UNIVERSITAT DE BARCELONA

A New Food Frequency Questionnaire to Assess Cocoa Consumption and its relationship with Health in University Students

Filipa Vicente

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UNIVERSITAT DE BARCELONA

Facultat de Farmàcia

Departament de Fisiologia

A NEW FOOD FREQUENCY QUESTIONNAIRE TO ASSESS COCOA CONSUMPTION AND ITS RELATIONSHIP WITH HEALTH IN UNIVERSITY STUDENTS

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A NEW FOOD FREQUENCY QUESTIONNAIRE TO ASSESS COCOA CONSUMPTION AND ITS RELATIONSHIP WITH HEALTH IN UNIVERSITY STUDENTS

Memòria presentada per **Filipa Vicente** per optar al títol de doctor per la Universitat de Barcelona

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INFORMEN

Que la memoria titulada ***A new food frequency questionnaire to assess cocoa consumption and its relationship with health in University students*** presentada per **FILIPA VICENTE** per optar al Grau de Doctor per la Universitat de Barcelona, ha estat realitzada sota la nostra direcció al Departament de Fisiologia, i sota la supervisió de la **Dra. Paula Pereira** a la Universitat Egas Moniz de Portugal, i considerant-la conclosa, autoritzem la seva presentació per ser jutjada pel tribunal corresponent.

I perquè així consti, signem el present a

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I dedicate this thesis
to my grandparents

João Vicente (1919-2001)
Margarida Baltazar Vicente (1920-)

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LIST OF ABBREVIATIONS

24HDR – 24 Hours Dietary Recall

AA – Adjuvant Arthritis

ACE - Angiotensin Converting Enzyme (ACE)

BMI – Body Mass Index

CIA – Collagen induced arthritis

CD – Cluster of differentiation

C-FFQ – Food Frequency Questionnaire in Spanish (applied in Cataluña)

COX-2 - Ciclo-oxigenase-2

CYP - Cytochrome P450

DC - Dendritic cells

DSS – Dextrane Sodium Sulphate

ENCAT – Enquesta nutricional de la població Catalana (Catalan Nutritional Questionnaire)

EFSA – European Food Safety Authority

EFSA-Q - European Food Safety Authority Questionnaire

ERK - Extracellular Signal-regulated Kinases

FFQ – Food Frequency Questionnaire

GALT - Gut-Associated Lymphoid Tissue

H_2O_2 – Hydrogen peroxide

HC – High consumers

HDL – High Density Lipoprotein

Ig – Immunoglobulin

IL – Interleuquin

i.p. – Intraperitoneal

JNK - c-Jun N-terminal kinase

LC – Low consumers

LDL – Low Density Lipoprotein

LPS – Lipopolysacharide

MAPK - Mitogen-activated Protein Kinase

MC – Medium consumers

MCP-1 – Monocyte Chemoattractant Protein-1

METs – Metabolic Equivalent of Task

MLNs – Mesenteric lymph nodes

NO – Nitric Oxide

NOS - Nitric Oxide Synthase

NF-κB – Nuclear Factor-κB

OVA – Ovalbumin

PAMPs – Molecular patterns associated to pathogens

P-FFQ – Food Frequency Questionnaire in Portuguese

PGE2 - Prostaglandin E2

PHA - Phytohaemagglutinin

PMBC - Peripheral Blood Mononuclear Cell

PPs - Peyer Patches

RA - Rheumatoid Arthritis

ROS - Reactive Oxygen Species

sIgA – Secretory Immunoglobulin A

SOD - Superoxide Dismutase

TAC - Total Antioxidant Capacity

TCR - T-cell receptor

Tc-cells – T-cytotoxic (Tc) cells

Th-cells - T-helper (Th) cells

TLRs – Toll Like Receptors

TNF-α – Tumor Necrosis Factor

ZDF – Zucker Diabetic Fatty

1. INTRODUCTION

1.1. COCOA AND CHOCOLATE

Cocoa, an important raw material for one of the most loved foods –chocolate-, is the product of the tree *Theobroma cacao*. This very special crop can be found in very limited regions of the globe, within latitudes of 10 degrees North to 10 degrees South of the Equator, altitudes below 294 m and it needs very special conditions only possible in the Tropics. The climate in this area is characterized by temperatures between 18 and 30ºC and a yearly precipitation around 1500 mm, crucial for the Cacao tree which also needs the shadow of other trees like banana and cassava in order to fulfil proper growth and development. One cacao tree produces 20 to 50 cabossides, as the fruits are called, per year and this pod consists of five loculli structures with 5 to 12 seeds within a soft and acid pulp. These seeds are the real valuable raw material, used for commerce, the cocoa beans. It is estimated that 10 kg cabossides are needed to produce 1 kg of cocoa [1].

The cocoa beans are removed from the pods and are covered with specific tree leaves (e.g. banana) in order to ferment. During fermentation, the sugars present in the seeds are converted into important acids responsible for flavour, colour and bitter taste. The last stage the seeds suffer consists of a drying process, basked at the sun or dry with hot air, which reduces half their weight and preserves them to be shipped and transported worldwide (**Figure 1**).

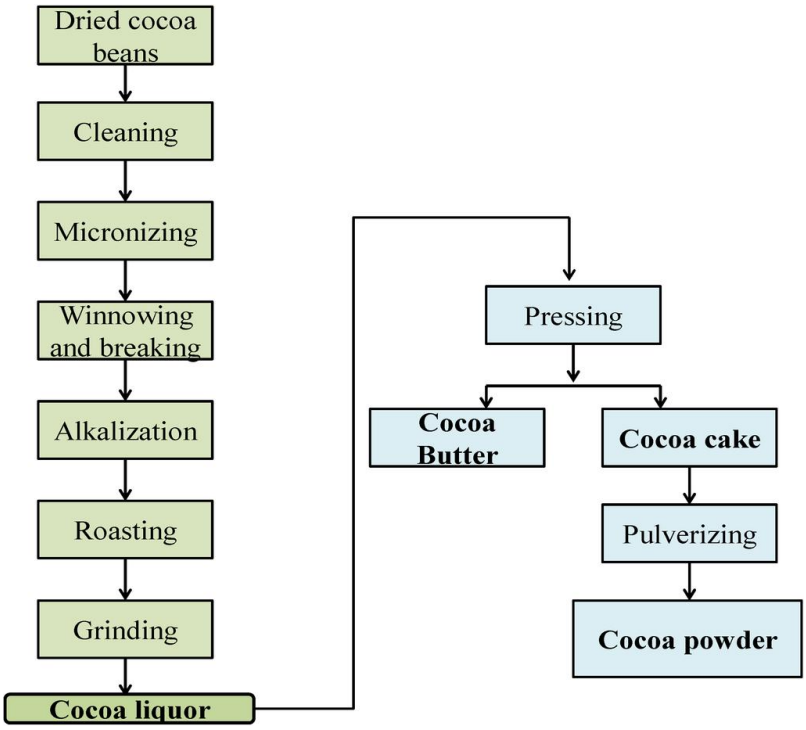

Figure 1: Manufacturing processes of cocoa beans [2].

The first step in the processing chain of the dried cocoa beans is the raw-bean cleaning that removes fibre, stones, grit, metal, other dross and immature beans [3]. A winnowing

process completes the raw material cleaning and initial processing. In some dark chocolate, cocoa drinks and cocoa powder, this step is followed by an alkalization which can affects flavour although is not indispensable.

The roasting procedure is essential for flavour development, time can vary from 5 to 120 min and temperature range is within 120 and 150ºC. These variables are different among chocolate types [4].

Once dried, fermented and roasted, the cocoa beans can be processed to produce specific sub products such as cocoa butter that results directly when the beans are grind. Cocoa butter is the only cocoa ingredient in white chocolate, and it is incorporated in several proportions in other chocolates and it is a by-product with specific usages in cosmetics [3].

In fact, chocolate and chocolate-based products production is probably the main destination of cocoa beans worldwide. The cocoa content in these food products can be quite different which plenty justifies their complex nutritional composition. However, along history, cocoa beans have been used in a most pure form not as a food product but mainly as a medicine explaining the potential protective role now attributed to an ingredient known since the Mayas.

1.1.1. HISTORICAL FRAMEWORK ABOUT THE MEDICINAL PROPERTIES OF COCOA

In spite most relevant documents refer to the history of cocoa (**Table 1**), the text commonly mentions chocolate a term derived from *xocolatl*, a thick and consistent drink made from the cocoa beans which was added with sugar and in some cases maize [5,6]. Invigorating and strengthening effects have been attributed to this beverage justifying the grown reputation among the European voyagers arriving the New World in the XV century.

The entry of cocoa in Europe was not free of controversy and its use was firstly limited to medicinal reasons. In fact, it is possible to describe the use of cocoa as a medicine from two points of view. In one hand, it was possible to prove the potential therapeutic usages described in archaeological evidence like paintings and rich texts found from Mesoamerican inhabitants, such as the Badianus manuscript. On the other hand, the Hippocratic-Galenic Medicine theory identified the effect of the different forms of cocoa (hot/cold) in specific symptom relief [6].

Although apparently separated in context, both theories have defended that the consumption of cocoa beans which was prepared mainly as a drink would have benefits in cardiovascular, digestive, respiratory, circulatory and even urinary system making this ingredient one of the very few natural products with the potential to treat several disorders [7]. This have probably justified that Carl Linnaeus named the chocolate tree *Theobroma cacao* in 1753, which means “the food of the gods” [3,5–7].

However, the medicinal use of cocoa suffered a major attack with the massive production of chocolate in 1847, when Joseph Fry & sons made the first solid bar. The addition of

sugar, milk, cocoa butter and other ingredients changed the consumption of cocoa and quite reduced the importance given to the chocolate consumption benefits [6].

The interest about the use of cocoa and/or chocolate diminished from the 19th Century although Ancel Keys recognized its moral improvement and energy boost effects and included it in soldiers rations [6,7].

Table 1: Summary of the main events in the history of chocolate and cocoa

300 – 900 A.D.	Mayans prepared a hot drink with milk and corn (*xocolatl)*
1502	Columbus discovers the New America Cocoa beans were being used as currency
1519-1560	Díaz del Castillo describes a drink made of the cocoa plant which King Montezuma in Mexico was using to give him energy
1552	It is created the Badianus Manuscript which describes the Mexican medicinal use of cacao
1577	Bernardino de Sahagún compiles information in the Florentine Codex explaining the different beverages made with cocoa, the dosages and effects
	Hernández classified the chocolate drink as cold and humid which would make it adequate to treat "hot diseases" such as liver abnormalities and fever
1624	Santiago de Valverde proposed in fact that cocoa was more adequate for cold and wet diseases and when people were healthy it was necessary to balance its hot nature
1662	Henry Stubb proposed a drink made with cocoa paste, mixed with sugar and melt in water to purify the blood and re-establishing the balance of body fluids
1753	Carl Linnaeus named the chocolate tree *Theobroma cacao* which means "the food of the gods"
1796	Antonio Lavedan wrote "Tratado de los Usos, Abusos, Propiedades y Virtudes del Tabaco, Café, Té y Chocolate" where cocoa was proposed as a drink which should be drunk in the morning to reinforce and strengthening the body.
1847	Joseph Fry & sons discovered a way to make a solid chocolate bar and changed the history, reducing the medicinal interesting and promoting for good the food use of cocoa

Only more recently, science has raised the interest on the health benefits of the so-called dark chocolate, rich in cocoa and with a lower sugar and milk content. Several data have demonstrated that chocolate can have diverse protective and preventive effects in human health in addition to its nutritive role but specifically in what refers to high cocoa content chocolate products [8,9].

1.1.2. THE NUTRITIVE VALUE OF COCOA

The common term cocoa refers to the non-fat component of the finely ground cocoa beans which is used in chocolate making but also as a cocoa powder product. This food product is an important source of fibre (26-40%) but also supplies 20% protein, 15% carbohydrates and 10-12% fat in addition to several vitamins (A, E, B and folic acid) as

well as minerals such as magnesium, phosphorus and potassium [10,11]. The overall nutritional composition of cocoa powder is presented in the **Table 2**.

Other minerals in cocoa powder deserve further attention. Some data have proved that cocoa and its products can contribute significantly for daily copper intake, which is a vital mineral for metalloenzymes like superoxide dismutase. Additionally it also supplies selenium, a crucial cofactor in the formation in glutathione peroxidases, iodothyronine deiodinases and several selenoproteins involved in antioxidant and detoxifying processes [12]. In addition to its macro and micronutrient composition, cocoa has been known because of its richness in phytochemicals namely polyphenols from flavonoid and non-flavonoid nature [10,13].

Table 2: Nutritional information of cocoa powder per 100 g [14]

Nutrient	Content (in 100 g)
Energy (kcal)	200
Protein (g)	21
Carbohydrate (g)	16
Sugars (g)	3
Lipids (g)	10.4
Fibre (g)	32.7
Potassium (g)	4.2
Calcium (mg)	150
Phosphorus (mg)	700
Magnesium (mg)	550
Zinc (mg)	7
Copper (mg)	4

Cocoa is also an important source of methylxanthines, mainly theobromine [15]. It represents 4% of the non-fat portion of cocoa. Similarly to caffeine, present in coffee, it is a adenosine receptor antagonist which can exert psychoactive phenomena [16,17] but differences have been found in the effects of theobromine and potential health benefits have been attributed to this methylxanthine. Experimental and some pilot studies data have shown antitumoral [18], anti-inflammatory [19] and cardiovascular protection [20] effects without the secondary effects of caffeine in the central nervous system. Recent research have also proposed a potential beneficial effect from cocoa theobromine [21,22] which adds up to its role as a phytochemical compound.

1.1.2.1. COCOA POLYPHENOL COMPOUNDS

Polyphenols are natural occurring compounds in vegetal food products such as fruits, vegetables, cereals and also in specific beverages such as wine, tea and coffee [23–25]. There are more than 8000 polyphenolic compounds identified and they can be classified into four classes according to the number of phenol rings and the structural elements that bind these rings. These are: phenolic acids, stilbenes, lignans and flavonoids, being this last one the most studied group [26].

Flavonoids consist of a basic structure with two aromatic rings bound by three carbon atoms forming an oxygenated heterocycle. Within the 4000 varieties of flavonoid compounds, six subclasses can be considered: flavonols, flavones, flavonones, flavanols, anthocyanins and isoflavones as presented in **Figure 2** [26,27].

Flavonoid compounds in cocoa include several subgroups but especially flavanols and anthocyanidins [28,29]. The main phytochemical compounds found in the cocoa are:

- Catechins which constitute around 37%
- Anthocyanidins which accounts for approximately 4%
- Proanthocyanidins which achieve 58% [8,28]

Some data have suggested that polyphenols constitute 12-18% of the whole beans dry weight [30] and some authors have proposed that cocoa is one of the most polyphenol rich foods along with tea and wine [31,32].

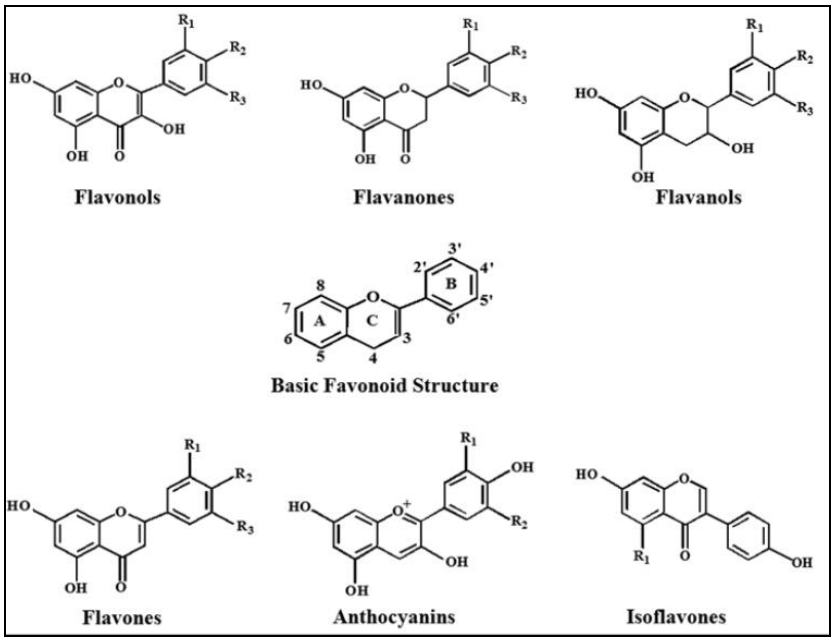

Figure 2: Flavonoid family compounds structure [26]

Catechins are also important compounds in tea, especially green tea, to which several health benefits had been attributed [33,34]. Although there is not an extensive research on the potential protective role of cocoa catechins, it is known that these compounds have multiple actions and can exert benefits such as antimicrobial activity [35–38], anti-inflammatory effects [39], antimutagenic and anticarcinogenic [40,41] actions as well as anticataract activities [42].

Cocoa is also a good source of anthocyanins which are natural pigments present in plant origin food products and responsible for natural bright colours such as blue, purple, red and orange in many fruits and vegetables [43] Several benefits in human health have been attributed to these colourful flavonoids, such as in the prevention of cardiovascular

[44,45], neurodegenerative [44,46] and metabolic [47,48], diseases as well as in cancer [49–52].

Additionally two groups of flavonol-based oligomers can be considered: the type B proanthocyanidins that are monomeric and oligomeric forms of catechin and epicatechin and procyanidins consisting only of epicatechin units. This group of compounds is quite diverse and the structural differences are based in the number of monomer of units involved as presented in **Figure 3** [53,54].

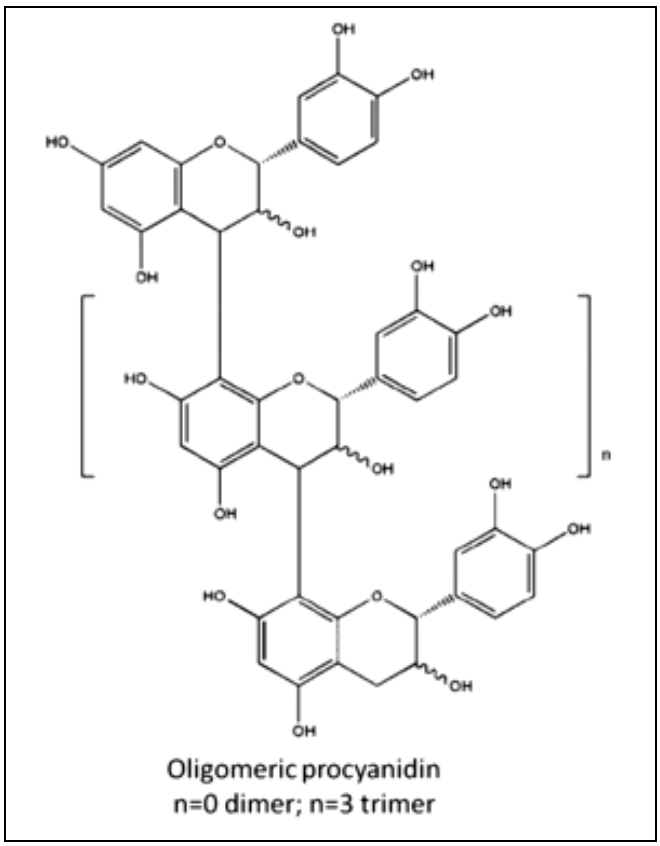

Figure 3: Proanthocyanidins structure [53]

However, it is known that processing affects cocoa and cocoa products composition, and therefore some differences can be found between cocoa powder and chocolate, especially when compared to the cocoa beans (**Table 3**).

The differences in nutritional composition among cocoa beans, powder, beverage and chocolate are due to the different cocoa content in several chocolate/cocoa products and this logically interferes with the potential protective benefits from their consumption. Moreover, the composition of cocoa and chocolate derived products can be significantly different due to the addition of sugar, milk and also cocoa butter. **Table 4** compares the nutritional composition of three types of chocolate bars classified according to its cocoa content and from cocoa powder (unsweetened).

Table 3: Polyphenol content in cocoa products and cocoa beans, distinction among several polyphenol compounds according to food product. Adapted from Kim *et al.* [28]

	Cocoa beans (mg/g)	Cocoa powder (mg/g)	Chocolate (mg/g)	Cocoa beverage (mg/100ml)
Procyanidin	n/a	18.35-27.75	1.081-85.36	106-111
(-)-Epicatechin	1.24-16.52	0.116-6.778	0.023-2.270	9.2-59
Catechins	0.05 -0.46	0.081-0.896	0.006-0.992	10.7-1.5
Epicatechin gallate	n/a	n/a	0.005-0.006	n/a
Catechin gallate	n/a	n/a	0.077-0.094	n/a
Epigallocatechin	n/a	n/a	0.032-0.119	n/a
Gallocatechin	n/a	n/a	0.164-0.231	n/a
Epigallocatechin gallate	n/a	n/a	0.437-0.462	n/a
Theobromine	11.1-24.0	15.2-25.0	1.20-6.67	128.9-222
Caffeine	2.0-2.9	0.907-2.5	0.170-0.778	8.6-14

In fact, seen as a snack or candy, chocolate consumption has been seen as a risk factor for weight gain because of the sugar and fat added [55,56]. A few studies in the first decade of XXI century started to explore the benefits from cocoa-rich chocolate, named dark chocolate, especially in what refers to cardiovascular health [23,24]

The most recent research confirms that cocoa polyphenols exert multiple benefits in human health as described in the following sections and dark chocolate and cocoa powder can be a valuable source.

Table 4: Nutritional composition of chocolate bars with different cocoa contents and cocoa powder [14].

	White chocolate	Milk chocolate	Dark chocolate[1]	Cocoa powder
Energy value (kcal)	539	535	547	228
Protein (g)	6	8	5	20
Fat (g)	32	30	32	14
Carbohydrates (g)	59	59	61	58
Sugars	59	51	46	2
Dietary fibre (g)	0	3	7	33

[1] No cocoa percentage reference

1.2. HEALTHY BENEFITS OF COCOA

Several reviews have addressed to the multiple benefits of cocoa polyphenols in human health. Published data point out that these compounds can exert several actions that can justify the potential protective effect in multiple pathologies. Experimental and clinical

studies confirm that several polyphenols present in cocoa and dark chocolate show antioxidant, cardioprotective, anti-inflammatory, immunomodulator, anticarcinogenic and neuroprotective actions in addition to a potential role in carbohydrate and lipoprotein metabolism regulation [8,28,59].

1.2.1. ANTIOXIDANTS EFFECTS OF COCOA

Many of the beneficial effects of cocoa are associated with its antioxidant effects due to its content in polyphenols [60–62]. Procyanidins, but also epicatechin and catechin, play an important antioxidant role [10]. It has been estimated that a serving of dark chocolate (40 g) provides an antioxidant capacity of 9100 Trolox equivalents [63] and can provide more phenolic antioxidants than beverages and fruits such as tea and blueberries, traditionally considered high in antioxidants [31,64]. Moreover, a cup-serving of cocoa possesses a total oxyradical scavenging capacity higher than that of jasmine and black tea [65]. One serving of dark chocolate is thought to impart a greater antioxidant capacity than the average amount of antioxidants consumed daily in the United States [63]. However, some studies reported a decrease of this activity when added to milk [66].

The antioxidant properties of cocoa can diminish during manufacturing processes. In this sense, flavanols levels in unfermented raw cacao beans are dramatically decreased during fermentation, roasting and alkalization [67] and new alternatives have been studied. Recently, cacao bean puffing has been described as an alternative to roasting to keep high antioxidant capacity [68].

The antioxidant effects of cocoa flavanols are attributed to their capacity to neutralize free radicals [69]. Epicatechin and catechin neutralize peroxyl, peroxynitrite, superoxide, and 1,1 diphenyl-2-picryl-hydrazyl (DPPH) [70]. Cocoa procyanidins and cocoa extract also scavenge radicals [71,72], such as peroxynitrites, showing an activity proportional to the number of monomeric units they contain [73].

Cocoa flavonoids are also able to chelate metals that enhance highly aggressive reactive oxygen species (ROS). In particular, epicatechin and catechin are very effective in chelating iron, and quercetin may also contribute to radical scavenging and metal ion chelation despite the fact that it is present in smaller proportions [74].

In addition, cocoa may exert its antioxidant activity by inhibiting enzymes responsible for ROS production (xanthine oxidase) [72], and upregulating or protecting antioxidant defence [75]. Epicatechin can regenerate the antioxidant α-tocopherol from its corresponding radical [76]. Moreover, quercetin at high doses increases glutathione concentration and superoxide dismutase (SOD) gene expression [77]. On the other hand, methylxanthines can also contribute to its antioxidant properties [78].

On the other hand, it has also been reported that ingested flavonoids produce no direct antioxidant effects *in vivo*, but modify protein kinases mediating signal transmission, thus inducing antioxidant gene expression or inhibiting oxidant gene expression [79]. However, despite these antioxidant characteristics, flavonoids in excess or in the presence of redox-active metals can become pro-oxidants [80,81].

The antioxidants properties of cocoa have been demonstrated by *in vitro* and *in vivo* experiments.

1.2.1.1. ANTIOXIDANT *IN VITRO* ACTIVITY

Numerous *in vitro* studies have demonstrated the antioxidant capacity of cocoa flavonoids and their metabolites. The isolated compounds epicatechin, catechin and procyanidin B_2 reduce oxidant-induced erythrocyte haemolysis [82,83], and also the calcium-dependent oxidation of a lymphocyte cell line [84]. Moreover, epicatechin and a cocoa extract reduce oxidative stress and increase the survival and enhances the activity of antioxidant enzymes in HepG2 cells under stress [85–87]. Epicatechin also protects Ins-1E pancreatic β cells against the induction of reactive oxygen species, carbonyl groups, p-JNK expression and cell death [88,89].

Cocoa procyanidins protect intestinal Caco-2 cell monolayers from the loss of integrity induced by a lipophilic oxidant [90]. In addition, cocoa polyphenolic extract inhibits superoxide anion formation and xanthine oxidase activity in stimulated myelocytic leukaemia HL-60 cells [72]. It has also been shown that cocoa procyanidins decrease ROS accumulation in rat pheochromocytoma cells [91], and cocoa extract and epicatechin reduce ROS production in a neuronal cell line under oxidative stress, thereby showing then a neuroprotective action [92].

1.2.1.2. PRECLINICAL STUDIES

Some studies have evidenced the antioxidant effects of cocoa *in vivo*. Thus, after hydrogen peroxide induced oxidative damage, a flavonoid-enriched cocoa powder, due to its high polyphenol content but also other cocoa components, exerts resistance to oxidative stress in the yeast *Saccharomyces cerevisae* and the worm *Caenorhabditis elegans* [93].

In mammals, cocoa intake increases the total antioxidant capacity (TAC) and decreases lipid oxidation products in plasma and tissues [51,94–96]. The plasma antioxidant effect reaches a maximum 2-6 h after cocoa intake [51]. Studies in rats and pigs have shown that a cocoa-enriched diet increases the antioxidant capacity of cells from thymus > spleen > liver [62] that can be attributed to different levels of flavonoid accumulation [97,98]. Cocoa intake promotes antioxidant defence by boosting catalase, SOD and glutathione peroxidise activities [96,99]. Furthermore, the intake of polyphenolic-rich fractions derived from cocoa powder increases the resistance of LDL to oxidation in rabbits [100].

Cocoa also improves antioxidant defences in oxidative stress situations. Thus, a long-term diet supplemented with cocoa fibre reduces lipid peroxidation in hypercholesterolemic rats [101], and cocoa supplementation reduces the oxidative stress associated with a chronic inflammatory pathology such as adjuvant arthritis [102]. In addition, in a model of type 2 diabetes (Zucker diabetic fatty rats), cocoa diet reduces ROS levels and carbonyl

content in the liver, improves the reduced SOD and suppresses total and phosphorylated nuclear factor erythroid-derived 2-like 2 (Nrf2), as well as p65-nuclear factor-κB enhanced levels in Zucker diabetic fatty rats (ZDF) rats [103].

1.2.1.3. CLINICAL STUDIES

The antioxidant power of cocoa and chocolate has also been assessed in humans by dietary intervention trials [104]. In the plasma of healthy volunteers consuming dark chocolate, increased TAC and decreased presence of lipid oxidation products have been reported [105]. Similar results were found in volunteers who consumed procyanidin-rich chocolate [51]. In these studies, TAC level was the highest 2 h after chocolate ingestion and returned to basal values 6 h after cocoa intake, probably because of the short plasma half-life of flavonoids and their uptake in cells.

A crossover trial on 12 healthy volunteers consuming dark chocolate, dark chocolate with full-fat milk, or milk chocolate showed that 1 h after the ingestion of dark chocolate plasma TAC values increased by 20%, whereas no changes were reported after ingestion of dark chocolate with milk or milk chocolate [106]. Similarly, it has been demonstrated the antioxidant properties of cocoa on prooxidant situations. Indeed, the consumption, over two weeks, of 105 g of chocolate containing about 170 mg of flavanols counteracts oxidative stress in soccer players [107].

1.2.2. EFFECTS ON CARBOHYDRATES AND LIPOPROTEIN METABOLISM

It has been suggested that different polyphenols from cocoa have multiple actions in carbohydrate metabolism [108]. These actions include mostly an improvement in glycemic response, insulin secretion and sensitivity contributing to a glucose homeostasis. On the other hand, cocoa also interact with cholesterol and lipid metabolism.

In vitro assays demonstrate the cocoa properties inhibiting α-amylase and α-glucosidase [109]. Furthermore, preclinical studies in ZDF rats demonstrate that cocoa feeding during the prediabetic state attenuates hyperglycaemia, reduces insulin resistant, and increases β cell mass and function, whereas, at the molecular level, cocoa-rich diet prevents β cell apoptosis by increasing the levels of Bcl-xL and decreasing Bax levels and caspase-3 activity [110].

On the other hand, *in vitro* studies have shown that cocoa phenolics increase apolipoprotein A1 and decreases apoB which is possibly due to the upregulation of the mature form of sterol regulatory element binding proteins (SREBPs) and to the increased activity of Low Density Lipoprotein (LDL) receptor [111]. Some results from *in vivo* studies have shown that cocoa consumption protects against LDL oxidation [112] and increases High Density Lipoprotein (HDL) levels [113–117]. Thus, the consumption of cocoa in hypercholesterolemic rabbits is able to slow down the oxidation of LDL and to reduce the area of atherosclerotic lesions in the aorta [100]. Furthermore, lower cholesterol,

triglycerides and LDL levels have been described in a model atherosclerosis in hamsters that were administered to cocoa for 10 weeks [64]. In addition, rats fed a fat diet supplemented with cocoa fibre for 3 weeks underwent a hypocholesterolemic effect and a reduction in lipid peroxidation [101,118]. Likewise, in rats fed a hypercholesterolemic diet, cocoa intake for 4 weeks reduces the concentration of plasma cholesterol and increases the cholesterol and bile acids in faeces. However, the effect was not so evident when the diet contained a mixture of epicatechin and catechin demonstrating the involvement of other substances in cocoa [119].

Some clinical trials demonstrate that individuals taking flavanol-rich dark chocolate have shown less insulin resistance and improved insulin sensitivity [108,116,120]. In a randomized clinical control trial in type 2 diabetic patients, cocoa intake (10 g, twice per day and for 6 weeks) lowered blood cholesterol, triglyceride, and LDL-cholesterol, together with TNF-α, hs-CRP, IL-6 [121]. In addition, some data show that chocolate consumption can be associated with lower incidence or risk of diabetes [122,123].

In addition, studies in healthy volunteers indicate that the consumption of cocoa reduces the oxidation of LDL [90] and increases HDL cholesterol levels in plasma [125–127]. A decrease in triglycerides and plasma biomarkers of lipid peroxidation have been found in healthy individuals who had consumed chocolate or cocoa drinks [51,105]. On the contrary, other studies have indicated that the regular consumption of cocoa in healthy individuals does not affect the concentration of these biomarkers [125,128] and the oxidation of LDL is not modified by a daily consumption of 48 g chocolate or 18 g cocoa for 6 weeks in individuals with heart disease [129].

It is important to point out that in addition to polyphenols, cocoa is also a relevant source of dietary fibre [95] which can also be crucial in preventing diabetes and dyslipidemia due to its digestive and metabolic effects which are within the multiple benefits proven in human health [130,131]. In this context, the consumption of two 15 g servings per day of a product enriched cocoa fibre reduced lipid peroxidation and glucose levels in individuals with moderate hypercholesterolemia [132].

1.2.3. COCOA AND CARDIOVASCULAR PROTECTION

Firstly, epidemiological studies demonstrated that Kuna Indians, a native population living in San Blas Islands off the coast of Panama who daily consume enormous amounts of cocoa, are protected against the age-related increase in blood pressure [133–135]. Later, several studies demonstrated the protective effect of cocoa and derivatives and cardiovascular diseases [13,136–138].

The benefit from cocoa polyphenols on cardiovascular health can be exerted through different mechanisms known to be disease risk factors. Cocoa is able to reduce blood pressure [139–142] and modify platelet function [143–145]. Moreover, as stated before, cocoa influence on total cholesterol, HDL, LDL, fasting glucose, fasting insulin and insulin resistance that may confer it cardioprotective properties [115,124,126,146]. Cocoa polyphenols has also influence on other cardiovascular disease risk factors like insulin

resistance [115,116,120,147] and there is emerging evidence on the beneficial role of polyphenol-rich dark chocolate in reducing body weight and body fat [148]. Additionally the anti-inflammatory and antioxidant properties described along this section are also relevant as protective actions.

In vitro and *in vivo* studies allowed identifying cell and molecular mechanisms by means of them cocoa acts on cardiovascular system.

1.2.3.1. *IN VITRO* STUDIES

In vitro studies have reported the vasodilator action of cocoa extracts or flavonoids. For example, cocoa is able to inhibit the activity of angiotensin converting enzyme (ACE) [149,150], an enzyme involved in the formation of the vasoconstrictor angiotensin II. It has been reported that the addition of a cocoa extract (6.25 to 100 mg/mL) in endothelial cells from human umbilical vein decreases activity of ACE in a dose-dependent manner [151].

A similar study has demonstrated the inhibitory effects of epicatechin in the gene expression and activity of arginase 2, an enzyme involved in the synthesis of Nitric Oxide (NO) in the vascular wall [152]. These results correlate to a study carried out in aorta isolated from rabbits where the polymeric procyanidins of cocoa are able to induce endothelial relaxation through the activation of Nitric Oxide Synthase (NOS) [153]. In addition, as stated before, cocoa flavonoids are able to prevent LDL oxidation *in vitro*, thus decreasing the possibility to produce atherosclerosis' plaques [154].

1.2.3.2. PRECLINICAL STUDIES

It has also evaluated the effect of cocoa on blood pressure in animal models. It has been showed the protective effect of epicatechin in rat models of spontaneous hypertension [155] and in rats treated with a potent inhibitor of NOS [156]. A nutritional supplement of epicatechin allowed to maintain blood pressure in the optimal rank and also prevented the increase of oxidative stress biomarkers, besides recovering the levels of NO [155,156]. Similarly, a single oral administration of flavonoid-enriched cocoa extract reduces blood pressure in a spontaneously hypertensive rat model [157].

It has been also showed that the prolonged intake of cocoa soluble fibre in the drinking water is able to protect against hypertension in a rat model of spontaneous hypertension [158]. However, after finishing the diet, animals showed an increase in blood pressure [158]. Ried *et al*. [139] have discarded that cocoa theobromine played a role for this decrease in blood pressure despite its known activity as a vasodilator.

1.2.3.3. CLINICAL STUDIES

Clinical trials in hypertensive patients have shown that the consumption of high-polyphenol dark chocolate decreases blood pressure [115,116,120,139,140], and improve

vascular functions [159]. There are numerous studies about the impact of cocoa and chocolate consumption in young or adult people, overweight or with hypercholesterolemia, and with pre-hypertension or hypertension, that demonstrate the anti-hypertensive effect of cocoa and chocolate [139,160]. Among the possible mechanisms induced by cocoa intake on blood pressure, there is the cocoa ability to increase the production of NO [128] and prostacyclin, an inhibitor of platelet aggregation [161].

Subjects with untreated mild hypertension that had an intake 1052 mg of cocoa flavanols per day for 6 weeks reduced their blood pressure [142]. Similar results were reported in overweight adults who consumed 74 g of dark chocolate [162].

On the other hand, it has been shown an improvement in endothelial and platelet functions after the intake of dark chocolate (with 70% cocoa) in healthy individuals [143,163], in overweight subjects [162] and in patients with transplanted heart [143]. Besides an improvement in the endothelial function, healthy volunteers who daily consumed cocoa rich in flavanols (821 mg flavanols per day) for 5 days showed a peripheral vasodilatation [164].

On the other hand, it has been described that other components of cocoa, such as its fibre, can also exert a protective effect against cardiovascular diseases. In this context, the consumption of two 15 g servings per day of a product enriched cocoa fibre reduced blood pressure [132].

Due to preclinical and clinical studies support the beneficial effects of flavonoids present in cocoa at cardiovascular level, the European Food Safety Authority (EFSA) developed in 2010 and 2012 health claims about cocoa flavonoids in relation to the cardiovascular system [165,166].

1.2.4. THE INFLUENCE OF COCOA IN CANCER RISK

Cancer has been recognized as a multifactorial and etiological complex disease with multiple risk factors. Due to its nutritive richness, and taking into account the fibre and polyphenol content, cocoa can exert multiple protective actions in cancer prevention.

The anti-carcinogenic mechanisms involved by cocoa polyphenols are related to their influence in reducing the carcinogenesis process which, according to experimental studies, has been proven to be possibly related to several cellular effects that include mainly the modulation of redox status and regulation signal transduction pathways important in cell cycle: proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis [167]. The formation of ROS, intermediate reactive species of oxygen and nitrogen can damage DNA and/or interfere with its repair and lead to mutations that prevent the control of cell replication. Thus, given its antioxidant flavonoids, it is not surprising that cocoa intake is also associated with an improvement in carcinogenic processes.

1.2.4.1. *IN VITRO* STUDIES

This kind of studies has demonstrated the antiproliferative, proapoptotic, and chemopreventive ability of coca extracts, along with preventive action in the formation of ROS [167–171].

In a model of DNA cleavage by mitomycin C, a cocoa extract is capable of inhibiting DNA damage [169]. Moreover, it has been shown that cocoa flavonoids added to rat liver epithelial cells inhibit several processes induced by H_2O_2 associated with carcinogenesis [171]. Particularly, cocoa inhibits the formation gap junction intercellular communication, the phosphorylation and internalization of the connexin 43, the ROS accumulation and the activation of extracellular signal-regulated kinases (ERK) [171]. The antiproliferative effects of cocoa procyanidins have also been observed by blocking the phase G2/M of cell cycle, and by decreasing the activity of ornithine and descarboxiled S-adenosylmethionine, key enzymes in polyamine biosynthesis [168].

