Research Article

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Annurca (*Malus pumila* Miller cv. Annurca) apple as a functional food for the contribution to a healthy balance of plasma cholesterol levels: results of a randomized clinical trial

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Abstract

BACKGROUND: Recent human studies have evaluated the effect of daily apple consumption on plasma cholesterol level, which is recognized as an important risk factor for cardiovascular disease (CVD). Nevertheless, slightly significant effects have been generally registered although consuming more than two apples a day for several weeks.

RESULTS: This study describes the influence of daily consumption of Annurca apples on the cholesterol levels of mildly hypercholesterolaemic healthy subjects. A monocentric, randomized, parallel-group, placebo-controlled, 4-month study was conducted. The subjects ($n = 250$) were randomly assigned to five treatment groups (each one of 50 subjects: 28 men and 22 women). Four groups were administered one apple per day among the following: Red Delicious, Granny Smith, Fuji, Golden Delicious. The fifth group was asked to consume two Annurca apples per day, since the weight of this cultivar is on average half that of the commercial ones considered in this study. Comparing results, Annurca led to the most significant outcome, allowing a reduction in total and low-density lipoprotein cholesterol levels by 8.3% and 14.5%, respectively, and an increase in high-density lipoprotein cholesterol levels by 15.2% (all $P < 0.001$).

CONCLUSION: Our data would reasonably indicate Annurca apple as a useful tool to contribute to the prevention of CVD risk through normal diet.
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Keywords: Annurca apple PGI; clinical trial; total cholesterol; LDL cholesterol; HDL cholesterol

INTRODUCTION

A high concentration of serum total cholesterol (TC) has been well documented as an established risk factor for cardiovascular disease (CVD) in both men and women.[1] A reduction in baseline low-density lipoprotein cholesterol (LDL-C) levels (approximately 120–190 to 100–140 mg dL^{-1}) by statin treatment has been associated with a significant reduction (approximately 25–35%) in CVD events.[2,3] Nevertheless, despite reaching optimal LDL-C levels in treated patients, the residual risk of CVD events in some remains elevated. Prospective cohort studies suggest that plasma concentrations of high-density lipoprotein cholesterol (HDL-C) have a significant and independent inverse association with risk of CVD events.[4–6] In particular, each 1 mg dL^{-1} increment of HDL-C concentration is associated with a 2% decrease in CVD risk in men and a 3% decrease in women.[7] Low HDL-C is a component of the metabolic syndrome and is defined as HDL-C level < 40 mg dL^{-1}, according to current guidelines.[8] Enhancing HDL-C levels to reduce this residual risk has been a focus of great interest and a major challenge of modern medicine and healthy nutrition.[9]

A daily four- to five-fruit portion consumption has been associated with a significant lowering of plasma TC and LDL-C, and blood pressure, in human dietary interventions studies.[10–12] Soluble and insoluble fibre fractions have been indicated as the major components responsible for the beneficial effects on human lipid metabolism.[13,14] Polyphenols would be able to decrease plasma oxidative stress through induction of antioxidant enzymes, which could potentially lead to a reduced risk of CVD.[15,16] Actually, a direct efficacy of fruit polyphenols on serum

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cholesterol levels would be very unlikely. A recent meta-analysis of 19 randomized controlled trials with low-fibre fruit juices has highlighted no significant results with regard to the effects on plasma TC, LDL-C and HDL-C in adults.[17] Nevertheless, *in vitro* and *in vivo* experiments would indicate oligomeric procyanidins as the fruit polyphenolic compounds most effective on cholesterol metabolism, although their mechanism of action is still to be elucidated.[18–21]

Apples are among the most widely consumed fruits, especially in northern Europe and North America, and among the most deeply studied fruits. Some published human studies have been undertaken to investigate the TC-lowering effects of apples or apple products. Dietary interventions with whole fresh apples have provided the most appreciable results, whereas no significant effects have been registered with other apple products (juice, whole dried apples).[22–28] In particular, the decrease in plasma TC could be ascribed exclusively to an influence on LDL-C levels,[24,25,29] while contrasting or no significant data have been provided regarding the effects of whole apple and apple product consumption on plasma HDL-C concentrations.[22–31] Similarly to fruit in general, the fibre fraction, mainly pectin, would be the major apple component responsible for the reported effects on cholesterol metabolism.[13,32,33] Nevertheless, a recent clinical trial in which apple polyphenolic extracts were administered to mildly hypercholesterolaemic subjects for 1 month resulted in a significant lowering of TC (5%) and LDL-C (8%), and a significant increase in HDL-C levels (5%).[34]

Our research group has previously evaluated the antioxidant composition and *in vitro* effects on specific metabolic parameters of polyphenolic extracts from Annurca apple – the only apple cultivar native to the Campania region of southern Italy, listed as a Protected Geographical Indication (PGI) product (Commission Regulation (EC) No. 417/2006).[19,20] By comparing experimental data with those from more common commercial apple cultivars (Red Delicious, Pink Lady, Fuji, Golden Delicious), Annurca apple exhibited the highest polyphenolic concentration, while results obtained by incubating human hepatocellular liver carcinoma (HepG2) cell lines with apple extracts indicated Annurca apple as the most effective in reducing cell cholesterol uptake, enhancing LDL-C receptor binding activity and increasing cell medium Apo-AI concentration, a crucial protein in the formation of the nascent discoidal HDL.[19,20] No intervention studies with Annurca apple are available so far.

