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Original article

A 4-week consumption of light or dark roast unfiltered (Turkish) coffee affects cardiovascular risk parameters of homocysteine and cholesterol concentrations in healthy subjects: a randomized crossover clinical trial

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Summary. *Background/aim:* The aim of this study is to investigate the impact of boiled, unfiltered (Turkish) coffee consumption on the plasma cardiovascular risk parameters of healthy subjects. The study also explores whether two unfiltered coffee beverages that differ in content due to varying degrees of roasting will affect cardiovascular biomarkers differently. *Methods:* In this crossover intervention study, healthy, nonsmoking, habitual Turkish coffee drinkers (n=28) were randomized to consume at least 3 cups of Light (LR) or Dark (DR) roast Turkish coffee brews per day for 4 weeks after a washout period (WO) of 2 weeks. Subsequent to each coffee abstinence period, both groups received the alternative intervention. After the first WO and the coffee intervention periods, anthropometric measures, blood pressure, heart rate and 10 biochemical parameters were collected and dietary records were completed. *Results:* The consumption of 3 ≥ cups Turkish coffee/day for 4 weeks, compared with the results after 2 weeks of coffee abstinence, led to a significant increase in homocysteine levels of habitual Turkish coffee drinkers in both coffee interventions ($p<0.01$). Anthropometric measurements, fasting blood glucose, blood pressure and heart rate did not change during the coffee consumption phase in either of the Turkish coffee groups. Both roasts increased concentrations of serum lipids compared to WO. However, only DR Turkish coffee intake significantly increased total cholesterol levels ($p<0.05$). *Conclusion:* Moderate amounts of LR or DR Turkish coffee consumption for 4 weeks, although differing in content, largely show similar biological effects as demonstrated by the tested cardiovascular biomarkers.

Key words: cardiovascular disease, cholesterol, homocysteine, coffee, diterpenes

Introduction

Coffee is widely consumed everywhere in the world and is prepared using several different methods (1). Turkish-style coffee, which is consumed in Greece, Cyprus, the former Yugoslavia, Turkey and the Middle East including Israel, is boiled coffee with a unique brewing procedure (2,3). Epidemiological studies suggest that consumption of boiled, unfiltered coffee is related to elevated risk of cardiovascular disease (CVD) (4). However, considerable controversy exists regarding the link between coffee consumption and CVD risk (5).

It has been shown that different coffee preparation and brewing methods influence the concentration of compounds present in the final coffee brew (6). Different from the coffee usually consumed in the western world, Turkish coffee is not drip filtered, but rather its preparation involves slowly boiling water that is mixed with finely ground Arabica coffee beans (7). This method causes a greater amount of biologically active

components (caffeine and diterpenes) to remain in the liquid (7,8).

Recent studies have shown that the diterpene content of a standard cup of coffee is highest in unfiltered preparation methods such as Scandinavian-style boiled coffee, French press (cafetiere) and Turkish coffee (with up to 88.7 mg/L in some Turkish brews) (1). The diterpenes (cafestol and kahweol) are compounds found in the lipid fraction of the coffee, and are associated with an increase in blood cholesterol (8). It was stated that the consumption of 10 mg cafestol per day- the amount present in three cups of unfiltered Turkish coffee- for 4 weeks increases serum cholesterol level by 5.0 mg/dL, whereas the consumption of 10 mg kahweol only rises it by 0.9 mg/dL (3,9). Nevertheless, favorable health effects have also been ascribed to diterpenes and data present in literature shows antioxidant activity, hepatoprotective, anticarcinogenic, anti-iflammatory and anti-angiogenic functions (10). Diterpene concentration in roasted coffee beans can be affected by various factors such as species, the cultivar, year harvested and degree of roasting (8). There are several other ingredients within coffee, which contribute to the biological activity (4). In addition to preparation method, roasting process greatly affects the chemical composition of the coffee (11,12). Coffee types which contain different major constituents have distinct cardiovascular health effects (13,14). Furthermore, coffee dose is defined as a potential modifier on CVD outcomes and in the meantime, current literature supports an association with 3-5 cups/day (5,15,16).

