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O papel do Chocolate e da Teobromina na Prevenção da Doença de Alzheimer

The Role of Chocolate and Theobromine in the Prevention of Alzheimer's Disease

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This work is dedicated to Oliver Sacks, whose inspirational words and humanitarian ideas have pushed me to venture into the unknown borders of neurology and neuroscience.

ABSTRACT

Cocoa-related products like chocolate have taken an important place in our food habits and culture. Preliminary research data shows that theobromine, a methylxanthine present in high concentrations in cocoa, might have a protective effect against Alzheimer's Disease (AD). In this work, we aim to examine the relationship between chocolate consumption and cognitive decline in an elderly cognitively healthy population. Furthermore, we will discuss the current state of the art concerning the study of the cognitive effects of chocolate and theobromine. A cohort of 531 participants aged 65 and over with a Mini-Mental State Examination (MMSE) not compatible with cognitive impairment was selected for the present study. The median follow-up was 48 months. Dietary habits were evaluated at baseline. The MMSE was used to assess global cognitive function at baseline and at follow-up. Cognitive decline was defined by a decrease ≥ 2 points in the MMSE score between evaluations. Relative risk (RR) and 95% confidence interval (95%CI) estimates were adjusted for age, education, smoking, alcohol drinking, body mass index, hypertension, and diabetes. Chocolate intake was associated with a lower risk of cognitive decline (RR = 0.59, 95%CI 0.38 – 0.92). To our knowledge, this is the first study to have assessed the negative association between regular long-term chocolate consumption and cognitive decline in humans.

Keywords: chocolate, cognitive decline, theobromine, A_{2A} receptors, prevention, Alzheimer's Disease

RESUMO

Os produtos com conteúdo de cacau, tais como o chocolate, conquistaram um importante lugar nos nossos hábitos alimentares e na nossa cultura. Dados preliminares indicam que a teobromina, uma metilxantina presente em alta concentração nos produtos à base de cacau, pode apresentar efeitos protectores contra o desenvolvimento da Doença de Alzheimer. Ao longo deste trabalho, iremos examinar a relação entre o consumo de chocolate e o surgimento de declínio cognitivo numa população envelhecida e cognitivamente saudável. Adicionalmente, iremos discutir o estudo dos efeitos cognitivos do chocolate e da teobromina. Para este estudo, foi selecionado uma coorte de 531 participantes com idade igual ou superior a 65 anos e com um valor no *Mini-Mental State Examination* (MMSE) incompatível com défice cognitivo. O período médio de seguimento foi de 48 meses. Os hábitos dietéticos foram avaliadas na linha de base. O MMSE foi utilizado para avaliar o funcionamento cognitivo geral tanto na linha de base como após o seguimento. Para se afimar a existência de de declínio cognitivo, um indivíduo teria que ter um decréscimo de ≥ 2 pontos no MMSE entre as avaliações. As estimativas do Risco Relativo (RR) e do intervalo de confiança de 95% (95%IC) foram ajustadas para a idade, educação, hábitos tabágicos, consumo de álcool, índice de massa corporal, hipertensão e diabetes. O consumo de chocolate foi associado a um menor risco de declínio cognitivo (RR = 0.59, 95%IC 0.38 – 0.92). De acordo com a nossa pesquisa, este é o primeiro estudo a avaliar a associação negativa entre o consumo regular e prolongado de chocolate e o surgimento de declínio cognitivo em humanos.

Palavras-chave: chocolate, declínio cognitivo, teobromina, receptores A_{2A}, prevenção, Doença de Alzheimer

Introduction

Chocolate consumption is generally considered a healthy and pleasant widespread habit. Despite the drawbacks related to the high caloric intake associated with this practice, its numerous beneficial effects on different main systems in the human physiology have been largely studied (1,2). In fact, many of the potential advantages of chocolate consuming have been attributed to the antioxidative properties of its components, namely the polyphenolic structures named flavonols (3). Remarkably, a new insight to what might be in the source of such benefits has been proposed in the past few years, with special regard to the psychoactive and neuroprotective effects of the methylxanthines present in chocolate and their implication in the prevention of neurodegenerative diseases (1,4,5).

