2.7	ND
Avicularin (Quercetin 3-*O*-arabinofuranoside)	76.6 ± 4.1	70.2 ± 4.1	68.2 ± 3.6	ND
Quercetin-O-pentoside	43.8 ± 2.7	42.6 ± 3.2	40.0 ± 3.2	ND
Quercitrin (Quercetin-3-*O*-rhamnoside)	72.9 ± 3.6	65.6 ± 4.0	63.3 ± 4.0	ND
Phloretin-2-*O*-xyloglucoside	64.4 ± 2.5	62.2 ± 3.8	60.3 ± 3.8	0.06 ± 0.003
Phloridzin (phloretin-2-*O*-glucoside)	65.3 ± 3.5	61.5 ± 3.6	59.1 ± 3.6	0.06 ± 0.002

Results were expressed as μg/mg DW ± SD ($n = 3$).
Results were significantly different at a level of $P = 0.001$.
ND: not detected.

These results showed that the Annurca polyphenolic extract was richer in dimeric procyanidins than other extract analysed, thus probably these compouds are responsible of this in vitro effect. This concept could be confirmed by a significant statistical correlation (R^2 = 0.9511) between dimeric procyanidin content of apple extracts and the observed cholesterol precipitation (Figure 31).
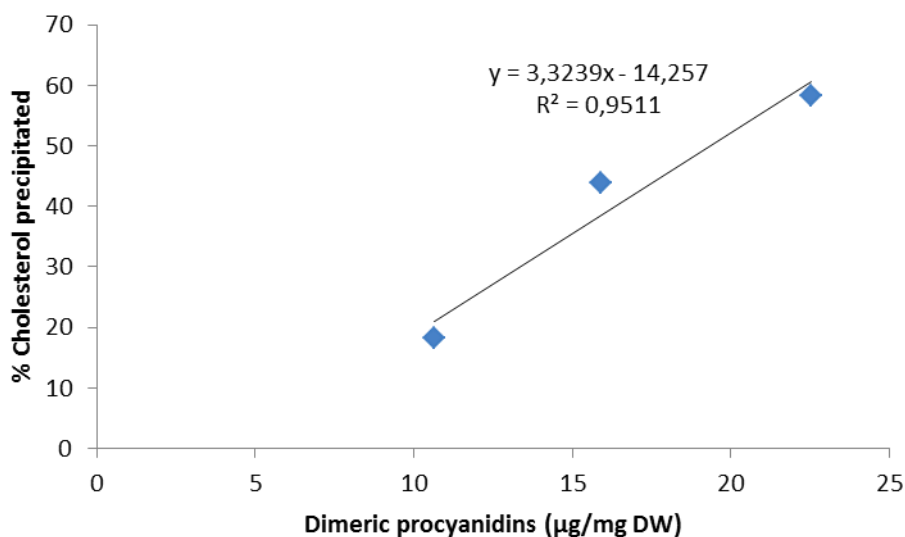

Fig. 31: Statistical correlation between dimeric procyanidin content of apple extracts and effect of apple extract solutions on *in vitro* solubility of micellar cholesterol.

Very interestingly, AMD exerted the strongest effects among all of the samples tested, although its much lower polyphenolic content than the other samples (Figure 29). A possible explanation could be found in its same technological formulation. In fact, AMD consists of Annurca polyphenolic extract microencapsulated in maltodextrins. Such vehicle may facilitate the water dispersion of polyphenols which are well known to be characterised by a quite low solubility in an aqueous medium. It could be hypothesized that the three apple extracts, although much richer in polyphenolic compounds, allow a very low water accessibility of their constituents to the cholesterol vesicles, while the polyphenolic aliquot provided by AMD could be much more effective than that of the other samples. However, this aspect needs to be further clarified.

