Effect of moderate beer consumption on post-menopausal women's health

New insights towards more comprehensive nutritional research and a personalitzed nutrition perspective

Marta Trius Soler

UNIVERSITAT DE BARCELONA

Universitat de Barcelona

Facultat de Farmàcia i Ciències de l'Alimentació

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Directors:
Dr. Rosa María Lamuela Raventós
Dr. Juan José Moreno Aznárez
Doctoranda:
Marta Trius Soler

Marta Trius Soler
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ABSTRACT

Beer is one of the most widely consumed alcoholic beverages in the world and has been crafted for many years, while the enhancement of post-menopausal women's health through the bioactive compounds of this beverage needs a more comprehensive approach. Therefore, the present dissertation aims to study the effects of moderate daily beer consumption on post-menopausal women's health, integrating new promising nutrition research approaches and supporting the view of personalized nutrition. To achieve this aim, a parallel controlled clinical trial was carried out and changes in menopausal symptoms and sex-hormone profile were evaluated at 6 months of intervention. Furthermore, its effects on bone tissue and cardiovascular health were evaluated at 2-years of follow-up. Results suggest that moderate daily beer (with and without ethanol) consumption could be a promising and safe strategy to optimize early post-menopausal women's quality of life and minimize the cardiometabolic alterations related to the onset of menopause, but not to improve bone health.

Additionally, the inter-individual variability of taste sensitivity was studied in a young-aged population and across the overmentioned study cohort. Findings bring new insights into the physiological implications of the gustatory function; being aging, sex, and metabolic alterations factors correlated with taste acuity. On the other hand, the extensive systematic review of the current evidence on biomarkers of alcohol intake pointed out that common biomarkers of alcohol intake, e.g., ethyl glucuronide, ethyl sulfate, fatty acid ethyl esters, and phosphatidyl ethanol, have a considerable inter-individual variance, especially at low to moderate intakes, while the current beer and wine intake biomarkers are highly promising for an accurate intake assessment of these beverages.

The results of this dissertation add knowledge regarding the effect of moderate daily beer (with and without ethanol) intake on post-menopausal women's health and encourage the scientific community to run well-designed clinical trials to prove alcoholic beverage consumption's effect on concrete health parameters and a specific study population.

RESUM

La cervesa és una de les begudes alcohòliques més consumides del món i la seva elaboració existeix des de fa molts anys, mentre que la millora de la salut de les dones postmenopàusiques a través dels compostos bioactius d'aquesta beguda necessita un enfocament més exhaustiu. Així doncs, la dissertació actual té com a objectiu estudiar els efectes del consum moderat i diari de cervesa en la salut de les dones postmenopàusiques, integrant nous enfocaments de recerca nutricional prometedors i donant suport a la visió de la nutrició personalitzada. Per a assolir aquest objectiu es va dur a terme un assaig clínic controlat i paral·lel i els canvis en els símptomes menopàusics i el perfil d'hormones sexuals van ser avaluats al cap de 6 mesos d'intervenció. A més, els efectes d'aquesta sobre el teixit ossi i la salut cardiovascular es van avaluar als 2 anys de seguiment. Els resultats suggereixen que el consum moderat i diari de cervesa (amb i sense etanol) podria ser una estratègia prometedora i segura per optimitzar la qualitat de vida de les dones postmenopàusiques primerenques i minimitzar les alteracions cardiometabòliques relacionades amb l'aparició de la menopausa, però no per a la millora de la salut òssia.

A més, la variabilitat interindividual de la sensibilitat gustatòria va ser estudiada en una població jove i a través de la cohort d'estudi anteriorment mencionada. Els descobriments aporten noves idees sobre les implicacions fisiològiques de la funció gustativa; sent l'envelliment, el sexe i les alteracions metabòliques factors relacionats amb l'agudesa gustativa. D'altra banda, l'extensa revisió sistemàtica de l'evidència actual sobre els biomarcadors de la ingesta d'alcohol va assenyalar que els biomarcadors comuns de la ingesta d'alcohol, ex., etil glucurònid, etil sulfat, èsters d'etil d'àcids grassos i fosfatidiletanol, tenen una considerable variància interindividual,

especialment entre ingestes baixes i moderades; mentre que els biomarcadors de cervesa i vi actuals són molt prometedors per a una avaluació precisa del consum d'aquestes begudes.

Els resultats d'aquesta dissertació afegeixen coneixement sobre l'efecte de la ingesta moderada i diària de cervesa (amb i sense etanol) en la salut de les dones postmenopàusiques i animen a la comunitat científica a realitzar assajos clínics ben dissenyats per demostrar l'efecte del consum de begudes alcohòliques sobre paràmetres de salut concrets i en una població d'estudi específica.

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LIST OF ABBREVIATIONS

8-PN: 8-prenylnaringenine
AB: alcoholic beer
ADH: alcohol dehydrogenase
ALDH: aldehyde dehydrogenase
BFI: biomarker of food intake
BFIRev: food intake biomarker reviews
BMD: body mineral density
CCK: cholecystokinin
CVD: cardiovascular disease
CVDRF: cardiovascular disease risk factor
CYP2E1: cytochrome P450 E1
DT: detection threshold
DXA: dual-energy X-ray absorptiometry
ERα: estrogen receptor α
ERβ: estrogen receptor β
EtG: ethyl glucuronide
EtS: ethyl sulfate
FAEEs: fatty acid ethyl esters
FSH: follicle-stimulating hormone
GLP-1: glucagon-like peptide-1
GPCR: G-protein-coupled receptors
HRT: Hormone replacement therapy
IX: isoxanthohumol
LDL-c: low-density lipoprotein cholesterol
HDL-c: high-density lipoprotein cholesterol
LH: luteinizing hormone
NAB: non-alcoholic beer
PEth: phosphatidyletahanols
PROP: 6-n-propylthiourail
PTC: phenylthiocarbamide
PYY: peptide YY
RT: recognition threshold
RCT: randomized controlled trial
SERMs: selective estrogen modulators
SGLT1: sodium/glucose cotransporter 1
SNP: single-nucleotide polymorphisms
STRAW+10: stages of reproductive aging workshop staging system
TB: taste buds
TC: total cholesterol
TR: taste receptor proteins
TRC: taste receptors cells
T1R: taste receptor type 1
T1R2: taste receptor type 1 member 2
T1R3: taste receptor type 1 member 3
T2R: taste receptor type 2
T2R1: taste receptor type 2 member 1
T2R14: taste receptor type 2 member 14
T2R38: taste receptor type 2 member 38
T2R40: taste receptor type 2 member 40

1. INTRODUCTION

1.1. Menopause

Menopause is the permanent cessation of spontaneous menses, which takes place as the finite store of ovarian follicles is depleted. Menopause is an expected natural life event for women in their middle years, although individual experiences vary [1].

1.1.1. Physiology of menopause

Menopause is characterized by a low output of ovarian estrogens and progesterone and a high production of pituitary gonadotropin hormones (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]). In the post-menopausal stage, estrogens are synthesized from androgens derived from the metabolism of estrone [2], and the release of pituitary-ovarian hormones is controlled through a negative feedback system [3,4]. Levels of LH and FSH can be secreted in tandem, with an increase for up to 5 and 7 years after the onset of menopause, respectively [5]. For its part, the term climacteric refers to an extended period and that includes the period before (perimenopause) and after (post-menopause) the onset of menopause [6].

Natural menopause is recognized after 12 consecutive months of amenorrhea not associated with a physiological (e.g., pregnancy, lactation) or pathological cause, thus it can also be induced by surgery, chemotherapy, or radiation. Menopause transition often begins when women are in their mid-to-late 40s and can last several years, most commonly 4-5 years [1,7]). The mean age of the onset of menopause is around the 50s [8] and a final menstruation cycle before 40 years of age is regarded as premature [1]. The Stages of Reproductive Aging Workshop staging system (STRAW+10) is considered the gold standard for assessing reproductive aging in research and clinical contexts (Figure 1) [9].

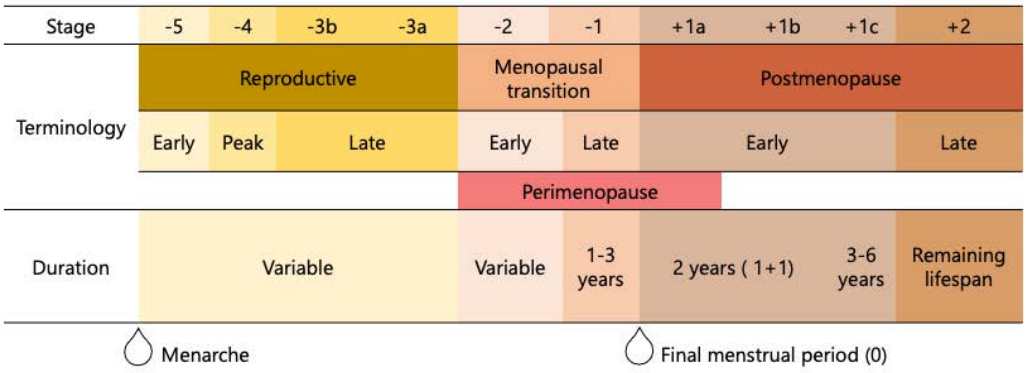

Figure 1. The Stages of Reproductive Aging Workshop + 10 staging for reproductive aging in women. Adapted from Harlow et al. (2012) [9].

The transition to menopause is a complex physiological process, often accompanied by the additional effects of aging and social adjustment [1]. A smooth transition through this challenging period is considered crucial for healthy and successful aging [10]. Due to the increase in life expectancy in the coming years, the number of women in this period of life will increase considerably. Menopause and climacteric should be one more stage in the life of every self-perceived woman, socially recognized and accompanied by health care, as a process towards positive and joyful maturity.

1.1.2. Post-menopausal women's health

Progressive hypoestrogenism due to menopause transition causes a negative impact on the quality of life of perimenopausal and post-menopausal women, due to the presence of climacteric symptoms (e.g., hot flashes, palpitations, insomnia, vaginal dryness) [1], readjustment of social and occupational considerations [11], changes on body composition and an increased risk of the metabolic syndrome and cardiovascular disease (CVD) [6,12]. On the other hand, menopause is also related to a high incidence of depression, irritability, and anxiety [13,14], an increased risk of sarcopenia [15], osteoporosis [16,17], and ovarian, endometrial, and breast cancer [18].

The distinction between clinical signs related specifically to the menopausal transition and those related to aging is commonly difficult. Moreover, many questions about it and the effect on women's health have not yet been adequately answered and patients' concerns sometimes have been underestimated. Effectiveness varies between therapies, and evidence of substantial clinical benefit exists only for a few [1]. Estrogen itself or in combination with progesterone has been for decades the therapy of choice for relieving menopausal symptoms. The inconclusive nature of hormonal replacement therapy's (HRT) risk and benefits [19–21] has generated great interest in alternative therapies, such as phytoestrogens, to relieve menopausal symptoms [22,23]. Most of longitudinal data that describe menopausal symptoms and the effectiveness of treatments have been collected among white women [7].

1.1.3. Specific nutritional needs of post-menopausal women

Inadequate food intake contributes to many health conditions, though nutritional prevention and mitigation strategies are needed to prevent or delay the onset of them and promote healthy aging [24]. Among health promotion and lifestyle aspects, nutritional habits are essential because they concern all women, can be modified, and impact both longevity and quality of life [25].

The Mediterranean diet pattern along with other healthy habits has a protective effect against bone, metabolic, and CVDs in the post-menopausal period. It consists of the use of a high intake of e.g., fruits, vegetables, legumes, whole grains, nuts, and olive oil, which have anti-inflammatory and antioxidant properties. It is also associated with a decrease in blood pressure, reduction of fat mass, improvement in cholesterol levels or risk of all-cause mortality [25–27].

Calcium is the most important nutrient for preventing and treating osteoporosis [28], while vitamin D interact to regulate the balance between blood calcium and phosphorus levels. It can be synthesized by the body with the help of sunlight or through the consumption of food sources such as oily fish, mushrooms, and some fortified dairy products [29,30]. In post-menopausal women, low blood levels of vitamin D are associated with alteration in bone turnover markers and metabolic syndrome [31]. In addition, results from several epidemiological and experimental studies indicate that dietary silicon may also increase bone mineral density (BMD) and reduce bone fragility [32,33].

On the other hand, phytoestrogens are plant derivatives that bear a structural similarity to 17-β-estradiol, thus have been proposed as natural selective estrogen modulators (SERMs) for the relief of menopausal symptoms, although the overall estimates of treatment efficacy from randomized clinical trials (RCTs) have provided conflicting results [23,34–38]. The main categories of phytoestrogens are flavonoids, lignans, and stilbenoids [39]. Common properties of most phytoestrogens include their metabolism by gut microbiota to additional microbiota metabolites with varying estrogenic activity, i.e., production of equol from isoflavones consumption [40]. Some described plants with potential estrogenic activities are soy, red clover, flaxseeds, pomegranate seeds, and hops [34,41–43].

Phytoestrogens can bind to either estrogen receptor α (ERα) or estrogen receptor β (ERβ), both principally involved in reproduction but not limited. Tissue distribution and the ratio of each ERs can vary. ERα has been suggested as the most important ER in the maintenance of lipid and carbohydrate metabolism and protection from bone loss, although both Ers seem to be able to protect against atherosclerosis and have been reported to alleviate vasomotor symptoms [44].

1.1.4. Gender-perspective on scientific research

Gender bias is defined as the differential medical treatment between men and women, which has led to the invisibility of women in science and a lack of valid results about women's health [45]. The health of women refers to all diseases and conditions that affect a woman and recognizes that individual-level biological factors interact with psychosocial aspects across a woman's life course [46]. Diseases' prevalence and biological determinants, as well as social and cultural, are essential for doing complex and realistic research with a gender-perspective.

Historically, much medical knowledge of menopause relied on convention rather than on rigorously designed studies, leading to a society and a health system being careless on this topic [1,7]. At times, serious symptoms are regarded as normal concomitants of women's aging and not addressed further, but at other mild symptoms are overmedicalized [1]. Indeed, menopause has been involved in the phenomenon of the medicalization of life, so this physiological process has been exposed to pharmacological treatment without balancing potential benefits and risks [8]. Therefore, advanced rigorous research on menopause is relevant to enhance the dissemination and implementation of evidence in women's care. Future direction lies with health education, a key factor to personal autonomy for health and well-being. In addition, focusing on the changes and increased risk of diseases during the menopause transition and post-menopause, as well as their specific nutritional needs, could lead to a better personalized diet design and health enhancement through bioactive compounds and lifestyle modifications.

1.2. Physiology of nutritional sensing

Chemosensory perception (taste, smell, and chemesthesis) is the detection of chemicals in the external environment and is essential for individual and species' survival [47]. The sense of taste is limited to the oral cavity and mainly the tongue, which can identify characteristics of many chemicals that comprise ingested foods [48].

Humans can perceive at least five taste qualities according to current knowledge, also referred to as basic tastes. Presumably, based on an evolutionary view, taste to sweetness permits the identification of carbohydrates to ensure high-energy nutrients intake; the umami taste allows the recognition of amino acids and ribonucleotides from cooked and aged meat to ensure adequate intake of protein; the salty taste ensures the proper dietary electrolyte balance; and the sour taste of vitamin C to maintain health as well as to recognize fermented, spoiled, or unripe foods. The bitter taste is assumed to be a warning against the intake of potentially noxious and/or poisonous chemicals [49], whereas not all bitter foods are toxic. In addition to these five taste modalities, fat/fatty acid taste or "oleogustus" has accumulated strong evidence during recent years [50], whereas other taste candidates are less consolidated (e.g., metallic [51], complex carbohydrates [52], calcium [53]). At present, taste has the added value of contributing to the overall pleasure and enjoyment of a meal [49].

1.2.1. Gustatory system

Taste buds (TBs) present in the gustatory epithelia in the oral cavity are the sensory organs of taste and the anatomical substrates and units of taste detection are taste receptors cells (TRCs) (Figure 2). Each human TB contains normally 50 and 100 closely

positioned TRCs. TRCs are small bipolar cells with no axon that project microvilli to the apical surface of the TB, where they gain access to the oral cavity forming a taste pore [48,49]. The taste perception process begins when taste-eliciting non-volatile compounds interact with the taste receptor proteins (TR) that are expressed in TRCs and come in many types (e.g., G-protein-coupled receptors (GPCRs), and ion channels). Upon exposure to an appropriate ligand, some TRs generate second messengers, whereas other taste stimuli themselves are transported into the cytoplasm of TRCs and activate downstream events [54]. It has been identified four morphologic subtypes of cells in TB: Type I or glial-like cells, which are half of the total number of cells in the TBs and can likely detect salty taste; Type II cells that express GPCRs to detect sweet, umami, and bitter tastes and are approximately one-third of cells in TB; Type III cells sense sour stimuli and represent 2-20% of cells in a TB; and Type IV cells likely represent stem or progenitor taste cells [55]. Each TB responds to more than on taste stimuli because they contain multiple type II cells of different taste GPCRs expression [54]. Contrary to the popular belief, sensory receptors for the different taste qualities are found across all areas of the tongue, but spatial differences in taste sensitivity have been described [56].

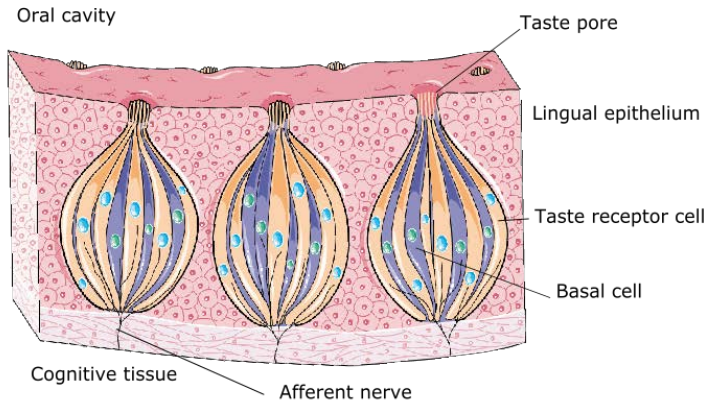

Figure 2. Illustration of multiple taste buds. Each taste bud contains multiple closely taste receptors cells, some of which synapse with afferent nerves (adapted from Mombaerts 2004) [57].

1.2.2. Sweet taste

Sweetness response is triggered in the taste receptor type 1 member 2 (T1R2) and T1R3 heterodimer (T1R2/T1R3), from the GPCRs family. The ligand selectivity (i.e., affinity) of T1R2/T1R3 dictates which compounds elicit sweet taste, thus it is responsible for sweet taste initial stimuli detection [54] and include both caloric and non-caloric molecules such as soluble carbohydrates, artificial sweeteners, D-amino acids, and sweet proteins [49,58].

T1R2/T1R3 heterodimer has three domains: 1) the Venus flytrap-like domain; 2) the cysteine-rich domain; and 3) the 7-transmembrane domain. Ligand binding to any of the domains is possible, and sucrose appears to bind to T1R2/T1R3 Venus flytrap domain [54,59]. Moreover, T1R3-independent mechanisms have been suggested for the detection of sugars and other sweeteners. For example, one postulated mechanism involves glucose transporter type 4 and sodium/glucose cotransporter 1 (SGLT1) [60,61]. The involvement of a Na^+-dependent transporter in transducing sugars brings a plausible explanation for the potentiation of sweet taste by Na^+ salts [62]. A modulatory effect on sour taste via communication between type II and type III TBCs has also been suggested [59]. Mammalian sweet and umami taste receptors are closely related and share the common subunit T1R3 [54].

1.2.3. Bitter taste

Bitter taste is also transmitted by GPCRs, which have shorter N termini and ligand-binding sites in their transmembrane segments than taste receptor type 1 (T1Rs). In humans, the perception of bitterness is mediated by at least 25 receptors of the type 2 receptor family (T2Rs). The different T2Rs interact with a very wide range of ligands, with varying specificity, which mainly includes plant products such as alkaloids,

phenols, and glycosides [63]. Furthermore, bitter compounds can interact with one or several T2Rs [64]. Some examples are shown in Table 1.

T2Rs are generally considered to function as monomers, although evidence suggests that are also formed as heterodimers [54,65]. T2Rs are co-expressed in TBs, allowing the possibility of receptor-receptor interactions [54]. Many T2Rs genes have polymorphisms, which are linked to differences in bitter taste perceptions. Bitter taste receptor 38 (T2R38) is the most studied case, which explains the different response to the synthetic compounds 6-*n*-propylthiourail (PROP) and phenylthiocarbamide (PTC) [66], as well as to other compounds with a thiocyanate (N-C=S) group such as isothiocyanates [67,68]. Three single-nucleotide polymorphisms (SNPs) in T2R38 have been identified, giving rise to five different haplotypes. The most common two haplotypes are PAV and AVI, being PAV homozygotes the individuals that perceive the greatest bitterness from PTC and PROP [69].

Table 1. List of some examples of bitter taste receptors and their ligands.

Bitter receptor	Ligands (examples)
T2R1	Gentian, XN, IX
T2R4	Quinine, epicatechin, naringenin
T2R5	Epicatechin, procyanidin C2
T2R7	Caffeine, quinine, malvidin-3-glucoside
T2R8	Oleuropein aglycon, oleuropein
T2R14	Caffein, quinine, genistein, naringenin, quercetin, XN, IX, resveratrol
T2R16	Sinigrin
T2R31 (T2R44)	Quinine, saccharin,
T2R38	Isothiocyanates, PTC, PROP, sinigrin
T2R40	Quinine, humulone, XN, IX

IX: isoxanthohumol; PROP: 6-*n*-propylthiouracil; PTC: phenylthiocarbamide; XN: xanthohumol. Examples of compounds reported from Tarragon *et al.* (2020) [70] and Cui *et al.* (2021) [71].

1.2.4. Taste sensitivity measurements

Threshold measurements are useful to study individual differences in taste acuity and bring to the researcher a non-subjective rating scale or sensory score. Nevertheless, thresholds represent only one point on a dose-response curve, so they tell little about how the sensory system behaves above them. Absolute or detection threshold (DT) is the lowest physical energy level of a stimuli or lowest chemical concentration that is perceivable. On the other hand, recognition threshold (RT) is the minimum level that takes on the characteristic taste or smell. Other interesting measurements to characterize sensory perception have been described in sensory evaluations (Figure 3) [72].

Chemical and physical methods have been proposed for threshold determination [73], and discrimination tests are used when determining whether two or more samples are perceptibly different. There are several different discrimination tests described, including paired comparison tests. A directional paired comparison test, also known as the 2-alternative forced choice, is commonly used to determine whether the two samples differ in a specified dimension. On the other hand, a differenced paired comparison test or same/different test is used to determine whether the two samples differ without specifying the dimension(s) of the potential difference [72]. In chemical gustometry, factors such as the tastant, the aqueous matrix characteristics (e.g., viscosity or mineral content), the amount of stimuli solutions, and the time between different solution administrations, among other variants, differ among experimental designs playing a role in the outcome of the threshold assessment [72,74–77].

Wide individual differences in taste sensitivity exist. DTs of PTC and PROP compounds, follow a bimodal distribution, while ratings for bitterness above the threshold test

allow identifying hypersensitive groups of "supertasters" [78]. Indeed, supertasters' status has been correlated with a higher number of papillae and TBs, as well as an enhancement of taste sensitivity and responsiveness [79]. Alternatively, trimodal distribution using DT or RTs by a rational strategy of following the expected frequency of homozygous tasters that are known to be in a specific population and categorize them as supertasters has also been proposed [78].

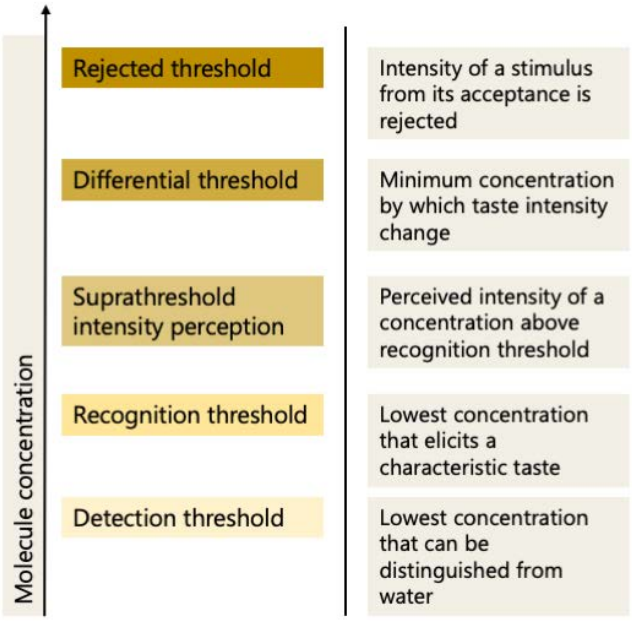

Figure 3. Taste sensitivity common parameters. Definitions taken from the *Sensory Evaluation of Food: Principles and Practices* book [72].

1.2.5. Applicability of sensory analysis on nutritional research and clinicians

TR signaling is not confined to TBs but occurs in a variety of extraoral tissues [80,81]. The expression of TRs in the gut, pancreas, brain, and adipose tissue suggests a physiological contribution of it to nutrient-sensing mechanisms and metabolism [82]. Knowing about the anatomy and physiological processes of the senses brings higher

understanding about the limits of sensory function, the correlations between sensations and how consumers and panelists interact with the products to stimulate their senses [72]. Due to the fast turnover rate of TB cells, and therefore TRs, the link between chronic conditions and chemosensory perception has been currently explored. Thus, taste acuity may serve as an indirect measurement of the impact of several environmental and physiological factors, although individual variability in taste sensitivity also has a genetic background [83].

In the context of health and pathology, several variables that could affect taste perception have been described. TR genotype, fungiform papillae density, and saliva (flow rate, buffering capacity, and molecular composition) are important factors associated with taste perception [84]. Other intrinsic factors (e.g., sex, age, smoking habit, medication) and clinical variables (e.g., dental cavities, lost teeth, sinusitis, rhinitis, body mass index [BMI]) may also impact taste sensitivity. In this sense, recent findings suggest that lower taste sensitivity may be associated with a higher risk of obesity [85,86], type 2 diabetes [87,88], or hypertension [89,90]. But beyond that, these thresholds may also vary depending on the development of certain pathologies. Therefore, taste threshold changes may be involved in pathogenic mechanisms and disease prognosis. In conclusion, for the presence and function of TRs in extraoral tissues, exciting new possibilities for targeted therapeutics against diseases or for the management of specific physiological conditions are both promising fields of research and modern health care [91].

1.3. Biomarkers of intake for alcohol and specific alcoholic beverages

Biomarkers are the biological and chemical test result of biospecimens, which permit to characterize objectively a particular exposure, susceptibility, or biological effect [92]. Concretely, biomarkers of exposure are chemical compounds or metabolites that are measured in biofluids after the exposure of the chemical or the metabolite precursor in the organism. Biomarkers of exposure or intake can be split again into food compound intake biomarkers, dietary pattern biomarkers, and biomarkers of food intake (BFIs) [93].

1.3.1. Biomarkers of food intake and its importance

BFIs are compounds measured in a biofluid collected after the consumption of a specific food, food component (e.g., ingredients), or food group; and can be used as an estimation of recent or average intakes of these entities. They can be a single metabolite or a combination of them. The combination of two or more metabolites may be done by: 1) including one of two or more BFIs; 2) summing up signals from one or more metabolites; 3) calculating the ratios of two BFIs; or 4) presenting a pattern of several metabolites specifying the rule for how BFIs in the pattern may be covered [93].

Biomarkers are important in nutritional research since the most used techniques of assessing the diet followed by individuals are known to be biased by several demographic, cultural, and individual factors [94]. Therefore, the use of exposure biomarkers in this field is a key factor in dietary interventions and compliance validation. Indeed, biomarkers are particularly useful in estimating the intake of specific bioactive compounds such as phytochemicals (e.g., polyphenols) [95,96].

Increasing the knowledge of food phytochemicals and updating food recommendations not only considering their macro- and micronutrient content but also their phytochemical content can be useful in understanding the role of specific food consumption on human health. The current pace of biomarker discovery and applications is growing due to the rapid development of "omics" technologies and data collection. This rapid development may reshape future research in nutrition and health, and therefore create a well-known consideration for biomarkers' applicability [97].

1.3.2. Validation of biomarkers of food intake

To support the development and use of BFIs, systematic reviews that attempt to validate them into trusted research tools are needed [97]. Furthermore, to cover the literature on BFIs in the most appropriate and consistent manner, a guideline that provides the basis for it has been published [98]. In that sense, a series of criteria are essential to validate these candidate BFIs and estimate the current level of validation of candidate BFIs too. Therefore, a validation scheme for the assessment by answering eight questions related to the analytical and biological aspects of the validation was published by the partners of the FoodBAll consortium [99]. The eight validation criteria as well as their description can be found in Table 2.

For qualitative markers validation, fewer validation criteria need to be fulfilled. For instance, dose-response and analytical validation do not need to be documented in detail. On the other hand, observational studies need additional validated criteria than those used as compliance biomarkers in experimental studies. As an example, the robustness criterion in clinical trials could be controlled through the experimental design.

Table 2. Factors to be considered for the validation and application of biomarkers.

Validation criterion	Description
1. Plausibility	Biomarkers should be specific to the food, and a chemical or experimentally explanation for why the food intake should increase the biomarker should be found.
2. Dose-response	The suitability of the biomarker over a range of intakes should be assessed (e.g., limit of detection, baseline habitual level, bioavailability, saturation effect).
3. Time-response	Evaluation of the degree to which the biomarker reflects the exposure (e.g., half-life, kinetics, long-term stability).
4. Robustness	Validation of the biomarker in controlled dietary interventions, as well as in cross-sectional studies (e.g., inter-individual variability, matrix effect, food interactions, population groups).
5. Reliability	Comparison of the biomarker and a gold standard or reference method and in different types of studies.
6. Stability	Suitable methodology protocols are appropriate (e.g., collection, processing, storage)
7. Analytical performance	Validation of the analytical method should be performed (e.g., precision, accuracy, intra- and inter-batch variation, limits of detection).
8. Reproducibility	Comparison of the data obtained and the validated analytical methods among the different laboratories

Reduced version of the information published by Dragsted et al. (2018) [99].

1.3.3. Clinical and epidemiological applicability of biomarkers of alcohol and specific alcoholic beverages intake

The accuracy of dietary intake reporting is essential to predict trustful epidemiological associations of diet-diseases and their determinants. In that sense, one of the complex considerations in alcohol research is to assess alcohol intake and compliance objectively. Biomarkers of both recent and longer-term alcohol intake are interesting to study the associated risks and benefits [97].

Several direct and indirect alcohol intake biomarkers have been proposed in forensic and clinical contexts. Among those, ethyl glucuronide (EtG), ethyl sulfate (EtS), in serum and urine, fatty acid ethyl esters (FAEEs) in hair, and phosphatidyletahanols (PEth) in serum have been considered the most promising direct biomarkers of alcohol intake. Beyond the overall alcohol consumption, there is also a considerable interest to discriminate between the different alcoholic beverages. In addition, factors such as the time lapse since last drink, the frequency of drinking, etc. become important questions in need of objective biomarker strategies.

Identification of biomarkers is hampered by the limited accuracy of self-reported dietary intake, while most of the data is reported as weekly or monthly averages, thus the habit of its consumption is hard to be well-reflected. In fact, alcohol consumption is mostly self-reported in observational studies, but its social and health implications lead to an underreporting of its intake [100]. Thus, misreporting bias of alcohol exposure should be considered in analyses, adding additional challenges on biomarker research [101,102]. Another key factor that must be assessed when alcohol biomarkers are wanted to be use as quantitative biomarkers is the intra and inter-individual differences in absorption, metabolism, distribution, and excretion of their metabolites. In this regard, a comprehensive review of biomarkers of alcohol intake is lacking.

1.4. Beer

Beer is one of the most widely consumed alcoholic beverage in the world. Traditionally, it mainly contains four ingredients: water, barley or wheat malt, hops, and yeast as raw materials; and their transformation products formed during malting and fermentation. It compromises thousands of compounds such as oligosaccharides, amino acids, nucleotides, fatty acids, and phenolic compounds, making beer a complex beverage matrix [103,104].

1.4.1. Beer and non-alcoholic beer biochemical composition

Water represents around 90% of the total composition, followed by ethanol and carbohydrates. Beer is also rich in vitamins of group B (e.g., thiamine, riboflavin, niacin, folic acid) and minerals (e.g., magnesium, potassium, silicon). The alcohol content in alcoholic lager beers varies on average from 3% to 5% alcohol by volume [104].

Some of the well-described beer bioactive compounds with health benefits are polyphenols, bitter acids, and silicon. Particular attention has been given to the polyphenols found in malt (75%) and hops (25%), due to their antioxidant and anti-inflammatory properties [105,106]. Moreover, upon wort boiling compounds from hops are isomerized and degraded forming other structures that are specifically from beer [107,108]. In addition, one of the main sources of dietary silicon in western diets is beer [109,110]. The large variety of compounds in the non-alcoholic fraction makes beer an important food research topic. The production of non-alcoholic beer (NAB) can be achieved by two approaches, yielding different matrix compositions and sensory profiles. Thus, the limitation of the alcoholic content of beer can be done (1) by limiting the fermentation process, and hence the alcohol production, or (2) by using physical methods to remove the alcohol at the end of the brewing process [111].

1.4.2. Beer polyphenols

An extensive variety of phenolic compounds had been described in beer including phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids, hydroxyphenylacetic acids), flavonoids (flavonols, flavonols, flavones), prenylflavonoids, indole-based compounds, and alkylmethoxyphenols [112,113].

Prenylflavonoids are a subfamily of compounds with oestrogen-like properties [23]. In beer, the major prenylflavonoid is named isoxanthohumol (IX), which comes from hops. Indeed, IX is produced from xanthohumol during the brewing process, making beer its main dietary source [107]. Following IX intake via beer consumption, this weakly oestrogenic compound can be mainly bioactivated to 8-prenylnaringenin (8-PN) (Figure 4). The biotransformation is leaded: (1) by the microorganisms inhabiting the gastrointestinal tract (80% of the total production); and (2) by a liver enzyme in minor amounts [114,115]. The 8-PN has a higher affinity for the oestrogen receptor α than β. The relative potency of 8-PN is almost equal to that of oestrone and is 70 times weaker than that of oestradiol [116]. In fact, the activity of 8-PN in beer is greater than the effects of phytoestrogens typically found in soya products [117].

The type of fermentation, classifying alcoholic beer (AB) into ale or lager, has been reported to not be associated with the prenylated flavonoid concentrations. On the other hand, the beer's alcoholic content seems to be positively correlated with the prenylated flavonoid concentration, contributing to yeast stability, and therefore enhancing the fermentation processes and the consequent increase of the alcohol content [113]. In the same direction, AB is richer in total polyphenol content compared to NAB [113], but no differences in the qualitative phenolic profile have been observed among them [112]. Additionally, the bioavailability of dietary polyphenols is of great importance in determining any association between their exposure and related health

effects. In that sense, ethanol might have a role in promoting the bioavailability of some phenolic compounds *in vivo* [118].

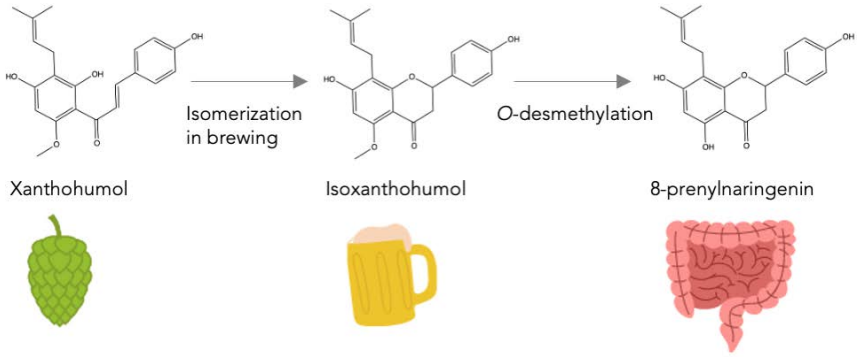

Figure 4. Transformation of prenylflavonoids from hops after wort boiling and gut microbiota metabolism.

1.4.3. Beer organoleptic characteristics

The food system and the concentration of each sensory stimuli define the relative contribution of the stimuli sensory properties. In this way, an unpleasant tastant sampled alone (e.g., a bitter compound in water) could make an important positive contribution to the flavour profile (odour, aroma, taste, and mouthfeel) of a food system when is present at an appropriate low concentration. As an example, bitterness sensory property adds to the appeal of popular foods such as chocolate, coffee, and beer [119].

Beer is a complex food matrix with sweet and bitter tastes as the main taste characteristics [120]. The appearance and flavour of the beer are largely affected not only by the type of cereal grain, but also by malting process, temperature, fermentation type, mashing, variety of hops and the aging process [121,122]. Kilning is the stage of the malting process that differentiate types of malt to be produced. The lager and ale malts, called white or enzymic malts, are those with sweet and nutty flavours. During the malting process, the amino acids, sugars, lipids, sulphur

compounds and phenols that are formed or released from the endosperm are precursors for flavours compounds [123].

Hop provides the most characteristic composition to beer and imparts bitterness and a distinguishing hoppy flavour. Additionally, hops provide some protection against bacterial spoilage and are fundamental for good foam formation [104]. The perceived bitterness in beer depends on the overall level of iso-α-acids and their relative proportions [123]. At the same time, sensitivity to taste stimuli and hedonic perception of it are not the same across individuals. Indeed, bitterness is the taste that arouses the least consensus [119]. Cultural factors and genetic predisposition to orosensory sensations appear to be the primary influences of bitter food choices and liking [119].

The properties of the beer depend very much on the yeast used too [123]. Yeasts produce ethanol, carbon dioxide and other compounds (higher alcohols, organic acids, esters, aldehydes, ketones, sulphur compounds, among others), which play a key role in beer taste perception [104]. Ethanol gives us a feeling of warmth and has its own aroma, easily found in pure alcohol [124]. From ethanol aroma, the intensity or quantity, as well as its quality, can also been assessed [123].

1.4.4. Beer bitter taste active compounds

Traditionally, the health effects of bioactive compounds of the diet have been explained based on their absorption and distribution in the body, as well as for their impact on the gut microbiota. The growing knowledge of the presence of TRs in the gut opens a new avenue for how these compounds can affect homeostasis and interfere with physiological mechanisms via endocrine signalling. Therefore, the binding of tastant molecules with their TRs may promote the secretion of peptide hormones or nerve signals directly to the brain centres that are responsible for

metabolic control, independently of their absorption, degradation or stability during transport and circulation to the relevant organs of the body. Thus, sensory properties of food products or food preparations could be important not only for their gastronomic aspect and organoleptic characteristics, but also for their function in promoting homeostasis and health care [125].

Trans and *cis*-iso-α-acids, generated from hop (*Humulus lupulus* L.) derived precursors, have been identified as the major contributors to the bitter taste of beer. Although 25 T2Rs have been detected in humans, only T2R1, T2R14 and T2R40 have been reported to mediate psychophysical responses to bitter hop-derived compounds [126]. Recently, T2Rs have been found to play a role in the digestive system and body metabolism. Specifically, T2R1, T1R14 and T2R40 are expressed in enteroendocrine cells responsible for incretin hormone secretion [81,127–129]. Furthermore, the α-subunit of gustducin, a specific G protein that mediates sweet and bitter gustatory signals, has been localized together with glucagon-like peptide-1 (GLP-1) in enteroendocrine cells of the human colon [130]. Thus, bitter compounds in beer may exert effects via the modulation of T2Rs in the gut and stimulating GLP-1 release from cells [81,127,128]. Indeed, a previous 3-treatment double-blind cross-over RCT found a suppressive energy intake effect and modified release of hormones involved in appetite and glycaemic regulation such as cholecystokinin (CCK), GLP-1 and peptide YY (PYY) after a gastric and duodenal delivery of a bitter hop extract [131]. Incretin hormones release promotes glucose-dependent insulin secretion and regulates glucose homeostasis. Identification of more bitter agonists with the corresponding T2R may afford candidate compounds with health effects and the associated pathways.

Finally, the relation between incretin hormones and the skeleton is increasingly recognized. The GLP-1 receptor has been reported in human osteoblastic cell lines,

increasing bone formation, and decreasing bone resorption [132,133]. Bitter taste compounds in beer and their threshold concentrations could regulate GLP-1 secretion and have a long-term role on health and chronic diseases, especially in metabolic disorders such as obesity and diabetes [131,134,135].

1.4.5. Alcohol consumption habit and its metabolism

Alcohol can be metabolized through different processes, and although is in the stomach where the first-pass metabolism of alcohol occurs, liver plays a major role in alcohol metabolism. It involves the conversion of ethanol to acetate in two steps: 1) liver alcohol dehydrogenase (ADH) converts ethanol to acetaldehyde, which has undesirable effect in the body; 2) the concentration of acetaldehyde is kept low through rapid oxidization to acetate by aldehyde dehydrogenase (ALDH). Parallelly, the oxidation of ethanol also occurs through catalase and cytochrome P450 E1 (CYP2E1) enzymes. Ethanol is not stored and remains in body water until eliminated, while acetate has a role in energy and regulation metabolism [136–138].

There is a 3-fold to 4-fold variability in the rate of alcohol elimination by humans, due to genetic (e.g., ADH and ALDH enzymes) and physiological factors (e.g., age, sex, body composition, nutritional status, gastrointestinal motility, and liver function) and the mode of ethanol intake (e.g., dose, time of administration, and food in which the ethanolic content is consumed). For example, ethanol is distributed from blood into all tissues and fluids proportionally to their relative water content. Females generally have a smaller volume of distribution for alcohol than males because of their higher percentage of fat [136–138].

Differences in the health effect among alcoholic beverages have been stated [139], as well as the importance of drinking patterns [140]. Additionality, some researchers

have stated that the amount of alcohol consumption is more important that the type of alcoholic beverage [141,142]. Mediterranean diet is a well-known dietary pattern consistently beneficial in the prevention of coronary heart diseases, as well as all-cause mortality [143]. One of their dietary elements is wine, consumed in moderation and mainly with meals [144]. Indeed, the rate of alcohol absorption depends on the concentration of alcohol and the rate of gastric emptying, being more rapid in the fasted state [136]. Sharing food and drinking in company, in moderation and respecting social beliefs, represents a social support and gives a sense of community, consideration that the Mediterranean diet takes also into account [145]. Gender differences in terms of alcohol intake habits have been described. Generally, men drink larger amounts of alcohol, more frequently and are more likely to be hazardous drinkers. Furthermore, men drink more in public places, less circumscribed and more often it is associated with aggressive behaviour. Women, instead, prefer mild types of alcoholic beverages and alcohol drinking is related to what is known as emotional drinking, being alcohol drinking related more often to depressive and negative mood [146]. Indeed, male young adults are the world's population consuming greater harmful amounts of alcohol [147]. Understanding sex differences of the reward-seeking behaviours and the development of substance use disorders will benefit both men and women [148].

Although it is well recognized that binge drinking and high alcohol use is a risk factor of death and disability, the eternal debate about the protective health effect of low to moderate alcohol consumption remains active across the scientific community. The epidemiological J-shaped relationship of alcohol consumption and coronary heart disease has been reported for decades [140]. More precisely, low to moderate drinking (up to 1 drink or 12.5 g alcohol/day for women and 2 drinks or 25 g alcohol/day for men) has been associated with lower rates of CVD [149], but it is not

uniformly protective for other conditions such as cancer [150]. Therefore, the discussion on how to integrate new scientific evidence into clinical practice and public health messages requires mention of all possible effects of alcohol and must point out that these effects may be distributed unequally across the population (i.e., injury risk affects more across younger individuals, whereas CVD mainly affects older adults [149]). In that sense, the association between low to moderate alcohol consumption and the incidence of cardiometabolic diseases is mainly seen in middle-aged men and post-menopausal women [151]. In addition, approaches to minimize individual-level risk due to alcohol consumption need to take into consideration not only alcohol use and specific health outcomes, but also interactions between genetic, environmental, and behavioural factors, as well as the societal and health system context of each. The last publication from the Global Burden of Disease 2020 alcohol collaborators [147] concluded that tailored guidelines and recommendations on alcohol consumption must take into consideration age and regions, but not incorporate sex-specific recommendations. It also highlighted that existing low consumption thresholds are too high for younger populations in all regions [147].

Some methodological problems that can affect the relationship between alcohol use and all-cause mortality have been also identified, such as those related to abstainers as the control group (e.g., former, and occasional drinkers counted in the abstainer group), and those related to the covariates (e.g., the comparative risk of different diseases varies across the life course). Moreover, the alcohol-heavy episodic pattern is normally not considered [152].

In conclusion, the interpretation of the J-shaped relationship has been criticized mainly due to potential confounding from the selected reference group and uncontrolled lifestyle factors. Well-designed, large-scale RCT are needed to prove alcohol consumption's effect on a specific health outcome and among a detailed

study population (e.g., age range, sex, ethnicity, social demographic index, health status, family health antecedents). Finally, due to changing consumer habits and the rising concern about alcohol abuse and health consequences, the NAB market is growing fast within the beverage industry [153].

1.4.6. Moderate beer intake (with and without ethanol) effects on post-menopausal women's health.

Clinical evidence about beer consumption effects needs to be more specific on sex and age-related differences and health outcomes. Indeed, some health effect of alcohol consumption might be different between sexes and/or genders. Beer, for their content in polyphenolic compounds, bitter acids, silicon, and ethanol has been described as an interesting beverage mitigating some of the health changes characteristics of menopause. However, due to the limited scientific evidence about the effect of beer consumption on women's health from interventional trials, strong conclusions are not currently available. Details of the published evidence up to date about three interesting health outcomes for post-menopausal women can be found in the publication titled "Effects of the Non-Alcoholic Fraction of Beer on Abdominal Fat, Osteoporosis, and Body Hydration in Women", presented below **(Publication 1).**

Publication 1

Effects of the Non-Alcoholic Fraction of Beer on Abdominal Fat, Osteoporosis, and Body Hydration in Women

Marta Trius-Soler, Arnau Vilas-Franquesa, Anna Tresserra-Rimbau, Gemma Sasot, Carolina E. Storniolo, Ramon Estruch, and Rosa M. Lamuela-Raventós

Molecules. 2020, p 3910. https://doi.org/10.3390/molecules25173910.

Abstract

Aim: The present review focuses on the effects of non-alcoholic components of beer on abdominal fat, osteoporosis, and body hydration in women, conditions selected for their relevance to health and aging.

Methods: Current and relevant evidence on the topic was selected and carefully reviewed.

Results: Although beer drinking is commonly believed to cause abdominal fat deposition, the available literature indicates this outcome is inconsistent in women. Additionally, the non-alcoholic beer fraction might improve bone health in post-menopausal women, and the effects of beer on body hydration, although still unconfirmed seem promising.

Conclusions: Most of the health benefits of beer are due to its bioactive compounds, mainly polyphenols, which are the most studied. As alcohol-free beer also contains these compounds, it may well offer a healthy alternative to beer consumers.