Caffeine and theobromine are two similar methylxanthines present in chocolate, coffee and tea. Whereas caffeine is the most abundant methylxanthine in coffee, theobromine has been found to be the main xanthine in all types of chocolate (4,6). The combination of caffeine and theobromine in the proportions found in cacao (the main compound of chocolate) has been shown to display psycho-stimulant effects (7,8). Besides, there is increasing evidence that dietary methylxanthines, such as those commonly found in chocolate products, may possess neuroprotective effects and are associated with significantly improved and/or preserved aspects of cognitive functioning (5). Caffeine, in particular, has been widely explored, in terms of its preventive effect on the development of two of the most prevalent neurodegenerative diseases, such as Parkinson's (9) and Alzheimer's Disease (AD) (10). Notably, a recent systematic review and meta-analysis of 11 observational epidemiological studies has pointed for a protective effect of chronical exposure to caffeine against dementia, AD, cognitive impairment and cognitive decline (11). However, the effects of theobromine have been less studied than those of caffeine (1,4,5), and the potential neuroprotective effect of theobromine is still poorly understood (6).

Despite their various mechanisms of action, both molecules seem to act mainly as nonspecific antagonists of adenosine receptors when considering a normal degree of consumption (1,4). In fact, the administration of adenosine A_{2A} receptor antagonists has been proven to prevent memory deficits in different animal AD models (12,13). In addition, both the blockade of A_{2A} receptors has also been shown to reduce the

production of amyloid-β, a peptide that accumulates in the brain of AD patients, and limit its synaptotoxic effect (13,14).

In the past years, biomarkers for AD diagnosis and early-detection have been intensively studied and the concentration of Aβ42 in the cerebrospinal has been found to be reduced in AD patients with different stages of the disease (15). Remarkably, the BIOMARKAPD project recently showed a significant positive correlation between the concentrations of theobromine in the cerebrospinal fluid and in the plasma with amyloid-β in the cerebrospinal fluid, suggesting that theobromine might also have a protective effect against AD (16).

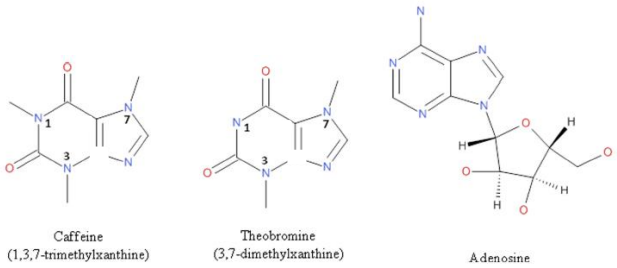

Fig. 1) Chemical structure of caffeine, theobromine and adenosine. Adapted from (1).

Animal studies on the cognitive effect of chocolate/cocoa

To date, there are only two published studies that address the effect of cocoa-related products on animal's cognitive function.

In 2007, Rozan *et al.* investigated the short-term effect of a cocoa polyphenolic extract (Acticoa powder) on free radicals produced by leucocytes after heat exposure and the extract's protective effects on subsequent cognitive impairments. The authors concluded that the 14-days oral administration of Acticoa powder had protected heat-exposed rats from cognitive impairments (by better performances in light extinction and Morris maze paradigms, meaning preserved short and long-term memory processes), potentially by counteracting the overproduction of free radicals and suggested that the cognitive outcomes could be related to preservation of brain function as a result of reduced inflammatory aggression, heat exposure, or enhanced brain plasticity (17).

Short after in 2008, Bisson *et al.* addressed the long-term effect on cognitive functions of the same polyphenolic extract, Acticoa powder, in old Wister-Unilever rats. During 12 months, they orally received supplementation at the dose 24 mg/kg/day, and

were also evaluated through light extinction and the Morris water maze, showing improved cognitive performance in terms of spatial memory and short- and long- term learning, increased lifespan by around 11% and preserved high urinary free dopamine levels, a neurotransmitter associated with important cognitive functions, such as efficient memory and problem-solving. In this light, the authors suggested that Acticoa powder could be beneficial in retarding age-related brain impairments, including cognitive deficits in normal ageing and perhaps neurodegenerative diseases (18).

Human studies on the cognitive effect of chocolate/cocoa

There is a wide range of data demonstrating beneficial cognitive effects of chocolate, cocoa and some of its components (1–6,19–22).

Despite not being focused solely on chocolate but also on the consumption of tea and coffee, a cross-sectional study involving 2031 subjects (aged 70-74 years) by Nurk *et al.* in 2009, based on the food habits reported in the previous year, concluded that habitual chocolate consumers had performed better in all cognitive tests applied and that the risk for poor test performance in most tests had been significantly reduced by chocolate consumption (23). The large population and the use of six different cognitive tests (including the MMSE) were major strengths of this study, but it is noteworthy to refer that the authors acknowledged some weaknesses, such as the study's retrospective cross-sectional design, the potential error in the estimates of nutrients and the volunteer sample, which might have been a source of recruitment bias.