3.3.2 NMR study of interaction of apple extracts and AMD with bile acid micelles

Figure 32 shows the ^{1}H NMR spectrum of the bile acid micelles alone, and after the addition of equal amounts (100 µg/mL) of three apple extracts and AMD. Interaction of both apple extracts and AMD with the bile salt micelles resulted in a shift of a few signals of the micelles, among those the most influenced was the signal of the trimethylammonium group in phosphatidylcholine (Figure 32).
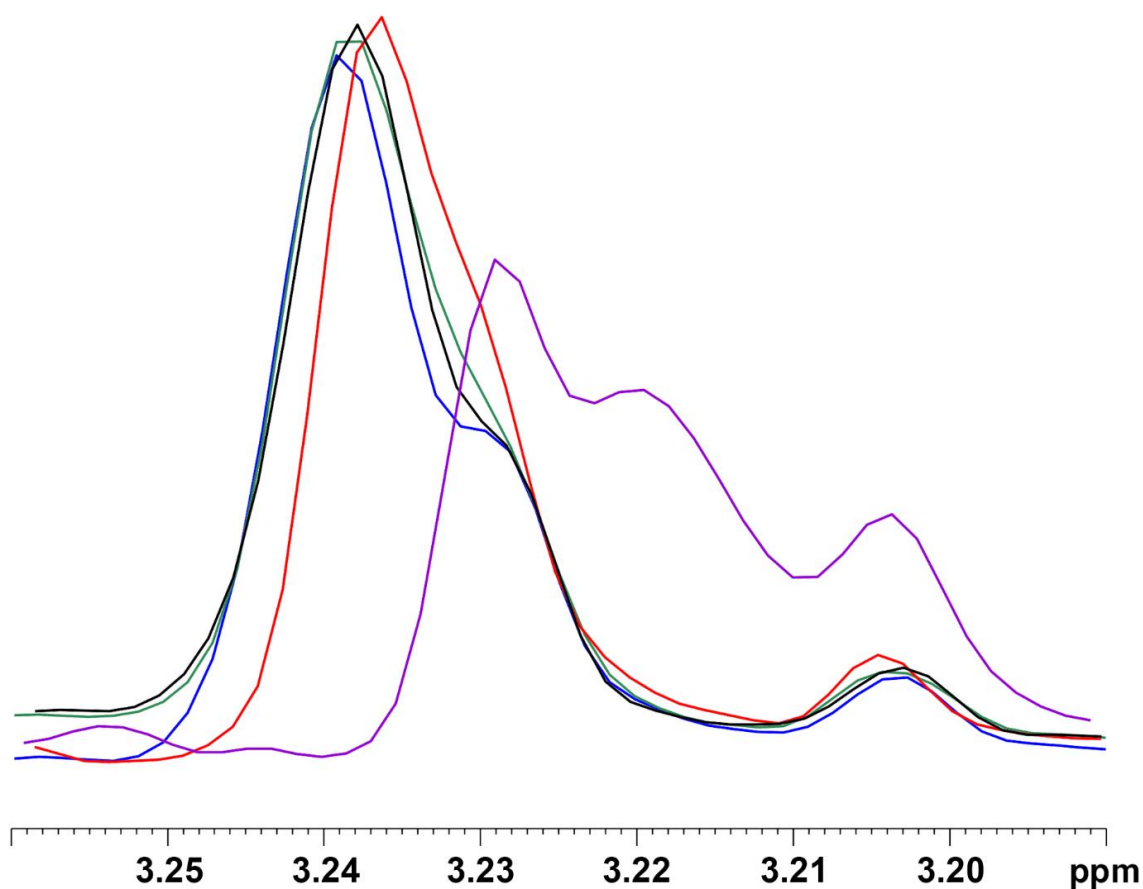
Fig. 32: Expanded region of the ^{1}H NMR spectrum of bile salt micelle (blue), after the addition of pink lady apple extracts (black), after the addition of golden apple extracts (green), after the addition of Annurca apple extracts (red), after the addition of AMD (violet).