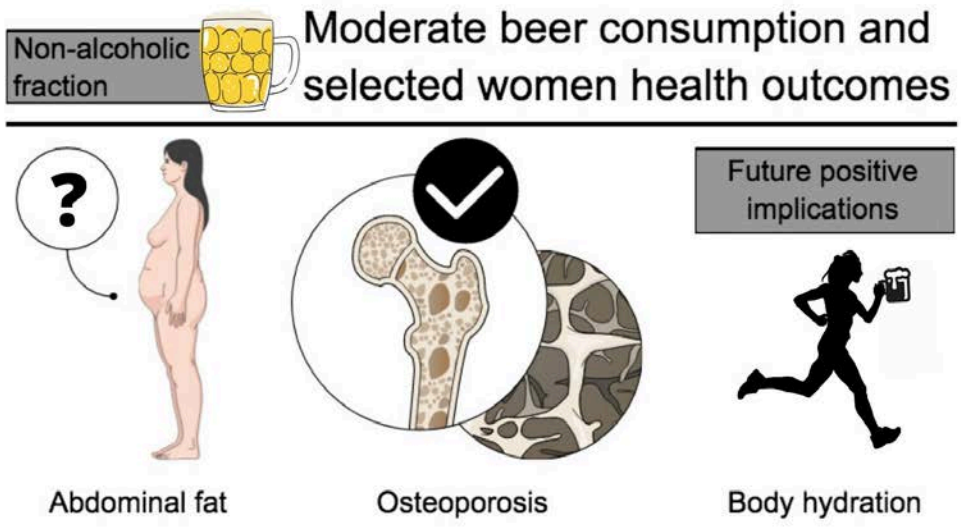

Figure 5. Graphical abstract of Publication 1.

molecules

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Review

Effects of the Non-Alcoholic Fraction of Beer on Abdominal Fat, Osteoporosis, and Body Hydration in Women

Marta Trius-Soler [1,2,3,†], Arnau Vilas-Franquesa [4,†], Anna Tresserra-Rimbau [1,2,3], Gemma Sasot [1,2], Carolina E. Storniolo [1,2,3], Ramon Estruch [3,5] and Rosa M. Lamuela-Raventós [1,2,3,*]

1 Department of Nutrition, Food Sciences and Gastronomy, XaRTA, School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; mtrius@ub.edu (M.T.-S.); annatresserra@ub.edu (A.T.-R.); gemmamsf@gmail.com (G.S.); carolina.e.storniolo@gmail.com (C.E.S.)
2 INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona, 08921 Santa Coloma de Gramanet, Spain
3 CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain; restruch@clinic.cat
4 Centre d'Innovació, Recerca i Transferència en Tecnologia dels Aliments (CIRTTA), XaRTA, TECNIO, MALTA-Consolider, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona (Cerdanyola del Vallès), 08193 Bellaterra, Spain; arnauvilas.nl@gmail.com
5 Department of Internal Medicine, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, 08036 Barcelona, Spain
* Correspondence: lamuela@ub.edu; Tel.: +34-934-034-843
† These authors contributed equally to this work.

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Abstract: Several studies have shown that binge drinking of alcoholic beverages leads to non-desirable outcomes, which have become a serious threat to public health. However, the bioactive compounds in some alcohol-containing beverages might mitigate the negative effects of alcohol. In beer, the variety and concentration of bioactive compounds in the non-alcoholic fraction suggests that its consumption at moderate levels may not only be harmless but could also positively contribute to an improvement of certain physiological states and be also useful in the prevention of different chronic diseases. The present review focuses on the effects of non-alcoholic components of beer on abdominal fat, osteoporosis, and body hydration in women, conditions selected for their relevance to health and aging. Although beer drinking is commonly believed to cause abdominal fat deposition, the available literature indicates this outcome is inconsistent in women. Additionally, the non-alcoholic beer fraction might improve bone health in postmenopausal women, and the effects of beer on body hydration, although still unconfirmed seem promising. Most of the health benefits of beer are due to its bioactive compounds, mainly polyphenols, which are the most studied. As alcohol-free beer also contains these compounds, it may well offer a healthy alternative to beer consumers.

Keywords: hops; malt; health; menopause; polyphenol; phytoestrogen; prenylnarigenin; humulones; ethanol; bioactives

1. Introduction

Beer, an alcoholic drink composed of four main ingredients (water, malt, hops, and yeast) [1], is one of the most consumed beverages in the world [2]. From a nutritional point of view, its main components are water (around 90%), followed by carbohydrates, ethanol, minerals, vitamins,

and bioactive compounds such as polyphenols and organic acids (iso-α-humulones). Beer composition, as well as its flavor, taste, and texture, differs considerably according to the ingredients and processing techniques [3]. Besides their health benefits, the bioactive compounds are also linked to the sensory characteristics of beer [4].

In view of the worldwide growth in beer consumption, studies investigating possible links between beer and different health outcomes are of utmost importance. Among others (i.e., liver disease), recently, one of the most important consequences of a high beer consumption is a greater risk of developing different site-specific cancers (e.g., colorectal [5], lung [6,7], prostate [8], and oral cavity, esophagus, and larynx cancer [9]). It is also known that high alcohol intake help to develop a dilated cardiomyopathy and also may trigger certain cardiovascular events [10,11]. Nevertheless, a moderate consumption of beer may also help to prevent these type of events [12,13].

Clinical evidence about beer consumption effects needs to be more specific on sex-related differences and health outcomes. Postmenopausal women due to the estrogen depletion suffer body changes [14] and there is an accumulation of abdominal fat [15], an increasing risk of osteoporosis [16] and a loss of body hydration [14] among other health issues. Interestingly, some studies have pointed out that bioactive compounds of beer may help to mitigate some of these adverse effects.

In a unit of beer the main bioactive compounds with health benefits described in several studies [9,17,18] are depicted in Table 1. Particular attention has been given to the polyphenols found in malt (75%) and hops (25%), due to their antioxidant and anti-inflammatory properties [19,20]. Polyphenols are also critical to the flavor, astringency, bitterness, haze, and body of beer [21,22], and their concentration varies according to the ingredients and processing [23,24]. Regular beer, both ale and lager beers, is richer in polyphenol content compared to alcohol-free beers [25].

Table 1. Mean content of selected bioactive compounds in a standard drink of regular beer.

Bioactive Compound	Avarege Level (mg/330 mL)
Phytoestrogens	
Xanthohumol	4.653×10^{-3}
6-Prenylnaringenin	8.547×10^{-3}
8-Prenylnaringenin	3.432×10^{-3}
Isoxanthohumol	0.132
Bitter acids	
α+β acids	0.891 [a]
Iso-α-humulones	9.207 [a]
Minerals	
Silicon	6.336
Sodium	14.883
Potassium	116.589

[a] mean value from three beer samples. Content of phytoestrogens from Rothwell et al. (2013) [26], bitter acids from Česlová et al. (2009) [27], silicon from Jugdaohsingh (2007) [28] and sodium and potassium derived from *the Food composition data of 16 European countries* via www.EuroFIR.org.

Among polyphenols, a particular group has attracted special interest for their estrogen-like properties [29]. Hops (*Humulus lupulus* L.) are a source of prenylflavonoids, a class of phytoestrogens, predominantly xanthohumol (XN), that during the brewing process isomerizes into isoxanthohumol (IX), 6-prenylnaringenine (6-PN), and 8-prenylnaringenine (8-PN) [30]. These compounds can mimic and modulate the action of estrogenic hormones by epigenetic mechanisms, via binding with cell surface receptors or by interacting with estrogen receptors (ERs). In particular, 8-PN has been described as the most estrogenic phytoestrogen, surpassing those typically found in soya products [31].

The aim of the present review is to summarize the available literature on the health outcomes of beer consumption in women, focusing on three specific health-related conditions: increased abdominal fat, osteoporosis, and overall body hydration. In particular, findings related to the beer bioactive compounds are discussed.

2. Beer Consumption Related to Health and Disease in Women

2.1. Beer, Abdominal Fat, and Weight Gain

A widely held belief is that beer consumption directly contributes to an increase in abdominal fat and ultimately leads to overweight and obesity. This assumption might be due to the nutritional value of beer, since it contains not only alcohol but also more carbohydrates than other alcoholic drinks [32]. In this section, we assess whether or not beer consumption can increase abdominal fat and site-specific adiposity in women, central obesity being the most relevant sign of metabolic syndrome (MetS) [33].

The type of alcoholic drink, as well as dose, frequency and time of consumption play a role in how alcohol drinking may change fat distribution [34,35]. Additional factors such as genetics, gender, and age may also be important determinants of central body fat [34]. Thus, for instance, drinking alcoholic beverages during meals was significantly more prevalent in females than in males in one study population [35]. In addition, it has been suggested that enlarged waist circumference (WC), known as "beer belly", commonly observed in regular beer consumers might be more due to unhealthy lifestyle factors and drinking patterns (e.g., physical inactivity and smoking) rather than to beer consumption alone [36].

Women seem to be more prone to fat deposition than men upon the consumption of high doses of alcohol [37]. In general, postmenopausal women have a higher total body fat mass and more abdominal fat than premenopausal women. More specifically, despite exhibiting a similar mean body mass index (BMI), postmenopausal women have a larger WC [15]. While both genders experience somatic changes with aging, in women they particularly affect the WC and waist-to-hip ratio (WHR) [33,38]. Interestingly, both visceral and subcutaneous adipocytes express estrogen and androgen receptors such as ER-α, a regulator of adipocyte activity and fat distribution responsible for these gender differences and hyperandrogenism in postmenopausal women [15,39]. As increased visceral abdominal fat deposition causes metabolic changes in fatty acid metabolism, it would be useful to know which foods and ingredients may be more effective for counteracting this fat accumulation in postmenopausal women [15].

Several studies have investigated the effects of gender in the relationship between beer consumption and abdominal adiposity [32,40]. A systematic review of observational studies published before November 2010 indicates that there is an inverse or no association between general obesity and moderate beer consumption in women, while findings referring to abdominal obesity seem to be inconsistent [40]. The authors pointed out that these conflicting observational data may be explained by the small proportion of women beer drinkers and their relatively low beer intake in the studies analyzed [40].

Alcohol or beer consumption and abdominal fat or weight gain have been described as having a U-shaped relationship, with the lowest BMI values observed in women who consumed an average of 6–24 g/day of alcohol [41]. In another study, women with a low beer consumption (maximum 1.32 L/week) also had the lowest WHR values, whereas non-consumers had the highest WC [33]. In the Third National Health and Nutrition Examination Survey (NHANES III), the lowest MetS and WC values were observed in the mild to moderate beer and wine drinkers [42]. Consequently, it can be stated that excessive beer intake may contribute to a higher WC and WHR, and even a higher overall BMI, yet the regular consumption of less than 0.5 L/day of beer (4% alcohol) seems unlikely to have this effect, according to the data available in cross-sectional and prospective observational studies [40]. Women studies evaluating the relationship between beer consumption and abdominal fat increase has been summarized in Table 2 [33,35–37,41,43–55].

In a study focused on the effects of a moderate beer intake on the body composition of healthy adults undergoing a high-intensity interval training, the group consuming alcohol-free beer experienced a significant decrease in visceral adipose tissue and WC, and a clear decreasing trend in the WHR. The other groups (consuming beer or water supplemented with vodka ethanol) did not show any changes in these variables [56].

Now, we should look for the compounds of regular and non-alcoholic beer responsible of these effects. The main bitter compounds of beer are iso-α-acids or iso-α-humulones, derived from the isomerization of α-acids in hops during brewing [57,58]. A study of mice fed with a high-fat diet (HFD) supplemented with iso-α-acids reported significantly reduced body weight, epididymal fat weight, and plasma triglyceride levels after the intervention, whereas in the control group the values increased [59]. As in other studies, it was concluded that iso-humulones might have a protective effect on internal organs damaged by obesity, making this a promising line of future research [59,60]. Iso-α-acids bind and activate both peroxisome proliferator-activated receptors α (PPARα) and γ (PPARγ), which exhibit anti-obesity and anti-inflammatory activities in vivo [59–61]. Regular beers contain 20–40 mg/L of iso-α-acids [27,62,63], and some bitter beers up to 50–80 mg/L [62].

A clinical trial with prediabetes subjects found that 32–48 mg/day of iso-humulones lowered the fasting blood glucose and hemoglobin A1c after 8 weeks, while the total fat and BMI in participants receiving 48 mg/day decreased at 12 weeks [62]. However, some effective concentrations of iso-humulones reported in the literature, such as 500 mg/kg body weight in mice, would be impossible to ingest through moderate or even high beer consumption [60]. Additionally, it would be difficult to formulate a food other than beer with 10–100 mg/L of iso-humulones and an effective dose of iso-α-acids because of their strong bitterness [57].

Matured hop bitter acids (MHBA) are components derived from α-acid oxidation and bear a β-tricarbonyl moiety in their structure such as α-, β-, and iso-α-acids. The bitterness of α-acid oxidation products is described as being more acceptable for the consumer compared to iso-α-acids, and some studies of the bioactive properties of MHBA have been carried out [57]. Weight gain in six-week-old male C57BL/6J mice, a model of MetS, was significantly suppressed when their high fat diet was supplemented with MHBA [64]. Additionally, MHBA administration induced cholecystokinin secretion and signal transduction in the rat gastrointestinal tract, resulting in an increase in the brown adipose tissue temperature. Moreover, MHBA may target TAS2 receptors (TAS2Rs) because they share a similar structure with iso-α-acid [57]. Although 25 TAS2 bitter taste receptors have been determined in humans, only TAS2R1, TAS2R14, and TAS2R40 have been reported to mediate psychophysical responses to bitter hop-derived compounds [65]. Specifically, TAS2R1 and TAS2R40 are expressed in enteroendocrine cells, responsible for incretin hormone secretion [66–68]. There is also interesting evidence that the consumption of mature hop extract significantly reduces abdominal visceral fat of healthy overweight subjects [58].

On the other hand, it has been found that a XN-rich hop extract (17.8% XN and 12.4% IX) prevents fat gain due to overnutrition by modulating preadipocyte differentiation in a 3T3-L1 mouse fibroblast cell line [69]. Furthermore, oral administration of 30 and 60 mg/kg/day of XN during 12-weeks in a C57BL/6J mice model improved markers of inflammation and MetS and decreased BMI in a dose-dependent manner. Nevertheless, the authors concluded that because XN concentrations found in beer are only about 0.2 mg/L, XN taken in the form of beer would be unlikely to have a protective effect against MetS [70]. Two other studies performed in the same C57BL/6J mice model demonstrated that XN derivatives [71] and IX [72] significantly changed the gut microbiota profile, constituting a potential mechanism against obesity and MetS [71,72].

Table 2. Women studies evaluating the relationship between beer consumption and abdominal fat increase.

Authors Year [Ref]	Type of Study	Study Population	Key Finding
Lapidus et al., 1989 [43]	Cross-sectional	1462 women 38–60 years-old	No correlation was found between WHR and beer consumption.
Slattery et al., 1992 [44]	Cross-sectional	1447 black women 1284 white women 18–30 years-old	Higher beer consumption was associated with a higher WHR among white and black women.
Kahn et al., 1997 [45]	Prospective observational	44080 women 40–54 years-old	OR of abdominal weight gain was positively associated in women drinking >0 to <5 days per week and no associated in women drinking <5 days per week versus non-drinkers
Dallongeville et al., 1998 [37]	Cross-sectional	11730 women 35–64 years-old	Beer & cider consumption was associated with a higher WHR.
Rosmond & Bjorntorp 1999 [46]	Cross-sectional	1137 women 40 years-old	Beer consumption was negatively correlated to WHR.
Machado & Sichieri 2002	Cross-sectional	1396 women 20–60 years-old	No trend association for OR for WHR >0.80 across beer consumption categories was found.
Vadstrup et al., 2003 [48]	Prospective observational	3970 women 20–83 years-old	Positive trend association was found for WC at follow-up across beer intake categories.
Bobak et al., 2003 [49]	Cross-sectional	1098 women 25–64 years-old	Beer intake was not associated with an increase in WHR.
Dorn et al., 2003 [35]	Cross-sectional	1322 women 53.3 ± 9.4 years-old	No trend association was found between sagittal abdominal diameter and beer consumption.
Halkjaer et al., 2004 [50]	Prospective observational	1131 women 30–60 years-old	Women consuming >4 drinks of beer per week have higher WC, while no significance increase in WC was found in the group drinking 1–3 drinks of beer per week compared to non-drinkers.
Deschamps et al., 2004 [52]	Cross-sectional	284 women 42.4 ± 4.6 years-old	Women drinking >1 glass of beer per day have a higher WRC than abstainers and those who drink <1 glass of beer per day. No trend association was found for WC.
Lukasiewicz et al., 2005 [53]	Cross-sectional	1268 women 47.7 ± 6.6 years-old	No trend association was found between beer consumption and WHC.
Halkjaer et al., 2006	Prospective observational	22570 women 55 (50–64) years-old	No trend association was found between ΔWC and beer consumption.
Krachler et al., 2006 [54]	Cross-sectional	3087 women 25–64 years-old	Increased beer consumption was not significantly associated to WC.
Tolstrup et al., 2008 [55]	Prospective observational	1610 women 50–65 years-old	Negative association was found for OR of WC across beer intake frequency categories among women who preferred beer.
Schütze et al. [36] 2009	Cross-sectional	2749 women 35–65 years-old	Positive trend association for ΔWC and ΔWHR was found across beer consumption categories.
Schütze et al., 2009 [36]	Prospective observational	12749 women 35–65 years-old	No trend association for WC was found across beer consumption categories.
Bergmann et al., 2011 [41]	Cross-sectional	158796 women 52.9 ± 9 years-old	Positive association was found for OR of WC and WHR for women drinking <6 versus ≤ 6 g per day of alcohol from beer.
Zugravu et al., 2019 [33]	Cross-sectional	784 women >18 years-old	No linear trend association was found between beer consumption and WC or WHR.

WC: waist circumference; WHR: waist-hip ratio.

2.2. *Beer and Osteoporosis*

Known as one of the most important health-related conditions of aging, osteoporosis is attributed to a decrease of bone mineral density (BMD), which ultimately leads to increased bone fragility [73]. Although common, the condition is underdiagnosed and undertreated, and clinical trials and public health strategies are needed to improve screening and management [74]. Nutrition, exercise and lifestyle are recognized as important aspects in osteoporosis prognosis [75], so modifiable environmental factors such as diet should be considered in its management [76].

Postmenopausal status has been described as a risk factor of BMD loss [16]. As a long-term consequence of the lack of estrogenic stimulation, menopausal bone loss has been linked to an accelerated bone turnover combined with an imbalance that favors bone resorption rather than formation [29,77]. The risk of osteoporosis is six times higher in postmenopausal versus premenopausal women [74]. One of the main mechanisms underlying the protective effect of estrogen against osteoporosis could be an enhanced expression of the vitamin D receptor in the duodenal mucosa and responsiveness to endogenous 1,25-dihydroxycolecalciferol [78].

Certain dietary factors, such as moderate alcohol consumption, have been positively associated with BMD values in postmenopausal women and in the general population [16,79,80]. A study found that women who consumed more than 1 drink of alcohol/day (i.e., 270 mL of beer, 100 mL of wine, or 27 mL of liquor) had a significantly higher femoral neck and lumbar spine BMD than non-alcohol consumers, in a lifestyle adjusted model [81]. Among alcoholic drink subtypes, only beer and low-alcohol beer (but not wine or liquors) seemed to have a significantly positive effect on lumbar spine BMD in older women [81,82]. Similarly, in a cohort of elderly men and women, the lowest hazard ratios for hip fracture tended to be among beer consumers [83]. Also, quantitative bone ultrasound values were higher in women who consumed beer compared to the non-beer or wine drinkers, independently of their gonadal status. This result could be explained by the phytoestrogen content and low grade of alcohol in beer [84]. In contrast, other studies have found positive associations between wine or wine preference and spine BMD in a postmenopausal population group, but not for beer or spirits [76,85]. Women studies evaluating the relationship between beer consumption and osteoporosis has been summarized in Table 3 [76,81,82,84].

In 2008, a systematic review and meta-analysis concluded that subjects consuming 0.5–1 drink/day, equivalent to 7–14 g alcohol/day, had a lower hip fracture risk than abstainers, whereas those consuming more than 2 drinks/day had a greater risk [86]. Thus, abstainers and heavy drinkers have a higher risk of hip fractures than light-moderate drinkers, with a U-shaped relationship between the variables [83,86]. Supporting these results, abnormal bone histology and decreasing bone formation and mineralization have been described in alcoholics [87]. The tendency of a higher association between BMD and beer or wine consumption compared to liquor suggests that other compounds besides ethanol may contribute to bone health [4].

Most of the positive effects of beer on osteoporosis in postmenopausal women have been attributed to the non-alcoholic fraction, specifically to polyphenols, silicon and α-acids. Among phenolic compounds, flavonoids have been inversely linked to bone resorption biomarkers in Scottish women aged 45–54 years. The flavonoids most consumed by the participants were catechins, demonstrating the significant contribution of these compounds to improving BMD [88,89]. The bioactive compounds in hops have been proposed as an alternative to conventional hormone replacement therapy. In particular, the phenolic phytoestrogens from hop extract seem to exhibit estrogen-like effects on bone metabolism [90]. A recent study in animals found that hop extract containing phytoestrogens and iso-α-acids attenuated bone loss and reversed high bone turnover in ovariectomy mice [91]. Furthermore, in vitro experiments demonstrate that hop phytoestrogens (XN, IX, 6-PN, and 8-PN) regulate both osteoblast and osteoclast activities, while α-acids exert a strong bone resorption inhibitory activity, however, the recommended dosage is still unclear [90–92].

The phytoestrogen XN inhibits the receptor activator for the nuclear factor κ B ligand (RANKL) signaling pathway, which has been identified as critical to osteoclast formation and bone resorption [93,94]. XN has also been reported to promote osteoblast differentiation, up-regulate alkaline phosphatase activity, and increase the expression of osteogenic marker genes in osteoblastic cell lines [95]. Interestingly, Prouillet et al. (2004) had previously suggested that one of the consequences of increased alkaline phosphatase activity could be an activation of the ER [94], and another study described an inhibitory resorption effect of XN in a dose-dependent manner [92]. Regarding 8-PN, a recent review of its therapeutic perspectives discusses plausible mechanisms for the anti-osteoporotic properties of this intestinal metabolite. 8-PN has preferential binding to ER-α, which is the prevailing

ER in bone tissue, and its prenyl group seems to be essential for the anti-osteoporotic mechanism [29]. In summary, the beneficial effects of 8-PN, promoting bone formation and inhibiting bone resorption, are mediated by ER-α instead of ER-β, and it is more potent than the isoflavones genistein and daidzein [96].

Silicon from malt has been reported to facilitate bone mineralization and regeneration [75,97], which are essential for bone formation [97]. Some alcoholic beverages such as beer or wine contain significant amounts of silicon [98], although due to the processing of barley and hops, beer is a better source than wine or other alcoholic beverages, with an average content of 19.2 mg/L and non-significant differences among different types of beer [28,75]. Moreover, silicon in beer has a high bioavailability [98,99]. Tucker et al. (2009) showed that adjustment for silicon intake mitigates the positive effect of beer consumption on BMD in older men and women [4].

To sum up, bone remodeling is a slow process and aging affects bone turnover [100]. The phenolic fraction of beer, including phytoestrogens and iso-α-acids from hops, and the silicon from malt seem to play a role in osteoporosis prevention. However, long-term clinical trials are needed to better predict the impact of beer consumption on bone mass, a major concern for postmenopausal women suffering from bone loss.

Table 3. Women studies evaluating the relationship between beer consumption and osteoporosis.

Authors Year [Ref]	Type of Study	Study Population	Key Finding
Pedrera-Zamorano et al., 2009 [86]	Cross-sectional	1697 women (710 premenopausal; 176 perimenopausal and 811 postmenopausal) 48.8 ± 12.59 years-old	Light or moderate consumption of beer was associated to higher bone mass in women independently on their gonadal status.
Fairweather-Tait et al., 2011 [76]	Cross-sectional	2464 postmenopausal women twins 56.3 ± 11.9 years-old	Beer consumption was not associated with higher BMD.
Yin et al., 2011 [82]	Cross-sectional	428 women 62.6 ± 7.2 years-old	Low alcohol beer consumption frequency was positively associated with BMD at lumbar spine.
Yin et al., 2011 [82]	Prospective observational	428 women 62.6 ± 7.2 years-old	No association between beer consumption frequency and BMD at hip was found.
McLenon et al., 2012 [81]	Prospective observational	3173 women 50–62 years-old	Moderate beer consumption had a positive significant effect on lumbar spine BMD after adjustment for lifestyle.
Kubo et al., 2013 [85]	Prospective observational	115,655 postmenopausal women 50–79 years-old	No association was observed between ≥ 1 servings of beer per week and risk of hip fracture.

BMD: bone mineral density.

2.3. Beer and Body Hydration

Hydration has a crucial impact on a variety of factors related to the correct functioning of the body and specific recommendations are needed for each population group. Female sex hormones affect the body water balance, although it is still unclear how the regulation of hydration in women may enhance wellness, safety, and mental and physical performance [101]. Estrogen and progesterone levels have been correlated with body fluid regulation and thermoregulation changes [101]. As more water is retained in the body when estrogen levels are high [102], hormonal depletion in menopause results in a loss of hydration, which should be carefully monitored. Current literature reports that estrogen therapy increases osmotic sensitivity and water retention, helping menopausal women to control diuresis and prevent dehydration [14]. The effect of estrogen on fluid regulation in older women seems to be related to sodium retention [102,103]. Not only the menopause but aging itself affects the fluid balance [14].

An estimated intake of 2.5 L of water/day is considered necessary under normal conditions or 3.5 L of water/day in hot weather or when exercising [104]. Perspiration while exercising may cause an important depletion of water and electrolytes [105], as well as part of the body's stored glycogen. Most recommendations for sustaining the nutritional state and optimizing water absorption during exercise include the intake of beverages containing carbohydrates and electrolytes, in particular glucose–fructose and sodium [106]. Besides the main components of water and carbohydrates, beer also contains electrolytes, which may play a role in maintaining water and electrolyte balance, although the ethanol content may counteract these positive effects.

The effect of beer consumption on the overall hydration status has been studied among men. Unfortunately, no studies on this issue have been performed in women. Hobson and Maughan (2010) investigated the effect of low-alcohol doses on induced euhydration or hypohydration [107], administering alcohol-free or alcoholic beer in each case to create four experimental conditions. In the euhydrated group, those consuming alcoholic beer produced more total urine in the 4 h after intake and for 3 h also exhibited considerably higher serum osmolality, a parameter associated with fluid balance, although the difference had disappeared at 4 h, the end of the monitoring period. The authors also mentioned that sodium excretion was notably lower in the alcohol consumers [108]. In an elderly population with more hydration problems, Polhuis et al. (2017) observed a temporary diuretic effect only after moderate consumption of stronger alcoholic beverages (wine, spirits), but not beer. This demonstrates that: (i) moderate consumption of beer and other weak alcoholic beverages may be safe in terms of hydration for the elderly and (ii) the diuretic effect was plainly triggered by the amount of alcohol in the beverage [108].

Several studies have investigated the effect of beer or its components in those practicing sports, monitoring hydration status, muscle performance, environmental conditions, and duration of exercise in male athletes [105,109,110]. The most controversial component of beer is ethanol. An early study from 1997 reported that the retention volume of the total fluid ingested was about 20% lower in those who consumed an alcohol-free beer supplemented with 4% alcohol compared to those who drank non-supplemented alcohol-free beer, following intermittent cycle ergometer exercises in the heat that induced dehydration of up to 2% of body mass [111]. Alcohol itself undoubtedly has a negative effect on exercise performance, although its extent may also depend on other factors, such as the mode and duration of exercise [109]. In extreme conditions, when the body requires greater hydration, any diuretic or anti-hydration effect of the ethanol in beer is more easily noted. Jiménez-Pavón et al. (2015) observed that consumption of 660 mL of regular beer (4% alcohol) after 1 h of running in hot conditions had no deleterious effect on any hydration marker [106]. Two other studies evaluated the effect of water, beer or alcohol-free beer on fluid and electrolyte homeostasis in male athletes or physically active men [112,113]. Castro-Sepulveda et al. (2016) reported that an intake of 700 mL of alcoholic beer before aerobic exercising increased plasma K^+ and decreased plasma Na^+ during the exercise activity, with a negative impact on athletic performance. Notably, this effect was not observed when alcohol-free beer was administered, to the extent that the decrease in plasma Na^+ during exercise was lower than after the ingestion of water. Accordingly, alcohol-free beer might be an effective sports drink for maintaining electrolyte homeostasis in males when taken before exercise [113]. In contrast, another study found that rehydration of young, healthy, and physically active males with non-alcoholic beer was not advantageous with regard to water [112]. A more recent study evaluated the effects of ingesting isotonic drinks or beer with different alcohol concentrations after mild dehydration or exercise among males. The net fluid balance was measured after a 5-hour observation period and the lowest rate of fluid retention (21%) was obtained for beer with 5% alcohol, whereas the highest (42%) was recorded for an isotonic sports drink [114]. Interestingly, the effects of modifying the sodium and alcohol content of beer have also been studied [115,116]. Participants consumed low-alcohol beer (2% alcohol + 25 or 50 mM/L of sodium) or normal beer (3.5% alcohol + 25 mM/L of sodium) and after exercise, the greatest fluid retention was observed in consumers of beer with the highest electrolyte content and the lowest concentration of alcohol (2% alcohol + 50 mM/L of sodium) [116].

While non-alcoholic beer has promising effects in terms of fluid homeostasis in the context of aerobic exercise, a low dose of alcohol (0.5 g/kg of body weight) consumed before muscle damage-inducing anaerobic exercise had no impact on the posterior muscle performance or related water loss in ten healthy young males [110].

Notably, all the aforementioned studies were performed in men. More research is needed to understand the effects of different types of drinks on the hydration state of female athletes, in order to improve performance and provide personalized supplementation recommendations [101].

3. Implications and Future Research

Most of the health benefits of beer are thought to be originated by its non-alcoholic components, mainly polyphenols. Although found in small quantities in the final product, the flavonoid XN (whose only source is hops) is of particular interest. Intestinal metabolites of related flavonoids, notably 8-PN, could also have an important role in human health. Other components, such as silicon or bitter acids, may help to explain other health effects of beer consumption, such as improvement in bone density. Nevertheless, the beneficial properties of beer components outlined in this review have not been extensively studied because of the adverse effects of ethanol. Human interventional trials are required to elucidate the real association between beer intake and health benefits in women, but the consumption of ethanol is an important obstacle for their development. We, therefore, suggest a directional change towards the non-alcoholic fraction of beer and its effect on the female population as an interesting target for future studies. With some authors already using this strategy, a greater focus on alcohol-free beer will lead to the emergence of more human trials and new evidence in this field. Finally, new long-term randomized trials on the effects of moderate alcoholic and non-alcoholic beer consumption (and other alcoholic beverages) on health and diseases, including cardiovascular disease, obesity, diabetes, cancer, cognitive decline, osteoporosis, and others in women (and also in women) are needed to better define the protective role (or not) of beer consumption, independent of other lifestyle factors, on the aforementioned conditions.

4. Conclusions

Although the results of studies on abdominal fat deposition in female beer consumers are inconsistent, moderate consumption appears not to have a significant effect on adiposity. Moderate beer intake has also been associated with improved bone health in elderly women in observational studies. Moreover, the non-alcoholic fraction of beer is of potential interest as a counteracting agent for bone mass loss after menopause.

In the elderly, beer intake does not seem to pose a risk for hydration. When ingested before exercise, beer with lower alcohol content has a better rehydration effect, and the consumption of alcohol-free beer may even have a positive impact on electrolyte homeostasis. However, the effects of beer on hydration in women still need to be investigated.

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2. HYPOTHESIS AND AIMS

HYPOTHESIS

This thesis is based on the hypothesis that due to its phenolic, silicon and ethanol content, moderate beer consumption may have relevant effects on post-menopausal women's health.

AIMS

The main aim of this thesis is to study the effects of moderate beer consumption on post-menopausal women's health, integrating new promising nutrition research approaches and supporting the view of personalized nutrition.

In order to achieve this general aim, the following specific aims were proposed:

Objective 1: To study the inter-individual variability of taste sensitivity.

1.1. To review the effect of physiological factors, pathologies, and acquired habits on the sweet taste detection and recognition threshold (Publication 2).

1.2. To investigate potential predictive variables for basic taste recognition thresholds (Publication 3).

1.3. To evaluate the influence of phenylthiocarbamide (PTC) phenotype on individual characteristics and on taste sensitivity (Publication 4).

1.4. To study the influence of taste sensitivity in a beer intervention clinical trial (Publication 8).

Objective 2: To validate biomarkers of alcoholic beverages and moderate alcohol intake.

2.1. To review the literature on biomarkers of alcoholic beverages and moderate alcohol intake (Publication 5).

2.2. To apply a biomarker of beer intake as a compliance intervention tool in a beer intervention clinical trial (Publication 6, 7, 8).

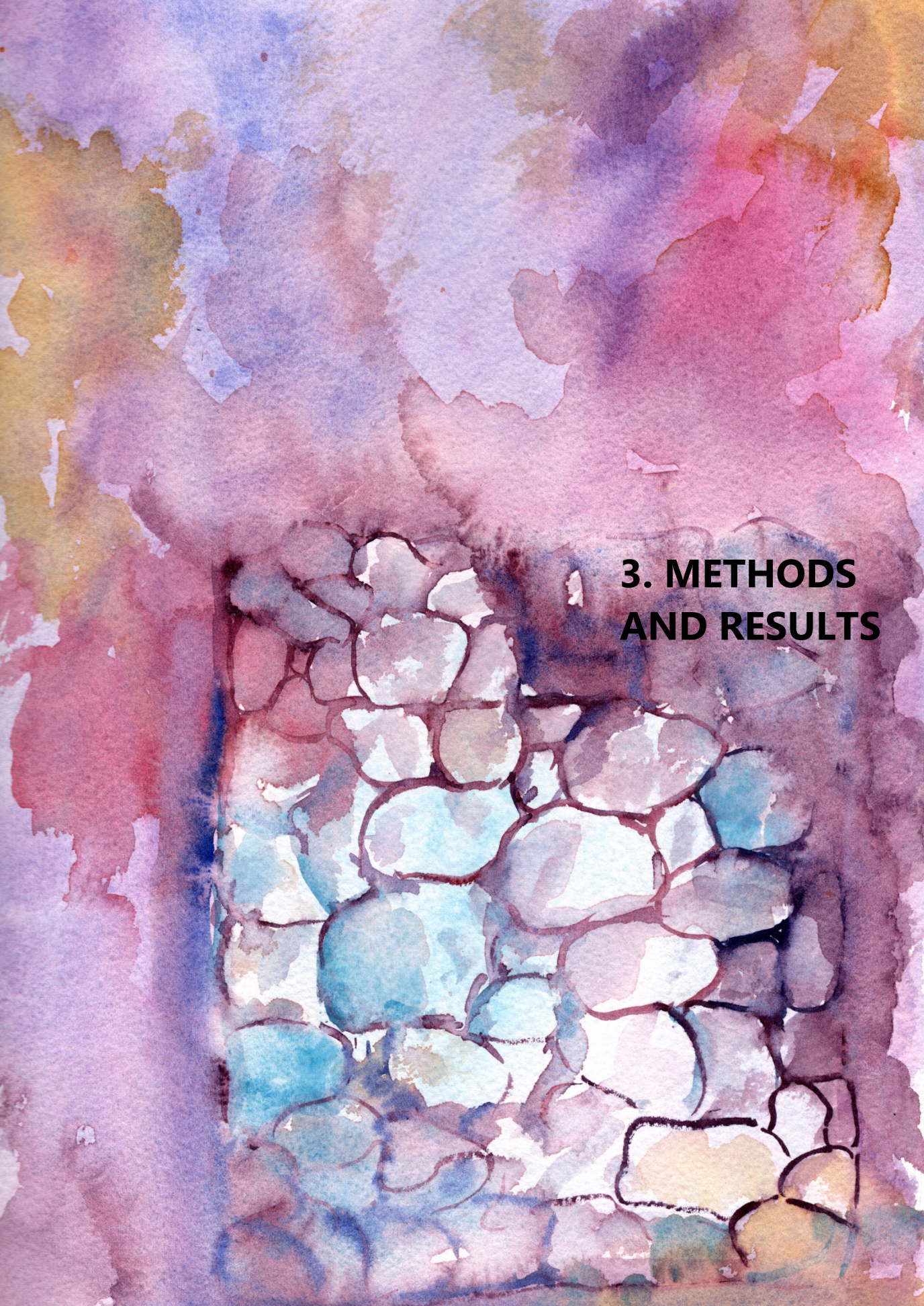
Objective 3: To study the effect of moderate daily consumption of beer (with and without ethanol) on three important menopausal women's health outcomes i.e., menopausal symptoms, bone tissue and cardiovascular health.

3.1. To review the scientific evidence about the effects of non-alcoholic components of beer on post-menopausal women's health (Publication 1)

3.2. To evaluate if a moderate daily intake of beer (with or without ethanol) could reduce menopausal symptoms in post-menopausal women (Publication 6).

3.3. To evaluate the effects of daily and moderate consumption of beer (with and without ethanol) on bone health in post-menopausal women (Publication 7).

3.4. To evaluate the effects of daily and moderate consumption of beer (with and without ethanol) on cardiovascular health in post-menopausal women (Publication 8).



3. METHODS AND RESULTS

3.1. Chemosensory perception and clinical implications

The specific **objective 1** of the present thesis focused on studying the inter-individual variability of taste sensitivity. Chemosensory perception is essential for individuals and species survival and vary between individuals and through life. The first aim was to study the influence of physiological factors, pathologies and acquired habits on sucrose DT and RT (Publication 2). The study of the inter-individual variability on taste sensitivity was also achieved by examining the possible effects of sociodemographic and clinical factors on basic taste sensitivities (Publication 3) and by evaluating the influence of PTC taste status in those specific outcomes and the consumption of bitter vegetables (Publication 4).

The first approach was accomplished by performing a systemic review and meta-analysis of the relevant literature available at that time. Research strategy, data extraction and evidence quality assessment were carried out. After a comprehensive search, a total of 48 studies were qualitatively considered and 44 were meta-analyzed. Age, type 2 diabetes, and BMI seem to be important variables to consider when assessing sweet (sucrose) taste sensitivity. Details are available in Publication 2.

In addition, a cross-sectional study was carried out in a large Spanish young cohort of college students, in which their basic taste RTs were determined, and sociodemographic, clinical, and dietary habit variables recorded. Results are displayed and discussed on the publications found below (Publication 3 and 4). The defined PTC super-taster sub-cohort could be differentiated from the non-tasters by variables related to weight control such as BMI and sucrose RT.

Lastly, the influence of taste sensitivity, intensity and hedonic perception in a beer intervention clinical trial was also studied. Results can be found in the specific **objective 3** section (Publication 8).

Publication 2

Effect of physiological factors, pathologies, and acquired habits on the sweet taste threshold: A systematic review and metanalysis

Marta Trius-Soler, Dimitri A. Santillán-Alarcón, Miriam Martínez-Huélamo, Rosa M. Lamuela-Raventós, and Juan J. Moreno

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Supplementary Material available in Annex 1.

Abstract

Aim: The present review aimed to study the influences of physiological factors (age and sex), pathologies (obesity and type 2 diabetes), and acquired habits (tobacco and alcohol consumption) on sucrose detection threshold (DT) and recognition threshold (RT).

Methods: A systematic review and meta-analysis of the relevant literature were performed.

Results: After a comprehensive search in the PubMed and Scopus databases, a total of 48 studies were qualitatively considered, and 44 were meta-analysed. The factors of aging (standard mean difference [SMD]: − 0.46; 95% confidence interval (CI), − 0.74 to − 0.19; I^2: 73%; Tau^2 : 0.18) and type 2 diabetes (SMD: 0.30; 95% CI, 0.06 to 0.55; I^2: 0%; Tau^2 : 0.00) were found to significantly increase the sucrose RT, whereas the DT only increased in subjects with a higher body mass index (SMD: 0.58; 95% CI, 0.35 to 0.82; I^2: 0%; Tau^2 : 0.00). No effects of sex and tobacco smoking were found, and associations with alcohol consumption could not be assessed, as it was included as a variable in only one study.

Conclusions: The present work provides insights into the variables that should be considered when assessing sweet taste sensitivity, discusses the mechanisms underlying differences in sweet taste, and highlights the need for further research in the field of personalized nutrition.

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COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

Comprehensive REVIEWS in Food Science and Food Safety WILEY

Effect of physiological factors, pathologies, and acquired habits on the sweet taste threshold: A systematic review and meta-analysis

Marta Trius-Soler[1,2,3,*] | **Dimitri A. Santillán-Alarcón**[1,*] | **Miriam Martínez-Huélamo**[1,2] | **Rosa M. Lamuela-Raventós**[1,2,3] | **Juan José Moreno**[1,2,3]

[1] Department of Nutrition, Food Sciences and Gastronomy, School of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain

[2] INSA-UB, Nutrition and Food Safety Research InstituteUniversity of Barcelona, Santa Coloma de Gramanet, Spain

[3] CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain

Correspondence
Juan José Moreno, Department of Nutrition, Food Sciences and Gastronomy, School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain.
Email: jjmoreno@ub.edu

*Both authors equally contributed.

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Abstract

Sweet taste perception is a key factor in the establishment of the food pattern with nonstatic thresholds. Indeed, taste sensitivity can be influenced by physiological factors (age and sex), pathologies (obesity and type 2 diabetes mellitus), and acquired habits (tobacco and alcohol consumption). In order to elucidate how these variables influence the sucrose detection threshold (DT) and recognition threshold (RT), a systematic review and meta-analysis of the relevant literature were performed. After a comprehensive search in the PubMed and Scopus databases, a total of 48 studies were qualitatively considered, and 44 were meta-analyzed. The factors of aging (standard mean difference [SMD]: −0.46; 95% confidence interval (CI), −0.74 to −0.19; I^2: 73%; Tau^2: 0.18) and type 2 diabetes mellitus (SMD: 0.30; 95% CI, 0.06 to 0.55; I^2: 0%; Tau^2: 0.00) were found to significantly increase the sucrose RT, whereas the DT only increased in subjects with a higher body mass index (SMD: 0.58; 95% CI, 0.35 to 0.82; I^2: 0%; Tau^2: 0.00). No effects of sex and tobacco smoking were found, and associations with alcohol consumption could not be assessed, as it was included as a variable in only one study. Feasible mechanisms underlying changes in sucrose thresholds include the modulation of hormones involved in energy and body weight homeostasis, taste bud abundance, taste brain signaling, and the gut–brain axis. The present work provides insights into the variables that should be considered when

Nomenclature: AFC, alternative forced choice; BMI, body mass index; CI, confidence interval; DB, Downs and Black; DT, detection threshold; GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like peptide 2; GLUT2, glucose transporter 2; GRADE, Grading of Recommendations Assessment, Development and Evaluation; IV, inverse variance; LAGB, laparoscopic adjustable gastric banding; LSG, laparoscopic sleeve gastrectomy; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis; PROP, 6-n-propylthiouracil; PTC, phenylthiocarbamide; RA, restrictive anorexia; RT, recognition threshold; RYGB, Roux-en-Y gastric bypass; SARS-COV-2, Severe Acute Respiratory Syndrome Coronavirus 2; SD, standard deviation; SE, standard error; SG, sleeve gastrectomy; SGLT1, Na+/glucose cotransporter 1; SMD, standard mean difference; SNP, single-nucleotide polymorphism; T1R2 or TAS1R2, taste 1 receptor member 2; T1R3 or TAS1R3, taste 1 receptor member 3; T2DM, type 2 diabetes mellitus; T2R105, taste 2 receptornonbreakingspacemember 105; TR, taste receptor.

assessing sweet taste sensitivity, discusses the mechanisms underlying differences in sweet taste, and highlights the need for further research in the field of personalized nutrition.

KEYWORDS
chemoperception, detection threshold, personalized nutrition, recognition threshold, sucrose

1 | INTRODUCTION

Chemosensory perception (taste, smell, and chemesthesis) is essential for individual and species survival (Hawkes, 2001). The human sense of taste, which is limited to the oral cavity and mainly the tongue, is capable of identifying a wide variety of tastes (Smith & Margolskee, 2001). The two taste receptors (TRs) that are responsible for sweet taste stimulus detection and ligand selectivity, taste 1 receptor member 2 (T1R2) and taste 1 receptor member 3 (T1R3), belong to the G protein-coupled receptor family (Adler et al., 2000; Hoon et al., 1999; Matsunami, Montmayeur, & Buck, 2000). Sweetness response is triggered in the T1R2/T1R3 heterodimer (Nelson et al., 2001) and sucrose appears to bind to the Venus flytrap domain of T1R2/T1R3 (Chandrashekar, Hoon, Ryba, & Zuker, 2006). Sweet taste allows the identification of high-energy nutrients and, in general terms, indicates the presence of soluble carbohydrates. Nevertheless, a wide diversity of noncarbohydrate molecules, such as D-amino acids (e.g., D-phenylalanine, D-alanine, and D-serine) (Chandrashekar et al., 2006) and sweet testing proteins (e.g., monellin, thaumatin, curcullin, and brazzein), or noncaloric molecules such as artificial sweeteners (e.g., saccharine, sucralose, and aspartame) (Jiang et al., 2005) are also sweet as a consequence of interaction with T1R2 and T1R3 (Gamble, 2017; Lindemann, 2001).

The minimum concentration of a taste agent in an aqueous solution at which the stimulus solution can be distinguished from distilled water is referred to as the detection threshold (DT), whereas the lowest concentration that elicits the characteristic of taste is the recognition threshold (RT). These definitions were initially established for salty taste thresholds (Richter & MacLean, 1939) and were then generalized to all taste stimuli (O'Mahony, Hobson, Garvey, Davies, & Birt, 1976). Although this systematic review and meta-analysis has only been focused on DT and RT measurements, other parameters are commonly used to define human sensory perception. The measure of the perceived intensity of a concentration above the RT is known as suprathreshold intensity perception (Weiffenbach, Fox, & Baum, 1986). The differential threshold is defined as the minimum stimulus concentration by which taste intensity must be changed in order to produce a significant change in sensory experience (Galindo-Cuspinera et al., 2009), whereas the intensity of a stimulus from which its acceptance is altered, based on the transition point between sensory acceptance and rejection, refers to the rejection threshold (Lima Filho, Minim, Silva, Della Lucia, & Minim, 2015).

Chemical and physical methods, such as three alternative forced choice (3AFC) and electrogustometry (EGM), respectively, have been proposed for threshold determination, although taste tests based upon chemical substances is the preferred method for assessing sweet taste thresholds (Snyder, Prescott, & Bartoshuk, 2006). In a chemical taste test method, different tastant solutions are presented and participants must determine if taste is perceived or not, or even describe its taste quality.