To the best of our knowledge, there are limited number of clinical trials investigating the relation between boiled coffee intake and cardiovascular health outcomes. Moreover, the effect of Turkish coffee has not yet been studied experimentally (3). This is the first study to investigate the impact of boiled, unfiltered (Turkish) coffee consumption on the plasma cardiovascular risk parameters of healthy subjects. The study also explores whether two boiled (Turkish) coffee beverages with different degrees of roasting produce distinctive cardiovascular biomarkers. Since Cyprus has a high number of regular boiled coffee drinkers, mean annual coffee consumption per capita is 6.1 kg which is higher than in the European community and United States (14). It would be interesting to see whether the effects on cardiovascular disease risk parameters would also be detectable in long-term habitual drinkers.

Materials and Methods

Study Subjects

Thirty healthy habitual Turkish coffee drinkers; Eastern Mediterranean University (EMU) staff, students or relatives who saw study information about the study published in the campus area or on the internet, were recruited to the study.

Eligibility criteria were: Regular Turkish coffee consumption of ≥ 1 cups/day, age 20-35 years, healthy, body mass index ((BMI) 18.5-24.9 kg/m2), non-smoker or former smoker (more than a year), willingness to abstain from coffee drinking and to consume ≥ 3 cups/day of Turkish coffee for 8 weeks.

Exclusion criteria were: Acute or chronic diseases, severe illness with in-patient treatment during the last 3 months, use of regular medication or any supplements, weight reduction >2 kg/week during the last month, pregnancy, breastfeeding, regular strong physical activity with ≥1h/day. We also excluded high intake of alcohol (defined as a weekly intake of >7 units for women and >14units for men), excess dietary consumption of total fat (>35% of daily calories), saturated fatty acid (>10% of daily calories) or cholesterol (>300mg/day).

Study Design

Ethical approval was obtained from the Faculty of Health Sciences of Eastern Mediterranean University (Famagusta, North Cyprus) and the study was registered as a clinical trial (NCT03495336). All subjects were informed about the aims of the study, agreed to participate and signed a consent form.

A survey was carried out to gather information regarding physical activity, medical condition and medication. Additionally to evaluate the inclusion to the study, a food frequency questionnaire (FFQ) and anthropometric measures (weight, height, body fat percent, fat free mass (FFM), waist circumference) were collected at the baseline interview. Our crossover clinical trial lasted 12 weeks (with two wash out and two coffee intervention periods (As shown in Figure)). After 2 weeks (wk)

washout period (coffee abstention phase), participants were assigned to one of the two coffee interventions (LR or DR) for 4-wk. At the end of this coffee phase subjects participated in an additional 2-wk wash out period and then for the next 4-wk period, they switched to the opposite/other coffee roast.

Participants were asked to maintain a stable exercise routine, sleep pattern and dietary habits throughout the study and were asked to avoid vitamin supplements, foods/beverages rich in caffeine (including coffee, cola beverages, cocoa, chocolate, energy drinks, and tea) and to keep 3-day food diaries (1 weekend day and 2 weekdays) prior to each measurement. Daily nutrient intake was calculated by using computer software (Ebispro, Stuttgart, Germany; Turkish version: BeBiS, Vers. 6.1). In order to assess physical activity levels, the validated Turkish version of the International Physical Activity Questionnaire (IPAQ)-short was administered (17).

Coffee Samples

Two commercially available Turkish Cypriot coffee blends differing in roasting were used in the study. Both coffee roasts were a blend of 100% Arabica (Coffea Arabica) and cultivated in the same geographic region (Brazil). They were vacuum-packed and provided by a different manufacturer. Roasting classification degree was measured according to "Roast Color Classification System" (Agtron/SCAA, Reno, NV,1995). The roasting processing time and temperatures were reported as 20 mins-200˚C and 40 mins-210 ˚C for LR and DR respectively. Coffee was distributed to participants in packages at the beginning of the each intervention. Subjects were instructed on how to prepare the beverage (5 g of Turkish coffee, 55 ml cold water) in a house hold Turkish coffee maker or cezve and were given standard cups and spoons. Participants were asked to drink at least 3 cups of the suggested Turkish coffee roast per day without a fixed schedule and to refrain from other types of coffee beverages throughout the study period. To control sugar intake only the customary (pre-study habitual) amount of Turkish coffee with sugar were allowed and the participants were asked to consume the rest of the coffee as plain (without sugar).