The shift was more intense in case of Annurca (-3 ppb) than GD extract (-2 ppb), while PL extract had no effect on micelle proton signals. This result indicates that Annurca extracts have a higher content of metabolites able to interact with the bile salt micelle compared to the other two apple species. Interestingly, AMD provided the highest signal shift, in

accordance with the results obtained from the in vitro cholesterol precipitation (Figure 29). The relatively intense upfield shift of the trimethylammonium group in phosphatidylcholine is in accordance with the results of Kobayashi et al. (2014) who reported that polyphenolic constituents of green tea, in particular epicatechin gallate and epigallocatechin gallate, interact with bile acid micelles through the trimethylammonium group in phosphatidylcholine (Kobayashi et al., 2014).

The addition of apple extracts and AMD to the micelles also caused the upfield shift of the same NMR signal.To identify extract components that bind the bile salt micelles, I used nuclear magnetic resonance saturation transfer difference (STD) spectroscopy. The method relies on the possibility to selectively saturate protons of macromolecular system (receptor) by irradiating the spectral region containing resonances of the macromolecular system which is also free of any smaller molecule signals. Due to effective spin diffusion saturation quickly propagates across the entire receptor. If the smaller molecule ligand binds the receptor, saturation will also spread onto the ligand. The result will be that intensity of the ligand signal will be attenuated. Subtraction of resulting spectrum from the reference spectrum without saturation yields the STD spectrum containing only signals of the binding ligands. STD experiment was acquired on the sample containing the Annurca extracts and the bile salt micelles (Figure 33)

On-resonance irradiation frequency was set at 1 ppm where only micelle resonances are present. To guarantee the absence of direct irradiation on extracts signals, an appropriate blank experiment was also performed. As shown in Figure 33, only a few signals were present in the STD spectrum among those observable in the original 1H NMR spectrum of the Annurca extracts in the presence of bile salt micelles. Among the others, signals assignable to chlorogenic acid and its analogues (6.2, 7.0, and 7.4 ppm) were not visible in the STD spectrum indicating that these compounds do not interact with the micelle. In contrast, signals around 5.9 and between 7.0 and 6.5 ppm are clearly visible in the STD spectrum. Considering the possible chemical constituents of the Annurca extracts these signals are fully compatible with (epi)catechin or procyanidins. For comparison purposes, also the proton NMR spectrum of Procyanidin B2 is reported.
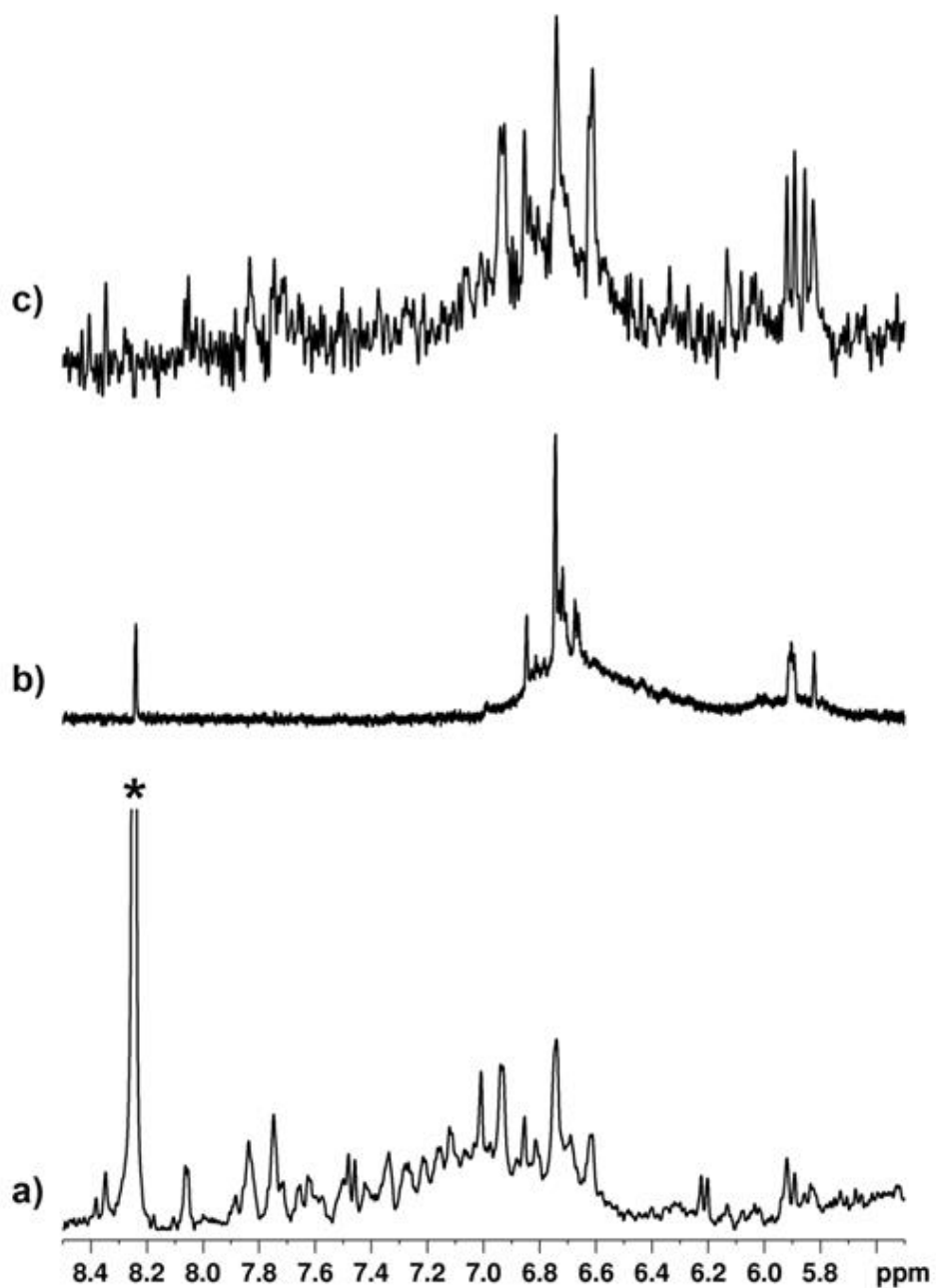
Fig. 33: Expanded region of the ^{1}H NMR spectrum of annurca extracts in the presence of bile salt micelle (a) and the corresponding STD spectrum (c). Expanded region of the ^{1}H NMR spectrum of AMD in the presence of bile salt micelle (b). The star indicates sample impurity.