Taste has the additional value of contributing to the overall pleasure and enjoyment of a meal (Chandrashekar et al., 2006). Moreover, sweet taste perception is an important phylogenetically preserved biological function (Kim, Wooding, Riaz, Jorde, & Drayna, 2006). In the context of genetics, health, and pathology, several variables that may affect the sweet taste and its perception have been described. They include T1R polymorphisms (Kim et al., 2006), age, sex, body mass index (BMI), smoking, consumption of alcohol, surgical interventions (Wasalathanthri, Hettiarachchi, & Prathapan, 2014), acute and chronic diseases such as otitis (Shin, Park, Kwon, & Yeo, 2011), cancer therapies with concomitant weight loss (Bolze, Fosmire, Stryker, Chung, & Flipse, 1982), chronic renal failure (Vreman, Venter, Leegwater, Oliver, & Weiner, 1980), and more recently, severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) infection (Lechien et al., 2020). Therefore, in the current study, we hypothesize that the sweet threshold in humans is not static, and it is influenced by physiological factors, pathologies, and acquired habits. A systematic review and meta-analysis on the influence of the usual descriptive physiological variables (e.g., age or sex), metabolic pathologies with the highest prevalence (e.g., obesity and type 2 diabetes mellitus (T2DM; Blüher, 2019; Glovaci, Fan, & Wong, 2019), and lifestyle habits most commonly described as perception modifiers (e.g., alcohol drinking and smoking habits; Da Ré et al., 2018; Silva et al., 2016) in sucrose DT and RT were performed, and the extent of the threshold differences was

discussed, with the aim of providing new evidence on this subject.

2 | MATERIALS AND METHODS

2.1 | Data sources and research method

Scientific literature was collected from the PubMed and Scopus databases (from the beginning of the database until July 2020). The search terms used were (sweet taste OR threshold) AND (T1R2 OR TAS1R2 OR T1R3 OR TAS1R3 OR sucrose). The search was restricted to the English language. In the PubMed database, the humans filter was used. In addition, manually selected reference articles and reviews were included. This work was conducted according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement (Supporting Information Table S1).

2.2 | Inclusion and exclusion criteria

The study selection was performed independently by two authors (M.T-S. and D.A.S-A.). Full-text articles were selected according to the following inclusion criteria: (1) original studies; (2) studies reporting the measurement of sucrose DT and/or RT by a chemical taste test; (3) studies including and comparing at least two groups of the variables studied (age, sex, BMI, T2DM, tobacco, or alcohol consumption); and (4) outcomes containing the mean sucrose threshold of the group with its respective measure of dispersion (95% confidence interval [CI], standard deviation [SD] or standard error [SE] or exact *P* value for group comparison). The exclusion criteria were (1) duplicated studies; (2) in vitro or animal studies; (3) ecological studies, editorials, reviews, and meta-analyses; and (4) thresholds assessed by a method other than the chemical taste test.

2.3 | Data extraction and management

Discrepancies in data information from selected papers were discussed by M.T-S. and D.A.S-A. If no consensus was reached, J.J.M. was consulted. For each study, the extracted variable was classified as DT or RT. The data for each study included in this systematic review and meta-analyses were the following: (1) author, year, and country of the study; (2) Downs and Black (DB) score (quality assessment); (3) outcome (DT and RT); (4) population sample tested; (5) sample size; (6) taste test and conditions of data collection; (7) sucrose range and number of solutions; and (8) key findings regarding the variable evaluated.

2.4 | Study quality assessment

The quality of each study was independently checked and discussed by M.T-S. and D.A.S-A. Any controversy regarding inclusion, data extraction, and/or quality assessment was resolved with the support of a third person (J.J.M.). To evaluate the risk of bias in individual studies, two validated scales were used: the DB score (Downs & Black, 1998) and the Cochrane risk of bias scale (Higgins et al., 2011).

The checklist of the DB scoring system, which is appropriate for assessing both randomized and nonrandomized studies of health care interventions, comprises 26 questions to evaluate reporting, external validity, and internal validity (bias and confounding). For the present work, five questions (questions 8, 17, 19, 23, and 24) were omitted because most sensory studies do not consider the study characteristics related to these questions. Finally, 21 questions from the DB checklist were used to evaluate the quality of the studies selected. The last question, concerning statistical power, was adapted to: "Did the authors of the study provide any information concerning a sample size calculation? Yes/No" (Downs & Black, 1998).

In the second risk of bias assessment, the Cochrane scale was used, including all the categories except one, as established by another study in the field (Tucker et al., 2017), in order to adapt the scale to the study design. Five domains (selection, performance, attrition, reporting, and other) were judged as having a high, low, or unclear risk of bias.

2.5 | Evidence quality assessment

The GRADE (Grading of Recommendations Assessment, Development, and Evaluation) scale was used to evaluate the overall strength of evidence for each outcome (Ryan & Hill, 2019). Starting with low evidence for the nonrandomized control trial design of studies included in this meta-analysis, outcomes were downgraded or upgraded depending on the GRADE criteria system.

2.6 | Statistical analysis

Before analyses, studies were classified by variable (e.g., age) and type of outcome (DT or RT). Each meta-analysis was performed by pooling the standard mean difference (SMD) derived from the difference in mean outcome between groups divided by the SD of outcomes among participants. Heterogeneity within studies was evaluated by the I^2 test, Tau^2, and 95% prediction intervals. Subgroup analyses were used to study heterogeneity in age, sex, and BMI variables. A random-effects model was used because of the nature of the studies, where the differences between populations or assessment of outcome may

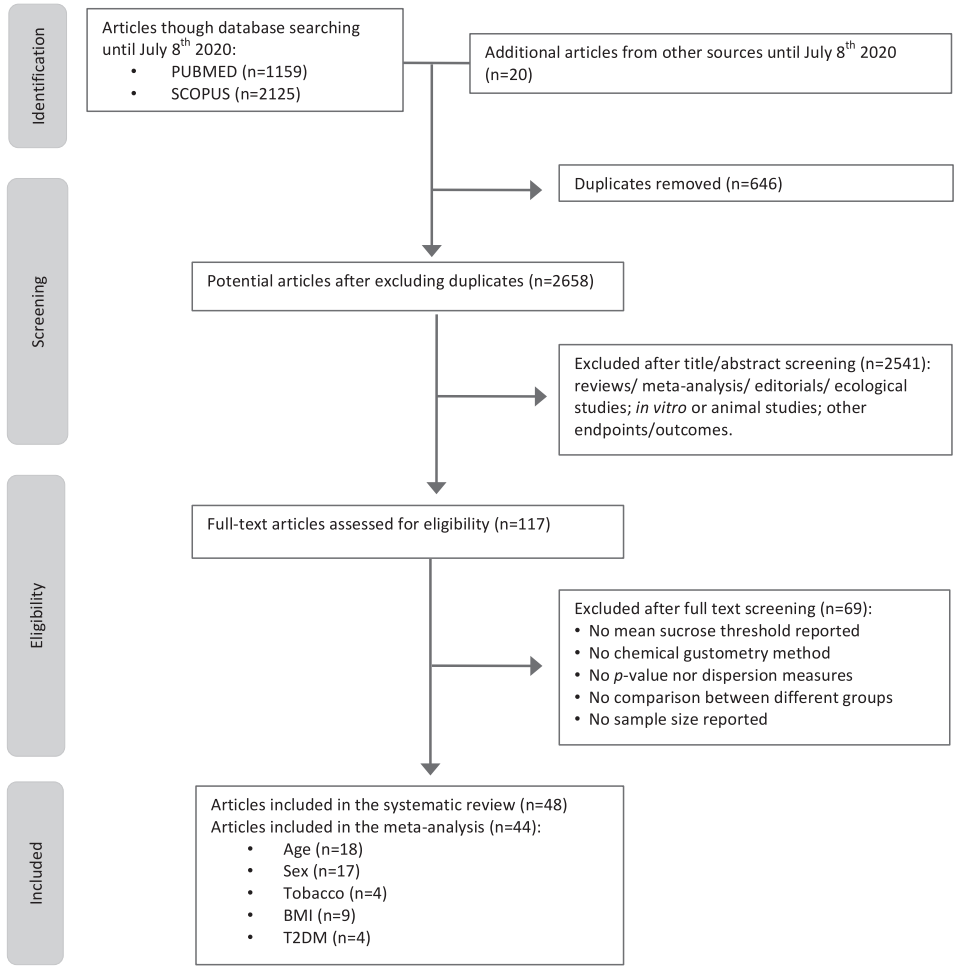

FIGURE 1 Flow chart of selected studies for the systematic review and meta-analyses

introduce variation between studies (Bouras, Tsilidis, Pounis, & Haidich, 2019). Meta-analyses and forest plots were performed with Review Manager (RevMan) Version 5.3 (The Cochrane Collaboration, 2014).

3 | RESULTS

3.1 | Literature search and study characteristics

Figure 1 depicts a flow diagram for article selection. A total of 3,284 articles from the two databases analyzed were identified, and 20 articles were included from other sources (manual searching and reviews). After removing duplicates, 2,658 papers were potentially eligible, whereas 2,541 studies were excluded based on inclusion and exclusion criteria after title and abstract screening. Thus, 117 articles were examined in detail, and, finally, 48 papers were included for the qualitative review and 44 of those were also included in the quantitative meta-analysis.

Based on the literature search and discussion, the variables considered for the present study were age, sex, tobacco smoking habit, alcohol consumption, BMI, and T2DM. Other settings, such as pathologies, including cancer and radiation treatment (Sandow, Hejrat-Yazdi, &

Heft, 2006), neurological diseases (e.g., Parkinson's and Alzheimer's disease; Sakai, Ikeda, Kazui, Shigenobu, & Nishikawa, 2016; Tarakad & Jankovic, 2017), otitis media (Snyder & Bartoshuk, 2016), or depression (Nagai, Matsumoto, Endo, Sakamoto, & Wada, 2015), among others, influence sweet taste thresholds, but fall outside of the scope of the present study, which is limited to sensory analysis with variables commonly controlled in nutrition and metabolism studies.

3.2 | Qualitative review: Thresholds and factors

The qualitative review of the studies included is summarized in Supporting Information Tables S2 to S7. Only four studies were included in the qualitative review (Eiber, Berlin, De Brettes, Foulon, & Guelfi, 2002; Nagai et al., 2015; Park et al., 2015; Than, Delay, & Maier, 1994). Although these studies fit the inclusion criteria, the comparison of their study subgroups could not be matched with the others (Eiber et al., 2002; Nagai et al., 2015; Than et al., 1994) or the outcome measure and its dispersion was only reported graphically (Park et al., 2015).

3.3 | Quantitative review: Thresholds and factors

3.3.1 | Age

Eighteen studies involving 1,450 participants were included in the meta-analyses. The data obtained allowed DT and RT to be divided by sex groups, creating subgroups for females, males, and both sexes.

In the case of DT (Figure 2a), eight comparisons found significantly higher sucrose thresholds in older versus younger participants (Bales, Steinman, Freeland-Graves, Stone, & Young, 1986; Da Silva et al., 2014; Fukunaga, Uematsu, & Sugimoto, 2005; Kennedy, Law, Methven, Mottram, & Gosney, 2010; Mojet, 2001; Moore, Nielsen, & Mistretta, 1982; Spitzer, 1988; Yamauchi, Endo, & Yoshimura, 2002b), two described the opposite (James, Laing, & Oram, 1997; Stevens, 1996), and five reported no significant differences (James et al., 1997; Mojet, 2001; Mojet, Christ-Hazelhof, & Heidema, 2005; Wardwell, Chapman-Novakofski, & Brewer, 2009; Wiriyawattana, Suwonsichon, & Suwonsichon, 2018). However, based on the overall effect, differences in DT between age groups were not significant.

The RT (Figure 2b) was significantly higher among older people (SMD: −0.46; 95% CI, −0.74 to −0.19; I^2: 73%; Tau^2: 0.18). This outcome was supported by the results of eight studies that reported a significant direct relation between aging and sucrose RT (Dye & Koziatek, 1981; Easterby-Smith, Besford, & Heath, 1994; Fukunaga et al., 2005; Kennedy et al., 2010; Richter & MacLean, 1939; Wardwell et al., 2009; Wiriyawattana et al., 2018; Yamauchi et al., 2002b). In fact, only one study reported the contrary (Wayler, Perlmuter, Cardello, Jones, & Chauncey, 1990) and another did not find any significant result (Kalantari, Kalantari, & Hashemipour, 2017). Although the result is significant, the prediction interval of the meta-analysis is expected to be nonsignificant in around 95% of the population (Supporting Information Table S8).

3.3.2 | Sex

Figure 3 summarizes the effect of sex on sweet thresholds. Seventeen studies including a total of 2,347 participants were meta-analyzed, including 15 articles on the DT and nine on the RT. Subgroups were defined according to age, in which participants under 18 years were classified as "children," those aged 18 to 60 were "young adults" and "older adults" were over 60 years old. The age of 60 was used as the cutoff for older adults because of age-related losses and health conditions (de Carvalho, Epping-Jordan, & Beard, 2019).

No difference was found in the DT between males and females in the children subgroup, based on only four studies (Fogel & Blissett, 2019; James et al., 1997; Joseph, Reed, & Mennella, 2016; Yamauchi et al., 2002b). The DT was significantly higher in adult males in one study (Da Silva et al., 2014), whereas another study reported the opposite (Wardwell et al., 2009). Regarding the RT results, a significantly higher RT was found in males in three studies (Hong et al., 2005; Sanematsu, Nakamura, Nomura, Shigemura, & Ninomiya, 2018; Wardwell et al., 2009). The results of the study by Yamauchi et al. (2002b) differed among study subgroups, and other previously unmentioned studies did not report any significant findings (Chang, Chung, Kim, Chung, & Kho, 2006; Fogel & Blissett, 2019; Horio & Kawamura, 1990; Hwang et al., 2018; Kalantari et al., 2017; Kunka, Doty, & Settle, 1981; Mojet, 2001; Vreman et al., 1980; Yamauchi, Endo, Sakai, & Yoshimura, 2002a). To sum up, neither total nor age subgroups showed significant differences in DT and RT between sexes (Figure 3a and 3b).

3.3.3 | Tobacco consumption

A meta-analysis of both sucrose DT and RT was performed with the results of four studies including 645 participants. One of the studies divided the comparisons

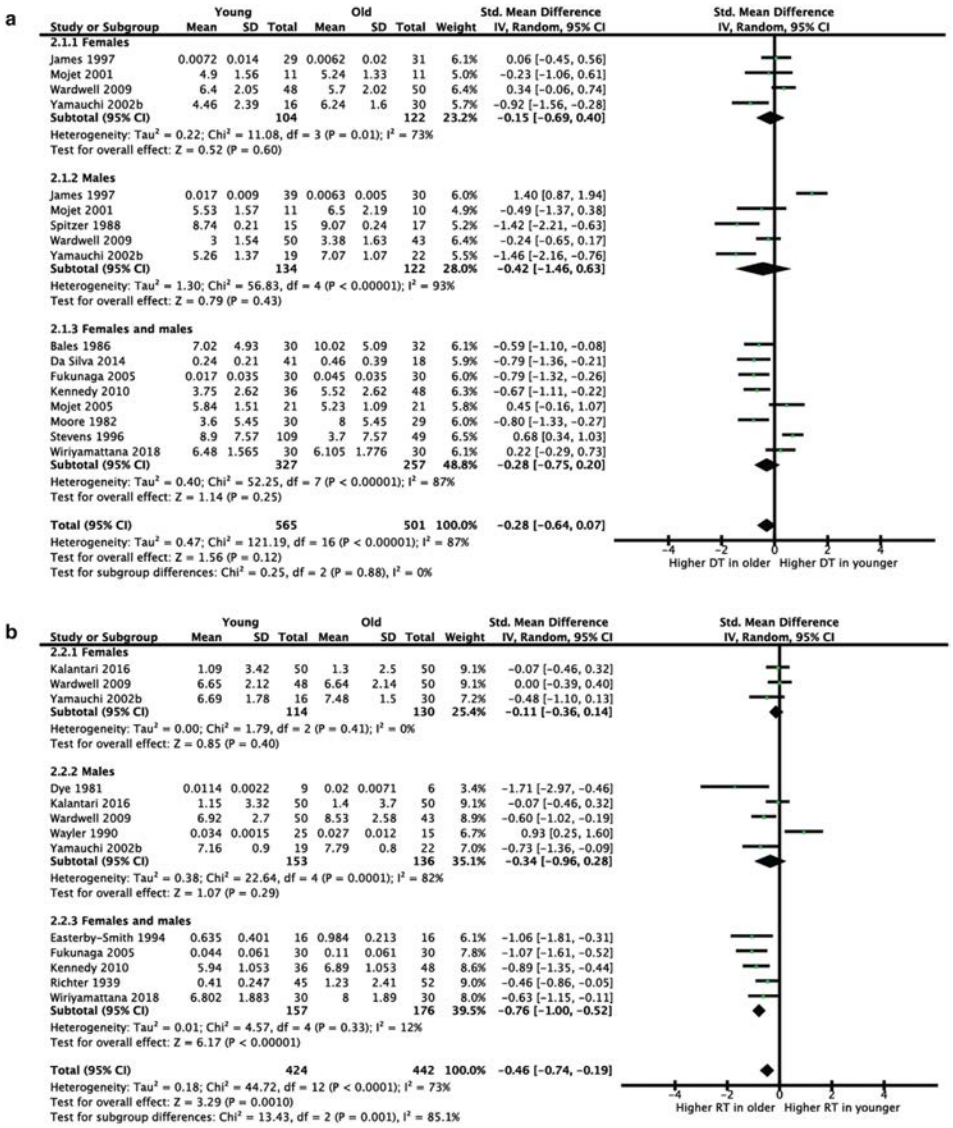

FIGURE 2 Forest plot of studies investigating the association between age and sucrose taste thresholds. SMD and 95% CI from the random model. (A) DT (B) RT. F: females; IV: inverse variance; M: males; SD: standard deviation. *Comparisons between higher versus lower. >18 years old noninstitutionalized age group categories were made when more than two study groups were available

into age groups (Yamauchi et al., 2002b). For the DT outcome (Figure 4a), 449 participants from two different studies were included in the meta-analysis. The study reporting single data found a significantly higher DT in 21- to 40-year-old women smokers (Pepino & Mennella, 2007), whereas DT differences between smokers and nonsmokers increased in parallel with age among the age subgroups (Yamauchi et al., 2002b). Nevertheless, no

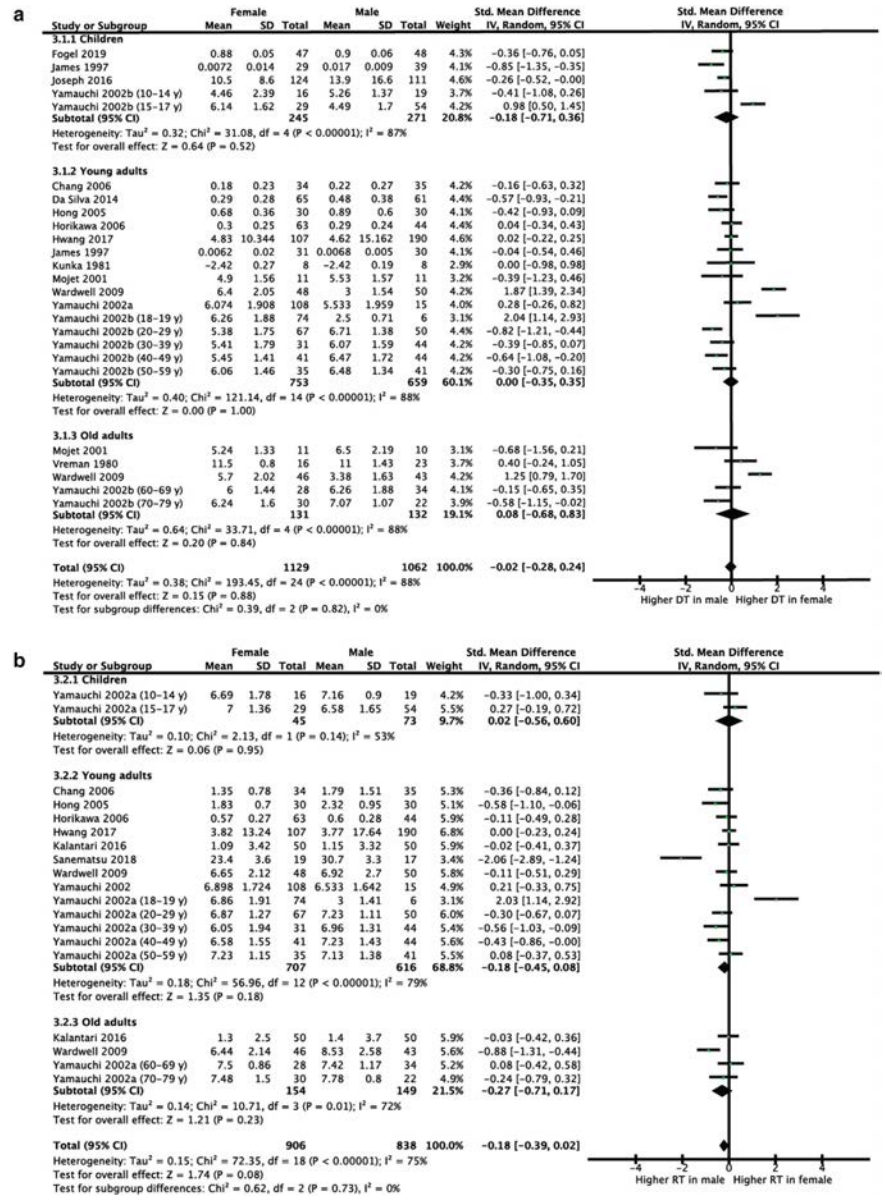

FIGURE 3 Forest plot of studies investigating the association between sex and sucrose taste thresholds. SMD and 95% CI from the random model. (A) DT and (B) RT. IV: inverse variance; SD: standard deviation; y: years old

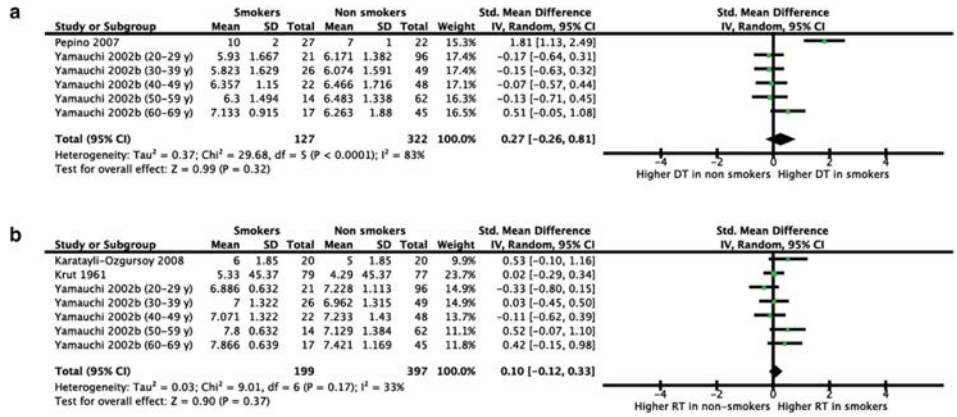

FIGURE 4 Forest plot of studies investigating the association between smoking and sucrose taste thresholds. SMD and 95% CI from random model. (A) DT and (B) RT. IV: inverse variance; SD: standard deviation; y: years old

significant differences were observed in the global result. The RT meta-analysis (Figure 4b) included three studies involving 596 participants (Karatayli-Ozgursoy, Ozgursoy, Muz, Kesici, & Akiner, 2009; Krut, Perrin, & Bronte-Stewart, 1961; Yamauchi et al., 2002b), and similarly, no significant differences were observed between groups.

3.3.4 | Alcohol intake

A meta-analysis could not be carried out as only one study reporting chemically assessed thresholds in alcohol drinkers and nondrinkers was found. Furthermore, no significant differences between groups were reported (Than et al., 1994).

3.3.5 | BMI

Nine studies involving 343 participants were included in this meta-analysis. The BMI was used as an indicator of the degree of obesity. Although waist circumference and the waist-to-height ratio are better predictors of obesity (Bosello, Donataccio, & Cuzzolaro, 2016), the BMI was more extensively determined. Participants of the articles included were candidates for bariatric surgery, patients with metabolic syndrome, or were even described as obese in the original article.

Regarding the DT (Figure 5a), subgroup analyses were defined according to weight loss after nonsurgical intervention (Umabiki et al., 2010), bariatric surgery (Abdeen, Miras, Alqhatani, & le Roux, 2018; Bueter et al., 2011; Nance, Eagon, Klein, & Pepino, 2017; Nishihara et al., 2019; Pepino et al., 2014), or by two parallel comparison groups (Bueter et al., 2011). In the studies in which the variable studied was weight loss, the participants constituted their own comparative group. Two studies revealed a significantly higher threshold in subjects with a higher BMI (Umabiki et al., 2010), whereas the remaining studies did not report any significant differences. Nonetheless, the overall outcome was that the sucrose DT increased with the BMI (SMD: 0.58; 95% CI, 0.35 to 0.82; I^2: 0%; Tau^2: 0.00). Indeed, the true size effect in about 95% of the population is predicted to range from 0.30 to 0.86 and thus remains significant (Supporting Information Table S8).

Although two studies described a significantly higher RT in subjects with a lower BMI (Hardikar, Höchenberger, Villringer, & Ohla, 2017; Pasquet, Frelut, Simmen, Hladik, & Monneuse, 2007) and one did not observe any significant difference (Green, Jacobson, Haase, & Murphy, 2015), the total effect indicated no significant differences in sucrose RT among BMI groups (Figure 5b).

3.3.6 | T2DM

The outcomes of the four studies, including 263 participants, allowed comparison of sucrose RT (Figure 6). Although only one (De Carli et al., 2018) of the four studies (De Carli et al., 2018; Dye & Koziatek, 1981; Wasalathanthri et al., 2014; Yazla et al., 2018) reported a significant difference between groups, the global effect showed that patients with T2DM have a significantly higher RT than nondiabetic subjects (SMD 0.30; 95% CI, 0.06 to 0.55; I^2: 0%; Tau^2: 0.00). However, the prediction interval is expected not to be significant in about 95% of the whole population (Supporting Information Table S8).

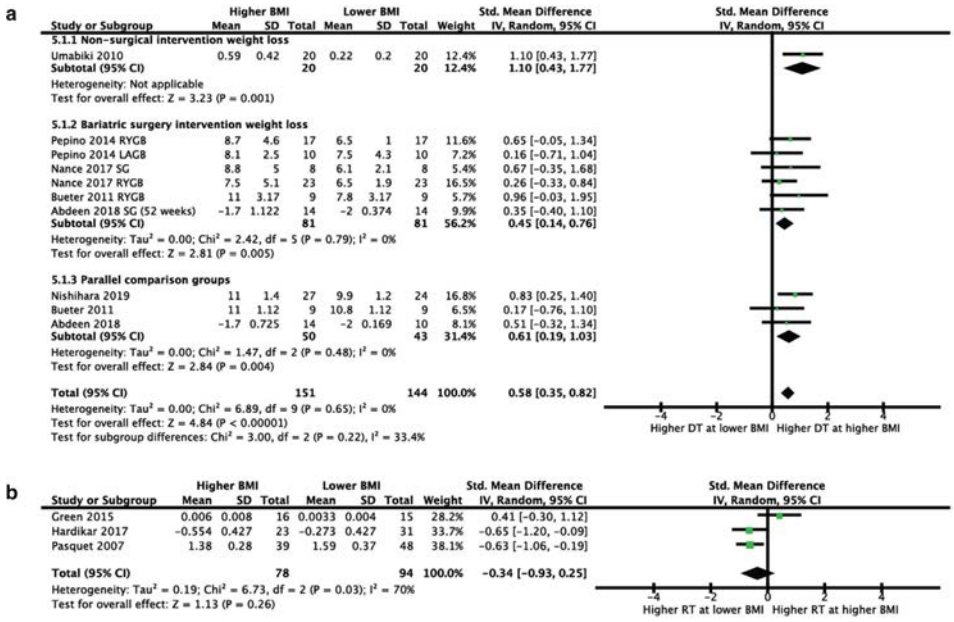

FIGURE 5 Forest plot of studies investigating the association between BMI and sucrose taste thresholds. SMD and 95% CI from random model. (A) DT and (B) RT. IV: inverse variance; LAGB: laparoscopic adjustable gastric band; RYGB: Roux-en-Y gastric bypass; SD: standard deviation; SG: sleeve gastrectomy

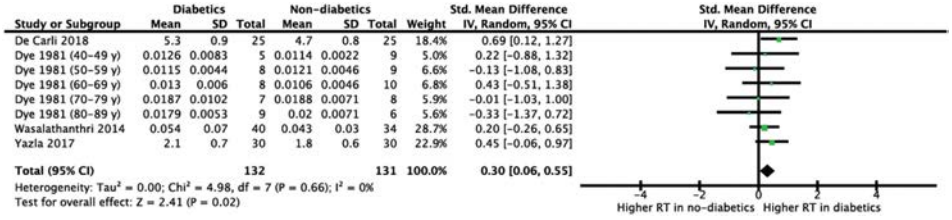

FIGURE 6 Forest plot of studies investigating the association between type II diabetes mellitus and sucrose taste RT. SMD and 95% CI from the random model. IV: inverse variance; SD: standard deviation; y: years old

3.4 | Study quality and overall strength of evidence

According to the DB scoring system, the quality of the individual studies ranged from 11 to 22 points out of a total possible score of 22. Many studies failed to blind the persons measuring the main outcomes and did not describe the staff, places, and facilities where the patients were treated. Information about a sample size calculation was also missing in most of the studies included. Additionally, taste-testing studies are at risk of bias due to nonrandom subject selection and the inability to blind researchers and participants to the purpose of the study.

The risk of detection bias was high due to the characteristics of the sensory studies, in which the investigator usually knows the concentrations of the stimuli tested. On the contrary, attrition and reporting biases were low, whereas a few articles were judged to be highly biased in terms of selection, performance, and other aspects. Figures showing the risk of bias according to the Cochrane scale are provided in Supporting Information Figures S1 and S2.

Few studies including tobacco smoking, alcohol consumption, and T2DM outcomes were identified. Due to the low number of studies quantified in some analyses, high or moderate heterogeneity was observed in most of the meta-analyses performed. The risk of bias or indirectness was not detected. Thus, according to the GRADE scale, the evidence available for the association between the modifiable and nonmodifiable factors and the sucrose thresholds is of low certainty.

4 | DISCUSSION

4.1 | Principal findings

Sweet taste thresholds are a measure related to the first contact of high-energy nutrients with the subject's metabolism, and their assessment may be important within nutritional and general health settings. Our findings indicating that older people have a higher sucrose RT are in line with a previous meta-analysis that addressed the effect of age on thresholds of five tastes (Methven, Allen, Withers, & Gosney, 2012). Some determinants hypothesized to explain taste loss in the elderly are internal factors, such as a diminishing number of taste buds, shorter lifespan of sensorial cells, and lower hyposalivation flow rate, or external factors, such as smoking, pharmaceutical or denture use, dietary habits, and difficulties in maintaining oral health (Sergi, Bano, Pizzato, Veronese, & Manzato, 2017; Wiriyawattana et al., 2018).

The effect of age on taste has also been investigated in animal models. The mRNA expression of the bitter taste 2 receptor 105 (T2R105) and gustducin significantly decreased with aging in mice, although other molecules tested for other tastes did not show significant changes in expression (Narukawa, Kamiyoshihara, Kawae, Kohta, & Misaka, 2018). This situation may be relevant when multiple taste stimuli are presented together, with the expression of a TR being important in taste–taste interactions (Keast & Breslin, 2003; Mojet, Heidema, & Christ-Hazelhof, 2004). In addition, no significant differences in the turnover rates of taste bud cells were observed between older versus younger experimental groups (Narukawa et al., 2018). Similarly, the number of taste buds, in old and young monkeys, has been reported as not being significantly different (Bradley, Stedman, & Mistretta, 1985). These experimental results suggest that the changes in taste thresholds due to aging are caused by factors other than degenerative changes in lingual taste buds, such as aging-related changes in serum components or alterations in neural mechanisms (Bradley et al., 1985; Narukawa et al., 2018).

Anatomical differences of the gustatory system between sexes have also been found, with women having more fungiform papillae and more taste buds than men (Chang et al., 2006; Hong et al., 2005; Hwang et al., 2018). Notably, in a previous article, estrogens seemed to reduce the attraction of sucrose for rats, but only at low concentrations (Curtis, Stratford, & Contreras, 2005). In addition, brain responses to sweet stimuli do not differ under low (ovariectomized animals), moderate (diestrous), or high estrogen (pregnancy animals) circulating conditions, suggesting that female sex hormones have organized but not activated sweet gustatory processing (Di Lorenzo & Monroe, 1989). On the other hand, lower thresholds have been observed among women in the preovulation phase of the menstrual cycle (Than et al., 1994) and the effects of hormonal changes during menopause such as mucosal dryness, a burning sensation, and taste disorders have also been described (Kalantari et al., 2017). However, the sex factor is not associated with differences in sucrose taste thresholds. This result is in agreement with a recent mini-review by Martin and Sollars (2017). More studies assessing the effect of menstrual cycle on sweet taste thresholds are required to understand the implications of female sex hormones in sensory perception.

The effect of tobacco consumption on taste threshold changes has been studied, and it has been reported that there smoking may have a slight influence (Da Ré et al., 2018). It is thought that nicotine may alter the perception of quinine hydrochloride, a molecule commonly used as a bitter tastant (Krut et al., 1961), indicating that bitter taste is the taste type most likely affected (Chéruel, Jarlier, & Sancho-Garnier, 2017). A lower sensitivity in smokers might be due to poorer oral hygiene with a concomitant increased risk of periodontal diseases and whole mouth complaints (Taybos, 2003). Other nicotine-associated mechanisms have been described, such as the inhibition of neurons in the nucleus of the salivary tract and alterations in serotonin and consequent modulation of cellular responses of TRs. However, one study concluded that a higher sucrose DT in smokers is related to the smoking dose in packs per year rather than acute exposure to nicotine. Accordingly, the greater the dose, the lower is the sucrose sensitivity. Moreover, this study demonstrated that the cigarette dose in pack-years was the variable that best predicts the sucrose threshold in current smokers, more than the current age or the age at which regular smoking began (Pepino & Mennella, 2007). Nevertheless, our results suggest that sucrose thresholds do not differ between tobacco smokers and nonsmokers. Nevertheless, further investigation is needed, due to the lack of evidence. In fact, the study reporting significant results showed a higher score in the quality assessment, using the most

robust taste test method and a more homogeneous sample (Pepino & Mennella, 2007).

Interestingly, it has been suggested that taste is the primary signal for ethanol detection in a beverage (Mattes & DiMeglio, 2001). It is of note that sugar alcohols elicit sweet taste through T1R2/T1R3 activation (Feeney, O'Brien, Scannell, Markey, & Gibney, 2011). Consequently, a strong relation between alcohol beverages and the threshold index for sweet taste has been described (Silva et al., 2016).

Zinc is a component of gustin, a protein present only in the parotid saliva in humans (Silva et al., 2016). One underlying explanation for lower sweet sensitivity may be zinc deficiency caused by the excessive consumption of alcohol and subsequent atrophy of the taste buds, which leads to dysgeusia, glossodynia, and hypogeusia (Cerchiari et al., 2006). Moreover, a deterioration in taste discernment has been described in drinkers in comparison with nondrinkers, using different methods, such as EGM and chemical taste responses (Lelièvre, Le Floch, Perlemuter, & Peynègre, 1989). Different protein salivary concentrations have also been proposed as a contributor factor (Silva et al., 2016).

No statistical differences were reported with alcohol intake and sucrose RT in the study included, but other studies have described that the consumption of alcohol over a long period might negatively affect the perception of sweetness (Silva et al., 2016). Indeed, Silva et al. (2016) concluded that alcohol intake may lead to increased consumption of sweetened substances, thereby affecting the nutritional status and even contributing to thiamine deficiency and T2DM. A sensory preference for sweet taste has also been linked to alcoholism and is considered as a risk factor (Mennella, Pepino, Lehmann-Castor, & Yourshaw, 2010; Silva et al., 2016). These discrepancies between studies might be due to the lack of connection between absolute taste thresholds (DT/RT) and sensory perception up to the suprathreshold concentrations of alcoholic beverages. More research is needed about the relationship between DT/RT and alcohol consumption, in order to obtain conclusive results.

Several studies have evaluated the effect of weight, BMI, body fat mass, or obesity status on sucrose taste thresholds (Abdeen et al., 2018; Bueter et al., 2011; Nance et al., 2017; Pepino et al., 2014; Umabiki et al., 2010), including subjects with diseases such as anorexia and bulimia (Eiber et al., 2002). Studies on waist circumference, a strong predictor of obesity, and taste sensitivity have also been performed (Ileri-Gurel, Pehlivanoglu, & Dogan, 2013; Low, Lacy, McBride, & Keast, 2016, 2017). In fact, maltodextrin DT was not significantly correlated to BMI, whereas participants who were more sensitive to complex carbohydrates had a higher waist circumference (Low et al., 2017). Other studies did not find any association between sweet taste function and waist circumference (Low et al., 2016) or the waist-to-hip ratio (Ileri-Gurel et al., 2013). A recent study found an inverse association between taste intensity perception and body weight, as well as waist circumference, BMI, and obesity (Coltell et al., 2019).

Along the same line, impairment of taste sensation has been described in patients with T2DM, especially in relation to sweetness (Wasalathanthri et al., 2014). Higher taste thresholds have been associated with hyperglycemia (Bustos-Saldaña et al., 2009), with a significant correlation between the sweet taste threshold and the blood glucose concentrations, suggesting diminished sweet taste response in patients with T2DM (Gondivkar, Indurkar, Degwekar, & Bhowate, 2009). However, although a direct relationship has been reported between blood glucose levels and sweet taste thresholds, other older studies concluded the contrary (Chochinov, Ullyot, & Moorhouse, 1972; Perros, MacFarlane, Counsell, & Frier, 1996). In the euglycemia state, T1R2 expression in humans increased in both healthy and diabetic subjects after intraduodenal glucose infusion, whereas during hyperglycemia, lower T1R2 expression was observed in healthy controls, and in diabetics there were no variations (Young et al., 2009). More recently, one study performed in 2020 reported significant differences in the ability to recognize sweet taste between T2DM patients and healthy controls, independently of their sex, glycemic control, and time since diagnosis (Pugnaloni et al., 2020).

The results of this meta-analysis show that a higher BMI and T2DM are linked with a higher sucrose DT and RT, respectively. On the other hand, differences in sucrose RT between subjects with higher and lower BMI are not conclusive, possibly because of the low number of studies and their heterogeneity.

Feasible mechanisms underlying changes in the sucrose DT include the modulation of incretin secretion with anorexigenic and glucose-regulatory effects triggered by T1R2/T1R3 or a reduction in taste bud abundance, among others (Kaufman, Choo, Koh, & Dando, 2018; K. R. Smith et al., 2016). The T1R2/T1R3, which mediate sweet taste sensing in the tongue, are also expressed in the gut, pancreas, and adipose tissue, suggesting a physiological contribution to whole body nutrient sensing and metabolism (Smith et al., 2016). In the digestive tube, sugars act through α-gustducin on the T1R2/T1R3 of neuroendocrine K cells, which release glucagon-like peptides (GLP-1 and GLP-2) and the peptide tyrosine–tyrosine. They also act on L cells that release glucose-dependent insulinotropic polypeptide (Jang et al., 2007; Raka, Farr, Kelly, Stoianov, & Adeli, 2019), thereby regulating energy homeostasis. Notably, sucralose can also induce GLP-1 secretion (Margolskee et al., 2007), and together with saccharin and

stevia it can modify the microbiota of consumers (Ruiz-Ojeda, Plaza-Díaz, Sáez-Lara, & Gil, 2019), with these events being involved in obesity and T2DM (Górowska-Kowolik & Chobot, 2019). It should be noted that the regulation of the gut–brain neuroendocrine axis involves other molecules and receptors besides the activation of T1R2/T1R3. Indeed, satiety induced by protein intake could be a main contributor to weight maintenance due to the release of satiety hormones such as peptide tyrosine–tyrosine, cholecystokinin, and GLP-1 (Raka et al., 2019).

In addition, chronic low-grade inflammatory response associated with obesity was found to reduce the density of taste buds in gustatory tissues of mice (Kaufman et al., 2018), explaining taste dysfunction in obese populations. The results of a longitudinal human study demonstrated that human fungiform papillae, the structures housing taste buds, decrease in abundance with increasing adiposity (Kaufman, Kim, Noel, & Dando, 2020).

Another plausible mechanism described for a reduction in taste sensitivity in obesity has been the influence of diet-induced obesity on the reduction of responsiveness to sweet taste stimuli in the peripheral taste cells, and thus, changes in the central taste system (Maliphol, Garth, & Medler, 2013). Glucose sensors are present in the brain, and T1Rs expression is regulated by nutritional status (Calvo & Egan, 2015). In comparison, the levels of T1Rs expression in hypothalamus neurons of obese mice were lower than those in lean mice (Laubach, Pierce, Shuler, & Hopkins, 2009), whereas nutrient deprivation has been linked to increased T2Rs expression (Calvo & Egan, 2015).

Glucose absorption also seems to be controlled by gastrointestinal nutrient-sensing mechanisms involving the Na^+/glucose cotransporter-1 (SGLT1) and the glucose transporter 2 (GLUT2), which are the two main mediators of dietary glucose absorption at the apical membrane of enterocytes (Gorboulev et al., 2012). SGLT1 expression has been shown to be regulated by intestinal expression of T1R2/T1R3 in response to glucose delivery (Shirazi-Beechey, Daly, Al-Rammahi, Moran, & Bravo, 2014). When glucose is sensed by intestinal T1R2/T1R3, GLP-2 is secreted from L cells to mediate increased SGLT1 expression in adjacent enterocytes (Sangild et al., 2006; Shirazi-Beechey et al., 2014; Tsai, Hill, Asa, Brubaker, & Drucker, 1997). GLUT2 is also upregulated in the presence of luminal sugars or sweeteners, but not in knockout mice lacking T1R3 and α-gustducin (Mace, Affleck, Patel, & Kellett, 2007; Margolskee et al., 2007). Thus, gastrointestinal sweet sensing seems to be a critical regulator of SGLT1 and GLUT2 expression and glucose uptake (Mace et al., 2007; Margolskee, 2007; Raka et al., 2019).

Leptin, another molecule involved in satiety, seems to be related to threshold differences between normal versus overweight subjects. Leptin levels significantly decrease after weight loss in obese females, and may be associated with decreasing sweet taste thresholds (Umabiki et al., 2010). It has been shown that leptin receptors in taste cells respond to systemic leptin, causing a decrease in responsiveness to sweet stimuli without affecting responses to sour, salty, and bitter substances. This suggests that post-ingestion hormone release is capable of regulating the peripheral gustatory apparatus by modulating the responsiveness of sweet stimuli (Depoortere, 2014). Receptors of adiponectin, a metabolic hormone that mediates insulin sensitivity, adipocyte development, and fatty acid oxidation, have also been found to be expressed in T1Rs, suggesting that adiponectin signaling could also impact sweet signaling (Crosson et al., 2019).

4.2 | Strengths and limitations

The general search term criterion used constitutes one of the strengths of the present study, as it allowed the identification of a large number of relevant papers and minimized the exclusion of potentially eligible studies. The manual search for papers based on the bibliography of reviews and articles further reduced the possibility of missing studies. However, studies with significant data but with a main goal other than sweet taste threshold evaluation or in which the abstract did not refer to threshold assessment may have been omitted.

Chemical taste response was the only sucrose threshold assessment method considered. Although EGM is especially suitable for testing the integrity of the whole taste sensory chain, including ionotropic transduction mechanisms, it excludes metabotropic transduction mechanisms that rely on sweet, bitter, or umami taste (Chaudhari & Roper, 2010). Additionally, the characteristics of the taste agent aqueous matrix (e.g., viscosity or mineral content), as well as the amount of stimulus solution and the time between solution administration, together with other factors, differ among studies and may bias the outcome of threshold assessment (Gonázlez Viñas, Salvador, & Martin-Alvarez, 1998; Murphy, Cardello, & Brand, 1981; Stone & Oliver, 1966; Whelton, Dietrich, Burlingame, Schechs, & Duncan, 2007). Although standardized methods for chemical taste threshold assessment are available (e.g., British Standard ISO), their use is limited to a few studies. Despite the wide variety of assessing methods, SMDs were used to standardize the results of the studies into a uniform scale before meta-analysis. However, methodological differences may have a direct impact on the individual results.

One of the influencing factors analyzed was diabetes, but only studies on T2DM were included, thereby

excluding the possible effects of type 1 diabetes mellitus on sweet taste thresholds. Regarding the effect of age, the groups were not identical among studies, which may have influenced the results. In addition, the study by taste sensory analysis in any pathological condition has a higher potential bias related to the difference in the duration, control, and treatment of the pathology among the study samples of the studies meta-analyzed.

Moreover, as the scope of this work was limited to physiopathological conditions related to the field of nutrition, other reported modifiers of the sweet taste threshold, such as nonnutritional-related pathologies, were not considered.

Threshold values were used because they allow comparability among studies. Nevertheless, as thresholds do not provide information about sensory perception across the full dynamic range of sensation, it has been argued that suprathreshold scales provide a more realistic perspective of sensory function (Snyder et al., 2006). Although each sweetener has its own affinity to heterodimer T1R2/T1R3, a strong correlation has already been described between DT/RT and caloric sweeteners across people (Low, McBride, Lacy, & Keast, 2017). Thus, the authors believe that the result of this systematic review and meta-analysis should be the same even with glucose or other caloric sweeteners.

In conclusion, the present study provides significant findings, although the assessment of biases, absence of randomized clinical trials, the small sample size, and heterogeneity may have obscured more consistent relationships between sucrose thresholds and the variables analyzed.

4.3 | Implication for sensory analysis and clinicians

Some authors have reported that DT/RT has limited utility in the prediction of food behavior and hedonic liking, when most of the sweet foods are within the sweetness-intensity perception range (Jayasinghe et al., 2017; Low et al., 2016). Moreover, the use of sucrose concentrations above the threshold cannot reveal a direct relation between DT/RT and sweet taste intensity (Jayasinghe et al., 2017). Thus, the relation between DT/RT and intensity perception in suprathreshold concentrations and sweet dietary intake is still not clearly defined (Hardikar et al., 2017; Tan & Tucker, 2019). However, alimentary patterns and enhanced hedonic liking of sweetness at high concentrations may result in a higher consumption of sweet food (dos Santos, Marreiros, da Silva, de Oliveira, & Cruz, 2017; Duffy, Hayes, Sullivan, & Faghri, 2009). Additionally, a dose-dependent relationship has been described between suprathreshold intensity perception and hedonic liking (Jayasinghe et al., 2017). On the contrary, a recent study reported a significantly negative correlation between sucrose DT and suprathreshold sensitivity, whereas sucrose DT and sweet preference had a weak positive correlation, eliciting hedonic liking (Chamoun et al., 2019). More studies are needed to elucidate evidence in taste sensitivity and food preferences and eating behavior.

It is notable that the decline in taste sensitivity with age occurs to a greater extent with sour, salty, and bitter than with sweet flavors, indicating that sweet taste sensitivity is a robustly preserved function over the lifetime (Yoshinaka et al., 2015). Although the exact mechanisms by which taste sensitivity decreases with age are still unknown, its measurement is a useful tool in personalized nutrition. Knowledge on how to overcome alterations in taste senses could have implications in the health-related quality of life of elderly people and may also be useful in the new food industry. Moreover, the identification of sensory loss is important as a predictive factor for neurodegenerative diseases (e.g., Parkinson's and Alzheimer's disease) and other conditions (Da Silva et al., 2014).

