A nutraceutical formulation based on Annurca apple polyphenolic extract is effective on intestinal cholesterol absorption: A randomised, placebo-controlled, crossover study

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ABSTRACT

Complementary and/or alternative safe substances, able to correct impaired lipid profile in humans, are still in great demand. The objective of the present work was to evaluate the in vitro and clinical effects of a novel nutraceutical product (AMD), formulated with Annurca apple polyphenolic extracts, on the intestinal cholesterol micellar solubility. AMD was able to decrease in vitro cholesterol micellar solubility by about 85.7%, while Nuclear Magnetic Resonance experiments allowed to hypothesise dimeric procyanidins as potential responsible compounds for this effect. Then, a randomised, double blind, single centre, placebo-controlled, crossover study, was designed to evaluate the effect of AMD on the fecal cholesterol excretion. Clinical data indicated that fecal cholesterol excretion was significantly increased (about +35%) in the AMD period compared with placebo period (P < 0.01). AMD may be regarded as a novel complementary and/or alternative safe remedy with clinical relevance in the primary cardiovascular disease prevention.

1. Introduction

Cholesterol in the intestinal lumen typically consists of one third dietary cholesterol and two-thirds biliary cholesterol. The average daily diet contains 300–500 mg of cholesterol obtained from animal products. The bile provides an additional 800-1200 mg of cholesterol throughout each day as gallbladder contractions produce a flow of bile acids, cholesterol, and phospholipids to facilitate lipid digestion and absorption [1,2]. Dietary cholesterol is a mixture of free and esterified cholesterol whereas biliary cholesterol is non-esterified and is introduced into the small intestine as a cholesterol–bile salt–phospholipid water-soluble complex. Dietary cholesterol enters the small intestine solubilized in the oil phase of the stomach digest, whereas the biliary cholesterol enters in the micelle phase of the bile [1–3]. Experimental evidence indicates that biliary cholesterol and dietary cholesterol are absorbed equally; however, the pattern of exogenous and endogenous cholesterol absorption differs along the length of the intestinal lumen [1,3,4]. In the absence of bile secretion, or in the presence of bile acid-binding compounds, there is virtually no intestinal absorption of cholesterol [1,3,4].

Ezetimibe (Zetia™ or Ezetrol™; Merck Sharp & Dohme Ltd, NJ, USA) is presently used to reduce absorption of dietary and biliary cholesterol, inhibiting its transport across the intestinal wall by binding to the duodenal Niemann-Pick C-like protein 1 L1 (NPC1L1) [5]. Actually, the enterocyte takes up both cholesterol and phytosterols from the intestinal lumen by NPC1L1 protein, which appears to be a common sterol transporter or permease in the brush border membrane. Nevertheless, the selectivity of this process accounts for the higher absorptions rates of cholesterol (50–60%) compared to the phytosterols, which are very poorly absorbed [5,6]. Although ezetimibe reduces LDL-C concentrations, the data on reducing adverse cardiovascular outcomes remain equivocal, with the controversy provoked further by some clinical trials of inadequate design to assess relevant clinical questions [7,8]. In addition, very recent studies have pointed out the attention on potential adverse clinical effects [9].

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 [10-15]. 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. Data indicated the ability of epigallocatechin gallate (EGCG) to lower the solubility of phosphatidylycholine (PC) and cholesterol in micellar solutions due to their elimination from the micelles by interaction between taurocholic acids and EGCG [16].

Annurca is the only apple cultivar native to Southern Italy, listed as a Protected Geographical Indication (PGI) product from the European Council (Commission Regulation (EC) No. 417/2006). 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 human hepatocellular liver carcinoma cells (HepG2, HB-8065) [17,18]. 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 [19]. 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.

Apple procyanidins are oligomeric compounds consisting of catechin monomeric units. It can be hypothesized that the lower molecular weight compounds (mainly dimers) may have a similar mechanism of action to that of monomeric catechins as regards their effects on micellar cholesterol solubility. To clarify this aspect, a first aim of the present work was to evaluate the in vitro effect of a nutraceutical product formulated by using Annurca water extract micro-encapsulated in maltodextrins (AMD) on micellar cholesterol solubility in a model reproducing the duodenal environment. Specifically, a main goal was to elucidate the molecular mechanism of action by performing an NMR study of the interaction between Annurca polyphenols and bile acids. Then, this formulation was tested for its potential effects on human fecal cholesterol excretion through a randomised clinical trial.