In 2010 Scholey and colleges designed a randomized, double-blind, controlled trial to examine the acute cognitive and subjective effects of cocoa flavanols consumption. In this study, 30 healthy young adults consumed high-flavanol cocoa drinks and a matched control drink. Over a 1 h testing period, participants repeatedly performed 10-min cycles of a Cognitive Demand Battery, a Rapid Visual Information Processing (RVIP) task, and a "mental fatigue" scale. Among other findings, the 994-mg beverage yielded speeded RVIP responses and the 520-mg beverage attenuated self-reported "mental fatigue". This was the first report on immediate improvements of cognitive function following high-flavanol cocoa consumption in healthy young adults (24).

In a recent systematic review by Scholey *et al.* published in 2013, of the eight studies that met the criteria for inclusion, three revealed clear evidence of cognitive enhancement following cocoa flavanols and methylxanthine consumption (19). All studies examined the cognitive effects of potentially psychoactive fractions of chocolate: five studies focused on cocoa flavonol fractions of cocoa (22,24–27) and three (from two articles) on combinations of the methylxanthines: caffeine and theobromine (7,28). In summary, this review concluded that there is a growing body of evidence indicating improved cognitive function with acute administration of cocoa flavonols, caffeine and theobromine, in isolation and in combination. It also stated that, despite seemingly strong evidence from epidemiological studies (23), no study had yet found cognitive effects in randomized controlled trials using sub-acute or chronic administration of chocolate and its components in humans. The dosing regimens periods varied from 5 days to 6 weeks and it was hypothesized that the lack of positive outcomes was related to possible methodological aspects (19). For example, in 2008 Crews *et al.* published an article concerning a double-blind, placebo-controlled, fixed-dose, parallel group clinical trial in which 101 healthy subjects were randomly assigned to receive a 37-g dark chocolate bar and 237 mL of an artificially sweetened cocoa beverage or similar placebo products each day for 6 weeks. The results failed to support the predicted sub-acute enhancing effects of dark chocolate and cocoa consumption on neuropsychological status. Again, the authors stated that there were recognized difficulties that could have contributed to the negative findings, such as the apparent cognitive intact profile and educational level (>15 years) of the subjects at baseline, the small sample size which may not have been large enough to detect significant group differences and the short duration of the treatment or the low quantity of chocolate/cocoa administered to subjects (26).

Probably because the electronic databases were searched in early-2012 for the review of Scholey *et al.* (19), a later article by Desideri and collaborators published in mid-2012 was not addressed. Included in the Cocoa, Cognition, and Aging (CoCoA) Study, this double-blind, parallel arm study in which 90 elderly individuals were set to consume cocoa drinks once daily for 8 weeks provided the first evidence that regular cocoa flavanol consumption could positively enhance cognitive function in older adults with mild cognitive impairment (29).

More recently, two randomized controlled trials revealed contradictory results concerning the effect of cocoa on cognitive function. Published in 2015 and also based on the Cocoa, Cognition, and Aging (CoCoA) Study, one of these randomized controlled trials found that daily cocoa consumption for 8 weeks had improved specific aspects of cognitive performance in a group of cognitively intact older adults (30). As in other studies, it was focused on the flavonoid content of cocoa, but it is interesting to notice that the results on the MMSE did not differ between the low and high-flavonoid intake groups and that even the low-flavonoid intake group had a significant improvement on the Verbal Fluency Test. As the authors hypothesize, given this drink's very low amount of flavonoids, it is possible that this improvement might have been attributed to the effect of the methylxanthines present in the ingested products (30). The second study, also published in 2015, was a randomized, placebo-controlled, double-blind clinical trial comprising 40 subjects, conducted to investigate the effects of both acute and sub-acute (four-weeks) 3058 mg *Theobroma cacao* seed extract daily supplementation on mood and mental fatigue, cognitive performance and cardiovascular functioning in young, healthy adults (31). Similarly to Crews *et al.* study of 2008 (26), supplementation with cocoa extract did not result in significant sub-acute positive effects.

In spite of these negative results, it is still possible that the long-term cumulative supplementation or regular ingestion of cocoa or chocolate may result in cognitive protection or enhancement. Along with the present work, more studies regarding the chronic effect of chocolate and its components on cognition are needed to address this issue.

Studies on the cognitive effect of caffeine and/or theobromine

Studies that address the effect of caffeine on cognitive function are abundant, both in animals (32) and in humans. Not only are there studies focused on the short-term effect of this methylxanthine (33,34), there are also various studies and reviews on the long-term effects of caffeine (11,14,35–37).

