

# Randomized, double blind placebo-controlled pilot study of the antihypertensive effects of Grana Padano D.O.P. cheese consumption in mild - moderate hypertensive subjects

G. CRIPPA<sup>1</sup>, D. ZABZUNI<sup>1</sup>, E. BRAVI<sup>1</sup>, G. PIVA<sup>2</sup>, I. DE NONI<sup>3</sup>, E. BIGHI<sup>2</sup>, F. ROSSI<sup>4</sup>

<sup>1</sup>Hypertension Unit, "Guglielmo da Saliceto" Hospital, Piacenza, Italy

<sup>2</sup>Institute of Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, Piacenza, Italy

<sup>3</sup>Department of Food, Environmental and Nutritional Sciences, Università degli Studi, Milan, Italy

<sup>4</sup>Institute of Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, Piacenza, Italy

**Abstract.** – **OBJECTIVE:** Grana Padano, an Italian protected designation of origin (PDO) semi-fat cheese, undergoes a long ripening period during which the proteolysis carried out by natural starter lactic acid bacteria releases peptides having sustained angiotensin-converting enzyme (ACE)-inhibitory activity. The length (generally 3-8 amino acid residues) and the sequence of these peptides are responsible for their ability to elicit ACE-inhibitory activity. The aim of this study has been the evaluation of the effect of a daily dietary supplement consisting in a small amount (30 g/day) of Grana Padano cheese, in terms of the lowering of the blood pressure (BP) of mild - moderate hypertensive subjects.

**PATIENTS AND METHODS:** Thirty mild - moderate hypertensive patients, with BP values not on target (> 140 and/or > 90 mmHg) after at least 3 months of stable treatment were considered in this randomized, double-blind placebo-controlled cross-over study. All patients randomly received a dietary integration (30 g/day) of Grana Padano cheese or a placebo (made from flavored grated bread mixed with fats and salts in concentrations equal to those of the cheese). BP was evaluated at baseline and at the end of the active and placebo treatments (2 months each) by:

- Office BP (OBP);
- Automated Office BP (AOBP) using the BpTRU<sup>®</sup>, an automated oscillometric device that provides the average of multiple (n=6) blood pressure measurements;
- Ambulatory Blood Pressure (ABP) 24 hour monitoring.

**RESULTS:** Dietary integration with Grana Padano cheese resulted in a significant decrease in Office, Automated Office and Ambulatory BP. The mean decrease (vs. placebo) for

24-hour ABP was  $-3.5$  mmHg for systolic and  $-2.4$  mmHg for diastolic BP ( $p = 0.0063$  and  $p = 0.0065$ , respectively).

**CONCLUSIONS:** Daily dietary integration with 30 g of Grana Padano DOP cheese effectively reduces BP and may help mild-to-moderate hypertensive patients to reach a target BP.

*Key Words:*

Hypertension, Grana Padano cheese, ACE-inhibiting peptides.

## Introduction

Hypertension is the major risk factor for stroke, cardiovascular disease, end-stage renal failure and overall mortality worldwide<sup>1,2</sup>. It is well established that blood pressure lowering intervention (i.e. lifestyle modification and pharmacological treatment) prevents cardiovascular events<sup>3</sup>. Even a small decrease in systolic blood pressure (BP), as low as 2 mmHg, is associated with lowering cardiovascular disease and stroke death rates by 4-5%<sup>4</sup>. However, the absence of a decline in the prevalence of hypertension in the general population underlines the need for effective lifestyle modifications (mainly through adoption of a healthy diet and physical activity) for the prevention and treatment of hypertension and related cardiovascular disease. The Dietary Approaches to Stop Hypertension (DASH) study indicates unequivocally that reduced sodium intake and a "healthy" diet (fruit, vegetables, low-fat dairy products) both ameliorate BP control in