In the light of these considerations, the present study evaluated the effects of daily Annurca apple consumption for 8 weeks on plasma TC, LDL-C and HDL-C in healthy, mildly hypercholesterolaemic, human subjects. Groups of subjects, matching the same criteria and consuming more conventional apple cultivars (Red Delicious, Granny Smith, Fuji, Golden Delicious), were included for a comparative analysis.

MATERIALS AND METHODS

Apple samples

Annurca (*Malus pumila* Miller cv. Annurca) apple fruits (each weighing about 100 g) were collected in Valle di Maddaloni (Caserta, Italy) in October, when fruits had just been harvested (green peel). Fruits were reddened following the typical treatment[35] for about 30 days and then analysed. Four other apple varieties (each weighing about 200 g) analysed in this study – *M. pumila* Miller cv. Red Delicious (RD), *M. pumila* Miller cv. Granny Smith (GS), *M. pumila* Miller cv. Fuji (F) and *M. pumila* Miller cv. Golden Delicious (GD) – were acquired from a local supermarket.

Study population and protocol

The study is listed on the ISRCTN registry with ID ISRCTN15653394. Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in November 2015. Patients aged 18–83 years were eligible for enrolment if they had the following values of serum cholesterol parameters at baseline: TC 200–260 mg dL^{-1}; HDL-C 30–45 mg dL^{-1}; LDL-C 189–206 mg dL^{-1}. The subjects were asked to keep their dietary habits unchanged throughout the entire study.

Exclusion criteria were: smoking, obesity (body mass index (BMI) >30 kg m^{-2}), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, drug therapy or supplement intake for hypercholesterolemia, drug therapy or supplement intake containing apple polyphenols, heavy physical exercise (>10 h per 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 14307 of 17 July 2015) 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, randomized, single-blind, placebo-controlled trial, conducted at the Samnium Medical Cooperative (Benevento, Italy).

The study duration was 16 weeks: the same group underwent 4 weeks of placebo treatment, consisting of the habitual diet without apples, followed by 8 weeks of intervention study and 4 weeks of follow-up. Both the examinations and the intervention study were performed in an outpatient setting. Clinic visits and blood sampling were performed after 12 h of fasting at weeks 0, 4, 8, 12 and 16. Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected in 10 mL EDTA-coated tubes (Becton-Dickinson, Plymouth, UK) and plasma was isolated by centrifugation (20 min, $2200 \times g$, 4 °C). All samples were stored at −80 °C until analysis. Plasma TC, HDL-C and LDL-C levels were determined using commercially available kits from Diacron International (Grosseto, Italy). Analyses were performed on a Diacron International Free Carpe Diem, and intra- and inter-day variations were 1.4% and 1.6% for TC, 1.6% and 2.2% for LDL-C, and 2.0% and 2.3% for HDL-C, respectively. In addition to these four meetings, six standardized telephone interviews were performed every 14 days 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).

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

Randomization, concealment and blinding

A total of 250 eligible patients were randomly assigned to five groups in order to receive apple samples. On the first day of week 0 and once a week during the 16 weeks, subjects were supplied with apples to be consumed during the following week. Apples were coded with different colours and given in random order. The code was not broken until all analyses were completed and the results were analysed statistically. If a patient dropped out before receiving apples, he or she was replaced by the next eligible patient enrolled at the same centre. The concealed allocation was performed by an Internet-based 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.

Study treatments

A group of 250 patients (168 men and 132 women, 18–69 years of age) was randomly divided into five subgroups (each one of 50 subjects: 28 men and 22 women). The volunteers enrolled in this study had the following values of serum cholesterol parameters at baseline: TC 200–260 mg dL^{-1}; HDL-C 31–45 mg dL^{-1}; LDL-C 179–206 mg dL^{-1}. Four groups were assigned to consume one apple per day among the following: RD, GS, F, GD. The fifth group was asked to consume two Annurca apples per day, since the weight of this cultivar is on average half that of the commercial varieties considered in this study.

