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Reduction by coffee consumption of prostate cancer risk: Evidence from the Moli-sani cohort and cellular models

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Meta-analytic data on the effect of coffee in prostate cancer risk are controversial. Caffeine as a bioactive compound of coffee has not yet been studied in deep *in vitro*. Our study aimed at evaluating in a population cohort the effect of Italian-style coffee consumption on prostate cancer risk and at investigating *in vitro* the potential antiproliferative and antimetastatic activity of caffeine on prostate cancer cell lines. 6,989 men of the Moli-sani cohort aged ≥50 years were followed for a mean of 4.24 ± 1.35 years and 100 new prostate cancer cases were identified. The European Prospective Investigation into Cancer and Nutrition-Food Frequency Questionnaire was used for the dietary assessment and the evaluation of Italian-style coffee consumption. Two human prostate cancer cell lines, PC-3 and DU145, were tested with increasing concentrations of caffeine, and their proliferative/metastatic features were evaluated. The newly diagnosed prostate cancer participants presented lower coffee consumption (60.1 ± 51.3 g/day) compared to the disease-free population (74.0 ± 51.7 g/day) ($p < 0.05$). Multiadjusted analysis showed that the subjects at highest consumption (>3 cups/day) had 53% lower prostate cancer risk as compared to participants at the lowest consumption (0–2 cups/day) ($p = 0.02$). Both human prostate cancer cell lines treated with caffeine showed a significant reduction in their proliferative and metastatic behaviors ($p < 0.05$). In conclusion, reduction by Italian-style coffee consumption of prostate cancer risk (>3 cups/day) was observed in epidemiological level. Caffeine appeared to exert both antiproliferative and antimetastatic activity on two prostate cancer cell lines, thus providing a cellular confirmation for the cohort study results.

Regular "Italian-style" coffee consumption is traditional in Italy, and during the past decades, other populations tend to adopt this dietary habit. "Caffè espresso" and "moka" and their combinations with milk "caffè latte," "cappuccino" and "macchiato" are the most widely used recipes for preparing ground coffee in Italy.

Consumption of coffee as a beverage, prepared with different methodologies, has been associated with reduction in the risk of diseases,[1–ô6] suicide risk[7,8] and depression[9] while the study of coffee drinking and cancer risk remains open to investigation. Numerous bioactive compounds are contained in coffee[10] and caffeine (1,3,7-trimethylxanthine; caffeine) catechins and other phenolic compounds have been proposed to exert antitumor effects.[11–ô16]

Among different types of cancers occurring with different etiology and pathophysiology, prostate cancer is one of the most common hormone dependent cancers in men.[17,18] It is more frequently diagnosed after 50 years of life, and its burden shows large variations worldwide;[17,18] in Italy the incidence is one of the lowest in the European Union.[17,19,20] The etiology remains unclear and the discussion about potential risk factors associated with the disease remain open.

The potential effect of coffee consumption on prostate cancer risk has been studied at epidemiological and meta-analytic level[14,15,21] but a serious debate remains ongoing.

The lack of certainty has been confirmed by the recent report of the World Cancer Research Fund International on diet, nutrition, physical activity and prostate cancer.[22] The expert panel stated that data on the effect of coffee consumption on prostate cancer are limited and no conclusion could be derived; while there is no mention on caffeine intake.

Recently, Taylor *et al.*[23] conducted a Mendelian randomization analysis to investigate the causal effects of coffee consumption on prostate cancer risk and progression, in a

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What's new?

Despite the relevance for public health of the prevention of prostate cancer through lifestyle modifications, so far the epidemiological results assessing the impact of coffee consumption on the incidence of the disease are still under debate. Our study aimed at evaluating the effect of Italian-style coffee consumption on prostate cancer risk. Reduction by Italian-style coffee consumption (>3 cups/day) of prostate cancer risk was observed at the epidemiological level in the Moli-sani cohort. Caffeine also appeared to exert both antiproliferative and antimetastatic activity on two prostate cancer cell lines, thus providing evidence at the cellular level supporting the cohort study results.

sample of 46,687 men of European ancestry from 25 studies in the PRACTICAL consortium. A weak positive association between coffee genetic risk score and increased risk of nonlocalized disease was suggested. The need for further studies of the causality of the association of coffee consumption and prostate cancer risk in prospective settings is further supported by these findings.

In the analysis of coffee drinking and prostate cancer risk, it is also important to consider that the methods of preparing coffee vary geographically and culturally and could result in different nutrient composition.[10,24,25] The use of filters is also very common and could result in a significant nutrient variation in the consumed beverage since the part of the ground coffee that does not overcome the filtration process includes significant amounts of different dietary compounds.[10,24,25]

On the contrary, Italian-style coffee is prepared using unfiltered methodologies with high-pressure hot water (about 90°C, "caffè Espresso") or boiling water ("caffè moka")[24] that may imply lower loss of bioactive compounds. This hypothesis increases the need for the study of Italian-style coffee in association with prostate cancer risk.

Thus, this work aimed at evaluating at epidemiological level the association of Italian-style coffee and incidence of prostate cancer in a traditional Italian sample population. Considering the uncertainty provided by the recent meta-analysis[14,15,21] and health reports[22] toward a clear-cut conclusion and trying to identify a possible mechanism of action of coffee constituents, we integrated within the same work results of *in vitro* experiments on the potential antiproliferative and antimetastatic activity of caffeine on PC-3 and DU145 prostate cancer cell lines.

Material and Methods

Moli-sani cohort

Study population. The cohort of the Moli-sani Project was recruited in the Molise region (Italy) from city hall registries by a multistage sampling, as previously described.[26,27] Between March 2005 and April 2010, 11,702 men were recruited and followed up for incident prostate cancer cases. Participants who had a history of cancer ($n = 364$), follow-up time <6 months and nonaccurate follow-up data ($n = 760$) or incomplete dietary data ($n = 499$) or were <50 years old ($n = 3,953$) were excluded from the analysis.