3.4 Conclusions

In vitro experimental results have demonstrated that apple polyphenolic extracts are able to decrease the cholesterol solubility in a model of duodenal bile micelle solution. This effect seems to be statistically correlated with the dimeric procyanidin content of the apple extracts, so that Annurca sample, being characterised by the highest concentration of these polyphenolic compounds, is able to exert the strongest effect on the micellar cholesterol solubility among the other apple extract samples. The ^{1}H NMR experiments have indicated that PC in the bile micelles could be the preferential target of the apple polyphenolic extracts, considering the evident shifts of the PC signals in the NMR spectrum. STD spectroscopy have allowed to hypothesise that the polyphenols responsible for these shifts are mainly (epi)catechin or dimeric procyanidins, confirming what already reported in the literature for the galloylated green tea catechins, compounds structurally similar to dimeric procyanidins.

The same i*n vitro* and NMR experiments have indicated for AMD much higher effects than the apple extract samples, in terms of both cholesterol precipitation and PC signal shifts in the NMR spectrum. Although its lower polyphenolic content at the same concentration in the solutions tested, AMD could be expected to better vehicle polyphenols to the micelle targets, thanks to its polar microencapsulated formulation. This product may be regarded as a powerful tool to decrease cholesterol solubility at the duodenal tract and, thus, its intestinal bioavailability. Therefore, the present work proposes AMD as an effective device to lower cholesterol absorption in mildly hypercholesterolemic healthy subjects.

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