2. Materials and methods

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, (2S,4R)-epicatechin, isoferulic acid, myricetin, phloretin, phloridzin (phloretin-2-O-glucoside), procyanidin B2, quercetin, quercitrin (quercetin-3-O-rhamnoside), rutin (quercetin-3-O-rutinoside), isorhamnetin, isorhamnetin-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.

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 been harvested (green peel). Fruits were reddened, following the typical treatment for about 30 days, and then analysed [20]. 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.

2.3. Industrial preparation of Annurca nutraceutical product (AMD)

AMD consisted of Annurca apple extract microencapsulated in maltodextrins. Large-scale production of AMD 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.

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 a photodiode array detector (DAD). The column selected was a Kinetex® C18 column (250 mm x 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, flavanones 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/mL 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 Advinax Expression mass spectrometer (Advinox 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.

2.5. GC–MS analysis

The effects of purified apple polyphenolic extracts and AMD on the micellar solubility of cholesterol were examined as described by previous authors [21]. 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 AMD (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. 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-Mass Spectrometry (GC–MS), as follows. GC–MS was performed with an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975C MSD (Agilent Technologies, Palo Alto, CA). The electron energy was 70 eV and the ion source temperature was 230 °C. Each sample was injected in split mode (10:1) at 280 °C and separated through a MXT-1 cross-linked dimethylpolysiloxane capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness, Silicosteel-treated stainless steel; Restek, Bellefonte, PA). The oven temperature was held initially at 260 °C for 3 min, ramped to 320 °C at 10 °C/min, increased to 330 °C at 2 °C/min (held for 8 min), and finally increased to 380 °C at 30 °C/m and held for 3 min. The carrier gas was ultra-high-purity helium at a column head pressure of 75.8 kPa (14.2 ps; column flow: 1.1 ml/min at an oven temperature of 260 °C). For quantitative analysis, the characteristic ions of each compound were determined as their TMS derivatives. Peak identification was achieved by comparing the retention time and matching the height ratios of the characteristic ions.

2.6. NMR Spectroscopy

All NMR experiments were carried out at 298 K with an Inova 700 MHz spectrometer (Varian Inc., Palo Alto, CA, USA), equipped with a cryogenic probe that was optimized for 1H detection. For interaction studies of extracts with the bile salt micelles, 30 μL from 10 mM stock solution in DMSO-d6 of the apple extracts or AMD, both prepared as described above, were added to 510 μL of micelle solution (6.6 mM L-sodium taurocholate, 0.6 mM/L PC, 0.5 mM/L cholesterol, 132 mM/L NaCl, and 15 mM/L sodium phosphate) and 60 μL of D2O. 1H and STD NMR spectra of extracts alone or bile salt micelles alone were acquired as reference. STD spectrum was acquired with 4096 scans with on-resonance irradiation at 1.07 ppm for selective saturation of micelle resonances and off-resonance irradiation at 30 ppm for reference spectra. A train of 40 Gaussian shaped pulses of 50 ms with 1 ms delay between pulses were used, for a total saturation time of 2 s. STD spectrum was obtained by internal subtraction of the saturated spectrum from the reference spectrum by phase cycling with a spectral width of 9000–90 Hz, relaxation delay 1.0 s, 8 k data points for acquisition, and 16 k for transformation.

2.7. Study population and protocol

Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in November 2017. Patients aged 18–83 years were eligible for enrolment if they had the following values of serum parameters at baseline: TC, 200–260 mg/dL; HDL-C, 30–45 mg/dL; LDL-C, 189–206 mg/dL; TG, 170–280 mg/dL.

Exclusion criteria were: smoking, obesity (BMI > 30 kg/m2), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, drug therapy or supplement intake for hypercholesterolemia, drug therapy or supplement intake containing apple polyphenols, heavy physical exercise (> 10 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 (protocol 150,061 of 15/11/2017), 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 randomised, double blind, single centre, placebo-controlled, crossover trial conducted at the Samnium Medical Cooperative (Benevento, Italy).