Within a final sample of 6,989 men followed for a mean of 4.24 ± 1.35 years, 100 new prostate cancer cases were identified. Incident cases of prostate cancer were ascertained by direct linkage with hospital discharge forms according to the ICD-9-CM code: 185. Events were validated through medical records when prostate cancer was mentioned in the diagnosis and confirmed by histological analyses. Information on tumor grade and stage at diagnosis was also extracted from medical records. The Gleason score was collected from histopathological reports. Moreover, six fatal cases were assessed by the Italian mortality registry (ReNCaM registry) and validated by Italian death certificates (ISTAT form). A critical evaluation of the diagnosis and the ascertainment of cases was conducted by qualified medical personnel and blinded to the present analyses.

The Moli-sani project was approved by the Catholic University ethical committee. All participants provided written informed consent.

Dietary assessment and definition of other factors. The European Prospective Investigation into Cancer and Nutrition—food frequency questionnaire (EPIC-FFQ) specifically adapted for Italian population was used to determine usual nutritional intakes consumed in the past year.[28] A computer program, Nutrition Analysis of FFQ (NAF)[29] was developed by the Epidemiology and Prevention Unit, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan to convert dietary questionnaire data into frequencies of consumption and average total daily quantity of coffee through different Italian-style recipes (i.e., caffè espresso, moka, caffè latte, cappuccino and macchiato) coffee (g/day) and energy intake (kcal/day). NAF was linked to the Italian food composition tables (FTC) for the energy assessment.[30] In the absence of accurate data on the caffeine content of food in Italy, caffeine intake was calculated using food composition data from National Nutrient Database for Standard Reference (Release 27) of the United States Department of Agriculture.[10]

Subjects were classified as "nonsmokers" if they had smoked <100 cigarettes in their lifetime or they had never smoked cigarettes, "former smokers" if they had smoked cigarettes in the past and had stopped smoking from at least 1 year, and "current smokers" those who reported having smoked at least 100 cigarettes in their lifetime and still smoked or had quit smoking within the preceding year.[31] Weight and height were measured while the subjects wore no shoes and light underwear and body mass index (BMI, kg/m^2) was calculated.

In vitro experiments

Reagents. Roswell Park Memorial Institute medium (RPMI-1640), glutamine, penicillin (10,000 UI/mL) and streptomycin (10,000 μg/mL) were from Eurobio Laboratoires (Le Ulis Cedex, France). Fetal calf serum (FCS) was from Hy Clone (South Logan, UT). Matrigel® (MG) was from Becton Dickinson (Oxford, UK). Caffeine, bovine serum albumin (BSA) and all reagents were from Sigma Chemicals (St. Louis, MO) unless stated otherwise. For *in vitro* studies caffeine was dissolved in phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+} to a final concentration of 100 mM and stored at −20°C.

Cell culture. The human PC-3 and DU145 prostate cancer cell lines were from ATCC. Cells were grown in RPMI-1640 culture medium supplemented with 10% FCS, 0.05% L-glutamine, 1% penicillin and streptomycin and maintained at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Caffeinated and decaffeinated coffee sample preparation. Regular (caffeine containing) and decaffeinated pure powdered coffees were purchased from vendor at the Istituto Superiore di Sanità. Aqueous extracts were prepared as described:[12] in brief, bidistilled water (80°C, 100 mL) was added to 10 g of regular or decaffeinated coffee and stirred for 5 min, then the solutions were sterilized using a 0.22 μm filter (Millipore, Milano, Italy) and stored into cryovials. For testing on cancer cell lines, samples were diluted (1:100) in complete culture medium (vol/vol) before use.

Proliferation assay. For proliferation assay, cells (3×10^4 cells/well) were seeded in triplicate in 12-well plastic plates and allowed to grow for 24 hr in a complete medium. Cells were then incubated with different concentrations (0.5, 1 and 2 mM) of caffeine for 24, 48 and 72 hr or with aqueous extracts (as described above) for 72 hr. Cells were harvested and counted with a Neubauer modified chamber.

Cell cycle analysis. Cells were exposed to 2 mM caffeine for 24, 48 and 72 hr. Floating and adherent cells were harvested and fixed in 80% cold ethanol. Fixed cells were washed and incubated with 200 μg/mL ribonuclease A (Life Technologies) for 30 min at 37°C and 50 μg/mL propidium iodide (PI) as described.[32] Samples were analyzed with a FACSCanto Becton Dickinson Instrument (Becton Dickinson, CA) and FACSDiva software (5.0.3 version).

Adhesion assay. The adhesion assays were performed on 24-well plastic plates coated with 50 μg of MG. Unbound surfaces were blocked with 3% BSA in RPMI-1640 for 30 min at 37°C, and then aspirated prior to the addition of cells. Control and 2 mM caffeine-treated cells (72 hr of exposure) were harvested and resuspended in 0.02% BSA in RPMI-1640. 8×10^4 cells/well were incubated for 1 hr at 37°C then cells were detached and counted.

Wound healing assay. Cells were allowed to grow to confluence in 12-well plates and wounds were made with a sterile plastic tip. Cells were further incubated for 24 hr with or without 2 mM caffeine in RPMI-1640 (without FCS to avoid the influence of cell growth rate on the healing process). Wound healing was recorded at 0 and 24 h after scratching under microscope and digital camera. The rate of migration was quantified with ImageJ® software (Wayne Rasband, National Institutes of Health, Bethesda, MD) and expressed as percentage of the control (100%).

Migration assay. The modified Boyden chamber migration assay was used as described previously[33] with slight modifications. DU145 and PC3 cells (1×10^5) in a serum-free medium with or without 2 mM caffeine were placed in the upper chamber with an 8-μm pore size polycarbonate filter (Millipore, Milano, Italy). The bottom chambers were filled with complete medium (10% FCS). Cells were allowed to migrate for 16 hr at 37°C. After incubation, membranes were fixed by 3.7% formaldehyde in PBS for 10 min. Cells on the top surface of the membrane (nonmigrated cells) were scraped with a cotton swab, whereas cells on the bottom side of the membrane (migrated cells) were stained with 5% Giemsa solution and then mounted on a glass slide. Six randomly selected fields were photographed under light microscope and quantitative evaluation of migration was performed by means of ImageJ software and expressed as percentage of the control (100%).

Statistical analysis

Moli-sani data. Data on the daily Italian-style coffee and caffeine intake (g/day) are presented as mean and standard deviation separated for newly diagnosed prostate cancer patients and prostate cancer-free participants. Differences in coffee consumption between these two groups were tested using Student's *t* test.