In 2007, Chen and collaborators published a review in which they highlighted A_{2A} receptors ability to fine tune neuronal and glial functions to produce neuroprotective effects. Modulation of A_{2A} receptors could facilitate the

neurophysiological mechanisms of learning and memory, by interfering with the pathological changes of AD or strengthening the resistance of neuronal cells to insults. This effect has been proposed to decrease the probability of emergence of AD in healthy subjects, or attenuate the disease progression in patients already affected (38). In fact, plasma and brain amyloid-β levels have been found to be reduced by acute or chronic caffeine administration in several AD transgenic lines and ages (33).

However and to our knowledge, there are still no randomized controlled trials on humans that address the long-term effect of theobromine on cognition and there are only a very few early publications to have reported individual and combined effects of caffeine and theobromine (6). In fact, theobromine has been generally ignored, partially due to the prevalent idea that it is a very low central nervous system stimulant (39,40).

Apart from some evidence provided by Ott in 1985 (considered to be "anecdotal" by Smit *et al.* (6)) who replaced his dietary caffeine intake with a daily dose of 600 mg theobromine and documented a similar behavior to caffeine in terms of its capacity to produce withdrawal symptoms, theobromine has only been showed to produce very minor subjective effects compared with caffeine (41).

A study by Smit and collaborators (2004) showed that both cocoa powder and a methylxanthine mixture containing a combination of caffeine and theobromine had significant similar effects on reaction time, energetic arousal and information processing. They concluded that the psychostimulant effect of chocolate is mainly due to the methylxanthines present in chocolate (7), an allegation that was considered to be "premature" in the review by Scholey *et al.*, knowing of the demonstrable acute psychoactive effects of other components of chocolate, such as cocoa flavonols (19).

Nonetheless, one possible explanation on the different effects of caffeine and theobromine may lay on their distinct pharmacokinetic and pharmacodynamic profiles.

Pharmacokinetics and pharmacodynamics of caffeine and theobromine

The pharmacokinetics of caffeine and theobromine have been thoroughly studied (42–45).

Theobromine absorption from the digestive tract is slow, with an estimated peak plasma time of 2.5 h (compared with 0.5 h for caffeine) (42). The clearance rate for acutely administered theobromine is 1.2 ml/min/kg, around half of that of caffeine (2.1 ml/min/kg). This contributes to a higher half-life for theobromine compared to caffeine (7.2 h against 4.1 h) (45). It has also been stated that inter-individual differences in theobromine clearance rates may be substantial, as is the case for caffeine (46). Additionally, it is important to be aware of the different conditions that may alter the clearance rate of these compounds, either by its decrease or enhancement (i.e. tobacco-smoking habits (47,48)).

	Caffeine	Theobromine
Peak plasma time (t max)	0.5 h	2.5 h
Clearance rate	2.1 ml/min/kg	1.2 ml/min/kg
Half-life	4.1 h	7.2 h

Table 1) Pharmacokinetic profiles of caffeine and theobromine. Adapted from (42,45).

In humans, methylxanthines are metabolized by demethylation by the enzyme superfamily cytochrome P450, especially its member CYP1A2 which is responsible for more than 95% of the primary metabolism of caffeine, making caffeine an important probe drug to monitor this enzyme's activity (49). Caffeine is metabolized to different compounds, including theobromine. Theobromine does not metabolize into other dimethylxanthines (i.e. theophylline or paraxanthine), nor does it "upgrade" to the trimethylxanthine caffeine. Because of this, the consumption of cocoa-related products exposes humans both to theobromine from the demethylation of caffeine, in addition to the direct ingestion of theobromine contained in these products (6).

Regarding the pharmacodynamic profile of the different methylxanthines, these have been show to display different affinities for the different receptor types (6). While theobromine is only 2- to 3-fold less active than caffeine as an antagonist of the adenosine A_1 receptors in rat brain, it is at least 10-fold less active than caffeine as an antagonist of A_{2A} receptors (50). Similar results were documented by Fredholm and Lindström in 1999, but these authors also provided evidence on the different caffeine-to-theobromine affinity ratios measured for striatum compared with cortex A_1 receptor

antagonism (theobromine was found to be 4.7 and 11.8 times less active than caffeine, respectively). With this work, the authors concluded that neither of the methylxanthines studied had showed any selectivity for a specific type of adenosine receptor (51). Finally it is also important to acknowledge that theobromine penetrates the brain to a lesser extent than caffeine (39).