Study outcomes and data collection

Primary and secondary efficacy outcomes

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

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

Safety

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

Statistics

Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalized linear mixed-effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR)[36] but also provides reasonably stable results even when the assumption of MAR is violated.[37,38] 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.[39] This approach is statistically efficient without using multiple imputation techniques.[40] Data retrieved after withdrawal of randomized 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 using Student's *t*-test or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed using Cochran's test ($P < 0.1$). The I^2 statistic was also calculated, and $I^2 > 50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. Percentage change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% CIs, *P*-values, or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration and Jadad score. Treatment effects were analysed using PROC MIXED with treatment (placebo, fresh apples) and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in serum cholesterol and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levene's test) and normal distribution (determined by residual plot examination and Shapiro–Wilk test) even after log transformation were analysed using a nonparametric test (Friedman). The level of significance (α-value) was 95% in all cases ($P < 0.05$).

Analysis sets

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

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Determination of sample size

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

Patient involvement

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

RESULTS

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 randomized. The most common reason was that patients did not meet the inclusion criteria regarding values of serum cholesterol parameters 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 consuming apples: they were divided into five subgroups (each one made up of 50 patients), according to the five different apple cultivars chosen for this study. Patients of all groups and subgroups underwent a placebo period during the first 4 weeks before the treatment period of 8 weeks. Follow-up period lasted a further 4 weeks. Figure 1 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 1 follows the CONSORT PRO reporting guideline[41] and reveals that, within the assessment period, the following percentage of patients for each group and subgroup provided data for the primary endpoint: subgroup RD, 88.6% (39 of 44 patients); GS, 97.8% (40 of 45 patients); F, 89.4% (42 of 47 patients); GD, 86.7% (39 of 45 patients); Annurca, 86.7% (39 of 45 patients). In each group, a few patients did not submit any diaries, giving no specific reason for this. Completeness of the patient diaries did not differ between the treatment groups.

Participants' baseline characteristics

Table 1 shows the demographic and clinical characteristics assessed at the baseline visit of all 250 patients randomized. Overall, about half of the randomized patients were female; the total age range was 18–83 years. The groups were well balanced for demographics and clinical factors.

Primary efficacy outcome measures

The approximate daily amounts of procyanidins taken through whole apples by subjects in each intervention subgroup were as follows: Annurca group, 85.09 mg d^{-1}; RD group, 42.12 mg d^{-1}; GS group, 59.82 mg d^{-1}; F group, 26.26 mg d^{-1}; GD group, 16.61 mg d^{-1}. No significant variation of plasma TC, LDL-C and HDL-C with respect to the baseline values was registered at the end of the placebo period in subjects belonging to apple-supplied groups (Table 1). Analysing the results, we can assert that the administration of apples led, in all groups, to a statistically significant variation of plasma TC, LDL-C and HDL-C levels. As shown in Table 2, Annurca exerted the highest hypocholesterolaemic effect, decreasing the TC by 8.3% (95% CI −2.54, $P = 0.0095$), followed by GS and RD, which led the TC to decrease by −4.4% and −3.0%, respectively, while F and GD apples showed the lowest hypolipidaemic effects (−2.0% and −1.2%, respectively). Analysing the LDL-C values in patients supplied with the different apple cultivars, we can assert that Annurca was approximately 1.7-fold more effective in reducing the LDL-C (−14.5%, 95% CI −3.41, $P = 0.0019$) than GS (−8.3%) which was the second most active among all the apples tested. The same trend was maintained when the HDL-C was taken into account. In fact, in line with our *in vitro* data concerning the capability of apple extracts to increase the Apo-AI levels in cellular media or to affect cholesterol metabolism,[19,20] all apples were generally more effective on HDL-C than on LDL-C. Once again, Annurca apple displayed the greatest effect (+15.2%, 95% CI −1.22, $P =$ 0.0042) with respect to GS (+4.5%), RD (+4.2%), F (+4.1%) or GD (+2.6%). Of note, all these results concerning the lipid profiles of patients were achieved already after 1 month of intervention study and were confirmed at the end of the second month. Comparing the *in vivo* hypolipidaemic effect of each single apple cultivar with their procyanidin content, an impressive correlation was found. In fact, Annurca, exhibiting the higher polyphenol amount, was the most effective *in vivo*, while F and GD, being the least rich in polyphenols, showed the poorest effect. However, beyond the hypolipidaemic effects shown by all apples here considered, the present clinical trial highlighted that the daily consumption of apples led the plasma glucose and triglyceride levels to a significant increase (Table 2). Specifically, on average, RD increased the glucose level by +13.1% (95% CI −5.68, $P = 0.0042$), while the least effective on plasma glucose was GS (+2.3%, 95% CI −4.21, P $= 0.0032$). Conversely, regarding the triglyceride levels, the major rise occurred in the GS-supplied patient group, where the increase was an average of +12.7% (95% CI −2.21, $P = 0.0032$). All the other apple cultivars impacted the triglyceride levels to a lesser extent (F, +10.8%, 95% CI −3.54, $P = 0.0036$; GD, +9.3%, 95% CI −5.13, $P =$ 0.0029; RD, +8.3%, 95% CI −5.06, $P = 0.0012$; Annurca, +6.1%, 95% CI −2.46, $P = 0.0027$).