Kaplan–Meier curves with outcome the newly diagnosed prostate cancer cases were used to illustrate the nonparametric incidence estimates according to groups of total Italian-style coffee intake.

Unadjusted and multiadjusted cox regression analyses were performed to evaluate the effect of Italian-style coffee intake and caffeine with prostate cancer incidence. Crude models or adjusted for either of the following factors: age, energy intake, smoking habits and BMI were generated with main outcome the prostate cancer incidence and independent variable the total Italian-style coffee and caffeine intake. The choice of the potential lifestyle confounders was firstly based on the report of World Cancer Research Fund International on diet, nutrition, physical activity and prostate cancer,[22] which described the evidence-based risk factors for prostate cancer. The selection was also confirmed by the test of the associations of the possible confounders with both the outcome and the coffee or caffeine intake. The assessment of potential interactions was also performed for the same factors, and no significant result was identified.

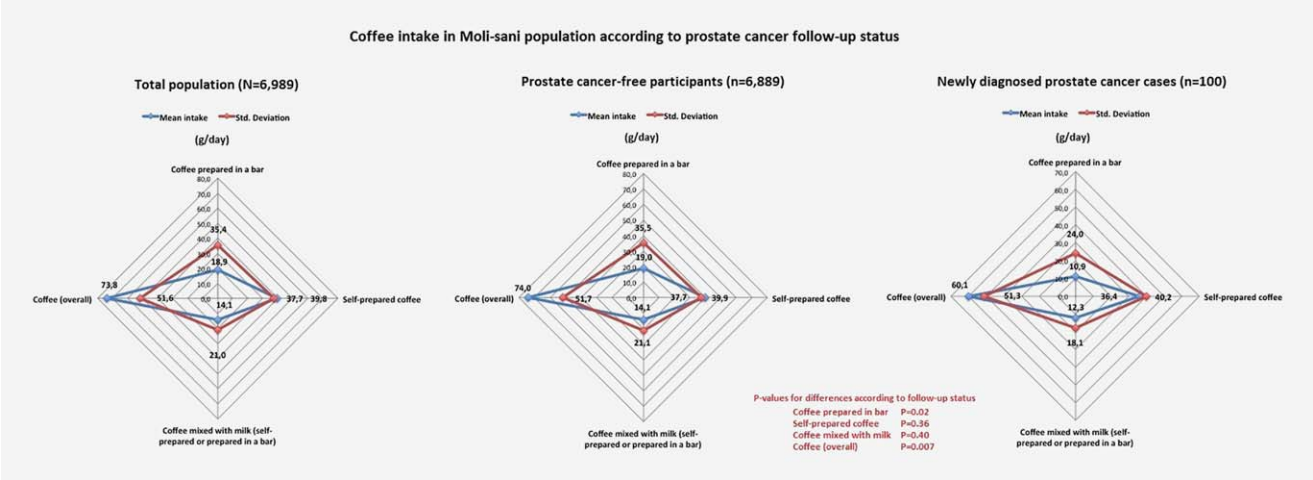

Figure 1. Mean consumption of various kinds of coffee according to follow-up status. [Color figure can be viewed at wileyonlinelibrary.com]

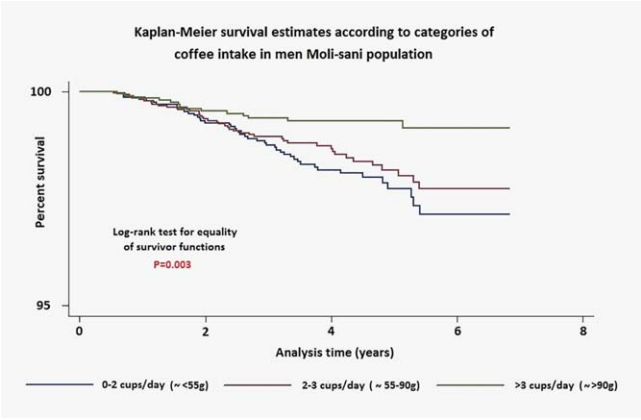

Figure 2. Kaplan–Meier nonparametric prostate cancer incidence estimates according to groups of total Italian-style coffee intake. [Color figure can be viewed at wileyonlinelibrary.com]

The proportionality of hazards was checked graphically and there was no evidence for nonproportional hazards. Results are presented as hazard ratios (HRs) and 95% confidence interval (CI).

Two-sided p-values < 0.05 was considered as statistically significant. STATA version 9 software was used for all calculations (STATA Corp., College Station, TX).

Experimental data. All experiments were performed in triplicate and the results are expressed as the mean ± SD of three different determinations. Differences between groups were assessed using Student's t test. Two-sided p-values < 0.05 was considered as statistically significant.

Results

Moli-sani cohort

In a mean of 4.24 ± 1.35 years of follow-up, 100 new prostate cancer cases were identified and the 50% of them had a total Gleason score over 7. Metastasis was evident in 8 cases and regional metastasis in other 6. Patients were aged in average 67 ± 8 years while prostate cancer-free participants were aged 63 ± 9 years ($p < 0.001$). The coffee consumers represented the 90.5% of the whole sample and the percentage did not differ among patients and the disease-free population ($p = 0.86$).

Figure 1 illustrates the mean consumption of various kinds of coffee according to follow-up status. The newly diagnosed prostate cancer participants presented lower intakes of coffee prepared in bar (10.9 ± 24.0 g/day) and total coffee consumption (60.1 ± 51.3 g/day) compared to the disease-free population (19.0 ± 35.5 and 74.0 ± 51.7 g/day, correspondingly) (p for both < 0.05). In addition, 28.4% of prostate cancer-free participants presented high coffee intake (i.e., >3 cups of coffee meaning > 90 g/day) as compared to 14% in patients ($p = 0.004$). Mean caffeine consumption was also lower in patients (133 ± 95 mg/day) as compared to healthy individuals (163 ± 110 mg/day) ($p = 0.008$). No significant difference was observed for coffee and caffeine intake among patients with high (>7) or low (≤7) total Gleason score (p for both > 0.05).