The study duration was 35 days. The subjects were randomly divided into two groups. They were followed by a crossover design including 7-day washout periods and 10-day intervention periods. The different menus were prepared by a nutritionist at each meal and each day; however, they were same between the two washout periods and also the two intervention periods (Table 1). During the intervention periods, each group was given about 370 mg cholesterol from two boiled eggs (about 50 g weight each) (185 mg cholesterol each within 30 min after lunch and dinner) and total 1000 mg AMD in gastro-resistant capsules or placebo (consisting in the administration of identically appearing capsules containing only maltodextrin) at two meals (one 500 mg capsule at each meal). Subjects were allowed to take water freely. They were provided with a food diary on which annotate their daily dietary habits and were instructed to maintain their habitual patterns of physical activity throughout the entire study period.

Both the examinations and the study treatments were performed in an outpatient setting. Clinic visits and blood sampling were performed after 12 h of fasting at days 8, 18, 25 and 35. 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 TC, HDL-C, LDL-C, glucose, and TG levels were determined using commercially available kits from Diacon International (Groseto, Italy). Analyses were performed on a Diacon International Free Carpe Dein, and intra- and inter-day variations were 1.4 and 1.6% for TC, 1.6 and 2.2% for LDL-C, 2.0 and 2.3% for HDL-C, 1.1 and 1.7% for glucose, and 1.3 and 1.8% for TG, respectively. Fecal samples were collected during the final 3 days of each intervention period. They were weighed, oven-dried, homogenized and stored at −20 °C. Then, they were subjected to lipid extraction according to AOAC (1995) method 948.16 [22], by using a 6-place units Extraction Unit E-S816 Soxhlet (Buchi, Flawil, Switzerland). After centrifugation at 3000 g for 5 min, supernatants were transferred into a preweighed scintillation vial, and dried under nitrogen. The concentration of fecal cholesterol was measured by the Lieberman-Burchard method [23], consisting in sulfonation, desaturation, and rearrangement of cholesterol in acid, with the formation of blue coloured halochromic sulphate of ketone which is able to absorb at λ, 550 or 610 nm.

In addition to these clinic visits, six standardised telephone

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean daily intake of energy and macro-nutrients during washout and intervention periods in male and female subjects.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Energy (kcal)</td>
</tr>
<tr>
<td><strong>Male (n = 30)</strong></td>
<td></td>
</tr>
<tr>
<td>Washout periods</td>
<td></td>
</tr>
<tr>
<td>Meal</td>
<td>1962.2</td>
</tr>
<tr>
<td>Intervention</td>
<td>2019.8</td>
</tr>
<tr>
<td>Eggs*</td>
<td>150.6</td>
</tr>
<tr>
<td>Eggs + eggs*</td>
<td>2170.4</td>
</tr>
<tr>
<td><strong>Female (n = 20)</strong></td>
<td></td>
</tr>
<tr>
<td>Washout periods</td>
<td></td>
</tr>
<tr>
<td>Meal</td>
<td>1549.3</td>
</tr>
<tr>
<td>Intervention</td>
<td>1574.3</td>
</tr>
<tr>
<td>Eggs*</td>
<td>150.6</td>
</tr>
<tr>
<td>Eggs + eggs*</td>
<td>1724.9</td>
</tr>
</tbody>
</table>

*Two boiled eggs (about 50 g weight each) have been consumed each within 30 min after lunch and dinner.
interviews were performed every 5 days starting from the first clinic visit, 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 treatment discontinuation, or adverse events they might have experienced in the meantime (which were also documented regularly on the case report forms during each telephone and clinic visit). All patients underwent a standardised physical examination, assessment of medical history (for up to five years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. Body mass index (BMI) was calculated from body height and body weight. Body fat percentage was measured using a body composition analyzer (TFB-310, Tanita Corp., Tokyo, Japan) and systolic blood pressure, diastolic blood pressure, and heart rate were measured using a HBP-9020 (OMRON COLIN Corp., Tokyo, Japan). 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.

2.8. Randomisation, concealment, and blinding

A total of 50 eligible patients (30 men and 20 women, 18–83 years of age) were randomly assigned to two sub-groups (each one of 25 subjects). If a patient dropped out before the intervention period, 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.