Regarding antimutagenic and chemopreventive effects, Ohno et al. [172] have found that a cocoa extract inhibits a chemical mutagen, benzo[α]pyrene, that causes DNA damage when it is activated by cytochrome P450 (CYP). In tumour cells of breast cancer, a cocoa extract causes CYP overexpression activating the benzo[α]pyrene metabolism which promotes the metabolism and the conversion of estrogen metabolites towards not genotoxic forms. This fact causes a decrease in the estrogen levels present in breast tumours and contributes to the cytotoxic activity of tamoxifen, an antiestrogen drug with cytotoxic effects [139].

1.2.4.2. *IN VIVO* STUDIES

Animal studies have shown favourable influences of cocoa phenolic components on prostate [174], liver [85] and colon cancer [168] mainly through an effect in slowing down or preventing the initiation-progression of cancer cells.

The effects of a cocoa liquor extract have been established on the enzyme activity of tumour markers such as alkaline phosphatase, the γ-glutamyl transpeptidase, the glutathione-S-transferase and glutathione reductase, in both plasma and liver obtained from rats with hepatocellular carcinoma. The results show that the daily administration of the cocoa liquor extract for 2 weeks reduces the activity of tumour markers in both compartments, demonstrating thus their potential in reducing the hepatocarcinogenesis severity [175].

It has also been evaluated the effect of an extract of cocoa powder (Acticoa) in the incidence of prostate cancer in Wistar rats. After causing cancer, the animals were fed with Acticoa (24 or 48 mg/kg). After 9 months of this diet, the group with the lowest dose showed a lower incidence of prostate tumours and a higher survival rate, becoming similar to that of healthy group [174]. Similarly, it has also described the protective effect of cocoa liquor on lung cancer in a model of multiple carcinogenesis in rats F344 and on initial stages of a pancreatic carcinogenesis model in Sprague-Dawley rats [176,177].

On the other hand, a cocoa-enriched diet exerts antiproliferative effects in Wistar rats with colon cancer through reducing the levels of ERK, protein kinase B and cyclin D, molecules involved in signalling processes during the cell division and cell survival by inhibiting apoptotic processes. In addition, the diet showed proapoptotic effects because reduced the levels of Bcl-XL and increased levels of Bax and caspase-3 activity [178].

Regarding human studies concerning the effect of cocoa in cancer, there are few data supporting cocoa as a potential protective food. The disease complexity and the multiple food components that can influence its risk deserve further analyses. Observational studies have reported a lower incidence of different types of cancer due to the ingestion of a diet rich in flavonoids, including those from cocoa [179]. In fact, the native Kuna Indians also showed a lower frequency of cancer death than in the population living in Panama, which could be explained, at least in part, by the high cocoa intake of Indians [180].

1.2.5. COCOA AND INFLAMMATION

Inflammation consists of a complex biological response of vascular tissues to harmful stimuli (i.e. infection, tissue injury, etc) with the objective of removing the injurious agent as well as initiate the reparation process of the tissue [181]. The first stage of inflammation includes sensing the inducers of the process, which may be exogenous, either microbial (molecular patterns associated with pathogens or PAMPs) or non-microbial (allergens, irritants or toxic compounds), or endogenous such as ROS production. All these inducers are detected by toll–like receptors (TLR) and NOD-like receptors (NLR); some of the last ones forming cytoplasmic complexes called inflammasomes [182,183] which are present in tissue resident cells from the innate immune system. Secondly, this recognition leads to a coordinated production of a variety of inflammatory mediators released from activated cells (mastocytes, neutrophils, macrophages…) or derived from inactive plasmatic proteins. Among these mediators there are chemokines, complement fragments, cytokines, vasoactive compounds, lipid mediators and inflammatory caspases and proteolytic enzymes, among others [181]. In the third stage, the released compounds induce vasodilatation and the selective extravasation of plasma proteins and recruitment of leukocytes to the site of the injury. Among these cells there are, principally, neutrophils, which become activated and produce ROS and reactive nitrogen species (RNS) to fight against the inducer pathogen. These compounds, however, may also affect host tissue integrity by causing damage to cellular components such as polyunsaturated acids, proteins, carbohydrates and nucleic acids in the surrounding tissue [184]. In the following stage, recruited macrophages and also tissue-resident cells and cells involved in a specific adaptive response (B and T lymphocytes) eliminate the pathogen completely [185]. The last stages of the inflammatory process consist of its resolution and reparation, phases in which an important change in the ratio between the lipid mediators prostaglandins and lipotoxins takes place [181]. In summary, during the inflammatory process there is a release of different products with a myriad of physiological effects.

The anti-inflammatory potential of cocoa is reported in isolated cells and there are also few preclinical and clinical studies trying to explore the prevention of clinical inflammation [186].

1.2.5.1. *IN VITRO* EFFECTS

The effects of cocoa on inflammatory response are mainly described *in vitro*. Most studies use cocoa flavonoids, such as monomers (epicatechin, catechin) or polymers (procyanidins), and are focused on the secretion of inflammatory mediators by macrophages and other leukocytes. These studies produce controversial results (**Table 5**).

Table 5: *In vitro* studies performed with cocoa on inflammatory cytokine and chemokine secretion

Cytokine/chemokine	effect	Target cells (stimulus)	Treatment	Reference
IL-1β	↑	PBMC (LPS)	monomer-pentamer procyanidins hexamer-decamer procyanidins	[187]
TNFα	↑	PBMC (PHA)	tetramer-octamer procyanidins	[188]
	↓	PBMC (PHA)	dimer procyanidins	
	↑	PBMC (LPS)	catechin epicatechin	[189]
	=	PBMC (LPS)	dimer procyanidins	[189]
	↑	PBMC (LPS)	monomer-pentamer procyanidins hexamer-decamer procyanidins	[187]
	↓	NR8383 -rat macrophage line-(LPS)	cocoa extract epicatechin isoquercitrin	[190]
IL-6	↑	PBMC (LPS)	monomer-pentamer procyanidins	[187]
	=	PBMC (LPS)	hexamer-decamer procyanidins	[187]
IFN-γ	↓	PBMC (PHA)	cocoa extract	[191]
MCP-1	↓	NR8383 -rat macrophage line- (LPS)	cocoa extract epicatechin isoquercitrin	[190]

IL-1β is a cytokine produced by inflammatory macrophages. Cocoa flavonols increase the secretion of this cytokine by Lipopolysacharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) when added to culture both as monomer-pentamer units and as longer chain fractions [187]. Similar results are found when quantifying the TNF-α and IL-6 secretion [187–189]. However, an entire cocoa flavonoid-enriched extract and also the monomers epicatechin and isoquercitrin produce a decrease in TNF-α production by macrophages under LPS-stimulus [190]. In these conditions there is also a decrease in the secretion of the chemokine Monocyte Chemoattractant Protein (MCP)-1 [190]. Similarly, epicatechin in stimulated whole blood cells culture suppresses the production of IL-6 and IL-8 [192].

The *in vitro* anti-inflammatory effect of cocoa depends on the experimental design. Thus, the influence of epicatechin on decreasing TNF-α secretion is milder when added 14 h before stimulation [190], which is attributed to monomer degradation. However, the effect of cocoa is much stronger when it is added 14 h prior to LPS-stimulation [190], which could indicate that cocoa needs this period to hydrolyze procyanidins to monomeric and dimeric compounds to be taken up by macrophages.

The effects of cocoa on inflammatory cells are also described by the cocoa ability to inhibit the surface expression of Cluster of Differentiation (CD)11b, a leukocyte activation marker [193]. A cytokine released by lymphocytes boosting inflammation is IFN-γ, and a cocoa extract suppresses IFN-γ production by PHA-stimulated PBMC [191].

Other anti-inflammatory evidences of cocoa flavonoids come from arachidonic acid metabolism. Inhibition of inflammatory enzymes, such as 5-, 12- and 15-lypoxygenases, has been demonstrated by epicatechin [194].

In addition, flavonoid-rich cocoa extract added to culture, decreases secretion of NO from LPS-stimulated macrophages [190,195]. Similarly, epicatechin, procyanidin B1, procyanidin B2, and more intensely quercetin are able to reduce the NO production in stimulated macrophages [160]. Apart from NO synthesis, other studies show that cells from different origins treated with cocoa fractions or flavonoids alone decrease the production of ROS in a dose-dependent manner [89,91,195].

The reports above described show the effect of different fractions of cocoa acting on cells cultured *in vitro* on some aspects of the inflammatory response. Nevertheless, these compounds are generally not found in plasma after gut absorption of cocoa [196], and then it is difficult to extrapolate what will really happen *in vivo*. Interestingly, it has been reported the effect of a cocoa diet on the secretion of inflammatory mediators from peritoneal macrophage culture *ex vivo* [51,197]. This approach allows us to consider the effects of cocoa compounds physiologically absorbed and metabolized on these cells. Peritoneal macrophages from rats fed with cocoa produce minor amounts of TNF-α, IL-6, NO and ROS after LPS stimulation [51,197].

1.2.5.2. PRECLINICAL STUDIES

A few studies have focused on the *in vivo* effects of cocoa on inflammation. With regards to isolated flavonoids administered *in vivo* by non-oral route, it has been reported that two subcutaneous or intravenous injections of catechin and epicatechin produced a significant reduction of paw oedema induced by carrageenin in rats [198].

It has been reported that oral administration of a cocoa polyphenolic fraction to mice can inhibit the ear oedema in a dose-dependent manner [72]. Moreover, a high cocoa intake can decrease the inflammatory response induced by carrageenin in the rat hind paw [197]. This model, widely applied for the screening of anti-inflammatory drugs, provokes a progressive local oedema over 4-6 h. Rats that received cocoa for a week (doses of 2.4, 4.8 or 9.6 g/kg/day) developed a lower paw oedema than reference animals. This effect was seen in the first hour and remained until the end of the study (6 h) in the higher

doses, but the lowest dose only showed a protective effect during the first hour [197]. Similarly, the effect of cocoa intake has been tested in rats in other local acute inflammatory models such as histamine, serotonin, bradikinin and PGE_2-induced hind paw swelling. Interestingly, rats receiving 2.4 g/kg/day of cocoa developed a reduction of paw oedema induced by bradykinin [197]. This effect may be partially attributed to the fact that flavonoids are able to bind bradykinin [199]. However, cocoa intake had no protective effect on oedema induced by histamine or serotonin [197]. These results could suggest that cocoa compounds did not counteract the actions of vasoactive amine. Moreover, it must be remembered that cocoa has a vasodilator effect [200] that would add to that of histamine. Similarly, serum concentration of MCP-1 decreased after a cocoa diet in rats [201].

Although quercetin seems to inhibit cyclooxigenase pathways and Prostaglandi E_2 (PGE_2) synthesis [201] and Ciclo-oxigenase-2 (COX-2) activity [202], cocoa ingestion produced no inhibition on PGE_2-induced inflammation [197], which means that cocoa cannot counteract PGE_2 effects when it is injected, but it remains to be seen if it could regulate its synthesis.

The anti-inflammatory activity of cocoa has also been extended to inflammatory bowel disease (IBD). Using IBD models, a number of flavonoids, such as quercitrin, rutin, diosmin, hesperidin, morin and silymarin have demonstrated anti-inflammatory activity [203]. However, a study using a cocoa diet in a dextrane sodium sulphate (DSS) model demonstrated that cocoa intake does not improve clinical colitis, although it certainly contributes to reducing colonic oxidative activity and serum inflammatory mediator concentrations [204]. More interestingly, it has recently been reported that a polyphenol-enriched cocoa extract was able to decrease acute DSS colitis in mice [205], thus evidencing the need for a high polyphenol content in the cocoa to achieve anti-inflammatory activity in the IBD.

The effect of a cocoa diet on *in vivo* models of neuronal inflammation has been reported. Rats fed a diet enriched with cocoa produced a decrease in the inflammatory response to an acute and chronic noxious stimulus of trigeminal ganglion neurons [206]. Likewise, systemic chronic inflammation such as adjuvant arthritis (AA) and collagen-induced arthritis (CIA) are also modulated by cocoa feeding. In AA, a cocoa-enriched diet was able to decrease the synthesis of antibodies against the pathology inducer, and to reduce the proportion of Th lymphocytes in blood and regional lymphoid tissues, but the cocoa diet produced only a tendency to modulate hind-paw swelling [207]. It must be added that the oral administration of some flavonoids such as quercetin and hesperidin were only able to partially reduce AA swelling [208,209] or only slightly decreased this chronic inflammatory model [210]. However, a cocoa diet was able to reduce the oxidative stress associated with AA [102]. Concerning the CIA model in rats, a diet enriched in cocoa beginning two weeks before CIA induction and given throughout the process, has been applied. Although arthritic cocoa-fed rats decreased specific autoantibody titres, the production of pro-inflammatory mediators from peritoneal macrophages, and the Th proportion in lymph nodes, they developed a similar hind-paw swelling as the reference arthritic animals [201]. On the contrary, Miyake et al. [211] reported that the oral

administration of highly oligomeric procyanidins isolated from Jatoba (*Hymenaea courbaril*) ameliorated CIA in mice and also decreased the serum concentrations of some specific autoantibodies.

1.2.5.3. CLINICAL STUDIES

With regard to studies in humans, although it has been reported that a supplementation with cocoa products in healthy humans did not affect inflammation markers [212], a cross-sectional analysis showed that the regular intake of dark chocolate by a healthy population in Southern Italy was inversely related to serum C-reactive protein concentration [213]. In addition, cocoa consumption for 4 weeks decreased some adhesion molecules involved in the recruitment of inflammatory cells [214]. Likewise, leukocytes from healthy volunteers showed a decrease in the activation of Nuclear Factor-κB (NF-κB) and also in the serum concentrations of some adhesion molecules, such as ICAM-1 and E-selectin, 6 hours after receiving 40 g of cocoa powder [215].

1.2.5.4. INTRACELLULAR ANTI-INFLAMMATORY MECHANISMS INDUCED BY COCOA COMPOUNDS

The exact mechanism by which cocoa could modulate inflammatory response and whether this action is attributed to its flavonoid content remain unclear. Some modulator effects of cocoa flavonoids on inflammatory cytokine secretion are often shown as being associated to a downregulatory action on the mRNA expression [188,216]. Nevertheless, the events that induce these changes are mainly unknown.

As indicated above, cocoa contains polyphenolic antioxidants that might promote changes in redox-sensitive signalling pathways (NOD-like receptors and inflammasomes) involved in inflammatory response. Moreover, it is also possible that cocoa flavonoids exert direct effects on particular targets of the protein or lipid kinases involved in those pathways [217]. Evidences obtained from *in vitro* studies using flavonoids from cocoa or other natural products, suggest several mechanisms to explain cocoa anti-inflammatory action regardless of the antioxidant activity. These hypotheses are summarized in **Figure 4** [186].

Flavonoids may modulate the receptor pathways involved in sensing the microbial molecules (PAMPs) inducers of the process recognized by TLR and subsequent MyD88 activation and TRIF-dependent signalling pathways present in resident immune tissue cells (**Figure 4, point 1**). This mechanism has been ascribed to tea flavonoids, closely related to cocoa flavonoids [218] and also to luteolin, a flavone present in cocoa [219]. Furthermore, oxidative stress accompanying inflammation may lead to inducing inflammasome activation or mitogen-activated protein kinase (MAPK) pathways (i.e. p38 MAPK) (**Figure 4, point 2**). These pathways may be controlled by the direct action of cocoa flavonoids, by their scavenging action or by direct interaction with kinases involved downstream [90,220]. The inhibition of MAPK involves extracellular signal-regulated kinases 1 and 2 (ERK 1 and 2) and c-Jun N-terminal kinase (JNK) and p38 MAPK linked to stress stimuli (**Figure 4, point 3**), such as ROS overproduction or inflammation. Among

other actions, this stimulation can lead to the phosphorylation of inhibitor proteins such as IκBs. The modulation of the activity of pro-inflammatory transcription factors such as nuclear factor-κB (NF-κB) is one of the clearest effects of cocoa flavonoids (**Figure 4, point 4**). NF-κB is redox-sensitive and triggers the expression of over 100 genes, many of them involved in the inflammatory response [221]. NF-κB is found in the cytoplasm of non-stimulated cells bound to κB inhibitor proteins (IκBs) which are modulated by MAPK action as described above. Flavonoids contained in cocoa, such as epicatechin, catechin, quercetin and also procyanidins, are known to inhibit the NF-κB pathway [222–225]. Mackenzie *et al* [225] shed light on the regulatory role of cocoa flavonoids on the NF-κB pathway. Epicatechin, catechin and dimeric procyanidins B accumulated in the cytosol can act at early stages of NF-κB activation, regulating oxidant levels and reducing IκBs phosphorylation. Moreover, at later stages, flavonoids, mainly dimeric procyanidins, can penetrate into the nuclei and selectively prevent NF-κB binding to their consensus sequence [225]. In addition, cocoa procyanidins inhibit the kinase activity of MEK1 thus attenuating the activation of NF-κB and also AP-1 and STAT-4 [220,226].

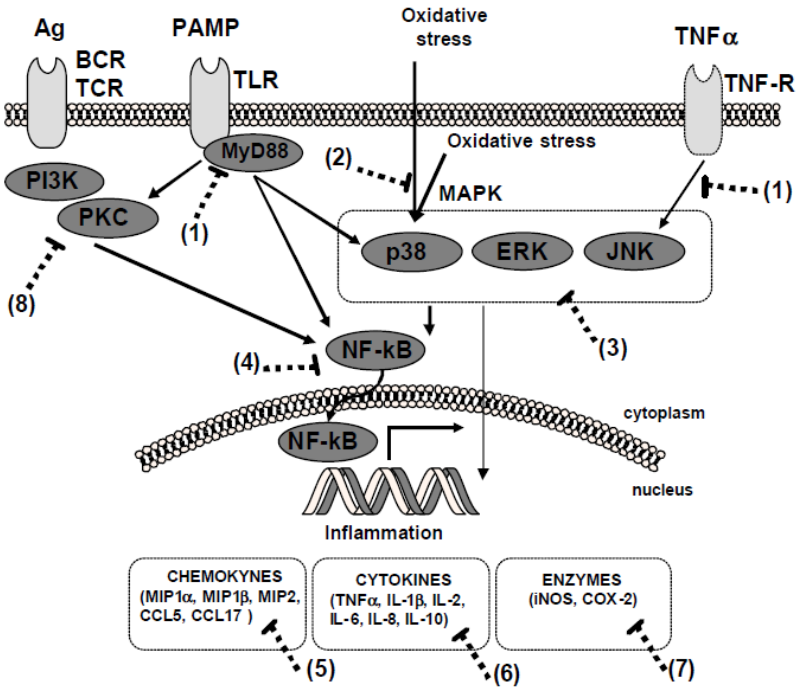

Figure 4: Potential targets of cocoa, cocoa extract and related flavonoids in inflammatory cells (from [186]). Points 1-8 explained in the text.

The inhibition of NF-κB activation or upstream signalling pathways (i.e. MAPK) may be responsible for the anti-inflammatory effects of cocoa reported above, such as reduction of chemokine (**Figure 4 point 5**) and cytokine production (**Figure 4 point 6**) [154,163]. Furthermore, the same targets can explain the inhibitory action of cocoa flavonoids on the expression of inducible nitric oxide synthase (iNOS), COX-2 and RCP gene (**Figure 4**

point 7) [191,202], with a subsequent decrease in nitric oxide (NO), prostaglandins (PGs) and RCP synthesis, respectively.

Finally, cocoa may modulate some pathways involved in antigen recognition in the adaptive response (**Figure 4 point 8**) by inhibition of the activation of PI3K and PKC, as shown in non-immune cells [217], which is also involved in T-cell receptor and B-cell receptor pathways after antigen interaction.

1.2.6. COCOA AS IMMUNOMODULATOR

The immunomodulator role of cocoa polyphenols has been extensively studied on the last years [226]. Studies conducted in experimental models have shown an effect of a cocoa enriched diet in lymphoid tissue composition, and systemic and intestinal immunity.

1.2.6.1. COCOA AND LYMPHOID TISSUES

Primary and secondary lymphoid tissues constitute two major categories of lymphoid organs. The formation of the primary repertoire of lymphocytes takes place in the primary tissues such as thymus and bone marrow. Secondary lymphoid tissues are responsible for the coordination of immune responses [228]. By means of preclinical studies in rats, it has been evidenced that a cocoa diet can induce changes in the cell composition of both primary and secondary lymphoid organs. In particular, a cocoa diet has an influence on the proportion of B lymphocytes and T-cell subsets i.e., T-cell receptor (TCR) $\alpha\beta$+ cells, TCR$\gamma\delta$+ cells, T-helper (Th) cells and T-cytotoxic (Tc) cells (**Figure 5**).

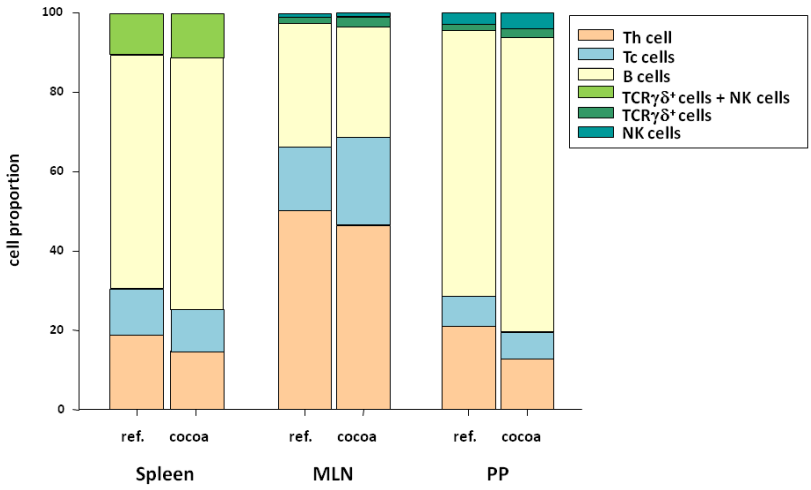

Figure 5: Summary of the effects of a 10% cocoa diet in rats on lymphocyte proportion in secondary lymphoid tissues [226]. MLN, mesenteric lymph nodes; PP, Peyer's patches.

A cocoa diet also influences the cell composition of rat thymus. A diet containing 10% cocoa in rats promoted the progression of immature thymocytes (double negative TCR$\alpha\beta^{low}$ and double positive TCR$\alpha\beta^{low}$ cells) towards more mature T-cell stages (CD4+CD8- TCR$\alpha\beta^{high}$ cells) [96]. Similarly, a diet with 10% cocoa is able to influence a secondary lymphoid tissue such as the rat spleen and lymph nodes (**Figure 5**). Young rats fed cocoa decreased the spleen percentage of Th cells while increasing that of B cells [229]. Additionally, adult Louvain rats fed 10% cocoa for 6 weeks reduced the proportion of TCR$\alpha\beta$+ cells in inguinal lymph nodes [201]. Likewise, the percentage of Th cells was reduced in mesenteric lymph nodes (MLNs) at the expense of Tc cells that increased in young Wistar rats fed 10% cocoa for 3 weeks [230] (**Figure 5**). Similarly, a high cocoa diet also affects the lymphocyte composition of intestinal Peyer's patches (PPs). In particular, cocoa intake reduced the TCR$\alpha\beta$+ cell percentage, mainly due to a decrease in the Th-cell proportion, and increased B-cell and TCR$\gamma\delta$+ cell percentages [230] (**Figure 5**). The increase in TCR$\gamma\delta$+ cell percentages in PPs and MLNs induced by cocoa is similar to the effects of apple polyphenol intake in healthy mice [231].

1.2.6.2. COCOA ON ACQUIRED SYSTEMIC IMMUNITY

The acquired immune response is an intricate reaction comprising a number of intracellular and intercellular events from the antigen entry until the development of effector mechanisms. Dendritic cells (DC) are antigen-presenting cells that take up, process and present antigen to TCR-specific Th lymphocytes. The interaction between DC and Th cells involves a lot of co-stimulatory molecules thus forming the immune synapses [232]. Specific recognition of antigenic peptide by TCR together with co-stimulatory molecules causes production of IL-2 (**Figure 6**) [233]. IL-2 binds to a receptor consisting of the subunits α, β and γ (CD25, CD122 and CD132, respectively) to produce cell proliferation [234]. CD25 is expressed after Th-cell activation. The binding of IL-2 to its receptor leads to the stimulation of complex transduction signals involving MAPK, JAK/STAT, and PI3K/Akt pathways that eventually mediates multiple biological processes including T-cell and B-cell growth and differentiation [233,234]. IL-2 and CD25 have been became markers of early lymphocyte T activation.

Some *in vitro* studies have reported the effect of isolated cocoa flavonoids and cocoa extracts on the synthesis of IL-2. Sanbongi et al. [235] found that cocoa liquor polyphenols inhibited both IL-2 gene and protein expression in human blood T cells. Likewise, a crude cocoa extract and pentamer, hexamer and heptamer procyanidins from cocoa also reduced IL-2 transcription in phytohaemagglutinin (PHA)-stimulated human PBMC [236]. Similarly, in a lymphoid cell line activated with phorbol 12-myristate 13-acetate (PMA) and IL-1 and cultured in the presence of epicatechin or a cocoa extract, it has been established that cocoa flavonoids were able to decrease the expression of surface CD25 and to diminish IL-2 secretion [237]. The ability of the cocoa extract to decrease CD25 expression was higher than that of epicatechin alone, which may be due to the effect of other cocoa flavanols [237]. Overall, these *in vitro* studies agree that cocoa flavonoids can decrease IL-2 production in Th cells. How cocoa flavonoids

modulate IL-2 gene is not known but it has been demonstrated that epicatechin and dimeric procyanidins decrease NF-κB activation on PMA-activated Jurkat cells, a lymphoid cell line [225]. The inhibition of NF-κB might mediate the downregulation of both IL-2 and CD25 in a similar way to that of the decrease in pro-inflammatory mediators.

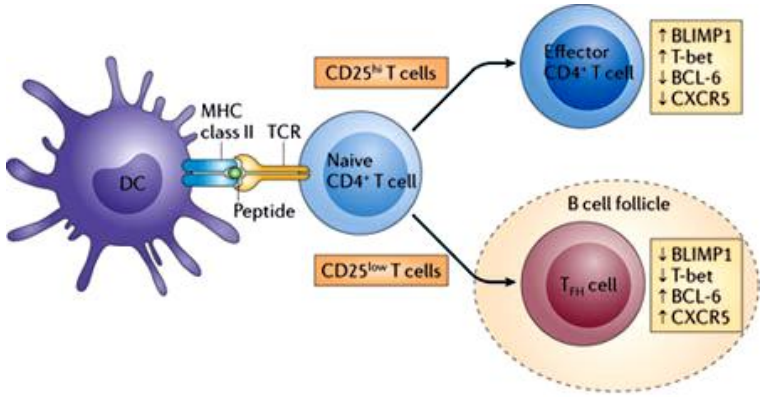

Figure 6: Interaction between dendritic cell (DC) that express peptide–MHC class II complexes together with co-stimulatory molecules (not shown) with naive Th (CD4+) cells that will become activated [234].

In spite of the *in vitro* results, *in vivo* studies do not confirm the downregulation of IL-2 by cocoa flavonoids. Some studies have showed the effect of a diet containing cocoa in rats on the functionality of immune cells isolated from spleen or lymph nodes. Splenocytes from rats fed cocoa diet (with either 4% or 10% defatted cocoa) did not decrease IL-2 production or CD25 surface expression after stimulation with PMA plus ionomycin [229,238]. Likewise, these cells showed a similar or even higher proliferative response [229]. In the same way, lymphocytes from cocoa-fed rat MLN produced higher or equal amounts of IL-2 [207,230,238].

After naïve Th-cell activation and proliferation, effector Th lymphocytes appear (**Figure 7**). Depending on the cytokines released to the medium, which are eventually related to the antigen that trigger the immune response, activated Th1 cells, Th2 cells, Th17 cells or regulatory T cells result [239].

Th1 cells direct cell-mediated immunity against intracellular pathogens by means of the synthesis and release of IFN-γ and TNF-α, among others. These cytokines promote phagocytosis and cytotoxicity recruiting macrophages, NK cells, Tc cells, and also the enhancement of complement-activating antibodies synthesis. Th1 activity is usually associated with inflammation. Th2 cells are designed to fight against extracellular pathogens, activating mast cells and eosinophils, and the production of antibodies which are not able to activate the complement system. Th2 cells are involved in the humoral immunity and allergic reactions [240]. The Th2 subset produces cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate and IL-10 with anti-inflammatory properties. IL-4 is mainly produced by activated Th2 cells and plays an important role in

regulating Th1/Th2 balance [240]. Recently, the effectors Th-cell family expanded with the discovery of Th17 cells. These cells produce IL-17 and exhibit effector functions distinct from Th1 and Th2 cells. The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells and they are potent inducers of tissue inflammation [239].

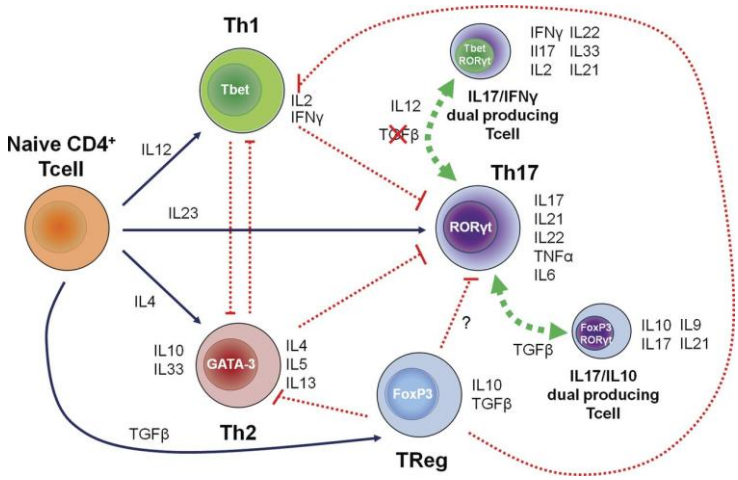

Figure 7: Naive CD4+T cells differentiate into distinct functional subsets: Th1, Th2 and Th17 cells. FoxP3, Forkhead box P3; IFN, interferon; IL, interleukin; RORγt, retinoic acid orphan receptor-γ thymus; T-bet, T-box expressed in T cell; TNF, tumour necrosis factor [241]

The effect of cocoa diets in rats on the cytokine production by Th1 and Th2 cells has been reported. The secretion of IFN-γ, the main cytokine related to Th1 activity, has been quantified in cells isolated from the spleen and lymph nodes of rats fed a cocoa diet. No changes in the secretion of this cytokine were observed in splenocytes [207,229], although others found increased values [238], and *in vitro* studies demonstrated a suppression of IFN-γ production by PHA-stimulated PBMC [191]. More interestingly, a cocoa diet in rats produced a lower IL-4 secretion in isolated splenocytes [229,238] and MLN cells [230]. However, IL-10 secretion was not modified in rats fed a cocoa diet [229,230]. The results obtained in these *in vivo* experiments did not exactly fit with those obtained in *in vitro* studies with cocoa flavonoids. Thus, an increase in IL-4 secretion after epicatechin addition in a lymphoid cell line and PBMC has been reported [236,237], whereas hexamer to octamer cocoa procyanidins presented an inhibitory effect on this cytokine [236].

As stated before, an increase in the percentage of B cells in spleen was observed in rats fed cocoa (**Figure 5**) [226]. However, the antibody response of these cells has been found to be attenuated. Thus, the ability to produce IgG, IgM and IgA by splenocytes from rats fed cocoa was depressed [229]. This effect was also reflected in serum immunoglobulin concentrations [229,242]. However, when the cocoa diet began later and the dose was lower, the effect was not so patent [243].

On the other hand, when animals were fed cocoa before and during an immunization process, the synthesis of specific antibodies and the number of IgG-secreting cells decreased [238]. The analysis of antibodies demonstrated that the impact on humoral response did not affect all antibody isotypes equally. The most attenuated isotypes were specific IgM, IgG1, IgG2a and IgG2c whereas anti-Ovoalbumin (OVA) IgG2b concentrations held steady or increased with the 10% cocoa diet. IgG isotypes can be associated with Th1 or Th2 immunity. In the rat, IgG1 and IgG2a are related to the Th2 response, while IgG2b depends on the Th1 response [244]. These results agree with others that evaluated certain food polyphenols, such as those from apple or soybean [231,245]. From all these results, it has been suggested that a cocoa diet mainly downregulates the Th2 immune response, whereas it maintains Th1 immunity. This hypothesis was supported by a lower IL-4 secretion from splenocytes and a higher production of IFN-γ from lymph node cells [238].

Because of a cocoa diet seems to attenuate antibody synthesis, it has been tested in experimental disease models in which antibodies play a pathogenic role, such as autoimmune diseases and allergic processes. Rheumatoid arthritis (RA) is a systemic autoimmune disease in which chronic inflammation of synovial joints results in joint destruction, pain, disability, and a reduced life expectancy [246]. The pathology of the RA is mediated by specific autoantibodies, mainly against citrullinated proteins such as collagen type II [246]. In consequence, CIA in rats or mice is the gold standard *in vivo* model for RA studies [247]. In such rat experimental model, the influence of a cocoa diet on joint inflammation and autoantibody titres has been reported [201,207]. Louvain rats fed cocoa from 2 weeks before arthritis induction, and during the disease period studied (4 weeks), reduced the synthesis of specific antibodies against type-II collagen, but this effect was not enough to mitigate the hind-paw swelling in arthritic animals during the study period [201].

Allergic reactions are mainly caused by IgE-mediated hypersensitivity. In allergic patients, the immune system reacts to innocuous substances by producing IgE. These antibodies bind to mast cells and, after allergen reaction, produce degranulation of mast cell mediators with a subsequent generation of allergic manifestations [248]. The effect of cocoa in an allergy model has been preclinically studied. A diet containing 10% cocoa prevented the synthesis of antibodies involved in allergic reaction in young rats, in particular, rats fed a cocoa diet showed lower titres of specific IgG1, IgG2a and a decrease of specific IgE of about 60-70% [249]. In addition, in a model of food allergy recently set up [250], a cocoa diet inhibits the synthesis of specific IgE and other Th2-related antibodies, and, partially, the release of mast cell mediator after anaphylaxis, although other variables were not modified [251]. The cocoa intake diet also attenuated the increase of some Th2-related cytokines released from mesenteric lymph node and spleen cells, and modulated the intestinal gene expression of molecules involved in allergic response. These effects are partially attributed to flavonoids (**Figure 8**) but other components might also play a role in cocoa's action [251]. In this context, the modulation of specific IgE and some allergic symptoms were also observed in allergy models after treatment with flavonoids. This is the case in treatment with baicalin [252], biochanin A

[253], quercetin [254], myricetin [255] and hesperidin [256]. In addition, it can be added that clinical trials applying a treatment with Pycnogenol, an extract of *Pinus maritime* containing procyanidins, demonstrated the efficacy of such intervention in reducing some signs of allergic asthma [257,258].

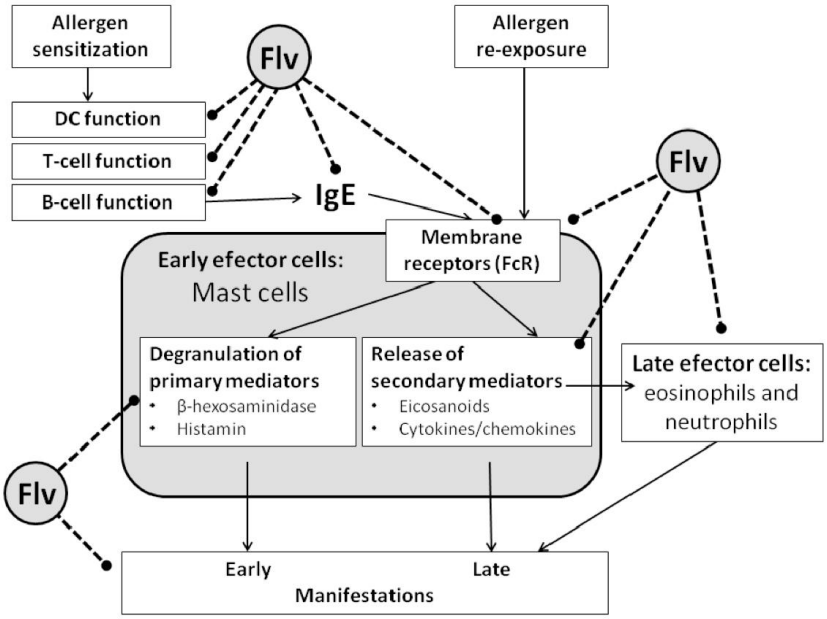

Figure 8: Main targets of flavonoids, such as those present in cocoa, in allergy reaction.

1.2.6.3. COCOA ON INTESTINAL IMMUNITY

The digestive system is the first compartment reached by dietary compounds. Bacteria, epithelial cells and immune cells in the intestine are the first ones to be affected by diet. Every day, the gut-associated lymphoid tissue (GALT) receives a huge antigenic load and has to distinguish between invasive pathogens and innocuous antigens from food and commensal bacteria. Briefly, the intestinal immune response is initiated in the M cells from PPs which uptake luminal antigens and transport them towards DC, which interact with interfollicular T lymphocytes or migrate towards MLN [259]. This process induces differentiation and maturation of B cells, which become IgA+ cells and later IgA-secreting cells [260]. The main resulting product of the GALT is the secretory-IgA (S-IgA) [261]. This immunoglobulin constitutes the first line of non-inflammatory immune protection at mucosal surfaces by neutralizing microbial pathogens and exotoxins and by interacting with innocuous dietary antigens and commensal microbes [262].

Some studies have addressed the dietary effects of cocoa on GALT function in healthy animals or humans. Dietary intervention with cocoa does not morphologically affect the intestinal structure [230], but is capable of modifying some important aspects of the GALT composition and functionality in rats as next detailed [230,242,243] and summarized in **Figure 9** [226].

An interventional nutrition study with a cocoa diet in rats modulates MLN lymphocyte activation. Isolated MLN cells from young rats fed 10% cocoa for 3 weeks strongly enhanced IL-2 secretion [230]. Nevertheless, isolated MLN lymphocytes from rats fed a long-term cocoa diet (9 weeks) did not change IL-2 production [238].