Coffee was brewed as instructed to the participants and analyzed for the caffeine and diterpenes content. Caffeine was determined by high-performance liquid chromatography (HPLC, Aligent Technologies, USA) with diode-array detector (DAD) and mass spectrometer. Cafestol and kahweol were analyzed in the unsaponified matter by HPLC-DAD.

Blood Sample Analysis

Subjects were advised to avoid the use of alcohol 72 hours before the blood sample collection. After an overnight fasting (min. 8 – max. 12 hours), blood samples were obtained at the end of the initial wash out period and after each 4-wk coffee intervention period, and sera were stored at –30˚ C, until analytical measurements were performed. The levels of serum glucose, triglycerides (TG), total cholesterol (TC), and high-density lipoprotein (HDL-C) cholesterol were determined using a Dimension Xpand Plus integrated clinical chemistry autoanalyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The serum levels of low-density lipoprotein (LDL-C) cholesterol were calculated using Friedewald's equation. EDTA-treated blood samples for total homocysteine analysis were immediately refrigerated (placed on ice) until the plasma was separated by centrifugation. All the samples were assayed for homocysteine by using High Performance Liquid Chromatography with fluorescent detection technique (HPLC-FLD). Serum Malondialdehyde (MDA) levels were determined with a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the procedures provided by the manufacturer.

Assessment of other Measurements

Weight, height, abdominal circumference, body fat and blood pressure were measured after the washout period and after each intervention. Weight (in kilograms) was measured in light clothing, without shoes, to the nearest 0.1 kg; height was measured using a stadiometer to the nearest centimeter; and BMI was calculated (weight/height squared; in kilograms per square meter). The percentage of body fat and FFM was measured by Tanita Segmental Body Composition Analyzer BC-418 MA (Tanita Corp. Tokyo, Japan) and waist circumferences (midway between the rib cage and the iliac crest) were measured using a flexible tape. Blood pressure (BP) and heart rate was monitored using an automatic arm sphygmomanom-

eter (Pic Indolor Diagnostic, BS 150, Artsana, Italy) after a 5-min rest in a sitting position.

Statistical analysis

The results are expressed as means. Caffeine and diterpenes content of the two coffee beverages were reported as mean±standard deviation (SD) and Kruskal–Wallis test was used for statistical comparison of coffee roasts. Differences in human variables were analyzed by repeated-measures analysis of variance (ANOVA) for comparisons of LR coffee intake with DR and of each roast with the washout. All analyses were performed using SPSS 24.0 (IBM-SPSS Inc., Chicago, IL, USA). A two-tailed $P < 0.05$ was considered significant.

Results

Subject characteristics

Out of thirty healthy volunteers who were recruited for the study, one woman and a man dropped out during washout period because of a severe illness (this had been predefined as exclusion criteria). Thus, twenty-eight healthy nonsmoker habitual Turkish coffee drinkers (27. 50±5.30 y, range 20-35 y) were evaluated in the study. Half of the participants were female and all the subjects were sedentary (physical activity less than 1h/day, PAL: 1.52±0.12). According to the questionnaire results, the daily habitual Turkish coffee consumption of the subjects was 2.44±1.19 cups per day (min. – max. = 1- 5 cups/day). Participants' baseline characteristics are summarized in Table 1. During the first 4-wk intervention period half of the participants (n=14, 50% women) ingested LR coffee and other half (50% women) consumed DR coffee and in the next 4-wk period, they switched to the opposite coffee roast (Fig.1).