2.9. Study outcomes and data collection

2.9.1. Primary and secondary efficacy outcomes

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

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

2.9.2. Safety

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 days 8, 18, 25 and 35, including adverse events occurring in the first three weeks after cessation of treatments.

2.10. Statistics

2.10.1. Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalised linear mixed effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR) [24], but also provides reasonably stable results even when the assumption of MAR is violated [25,26]. 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 [27]. This approach is statistically efficient without using multiple imputation techniques [28]. Data retrieved after withdrawal of randomised study treatment were also included in the analysis.

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student’s t test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed by using Cochran’s test (p < 0.1). The F statistic was also calculated, and F > 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 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 fecal and 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).

2.10.2. 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 pre-specified minimal exposure to the treatment regimen). Hence, patients who prematurely discontinued the study or treatment were excluded from the per protocol sample.

2.11. 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.

3. Results

3.1. AMD composition

AMD was formulated by spray-drying Annuca apple polyphenolic extract in combination with maltodextrins, with a maltodextrins/extract ratio 4:1. LC–MS analysis indicated a peculiar composition of AMD product respect to the apple extract samples, not only in terms of lower total polyphenolic concentration, but also as regards its qualitative profile (Table 2). In fact, the main constituents were represented by [+]catechin, [−]epicatechin, and procyanidin compounds, followed by traces of chlorogenic acid, rutin, phloretin-2-O-xylloglucoside, and phlorizin.
3.3. NMR experiments

Among all of the samples tested, NMR experiments revealed the strongest effect on cholesterol solubility (about 85.7% of cholesterol precipitated) for Annurca apple sample extracts, while AMD reared the most intense field shift of a few signals of the micelles, among which, the trimethylammonium signal was also clearly doubled.

3.4. Enrolment and subject attrition

Patients were enrolled in November 2017. A total of 68 patients were screened for eligibility; 18 patients (26.5%) did not pass the screening stage; 50 patients were randomised. The most common reason was that patients did not meet the inclusion criteria regarding values of serum parameters at baseline (n = 7), followed by fulfilment of exclusion criteria (n = 6), and concerns about the protocol, especially fear of placebo (n = 2). Some refused to participate for no specific reasons (n = 3).

Overall, 50 patients were assigned to the group of intervention study: they were divided into two subgroups (each one made of 25 patients). Patients of subgroups underwent a washout period of 7 days before the intervention period of 10 days, according to a crossover plan. Fig. 3 shows the flow of participants through the trial together with the completeness of diary information over the entire treatment period. No patient prematurely terminated study participation. Fig. 3 follows the CONSORT PRO reporting guideline [29] and reveals that the trimethylammonium signal was also clearly doubled.

3.5. Primary efficacy outcome measures: effect on fecal cholesterol excretion

Table 3 shows that fecal cholesterol excretion was significantly increased (about +35%) in the AMD period compared with placebo period (P < 0.01). No significant differences between the two periods

Table 2
Polyphenolic composition of apple extracts and Annurca nutraceutical formulation (AMD) determined by HPLC.