Early studies suggested that the frequency of phenylthiocarbamide (PTC) and 6-*n*-propylthiouracil (PROP) tasters is higher in females than in males (Martin & Sollars, 2017; Prutkin et al., 2000) and among nonsmokers (Ye et al., 2011). As the PTC/PROP tasting mechanism has been linked to sweet liking (Kaczor-Urbanowicz et al., 2017; Yeomans, Tepper, Rietzschel, & Prescott, 2007), unequal distribution of this taste phenotype could be a potential explanation (Gondivkar et al., 2009) for different sweet-eating behaviors. Indeed, these differences between sexes could explain different dietary habits, as well as smoking behavior and alcohol consumption (Chang et al., 2006; Hong et al., 2005).

This meta-analysis demonstrates that a decrease in BMI after bariatric surgery or a behavioral intervention is associated with a reduction in sucrose DT. This result brings to light the idea that intraindividual changes in DT/RT during weight loss can be a potential consideration in the monitoring of obesity treatment. In addition, T2DM increases the sucrose RT, and this effect seems to increase among uncontrolled diabetic patients (Gondivkar et al., 2009; Yazla et al., 2018). To sum up, sucrose threshold measurement and its changes might be a marker of the severity of obesity and T2DM, independent of their influence or not in hedonic liking or dietary patterns, and a useful tool in personalized nutrition in the treatment of these disorders. However, to prove causality, prospective controlled studies need to be performed.

Although environmental factors have an influential role in sweet thresholds, T1R genes present multiple polymorphisms, which are thought to be associated with variations

in sweet taste perception (Kim et al., 2006; Tarragon & Moreno, 2018). Indeed, T1R2 is within the top 5% to 10% of all human genes with regard to the reported number of polymorphisms (Kim et al., 2006), and geographical and evolutionary differences in the distribution of genetic variants such as single-nucleotide polymorphism have been established (Yamauchi et al., 2002b). For example, T1R3 gene promoters rs307355 and rs3574481 explain about 16% of the variability in taste sensitivity and have different frequencies according to the population and geographical location (Fushan, Simons, Slack, Manichaikul, & Drayna, 2009).

5 | CONCLUSIONS

Chemosensory perception is not a static measurement due to environmental, physiological, and genetic factors. Indeed, our results suggest that aging and T2DM are factors that significantly increase the sucrose RT, whereas only subjects with a higher BMI have a higher DT. Sex and smoking showed no effect, whereas the effects of alcohol consumption or even alcohol abuse are still unknown.

Because TR may be involved in the release of orexigenic/anorexigenic and energy-metabolism-modulator molecules, further studies are required to relate sucrose thresholds with the levels of the hormones involved in energy homeostasis. Knowledge as to how the sweet DT and RT are affected by physiological and pathophysiological factors may be of interest when analyzing their roles in the pathogenesis of high prevalence pathologies such as obesity and T2DM, as has been recently reported with T2Rs (Tarragon & Moreno, 2020).

Although more research is needed, these results imply the appearance of a new way of optimizing the clinical practice of nutritionists and understanding the complexity of dietary practice and human beings.

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AUTHOR CONTRIBUTIONS

J. J. Moreno designed the study. M. Trius-Soler and D. A. Santillán-Alarcón collected data, performed statistical analysis, interpreted results, and drafted the manuscript. J. J. Moreno, M. Martínez-Huélamo, and R. M. Lamuela-Raventós interpreted the study results and performed the writing–review of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest in relation to this manuscript.

ORCID

Marta Trius-Soler https://orcid.org/0000-0003-2868-7142

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Publication 3

Inter-individual characteristics on basic taste recognition thresholds in a college-aged cohort: potential predictive factors

Marta Trius-Soler, Emily P. Laveriano-Santos, Clara Góngora, and Juan J. Moreno

Supplementary Material available in Annex 2. Submitted in Food & Function

Abstract

Aim: We investigated the possible influence of sociodemographic (sex and smoking habit) and clinical variables (dental cavities, missing teeth, sinusitis, rhinitis, body mass index, metabolic high prevalence family antecedent diseases) on tastant (sucrose, monosodium glutamate, sodium chloride, citric acid, quinine, sinigrin, phenylthiocarbamide) recognition thresholds (RTs).

Methods: RTs were determined using a same-different task approach in a college-aged cohort (n= 397). Predictive models for higher or lower tastant RTs were build.

Results: higher sucrose RT was found in females than in males, while sinusitis and rhinitis had a significant effect on sucrose and sodium chloride RTs. Smoking habit was not an important predictive factor of taste sensitivity, although its long effect on RTs remain unclear. Additionally, a positive correlation was found between all the tastant RTs studied.

Conclusions: Although the results did not show a clear pattern, the statistical approach employed should prove useful in future studies of predictors of taste sensitivity.

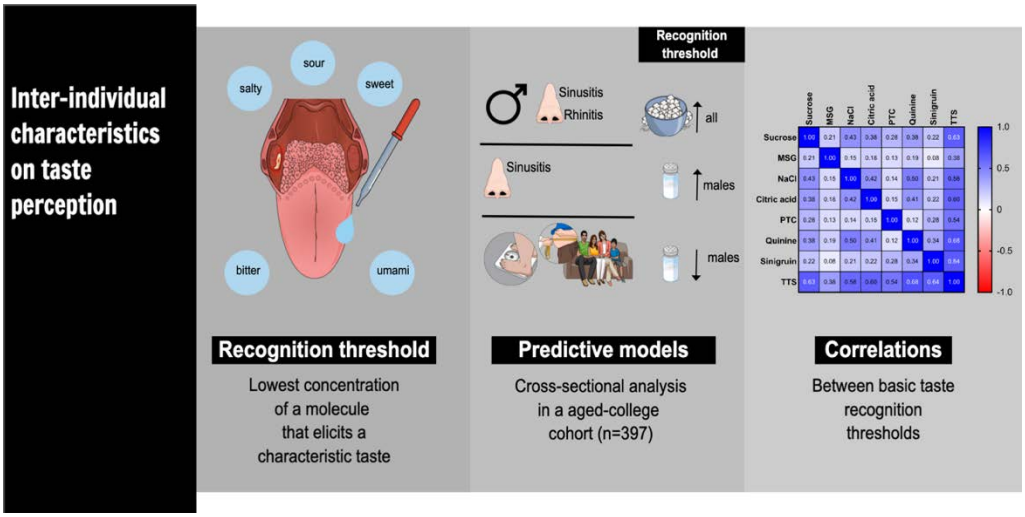

Figure 6. Graphical abstract Publication 3.

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Complete List of Authors:	Trius-Soler, Marta; University of Barcelona, Department of Nutrition, Food Sciences and Gastronomy, XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; ; INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona; University of Barcelona, Department of Nutrition, Food Sciences and Gastronomy, XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; Laveriano-Santos, Emily; University of Barcelona, Department of Nutrition, Food Sciences and Gastronomy, XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; ; INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona; CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III Góngora, Clara; University of Barcelona, Department of Nutrition, Food Sciences and Gastronomy, XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; Moreno, Juan; University of Barcelona, Department of Nutrition, Food Sciences and Gastronomy, XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; ; INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona; CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III

ARTICLE

Inter-individual characteristics on basic taste recognition thresholds in a college-aged cohort: potential predictive factors

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Marta Trius-Soler [a,b,c], Emily P. Laveriano-Santos [a,b,c], Clara Góngora [a], and Juan J. Moreno [a,b,c]†

Studying nutritional status from the perspective of taste sensitivity, rather than only dietary patterns, may provide new insights into the role of taste receptor signaling in the development of metabolic-associated diseases. In this cross-sectional study, we investigated possible influence of sociodemographic (sex and smoking habit) and clinical variables (dental cavities, missing teeth, sinusitis, rhinitis, body mass index, metabolic high prevalence family antecedent diseases) on tastant (sucrose, monosodium glutamate, sodium chloride, citric acid, quinine, sinigrin, phenylthiocarbamide) recognition thresholds (RTs) in a college-aged cohort (n= 397). Predictive models for the tastant RTs were generated and a higher sucrose RT was found in females than in males, while sinusitis and rhinitis had a significant effect on sucrose and sodium chloride RTs. Smoking habit was not an important predictive factor of taste sensitivity, although its long-effect on RTs remain unclear. Additionally, a positive correlation was found between all the tastant RTs studied. Although the results did not show a clear pattern, the statistical approach employed should prove useful in future studies of predictors of taste sensitivity.

Introduction

Humans can distinguish across five basic tastes, namely sweet, umami, salty, sour, and bitter. The sense of taste is essential for survival as it allows the identification of essential nutrients and can avoid the ingestion of potentially spoiled foods or toxins (1). Humans have approximately 5,000 taste buds in the oral cavity, which are the sensory organs of taste (2). Each taste bud contains 50-100 closely positioned taste receptor cells with an average life span in adults of 1-2 weeks (range between 3-24 days), some of which are synapsed with nerve fibres (3,4). Although the sense of taste is limited to the oral cavity, taste receptor (TR) signalling is not confined to taste buds but occurs in a variety of extraoral tissues (5,6). The expression of TRs in the gut, pancreas, brain, and adipose tissue suggests a physiological contribution in nutrient-sensing mechanisms and metabolism)7(. Moreover, due to the fast turnover rate of taste cells, and therefore TRs, taste acuity may serve as an objective measure of several environmental and physiological factors, although individual variability in taste sensitivity has a genetic background)8(.

[a] *Department of Nutrition, Food Sciences and Gastronomy, XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain.*
[b] *INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona, 08921 Santa Coloma de Gramanet, Spain.*
[c] *CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain.*
† Correspondence author
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TRs can be divided into G-protein-coupled and channel-type receptors. Type-1 taste receptors (T1Rs) are a family of G-protein-coupled receptors with three members (T1R1, T1R2, T1R3). The heterodimer complexes T1R2/T1R3 and T1R1/T1R3 function as sweet and umami receptors, respectively (9). In addition, glucose transporters such as sodium-glucose transporters (10) or metabotropic glutamic acid receptors 1 and 4 (11) may play a role in the detection of sweet or umami tastes, respectively. Channel-type, such as epithelial Na+ channels, are responsible for salty taste (12), while transduction of sour involves permeation of H^+ through an apical ion channel identified as Otopretin1 (5). Multiple channel-type receptors are reported to mediate sour taste, including polycystic-kidney-disease 2-like 1 (PKD2L1), polycystic-kidney-disease 1-like 3 (PKD1L3), acid-sensing ion channels (ASIC), or hyperpolarization-activated cyclic nucleotide-gated K+ channels (HCN) (13–15). In contrast to the above-mentioned tastes, the perception of bitterness is mediated by receptors of the type-2 receptor family (T2Rs). T2Rs have a very wide range of ligands, which mainly include plant products such as alkaloids, phenols, and glycosides (16). Moreover, there had been research around across taste sensitivity correlations within some tastants (17,18).

The recognition threshold (RT) refers to the lowest concentration of a stimulus for which the taste quality can be recognized and is a parameter commonly used to define taste acuity, among other measurements (19). In addition to age and sex, several factors can influence RTs, although their relative effects are poorly understood. Habitual smoking as well as mouth and nose complaints have been linked with a significant reduction in taste acuity (20,21). However, a recent systematic review and meta-analysis found that the RT for sucrose was not

affected by sex and tobacco smoking, whereas a higher RT was associated with aging and type 2 diabetes (22). This review also observed that most of the meta-analysed studies had a limited number of participants and that further research with larger cohorts would help to clarify the functions of the gustatory system.

Available data suggest that taste pathways throughout the body, including in the gut and nervous system, could act as nutrient sensors, regulating energy balance, glucose homeostasis, and food intake. Interestingly, the interaction between peripheral and central pathways can be precisely regulated by diverse mediators such as leptin, ghrelin, insulin, glucagon-like peptide 1 (GLP-1) and endocannabinoids (7,23,24). Studying nutritional status from the perspective of taste sensitivity, and not only dietary patterns, may shed new light on the role of the development of metabolic-associated diseases on TR signalling, and vice versa. Recent findings suggest that higher risk of obesity (25,26), type 2 diabetes (27,28), or hypertension (29,30) may be associated with a lower taste sensitivity (or high RTs).

On the other hand, diseases related to metabolic syndrome in adults often have their origins in childhood (31,32), so it is of interest to find predictive markers of risk in healthy young adults (33). In the present study, participants RTs were studied based on a family history of obesity, hypertension, and diabetes, a risk factor for the development of these diseases (34–37).

Knowledge of individual gustatory sensations might be critical to understand the origin, causality, physiological processes, and significance of changes in taste acuity. Therefore, the aim of the present cross-sectional study was to investigate the possible influence of sociodemographic (sex and smoking habit) and clinical factors (dental cavities, missing teeth, sinusitis, rhinitis, body mass index (BMI), and metabolic high prevalence family antecedent diseases) on basic taste sensitivities in a college-aged cohort. Furthermore, we studied the inter-individual relationship between seven tastant RTs representative for the five basic tastes.

Materials & methods

Chemicals

Sucrose, monosodium glutamate (MSG), sodium chloride (NaCl), citric acid, phenylthiocarbamide (PTC), quinine and sinigrin were supplied by Sigma Aldrich (St. Louis, MO, USA). Distilled water was used as the solvent to prepare the corresponding dilutions.

Study design and participants

Students taking degrees in Culinary and Gastronomy Sciences, Food Science and Technology, and Nutrition and Dietetics at the Torribera Campus of Barcelona University were recruited for the study, which took place between October 2017 and April 2019. From a total sample of 404 students, 397 healthy subjects (63% females) aged from 17 to 29 years were included in the analysis. Subjects with missing data on age were assumed to be in the same range and also included in the analysis. Anyone else that were outside the age range were excluded (n = 7).

All participants were informed about the objectives and benefits of the research and formalized their acceptance by signing the informed consent. The study (Torribera Students Taste Study) was approved by the Ethics Committee of the University of Barcelona (Institutional Review Board: IRB 00003099) and conducted according to the declaration of Helsinki for Medical Research involving Human Subjects (WMA, 2001).

Outcome assessment: recognition threshold and stimulus concentrations

RTs were determined in a using a same-different task approach (38). Participants were successively provided with sets of one blank sample (water) and one target sample (chemical dilutions). For each pair set, participants indicated if the two samples tasted different or not, and also recognize the corresponding basic taste. Participants were also requested not to smoke, chew gum, or eat any product for 2 h prior to the test. Sets for each prototypic tastant were presented in ascending concentrations *(Table 1)*. A wide concentration range for each tastant (molecule) was used, considering the bibliography about the topic (39–42). The experimenter placed 0.5 mL of each sample, administered at room temperature, on the tip of the tongue and after 5-10 seconds of regional stimulation, the participant washed out their mouth with water, and waited for 20 seconds before tasting the next test solution. When the subject could not identify the target sample, they were given another set with an increased concentration. Otherwise, if the subject recognized the target sample, the procedure was repeated five minutes later. The assay was concluded when the participant correctly recognized the target sample at a given concentration twice consecutively. The concentration at which the procedure stopped was considered the tastant RT. Before each new taste test, participants rinsed their mouths with water. The order of sensory testing across taste qualities was the same among all participants.

Table 1. Concentrations of tastant test solutions.

Score	Sweet	Umami	Salty	Sour	Bitter		
	Sucrose (mM)	MSG (mM)	NaCl (mM)	Citric acid (mM)	PTC (μM)	Quinine (μM)	Sinigrin (μM)
1	1.2	3.0	3.9	1.2	0.7	9.4	50
2	2.3	7.5	7.8	2.3	3.5	18.7	100
3	4.7	15.0	15.6	4.7	14	37.5	300
4	9.4	30.0	31.3	9.4	56.2	75	600
5	18.8	60.0	62.5	18.7	112.5	150	-

6	37.5	120.0	125.0	37.5	225	300	-
7	75.0	-	250.0	75.0	900	-	-
8	150.0	-	500.0	-	-	-	-

MSG: monosodium glutamate, NaCl: sodium chloride, PTC: phenylthiocarbamide.

Predictor assessment: data collection

Data on age, sex, smoking habits as well as mouth (dental cavities and missing teeth) and nose (sinusitis and rhinitis) complaints were collected through a brief structured self-reported questionnaire. First- and second-degree family histories of overweight-obesity, diabetes, and hypertension were also recorded. Height and body weight of participants wearing light clothes and no shoes were measured following the International Standard of Anthropometric evaluation (43). BMI was calculated as weight/height squared (kg/m^2).

Statistical analysis

The descriptive characteristics of the male and female participants were compared by applying the Student's t test with Welch's approximation to compare continuous variables. The Chi-square test was used to compare proportions among categorical variables of the total study sample and the subsamples.

RTs for basic tastes were scaled in multiples of 1 standard deviation *(Table 1)*. Sequentially, the individual RT scores for each tastant were normalized using Min-Max scaling to transform the data to the same scale, following the general formula:

$$x' = \frac{x - \min(x)}{\max(x) - \min(x)}$$

Once the data were normalized, sucrose was selected for estimating the RT for sweet, MSG for umami, NaCl for salty, citric acid for sour and PTC, quinine, and sinigrin for bitter. Taking into account these three molecules, 11 out of at least 25 T2Rs were considered (44), coming up with a more global information of bitter chemosensory acuity for each participant. The total taste score (TTS), ranging from 0 to 1, was calculated by adding the respective normalized RT scores obtained for the five basic tastes and divided by five. The TTS was proposed as a measure of the overall taste sensitivity of the participants. Internal reliability for the TTS was evaluated through the use of Cronbach's α (α: 0.671). Spearman correlation coefficients were estimated to study linear associations between basic taste RTs and the TTS.

The association between descriptive variables and taste RTs was statistically assessed in three steps. First, an exploratory principal component analysis (PCA) was performed in which the 11 descriptive variables were considered as candidate factors (sex, smoking, dental cavities, missing teeth, sinusitis, rhinitis, BMI, family antecedents of diabetes, family antecedents of hypertension, family antecedents of overweight/obesity, any of the previous family antecedents). Factors with an eigenvalue higher than 1 were retained. Four factors were extracted, explaining 57.4% of the total variance of the whole study cohort. Additionally, four factors (62.6% of the female cohort variance) and five factors (69.0% of the male cohort variance) were extracted from the female and male cohorts, respectively *(Supplementary Table 1 and 2)*. To analyze the association of each extracted PCA factor with each molecule RT, ordinal logistic models were fitted. On the other hand, to analyze the association of each extracted PCA factor with the TTS, linear regression models were fitted.

After identifying PCA factors significantly associated with tastant (molecule) RTs and TTS, the correlation between the relevant descriptive variables represented in these PCA factors and the tastant RTs or the TTS were evaluated. Descriptive variables with absolute loadings > 0.3 were considered significant. As some tastant RTs were not significantly correlated with any PC factor, it was not possible to build a predictive model (e.g., quinine, sinigrin, TTS). Models were predicted for all sample cohort, as well as for the female and the male cohorts separately.

In the final step, the significant descriptive variables (absolute loadings > 0.3) were combined into increasing and decreasing scores based on their association with higher or lower tastant RTs. Then, the effects of each increasing or decreasing score on the different taste RTs were analyzed by applying ordinal logistic regressions or simple linear regressions. In few words, ordinal logistic regression is a statistical analysis method for studying the relationship between an ordinal response variable (e.g., molecule RT) and one or more explanatory variable (e.g., increasing or decreasing RT scores). On the other hand, simple regression model approach is used for modelling linear predictor functions. Ordinal logistic models were calculated for each tastant RT. Both increasing or decreasing RT scores were introduced in the model as continuous variables. Predicted models *p*-values were corrected for multiple testing when build, by the procedure previously described by Simes (1986) (45).

Due to the large dataset and the small percentage of missing values, no data imputation was applied. All statistical analyses were conducted using the Stata statistical software package version 16.0 (StataCorp, College Station, TX, USA). Statistical tests were two-sided, and *p*-values below 0.05 were considered significant. Data were visualized using GraphPad Prism 9 (GraphPad Prism Software, Inc. La Jolla, CA).

Results and discussion

Participant characteristic

Table 2 presents the baseline sociodemographic and clinical data for all participants and by sex. Participants had a mean age of 18.9 ± 1.7 years, more than half were females (63%), and the majority were non-smokers (80.9%). Smoking was significantly more prevalent among males. There were non-significant differences between males and females in mouth health (dental cavities, and missing teeth) and nose complaints (rhinitis, and sinusitis).

The mean BMI of the participants was within the normal weight criterion of the World Health Organization (18.5-24.9 kg/m2),

and males had a significantly higher BMI than females (p-value: <0.001). A family history of diabetes was significantly more prevalent in females (36.4%) in this study cohor

Table 2. Characteristics of the study participants.

	All	Females	Males	*p*-value
Population, *n (%)*	397 (100)	250 (63.0)	147 (37.0)	
[1]Age, *years*	18.9 ± 1.7	18.8 ± 1.7	19.2 ± 1.9	0.097
Smoking, *n (%)*				
Non-smokers	321 (80.9)	211 (84.4)	110 (74.8)	**0.018**
< 10 cigarettes/day	61 (15.4)	34 (13.6)	27 (18.4)	
≥ 10 cigarettes/day	15 (3.8)	5 (2.0)	10 (6.8)	
Dental cavities, *n (%)*	226 (56.9)	148 (59.2)	78 (53.1)	0.233
Missing teeth, *n (%)*	17 (4.3)	7 (2.8)	10 (6.8)	0.057
Rhinitis, *n (%)*	26 (6.6)	16 (6.4)	10 (6.8)	0.876
Sinusitis, *n (%)*	26 (6.6)	15 (6.0)	11 (7.5)	0.564
[2] BMI, kg/m^2	22.1 ± 2.9	21.4 ± 2.7	23.3 ± 2.8	**<0.001**
BMI diagnosis, *n (%)*				
Underweight	33 (8.5)	28 (8.5)	5 (3.5)	**<0.001**
Normal weight	289 (74.3)	189 (74.3)	103 (71.0)	
Overweight-obese	67 (17.2)	30 (12.3)	37 (25.5)	
Family antecedents, *n (%)*				
Diabetes	130 (32.8)	91 (36.4)	39 (26.7)	**0.048**
Hypertension	155 (39.0)	97 (38.8)	58 (39.5)	0.897
Overweight-obesity	77 (19.4)	51 (20.4)	26 (17.7)	0.509
Any of the previous	235 (59.2)	143 (57.2)	92 (62.6)	0.292

[1] n= 357 (40 missing values); 2 n= 389 (8 missing values). BMI: body mass index. Continuous variables are expressed as mean ± standard deviation (SD) and categorical variables as n (%). Statistical analyses were undertaken using the Student t-test when comparing continuous variables using Welch's approximation. A Chi-square test was used for categorical variables. Values shown in bold are statistically significant. *p*-value <0.05.

Distribution and sex-related differences in the taste recognition thresholds

Figure 1 illustrates the cumulative frequency curves for taste RTs in each sex, showing percentage of individuals that recognized the specific taste within a given tastant concentration interval. The beginning and end of the curves correspond to the lowest and highest RT (plateau point) recorded, respectively. The steepness of the curve is inversely correlated with the variance in each tastant RT score. Four differently shaped curves were obtained considering the concentration steps used in this study: 1) sucrose, NaCl and citric acid have quite a symmetrical and close to normal distribution; 2) that of MSG and quinine is skewed to the right; 3) sinigrin has a unimodal distribution with a skew to the left; and 4) PTC shows a characteristic tripartite grouping. More precisely, the MSG curve had a starting point at score 2 and a plateau point at score 4, and the cumulative frequency curve was less steep. In general, the shape of the cumulative frequency curves was similar between both sexes.

Overall, females and males did not have significantly different taste RT scores, except for higher sucrose RTs in females (female sucrose RT: 5.2 ± 1.2; male sucrose RT: 4.9 ± 1.5; *p*-value: 0.013). Females had a higher mean score for sucrose but the same standard deviation.

The same tendency for sucrose RT was previously reported by Nagai *et al.* (2015) (46). On the contrary, Hong *et al.* (2005) found that the sucrose RT was lower in females than in males (39). Overall, results from previous studies show no significant differences in taste acuity between the sexes during youth, or according to age groups (40,47–51). Previous findings suggest that the gustatory system matures later in boys, but that the difference between the sexes during childhood disappears in young adults (49). On the other hand, the subsequent drop in taste acuity associated with aging is reported to occur more sharply in men than in women (18,47), which might explain the sex-related differences reported by some researchers (22). Indeed, in a previous systematic review and meta-analysis about sucrose RT, it was concluded that no significant differences can be observed between males and females (22), whereas the age of the cohort seems to be a major factor.

The mean RT for the tastants in the entire study population, expressed as real taste solution concentrations, were the following: 29.5 ± 32.3 mM sucrose; 15.3 ± 16.5 mM MSG; 56.9 ± 62.0 mM NaCl; 8.2 ± 7.8 mM citric acid; 70.2 ± 199.0 µM PTC; 57.2 ± 64.8 µM quinine; and 382.5 ± 218.2 µM sinigrin. The mean ± SD of RT scores for each tastant by sex can be found in *Supplementary Table 3*.

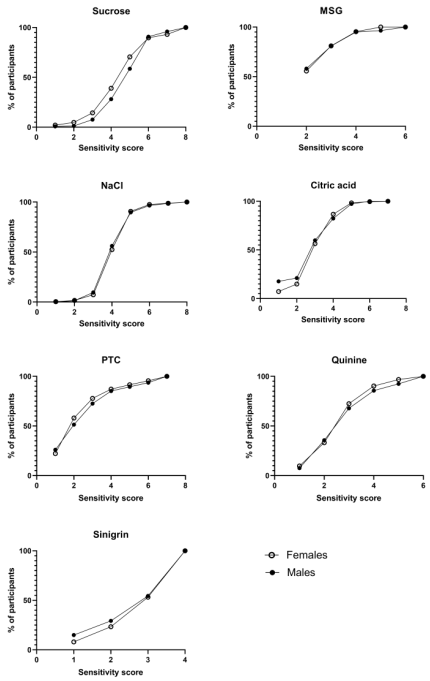

Figure 1. Cumulative frequency curves of the recognition threshold score for the analyzed tastants (sucrose, monosodium glutamate (MSG), sodium chloride (NaCl), citric acid, phenylthiocarbamide (PTC), quinine and sinigrin) by sex.

Association between different taste recognition thresholds

Associations between the perceptions of different tastes can shed light on the mechanisms underlying taste and their interactions. Significant positive correlations were found between tastant RTs representing the five basic tastes and the TTS, indicating that the ability to recognize a given taste is related to the ability to recognize the others.

Figure 2 depicts the association between the RTs for each administered tastant, and between the RTs and the TTS in the whole study population. When considering all the participants, the strongest positive correlation was between the RT scores for NaCl and quinine (*r*: 0.50; *p*-value: < 0.001), followed by NaCl and sucrose (*r*: 0.43; *p*-value: < 0.001); NaCl and citric acid (*r*: 0.42; *p*-value: < 0.001); and citric acid and quinine (*r*: 0.41; *p*-value: < 0.001). The TTS had a fair to moderate positive correlation coefficient with all the tastant RT scores (*p*-value: < 0.001).

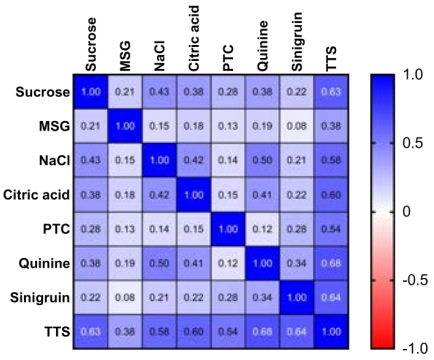

Figure 2. Spearman correlation coefficients between the recognition thresholds for tastants (sucrose, monosodium glutamate (MSG), sodium chloride (NaCl), citric acid, phenylthiocarbamide (PTC), quinine and sinigrin) and the total taste score (TTS). Sample size *n*=360)

A previous study found detection thresholds for four tastes (sweet, salty, sour, and bitter) highly correlated, but not particularly high between RT among healthy young adults (17); while positive associations between tastes that share common features in the transduction mechanisms were recently described (52). The PTC RT was significant but fairly correlated with the other molecule RTs in this study, as well as the results reported for PTC tasters of other studies, classified by a PTC threshold lower than 1.8 µM (39,51). Further research is needed to understand gustatory perceptions in terms of taste thresholds.

Predictor variables for recognition thresholds

Table 3 summarizes the association between tastant RT score, and their direct predictor variables previously identified in the PCA factor. Females and sinusitis and rhinitis conditions make up a had a significant predictive model for higher sucrose RT (adjusted *p*-value: 0.035). Additionally, a significant direct relationship between sinusitis and the RT for NaCl was observed in males (adjusted *p*-value: 0.040).

Table 3. Predictive models for higher taste recognition thresholds and total taste scores.

Tastant	Components of the predictor model	B (95% CI)	*p*-value	[1]*p*-value
Sucrose *All (n= 395)*	Females, sinusitis, rhinitis	1.03 (0.28; 1.78)	**0.007**	**0.035**
NaCl *Males (n=146)*	Sinusitis	1.00 (0.22; 1.78)	**0.012**	**0.040**
Citric acid *Males (n=147)*	Smoking	1.00 (-0.26; 2.03)	0.056	0.093
PTC *Males (n= 142)*	BMI, family antecedents of overweight/obesity	0.71 (-031; 1.73)	0.173	0.247

B: regression coefficient. CI: confidence interval. BMI: body mass index (Kg/m^2). NaCl: sodium chloride. PTC: phenylthiocarbamide. Ordinal logistic regression applied for the predictor models. [1]p-value adjusted for multiple comparisons.

On the other hand, the predictive models outlined in *Table 4* were applied to study the association between RT scores and a summed score of descriptive variables directly associated with lower RTs. Males with family antecedents of overweight-obesity and higher BMI had a significantly lower RT for NaCl (adjusted *p*-value: 0.035).

Table 4. Predictive models for lower taste recognition thresholds and total taste scores

Tastant	Components of the predictor model	B (95% CI)	*p*-value	adjusted *p*-value[1]
Sucrose				
All (n= 395)	BMI	0.20 (-1.83; 2.22)	0.850	0.882
NaCl				
Males (n=146)	BMI, family antecedents of overweight /obesity	-0.98 (-1.67; -0.28)	**0.006**	**0.035**
Citric acid				
All (n= 395)	Dental cavities, missing teeth	-0.93 (-1.80; -0.07)	**0.035**	0.070
Females *(n=248)*	Dental cavities, missing teeth	-1.05 (-2.00; -0.10)	**0.030**	0.070
Males (n=147)	Missing teeth, rhinitis	-0.91 (-2.83; 1.01)	0.352	0.440
PTC				
Males (n= 142)	Sinusitis	-1.00 (-14.24; 12.24)	0.882	0.882

B: regression coefficient. CI: confidence interval. BMI: body mass index (Kg/m^2). NaCl: sodium chloride. PTC: phenylthiocarbamide. Ordinal logistic regression applied for the predictor models. [1]*p*-value adjusted for multiple comparisons.

The descriptive variables studied as potential predictors of taste RTs did not show any clear pattern of association with the bitter tastants (PTC, quinine, and sinigrin) and neither for MSG. No significant predictive model could be built for TTS either, while predicted models for citric acid were not significant after multiple comparison analyses.

Oral and nasal disorders, as well as sex and BMI appear to be better predictors of sensitivity to tastes related to TR1s (sweet and umami) and channel-type receptors (salty and sour) than bitter receptors (T2Rs). Indeed, the RTs for bitter, sweet, and umami might be inherited, or associated with other descriptive variables not included in this study (53–55). Moreover, the lack of significance in the results could be due to the homogeneity of the study population. Besides the well-studied T2R38 polymorphisms, at least 10 bitter TRs contain single nucleotide polymorphisms, one of them supporting a genetic difference related to quinine bitterness (8,56). As an example, a single nucleotide polymorphism in the sweet taste receptor subunit T1R2 and glucose transporter 2 genotypes were found to be associated, individually and in combination, with the prevalence of dental caries and a plausible dietary preference for sweet food (57).

Findings from the present study suggest that sinusitis and rhinitis might be important clinical variable predictors of sucrose and NaCl RTs in young adults. Although the cause of sinusitis and rhinitis was not specified, which hampered the analysis, studies on viral, allergic, or chronic sinusitis/rhinitis all indicate that chemosensory impairment is prevalent in afflicted individuals, especially regarding salty taste (58,59). PTC sensitivity has been previously described as predictive of sinonasal innate response and nasal symptoms in healthy individuals (60). Indeed, T2R38 receptors expressed in the nasal epithelium detect bacterial quorum-sensing molecules, activation the innate immune response that protects against bacterial invasion (61). Thus, those with higher PTC sensitivity might have less frequent sinus infections (60). Other investigations have demonstrated that patients with chronic rhinosinusitis and nasal polyps are significantly less sensitive to the bitter taste of quinine (20). However, results from this study were not in line with those previously mentioned.

BMI and family antecedents of overweight/obesity predicted significant lower NaCl in our male cohort. Obesity is characterized by a chronic low-grade inflammation, whose effects can accumulate gradually over time. Interestingly, available evidence indicates a change in taste perception following bariatric surgery, especially for salt, sweet and sour taste, suggesting a link between taste perception and the development of obesity (22,62,63). On the contrary of the present results, Fernández-García *et al.* (2017) showed for the first time that sensitivity to taste (sweet, salty, and sour) is inversely associated with BMI-related variables such as body fat mass (25). Likely mechanisms responsible for changes in taste sensitivity include the modulation of hormones involved in energy and body weight homeostasis, taste bud abundance, taste receptor signals to the brain, changes in diet, or psychological consequences of weight loss interventions (22,62,64,65).

Cigarette smoking did not can affect RTs in our study, although smoke can irritate the oral mucosa leading to atrophy of the tongue papilla [21]. However, the scientific evidence for the potential effect of smoking on taste perception is inconsistent. In line with our results, smoking generated an unaltered salty taste response (48,66–69). Contrary to our findings, a tendency towards a decrease in salt taste sensitivity in smokers has been reported (47,70–73).The effect on sweet and sour is even less clear (47,48,66–69,71–76). The conflicting or inconclusive results could likely be explained by covariates that are usually mentioned by the researchers in the discussion but not considered in the analyses (e.g., smoking dose, dose in pack-years, type of cigarette, specific taste, the site of the oral cavity, or sex of the subject) (66,73,74,77).

Strength and limitations

To our knowledge, this is the first time that all the aforementioned variables have been screened in one observational study in search of predictive factors of taste sensitivity. The study population, although large, was a

homogenous cohort of healthy college-aged Spanish females and males, which could explain some of the non-significant results. The percentages of smokers were in concordance with the National Health Survey of Spain 2017, being around ≈20% in 15-24-year-olds and higher in men than in women (78). A factor that differentiates our study from others is the size of the sample, as for the first time more than a hundred young females and males were compared for this purpose, whereas in previous work the maximum number of subjects in each group was around fifty (17,79–81).

However, the study has some limitations that may have influenced the lack of relationships between independent measurements of taste function. The main weak point is that taste perception, a multi-faceted process, was assessed based only on measuring RTs. A more complete analysis would also require monitoring of the detection threshold, suprathreshold intensity, and hedonic perception, among other factors. Additionally, the estimation of the RT values by a same-different task methodology could have been more precise: 1) the order of the tastants should be randomized and blinded for the participant; 2) the samples in the series should all have been tasted preferably twice on different days; and 3) the volume used for the stimulus was low in comparison with common approaches. Moreover, although the menstrual cycle has been described as a modifier of taste sensitivity (82,83), the phase of the menstrual cycle at the time of the taste assessment was not recorded. Nevertheless, the sample size for females was large enough to represent the entire menstrual cycle and therefore reduce its implications. Participants medication was neither recorded.

Conclusions

To sum up, predictive models of RTs for tastant molecules representative of basic tastes in association with sociodemographic and clinical factors were determined in a large sample of young adults as part of a cross-sectional study. Predictive models for PTC, sinigrin, quinine, and TTS could not be built. A higher sucrose RT was found in females than in males, while sinusitis and rhinitis were factors for predictive models for higher sensitivity to sucrose and NaCl RTs. Smoking habit did not have any influence, although its long-effect on taste sensitivity is still unclear. Additionally, a positive correlation was found across all the tastant RTs. Although the results from the generated models did not show a clear pattern that might be explained by the short exposure of some predicted factors due to the young age of the cohort population, this new statistical predictive approach may be fruitfully used in further studies on how environmental/physiological/physiopathological factors can influence taste acuity. Further research is needed to understand the physiological implications of the gustatory function and predictors of taste sensitivity. Such insights into individual taste perception could be applied in the design of individualized dietary treatments, and clinical interventions.

Author Contributions

Marta Trius-Soler: Conceptualization, Methodology, Data curation, Software, Formal analysis, Writing-Original draft preparation. Emily P. Laveriano-Santos: Formal analysis, Writing-Reviewing and Editing. Clara Góngora: Data curation, Writing-Reviewing and Editing. Juan J. Moreno: Conceptualization, Methodology, Investigation, Data curation, Visualization, Supervision, Writing-Original draft preparation, Project administration.

Conflicts of interest

There are no conflicts to declare.

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Publication 4

Association of phenylthiocarbamide perception with anthropometric variables and intake and liking for bitter vegetables

Marta Trius-Soler, Paz A. Bersano-Reyes, Clara Góngora, Rosa M. Lamuela-Raventós, Gema Nieto, and Juan J. Moreno

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Supplementary Material available in Annex 3.

Abstract

Aim: The aim of this cross-sectional study was to evaluate the influence of phenylthiocarbamide (PTC) phenotypes on (1) individual anthropometric and clinical history variables; (2) other basic taste recognition thresholds (RTs), and (3) the hedonic perception and habitual intake of Brassicaceae vegetables in a young adult population (18.9 ± 1.7 years old).

Methods: The PTC phenotype was determined by the quantitative measure of the PTC recognition threshold (non-tasters, 24.1%; tasters, 52.3%; and super tasters, 23.6%).

Results: No significant differences in smoking habits, oral and nasal disorders, family antecedents of diseases related to metabolic syndrome, and Brassicaceae vegetable hedonic perception and consumption were found between the PTC phenotype groups. The average BMI of super-taster females and males was significantly lower compared to non-tasters. In addition, the PTC taster status was a predictor of lower scores for other basic taste RTs.

Conclusions: Overall, the defined PTC super-taster cohort could be differentiated from the non-tasters by variables related to weight control such as BMI and sucrose RT.

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Association of phenylthiocarbamide perception with anthropometric variables and intake and liking for bitter vegetables

Marta Trius-Soler[1,2,3], Paz A. Bersano-Reyes[4], Clara Góngora[1], Rosa M. Lamuela-Raventós[1,2,3], Gema Nieto[4] and Juan J. Moreno[1,2,3*]

Abstract

Phenylthiocarbamide (PTC) sensitivity, a sensory trait mediated by the bitter taste receptor 38 (TAS2R38), has been described as a promising biomarker of health status or disease risk. The aim of this cross-sectional study was to evaluate the influence of PTC phenotypes on (1) individual anthropometric and clinical history variables; (2) other basic taste recognition thresholds (RTs), and (3) the hedonic perception and habitual intake of *Brassicaceae* vegetables in a young adult population (18.9 ± 1.7 years old). The PTC phenotype was determined by the quantitative measure of the PTC recognition threshold (non-tasters, 24.1%; tasters, 52.3%; and super tasters, 23.6%). No significant differences in smoking habits, oral and nasal disorders, family antecedents of diseases related to metabolic syndrome, and *Brassicaceae* vegetable hedonic perception and consumption were found between the PTC phenotype groups. The average BMI of super-taster females and males was significantly lower compared to non-tasters. In addition, the PTC taster status was a predictor of lower scores for other basic taste RTs. Overall, the defined PTC super-taster cohort could be differentiated from the non-tasters by variables related to weight control such as BMI and sucrose RT.

Keywords: Recognition threshold, Non-tasters, Super-tasters, Body mass index, Brassicaceae, Bitter taste

Introduction

Mammals have complex sensory transduction pathways to distinguish the quality and safety of food, and they can differentiate between at least five basic tastes: sweet, bitter, salty, sour and umami. The chemosensory perception of taste is complemented by the olfactory system [1]. In particular, bitter taste is believed to be a protective sensory response to prevent the ingestion of potentially toxic compounds, although not all bitter foods are toxic [2]. The genetic background of sensitivity to bitter taste has been extensively researched. To date, the majority of genotype-phenotype studies in this field have focused on the polymorphisms of the gene encoding the bitter taste receptor 38 (T2R38), which are responsible for the different phenotypes of people who are insensitive or taste-blind (non-tasters), moderately sensitive (tasters) and highly sensitive (super-tasters) to certain bitter substances [3, 4]. This discovery was made in 1931 with an artificial compound, phenylthiocarbamide (PTC) [5]. It is now known that phenotypic variation in perception of PTC bitterness, including super-tasting, can be explained to a large extent, but not fully, by T2R38 polymorphisms [6, 7].

The phenotypic variation in sensitivity to PTC and structurally related compounds has been associated with indicators of obesity [8–10], as well as with an unequal response to a weight loss intervention [11]. In particular, non-tasters females have been reported to have a higher body mass index (BMI) than tasters and super-tasters

*Correspondence: jjmoreno@ub.edu

[3] CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain
Full list of author information is available at the end of the article

[10, 12]. However, only a few studies have analyzed BMI as a continuous variable or its relationship with PTC/PROP sensitivity [13–15].

Interestingly, super-tasters are reported to have a higher density of fungiform papillae on the tongue compared to tasters and non-tasters [4]. Furthermore, high sensitivity to bitter taste has been linked to an enhanced perception of sweetness [16]. However, the relationship between bitter and the other basic taste sensitivities still requires more in-depth research.

The synthetic compound PTC contains a thiocyanate (N-C=S) group that is also found in 6-n-propylthiourail (PROP) and isothiocyanates. The main dietary sources of isothiocyanates belong to the *Brassicaceae* family (e.g., cruciferous vegetables, mustard, and arugula) [17, 18] and their intake has been hypothesized to be lower among PTC tasters in some studies [19–21]. Although a wide range of factors can influence food choices and preferences, taste is held to one of the most important [22, 23]. Age and sex are reported to modify the relationship between food choices and PROP/PTC taster status [4].

Understanding the underlying characteristics of the PTC phenotype is a critical first step in harnessing its usefulness as a biomarker of health and disease. Therefore, the present study aimed to evaluate if PTC taste status has an influence on (1) individual anthropometry and clinical history; (2) the basic taste recognition thresholds (RTs) and estimated total taste acuity; (3) and the consequent possible differences in liking and/or consumption of vegetables with a PTC-related bitter taste. This cross-sectional study was carried out in a large Spanish young adult cohort of college students and represents the first investigation of these research questions in this specific population.

Results

Anthropometric and clinical differences between PTC super-tasters, tasters, and non-tasters by sex

The differences in anthropometric and clinical variables between PTC non-tasters, tasters, and super-tasters by sex are shown in Table 1. Among all the participants, 24.1% were non-tasters, 52.3% were tasters, and 23.6% were super-tasters. By sex, 22.2% of women and 27.5% of men were non-tasters, 55.7% and 46.5% were tasters, and 22.2% and 26.1% were super-tasters, respectively.

The three well-established PTC phenotypes did not differ significantly in tobacco smoking habits, oral and nasal disorders, and first- and second-degree metabolic syndrome-related diseases. Among women, super-tasters had a significantly lower mean BMI compared to non-tasters, and among men, super-tasters also had the lowest mean BMI (Fig. 1, Table 1).

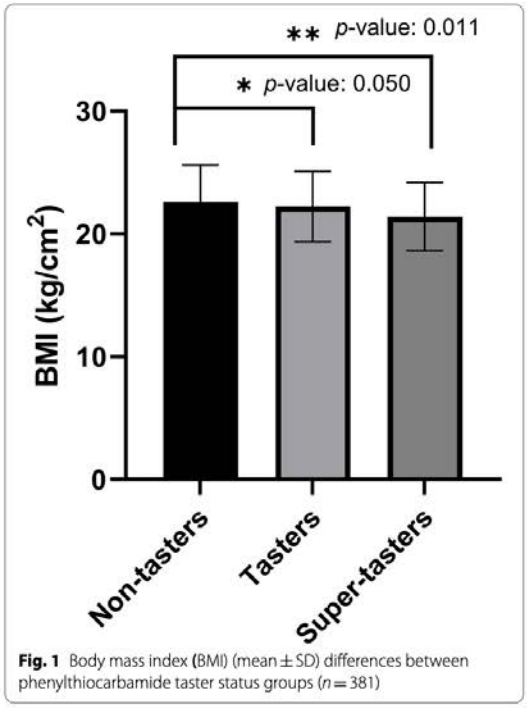

Fig. 1 Body mass index (BMI) (mean ± SD) differences between phenylthiocarbamide taster status groups ($n = 381$)

Influence of PTC tasting status on taste recognition thresholds

The influence of PTC tasting status on basic taste RTs, as well as on the total taste score (TTS), was investigated. Among women, the PTC super-taster group had significantly lower RTs for sucrose and sinigrin as well as TTS compared to the non-tasters. Among males, basic taste RTs were systematically lower in super-tasters compared to tasters and non-tasters. Overall, a higher proportion of PTC super-tasters could detect saccharin bitterness (91.9% of super-taster males and 74.6% of super-taster females). Quinine was the only tested substance without significant differences in RT between the PTC super-tasters and non-tasters. Thus, individuals who were most sensitive to PTC bitterness were also the most sensitive to the other basic tastes. These results were more pronounced in the male cohort (Table 2).

Influence of PTC tasting status on the liking and consumption of bitter vegetables

Participants were asked if they like and consume cruciferous vegetables, mustard, or bitter leaves (endive and arugula). After the statistical analysis, no differences were found between the PTC tasting groups stratified by sex

Table 1 Anthropometric and clinical differences between phenylthiocarbamide taster status groups by sex

	Sex	Non-tasters ($n=94$)	Tasters ($n=204$)	Super-tasters ($n=92$)	*p*-value
Smokers, *n (%)*	F	8 (14.6)	22 (15.9)	8 (14.6)	0.955
	M	12 (30.77)	17 (25.8)	7 (18.9)	0.492
Caries, *n (%)*	F	36 (65.5)	82 (59.4)	29 (52.7)	0.397
	M	19 (48.7)	40 (60.6)	19 (51.4)	0.436
Missing teeth, *n (%)*	F	2 (3.6)	4 (2.9)	1 (1.8)	0.845
	M	1 (2.6)	6 (9.1)	3 (8.1)	0.431
Sinusitis, *n (%)*	F	3 (5.5)	10 (7.3)	2 (3.6)	0.623
	M	4 (10.3)	3 (4.6)	4 (10.8)	0.411
Rhinitis, *n (%)*	F	5 (9.1)	9 (6.5)	2 (3.6)	0.507
	M	5 (12.8)	2 (3.0)	3 (8.1)	0.159
BMI, kg/m^2	F	21.8 ± 2.9^{a}	21.5 ± 2.6^{ab}	20.7 ± 2.7^{b}	**0.043**
	M	23.6 ± 2.8^{ab}	23.6 ± 2.9^{a}	22.4 ± 2.6^{b}	0.099
BMI diagnosis, *n (%)*					
Underweight	F	4 (7.6)	14 (10.4)	10 (18.5)	0.133
Normal weight		39 (73.6)	104 (77.0)	41 (75.9)	
Overweight-obese		10 (18.9)	17 (12.6)	3 (5.6)	
Underweight	M	1 (2.6)	1 (1.5)	3 (8.3)	0.351
Normal weight		28 (73.7)	45 (68.2)	26 (72.2)	
Overweight-obese		9 (23.7)	20 (30.3)	7 (19.4)	
Family antecedents, n (%)					
Diabetes	F	16 (29.1)	55 (39.9)	20 (36.4)	0.374
Hypertension		14 (25.5)	58 (42.0)	24 (43.6)	0.071
Obesity		13 (21.8)	29 (21.0)	9 (16.4)	0.723
Diabetes	M	12 (30.8)	15 (23.1)	11 (29.7)	0.628
Hypertension		13 (33.3)	26 (39.4)	18 (46.7)	0.390
Obesity		9 (23.1)	14 (21.2)	3 (8.1)	0.170

BMI body mass index, *F* females, *M* males

Continuous variables are expressed as mean ± SD and categorical variables as n (%). Statistical analyses were carried out using the Kruskal-Wallis rank-sum test (post-hoc Dunn test) when comparing quantitative variables. A chi-square test was used for categorical variables. Different letters indicate significant differences (*p*-value <0.05) among groups. Values shown in bold are statistically significant *p*-value < 0.05

One participant had missing data on family antecedents of diabetes, and three, missing data on BMI

(Table 3). In the three groups, 36.4–61.8% of the individuals affirmed that they liked and consumed bitter vegetables. Moreover, the majority liked and consumed one or two out of the three options, resulting in a mean sum of bitter food intake of 1.5 ± 0.8 out of a possible total score of 3.