Coffee analysis

Coffee gave 1.71±0.07 mg of caffeine per mL in LR (94.05 mg per cup/55 mL) and 1.97±0.03 mg per mL in DR (108.35 mg per cup/55 mL). The mean concentrations of cafestol and kahweol were 1.79± 0.09 mg and 1.67±0.073 mg per cup/55 mL in LR and 6.83±0.27 and 6.17±0.12 mg per cup/55 mL in DR, respectively ($p=0.001$). DR provided more cafestol and kahweol than LR ($p<0.05$). Table 2 shows mean

Table 1. Baseline characteristics of the participants (N=28) [1]

Gender (male/female, n)	14/14, 28
Age (year)	27.50±5.30
Body mass index (kg/m²)	23.32±3.44
Fat mass (male/female, %)	20.74±5.36 (20,16 ± 4,53/21,32 ± 6,19)
Waist circumference (male/female, cm)	83,02±13,13 (92,00 ±10,8/73,35 ±7,20)
Habitual Turkish coffee consumption before the trial (cups/day)	2,44±1,19
Instant coffee(cups/day) (n=15)	1,67±0,82
Filter coffee(cups/day)(n=2)	1,00±0,00
Other coffee types	0
Carbohydrate (energy%)	47.11±6.82
Protein (energy%)	17.96±3.78
Total fat (energy%)	34.11±6.80
Cholesterol (mg/d)	372.99 ±244.61
Saturated fat (g/d)	33,93 ±18,44
Fiber (g/d)	20.81±8.20
Physical activity level (PAL)	1.52±0.12

[1] *Data expressed as mean ± standard deviation, numbers or %*

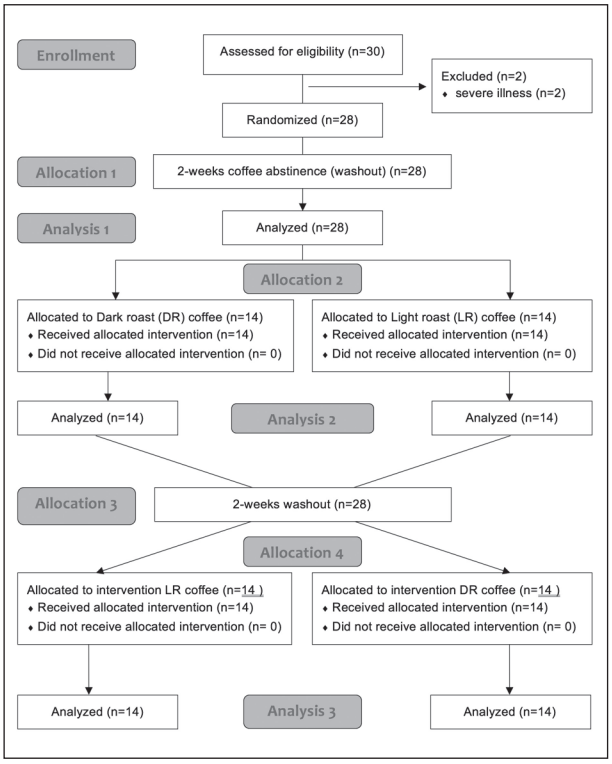

Figure 1. Study design.

Table 2. Mean ± SD concentrations of selected constituents in the commonly consumed Turkish Cypriot market blend Light roast (LR) and Dark roast (DR) Turkish Coffees.[2]

Compound	LR	DR
Caffeine (mg/mL)	1.71± 0.07	1.97±0.03
Cafestol (mg/55mL)	1.79±0.09[a]	6.83±0.27[b]
Kahweol (mg/55mL)	1.67±0.07[a]	6.17±0.12[b]

[2] Concentrations of components have been described as mean from duplicate measurements from five independent samples. Kruskal–Wallis test was used to analyze whether the repeated measurements within groups demonstrate any difference from the median. Different letters on the same line indicate significant difference (p<0.05).

± SD concentrations of selected constituents in the market blend Light roast (LR) and Dark roast (DR) coffee. Subjects consumed two different coffee blends with almost similar caffeine but substantially different diterpen (cafestol and kahweol) contents. The mean number of cups of coffee per day did not significantly differ between both groups.