Further analysis on the effect of Italian-style coffee intake on prostate cancer incidence is illustrated in Figure 2. Kaplan–Meier nonparametric incidence estimates according to groups of total coffee intake showed lower incidence rates for the population at highest consumption (>3 cups/day) ($p = 0.003$).

Results derived through crude and multiadjusted cox regression analysis are presented in Table 1. The findings of Kaplan–Meier curves were confirmed since an inverse effect of high Italian-style coffee consumption on prostate cancer incidence was observed. In fact, multiadjusted analysis showed that the subjects with the highest consumption(>3 cups/day) had 53% lower prostate cancer risk compared to participants with those with the lowest consumption (0–2 cups/day) ($p = 0.02$).

Caffeine consumption showed almost the same effect on prostate cancer risk as coffee, also in terms of HRs (Table 1).

Table 1. Cox regression analysis evaluating the effect of Italian-style coffee and caffeine consumption on prostate cancer incidence

Num. of cases: 100 Num. at risk: 6,989	Crude models		Models adjusted for energy intake		Models adjusted for energy intake and smoking habits		Models adjusted for energy intake, smoking habits, age and BMI	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Coffee intake (g/day)[1]	0.94 (0.90, 0.98)	0.005	0.94 (0.90, 0.98)	0.008	0.94 (0.90, 0.99)	0.01	0.96 (0.91, 1.005)	0.08
Coffee intake								
0–2 cups (0–55 g) (num. cases: 45)	Reference category		Reference category		Reference category		Reference category	
2–3 cups (55–90 g) (num. cases: 41)	0.80 (0.52, 1.22)	0.30	0.82 (0.54, 1.26)	0.37	0.83 (0.54, 1.28)	0.40	0.91 (0.59, 1.41)	0.68
>3 cups (>90 g) (num. cases: 14)	0.36 (0.20, 0.66)	0.001	0.37 (0.20, 0.69)	0.002	0.38 (0.21, 0.71)	0.002	0.47 (0.25, 0.87)	0.02
Caffeine intake (mg/day)[1]	0.97 (0.95, 0.99)	0.005	0.97 (0.95, 0.99)	0.007	0.97 (0.95, 0.99)	0.01	0.98 (0.96, 1.001)	0.07
Caffeine intake								
<124 mg/day (num. cases: 44)	Reference category		Reference category		Reference category		Reference category	
114–193 mg/day (num. cases: 37)	0.83 (0.54, 1.28)	0.40	0.83 (0.54, 1.29)	0.42	0.84 (0.54, 1.30)	0.44	0.90 (0.58, 1.41)	0.66
>193 mg/day (num. cases: 19)	0.40 (0.23, 0.69)	0.001	0.40 (0.23, 0.71)	0.001	0.41 (0.23, 0.73)	0.002	0.51 (0.29, 0.90)	0.02

[1]Hazard ratios for 10 g/day increase in coffee consumption and 10 mg/day increase in caffeine intake.

Effects of regular and decaffeinated coffee on prostate cancer cells

To understand the mechanisms by which Italian-style coffee exerts such protective effect, the antiproliferative activity of aqueous extracts of regular (caffeine containing) and decaffeinated coffee on DU145 and PC-3 human prostate cells was first examined, to assess the potential antineoplastic role of caffeine. Both cancer cells exposed to regular coffee extract for 72 hr exhibited a significant reduction in proliferation rate as compared to cells treated with the decaffeinated extract (Fig. 3).

Caffeine affects prostate cancer cell growth

To further investigate the effect of caffeine on human prostate cancer cell proliferation, DU145 and PC-3 cells were treated with different concentrations of this methylxantine for 24, 48 and 72 hr. As shown in Figure 4*a*, caffeine showed a dose- and time-dependent inhibitory effect on the growth of prostate cancer cells. In particular, after 72 hr of 2 mM caffeine treatment, the values of reduction in cell growth were about 73% in DU145 and 57% in PC-3, with respect to the control ($p < 0.05$). Preheated caffeine was also tested, to make the observed effects of the purified component more comparable to the modality of coffee consumption in the population, above all with respect to the protocol usually applied, in cafeteria or at home, to prepare the Italian-style coffee, in both cases requiring hot water. No difference was observed in proliferation of prostate cancer cells (data not shown) between preheated (80°C for 5 min) or nonheated caffeine effects.

The mechanism of cell growth inhibition by caffeine was then investigated, and DNA content was measured using flow cytometry after PI staining of nuclei. Exposure to 2 mM caffeine resulted in an alteration of cell cycle distribution in DU145 and PC-3 cells (Fig. 4*b*), with an accumulation of cells in G_0/G_1 and subsequent reduction in S and G_2/M phases (Fig. 4*c*). A slight induction of cell death (sub-G_1 population) in DU145 cells after 72 hr of caffeine treatment was observed.

Caffeine inhibits cell adhesion and motility of prostate cancer cells

Prostate cancer cells are characterized by a significant ability to spread from the prostate to other parts of the body (namely bones and lymph nodes); therefore, we examined the effects of caffeine on some crucial steps of the metastatic cascade. The interaction of tumor cells with basement membrane proteins plays a pivotal role in tumor cell metastatization. Figure 5*a* illustrates the adhesion pattern of DU145 and PC-3 cells over MG-coated substrates. As shown, caffeine reduces the adhesion ability of cancer cells by about 40%, with respect to the control, in both cell lines. We next examined the effects of caffeine on cell motility and migration ability through the *in vitro* wound healing assay. As shown in Figure 5*b*, a difference in wound

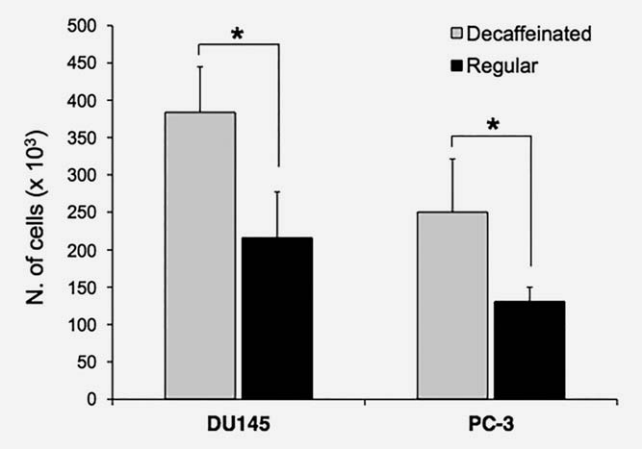

Figure 3. Antiproliferative effect of decaffeinated and regular (caffeine-containing) coffees aqueous extracts on DU145 and PC-3 prostate cancer cells. Data represent the mean of three different determinations ± SD (*$p < 0.05$).