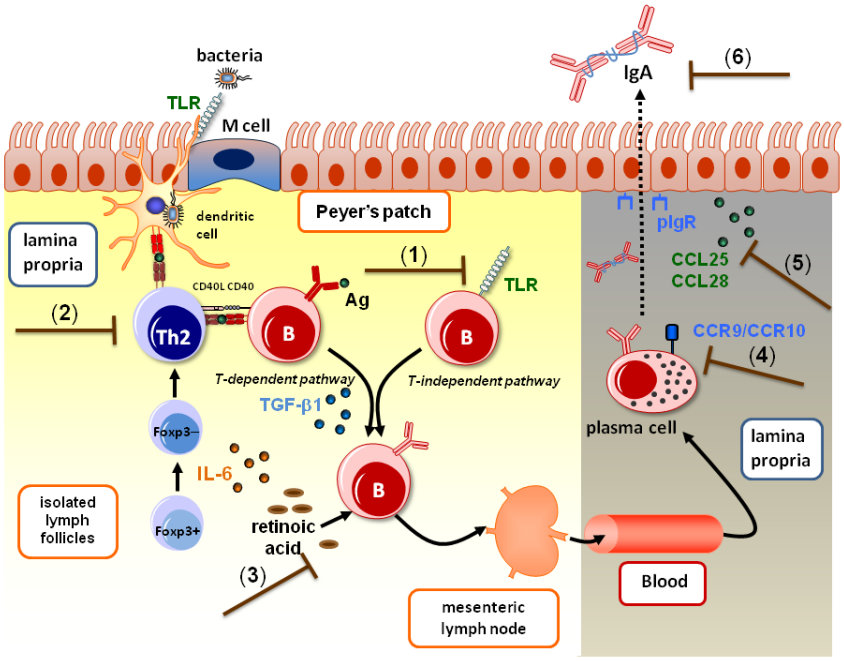

Figure 9: Summary of the effects of a 10% cocoa diet in rat's gut-associated lymphoid tissue [226]

To ascertain whether cocoa modified Th effector cell functionality in the GALT, IFN-γ, IL-4 and IL-10 cytokine production was studied in stimulated MLN cells isolated from animals fed a cocoa diet. The nutritional 10% cocoa intervention for 3 weeks resulted in a lower IL-4 secretion, IL-10 secretion tended to decrease whereas that of IFN-γ tended to increase [230]. These results suggest that high cocoa diets, similarly to the results found in the systemic compartment, downregulate Th2 responses.

S-IgA plays a key role in the maintenance of gut homeostasis and oral tolerance and its function and production are tightly regulated [259]. The relationship between a cocoa diet and S-IgA has been demonstrated in different experimental designs using rats, where the effect of varied proportions of cocoa diets (2%, 4%, 5% and 10%), different age at the beginning of the dietary nutritional (3 or 6 weeks of age) and length of diet (3, 6 or 9 weeks) have been analyzed [230,242,263,264]. Cocoa reduces S-IgA protein and gene expression which conducts a different pattern of IgA-coating bacteria. The effect of cocoa on S-IgA might be due to the influence of cocoa on genes related to Th maturation, Th-B cell interactions, and IgA+ B cell gut homing, among others [242,243] (**Figure 9**).

The downmodulatory effect of cocoa on S-IgA was firstly established in faecal samples after 2 weeks of 10% cocoa intake in young rats. The decrease in faecal IgA correlated with a lower concentration of S-IgA and S-IgM in gut washes [230]. These results were confirmed in a study that extended the dietary intervention with a 10% cocoa diet up to 6 weeks [242]. This effect was associated with a lower number of PP cells with a high capacity to secrete IgA [230].

In order to look further into the downregulation of S-IgA through a cocoa diet in rats, the gene expression of several molecules involved in intestinal immune response was established using different cocoa proportions (2%, 5% and 10%), supplementation periods (3 or 6 weeks) and initial age of rats (3 or 6 weeks) [242,243]. A pathway for B cells to become IgA-secretory cells is a T-cell-dependent process located in either PPs or MLNs, inductive sites of the intestinal immune system [260,265]. The maturation of mucosal Th cells depends on IL-6, among others; the interaction between activated Th cells and B cells requires the interaction of CD40 ligand with CD40 [266,267], and the differentiation of B cells into IgA+ B cells involves transforming growth factor-β1 (TGF-β1), IL-5, IL-6, IL 10 and IL-21 [268,269]. The study of a 10% cocoa diet for 3 or 6 weeks in rats on the mechanisms of S-IgA secretion revealed that the cocoa diet did not modify TGF-β1 gene expression in PPs, MLNs, or the small intestine; however, IL-6 gene expression was reduced ~95% in MLNs after 6 weeks of a 10% cocoa diet [242] but not earlier [243]. Likewise, cocoa intake did not modify CD40 gene expression either in PPs or in MLNs [242], which is in accordance with previous studies that have shown that a cocoa diet increased the proportion of B cells in PPs [230]; however, a 10% cocoa diet (but not lower proportions) for 6 weeks (but not in a shorter period), reduced CD40 gene expression in the small intestine [242,243].

When IgA+ B-cells become activated they leave PPs, go to the bloodstream and come back to the intestine or other mucosa [261,270]. The gut-homing system requires the integrin $\alpha_4\beta_7$ and some chemokine receptors on activated gut lymphocytes [265]. Chemokines produced by epithelial cells such as CCL25 and CCL28 interact with the chemokine receptors CCR9 and CCR10 respectively, to recruit IgA+ B-cells [271]. The CCR9 expression on IgA+ B cells is induced by retinoic acid [272] through its ligation to nuclear retinoic acid receptors (RAR) [273]. Diets containing 2%, 5% or 10% cocoa for 3 weeks in 6-week-old animals did not affect the gene expression of CCR9, CCL25, RARα and RARβ but increased the CCL28 gene expression in the small intestine wall [243]. When the cocoa diet began earlier and lasted longer (3-week-old animals fed 10% cocoa for 6 weeks), the gene expression of gut-homing molecules such as RAR, CCR9 and CCL28, but not CCL25, was downregulated in the small intestine [242]. Overall, these results demonstrate the longer the cocoa intake the greater the sensitivity of gut-homing mechanisms in the intestine. When IgA+ B-cells reach the intestine, they differentiate into IgA-secreting cells mainly releasing dimers of IgA. This immunoglobulin is actively secreted to the apical surface of epithelial cells by a polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of epithelial cells [259]. The gene expression of pIgR was not modified by any cocoa diet given for 3 or 6 weeks [242,243].

In summary, a high cocoa diet induces a lower number of IgA+ B cells reaching the intestinal lamina propria by downregulating either the expression of chemokine or that of their receptors (**Figure 9**). However, in the gut lamina propria some other mechanisms remain working efficiently.

On the other hand, the GALT maintains mucosal homeostasis by inducing a state of non-responsiveness to innocuous antigens, such as commensal bacteria, or by responding actively to counteract pathogens [274]. In this regard, toll-like receptors (TLRs), through

the recognition of conserved molecular motifs on microorganisms, are important molecules involved in the cross-talk between microorganisms and gut epithelial and immune cells [275]. It has been reported that the generation of IgA+ B cells can be independent of Th cells and involve TLR non-specific recognition [274]. In this context, cocoa diets in rats have shown differential TLR expression patterns for TLR2, TLR4, TLR7 and TLR9 in PPs, MLNs, the small intestine and colon [242,243,263].

A high (10%) and continuous cocoa diet produced an upregulation of TLR4 and TLR9 and a downregulation of TLR2 and TLR7 in PPs and MLNs (inductor sites of intestinal immune response) [242,243]. Conversely, in the small intestine and the colon, cocoa-fed animals showed lower TLR4 and TLR9 and higher TLR2 and TLR7 gene expression [242,263]. TLR4 expression is positively correlated to the number of IgA-secreting cells in the lamina propria and their recruitment through CCL28 [275,276]. In consequence, the downregulation of TLR4 through a cocoa diet in the intestine (as effector site) could be associated with the decrease in S-IgA in faeces.

TLRs are expressed preferentially in tissues that are in constant contact with microorganisms [275,277]. Therefore, changes in the TLR expression could reflect changes in the intestinal microbiota and/or its relation to intestinal immune cells [278]. This is possible due that cocoa flavonoids reaching the colon can interact with intestinal microbiota through a bidirectional relationship. Thus, bacteria can be involved in the polyphenol metabolism, and flavonoids can influence microbiota growth and composition [279]. In this context, changes in intestinal microbiota composition may influence the immune system as well as the compounds originated by the bacterial metabolism.

Cocoa flavonoids have a particular bacterial metabolism due to the high degree of polymerization of its flavanols. After cocoa intake, monomers (i.e. catechin and epicatechin) are rapidly absorbed in the small intestine, while the largest proportion of dietary polyphenols (90-95%) in the form of oligomers and polymers (i.e. cocoa procyanidins) pass intact through the gastrointestinal tract, reaching the colon [280]. This fact allows them to be metabolized by the intestinal microbiota. Colonic bacteria is composed of more than 500 species and a bacterial load of approximately 10^{11}-10^{12} bacteria/g of colonic contents [281]. It is known that microbiota has the ability to metabolize polyphenols to simpler metabolites and this conversion is often essential for absorption and modulates the biological activities of these compounds which are more beneficial than the original forms found in food [280,282–284].

Cocoa polyphenols are extensively degraded in the colon by a broad range of reactions able to generate various phenolic acids, mainly including phenylpropionic, phenylacetic and benzoic acid derivates [282,285–288]. Later, colon bacterial metabolites are absorbed into the bloodstream, providing another source of potentially bioactive compounds [289]. Once absorbed, the microbial metabolites from flavanols are mainly metabolized in the liver by phase-II enzymes as hepatic conjugated derivatives that are subsequently eliminated in urine [284]. In particular, the presence of 5-(3',4',5'-trihydroxyphenol)-γ-valerolactone and 5-(3',4'-dihydroxyphenol)-γ-valerolactone in urine

is considered to be a potential biomarker of flavan-3-ols consumption in humans after cocoa products intake [290]. At the same time, a portion of microbial metabolites (non-conjugated microbial metabolites) is eliminated in the faeces. The excretion of microbial metabolites varies markedly between subjects and, for some individuals, it may also vary with the substrate [280].

Regarding the intestinal bacteria with the ability to catabolize flavanols, a limited number of bacterial species have been identified as being involved in the polyphenols catabolism. Interestingly, the majority of the bacteria characterized belong to the Clostridia group, which is a large component of the gut microbiota [282,291].

It is known that unabsorbed dietary phenolics and their metabolites can exert significant effects on the intestinal environment by modulation of the microbiota [292]. Although there is limited information concerning the ability of (+)-catechin and (-)-epicatechin, the main monomers present in cocoa, to promote or inhibit the growth of selected intestinal bacteria, there are some *in vitro*, preclinical and clinical studies regarding this subject [263,282,293].

In vitro studies have shown the antimicrobial properties of some polyphenols [292,294]. To date, Tzounis et al. [282] showed that (+)-catechin induced an inhibitory effect in the growth of the *Clostridium histolyticum* group using the batch culture approach, at the same time that both (+)-catechin and (-)-epicatechin enhanced the growth rate of the beneficial bacteria group, *Eubacterium rectale* - *C. coccoides*. Furthermore, there were increases in both Lactobacillus spp. and *Bifidobacterium* spp. following (+) catechin exposure, as well as a small but significant increase in the growth of *E. coli* after (+)-catechin incubation [282].

The effects of cocoa polyphenols observed in animal models are partially in line with these results. Young rats receiving a 10% cocoa intake for 6 weeks showed a significant decrease in the proportion of *Bacteroides, Staphylococcus* genus and *C. histolyticum* subgroup [263].

With regard to human studies, evidence of the effects of cocoa or cocoa products intake on microbiota composition is scarce. A human intervention study evaluated the high-cocoa flavanol consumption effect on microbiota composition from healthy volunteers [293]. The results showed that a 4 weeks daily ingestion of a high-cocoa flavanol beverage containing 494 mg flavanols increased the growth of *Lactobacillus* spp., and *Bifidobacterium* spp. in comparison with a control low-cocoa flavanol drink that contained only 29 mg flavanols [293]. Overall, all these findings strengthen the evidence that cocoa polyphenols can have significant effects on the growth of select gut microbiota.

1.3. CHOCOLATE AND COCOA PRODUCT CONSUMPTION – THE NEED TO QUANTIFY

Although the benefits of cocoa consumption in human health have been proven, there are very few data addressed the cocoa consumption in specific populations. Moreover, these data mostly refer to country annual consumption per capita.

Seligson et al. [295] have shown results referring to chocolate candy consumption in 1991 and, according to this study, Switzerland was placed the first with an estimated intake of 9.9 kg/year and Spain was the last (1.5 kg/year). More recently, it has been established an interesting correlation within chocolate consumption and Nobel Prize winning [296]. According to this analysis, Switzerland has shown the highest chocolate consumption, over 10 kg/year/capita, and more Nobel Laureaates per 10 million population. Other countries such as Germany, Norway, Denmark and United Kingdom have lower chocolate consumption (7 to 8 kg/year/capita) but also a high number of Nobel prizes as well as Sweden, which competes with Switzerland in Nobel Laureates but with a considerably lower chocolate consumption. It is important to highlight that this statistics only refers to chocolate consumption and do not distinguish either the type of chocolate consumed and if there are other cocoa sources in the diet. In spite chocolate bars can be considered the most direct source of cocoa, its content can be quite variable as well as the nutritional content of chocolate products thus polyphenol amount is also quite variable as showed in Section 1.1.2.

In addition, due to the like for the chocolate taste, several food products include it as an ingredient, and others include cocoa. Thus, they can add a possible influence to the total daily intake of cocoa, which can justify where there is not clear data on the total cocoa consumption in a normal diet. Additionally, in spite cocoa is plenty recognized as a polyphenol source, the results from studies regarding polyphenol intake have not been conclusive. Cocoa has not often been found within the top sources of polyphenol intake [297,298] and some data reveals that cocoa accounts for less than 10% of polyphenol intake. These results can be explained by the methodologies used, specifically most assess polyphenol intake based on data from a food frequency questionnaire (FFQ) which include fruits and vegetables but poorly distinguishes the several chocolate and cocoa sources other than chocolate bars [299].

On the other hand, apart of non considering the cocoa/chocolate products other than bars, the cocoa percentage in chocolate bars is not always inquired about, though the presence of polyphenols is totally different among white, milk or the different types of dark chocolate [300,301].

According to Cooper et al. [302] the non-fat cocoa solids amount is a good marker to determine the total phenolics in the product and Miller et al. [303] has proven to be higher in cocoa powder (72-87%) and lowest in milk chocolate (5-7%). The presence of polyphenols could additionally be influenced by specific manufacturing processes like alkalization or ditching of cocoa powder [63,304].

Considering all these variables and the liking for chocolate flavour in multiple food products, the quantification of cocoa intake and in the population seems to be underestimated justifying the need for the development of a more specific FFQ.

1.3.1. THE USE OF FFQ TO ASSESS COCOA INTAKE

The study of a population diet can be conducted using several tools, which can be globally classified as dietary surveys, diet story method or FFQ [305]. The choice tool depends mostly on data application need and specific differences determine which is more appropriate for the aim of the study. Sample characteristics can be also relevant in this choice.

The most frequently used resource in epidemiologic studies is FFQ. Although direct tools such as 24 hours dietary recalls (24HDR) and food records (3 to 7 days) are more accurate, they would implicate a considerable elaborate logistics with personal and funds available that would not be feasible in large population studies [306,307].

When choosing the assessment tool, the strengths and limitations of each possibility should be considered. Most studies use one or more of the following three: food records, 24HDR and the FFQ. **Table 6** summarizes the most relevant strengths, limitations and features of each one of this assessment tools.

Food record is a valuable and quite accurate tool to assess food intake. A food record consists in a complete log of the foods and drinks ingested over 3-7 days. Besides the large variety of data to be analysed and processed, it is also crucial to ensure that participants are trained previously considering that the accuracy of the data depends mostly on them [308].

When food records are not logistically possible for any reason, the 24HDR is frequently seen as an alternative. However, the data collection and completeness also depends on the effort of the individual, the interviewer technique can be determinant for a correct methodology. In the 24HDR, the participant is asked to describe what foods and drinks have been ingested in the previous 24 hours. This questionnaire can be repeated if necessary in order to ensure that the information is more representative of the usual intake [309,310].

However, both these two assessment tools require logistic means that are not always feasible in large population studies. In addition to the time spent in the data collection and processing, there is a need to train people to explain procedures or apply the questionnaires.

The FFQ is a valuable, cost-effective and easily applied dietary assessment tool and can be applied in a large population; it is especially adequate to estimate the intake of a food, food group and/or food component in a population and to study its association with health parameters or disease risk [311].

Table 6: Food assessment tools main features, strengths and limitations

	Food records	24HDR	FFQ
Procedure	The participant is trained and instructed to log the food and beverage intake over 3-7 days	The interviewer asks about the food and beverage intake in the last 24h	The participant answers a FFQ which consists of a food checklist
Type of Data	Quantitative data about food intake		Food consumption frequency
Strengths	Detailed data No interviewer is needed No recall bias	No literary is needed Detailed data	Cost-effective Time saving
Limitations	The participant must be trained Possible under-reporting Literary and motivation are required	The interviewer must be trained Last 24 h may not be representative Possible recall *bias*	Possible recall bias Can be limited due to the closed questions Lower accuracy for direct measures

When adopting a FFQ as a dietary assessment tool in their study, investigators should made an option between adopting one previously validated FFQ or developing and validating a new FFQ. Some FFQ have been pointed out as valid options and used in other further studies, the most known examples are: the Harvard FFQ developed by Walter Willett in 1985; the National Cancer Institute-Block Health Habits and History Questionnaire, developed in 1986; and the Epic Norfolk FFQ from the University of Cambridge. Based on this methodology it is also possible to find FFQ developed in specific countries or even regions such as the Portuguese FFQ developed in the University of Oporto [312,313] and the ENCAT-2003 from Catalonia Health Department [314].

In fact, the nationality, ethnical and cultural aspects are an important factor which should be considered in the choice and application of a FFQ. The development of specific questionnaire for the purpose and sample of each study is the most appropriate way to ensure that the tool fulfils the intended goal [315].

Within several critical points, one matter of concern in the use of FFQ is its design. There is not any perfect FFQ to be used in every study independently of its purpose, in spite most FFQ are developed based on previously validated FFQ. There are several aspects to consider in the design of a new FFQ but the food list is probably the most important start point, it should be comprehensive and answer the aim of the study but not too much exhaustive [316]. It should also be prepared according to the target population of the study and some preliminary information should be obtained through other dietary assessment tools or observation [317].

Previous questionnaires designed to estimate polyphenol intake included a quite varied list of fruits and vegetables as well as drinks such as tea, coffee and wine but very few products of cocoa because it was not the aim of the study [297,298,318,319].

Similarly, national FFQ developed to study the population frequency of consumption within specific food groups do not list with detail cocoa products, including only cocoa powder and chocolate bars, some also include chocolate cookies [312,314].

The method of administration can also be a subject of some debate. The ideal way would be a face-to-face interview: a trained investigator would apply the questionnaire to the participant, but this raises logistical problems and increases the time length and the costs of application. A considerable proportion of FFQ are designed to be self-administered [317] but in order to overcome errors from this, it is crucial that the participant received some preliminary instruction about the questionnaire, a member of the investigation group must be able to answer any question during the procedure and at the end the questionnaire to verify for completeness [320,321].

The step next to FFQ development is the validation process and this is crucial to ensure complete agreement with the purpose of the study. The validation process can be conducted according at least two methodologies. A possibility is using a biomarker that should be adequate to the food component studied in the questionnaire and can be obtained in biological materials (e.g. blood, saliva). Other possibility is using a reference tool such as the 24HDR or a food record. In spite, some guidelines can be considered there is not any considered perfect methodology for the validation procedure and it could be due also to the subject and nature of the food component studied [316] .

The use of biomarkers is quite difficult; it requires the existence of a biomarker for a specific food and this is not always possible and the use of a specific biological fluid adequate for the desired compound. In the specific case of flavonoids, their short plasma half-live difficult the quantification in plasma, and 24 h urine samples are considered the better approach in small-scale samples but not in large populations. The use of punctual urine samples would be easier but it is not possible to ensure a correct quantification in this case for daily intake [322].

Thus, most studies are validated using the most logistically feasible procedure and following Cade [316] recommendations, the new designed FFQ is compared to a validated FFQ, considered as a gold standard [323–325], and especially to a reference method like a 3 to 7 day record [326–328] or 24HDR [329–331]. If there is a good agreement, the questionnaire can be considered valid for the purpose it was designed.

1.3.2. DATA ON CHOCOLATE AND COCOA PRODUCTS AMONG UNIVERSITY STUDENTS

The entry at the university is a crucial period in a teenager life and it is known that this can influence dietary habits, especially in students living away from home.

Studies evaluating the dietary patterns in this age group have shown a lower consumption of fruits and vegetables, higher frequency of meal skipping but also higher

frequency of snacking with an increased prevalence for sugary foods including candy [332,333]. Within these, chocolate and chocolate products are mostly included in candy or sugary products category thus considered undesirable. Thus, chocolate consumption among this population is not frequently seen as a desired food choice and some data suggests it was more frequently consumed when individuals were under stress [334].

Some other studies have evaluated the role of chocolate bars as caffeine source. In their study Bühler *et al.* [335] show that chocolate bars are important sources of caffeine for 11-23 year-old students contributing with almost 15% of daily caffeine intake in male and around 20% of caffeine intake in male students. Similar results were obtained by Tannous and Kalash [336] which demonstrated that chocolate drinks contributes with 20% of the daily caffeine intake in North Lebanon university students.

There is no clear data on the consumption of cocoa consumption in university students. Results from Spanos and Hankey [333] have shown that around 25% of the individuals do not eat chocolate bars often but 16% referred they were eating once or more times daily.

A Portuguese study conducted in Oporto city have reported that almost one third (30%) of the individuals in the age group from 18 to 31 years do not consume chocolate bars regularly and only 5% eats twice or three times daily [337].

Once more, most questionnaires applied for university students food habits are quite limited in chocolate / cocoa products and underestimate cocoa intake or put chocolate in a negative image of the food what can be a significant bias in the study.

Nevertheless, to date no consistent information can be found in food frequency of consumption in university students in different countries. Mostly these studies address the fact that individuals have left parents house or are living away from home and its influence in their habits and food choices.

2. OBJECTIVES

As stated in the Introduction, on the one hand, cocoa polyphenol content is plenty recognized as well as the benefits from its consumption in human health. However, the contribution of cocoa to daily polyphenol intake is not totally clear due to the lack of studies estimating the exact cocoa consumption. On the other hand, preclinical and clinical data associating flavonoid intake and allergy amelioration as well as preclinical data demonstrating cocoa intake and allergy prevention suggest the role of this food on such disease.

In addition, there are not observational studies that allow correlate the cocoa consumption with a healthy status (i.e. focusing in diseases such as allergies), and also with physical activity.

Taking these facts into account, our global hypothesis is that cocoa consumption is underestimated in overall studies and therefore a new tool for this purpose should be developed because consumption of cocoa could have an impact on immune mediated diseases in which preclinical evidences exist, such as on allergies.

Therefore, the present thesis has been defined based on the following aims:

- To develop and validate a Food frequency questionnaire (FFQ) including the main cocoa food products common in Catalan and Portuguese dietary habits in a young population.
- To establish the consumption of cocoa in university students of Catalonia and Portugal.
- To establish the relationship between cocoa intake and healthy status, including the presence of allergies, and healthy lifestyle practices.

3.MATERIAL AND METHODS

3.1. STUDY DESIGN

The study includes two Validation Studies with a small number of participants and a Main Study using the validated questionnaires in a larger cohort. The flow diagram in **Figure 10** shows the steps performed to achieve the objectives of this thesis.

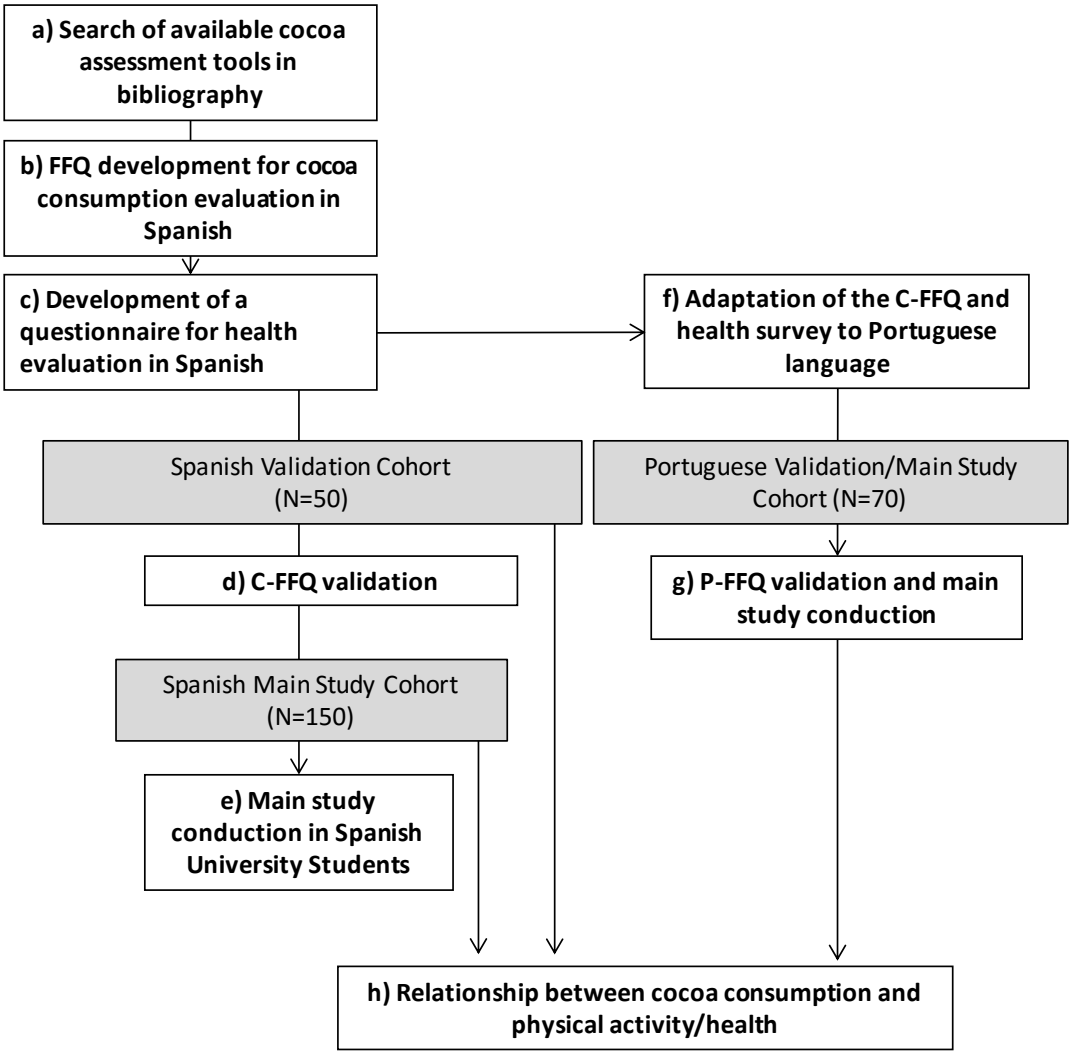

Figure 10: Flow diagram showing the steps performed to achieve the objectives of this thesis.

A brief description of the steps from the process is detailed below:

a) Search of available cocoa assessment tools in bibliography

To develop the main objective of the thesis, an extensive dietary assessment about cocoa consumption was required. Thus, firstly, we evaluated in the current dietary assessments (published food frequency questionnaires, FFQ)) if the questions regarding cocoa consumption were enough to obtain the required data. Even though we found extensive

FFQ including some questions about cocoa products, the specific number of items for this type of products was very limited. Thus, a development of a new FFQ for this particular purpose became then the first step of this thesis.

b) FFQ development for cocoa consumption evaluation in Spanish

A specific FFQ was built in order to assess the precise cocoa consumption in Spanish language (C-FFQ) (*Section 3.4*).

c) Development of a questionnaire for health evaluation in Spanish

In order to achieve the final objective, we developed an *ad hoc* questionnaire for that purpose in Spanish language (*Section 3.7.2*).

d) C-FFQ validation

To validate the C-FFQ, it was applied in a small sample population of students (N=50) from the University of Barcelona, together with a 24 hours dairy recall (24HDR) and a validated FFQ from the European Food and Security Authority (EFSA), which includes some items in common.

In addition, the consumption frequencies of the diverse cocoa products were transformed to the amount of cocoa ingested through the elaboration of a table with the serving size and cocoa amount for each product (*Section 5*).

This part was conducted in the Faculty of Pharmacy at the University of Barcelona between April and June 2014.

e) Main Study conduction in students from the University of Barcelona

In order to establish the relationship between the cocoa consumption and the healthy status and the physical activity, the C-FFQ was applied to a larger population (N=200) together with a validated survey about physical activity (IPAQ) and the health questionnaire developed.

f) Adaptation of the C-FFQ to Portuguese language

In order to perform the study also in Portugal, the C-FFQ was translated to Portuguese, generating the so-called P-FFQ.

g) Validation of the P-FFQ and Main Study conduction

A Validation Study was then conducted in a Portuguese cohort (*Section 3.5*) between July and October 2014. In this case, the participants were University students from the Egas Moniz Health Sciences Institute (N=70). These students also answered to the physical activity survey (IPAQ) and the health status questionnaire developed (translated from Spanish to Portuguese).

The participants involved in the Portuguese Validation study and the Portuguese arm of the Main study were the same.

h) Relationship between cocoa consumption and physical activity/health

All the FFQ data from the Spanish and the Portuguese cohorts were analyzed together in order to obtain an overall relationship between cocoa consumption and physical activity/health (*Section 3.7.3*).

3.2. ETHICAL ASPECTS

The study was conducted respecting the guidelines laid down in the Declaration of Helsinki. Because of the study was carried out in two different universities, it was submitted to the evaluation of both the Ethical Committee of the University of Barcelona (IRB 00003099) and to the Egas Moniz Ethical Commission. Both approvals were obtained.

Following the Ethical Committee guidelines, participants were informed about the aim and the procedures of the project by a member of the team previously to their participation. In this sense, at the beginning of the group meeting, a document stating the main aspects of the process was distributed. The participant had to confirm in that document that he/she received enough information of the project, that he/she had the opportunity to make any question about, that he/she was answered if asked, that he/she was free of leaving the room if desired, and that he/she agreed to participate without any compensation for answering the questionnaire. After signing the document, it constituted the written consent. This document signed by the participant was also signed by the responsible for the research, scanned and a copy was sent to the student by e-mail in order to provide him/her an evidence of the compromise established.

The questionnaires were assigned to a code. The specific information about the person received the same code and was located in a private folder from the responsible for the research. Thus, all data was used in a blinded manner and no information regarding the identity of the participants was not and will not be included in the data management or publications.

3.3. PARTICIPANTS

The Spanish sample, both from the Validation (N=50) and the Main Cohort (N=150) Studies, consisted of male and female students recruited from among students from several health science graduation and post-graduation programmes at the University of Barcelona.

In the Validation Cohort, the participants were recruited via an email or by means of direct invitation in the classroom in the Faculty of Pharmacy. Thus, all students from this sample belong to studies organised by or conducted in this Faculty. Once validated the C-FFQ, an additional number of 150 students were recruited for the Main Cohort study.

Part of these students also belonged to the Faculty of Pharmacy programmes, and other students belonged to the Degree of Biomedical Sciences, Odontology and Medicine from the Bellvitge Campus, all of them students from the University of Barcelona.

The Portuguese sample consisted of male and female students from several health sciences disciplines such as Pharmacy, Nutrition, Dentistry, Nutrition and Psychology from the Egas Moniz Health Sciences Institute in Monte Caparica (Portugal). The participants were recruited by direct invitation in the classroom.

All the participants in Spain and Portugal were called for group meetings where they were informed about the objectives and procedures of the study. Although, C-FFQ and P-FFQ were designed to be self-administered, it was conducted in groups in the presence of at least one member of the research team in order to clarify any queries. Written informed consent was obtained from all participants (*Section 3.2*).

3.4. FFQ DEVELOPMENT

The FFQ was developed based on the ENCAT-2003, a validated FFQ previously applied in Catalonia in order to study food habits and to evaluate their association with health status [314]. Based on this, the FFQ was designed by increasing the number of questions referring to cocoa consumption and reducing others present in that survey.

The FFQ was written in Spanish (C-FFQ) and later translated to Portuguese (P-FFQ). A full version of both FFQ is published in the internal UB Repository (Dipòsit Digital de la UB). The C-FFQ can be downloaded at http://hdl.handle.net/2445/60475 and the P-FFQ can be found in http://hdl.handle.net/2445/65520. Both documents can also be found in the **Appendix 1**.

The FFQ included 90 food items that potentially contain polyphenols (fruits, vegetables, beverages and other type of food sources) and specially those containing cocoa. Following Cade's recommendations [317], related items were clustered together.

Firstly, information regarding the consumption of chocolate with cereals was required. Then, the FFQ inquired about the 12 most commonly consumed fresh fruits in Catalonia (apples, pears, oranges, tangerines, lemons, bananas, peaches, pineapples, strawberries, berries, melons, watermelons and others), as well as the intake of fruit juices, jam and canned fruit. In addition, information about the intake of vegetables (cooked vegetables, tomatoes in several forms, onions, peppers, carrots and others) was asked. Besides the presence of chocolate in the cereals as mentioned before, the FFQ also included several questions regarding other sources of cocoa. This part was the most developed in this FFQ. First, it asked about dairy products that can contain cocoa/chocolate (milk, flan, custard, yogurt, ice cream). Then, there were items related to confectionery that can include chocolate, such as pastries and snacks. Each one of this item was also differentiated in basis of its content of chocolate, including stuffed or covered by chocolate. Six other items were devoted to asking which kind of chocolate bars the participant consumed more frequently. As the main objective was not the chocolate bar consumption itself but the cocoa derived from such intake, this item was divided in

several categories: white chocolate bars, milk chocolate bars, and those with <60%, 60-70%, 70-85% and >85% of cocoa content. Next, there were items related to cocoa/chocolate beverages and spreads. Finally, information relating to the intake of coffee, tea, infusions, wine (red, white or rosé), beer and other alcoholic beverages was requested.

The frequency of consumption of the 90 food items was assessed using 12 categories ranging from 1 to 12, with '1' meaning never, '2' 1-6 times per year, and then increasing progressively as far as '12', which meant 3 or more times per day. The quantity of food items was specified as one portion or piece, and for items such as chocolate bars, a 100 g bar was used as a reference (although later converted in portions for further calculations). Both the C-FFQ and the P-FFQ referred to the consumption average in the last 12 months.

Apart from the information about polyphenols consumption, in the first section of the questionnaire, the participants had to provide general and sociodemographic information such as date of birth, gender, people living with the participant, and educational level and labour status.

3.5. C-FFQ AND P-FFQ VALIDATION PROCEDURE

In order to validate the developed C-FFQ and P-FFQ, the documentation provided to the participants in the Spanish and Portuguese Validation Cohorts also included two additional sections: a 24-hour dietary recall (24HDR) and the EFSA questionnaire. The EFSA questionnaire includes questions regarding the average consumption of coffee, tea, chocolate bars and chocolate snacks over the last year.

3.5.1. REFERENCE METHOD – THE 24 HOURS DIETARY RECALL (24HDR)

Following Cade *et al*. guidelines [317], the 24HDR was chosen as a reference method. The students were instructed to describe what they ate, at what time and to estimate the amounts eaten (**Appendix 2**). This questionnaire was applied on the same day of the FFQ. In order to avoid the weekend effect, the FFQ was conducted from Tuesdays to Fridays.

In Spain, a unique 24HDR was applied while the Portuguese students filled a 24HDR on the day of the FFQ and two more in the following weeks. Data from the three 24HDR Portuguese students were averaged to carry out the validation procedure.

3.5.2. THE GOLDEN STANDARD QUESTIONNAIRE

The EFSA questionnaire (EFSA-Q-2011-00309, henceforth called the EFSA-Q) entitled "Gathering consumption data on specific consumer groups of energy drinks – Adults 18-65" [338] was used as gold standard. This document can be downloaded at http://www.efsa.europa.eu/en/supporting/doc/394eax1.pdf, and can also be found in the **Appendix 3**. This questionnaire included several questions about the frequency of consumption of chocolate and cocoa products such as chocolate bars, chocolate snacks

and hot chocolate in addition to coffee, coffee drinks and tea, which are also present in the FFQ developed in this study. The frequency of consumption was assessed differently according to each food product.

In order to do not alter the meaning of this validated document, the questionnaire was presented to the participants in its original language, English, just after the FFQ and the 24HDR.

3.5.3. FFQ DATA PROCESSING

After data entry, one of the first stages of data processing consisted on the conversion of the frequencies established into portions per day. In this sense, if the frequencies consisted in an interval, not defining a specific number, average values were considered. Thus, for example, for frequencies of 1-6 times/year we considered the mean per year (3.5 times/year), and this was transformed into times per day by dividing it by 365 days (0.00958 times/day).

Overall, considering the several frequencies used in the FFQ, the conversion done as included in the **Table 7**.

Table 7: Daily frequency conversion derived from codes used in the FFQ.

Code	Frequency	Number of daily portions
1	0	0
2	1-6 per year	0.009589041
3	7-11 per year	0.024657534
4	1 per month	0.033333333
5	2-3 per month	0.083333333
6	1 per week	0.142857143
7	2 per week	0.285714286
8	3-4 per week	0.5
9	5-6 per week	0.785714286
10	1 per day	1
11	2 per day	2
12	3 or more per day	3

3.5.4. VALIDATION PROCESS OF FFQ

In order to validate both C-FFQ and P-FFQ, their data were compared with those obtained from the consumption frequencies from the EFSA-Q and 24HDR. Data obtained from FFQ items referred to coffee with milk (caffè latte) and espresso, enabled the portions of coffee per day to be calculated. These results were also used to study the validity of the developed FFQ when comparing with the EFSA-Q and 24HDR data.

From the EFSA-Q, we took into consideration the answers to the questions referring to general consumption habits. In particular, we considered the answers to questions 28-30 (devoted to drinking coffee or beverages with coffee), 31-33 (dedicated to tea) and 34-37

(concerning hot chocolate beverages, chocolate bars and snacks). The frequency in the EFSA-Q was asked in a scale from 1 to 6 in a decreasing frequency per week (from every day, to rarely and finally never), and next, in the case of coffee, tea and hot chocolate, the EFSA-Q inquired about the number of cups per day. From these data, we calculated the number of portions of coffee, tea, hot chocolate, chocolate bars and chocolate snacks consumed per day.