Multivariate analysis of the inter-individual differences across stimuli

To understand how the different RTs, clinical history, and liking and consumption of bitter vegetables of individuals are correlated, a PCA (Principal Component Analysis) plot of the analytical data was generated, which reduced the dimension of the dataset while retaining as much information from the data as possible. A PCA was applied to the matrix of 335 subjects × 23 parameters. Principal components (PCs) were computed from participant inter-individual differences in each PCA model. In the PCA of the Imax matrix, the first two PCs were selected. Fig. 2 shows the bi-dimensional plot of PC 2 vs. PC 1. Vectors of the original variables are plotted according to their factor loadings. The PCA biplot accounted for about 24.9% of the variation within the data set. The first major observations are that all stimuli are distributed on the negative side of PC 1 (15.3% of variation) and was strongly correlated with TTS (values close to −1). A negative loading in PC 1 grouped the RTs close together, indicating that the RT scores are positively correlated. Another interesting observation is that bitter food liking and consumption are located on the positive side of PC 2, explaining 9.6% of the total inter-variability.

On the other hand, the PC score graph shows a tendency of data points separation between super-tasters and non-tasters of PTC, however it appears to be

Table 2 Influence of phenylthiocarbamide taster status on basic taste recognition thresholds, total bitter score, and total taste score of the studied taste stimuli by sex

	Sex	Non-tasters	Tasters	Super-tasters	*p*-value
Sucrose	F	5.7 ± 1.1^{a}	5.2 ± 1.2^{a}	4.7 ± 1.2^{b}	**<0.001**
	M	5.1 ± 1.7^{a}	5.2 ± 1.3^{a}	4.1 ± 1.2^{b}	**<0.001**
Monosodium glutamate	F	2.8 ± 1.0^{a}	2.7 ± 0.9^{a}	2.5 ± 0.8^{a}	0.347
	M	3.1 ± 1.4^{a}	2.7 ± 0.9^{ab}	2.3 ± 0.5^{b}	0.062
Sodium chloride	F	4.6 ± 1.1^{a}	4.6 ± 0.9^{a}	4.3 ± 0.8^{a}	0.106
	M	4.6 ± 1.1^{a}	4.6 ± 0.9^{a}	4.1 ± 0.6^{b}	**0.007**
Citric acid	F	3.5 ± 1.1^{a}	3.4 ± 1.0^{a}	3.2 ± 1.2^{a}	0.204
	M	3.5 ± 1.4^{a}	3.4 ± 1.2^{a}	2.5 ± 1.3^{b}	**0.001**
Quinine	F	3.1 ± 1.3^{ab}	3.0 ± 1.2^{a}	2.9 ± 0.9^{b}	0.514
	M	3.2 ± 1.7^{ab}	3.3 ± 1.2^{a}	2.6 ± 0.9^{b}	**0.031**
Sinigrin	F	3.7 ± 0.7^{a}	3.0 ± 0.9^{b}	2.9 ± 1.0^{b}	**<0.001**
	M	3.2 ± 1.1^{a}	3.3 ± 0.8^{a}	2.3 ± 1.2^{b}	**<0.001**
TTS	F	0.504 ± 0.108^{a}	0.443 ± 0.098^{b}	0.399 ± 0.097^{c}	**<0.001**
	M	0.487 ± 0.162^{a}	0.462 ± 0.104^{a}	0.326 ± 0.106^{b}	**<0.001**
Saccharin	F	30 (52.6)	95 (69.3)	41 (74.6)	**0.030**
(bitterness detected)	M	17 (39.5)	46 (69.7)	34 (91.9)	**<0.001**

F females, *M* males, *TTS* total taste score

Quantitative variables are expressed as mean ± SD and categorical variables as n (%). Statistical analyses were undertaken using the Kruskal Wallis rank-sum test (post-hoc Dunn test) when comparing quantitative variables. A chi-square test was used for categorical variables. Different letters indicate significant differences (*p*-value <0.05) among groups. Values shown in bold are statistically significant, *p*-value < 0.05

Table 3 Differences in the proportions of participants liking and consuming bitter foods among phenylthiocarbamide taster status groups

	Sex	Non-tasters	Tasters	Super-tasters	*p*-value
Cruciferous	F	33 (58.9)	82 (60.7)	34 (61.8)	0.951
	M	20 (45.5)	27 (42.2)	17 (50.0)	0.759
Mustard	F	24 (42.7)	72 (53.3)	29 (53.7)	0.379
	M	16 (36.4)	26 (41.3)	14 (40.0)	0.875
Bitter leaves	F	29 (50.9)	77 (56.2)	24 (43.6)	0.281
(Endive and arugula)	M	19 (44.2)	29 (44.6)	19 (54.3)	0.597
Sum of bitter food intake	F	1.4 ± 0.8	1.5 ± 0.9	1.6 ± 0.8	0.444
	M	1.3 ± 0.8	1.3 ± 0.8	1.4 ± 0.9	0.739

F females, *M* males

Categorical variables as n (%). A chi-square test was applied

overlapped. From left to right and from bottom to top, it can be observed that PTC super-tasters are located more towards the bottom-left quadrant of the graph, while non-tasters are near the top-right quadrant. Tasters are located between the two other PTC taster status groups.

Discussion

T2R38 is a member of the T2R bitter taste receptor gene family, which in humans includes 25 functional genes and 11 pseudogenes with several genetic signatures of natural selection [24–26]. The association between T2R38 gene variation and sensitivity to thiourea bitterness has been well characterized by numerous research teams [27, 28]. Therefore, the present study evaluated the applicability of this phenotypic variation in PTC perception as a biomarker of health-related factors, global taste sensitivity and hedonic perception and intake of *Brassicaceae* vegetables. Interestingly, it was observed that PTC tasting status is related to other basic taste RTs and BMI. Conversely, PTC RTs were not correlated with differences in hedonic perception and consumption of bitter vegetables.

A primary aim of the current work was to identify if health-related factors had a variable distribution among PTC taster status groups, as might be expected according

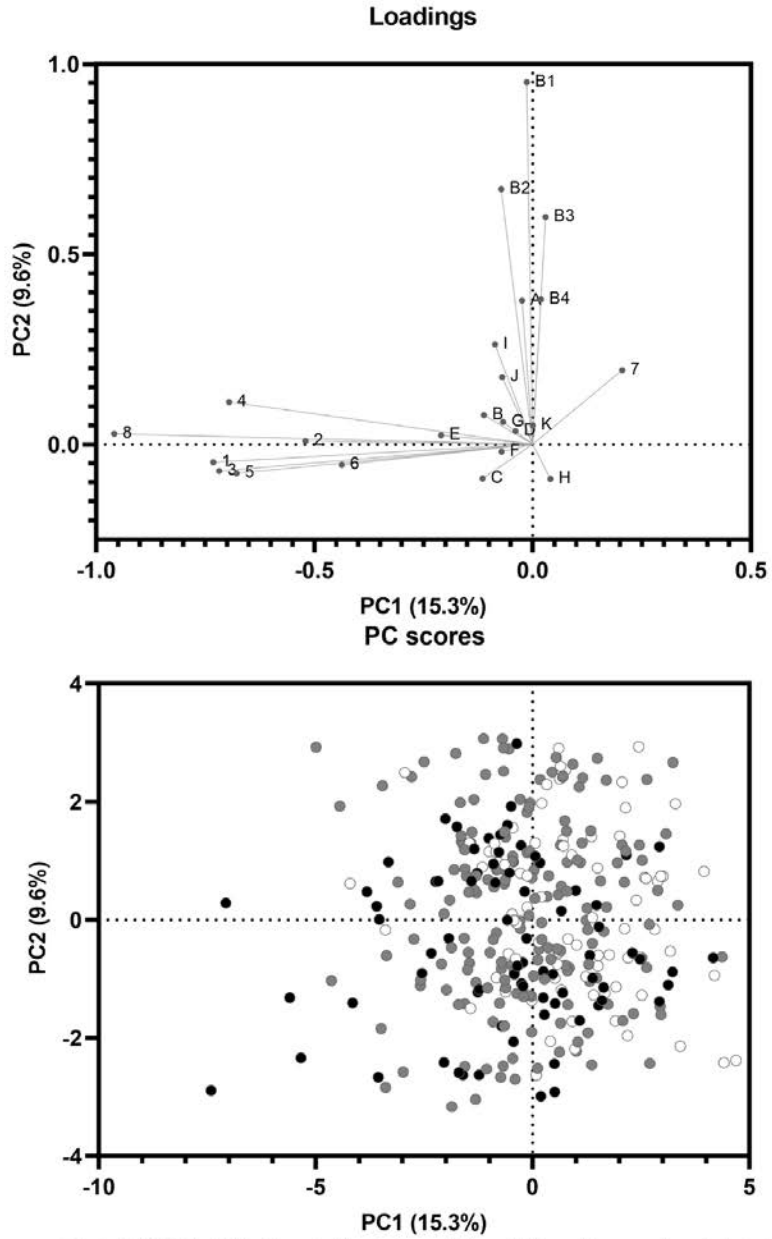

Fig. 2 Principal Component Analysis (PCA) of individual characteristics, taste sensitivity, and liking and consumption of selected bitter vegetables. Black points indicate the non-taster phenylthiocarbamide (PTC) group; gray points indicate the taster PTC group, and white points indicate the super-taster PTC group. A, sex; B, age; C, smoking habit; D, body mass index; E, sinusitis; F, rhinitis; G, caries; H, missing teeth; I, family antecedents of diabetes; J, family antecedents of hypertension; K, family antecedents of obesity; B1, liking and total consumption of bitter food; B2, liking and consumption of cruciferous vegetables; B3, liking and consumption of endive and arugula; B4, liking and consumption of mustard; 1, sucrose recognition threshold; 2, monosodium glutamate recognition threshold; 3, sodium chloride recognition threshold; 4, citric acid recognition threshold; 5, quinine recognition threshold; 6, sinigrin recognition threshold; 7, saccharin bitterness perception; 8, total taste score

to current evidence. For example, polymorphisms of the TAS2R38 gene are linked to significant differences in the ability of upper respiratory cells to clear and kill bacteria [29], and consequently may be involved in susceptibility to upper respiratory infection and recalcitrant sinusitis [30]. However, our results did not show a different incidence of sinusitis and rhinitis between PTC non-tasters, tasters, and super-tasters. Nor did PTC non-tasters and tasters differ in smoking habits, in contrast with other studies that have observed effects of smoking on PTC thresholds [15, 31]. Regarding sex, although it has been suggested that a higher frequency of super-tasters are female [32], no differences were found in our cohort. These results might be due to the age range of the studied population, which supports the possibility that the pattern of sex difference can differ according to the age group.

In this cohort of healthy Spanish young adult college students, the super-tasters had a lower BMI than tasters and non-tasters. The same finding was also reported by Padiglia *et al.* (2010) in an Italian cohort of normal weight 20–29-year-old females and males [12]. Studies carried out in older subjects also report that non-tasters are more likely to have a higher BMI than super-tasters [10, 15, 33]. However, other studies have not found any evidence of a relationship between BMI and PTC taster status [11, 34].

Regarding T2R38 phenotypes, Tepper *et al.* (2008) found that a high PROP sensitivity was associated with a lower BMI when demographic variables and the PROP dietary restraint interaction term were included in the model in females but not males, describing PROP as a marker for susceptibility to weight gain only in females [14]. On the other hand, the PROP detection threshold was positively correlated with BMI in both obese and normal weight groups in another study [13]. Additionally, the PROP phenotype was described as a better predictor of variation in body weight compared to the T2R38 genotype [14].

Interestingly, the results of the present study showed that basic taste RTs of the PTC non-tasters were higher compared to the super-tasters for sucrose and sinigrin, and they also had a higher TTS. In PTC super-taster males, hypergeusia was also observed for other evaluated stimuli, and lower RTs were recorded for sodium chloride (NaCl), citric acid and quinine. These findings are consistent with the results of previous studies based on taste thresholds [35, 36] and suprathreshold intensity scaling [2, 6]. Anatomical data also support these differences between PTC taster groups, as super-tasters reportedly have more fungiform papillae and taste buds than non-tasters [37]. Although the data about PTC tasting refer specifically to the perceived intensity of bitterness, differences in other RTs between the PTC taster groups are also of interest, as they reflect the ability to distinguish between the five basic tastes.

PTC non-taster status has been associated with a higher accumulation of adiposity [10, 14], and unhealthy food preferences and dietary habits (e.g., a higher consumption and acceptance of fat) [33, 38] that favor the development of chronic non-communicable diseases (e.g., diabetes, obesity) and certain types of cancer. Interestingly, our findings suggest that these health-impacting tendencies can also be related to a lower sensitivity to other basic tastes involved in calory intake, such as sweetness, as reported previously by our group [39], and umami. Furthermore, the effects of weight loss interventions or dietary restraint on BMI may vary according to the PTC phenotype [11, 40]. Intestinal type 1 taste receptors (T1Rs), which are also responsible for sweet and umami tastes, are associated with the secretion of gut hormones, interfering with sodium-dependent glucose transport after sugar ingestion [41]. Research on the physiological implications of the gustatory function is currently growing, and new data are emerging on its ability to predict health status and the role of taste sensitivity in disease prognosis [39, 42, 43]. These discoveries contribute to the body of evidence supporting the hypothesized relationship between PTC taste perception and body weight homeostasis in young healthy individuals.

The discovery of extra-oral T2Rs in several metabolically active tissues has generated intense interest in their physiological significance and potential health impact [44]. T2Rs, which are expressed in enteroendocrine cells, can be involved in nutrient-gut interactions that modulate the secretion of gut hormones such as ghrelin, cholecystokinin, and glucagon-like peptide 1, thereby influencing gastrointestinal motility, appetite, and glycemia [41]. Consequently, a T2R38 genotype could have important consequences for weight homeostasis involving some of these mechanisms, as suggested recently [45, 46]. These bitter taste receptors in the intestinal tract were found to be upregulated in overweight/obese subjects, indicating that sensory receptors are involved in diet-related weight increases [8, 47]. Interestingly, T2R38, at both RNA and protein levels, has been recently described in human adipocytes, with higher expression levels in the adipose tissue of obese compared to lean individuals. Moreover, the in vitro stimulation of T2R38 by PROP induced a decrease in lipid accumulation, suggesting that T2R38 expression can modulate adipocyte functions. T2R38 gene variants did not influence the expression levels of the receptor [48].

Although neither PTC nor PROP are found in foods, other thiourea-containing compounds such as glucosinolates are present in cruciferous vegetables. In the

present study, PTC taster status did not affect the liking or consumption of cruciferous vegetables, mustard, and bitter leaves. Scientific evidence for the relationship between the ability to taste PTC and reported preferences for bitter vegetables is inconsistent. Food choices are influenced by multiple factors, making it difficult to predict a habitual diet if physical activity, cultural practices, socioeconomic status, and food access are not taken into consideration. Current results indicate that a single marker, be it phenotypic or genotypic, is insufficient to fully characterize orosensory responses related to diet and health [32, 49].

This study was carried out in a mixed-sex cohort aged 18–30 years representative of the Spanish college student population. The methodology applied was low cost and non-invasive, and the sample size was large in comparison with previous studies. Height and weight were measured to eliminate the risk of false self-reporting. Furthermore, a quantitative determination of basic taste sensitivity was performed, in contrast with the majority of studies in this field, which are based on qualitative/semi-quantitative estimations. However, the study also has some limitations. First, BMI was used as a marker of obesity, although this has been questioned as a criterion for classifying fatness in college athletes and non-athletes [50]. Second, the estimation of RT values by same-different task methodology (discriminating test type) could have been more precise. Indeed, the lack of standardized screening methods for PTC/PROP taste sensitivity hinder valid across-study comparisons, although 3-alternative forced choice (3-AFC) with reversal might be the more adequate method, it would have been at the same time too tedious and tiring to the participants due to the high number of solutions tasted. Finally, few data about dietary intake were collected (e.g., no food frequency questionnaires or 24-h records) and data on bitter vegetable liking and consumption were only descriptive, based on dichotomous questions (yes/no).

Conclusion

The relationship of the three well-known phenotypes of PTC perception with anthropometric and clinical history variables, taste acuity determined by measuring basic taste RTs, and differences in the liking and consumption of *Brassicaceae* bitter vegetables was investigated in a large sample of young adults as part of a cross-sectional study. In this healthy homogeneous young population, only PTC super-tasters were differentiated from non-tasters and tasters by BMI, a factor related to weight control. Furthermore, PTC non-taster status was able to predict higher scores (low sensitivity) in other basic taste RTs and TTS. On the contrary, PTC taster status was not associated with liking perception and/or consumption of vegetables with a PTC-related bitter taste. Further investigations on super-tasting should be conducted to evaluate and confirm these associations and analyze the mechanisms involved.

Material and methods

Chemicals

Sucrose, monosodium glutamate (MSG), NaCl, citric acid, PTC, quinine, sinigrin and saccharine were supplied by Sigma Aldrich (St. Louis, MO, USA). Distilled water was used as the solvent to prepare the corresponding solutions.

Study design and participants

A total of 403 students were recruited between October 2017 and April 2019. Participants older than 30 years were excluded from the analysis ($n=6$). Missing PTC RT data was also an exclusion criterion ($n=7$). Among the participants in the study, 357 were aged between 17 and 29 years (mean age: 18.9 ± 1.7 years), but other students with missing age data were included on the assumption they belonged to the same age range. Thus, the final cohort consisted of 390 young adults, all of whom were graduate students in Culinary and Gastronomic Sciences, Food Science and Technology, or Human Nutrition and Dietetics at the Torribera Campus of the University of Barcelona.

The research (Torribera Students Taste Study) was carried out according to the Declaration of Helsinki for Medical Research on Human Beings (WMA, 2001), and approved by the Ethics Committee of the University of Barcelona (Institutional Review Board: IRB 00003099). All the students were given information about the study, knew the objectives and benefits, accepted, and formalized their participation by signing the informed consent.

Recognition threshold and stimulus concentrations

RT is the lowest concentration of a tastant that elicits a particular taste response [51]. The RT assessment methodology used in this study was a same-different task approach [52]. Participants were provided with successive sets of two samples. Each set contained one blank sample (water) and one stimulus sample (chemical dilutions). For each set, participants indicated if the samples tasted different and if they could recognize the taste. The regional stimulation was done by dropping 0.5 mL of sample at room temperature on the tip of the tongue for 5–10 s. Before tasting the next test solution, participants rinsed out their mouths with water and waited for 20 s.

Sets were presented in ascending concentrations (Table 4). The assay stopped when the participant correctly recognized the stimulus sample at a given concentration twice consecutively. The concentration at which

Table 4 Concentrations of taste test solutions

	Sweet	Umami	Salty	Sour	Bitter		
Score	Sucrose (mM)	MSG (mM)	Sodium chloride (mM)	Citric acid (mM)	PTC (μM)	Quinine (μM)	Sinigrin (μM)
1	1.2	3.0	3.9	1.2	0.7	9.4	50
2	2.3	7.5	7.8	2.3	3.5	18.7	100
3	4.7	15.0	15.6	4.7	14	37.5	300
4	9.4	30.0	31.3	9.4	56.2	75	600
5	18.8	60.0	62.5	18.7	112.5	150	–
6	37.5	120.0	125.0	37.5	225	300	–
7	75.0	–	250.0	75.0	900	–	–
8	150.0	–	500.0	–	–	–	–

MSG monosodium glutamate, *PTC* phenylthiocarbamide

the procedure stopped was considered the stimulus RT. We used a wide concentration range for each molecule, considering the bibliography about the topic [35, 53–55]. The order of sensory testing across taste qualities was the same among all participants and they were tested during the same test session. Before each new taste test, participants rinsed their mouths with water. Participants were asked not to chew gum, eat any product, or smoke for 2 h before the test.

Determination of PTC taster status

PTC taster status was determined on the theoretical assumption that the PTC phenotype is a marker of a variety of chemosensory experiences, including the RT [56]. Participants with a PTC RT score of 1 ($\leq$ 0.7 μM) were classified as super-tasters; those with a score of 2 or 3 (3.5–14 μM) were classified as tasters; finally, those with a score higher than 3 (> 14 μM) were in the non-taster group. The three PTC taster status groups correspond to the three phenotypic groups with a known distribution in the Caucasian population (approximately 25% super-tasters, 50% tasters and 25% non-staters) [2]. In parallel, taster status distribution was studied by calculating the ratio of the PTC RT scores divided by the NaCl RT scores. The proportions of the generated groups were very similar, and the PTC taster groups obtained by the two approaches had a Spearman coefficient correlation of 0.896. Moreover, the cumulative RT frequency curve for PTC also showed a trimodal distribution (Supplementary Fig. 1), corresponding to the cut-off used. Although we cannot eliminate all classification errors, the three rational strategies came with a similar conclusion.

Elderly tasters are reported to have a higher mean PTC RT than young tasters [57]. However, few studies have used PTC RT as a method of classifying individuals into groups of PTC taster status [4], and limited data were available in the literature to establish PTC cut-off scores for the present study, considering the age of the participants and the method used for evaluating taste function.

Predictor assessment: data collection

All participants filled in a brief structured self-reported questionnaire (paper) in person, which provided us with data about their clinical history and preference for and consumption of bitter vegetables. Accordingly, data about age, sex, smoking habits, discomfort in the mouth (caries and missing teeth) and nose-related complaints (sinusitis, rhinitis) were recorded. As only fifteen subjects had a smoking habit of more than 10 cigarettes/day, smoking habit was not a dividing factor in the analysis. First- and second-degree family histories of diabetes, hypertension, and obesity were also recorded. Liking and consumption of cruciferous vegetables, endives, arugula, and mustard were verified by asking a dichotomous question (yes/no). To establish perception of saccharin bitterness, the participants were administered a test solution (10 mM) and asked if they detected it or not.

Height and body weight of participants wearing light clothes and no shoes were measured following the International Standards for Anthropometric Assessments [58]. BMI was calculated as weight/height squared (kg/m^2) and classified as "low weight" if < 18.5 kg/m^2, "normal weight" if 18.5–24.9 kg/m^2 and "overweight-obesity" if $\geq$25 kg/m^2 [59].

Statistical analysis

Statistical analyses were carried out to compare the PTC taster status groups of non-tasters, tasters, and super-tasters. The Kruskal-Wallis rank-sum test was applied for continuous variables, Dunn's post-hoc correction for multiple comparisons, and the chi-square test was used to compare proportions of categorical variables among the PTC taster groups. Standardized PCA was applied, and PCs were selected based on a

parallel analysis. Sex stratification analyses were performed to ascertain the impact of sex on the results.

Basic taste RTs were scaled in multiples of 1 standard deviation (Table 4). The TTS was calculated by adding the normalized RT scores for the five basic tastes and dividing by five, resulting in a new variable ranging from 0 to 1. To estimate the different RTs, the following tastants were used: sucrose for sweet; MSG for umami; NaCl for salty, and citric acid for sour. The score for the bitter RT was calculated by adding together the respective normalized scores obtained for the three bitter tastants (PTC, quinine, and sinigrin) and dividing by three, which resulted in a representative bitterness sensitivity score ranging from 0 to 1. Variables were normalized using Min-Max scaling to range the data into the same scale, following the general formula:

$$x' = \frac{x - \min(x)}{\max(x) - \min(x)}$$

Due to the large dataset and the small number of participants with missing information, no data imputation was applied in the statistical comparisons. However, the mean age was imputed for the PCA, resulting in a total analysed PCA sample of 335 participants. Statistical tests were two-sided, and p-values below 0.05 were considered significant. All statistical analyses were conducted using the Stata statistical software package version 16.0 (StataCorp, College Station, TX, USA). Data were visualized using GraphPad Prism 9 (GraphPad Prism Software, Inc. La Jolla, CA).

Abbreviations

BMI: Body mass index; WHO: World Health Organization; SD: Standard deviation; PTC: Phenylthiocarbamide; TTS: Total taste score; PROP: 6-n-propylthiourail.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12263-022-00715-w.

Additional file 1: Cumulative frequency curves of the PTC recognition threshold scores.

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Authors' contributions

M.T-S: Conceptualization, methodology, data curation, software, formal analysis, and writing—original draft preparation. P.A. B-R: Methodology, data curation, software, formal analysis, and writing—original draft preparation. C. G.: Data curation and writing—reviewing and editing. R.M. L-R and G. N.: Supervision and writing—reviewing and editing. J.J. M: Conceptualization, methodology, investigation, visualization, supervision, writing-reviewing, and project administration. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available upon request to J.J. M.

Declarations

Ethics approval and consent to participate

The research (Torribera Students Taste Study) was carried out according to the Declaration of Helsinki for Medical Research on Human Beings (WMA, 2001) and approved by the Ethics Committee of the University of Barcelona (Institutional Review Board: IRB 00003099). All the students were given information about the study, knew the objectives and benefits, accepted, and formalized their participation by signing the informed consent.

Consent for publication

All the authors have consented to publication.

Competing interests

The authors declare they have no competing interests.

Author details

[1]Department of Nutrition, Food Sciences and Gastronomy, XIA, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain. [2]INSA-UB, Instituto de Investigación en Nutrición y Seguridad Alimentaria, Universidad de Barcelona, 08921 Santa Coloma de Gramenet, Spain. [3]CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain. [4]Department of Food Technology, Food Science and Nutrition, Faculty of Veterinary Sciences, Regional Campus of International Excellence "Campus Mare Nostrum", Espinardo, 30071 Murcia, Spain.

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Publisher's Note

3.2. Validation of biomarkers of intake of alcohol and specific alcoholic beverages

The specific **objective 2** of the present thesis was to validate biomarkers of ethanol intake *per se* and of those related to most of the larger categories of alcoholic beverages consumed.

To achieve this objective, a systematic review was carried out following the guidelines for biomarker for food intake reviews (BFIRev) [99]. A primary and a secondary literature search was performed to select articles that identified potential BFIs. Then, the status of validation of each candidate BFI was assessed, and future steps needed to reach the full validation proposed. The results obtained leads to the conclusion that biomarkers of alcohol, beer and wine intake cover recent high or moderate intakes reasonably well, while low intakes may go unnoticed, especially for ethanol per se. Therefore, markers sensitive to low alcohol intakes, smart biomarker combinations to discriminate different recent or longer-term intake scenarios and potentially better sampling methods to cover intermittent intakes are still needed. Results are shown and discussed specific and globally in Publication 5.

In addition, the biomarker of beer intake IX was applied as a compliance intervention tool in a beer intervention clinical trial. This information can be found in the publications belonging to the specific **objective 3** section (Publication 6, 7, 8).

Publication 5

Biomarkers for alcoholic beverages and moderate alcohol intake: a systematic literature review

Marta Trius-Soler, Giulia Praticò, Gözde Gürdeniz, Mar Garcia-Aloy, Raffaella Canali, Natella Fausta, Elske M. Brouwer-Brolsma, Cristina Andrés-Lacueva, Lars Ove Dragsted

Supplementary Material available in Annex 4. Submitted in Genes & Nutrition.

Abstract

Aim: The aim of this systematic review is to list and validate biomarkers of ethanol intake per se excluding markers of abuse, but including biomarkers related to common categories of alcoholic beverages.

Methods: Validation of the proposed candidate biomarker(s) was done according to the published guideline for biomarker reviews (BFIRev).

Results: The search resulted in more than 20,000 titles of which ~170 papers reported directly on biomarkers and applications in human studies. Biomarkers of alcohol, beer and wine intake cover recent high or moderate intakes reasonably well, while low intakes may go unnoticed. Inter-individual variation, variability in drinking patterns and variability in the beverage production processes all contribute as factors causing quantitative uncertainty regarding intakes while qualitative methods to discriminate no intake from moderate or high intakes are generally more reliable.

Conclusions: Common biomarkers of alcohol intake, e.g., as ethyl glucuronide, ethyl sulfate, fatty acid ethyl esters and phosphatidyl ethanol show considerable inter-individual response, especially at low to moderate intakes, and need further development and improved validation, while food intake biomarkers for beer and wine are highly promising and may help in more accurate intake assessments for these specific beverages.

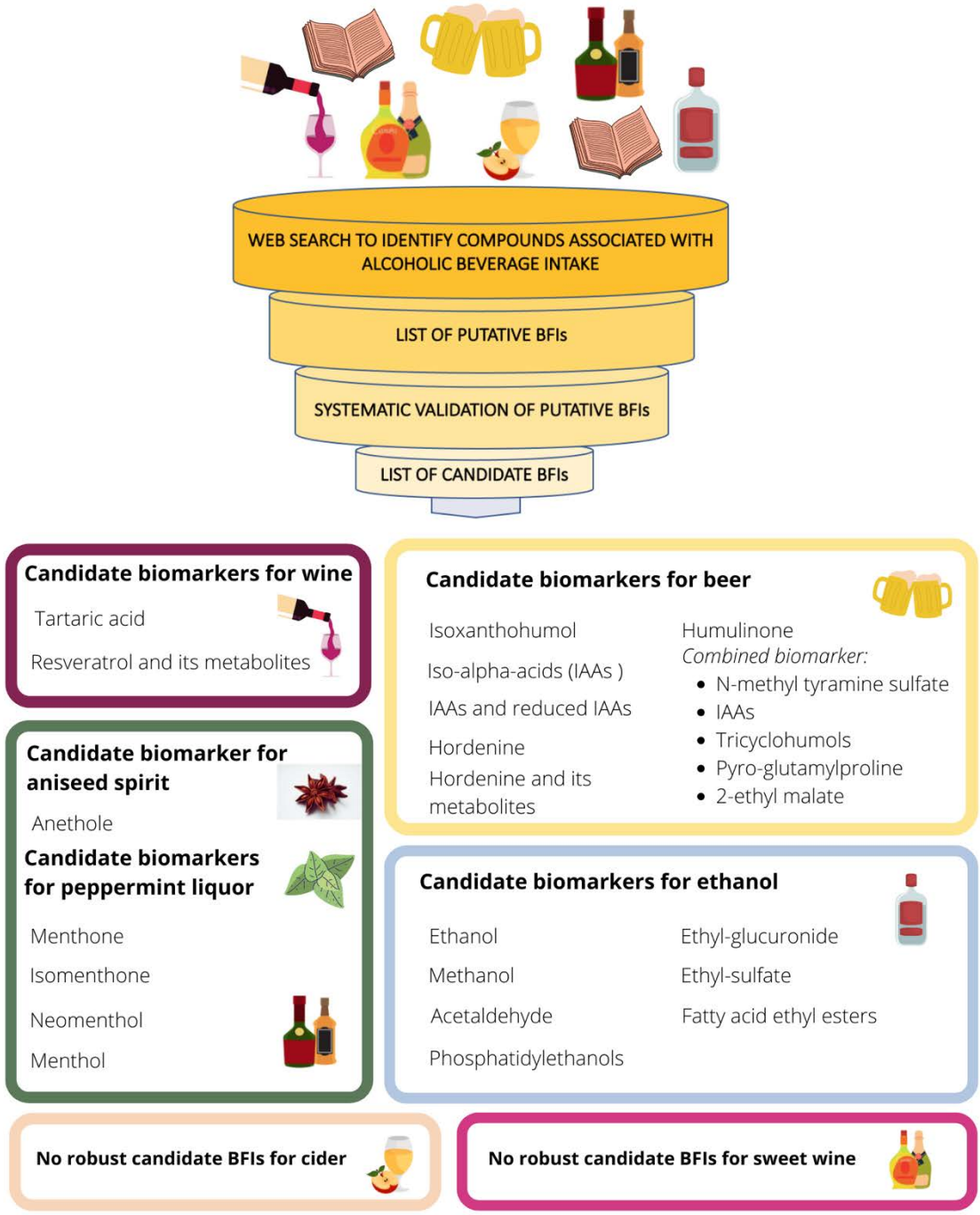

Figure 7. Summary of the candidate biomarkers for alcohol and specific alcoholic beverages (Figure 3 of the Publication 8).

16th October 2022

Dear Editor,

We would like to submit the manuscript entitled "Biomarkers of moderate alcohol and alcoholic beverages: a systematic literature review" to be considered for publication in Genes &Nutrition.

Numerous tools have been developed in order to assess alcohol intake including questionnaires, physiological measures, and biochemical assays on samples, and among them, objective measures have been a subject of considerable technical interest. In this context, the first objective of this systematic literature review was to use the BFIRev guidelines to inventory the potential biomarkers to estimate alcoholic beverage intakes while omitting the extensive literature on alcohol abuse. The second objective was to evaluate how well the identified intake biomarkers have been validated, and also what still needs to be done to further develop this area. For the present review, five subgroups of alcoholic beverages including the most widely consumed (beer, cider, wine, sweet wine, and spirits/distillates) were selected. Biomarkers were also assessed for general alcohol/ethanol consumption.

The results obtained leads to the conclusion that biomarkers of alcohol, beer and wine intake cover recent high or moderate intakes reasonably well, while low intakes may go unnoticed, especially for total alcohol. Therefore, markers sensitive to low alcohol intakes, smart biomarker combinations to discriminate different recent or longer-term intake scenarios and potentially better sampling methods to cover intermittent intakes are still needed.

We confirm that no part of the work is currently under consideration for publication elsewhere.

Looking forward to hearing from you,

Yours sincerely,

Dr. Lars Ove Dragsted
Department of Nutrition, Exercise and Sports, Faculty of Science
University of Copenhagen
1958 Frederiksberg C
Denmark

Biomarkers of moderate alcohol intake and alcoholic beverages: systematic literature review

Marta Trius-Soler[1-4], Giulia Praticò[1], Gözde Gürdeniz[1], Mar Garcia-Aloy[5,6], Raffaella Cana Natella Fausta[7], Elske M. Brouwer-Brolsma[8], Cristina Andrés-Lacueva[3,5,9], Lars C Dragsted[1]

[1]Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhag 1958 Frederiksberg C, Denmark

[2] Polyphenol Research Laboratory, Department of Nutrition, Food Sciences and Gastronor XIA School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Sp

[3]INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona, 089 Santa Coloma de Gramanet, Spain.

[4]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutric (CIBEROBN), Instituto de Salud Carlos III, Madrid, 28029, Spain.

[5]Biomarker & Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences a Gastronomy, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08(Barcelona, Spain.

[6]Metabolomics Unit, Research and Innovation Centre, Fondazione Edmund Mach, S Michele all'Adige, Italy.

[7]Research Centre for Food and Nutrition, Consiglio per la ricerca in agricoltura e l'ana dell'economia agraria (CREA), Rome, Italy

[8]Division of Human Nutrition and Health, Department Agrotechnology and Food Scienc Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, The Netherlan

[9]Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saluda (CIBERFES), Instituto de Salud Carlos III, Madrid, 28029, Spain

* Corresponding author: E-mail address: ldra@nexs.ku.dk; Tel: (+45)35332

Abstract

The predominant source of alcohol in the diet are alcoholic beverages, including beer, wine, spirits and liquors, sweet wine, and ciders. Self-reported alcohol intakes are likely to be influenced by measurement error, and thus affecting the accuracy and precision of currently established epidemiological associations between alcohol itself, alcoholic beverage consumption, and health or disease. Therefore, a more objective assessment of alcohol intake would be very valuable, which may be established through biomarkers of food intake (BFIs). Several direct and indirect alcohol intake biomarkers have been proposed in forensic and clinical contexts to assess recent or longer-term intakes. Protocols for performing systematic reviews in this field, as well as for assessing the validity of candidate BFIs have been developed within the Food Biomarker Alliance (FoodBAll) project. The aim of this systematic review is to list and validate biomarkers of ethanol intake *per se* excluding markers of abuse, but including biomarkers related to common categories of alcoholic beverages. Validation of the proposed candidate biomarker(s) for alcohol itself and for each alcoholic beverage was done according to the published guideline for biomarker reviews. In conclusion, common biomarkers of alcohol intake, e.g., as ethyl glucuronide, ethyl sulfate, fatty acid ethyl esters and phosphatidyl ethanol show considerable inter-individual response, especially at low to moderate intakes, and need further development and improved validation, while BFIs for beer and wine are highly promising and may help in more accurate intake assessments for these specific beverages.

Keywords: Biomarkers of food intake, alcohol, ethanol, alcoholic beverages

1. Introduction

Ethanol ('alcohol') intake ('drinking') has been associated with numerous adverse effects on health and on the quality of life, whereas light to moderate drinking, typically 1-2 drinks/day in western countries, has been associated with beneficial health effects [1,2]. In most countries alcohol intake is not recommended, whereas upper limits for moderate alcohol intake have been set at 1 or 2 units a day. The amount of alcohol in a 'unit' or a standard 'drink' varies from around 8-14 g (10-17.7 mL) between different countries, the lowest currently in the United Kingdom (UK) and the highest in the United States (US) [3,4]. Assessing alcohol intake is important for health and societal research, but also for forensic and other legal causes to investigate abuse/misuse of alcohol or to monitor abstinence when drinking is prohibited [5–7]. Numerous tools have therefore been developed in order to assess alcohol intake, including questionnaires, physiological measures, and biochemical assays on samples such as blood, urine, or hair [8,9]. However, the subjective tools (i.e., questionnaires) to assess alcohol intake are known to be biased by social and personal attitudes to drinking [10] and objective measures have therefore been a subject of considerable technical interest [11]. These objective measures may largely be divided into (a) direct measures relating to alcohol metabolites and (b) indirect measures relating more to the physiological and biochemical effects of drinking. Indirect markers are dominating research on risks and abuse of alcohol intake (i.e., longer-term intakes); while direct markers are used most often used to measure recent intake.

For the purpose of nutritional assessment there are interests in biomarkers of both recent and longer-term alcohol intake to study associated risks and potential benefits [12]. Moreover, there is considerable interest to discriminate between the different alcoholic beverages; that is, to objectively assess the type of alcoholic beverage consumed, for instance physiological or health effects specifically related to red wine or beer have recently been reviewed [13–15]. Assessing compliance is also important and demands objective tools to assess alcohol consumption;

factors such as the time lapse since last drink, the frequency of drinking, the different beverages consumed, etc. are also important questions in need of objective biomarker strategies.

The predominant source of alcohol in the diet are alcoholic beverages, including commonly consumed products such as beer, wine, spirits and liquors, sweet wine, ciders, as well as various niche products, e.g., kombucha. Besides, alcohol is also formed in several food fermentation processes and may exist as residuals in some foods [16] or may even be inhaled from environmental sources or formed to a variable extent in the human body [17]. While oral intake constitutes quantitatively close to 100% of relevant exposures in nutrition, some examples of other routes exist and have been of importance in forensic cases [18]. For the purpose of nutritional intake biomarkers of alcoholic beverages, the source, timing, frequency, and amount are all among the relevant variables to consider when assessing biomarker quality and use [19]. The aims of the current systematic review are a) to list all putative markers suitable for measurement of moderate alcohol intakes, and b) to validate these markers according to common guidelines, thereby pointing out what evidence is still missing in the scientific literature. In the following sections we report a systematic assessment of the literature on biomarkers of ethanol intake *per se* and of biomarkers related to most of the categories of alcoholic beverages, which contribute most to overall alcohol production. The review explicitly excludes biomarkers related only to intakes above moderation but has an additional focus on inter-individual response variability as well as any natural background levels of the biomarkers in subjects with no intake. What constitutes moderate intake is historically and geographically diverse and we have therefore covered the studies on biomarkers within the ranges reported as common social drinking, thereby excluding chronic abuse. Narrative reviews on alcohol intake biomarkers in relation to forensic and clinical studies have been published recently [15,18].

2. Methods

2.1 Selection of food groups

For the present review, five subgroups of alcoholic beverages including the most widely consumed (beer, cider, wine, sweet wine, and spirits/distillates) were selected. Biomarkers were also assessed for general alcohol/ethanol consumption. A systematic literature search was carried out separately for each alcoholic beverage subgroup and for alcohol/ethanol as detailed below.

2.2 Primary literature search

The reviewing process was performed following the guidelines for food intake biomarker reviews (BFIRev) previously proposed by the FoodBAll consortium [20]. Briefly, a primary research was carried out in three databases (PubMed, Scopus and the ISI Web of Science) using a combination of common search terms: (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation) AND (trial OR experiment OR study OR intervention) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (urine OR plasma OR serum OR blood OR hair OR excretion) AND (intake OR meal OR diet OR ingestion OR consumption OR drink* OR administration) along with the specific keywords for each alcoholic beverage subgroup (Supplementary Table 1). The fields used as a default for each of the databases were [All Fields] for PubMed, [Article Title/ Abstract/ Keywords] for Scopus, and [Topic] for ISI Web of Science. Breath alcohol was not systematically covered in the primary search but papers including data on breath ethanol levels were kept.

The last search was carried out in March 2022. It was limited to papers in the English language, while no restriction was applied for the publication dates. The research papers identifying or using potential biomarkers of intake for each alcoholic beverage subgroup and for total alcohol consumption were selected according to the process outlined in Figure 1. Articles showing use of the markers in human observational, or intervention studies were considered eligible. Additional papers were identified from reference lists of these papers and from reviews or book chapters identified through the literature search. Exclusion criteria for the primary search were

articles focused on the following effects of alcoholic beverage subgroups or ethanol/alcohol intake, while not using a biomarker of intake: (1) cholesterol, plasma lipids, inflammatory biomarkers, or blood pressure; (2) cardiovascular diseases, diabetes, or gout; (3) high alcohol consumption in relation to alcoholism; (4) other biomarkers (e.g., contaminants and effect markers), or (5) animal, *in vivo* and *in vitro* studies. Papers considering biomarkers of relevance only to alcohol abuse were omitted, except if they provided important information on e.g., kinetics .

2.3 Secondary literature search

For each identified potential biomarker of food intake (BFI) identified, a second search step was performed to evaluate its specificity using the same databases (PubMed, Scopus, and the ISI Web of Science). The search was conducted with ("the name and synonyms of the compound" OR "the name and synonyms of any parent compound") AND (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation OR pharmacokinetics) in order to identify other potential foods containing the biomarker or its precursor. Specific as well as non-specific biomarkers were selected for discussion in the text, while only the most plausible candidate BFIs have been tabulated, including the information related to the study designs and the analytical methods.

2.4 Marker validation

To evaluate the current status of validation of candidate BFIs and to suggest the additional steps that are needed to reach the full validation, a set of validation criteria [19] was applied for each candidate BFI. The assessment was performed by answering 8 questions related to the analytical and biological aspects of the validation together with a comment indicating the conditions under which the BFI is valid (see explanation under Table 1). The questions were answered with Y (yes, if questions were fulfilled under any study conditions), N (no, if

questions had been investigated but they were not fulfilled under any conditions), or U (unknown, if questions had not been investigated or answers were contradictory) according to the current literature.

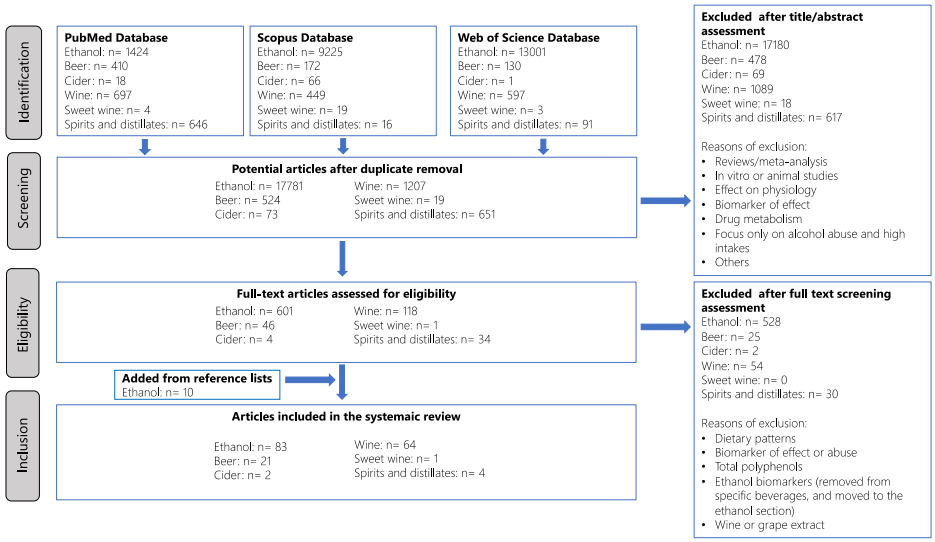

Figure 1. Flow-chart of the study selection according to guidelines for biomarker of food intake reviews (BFIRev) procedure.

3. Results

3.1 Alcohol/ethanol intake

The search for references to alcohol intake biomarkers resulted in 20,255 potentially relevant papers covering intakes of ethanol, beer, wines, spirits, and liqueurs; however, most of these were not related to biomarker development or validation but to many other fields within alcohol research, especially alcoholism (n=19,451), see Figure 1. In Table 1 there is a list of the candidate biomarkers identified for alcohol intake representing all the identified studies, along with data for their validation as biomarkers at low to moderate alcohol intakes. Table 1 builds upon the identified studies listed in Supplementary Table 2. The samples used include blood, urine, breath, and hair. The direct alcohol intake biomarkers in these various samples are almost all metabolites of alcohol, i.e., ethanol itself, acetaldehyde, or their adducts with other

biomolecules (Figure 2). For some beverages, especially beer and wine, characteristic compounds were observed as biomarkers. The proposed candidate biomarkers reflecting alcohol and specific alcoholic beverages intake are shown in Figure 3.

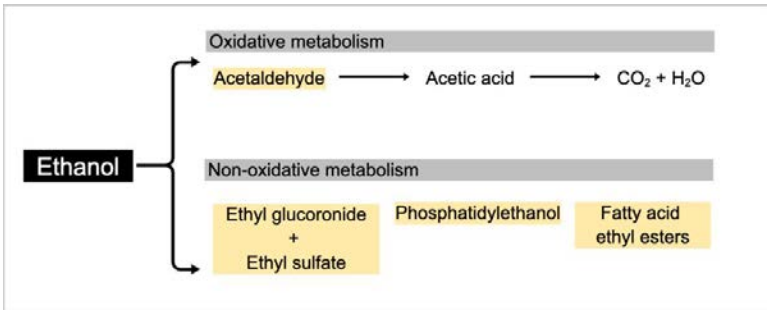

Figure 2. The metabolism excretion of ethanol in the human body.