Cardiovascular risk biomarkers

Cardiovascular effects of Turkish coffee consumption on food intake, anthropometric measures, physical activity, blood pressure and blood glucose

Self-reported diets (a 3-d food diary) showed that none of the participants consumed a significant amount of caffeine foods other than coffee during the study and the nutritional intake was similar before (during washout) and after each intervention period for all participants (P > 0.05). None of the dietary intake parameters showed statistical differences (Table 3) and volunteers did not report any changes in their physical activity throughout the study (data not shown).

Compared to no-coffee periods, subjects drank 3 ≥ cups/d (165 mL/d or more) of Turkish coffee in each 4-wk coffee periods. No adverse or side effects were seen in any of the coffee intervention groups.

There were no significant differences found between the coffee abstention period and the two coffee groups for primary anthropometric measures (body weight, body fat mass, FFM, BMI and waist circumference) (Table 4). However, notably, the body fat percent of subjects was nonsignificantly reduced following consumption of each coffee roast for 4-wk, by 1% (from 21.73±5.37 to20.63±5.38 in the LR and 20.66±5.47 in the DR p=0.057 each, Table 4). No significant change was observed for diastolic or systolic blood pressure, heart rate and fasting blood glucose (p>0.05).

Plasma Total Homocysteine (tHcy) Levels

The consumption of Turkish coffee (3 ≥ cups/d) for a 4-week period compared with the results after a 2-wk coffee abstinence, led to a significant increase in homocysteine (μmol/L) levels. The mean concentration of plasma homocysteine was 9.66±2.24 μmol/L at the end of the no-coffee period, 12.03±3.08 μmol/L at

Table 3. Average daily nutrient intake of volunteers on the basis of 3-day food records during the study.[3]

Energy and Nutrient Intake	WO	LR	DR
Energy (Kcal)	2103. 47±771.12	2102.36±763.50	2083.62±760.28
Proteins (g)	93.52 ±43.26	89.95 ±41.58	91.25 ±43.79
Lipids (g)	115.15±52.06	116.24±50.12	114.58±51.64
Saturated fatty acid (FA) (g)	32.98±17.25	33.83±18.45	33.60±17.46
Monounsaturated FA (g)	39.62± 18.72	36. 21± 18.51	36. 17± 18.70
Polyunsaturated FA (g)	22.52±11.79	22.80±11.64	23.12±11.69
Omega-3 (g)	2.26±1.40	2.35±2.42	2.41±1.85
Omega-6 (g)	20.26±10.87	20.45±12.08	20.69±9.68
Cholesterol (mg)	357.99±254.61	364.69±248.29	364.79±251.17
Carbohydrates (g)	173.26±59.39	174.10±54.91	171.85±57.13
Dietary fibre (g)	20.98±8.40	21.14±8.63	21.35±7.96

[3]Data are expressed as mean ± SD (N=28). Group comparisons were analyzed by repeated-measure ANOVA, followed by the Friedman test. No significant differences were detected between groups (p>0.05).

Table 4. Concentration of serum lipids, plasma total homocysteine and other cardiovascular risk parameters in coffee-free period and changes after 4 weeks of LR or DR coffee ingestion.[4]