patients with hypertension<sup>5,6</sup>. There is a growing body of evidence indicating that the intake of dairy products is inversely related with the risk of hypertension<sup>7</sup>, suggesting that these products have a potential role in the prevention of hypertension and its cardiovascular complications<sup>8-10</sup>. A moderate consumption of dairy foods is also a characteristic of the Mediterranean Diet, which has shown to be protective against hypertension<sup>11,12</sup>. The blood pressure lowering effect of milk derivatives could be ascribed to their high calcium content<sup>13</sup>, and also to the presence of angiotensin-converting enzyme (ACE)-inhibiting peptides<sup>14</sup>; both nutrients are abundantly present in dairy products<sup>14</sup>. ACE-inhibiting peptides in cheese derive from the proteolysis of milk protein, mainly casein, during ripening. The tripeptides Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP) were the first potent inhibitory peptides isolated from hydrolyzed bovine casein, while in more recent years, other peptides have been identified: Arg-Tyr-Leu-Gly (RYLG), Arg-Tyr-Leu-Gly-Tyr (RYLGY) and Ala-Tyr-Phe-Tyr-Pro-Glu-Leu15 (AYFYPEL), Leu-His-Leu-Pro-Leu-Pro (LHLPLP) and His-Leu-Pro-Leu-Pro (HLPLP)<sup>16,17</sup>. The level of these peptides in cheese is affected by several factors (e.g. proteolytic activity of coagulant, milk and, mainly, microbial culture, length of ripening, manufacturing practices), and for these reasons there are many differences among different cheeses as far as ACE-inhibiting peptide concentration is concerned<sup>18</sup>. Generally, the level of these peptides is low at the beginning of cheese ripening, but it increases with time. However, the relationship between length of ripening and level of ACE-inhibiting peptides is not linear, because these peptides can be further degraded by proteolytic activity in cheese. Grana Padano is a semi-fat, hard, protected designation of origin (PDO) Italian cheese made from raw bovine milk and containing 700 mg/100 g of sodium, on average<sup>19</sup>. Several ACE-inhibiting peptides have been found in this cheese, especially in its *in vitro* gastrointestinal digestates<sup>20</sup>. Preliminary data from an open controlled study carried out on hypertensive patients<sup>21</sup> indicated a possible role for Grana Padano consumption in the reduction of BP in hypertensive individuals. However, the most recently available meta-analysis performed on the effect of dairy ACE-inhibiting peptides on BP<sup>14</sup> has yielded conflicting results. Significant heterogeneity was found between studies carried out on Asian and on European subjects: in Asian individuals, data revealed a significant reduction in

SBP and DBP related to dairy product consumption, while only a small and non-significant effect of Val-Pro-Pro and Ile-Pro-Pro peptides on BP was found in European studies performed using Caucasian subjects. The aim of this study was to assess whether dietary supplementation with 30 g/day of Grana Padano PDO cheese was able to lower BP in subjects affected with mild-to-moderate hypertension.

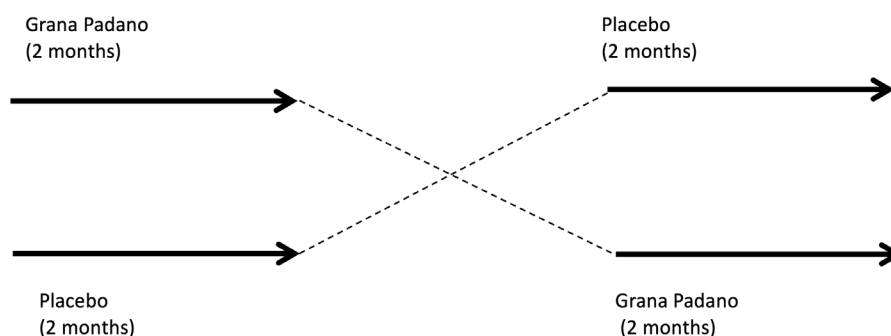
## Materials and Methods

### *Cheese Characteristics*

Grana Padano cheese, aged 12 months, was provided by the Consortium of Grana Padano cheese (Desenzano del Garda, Brescia, Italy). Moisture, fat, protein, NaCl and cholesterol contents were determined using the methods proposed by the International Dairy Federation (IDF)<sup>22-26</sup>. Levels of the ACE-inhibiting peptides were determined in the water-soluble extract (<3 kDa) of Grana Padano using the Ultra Performance Liquid Chromatography/High Resolution Mass Spectrometry (UPLC/HR-MS) method<sup>20</sup>.