<table>
<thead>
<tr>
<th>Polyphenolic Component</th>
<th>Annurca</th>
<th>Pink Lady</th>
<th>Golden Delicious</th>
<th>AMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>106.2 ± 10.2</td>
<td>100.4 ± 8.7</td>
<td>104.2 ± 7.2</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>p-Coumaroylquinic acid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(-)-Catechin</td>
<td>62.4 ± 4.7</td>
<td>59.7 ± 4.2</td>
<td>57.2 ± 3.7</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>(-)-Epicathecin</td>
<td>68.1 ± 5.2</td>
<td>60.3 ± 4.6</td>
<td>59.4 ± 4.0</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Procyanidin B3</td>
<td>6.5 ± 0.5</td>
<td>6.2 ± 0.7</td>
<td>5.71 ± 0.9</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>12.6 ± 1.9</td>
<td>53.8 ± 3.9</td>
<td>10.1 ± 1.7</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>16.0 ± 1.8</td>
<td>6.01 ± 0.8</td>
<td>10.2 ± 1.7</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>Procyanidin trimmer (isomer)</td>
<td>21.3 ± 2.1</td>
<td>63.7 ± 5.1</td>
<td>16.1 ± 1.8</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.41 ± 0.04</td>
<td>0.41 ± 0.04</td>
<td>0.41 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>Rutin (Quercetin-3-O-rutinoside)</td>
<td>42.7 ± 3.5</td>
<td>38.4 ± 2.8</td>
<td>36.1 ± 2.2</td>
<td>0.06 ± 0.002</td>
</tr>
<tr>
<td>Hyperin (Quercetin-3-O-galactoside)</td>
<td>109.1 ± 11.3</td>
<td>100.4 ± 9.2</td>
<td>98.1 ± 8.1</td>
<td>ND</td>
</tr>
<tr>
<td>Isoquercitrin (Quercetin-3-O-glucoside)</td>
<td>72.6 ± 4.3</td>
<td>69.5 ± 4.4</td>
<td>67.1 ± 3.5</td>
<td>ND</td>
</tr>
<tr>
<td>Reynoutrin (Quercetin-3-O-xylloside)</td>
<td>76.8 ± 3.8</td>
<td>70.0 ± 4.5</td>
<td>68.4 ± 3.4</td>
<td>ND</td>
</tr>
<tr>
<td>Guaiaverin (Quercetin 3-O-arabinopyranoside)</td>
<td>72.5 ± 3.7</td>
<td>67.2 ± 3.8</td>
<td>64.2 ± 2.7</td>
<td>ND</td>
</tr>
<tr>
<td>Aviceularin (Quercetin 3-O-arabinofuranoside)</td>
<td>76.6 ± 4.1</td>
<td>70.2 ± 4.1</td>
<td>68.2 ± 3.6</td>
<td>ND</td>
</tr>
<tr>
<td>Quercetin-O-pentoside</td>
<td>43.8 ± 2.7</td>
<td>42.6 ± 3.2</td>
<td>40.0 ± 3.2</td>
<td>ND</td>
</tr>
<tr>
<td>Quercitrin (Quercetin-3-O-rhamnoside)</td>
<td>72.9 ± 3.6</td>
<td>65.6 ± 4.0</td>
<td>63.3 ± 4.0</td>
<td>ND</td>
</tr>
<tr>
<td>Phloretin-2-O-xylloglucoside</td>
<td>64.4 ± 2.5</td>
<td>62.2 ± 3.8</td>
<td>60.3 ± 3.8</td>
<td>0.06 ± 0.003</td>
</tr>
<tr>
<td>Filflavolin (phloretin-2-O-glucoside)</td>
<td>65.3 ± 3.5</td>
<td>61.5 ± 3.6</td>
<td>59.1 ± 3.6</td>
<td>0.06 ± 0.002</td>
</tr>
</tbody>
</table>

Results were expressed as μg/mg DW ± SD (n = 3).

Abbreviations: Ann: Annurca; GD: Golden Delicious; PL: Pink Lady.

Data are means ± SE of triplicate experiments.

No significant differences were detected by the Tukey – Kramer multiple comparison test.
Fig. 2. (A) Aliphatic region of the $^1$H NMR spectrum of bile salt micelle (blue) after the addition of: Pink Lady apple extracts (black); Golden Delicious apple extracts (green); Annurca apple extracts (red); AMD (violet).

(B) Expanded region of the $^1$H NMR spectrum of Annurca extracts in the presence of bile salt micelle (a) and the corresponding STD spectrum (b). The star indicates sample impurity.

Fig. 3. Study flowchart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment 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. AMD = Annurca nutraceutical product; FAS = full analysis set.
Significantly different between the placebo and AMD periods; \(^{*} P < 0.01\).

were detected as regards the other parameters.

3.6. Secondary efficacy outcome measures: effect on blood biochemical parameters

Results of the blood biochemical analyses are reported in Table 4. Plasma total cholesterol concentration was the only parameter to result significantly different from the initial values within the same treatment period \((P < 0.01)\), but also significantly different between the placebo and AMD periods \((P < 0.05)\). No other differences between initial and final values in all parameters in both placebo and AMD periods were found.