From data obtained from the 24HDR questionnaire, we estimated the consumption frequency of coffee, tea and cocoa/chocolate-derived products. Because data from the 24HDR included portion size, the daily frequency of chocolate bars (100 g) was calculated assuming that a portion size is 20 g (see *Section 3.6*).

Once we had the consumption frequencies for each product of interest in the FFQ, we compared the similarity of their results with those from the EFSA and 24HDR by several approaches.

a) Direct consumption frequency comparison

The average of consumption frequency from the three tools was compared through Wilcoxon test for each of the products containing cocoa and some other used as internal standards such as coffee and tea.

b) Correlation between data from different questionnaires

To study the agreement between the new FFQ and each of the other two assessments (the EFSA-Q and the 24HDR), the correlation between data was calculated by Spearman's test, after Kolmogorov–Smirnov normality test.

c) Bland-Altman plots

To better study the agreement between the C-FFQ and the two other methods in a graphical form, Bland-Altman plots were also included. The Bland–Altman method calculates the mean difference between two methods of measurement (the 'bias'), and 95% limits of agreement of the mean difference (2 standard deviations). It was expected that the 95% limits include 95% of differences between the two measurement methods. The Bland–Altman method can even include estimation of confidence intervals for the bias and limits of agreement. Overall, the presentation of the 95% limits of agreement is for visual judgement of how well two methods of measurement agree. The smaller the range between these two limits the better the agreement is.

In our case, for better comparisons, Bland–Altman plots were obtained for particular cocoa products as well as for pooled products in the same category (i.e. chocolate bars) and even for the total cocoa products consumption frequencies.

d) Quintile classification

The quintile classification is a well-known data classification method of a population from a study using equal intervals. If we define the number of intervals into five, the

classification scheme divides the range of attributed values into five equal-sized subranges (quintiles).

Therefore, to compare the C-FFQ/P-FFQ and the EFSA-Q/24HDR results, study participants were classified into quintile categories of either cocoa, tea or coffee consumers based on the distribution of data obtained from these surveys. Proportions of subjects classified into the same/adjacent or grossly classified (or even in opposite quintiles) were derived for each classification.

3.6. ASSESSMENT OF COCOA CONTENT IN FOOD PRODUCTS

To estimate the total cocoa intake of each participant, the amount of cocoa per portion within each inquired item in the FFQ was calculated. For that, two aspects were taken into account: first, the portion size for each product, and secondly, the cocoa content of each product. For that, food labels in 5-7 food products (from Spain and Portugal) in each food category included in the developed FFQ were analyzed. From that, we obtained the portion size and the cocoa content (**Table 8**).

Regarding portions size, all data from food labels were compared to established portions [312,339]. The portion size from food labels was only used when no other available data were found.

Due to the lack of information of cocoa content for most of the sources, the analysis of the food labels of the 5-7 products per item allowed us to calculate the amount of cocoa per portion. Specific raw cocoa ingredients were considered in order to estimate the amount of cocoa in each product, such as “chocolate powder”, ”cocoa powder” and “chocolate” or even “chocolate chips”. The amount of each one of these ingredients were converted in cocoa amount in 100 g of each food. Some ingredients were in fact part of the items asked in the FFQ.

For other products such as “chocolate cake”, the sum of the cocoa content in each ingredient was considered, and this result was divided by the number of portions. With these data compiled, it was possible to establish the cocoa content of each cocoa product included in the FFQ. Moreover, references from previous works in this regard have been also considered [339].

Table 8: Cocoa content in cocoa and chocolate products inquired in the FFQ.

Product	Cocoa (g) content in 100 g	Portion size	Cocoa (g) per portion
Breakfast cereals with chocolate	5.55 g	35 g	1.94 g
Muesli cereals with chocolate	4.88 g	35 g	1.71 g
Chocolate/cocoa powder (to add to milk)	22.77 g	20 g	4.55 g
Chocolate soy drink	1.45 g	220 g	3.19 g
Chocolate pudding	1.70 g	100 g	1.70 g
Creamy dessert with chocolate	1.83 g	100 g	1.83 g
Chocolate yogurt	1.17 g	125g	1.46 g
Chocolate ice cream	5.88 g	100 g	5.88 g
Ice cream with chocolate parts	2.80 g	100 g	2.80 g
Chocolate covered pastry (e.g. Palmier)	4.26 g	35 g	1.49 g
Chocolate filled pastry (e.g. Croissants)	5.03 g	35 g	1.76 g
Filled chocolate cookies	5.51 g	21 g	1.16 g
Covered chocolate cookies	12.03 g	21 g	2.53 g
Chocolate cookies	4.66 g	21 g	0.98 g
Chocolate chips cookies	5.52 g	21 g	1.16 g
Chocolate cake	7.17 g	100 g	7.17 g
Cake with chocolate parts	3.20 g	100 g	3.20 g
Chocolate nougat	23.80 g	20 g	4.76 g
Bonbons	33.79 g	20 g	6.76 g
Milk chocolate	29.64 g	20 g	5.93 g
Dark chocolate (<60% cocoa)	50.00 g	20 g	10.00 g
Dark chocolate bars (60 - 70% cocoa)	69.00 g	20 g	13.80 g
Dark chocolate bars (70-85% cocoa)	72.13 g	20 g	14.43 g
Dark chocolate bars (>85% cocoa)	89.75 g	20 g	17.95 g
Chocolate milkshake	1.23 g	220 g	2.71 g
Hot chocolate	25.30 g	38 g	9.61 g
Chocolate spreads (e.g. Nutella)	7.18 g	20 g	1.44 g

3.7. ASSOCIATION BETWEEN COCOA CONSUMPTION, LIFESTYLE, HEALTH STATUS AND IMMUNE FUNCTION

After the validation process, the FFQ questionnaires were applied to new participants achieving a total of 270 individuals from both Universities. In all cases, in addition to the cocoa consumption, participants also answered some questions about physical activity, lifestyle and health status, through different sections of questionnaires.

3.7.1. PHYSICAL ACTIVITY QUESTIONNAIRE

Physical activity was assessed through the short version of the International Physical Activity Questionnaire (IPAQ). This questionnaire consists in 4 general questions and is available for use in a self-administered way. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data related to health related physical activity. The development of an international measure for physical activity started in Geneva in 1998 and was followed by extensive examination of reliability and validity in 12 countries (14 sites) in 2000. The final results suggest that these measures have acceptable measurement properties for use in different places and in different languages, and are suitable for national population prevalence studies of participation in physical activity.

In our case, we were interested in knowing about the type of physical activity that participants do as part of their daily lives. The questions concern about the time they spent being physically active in the last seven days. Participants were asked to think about the activities they do as part of the work in the garden and in the house, to get from one place to another, and in his spare time off, exercise or sport.

The data obtained from the IPAQ short form included two types of results: categorical and continuous indicators of physical activity.

- The categorical data allow classifying individuals among three categories of physical activity: inactive, minimally inactive and a separate category labelled “HEPA” level, which is a more active category (**Figure 11**). This last one can be computed for people who exceed the minimum public health physical activity recommendations, and are accumulating activity enough for a healthy lifestyle.
- The continuous indicator was presented as median minutes or median metabolic equivalent of task (METs) per minutes. Median values can be computed for walking (W), moderate-intensity activities (M), and vigorous-intensity activities (V). The continuous score is expressed as METs-min/week for each activity (W, M or V), assessed from METs level multiplied per minutes of activity multiplied per events per week. For example, in an individual that do 30 min of W, M and V, 5 times/week:

- Walking (3.3 METs)= 3.3 x 30 x 5 = 495 MET-min/week
- Moderate Intensity (4.0 METs)= 4.0 x 30 x 5 = 600 MET-min/week
- Vigorous Intensity (8.0 METs) = 8.0 x 30 x 5 = 1,200 MET-min/week
- Total MET-min/week = (W METs x min x days) + (M METs x min x days) + (V METs x min x days) = 2,295 MET-min/week

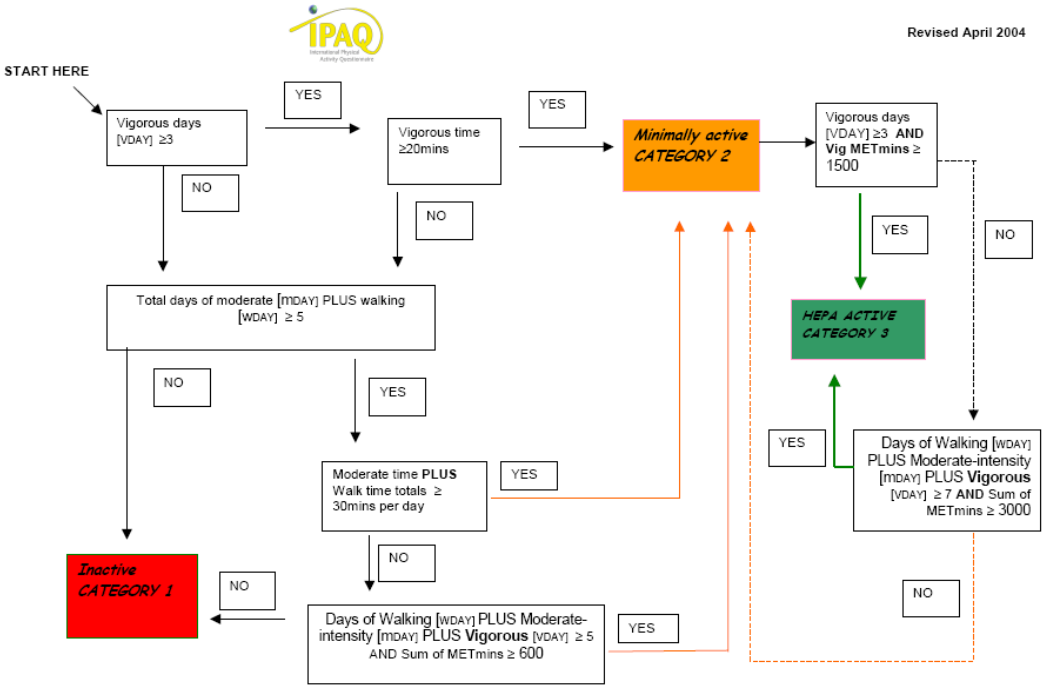

Figure 11: Flow chart algorithm for the analysis of IPAQ short form.

3.7.2. LIFESTYLE AND HEALTH STATUS QUESTIONNAIRE

In addition to the FFQ, all the participants in the three studies (Spanish validation, Portuguese Validation and Main Cohort, N=270) answered to a more complete questionnaire providing information regarding their life style and health status.

The questionnaire, created *ad hoc* for this purpose, was partially based in that used in the "Enquesta nutricional de la població Catalana (ENCAT)" 2003 [314] but in our case it had the following sections (**Appendix 4**):

- section 1: questions related to anthropometrical values.
- section 2: questions about lifestyle habits, the self-evaluation of health status, usual medication and nutritional supplements.
- section 3: questions about the immune function assessment, including the presence of allergies and intolerances as well as the frequency of symptoms of this phenomenon.

Anthropometrical values included weight and height. The second section included one question about the self-evaluation of health status, two questions about tobacco behaviour, a question about blood pressure, a list of chronic diseases to select as well as the days having fever or being in bed in the last month. Moreover, they were asked to select from a list of 24 different drugs used in the last week and the person prescribing it was also inquired.

As it seems that cocoa has immunomodulator activity and may have a role in allergy prevention, thus third section was devoted to ask about infections and allergies. For example, the questionnaire included questions about having the common cold in the last period, if they have any allergies and if they require any special drug treatment for that (**Appendix 4**).

3.7.3 CORRELATION BETWEEN COCOA CONSUMPTION, PHYSICAL ACTIVITY, LIFESTYLE AND HEALTH STATUS INDICATORS

The cocoa intake was estimated for the 270 participants using the same methodology as in the previous steps in the Validation process.

After finishing the individual total cocoa intake assessment, the 270 individuals sample was divided into three groups according to cocoa consumption. The first group included the individuals ingesting less than 7 g of cocoa daily, the second group referred a consumption within 7 and 15 g and the third group included individuals consuming the highest proportion (>15g). After this, the next goal was to evaluate if there was any association with lifestyle and health status variables.

Firstly, a descriptive statistics analysis was conducted for continuous variables such as weight, height, body mass index, total METs/week, sitting time and blood pressure. Additionally, as previously referred, the health status questionnaire also included questions referring to the duration of fever symptoms, days feeling sick and the frequency of allergy symptoms. These were also analysed as continuous variables. In all the cases, after assessing normality through Kolmogorov-Smirnov test, the Spearman correlation analysis was applied to establish correlations between cocoa and the variables. The paired sample Wilcoxon test was also applied to establish differences between groups.

The other variables, considered as categorical, were analysed through a most detailed methodology. Specific categories were chosen to each variable in order to classify individuals according to each one, in “yes” or “no”. For example, as presented in **Table 9**, individuals were classified according to gender, lifestyle (living with own family or flatmates), if they were working or not and if they were working more than 10 hours/week. The frequency of each categorical variable was assessed in the three groups of cocoa consumption and the chi-square test was conducted in order to establish if there were significant differences in these frequencies. Additionally, the linear by linear association was established in order to evaluate if there was a tendency between the three groups for each variable.

Table 9: Categories of the studied variables used to classify population of study.

General Variable	Categories
Gender	Female Male
Lifestyle	Living alone Living with family (parents, brothers, etc.) Living with own family Living with flatmates
Working	Yes /No
Working more than 10h/week	
Physical activity	Low active Moderate active Highly active
Body mass index	Normal weight Underweight Excessive weight
Health status evaluation	Good to excellent health Average or bad health
Smoking	Yes No Previously smoking Never smoked
High blood pressure	Yes No
Health related Variable	**Categories**
Existence of chronic disease	
To have had flu...	
To have felt sick ...	
To have had diarrhoea ...	
To have any type of allergy/intolerance...	Yes
To have an allergy...	No
To have intolerance...	
Type of symptoms ... (oropharynge, respiratory, gastrointestinal, etc.)	
To have missed work sessions due to allergies...	

3.8. STATISTIC ANALYSES

The statistical analysis included different procedures according to the nature of each variable.

In the sociodemographic information, variables such as age, weight and height have been analysed through descriptive statistics, mean and standard deviation of the mean have been calculated, as well as minimum and maximum values have been established for each category.

The consumption frequencies obtained through the FFQ applied (C-FFQ and P-FFQ), EFSA-Q and 24HDR, firstly the normality test Kolmogorov-Smirnov has been applied in order to test if data followed a normal distribution. Results from C-FFQ/P-FFQ have been

compared to EFSA-Q and 24HDR through the Wilcoxon test. The level of statistical significance was 95%. In addition, the Spearman rank correlation test has been conducted to evaluate the relationship between data from consumption frequencies according the developed FFQ and each one of the standards (24HDR or EFSA-Q).

The level of agreement has been also evaluated through Bland Altman plots after assessing the mean and the difference of C-FFQ/P-FFQ and EFSA/24HDR values.

To establish an association of cocoa intake with variables, the chi-square test has been applied in order to evaluate if there was a linear by linear association between the three tertiles of cocoa intake for each continuous variable. This test has been performed considering a level of significance of 95%.

Statistical analysis was performed using PASW Statistics version 18.

The statistical analysis has been verified by the Dr. Joan Salvador Vila Domènech from the Institut Hospital del Mar d'Investigació Mèdica (IMIM), specialist in statistics applied to medical and health sciences.

4. RESULTS

As described in the Material and Methods section, the present work has been conducted in three phases.

Therefore, this results section describes:

- The validation of the Spanish version of the developed FFQ (C-FFQ)
- The validation of the Portuguese version of the same questionnaire (P-FFQ)
- The application of C-FFQ and P-FFQ to a larger sample in addition to a physical activity (IPAQ-Q) and health status assessment questionnaire, and the relationship between variables.

4.1. VALIDATION OF THE C-FFQ

Firstly, the FFQ in Spanish (C-FFQ) was developed and validated. This part was conducted in the Faculty of Pharmacy at the University of Barcelona between April and June 2014.

4.1.1. GENERAL PARTICIPANT CHARACTERISTICS

A sample of 50 individuals completed the three questionnaires (C-FFQ, EFSA-Q and 24HDR). The **Table 10** shows the general characteristics of the study participants.

The participants comprised 8 males and 42 females, a representative gender proportion in the health sciences graduation programmes considered (1:5). Within this population, 56% were undergraduate students and 42% were enrolled in one of the master's or PhD studies within the several University of Barcelona programmes.

Participants were on average 24.10 ± 3.29 (mean ± SDM) years old; the youngest was 20 years old and the oldest 31 years old. The female undergraduate students were in average 22 years old and the graduate female students were in average 27 years old.

The anthropometric data demonstrate that most undergraduate and postgraduate students had normal weight values, with a BMI of 21.42 ± 2.27 kg/m^2. However, 4 students had BMI under 18.5 kg/m^2 and 6 participants had a BMI higher than 25 kg/m^2 showing overweight.

Most of the participants were single (86%) and a minority was married or living with their partner. A 63% of the undergraduate students and a 56% of the postgraduate ones were living with their family (60% of total population), whereas 26% of the undergraduate and 39% of the postgraduate students were living with flatmates (32% of total population). Only a few individuals were living in University residence facilities (4%) and were undergraduate students.

Table 10: Demographic characteristics of participants who completed the C-FFQ, EFSA-Q and 24HDR. Values are expressed as mean ± SDM, as well as the range and proportion for anthropometrical and demographic data, respectively.

	Undergraduate students		Graduate students		Total
	Men (N=7)	Women (N=20)	Men (N=1)	Women (N=22)	
Age (y)	22.29 ± 0.49	21.55 ± 1.99	24	27.00 ± 2.35	24.10 ± 3.29
range	[22–23]	[20–27]		[24–31]	[20–31]
Weight (kg)	69.93 ± 6.11	56.40 ± 6.25	64	59.20 ± 9.17	59.68 ± 8.70
range	[64–80]	[47–75]		[41–80]	[41–80]
Height (m)	1.75 ± 0.03	1.65± 0.05	1.73	1.65 ± 0.07	1.67 ± 0.07
range	[1.70–1.79]	[1.54–1.76]		[1.59–1.80]	[1.48–1.80]
BMI (kg/m^2)	22.79 ± 1.83	20.69 ± 1.92	21.38	21.65 ± 2.55	21.42 ± 2.27
range	[20.52–25.36]	[17.69–24.92]		[18.43–27.68]	[17.69–27.68]
Residential status, %					
- alone	0 %	2 %	0 %	2 %	4 %
- with family	12 %	22 %	0 %	26 %	60 %
- with flat mates	2 %	12 %	2 %	16 %	32 %
- in residence	0 %	4 %	0 %	0 %	4 %

4.1.2. ANALYSIS OF THE CONSUMPTION FREQUENCY

The consumption frequencies for tea, coffee, hot chocolate, chocolate bars and chocolate snacks were considered to validate the C-FFQ. The average consumption for these foods is summarized in **Tables 11** (C-FFQ and EFSA-Q) and **12** (C-FFQ and 24HDR).

For food groups, the consumption frequency of tea was about 2 cups/week when asked by both the C-FFQ and the EFSA-Q (**Table 11**). However, the 24HDR revealed a lower intake ($p<0.05$) (**Table 12**): less than one cup/week. Concerning coffee, the C-FFQ provided lower overall coffee consumption frequency than the EFSA-Q ($p<0.05$), whereas the 24HDR revealed a lower frequency of coffee consumption (i.e. a cup every 2 days) than the C-FFQ ($p<0.05$).

Focusing on cocoa- and chocolate-derived products, the results obtained from the three methods differed more substantially. The C-FFQ detected a lower consumption of hot chocolate than the EFSA-Q ($p<0.05$) (**Table 11**), whereas the intake frequency of chocolate snacks and some type of bars according to the C-FFQ was higher than the values from the EFSA-Q ($p<0.05$). University students consumed one chocolate snack every 3 days according to the C-FFQ whereas they consumed one per week based on the EFSA-Q results. When the C-FFQ was compared with the 24HDR (**Table 12**), a lower hot chocolate consumption and a similar consumption frequency of chocolate snacks were obtained. Nevertheless, the 24HDR revealed a lower chocolate bar intake ($p<0.05$).

The C-FFQ also evaluated the particular consumption of chocolate/cocoa in dairy, pastry, dessert, cereal and spread products ("Others" in **Table 12**), which were not included in the EFSA-Q. The results obtained showed that University students ate at least one of these products per day. As the 24HDR includes open questions, this type of chocolate

consumption is also covered, although it showed a significantly lower consumption of these products than the C-FFQ ($p<0.05$).

Table 11: Consumption frequency of a portion (times/day) of foods of interest estimated by the C-FFQ and EFSA-Q (mean values and standard deviations of the mean)

	C-FFQ		EFSA-Q	
	Mean	SDM	Mean	SDM
Tea	0.3364	0.4913	0.2955	0.3147
Coffee	0.8616	0.6211	1.1800*	1.0631
Cocoa/chocolate-derived products:				
1. Hot chocolate	0.0142	0.0175	0.0860*	0.2115
2. Chocolate snacks	0.3228	0.2678	0.1429*	0.1384
3. Chocolate bars:	0.8533	1.1915	0.7143	0.8537
3.1. White	0.0670	0.1296	0.0000*	0.0000
3.2. Milk	0.2565	0.3926	0.5000	0.8688
3.3. Dark	0.5298	1.1002	0.2143*	0.4389
Total 1+2+3	**1.1903**	**1.2979**	**0.9431***	**0.9595**

* Significant differences ($p<0.05$) when compared to C-FFQ data of the product in the same row by using the Wilcoxon paired test.

Table 12: Consumption frequency of a portion (times/day) of foods of interest estimated by the C-FFQ and 24HDR (mean values and standard deviations of the mean)

	C-FFQ		24HDR	
	Mean	SDM	Mean	SDM
Tea	0.3364	0.4913	0.1200*	0.3283
Coffee	0.8616	0.6211	0.4600*	0.6764
Cocoa/chocolate-derived products:				
1. Hot chocolate	0.0142	0.0175	0.0000*	0.0000
2. Chocolate snacks	0.3228	0.2678	0.3200	0.6207
3. Chocolate bars:	0.8533	1.1915	0.5000*	2.1452
3.1. White	0.0670	0.1296	0.0000*	0.0000
3.2. Milk	0.2565	0.3926	0.4400*	2.1348
3.3. Dark	0.5298	1.1002	0.0600*	0.3136
Total 1+2+3	**1.1903**	**1.2979**	**0.8200***	**2.1917**
4. Others:	1.3468	1.4903	0.7600*	1.0606
4.1. Dairy	0.6103	1.1278	0.3800*	0.7796
4.2. Pastries	0.2850	0.3431	0.0800*	0.2740
4.3. Desserts	0.1450	0.2890	0.0600*	0.2399
4.4. Cereals	0.2077	0.3821	0.1600*	0.4219
4.5. Spreads	0.0990	0.1871	0.0800*	0.2740
Total with others (1+2+3+4)	**2.5372**	**2.2500**	**1.5800***	**2.6349**

* Significant differences ($p<0.05$) when compared to C-FFQ data of the product in the same row by using the Wilcoxon paired test.

Overall, when considering the consumption of only hot chocolate, chocolate snacks and chocolate bars, its average consumption frequency obtained by the C-FFQ (1.1903) was higher than that of the EFSA-Q (0.9431) (**Table 11**) and 24HDR (0.8200) (**Table 12**) ($p<0.05$). However, the C-FFQ and the 24HDR enable the consumption of other chocolate food products to be estimated, and therefore, the consumption frequency of any type of cocoa product were twice higher than the above consumption estimation (2.5372 and 1.5800 for the C-FFQ and 24HDR, respectively).

4.1.3. RELATIONSHIP BETWEEN THE C-FFQ, EFSA-Q AND 24HDR

To explore the validity of the C-FFQ, we compared the obtained results to those from the EFSA-Q and the 24HDR in terms of Spearman's correlation coefficients of the frequency consumption obtained in each questionnaire.

Considering the results from the C-FFQ and the EFSA-Q (**Table 13**), weak to moderate positive correlations were found for all the considered common elements ($p<0.05$). Particularly, a strong correlation was found for tea consumption (0.841) and a moderate correlation was found for coffee (0.649). In chocolate/cocoa products, correlations were positive but not so strong, i.e., moderate correlations were found for chocolate snacks (0.479), chocolate milk bars (0.429) and dark chocolate bars (0.569). The correlation of the total frequency consumption of cocoa/chocolate between the EFSA-Q and C-FFQ results was calculated, and a low positive correlation was found (0.281).

Table 13: Correlations (Spearman's coefficient, ρ) between the food consumption frequency obtained from the C-FFQ and EFSA-Q.

	C-FFQ *vs* EFSA-Q	
	Spearman's coefficient (ρ)	p
Tea	0.841	0.000
Coffee	0.649	0.000
Cocoa/chocolate-derived products		
1. Hot chocolate	0.341	0.015
2. Chocolate snacks	0.479	0.000
3. Chocolate bars:	0.330	0.019
3.1. White	n.d.*	
3.2. Milk	0.429	0.002
3.3. Dark	0.569	0.000
Total C-FFQ (1-3) vs Q (1-3)	**0.281**	**0.048**

*n.d.: not possible to determine because values are equal to zero

Concerning the correlation between the C-FFQ and the 24HDR, similar significant results were found (**Table 14**). Correlations were higher in coffee than in tea and cocoa/chocolate-derived products. The consumption frequencies in both assessments

correlated significantly ($p<0.05$) when considering chocolate snacks, milk and dark chocolate bars and dairy products. However, the correlation in other products containing cocoa such as pastries, desserts, cereals and spreads did not achieve statistical significance.

Table 14: Correlations (Spearman's coefficient, ρ) between the food consumption frequency obtained from the C-FFQ and 24HDR.

	C-FFQ *vs* 24HDR	
	Spearman's coefficient (ρ)	p
Tea	0.328	0.020
Coffee	0.653	0.000
Cocoa/chocolate-derived products		
1. Hot chocolate	n.d.*	
2. Chocolate snacks	0.320	0.023
3. Chocolate bars:	0.279	0.050
3.1. White	n.d.*	
3.2. Milk	0.358	0.011
3.3. Dark	0.330	0.017
Total C-FFQ (1-3) *vs* Q (1-3)	**0.086**	**0.551**
4. Others:	0.447	0.001
4.1. Dairy	0.666	0.000
4.2. Pastries	0.216	0.132
4.3. Desserts	0.118	0.416
4.4. Cereals	0.192	0.182
4.5. Spreads	0.228	0.112
Total C-FFQ (1-4) *vs* Q (1-4)	**0. 475**	**0.000**

*n.d.: not possible to determine because values are equal to zero

According to the 24HDR, hot chocolate was not consumed by any participant the day before the assessment and no correlation could be calculated. Considering the total cocoa/chocolate consumption in the C-FFQ and 24HDR, a moderate positive correlation was only found in that consumption including any type of cocoa product (0.475).

The Bland-Altman analysis for main foods studied showed a similar pattern between the tested C-FFQ and the two validating methods (EFSA-Q and 24HDR) (**Figure 12**).

As it can be observed in all plots, the values of the difference in the daily intake frequency was almost zero meaning that no apparent change in the magnitude of between-measurement differences (C-FFQ *vs* EFSA-Q and C-FFQ *vs* 24HDR) appeared across mean values of the methods used. The mean difference of consumption frequency of total cocoa/chocolate products between C-FFQ and EFSA-Q (0.247) was similar to that found in the C-FFQ and 24HDR Bland-Altman plot (0.624).

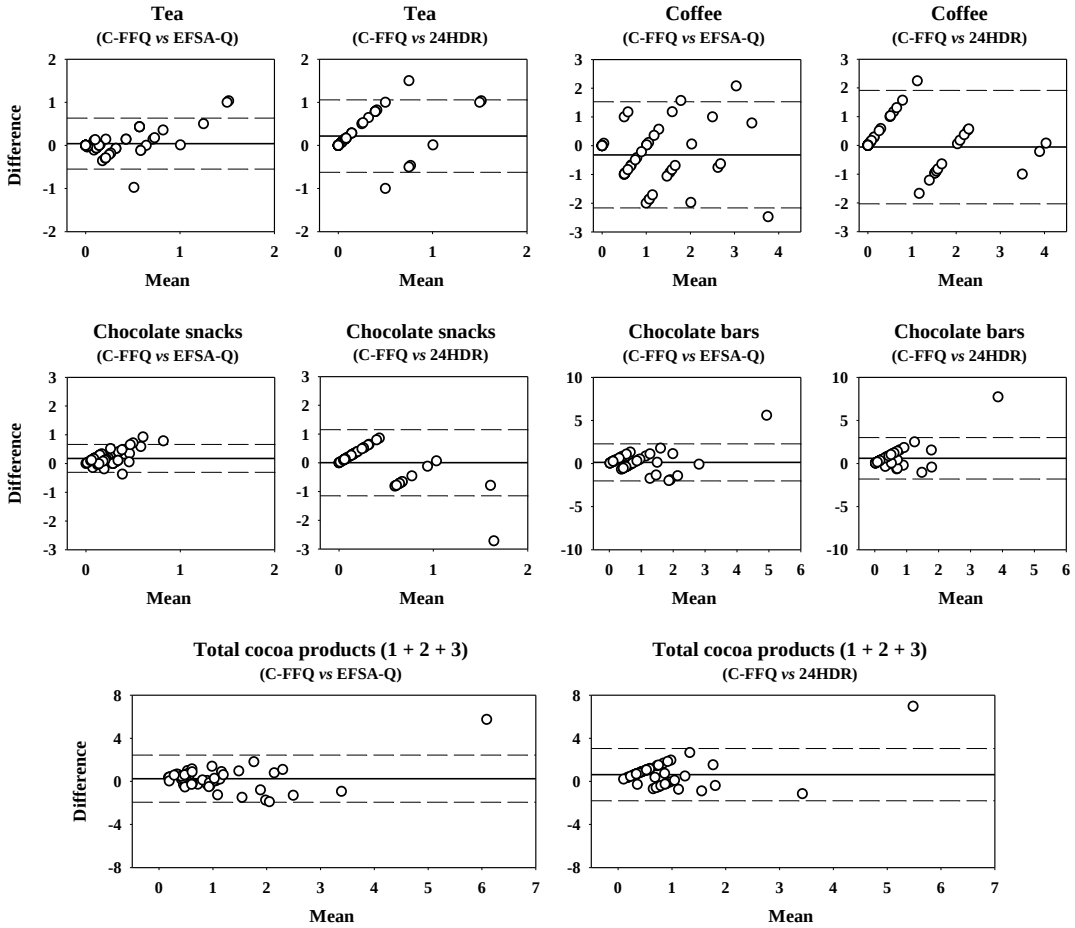

Figure 12: Bland-Altman plots showing the relationship between difference in the daily intake frequency of the issue at the top, and the corresponding mean of the daily intake frequency estimated by either C-FFQ and EFSA-Q or C-FFQ and 24HDR. Solid lines are the mean difference and dashed lines are lower and upper 95% limits of agreement.

4.1.4. SUBJECT-CATEGORIZED CONSUMPTION FOR THE THREE QUESTIONNAIRES

The degree of potential misclassification associated with categorized intakes assessed by the C-FFQ in comparison to the EFSA-Q (**Table 15**) or the 24HDR (**Table 16**) was examined as the proportion of participants classified in the same or adjacent quintile or grossly classified.

When considering percentile classification for the C-FFQ and the EFSA-Q (**Table 15**), the majority of the individuals were classified in the same/adjacent quintile, ranging from 68% for total chocolate bars to 98% for dark chocolate bars. More than 70% participants were in the same quintile for dark chocolate bars. Low proportion of grossly classified individuals (<15%) was found for tea (10%), hot chocolate (12%), chocolate snacks (10%) and dark chocolate bars (2%). Although the EFSA-Q did not include the same cocoa product variety, more than 70% participants were in the same/adjacent quintile for all cocoa/chocolate products inquired.

Table 15: Classification of individuals in same quintile, adjacent quintile and grossly classified for each of the foods considered of interest comparing the C-FFQ and the EFSA-Q.

	C-FFQ *vs* EFSA-Q		
	Same quintile	Adjacent quintile	Grossly classified
Tea	56%	34%	10%
Coffee	44%	38%	18%
Cocoa/chocolate-derived products			
1. Hot chocolate	46%	42%	12%
2. Chocolate snacks	36%	54%	10%
3. Chocolate bars:	32%	36%	32%
3.1. White		n.d.*	
3.2. Milk	28%	52%	20%
3.3. Dark	72%	26%	2%
Total C-FFQ vs Q (1-3)	38%	36%	26%

*n.d. not possible to determine because in the EFSA-Q no questions regarding other cocoa/chocolate-derived products were included

When comparing percentile distribution of subjects in the C-FFQ and the 24HDR (**Table 16**), similar results were obtained.

Table 16: Classification of individuals in same or adjacent quintile, and grossly classified for each of the foods considered of interest considered of interest comparing the C-FFQ and the 24HDR.

	C-FFQ *vs* 24HDR		
	Same quintile	Adjacent quintile	Grossly classified
Tea	28%	30%	42%
Coffee	46%	34%	20%
Cocoa/chocolate-derived products			
1. Hot chocolate		n.d.*	
2. Chocolate snacks	46%	30%	24%
3. Chocolate bars:	32%	34%	34%
3.1. White		n.d.*	
3.2. Milk	30%	46%	24%
3.3. Dark	76%	22%	2%)
Total C-FFQ (1-3) vs Q (1-3)	24%	38%	38%
4. Others:	48%	42%	10%
4.1. Dairy	92%	8%	0%
4.2. Pastries	84%	12%	4%
4.3. Desserts	94%	4%	2%
4.4. Cereals	78%	18%	4%
4.5. Spreads	88%	10%	2%
Total C-FFQ (1-4) vs Q (1-4)	34%	32%	34%

*n.d. not possible to determine because its consumption was not reported in the 24HDR.

The highest value for the same/adjacent quintile was 98% for dark chocolate bars and the lowest 58% for tea. The C-FFQ questionnaire included a large variety of chocolate products that was not considered in the EFSA-Q but could be referred to in dietary recalls. Within these other chocolate/cocoa products, it is important to point out that the proportion of grossly classified individuals was very low. Particularly, for dairy products, 92% of the sample was in the same quintile and the remaining 8% was in the adjacent quintile; moreover, for desserts, 94% students were also in the same quintile and 4% in the adjacent quintile.

4.1.5. CONSUMPTION OF COCOA/CHOCOLATE PRODUCTS ACCORDING THE C-FFQ IN 50 STUDENTS SAMPLE

Based on the data from the C-FFQ we further studied the frequencies of consumption referred to each quintile and the distribution of the 50 participants among three consumption categories of cocoa/chocolate products.

The consumption frequency of each cocoa/chocolate product family can be more easily examined graphically by using box plot representations considering the consumption frequency in each quintile classification (**Figure 13**). In general, these data showed that, logically, the consumption frequency (product/day) increased with the quintile studied, with clear differences among the five percentiles in the main categories studied. In most of the cocoa/chocolate products, the degree of dispersion for each quintile was very low, and the outliers, plotted as individual points, were much closer to the box data. Only in data concerning chocolate snacks, pastries and cereals, more spread data can be observed.

The consumption frequency observed for the participants in the lowest quintile was about 25 times lower than that obtained for the students when grouped in the highest quintile for chocolate bars. This ratio was about 10-15 times for chocolate snacks and other chocolate products.

Focusing on the subcategories included in the “other chocolate products”, it can be seen that only in the case of dairy products there was an increasing consumption frequency among the quintiles, whereas for chocolate desserts, pastries, cereals and spreads the four lowest quintiles had similar values, and differed from those in the highest quintile.

On the other hand, we distributed the participants among three categories of consumers: “high cocoa consumers” (i.e. eating more than or equal to one cocoa/chocolate product), “low cocoa consumers” (i.e. eating less than one cocoa/chocolate product) and “non-consumers”, expressed per week or day depending on the type of product. More than one snack per day or one bar per week was considered as high consumption (**Table 17**).

None of the participants was classified as a high consumer for hot chocolate as well as for spreads (no one consumed hot chocolate at least once per week and no one consumed spread per day). However, 42% of students were classified as high consumers of chocolate bars because they ate at least one 100 g bar per week. The number of high

consumers was higher for dark chocolate bars (24%) than for other bar types (2-14%), and it was similar to chocolate snacks high consumers (28%).

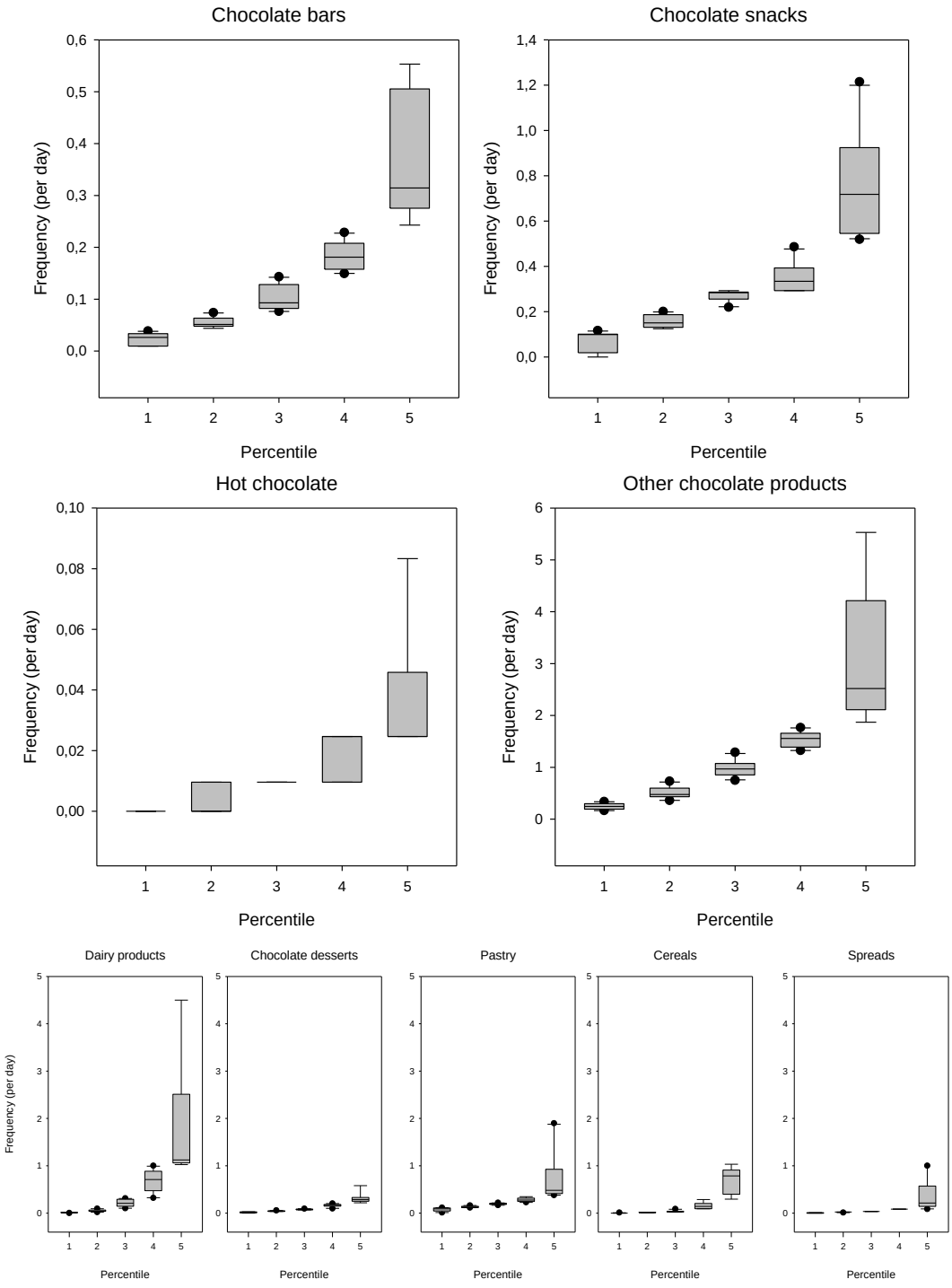

Figure 13: Classification of consumption frequencies (product/day) referred to in each quintile (percentiles 1-5) for the different cocoa/chocolate products studied.