### *Experimental Design*

A randomized, double blind, placebo-controlled crossover design was adopted. The study was carried out between September 2014 and June 2016 at the Hypertension Unit of Guglielmo da Saliceto Hospital (Piacenza, Italy) and conducted according to the Good Clinical Practice Guidelines and the latest version of the Declaration of Helsinki. The protocol was approved by the Local Ethics Committee (Prot. UC/UI 01/10) and monitored by the same institution. All patients signed written informed consent before participating in the study. Subjects were randomized (in a 1:1 ratio using a computer-generated randomization schedule) to receive 30 g of Grana Padano DOP cheese or a placebo. Patients who first received the active treatment were switched to the placebo after a study period of 2 months and vice versa (Figure 1). The sachets of Grana Padano and placebo were transparent and differences between the external appearance, the aroma and the taste were indiscernible, the placebo being made with grated bread and added purified Grana Padano cheese aroma. Salts of sodium and calcium, as well as fat (groundnut oil) were added to the grated bread in order to obtain the same levels of salts and fat in both the cheese and the placebo. Patients were provided with 2 lots (each comprising 35 sachets,



**Figure 1.** Experimental scheme adopted in this study.

**Table I.** Chemical composition (g/100 g) of Grana Padano and grated bread used as placebo.

Nutrients	Grana Padano	Placebo
Lipids	28.92	28.94
Proteins	31.43	12.65
Na	0.56	0.53
Ca	0.88	0.86

to be kept refrigerated) for each study period. The chemical characteristics of the Grana Padano and the placebo are reported in Table I.

### Patients

Thirty-four patients were originally involved in the study. Three individuals withdrew their consent before the final examination and one individual did not comply with the dietary supplementation protocol, leaving a total of 30 patients who completed the study. All dropouts were unrelated to side effects or changes in health conditions. The baseline characteristics of the 30 subjects

who completed the study are shown in Table II. The patients, aged  $52.1 \pm 12.5$  years, 13 of whom were females, all with mild-to-moderate essential hypertension, were on stable (3 months at least) non-pharmacological (9 individuals) and pharmacological (21 individuals) treatment before randomization. Pharmacological treatment consisted of angiotensin-converting-enzyme inhibitors, early-distal-tubule diuretics, angiotensin-receptor blockers, beta-adrenergic blockers, and Ca-channel blockers or their combinations. At baseline, all patients had office blood pressure (OBP) mean values not on target (i.e.  $> 140$  and/or  $90$  mmHg during 2 consecutive clinical examinations).

### Blood Pressure Measurements

The patients' blood pressure was monitored in 3 different ways:

- Office blood pressure (OBP);
- Automated office blood pressure (AOBP);
- 24-hour Ambulatory blood pressure (ABP) monitoring.

OBP was measured using a conventional mer-

**Table II.** The standard deviation must reported as  $\pm$  not with the sign +.

	Male	Female	Whole group
Number	13	17	30
Age (years)	$50.1 \pm 12.7$	$56.0 \pm 11.7$	$52.3 \pm 12.5$
BMI ( $\text{kg}/\text{m}^2$ )	$27.3 \pm 2.8$	$24.3 \pm 6.97$	$26.3 \pm 4.7$
SBP (mm Hg)	$137.8 \pm 11.6$	$138.7 \pm 4.7$	$138.2 \pm 9.52$
DBP (mm Hg)	$88.6 \pm 7.7$	$87.1 \pm 6.2$	$88.1 \pm 7.11$
Heart rate (bpm)	$71.9 \pm 5.8$	$78.2 \pm 7.2$	$74.2 \pm 6.92$
Creatinine (mg/100 ml)	$0.97 \pm 0.13$	$0.73 \pm 0.09$	$0.86 \pm 0.23$
Cholesterol (mg/100 ml)	$196.6 \pm 28.37$	$217.3 \pm 35.17$	$205.6 \pm 45.8$
Triglycerides (mg/100 ml)	$125.9 \pm 63.45$	$125.6 \pm 66.20$	$124.0 \pm 85.7$
Na (mEq/L)	$139.3 \pm 2.06$	$139.4 \pm 2.34$	$139.5 \pm 1.71$
K (mEq/L)	$4.23 \pm 0.28$	$4.37 \pm 0.59$	$4.1 \pm 0.77$
Cl (mEq/L)	$103.9 \pm 1.88$	$104.9 \pm 2.02$	$101.6 \pm 18.7$