Safety Issue, study strength and limitations

Although no specific toxicity studies have been performed herein, mutagenicity tests and acute/subacute toxicity studies have long since demonstrated the safety of polyphenol content of apples both in mice and humans. Other safety assessments, such as vital signs, blood pressure or electrocardiographic findings, were all periodically monitored and baseline values did not change substantially during or at the end of the trial. 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. Conversely, the main limitations of our study include short-term assessment for the treatment of a chronic condition and the choice of exclusively white race.

DISCUSSION

Although it is widely accepted that the beneficial effects of apple on human health are due to its peculiar phytocomplex rather

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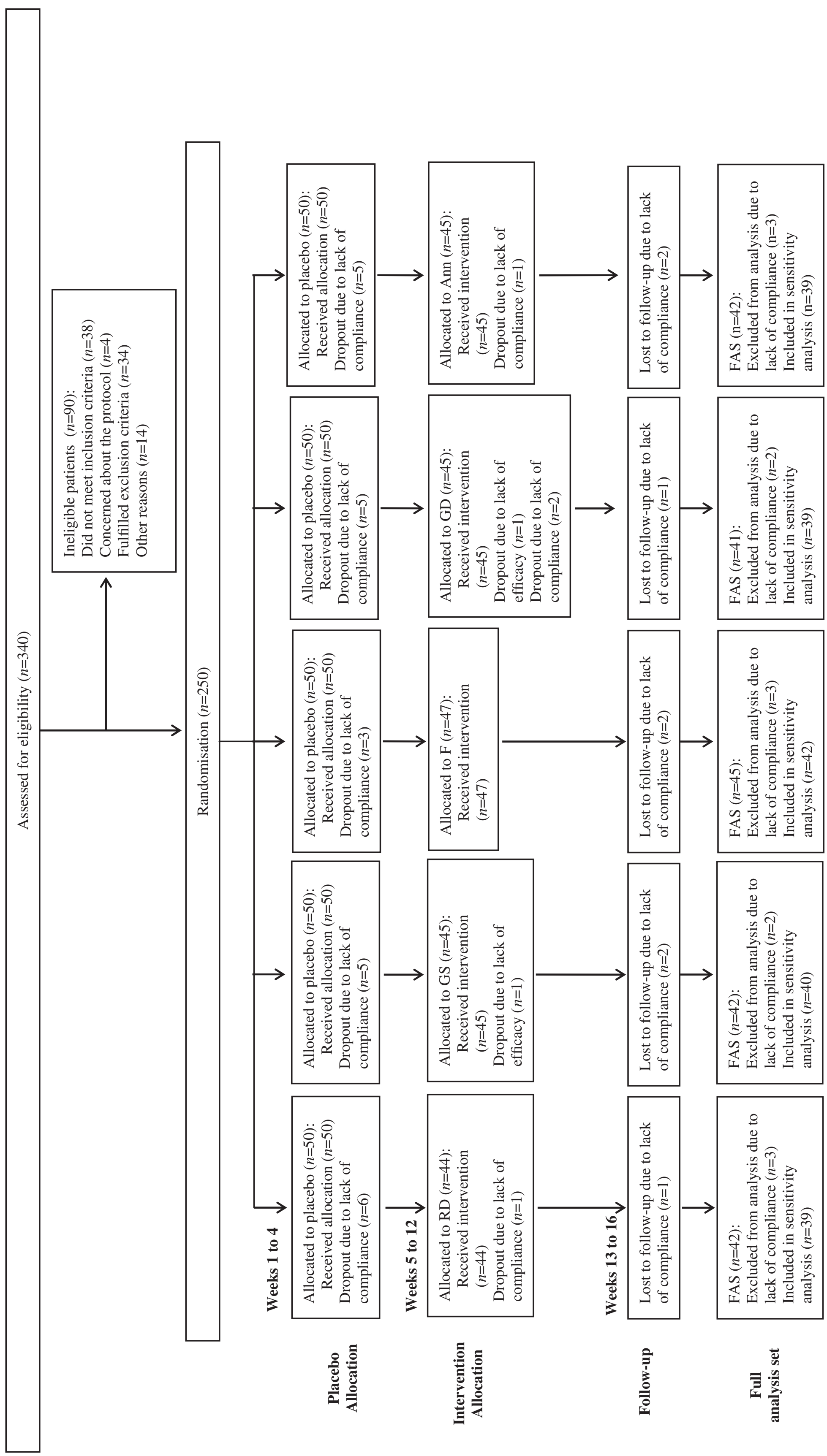

Figure 1. Study flow chart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from pre-screening to data collection, and the extent of exclusions, loss to follow-up and completeness of diary documentation available across the entire trial period. RD, Red Delicious apple; GS, Granny Smith apple; F, Fuji apple; GD, Golden Delicious apple; Ann, Annurca apple; FAS, full analysis set.