Table 17: Classification of individuals regarding their consumption frequency for each of the foods considered of interest in the C-FFQ in three categories: high consumers, low consumers and non-consumers

	High consumers (≥1)	Low consumers (<1)	Non-consumers (0)
Hot chocolate (product/wk)	0%	68%	32%
Chocolate bars (product/wk):	42%	58%	0%
- White	2%	46%	52%
- Milk	14%	72%	14%
- Dark	24%	62%	30%
Chocolate snacks (product/day)	28%	68%	4%
Others (product/day):	48%	52%	0%
- Dairy	20%	78%	2%
- Pastries	4%	96%	0%
- Desserts	2%	94%	4%
- Cereals	4%	78%	18%
- Spreads	0%	88%	12%

In addition, almost a half of the University students enrolled declared that they consumed a cocoa product classified as "others" per day, with dairy products being the most popular choice (20%). In this category, 18% students stated not to eat chocolate cereals, 12% students did not eat spreads and less than 5% recognized that they did not eat dairy products and desserts. Nobody said that never consumed chocolate pastries.

Only in three product categories the percentage of non-consumers was higher than or equal to 30% (hot chocolate, white chocolate bars and dark chocolate bars), but no one involved in the study declared that they never consumed chocolate bars.

4.1.6. CONSUMPTION DISTRIBUTION OF COCOA/CHOCOLATE PRODUCTS

In addition, we estimated the cocoa intake per day of each of the 50 Spanish participants by considering the amount of cocoa per portion (**Table 8**) within each inquired item in the C-FFQ (**Figure 14**).

The cocoa consumption average per day in the studied population was about 12 g. Chocolate bars mainly provided this amount (55%) but dairy products were also important (23%). Hot chocolate and spreads were the products that less contributed in cocoa consumption. It was not only due to their low content of cocoa, but also because the reported low consumption frequency.

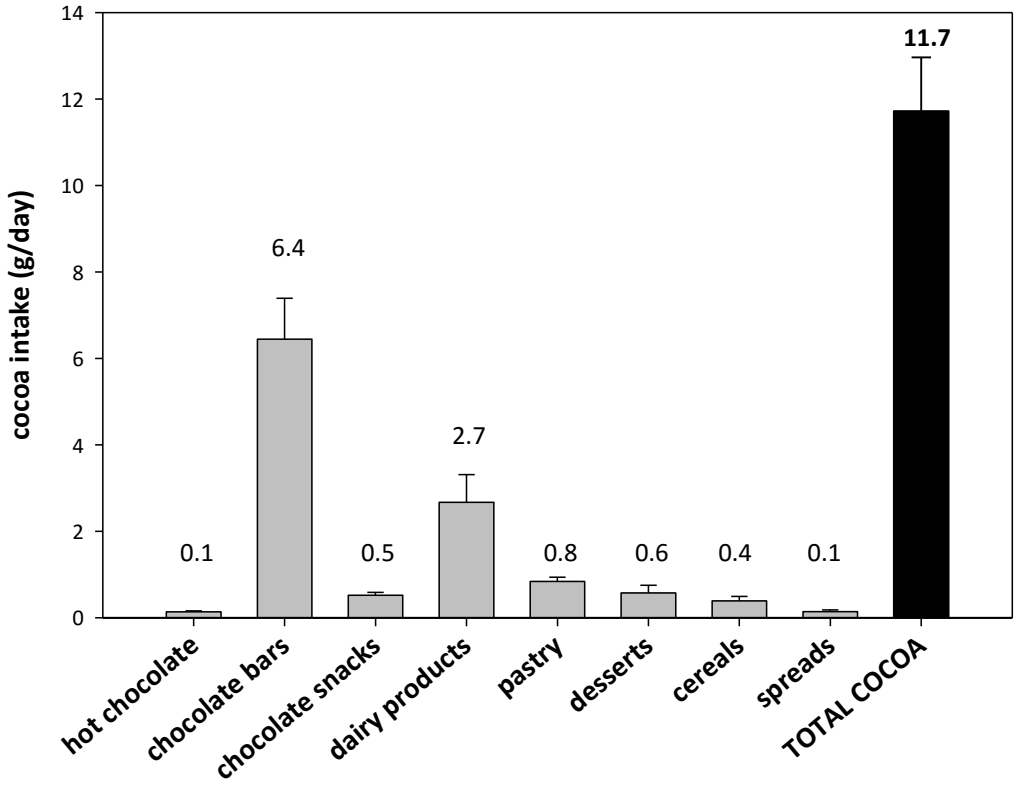

Figure 14: Estimation of cocoa intake (g/day) according to the sources included in C-FFQ. Results are expressed as mean ± standard error of the mean (N=50).

4.2. VALIDATION OF THE P-FFQ

After the validation of C-FFQ in the first cohort with students from the University of Barcelona, the C-FFQ was translated to Portuguese and it was applied to students from Egas Moniz Health Sciences Institute in order to also validate this FFQ in this context.

4.2.1. GENERAL PARTICIPANT CHARACTERISTICS

A sample of 70 individuals completed the two food frequency questionnaires (P-FFQ, EFSA-Q) and three 24HDR (one the same day of FFQs and the other two separated two weeks). The **Table 18** shows the general characteristics of the study participants.

Most of the participant consisted in women (70%) and undergraduate students (80%). The undergraduate students belong to the Nutrition, Pharmacy, Dentistry, Nursery and Health sciences studies, whereas graduate students were enrolled in Clinical nutrition, Sports nutrition, Dentistry and Pharmacy post-graduate programmes.

Participants were on average 24.74 ± 3.00 years old (mean ± SDM); the youngest was 19 years old and the oldest 32 years old. These values were very similar to those from the first cohort studied (Section 4.1.1).

Table 18: Demographic characteristics of participants who completed the P-FFQ, EFSA-Q and 24HDR. Values are expressed as mean ± SDM, as well as the range and proportion for anthropometrical and demographic data, respectively.

	Undergraduate students		Graduate students		
	Men (N=19)	Women (N=37)	Men (N=2)	Women (N=12)	Total
Age (y)	21.61 ± 3.29	20.97 ± 2.63	23.00 ± 0.00	24.33 ± 2.53	24.74 ± 3.00
range	[19-32]	[19-30]	[23–23]	[21–31]	[19–32]
Weight (kg)	65.21 ± 8.28	59.35 ± 11.25	77.00 ± 3.00	60.92 ± 6.40	62.02 ± 10.66
range	[52–87]	[35–100]	[74–80]	[51–75]	[35–100]
Height (m)	1.72 ± 0.08	1.65 ± 0.07	1.75 ± 0.02	1.67 ± 0.06	1.68 ± 0.08
range	[1.73–1.87]	[1.46–1.81]	[1.73–1.76]	[1.62–1.82]	[1.46–1.87]
BMI (kg/m^2)	22.10 ± 2.47	21.13 ± 4.63	25.28 ± 0.55	21.92 ± 1.93	22.04 ± 2.81
range	[17.8–29.1]	[16.4–32.6]	[24.7–25.6]	[17.8–26.3]	[16.4–32.6]
Residential status, %:					
- alone	5.7 %	2.9 %	0 %	0 %	8.6 %
- with family	18.6 %	45.7 %	2.9 %	12.9 %	75.7 %
- with flat mates	1.4 %	1.4 %	0 %	4.3 %	7.1 %
- in residence	1.4 %	2.9 %	0 %	0 %	4.3 %

The anthropometric data demonstrates that most Portuguese participants showed normal body weight data, with BMI of 22.04 ± 2.81 kg/m^2 on average; however, there

were 7 individuals with a BMI ranging between 25 and 30 kg/m^2, which is considered overweight, and 3 with a BMI under 18.5 kg/m^2.

An 80% of the undergraduate students and a 78% of the graduate ones were living with their family (76% of total population). Only a few individuals were living with flatmates (3.6% undergraduate and 21% graduate students), whereas 4% of whole students were living in University residence facilities.

4.2.2. ANALYSIS OF THE CONSUMPTION FREQUENCY

The consumption frequencies for chocolate and cocoa products estimated by P-FFQ and EFSA-Q are summarised in **Table 19.** The consumption frequencies for chocolate and cocoa products estimated by P-FFQ and the mean values of the three 24HDRs can be found in **Table 20**.

Table 19: Consumption frequency of a portion (times/day) of foods of interest estimated by P-FFQ and EFSA-Q (mean ± standard deviations of the mean).

	P-FFQ	EFSA-Q
1. Hot chocolate	0.088 ± 0.177	0.106 ± 0.224
2. Chocolate snacks	0.919 ± 1.980	0.312 ± 0.244*
3. Chocolate bars	0.769 ± 0.479	0.939 ± 0.750
3.1. White	0.121 ± 0.209	0.031 ± 0.188*
3.2. Milk	0.240 ± 0.241	0.602 ± 0.711*
3.3. Dark	0.407 ± 0.401	0.306 ± 0.668*
Total 1+2+3	1.775 ± 2.169	1.357 ± 0.889

* Significant differences have been found when compared to P-FFQ ($p<0.05$) in the Wilcoxon paired samples test

In general, the developed P-FFQ showed a higher frequency of cocoa/chocolate products than the EFSA-Q and 24HDR ($p<0.05$) (**Table 19**).

Focusing in each product, the consumption of hot chocolate, as detected in the first cohort, was very low: the students drank a cup of hot chocolate every 10 days according P-FFQ and EFSA-Q. However, University students consumed almost one chocolate snack per day according to the P-FFQ whereas they consumed one every 2-3 days based on the EFSA-Q. On the other hand, the total consumption of chocolate bars was similar between the P-FFQ and EFSA-Q, but both questionnaires did not agree regarding the type of bars which was the most consumed. Overall, the consumption frequency of a cocoa/chocolate product considered by the EFSA-Q was 1.4 portions per day, whereas according to the P-FFQ the frequency of consumption achieved 1.8 cocoa/chocolate pieces per day. There were no significant differences between both results.

When comparing the P-FFQ with the average of the three 24HDR, results were quite similar (**Table 20**). The P-FFQ showed higher consumption frequency for all the cocoa/chocolate products considered with the exception of hot chocolate, that was consumed every 5 days according the 24HDR and every 10 days according the P-FFQ, but these results were not statistically different. On the other hand, the 24HDR revealed a lower chocolate snacks and bar intake ($p<0.05$).

Table 20: Consumption frequency of a portion (times/day) of foods of interest estimated by C-FFQ and 24HDR (mean ± standard deviations of the mean).

	P-FFQ	24HDR[1]
1. Hot chocolate	0.088 ± 0.177	0.214 ± 0.361
2. Chocolate snacks	0.919 ± 1.980	0.243 ± 0.534*
3. Chocolate bars	0.769 ± 0.479	0.041 ± 0.113*
3.1. White	0.121 ± 0.209	0.000 ± 0.000*
3.2. Milk	0.240 ± 0.241	0.035 ± 0.108*
3.3. Dark	0.407 ± 0.401	0.007 ± 0.032*
Total 1+2+3	1.775 ± 2.169	0.499 ± 0.650*
4. Others	1.397 ± 1.585	0.321 ± 0.504*
4.1. Cereals	0.217 ± 0.369	0.048 ± 0.197*
4.2. Dairy	0.295 ± 0.451	0.143 ± 0.372*
4.3. Desserts	0.211 ± 0.487	0.010 ± 0.056*
4.4. Pastry	0.542 ± 0.840	0.098 ± 0.240*
4.5 Spreads	0.132 ± 0.315	0.024 ± 0.103*
Total with others (1+2+3+4)	**3.172 ± 3.470**	**0.820 ± 0.871***

[1]Average of the three 24HDR conducted in the presented study

* Significant differences have been found when compared to P-FFQ ($p<0.05$) in the Wilcoxon paired samples test

The 24HDR did allow to compare results regarding cereal, dairy, dessert, pastry and spread products ("Others" in **Table 20**), which were not included in the EFSA-Q. The results obtained showed that university students ate more than one of these products per day according the P-FFQ. The 24HDR results indicated a significantly lower consumption of these other cocoa/chocolate products ($p<0.05$).

Overall, and similarly as observed in the first cohort of 50 students from the University of Barcelona (Section 4.1.2.), the consumption average for the P-FFQ (3.172 portions/day) was higher that the 24HDR (0.820 portions/day) ($p<0.05$), and the consideration of "Other products" doubled the values of consumption frequency of cocoa/chocolate products.

4.2.3. Relationship between the P-FFQ, EFSA-Q and 24HDR

To explore the validity of the P-FFQ, we compared the results obtained to those by the EFSA-Q and the 24HDR in terms of Spearman's correlation coefficients of the frequency consumption obtained in each questionnaire.

Considering the results from the P-FFQ and the EFSA-Q, we found significant positive correlations between most these results ($p<0.05$) (**Table 21**). There were a very strong correlation for chocolate snacks consumption (0.932) and moderate correlations for hot chocolate (0.573) and chocolate milk bars (0.555). When total cocoa/chocolate products consumption frequency was considered, the correlation analysis has also proven to be positive with data from EFSA-Q (0.638).

Table 21: Correlations (Spearman's coefficient, ρ) between the food consumption frequency obtained from the P-FFQ and EFSA-Q

	P-FFQ *vs* EFSA-Q Spearman's coefficient (ρ)	p
1. Hot chocolate	0.573	0.000
2. Chocolate snacks	0.932	0.000
3. Chocolate bars:	0.499	0.000
3.1. White	-0.054	0.657
3.2. Milk	0.555	0.000
3.3. Dark	0.210	0.081
Total P-FFQ (1-3) *vs* EFSA-Q (1-3)	**0.638**	**0.000**

Considering the correlation between P-FFQ and 24HDR, weaker correlations were found (**Table 22**). The highest correlation coefficients were found for chocolate snacks (0.321), chocolate milk bars (0.353) and dairy products (0.333). However, the correlations for other products containing cocoa such as hot chocolate, dark chocolate bars, pastries, desserts and cereals did not achieve statistical significance.

Table 22: Correlations (Spearman's coefficient, ρ) between the food consumption frequency obtained from the P-FFQ and 24HDR.

	P-FFQ *vs* 24HDR[1]	
	Spearman's coefficient (ρ)	p
1. Hot chocolate	0.228	0.057
2. Chocolate snacks	0.321	0.007
3. Chocolate bars:	0.133	0.271
3.1. White	n.d.*	
3.2. Milk	0.353	0.003
3.3. Dark	0.133	0.272
Total P-FFQ (1-3) vs 24HDR (1-3)	**0.197**	**0.102**
4. Others:	0.170	0.160
4.1. Dairy	0.333	0.005
4.2. Pastries	-0.050	0.682
4.3. Desserts	0.096	0.428
4.4. Cereals	0.229	0.056
4.5. Spreads	0.297	0.013
Total P-FFQ (1-4) vs 24HDR (1-4)	**0.235**	**0.050**

[1]Average of the three 24HDR conducted in the presented study
*n.d.: not possible to determine because values are equal to zero

The agreement between P-FFQ, EFSA-Q and 24HDR was also established by Bland-Altman plots for chocolate snacks and bars, as well as for total cocoa products (**Figure 15**). As it can be observed in all plots, the values of the difference in the daily intake frequency were almost zero meaning that no apparent change in the magnitude of between-measurement (P-FFQ *vs* EFSA-Q and P-FFQ *vs* 24HDR) differences appeared across mean values of the methods used.

The mean difference of consumption frequency of total cocoa/chocolate products between P-FFQ and EFSA-Q (0.418) was lower to that found in the P-FFQ and 24HDR Bland-Altman plots (1.276).

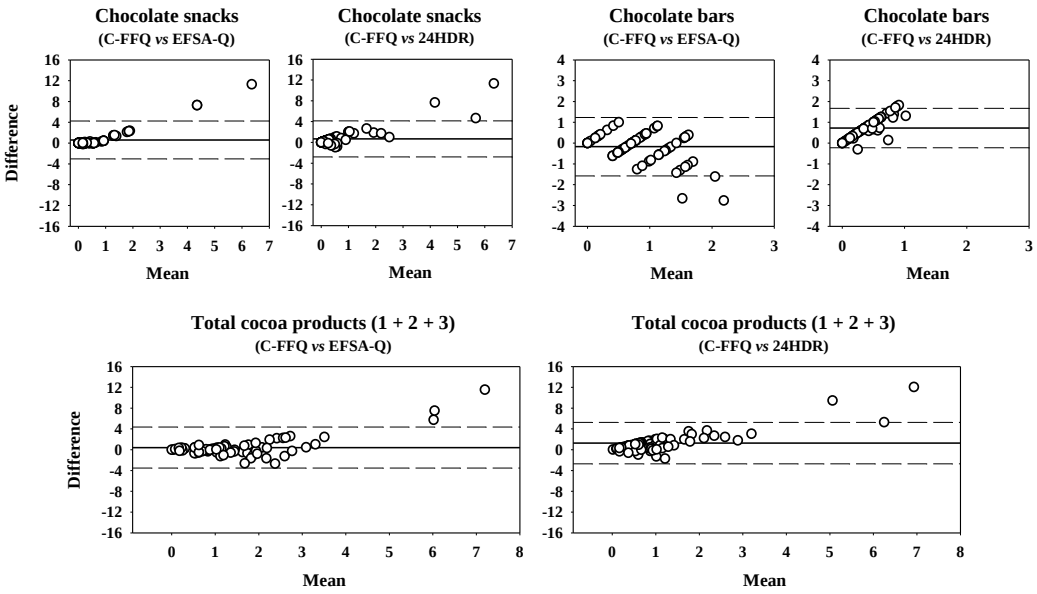

Figure 15: Bland-Altman plots showing the relationship between difference in the daily intake frequency of the issue at the top, and the corresponding mean of the daily intake frequency estimated by either P-FFQ and EFSA-Q or C-FFQ and 24HDR. Solid lines are the mean difference and dashed lines are lower and upper 95% limits of agreement.

4.2.4. SUBJECT-CATEGORIZED CONSUMPTION FOR THE THREE QUESTIONNAIRES

In a similar way to the previously described validation process for the C-FFQ, the individuals from the Egas Moniz Institute were classified into five categories (quintiles) according to cocoa/chocolate product intake assessed by the three questionnaires. The classification obtained with the P-FFQ was compared to the distribution by the EFSA-Q (**Table 23**) and the 24HDR (**Table 24**). The degree of potential misclassification was examined as the proportion of participants classified in the same or adjacent quintile, or grossly classified

When the percentile classification for the P-FFQ and the EFSA-Q was considered (**Table 23**), most individuals were classified into the same/adjacent quintile, ranging from 81% for total consumption to 99% for chocolate snacks. Even more than 90% participants were in the same quintile for chocolate snacks, as well as for white chocolate bars. Half or more than a half of students were in the same quintile for the consumption of hot chocolate, and milk and dark chocolate bars. Although the EFSA-Q did not include the same cocoa product variety, more than 80% participants were in the same/adjacent quintile for all cocoa/chocolate products inquired.

Table 23: Classification of individuals into the same or adjacent quintile, and grossly classified for each of the foods considered of interest when P-FFQ and EFSA-Q are compared.

	P-FFQ *vs* EFSA-Q		
	Same quintile	Adjacent quintile	Grossly classified
1. Hot chocolate	59%	30%	11%
2. Chocolate snacks	93%	5.7%	1.3%
3. Chocolate bars:	47%	37%	16%
3.1. White	90%	7.1%	2.9%
3.2. Milk	50%	34%	15%
3.3. Dark	51%	40%	9%
Total P-FFQ (1-3) *vs* EFSA-Q (1-3)	42%	39%	19%

Table 24: Classification of individuals in same or adjacent quintile, and grossly classified for each of the foods considered of interest when P-FFQ and 24HDR are compared.

	P-FFQ *vs* 24HDR		
	Same quintile	Adjacent quintile	Grossly classified
1. Hot chocolate	37%	43%	20%
2. Chocolate snacks	47%	41%	11%
3. Chocolate bars:	40%	39%	21%
3.1. White		n.d.*	
3.2. Milk	51%	36%	13%
3.3. Dark	49%	43%	8.6%
Total P-FFQ (1-3) *vs* 24HDR (1-3)	23%	35%	43%
4. Others:	26%	49%	26%
4.1. Dairy	86%	11%	2.9%
4.2. Pastries	43%	44%	13%
4.3. Desserts	93%	5.7%	1.4%
4.4. Cereals	93%	5.7%	1.4%
4.5. Spreads	97%	2.9%	0%
Total P-FFQ (1-4) *vs* 24HDR (1-4)	24%	34%	41%

*n.d. not possible to determine because its consumption was not reported in the 24HDR.

The classification of Portuguese students into five groups according the product consumption assessed by P-FFQ and 24HDR was summarized in **Table 24.** P-FFQ questionnaire included a large variety of chocolate products that were not considered in the EFSA-Q but could be referred to in 24HDR. More than 75% participants were distributed in the same/adjacent quintile for hot chocolate, chocolate snacks and chocolate bars (both milk and dark chocolate bars). However, when considering the total cocoa/chocolate consumption supplied by hot chocolate, chocolate snacks and chocolate bars, the percentage of individuals grossly classified was higher than 40%. Nevertheless, particular consumption of others products allow obtaining very low misclassification. More than 95% students were found in the same or adjacent quintile for the consumption of chocolate/cocoa-containing dairy, desserts and cereals.

Moreover, it must be highlighted that for chocolate spreads, all participants were classified into the same or adjacent quintile (**Table 24**).

4.2.5. CONSUMPTION OF COCOA/CHOCOLATE PRODUCTS ACCORDING THE P-FFQ IN 70 STUDENTS SAMPLE

Based on data from the P-FFQ, we further studied, as carried out in the first cohort of students from the University of Barcelona, the frequencies of consumption referred to in each quintile (**Figure 16**), and the 70 participants' distribution in three consumption categories (**Table 25**).

The consumption frequency of each cocoa/chocolate product can be easily observed by using box plot representations that consider the consumption frequencies in each quintile classification carried out as section 4.2.4 (**Figure 16**). In some products, such as hot chocolate, dairy products and spreads, the two lowest quintiles corresponded to non-consumers, but in the other products, logically, the consumption frequency increased with the quintile studied. In the case of chocolate bars consumption, there was low dispersion in each quintile. On the contrary, the highest quintile for the consumption of chocolate snacks, hot chocolate, chocolate desserts and pastry showed a considerable dispersion.

Similarly to the observations from the cocoa/chocolate consumption in the University of Barcelona cohort, for some "Other chocolate products" (dairy products, desserts, pastries, cereals and spreads) the four lowest quintiles had similar values. However, the consumption of chocolate bars was 10 times higher in the highest quintile with respect to that in the lowest quintile. This difference achieved values of more than 30 folds for "Other chocolate products", more than 50 folds for pastry products and more than 150 folds for chocolate snacks.

On the other hand, we distributed the participants as "high cocoa consumers" (i.e. eating more than or equal to one cocoa/chocolate product), "low cocoa consumers" (i.e. eating less than one cocoa/chocolate product) and "non-consumers", expressed per week or day depending on the type of product. More than one snack per day or one bar per week was considered as high consumption (**Table 25**).

Globally, most students were low consumers of the considered products. Around 40% Portuguese students referred non-consuming hot chocolate but, on the contrary, 19% of these students usually take a cup of hot chocolate per week. Considering chocolate bars, most students consumed more than one chocolate bar per week and only 4/70 stated not to eat chocolate bars. Comparing the three types of chocolate bars, very few individuals were high consumers of white chocolate bars and a half of the students did not use to eat this type of bar. Milk chocolate bars had the lowest percentage of non-consumers in comparison with the other bar consumers. However, a 24% studied population were high consumers of dark chocolate (**Table 25**).

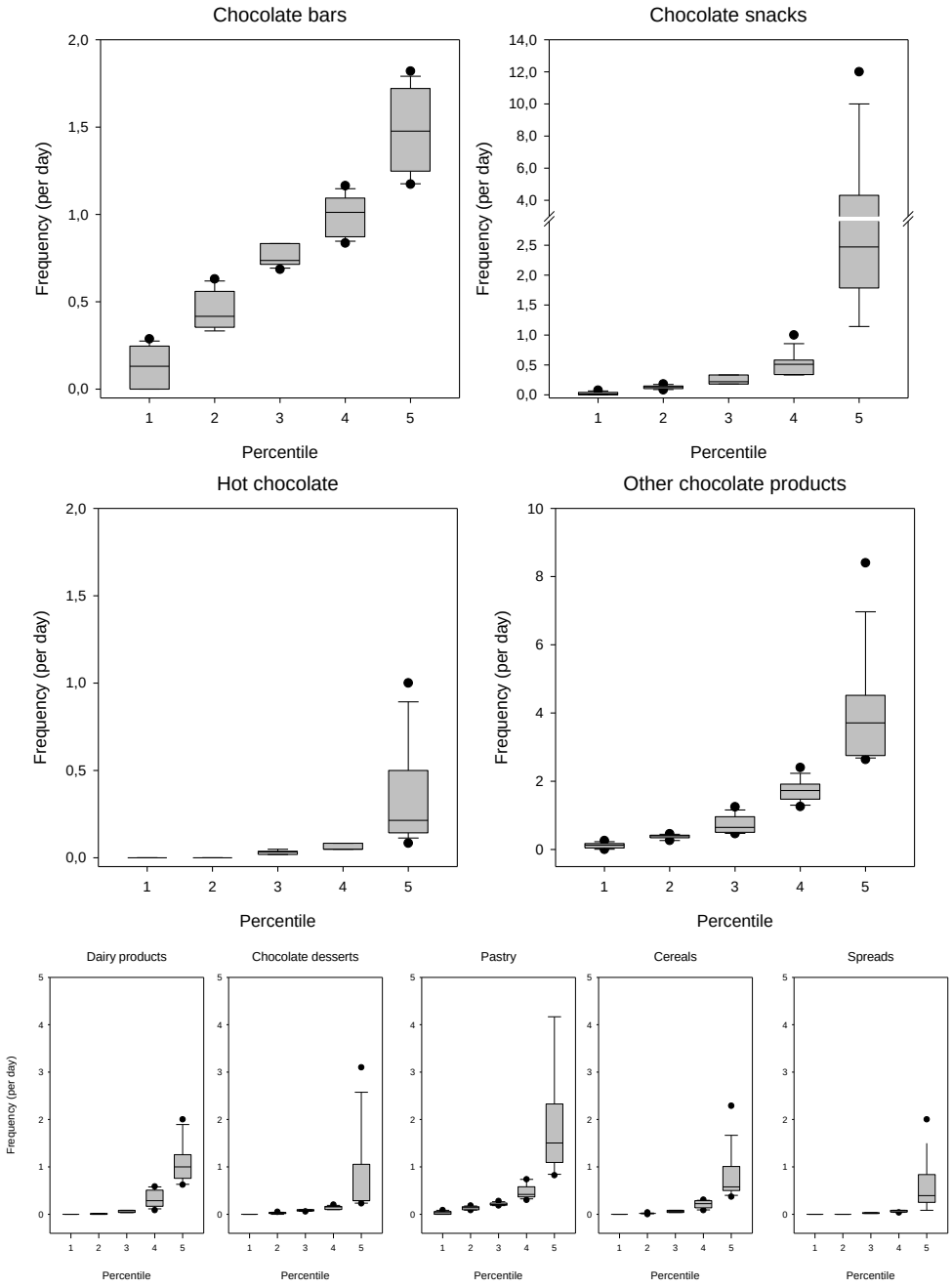

Figure 16: Classification of consumption frequencies (product/day) referred to in each quintile (percentiles 1-5) for the different cocoa/chocolate products studied.

More than 20% of the Portuguese students take a chocolate snack per day, and 10% of this population do not eat this type of chocolate product. However, only one student referred not to eat any other cocoa/chocolate product of the categories of dairy, pastry, dessert, cereal and spreads. Concerning these categories, cocoa/chocolate pastries have the highest percentage of high consumers and the lowest one of low consumers. On the other hand, spreads have the highest proportion of low consumers and the lowest values of high consumers.

Table 25: Classification of individuals regarding their consumption frequency for each of the foods considered of interest in the P-FFQ in three categories: high consumers, low consumers and non-consumers.

	High consumers (≥1)	Low consumers (<1)	Non-consumers (0)
Hot chocolate (product/wk)	19%	41%	40%
Chocolate bars (product/wk):	57%	37%	5.7%
- White chocolate	2.9%	47%	50%
- Milk chocolate	10%	73%	17%
- Dark chocolate	24%	62%	30%
Chocolate snacks (product/day)	21%	69%	10%
Others (product/day):	43%	56%	1.4%
- Dairy	11%	57%	31%
- Pastries	16%	77%	7.1%
- Desserts	4.3%	71%	24%
- Cereals	7.1%	71%	21%
- Spreads	4.3%	56%	40%

4.2.6. CONSUMPTION DISTRIBUTION OF COCOA/CHOCOLATE PRODUCTS

In addition, in the 70 Portuguese students sample, we estimated the cocoa intake per day of each participant considering the amount of cocoa per portion (**Table 8**) within each inquired item in the P-FFQ and the total consumption (**Figure 17**). The cocoa consumption average per day in the studied population was about 14 g. This amount was mainly provided by chocolate bars (51%), but other products were also important. In this sense, cocoa/chocolate pastry products contributed by 15% to the total cocoa intake, and the consumption of dairy products added an 11% to the total cocoa intake.

The average total cocoa intake in the Portuguese population (14 $\pm$ 1.42 g) did not statistically differ from the first cohort at the University of Barcelona students (12 $\pm$ 1.24 g). However, the contribution of pastry products for cocoa intake was higher for the Portuguese students (2.2 $\pm$ 0.4 g) than for the Spanish ones (0.4 $\pm$ 0.1 g) ($p<0.01$). In addition, the consumption of hot chocolate was also higher in the Portuguese students (0.79 $\pm$ 0.20 g *vs* 0.14 $\pm$ 0.02 g) ($p<0.01$).

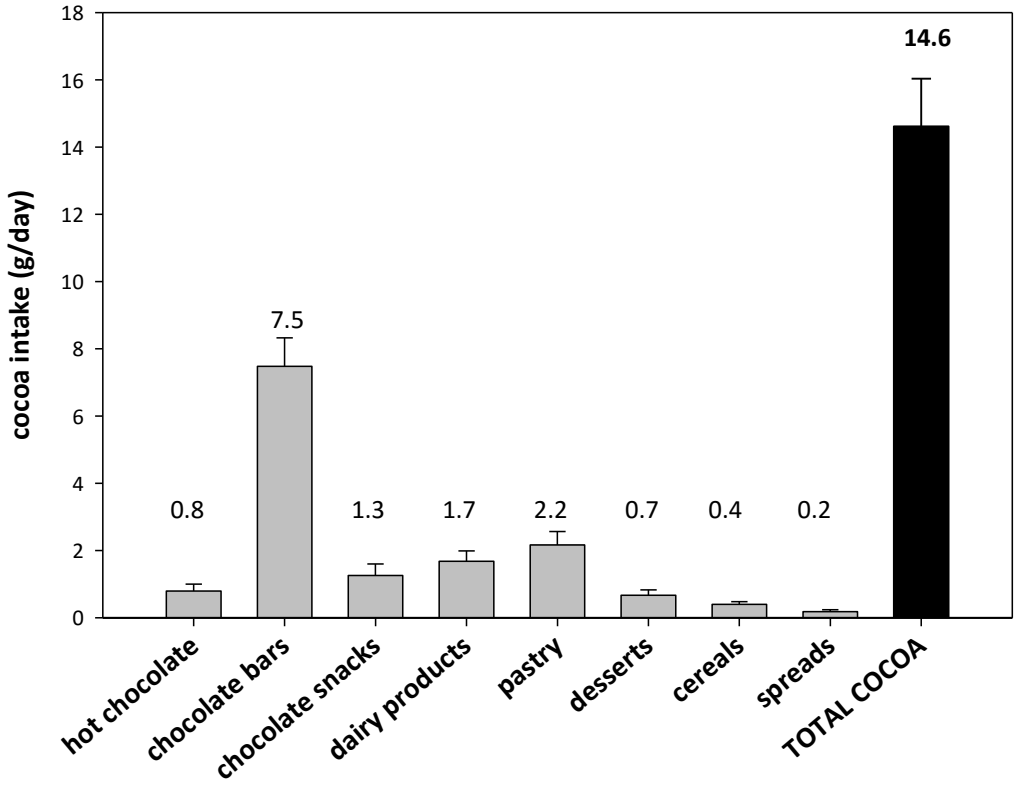

Figure 17: Estimation of cocoa intake (g/day) according to the sources included in P-FFQ. Results are expressed as the mean ± standard error of the mean (N=70).

4.3. COCOA INTAKE, LIFESTYLE, PHYSICAL ACTIVITY AND HEALTH STATUS

The developed questionnaire, in their C-FFQ and P-FFQ versions, has proven to give some credible data about cocoa and chocolate product consumption after applying to a cohort of 50 students from the University of Barcelona, and to a cohort of 70 students from the Egas Moniz Health Sciences Institute, in comparison with the 24HDR and the EFSA-FFQ. Therefore, after considering this FFQ as a valid option to estimate accurately the cocoa consumption in the University students' population, we increased the number of participants. The new objective was to establish a relationship between cocoa consumption and health, and in particular with physical activity as well as diseases related to immune system such as infections and allergies.

4.3.1. SAMPLE SOCIODEMOGRAPHIC CHARACTERIZATION

Overall, the questionnaire was applied to a sample of 270 individuals (200 Spanish students and 70 Portuguese students). The **Table 26** summarizes the main sociodemographic characteristics of the sample. The population comprised 71 males and 199 females, with a gender proportion of ~1:4 that is quite representative in the Health Sciences graduation programmes considered and similar to that found in the previous FFQ validation studies conducted in Spain (~1:5) and in Portugal (~1:3).

Most participants (75%) were undergraduates and only the remaining 25% of students were enrolled in one of the master's or PhD programmes conducted at any of the both Universities considered. Taking into consideration the gender distribution in undergraduate and postgraduate populations, this ratio was of about 1:3 and 1:6.5, respectively.

Participants were on average 22.5 ± 3.9 (mean ± SDM) years old; being the youngest 18-year-old and the oldest 42–years-old. The undergraduate students were in average 21.3year-old and the graduate students were in average 26.1-years-old, with no gender differences in both cases.

The anthropometric data demonstrated that most of the students in both subpopulations (undergraduate and graduate students) had normal weight and height values, being very similar if distributed by gender: ~70 kg and 1.75 m for males and ~59 kg and 1.65 m for females. The BMI was calculated and had a mean value of 21.9 ± 2.8 kg/m^2 was obtained for the entire population studied, although it was slightly lower in the case of the females (~22 kg/m^2) with respect to that of the males (~23 kg/m^2). Nevertheless, some undergraduate and postgraduate student's data revealed BMI values out the normal range, in particular 19/270 participants (7.0%) had a BMI lower than 18.5 kg/m^2 and 31/270 students (11.5%) had a BMI higher than 25 kg/m^2 .

Most of the participants were single (>90%) and a very low proportion was married or living with their partner. A ~62% of the students were living with their family, being the undergraduate students those with higher percentage (~84% and ~63%, males and

females, respectively) than the postgraduate ones (~44% and ~37%, males and females respectively). On the contrary, around a third of the participants (~28%) referred to live with flatmates, which is common at this age in University students. However, in these students, the distribution according to lifestyle was more diverse.

Table 26: Sociodemographic characteristics of participants who completed the C-FFQ or the P-FFQ. Values are expressed as mean ± standard deviations of the mean, as well as the range and proportion for anthropometrical and demographic data, respectively.

	Undergraduate students		Graduate students		Total (N=270)
	Men (N=63)	Women (N=140)	Men (N=8)	Women (N=59)	
Age (y)	21.53 ± 3.20	21.21 ± 3.21	25.22 ± 3.90	26.41 ± 3.21	22.55 ± 3.94
Range	[19-32]	[18-41]	[23-31]	[19-42]	[18-42]
Weight (kg)	70.67 ± 11.38	58.69 ± 9.35	69.50 ± 11.13	59.83 ± 3.21	62.05 ± 10.97
Range	[43-103]	[35-100]	[48-89]	[41-90]	[41-100]
Height (m)	1.75 ± 8.30	1.65 ± 6.29	1.73 ± 11.51	1.65 ± 3.21	1.67 ± 8.49
Range	[1.50-1.92]	[1.46-1.88]	[1.46-1.87]	[1.48-.1.86]	[1.46-1.92]
BMI (kg/m^2)	22.79 ± 2.83	21.49 ± 2.78	23.20 ± 2.50	21.93 ± 3.21	21.94 ± 2.77
Range	[15.98-29.07]	[16.42-32.66]	[20.73-29.06]	[16.16-28.46]	[15.98-32.66]
Residential status, %:					
- alone	1.6%	0.71%	22.2%	5.1%	2.6%
- with family	83.9%	62.9%	44.4%	37.3%	61.5%
- own family	4.8%	5.0%	0%	20.3%	8.1%
- flatmates	9.7%	31.4%	33.3%	37.3%	27.8%

4.3.2. CONSUMPTION OF COCOA PRODUCTS

Similarly to the validation cohorts, the consumption frequency of hot chocolate, chocolate snacks, chocolate bars, and also dairy, pastry, dessert, cereal and spread products containing cocoa/chocolate was determined in the cohort of 270 students (**Table 27**).