cury sphygmomanometer, according to the British Society of Hypertension recommendations (average of 3 consecutive readings in the sitting position, at intervals of at least 2 minutes). AOBP was obtained using the BpTRU® (VSM MedTech Ltd, Coquitlam, British Columbia, Canada), allowing multiple measurements to be taken without the presence of the physician in the room, to minimize observer-patient interaction and the anxiety experienced by many patients in this situation. This device is programmed to take the first reading (which is automatically discarded) with the physician in the consulting room. The device subsequently took 5 consecutive readings at 2-minute intervals and provided the average of the 5 unattended measurements. ABP monitoring was performed by means of a non-invasive, portable, validated SpaceLabs 90207-30 recorder (SpaceLabs Inc., Issaquah, WA, USA). Patients wore the device for 24 hours on all days except holidays and the recorder was set to take readings every 15 minutes during daytime (7 am to 11 pm) and every 30 minutes during nighttime (11 pm to 7 am). ABP recording was considered complete when 80% or more of the scheduled readings were available, with at least 2 valid readings per hour. Analysis of the ABP monitoring, and the evaluation of daytime, nighttime and circadian rhythm were performed according to the 2013 ESH-ESC guidelines<sup>27</sup>. Body weight and height were determined in the morning, with the subjects fasting and wearing light clothes, using a GIMA scale (0.1 kg) with stadiometer. In addition, fasting patients' blood and urine samples were obtained at the beginning and the end of each two-month period. Blood samples were evaluated for: hematic crisis, creatinine, glucose, triglycerides, total, HDL and LDL cholesterol, Na, K, Ca, Mg, urate. The bio-humoral analyses were carried out using an Au 500 Beckman Coulter Analyzer (Beckman Coulter Inc. Brea, CA, USA).

### Statistical Analysis

The primary outcome variables were the changes in mean systolic and diastolic ABP values

(mean 24-h BP, daytime BP and nighttime BP). The secondary outcome variable was the rate of BP normalization after intervention. Statistical software SAS 9.3 (SAS Institute, Cary, NC, USA) was used for the statistical analysis. Continuous variables are expressed as mean  $\pm$  SD, while qualitative variables are expressed as percentages. Changes in BP values (systolic and diastolic OBP, AOBP, ABP) were compared to placebo values by using the Student's *t*-test for paired samples (two-sided, alpha level  $p < 0.05$ ). To compare two qualitative variables with repeated measures, the non-parametric Wilcoxon test for paired samples was used. Differences in chemical and hematological parameters at baseline and after treatment were compared using Dunnett's test. We calculated a priori that a sample size of 25 subjects should give power of 0.80 to detect a change of at least 4 mmHg in mean systolic ambulatory blood pressure between active treatment and placebo (Student's *t*-test). We oversampled to 30 subjects (20% more).

## Results

### ACE-Inhibiting Peptides

The analysis of the cheese used for the study confirmed the presence of IPP and VPP peptides. Another three bioactive peptides were found: LHLPLP (which has shown an ACE-inhibiting activity similar to that of VPP and IPP), and HLPLP and RYLG (which are less effective in inhibiting ACE activity<sup>15-17</sup>). The concentrations of ACE-inhibiting peptides contained in the samples of Grana Padano cheese are shown in Table III.

### Office Blood Pressure (OBP)

Dietary integration with Grana Padano significantly reduced both SBP and DBP values at the end of the treatment period with respect to baseline and placebo. No significant effects were observed for (heart rate) HR. Office systolic BP dropped from  $138.2 \pm 9.5$  to  $133.4 \pm 6.2$  mmHg,

**Table III.** Concentration (mg/kg) of ACE-inhibiting peptides in in water soluble extracts of Grana Padano cheese.

ACE-inhibiting peptides							
VPP	IPP	RYLG	RYLGY	AYFYPE	HLPLP	AYFYPEL	LHLPLP
3.95	3.18	1.93	0	0	0.53	0	1.95

VPP = Val-Pro-Pro; IPP = Ile-Pro-Pro; RYLG(Y) = Arg-Tyr-Leu-Gly-(Tyr); AYFYPE(L) = Ala-Tyr-Phe-Tyr-Pro-Glu-(Leu); (L)HLPLP = (Leu)-His-Leu-Pro-Leu-Pro.