closure during 24 hr was detected both in control and treated cells. After 24 hr of exposure to methylxanthine, there was a significant reduction of cell migration compared to the control (Fig. 5*c*). In addition, also in the Boyden chamber migration assay (Fig. 5*d*), DU145 and PC-3 cells decreased significantly their ability to migrate by 41% ($p < 0.05$) and 68% ($p < 0.01$), respectively (Fig. 5*e*). Taken together, these data confirmed the potential ability of caffeine to markedly interfere with the first steps of the metastatic cascade.

Discussion

Despite the relevance for public health of the prevention of prostate cancer through lifestyle modifications, the epidemiological results assessing the impact of coffee consumption on the incidence of the disease are still under debate.[14,15,21,22] Our findings illustrate in a large observational study, a protective effect of high Italian-style coffee consumption on disease risk.

Prostate cancer is one of the most common chronic diseases occurring in men older than 50 years, and its rates present variations among different countries.[17,18] Its incidence in Italy has been reported as one of the lowest in the European Union[17,19,20] while other populations from developed countries showed relatively elevated prostate cancer incidences.[17,18]

Despite these recent data, the studies of lifestyle modifications that have a role in the prevention of prostate cancer are still limited. This is also confirmed by the recent report of the World Cancer Research Fund International on diet, nutrition, physical activity and prostate cancer.[22]

The expert panel, while stating that there is some limited evidence for prostate cancer risk increment by high consumption of dairy products, diets high in calcium, low plasma alpha-tocopherol concentrations and low plasma selenium, concluded that data on the effect of coffee consumption o were limited and not conclusive; furthermore there is no mention on caffeine intake.

The scientific community attributes the low incidence of prostate cancer observed in Italy to the spread of new diagnostic procedures (ultrasound-guided biopsy and prostate-specific antigen, PSA test).[20] This evidence is under investigation, since the elaboration of new diagnostic tests has been undertaken also in other developed countries with higher prostate cancer incidences.

The findings of this work indicate that a dietary habit followed for ages in Italy and described by the consumption of high Italian-style coffee has a protective effect on prostate cancer risk. Indeed, the consumption of >3 cups/day was associated with a 53% reduction in disease risk compared to the lower consumption of 0–2 cups/day.

The present results are in agreement with data from recent meta-analyses supporting a decreased disease risk with the consumption of different types of coffee.[15,16] Particularly, in one of them involving nine cohort studies and 455,123 subjects, researchers concluded that intake of >4 or 5 cups/day was associated with a reduced disease risk and lower rates of fatal cases.[14]

Data from Health Professionals Follow-up study including 5,035 prostate cancer cases in a population of 47,911 men indicated that the intake of >6 cups/day was associated with 60% lower risk of lethal and advanced prostate cancer than the nonconsumption.[34]

On the contrary, a meta-analysis of twelve epidemiological studies showed a significantly harmful effect of higher coffee consumption on prostate cancer rate analyzing data from seven case-control studies.[21] In addition, the same meta-analysis concluded that no significant effect was evident on disease risk by elaborating data of four cohort studies.[21]

A very recent analysis of 25 case–control studies in the PRACTICAL Consortium, used the Mendelian randomization approach to investigate the causal effects of coffee consumption on prostate cancer risk and progression, by using two genetic variants robustly associated with caffeine intake as proxies for coffee consumption. The resulting data were not consistent with a substantial effect of coffee consumption on reducing prostate cancer incidence, progression or mortality. However, the genetic proxy they used were not specific for coffee, while information on coffee consumption was missing for the majority of their populations that also presented evidence for selection bias.

In contrast, we analyzed a very homogeneous population thus confirming evidence coming from the more recent meta-analyses;[14,15] and we devoted experimental studies to investigate the possibly causal role of caffeine in reducing the proliferative and metastatic potential of prostate cancer cell lines. The antitumor effect of coffee intake has been shown also in other types of cancer such as bladder, breast, buccal and pharyngeal, colorectal, endometrial, esophageal, hepatocellular, leukemic, pancreatic[13] and liver cancer.[16] Most of the studies attribute these effects to different physiological actions of the bioactive compounds of coffee mainly to caffeine, diterpenes (cafestol and kahweal) and chlorogenic