Biomarkers	WO	LR	DR
Homocysteine (μmol/L)	9.66±2.24	12.03±3.08**	11.82±3.22**
Cysteine (μmol/L)	251±26.23	282.98±35.91**	289.69±41.33**
Cysteine/Homocysteine	27.05±5.42	24.99±6.8	25.67±5.18
Fasting blood glucose (mg/dL)	89.93±14,5	90.67±17.43	90.04±18.92
Total cholesterol (mg/dL)	182.93±33.87	190.63±29.02	192±31.94*
HDL cholesterol (mg/dL)	54.37±12.24	57.52±14.09	57.04±12.26
LDL cholesterol (mg/dL)	112.81±32.43	116.85±28.85	117.11±29.48
VLDL cholesterol (mg/dL)	3.94±1.8	4.31±1.35	4.55±1.61
Triglycerides (mg/dL)	78.74±35.99	86.15±27.04	91±32.15
Body weight (kg)	67.85±16.86	67.51±17.18	67.6±17.35
Body mass index (kg/m²)	23.25±3.57	23.16±3.61	23.18±3.65
Fat mass (%)	21.73±5.37	20.63±5.38	20.66±5.47
Systolic blood pressure (mmHg)	117.5±13.3	115.79±13.7	114.29±12.84
Diastolic blood pressure (mmHg)	76.54±9.19	75.61±7.8	76.04±8.83
Heart rate (beats/min)	78.75±11.15	80.96±13.73	81.5±11.89
Malondialdehyde (MDA) (μM)	28.16±35.9	27.92±25.34	18.02±12.74

[4] *ANOVA, analysis of variance;*
Data expressed as means ± SD (N=28).
***Significantly different from (coffee-free period) washout (ANOVA of repeated measures): P<0.01*
**Significantly different from washout (ANOVA of repeated measures): P<0.05*
§Significantly different from LR Turkish coffee (ANOVA of repeated measures): P<0.05

the end of the LR and 11.82±3.22 μmol/L at the end of the DR coffee periods. We thus observed an increase ($p<0.01$) in homocysteine concentrations after LR and DR intake with 24.5% (or 2.4 μmol/L) and 22.4% (or 2.2 μmol/L), respectively caused by unfiltered Turkish coffee. The significant effect of Turkish coffee on the homocysteine (μmol/L) and cysteine (μmol/L) concentration was seen during both coffee roast interventions. However, no differences between coffee effects on homocysteine and cysteine could be determined by group comparison. In addition, no noticeable changes were seen for the cysteine/homocysteine ratio.

Blood Lipid Concentrations

Although we observed a small rise in all serum lipid parameters after the consumption of either LR or DR coffee compared to abstention, there was no significant impact ($p>0.05$) on HDL-C, LDL-C, VLDL-C and TG plasma concentrations. Table 4 shows an increase in TC levels after each coffee roast intake compared to WO (from 182.93±33.87 mg/dL to 190.63±29.02 mg/dl in the LR-group and 192±31.94 mg/dl in the DR group). However, only DR intervention enhanced TC concentrations significantly (5.5%, $p<0.05$). Relative to baseline values, DR Turkish coffee raised mean TC concentrations by 10.0 mg/dL (0.56 mmol/L). No difference in TC levels between the coffee roasting intervention periods observed. In addition, no difference in other serum lipid parameters was monitored between coffee roasts.

Discussion

Previous randomized control trials reported that a high intake of unfiltered and filtered coffee (1 L/d) elevate plasma homocysteine concentrations (18,19). In our crossover study, although the coffee intake was lower (three-five cups) both coffee groups exhibited a greater increase of mean plasma total homocysteine (tHcy) levels compared to preceding control trials. In contrast, in a small clinical trial, Esposito et al. (20)

were unable to detect any significant rise in homocysteine levels, from drinking five cups of Italian style coffee per day for one week. Moreover, Mursu et al. (21) emphasized that consumption of coffee in a short-term did not increase tHcy levels. Possibly, in our study the intervention time of 4 weeks was long enough and/or the amount of coffee consumed was sufficient to promote changes within that parameter. Taken together, no difference between the effectiveness of both coffees on tHcy parameters was observed. Therefore, our results provide unequivocal evidence that Turkish coffee in moderate amounts would increase total plasma homocysteine concentrations, regardless of its roasting degree. In our study, the analysis of Turkish coffee blends showed different amounts of diterpenes (both cafestol and kahweol), but the concentrations of caffeine in both roasts were almost equal and the increase on tHcy levels were similar. This result might reflect the assumption that caffeine is one of the coffee constituents which increases plasma tHcy levels. In addition, Verhoef et al. (22) claimed that caffeine has been suggested to be partly responsible as the component in coffee, and coffee but not caffeine itself, as the factor that influences the rise in homocysteine levels in the blood after consumption. Thus in the present study, the changes can be related to the increased amount of coffee and caffeine intake during interventions which may have led to a significant increase in homocysteine levels. Moreover with our results, question still remains as to whether healthy subjects with increased homocysteine levels affects the cardiovascular system in the same way as it would in prediagnosed patients (23). It can be concluded that drinking large quantities of Turkish coffee could raise homocysteine in plasma. However, whether this raises the risk of cardiovascular disease is not yet certain (19). As previously reported by other authors, an important finding of this study was that there was a notable positive dose-response relation between Turkish coffee consumption and plasma tHcy, which was stronger than the relation between coffee and total serum cholesterol increase (23,24).