3.7. Safety issues

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 AMD dose adopted for the trial was of 1997 established 1000 mg as maximum polyphenolic extract daily intake. Therefore, the Commission Regulation (EC) No. 258/1997 established 1000 mg as maximum polyphenolic extract daily intake in humans. Therefore, the AMD dose adopted for the trial was of 1997 established 1000 mg as maximum polyphenolic extract daily intake in humans.

Table 3
Fecal lipid excretion in 3 days \((n = 50)\).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Placebo</th>
<th>AMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet fecal weight (g)</td>
<td>231.4 ± 119.1</td>
<td>329.0 ± 100.1</td>
</tr>
<tr>
<td>Dry fecal weight (g)</td>
<td>76.2 ± 28.0</td>
<td>93.5 ± 21.4</td>
</tr>
<tr>
<td>Total cholesterol (mg)</td>
<td>74.1 ± 22.6</td>
<td>102.1 ± 23.9</td>
</tr>
</tbody>
</table>

Values are means ± SD \((n = 5)\).

Table 4
Initial and final values of physical and blood parameters of subjects administered with placebo and AMD during treatment periods \((n = 50)\).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Placebo</th>
<th>AMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex (No (%))</td>
<td>30 (60.0%)</td>
<td>–</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.5 ± 10.2</td>
<td>–</td>
</tr>
<tr>
<td>White ethnicity (No (%))</td>
<td>50 (100.0%)</td>
<td>–</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.8 ± 3.1</td>
<td>20.7 ± 3.1</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>106.2 ± 10.3</td>
<td>107.3 ± 12.1</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>67.3 ± 7.4</td>
<td>66.8 ± 6.9</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>239.9 ± 11.9</td>
<td>240.8 ± 12.7</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>39.4 ± 7.6</td>
<td>39.8 ± 6.9</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>179.3 ± 11.8</td>
<td>187.4 ± 10.7</td>
</tr>
<tr>
<td>AST (GOT) (U/L)</td>
<td>20.4 ± 8.6</td>
<td>21.1 ± 7.1</td>
</tr>
<tr>
<td>ALT (GPT) (U/L)</td>
<td>28.1 ± 7.3</td>
<td>27.1 ± 6.9</td>
</tr>
<tr>
<td>γ-GTP (U/L)</td>
<td>35.8 ± 9.4</td>
<td>37.2 ± 8.6</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>221.8 ± 14.9</td>
<td>224.8 ± 13.7</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>175.9 ± 10.1</td>
<td>172.0 ± 10.6</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.32 ± 0.9</td>
<td>4.11 ± 0.6</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.58 ± 0.08</td>
<td>0.56 ± 0.09</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.83 ± 0.06</td>
<td>0.84 ± 0.05</td>
</tr>
</tbody>
</table>

\(^*\)Initial refers to samples collected at days 8 and 25. Final refers to samples collected at days 18 and 35.

Values are means ± SD \((n = 5)\).

Significantly different from the initial values within the same treatment period; \(^{*} P < 0.01\).

Significantly different between the placebo and AMD periods; \(^{*} P < 0.05\).

4. Discussion

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

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 \([30]\). In contrast, green tea catechins without a galloyl moiety did not precipitate 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 \([31]\). 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 ECGG decreases the micellar solubility of cholesterol via specific interaction with PC.

Specifically, dimeric procyanidins are the most structurally similar to the green tea catechins with a galloyl moiety. 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 (Fig. 1). A significant statistical correlation ($R^2 = 0.9511$) between dimeric procyanidin content of apple extracts (Table 2) and the observed cholesterol precipitation (Fig. 1) supported our hypothesis.

Very interestingly, AMD exerted the strongest effects among all of the samples tested, although its much lower polyphenolic content than the other samples (Fig. 1). A possible explanation could be found in its 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 cultivars 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 in this sense. However, this aspect needs to be further clarified.

**4.2. NMR study of interaction of apple extracts and AMD with bile acid micelles**

To study the interactions between the apple extracts and the bile acid micelles we first analysed $^1$H NMR spectra of solutions containing both these constituents.