Table 1. Overview of the current level of validation[1] of candidate BFIs.

Food item	Metabolites	Biofluid Locations	Questions[2]							
			1	2	3	4	5	6	7	8
Alcohol	Ethanol	Breath/Blood/Urine	Y	Y	Y	Y[3]	Y	Y	Y	Y
	Methanol	Blood	Y	Y[4]	Y	N	N[4]	Y	Y	U
	Acetaldehyde	Blood/Urine	Y	U	U	U[3]	U	U	U	U
	Ethyl glucuronide	Blood/Urine	Y	Y[4]	Y	Y[3]	Y	Y	Y	Y
		Hair	Y	Y[4]	U	N[3]	Y	Y	Y	U
	Ethyl sulfate	Blood/Urine	Y	Y[4]	Y	Y[3]	Y	Y	Y	Y
		Hair	Y	U	U	U	U	U	U	U
	Fatty acid ethyl esters	Blood	Y	Y[4]	Y	Y[3]	Y	N	Y	Y
		Hair	Y	Y[4]	N	N	Y	U	U	U
	Phosphatidylethanols	Blood/Erythrocytes	Y	Y[4]	Y	Y	Y	Y	U	N
Beer	Iso-α-acids (IAAs)	Blood/Urine	Y	Y	Y	Y	Y	U	Y	Y
	IAAs + reduced IAAs	Blood/Urine	Y	U	Y	Y	Y	U	Y	U
	Isoxanthohumol	Urine	Y	Y/N[5]	Y	Y	Y	Y	Y	Y
	Hordenine and its metabolites	Blood/Urine	Y	U	Y	U	Y	U	Y	Y
	Combined marker[6]	Urine	Y	U	Y	Y	Y	U	U	U
	Humulinone	Urine	Y	U	U	U	U	U	U	U
Wine	Resveratrol and conjugated metabolites	Blood/Urine/LDL	Y	U	Y	Y	Y	Y	Y	Y
	Tartaric acid	Urine	Y[7]	Y	Y	Y	Y	Y	Y	Y
Aniseed spirit	Anethole	Blood	Y	Y	Y	Y	Y	U	Y	U
Pepper-mint liquor	Menthone	Blood	Y	Y	Y	U	N	U	Y	U
	Isomenthone	Blood	Y	Y	Y	U	N	U	Y	U
	Neomenthol	Blood	Y	Y	Y	U	N	U	Y	U
	Menthol	Blood	Y	Y	Y	U	N	U	Y	U

[1] The answers Y and N in this table mean that in specific situations the marker has shown validity for the aspect in question. For any specific use the marker validity has to be reconsidered carefully.

[2] 1) Plausibility; 2) Dose-response; 3) Time-response; 4) Robustness; 5) Reliability; 6) Stability; 7) Analytical performance; 8) Reproducibility.

[3] Unexplained background levels commonly reported

[4] Not well documented at intakes below 5-10g alcohol

[5] Y is for males, N for females.

[6] N-methyl tyramine sulfate, iso-α-acids, tricyclohumols, pyro-glutamyl proline, 2-ethyl malate.

[7] Not plausible as a unique marker of wine intake but as a general marker of grape products.

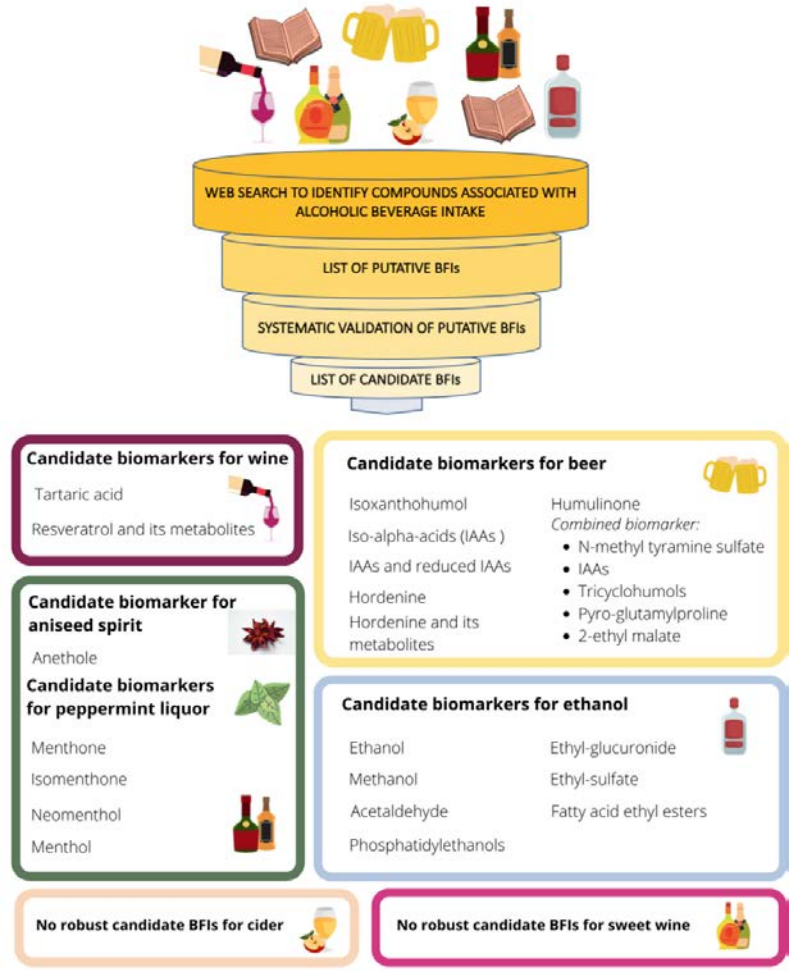

Figure 3. Summary of the candidate biomarkers for alcohol and specific alcoholic beverages.

Ethanol and methanol

Ethanol *per se* can be measured in breath, blood, serum, and plasma as well as in hair and urine, and all of these samples are commonly used to assess recent exposure in forensics. The most common marker used to assess recent alcohol intake is ethanol vapor in exhaled air, which is used routinely to test vehicle drivers, pilots, and other machine operators. The concentration of ethanol in blood, urine, hair, or tissue is used to assess recent exposure in forensics. Within 2-4 hours of moderate alcohol intake (1-2 drinks) and around 12 hours after high, acute alcohol

intake (binge drinking) ethanol itself cannot be measured any longer in breath, blood or freshly voided urine [21]. The presence of ethanol in human samples depends to a large extent on the exposure, the time since ingestion, and the genetics and lifestyle of the individual. Ethanol is metabolized by alcohol dehydrogenase (ADH, EC 1.1.1.1) to acetaldehyde and gene variants with very fast clearance result in fast removal, but these variants are rare in subjects of European or African descent but more common in the Middle East and Asia [22]. Most human subjects have zero-order clearance of ethanol from blood, meaning that the rate of metabolism is independent of the ethanol concentration with clearance at around 0.15 g/L/hour after 2 or more drinks, due to saturation of metabolism. Depending on body size and composition this means burning of around one unit of alcohol (10-15 g, depending on definition) in 1.25 (men) to 1.75 (women) hours, but women may have higher elimination rates than men, partially compensating for the difference in distribution volume [23]. At lower intakes when the major degradation pathway is no longer saturated, the rate gradually approaches first order kinetics, meaning that elimination becomes slower. High levels of ethanol inhibit the activity of ADH towards other alcohols, thereby causing accumulation of methanol and propanol. Ethanol is found at low levels in many foods, especially fermented foods and high endogenous production by fermentation (auto-brewing) is also known from rare cases in children as well as adults [24]. Low steady-state levels in subjects below 0.1 mg/dL have been reported by sensitive analyses (summarized in [25]).

Methanol is slowly formed during several endogenous metabolic processes and low levels are also coming from foods; the ethanol concentrations necessary for methanol accumulation may be observed already after a few hours of drinking. Therefore, measuring methanol in blood or urine is a useful marker within a day of alcohol intake to reveal a recent (binge) drinking episode or alcohol dependence (> 5 mg/L/day) [18]. It has recently been shown that methanol as well as 1-propanol are formed from ethanol in humans after acute intake of 40-90 g ethanol

and both compounds may therefore serve as potential markers of binge drinking [26]. The half-life of 1-propanol, which is also a potential microbial metabolite [27], is similar to that of ethanol, while methanol has a longer half-life making it useful for examining high drinking episodes within 1-2 days. However, moderate alcohol intakes may not inhibit ADH sufficiently to increase methanol levels and none of the alcohol congeners are therefore useful biomarkers of social (moderate) drinking.

The distribution volume for ethanol is mainly the water phase, meaning that subjects with a similar body weight will differ in blood ethanol concentration after exposure, depending on their fat mass. Thus, ethanol in blood, plasma and serum are useful biomarkers that will in most cases reflect recent intake in a dose-related manner. The concentration in breath is directly proportional to the concentration in blood at moderate intakes, so it will also reflect both dose and distribution volume. However, the breath test has limitations and must be confirmed by other biomarkers, especially in heavy drinkers [28,29].

Acetaldehyde

The primary metabolic product of ethanol is acetaldehyde formed by ADH [30], which may also be directly quantified in blood and urine samples. However, due to its reactivity with amino groups in proteins, acetaldehyde is reversibly or irreversibly bound to proteins. Acetaldehyde is further metabolized to acetate by aldehyde dehydrogenase (ALDH, EC 1.2.1.3), which is also polymorphic. In a recent study, acetaldehyde in whole blood was measured in wild type homozygous and ALDH-heterozygous Koreans by dinitrophenylhydrazine derivatization and liquid chromatography–mass spectrometry (LC-MS/MS) after a single challenge (0.8 g/kg body weight) with approximately 4 units of vodka [31]. No background was observed before the challenge, and blood levels were low in wild type homozygous volunteers, but peaked at 15 times higher levels in the heterozygotes ½-1 hours after the drink and was still detectable at 6 hours. Further validation of the method was

not reported. Blood alcohol concentration (BAC) was higher in the heterozygotes, indicating that there may be feedback inhibition of ADH by acetaldehyde [31]. In a recent paper on the carbonyl metabolome no acetaldehyde was reported in urine after derivatization with danzyl hydrazine [32]. No information was provided on the human donor, or the collection of the urine sample analyzed in this methods paper.

Protein adducts of acetaldehyde have been used to assess the average alcohol intake over the lifetime of the protein or cellular structure used for the assessment. For instance, acetaldehyde adducts in erythrocytes could theoretically be used to estimate intakes over its lifetime of around 120 days, while acetaldehyde in each centimeter of hair, starting from the scalp, might become a future method to measure average exposures per month [33].

Acetaldehyde binding to amino groups in proteins results in formation of Schiff bases. As long as these bases are not reduced, acetaldehyde can be released, and this is accelerated by acid and heat; this procedure was used already in 1987 to design a highly sensitive assay using plasma proteins or hemoglobin, and the method was later validated and widely used by insurance companies in the US to identify subjects at high risk of being alcohol abusers [34,35]. The method has a relatively high background in teetotalers for both plasma protein and hemoglobin adducts of acetaldehyde, overlapping with levels observed in alcoholics [34]. This would indicate that background metabolic processes leading to acetaldehyde formation are quite common and active. These methods have so far not been used to report levels in low or moderate alcohol users. Other methods to determine acetaldehyde have been developed using capillary electrophoresis (CE) or gas chromatography (GC) coupled with MS to identify acetaldehyde-protein adducts [36,37]. In the CE-based study an investigation of levels in three moderate drinkers (< 2 units/day) and one non-drinker were compared, showing apparent acetaldehyde-hemoglobin peaks only in the three drinkers [36]. In the GC-MS based study 20 human samples were also analyzed and in this case no overlap between levels in 10 non-

drinkers and 10 alcoholics was observed. However, background levels in non-drinkers were quite high and variable. The levels observed in this small sample set was apparently independent of age, smoking, ADH and ALDH genotypes, or body mass index [37]. Larger studies are needed to confirm this and to address other aspects of method validation (Table 1). Additional methods have been proposed e.g., the formation of a cysteinyl-glycine adduct measurable in rat urine has been reported [38]. A new method for measuring free cysteine- and cysteinyl-glycine adducts of acetaldehyde in urine and plasma has recently been published but adducts were not found in humans after acetaldehyde exposure due to too high background levels [39]. However, these adducts are not stable over time in serum and were found to be destabilized in the presence of strong nucleophiles [40].

Acetaldehyde is genotoxic and reacts directly with DNA bases, to form e.g., N^2-ethyl-deoxyguanosine residues and several other DNA-adducts [41,42]. These may be measured directly in tissue DNA, or they may be repaired, forming excretion products to be measured in urine. The adducts measured in DNA have been used as markers of alcohol dose in investigations on ethanol intake and show dose-dependence and time course of repair and elimination in oral cavity exfoliated epithelium. Single moderate alcohol doses lead to measurable acetaldehyde in saliva and in exfoliated oral cells [43]. The oral cavity adducts may therefore be candidate biomarkers of recent alcohol intake, especially for liqueurs providing high local concentrations. However, the effect was only observed locally; acetaldehyde adduct formation in lymphocytes and granulocytes were not affected by three single moderate doses provided in the same pilot study [41]. In conclusion, acetaldehyde forms adducts with proteins and DNA and moderate exposures may lead to increases, however relatively high background levels are often observed potentially limiting usefulness, and thorough validation will be needed for these methods to translate into useful biomarkers of moderate alcohol intake.

Ethyl glucuronide

Ethanol is conjugated by UDP-glucuronosyl transferases (UDPGT; EC 2.4.1.17) to a low extent by phase II metabolism into ethyl glucuronide (EtG). EtG was first observed and later isolated from urine of ethanol exposed rabbits [44,45]. The first quantification in human urine was not until 1995 [46] and soon after it was suggested as a biomarker of alcohol intake in forensics [47]. For about 20 years now, EtG has become widely used in forensic studies due to its sensitivity and reliability. However, most studies are related to abuse and therefore beyond the scope of this review. Ever since the earliest findings in animal studies it is clear that several UDPGT isozymes in rabbits and in rodents [48] can conjugate ethanol. The Km for the most active human UDPGT isozymes is on the order of 8 mM [49]. This corresponds to the peak blood alcohol concentration after intake of around 10 g alcohol and the rate of formation of EtG is therefore expected to be lower at low intakes and to increase at higher intakes. This has been confirmed in several studies in humans, where non-linear dose-concentration and dose-excretion curves for EtG have been observed showing increased fractional levels with the administered dose [25,50,51]. In Measurements of EtG during pregnancy to reveal sporadic social drinking have also been investigated in forensics showing variable frequencies of positive samples in different populations, with many cases among women reporting total abstinence [52,53]. Characteristic individual EtG background levels in urine have been observed in alcoholics housed for weeks in a closed ward by repeated daily sampling during abstinence [25]. These results indicate that low levels of EtG may occur even without alcohol intake but long-term fully controlled studies to confirm this are needed.

EtG is quite water soluble and is therefore often assessed by GC-MS or LC-MS/MS in blood or urine samples collected within hours of exposure. The elimination kinetics are slower than for ethanol itself and the ability to measure recent alcohol exposure by this marker may therefore extend beyond 12 hours in blood [54] and 24 hours or more in urine, depending on the dose and the sensitivity of the analysis [46]. The time windows for measuring blood EtG

and excretion of EtG in urine are important for assessing recent intakes based on spot samples. In several studies serial blood samples have been collected to compare EtG with BAC or controlled alcohol intakes (n=1-54) [55–57]. The useful time window for EtG measurement after a single ethanol dose was reported to be at least 10 hours in blood and 24 hours in urine after a peak BAC of 0.12 g/L (n=10) [55]. In another study showing dose-response, the apparent time window for EtG in serum was 25-50 hours, depending on alcohol dose, with the lowest dose tested being ~25 g (2-2½ units) [57]; no background level above the method cut-off for EtG could be measured after 1 week of abstaining. In a recent study there was a high variability in the peak level and total EtG excretion in 24 volunteers after drinking 48 g of alcohol as beer [58]. Inter-individual variation in peak serum levels of EtG (at 10-20 hours) and time to reach plasma levels below LOQ (range: 35-100 hours) has been reported after binge-drinking of 64-172 g alcohol within 6 hours [59,60]. Since EtG in urine depends on the diuresis it is often recommended to correct EtG for creatinine excretion; this method improves analyses of excretion kinetics [56]. The limit of quantification (LOQ) for EtG has been reported to be as low as 0.02 mg/L [61], well below the widely accepted cut-off at 0.1 mg/L, which corresponds to a level typically observed in a spot urine sample collected around 24 hours after intake of 10 g of alcohol. A few documented cases exist of measurable EtG in urine above this level from non-drinkers, including pregnant women and children, indicating that sources of alcohol or EtG exposure are likely to exist in non-drinkers; these sources may include the use of hand-sanitizers, gut microbial fermentation, and possibly consumption of fermented foods [25] [53]. EtG is stable in the autoanalyzer at 4 ºC for up to 96 hours [62]. In a study of EtG-free blood samples spiked with ethanol, EtG formation was observed at 37 ºC after 3 days; degradation of the EtG in positive blood samples was observed during storage at 25 for >3d or at 37 ºC for > 1day, but EtG was stable at 4 ºC or -20 ºC [63]. Measurement of EtG with a dip-stick has been shown to be insufficiently sensitive for routine use [54], but in a prospective

cohort study among subjects with mild symptoms of kidney disease values measured by dip-sticks correlated well with self-reported alcohol intake (r=0.68, *p*-value < 0.001) [64]. However, a large part of the subjects reporting no intake (~50%) exhibited EtG-values above the 0.1 mg/L cut-off, suggesting potential effects of kidney disease or its etiological factors on EtG formation or excretion.

EtG also accumulates in hair, making hair samples an attractive means of potentially assessing past exposures [65,66]; the method seems specific for heavy drinking, but sensitivity issues and possibly also inter-individual variation may render it less useful for determination of intake levels within light to moderate drinking [67,68]. Improved methods for extraction and milling of the hair samples increase the sensitivity [69] but only few studies have experimentally investigated relevant hair EtG levels at different levels of social drinking. In a study of 15 students excessive drinkers were clearly identified while there was an overlap between levels observed in students reporting moderate intakes or abstinence and only one of five abstainers had levels below detection [62]. In a study of a few teetotalers (children) and social drinkers (up to 20 g/day), all samples were negative (<LOD of 2 pg/mg hair) [70]. At intakes of 0, 1 or 2 drinks/day for 12 weeks, both dose-response and time response were observed at the group level using standardized protocols for hair analysis [71] these protocols have been debated and could possibly be improved [72,73]. Standardized cut-offs for very low or no drinking and for heavy drinking have been agreed at 7 and 30 pg/mg hair, respectively [74]. Background levels are still occasionally found in abstainers [75] and levels tend in general to be higher with high body mass index or in subjects with kidney damage [75,76]. Hair EtG measurements may also be less sensitive at low alcohol intakes (≤ one drink per day) [77]

In conclusion, EtG measured by LC-MS/MS in blood or urine are short-term markers of alcohol intake with a time window exceeding that of BAC, with well-known time- and dose-response, and with legal cut-off levels for background exposures that are rarely exceeded in non-drinkers.

However, levels between the suggested cut-offs of 0.1 and 0.5 mg/L have been observed repeatedly in non-drinkers and intakes below 10 g alcohol may occasionally overlap with background levels in a time window of 24 hours. In hair EtG by LC-MS/MS is a well validated marker for high alcohol consumption, however it is highly variable and less sensitive in subjects with lower intakes.

Ethyl sulfate

Ethyl sulfate (EtS) is another common, low-abundance phase II metabolite of ethanol with characteristics very similar to EtG. The first data on its formation also came more than 70 years ago from animal studies (i.e., rats) [78] and the first human urine identification and first legal method were published during 2004 [6,79]. Already some of the earliest studies confirm EtS as a plausible marker since several aliphatic alcohols were substrates of mammalian sulfotransferases (EC 2.8.2.2) [78]. Several human isoenzymes can perform the sulfation of ethanol *in vitro* with quite variable conjugation rates as already shown in 2004, but in 10 volunteers provided with 0.1 or 0.5 g ethanol per kg body weight (3-27 g) the excreted amount varied only by a factor of 3 within as well as between subjects, independent of sex [80]. Variability in human absorption and excretion kinetic constants in 13 male volunteers after a dose of 30-60 g ethanol was also reported to be only around 2 for each [81]. The time-response in 13 volunteers was also investigated after consumption of a low alcohol dose (0.1 g/kg body weight) showing a peak at 2-5 hours and a time window of detection of 6-10 hours; preliminary indication was also shown of a higher fractional as well as total excretion at a 5 times higher dose (time window ≥ 24 hours) [80]. In a recent study in 24 male and female volunteers provided with 47.5 g alcohol (beer) within 15 minutes, the inter-individual variability in EtS excreted over 10.5 hours was more than 100-fold at the excretion peak apex and with a variable peak time of 2.5-8.5 hours [58]. EtS showed considerable correlation with measured levels of EtG before as well as after the drink. In analogy with EtG, background levels of EtS are only

observed by more recent, sensitive analyses [57]. Background levels in most volunteers after 3 days of abstaining was high (>1 mg/L for EtS and 1.8 mg/L for EtG) with reasonable correlation between markers ($r^2 = 0.56$). In this study one of the volunteers hardly produced any EtS or EtG after drinking 47 g alcohol in 15 minutes, while a few others only showed very low levels, indicating that these markers may miss a small percentage of drinkers. ADH genotyping was not provided, but the authors suggest polymorphic phase 2 enzymes to be the main cause of this variability [58]. however, this is less likely considering the high correlation between the EtS and EtG markers. BAC at 30 minutes after the drink was apparently not associated with low EtG or EtS excretion and further investigation to identify the causes of such marker variability is needed in order to use EtS (and EtG) in routine analysis at low intakes. The higher fractional excretion of EtS at higher doses indicates a relatively high Km in analogy with EtG [80]. A 25-fold higher Km for formation of human EtS than for EtG *in vitro* [49] but this does not seem to correspond with the observed EtS and EtG formation in humans showing similar dose- and time-response compared with EtG [57]. Additional study of Km for the human sulfotransferases forming EtS is therefore needed. In a study of human blood samples that were blank, alcohol-spiked, or positive for EtS no formation or degradation of EtS was observed over 7 days in any samples at temperatures from -20 ºC to 37 ºC [82]. EtS is also stable in a standardized anaerobic bacterial incubation while < 20% were lost under aerobic conditions over 28 days at 20 ºC in the dark [83].

Only a single publication has so far evaluated EtS in hair as a marker of alcohol intake and it was reported that it may actually compare favorably with hair EtG, however more studies are needed before it can be validated as a biomarker of low or moderate alcohol intakes [84].

In conclusion, EtS in serum or urine is a well validated biomarker of recent alcohol intake, comparable with EtG. Likewise, EtS measurements are accurate and precise, show dose- and time-response even at quite low intakes but some subjects produce very little while others have

measurable background levels after abstinence. Care must therefore be exercised in interpretation of individual levels in the lower range. Hair EtS has not been extensively validated and needs further investigation.

Phosphatidylethanols

Phosphatidylethanols (PEths) are polar fatty acid esters, known to be formed enzymatically by phospholipase D in red blood cells, especially at high blood alcohol levels [85]. *In vitro* studies also indicate that relatively high blood ethanol concentrations are needed for PEth formation, with PEth 16:0/18:1 as the most abundant species [86]. PEth has therefore been historically regarded as a useful marker of high alcohol intake, e.g., in forensics [87]. However, the levels observed at lower intakes have not been well studied until recently; studies on alcoholics have indicated variable levels even at intakes below 40 g/day during less intense drinking periods, overlapping with levels observed at much higher intakes [87]. PEth levels in dried blood spots were shown not to differ from those in fresh blood samples in a group of 40 alcohol detoxification patients attending a ward; all patients had levels indicating problem drinking but the levels varied approximately 100-fold [88].

Some studies have investigated the PEth blood levels over time in abstainers, after withdrawal from heavy intakes, or during experimentally controlled multiple or single moderate alcohol doses or abstaining [89–91]. One study investigated PEth over time during abstention [90]; in this study of 56 alcoholic withdrawal patients and 35 non-drinking in-patients PEth was measured after 4 weeks without alcohol intake. The non-drinkers had blood PEth < 0.3 µM (LOQ for detection by an older light-scattering technique) throughout, and the two groups were easily differentiated with 100% specificity (the area under the receiver operating characteristics curve (AUROC) = 0.97) using a cut-off at 0.36 µM. Some withdrawal patients had levels below the cut-off despite measurable BAC at admission. This study demonstrates that abstainers and heavy abusers can mostly be discriminated by PEth after 1-4 weeks [90] but that inter-

individual differences in formation and response levels exist and may complicate judgement in individual cases. [85,86]. Another study that included 36 subjects (32-83 years old) evaluated the change in PEth levels at 3-4 weeks intervals in subjects attending outpatient treatment to reduce drinking. Comparison of individual changes in PEth concentration *vs.* past 2-week alcohol consumption between two successive tests revealed that an increased ethanol intake by ~ 20 g/day (1-2 drinks) elevated the PEth concentration by on average ~ 0.10 µM, and *vice versa* for decreased drinking [92]. The elimination characteristics of three PEth homologs have been studied in 47 heavy drinkers during approximately 2 weeks of alcohol detoxification at hospital. During abstinence, the elimination half-life values ranged between 3.5–9.8 days for total PEth, 3.7–10.4 days for PEth 16:0/18:1, 2.7–8.5 days for PEth 16:0/18:2 and 2.3–8.4 days for PEth 16:0/20:4. Individual significant difference in the elimination rates between different PEth forms were also found, indicating that the sum may be the best biomarker [93].

In a randomized parallel intervention study PEth during abstention or moderate alcohol intakes (16 g/day for women and 32 g/day for men) were compared in 44 volunteers over a period of 3 months [89]. In the abstaining group, PEth decreased on average to below LOQ for the sensitive method applied (0.005 µM) and only 6 of 23 subjects still had measurable levels (all < 0.04 µM). In the group randomized to drinking, all subjects had levels > LOQ after 3 months but average PEth did not change despite higher intakes by a factor of 1.6-56 according to baseline interviews. AUROC for qualitatively discriminating between the two groups at 3 months was 92% (82-100%). This study shows that PEth has a good ability to discriminate abstainers from moderate drinkers and that 0.05 µM is a reasonable cut-off although larger studies would be needed to ascertain that higher levels are not observed in a small minority of abstainers [89], especially among subjects with reduced kidney function. Along the same line, studies from Sweden categorize subjects with levels below 0.05 µM in blood as “abstainer”

0.05-0.3 µM as "moderate drinkers", and > 0.3 µM as "overconsumer" [94,95]. Current evidence does not indicate that PEth is formed at different rates in men and women [96,97].

In a recent randomized and highly controlled experimental study, healthy volunteers were provided with either 0.25 or 0.5 g ethanol/kg body weight (1-3 drinks in 15 minutes) after only one week of abstaining; measurable levels in whole blood were evident in all volunteers after alcohol intake and was observable until 14 days later in most subjects [91]. In a similar study done by the same research group, doses of 0.4 or 0.8 g ethanol/kg body weight were administered (2-5 drinks in 15 minutes) [96]. Background levels and a proportional dose-response increase were observed, no sex difference in PEth homologues pharmacokinetics were found and PEth 16:0/18:2 synthesis was higher than PEth 16.0/18.1 at both doses; however, the mean half-life of PEth 16.0/18.1 was longer than that of 16.0/18.2 (7.8 ± 3.3 days and 6.4 ± 5.0 days, respectively) [96]. These studies indicate that moderate alcohol intakes over a short period affect PEth in all subjects but with large variations between individuals, especially at the higher doses. This was also reported previously by others [98] and has even been observed experimentally in primates [99]. Individual measurements may therefore not accurately reflect the consumed amount of alcohol, even in a very controlled setting of high intakes over a limited time span.

Quantitation of PEth has improved much in sensitivity in recent years and several studies have investigated levels even in pregnant women. In three studies, 1.4-40% may not be abstinent as determined by PEth at the end of the first trimester, depending on the population and analytical sensitivity [100–102]. Few studies exist at low to moderate consumption levels using high sensitivity analytics, but subject-reported intakes correlate with blood PEth [91,101]. In a study using a new highly efficient ultrasound-assisted dispersive liquid-liquid microextraction procedure, PEth dose-response was observed in groups reporting alcohol intake levels from 14-98 g/week, 98-210 g/week, or > 210 g/week. Dose-response was presented as differences

between the three group averages and indicate considerable overlap between individual levels at these three intake levels [103]. While abstainers are often below the detection or cut-off level for PEth [104], and many social drinkers have non-detectable PEth with current methods [98,104] up to a few per cent of subjects reporting to be abstaining seem to have low but measurable levels of PEth in their samples [100]. This is likely due to incorrect reporting of intakes. Recent PEth measurements have a good concordance with other biomarkers at chronic high alcohol intakes and seems more sensitive than older methods [87,90]. High PEth (> 0.3 µM), indicating heavy alcohol consumption, is also 95% concordant with blood EtG > 100 ng/mL; however, at PEth levels indicating moderate alcohol intakes (0.05-0.3 µM), concordance with EtG (>1 mg/L) is only 56% [105]. Formation and degradation of PEth has been investigated over 7 days with blood samples that were either negative for PEth, added with ethanol, or positive for PEth, [63]. Formation of PEth was observed at 37 ºC and -20 ºC, peaking after 4 days and then decreasing, while a linear loss of PEth with time was observed at 25 ºC, reaching approximately 40% at 7 days. Stable levels over 7 days were observed at 4 ºC. Further studies are needed to investigate potential loss of PEth during long-term sample storage at -20 ºC or -80 ºC.

In conclusion, with highly sensitive analytical methods PEth is a sensitive and specific marker of ethanol intake at levels as low as a single alcoholic drink with an extended time window of days or weeks after intake, but inter-individual variations are high after single as well as repeated doses. PEth seems useful in studies of high drinking levels, but may also prove useful for estimating average intakes in groups of social drinkers; further studies to verify this should include additional repeated sampling in a controlled study of low-responders to PEth and of reported alcohol abstainers having positive blood PEth.

Fatty acid ethyl esters

Alcohol also interferes with lipase metabolism substituting for aliphatic alcohols that esterify fatty acids; in neutral lipids this results in formation of fatty acid ethyl esters (FAEEs) [106]. FAEEs are formed by cellular synthesis, e.g., by mononuclear blood cells, directly from ethanol at physiological doses [107] and formation is likely to be directly proportional to the individual total BAC over time, given by the area under the BAC curve (AUC) [108]. FAEEs are stable at 4 ºC or below for at least 48 hours [108]. FAEE stability has been investigated in a 7-day storage experiment with blood samples that were either negative for FAEE, negative but added with ethanol, or positive for FAEE [63]. In the negative samples FAEE was formed at 25 ºC and 37 ºC. Addition of ethanol to negative samples strongly increased FAEE formation at these temperatures. Formation of FAEE was also observed in the positive samples where FAEE increased at 37 ºC up to 5 days, followed by degradation. Formation increased also up to 4 days at 25 ºC and remained stable until 7 days, while FAEE in the positive samples was stable at 4º C and -20 ºC for 7 days. These results indicate that sampling and storage are crucial for the analysis of FAEEs and that formation as well as degradation may distort results.

Peak serum FAEE concentrations may be around twice as high in men compared to women at the same blood alcohol concentration, indicating that the AUC for BAC rather than peak BAC reflects FAEE formation, while dosing rates (drinking within 2-90 min) had little effect on kinetics [109,110]. In a single-dose study with alcohol doses from 6-42 g in healthy young men, the characteristics of the most abundant FAEEs (palmitic-, oleic- and stearic acid ethyl esters) were showing initial kinetic properties similar to plasma EtG with peak formation within 30-60 minutes, clear time- and dose-response relationships, and a time window for detection in blood plasma of 3-6 hours [45]. The fractional formation (or rate of degradation) of FAEEs was dependent on the dose, indicating non-saturated kinetics for the enzymes involved in FAEE metabolism; while C_{max} for FAEE was almost linear after single doses of 6-42 g alcohol, the AUC was almost 4-fold higher on average at the highest compared with the lowest dose,

and inter-individual variation also increased with dose [51]. These results would indicate that FAEE degradation rather than its formation may be affected by saturation kinetics. After binge-drinking 64-172 g alcohol, background serum FAEE was reached 15-40 hours later [59,60]. Again, inter-individual variation was large [59]. After chronic high intakes FAEEs can be observed in blood for a much more extended period [111], even up to 99 hours [60]. This may be seen as additional evidence that FAEE elimination or excretion may show saturation kinetics, being compromised in alcoholics; this might be due to alcohol-induced effect on blood lipids, but studies differ on whether other blood lipids do [109] or do not [60] affect FAEE. Serum albumin has been shown to affect FAEE levels significantly, possibly by affecting FAEE transport [112]. FAEE above background levels may also be measured in dried blood spots collected up to 6 hours after high doses of alcohol [113], however this technique has not been investigated at moderate or low doses.

FAEE in hair has been investigated to a considerable extent. Levels increase with chronic intake levels [70,114], however individual variation in hair FAEE is considerable with a large overlap between subjects claiming no, moderate, or high habitual intakes [70,114,115]. This variability includes null as well as high levels in hair from some subjects in all three groups. Analysis of hair segments indicate similar but highly individual profiles; further comparison of FAEEs on the hair surface or the inner parts of hair indicate that FAEE enter into the hair from hair sebum [114]. FAEE in hair from different body locations has been shown to correlate, albeit with large variations within and between subjects [116]. In one study the authors found no correlation between FAEE and EtG in hair [70], indicating that incorporation of these compounds may be affected by different biochemical or physiological processes. FAEE was measurable in all hair samples using sensitive analytical techniques, even in children's hair [70]. FAEE has also been detected in sebum collected by skin wipe tests showing that teetotalers and social drinkers were not different, however heavy drinking affected skin sebum

levels [117]. These findings indicate that endogenous formation pathways for FAEE may potentially exist.

FAEEs are sensitive to hair products containing alcohol [118], and a negative test for FAEE in serum or EtG in serum or urine along with positive FAEE or EtG in hair is regarded as reflective of hair product use [115]. In 8% of cases negative for FAEE, EtG may be measured in hair, which is likely to reflect the potential presence also of EtG in some hair products [115]; this might indicate that a non-trivial percentage of cases positive for both EtG and FAEE in hair might be artifacts due to the use of several hair products and hence, not reflective of alcohol use. Hair FAEE may also be affected negatively by shampooing and potentially by other hair products, which could potentially extract FAEE from the hair [118]. However, in large cross-sectional studies among forensic cases, neither body composition nor any use of hair wax, grease, oil, gel, or spray had any major effects on hair FAEE [119,120]; instead, bleaching and/or dyeing reduced hair FAEE. Higher levels of FAEE as well as EtG was observed in abstainers than in moderate drinkers within this target group; this observation was ascribed to misreporting [120].

In conclusion, FAEE is formed readily from ethanol by lipases, apparently in a dose-response fashion related to the area under the BAC curve; this curve is known to vary between individuals but transport, degradation, and excretion of FAEE may also depend on blood levels and on drinking habits, leading to large inter-individual differences in kinetic behavior of FAEE measurements. Heavy drinking leads to delayed FAEE clearance, however in moderate drinkers, plasma, or serum FAEE levels decrease to baseline at a time point between those of BAC and EtG. Hair FAEE seems to be observed at levels above LOQ more readily than hair EtG, and is practically always detected by sensitive methods, even for teetotalers, including children. This might indicate the presence of external or endogenous sources or of measurement errors that are still not explained. However, a large, strictly controlled study is

still missing on FAEE in blood as well as hair, especially investigating the levels in teetotalers and light to moderate drinkers.

5-Hydroxytryptophol and related metabolites

A few other markers should be mentioned here since they have been applied for 'direct' measurement of steady-state alcohol intake. These are metabolites formed at an altered rate following high ethanol intake, namely a decrease in 5-hydroxyindole-3-acetate (5-HIAA) and an increase in 5-hydroxytryptophol (5-HTOL); the latter is measured in more recent studies as its glucuronide (5-HTOLG), which is more abundant [121,122] in the urine. The ratios of 5-HTOL:5-HIAA or 5-HTOLG:5-HIAA as well as the ratio 5-HTOL to creatinine in urine have been shown to peak 4-6 hours after a single dose of 0.8 g/kg alcohol (high intake). The ratios stayed above baseline until 16-26 hours later [123] thereby forming a marker of recent high alcohol intake with an excretion time window of urine ranging between that of ethanol and of EtS or EtG [50]. Little investigation has been done on 5-HTOL at low to moderate intakes of alcohol, or on the detailed kinetics of the marker at single or chronic intakes. The markers can therefore not be validated at moderate alcohol intakes.

Metabolomics investigations

Several studies have applied untargeted metabolomics (metabolite profiling) to discover and validate biomarkers of general alcohol intake by comparison with dietary instruments such as food frequency questionnaires [124]. In a study of 3,559 female twins from the UK, who reported their alcohol intake by food frequency questionnaire (FFQ), increased levels of hydroxyvalerate, androgen sulfate metabolites and several other endogenous metabolites were associated with alcohol, but no direct markers of alcohol intake were observed by the profiling technique [125]. In an NMR metabolomics study from Finland, 9,778 young adults (53% women) with moderate alcohol intakes according to questionnaires were investigated; no direct

markers of alcohol intake were observable but lipoprotein markers (e.g., HDL), phospholipids, androgens and branched-chain amino acids associated with alcohol intake corroborating findings in other studies [126].

In other observational studies using metabolic profiling to investigate alcohol intake, EtG is frequently observed along with other metabolites associated with alcohol intake. In the Lung, Colorectal and Ovarian Cancer Screening Trial, FFQ data from 1,127 postmenopausal women (50% having breast cancer) were used to find serum metabolites associated with alcohol intake [127]; these included EtG and a large number of androgen steroid hormone metabolites as well as hydroxyisovalerate and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) (a fish intake marker). A metabolite profiling study of 849 males and females from the PopGen study in Kiel, Germany, confirmed most findings from previous studies in UK and US, showing EtG along with hydroxyvalerates, androgenic metabolites and CMPF to be significantly associated with alcohol intake [128].

Some of the associations with alcoholic beverages intake may reflect biological effects of alcohol, e.g., on lipoproteins and several lipid classes [129–131], or on steroid metabolism affecting androgens and estrogens [126–128,132]. The associations may also reflect apparent confounders of alcohol intake such as fish [128,130] coffee [130] or tobacco [133] related metabolites, or with specific alcoholic beverages (covered later in this review), but few besides EtG are likely to directly reflect alcohol intake. This is supported by the country- or sex-specific nature of the associations, for instance none of the previously mentioned metabolite associations were observed in Japanese cohorts, where only men were included in the analysis [134,135].

Mono- and dihydroxy-valeric acids have been observed in several studies [128,130], however the cause of their association with alcohol has not been investigated extensively. Two reasonable explanations may be proposed; a) some shorter- or branched-chain hydroxylated,

and branched chained acids are oxidized metabolites of the side products (fusel) commonly formed during alcoholic fermentations; or b) alcohol intake affects branched-chain amino acid metabolism [136], leading to higher postprandial plasma levels and increased degradation into hydroxyvalerates. Further studies are needed in order to investigate these possibilities; if hydroxy-valerates result from fusel, they may prove useful in future combined markers to estimate intakes of specific alcoholic beverages.

Indirect measures of alcohol intake

Although these markers are not the primary subject of this review, they are shortly mentioned here because they are often used in assessment of alcohol intake. Some indirect markers are in reality efficacy markers that may be affected by high, chronic alcohol intake.

Alcohol is acutely as well as chronically toxic to the liver and hepatic enzymes such as gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate transaminase (AST) therefore leak into the blood as part of the toxic response to high alcohol intakes [18]. This toxic response is useful to assess whether hepatic effects are found in association with alcohol intake, but the tests are not specific to alcohol since most other liver conditions also increase GGT, ALT and AST [137].

Three markers of common use in alcohol research are the mean corpuscular volume of the erythrocyte (MCV), carbohydrate deficient transferrin (CDT) and plasma sialic acid index of apolipoprotein J, all measured in blood. Among these, the sialic acid seems to compare with liver enzymes [138,139] while MCV is related to nutritional status [137], but none of them are relevant at moderate intake levels.

Daily use of alcohol is also associated with a number of more general biochemical and physiological effects even at light to moderate intakes (< 20 g/day), including an increase in high-density lipoproteins (HDL) and adiponectin, and at high doses also increased heart rate

and higher blood pressure [140]. The most widely used marker among these is the increase in HDL cholesterol with alcohol intake, and this marker as well as its main apolipoprotein A1 (ApoA1) seem sufficiently sensitive at the group level to pick up contrasts of a single drink a day versus abstaining [141]. However, since not all subjects may react by increasing their HDL and since many other factors affect the level of this lipoprotein, the marker is most useful at the group level, i.e., to assess whether a change in alcohol intake is taking place in a group of subjects. None of the HDL subfractions seem to respond differently compared with total HDL or total ApoA1 [141].

While none of the indirect measures of alcohol intake are specific or very sensitive, attempts have been made to combine them into a multivariate model to predict moderate *vs*. high intakes of alcohol. The so far best investigated model is the Early Detection of Alcohol Consumption (EDAC) score combining 36 routine clinical chemistry and hematology markers that may to some extent be affected by daily alcohol intake. The specificity for detecting problematic daily alcohol intake levels was found to be above 90% for both males and females by EDAC, however the sensitivity in the first published study was quite low, below 50% [142]. subsequent testing in much larger sample materials have confirmed higher specificity and report sensitivities of 70-85%, resulting in overall AUROC values ranging from 80-95% [143,144]. The EDAC score is well validated with receiver operating characteristics (ROC) of around 0.95 for identifying heavy drinkers [35]. However, this categorical tool cannot be used for more accurate assessment of recent or longer-term light or moderate alcohol intake and is not useful for alcohol intake assessment in nutrition studies.

In conclusion, these markers and classification tools are not tabulated as valid biomarkers within moderate intakes in Table 1 but are listed among disregarded markers in Supplementary Table 3.

Marker validation

Candidate and established markers of moderate alcohol intake are listed in Table 1 along with their validation by eight validation criteria, while markers that are not able to reflect such intakes are listed in Supplementary Table 3. Among ethanol/alcohol biomarkers, ethanol has been validated for dose- and time response and is also broadly used due to good analytical performance, robustness, reproducibility, reasonable stability, and reliability. The drawbacks are considerable inter-individual variability in response after a given dose, and a short half-life resulting in a narrow time window of detection. Methanol is formed by several endogenous processes and degradation is inhibited by ethanol at higher doses. Dose- and time-response is therefore only seen at higher chronic intake levels or after binge drinking, and methanol is not a valid marker for moderate doses of ethanol. The robustness is weak due to variable other sources of exposure but analytical performance by GC is well established and reproducible.

Acetaldehyde might potentially be an ideal marker of long-term intake but is not extensively investigated. iAs a primary metabolite of ethanol it is plausible but there are no established and validated analytical methods, dose- and time-responses are not well known, robustness is challenged by exposures from other sources, including endogenous formation; moreover, acetaldehyde stability, reliability and reproducibility seem to depend on the analytical approach or are simply unknown.

EtG in blood or urine is analytically well established, quite reliable, and reproducible, however formation kinetics varies between individuals. It is stable at low temperatures, robust, and dose- and time-response are well validated at moderate and high single or repeated doses. The major weakness of this marker is the large variability in response at low alcohol intakes and an unknown source of background in some subjects. EtG in hair is more variable between subjects having similar intakes than blood or urine EtG, and its robustness is affected by hair products; dose-response seems fair at higher intakes, but time-response is complex due to hair growth

and loss of EtG due to wear and tear, including hair wash. The analytical performance is well documented.

EtS is another direct phase 2 metabolite of ethanol (hence plausible) and very similar to EtG in terms of all performance parameters, but causes of low formation in some subjects is unexplained. EtS may be observed at slightly lower alcohol intakes compared to EtG, but this needs further verification. EtS in hair is not yet well documented.

FAEEs in blood are apparently proportional to the AUC for alcohol in blood, however formation seems higher in men than in women. The rate of FAEE degradation in the blood varies between individuals, and FAEE is also unstable in blood samples at temperatures above 4ºC. Biological degradation is much delayed in heavy drinkers, strongly distorting the time-response curve at higher regular intakes; this may be used to identify problem drinking but reduces the applicability of the marker as a BFI for alcohol intake in studies where alcohol abusers may be among participants. Due to the high inter-individual variation, FAEE dose-response only gives a rough estimate of the intake level with considerable misclassification at the individual level. FAEE in hair is a promising marker for estimation of longer-term intake levels, however the variation between individuals seems even larger, and background levels are therefore highly variable, so more investigation will be needed in order to understand the biology behind high variability and background levels to further develop and evaluate the appropriate use of this marker.

Blood and dried blood spot PEth are still methods under development, resulting in some heterogeneity in the literature regarding the levels observed [145,146]. PEth is clearly dependent on the activity of phospholipase D, leading to considerable inter-individual variation. PEth stability and formation in the samples may be an issue, and so are effects of drying the blood and keeping the blood spots at room temperature [82,147,148]. The most sensitive methods for PEth analysis also reveal individual variability but at the same time

indicate that background levels are low for the majority of subjects. Individual levels after extended periods of abstaining or low intakes are still missing in the literature and reliability in terms of relationships with actual doses are not sufficiently investigated at lower doses.

3.2 Beer

Beer is one of the world's oldest drinks [149] and the most widely consumed alcoholic beverage [150]. It is a very complex beverage comprised of thousands of compounds such as oligosaccharides, amino acids, nucleotides, fatty acids and phenolic compounds [151,152]. Traditionally, basic ingredients of beer are water, sprouting cereal grains, yeast and boiled hops (wort) as raw materials; their transformation products formed during malting and fermentation are suggested as a source of potential candidate beer intake biomarkers. Barley is the most commonly used cereal, though wheat, maize and rice are also used, mainly as addition to barley. The appearance and flavor of the beer is affected not only by the type of cereal grain but also by many other parameters such as type of malting process, temperature, fermentation type, mashing and the variety of hops used for the wort. The wort provides highly characteristic components to the beer imparting bitterness, odor, and aroma. Some of the characteristic phytochemical constituents of hops are α-acids, β-acids and prenylated chalcones such as xanthohumol (XN) [153]. These compounds may not be specific to beer intake since hops products are also consumed as herbal remedies, however upon boiling of the wort the α-acids are isomerized and degraded forming other chemical structures, iso-α-acids (IAAs), that are only found in beer. Therefore, compounds produced from rearrangement of hop constituents can be suggested as plausible candidate beer intake biomarkers.

Iso-α-acids and reduced iso-α-acids

IAAs exist in three predominant analogue forms (isohumulones, isocohumulones, and isoadhumulones) and each of them are also present as diastereoisomers [154]. The *cis:trans*

ratios of IAA (usually ~2.2:1) is influencing beer bitterness [155,156]. Rodda *et al.* (2013) suggested IAAs and reduced IAAs as biomarkers of beer intake [154]. They could quantify *trans*-IAAs and qualitatively monitor *cis*-IAAs in plasma at 0.5 hours and up to 2 hours after beer intake in a pilot study with one subject [154]. Postprandial studies investigating the excretion profile of IAAs after beer intake revealed a rapid absorption of IAAs into plasma (T_{max} 30-45 minutes), compared to the excretion profile in urine that typically shows a peak between 90 minutes and 3 hours [157,158].