The results consistently show that unfiltered coffee has a negative effect on plasma serum lipid levels (25-27). In accordance with this, one of the key findings of the present study is that both Turkish coffee roasts increased serum lipid levels whereas only DR Turkish coffee intake significantly increased TC levels. This is believed to be due to the diterpenes (26,27) because the DR coffee contains higher amounts of cafestol and kahweol (ca. three times more of both) and is found more effective in significantly increasing serum TC than the LR coffee. It cannot be ruled out that the observed significant TC increase in our study was due to caffeine as seen in other studies (5,28) because both of the coffee roasts had similar amounts but exhibit different effects. In other words, we here demonstrated for the first time that roasting might affect the total diterpene profile of Turkish coffee (both cafestol and kahweol) profile and in turn, the TC levels of subjects. In several studies, specifically cafestol has been stated to be the most potent cholesterol raising compound of a coffee brew (5,26,27). The reason for the increase of plasma concentration due to the effect of cafestol is not entirely known (5). However, recently a mechanism that could explain this effect was proposed. It was stated that cafestol elevates serum cholesterol levels by activating farsenoid X and pregnane X receptors in the intestine causing the body to send a signal to the liver to stop the breakdown of cholesterol. As a result cholesterol accumulates in the serum and leads to an increase in concentrations (26).

An interesting finding that should be mentioned is that we did not observe a significant increase in serum LDL-C and TG levels, a cafestol consumption-related effect which was also seen in several studies (26,28, 29,30). Our potential explanation for this is the quantity of Turkish coffee intake and the relatively taken diterpen concentrations whereas the aforementioned increase in most of the literature data was observed with a marginal amount of unfiltered coffee (3,25,28,30). This would be in agreement with the results of a recent study where the daily volumes of (62.3±40.60 ml; 0.7±0.50 cup) Turkish coffee consumption caused no significant alteration in the serum lipid levels among the study population (13).

In addition to the relationship between the amounts of coffee consumed, the method of coffee preparation is also an important determinant of serum lipid levels (28,30). According to previous studies, shorter contact with hot water and retention of diterpenes by filter paper are the reasons for poor or no influence of filtered coffee on serum levels when com-

pared to boiled coffee (29,31,32). However, in a recent randomized clinical trial Corrêa et al. (5) found that drinking 3–4 cups of light- or medium-roasted paper-filtered coffee per day for 4 weeks, compared to our study, exhibited a stronger increase in TC and LDL-C levels in both groups even though the cafestol content is almost similar and has a lower-level of kahweol per cup. It was reported that the difference in results might be related to the higher caffeine intake (28,33) since it was almost twice as much compared to ours (28, 33). Therefore, in addition to the primary effect of diterpenes on TC levels, caffeine might have an impact in a dose dependent manner. Further studies should be conducted in order to investigate and observe the required doses.