The mean value of hot chocolate consumption was lower than 0.05 portions/day that means that, on average, students took a cup of hot chocolate every 3 weeks. However, they ate a chocolate snack every 2 days and more than a portion of chocolate bar per day. On the other hand, the consumption of cocoa/chocolate dairy products was almost one per day. Pastries were also a good source of cocoa/chocolate intake because they provided almost one portion every two days. Nevertheless, desserts, cereals and spreads were consumed with low frequency having values that ranged between one portion every 3 days (cereals) to one portion every week (spreads). Overall, the University students used to eat more than 3 portions of products containing cocoa per day.

Table 27: Consumption frequency of a portion (times/day) of foods containing cocoa or chocolate estimated by the C-FFQ or the P-FFQ (mean values and standard deviations of the mean, N=270).

	C-FFQ / P-FFQ	
	Mean	SDM
Hot chocolate	0.0445	0.1268
Chocolate snacks	0.5552	1.1164
Chocolate bars	1.1961	1.9876
Dairy	0.7429	1.1471
Pastries	0.4149	0.6196
Desserts	0.1984	0.3907
Cereals	0.2779	0.4450
Spreads	0.1407	0.2701
TOTAL	**3.5706**	**3.5894**

In addition, from the frequency of consumption and the amount of cocoa per portion of each product (**Table 8**), the cocoa intake per day of each participant was estimated. Results are summarized in the **Figure 18**. On average, each student ate about 13.19 g of cocoa per day. Almost a half of this cocoa was provided by chocolate bars. Dairy products represented more than 20% of the total cocoa intake and then pastries delivered about 10% of total cocoa intake. The rest of products provided 1.5-6.5 % of total cocoa intake.

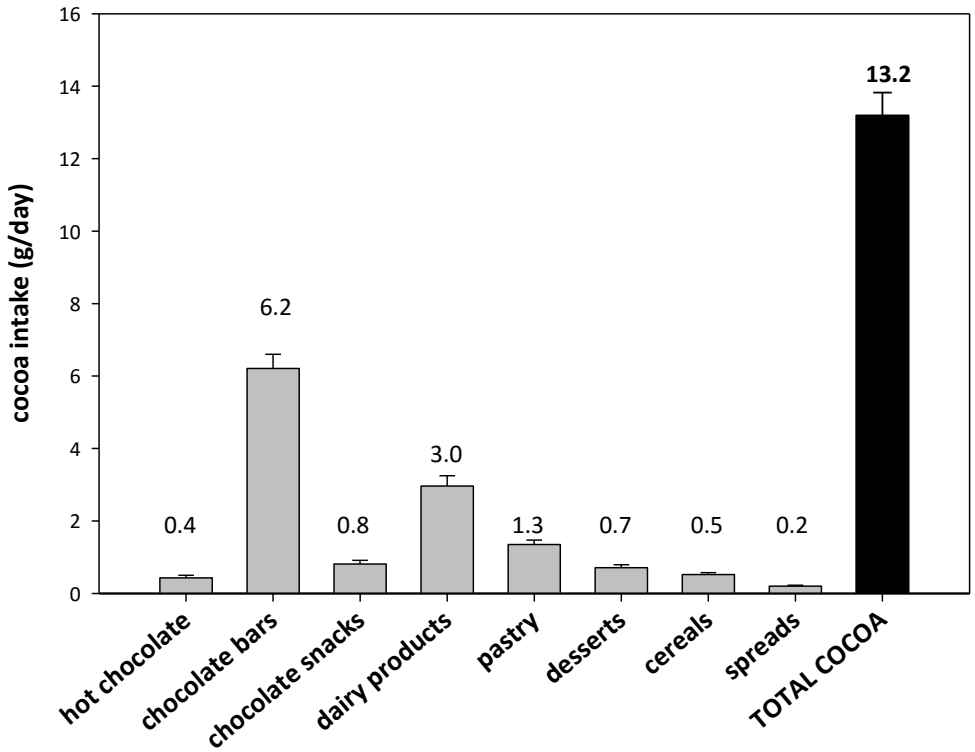

Figure 18: Estimation of cocoa intake (g/day) according to the sources included in C-FFQ. Results are expressed as average ± standard error of the mean (N=270).

4.3.3. POPULATION DISTRIBUTION ACCORDING TO COCOA CONSUMPTION

After calculating the cocoa consumption of the 270 students, individuals were classified into three subgroups according to cocoa consumption distribution. To better adjust the cocoa consumption of each group, the population was classified in not exact tertiles (each exact tertile should include 90 students) but almost: first "tertile" included 87 participants, second "tertile" was made by 91 participants, and last "tertile" was a group with 92 students. With this distribution, the sample in the subgroup of 87 participants (first "tertile") consumed less than 7 g of cocoa/day and they were considered as "Low consumers" (LC). The second subgroup of 91 participants (second "tertile") consumed an amount of cocoa comprised between 7-15 g of cocoa/day and they were considered as "Moderate consumers" (MC). Finally, the last subgroup of 92 participants (third "tertile") consumed more than 15 g of cocoa/day and were considered as "High consumers" (HC) (**Figure 19**).

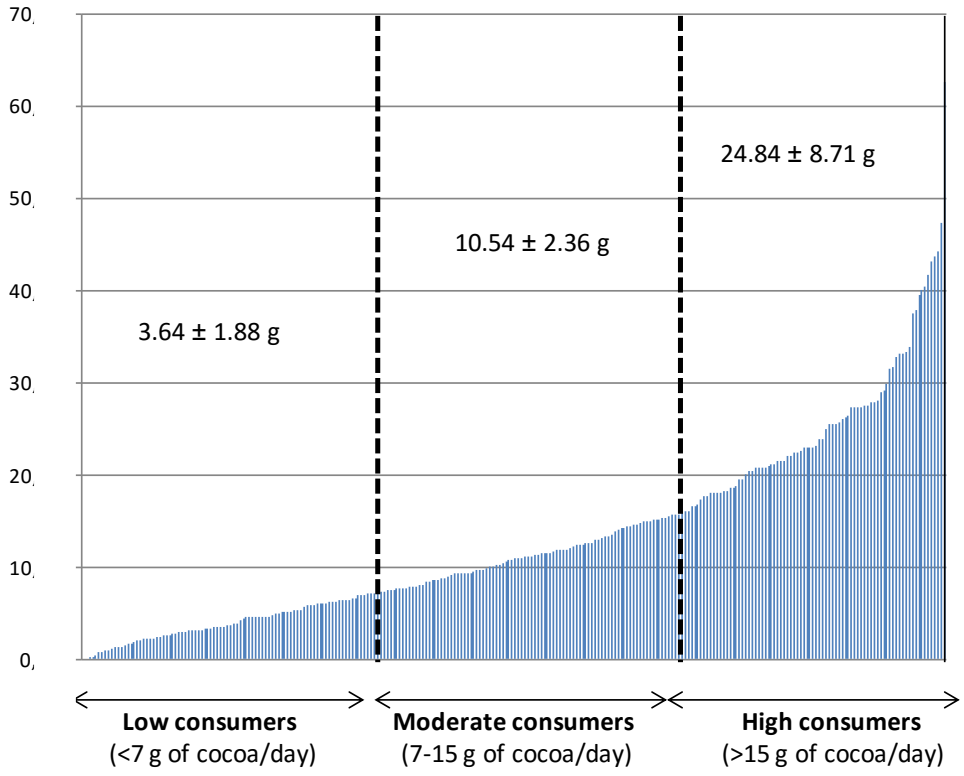

Figure 19: Population distribution according to its daily cocoa consumption. Values of mean consumption in each subgroup are expressed as mean ± standard deviation of the mean.

As mentioned above, the overall consumption of cocoa in the entire sample was of about 13 g of cocoa/day. However, the consumption in each subgroup was ~3.7 g for LC, ~10.6 g for MC, and ~24.9 g for HC (**Figure 19**). This approximation allowed to distribute participants in three groups that are 2.5 times fold difference each (i.e.: LC consumption x 2.5 = MC consumption, and MC consumption x 2.5 = HC consumption). This distribution

may allow observing differences in health variables associated to this differential pattern in cocoa consumption.

4.3.4. LIFESTYLE CHARACTERIZATION ACCORDING TO THE COCOA CONSUMPTION

After classifying the 270 students according to their cocoa consumption, the sociodemographic characteristics of each group were taken into account. The **Table 28** summarizes the characteristics of the three LC, MC and HC students. The gender distribution of individuals among the three groups did not differ statistically, although the HC group contained more proportion of female students. Similarly, no differences concerning the age of each group were found. However, the marital status differed between high and low consumers, in such a way that the percentage of singles LC was higher than that in the HC group (97% vs 90%).

Table 28. Lifestyle features characterization of low, moderate and high cocoa consumers. Statistical differences according Student's t test or Chi square test (different letters mean statistical difference, N=270)

	Low consumers (<7g) N=87	Moderate consumers (7 – 15g) N=91	High consumers (>15g) N=92
Cocoa intake (g)	3.64 ± 1.88 [a]	10.54 ± 2.36 [b]	24.84 ± 8.71 [c]
Gender			
Female %	73.6% [a]	70.3% [a]	77.2% [a]
Male %	26.4% [a]	29.7% [a]	22.8% [a]
Age (years)	22.84 ± 3.49 [a]	22.52 ± 4.12 [a]	22.32 ± 4.14 [a]
Single (%)	96.6% [a]	91.2% [a,b]	90.2% [b]
Residential status			
Alone %	2.3% [a, b]	5.5% [a]	0% [b]
With family %	57.5% [a]	58.2% [a]	68.5% [b]
Own family %	11.5% [a]	5.5% [b]	7.6% [a,b]
Flatmates %	28.7% [a]	30.8% [a]	23.9% [a]
Work (%)	16.19% [a]	16.6% [a]	18.5% [a]
more than 10h/week %	5.7% [a]	5.5% [a]	8.7% [a]
Smokers (%)	20.7% [a]	19.8% [a]	21.7% [a]
Never smoked %	74.7% [a]	68.1% [a]	68.5% [a]
Ex-smokers %	5.7% [a]	15.1% [b,c]	12.5% [c]

When analysing the residential status, some differences also raised. Thus, although the number of students living alone was very low, the percentage was higher in the MC group than in the HC group. Likewise, the percentage of people living with family was higher in the HC group (68%) than in the other two groups (about 58% in LC and MC groups). The

highest proportion of students living with their own family was found in the LC group (11% *vs* 5.5-7.6 in the MC and HC groups, respectively).

No differences in the relative number of students with work were found although there was a tendency to be the highest percentage in the HC group. Therefore, it seems that having economical inputs did not produce more cocoa consumption.

Concerning smoking, no differences appeared when considering the current smokers and the students that never smoked. However, when considering ex-smokers, the proportion was higher in the MC and HC groups (15.1% and 12.5%, respectively) than in the LC group (5.7%) ($p<0.05$). These results suggest that leaving tobacco could produce an increase in cocoa consumption.

4.3.5. COCOA CONSUMPTION AND PHYSICAL ACTIVITY

Physical activity is usually included as an activity influencing on the healthy lifestyle, and for this reason, we included such evaluation in this study. As mentioned in Material and Methods section we used the short IPAQ questionnaire. This tool allows to divide a population by its physical activity measured in METs or to categorize them into three groups depending on its activity (low activity, moderate activity or high activity).

The physical activity in the whole student population (N=270) was 2692.18 ± 2549.50 METs/week (mean ± SDM). This activity was very variable among students ranging from 0 to 15960 METs/week. We found that 37/270 (~14%) had a sedentary lifestyle (<600 METs/week) and 110/270 (~40%) of the students did not reach the physical activity levels established by the recommendations guidelines for health-promotion (value of 1500 METs/week) [340]. However, when we categorized the students according to the three physical activity levels taking into account IPAQ, we found that the whole population of students were distributed as follows: 18.15% as low active, 38.88% as moderate active, and 41.85% as highly active.

Figure 20 shows the correlation between cocoa consumption and physical activity measured in METs/week obtained by the IPAQ questionnaire. An inverse correlation was found between cocoa consumption and physical activity, in such a way the more cocoa they ate, the less exercise they did. This weak association was not significant after the Spearman analysis of the data, although a p value of 0.087 was obtained.

This same inverse tendency could also be observed when the particular cocoa product families included in the FFQ, such as hot chocolate, chocolate snacks, chocolate bars and also other products such as dairy and pastry were analyzed (**Figure 20**). The bars seem to be lower associated with this behaviour with respect the other types of products.

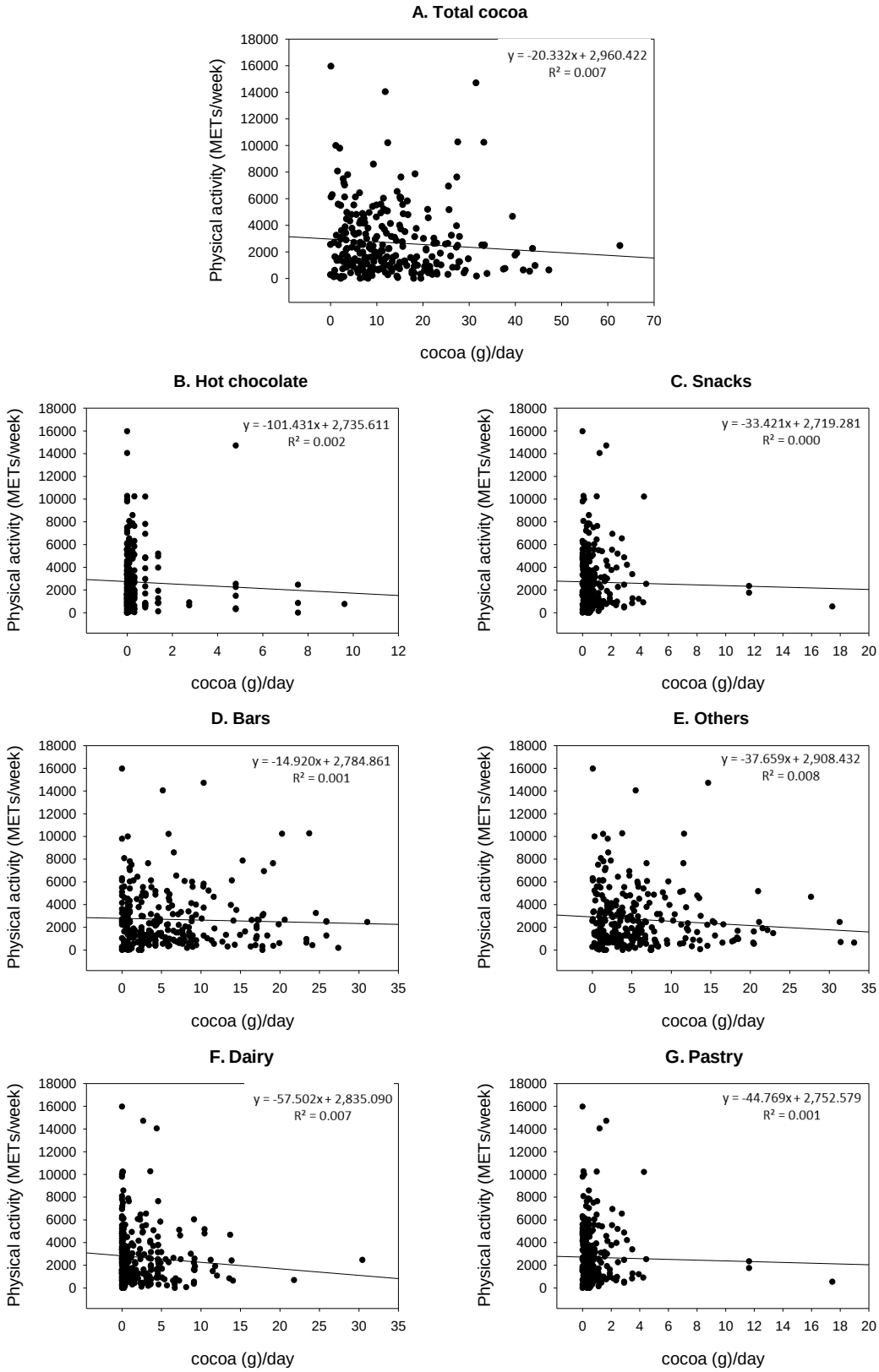

Figure 20: Relationship between total and specific cocoa products consumption (X axis) and physical activity (Y axis) of the total amount of participants in the study (N=270): **A)** total cocoa, **B**) hot chocolate, **C**) chocolate snacks, **D**) chocolate bars, **E**) other products, **F**) dairy, **G**) pastry. Linearity tendency formula and regression value are located at the upper right quadrant of the figure.

Regardless the non significant low tendencies when all population were analyzed together, the METs/week and the categorical activity classification (low, moderate and high activity) in each cocoa consumption group (LC, MC and HC) were studied. As it can be observed in **Table 29**, the categorised results were consistent with the previous correlations. In general, cocoa LC reported a higher physical activity (~3000 METs/week) than MC and HC groups (~2500 METs/week) considering the total METs spent per week, although no statistically significant differences were obtained probably due to the high dispersion.

Table 29: Physical activity data obtained for the three cocoa consumption subgroups. Values from continuous variables (METs/week and sitting time/day) are expressed as mean ± standard deviation of the mean, and percentages as proportions in each subgroup. Statistical differences according to Student's t test (continuous variables) or Chi square test (proportions) are shown with different letters.

	Low consumers (<7 g) N=87	Moderate consumers (7 – 15 g) N=91	High consumers (>15 g) N=92
METs/week	3002.40 ± 2735.29 [a]	2515.93 ± 2279.97 [a]	2573.15 ± 2580.11 [a]
Physical activity category			
Low activity, %	13.79% [a]	18.68% [a,b]	22.83% [b]
Moderate activity, %	36.78% [a]	40.66% [a]	40.22% [a]
High activity, %	49.43% [a]	40.66% [a,b]	36.96% [b]
Sitting time/day (h)	6.68 ± 1.62 [a]	6.41 ± 2.50 [a]	7.05 ± 3.41 [a]

Regarding physical activity category classification (**Table 29 and Figure 21**), the LC group was the one with the highest proportion of active students (~50%) and with the lowest proportion of inactive students (~14%), whereas the HC group showed the lowest proportion of highly active participants (~37%) and the highest proportion of students with low activity (~23%). The proportion of students with high and low activity differed significantly according they belong to low and high consumer groups ($p<0.05$).

In the IPAQ questionnaire, the students were also inquired about their sitting time along the day. This value was similar among the three groups and varied about 6-7 h/day (**Table 29**).

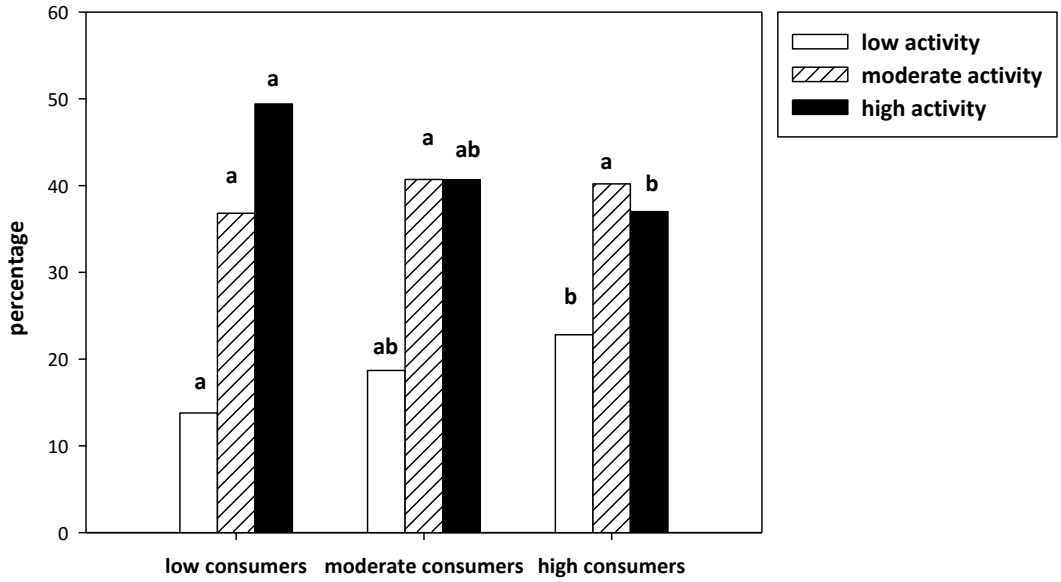

Figure 21: Percentage of students with low, moderate and high activity after classifying students according the cocoa consumption. Statistical differences according to Chi square test are shown with different letters.

4.3.6. COCOA CONSUMPTION, BMI AND BLOOD PRESSURE

Health status assessment was evaluated according to several criteria. The anthropometric data reported revealed a weak inverse tendency correlating cocoa consumption and BMI, although it was not found a statistical association by Spearman's test (**Figure 22**). Thus, it can be suggested that high cocoa consumption was not accompanied by higher BMI.

A similar tendency could also be observed when the particular cocoa product families included in the FFQ, such as hot chocolate, chocolate snacks, chocolate bars and also other products such as dairy and pastry were analyzed (**Figure 22**).

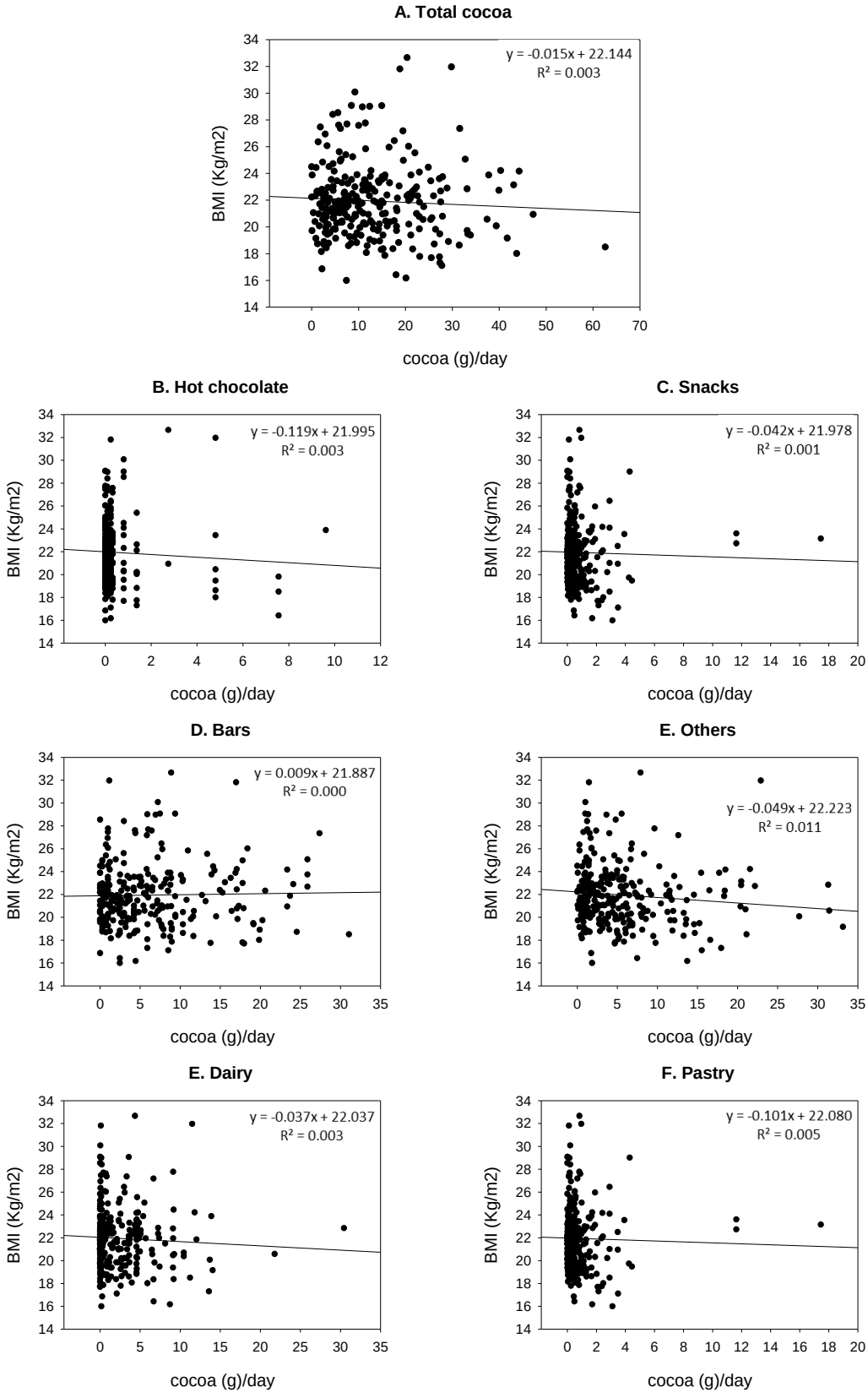

Figure 22: Relationship between total and specific cocoa products consumption (X axis) and BMI (Y axis) of the total amount of participants in the study (N=270): **A)** total cocoa, **B**) hot chocolate, **C**) chocolate snacks, **D**) chocolate bars, **E**) other products, **F**) dairy, **G**) pastry. Linearity tendency formula and regression value are located at the upper right quadrant of the figure.

Regardless the lack of significant correlation, the BMI was calculated for each subgroup. In this case, non-significant differences were found in the mean values of BMI in each subgroup (LC, MC and HC) (**Table 30**). However, when the number of underweight (BMI<18.5) and overweight (BMI>25) participants were assessed [341], a significantly higher number of underweight students was found in the HC group in comparison the LC group ($p<0.05$). In addition, the lowest proportion of overweight individuals was found in the MC group, and this value was statistically lower than that in the LC group ($p<0.05$) (**Table 30**). When the ratio between underweight and overweight students was assessed in each cocoa consumption group, the HC and MC groups achieved a higher ratio than LC group ($p<0.05$).

Table 30. Anthropometric indicators of health for the three cocoa consumption subgroups. Values from continuous variables are expressed as mean ± standard deviation of the mean, and percentages as proportions in each subgroup. Statistical differences according Student's t test (continuous variables) or Chi square test (proportions) are shown with different letters.

	Low consumers (<7g)	Moderate consumers (7 – 15g)	High consumers (>15g)
Anthropometric measures			
BMI (Kg/m^2)	22.16 ± 3.02 ^{a}	21.85 ± 2.63 ^{a}	21.84 ± 2.65 ^{a}
underweight (%)	4.60 % ^{a}	6.59 % ^{a,b}	10.87 % ^{b}
overweight (%)	14.94% ^{a}	8.79 % ^{b}	10.87 % ^{ab}
under/overweight ratio	0.31 ^{a}	0.75 ^{b}	1.00 ^{b}
Blood pressure			
mean blood pressure (mmHg)	87.19 ± 9.53 ^{a}	87.01 ± 7.69 ^{a}	86.84 ± 9.37 ^{a}
high blood pressure (%)	2.29 % ^{a}	3.29 % ^{a}	1.09 % ^{a}

On the other hand, blood pressure is a variable that has been reported to be modulated by cocoa components [138]. For this reason, participants were asked about its usual values of blood pressure. From these values, the mean blood pressure was calculated, and the analysis of correlation between blood pressure and cocoa consumption did not show any significant tendency (**Figure 23**). A similar behaviour could be observed when the particular cocoa product families included in the FFQ, such as hot chocolate, chocolate snacks, chocolate bars and also other products such as dairy and pastry were analyzed (**Figure 23**). The chocolate snacks consumption seems to have the highest association with a lower blood pressure.

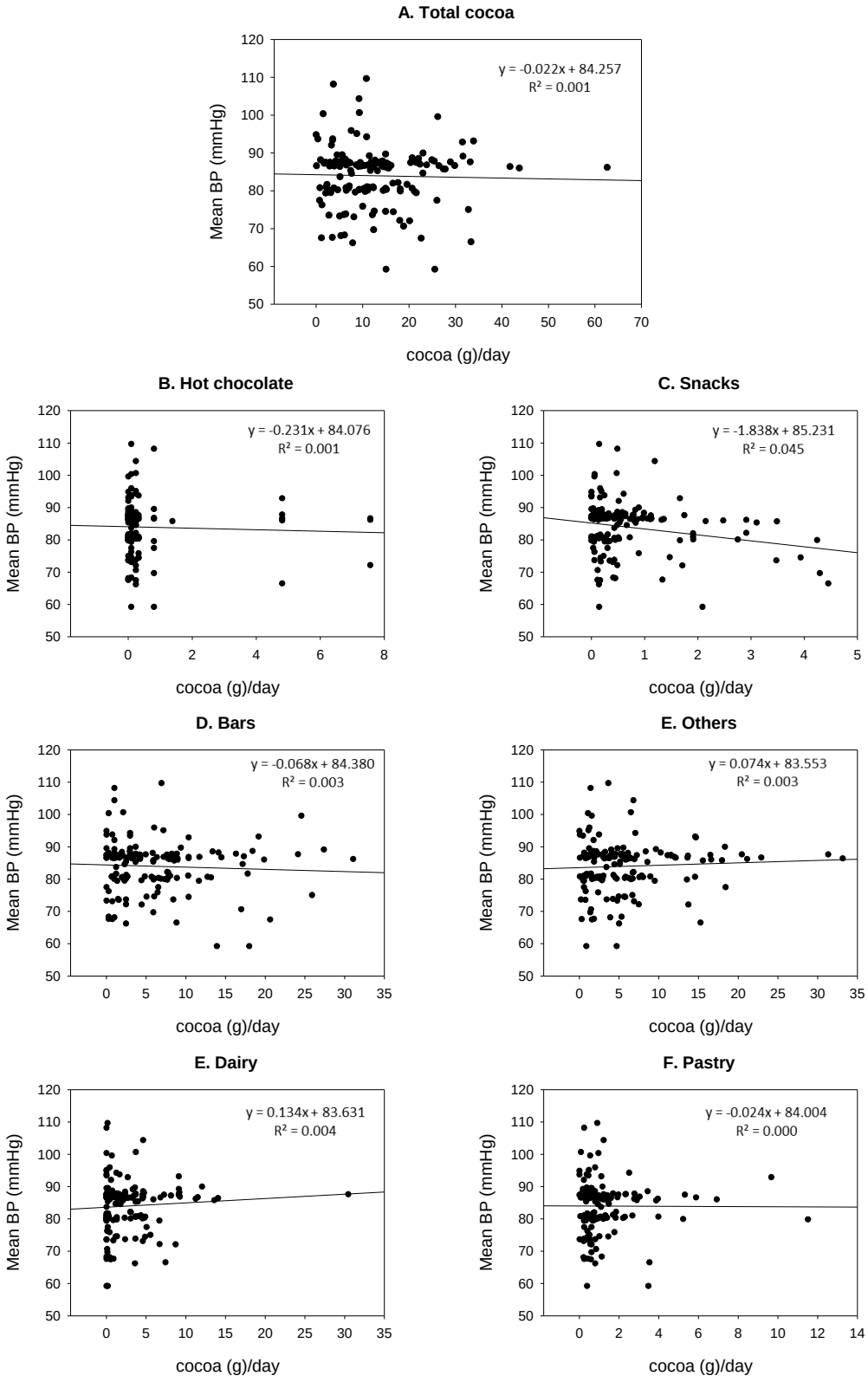

Figure 23: Relationship between total and specific cocoa products consumption (X axis) and mean blood pressure (Y axis) of the total amount of participants in the study (N=270): **A)** total cocoa, **B**) hot chocolate, **C**) chocolate snacks, **D**) chocolate bars, **E**) other products, **F**) dairy, **G**) pastry. Linearity tendency formula and regression value are located at the upper right quadrant of the figure.

The mean blood pressure and the proportion with high blood pressure were also studied according the cocoa consumers (LC, MC and HC) (**Table 30**). The mean value of blood pressure was of about 87 mmHg for all groups without statistical differences among them. Moreover, very few individuals declared high blood pressure (<4%), independently from cocoa intake.

4.3.7. COCOA CONSUMPTION AND DISEASE

Because the cocoa diet has been attributed immunomodulator and anti-inflammatory properties [226] , the participants were inquired about the diseases they usually suffer. In this context, they were asked about chronic diseases but also about the episodes of fever, flu, diarrhoea, or being ill in general during the last month. The results are shown in **Table 31**.

More than 50% students declared to have a chronic disease, being the highest proportion found in the LC subgroup when compared to those groups eating higher amounts of cocoa. However only the percentage of MC suffering from chronic disease (50.55%) was statistically lower than that in the LC (64.37%).

On the other hand, although without reaching statistical significance, MC group reported less cases of fever or illness in general than the other subgroups. Specifically, they reached a value around 2% lesser than these proportions in the LC but also in the HC.
Finally, around 30% of students referred to have had flu in the inquired period. However, when its presence was analyzed in each cocoa consumption subgroup, it could be observed that the proportion of students suffering of flu in the HC subgroup (29.3%) was lower when comparing to moderate consumers (31.87%, $p<0.05$) but not with respect LC. No statistical differences were found regarding diarrhoea or illness in general.

Table 31: Indicators of disease for the three cocoa consumption subgroups. Values are expressed as proportions in each subgroup. Statistical differences according to Chi square test are shown with different letters.

	Low consumers (<7g) N=87	Moderate consumers (7 – 15g) N=91	High consumers (>15g) N=92
Chronic diseases (%)	64.37% [a]	50.55% [b]	58.70% [a,b]
Fever (%)	8.05% [a]	5.49% [a]	10.87% [a]
Flu (%)	32.18% [a,b]	31.87% [a]	29.35% [b]
Diarrhoea (%)	0.00% [a]	1.10% [a]	1.09% [a]
Ill (%)	9.20% [a]	6.59% [a]	9.78% [a]

4.3.8. COCOA CONSUMPTION AND ALLERGY

The questions included in the FFQ also inquired about the presence and frequency of allergy symptoms. The results were evaluated and compared within low, medium and high consumers.

The relative number of students with intolerance does not differ between groups and was about 5.5-7%. However, approximately 20% of the total student population declared to suffer some allergy and statistical differences have been found between LC, MC and HC groups. Results are shown in **Figure 24**. Specifically, 24 students in the LC group reported allergy (27.6%) which was a higher percentage than that obtained in the MC and HC groups (about 13 and 19%, respectively) ($p<0.05$ in both cases).

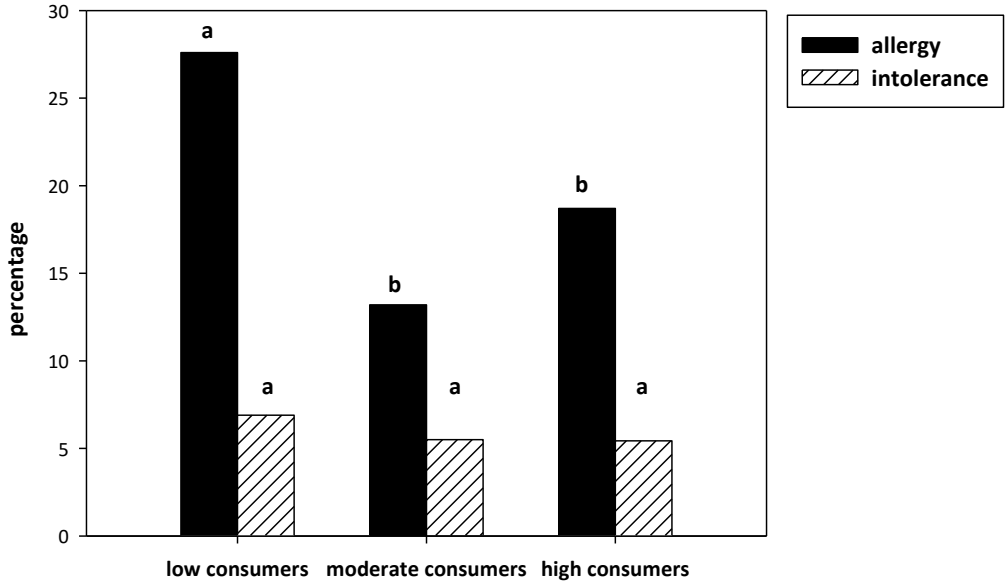

Figure 24: Percentage of students with allergy and with intolerance after classifying students according the cocoa consumption. Statistical differences according to Chi square test are shown with different letters.

Considering only allergic people of each group, frequency of allergy symptoms (days/month) was studied (**Table 32**). Although the LC group had the highest frequency, it had a big dispersion and statistical significant differences were not found when this result was compared to those from MC and HC groups.

Students were also inquired about allergic symptoms at least once a month. Half of the allergic students of the LC group declared to suffer from allergy once a month, whereas this proportion was significantly lower in allergic MC group (about 28%, $p<0.05$). However, the percentage of students with allergy once a month in the HC group was the highest (about 59%, $p<0.05$ *vs* LC and MC groups).

In addition, the FFQ inquired about the type of allergic symptoms suffered by the allergic students (**Table 32**). More than 40% of the allergic students in the LC group reported to develop cutaneous symptoms such as hives, redness or itching. This proportion was significantly higher than that in the MC students with allergy (28%, $p<0.05$) and did not

differ from that of the HC allergic students. Some students reported to develop rhinitis, sneezing or mouth itching, and considering the proportion in each group of cocoa consumers, the LC group had the highest proportion that was significantly higher than that in the MC group ($p<0.05$). No differences were found in this sense between LC and HC students.

The number of allergic students with respiratory symptoms such as asthma was very low (0-17% of allergic students) and again the lowest proportion was found in the MC group. No students stated to suffer from digestive symptoms of allergy in any of the groups.

Table 32: Characteristics of allergy in students of the three groups of students according the cocoa intake. Statistical differences according Chi square test are shown with different letters.