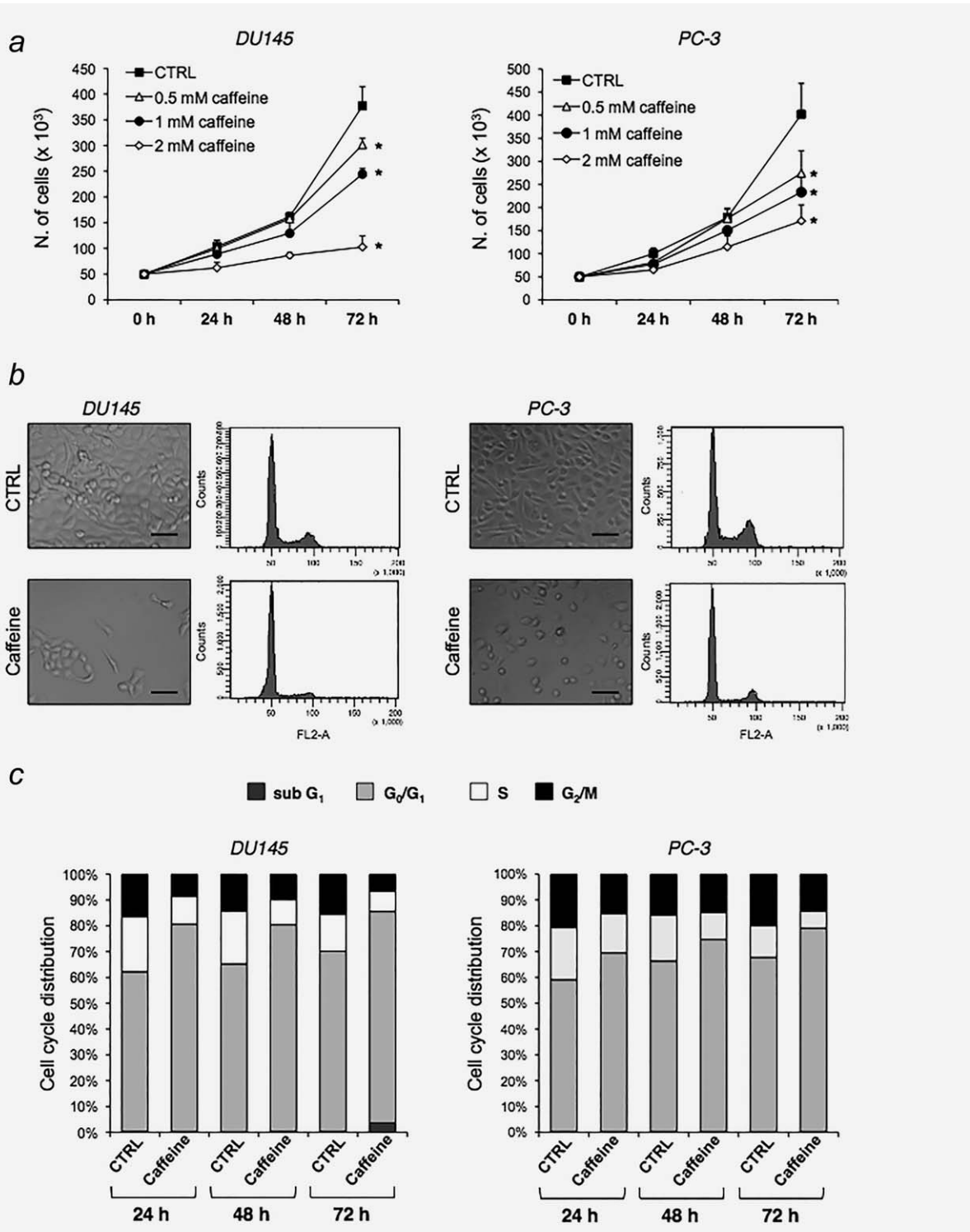

Figure 4. Effects of caffeine on prostate cancer cell growth and cell cycle distribution. Caffeine reduces cell proliferation in a time- and dose-dependent manner. (*a*) Cell growth curve of DU145 and PC-3 cells treated with 0.5, 1 and 2 mM caffeine for 24, 48 and 72 hr. Control cells were incubated with phosphate buffer saline only. Caffeine induces cell cycle arrest in G_0/G_1 phase. (*b*) Representative images showing the cell density and cell cycle distribution of DU145 and PC-3 after 72 hr of treatment with 2 mM caffeine. Scale bar: 50 μm. (*c*) Flow cytometric analysis of floating and adherent cells. Graph shows cell-cycle distribution of DU145 and PC-3 cells. Statistical significance versus control: $*p < 0.05$.