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Table 1. Baseline characteristics of intention-to-treat sample according to study treatment

Placebo					
Characteristics	Ann (*n* = 50)	RD (*n* = 50)	GS (*n* = 50)	F (*n* = 50)	GD (*n* = 50)
Demographics					
Age (years)	42.4 ± 10.6	41.8 ± 10.8	43.8 ± 10.5	44.8 ± 11.2	42.7 ± 11.2
Male sex (No. (%))	28 (56%)	26 (52%)	27 (54%)	25 (50%)	26 (56%)
White ethnicity (No. (%))	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)
Clinical parameters					
TC (mg dL^{-1})	240.2 ± 11.5	236.3 ± 12.4	239.1 ± 13.4	233.5 ± 12.6	236.1 ± 13.8
LDL-C (mg dL^{-1})	187.9 ± 12.1	185.2 ± 11.9	191.0 ± 11.3	184.1 ± 12.3	187.8 ± 13.1
HDL-C (mg dL^{-1})	39.9 ± 6.8	36.2 ± 7.7	36.8 ± 6.1	36.3 ± 7.2	32.9 ± 7.3
Glucose (mg dL^{-1})	111.2 ± 9.2	88.7 ± 2.2	99.5 ± 8.1	91.4 ± 9.4	100.1 ± 7.3
Triglycerides (mg dL^{-1})	87.1 ± 9.2	102.8 ± 12.1	93.6 ± 11.8	92.4 ± 10.2	102.3 ± 12.1
Treatment					
Characteristics	Ann (*n* = 45)	RD (*n* = 44)	GS (*n* = 45)	F (*n* = 47)	GD (*n* = 45)
Demographics					
Age (years)	43.5 ± 10.2	42.1 ± 11.1	44.2 ± 10.6	45.5 ± 10.7	43.8 ± 12.2
Male sex (No. (%))	25 (55.5%)	23 (52.3%)	24 (53.3%)	23 (48.9%)	23 (51.1%)
White ethnicity (No. (%))	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)
Clinical parameters					
TC (mg dL^{-1})	239.1 ± 11.9	235.9 ± 13.7	237.6 ± 14.3	235.5 ± 13.3	238.4 ± 15.3
LDL-C (mg dL^{-1})	189.6 ± 11.7	183.7 ± 12.3	189.1 ± 11.6	185.7 ± 11.1	189.1 ± 11.6
HDL-C (mg dL^{-1})	38.5 ± 7.2	37.9 ± 8.1	35.4 ± 6.7	36.7 ± 7.5	34.2 ± 6.9
Glucose (mg dL^{-1})	109.3 ± 8.9	89.5 ± 2.7	98.6 ± 8.5	90.5 ± 10.0	101.2 ± 9.3
Triglycerides (mg dL^{-1})	85.4 ± 8.3	101.5 ± 16.8	95.5 ± 9.0	90.3 ± 10.4	100.5 ± 11.8

Value are means ± SD.
Ann, Annurca apple; RD, Red Delicious apple; GS, Granny Smith apple; F, Fuji apple; GD, Golden Delicious apple.

than to individual constituents, it is somehow reasonable to think that certain individual components, present in high amounts in apples, may contribute more than others to a certain healthy effect. In this scenario, with the aim of investigating whether other apple ingredients, besides the well-studied pectin or phytosterols, significantly contribute to apple's hypolipidaemic properties, we focused our attention on polyphenolic compounds, and especially on the procyanidin fraction, as the latter has been previously linked to the hypocholesterolaemic effect of green tea, grape seeds or cocoa.[18,42,43]

In this respect, we demonstrated that among five apple cultivars Annurca polyphenols are unequivocally more effective on cholesterol metabolism, in a model of human liver carcinoma cell (HepG2) culture, than the rest of the common commercial cultivars.[19,20] Herein, to understand the reasons behind the latter finding, we have profiled the polyphenolic content of Annurca flesh and compared it with those of other apple cultivars. Interestingly, we found that Annurca possesses the highest total amount of polyphenols, with a procyanidin level far above those of all the other cultivars analysed.[19] Indeed, Annurca contains from two to four times more procyanidins than the other apple samples, while the other polyphenolic components, such as dihydrochalcones, flavonols and anthocyanins, are present in an amount close to the average value found for the five apples species considered. As things stand, it is reasonable to think that, if Annurca polyphenolic extract is the most active in affecting cholesterol metabolism, and if its major constituent is represented by procyanidins, the latter compounds must play a role in this healthy effect, similar to what has been found for green tea, grape seeds or cocoa.