	Low consumers (<7g)	Moderate consumers (7 – 15g)	High consumers (>15g)
Frequency of allergic symptoms (days/month)	5.69 ± 9.91 [a]	1.58 ± 2.54 [a]	3.95 ± 6.34 [a]
People with allergy once a month (%)	50.0% [a]	27.8% [b]	58.8% [c]
Cutaneous symptoms (%)	41.7% [a]	25.0% [b]	35.3% [a,b]
Oropharynx symptoms %	37.5% [a]	25.0% [b]	35.3% [a,b]
Respiratory symptoms %	16.7% [a]	0.00% [b]	11.8% [b]
Digestive symptoms %	0.00% [a]	0.00% [a]	0.00% [a]
Days of allergy-produced absenteeism (mean ± SDM)	0.25 ± 0.74 [a]	0.00 ± 0.00 [a]	0.12 ± 0.33 [a]
People with allergy treatment %	54.2% [a]	33.3% [b]	58.8% [a,b]

Finally, the absenteeism consequent to allergy and those students under allergy treatment in the last year was inquired. From the 24 allergic subjects in the LC group, only three reported that they had to remain at home for 1-3 days due to the allergy. From the 12 allergic students in the MC group, no one declared to have been absent by allergy, and in the HC group, 2/15 students reported to remain at home for 1 day. There were no statistical differences between these values. It is important to point out that more than a half of students consuming the lowest cocoa amounts and the highest cocoa amounts were taking anti-allergic treatment. This percentage was only of 33% in the moderate consumers ($p<0.05$ *vs* LC group).

5. DISCUSSION

Cocoa has been previously recognized as a valuable source of polyphenol compounds with multiple benefits in human health [8,9,59]. spite of this, studies evaluating polyphenol intake underestimate the contribution of cocoa, which can be due to the fact that few chocolate and cocoa products are considered in the food list from the questionnaires used [297]. In most cases, consumption is based just from questions regarding chocolate snacks and bars, but no other sources are inquired. Additionally, there is no known data on cocoa intake in a very specific population such as University students. Chocolate, the potential main source of cocoa, is commonly seen as a candy and just only in the last few years, dark chocolate has gained popularity [342,343].

Considering this, the present thesis is based in two hypotheses: first, that cocoa consumption is underestimated in overall studies and therefore a new tool for this purpose should be generated, and secondly, that the consumption of cocoa has an impact on immune mediated diseases in which preclinical evidences exist [344]. Therefore, in order to test these hypotheses, this thesis includes three specific objectives: 1) to develop and validate a food frequency questionnaire (FFQ) including the main cocoa food products common in Catalan and Portuguese dietary habits in a young population, 2) to establish the consumption of cocoa in the University students of Catalonia and Portugal, and 3) to establish the relationship between cocoa intake and healthy status, including the presence of allergies, and healthy lifestyle practices.

The first objective of this thesis consisted in developing a FFQ that included the main cocoa food products. Although the 24HDR allows any cocoa form of the diet to be considered, it is very common to perform a FFQ instead of a dietary recall as it allows a broad food list to be included, and regular and occasional intake to be assessed more easily. Therefore, FFQ becomes an important tool for evaluating the consumption frequency of foods, and it is very useful for studying a considerable population [316]. Moreover, FFQs are especially important when dietary assessment pretends to establish possible associations, mainly aiming a specific food or food component with the disease risk or other health marker in a large sample [315–317]. Previous studies conducted in the University students about food habits have also used FFQ [345–347]. Some FFQs have been used for evaluating general or particular food consumption and among the products inquired about, they included also those containing cocoa [339,348–350]. However, questions about cocoa/chocolate consumption do not include all possible sources and do not deep enough into the type of chocolate, its cocoa percentage and, consequently, its polyphenol content. For this reason, we develop a FFQ designed for the precise evaluation of cocoa/chocolate product intake.

The designed FFQ has been built based on a validated FFQ, the ENCAT-2003 [314]. In order to fulfil the purpose of the present study, the food list was defined based on a comprehensive choice of cocoa/chocolate derived products common in Spanish and

Portuguese food habits after the several observations from food product diversity. Thus, a 90-item food list was considered including several chocolate and cocoa products grouped according to product categories (breakfast cereals, dairy products, confectionery -pastry and snacks-, chocolate bars and spreads) that can contain cocoa. Additionally, the new FFQ included other food sources of polyphenols such as fruits and vegetables, tea, coffee and wine. The new FFQ inquired for the average intake over the last 12 months. The frequency of consumption of the 90 food items was assessed using 12 categories ranging from 1 ('never') to 12 ('3 or more times per day'). The quantity of food items was specified as one portion or a piece.

One of the new additions in this questionnaire was the classification of chocolate bars according to cocoa content. It is known that dark chocolate presents more benefits than milk chocolate; however, most of the studies concerning dark chocolate do not specify clearly which was the proportion of cocoa in chocolate bars [112,125,144,213,351]. This could be one of the main strengths of the present FFQ because a frequent dark chocolate consumer would be able to identify the type of chocolate that consumes more frequently.

The first FFQ developed was written in Spanish and was called C-FFQ and later it was translated to Portuguese and called P-FFQ. To evaluate the new FFQ validity in each of both languages, bibliographical recommendations [316] and other similar studies were considered. The developed questionnaire was compared with two other approaches. On the one hand, we used a validated questionnaire designed by the EFSA for gathering specific consumption that included some questions about cocoa (EFSA-Q). On the other hand, a 24HDR was also used a tool to compare the new FFQ, similar to performed in other validation studies. Both approximations have been widely used as reference methods for developing new FFQ [309,316,323,352,353].

Results from the new FFQ, the EFSA-Q and the 24HDR were converted into consumption frequency (portions/day) for categories. The common categories between the new FFQ and the EFSA-Q were hot chocolate, chocolate snacks and chocolate bars (including white, milk and dark chocolate). The new FFQ and 24HDR added the categories of dairy, pastries, desserts, cereals and spreads, because they were inquired in the new FFQ and could be also present in the 24HDR. In addition, results from tea and coffee consumption were also analyzed in both comparative studies as a common food to be considered as an internal parameter to evaluate similarities. The relationship between the consumption frequency of cocoa derived from the new FFQ, the EFSA-Q and the 24HDR was established by comparing the results obtained (Wilcoxon paired test), analysing their correlation (Spearman's coefficient), studying their differences (Bland-Altman plots) and analysing the misclassification of the participants after their categorization (quintiles classification).

The Spanish version of the new FFQ was first applied in a small sample in Barcelona in order to evaluate its validity. For that, the results from the C-FFQ, EFSA-Q and 24HDR were obtained from a sample of 50 students from the University of Barcelona. Once

applied the C-FFQ in students from the University of Barcelona, the Portuguese version (P-FFQ) was used to inquiry a sample of 70 students from the Egas Moniz Health Science Institute in Portugal. The evaluation of its validity was performed similarly to that previously used in Spain, using also the EFSA-Q and the 24HDR approaches to compare.

In both validation studies, the sample characteristics were in accordance to the population to which this questionnaire was built, University students. The proportion of male/female was representative of gender distribution in health sciences University programmes [354]. Following recommendations from Cade et al. [316,317] and similarly to other studies [355,356], in the present research some specific attention has also been given to the process of FFQ administration. In this sense, the FFQ was designed to be self-administered, but it was applied in groups in presence of a member of research team in order to clarify any doubt.

Regarding the comparison of our new FFQ with the EFSA one, the cocoa consumption frequencies showed significantly higher overall values estimated by the C-FFQ and the P-FFQ than that obtained from the EFSA-Q in both populations, Spanish and Portuguese students, respectively. However, the comparison of cocoa consumption frequencies obtained from the new FFQ in both languages and the EFSA-Q showed a good correlation both in individual products and also in overall cocoa products consumption.

Some differences rose when we compared the food products in common between the new developed FFQ and the EFSA-Q. The cocoa consumption frequency declared by the students for the common items in both methods was higher in the C-FFQ/P-FFQ than in the EFSA-Q. In particular, the products present in EFSA-Q common with the new FFQ were chocolate bars, chocolate snacks and hot chocolate. Moreover, the EFSA-Q included several questions with eliminatory answers especially for chocolate bar type, and therefore, participants could only answer about the most frequently consumed type of chocolate bar (white, milk or dark) without detailing the cocoa content of each. On the contrary, C-FFQ and P-FFQ allow choosing the frequency of consumption for each chocolate bar type and several cocoa contents. The differential way of the new developed tool to ask about same products may have facilitated the student to answer the question, and therefore it seems to have an influence on the particular product consumption.

On the other hand, the new tool includes a quite considerable number of foods with cocoa that are absent in the EFSA-Q and also in other questionnaires, even with more items [323,339,348]. Dairy, pastries, desserts, cereals and spreads containing cocoa are taken into account in the C-FFQ/P-FFQ, therefore the cocoa consumption derived from these products had to be added to the previous one from the common products in both questionnaires. These results partially confirm our first hypothesis that cocoa consumption was underestimated.

Regardless of significant differences in the consumption frequency of overall or particular products, both the results using the C-FFQ and the P-FFQ, and those using the EFSA-Q

were derived from a similar pattern of consumption, had a moderate correlation and good quintile classification and, in addition, Bland-Altman analyses agreement was clear. All this data suggest that the new tool deserves to be considered as a new validated FFQ for evaluating cocoa consumption.

Data from the C-FFQ/P-FFQ was also compared to the 24HDR. The cocoa consumption frequencies were also significantly higher when were estimated by the C-FFQ and P-FFQ than that obtained from the 24HDR in the both Spanish and Portuguese cohorts, respectively. Similarly as in the case of the comparison with the EFSA-Q, the relationship of cocoa consumption frequencies obtained from the C-FFQ/P-FFQ and the 24HDR showed a good correlation in individual products and also in overall cocoa products consumption. Moreover, again, a similar pattern of consumption for both tools was observed, as well as moderate correlation and good quintile classification and, Bland-Altman analyses agreement. These last results confirmed the validation of the developed new tool for cocoa assessment.

It has to be taken into account that, the 24HDR refers to the reporting of food intake in a single period of time, while the FFQ depends on the individual's memory. Perhaps this is why the consumption frequency of all the cocoa products included in the "others" category from the FFQ (dairy, pastries, desserts, cereals and spreads) was higher than that in the 24HDR in both the validation cohorts studied. In a 24HDR, the participant has to describe his/her intake in the day before from scratch without a food list as a memory helper. Thus, this is a limitation of this method, it depends on the participant's memory and it refers to a limited period of time while the FFQ refers to the estimation of intake frequency during a longer period [315,357]. However, we cannot dismiss an overestimation of cocoa products found in the C-FFQ/P-FFQ due to a misjudgement in averaging the intake over one year. On the other hand, 24HDR data could also underestimate chocolate consumption because sometimes chocolate is a special food for celebrations, including Sundays, which were avoided in the current 24HDR. Although this difference, Bland-Altman analyses of total and also particular cocoa/chocolate products consumption frequency showed a relatively good agreement between methods, including the "others" cocoa products.

It is also important to state that some authors have suggested to use several 24HDR to assess frequency consumption and that the application of only one 24HDR could be a limitation. For this reason, after the validation process using a single 24HDR in the Spanish sample, two additional 24HDR were applied after one week each one in the Portuguese cohort. Although this variation in the validation process, results from Portugal showed similar agreement of P-FFQ with the average of the three 24HDR in a similar way than the results from the validation process using just one 24HDR in the case of the C-FFQ.

The correlation coefficients found between the C-FFQ/P-FFQ and EFSA-Q or between the C-FFQ/P-FFQ and 24HDR were comparable with other similar validation studies [358–360]. In addition, the classification ability using the extreme misclassification method was

similar to or even better than that in other similar approaches [339,348,352,361]. Therefore, again it could be defended that the developed FFQ should be considered as a valid assessment tool.

With regard to differential consumption frequencies between both cohorts, we have to mention that cocoa dairy products consumption frequency in the Spanish cohort from the validation study was twice the consumption frequency found in the Portuguese cohort. On the contrary, the consumption frequency of pastries was two-times lower. The differential habits will have an impact in the overall cocoa consumption. This differential pattern could be associated with data collection period. P-FFQ and C-FFQ were conducted in different months, which can influence cocoa consumption. According to Seligson [295], people tend to eat more chocolate during winter and autumn than during summer, and values for spring are intermediate within autumn and summer. However, this mentioned study refers mostly to chocolate as a candy, and once more this product choice is quite limited in the study and can influence participant answer. The differential chocolate pastry consumption in Portuguese and Spanish students is not reflected in other studies considering general population in which the consumption frequency is quite similar (1.8 and 1.5 kg/year, respectively) [362].

The second objective of the thesis consisted in establishing the consumption of cocoa in University students of Catalonia and Portugal. For that, we converted cocoa product frequency consumption in cocoa consumed by using the amount of cocoa present in a portion of such product.

We first used the validation cohorts in both countries to evaluate cocoa consumption. The average total cocoa intake in the students enrolled in the University of Barcelona was around 12 g and was not statistically different from that calculated from the Egas Moniz students evaluated which was of about 14 g. In both, the C-FFQ and P-FFQ, chocolate bars were relevant contributors to cocoa intake (>50%) followed by dairy and pastry products in Spain (23 and 5%, respectively) whereas in Portugal, bars intake was followed by pastry and dairy products (15 and 11%, respectively). Statistical differences appeared when comparing chocolate pastry and hot chocolate (both higher in Portuguese students) between both cohorts. Anyway, in both cases, chocolate snacks, item usually asked in FFQ assessing cocoa consumption, contributes in lower percentage to the total cocoa intake. Moreover, dairy products containing cocoa are not included in most questionnaires asking for flavonoids intake. This fact, again reinforce or hypothesis that cocoa consumption is nowadays underestimated.

Additionally, once validated the FFQ in both languages more individuals were included in the study and cocoa consumption was estimated from a total of 270 University students. Cocoa consumption was on average 13 g of cocoa per day. This amount of cocoa is in line

with other published studies [295,296]. Almost half of this cocoa was provided by chocolate bars, in spite dairy products represented more than 20% of the total cocoa intake. Moreover, pastries delivered about 10% and the rest of products provided 1.5-6.5 % of total cocoa intake. The high contribution of dairy products to cocoa consumption in this larger sample was due that the 150 new enrolled people were Spanish, which in basis of previous results, ate cocoa dairy products more frequently than Portuguese ones. This overall result again reinforces the fact of the need of the developed FFQ that inquires about this type of products.

If we focus on the results regarding cocoa consumption and cocoa consumption frequencies from the C-FFQ/P-FFQ and we compare them with published data, we can see some disagreements. The FFQs usually applied in those studies do not have so many items related to cocoa, they are conducted in a global or older population, and most of them collect data from different countries. This last fact is of great importance because the FLAVIOLA study has shown that the Spanish population is the highest consumer of flavanols derived from cocoa products in comparison with other European countries [363]. In this context, the Lothian birth cohort 1936 study reported that 84% of the population are chocolate consumers at a frequency of 2-3 times per week [364], which agree with our cohorts whose the mean consumption frequency was 3.5 pieces per day. The amount of cocoa intake in the considered students (13 g/day) was much higher than in other studies reporting, for example, a 1.5 g/day in 50-year-old Polish men [350]. However, data from a similar population in Spain indicate chocolate consumption of 42.6 g/day in the higher tertile [365], whereas when we calculated the consumption in the same tertile of our population, was of about 22 g/day. Additionally, in the results from P-FFQ, it is important to highlight that 57% of the individuals referred to a eat one chocolate bar more than once per week which in part is in accordance with the results obtained in another Portuguese study [313].

The second hypothesis of this thesis supports the idea that cocoa consumption has an impact on health and in immune mediated diseases in which preclinical evidences exist [201,209,210,366]. In order to test this hypothesis we planned the third objective of this thesis: to establish the relationship between cocoa intake and healthy status, including the presence of allergies, and healthy lifestyle practices. For that, we used the total number of students enrolled in the study in both countries (N=270). From those individuals, besides the cocoa consumption obtained through the new validated FFQ, we also obtained data from their physical activity (IPAQ questionnaire) or health status (*ad hoc* questionnaire). This last questionnaire was prepared to follow some recommendations addressed by Terwee *et al*. [367]. In this sense, students were asked to self-evaluate their health status and very direct and concrete questions addressed the presence or absence and related aspects of chronic diseases such as allergies. After having these data, students were distributed in “tertiles” according to their cocoa

consumption, being the low consumers those eating <7 g/day, the moderate consumers eating 7-15 g/day and the high consumers those having an intake >15 g/day. The health variable differences between each “tertile” were studied, as explained in the Material and Methods section.

The sociodemographic characterization of the sample enrolled reflected the type of population studied: University students in health sciences graduations and masters programs. The students have in average ~22-years-old, ~1:4 male/female proportion and mostly living with their family. When analysing these variables in relation with their cocoa consumption, we observed that males and females and people with similar age were distributed in the three “tertiles” without any significant difference among them. Moreover, although particular differences rose in the residential status according to cocoa consumption, we can suggest that the intake of cocoa is not clearly associated to a specific age, gender or type of residence. This lack of statistical difference cannot be extrapolated to general population, because in our case, the sample included only University students, and then it was very homogeneous. It is of interest that the interaction of smoking with cocoa products consumption was also studied and the results obtained were quite particular. Although no differences were found when considering the current smokers and the students that never had smoked, the percentage of ex-smokers in moderate and high cocoa consumers was 2-3 times higher than that in low cocoa consumers. It can be suggested the role of cocoa as a candy to substitute the tobacco habit. In this line, the ex-smoker may be looking for a sensory property lost after leaving tobacco, which can be found in cocoa because cocoa is widely applied to cigarettes and has been used by the tobacco industry as an additive since the early 20th century [368]. On the other hand, there is consistent evidence that smoking cessation is associated with increases in body weight and BMI [369], however this is not the case in our study as will be discussed later.

Health status assessment was evaluated according to several criteria, including anthropometrical data declared by the students in the questionnaire. Excessive chocolate consumption is regularly seen as a hazard for weight control [370,371], however our results from the FFQ suggest the opposite effect. Particularly, although a weak inverse tendency correlating BMI and cocoa intake (globally but also in specific products) and not significant differences in BMI were found among “tertiles”, some cocoa effect was found in the percentage of overweight individuals [341] in each cocoa group and in the underweight/overweight ratio. Specifically, moderate and high cocoa consumers included lower percentage of underweight people and had an underweight/overweight ratio two- and three-times statistically higher than that in the low consumer group. These results are in line with the preclinical data obtained by our group of research in rats from different strains, age and health condition [263,264,372] after receiving a 10% cocoa diet, in which a reduction of body weight of the animals have been consistently observed. Moreover, other studies in humans also reflect this behaviour. In this sense, in a younger population than in our study, the HELENA study comprising 1458 adolescents, it was shown that higher chocolate consumption was associated with lower levels of BMI and

total and central fatness [365]. In addition, in an older population than in our study, comprising 1018 adults in California, it has been demonstrated that those who consumed chocolate more frequently had a lower BMI than those who consumed it less often [373]. Following the comparison with results from older population, a study in 2013 showed that by a prospective analysis from a large cohort in United States, chocolate habit was associated with long-term weight gain, in a dose response-manner; however, after a cross-sectional approach chocolate was associated with lower body weight only applied to participants with pre-existing serious obesity-related illness [56]. This last effect is in line with the reduction in the percentage of overweight subjects in the high cocoa consumers described in our study.

This questionnaire also included questions about blood pressure. It is widely described the effect of cocoa flavanols or cocoa intake to modulate high blood pressure [105–108]. However, most of these studies have been performed in individuals with hypertension [139,140,142] and therefore the anti-hypertensive effect of cocoa has been demonstrated. In our study very few individuals (<3%) have reported high blood pressure, which was expected as the sample was formed by young healthy subjects. Due to this fact, we have not been able to find any association between cocoa consumption and blood pressure in our study. However, a tendency to reduce the proportion of hypertensive students in the high cocoa consumers group was observed. It is suggested that, the cocoa effect in blood pressure seem to be associated to the relaxation effects of procyanidins [152,153]. Procyanidins are in fact one of the compounds present in cocoa/chocolate products which can exert this protective action in cardiovascular health. Potassium and magnesium, important micronutrients present in cocoa and in chocolate are also relevant to control blood pressure [374,375].

Focusing in healthy lifestyle practices, FFQ was applied together with a validated questionnaire of physical activity, in particular, the short version of the IPAQ for adult populations. The IPAQ questionnaire allows a very concrete and precise evaluation of the physical activity level by considering several forms of physical activity, including walking and also the time of being seated, besides sports practice and/or programmed exercise. The IPAQ was able to standardize measures of health related physical activity behaviours of individuals from multiple countries and socio-cultural contexts [376]. Two meta-analysis have concluded that this tool is a valid and congruent questionnaire to evaluate physical activity in young and adult populations [377,378]. IPAQ has been used previously in young adults and especially in University students [379–381]. For these reasons, IPAQ was chosen in the current study.

From the results of IPAQ questionnaire, it can be concluded that the range of physical activity in the students' populations was very wide. In this sense, it is important to highlight that there were students that reported a very low physical activity that would be hazard for health promotion and disease prevention [382,383]. In addition a 14% had a sedentary lifestyle (<600 METs/week) and even 40% of the students did not reach the physical activity levels established by the recommendations guidelines for health-promotion (value of 1500 METs/week) [340]. However, these results are more favourable

than that reported by Varela-Mato et al. [381] using the same IPAQ questionnaire in a sample from the University of Vigo (Spain). In addition, the results obtained in the current Spanish and Portuguese university students sample were better than those reported by Haase et al. [384] focused in several worldwide countries in which sedentarism is followed by 46% of Mediterranean university women. Interestingly, the current results agree with results reported for university women from North-Western Europe and United States (24% of inactive students) [384].

The study of the profile of physical activity according to the consumption of cocoa provided an inverse correlation between the cocoa consumption and the physical activity, in such a way that the more cocoa they ate, the less exercise they did. This fact could also be seen when studying the proportion of people with low and high physical activity in the groups of students with low and high consumption of cocoa. This analysis allows realizing that in the higher consumer group, the percentage of students with high activity was lower than that in the lower consumer group, and the proportion of students with low activity was higher than that in group consuming less cocoa.

These results do not match with the scientific evidence that cocoa flavonoids are beneficial on physical activity. In this sense, preclinical studies show that cocoa flavonoid intake enhance fatigue resistance [385], and attenuate muscle damage after physical performance [386]. In addition, studies in humans have demonstrated that chocolate intake is an effective recovery aid after exhausting exercise [387]. The beneficial effects on exercise have been associated with the antioxidant properties of cocoa [388–390].

Taking into account the obtained results associating the physical activity with cocoa consumption we can suggest that the most active students within this sample do not recognize cocoa as an ergogenic aid, therefore further recommendations should be addressed to these individuals.

Besides anthropometrical data, blood pressure and physical activity results, the health status assessment of the individuals taking part of this study was also inquired, in particular it was asked for common diseases they usually had. In this sense, results from the health questionnaire showed a low frequency of chronic conditions in this sample as expected considering the age group of these participants. However, some relevant facts should be highlighted, the proportion of individuals showing chronic diseases was the highest in the low cocoa consumer group, which may suggest a role of cocoa in this sense. It has to be taken into account that only moderate consumption group has significantly less chronic diseases frequency. Considering the multiple benefits suggested from cocoa consumption these data reinforces, at least, the recommendation of a moderate cocoa intake. The most pointed out chronic conditions reported in this questionnaire were headaches and migraines. In spite there is some controversy on the effect of chocolate in migraine and headache origins [391], there is a hypothesis suggesting that they can derive from food hypersensitivity IgE and IgG-mediated, which can be ameliorated by cocoa polyphenols immunomodulator effects [392]. A high chocolate consumption can be also a food trigger for migraines and headaches [392] in

spite no biological mechanisms have been clearly identified and there is no solid scientific evidence [393]. Regarding other indicators of disease in which the immune response could be involved, and therefore expecting some effect due to the high immunomodulatory action of cocoa, we failed to observe an association with cocoa consumption (i.e. fever, flu, diarrhoea, or illness in general).

The possible relationship between cocoa consumption and allergy modulation is sustained in the evidences about flavonoid role on allergies [394] and in previous studies developed in our group of research involving cocoa on several animal models of allergy [249–251,395]. Regarding flavonoid effects on allergies, preclinical studies mainly carried out in rodents suggest that they may have a role in the prevention of IgE synthesis and mast cell degranulation. Using animal models with allergic asthma, it can be concluded that preventive treatment with particular flavonoid classes can reduce airway hyperresponsiveness, which is accompanied by lowered inflammatory mediators such as histamine and cytokines as well as cell infiltration [394]. In addition, there are some clinical trials in patients with allergic asthma or rhinitis that offer promising results with regard to these natural compounds. Among the mechanisms involved, there is a lower expression of IgE receptor or other membrane receptors, the modulation of calcium influx, and the downregulation of particular signaling pathways that eventually produces lower primary and secondary mediator release. Globally, some particular flavonoids could be an alternative or complementary therapy in the prevention and treatment of some allergies. Nevertheless, an increased number of clinical trials seem to be required in order to confirm the therapeutic role of flavonoids [394].

As previously mentioned, cocoa effects on allergies at preclinical level has been also established as previously mentioned. Studies in young rats reported the impact of cocoa intake on healthy immune status and allow suggesting its role in the prevention of some immune-mediated diseases. Specifically, it has been demonstrated that cocoa intake decreased Th2 immune-related antibodies in rats [229,230,238]. In addition, its effect on a rat model of allergy [249] and on food allergy and an anaphylactic response [251] has been established. In the first study, a cocoa-enriched diet in young rats produced an immunomodulatory effect that prevented the anti-allergen IgE synthesis [249]. In the food allergy approach, the synthesis of anti- OVA IgE and other Th2-related antibodies, Th2-related cytokines released from mesenteric lymph node and spleen cells, and intestinal gene expression of molecules involved in allergic response were modulated by cocoa diet, and the release of mast cell protease II after anaphylaxis was partially prevented [251].

In this context, the relationship of cocoa consumption and allergy was studied after classifying the students into three groups depending on their cocoa intake and the answers to the questionnaire related to this disease. Globally, a 19.6% of the total student population declared to suffer from some allergy. This self-reported value agree with an observational study performed in Spanish adult population which describes an overall 21,6% prevalence of allergy: 24.6% in women, 18.3% in men, and 26.9% in individuals from 18 to 24 years old [396]. Regardless the number of allergic subjects in all

the study, its distribution was different depending on their cocoa consumption. Particularly, the percentage of allergic people in the moderate and high consumers groups (13 and 19%, respectively) resulted statistically different than the 27.6% of allergic students in the low consumer group. Moreover, the cocoa intake, especially moderate consumption, was also associated with lower presence of symptoms. All these results allow suggesting that the habit of cocoa consumption in order to prevent or ameliorate the health imbalance induced by allergic processes and therefore allow confirming our initial second hypothesis. This is the first time to our knowledge that an inverse relationship between cocoa consumption in humans and allergy has been established. In this sense, although further investigation is required, it can be also suggested that an appropriate intake of flavonoids, not just only cocoa, may play a role in the prevention and eventually in the management of allergic diseases. This fact it is of importance because it would mean that an appropriate dietary habit, including cocoa products, might substantially prevent the onset of allergic diseases and ameliorate allergic symptoms. On the contrary, no association was found between cocoa consumption and intolerances, which do not include the immune mechanisms modulated by cocoa.

In summary, the present study evidences the underestimation of cocoa consumption due to approach limitations and has generated a valid tool for estimating real cocoa consumption. Cocoa intake in University students has been assessed, and different patterns of consumption have been found between Portuguese and Spanish university students regarding the type of cocoa source more frequently consumed. Finally, some clear associations between cocoa consumption and health status have been found. Therefore, this study can be the first part of a new study using the same methodology in a higher number of participants or different type of population. Alternatively, this health improvement by cocoa consumption showed here can be confirmed by an interventional clinical study.

6. CONCLUSIONS

The results obtained from this study have enabled us to draw the following conclusions:

Concerning Objective 1:

- It has been developed a new tool devoted to evaluate the cocoa consumption based in a FFQ with 90-items, which considers a wide range of products that are not included in other FFQs (dairy, pastry, desserts, cereals and spreads). The new FFQ has been validated in University students in Spanish and Portuguese languages through its comparison with two reference methods -a validated FFQ and a 24HDR assessment- by obtaining moderate correlations and good quintile classification.

- The new FFQ allows assessing cocoa consumption in a more detailed manner and, moreover, with a higher number of products resulting in a higher cocoa consumption than other current methods, such as the EFSA-Q or the 24HDR. This fact permits to affirm that cocoa intake assessment with current methodologies is usually underestimated.

Concerning Objective 2:

- Cocoa consumption has been assessed by this new FFQ resulting in an intake of about 12 g or 2.5 cocoa products per day for Spanish students (N=50), 14 g or 3 cocoa products per day for Portuguese students (N=70) and 13 g or 3.5 cocoa products per day for the large cohort studied (N=270).

- Differential cocoa consumption patterns between both cohorts have been found. Although in both cohorts the chocolate bars are the main source of cocoa, later the dairy and pastries, followed by snacks as the fourth source type; the second cocoa provider is cocoa dairy products in the Spanish cohort whereas it is the cocoa pastry in the case of the Portuguese cohort.

Concerning Objective 3:

- The sociodemographic characteristics of the University individuals enrolled in the study constituted a very homogeneous population of young healthy individuals. Due to this similarities and healthy status of participants, with exception of some particular situations as found in the ex-smokers subgroup, the cocoa intake has not been associated with a specific age or lifestyle pattern. This fact is evidenced in the lack of significant effect of cocoa on mean blood pressure.

- Although no association of BMI with cocoa intake was found, a reduction in the percentage of overweight subjects and the underweight/overweight ratio appeared in the moderate and high cocoa consumers group.

- High cocoa consumption can be associated with low physical activity because the proportion of low activity and high activity people in the high cocoa consumer group are significantly increased and reduced, respectively, with respect to the low cocoa consumer group.

- Moderate cocoa consumption has been associated with lower presence of self-reported chronic disease in the University student's sample.

- The proportion of allergic people in the moderate and high cocoa consumers resulted statistically lower than that in the students in the low cocoa consumer group. Moreover, the cocoa intake, especially moderate consumption, was also associated with lower presence of allergic derived symptoms.

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APPENDICES

Appendix I
C-FFQ and P-FFQ

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DATOS SOCIOECONÓMICOS

Objetivo: Es necesario conocer ciertos datos sociodemográficos que permitan una mejor interpretación de la información posterior.

1. Datos personales del encuestado (rellenar casillas)

Nombre	
Apellidos	
Sexo	
Año de nacimiento	
Lugar de nacimiento	
Dirección actual	
Teléfono	
E-mail	

2. Estado civil/legal (marcar con una cruz):

Soltero/a	
Casado/a o vivo en pareja	
Separado/a o Divorciado/a	
Viudo/a	

3. ¿Con quién convive actualmente? (si la residencia es diferente durante la semana y el fin de semana especificar la situación durante la semana)

Solo/a	
Padres y hermanos	
Padres	
Padre o madre	
Hermanos	
Pareja / cónyuge	
Pareja e hijos	

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Hijos	
Compañeros de piso	
Residencia o similar	
Otros (especificar)	

4. ¿Cuántas personas conviven en su casa?

Solo/a	
2	
3	
4	
>4	

5. ¿Qué tipos de estudios está cursando?

Primaria	
Secundaria	
Formación profesional I o ciclos formativos de grado medio	
Formación profesional II o ciclos formativos de grado superior	
Bachillerato o similar	
Estudios universitarios de primer ciclo: diplomatura	
Estudios universitarios de segundo ciclo: grado o licenciatura	
Estudios universitarios de tercer ciclo: postgrado, máster, doctorado o similar.	
Otros (especificar)	

6. ¿Además de estudiar, también trabaja?

SI	
NO	

7. Si también trabaja, ¿cuántas horas de promedio a la semana?

< 2 h	
2-5 h	
5-10 h	
10-15 h	
>15h	

8. Si también trabaja, ¿Qué horario realiza?

Jornada partida	
Jornada continua, principalmente mañanas	
Jornada continua, principalmente tardes	
Jornada continua, principalmente noches	
Turnos	
Jornada irregular o variable según los días	

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Otros (especificar)	

CUESTIONARIO DE FRECUENCIA DE CONSUMO DE ALIMENTOS

A continuación indique con una **X** si acostumbra a tomar los alimentos del siguiente listado y la frecuencia con la cual los acostumbra a tomar

	1. Nunca	**2.** 1-6 veces al año	**3.** 7-11 veces al año	**4.** 1 vez al mes	**5.** 2-3 veces al mes	**6.** 1 vez por semana	**7.** 2 veces por semana	**8.** 3-4 veces por semana	**9.** 5-6 veces por semana	**10.** 1 vez al día	**11.** 2 veces al día	**12.** 3 o más veces al día
Cereales (tipo almuerzo o en barras)												
1. Cereales con chocolate												
2. Cereales sin chocolate												
Müesli												
3. Müesli con chocolate												
4. Müesli sin chocolate												
Frutas (naturales)												
5. Manzana												
6. Pera												
7. Naranja												
8. Mandarina												
9. Limón												
10. Plátano												
11. Melocotón												
12. Piña												
13. Fresa o fresón												
14. Frutas rojas (moras, …)												
15. Melón												
16. Sandía												
17. Otras frutas												
Otros preparados de fruta												
18. Zumo de frutas												
19. Mermelada												
20. Fruta en conserva												

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		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
		Nunca	1-6 veces al año	7-11 veces al año	1 vez al mes	2-3 veces al mes	1 vez por semana	2 veces por semana	3-4 veces por semana	5-6 veces por semana	1 vez al día	2 veces al día	3 o más veces al día
Verduras y hortalizas													
21. Verduras cocidas (espinacas, judías,...)													
22. Tomate													
23. Tomate frito													
24. Pan con tomate													
25. Cebolla													
26. Pimiento													
27. Zanahorias													
28. Otras hortalizas													
Productos lácteos y similares													
Leche entera	29. con café												
	30. con café descafeinado												
	31. con cacao												
	32. sola												
Leche semidesnatada	33. con café												
	34. con café descafeinado												
	35. con cacao												
	36. sola												
Leche desnatada	37. con café												
	38. con café descafeinado												
	39. con cacao												
	40. sola												
Batido de soja	41. con chocolate												
	42. sin chocolate												
Flan	43. de chocolate												
	44. sin chocolate												
Natillas	45. de chocolate												
	46. sin chocolate												
Yogur	47. de chocolate												
	48. con fruta												
	49. natural o de sabores												
Helado	50. de chocolate												

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	51. con trozos de chocolate											
	52. sin chocolate											
		1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**
		Nunca	1-6 veces al año	7-11 veces al año	1 vez al mes	2-3 veces al mes	1 vez por semana	2 veces por semana	3-4 veces por semana	5-6 veces por semana	1 vez al día	2 veces al día	3 o más veces al día
Repostería y pastelería													
Repostería (croissant, donuts, palmeras, bollicao…)	53. sin chocolate												
	54. cubierta con chocolate												
	55. rellena de chocolate												
Galletas	56. sin chocolate												
	57. rellenas de chocolate (Príncipe..)												
	58. recubiertas de chocolate (Kit kat, Huesitos, Filipinos, Petit ecolier, Pims)												
	59. con chocolate (Oreo)												
	60. con trocitos de chocolate (Chips Ahoy,…)												
Pastel (una ración)	61. de chocolate												
	62. con partes de chocolate												
	63. sin chocolate												
Polvorones / mantecados	64. de chocolate												
	65. sin chocolate												
Turrón	66. de chocolate												
	67. sin chocolate												
68. Bombones													
Chocolate (considera el tiempo en que consumes una tableta entera)													
69. Chocolate blanco													
70. Chocolate con leche													
71. Chocolate negro (<60% cacao)													
72. Chocolate negro (60-70% cacao)													
73. Chocolate negro (70-85% cacao)													
74. Chocolate negro (> 85% cacao)													
Chocolate en vaso o taza (1 ración)													
75. Batido de chocolate (Cacaolat, o similar…)													
76. Chocolate soluble en agua													
77. Chocolate caliente a la taza (Paladin, Ram, Valor, Torres,,…)													

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	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
	Nunca	1-6 veces al año	7-11 veces al año	1 vez al mes	2-3 veces al mes	1 vez por semana	2 veces por semana	3-4 veces por semana	5-6 veces por semana	1 vez al día	2 veces al día	3 o más veces al día
Crema de chocolate y otros												
78. Crema de cacao (Nocilla, Nutela,…)												
79. Café solo												
80. Café descafeinado solo												
81. Té solo												
82. Té con leche												
83. Otras infusiones												
Bebidas alcohólicas												
84. Vino tinto												
85. Vino blanco												
86. Vino rosado												
87. Cerveza												
88. Cava o similares												
89. Carajillos, vermut, chupitos y licores afrutados												
90. Whisky, coñac, ginebra, vodka, ron, aguardientes, combinados y similares												

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FOOD FREQUENCY QUESTIONAIRE FOR COCOA AND CHOCOLATE CONSUMPTION

Cocoa has been highlighted as a food with potential benefits to human health due to it being an important source of polyphenols. However, few studies show the contribution of cocoa and chocolate products in polyphenol intake. The aim of this work was to develop and validate a food frequency questionnaire (FFQ) evaluating the intake of food products containing cocoa (C-FFQ). A sample of university students was recruited to complete the 90-item questionnaire in Spain and therefore in Spanish (http://diposit.ub.edu/dspace/handle/2445/60475) and in Portugal in a Portuguese version, which is the present document. The developed FFQ questionnaire can be considered as a valid option for assessing the consumption frequency of cocoa- and chocolate-derived products, thereby allowing the evaluation of cocoa polyphenol intake in further studies.

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Questionário de frequência de ingestão dos alimentos

Na tabela que se segue, indique com um X se costuma ingerir os alimentos da seguinte lista e a frequência com o que costuma fazer.