	A_{2A} Striatum	A_1 Striatum	A_1 Cortex
Caffeine	8,556 (6,468-11,312)	20,490 (11,809-34,837)	16,643 (9,871-27,962)
Theobromine	109,048 (19,483-577,561)	196,505 (66,964-158,474)	197,000 (167,600-234,300)

Table 2) Potency of caffeine and theobromine to displace CGS 21680 (adenosine receptor agonist) from adenosine receptors. Results are given as Ki values in nM (mean and 95% confidence interval). Adapted from (51).

These different affinities for different receptor types may then explain the alternative effects of caffeine and theobromine.

Given all this evidence, the role theobromine on chocolate consumption cognitive effects seems to be rather limited. However, the identification of potential risk and protective factors, such as the consumption of theobromine-enriched products like chocolate, should be targeted as vital for the prevention of neurodegenerative disorders.

Main goal

In this present study we aim to assess the association between chocolate consumption and cognitive decline. The hypothesis that chocolate could decrease the incidence of cognitive decline by at least 2 points in the score of a widely used cognitive test, the MMSE, was specifically tested in participants aged 65 and over.[1]

[1] This work is based on the data already collected for a wider research, the EPIPorto study. My personal contribution focused on the aspects mentioned above and was adapted to match the demands of the Medicine Master's Final Essay.

Methods

Study population

This study was based on the evaluation of a cohort of adults living in Porto, as previously described (36). Briefly, a total of 2485 participants were recruited between 1999 and 2003, by random digit dialling having households as the sampling unit; when a household was selected, all residents were identified by age and gender, and one resident (aged 18 or more years) was randomly selected as the respondent, without replacement if there was a refusal. The participation rate was 70%. A visit to the Department of Clinical Epidemiology, Predictive Medicine and Public Health of Porto Medical School (former Department of Hygiene and Epidemiology of Porto Medical School) was scheduled by telephone according to the participant's convenience. A personal interview, using a structured questionnaire comprising data on socio-demographic, clinical, and lifestyle exposures, and a physical examination was performed by trained interviewers. From the whole cohort, 648 participants were aged 65 and over and 531 were selected for the present study, after exclusion of 62 cognitively impaired at baseline (the criteria used to define cognitive impairment is defined below), 32 for whom there was no baseline MMSE, and 23 for whom there was no information on chocolate or caffeine intake.

Follow-up evaluation

The follow-up evaluation of the cohort took place between 2005 and 2008. A visit to the Porto Medical School was scheduled, for questionnaire evaluation and physical examination of the participants.

Cognitive testing

The MMSE (52,53) was used to assess global cognitive function at baseline and at follow-up. The MMSE, which includes questions on orientation, registration, attention and calculation, recall, language and visual construction, was originally designed for clinical practice, but is now extensively used in epidemiological studies. Although it does not assess executive function, a major feature of cognitive decline

(52), the MMSE is a reliable and valid test for cognitive impairment, has high test-retest reliability, and is a good indicator of clinically significant cognitive decline (54). The cut-off values adjusted for education levels were used as proposed in other studies (55,56). The normative cut-off values of MMSE adjusted for education for the Portuguese population were used (53). Subjects that had a MMSE score below cutoff at baseline were considered to be cognitively impaired and therefore excluded. Participants had to score above 15 if they were illiterate, above 22 if they had ≤ 11 years of education, and above 27 if they had > 11 years of education.

A decline of at least 2 points in the score of the MMSE from baseline to the follow-up visit was considered meaningful from a clinical point of view (35).

Chocolate dietary intake

Dietary habits in the 12 months preceding the baseline interview were evaluated using a semi-quantitative food frequency questionnaire (FFQ) comprising 82 food and beverage items or groups. It was designed according to Willett *et al.* (57), and was adapted by inclusion of a variety of typical Portuguese food items and validated as previously described. For each FFQ item, subjects were asked the average frequency of consumption and the portion size usually consumed (based on a photograph manual with small, medium and large portion sizes). This information was used to estimate the average daily intake of each item by multiplying the usual frequency of intake per day by the average portion size of the corresponding item. The food items/groups of the FFQ used to address chocolate overall consumption were chocolate bars, chocolate snacks and cocoa powder.

Socio-demographic, clinical and other behavioural factors

The assessment of socio-demographic, clinical and other behavioural factors was previously described in detail (36) and is briefly described in footnotes of table 1.

Statistical analysis

Data analysis was conducted in 309 subjects who at baseline were aged 65 or more and had a MMSE not compatible with cognitive impairment, and who were re-evaluated. Comparison of the baseline characteristics between subjects that were followed, died or were lost to follow up was done using the Chi-Square or the Kruskal-Wallis tests, as appropriate, to compare all groups.