Most of the interventions failed to demonstrate a significant decrease in lipid damage marker MDA with the exception of results found by Sirota et al. (34) who stated that consumption of 200 mL Turkish roasted coffee during a meal based on red-meat cutlets resulted in a significant inhibition of postprandial plasma MDA. In addition, Yukawa et al. (35) found a modest reduction of LDL oxidation susceptibility and a significant decrease in serum cholesterol, LDL cholesterol and MDA levels after consumption of 150 mL (8g Arabica) coffee 3 times per day for a week (36). No effect between treatments and control/placebo were found by other authors (21,37,38,39) which was consistent with our results. However, Leelarungrayub et al. (31) reports a significant higher level of MDA in men consuming 3 cups of caffeinated coffee (150 ml coffee, 8 g Arabica/cup) for a week, when compared to decaffeinated coffee or control, followed by a submaximal exercise test. This study is particularly relevant as similar results were found in previous tests where increased intramuscular fat oxidation was observed after caffeine-rich foods were consumed (31). However, it is not yet clear why there are certain discrepancies with a few of the previous reports. It may be due to the differences between study designs since especially, other study trials have observed a relatively large amounts of coffee consumption in contrast to this study.

None of the anthropometric measures were changed in either of the roasting groups after the consumption of coffee for 4-weeks. A similar finding has been reported in a recent study in overweight adults (40). An important finding of our study is that even though the body weight remained unchanged, we observed a slight loss in body fat percentage. In a crossover study consumption of coffee for 4 weeks resulted in a significant reduction in body fat levels of healthy subjects irrespective of roasting degree. In addition, it was demonstrated that coffee constituents other than those associated with roasting involved a significant, but weaker reduction in body fat content (41). Bakuradze et al. stated that the significant change in body fat and unvaried change in weight may be explained by the particularly counterbalancing effect of some gain in FFM (42). However, this result is not in accordance with our finding where there was no change observed in FFM after coffee intake.

The relation between regular coffee consumption and blood pressure (BP) was demonstrated with a J–shaped curve (43). Simply, it was stated that a moderate consumption of coffee may have a protective effect on BP, whereas high intake could increase the risk of hypertension (33,43). In normotensive habitual Turkish coffee drinkers in our study, we did not observe an increase in BP in 4 weeks. A Similar finding in a recent randomized control trial (33) explained that in habitual coffee drinkers a partial tolerance to caffeine might be developed as a result of the duration of consumption.

There are several strengths and limitations to be noted. Firstly, it can be stated that in our study, a placebo/control group was lacking. Secondly despite the subjects' coffee refinement between the interventions, the absence of the measurements for the second washout period is a potential limitation. Additionally our study has comprised a small sample size, which included a continued participation period of 12 weeks and a fixed dose of coffee intake. However, strength of this study lies in the amount of coffee used and the method of preparation which are similar to those used by the study population in real life. Therefore, to recommend consuming coffee in line with the amounts and preparation method used in this study is a suitable way to observe the desirable effects of Turkish coffee in real-life circumstances. Moreover, the crossover design of our study and the distribution of subjects eliminated gender differences as a cofounding factor. In addition, another limitation of this study is the possibility of a

discrepancy between the number of coffee pads given to participants and the mean number of cups of coffee they reported to have consumed during coffee intervention. For future studies in order to handle this problem at baseline and during coffee intervention we should recommend analyzing serum diterpene (cafestol and kahweol) and caffeine concentrations as objective measures for coffee consumption. Finally, another limitation of this study was that other metabolites as well as other components responsible for coffee's total effect on findings were not measured. This is the first clinical trial to show an increase in homocysteine and blood lipids of healthy habitual Turkish coffee drinkers after coffee consumption and to compare the effects of two different coffee roasts on CV disease risk parameters.

Moderate amount of Turkish coffee consumption has significant undesirable effects on total plasma homocysteine biomarkers, regardless of its roasting degree. This can be attributed to the changes related to the increased amount of coffee and caffeine intake during interventions. Although significant increase in cholesterol levels were only seen in the DR coffee intervention group, most likely because of higher cholesterol-raising diterpenes content, a rise in all lipid parameters was observed in both groups. To sum up, the results of this intervention study indicate that 3-5 cups/d of LR or DR Turkish coffee consumption for 4 wks., although differing in contents, largely exert similar biological effects as demonstrated by the cardiovascular biomarkers tested. More interventional studies and the evaluation of other coffee components are needed to clarify the effects of coffee on CV risk factors.

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