**Table IV.** The abbreviation OBP must be placed in the main part of the table (white), not on the grey one. OBP must be placed in the first row, just above SBP.

OBP	Grana Padano	Placebo	GP vs. placebo		
			$\Delta$ BP	<i>p</i> -value	RMSE
SBP (mm Hg)	- 6.60*	- 1.77	4.83	0.0085	6.8764
DBP (mm Hg)	-5.33*	-1.77	3.56	0.0126	5.3660
HR (bpm)	-1.80	-0.30	1.50	0.4714	8.0141
AOBP					
SBP (mm Hg)	- 6.77*	- 1.10	5.67	0.0040	7.3109
DBP (mm Hg)	- 2.53	+ 0.07	2.60	0.0964	5.9587
HR (bpm)	1.70	3.47	1.77	0.3421	7.1435
ABP 24 hours					
SBP (mm Hg)	- 5.73*	- 1.33	4.40	0.0405	8.1310
DBP (mm Hg)	- 4.77*	- 1.07	3.70	0.0163	5.7912
HR (bpm)	-1.13	+ 0.03	1.16	0.3667	4.9661
ABP Daytime					
SBP (mm Hg)	- 6.60*	- 1.77	4.83	0.0398	8.9004
DBP (mm Hg)	-5.40*	-1.90	3.50	0.0371	6.3543
HR (bpm)	-1.23	+ 0.03	1.26	0.4360	6.2537
ABP Nighttime					
SBP (mm Hg)	-2.17	+ 0.47	2.64	0.2958	9.6665
DBP (mm Hg)	-2.30	+ 0.37	2.67	0.0557	5.2903
HR (bpm)	-0.50	+ 0.40	0.90	0.9485	5.9664

\*significantly different from baseline ( $p < 0.05$ ). RMSE = Root mean square error

while diastolic office B decreased from  $88.1 \pm 7.1$  to  $84.5 \pm 5.4$  mmHg.

#### ***Automated Office BP (AOBP)***

Unattended, automated BP measurements showed a significant reduction only for SBP (5.7 mmHg vs. placebo), while the diastolic BP decrease did not reach statistical significance ( $p$  0.096). In this case too, HR was not affected by dietary integration or placebo.