The association between chocolate intake and the development of cognitive impairment was quantified through crude and age-, education-, body mass index, diabetes-, hypertension-, smoking- and alcohol drinking-adjusted relative risks (RR) and respective 95% confidence intervals (95%CI) using Poisson regression. Data were analysed using STATA®, version 11.

Decline in cognitive performance was defined as a decrease of at least two points on the MMSE from the baseline assessment to follow up and impairment in cognitive performance was defined based on normative cut-off values of MMSE adjusted for education for the Portuguese population.

Ethics

This study was approved by a local Ethics Committee, and all participants gave written informed consent.

Results

Among the 531 participants that were eligible, 309 (58.2%) completed the follow-up evaluation (median follow-up: 48 months), 58 (10.9%) died before follow-up could be accomplished and there were 164 (30.9%) losses to follow-up. Participants who died during the follow up period were more likely to be older, hypertensive, to have a lower BMI, and worse MMSE score. No statistically significant differences between the groups were found regarding education, smoking, alcohol, diabetes and caffeine or chocolate consumption (Table 3).

Chocolate intake was independently associated with a nearly 40% lower risk of cognitive decline, defined as $\Delta MMSE \leq -2$ between baseline and follow-up. The RR estimates were similar for the different levels of chocolate consumption analysed, though only statistically significant for participants with an average weekly consumption of chocolate lower than one standard portion, corresponding to three pieces of chocolate bar, one chocolate snack or one table spoon of cocoa powder (Table 4). Chocolate intake was not significantly associated with a decreased risk of cognitive impairment defined as abnormal MMSE score at follow-up (Table 4).

Table 3) Socio-demographic, clinical and behavioural characteristics of the cohort.

	Followed in 2005-2008 (n=309)	Deceased during follow-up (n=58)	Not followed in 2005-2008 (n=164)	P
Sex (% women)	58.6	44.8	63.4	**0.047**
Age (years)*	70 (67-74)	74 (69-79)	72.5 (69-76)	**<0.001**
Age (% ≥75 years)	20.71	46.55	34.76	**<0.001**
Education (years)*	4 (4-8)	4 (4-7)	4 (3-7)	0.3459
Education (%)				
Illiterate	6.5	5.1	9.1	
<12 years	79.2	81.0	78.1	0.801
≥12 years	14.2	13.8	12.8	
Body mass index (Kg/m^2)*	27.8 (25.0-29.9)	26.2 (22.4-30.1)	27.5 (24.7-30.7)	0.1651
Body mass index (%)				
≤25.0 Kg/m^2	24.59	41.07	25.79	
25.0-29.9 Kg/m^2	50.49	33.94	40.88	**0.019**
≥30.0 Kg/m^2	24.92	25.00	33.33	
Smoking (% ever smokers)	34.0	43.1	26.2	0.055
Alcohol drinking (% ever drinkers)	86.1	82.8	85.9	0.796
Hypertension (%)	79.7	90.7	90.5	**0.004**
Diabetes (%)	12.0	17.5	13.4	0.515
MMSE*	28 (27-29)	28 (25-29)	27.5 (25.5-29)	**0.008**
Caffeine intake (mg/day)*	32.8 (10.6-78.8)	33.9 (13.9-78.9)	32.1 (13.6-78.8)	0.840
Chocolate intake (% consumers)	40.1	41.4	39.6	0.973

* Results are presented as median (percentile 25-percentile 75).

Table 4) Association between chocolate consumption and cognitive decline (ΔMMSE≤ -2) or development of cognitive impairment*.

	Δ MMSE≤ -2				Cognitive impairment*			
	Follow-up		RR (95% CI)		Follow-up		RR (95% CI)	
	PM	No. subjects with outcome	Crude	Adjusted [a]	PM	No. subjects with outcome	Crude	Adjusted [a]
Chocolate consumer								
No	9516	72	1 [reference]	1 [reference]	9516	13	1 [reference]	1 [reference]
Yes	7128	31	0.57 (0.37-0.87)	**0.59 (0.38-0.92)**	7128	9	0.92 (0.39-2.16)	0.96 (0.39-2.35)
Chocolate intake								
No intake	9516	72	1 [reference]	1 [reference]	9516	13	1 [reference]	1 [reference]
< 1 portion† / week	4668	21	0.59 (0.37-0.97)	**0.57 (0.34-0.94)**	4668	6	0.94 (0.36-2.48)	1.03 (0.37-2.82)
≥ 1 portion† / week	2460	10	0.54 (0.28-1.04)	0.65 (0.33-1.31)	2460	3	0.89 (0.25-3.13)	0.85 (0.22-3.22)

RR – Relative risk; 95% CI – 95% Confidence interval; PM – Person months; MMSE – Mini-Mental State Examination; Δ – MMSE at follow-up - MMSE at baseline.