Thus, to verify if these *in vitro* properties of Annurca apple would be maintained *in vivo*, we designed a 3-month clinical trial. Specifically, we were interested in knowing if the *in vivo* hypocholesterolaemic effect of Annurca daily consumption was really superior to those of other cultivars regularly consumed, as our *in vitro* data have suggested. Nevertheless, differently from our previous *in vitro* studies, we decided to use *in vivo* the Granny Smith cultivar in place of Pink Lady, for two main reasons. Firstly, since both cultivars are characterized by a similar polyphenolic composition, an equivalent *in vivo* effect of the two cultivars could be reasonably hypothesized, whereas Granny Smith is unquestionably a more widespread and commercially interesting apple. Secondly, Granny Smith apple is the only cultivar that has been employed for a nutraceutical application (Applephenon®).[34] This product, formulated using Granny Smith apple polyphenolic extract, is the only apple-based nutraceutical for which clinical study results, regarding the effects on plasma cholesterol levels, are available so far.[34] Therefore, if in a clinical trial with whole apples a specific cultivar should have equal or even greater effects on plasma cholesterol levels than those of Granny Smith apple, that cultivar could be an ideal candidate for the formulation of alternative apple-based nutraceutical products for testing in a future human study. Thus we planned groups of patients who, after a 1-month placebo period, were each asked to consume a different apple cultivar (among Annurca, RD, GS, F, GD), one a day (or two apples per day in the case of Annurca, since the weight of this cultivar

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Table 2. Effects of apple samples on plasma cholesterol, glucose and triglyceride metabolism

		Annurca	Δ (%)	RD	Δ (%)	GS	Δ (%)	F	Δ (%)	GD	Δ (%)
TC (mg dL^{-1})	*t* 0	239.1 ± 11.9		235.9 ± 3.7		237.6 ± 4.3		235.5 ± 3.3		238.4 ± 5.3	
	t 30	219.2 ± 12.8	−8.3	228.5 ± 4.2	−3.1	227.2 ± 3.5	−4.4	230.8 ± 4.1	−2.0	235.3 ± 1.9	−1.3
	t 60	218.9 ± 12.1	−8.4	229.0 ± 3.6	−2.9	226.9 ± 3.8	−4.5	230.1 ± 3.4	−2.3	235.8 ± 2.0	−1.1
LDL-C (mg dL^{-1})	*t* 0	189.6 ± 11.7		183.7 ± 2.3		189.1 ± 1.6		185.7 ± 1.1		189.1 ± 1.6	
	t 30	161.6 ± 10.9	−14.7	171.7 ± 1.3	−6.5	172.4 ± 0.8	−8.8	177.6 ± 1.7	−4.4	184.1 ± 2.0	−2.6
	t 60	161.9 ± 11.2	−14.5	173.1 ± 1.6	−5.8	173.4 ± 1.2	−8.3	176.9 ± 2.1	−4.7	184.1 ± 2.3	−2.6
HDL-C (mg dL^{-1})	*t* 0	38.5 ± 7.2		37.9 ± 8.1		35.4 ± 6.7		36.7 ± 7.5		34.2 ± 6.9	
	t 30	44.3 ± 7.9	+14.0	39.5 ± 7.5	+4.2	37.6 ± 8.0	+6.2	37.7 ± 6.8	+2.7	34.7 ± 6.4	+1.5
	t 60	43.9 ± 8.1	+15.2	39.9 ± 7.0	+4.2	37.0 ± 8.3	+4.5	38.2 ± 6.0	+4.1	35.1 ± 5.6	+2.6
Glucose (mg dL^{-1})	*t* 0	109.3 ± 8.9		89.5 ± 2.7		98.6 ± 8.5		90.5 ± 10.0		101.2 ± 9.3	
	t 30	118.3 ± 12.0	+10.1	103.4 ± 10.8	+15.5	100.9 ± 12.4	+6.9	99.4 ± 8.3	+15.5	111.3 ± 10.1	+10.1
	t 60	120.3 ± 13.1	+9.1	101.2 ± 11.6	+13.1	105.4 ± 10.3	+2.3	104.5 ± 10.1	+9.8	109.3 ± 9.8	+8.0
Triglycerides (mg dL^{-1})	*t* 0	85.4 ± 15.2		101.5 ± 16.8		95.5 ± 13.0		90.3 ± 18.4		100.5 ± 11.8	
	t 30	91.5 ± 16.4	+7.2	111.3 ± 19.3	+9.7	111.2 ± 21.1	+16.5	102.7 ± 16.7	+13.7	107.7 ± 13.4	+7.2
	t 60	90.6 ± 17.3	+6.1	109.9 ± 14.7	+8.3	107.6 ± 15.2	+12.7	100.0 ± 14.2	+10.8	109.8 ± 16.5	+9.3