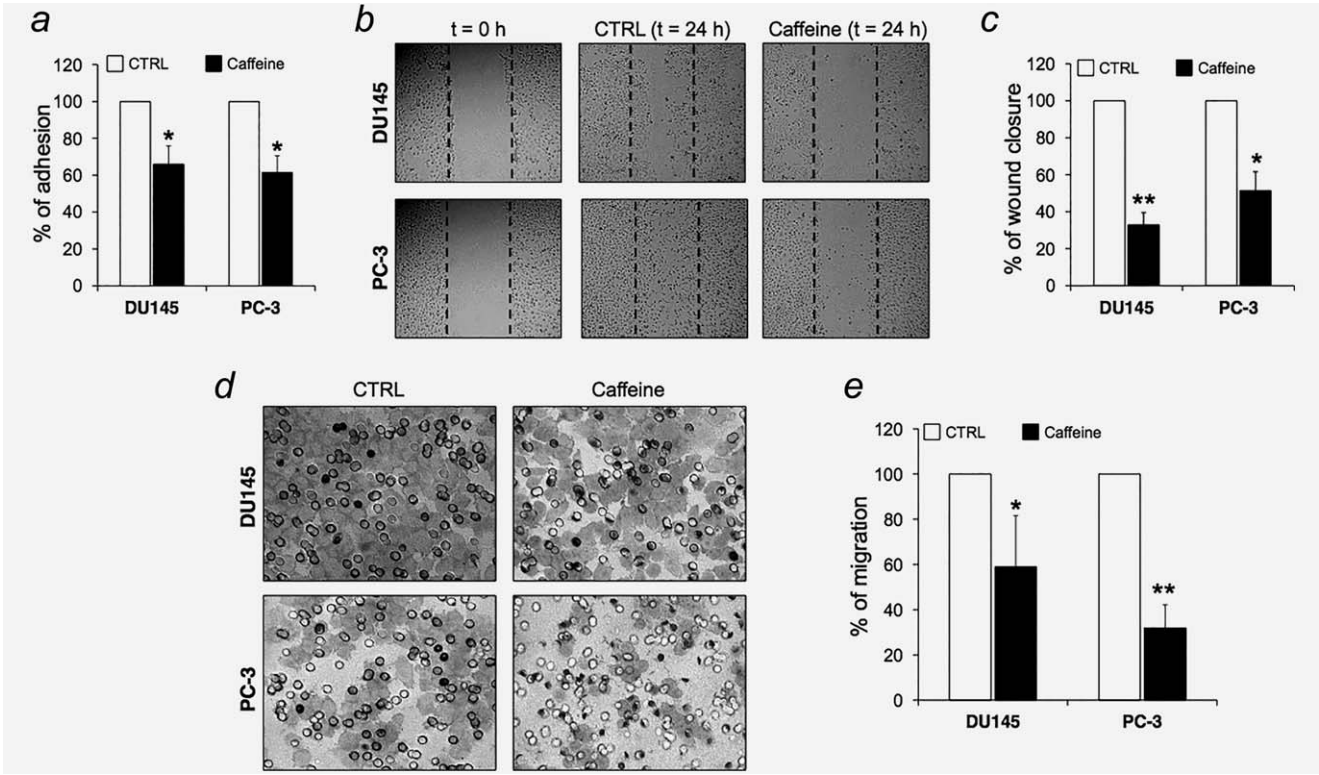

Figure 5. Caffeine interferes with adhesion and migration abilities of prostate cancer cells. (*a*) Effects of 2 mM caffeine on adhesion to MG (reconstituted basement membrane). (*b*) Representative images of wound healing assay (original magnification ×100). (*c*) Significant inhibition in wound coverage was observed after 72 hr of treatment with methylxanthine (2 mM). Values were calculated as percentage of the control, expressed as 100%. A Boyden chamber migration assay confirms the reduction of migration ability of prostate cancer cells after caffeine treatment. (*d*) Representative micrographs showing cells migrated to the lower surface of the filter in the Boyden chamber assay (original magnification ×100). (*e*) The percentage of migration (control 100%) calculated (ImageJ software) from multiple fields is plotted. $*p < 0.05$, $**p < 0.01$ compared to controls.

acid.[14] In summary, caffeine presents antioxidant properties, prevents oxidative DNA damage and modifies the apoptotic response; diterpenes trigger biochemical responses in human metabolism that contribute to reduction in the genotoxicity of several carcinogens and chlorogenic acid share effects in reducing oxidative stress and protect against environmental carcinogenesis.[14]

All these bioactive components reveal their physiological actions in relatively high concentrations that could be achieved only with high intake of coffee. This could explain the protective effect of coffee intake in prostate cancer risk only after long-term consumption of >4 cups/day, as indicated by most of the recent meta-analyses[14,15] and not in lower consumption categories.

Our data have shown a protective effect by the long-term consumption of >3 cups corresponding to >90 g of Italian-style coffee/day. It should be considered that the preparation of Italian-style coffee in high-pressure hot water (about 90°C) or boiling water and without the use of filters compared to other studied populations might result in lower loss of bioactive compounds and greater concentrations in the finally consumed food.

Considering that caffeine has been proposed as one of the main bioactive compounds and given the above mentioned limitations, further *in vitro* experiments on the antiproliferative and antimetastatic activity of caffeine on human prostate cancer cell lines were performed and presented in this work. It should be also reported that the consumption of decaffeinated coffee in the present population was remarkably low and made the comparison of decaffeinated or nondecaffeinated coffee consumers impossible.

Experiments in cellular models demonstrated that treatment with regular coffee extract led to a major decrease in cancer cell proliferation with respect to decaffeinated extract. These data underline the efficacy of caffeine to reduce cancer cell growth compared to other phytochemicals present in coffee extracts. Several studies have described caffeine as anticancer agents by *in vitro* and *in vivo* models.[35–ô37] For instance, caffeine suppressed the growth of breast cancer cells impairing cell-cycle progression and enhancing cell death.[38] Furthermore, caffeine exhibited antineoplastic activity also against other cancer cells, e.g., melanoma cells.[39] On the other hand, studies with caffeine could not perfectly support evidences derived from epidemiologic studies in which a population of italian-coffee consumers is analyzed, due to the presence of many additional compounds (e.g., catechins and other phenolic ones) in these beverages.

In the present study, the observation that a significant fraction of the observed effects on cells was lost when a

decaffeinated was compared to a caffeinated preparation, suggests that a significant portion of the observed antineoplastic effect may be due to caffeine. Further, data obtained when preheated coffee was compared to nonheated coffee, in which both extracts showed the same antineoplastic effects, strongly support the concept that the effects observed in epidemiological studies are due to caffeine and not related to possibly different methods of coffee extraction.

In the present study, the antiproliferative activity of caffeine against human PC-3 and DU145 prostate cells was assessed. Firstly, it was reported that caffeine inhibited proliferation in a dose- and time-dependent manner. The caffeine-mediated inhibition of cell growth was associated with impairment of G_1 to S cell-cycle transition. These data were in agreement with previous studies, which reported caffeine-induced accumulation in G_0/G_1 cell cycle phase in DU145 prostate[40] and in MCF-7 and MDA-MB-231 breast cancer cells.[37]

Our data also revealed that caffeine inhibited metastatic behavior of PC-3 and DU145 cells. On these cellular models, caffeine exerted strong effects on adhesion and migration, measured both as a single cell (Boyden chamber model) and as a collective multicellular, tissue type phenomenon (scratch test). The observed effects with caffeine treatment of cell cultures may be taken into consideration in close connection with those suggested by epidemiologic studies on the Moli-sani cohort. Regarding the mechanisms possibly involved in the antineoplastic activity played by caffeine, it is noteworthy that this methylxanthineis anon-specific antagonist of adenosine receptors (ARs) (a type of purinergic receptors).[41] Caffeine therefore may trigger or inhibit the adenosine pathway, depending on the type of purinergic receptor involved. It has been demonstrated that adenosine can be accumulated in hypoxic tissues, including the tumor microenvironment[42] and that it plays an important role in the regulation of processes as immunosuppression, angiogenesis, proliferation, vascular tone, endothelial permeability and inflammation[43,44] acting on ARs. The AR antagonism activity requires about 20 times lower concentration of caffeine than the concentration required for other known actions of this methylxantine, e.g., inhibition of phosphodiesterase. This evidence indicates that the effects of caffeine in human subjects who regularly consume coffee or beverages containing caffeine are mainly mediated by its ability to antagonize the ARs.[45] The latter would be present at higher levels in prostate cancer tissues than in control ones; one of them, the A_{2B}, was reported to specifically promote prostate cancer cell growth.[44] This pathway therefore can be proposed as a novel target for prostate cancer treatment and represents a possible mechanism to explain the protective role of caffeine in the present study.

Limitations

Beyond the relevance of the findings of the present work, some limitations still exist. First, although accurate from a methodological epidemiologic perspective, the number of newly diagnosed prostate cancer cases in Moli-sani cohort during the follow-up period was relatively small. Thus, the power of the observed associations in the present study is somehow limited. Moreover, the duration of the follow-up (i.e., 4.24 ± 1.35 years) was rather short and may still raise the potential of reverse causation, despite the longitudinal study design.