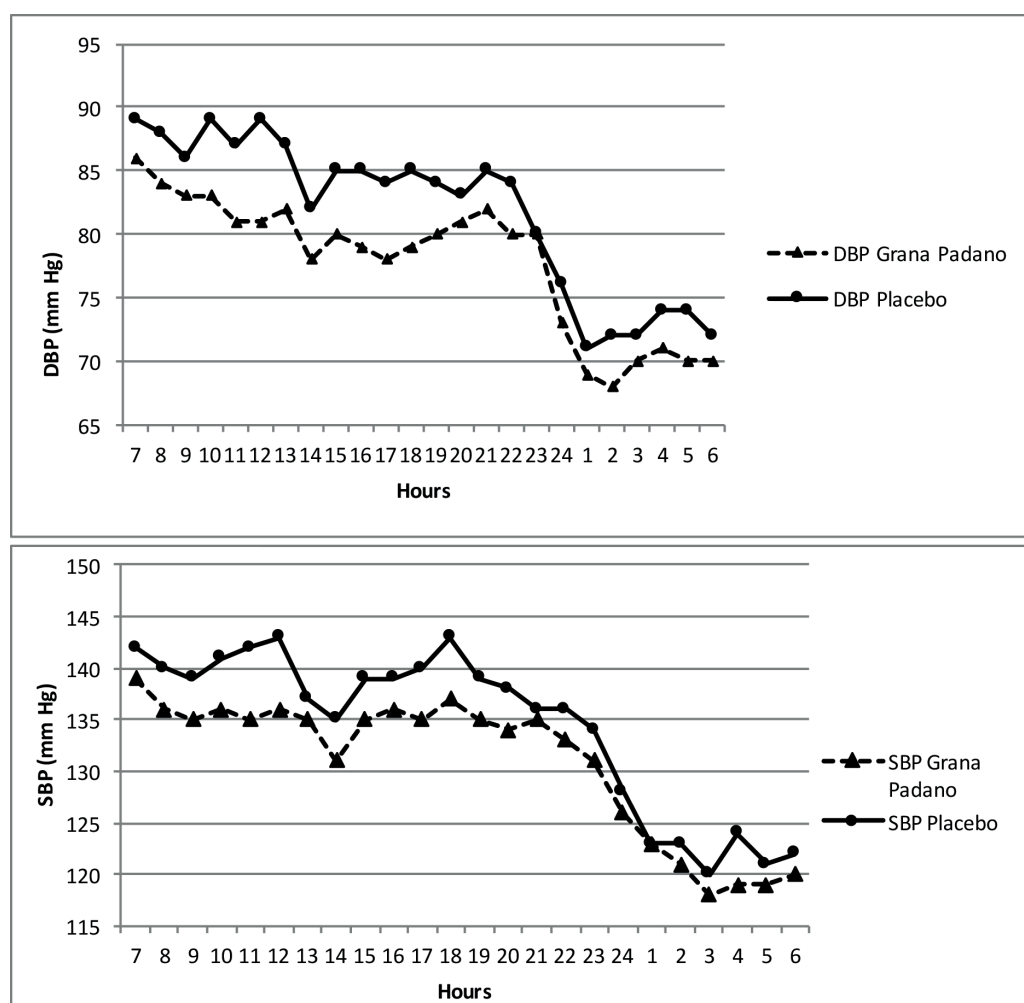
#### ***Ambulatory BP (ABP)***

The systolic and diastolic daytime, nighttime and 24-h ambulatory blood pressures observed at the baseline and at the end of the placebo period were very similar. A certain degree of placebo effect on all BP values is discernible but it was not statistically significant. Conversely, dietary integration with Grana Padano cheese was accompanied by a sustained reduction in ambulatory blood pressure values and particularly in 24-hour systolic and diastolic BP with respect to placebo. Circadian rhythm was not substantially influenced by treatment with Grana Padano cheese, although the BP lowering effect was observed to be more pronounced during daytime. In fact, during nighttime the fall in BP was less evident, and neither systolic nor diastolic ABP were si-

gnificantly changed after dietary intervention. Among the 30 patients considered in the study, 4 patients showed the non-dipper pattern, according to the international definition (less than 10% drop in systolic BP during the night). However, all 4 patients experienced a significant reduction in nocturnal BP according to 24-h ABP analysis, and none showed a tendency to rise. Active treatment did not modify the circadian rhythm, although the BP reduction observed after dietary integration was lower during the night with respect to the daytime. Table IV shows the changes in clinic, automated office and ambulatory BP values for the whole day, and split into daytime and nighttime. Hourly mean systolic and diastolic ABP values after 2 months of dietary integration or placebo are reported in Figure 2.

#### ***Biochemical Data***

Mean blood glucose and serum sodium, potassium, magnesium, calcium, uric acid, creatinine, hemoglobin concentrations, white blood cell and platelet counts were within the normal range at the baseline, while mean total cholesterol levels were slightly above the normal range (Table II). No significant changes were observed either after dietary integration or after placebo for all the examined variables, including body weight.



**Figure 2.** Pattern of hourly diastolic (DSB) and systolic (SBP) ABP values after 2 months of dietary integration with Grana Padano or placebo.

## Discussion

The pattern of ACE inhibiting peptides is similar to that found by Stuknytė et al<sup>20</sup>, with VPP and IPP having higher concentrations. The other ACE-inhibiting peptides recovered had previously showed significant anti-ACE activity, particularly the LHLPLP peptide<sup>20</sup>. It is interesting to note that the level of this peptide increases to 306 mg/kg after in vitro gastrointestinal digestion of Grana Padano<sup>20</sup>. This randomized, double-blind, placebo-controlled, cross-over study has confirmed the significant antihypertensive effect of a 2-month daily dietary integration with 30 g per day of Grana Padano cheese in mild-to-moderate hypertensive subjects with BP level not on target (i.e. > 140 and/or 90 mmHg) despite pharmacological and/or non-pharmacological treatment. Sy-

stolic and diastolic office and ambulatory BP decreased significantly after 2 months of treatment. The magnitude of the BP lowering effect we observed was similar to that reported by Pripp et al<sup>28</sup> (2008), but is higher than the average BP reduction in the European subjects reported by Cicero et al<sup>14</sup> in which other dairy products or isolated tripeptides were tested. However, in both meta-analyses the data refer mainly to BP values measured by conventional measurement (OBP) and the studies considered for data extraction also refer to normotensive subjects. In the present study, all the patients considered were hypertensive subjects and had base-line values of automated (AO or ABP) BP above the normal range, a condition in which the effect of ACE-inhibiting peptides is expected to be more relevant<sup>28</sup>. The observed reduction in BP could be attributed to other nutrients present