Subjects consumed one apple per day (two Annurca apples per day) for 2 months.
Value are means ± SD ($n = 5$).
Results are significantly different at a level of $P = 0.001$.
RD, Red Delicious; GS, Granny Smith; F, Fuji; GD, Golden Delicious.

is on average half that of the commercial varieties considered in this study) for 2 months. Analysing results, we can assert that the administration of apples led, in all groups, to a certain variation of plasma TC levels. As shown in Table 2, Annurca showed the highest hypocholesterolaemic effect, decreasing the TC by 8.3%, followed by GS and RD (−4.4% and −3.1%, respectively), while F and GD apples showed the least effect (−2.0% and −1.3%, respectively).

Some published human studies have recently been undertaken to investigate the TC-lowering effects of apples or apple products. Dietary intervention with whole fresh apples provided the most appreciable results, while no significant effects were registered with other apple products (juice, whole dried apples). Gormley *et al.*[22] conducted a study in which 76 mildly hypercholesterolaemic men participated in a time-course, parallel design. The intervention group was assigned to eat an average of three apples (Irish Golden Delicious) a day, and the control group was allowed to eat a maximum of three apples per week, both over 4 months. The serum TC levels of the intervention group decreased by 8.1%. Two additional studies used a design without comparisons to controls, and consisted of a single intervention with supplementation of two to three apples (Red or Golden Delicious) per day.[23,24] In the study by Sable-Amplis *et al.*,[23] a total of 30 hypercholesterolaemic men and women were instructed to maintain their habitual diets, while adding apples as prescribed. Their TC levels were measured before and after 1 month of intervention. A significant reduction in TC (by 9–12%) was observed for both groups. TC-lowering effects were also observed in the study by Girault *et al.*[24] A total of 235 men and women participated in this study, and 109 of these subjects were overweight or hypercholesterolaemic. Significant results were found only among the hypercholesterolaemic subjects. In this group, the TC levels decreased by 5–6%. The intervention lasted 2 months, and there were no inclusion or exclusion criteria. The more recent single-blinded, randomized, cross-over human study by Ravn-Haren *et al.*[25] evaluated the effect on plasma TC in 34 healthy men and women who were each assigned to a period of 4 weeks with 550 g of whole apples (Shampion cultivar) per day. A slight reduction (5.6%) in plasma TC levels was found. The other available human studies with fresh apples reported an insignificant variation of plasma TC.[26–28]

All of the apple constituents have been reported to affect plasma TC levels, although high amounts of individual components have been indicated as necessary to provide significant effects in humans. These studies would prove that a combination of such constituents, rather than taken alone, would be more effective on plasma TC levels. Data from human studies demonstrate that pectin at a minimum dose of 6 g d^{-1}, corresponding to seven to eight medium-sized apples per day, was necessary to decrease TC by 6%,[13,32,33] while phytosterols at a dose of 2–3 g d^{-1}, corresponding to at least 120 apples per day, led to a similar result.[44] Although similar results have been reported for apple polyphenolic constituents,[34,45] attention has been given to these compounds because, apart from directly regulating plasma TC levels, they can also exert an important antioxidant protection function in both LDL and HDL structures, thus contributing to the maintenance of a healthy balance of plasma cholesterol lipoproteins.[46,47]

Although the molecular mechanism of action is still to be clarified, previous studies have hypothesized that apple oligomeric procyanidins, a peculiar apple polyphenolic fraction, would play a major role in regulating plasma TC levels. In particular, Tenore *et al.*[19] have positively correlated the oligomeric procyanidin content of apple polyphenolic extracts with their capacity to inhibit cholesterol uptake in HepG2 cell lines. Actually, a previous study had already indicated grape seed oligomeric procyanidins as capable of acting like β-cyclodextrins by promoting the formation of micelle-like complexes, in which cholesterol is selectively and intensively incorporated, thus preventing their uptake and giving a false impression of inhibited cholesterol membrane transport in these cells.[18]

Table 2 clearly indicates that the same trend (Annurca > GS > RD > F > GD) found for TC is maintained as regards the apple LDL-C lowering and HDL-C raising effects. On average, consumption of one apple (or two Annurca apples) per day caused a significant variation of plasma LDL-C levels. Interestingly, Annurca showed the highest effects, favouring a decrease in LDL-C by 12.1% (Table 2).