Despite their specificity for beer, potential applicability of IAAs as quantitative biomarkers of beer intake is limited by their instability since their quantity varies during storage [153,159]. The degradation is strongly dependent on the stereochemistry of the IAAs. *Trans*-IAAs are degrading faster than *cis*-IAAs, leading to the formation of tri- and tetra-cyclic compounds during storage. In urine, oxidized degradation products such as mono- and di-hydroxylated humulones have been observed both for *cis*- and *trans*-IAAs [160]. An untargeted LC-MS based metabolomics study revealed many of the oxidized excretion products in urine following a single drink of alcoholic or non-alcoholic beer in a cross-over design. None of the IAAs were detected in a pilot validation study with a low-hopped beer variety, underpinning the limitation of IAA metabolites as a reliable marker only for hopped beer intake [157]. This suggests that the IAAs in low-hopped beers are complete degraded or present at too low levels for detection and use as BFI.

Reduced IAAs, namely rho-IAA, tetrahydro-IAA, and hexahydro-IAA, have also been proposed as promising beer biomarkers. Reduced IAAs are light-stable synthetic derivatives of IAAs; they are usually added to hops to avoid light-induced degradation of IAAs resulting in undesirable (stall) aroma of beers bottled in clear or green bottles and hence, subject to light exposure [161]. In one study, the levels of IAAs were found to be lower or insignificant for clear (or green) bottled beers [162]; measures to stabilize their flavor and bitterness can

therefore be taken, such as the addition of reduced IAAs or a high content of isocohumulone [163]. The total level of IAAs together with reduced IAAs has been suggested as a combined qualitative beer intake biomarker with a specificity of 86% in plasma of post-mortem specimens [162]. However, further validation studies are needed for more general use.

In addition to IAAs and reduced IAAs, an oxidation product of α-acids called humulinone has been proposed as a biomarker of beer intake based on LC-MS profiles of urine collected after 4 weeks of a beer consumption in an intervention study [164]. Even so, humulinones are not only minor biotransformation products of α-acids but their concentration in beer is also shown to be diminished with longer-term storage, leading to the formation of other compounds [153,165]. This might reduce the potential usefulness of these compounds as biomarkers.

In terms of bioavailability, oral administration of IAAs to rabbits leads to recovery of less than 6% of the dose in urine and feces, suggesting that their metabolism potentially goes through phase I and II reactions [166]. Incubation of IAA with rabbit microsomes demonstrated cytochrome P450 catalyzed oxidation and transformations of IAA with formation of many compounds. Oral administration of IAAs to rabbits did not show any indication of direct glucuronidation or sulfation [167], yet phase II metabolism takes place through cysteine and methyl conjugation of oxygenated IAAs as demonstrated in urine metabolic profiles following beer consumption [157].

Isoxanthohumol

Other hop components, named prenylated phenols (isoxanthohumol (IX), 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN), and XN), have been widely investigated due to their biological activity and potential health effects [168–170]. In line with the formation of IAAs, IX is formed through cyclization of XN during wort boiling. The most abundant prenylated flavonoid in beer is IX (3-6 μmol/L) whereas XN, 6-PN and 8-PN are comparably minor

constituents (~0.03 µmol/L) [153]. More importantly, 8-PN is also formed through the conversion of IX by the intestinal microbiota [171] or through the cytochrome P450 catalyzed O-demethylation [172]. Therefore, the concentrations of 8-PN and IX in body fluids depend not only on their amount in beer consumed but also on host factors, i.e., their potential biotransformation [173,174].

IX is not yet documented to come from any other dietary source than beer or hop extracts. Quifer-Rada *et al.* (2013) developed a LC-MS method for analysis of IX, XN and 8-PN to qualify beer consumption in a single-dose drinking study with 10 subjects [175]. Eight hours after the consumption of a single moderate dose of beer, spot urine samples showed a significant increase only for the IX concentration in all subjects. Surprisingly, 8-PN was also detected in a spot urine after 4-days of a wash-out period in all subjects. Therefore, a delayed conversion of IX to 8-PN has been proposed [18, 19] and may indicate the usefulness of these compounds to assess either very recent (IX) or past intakes (8-PN) within several days; further studies are needed to investigate the kinetics of 8-PN excretion.

IX has also been evaluated as a urinary BFI for beer in three different trials [176]. In a dose-response, randomized, cross-over clinical trial a linear association between beer dose and IX was observed in male volunteers, while IX among females showed individual saturation kinetics of excretion. Inter-individual differences in conversion of IX to 8-PN by the intestinal microbiota has been previously reported [170] and could be an influencing factor contributing to the saturation kinetics in females. In a second randomized cross-over intervention trial with 33 males consuming beer, non-alcoholic beer, or gin for 4 weeks, suitability of IX as a qualitative biomarker of beer intake in men was evaluated. The prediction of beer intake (beer and non-alcoholic beer *vs.* gin) achieved a sensitivity and a specificity of 98% and 96%, respectively. Lastly, beer intake data, recorded by a validated food frequency questionnaire, from a randomly selected subgroup of 46 volunteers participating in the PREDIMED cohort

was assessed resulting in a 67% sensitivity and a 100% specificity. The low sensitivity was justified by the large range of beer intakes (22-825 mL/day), although some low volume drinkers in the group could also have been misclassified as non-beer drinkers. The analytical method has subsequently been used to assess volunteer's compliance in two additional beer interventions [164,169]. The authors reported an increase of IX in 93.5% of collected urine samples from both intervention groups, drinking beer or non-alcoholic beer, respectively [169].

In a subsequent paper, Quifer-Rada *et al.* (2014) concluded that IX is a specific and accurate biomarker of beer intake [176], however others have pointed out that this result did not take into account the previously demonstrated extensive glucuronidation of prenylflavanoids [177]; other authors applied hydrolysis of glucuronides in the urine prior to analysis to calculate the total prenylflavanoids excreted [178,179]. Recently, Daimiel *et al.* (2021) measured plasma and urinary levels of IX and 8-PN by treating the samples with a mixture of β-glucuronidase and arylsulfatase to liberate any conjugated IX and 8-PN [180]. As expected, urine IX concentration was higher after beer and non-alcoholic beer intake compared with both washout periods, while an increase in plasma IX was only found after alcoholic beer intake. Furthermore, stability of 8-PN in urine after beer consumption and in plasma after beer and non-alcoholic beer interventions suggests that the compound is useful as a beer intake biomarker. Breemen *et al.* (2014) studied the profiles of 8-PN, 6-PN, IX, and XN and their conjugates in serum and in 24 hours urine samples from 5 women following a boiled spent hops extract intake [177]. In serum, the half-life of IX and 8-PN (free and glucuronide conjugated) are up to 24 hours and >24 hours, respectively in different individuals [177]. One of the findings was a large inter-individual variability in the excretion profiles related to the conversion of IX to 8-PN. This may complicate the applicability of IX as a single quantitative biomarker of beer intake for both men and women. Furthermore, prenylflavanoids behave differently from most polyphenols since they are unstable at acidic pH. Therefore, a specific

analytical method must be applied to determine them in biofluid samples after beer consumption [175], potentially complicating the use of these markers in multi-marker methods.

Hordenine and its conjugates

Besides compounds originating from hops, germinated barley contains hordenine (N,N-dimethyltyramine), which has also been suggested as a biomarker of beer intake [181]. Hordenine is produced during germination of barley and transferred to beer from malted barley. The appearance of tyramine methyltransferase activity during germination associates with the accumulation of N-methyltyramine, a precursor of hordenine [182]. Thus, products made with ungerminated barley such as barley bread do not contain hordenine. Steiner *et al.* (2016) [181] developed a LC-MS method for quantification of hordenine in a drinking study with 10 subjects drinking either beer or wine. The results demonstrated detection of hordenine in serum samples only after beer consumption. Hordenine concentration in serum varied according to the type of beer consumed and its hordenine content. After beer intake, the serum profile implied total removal of hordenine by 2.5 hours, but only one subject was profiled [181]. Sommer *et al.* (2020) also evaluated free hordenine and its conjugates in plasma as beer intake biomarkers [183]. The concentration of free hordenine reached its peak at 30-90 min after the beginning of the exposure and then rapidly decreased. Part of the free hordenine was biotransformed into glucuronide and sulfate conjugates immediately after its absorption. Hordenine sulfate $T_{max.}$ was between 90-150 min, while hordenine glucuronide T_{max} was 150-210 min in plasma. Urinary excretion peaked at 2-3.5 h after beer consumption but was still detected after 24h [183]. In another study, hordenine in urine reached its maximum excretion into urine already at 0-1.5 h following beer intake [157]. However, hordenine was also detected prior to beer intake in some subjects, albeit at lower levels, indicating non-compliance, very long excretion half-life for some subjects, or intake of hordenine through consumption of other barley germ containing foods or other food sources [157,184]. Further studies are needed to evaluate the

potential use of hordenine as a biomarker of beer intake. In particular, it should be assessed whether the concentration is sufficiently high for beer intake compared to the consumption of other foods, potentially containing barley germs or other confounding food sources, such as bitter orange or certain dietary supplements [5,185].

Metabolomics investigations

Quifer-Rada *et al.* (2017) [164] investigated urinary metabolomics profiles following 4 weeks of intervention with beer, non-alcoholic beer or gin. The authors proposed humulinone and 2,3-dihydroxy-3-methylvaleric acid as potential novel biomarkers. However, based on the established standard procedure for identifications of metabolites in untargeted metabolomic studies [186], the identification of the latter was at level 2. The authors suggested that 2,3-dihydroxy-3-methylvaleric acid may be a product of fermentation i.e., a *Saccharomyces cerevisiae* metabolite, and this is corroborated by several observational metabolomics profiling studies; however, also wine is fermented by *Saccharomyces cerevisiae* and several hydroxy-valerates have been found to associate with intakes of beer, wines, and total alcohol [128,130]. Therefore, further studies are needed to confirm the specificity of 2,3-dihydroxy-3-methylvalerate as a biomarker of beer intake.

Another untargeted metabolomic study investigated the immediate effect of beer intake on urinary and plasma LC-MS profiles [157]. Many of the compounds associated with beer were originating from hops, yet those were either oxidation products or IAAs and as mentioned previously their level may change with storage. Other compounds were originating from wort, fermentation, or human metabolism of IAAs. Although those were clearly upregulated with beer intake, they were also present at least in some of the baseline samples. Therefore, a combined biomarker model was proposed [157]. For the aggregated beer intake biomarker, IAAs, and their major degradation products, tricyclohumols, were selected as hop metabolites, a sulfate conjugate of N-methyl tyramine (a hordenine precursor) as a barley metabolite, pyro-

glutamyl proline as a product from the malting process and a compound putatively identified as 2-ethyl malate, as a known product from fermentation. The combined biomarker model from 24 hours pooled urine samples of 19 subjects was validated against an independent study with four subjects in which they consumed two different types of beer. The biomarker model predicted all the samples collected up to 12 hours correctly (AUC = 1). This proposed biomarker model still needs to be validated in other studies with an observational setting to confirm robustness.

Marker validation

Among the beer biomarker candidates, IX has been investigated for many different aspects of validation. The major issues for the potential application of IX as a biomarker of beer intake is its conversion to 8-PN in the gut, the extensive glucuronidation and the interindividual and potentially sex-dependent variation in excretion kinetics. Instead of using only IX, a combination of IX, 8-PN and their conjugates might be a promising approach as a qualitative biomarker of beer intake. The stability is the main concern for IAAs, therefore their combination with the reduced IAAs is also promising. Hordenine may not be specific to beer, thus further studies are required to evaluate its excretion in relation to other foods. The combined biomarkers approach is a highly promising tool for beer intake but still needs validation in observational studies. The assessment of the candidate beer intake biomarkers by the full set of validation criteria can be found in Table 1.

Cider

Cider is a beverage obtained from alcoholic fermentation of apples or pears. It is very popular in the UK, which is also the largest producer and consumer in the world. Cider is also consumed in other European countries, such as Spain, France, Ireland, and Germany, and low- or non-alcoholic versions are common soft drinks in some countries, including Sweden. According to

the European Cider Trends 2020, the cider consumption in Europe from 2015 to 2019 is roughly 4 L/capita/year (from 0.15 L/capita/year in Russia to 14 in UK) [187]. In recent years there is a gradual but constant increase in cider consumption [187], probably due to the consumers' appreciation of its low alcoholic content and because it is perceived as natural, genuine, and healthy.

To date, there are no untargeted metabolomics studies investigating the metabolic effect of cider consumption, while two pilot studies have used targeted approaches to identify specific cider polyphenolic metabolites [188,189]. In the first study, 6 human subjects consumed a high single dose of cider (1.1 L), and polyphenolic metabolites were searched in plasma and urine samples after β-glucuronidase and sulfatase treatment [188]. Low levels of isorhamnetin (3'-methyl quercetin), tamarixetin (4'-methyl quercetin) and caffeic acid derivatives were found in human plasma after hydrolysis of conjugates, while hippuric acid and phloretin were found in urine. The second study was focused on the metabolism of dihydrochalcones, which are phenolic compounds distinctive of apple and apple products [189]. In this study, 9 healthy subjects (21-42 years old) and 5 subjects with ileostomy (40-54 years old) received a single dose of cider (500 mL) and the main metabolites found in plasma, urine, and ileal fluid were phloretin-glucuronides and phloretin-glucuronide-sulfates. The main metabolite in all biological samples was phloretin-2'-*O*-glucuronide, having a T_{max} in plasma of 0.6 hours and accounting for 84% of the cider-related metabolites found in the urine of the volunteers [189].

With the exception of phloretin derivatives, which are specific to apple products, other putative biomarkers identified such as hippuric acid and quercetin metabolites are unspecific and relate to almost any intake of fruit or vegetables. In fact, they have been already identified after consumption of other foods rich in polyphenols [190–193] and have been suggested as possible dietary biomarkers of total fruit and/or vegetable consumption [194,195]. Phloretin and phloretin conjugates are found in urine after consumption of apples and apple products [196],

including cider [197]. Human supplementation studies demonstrate that single doses of apple or apple juice, as well as cider, determine the appearance of phloretin derivatives in plasma and urine [198]. However, phloretin derivatives have been detected in human urine also after grapefruit juice and orange juice consumption, either as a result of naringenin metabolism or adulteration [199]. Moreover, phloretin excretion, determined in 24 hours, overnight or in morning spot urine samples, has been suggested as a short-term dietary biomarker of all fruits, of fruit juice consumption, and/or apple consumption [200–204]. Without additional markers representing the apple fermentation or the ethanol content to form a combined biomarker, the phloretin metabolites would not seem generally suitable as biomarkers of cider intake. In conclusion, there are not many studies investigating biomarkers of cider intake and none of the suggested biomarkers appear to be adequate or specific to cider intake.

3.3 Wine

Wine is a common beverage consumed in Mediterranean countries, obtained through the fermentation of grape must. Mediterranean diet has been defined by low to moderate amounts of red wine often accompanying main meals, among other dietary factors [205,206]. Basic ingredients of wine are water, grapes and yeast as raw materials and their transformation products formed during maceration and fermentation [207]. Generally, the ethanol concentration in wine ranges between 10-13%. More than 500 compounds have been found in wine, derived primarily from the few compounds that occur individually at high concentrations. The main compounds that occur at high concentration are water, ethanol, organic acids, sugars, and glycerol. Those are primarily responsible for the taste and mouth-feel. Besides, phenolic compounds are an additional large and complex group of compounds of particular importance to the characteristics and quality of wine [208]. Polyphenols from wine can be divided into two primary groups: the flavonoids and non-flavonoids. Red wine is around 10-fold higher in polyphenolic content than white wine [206]. Due to the maceration during red wine production,

extraction of color and other substances from grape skin and seed occurs, so that polyphenolic compounds in red wine increase. Colorless and filtered grape juice is used during white wine alcoholic fermentation, so that contact with grape skin is avoided [207].

Resveratrol and its conjugates

3,4',5-Trihydroxystilbene, commonly known as resveratrol (RV), is a natural stilbene present in grape and grape products. They are the primary sources of dietary stilbenes, especially in red wine [193]. During the red wine making process, skin, and seeds, which are the RV richest parts of the grape, are macerated and stay in contact with the alcohol formed during the fermentation. Both processes facilitate the extraction of RV and explain why red wine contains more stilbenes and other polyphenols than white wine [209]. RV and its derivatives can also be found in minor concentrations in some nuts (e.g., peanuts, pistachios), berries, and in dark chocolate [210].

RV can be found as diastereoisomers that coexist in plants as well as in wine, although the *trans* isomer appears to be the more predominant and stable natural form [209,211]. RV has been widely studied for being a biologically active molecule, however its bioavailability is limited due to rapid metabolism after absorption [212]. Indeed, metabolites are the primary circulating forms [213]. Metabolism of RV in humans involves the formation of glucuronides and sulfate conjugates of the RV absorbed in the small intestine [212,214]. The unabsorbed RV reaches the colon and is converted into dehydroresveratrol (DHRV) by the microbiota [215]. Total RV glucuronides have been reported to be a putative intake biomarker of wine consumption [216], but ignoring part of RV metabolism with this approach may limit its applicability. Other authors have used enzymatic hydrolysis of conjugates to liberate RV as wine intake biomarker [217–219].

Strategies to increase RV bioavailability have been evaluated in several single dose studies [214,216,217,220,221]. In these studies, RV conjugates have been confirmed in plasma, serum, and urine after wine consumption [214,216,217,220–222]. Rotches *et al.* (2012) reported 17 metabolites including conjugates of RV, piceid and DHRV in human biological samples after red wine intake [223]. The main RV phase II metabolite found in plasma and urine was *cis*-RV-O-glucuronide, with a C_{max} ~2-6 times higher than the other glucuronides at 2-2.5 hours after the wine consumption. RV glucosides were rapidly absorbed and appeared around 1h after intervention, while phase II and microbial metabolites appeared between 0-8 hours and 4-12 hours, respectively [221]. Additionally, a high inter-individual variability was found in C_{max} and AUC of DHRV glucuronides, most likely due to a high heterogeneity in the microbiota between the participants [221]. RV metabolites have also been observed in human LDL particles after a single dose of 250 mL of red wine, indicating affinity for lipoprotein particles [223].

Randomized, controlled, cross-over intervention trials over periods of 3-4 weeks have been performed to compare effects of red wine, dealcoholized red wine and gin [213,219,224]. Phase II derivatives of RV and microbiota derived DHRV metabolites in 24-hour urine samples were sensitive and specific to wine consumption, being a useful tool to evaluate compliance in the clinical studies thereby having a potential applicability for making associations between the intake of wine and biological effects [213,219,224]. In a comparative study between the 4 weeks consumption of red wine or dealcoholized red wine, no differences between the interventions were observed in terms of concentrations of RV metabolites excreted [213]. More precisely, several combinations of different phenolic metabolites (mainly gallates) and RV metabolites (host and microbial) were shown to predict wine consumption with an AUC of up to 98% for urine samples and 91% for plasma samples with 4 weeks of red wine, gin or dealcoholized red wine intake [219]. However, the combined biomarkers have not been

evaluated for robustness or for high and low red wine intake levels in cross-sectional studies, so further validation is needed. The marker combinations are independent of alcohol since dealcoholized red wine was detected just as well as the alcohol containing wine.

Recently, González-Domínguez *et al.* (2020) optimized a multi-targeted metabolomic platform for the quantitative analysis of 450 food-derived metabolites by ultra-high performance liquid chromatography–- tandem mass spectrometry (UHPLC-MS/MS) [225]. The putative biomarkers were validated by a 1-month intervention trial with Mediterranean diet supplemented with 270 mL/day of red wine. The consumption of red wine was reflected by the detection of a significant increase in plasma of *cis*-RV-4'-sulfate, DHRV-3-sulfate and ethyl sulfate [225]. Furthermore, differences between the changes observed in urinary RV concentrations after intake of red and white wines have been the subject of several studies [226–228]. The biomarkers were significantly better at detecting red than white wines, showing a limitation in the combined marker applicability for general wine consumption [226–228]. Additionally, urinary anthocyanin concentrations significantly increased after red wine but remained practically unchanged after white wine intake, being a specific measure of red wine intake and a promising group of biomarkers to differentiate red and white wine consumption [226]. However, anthocyanins are also found in other foods, particularly red and blue berries and intake of these foods may affect specificity of combined measurements of RV metabolites and anthocyanins.

RV metabolites have also been tested as wine biomarkers in two large cohorts [228–231]. In the EPIC cohort, dietary RV and RV-3-O-glucoside intakes were estimated based on 24 hours dietary recalls using the food content values of these two compounds reported in the Phenol-Explorer database [193], and compared to the measured levels in 24 hours-urine samples collected on the recall days. Urinary excretion of RV was significantly and positively associated with wine intake [229]. In addition, using a metabolomic approach, red wine

consumption was predicted with an AUC of 86.9% for DHRV glucuronide among a sub-sample of 418 subjects from the EPIC study [231]. As another example of a cross-sectional study, the correlation between a 137-item validated FFQ in 1,000 subjects from the PREDIMED study and the concentration of RV metabolites excreted in morning urine has been studied. Drinkers and non-drinkers could be discriminated with a sensitivity of 93.3% and a specificity of 92.1%, and one drink of wine per week could be detected. Moreover, the concentrations of urinary RV metabolites of consumers of 3 glasses of wine/week were higher than those of the 1 glass/week consumers [230] at the group level. In a smaller study with 52 participants from the same cohort, those who reported wine consumption had significantly higher urinary concentrations of *trans*- and *cis*-RV-3-O-glucuronide than those who did not consume wine, and wine intake was predicted based on this marker with a sensitivity of 72% and a specificity of 94%. The percentage of false negatives was higher in those consuming wine intermittently than in those consuming it daily (43% and 24%, respectively) [228]. In another study, no correlation was found between data from a FFQ and the determination of free RV in plasma in a cross-sectional study with only 25 volunteers [232]. However, free RV is known to be rapidly absorbed and biotransformed. Therefore, RV metabolites seem to be a more precise objective measure of wine consumption in epidemiologic studies.

Tartaric acid

Tartaric acid or tartrate is one of the major components of red and white wine and the main component responsible for wine acidity [233]. Although it can be also found in other fruits, tartrate concentration is much higher in grapes or wine [234]. Indeed, tartaric acid has been proposed as candidate BFIs of grapes [235]. The only food source that presents similar amounts of tartaric acid is tamarind, a tropical sour fruit not commonly consumed in western countries [236]. Tartrate is mainly found in the grape pulp and in much higher concentration compared to RV, leading to 14-20% of the ingested dose of tartrate excreted unchanged [237]. The

applicability of tartrate as a BFI for wine consumption has therefore been assessed in wine interventions and observational studies.

Regueiro *et al.* (2013) developed a LC-MS method for analysis of wine organic acids to qualify wine consumption in a single dose drinking study with 5 subjects [238]. Ten hours after the consumption of 200 mL of red wine, spot urine samples showed a significant increase in tartaric acid concentration in all subjects [238]. Furthermore, a dose-response study has been conducted, showing that urinary tartaric acid concentration reflects the amount of wine consumed, and therefore allow to discriminate among levels of consumption in a male population [239].

Tartaric acid has also been evaluated by ^{1}H-NMR, showing that it is the most discriminating metabolite in urine after dealcoholized wine as well as regular wine consumption in the setting of a prospective, randomized, controlled, cross-over trial [240,241]. Additionally, 24-hour urine excretion of tartaric acid after white wine consumption has been reported as useful in an intervention to evaluate compliance [242].

Recently, tartaric acid has been applied as an objective measure for wine consumption in a cross-sectional study of a sub-sample of postmenopausal women (60-80 years old) from the PREDIMED study [243]. After adjustments for several covariates (e.g., consumption of fruits, raisins), women who consumed more wine presented higher concentrations of tartaric acid in urine. [243]. Those who reported not consuming wine were excluded from the analysis, so background levels of tartaric acid were not reported[243]. However, a certain background of tartrate is commonly seen in a method validation study, 80 urine samples from 4 different subjects were analyzed in order to test the method. Tartaric acid was detected in 71 samples (67 above the limit of quantification, 68 ng/mL, but still very low) after a beer intervention study during which the volunteers were asked to abstain from other alcoholic beverages [244]. The background levels observed were only 0.1 % of the average excretion seen in the previous

studies after intake of 300 mL of aged white wine [245]. In contrast, RV was not detected in any urine sample after the beer intervention [244]. Therefore, tartaric acid seems to be a promising quantitative biomarker of wine intake in epidemiological studies, although some noise can be expected due to the ingestion of low doses of this compound from other food sources.

Other authors proposed hydroxycinnamic acids that occur in white wine conjugated with tartaric acid (e.g., caftaric, fertaric) as putative BFIs. However, those compounds were detected in very low or undetectable levels in plasma [246], possibly due to fast hydrolysis in the human gastrointestinal tract.

Metabolomic investigations

Some studies have applied an untargeted metabolomic approach to obtain a holistic view of the metabolites associated with the intake of wine [128,231,240,241,247–249]. Other authors have opted for a targeted analysis to detect precursor wine compounds, intermediate metabolites and end-products [218,219,250,251]. Those studies reported a wide urinary and blood metabolomic fingerprint of anthocyanins (e.g., malvidin glucoside), phenolic acids (e.g., gallic acid sulfate), hydroxybenzoic acids (e.g., methylgallic sulfate), stilbenes (e.g., RV metabolites), flavan-3-ols (e.g., epicatechin glucuronide), phenyl alcohol (e.g., hydroxytyrosol), or hydroxyphenylvalerolactones after wine consumption. In addition, syringic acid and 3-hydroxyphenylacetic acid in feces were correlated with red wine intake by a UPLC-ESI-MS/MS analysis in samples from 74 volunteers [251]. None of these are fecal markers are regarded as promising biomarkers, see Supplementary Table 3.

Vázquez-Fresno et al. (2015) investigated urinary metabolomics profiles following a wine intervention study and also evaluated urinary metabolomics profiles associated with wine consumption in a free-living population [247]. A combined biomarker model using tartaric acid

and EtG, showed an AUC of 90.7% and 92.4% in the intervention and in the observational study, respectively. Moreover, this combined wine biomarker model was applied to assess the time-response criterion, defining a timeframe of 2-3 days after the last glass of wine consumed to detect significantly higher amounts of both markers in wine drinkers in comparison to non-wine consumers [247]. This would indicate that tartrate together with EtG may be seen as an intermediate-term biomarker of wine intake, with good prospects for use in observational studies.

To determine the impact of moderate wine consumption in the overall urinary metabolome, samples from a red-wine intervention study (250 mL/day, 28 days) were also investigated by Esteban-Fernández et al. (2018) [248]. The 24-hours urine was collected before and after intervention and analyzed by an untargeted UHPLC-QTOF-MS metabolomics approach. A total of 94 compounds linked to wine consumption, including specific wine components (tartaric acid), microbial-derived phenolic metabolites (5-(dihydroxyphenyl)-γ-valerolactones and 4-hydroxyl-5-(phenyl)-valeric acids), and several endogenous compounds with changed excretion levels in the urine [248].

Marker validation

Among the wine biomarker candidates, RV conjugates and tartaric acid have been investigated for many different aspects of validation. The major issues for the application of RV and its conjugates as wine intake biomarkers are that the content in wine is subject is highly variable and that human metabolism is showing inter-individual differences. However, dose-response in agreement with dietary instruments has been observed in observational studies indicating validity of RV. Combining RV with anthocyanins might improve specificity for red wine but this needs further study. Tartaric acid seems to fulfill all the criteria for full validation, although a cut-off or correction may be needed for studies in subjects consuming other grape products, including raisins and fresh grapes. Both tartrate and RV metabolites may also be applied as

grape and grape product BFIs and therefore will inherently lead to some misclassification when used as wine intake biomarkers. Another concern might be the presence of tartaric acid in some processed foods at relatively modest concentrations, due to addition as an acidifying agent, and in high amounts in tamarind. Thus, its applicability might be limited to those countries where tamarind is not a commonly eaten food; the ratio of tartaric acid to RV might be an approach worth pursuing in future studies to separate alcoholic wine consumption from intakes of potentially interfering foods. For regular wine, addition of alcohol biomarkers would further help discrimination from other grape products. Both RV and tartaric acid have been validated in wine interventions and in observational studies. The full validation criteria can be found in Table 1.

3.4 Sweet wine

Dessert wine is any sweet wine, which is made by naturally fermented juice from fruit, generally grapes, and usually fortified with alcohol [252]. Sweet wine is often served with dessert or is the dessert itself. Some examples of dessert wines are sherry, port wine, some sweet sparkling wines, and sweet wines from Riesling grapes, picked late in the season to increase their sugar content. The percentage of alcohol is between 10-20% [253]. The higher levels of sugar and alcohol are obtained by different ways: a) some grape varieties naturally produce high amounts of sugar; b) by directly adding sugar or honey; c) by adding alcohol, a process known as fortification; or d) by removing water to concentrate the sugar [252].

Only three papers related to dessert wine intake biomarkers were found. In two studies, a sweet sparkling wine was used with the aim to identify general markers of alcohol consumption (i.e., EtG, EtS) and not specifically to find markers for intake of sparkling wines [6,254]. In order to determine biomarkers of wine consumption, including sparkling wine, measurement of *cis* and *trans*-RV-3-*O*-glucuronides was performed in urine and serum after supplementation of 10 healthy young men with 300 mL/day of sparkling wine for 4 weeks [228]. A significant

increase of both isomers and of total RV metabolites was observed in urine (but not in serum), while RV aglycone, RV-3-*O*-glucoside and sulfate conjugates were undetected. The presence of *cis*- and *trans*-RV-3-O-glucuronides was also found after supplementation of white and red wine (200 mL/day) but not after gin supplementation (100 mL/day) suggesting that these metabolites can be considered specific biomarkers of grape wine intake in general, including the sweet grape wines.

In conclusion, the compounds so far identified represent very unspecific biomarkers. EtG and EtS are general biomarkers of alcohol intake [18] while RV is a biomarker of wine consumption [213,219,221,227,250] or of grape juice intake [255].

3.5 Distillates and spirits

Spirit-based beverages are alcoholic drinks that contain at least 15% of alcohol. Such drinks can be produced directly by distillation of naturally fermented products with or without aromatizing substances; or indirectly by the addition of other alcoholic beverages, ethyl alcohol, or a non-alcoholic drink to the spirit-based beverage. Many categories of spirit-based beverages with clearly defined characteristics exist, as well as a classification based on their geographical origin. Most are distillates based on fermentation products of almost any carbohydrate-rich crop, including brandies (cognac and fruit brandies), vodka (originally from distilled beer), aquavit or schnapps (based on fermented potato), whisky (from fermented roasted barley), rum (from sugar cane), gin (from a re-distilled grain mash and juniper) and tequilas (from agave cactus). In addition, some distillates exist as sugar sweetened liquors; alcoholic spirits exist both unsweetened and as sweetened products often spiced with anise (ouzo, pastis, etc.), or with alcoholic extracts of fruit (fruit liquors) [256].

Although these kinds of alcoholic beverages are commonly used as alcohol control beverages in biomarker studies on wine and beer [181], and in clinical trials [176,242,248,257] only few

studies have aimed to identify candidate biomarkers of intake of distillates and spirits. Only two studies have investigated plausible intake biomarkers of aniseed spirit and peppermint liquor, respectively [258,259].

Ouzo, raki, pastis, sambuca and mistra are alcoholic beverages with relatively high concentration of anethole [260]. Furthermore, anethole is also present in anise and fennel tea, as well as in some drugs (e.g., expectorants, antitussive, antispasmodic) and in perfumery, although their dosages are much lower than what results from moderate consumption of anise spiced spirits [258,261]. Anethole has therefore been described as a characteristic marker for the consumption of aniseed spirits. This compound has serum pharmacokinetics being useful in verifications of post-offence drinking claims. Three hours after drinking 120 mL of ouzo and 7 hours after consumption of 360 mL of ouzo, anethole levels in serum were still detectable [258]. As a note, this intake level would also allow detection of general alcohol intake biomarkers, and even for a more extended period.

Menthone occurs in four optically active stereoisomers, while menthol occurs in eight. Menthol is commonly used in toothpaste, mouthwash and pharmaceutical preparations [262]. It has been detected also in tobacco products [263], Chinese medicinal herbs [264] and honey [265]. Menthone, isomenthone, neomenthol and menthol have been proposed as peppermint liquor biomarkers [259]. The kinetic profiles of these compounds in serum have been established after conducting three dose-response drinking experiments [259]. The concentration changes indicated rapid absorption, similar to the blood-alcohol concentration peak. Determination of menthone, isomenthone, neomenthol and menthol within an approximate time frame of 30 minutes to 4 hours in serum makes them very suitable biomarkers of recent intake of spirits containing these flavor materials such as peppermint liquor, mint liquor and digestif bitters. However, as serum menthol and neomenthol levels may be also altered through consumption or use of pharmaceutical and dental products, peppermint sweets and teas, they cannot be

regarded as specific individual markers. However, menthol and neomenthol may be specific in combination with alcohol intake biomarkers [259].

In a prospective, randomized, controlled cross-over trial with 61 subjects at high cardiovascular risk, comparison of markers of three different beverages (gin, red wine, and dealcoholized red wine) showed two significantly correlated (unidentified) urinary compounds following the consumption of gin. However, these unknown potential gin intake biomarkers were also present in some baseline samples, and present in all urinary metabolomes following intake of gin [240]. Others studied the effect of alcohol on urinary excretion of the disulfide, 2-thiothiazolidine-4-carboxylic acid (TTCA), among non-exposed subjects, and showed that high liquor intakes (150-250 mL) may interfere with the levels of urinary TTCA [266]. However, TTCA levels have also been proposed to reflect crucifer intake [267]. In some other studies, spirits and distillates have been used with the aim to identify general markers for alcohol consumption (e.g., PEth, EtG, FAEE) and not for finding specific intake biomarkers of spirits or distillates [132,268–272].

In a recent study, the α-glucoside of ethanol was identified in Japanese sake, wine, and beer and in human blood and urine from seven autopsy cases [273]. In red and white wine mainly the β-isomer was observed while beer had equal levels of both isomers. Sake differed from wine and beer in having only the α-isomer of ethyl glucoside. The origin of the autopsy cases was not revealed but based on very high levels of the α- (26-837 µg/mL in urine and 1.4-33 µg/mL in blood) compared with the β-isomer (0-3.2 µg/mL in urine and 0-3 µg/mL in blood) it might be assumed they were heavy users of sake. Commercial drug free reference samples of blood and urine were measured as reference (levels < 0.3 µg/mL in blood and <0.6 µug/mL in urine), however the actual origin of the compounds in the autopsy samples and whether the compounds might exist in other fermented foods than alcoholic beverages are still unknown.

However, the two ethyl glucoside isomers show potential promise as future markers to discriminate sake drinkers, wine drinkers and beer drinkers.

Marker validation

Among the candidate biomarkers of distillates and spirits, anethole has been investigated for aniseed spirits, while menthone, isomenthone, neomenthol and menthol have been proposed as peppermint liquor intake biomarkers. Anethole seems to be a promising biomarker for this type of distillate, but still needs to be validated by independent verification and by measurement in controlled cross-sectional studies to confirm its reproducibility. The potential application of menthol is currently still hampered by the lack of robustness due to the common use of this flavoring. Combining menthol with an ethanol biomarker might decrease the level of misclassification, while more research is also needed in terms of analytical performance, robustness, and reproducibility. The full validation criteria can be found in Table 1.

4. Discussion

In this extensive literature review we have used the BFIRev guidelines to cover all reports on biomarkers related to moderate alcohol intake and use of alcoholic beverages. The search resulted in more than 20,000 titles of which ~170 papers reported directly on biomarkers and applications in human studies. These markers include five main direct markers of alcohol intake, ethanol, EtG, EtS, FAEEs and PEth; two main markers of wine intake, RV metabolites and tartrate; three main groups of markers of beer intake, xanthohumol metabolites, IAA metabolites, and hordenine-related metabolites. Few of these are perfect markers, but in combinations also including some other compounds, they attain good or very good performance for assessing intakes. These results point at the necessary future work needed to identify the best biomarker combinations and to validate them according to guidelines.

Compared with other food group biomarkers, BFIs of alcoholic beverages are among the most extensively investigated and several markers of alcohol intake are in common legal use. Therefore, biomarkers for alcohol intake are a showcase for the development of BFIs in general; it illustrates the usefulness and promise of the area, as well as the caveats and limitations, and hence the need for further development of the theory and technology for this area and for biomarkers in general.

Ethanol itself is the most obvious biomarker and has been extensively used for decades. However, as ethanol is also relatively quickly eliminated from the body it has a narrow detection time window [21]. Other commonly-known biomarkers for total alcohol intake include some liver enzymes, MCV, CDT and others, which have been mainly used for testing alcohol abuse and cannot be used at moderate intake levels. Some of the best markers of alcohol intake listed here can distinguish intake levels above and below moderation at the group level but have some caveats at the individual level related to analytical background, inter-individual variability in response, and kinetics; for instance, they do not yet allow to distinguish between recent intakes, chronic intakes and the timing since last intake. Despite the fast elimination from the body, breath ethanol remains an important marker of recent intake, especially in relation to traffic offences. Breath ethanol is a good reflection of blood alcohol levels and of the impact of alcohol on cognitive judgement and control of motor function, but longer-term markers are needed to reveal high intakes of alcohol within the last day or two. Potentially promising makers here include EtG, EtS, PEth and FAEE. Plasma or urine levels of acetaldehyde could also potentially be developed to serve this purpose [18,31,36].

EtG and EtS have considerably longer half-lives in plasma than ethanol by covering moderate alcohol intake ≥ 24 hours [46,57], but inter-individual variability may be high [58]. EtG can also be detected in hair and provide insight on longer-term average intakes, but again inter-individual variability may be high, and analysis may be disturbed by hair products and by the

sampling method [67,68]. Although the application of EtG in hair is still not fully validated at low to moderate intake levels, hair EtG analyses are already common in legal use. Hair FAEE is a promising marker used to verify hair EtG but also suffers from high analytical background levels and variable individual responses at similar intake levels. Plasma FAEE and blood or erythrocyte PEth are useful and reliable markers of alcohol abuse [87]. FAEE seems also promising as a marker of chronic alcohol abuse with apparently increased half-life after chronic high intakes, thereby potentially discriminating occasional binge drinkers from chronic abusers [60,111].

In terms of the central theme of this review – i.e., identification of biomarkers to quantify moderate or low alcohol intakes for the purpose of dietary and nutrition studies – the four primary alcohol markers are useful, but their validation at this intake is still not complete. The definition of what constitutes moderate alcohol intake has been changing over time with previous upper bounds of 40-60 g/day that are nowadays more commonly set at 10-30 g/day [3,4]. At the group level, these intake ranges are relatively well studied for EtG, EtS, FAEE and PEth, and all of them can discriminate between low, moderate, and high intakes. At the individual level there is often some overlap between the ranges observed for each of these three intake levels, most likely due to inter-individual differences in the activities and kinetics of the enzymes involved in ethanol metabolism, and inter-individual differences in biomarker degradation and excretion. Further investigation of these factors is needed to fully validate the markers for detecting and quantifying individual low intakes.

Studies combining information from two or more of these markers indicate that improved classification of individuals' recent intakes can be achieved [115]. None of the individual markers are able to discriminate non-consumers from subjects with sporadic or daily low alcohol intakes. Available studies indicate EtS and EtG are currently the most promising biomarkers for alcohol intake. However, it should also be noted that the fractional formation

of EtS and EtG is low at intake levels below one unit [25,51,57]. When the alcohol is not consumed within a short time span (10-30 minutes) the peak blood alcohol concentration will not exceed the Km for the involved enzymes, leading to an even lower response [57]. The high Km of formation, the variability in EtG and EtS formation between subjects due to polymorphisms, and the competition for the enzymes by other substrates are all factors making it difficult to measure accurately the individual low or null intakes [58]. The Km values for FAEE and PEth formation need to be investigated but are likely much lower. PEth shows considerable promise but needs further validation, and assessing the analytical background levels in human blood samples would improve our understanding of how to use this marker at low intakes [58]. Even non-consumers (e.g., children) sometimes have non-zero levels of all alcohol biomarkers when sensitive analytical methods are used, and the cause of this apparent background exposure has not been studied in much detail [25]. Apart from non-compliance, which may not explain all the documented cases, some other factors could affect the biomarkers. One of these is the 'hidden' alcohol in many common foods, e.g., in many fermented foods (bread, dairy), in fruit and fruit juices, and others; the exposure levels from these sources are low but potentially variable and high intakes of some of these foods may cause non-zero biomarker levels with sensitive analyses. Another explanation is the potential endogenous alcohol formation from human or microbial metabolism; the latter is well known to cause incidences of the auto-brewing syndrome where non-drinking victims have biomarker levels usually associated with alcohol abuse, i.e., alcohol is formed faster than it is degraded, leading to build-up of intoxication [24]. It is unknown whether much lower levels of auto-brewing may be a common phenomenon, explaining non-zero background levels of the biomarkers. Due to the relatively high Km of the alcohol intake biomarkers, EtG and EtS, they will only be formed at trivial levels if auto-brewing would take place at half a unit an hour or less, and the ethanol production would therefore go undetected, unless measured as ethanol

itself by GC-MS; FAEE and PEth may be formed with lower enzymatic Km values and for these markers, low-level auto-brewing might result in background levels that would vary considerably between subjects, as also observed for most sample types [100,114,274]. Other factors such as polymorphisms, environmental factors or misreporting might also play a role [93,96]. Endogenous background formation of alcohol would be expected to increase biomarker levels in subject groups at increased risk of gut microbial dysbiosis, e.g., subjects suffering from small bowel microbial overgrowth, diabetes, or obesity. However, more direct investigations and evidence are needed to verify whether auto-brewing plays a role in forming a background exposure to alcohol, which may also add a new perspective to the commonly observed J-shaped association between alcohol intake and risk of cardiovascular disease or diabetes [275].

Additional evidence for low or moderate intake levels may come from biomarkers specific to the beverages commonly consumed, especially from beer or wine. There are several markers for beer and wine intake related to typical constituents, e.g., IX [176] or iso-α -acid metabolites [157,158] from wort, N-methylated tyramine metabolites from barley sprouts [183], or RV metabolites [213,219,224] and tartrate [243] from grapes. In addition, the yeast fermentation used to brew these beverages leads to several metabolites, including hydroxyvalerates [125] and ethyl malate [157]. These and other markers are sufficiently well validated to identify intakes with good confidence and may therefore support the alcohol markers. However, most of these markers are only useful within 24 hours of intake, while no markers exist to quantify longer-term intakes. Therefore, considerable work is still needed to develop and validate combined BFIs for each exposure scenario as well as developing of additional sample types or sampling techniques to provide reliable biomarkers for shorter- and longer-term low or moderate intakes of alcoholic beverages. Since beer and wine exist in very many forms and as

non-alcoholic beverages, the known biomarkers will need additional validation and development to discriminate exposures in more detail.

The current review has used the BFIRev [20] approach in analogy with multiple previous reviews for various food groups. By this approach some papers may have been missed or misinterpreted since only one author has been selecting the papers relevant for each biomarker. Moreover, our method evaluation is less stringent. Specifically, the overall classification by the validation criteria is based on a judgement on whether there is any evidence that the criterion may be fulfilled under certain conditions. In the case of alcohol biomarkers, where the majority of papers are concerned with identification of problem drinkers, this tends to make the overall evaluation of most markers more favourable while their use for estimating lower intakes may be compromised. In forensics the interest in abstinence has increased in recent years, especially as the adverse effects of alcohol intake during pregnancy has become clearer and legislation is emerging in some countries incriminating such alcohol use in order to protect the child. Consequently, the detection of abstinence is becoming more important and potential sources of error in estimates of null and minimal intakes have become more urgent to identify. In the current review the critical assessment of low intakes by the available BFIs for alcohol is the major point of focus and the caveats identified here may therefore spur new research to settle uncertainties, thereby also improving the legal assessment of cases where only abstinence is accepted.

In nutrition research alcohol intake is usually ignored, i.e., the contribution of alcohol to energy intake and to nutrition related health is rarely included in experimental studies on dietary effects. Despite the high energy density of alcohol, current evidence indicates that moderate alcohol intake does not contribute to weight gain [276] but measurements of energy intake may still be offset. In the Mediterranean diet, low-dose wine intake is included and even recommended in dietary pattern interventions [277]. The trust in dietary assessment

instruments when it comes to alcohol intake is debated and it is sometimes assumed that a large proportion of subjects' misreport, especially those who report abstinence or low-drinking [10]. Reliable biomarkers to discriminate between abstinence and low or moderate intakes, both long- and short-term markers, could therefore have considerable impact on future nutrition and health research.

5. Conclusion

Biomarkers covering the intake of alcoholic beverages rank among the most well investigated and validated biomarkers of food and beverage intake. Biomarkers of alcohol, beer and wine intake cover recent high or moderate intakes reasonably well, while low intakes may go unnoticed. Inter-individual variation, variability in drinking patterns and variability in the beverage production processes all contribute as factors causing quantitative uncertainty regarding intakes while qualitative methods to discriminate no intake from moderate or high intakes are generally more reliable. Classification of no intake *vs.* low intakes is still only fair at best, which is unfortunate since the major controversy in research on moderate alcohol intake and health is the effects of abstention vs. low intakes. Several developments in biomarkers for alcoholic beverages and their non-alcoholic counterparts are therefore still needed, especially markers sensitive to low alcohol intakes, smart biomarker combinations to discriminate different recent or longer-term intake scenarios and potentially better sampling methods to cover intermittent intakes.

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Authors' contributions: GP and LOD designed the research. All authors conducted one or more literature searches for specific beverages, extracted the information and drafted the corresponding section of the paper. MTS and LOD edited the manuscript and updated the literature searches, tables, and figures. All authors read, commented on and approved the final manuscript.

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Supplementary material: Supplementary data associated with this article can be found, in the online version, at xxx

Figures captions:

Figure 1. Flow-chart of the study selection according to guidelines for biomarker of food intake reviews (BFIRev) procedure.
Figure 2. The metabolism excretion of ethanol in the human body.
Figure 3. Summary of the candidate biomarkers for alcohol and specific alcoholic beverages.

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3.3. Effects of moderate daily beer consumption on post-menopausal women health

The specific **objective 3** of the present thesis was to study the health effect of a moderate daily beer consumption in a post-menopausal women sample. A non-randomized controlled clinical trial was carried out and changes on menopausal symptoms and sex-hormone profile evaluated at 6 months of follow-up (Publication 6). Furthermore, intervention effect on bone tissue (Publication 7) and cardiovascular risk factors (CVDRF) were evaluated (Publication 8) at 2-years of follow-up.

Post-menopausal women aged 45-70 years old were recruited into the study between April 2017 and June 2019. Post-menopausal status was evaluated as inclusion criteria. Women using estrogen hormone therapy, silicon and polyphenols supplements were excluded. Therefore, a total of 37 volunteers were enrolled in the clinical trial and allocated in three different study arms. One participant dropped out at 3-months, while two at 6-months of follow-up. Three participants were excluded from the bone tissue statistical analysis due to drugs affecting bone metabolism use, resulting in a total sample size of 31 post-menopausal women in that specific analysis. Details of the study subjects, study design, intervention characterization and controlled variables can be found in the publications belonging to this section.