* The normative cut-off values of MMSE adjusted to the education for the Portuguese population were used. Subjects were classified as cognitively impaired at follow-up when having a MMSE score below 16 if they were illiterate, 23 if they had ≤ 11 years of education, 28 if they had > 11 years of education.

[a] adjusted for age (continuous), education (continuous), body mass index – BMI (continuous), diabetes, hypertension, smoking (never/ever), and alcohol drinking (never/ever). Hypertension, diabetes, IMC, tobacco and alcohol consumption were assessed as previously described (36). Arterial hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or current antihypertensive drug therapy (58). Participants on anti-diabetic therapy and/or with fasting plasma glucose concentrations ≥126 mg/dL and/or diagnosed with diabetes by a health professional were considered to have diabetes mellitus (59). Body mass index (BMI) was calculated as weight (kg) divided by squared height (m^2), and further divided into the following categories (60): obese (≥30 kg/m^2), overweight (25.0–29.9 kg/m^2), normal and underweight (<24.9 kg/m^2). Regarding smoking habits and the consumption of alcoholic beverages, subjects were categorized in never- and ever-smokers and never- and ever-drinkers.

† one standard portion corresponds to three pieces of chocolate bar, one chocolate snack or one table spoon of cocoa powder.

Discussion

Our results suggest that the consumption of chocolate has a protective effect on cognitive decline in elderly patients, as defined by the decrease in two or more points in the MMSE.

To the best of our knowledge, no other observational studies have specifically addressed the long-term effect of chocolate consumption on cognitive decline in humans. As stated before, most studies are generally focused on the intake of antioxidant and flavonoid compounds present in chocolate or cocoa, rather than chocolate intake itself or its methylxanthine content (21,23,61–63), making it hard to establish direct relations between this and other studies. Furthermore, most studies deal with the acute (22,24,27,31) and sub-acute (26,30,31) cognitive enhancing effects of both flavonoids and methylxanthines and not the potential protective chronic results we addressed in the present study. Outcomes on cognitive function were also various across all studies, and only some have utilized the MMSE (23,30). Nonetheless, it is possible to relate the conclusions on the present work to some of these studies, since higher content of cocoa in chocolate products relates both to its flavonoid, methylxanthine and theobromine concentration.

There are many possible explanations for the positive effects that have been documented and proposed until this day and some of those may still hold theobromine at a key spot.

Alternative mechanisms of action

Despite acting primarily as an adenosine receptor antagonist, theobromine participates in three other mechanisms: regulation of intracellular calcium level, phosphodiesterase inhibition and modulation of $GABA_A$ receptor activity. In fact, high doses of methylxanthines, probably achieved through the use of supplements, have different actions in other targets, such as interacting with DNA and altering its structure (64). However, in the doses normally consumed, these effects are probably irrelevant (4).

Different susceptibility to methylxanthine effects may also be important in this context. Unfortunately, current studies have failed to validate clear relationships between gene variants, caffeine intake, and phenotypes, information that might help solve this question (65).

One other alternative mechanism of action is the interaction of theobromine with A3 receptors. There is no record of theobromine being tested on A3 receptors, but similar compounds such as caffeine and theophylline seem to display very low affinity for these targets (66).

It is also interesting to note that antagonists may have different blockade potencies for the same type of receptors. This usually depends on their location in specific cells or in specific locations inside the cells (67). One possible explanation for theobromine effect is that it acts preferably in specific adenosine receptors, which not only might explain its effect on cognition, but also its differential effects compared to caffeine (for example, its pro-sleep effect against caffeine's insomnia effect (68)).

Finally, the positive interaction with other compounds should not be excluded. For example, the interaction with flavonoids is certainly of the most high interest, as Jalil *et al.* recognized when they suggested that there may be direct or indirect synergism between flavonoids and methylxanthines (69).

Limitations of this study

As in other studies, some methodological issues oblige us to be cautious about the conclusions we presented in this work. We will discuss some of them and suggest improvements for better study modeling in the future.

Initially, we desired to relate theobromine intake to cognitive decline, but this was not possible. The necessity of successive estimates for the calculation of theobromine intake (based on the data collected from the FFQ) was perceived to result in a probably excessive margin of error. Because of this limitation, we decided to focus on chocolate intake, data we could access directly from the FFQ.