Results, illustrated in Figure 2A, lead to hypothesize that Annurca extracts have a higher content of metabolites able to interact with the bile salt micelle compared to the other two apple cultivars. The relatively intense upfield shift of the trimethylammonium group in phosphatidylcholine and its doubling was in accordance with the results of Kohayaishi et al. (2014) who reported that polyphenolic constituents of green tea, in particular ECG and ECGG, interact with bile acid micelles through the trimethylammonium group in phosphatidylcholine [31]. The same authors also observed an intermolecular nuclear Overhauser effect (NOE) between PC and EGCG in bile salt micelles with EGC added, but not between cholesterol and EGCG, which strongly suggested that EGCG decreases the micellar solubility of cholesterol via specific interaction with PC. In our case, relatively low concentration of polyphenols in the samples did not allow the observation of NOE cross peaks between aromatic and micelle components signals in the NOESY spectra (data not shown).

To identify extract components that bind the bile salt micelles, we used nuclear magnetic resonance saturation transfer difference (STD) spectroscopy [32]. 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. As shown in Figure 2B, signals assignable to chlorogenic acid and its analogues (6.2, and 7.5 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 [33], these signals are fully compatible with (epi)catechin or procyanidins. Interestingly, the number of signals at about 5.9 ppm, assignable to the H6 and H8 protons of a procyanidin moiety, indicate that the mostly interacting molecule in the extract is a dimeric procyanidin since four signals of similar intensity are clearly detectable.

**4.3. Clinical effects on intestinal cholesterol absorption**

Inhibiting intestinal cholesterol absorption represents an alternative approach to statins for LDL cholesterol lowering, which also affects whole body cholesterol metabolism. The drug ezetimibe reduces intestinal cholesterol absorption by targeting NPC1L1 protein, a sterol transporter expressed in the apical membrane of enterocytes [5]. As a result, less cholesterol is delivered to the liver, thereby upregulating LDL receptors and reducing LDL cholesterol [34]. When given to patients with primary hypercholesterolemia, ezetimibe (10 mg/day) reduces LDL cholesterol up to 20% [35,36]. Previous clinical studies have examined changes in body cholesterol metabolism associated with ezetimibe treatment; all support the idea that ezetimibe increases endogenous cholesterol excretion [37,38]. However, this conclusion is tempered by several limitations of these studies, including lack of a control group, lack of concurrent ezetimibe treatment during metabolic measurements, collection of stool samples without control of either dietary cholesterol or phytosterol intake, lack of measurement of intestinal cholesterol absorption efficiency or total endogenous cholesterol excretion, and a focus on analyses performed during rapid changes in cholesterol enrichment, where repeatability of calculated parameters is limited. More importantly, recent surveys have highlighted potential side effects related to the chronical use of this drug. Specifically, a pharmacoepidemiological analysis over 11 years of dispensed pre-scriptions from Medicare Australia has collected numerous cases regarding adverse event data [9]. Between 2004 and 2015, there were 575 adverse event case reports for all ezetimibe products (456 for ezetimibe only, 112 for ezetimibe plus simvastatin, 6x for ezetimibe plus atorvastatin, and one for ezetimibe plus rosvastatin). For ezetimibe-only products, musculoskeletal, connective tissue, and gastrointestinal disorders were the most often reported adverse events. Myalgia was the predominant musculoskeletal condition (in 42% of cases), followed by arthralgia (10%) and muscle spasms (9%). Nausea was the most widespread gastrointestinal adverse event (19%), followed by diarrhea (11%) and abdominal pain (10%). For ezetimibe plus simvastatin products, musculoskeletal and connective tissue disorders (in 76% of cases), then nervous disorders (24%, eg, seizures and amnesia), general disorders and administration site conditions (24%, eg, pain and asthenia) were the most common adverse events. Rhabdomyolysis was the most commonly reported musculoskeletal adverse event reported (31%), followed by myalgia (25%) and myopathy (10%).