Additionally, the administration of a FFQ for the evaluation of dietary habits in Moli-sani population is less accurate at the individual level than other dietary measurement methods. Possible errors because of misreporting by the participating subjects should also be considered. Furthermore, coffee intake might represent a marker for general healthier lifestyle. Related biases always exist in the study of dietary habits and health in epidemiological (i.e., not clinical) environment. To rule out as much biases as possible the multiadjusted models have been adjusted for age, smoking habits, energy intake and BMI that are lifestyle factors that have also been recognized[22] as evidence-based risk factors for prostate cancer. However, the present analysis might also be weakened by possible unknown confounding factors or selection bias related to the study recruitment procedure.

Epidemiologic studies in which a population of Italian-coffee consumers was analyzed, could not be completely fitting with the caffeine experiments, due to the presence of many additional compounds (e.g., catechins and other phenolic ones) in these beverages. Nevertheless, the observation that a significant fraction of the observed effects on cells was lost with a decaffeinated-preparation, suggests that a substantial portion of the observed antineoplastic effect may be due to caffeine.

Conclusions

A protective effect of high Italian-style coffee consumption (>3 cups/day) on prostate cancer risk was observed at epidemiological level. Considering the importance of prostate cancer prevention through healthy lifestyles, this evidence gains important meaning in public health perspectives. A possible biological plausibility of these observations was provided by a cellular counterpart, two prostate cancer cell lines, found responsive to the antiproliferative and antimetastatic effects of caffeine. Altogether, our study provides an integrated approach, with epidemiological and cellular models, to the debated issue of coffee consumption impact on prostate cancer risk.

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Appendix: Moli-Sani Study Investigators

The enrolment phase of the Moli-sani study was conducted at the Research Laboratories of the Catholic University in Campobasso (Italy), the follow up of the MOLI-SANI cohort is being conducted at the IRCCS Neuromed, Pozzilli, Italy.

Steering committee: Licia Iacoviello (Neuromed, Pozzilli, Italy), Chairperson, Maria Benedetta Donati and Giovanni de Gaetano (Neuromed, Pozzilli, Italy).

Safety and data monitoring committee: Jos Vermylen (Catholic Univesity, Leuven, Belgio), Chairman, Ignacio De Paula Carrasco (Accademia Pontificia Pro Vita, Roma, Italy), Simona Giampaoli (Istituto Superiore di Sanità, Roma, Italy), Antonio Spagnuolo (Catholic University, Roma, Italy) .

Event adjudicating committee: Deodato Assanelli (Brescia, Italy), Vincenzo Centritto (Campobasso, Italy), Pasquale Spagnuolo and Dante Staniscia (Termoli, Italy).

Scientific and organizing secretariat: Francesco Zito (Coordinator), Americo Bonanni, Chiara Cerletti, Amalia De Curtis, Augusto Di Castelnuovo, Licia Iacoviello, Roberto Lorenzet, Antonio Mascioli, Marco Olivieri and Domenico Rotilio.

Data management and analysis: Augusto Di Castelnuovo (Coordinator), Marialaura Bonaccio, Simona Costanzo and Francesco Gianfagna.

Informatics: Marco Olivieri (Coordinator), Maurizio Giacci, Antonella Padulo and Dario Petraroia.

Biobank and biomedical analyses: Amalia De Curtis (Coordinator), Sara Magnacca, Federico Marracino, Maria Spinelli, Christian Silvestri, Giuseppe dell'Elba, Claudio Grippi.

Communication and press office: Americo Bonanni (Coordinator), Marialaura Bonaccio and Francesca De Lucia.

Moli-family project: Francesco Gianfagna, Branislav Vohnout.

Recruitment staff: Franco Zito (General Coordinator); *Secretariat*: Mariarosaria Persichillo (Coordinator), Angelita Verna, Maura Di Lillo, Irene Di Stefano; *Blood sample*: Agnieszka Pampuch; Branislav Vohnout, Agostino Pannichella, Antonio Rinaldo Vizzarri; *Spirometry*: Antonella Arcari (Coordinator), Daniela Barbato, Francesca Bracone, Simona Costanzo, Carmine Di Giorgio, Sara Magnacca, Simona Panebianco, Antonello Chiovitti, Federico Marracino, Sergio Caccamo, Vanesa Caruso; *Electrocardiograms*: Livia Rago (Coordinator), Daniela Cugino, Francesco Zito, Francesco Gianfagna, Alessandra Ferri, Concetta Castaldi, Marcella Mignogna, Tomasz Guszcz; *Questionnaires*: Romina di Giuseppe (Coordinator), Paola Barisciano, Lorena Buonaccorsi, Floriana Centritto, Antonella Cutrone, Francesca De Lucia, Francesca Fanelli, Iolanda Santimone, Anna Sciarretta, Maura Di Lillo, Isabella Sorella, Irene Di Stefano, Emanuela Plescia, Alessandra Molinaro and Christiana Cavone.

Call center: Giovanna Galuppo, Maura Di Lillo, Concetta Castaldi, Dolores D'Angelo and Rosanna Ramacciato.

Follow-up: Simona Costanzo (Coordinator); Data management: Simona Costanzo, Marco Olivieri; Event adjudication: Livia Rago (Coordinator), Simona Costanzo, Amalia de Curtis, Licia Iacoviello, Mariarosaria Persichillo.

Regional health institutions: Azienda Sanitaria Regionale del Molise (ASReM, Campobasso, Italy), UOC Servizio Igiene e Sanità Pubblica – Dipartimento di Prevenzione; Offices of vital statistics of the Molise region and Molise Dati Spa (Campobasso, Italy).

Hospitals: Presidi Ospedalieri ASReM (Presidio Ospedaliero A. Cardarelli – Campobasso, Ospedale F. Veneziale – Isernia, Ospedale San Timoteo – Termoli (CB), Ospedale Ss. Rosario – Venafro (IS), Ospedale Vietri – Larino (CB), Ospedale San Francesco Caracciolo – Agnone (IS); Istituto di cura Villa Maria – Campobasso; Fondazione di Ricerca e Cura Giovanni Paolo II – Campobasso; IRCCS Neuromed – Pozzilli (IS).

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