in Grana Padano cheese. This cheese contains a considerable amount of calcium, and the possible role of calcium supplementation in reducing BP is known from epidemiological and intervention trials<sup>29</sup>. However, the same amount of calcium as that contained in the cheese was provided with the placebo, and thus the antihypertensive effect observed cannot be attributed to increased dietary calcium intake. Some patients included in the study were on stable treatment with dihydropyridine Ca channel blockers (amlodipine 3 patients and barnidipine 5 patients). The effect of dietary Ca intake on BP is independent from Ca channel blockers due to dihydropyridine Ca antagonist agents. The mechanism by which increased calcium intake could lower BP has not been identified, but numerous possibilities have been proposed. A membrane-stabilizing effect wrought by an increase in extracellular Ca, which is the main mechanism of the antihypertensive effect of Ca channel blockers, would appear unlikely, since increases in extracellular Ca concentration due to increased intake are minimal. Many of the proposals have attempted to relate dietary Ca to neural, hormonal, and renal effects of dietary Ca. In any case, no interference between Ca intake and Ca blockers has ever been demonstrated in experimental animals or in humans<sup>30</sup>. All dairy products are a potential source of ACE-inhibiting peptides, but as reported in Table III, Grana Padano cheese is a source of these peptides, which effectively inhibit angiotensin converting enzyme<sup>20,31</sup>, and their long-term administration has been shown to lower BP in mildly hypertensive subjects<sup>32-34</sup>. Moreover, among the factors that can affect the response to these peptides, the action of gastric and gut proteases, which may inactivate some peptides (but can also produce other effective ACE-inhibiting peptides)<sup>20</sup> should be considered. In any case, the activity of the ACE-inhibiting peptides present in Grana Padano cheese is clearly preserved, VPP, IPP and LHLPLP peptides having a lower IC<sub>50</sub>, which indicates the high biological activity of these peptides<sup>20</sup>. Finally, dairy products could also influence blood pressure through mechanisms that are independent of ACE inhibition. These include the vascular release of endogenous vasodilators, such as prostaglandin I<sup>35</sup> nitric oxide (NO)<sup>36</sup> and carbon monoxide (CO)<sup>37</sup> and even an lactotropine- $\alpha$  mediated activation of opioid receptors<sup>38</sup>. The magnitude of the fall in BP (office BP -4,8/-3,5 mmHg) observed in this study is remarkable, bearing in mind that a decrease in SBP by 3 mmHg would reduce the risk of stroke

by 10-13% and the risk of myocardial infarction by 7%, over a 5-year period<sup>39</sup>. Moreover, the BP reduction obtained with this dietary integration appears relevant since other non-pharmacological treatments, such as dietary sodium curtailment in individuals with mild hypertension, have been shown to lower SBP by 2-5 mmHg, and DBP by 1-2 mmHg<sup>40,41</sup>. From a clinical point of view the BP reduction obtained through dietary integration with Grana Padano cheese seems remarkable, since 20 subjects (67% of the studied population) achieved normalized BP values by the end of the 2-month period of active treatment.

## Conclusions

The consumption of dairy foods, and Grana Padano in particular, should be considered as a new opportunity and a feasible strategy for hypertension prevention and treatment in large populations. Considering the wide availability of this food, its significant efficacy and the lack of side effects, it may represent an alternative to drugs in some patients and the opportunity to reduce medications in treated individuals. ACE-inhibiting peptides could explain, at least partially, the anti-hypertensive effects of Grana Padano consumption. In order to optimize the use of these milk-derived bioactive peptides, more studies are needed to better understand the effects of digestive enzymes on their concentration, the molecular mechanisms involved in their actions and their biological activity. In conclusion, the results of this double blind controlled study suggest that daily supplementation with Grana Padano cheese can be useful for reducing blood pressure and consequently the risk of cardiovascular disease in hypertensive patients.

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## Conflict of Interest

The Authors declare that they have no conflict of interest.

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