Our clinical data demonstrated that AMD was able to increase fecal cholesterol excretion when healthy subjects took high-cholesterol diet. This is the first report of the effects of an apple polyphenolic extract on the intestinal cholesterol absorption. A crossover design, to study the differences in treatments, generally yields a more efficient comparison of treatments than a parallel design. Subjects are on their own controls. The within-patient variation is less than between-patient variation. Crossover design requires a smaller sample size than a parallel design. Subjects are on their own controls. Differences in treatments, generally yields a more efficient comparison of treatments than a parallel design. Subjects are on their own controls. This is the concern of crossover studies are represented by the carryover (or residual) effects, defined as the effect of the treatment from the previous time period on the response at the current time period. It occurs when the effect of a treatment given in the first time period persists into the second period and distorts the effect of the second treatment. Nevertheless, the incorporation of a washout period in the design can diminish the impact of carryover effects. In our study, no residual effects of AMD polyphenols were expected to interfere with the second period because of the well-known very short half-lives of catechin derivatives (about 4–5 hours) [39–42]. As regards the possible carryover effect of cholesterol from eggs, previous authors have reported that the daily consumption of up to three eggs, prolonged for several days, would not significantly influence plasma total cholesterol profile [43].
Thus, the first time intervention period, allocating subjects to eggs and placebo, would not distort the effect of the second treatment.

As regards the possible molecular mechanism of action at the bases of the effects of AMD on the fecal cholesterol excretion, our in vitro data are a first effort corroborating the hypothesis that apple dimeric procyanidins are able to decrease the intestinal cholesterol micellar solubility (Figs. 1 and 2). To date, such effects have been recently elucidated both in vitro and in vivo for tea catechins, particularly as concerns EGCG. Specifically, recent evidences confirm their capacity to decrease intestinal cholesterol absorption in animals by interfering with the emulsification, digestion, and micellar solubilization of lipids, critical steps involved in the intestinal absorption of dietary fat, cholesterol, and other lipids [12,21,44]. In a previous clinical study, Hsu et al. (2006) have demonstrated that polyphenol-enriched oolong tea could increase cholesterol excretion into feces when subjects took high-lipid diet [45]. Galloylated catechins are structurally similar to apple dimeric procyanidins, so that it could be hypothesized a common mechanism of action on in vivo micellar cholesterol solubility. We formulated gastric-resistant capsules due to previous knowledge about gastric digestion effects on some polyphenolic components. Procyanidins have been usually reported to be quite susceptible to gastric pH conditions, being almost completely degraded to monomeric (−)-epicatechin within 1.5 h [46]. Actually, available literature data refer to dimeric compounds, mainly procyanidin B1, B2 and B5. In vivo studies have demonstrated that procyanidins having a higher degree of polymerization than dimeric compounds, such as those from grapeseeds and cocoa, would not be degraded to more readily absorbable monomers [47]. Moreover, the specific technological formulation of AMD product, consisting of Annurca extract microencapsulated in maltodextrins, would possibly account for a better solubility of apple polyphenols in the intestinal aqueous medium and, thus, an improved accessibility of AMD constituents to the cholesterol vesicles, as corroborated by our in vitro results.

5. Conclusion

Our clinical results indicated AMD as effective in favouring the fecal cholesterol excretion in healthy subjects fed a high cholesterol diet. According to our in vitro data, AMD would act by decreasing the intestinal micellar solubility of cholesterol in a dose-dependent manner. NMR experiments would indicate dimeric procyanidins as the main AMD constituents able to interact with bile acid micelles through the trimethylammonium group of phosphatidylcholine (PC), thus, leading cholesterol to precipitation. The same in vitro and NMR experiments have indicated for AMD much higher effects than raw 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 dimeric procyanidins 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 remedy to lower cholesterol absorption in healthy subjects.

Contributors

GCT, AC, DC, GB, MDA, and EN were responsible for study concept and design. GCT, DC, GB, MDA, RC, and EN acquired the clinical data. GCT, RC, and EN acquired the in vitro data. AC and DB designed, acquired and analysed the NMR experiments. All authors analysed and interpreted the data and drafted the manuscript. All authors critically revised the manuscript for important intellectual content. EN obtained funding. EN provided administrative, technical, or material support. All authors supervised the study. All authors, external and internal, had full access to all of the data. EN is the guarantor.

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Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: EN has received research grants from Regione Campania under POR Campania FESR 2007–2013 · O.O. 2.1 (FarmaBioNet); no other relationships of activities that could appear to have influenced the submitted work.

Ethical approval

The study was approved by the ethics committee at the Hospital AO Rummo of Benevento, Italy.

Transparency statement

The lead author (the manuscript’s guarantor) affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

Data sharing

No additional data available.

Conflict of interest

None.

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