	1. Nunca	2. 1-6 vezes por ano	3. 7-11 vezes por ano	4. 1 vez por mês	5. 2-3 vezes por mês	6. 1 vez por semana	7. 2 vezes por semana	8. 3-4 vezes por semana	9. 5-6 vezes por semana	10. 1 vez por dia	11. 2 vezes por dia	12. 3 ou mais vezes por dia
Cereais (tipo pequeno-almoço ou em barras)												
1. Cereais com chocolate												
2. Cereais sem chocolate												
Muesli												
3. Muesli com chocolate												
4. Muesli sem chocolate												
Frutas (naturais)												
5. Maçã												
6. Pera												
7. Laranja												
8. Tangerina												
9. Limão												
10. Banana												
11. Pêssego												
12. Ananás												
13. Morangos												
14. Frutos vermelhos (amoras, etc.)												
15. Melão												
16. Melancia												
17. Outras frutas												
Outros preparados de fruta												
18. Sumo de frutas												
19. Marmelada												
20. Fruta em conserva												

CC BY NC

		1. Nunca	2. 1-6 vezes por ano	3. 7-11 vezes por ano	4. 1 vez por mês	5. 2-3 vezes por mês	6. 1 vez por semana	7. 2 vezes por semana	8. 3-4 vezes por semana	9. 5-6 vezes por semana	10. 1 vez por dia	11. 2 vezes por dia	12. 3 ou mais vezes por dia
Verduras e hortaliças													
21. Verduras cozidas (espinafres, feijão-verde, brócolos)													
22. Tomate													
23. Tomate frito													
24. Pão com tomate													
25. Cebola													
26. Pimento													
27. Cenoura													
28. Outras hortaliças													
Produtos lácteos e similares													
Leite gordo	29. com café												
	30. com café descafeinado												
	31. com cacau												
	32. simples												
Leite meio-gordo	33. com café												
	34. com café descafeinado												
	35. com cacau												
	36. simples												
Leite magro	37. com café												
	38. com café descafeinado												
	39. com cacau												
	40. simples												
Bebida de soja	41. com chocolate												
	42. sem chocolate												
Flan	43. de chocolate												
	44. sem chocolate												
Leite-creme	45. de chocolate												
	46. sem chocolate												
Iogurte	47. de chocolate												
	48. com fruta												
	49. natural ou de sabores												
Gelado	50. de chocolate												
	51. com pedaços de chocolate												

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	52. sem chocolate											
		1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**
		Nunca	1-6 vezes por ano	7-11 vezes por ano	1 vez por mês	2-3 vezes por mês	1 vez por semana	2 vezes por semana	3-4 vezes por semana	5-6 vezes por semana	1 vez por dia	2 vezes por dia	3 ou mais vezes por dia
Mercearia doce e Pastelaria													
Pastelaria (croissant, donuts, palmiers, bollicao…)	53. sem chocolate												
	54. coberta com chocolate												
	55. recheada com chocolate												
Bolachas	56. sem chocolate												
	57. recheadas de chocolate (Ex:. Príncipe..)												
	58. cobertas de chocolate (Ex:. Kit kat, Ossitos, Filipinos, Petit ecolier, Pims)												
	59. com chocolate (Ex:. Oreo)												
	60. com pedaços de chocolate (Ex:. Chips Ahoy,…)												
Bolo (uma porção)	61. de chocolate												
	62. com partes de chocolate												
	63. sem chocolate												
Biscoitos secos/amanteigados	64. de chocolate												
	65. sem chocolate												
Turrão	66. de chocolate												
	67. sem chocolate												
68. Bombons													
Chocolate (considere a frequência com que ingere uma tablete inteira)													
69. Chocolate branco													
70. Chocolate com leite													
71. Chocolate negro (<60% cacau)													
72. Chocolate negro (60-70% cacau)													
73. Chocolate negro (70-85% cacau)													
74. Chocolate negro (> 85% cacau)													
Chocolate em copo ou caneca (1 ração)													
75. Batido de chocolate (ex:. Colacao, Ucal, etc.)													
76. Chocolate solúvel em água													
77. Chocolate quente na caneca (ex:. Royal, Nestlé, etc.)													

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
	Nunca	1-6 vezes por ano	7-11 vezes por ano	1 vez por mês	2-3 vezes por mês	1 vez por semana	2 vezes por semana	3-4 vezes por semana	5-6 vezes por semana	1 vez por dia	2 vezes por dia	3 ou mais vezes por dia
Creme de chocolate e outros												
78. Creme de cacau (Ex:. Tulicreme, Nutella,...)												
79. Café												
80. Café descafeinado simples												
81. Chá simples												
82. Chá com leite												
83. Outras infusões												
Bebidas alcoólicas												
84. Vinho tinto												
85. Vinho branco												
86. Vinho rosé												
87. Cerveja												
88. Vinho cava ou similares												
89. Café com bebida espirituosa, vermute, licores, shot												
90. Whisky, cognac, vodka, rum, aguardentes, combinados e similares												

Appendix II
24HDR

RECORDATORIO DE 24 HORAS

En la siguiente tabla indique qué comió ayer. Clasifique las comidas según las acciones de la primera columna e indique la hora en que lo hizo.

En el apartado de ALIMENTO Y PREPARACIÓN, ponga todo lo que comió (primer plato, segundo plato, postre) y en la CANTIDAD utilice términos como ración, plato, vaso, taza, bol, etc. También estime la cantidad como una ración, media ración, doble ración, etc

ACCIÓN	HORA	ALIMENTO, PREPARACIÓN	CANTIDAD
Al levantarse			
Desayuno			
Media mañana			
Comida			
Merienda			
Cena			
Entre horas			

QUESTIONÁRIO DAS 24H ANTERIORES

Indique o que comeu e bebeu no dia de ontem na tabela que se segue. Indique a hora de cada refeição.

Na coluna Alimento e preparação, ponha todos os alimentos ingeridos (entrada, sopa, 1º e 2º prato, sobremesa) e a quantidade. Utilize medidas como prato, copo, chávenas, tigelas e colheres para estimar a porção assim como termos como "meia dose" ou "uma dose", etc.

	HORA	ALIMENTO, PREPARAÇÃO	QUANTIDADE
Ao acordar			
Pequeno-almoço			
Merenda da manhã			
Almoço			
Lanche			
Jantar			
Ceia			

Appendix III

EFSA-Q

GATHERING CONSUMPTION DATA ON SPECIFIC CONSUMER GROUPS OF ENERGY DRINKS

QUESTIONNAIRE

ADULTS 18-65

March 2012

Good morning.

The Consortium **Nomisma-Areté** was awarded by the **European Food Safety Authority (EFSA)** to carry out a study on consumption habits, with specific regards to beverages. We kindly ask you if you could fill in the following questionnaire. Answering this questionnaire will not take you more than 5 minutes.

Please consider that the data will be collected anonymously and be disseminated only in aggregated form.

Thank you for your collaboration.

RESPONDENT INFORMATION

1. Year of birth

_____ → **IF > 1994 THE TARGET IS NOT ELIGIBLE (< 18 years old)**

→ **IF < 1947 THE TARGET IS NOT ELIGIBLE (> 65 years old)**

2. Gender of respondent

Male ❑

Female ❑

3. Level of education

None ❑
Primary school ❑
Lower secondary school ❑
Upper secondary school ❑
Degree ❑
Master/PhD ❑

4. Are you a

Student ❑
Worker ❑
Other ❑ Specify _____

5. Do you live in a…

Rural area ❑→ **QUESTION 7**
Urban area ❑→ **QUESTION 6**

6. (IF YOU LIVE IN AN URBAN AREA) – Approximately, how many inhabitants does your city have?

< 5.000 ❑
5.000-20.000 ❑
20.000-100.000 ❑
100.000-500.000 ❑
> 500.000 ❑

DRINKS CONSUMPTION

7. Which of the following beverages do you drink most often ...

Please choose your favourite (only 1) for each column	**Usually during the day**	**During sport**	**Entertainment**
Water (bottled/flavoured)	❑	❑	❑
Fizzy drinks (e.g. Coca-Cola, Fanta, soda)	❑	❑	❑
Fruit juices, smoothies ...	❑	❑	❑
Hot chocolate	❑	❑	❑
Coffee/cappuccino	❑	❑	❑
Tea/Ice tea	❑	❑	❑
Energy drinks (e.g. Red Bull, Monster, Burn...)	❑	❑	❑
Sport Drinks (e.g. Gatorade, Lucozade, Aquarious...)	❑	❑	❑
Wine	❑	❑	❑
Beer	❑	❑	❑
Spirits (e.g. vodka, rum, ...)	❑	❑	❑
Other drinks Specify _____	❑	❑	❑

ENERGY DRINKS CONSUMPTION

Furthermore, we will ask you some brief questions on the consumption of Energy Drinks, namely products such as Red Bull, Monster, Burn, etc.

8. During the past 3 days, have you been drinking any energy drink?

Yes ❑

No ❑

9. Over the last year, have you been drinking energy drinks in at least one occasion?

Yes ❑ → **QUESTION 10**

No ❑ → **THANK YOU FOR YOUR COLLABORATION. THE INTERVIEW IS FINISHED**

10. Where and how often do you drink energy drinks?

Please indicate the answer for each place/situation

	Usually	Sometimes	Never
At home, in ordinary situations	❑	❑	❑
At home, with friends during parties	❑	❑	❑
Bar/Pub	❑	❑	❑
Disco	❑	❑	❑
Associated to sport and physical exercise	❑	❑	❑
At work	❑	❑	❑
Other Specify _____	❑	❑	❑

11. In an average month, over the last year, have you been drinking energy drinks about…

Every day ❑
4-5 days a week ❑
2-3 days a week ❑
Once a week ❑
Once-twice a month ❑
Rarely ❑

12. What is the usual size of energy drinks you consume?

Can: 250 ml ❑
Can: 355 ml ❑
Energy shot: 50 ml ❑
Other ❑ Specify format and indicate volume in ml _____

13. In an average month, over the last year, how many cans of energy drinks have you been drinking?

1 or less ❑
2 to 4 cans ❑
5 to 10 cans ❑
11 to 20 cans ❑
More than 20 cans ❑ Specify the average number _____

14. Over the last year, how many cans of energy drinks have you been drinking in a single session (time frame of a couple of hours e.g. a night out, during study or sport session)?

Only 1 ❑
2 ❑
3 ❑
4 ❑
More than 4 ❑ Specify the average number _____

15. Why do you usually drink energy drinks?

Please choose one answer for each column. The first column indicates the first choice in order of importance, the second column the second choice and the third column the third choice (1st choice, 2nd choice, 3rd choice)

	1st choice	2nd choice	3rd choice
Need energy (in general)	❑	❑	❑
Stay awake	❑	❑	❑
I like their taste	❑	❑	❑
Concentration augmenting (Studying/Working)	❑	❑	❑
Long time driving	❑	❑	❑
Treat hangover	❑	❑	❑
Enhance sport performance	❑	❑	❑
Stimulate my metabolism	❑	❑	❑
Other Specify _____	❑	❑	❑

16. Which brand of energy drinks do you usually drink?

Please choose one answer for each column, based on the number of cans consumed. The first column indicates the first choice in order of importance, the second column the second choice and the third column the third choice.

	1st choice	2nd choice	3rd choice
Red Bull	❑	❑	❑
Burn	❑	❑	❑
Shark	❑	❑	❑
Kx	❑	❑	❑
Monster	❑	❑	❑
Battery	❑	❑	❑
Relentless	❑	❑	❑
Rockstar	❑	❑	❑
Fullthrottle	❑	❑	❑
Other brand Specify _____	❑	❑	❑

17. Considering your favourite brand, what percentage of your total consumption of energy drinks does it represent?

If you drink only 1 brand, your answer will be 100%

Specify _____ %

18. Do you usually prefer energy drinks…

With sugar ❑
Sugar free ❑

ENERGY DRINKS & ALCOHOL CONSUMPTION

Now we would need you to answer some questions on consuming energy drinks and alcohol in a single session. Please consider for "single session" a situation in a defined time frame of about a couple of hours (e.g. nights out, study or sport session).

19. Over the last year, at least once, have you been drinking energy drinks and alcohol in a single session?

Multiple-choice answer

Yes, mixed at the moment of consumption in the same glass (e.g. cocktail: energy drink and alcohol) ❑
Yes, pre-mixed alcoholic energy drinks (e.g. can/bottle ready to drink containing alcohol) ❑
Yes, but consumed in different moments (e.g. consumption of alcohol preceded/followed by consumption of an energy drink) ❑
No, never ❑ → **Q. 24**

20. In an average month, over the last year, have you been drinking energy drinks and alcohol (mixed together or consumed in different moments during a single session) about…

Every day ❑
4-5 days a week ❑
2-3 days a week ❑
Once a week ❑
Once-twice a month ❑
Rarely ❑

21. You usually drink energy drinks and alcohol …

About every time ❑
Often (approx. 1 out of 2 intakes of energy drinks are mixed with alcohol) ❑
Sometimes (approx. 1 out of 4 intakes of energy drinks are mixed with alcohol) ❑
Rarely ❑

22. What kind of energy drinks do you usually mix with alcohol?

Please choose one answer for each column, based on the number of cans consumed. The first column indicates the first choice in order of importance, the second column the second choice and the third column the third choice

	1st choice	2nd choice	3rd choice
Red Bull	❑	❑	❑
Burn	❑	❑	❑
Shark	❑	❑	❑
Kx	❑	❑	❑
Monster	❑	❑	❑
Relentless	❑	❑	❑
Full Throttle	❑	❑	❑
Pre-mixed alcoholic energy drinks Specify _____	❑	❑	❑
Other Specify _____	❑	❑	❑

23. In an average month, over the last year, how many cocktails of energy drinks mixed with alcohol have you been drinking in a single session?

0 (None) ❑
Only 1 ❑
2 ❑
3 ❑
4 ❑
More than 4 ❑ Specify the average number _____

ENERGY DRINKS & SPORTS

Now we would need you to answer some questions on consumption of energy drinks associated with sport and physical exercise.

24. Over the last year, have you usually practised sport or physical exercise (jogging included) about …

Every day	❑
3-4 times a week	❑
Twice a week	❑
Once a week	❑
Rarely	❑ → **QUESTION 28**
Never	❑ → **QUESTION 28**

25. You usually drink energy drinks before/in association with/after sport or physical exercise …

About every time	❑
Often (approx. on 1 out of 2 sport session you drink energy drinks – 50%)	❑
Sometimes (approx. on 1 out of 4 sport session you drink energy drinks – 25%)	❑
Never	❑ → **QUESTION 28**

26. In an average month, over the last year, how many cans of energy drinks have you been drinking in a single sport/physical exercise session?

Only 1	❑
2	❑
3	❑
4	❑
More than 5	❑ Specify the average number _____

27. You usually drink energy drinks before/during/after sport in order to increase ….

Please choose one answer for each column. The first column indicates the first choice in order of importance, the second column the second choice and the third column the third choice

	1st choice	2nd choice	3rd choice
Endurance time at the maximum intensity	❑	❑	❑
Aerobic endurance	❑	❑	❑
Vitality	❑	❑	❑
Concentration	❑	❑	❑
Power	❑	❑	❑
Other Specify _____	❑	❑	❑

GENERAL CONSUMPTION HABITS

Let's continue with some questions on your consumption habits of other beverages and chocolate.

28. In an average WEEK, over the last year, have you been drinking COFFEE or BEVERAGES WITH COFFEE (e.g. Cappuccino) about…

Every day ❑
4-5 days a week ❑
2-3 days a week ❑
Once a week ❑
Rarely ❑
Never ❑ → **QUESTION 31**

29. When you drank it, in an average DAY, how many cups/mugs have you been drinking?

Please consider a regular cup/mug

	None	1 cup	2 cups	3 cups	4 cups	More than 4 cups
Espresso coffee	❑	❑	❑	❑	❑	❑ Specify the average number ____
Coffee (instant, ground, ice-coffee …)	❑	❑	❑	❑	❑	❑ Specify the average number ____
Cappuccino	❑	❑	❑	❑	❑	❑ Specify the average number ____

30. Do you usually drink COFFEE/BEVERAGES WITH COFFEE (e.g. Cappuccino) …

Caffeinated ❑
Decaffeinated ❑

31. In an average WEEK, over the last year, have you been drinking TEA (INSTANT, TEA BAG, ICE-TEA …) about…

Every day ❑
4-5 days a week ❑
2-3 days a week ❑
Once a week ❑
Rarely ❑
Never ❑ → **QUESTION 34**

32. When you drank it, in an average DAY, how many cups of TEA (INSTANT, TEA BAG, ICE-TEA …) have you been drinking?

Please consider a regular cup/glass

1 ❑
2 ❑
3 ❑
4 ❑
More than 4 ❑ Specify the average number _____

33. Do you usually drink TEA …

Standard ❑
Decaffeinated ❑

34. In an average WEEK, over the last year, have you been drinking HOT CHOCOLATE about…

Every day ❑
4-5 days a week ❑
2-3 days a week ❑
Once a week ❑
Rarely ❑
Never ❑ → **QUESTION 36**

35. When you drank it, in an average DAY, how many cups of HOT CHOCOLATE have you been drinking?

Please consider a regular cup/mug

1 ❑
2 ❑
3 ❑
4 ❑
More than 4 ❑ Specify the average number _____

36. In an average WEEK, over the last year, how many CHOCOLATE BARS have you been eating?

Please consider a 100 grams chocolate bar

None ❑
1 ❑
2 ❑
3 ❑
4 ❑
More than 4 ❑ Specify the average number _____

37. In an average WEEK, over the last year, how many CHOCOLATE SNACKS (e.g. Mars, Kit kat..) have you been eating?

None ❑
1 ❑
2 ❑
3 ❑
4 ❑
More than 4 ❑ Specify the average number _____

38. Do you usually prefer BARS/ SNACKS with …

Dark chocolate ❑
Milk chocolate ❑
White chocolate ❑

39. In an average WEEK, over the last year, have you been drinking COLAS (e.g. Coca-Cola, Pepsi) about…

Every day	❑
4-5 days a week	❑
2-3 days a week	❑
Once a week	❑
Rarely	❑
Never	❑ → **QUESTION 42**

40. In an average WEEK, over the last year, how many cans of COLAS (e.g. Coca-Cola, Pepsi) have you been drinking?

Less than 1 can (0,33 l/week)	❑	
Between 1 and 2 cans (0,33- 0,66 l/week)	❑	
Approximately 3 cans (1 l/week)	❑	
Between 4 and 6 cans (1,32- 2 l/week)	❑	
Between 7 and 10 cans (2,31- 3 l/week)	❑	
More than 10 cans a week	❑	Specify the average number _____

41. Do you usually drink COLA …

With caffeine	❑
Decaffeinated	❑

CONSUMER PROFILE

The interview is nearly completed. We ask you to answer to some final questions on your lifestyle.

42. On average, how many hours do you sleep every night?

Specify the average number _____

43. Are you a regular smoker?

Yes	❑
No	❑

44. Height:

Specify (cm)_____

45. Weight:

Specify (Kg)_____

46. Characteristics of your family unit:

I am single and live with my parents	❑	
I am single and live on my own	❑	
I live with my partner and we have no children	❑	
I live with my partner and we have children	❑	
Other	❑	Specify _____

Appendix IV
IPAQ Short Form

3. DATOS DE ACTIVIDAD FÍSICA

***Introducción**: El siguiente **Cuestionario Internacional de Actividad Física** (IPAQ, por sus siglas en inglés) es una versión corta (4 preguntas generales) disponibles para ser usadas de forma autoadministrada, entre otros. El propósito de los cuestionarios es proveer instrumentos comunes que pueden ser usados para obtener datos internacionalmente comparables relacionados con actividad física relacionada con salud.*

El desarrollo de una medida internacional para actividad física comenzó en Ginebra en 1998 y fue seguida de un extensivo examen de confiabilidad y validez hecho en 12 países (14 sitios) en el año 2000. Los resultados finales sugieren que estas medidas tienen aceptables propiedades de medición para usarse en diferentes lugares y en diferentes idiomas, y que son apropiadas para estudios nacionales poblacionales de prevalencia de participación en actividad física.

Objetivo: Estamos interesados en saber acerca de la clase de actividad física que la población hace como parte de su vida diaria. Las preguntas se referirán acerca del tiempo que usted utilizó siendo físicamente activo en los **últimos 7 días**. Por favor responda cada pregunta aún si usted no se considera una persona activa. Por favor piense en aquellas actividades que usted hace como parte del trabajo, en el jardín y en la casa, para ir de un sitio a otro, y en su tiempo libre de descanso, ejercicio o deporte.

SECCIÓN 1: Piense acerca de todas aquellas **actividades vigorosas** que usted realizó en los **últimos 7 días**. Actividades **vigorosas** son las que requieren un esfuerzo físico fuerte y le hacen respirar mucho más fuerte que lo normal. Piense *solamente* en esas actividades que usted hizo por lo menos 10 minutos continuos.

1. Durante los **últimos 7 días**, ¿Cuántos días realizó usted actividades físicas **vigorosas** como levantar objetos pesados, excavar, aeróbicos, o pedalear rápido en bicicleta?

Días por semana	
Ninguna actividad física vigorosa (Pase a la pregunta 3)	

2. ¿Cuánto tiempo en total usualmente le tomó realizar actividades físicas **vigorosas** en uno de esos días que las realizó?

Horas por día	
Minutos por día	
No sabe/No está seguro	

SECCIÓN 2: Piense acerca de todas aquellas **actividades moderadas** que usted realizó en los **últimos 7 días** Actividades **moderadas** son aquellas que requieren un esfuerzo

físico moderado y le hace respirar algo más fuerte que lo normal. Piense *solamente* en esas actividades que usted hizo por lo menos 10 minutos continuos.

3. Durante los **últimos 7 días**, ¿Cuántos días hizo usted actividades físicas **moderadas** tal como cargar objetos livianos, pedalear en bicicleta a paso regular, o jugar dobles de tenis? No incluya caminatas.

Días por semana	
Ninguna actividad física moderada (Pase a la pregunta 5)	

4. Usualmente, ¿Cuánto tiempo dedica usted en uno de esos días haciendo actividades físicas **moderadas**?

Horas por día	
Minutos por día	
No sabe/No está seguro	

SECCIÓN 3: Piense acerca del tiempo que usted dedicó a **caminar** en los **últimos 7 días**. Esto incluye tareas domésticas, caminatas para ir de un sitio a otro, o cualquier otra caminata que usted hizo únicamente por recreación, deporte, ejercicio, o placer.

5. Durante los **últimos 7 días**, ¿Cuántos días caminó usted por al menos 10 minutos continuos?

Días por semana	
No caminó (Pase a la pregunta 7)	

6. Usualmente, ¿Cuánto tiempo gastó usted en uno de esos días **caminando**?

Horas por día	
Minutos por día	
No sabe/No está seguro	

SECCIÓN 4: La última pregunta se refiere al tiempo que usted permaneció **sentado(a)** en la semana en los **últimos 7 días**. Incluya el tiempo sentado(a) en el trabajo, la casa, estudiando, y en su tiempo libre. Esto puede incluir tiempo sentado(a) en un escritorio,

visitando amigos(as), leyendo o permanecer sentado(a) o acostado(a) mirando televisión.

7. Durante los **últimos 7 días**, ¿Cuánto tiempo permaneció **sentado(a)** en un **día laborable**?

Horas por día	
Minutos por día	
No sabe/No está seguro	

3. Dados de atividade física

Introdução: O questionário que se segue, o **Questionário Internacional de Atividade física** (IPAQ pelas suas suas siglas) é uma versão curta (4 perguntas gerais) disponível para poder ser autoadministrada. O propósito deste questionário passa por permitir criar um instrumento para obter dados internacionalmente comparáveis de atividade física relacionada com a saúde.

O desenvolvimento de uma medida internacional para a atividade física começou em Genebra no ano de 1998 e foi seguida por um sistema extensivo de fiabilidade e validade feito em 12 países (14 sítios) no ano de 2000. Os resultados finais sugerem que estas medidas têm propiedades aceitáveis de medição para serem usadas em diferentes lugares e diferentes idiomas e que são apropriadas para estudos nacionais e populacionais para a prevalência de participação em atividade física.

Objetivo: Estamos interesados em saber sobre o tipo de atividade física que a população faz habitualmente na sua vida diária. As perguntas referem-se ao tempo que se dedicou a ser físicamente ativo nos **últimos 7 dias.** Por favor responda a cada pregunta mesmo que não se considere uma pessoa ativa. Por favor pense nas atividades que podem fazer parte do seu trabalho, jardín e casa, assim como de ir para um sítio a outro, ou no seu tempo livre de descanso, exercício ou desporto.

SECÇÃO 1: Pense em todas as **atividades vigorosas** que realizou nos **últimos 7 dias**. Atividades vigorosas são as que requerem um esforço físico forte ou que acarretam algum esforço respiratorio em relação ao normal. Pense apenas nessas atividades que realizou durante pelo menos 10 minutos consecutivos.

1. Durante os **últimos 7 días**, quantos días realizou atividades físicas **vigorosas** como levantar objetivos pesados, escavar, exercício cardiovascular ou pedalar rápido na bicicleta?

Dias por semana	
Nenhuma atividade vigorosa (Passe para a pregunta 3)	

2. Quanto tempo passou em média a realizar atividades **vigorosas** nos días em que as realizou?

Horas por dia	
Minutos por dia	
Não sabe/Não tem a certeza	

SECÇÃO 2: Pense em todas as atividades moderadas que realizou nos últimos 7 dias. São atividades moderadas as que requerem um esforço físico moderado e que fazem respirar um pouco mais forte do que o normal. Pense apenas nas atividades moderadas que fez durante pelo menos 10 minutos consecutivos.

3. Durante os **últimos 7 días**, quantos días realizou atividades físicas **moderadas** como carregar objetivos leves, pedalar a um ritmo médio, jogar ténis em duplas? Não inclua caminhadas.

Días por semana	
Nenhuma actividad física moderada (Pase a la pregunta 5)	

4. Quanto tempo passou em média a realizar atividades **moderadas** nos días em que as realizou?

Horas por dia	
Minutos por dia	
Não sabe/Não tem a certeza	

SECÇÃO 3: Pense sobre o tempo que dedicou a caminhar nos últimos 7 dias. Isto inclui tarefas domésticas, caminhadas para ir de um sítio a outro, ou a qualquer outra caminhada que fez únicamente por lazer, desporto, exercício ou prazer.

5. Durante os **últimos 7 días**, quantos días caminhou durante pelo menos 10 minutos consecutivos?

Dias por semana	
Não caminhou (Passe para a pergunta 7)	

6. Durante quanto tempo **caminhou** habitualmente durante esses días?

Horas por dia	
Minutos por dia	
Não sabe/Não tem a certeza	

SECÇÃO 4: A última pergunta refere-se ao tempo que permaneceu sentado(a) por semana nos últimos 7 dias. Inclua o tempo sentado(a) no local de trabalho, em casa, a estudar e no seu tempo livre. Isto pode incluir tempo sentado(a) num escritório, a visitar amigos(as), a ler ou permanecendo sentado(a) ou deitado(a) a ver televisão.

7. Durante os últimos **7 dias**, quanto tempo permaneceu sentado(a) durante no **dia útil da semana**?

Horas por dia	
Minutos por dia	
Não sabe/Não tem a certeza	

Appendix V

Health Assessment Questionnaire

4. DATOS DE SALUD

Objetivo: Estamos interesados en obtener información sobre su estado general de salud y estilo de vida.

SECCIÓN 1: A continuación se le pregunta por ciertos **marcadores antropométricos**.

1. Nos podría decir cuál es su:

Altura actual (cm)	
Peso actual (Kg)	
Peso habitual (Kg)	
Peso máximo (Kg)	

SECCIÓN 2: A continuación se le pregunta por su **estado general de salud** y morbilidad.

2. ¿Cómo diría usted que es su salud en general?

Excelente	
Muy buena	
Buena	
Regular	
Mala	

3. De las siguientes situaciones, ¿cuál describe mejor su comportamiento respecto al tabaco?

Actualmente no fumo nada	
Actualmente fumo ocasionalmente (menos de una vez al día)	
Actualmente fumo cada día	

4. Antes, ¿fumaba usted?

No he fumado nunca	

Había fumado menos de una vez al día durante 6 meses o más	
Había fumado menos de una vez al día durante menos 6 meses	
Había fumado diariamente durante 6 meses o más	
Había fumado diariamente durante menos de 6 meses	

5. ¿Cuál suele ser su presión arterial?

Pa (sistólica/diastólica)		

6. ¿Nos podría decir si padece o ha padecido alguno de los trastornos crónicos que constan a continuación?

1. Presión alta	
2. Colesterol elevado	
3. Diabetes o azúcar en la sangre	
4. Anemia	
5. Alergias crónicas	
6. Asma	
7. Bronquitis crónica	
8. Varices en las piernas	
9. Trastornos alimentarios (anorexia, bulimia, …)	
10. Migraña o dolores de cabeza frecuentes	
11. Cataratas	
12. Dolor de espalda crónico cervical	
13. Dolor de espalda crónico lumbar o dorsal	
14. Artrosis, artritis o reumatismos	
15. Osteoporosis	
16. Problemas de próstata	
17. Incontinencia urinaria	
18. Estreñimiento crónico	
19. Hemorroides	
20. Úlcera de estómago o duodeno	
21. Problemas crónicos de piel	
22. Problemas de tiroides	
23. Depresión / ansiedad	
24. Otros trastornos mentales (especificar)	
25. Embolia/apoplejía	

26. Infarto de miocardio	
27. Otras enfermedades del corazón	
28. Tumores malignos	
29. Otros trastornos crónicos (especificar):	

7. ¿Ha tenido fiebre en las últimas 4 semanas?

No	
Si (días)	/

8. ¿Ha estado en cama por enfermedad en las últimas 4 semanas?

No	
Si (días)	/

9. De la siguiente lista, ¿qué medicamentos ha tomado durante la última semana? En caso afirmativo, ¿le ha sido recetado por el médico, se lo ha aconsejado el farmacéutico o lo ha tomado por iniciativa propia? (marque tan sólo aquellas casillas en las que la respuesta sea afirmativa)

	NO	**Si** (recetado por el médico)	**Si** (aconsejado por el farmacéutico)	**Si** (por iniciativa propia)
1. Analgésicos y/o antiinflamatorios				
2. Vitaminas o minerales				
3. Tranquilizantes, sedantes				
4. Medicamentos para la alergia				
5. Medicamentos para la tos o el resfriado				
6. Antibióticos				
7. Medicamentos para el asma				
8. Medicamentos antidepresivos				
9. Medicamentos para la presión arterial				
10. Medicamentos para el colesterol				
11. Medicamentos para el corazón				
12. Insulina o medicamentos para la diabetes				
13. Medicamentos para dormir				
14. Medicamentos para problemas de piel				
15. Medicamentos para el estómago				
16. Laxantes				
17. Medicamentos para la osteoporosis				
18. Anticonceptivos orales				
19. Medicamentos para problemas de los				

ojos				
20. Medicamentos para problemas del oído				
21. Medicamentos para adelgazar				
22. Productos homeopáticos				
23. Plantas medicinales				
24. Otros (especificar):				

SECCIÓN 3: A continuación se le pregunta por los posibles **procesos infecciosos** (resfriado, gripe, etc…) y **alergias**

10. ¿Ha estado resfriado o con gripe en las últimas 4 semanas?

No	
Si (días)	/

11. ¿Está vacunado de la gripe?

No	
Si	

12. ¿Ha presentado diarrea en las últimas 4 semanas para la que el médico le ha recetado antibiótico?

No	
Si días)	/

13. ¿Tiene algún tipo de alergia o intolerancia?

SI, intolerancia alimentaria	
SI, alergia alimentaria	
Si, alergia respiratoria	
NO (ha finalizado la encuesta)	

14. ¿Tiene antecedentes familiares de alergia?

SI	
NO	

En caso afirmativo, señale el parentesco (marque varios, si es el caso):

Padre	
Madre	
Un hermano	
Más de un hermano	
Otros (especificar):	

15. En caso afirmativo a las preguntas anteriores, ¿quién le ha diagnosticado?

Por experiencia propia (no he ido al médico)	
Médico general	

Alergólogo	
Nutricionista	
Farmacéutico	
Otro especialista (especificar):	

16. ¿Cuántas veces presenta manifestaciones alérgicas a lo largo del año?

	Nunca	1-6 veces al año	7-11 veces al año	1 vez al mes	2-3 veces al mes	1 vez por semana	2 veces por semana	semana	semana	1 vez al día	2 veces al día	3 o más veces al día
encia												

17. ¿Qué le produce alergia o intolerancia? Marcar en caso afirmativo y especificar si se conoce:

Alérgeno	**Alergia**	**Intolerancia**	**Especificar si se conoce**
Polen			
Polvo			
Frutos secos			
Fruta fresca			
Leche			
Huevo			
Pescado			
Marisco			
Legumbres			
Otros alimentos (especificar)			
Medicamentos			

Otros (especificar)			

18. ¿Cómo nota su alergia?

Granitos	
Enrojecimiento	
Estornudos	
Mucosidad nasal	
Irritación de los ojos	
Picor en la boca	
Hinchazón de los labios	
Asma	
Diarrea	
Vómito	
Maldigestión	
Disconfort/dolor abdominal	
Choque anafiláctico	
Otros (especificar)	

19. ¿Se ha ausentado del trabajo o lugar de estudios por la alergia en el último año?

No, nunca	
Si (días)	/

20. ¿Se medica para controlar su alergia?

No	
Si, continuamente para prevenir su aparición (especificar medicamento)	/
Si, tan sólo cuando tengo manifestaciones (especificar medicamento)	/

4. Dados de saúde

Objetivo: Estamos interesados em obter informação sobre o seu estado geral de saúde e estilo de vida

SECÇÃO 1: Seguidamente perguntamos por alguns **marcadores antropométricos**

1. Pode dizer-nos os seguintes dados

Altura actual (cm)	
Peso actual (Kg)	
Peso habitual (Kg)	
Peso máximo (Kg)	

SECÇÃO 2: Gostaríamos de saber o seguinte sobre o seu **estado geral de saúde** e morbilidade.

2. Como considera a sua saúde no geral?

Excelente	
Muito boa	
Boa	
Suficiente	
Má	

3. Nas seguintes situações, como descreve o seu comportamento relativamente ao tabaco?

Atualmente não fumo nada	
Atualmente fumo ocasionalmente (menos de uma vez por dia)	
Atualmente fumo diariamente	

4. Fumava antes?

Nunca tinha fumado	

Tinha fumado pelo menos uma vez por dia durante 6 ou mais meses	
Tinha fumado pelo menos uma vez por dia durante menos de 6 meses	
Tinha fumado diariamente durante 6 meses ou mais	
Tinha fumado diariamente durante menos de 6 meses	

5. Quanto costuma ter de tensão arterial?

Pa (sistólica/diastólica)		

6. Pode dizer-nos se sofre ou se já sofreu de alguma das patologías crónicas que se seguem?

1. Hipertensão arterial	
2. Colesterol elevado	
3. Diabetes ou Hiperglicemia	
4. Anemia	
5. Alergias crónicas	
6. Asma	
7. Bronquite crónica	
8. Varizes nas pernas	
9. Doenças do comportamento alimentar(anorexia, bulimia, …)	
10. Enxaquecas ou dores de cabeça frequentes	
11. Cataratas	
12. Dor de costas crónica na zona cervical	
13. Dor de costas crónica na zona lombar ou dorsal	
14. Artrose, artrite ou reumatismo	
15. Osteoporose	
16. Problemas de próstata	
17. Incontinência urinaria	
18. Obstipação crónica	
19. Hemorróidas	
20. úlcera no estômago ou duodeno	
21. Problemas de pele crónicos	
22. Problemas da tiróide	
23. Depressão/ansiedade	
24. Outros transtornos psicológicos (especificar)	
25. Embolia/Apoplexia	

26. Enfarte do miocárdio	
27. Outras formas de doença cardiovascular	
28. Tumores malignos	
29. Outras patologías crónicas (especificar):	

7. Sentiu febre nas últimas 4 semanas?

No	
Sim (dias)	/

8. Esteve acamado nas últimas 4 semanas?

No	
Sim (dias)	/

9. Da lista que se segue, que medicamentos tomo una última semana? Se tomou, indique se foram receitados por um médico, aconselhados pelo farmacéutico ou se tomou por inciativa própria. (marque apenas na opção que corresponda à resposta correspondente).

	NO	**Si** (recetado por el médico)	**Si** (aconsejado por el farmacéutico)	**Si** (por iniciativa propia)
1. Analgésicos e/ou anti-inflamatórios				
2. Vitaminas ou minerais				
3. Tranquilizantes e sedativos				
4. Medicamentos para alergias				
5. Medicamentos para a tosse ou resfriado				
6. Antibióticos				
7. Medicamentos para a asma				
8. Medicamentos antidepressivos				
9. Medicamentos para a tensão arterial				
10. Medicamentos para o colesterol				
11. Medicamentos para o coração				
12. Insulina ou medicamentos a diabetes				
13. Medicamentos para dormir				
14. Medicamentos para problemas de pele				
15. Medicamentos para o estômago				
16. Laxantes				
17. Medicamentos para a osteoporose				
18. Anticontraceptivos orais				
19. Medicamentos para problemas oculares				

20. Medicamentos para problemas nos ouvidos				
21. Medicamentos para emagrecer				
22. Produtos homeopáticos				
23. Plantas medicinais				
24. Outros (especificar):				

SECÇÃO 3: Em seguida perguntamos por possíveis procesos infecciosos (constipação, gripe, etc.) e alergias.

10. Esteve constipado nas últimas 4 semanas

Não	
Sim (días)	/

11. Está vacinado para a gripe?

Não	
Sim	

12. Teve diarreia nas últimas 4 semanas para que tenha sido passado algum antibiótico pelo médico?

Não	
Sim (días)	/

13. Tem algum tipo de alergia ou intolerancia?

SIM, intolerância alimentar	
SIM, alergia alimentar	
SIM, alergia respiratória	
Não (termina aqui o questionário)	

14. Tem antecedentes familiares de alergia?

SIM	
NÃO	

Se sim, assinale o parentesco (marque vários se for o caso)

Pai	
Mãe	
Um irmão	
Mais de um irmão	
Outros (especificar)	

15. Em caso afirmativo nas questões anteriores, quem fez o diagnóstico?

Por experiencia própria (não fui ao médico)	
Médico de clínica geral	
Alergologista	

Nutricionista	
Farmacêutico	
Outro especialista (especificar):	

16. Quantas vezes por ano apresenta manifestações alérgicas ao longo do ano?

	Nunca	1-6 veces al año	7-11 veces al año	1 vez al mes	2-3 veces al mes	1 vez por semana	2 veces por semana	semana	semana	1 vez al día	2 veces al día	3 o más veces al día
encia												

17. O que produz alergia ou intolerancia? Marcar apenas em caso afirmativo e especificar se souber.

Alergéneo	**Alergia**	**Intolerancia**	**Especificar se souber**
Polen			
Polvo			
Frutos secos			
Fruta fresca			
Leite			
Ovo			
Peixe			
Marisco			
Leguminosas			
Outros alimentos (especificar)			
Medicamentos			
Outros (especificar)			

18. Como nota a sua alergia?

Borbulhas	
Vermelhidão	
Espirros	
Mucosidade nasal	
Irritação ocular	
Comichão na boca	
Inchaço dos lábios	
Asma	
Diarreia	
Vómito	
Má digestão	
Desconforto/Dor Abdominal	
Choque anafiláctico	
Outros (especificar)	

19. Ausentou-se do seu local de trabalho ou estudo devido à alergia no último ano?

Não, nunca	
Sim (días)	/

20. Medica-se para controlar a alergia?

Não	
Sim, continuamente para prevenir o seu aparecimento (especificar o medicamento)	/
Sim, apenas quando tenho sintomas (especificar medicamento)	/