In fact, a common limitation in dietary studies is the error in the estimates of nutrients. If the types of certain foods are not specified (i.e. dark chocolate vs milk

chocolate), it is possible to over or underestimate true associations with outcomes (70). In the case of chocolate, there is a wide variety on theobromine concentration among different types of chocolate and the theobromine/caffeine ratio has also been observed to be highly variable: from 1,9 to 10,6 (71). One other limitation was that the FFQ was not designed specifically for the assessment of chocolate and theobromine consumption. Future studies might need to have a more focused design, with a better FFQ that is able to address more precisely and accurately the consumption of cocoa-related products.

Product	Portion size[a]	Concentration (mg per portion)
Chocolate, dark	50 g	378 (237 519)[b]; 221[c]
Chocolate, milk	50 g	95 (65 160)[b]; 94[c]
Cocoa powder	10 g	189 (146 266)[b]; 203[c]; 260[d] 206 (178 240)[e] 263 (219 284)[f]
Tea (regular, bag)	230 ml	3.1 (1.4 4.4)[b, g]
Coffee (filter/percolated)	7.6 g/200 ml	0.3 (0.3 0.3)[b]
Coffee (instant)	1.6 g/200 ml	0.2 (0.1 0.5)[b]
Cola drinks	Can (330 ml)	ND[h]

ND not detected
[a]MAFF (1988)
[b]MAFF (1998); figures recalculated using comments in Annex C of this reference where appro priate
[c]Craig and Nguyen (1984)
[d]Risner (2008)
[e]Bonvehí and Coll (2000)
[f]De Vries et al. (1981)
[g]This is in accord with values of first brew in Hicks et al. (1996).
[h]Dried kola nut contains 0.05 0.10% theobromine (Souci et al. 1981; see also Duke 1992 in Burdock et al. 2009)

Table 5) Theobromine content of various products. From (6).

Our results only pointed for a protective effect of chocolate consumption in subjects with an average weekly consumption of chocolate lower than one standard portion. Despite the relatively high number of participants, is possible that a higher level of participation could have contributed for the better understanding of the dose-effect relation. In fact, this is an inherent problem of observational studies of this kind: for them to reveal strong conclusions, they require a large number of subjects and a long follow-up period.

MMSE is a well-know and established method to address cognitive functioning. However, it might be interesting to explore other ways of assessing this outcome and

the effects of the different methylxanthine compounds. For example, electrophysiological outcomes such as Cortical Silent Period has been proposed to work as surrogate marker of the effect of caffeine in the brain (72). Not only may this improved outcome assessment, it may also improve the selection of participants for future studies, through the use of specific markers that signal individual susceptibility to develop certain pathological processes.

Finally, it is also important to acknowledge the variety of confounding unknown lifestyle factors and the possible presence of substances that alter the effects we have proposed to measure. This topic deserves special attention, and must be addressed with thorough study design. For example, as alerted in other studies, moderate coffee drinking may be related with healthy and social lifestyles that confer part of the protective effects or some medical conditions may be overrepresented and negatively affect cognitive functions (73).

Conclusions and insights for the future

The present work opens a pathway for more deep and focused research on the effects of chocolate consumption and theobromine intake on the prevention of cognitive decline. More and better studies for the short and long term effect of chocolate consumption are needed.

Larger-scale, well designed studies that enlarge the body of knowledge available are to be promoted. The assessment of different populations and of alternative dosage regimens that vary in terms of their flavonoid and methylxanthine content should allow for the expansion of the evidence on this topic, such as the possible synergic interaction between these two groups of compounds. The development of consensual criteria for the definition of outcomes as well as the creation of categories and types of exposure might be useful for the better design of future epidemiological studies.

The findings in the present work should also be confirmed through bigger prospective cohort studies, which could detect a possible dose-dependent relation of the protective effect of chocolate consumption and/or theobromine.

In conclusion, over the last decades our understanding of chocolate effect on both physical and mental health has progressed remarkably. Methylxanthines have shown to play a key role on the neurocognitive aspects regarding the consumption of cocoa-related products. Theobromine, once a widely dismissed compound, has now become a potential player on the subject and has deserved a new found interest on its neuropharmacological effects. With chocolate being generally the main supplier of theobromine to the human diet, there is a clear incentive for the study of alternative way of delivering its active compounds and for the thorough research on its effect on the prevention of cognitive decline and even the development of neurodegenerative diseases such as AD.

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