Published human studies available in the literature have reported only slight plasma LDL-C-lowering capacities of whole fresh apples, while no significant effects have been reported with apple products such as juice or whole dried apples. The above-mentioned human study by Girault *et al.*[24] indicated a decrease in plasma LDL-C by 5.5%. An unblinded, randomized, crossover human study[29] evaluated the effect on plasma total cholesterol in 25 healthy men and women who were each assigned to a period of 6 weeks with 340 g of whole apples per day. A small reduction (5%) in plasma LDL-C levels was found. The above-mentioned human study by Ravn-Haren *et al.*[25] reported a decrease by 6.7% in plasma LDL-C.

As stated above, pectin at a minimum dose of 6 g d^{-1} led to a significant effect (−6%) on total plasma cholesterol in humans.[13,32,33] Since no effect was detected on HDL cholesterol and triglycerides, it was reasonable to expect that LDL and VLDL were the main plasma cholesterol fractions affected. Similarly, the decrease in plasma TC reported in an intervention study with phytosterols was ascribed exclusively to an influence on LDL-C levels.[44] No previous data are available regarding which polyphenolic compound in apples could play a major role in the apple effects on LDL metabolism. It is reported that the addition of increasing amounts of tea catechins would be able to upregulate the LDL receptor binding activity of HepG2 cells in a dose-dependent manner.[48] Procyanidins – oligomeric compounds consisting of catechin and epicatechin monomers – may be expected to act through a similar mechanism. Interestingly, Tenore *et al.*[20] have observed a good linear correlation between apple peel and flesh procyanidin content and *in vitro* LDL receptor binding upregulation ($R = 0.9488$ for peel; $R = 0.9887$ for flesh).

Our experimental data reported in Table 2 reveal that consumption of one apple (or two Annurca apples) per day caused a significant variation of plasma HDL-C levels. Very interestingly, Annurca showed the highest efficacy, favouring an increase in HDL-C values by 15.2% (Table 2).

Human dietary intervention studies with both whole fresh apples and apple products have provided contrasting results regarding the effects on plasma HDL-C levels. The above-mentioned studies by Gormley *et al.*[22] and Girault *et al.*,[24] both with apples, registered an increase by 12.5% and a decrease by 5.8%, respectively, of HDL-C. The more recent study by Mee and Gee[30] involved administering 568 mL filtered apple juice per day to 25 hypercholesterolaemic men. A significant decrease (−9%) in HDL-C from the baseline was observed in a 6-week crossover study.[30] The other available human studies with both fresh apples and apple products reported an insignificant variation of plasma HDL-C.[23,25–29,31] No previous data are available regarding which polyphenolic compound in apples could play a major role in the effects of apple on HDL-C metabolism. Studies conducted on animals (hyperlipidaemic rats) and humans (subjects with obesity and metabolic syndrome) have tested the effectiveness of tea catechins in enhancing plasma HDL-C levels;[49,50] catechin and epicatechin oligomeric compounds may be hypothesized to follow a similar mechanism of action. Actually, a recent study[20] has indicated that apple polyphenolic extracts are able to increase ApoAI levels – the major protein component of HDL particles in plasma – in HepG2 cell lines. A generally significant correlation between apple peel and flesh procyanidin content and increase in ApoA1 cell expression was revealed ($R = 0.8946$ for peel; $R = 0.9772$ for flesh).[20]

Along with these hypocholesterolaemic effects, a significant rise in glucose and triglycerides in all groups supplied with apples has been found (Table 2). Actually, this is not surprising considering that, although apple sugar content is lower than the average of most fruits, almost 90% of the energy from an apple is derived from its carbohydrates, mainly sugars, of which fructose is the dominant form, followed by glucose. Moreover, fructose is lipogenic, as its breakdown products can either enter the citric acid cycle or can serve as substrates for the formation of mevalonate and triglycerides.[51]

It has to be said that a thorough comparison of our clinical trial results with others previously published is not really feasible as either the number of consumed apples per day or the apple species investigated is diverse; nonetheless, the results obtained within the group supplied with Annurca (−8.3% TC, −14.5% LDL-C, +15.2% HDL-C) were beyond all expectations and represent an unprecedented result, never obtained with any other food until now.

CONCLUSION

The present study shows that, according to the literature, the effect of daily apple consumption on plasma cholesterol levels is strictly dependent on the kind of apple cultivar and its polyphenolic composition. Daily consumption of two Annurca apples beneficially affects serum cholesterol levels in healthy subjects. In particular, among the different apple cultivars tested, and according to our previous *in vitro* data, Annurca apple has provided the most appreciable results, decreasing TC and LDL-C by 8.3% and 12.1%, respectively, and increasing HDL-C by 15.2%. The effect on HDL-C is the most promising since very few natural or pharmaceutical substances have been reported to exert an appreciable positive influence on the so called ‘good cholesterol’. Our data would reasonably indicate Annurca apple to be a functional food able to contribute to the maintenance of a healthy balance of plasma cholesterol levels, and thus may be regarded as a useful tool for the prevention of CVD risk through normal diet.

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