The study was conducted in compliance with the Declaration of Helsinki. All procedures were approved by the Bioethics Commission of the University of Barcelona (Institutional Review Board: IRB 00003099) in March 2017, and study protocols were registered at ISRCTN (ISRCTN14959650, ISRCTN13825020). All participants signed informed consent. In addition, those participants that finished the study underwent a sensory analysis evaluation to study their taste sensitivity and the applicability of it on nutritional research. Complementary sensory analysis protocol

was also approved by the Bioethics Commission of the University of Barcelona (Institutional Review Board: IRB 00003099) in July 2022.

AB and NAB interventions seem to decrease menopausal symptoms at 6 months of follow-up, while it might have a preventive effect against cardiometabolic alterations during menopause transition after 2-year intervention. At that follow-up and according to the findings of this pilot study, moderate daily beer intake, both AB and NAB, does not have a protective effect against bone loss in early post-menopausal women, although markers of bone formation increased. Beer liking was a significant factor in the study arm choice. Risk of the conducted intervention was assessed by monitoring liver enzymes, those remaining within normal values for the three arms throughout the study.

Publication 6

Moderate Consumption of Beer (with and without Ethanol) and Menopausal Symptoms: Results from a Parallel Clinical Trial in Post-menopausal Women

Marta Trius-Soler, María Marhuenda-Muñoz, Emily P. Laveriano-Santos, Miriam Martínez-Huélamo, Gemma Sasot, Carolina E. Storniolo, Ramon Estruch, Rosa M. Lamuela-Raventós, and Anna Tresserra-Rimbau

Nutrients 2021, 13 (7). https://doi.org/10.3390/nu13072278

Supplementary Material available in Annex 5.

Abstract

Aim: We evaluated if a moderate daily consumption of beer with (AB) and without alcohol (NAB) could improve menopausal symptoms and modify cardiovascular risk factors.

Methods: A total of 37 post-menopausal women were enrolled in a parallel controlled intervention trial and assigned to three study groups: 16 were administered AB (330 mL/day), 7 NAB (660 mL/day), and 14 were in the control group. After a 6-month follow-up, 34 participants finished the trial.

Results: AB and NAB significantly reduced the severity of the menopause-related symptoms (*p*-value AB *vs.* Control: 0.009; *p*-value NAB *vs.* Control: 0.033). In addition, AB had a beneficial net effect on psychological menopausal discomforts compared to the control group. The sex hormone profile did not differ significantly between the study groups and NAB was found to have a beneficial effect on low-density lipoprotein cholesterol, Apoliprotein A1, and diastolic blood pressure measurements,

Conclusions: A moderate daily AB and NAB consumption may provide an alternative approach for post-menopausal women seeking relief from mild to moderate climacteric symptoms. Furthermore, moderate NAB consumption improved the lipid profile and decreased blood pressure in post-menopausal women.

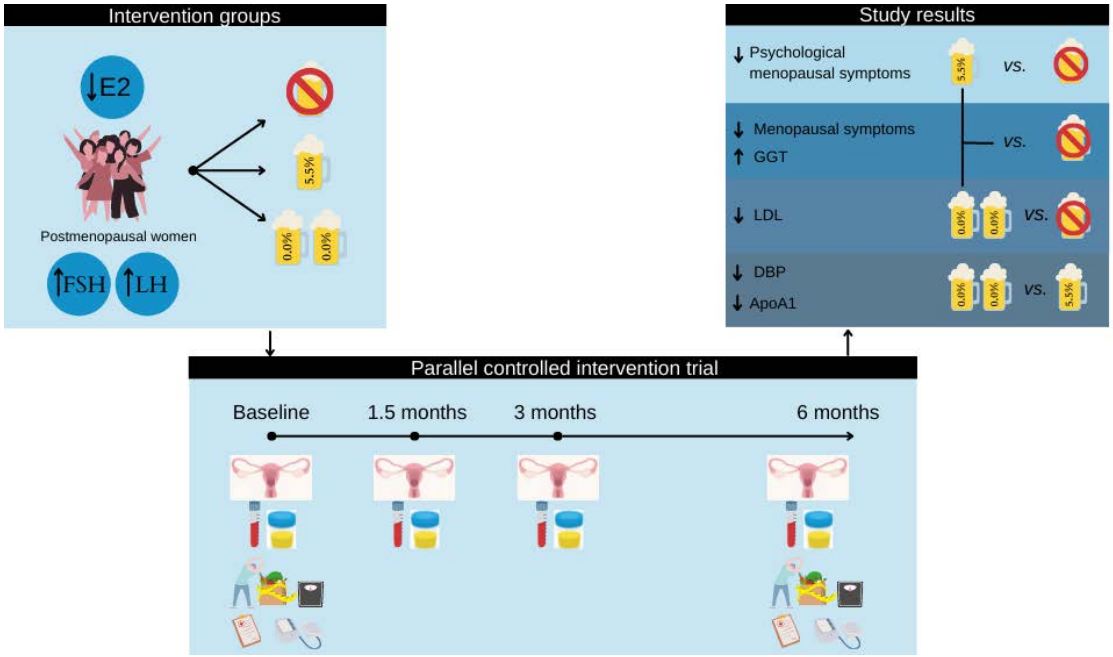

Figure 8. Graphical abstract Publication 6.

nutrients

MDPI

Article

Moderate Consumption of Beer (with and without Ethanol) and Menopausal Symptoms: Results from a Parallel Clinical Trial in Postmenopausal Women

Marta Trius-Soler [1,2,3], María Marhuenda-Muñoz [1,2,3], Emily P. Laveriano-Santos [1,2], Miriam Martínez-Huélamo [1,2], Gemma Sasot [1,2], Carolina E. Storniolo [1,2], Ramon Estruch [3,4], Rosa M. Lamuela-Raventós [1,2,3,*] and Anna Tresserra-Rimbau [1,2,3,*]

1 Department of Nutrition, Food Sciences and Gastronomy, XaRTA, School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; mtrius@ub.edu (M.T.-S.); mmarhuendam@ub.edu (M.M.-M.); elaversa21@alumnes.ub.edu (E.P.L.-S.); mmartinezh@ub.edu (M.M.-H.); gemmamsf@gmail.com (G.S.); carolina.e.storniolo@gmail.com (C.E.S.)
2 INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona, 08921 Santa Coloma de Gramanet, Spain
3 CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain; restruch@clinic.cat
4 Department of Internal Medicine, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, 08036 Barcelona, Spain
* Correspondence: lamuela@ub.edu (R.M.L.-R.); annatresserra@ub.edu (A.T.-R.); Tel.: +34-934-034-843 (R.M.L.-R. & A.T.-R.)

Abstract: The menopausal transition can be a challenging period for women's health and a trigger of uncomfortable symptoms. Beer is the main food source of isoxanthohumol, a precursor of 8-prenylnaringenin, the strongest phytoestrogen identified to date. As phytoestrogens are reported to reduce perimenopausal symptoms, we evaluated if a daily moderate consumption of beer with (AB) and without alcohol (NAB) could improve menopausal symptoms and modify cardiovascular risk factors. A total of 37 postmenopausal women were enrolled in a parallel controlled intervention trial and assigned to three study groups: 16 were administered AB (330 mL/day), 7 NAB (660 mL/day), and 14 were in the control group. After a 6-month follow-up of the 34 participants who finished the trial, both interventions (AB and NAB) significantly reduced the severity of the menopause-related symptoms (p-value AB vs. Control: 0.009; p-value NAB vs. Control: 0.033). Moreover, AB had a beneficial net effect on psychological menopausal discomforts compared to the control group. As the sex hormone profile did not differ significantly between the study groups, the effects of both types of beers (AB and NAB) are attributed to the non-alcoholic fraction of beer. Furthermore, moderate NAB consumption improved the lipid profile and decreased blood pressure in postmenopausal women.

Keywords: phytoestrogens; prenylflavonoids; polyphenols; health; menopause; alcohol; cardiovascular risk factors

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1. Introduction

Menopause is characterized by a low output of ovarian estrogens and a high production of pituitary gonadotropin hormones (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), which trigger uncomfortable symptoms such as hot flashes, night sweats, sleep disturbance, and vaginal dryness [1–3]. From a health point of view, estrogens are widely regarded as protectors against atherosclerosis, whereas progesterone and androgens may act as atherogenic factors in postmenopausal women [4]. A smooth transition through this challenging period is considered crucial for healthy and successful aging [2]. Although hormone replacement therapy effectively reduces vasomotor symptoms associated with the decrease of estrogen, its benefits do not outweigh the higher

risks of stroke and venous thromboembolism or the increased incidence of breast cancer and coronary heart events associated with the co-administration of estrogens and progestin [5]. A post-hoc analysis of data from the Women's Health Initiative and some new evidence indicate that hormone replacement therapy may have beneficial health effects for women below the age of 60, which calls for a reevaluation of the use of estrogen alone in younger postmenopausal women [6,7]. The inconclusive nature of these results has generated great interest in alternative therapies, such as phytoestrogens, to relieve menopausal symptoms [8,9].

Phytoestrogens are compounds with estrogen-like properties naturally found in plants [7,10,11]. Among flavonoids, isoflavones are the subclass with the highest phytoestrogen effect [12]. In recent years, prenylated chalcones, flavonoids present in hops (*Humulus lupulus* L.), have attracted considerable attention for their health benefits [13–18]. Beer is the main dietary source of isoxanthohumol (IX), which is produced from xanthohumol (XN) during the brewing process [19]. Once ingested, the weakly estrogenic IX can be bioactivated to 8-prenylnaringenin (8-PN), the strongest phytoestrogen identified to date [20,21], by microorganisms inhabiting the gastrointestinal tract [17,22] or converted in the liver in minor amounts [17,23,24]. In a previous intervention study with 36 menopausal women, Erkkola et al. (2010) observed that 100 μg/day of 8-PN from a hop extract relieved the symptoms of menopause and increased the quality of life of menopausal women [13]. Our research group has determined that Spanish beers contain around 500 μg/L of IX and 50 μg/L of 8-PN [25].

Accordingly, we conducted a six-month parallel, controlled clinical intervention trial to evaluate if a moderate daily intake of beer, with or without alcohol, could reduce menopausal discomforts in women going through the menopause transition. The effect of the intervention on the female sex hormone profile and cardiovascular risk factors (CVRF) was also monitored.

2. Materials and Methods

2.1. Study Population and Recruitment

Postmenopausal women aged 45–70 years were recruited into the study between April 2017 and June 2019 from the Outpatient Clinic of the Internal Medicine Department of the Hospital Clinic of Barcelona through poster boards in different settings and advertisements on the radio. Potential participants were screened according to the defined inclusion and exclusion criteria. The postmenopausal status of each participant was validated by the following criteria: (1) absence of menses for the last 12 months; (2) follicle-stimulating hormone (FSH) blood levels of 23–116 U/L, and (3) 17-β-estradiol (E2) blood levels < 37 pg/mL. Women using estrogen therapy or taking silicon or polyphenol supplements were excluded.

2.2. Study Design

The participants were assigned to one of the three study groups according to their preference, given that the intervention involved a medium alcohol consumption. The non-randomized design was chosen in accordance with ethical considerations but also reflecting the reality of participant lifestyle.

After a run-in period of 15 days, in which subjects were asked not to consume any alcoholic beverage, NAB or hop-related products, participants were allotted to a study group for 6 months. One group consumed 14 g of ethanol a day in the form of AB (330 mL/d) (AB group); another received NAB (660 mL/d) containing a similar amount of non-alcoholic compounds to AB (NAB group), and the third group did not receive any intervention and were instructed to refrain from consuming alcohol, NAB or hop-related products (control group). None of the participants were allowed to consume any other alcoholic beverages during the study.

For a parallel design, the sample size calculation indicated that to detect mean differences of 3 points in Menopause Rating Scale (MRS) total score with a standard deviation

(SD) of 3 points assuming a maximum loss of 10% of participants, 18 subjects per group were needed to complete the study ($\alpha = 0.05$; power = 80%).

All participants signed an informed consent. Eligible subjects were asked to come for four visits during the intervention period (baseline, and 1.5, 3, and 6 months). The study protocol was approved by the Bioethics Commission of the University of Barcelona (Institutional Review Board: IRB 00003099) in March 2017, registered at ISRCTN (ISRCTN14959650) and conducted in compliance with the Declaration of Helsinki.

2.3. Intervention-Phytoestrogen Dose

To standardize the daily phytoestrogen dose for each group, participants consumed the same brand of beer throughout the study and were encouraged to drink it during meals. They were supplied with beers every month as a measure of intervention compliance. The contents of prenylflavonoids IX, 8-PN and XN are detailed in Table 1. Due to the dietary nature of the intervention, neither the participants nor the researchers were blind to the intervention treatments. However, laboratory personnel and technicians were blinded to the intervention received by the participants. Intervention compliance was assessed by data obtained from structured questionnaires and by the measurement of IX, a validated beer intake biomarker, in 24-h urine samples collected at baseline, and 1.5, 3, and 6 months by solid phase extraction liquid chromatography coupled to mass spectrometry (LC-MS/MS) [25].

Table 1. Intervention-phytoestrogen content of the administered alcoholic (AB) and non-alcoholic beer (NAB).

Intervention Group	IX µg/Day	XN µg/Day	8-PN µg/Day	6-PN µg/Day	Total Amount µg/Day	Alcohol g/Day
AB	302.7 ± 16.8	27.9 ± 0.6	5.5 ± 0.4	22.8 ± 0.3	358.9 ± 17.4	14
NAB	104.7 ± 3.8	81.3 ± 4.0	10.3 ± 0.8	62.7 ± 2.2	259.0 ± 10.3	0.0

6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; IX, isoxanthohumol; XN, xanthohumol. Values are means of three analyses ± SDs.

Quantification of Prenylflavonoids in Beer Intervention Samples by LC-MS/MS

Qualitative and quantitative analyses of prenylflavonoids in beer samples were carried out according to the method of Quifer-Rada et al. (2013), with some modifications [26]. Briefly, the beer foam from AB and NAB was removed by agitation and ultrasonication. Then, the alcohol content from alcoholic beer was evaporated under a gentle stream of N_2 and was refilled with water. Samples were filtered through a 0.45-µm polytetrafluoroethylene filter and 600 ng/mL of taxifolin was added as an internal standard prior to the analysis. The identification and quantification of the selected analytes (IX, XN, 6-prenylnaringenin (6-PN), and 8-PN) was carried out using an Acquity UHPLC system equipped with a Waters binary pump (Waters, Milford, MA, USA). The UHPLC separation was performed with a Luna C18 column, 50 mm × 2.0 mm i.d., 5 µm (Phenomenex, Torrance, CA, USA), directly interfaced to an API 3000™triple quadrupole mass spectrometer (Sciex, Concord, ON, Canada) with a turbo ion spray source working in negative mode. The mobile phases used were 5 mM of ammonium bicarbonate buffer adjusted to pH 7.0 and acetonitrile and methanol (1:1), at a constant flow rate of 600 µL/min and a column temperature of 40 °C. Sample injection volume was 10 µL. Multiple reaction monitoring mode was used to identify and quantify the analytes. Calibration curves from 0 to 1000 ppb were prepared adding standards to pure water containing 20 mg/L of ascorbic acid. The reagents, materials, and MS/MS parameters were the same as reported in Quifer-Rada et al. (2013) [18].

2.4. Measurements and Outcome Assessment

2.4.1. Medical History

Individual information was collected at baseline and updated through the trial by face-to-face interviews. Interviews were based on a structured questionnaire that included medical and sociodemographic questions, with special attention given to menopausal symptoms and CVRF. Current and past consumption of alcohol, smoking and sleeping habits, daily life and work stress, time since the onset of menopause, and medication received were also recorded.

2.4.2. Climacteric Symptoms

The primary outcome was changes in menopausal symptomatology. Menopausal discomforts were quantified (frequency and severity) using the MRS questionnaire [1,27,28]. The MRS consists of three independent factorial dimensions, with four items in the somato-vegetative subscale, four items in the psychological subscale and three additional items in the urogenital subscale. Each of the eleven symptoms were rated from 0 (no complaints) to 4 (very severe symptoms) as perceived by the participant, with a total MRS score ranging from 0 to 44 points. The Spanish Validated Version of the MRS questionnaire was used and filled in for the purposes of this intervention trial at the four time points (baseline, and 1.5, 3, and 6 months [27].

2.4.3. Anthropometric Measurements

Anthropometric measurements were obtained at the beginning and end of the trial intervention period (visit at 6 months). Resting supine 12-lead electrocardiograms were recorded at baseline.

Diastolic and systolic blood pressure (DBP and SBP) and heart rate were measured in triplicate in resting and fasting conditions using a validated semiautomatic oscillometer (Omron HEM-705CP). Trained registered staff following anthropometric standardization protocols premeasured body weight, height, and waist circumference. Participants were weighed wearing light clothing and without shoes, using a high-quality calibrated scale. Height was measured with a wall-mounted stadiometer. Waist circumference was measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest [29]. Body mass index (kg/m^2) (BMI) was calculated as weight (kg) divided by the height squared (m^2).

2.4.4. Biological Samples and Biochemical Analyses

Overnight fasting blood samples were collected at baseline and 1.5, 3, and 6 months of intervention. Automated biochemical profiles were measured at the Biomedical Diagnostic Center of the Hospital Clinic (Table 2).

Table 2. Baseline anthropometric measurements and biochemical analysis according to the intervention group.

	Control (*n* = 14)	**AB (*n* = 16)**	**NAB (*n* = 7)**	***p*-Value**
Weight, kg	71.7 ± 13.0	64.7 ± 10.3	75.2 ± 20.3	0.324
BMI, kg/m^2	27.2 ± 4.4	25.3 ± 3.7	30.0 ± 9.0	0.634
WC, cm	89.4 ± 9.7	87.0 ± 10.3	90.6 ± 16.8	0.810
DBP, mmHg	74 ± 13	73 ± 6	74 ± 6	0.944
SBP, mmHg	121 ± 15	120 ± 14	120 ± 16	0.929
Heart rate, bpm	70 ± 12	68 ± 11	71 ± 7	0.657
Glucose, mg/dL	90.9 ± 6.2	93.9 ± 7.7	97.1 ± 11.5	0.376
Creatinine, mg/dL	0.69 ± 0.12	0.64 ± 0.10	0.70 ± 0.11	0.483
Uric acid, mg/dL	4.9 ± 0.7	4.6 ± 1.3	5.3 ± 1.7	0.322
Total cholesterol, mg/dL	185 ± 30	206 ± 22	208 ± 27	0.060
LDL-C, mg/dL	114 ± 23	135 ± 25	142 ± 18	0.063
HDL-C, mg/dL	56 ± 13	57 ± 8	56 ± 13	0.895

Table 2. *Cont.*

	Control (n = 14)	AB (n = 16)	NAB (n = 7)	p-Value
Triglycerides, mg/dL	69 ± 32	77 ± 25	66 ± 18	0.663
ApoA1, mg/dL	150 ± 19	163 ± 14	158 ± 22	0.058
ApoB mg/dL	94 ± 17	106 ± 20	105 ± 15	0.188
AST, U/L	21 ± 12 [a]	26 ± 10 [b]	22 ± 5 [ab]	0.025
ALT, U/L	19 ± 10	24 ± 14	18 ± 5	0.217
GGT, U/L	14 ± 5	22 ± 10	20 ± 15	0.057
Total proteins, g/L	70 ± 3	69 ± 5	71 ± 4	0.541
Albumin, g/L	42 ± 2	43 ± 3	42 ± 2	0.530
TSH, ng/mL	2.11 ± 1.50	2.63 ± 2.23	2.58 ± 0.50	0.290
FT4, ng/mL	1.16 ± 0.14	1.15 ± 0.16	1.07 ±0.07	0.318
T3, ng/mL	1.16 ± 0.21	1.15 ± 0.15	1.24 ± 0.40	0.961
Cortisol, µg/dL	13.3 ± 4.1	13.7 ± 5.1	14.2 ± 5.5	0.900
PTH, ng/mL	65.3 ± 24.2	61.6 ± 21.9	66.0 ± 17.8	0.653
25-hydroxy-vitamin D3, ng/mL	23.2 ± 4.8	27.4 ± 10.5	24.8 ± 13.5	0.656
FSH, U/L	66.7 ± 21.5 [a]	103.0 ± 44.4 [b]	55.8 ± 22.5 [a]	0.006
E2, pg/mL	24.1 ± 12.8	19.9 ± 8.1	22.5 ± 7.7	0.651

AB: alcoholic beer; ALT: alanine transaminase; ApoA1: Apolipoprotein A1; ApoB: Apolipoprotein B; AST: aspartate transaminase; BMI: body mass index; DBP: diastolic blood pressure; E2: 17-β-estradiol; FSH: follicle-stimulating hormone; FT4: thyroxine; GGT: gamma-glutamyl transferase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; NAB: non-alcoholic beer; PTH: parathyroid hormone; SBP: systolic blood pressure; T3: tri-iodothyronine; TSH: thyroid stimulating hormone; WC: waist circumference. Results are presented as mean ± THE. Kruskal Wallis test followed by post-hoc Dunn's test was used for statistical comparisons. Means within the same row carrying different superscripts ([a,b]) are significantly different. p-value < 0.05.

Blood from each visit was drawn into ethylenediaminetetraacetic acid (EDTA) collection tubes, and plasma was separated after centrifugation at 1500 g (RCF) for 15 min at 7 °C. 24-h urine samples were also collected at all visits. Plasma and 24-h urine samples were stored in aliquots at −80 °C until the day of analysis.

Stored plasma aliquots collected at the different time points were used to analyse sex hormones. FSH, LH, progesterone, E2, and sex hormone-binding globulin (SHBG) were measured by a chemiluminescent immunoassay using an Atellica instrument (Siemens), while total testosterone (T-total) was measured by a direct chemiluminescent immunoassay with a Cobas instrument (Roche). The free testosterone index (FTI) was defined as the ratio between testosterone levels and SHBG levels, multiplied by a constant. To study the bioavailable E2, the free oestradiol index (FEI) was calculated as the molar ratio of plasma E2 to the plasma SHBG level and multiplying by 100 [30]. The lower detection limits of plasma E2 and progesterone were 12 pg/mL and 0.21 ng/mL, respectively; levels below these limits were defined as 11 pg/mL of E2 and 0.20 ng/mL of progesterone.

2.4.5. Dietary Intake and Physical Activity Assessments

Dietary intake was recorded at baseline using a 151-item semi-quantitative food frequency questionnaire (FFQ) [31]. The total energy intake (kcal/day) and absolute consumption values of phytoestrogen-rich food subclasses (legumes, seeds, and whole grains) per day were estimated according to Spanish food composition tables and the Phenol-Explorer database [32]. Isoflavonoid intake (mg/day) was estimated from the consumption of plant-based milks, alcoholic beer, and non-alcoholic beer reported in the FFQ, multiplying the isoflavonoid content in food (mg/100g of food) by the daily consumption of each food (g/day) [31]. In addition, the 14-point Mediterranean Diet Adherence questionnaire was used as a healthy dietary index in order to evaluate differences between study groups at baseline [33]. The subjects also filled out a 7-day food record questionnaire at baseline and at the end of the intervention. These dietary registers were carefully checked and three representative days (two in the week and one at the weekend) were fully evaluated using nutrition analysis software, PCN Pro, developed at the University of Barcelona (Programa de Càlcul Nutricional Professional, Santa Coloma de Gramenet, Barcelona, Spain). Phys-

ical activity was registered at the beginning and end of the study using the Minnesota leisure-time questionnaire, previously validated in a population of Spanish women [34].

2.5. Statistical Analyses

Continuous variables are expressed as mean ±SD. Categorical variables are expressed as number (n) and proportion (%). Differences in the characteristics of volunteers between groups at baseline were tested by the chi-square test for categorical variables and the Kruskal–Wallis test with post-hoc Dunn's test for continuous variables.

The effect of beer interventions on climacteric symptoms, sex hormone profile, and CVRF was estimated by performing a generalized estimating equation (GEE) on Poisson regression models for repeated measures. Identity link function, independent correlation, and robust standard error parameters were specified due to the low number of clusters and the nature of the variables [35]. Adjusted differences and their corresponding 95% CI were computed using the increasing complexity models. Climacteric symptom models included time since the onset of menopause, the baseline exposure variable (stress/depression from daily life (score 1–5), FSH levels (continuous), and isoflavonoid consumption (continuous). Sex hormone profile models included the variables described above, without taking into consideration FSH levels. An interaction term of time-exposure allowed the evaluation of potential differences between exposure intervention groups in response to changes over time.

Intragroup differences in MRS questionnaire items were assessed by a Kruskal–Wallis test followed by post-hoc Dunn's test. Differences between baseline and 6 months in sex hormone levels and dietary patterns were analyzed by a non-parametric test for two related samples in each arm/group. A Wilcoxon matched-pair signed-rank test for small samples was applied to symmetric variables, while for asymmetric variables the sign test of matched pairs was used. Symmetry was studied by the skewness and kurtosis test for normality (control and AB group) or graphically (NAB group).

Percent changes (% changes) of hormone levels were calculated by dividing the differences between the final and the initial hormone values by the initial hormone concentration per 100. Spearman's correlation coefficient was estimated to study linear associations between different baseline hormone levels, different % changes in hormone levels, and between individual % changes and their corresponding baseline hormone concentration. Due to their theoretical relationship, associations between individual % changes in FTI and T-total or SHBG were not studied, nor in the FEI and E2 or SHBG. Correlation coefficient values were interpreted by Chan's guideline on the strength of the linear relationship [36].

All statistical analyses were conducted using the Stata statistical software package version 16.0 (StataCorp, College Station, TX, USA). Statistical tests were two-sided and *p*-values below 0.05 were considered significant.

3. Results

3.1. Study Subjects, Intervention, and Compliance

The recruitment and compliance of the study participants are detailed in a flow diagram (Figure 1). A total of 37 postmenopausal women were enrolled in the clinical trial and assigned to the three study groups: 16 chose the AB intervention, while 7 and 14 chose to be allocated to the NAB or the control group, respectively. Only 3 women dropped out and 34 participants finished the trial. As shown in Figure 1, two subjects from the control group and one from the NAB group dropped out due to difficulty in continuing visits or in complying with the intervention.

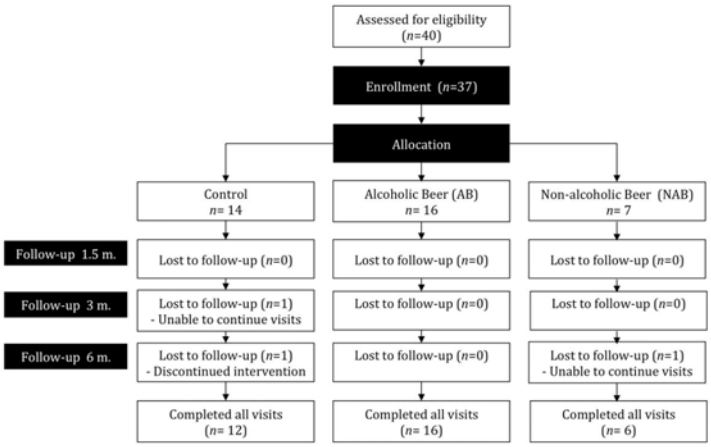

Figure 1. Flow diagram of participant recruitment and compliance in each phase of the intervention trial.

The prenylflavonoid contents of beers given to the AB and NAB groups are shown in Table 1. Subject compliance with the intervention was 100% according to dietary self-records at 6 months and the interviews in the different visits during the intervention. To confirm intervention compliance, IX concentrations were measured in the 24-h urine provided by the participants at all four visits. IX concentration was below the detection level <0.04 ppb at baseline [26], and increased in 93.5% of collected urine samples from both intervention groups.

3.2. Participant Characteristics at Baseline

The baseline characteristics of the participants are summarized in Tables 2 and 3. The age range of the participants was 49–66 years. Study groups revealed no significant differences in terms of age, smoking habits, sleeping hours, stress/depression from work, and time since the onset of menopause. The alcoholic drinking habit and stress/depression from daily life scores were the two baseline items that were significantly different between study arms. Women in the AB group drank alcoholic beverages more frequently and were more stressed than women from the other groups. Wine and beer were the two most preferred types of alcoholic beverages in all the study groups.

Table 3. Baseline characteristics of the participants according to intervention group.

	Control (*n* = 14)	**AB (*n* = 16)**	**NAB (*n* = 7)**	***p*-Value**
Medical records				
Age, *years*	55.6 ± 5.1	54.9 ± 3.6	56.4 ± 3.2	0.647
Smoking habit				
Current, *n (%)*	1 (7.1)	6 (37.5)	2 (28.6)	0.170
Former, *n (%)*	3 (21.4)	4 (25.0)	0 (0.0)	
Never, *n (%)*	10 (71.4)	6 (37.5)	5 (71.4)	
Sleeping time, *hours*	6.4 ± 0.9	7.0 ± 1.3	6.8 ± 1.0	0.448
Stress/depression from daily life[1]	2.6 ± 1.4[ab]	3.4 ± 1.0[a]	1.7± 1.1[b]	0.013
Stress/depression from work[1]	2.9 ± 1.5	2.7 ± 1.6	2.6 ± 1.3	0.881
Time since the onset of menopause, *months*	52.1 ± 35.5	36.7 ± 28.0	46.0 ± 55.5	0.432
Physical activity, *MET-min/day*	731 ± 449	681 ± 616	467 ± 118	0.587

Table 3. *Cont.*

	Control (n = 14)	AB (n = 16)	NAB (n = 7)	p-Value
Dietary history				
Total energy intake, *kcal/day*	2695 ± 517	2726 ± 673	2352 ± 264	0.189
MedDiet, *14-item score*	8.7 ± 1.8	7.4 ± 1.6	7.9 ± 2.4	0.170
Legumes, *g/day*	62 ± 31	53 ± 23	43 ± 24	0.586
Seeds, *g/day*	5.0 ± 8.4	0.9 ± 1.5	1.7 ± 1.9	0.208
Whole grains, g/day	51 ± 39	95 ± 83	72 ± 69	0.419
Isoflavonoids, *mg/day*	6.4 ± 13.2	2.3 ± 5.3	10.3 ± 17.6	0.079
Alcohol drinking habit				
Weekly, *n (%)*	1 (7.1)	9 (56.3)	1 (14.3)	0.025
Occasionally, *n (%)*	10 (71.4)	7 (43.8)	5 (71.4)	
Never, *n (%)*	3 (21.4)	0 (0.00)	1 (14.3)	
Type of alcohol preferred				
Beer, *n (%)*	3 (21.4)	8 (50.0)	3 (42.9)	0.482
Wine, *n (%)*	7 (50.0)	7 (43.8)	3 (42.9)	
Spirits, *n (%)*	1 (7.1)	1 (6.3)	0 (0.0)	
None, *n (%)*	3 (21.4)	0 (0.0)	1 (14.3)	
Medication				
Antihypertensive agents, *n (%)*	1 (7.1)	3 (18.7)	1 (14.3)	0.649
Lipid-lowering medication, *n (%)*	0 (0.0)	2 (12.5)	0 (0.0)	0.250
Antidepressants, sedatives, anxiety pills, *n (%)*	3 (21.4)	3 (18.8)	0 (0.0)	0.425
Sleeping pills, *n (%)*	2 (14.3)	2 (12.5)	0 (0.0)	0.585
Dietary supplements, *n (%)*	3 (21.4)	8 (50.0)	4 (57.1)	0.172

[1] score from 1–5. AB: alcoholic beer; MedDiet: Mediterranean Diet Adherence Screener 14-item score; NAB: non-alcoholic beer. Continuous variables are presented as mean ± SD. Categorical variables are expressed as number (n) and proportion (%). Chi-square test was applied to study differences in categorical variables. Kruskal–Wallis test followed by post-hoc Dunn's test was applied to study differences in continuous variables. Means within the same row carrying different superscripts [(a, b)] are significantly different. p-value < 0.05.

Most of the baseline anthropometric measurements and biochemical parameters of the three groups were balanced, as shown in Table 2. Specifically, the study arms were similar in body weight, BMI, blood pressure (BP), lipid and thyroid profiles, and other baseline clinical characteristics. Most participants were normoweight or overweight with an elevated waist circumference, and had normal BP, heart rate, and analytical values. However, women in the AB group had significantly higher levels of FSH and aspartate transaminase compared to the other study groups.

Covariates

For a more in-depth study of the intervention effects, differences at baseline and changes in dietary habits, related medication history and physical activity during the intervention, were evaluated between the study groups. No statistical differences in medication use and physical activity were observed at baseline or at the end of the intervention.

Food, nutrient, and energy intakes were derived from both food records and FFQs. At baseline, significant differences in fiber intake (p-value: 0.008) from food recalls were found between groups (Supplementary Table S1), whereas recorded information from the FFQs did not reveal any significant differences. All dietary parameters except for polyunsaturated fatty acids studied by food records correlated significantly with the FFQs (coefficients of correlation > 4000) (*data not shown*). Before the run-in period, alcohol consumption was significantly higher among women allocated to the AB group (Control: 1.9 ± 2.4 g/day; AB: 6.6 ± 4.1 g/day; NAB: 1.9 ± 2.1 g/day; p-value: <0.001). The analyses of macronutrients at baseline and the end of the study revealed a low percentage of carbohydrate intake (<45–60% kcal/day), and a high percentage of total fat (>20–35% kcal/day) and saturated fatty acid (>10% kcal/day) intakes [37], without differences between study groups.

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Individual changes during the study were also monitored. According to the 3-day food records, the control and NAB groups did not change their dietary habits. Furthermore, in the AB group only alcohol consumption was significantly higher in comparison to the washout period (p-value: <0.001)

3.3. Intervention Effects on Climacteric Symptoms

The MRS questionnaire was used to determine the effect of AB and NAB consumption on the severity of climacteric symptoms. Before the intervention, the mean scores of the total recorded symptoms did not significantly differ between the three study arms (Figure 2).

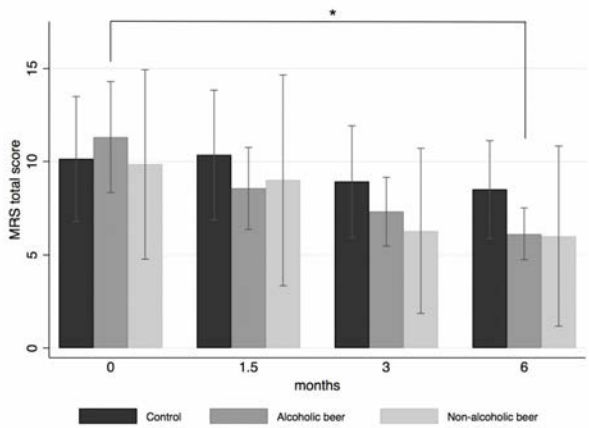

Figure 2. Evolution of total MRS score of the study groups during the intervention. Results are expressed as mean ± standard deviation. Means with (*) are significantly different. p-value <0.05.

The three most frequently experienced symptoms of the eleven composing the MRS were joint and muscular discomfort (70.3%), physical and mental exhaustion (70.3%), and sleep problems (64.9%). The mean scores for the eleven symptoms in all the study groups were between "absent" and "moderate" (0–2 points). However, as shown in Supplementary Table S2, the only significant change at 3 months in the control group was a diminished heart discomfort symptom (p-value: 0.028). Furthermore, women in the AB group reported a significant decrease in the total MRS score at the end of the follow-up. The NAB group did not show any statistically significant changes throughout the intervention period, although the MRS total score had a decreasing tendency of 4.2 ± 3.0. Over the intervention period, menopausal symptoms in the AB and NAB groups decreased in severity by an average of 46.0% and 42.4%, respectively, in comparison with 10.9% in the control group (Figure 2). While these results point to a positive effect of the interventions compared to the control, intra-individual differences in MRS scores as well as the time-exposure interaction provide a more precise indication of the intervention effect.

Table 4 shows the intervention effect on MRS subscales and total MRS scores during follow-up. Menopausal women that received AB and NAB experienced a significant reduction in climacteric symptoms in comparison with those in the control group at 6 months of the intervention. The time-exposure interaction measured linearly was found to be statistically significant when comparing the AB and control groups (p-trend: 0.011), consisting of an expected decrease of the adjusted differences in the total MRS score between the AB and control groups of -0.6 points (95% IC: −1.1, −0.1) for each three additional months of intervention (*data not shown*). Furthermore, mild psychological symptoms (depressive mood, irritability, anxiety, and physical and mental exhaustion) after 6 months of daily moderate beer consumption decreased significantly (adjusted difference: −2.1;

95% IC: −3.5, −0.6) compared to the control group, with a significant linear time–exposure interaction (adjusted difference: −0.3; 95% IC: −0.5, −0.1; *p*-trend: 0.010). However, there were no significant differences between groups in the urogenital and somatic domains after the 6-month treatment period. The alcoholic fraction derived from AB consumption did not entail a lower or increased climacteric symptom severity (AB vs. NAB group, Table 4).

Table 4. Intervention effect on somatic, psychological, and urogenital domain scores and total MRS (Menopausal Rating Scale) score at follow-up.

	AB vs. Control			NAB vs. Control			AB vs. NAB		
	Difference Time-Exposure (95% IC)	***p*-Value**	***p*-Trend**	**Difference Time-Exposure (95% IC)**	***p*-Value**	***p*-Trend**	**Difference Time-Exposure (95% IC)**	***p*-Value**	***p*-Trend**
Somatic									
Model 1	−1.1 (−2.7, 0.6)	0.199	0.223	−1.0 (−3.2, 1.2)	0.354	0.367	−0.0 (−1.9, 1.0)	0.988	0.993
Model 2	−1.2 (−2.7, 0.3)	0.120	0.126	−1.5 (−3.7, 0.7)	0.184	0.230	0.3 (−1.7, 2.2)	0.779	0.890
Model 3	−1.3 (−2.8, 0.2)	0.088	0.083	−1.7 (−4.0, 0.5)	0.128	0.138	0.5 (−1.6, 2.5)	0.665	0.737
Psychological									
Model 1	−2.1 (−3.8, −0.5)	0.011	0.021	−1.1 (−2.8, 0.6)	0.196	0.254	−1.0 (−2.9, 0.9)	0.290	0.377
Model 2	−2.1 (−3.5, −0.7)	0.004	0.007	−1.1 (−2.8, 0.7)	0.236	0.321	−1.0 (−3.1, 1.0)	0.313	0.331
Model 3	−2.1 (−3.5, −0.6)	0.004	0.010	−1.3 (−2.9, 0.3)	0.120	0.157	−0.8 (−2.6, 1.0)	0.396	0.415
Urogenital									
Model 1	−0.4 (−1.3, 0.5)	0.367	0.354	−0.1 (−1.2, 1.0)	0.861	0.898	−0.3 (−1.3, 0.7)	0.544	0.488
Model 2	−0.4 (−1.3, 0.4)	0.309	0.252	−0.5 (−1.7, 0.7)	0.397	0.459	0.1 (−1.1, 1.2)	0.915	0.949
Model 3	−0.5 (−1.4, 0.3)	0.214	0.170	−0.7 (−1.9, 9.5)	0.236	0.288	0.2 (−1.0, 1.3)	0.783	0.933
Total MRS									
Model 1	−3.5 (−6.8, −0.3)	0.031	0.041	−2.2 (−5.3, 0.9)	0.160	0.191	−1.3 (−4.2 1.6)	0.376	0.354
Model 2	−3.8 (−6.8, −0.9)	0.011	0.013	−3.0 (−5.9, −0.1)	0.040	0.073	−0.8 (−3.7, 2.2)	0.602	0.465
Model 3	−3.9 (−6.9, −1.0)	0.009	0.011	−3.1 (−6.0, −0.2)	0.033	0.062	−0.8 (−3.8, 2.2)	0.601	0.456

AB: alcoholic beer; NAB: non-alcoholic beer. Generalized estimating equation (GEE) models were used to estimate the effect (difference) of the intervention among study groups. Model 1: time since onset of menopause; Model 2: adjusted as in Model 1 plus stress/depression from daily life (score 1–5) and follicle-stimulating hormone concentration; Model 3: adjusted as in model 2 plus isoflavonoid consumption (mg/day) at baseline. *p*-value < 0.05.

3.4. Intervention Effects on Sex Hormone Profile

The hormone levels at baseline and after 6 months of intervention are shown in Supplementary Table S3. A significant change in FSH levels was found in the AB group (*p*-value: 0.038). Plasma levels of LH, E2, progesterone, T-total, FTI and SHBG did not change significantly during the study in any group, nor did the FEI or the T/E2 ratio.

The intervention effect on the sex hormone profile at follow-up can be seen in Table 5. The NAB intervention resulted in a significant decrease in the FTI (adjusted difference: −0.43; 95% IC: −0.86, −0.01; *p*-value: 0.046) compared to the control group. Furthermore, the reduction in FSH (adjusted difference: −10.01; 95% IC: −14.76, −1.36; *p*-value: 0.023), and LH (adjusted difference: −4.74; 95% IC: −8.92, −0.56; *p*-value: 0.026) values was significantly higher in the AB group in comparison with the NAB and control groups, respectively. SHBG levels in both AB and NAB groups were lower at the end of the intervention period, but not significantly.

To better appreciate the changing patterns in sex hormones and the influence of the interventions on the individual hormones, each hormone value was expressed as the % change in hormone concentration, and associations between these responses were studied. In general, % changes of LH and FSH were close to being significantly correlated (r: 0.327; *p*-value: 0.059). Maintaining the inverse correlation shown at baseline, the higher the % change in E2, the lower the % change in FSH (r: −0.360; *p*-value: < 0.037). Other significant correlations found between changes in individual hormones were the % changes of FSH values and the baseline levels of this hormone (r: −0.414, *p*-value: 0.015). Interestingly, participants who initially had a higher amount of SHBG were those who had a lower increment or even a decrease of this protein at the end of the study (r: −0.591, *p*-value: < 0.001). Indeed, individuals who had SHBG values above 80 nMol/L at baseline had lower levels at 6 months, regardless of their group (n = 7).

Table 5. Intervention effect on female sex hormone profile at follow-up.

	AB vs. Control		NAB vs. Control		AB vs. NAB	
	Difference Time-Exposure (95% IC)	***p*-Value**	**Difference Time-Exposure (95% IC)**	***p*-Value**	**Difference Time-Exposure (95% IC)**	***p*-Value**
LH						
Model 1	−5.02 (−9.30, −0.73)	0.022	−1.44 (−9.14, 6.27)	0.714	−3.55 (−11.26, 4.17)	0.367
Model 2	−4.77 (−9.03, −0.51)	0.028	−1.25 (−9.18, 6.68)	0.758	−3.52 (−11.35, 4.34)	0.382
Model 3	−4.74 (−8.92, −0.56)	0.026	−1.09 (−8.99, 6.80)	0.786	−3.71 (−11.68, 4.26)	0.361
FSH						
Model 1	−7.70 (−17.23, 1.83)	0.113	2.29 (−8.37, 12.95)	0.674	−9.99 (−18.80, −1.18)	0.026
Model 2	−7.20 (−16.85, 2.45)	0.144	2.89 (−7.88, 13.66)	0.599	−10.09 (−19.01, −1.17)	0.027
Model 3	−6.25 (−16.26, 3.76)	0.221	3.76 (−7.23, 14.76)	0.502	−10.01 (−14.76, −1.36)	0.023
E2						
Model 1	−2.61 (−17.88, 12.66)	0.738	−7.53 (−21.61, 6.55)	0.295	4.92 (−6.64, 16.47)	0.404
Model 2	−2.32 (−17.64, 13.00)	0.766	−7.39 (−21.55, 6.76)	0.306	5.07 (−6.47, 16.61)	0.389
Model 3	−2.22 (−17.40, 12.96)	0.774	−7.30 (−21.44, 6.84)	0.312	5.08 (−6.45, 16.60)	0.388
Progesterone						
Model 1	0.03 (−0.13, 0.18)	0.742	−0.00 (−0.17, 0.16)	0.984	0.03 (−0.05, 0.11)	0.502
Model 2	0.02 (−0.12, 0.16)	0.802	−0.01 (−0.17, 0.14)	0.861	0.03 (−0.05, 0.11)	0.446
Model 3	0.02 (−0.11, 0.15)	0.803	−0.02 (−0.16, 0.12)	0.822	0.03 (−0.05, 0.11)	0.414
SHBG						
Model 1	−10.08 (−20.77, 0.61)	0.065	−11.37 (−26.60, 3.86)	0.143	1.29 (−14.31, 16.90)	0.871
Model 2	−10.16 (−20.87, 0.55)	0.063	−11.28 (−26.72, 4.16)	0.152	1.11 (−14.64, 16.87)	0.890
Model 3	−10.00 (−20.82, 0.83)	0.070	−10.60 (−26.26, 5.07)	0.185	0.60 (−15.52, 16.72)	0.942
T-total						
Model 1	−2.91 (−8.72, 2.90)	0.327	−6.21 (−12.31, −0.11)	0.046	3.30 (−0.53, 7.13)	0.091
Model 2	−3.08 (−8.82, 2.66)	0.292	−6.70 (−12.75, −0.66)	0.030	3.62 (−0.16, 7,41)	0.061
Model 3	−2.70 (−8.31, 2.91)	0.345	−5.56 (−11.94, 0.82)	0.088	2.86 (−1.50, 7.21)	0.199
TFI						
Model 1	−0.23 (−0.71 0.24)	0.335	−0.42 (−0.86, 0.01)	0.054	0.19 (−0.14, 0.53)	0.259
Model 2	−0.25 (−0.72, 0.21)	0.282	−0.47 (−0.90, −0.05)	0.029	0.22 (−0.08, 0.52)	0.155
Model 3	−0.24 (−0.69, 0.22)	0.312	−0.43 (−0.86, 0.01)	0.046	0.20 (−0.10, 0.50)	0.194
FEI						
Model 1	0.004 (−0.113, 0.122)	0.943	−0.031 (−0.154, 0.092)	0.619	0.035 (−0.043, 0.114)	0.377
Model 2	0.011 (−0.109, 0.131)	0.857	−0.026 (−0.151, −0.100)	0.688	0.037 (−0.041, 0.114)	0.351
Model 3	0.011 (−0.108, 0.131)	0.852	−0.025 (−0.149, 0.099)	0.689	0.037 (−0.040, 0.113)	0.349

AB: alcoholic beer; FEI: free estrogen index; FSH: follicle-stimulating hormone; LH: Luteinizing hormone; NAB: non-alcoholic beer; SHBG: sex hormone-binding globulin; FTI: free testosterone index; T-Total: total testosterone. Generalized estimating equation (GEE) models were used to estimate the effect (difference) of the intervention among study groups. Model 1: time since onset of menopause; Model 2: adjusted as in Model 1 plus stress/depression from daily life (score 1-5) at baseline; Model 3: adjusted as in model 2 plus isoflavonoid consumption (mg/day) at baseline. *p*-value < 0.05.

3.5. Intervention Effects on CVRF

Changes in anthropometric and clinical variables were explored and the intervention effects on CVRF are shown in Table 6. Only mean aspartate transaminase levels differed between the control and AB groups at baseline, but all values fell within the normal range established for this enzyme (Table 2). Daily moderate AB and NAB consumption did not affect anthropometric variables after 6 months. However, DBP was found to diminish in the NAB group in comparison with the AB group (adjusted difference: −7.7; 95% IC: −13.3, −2.1; *p*-value: 0.007). Regarding the lipid profile, the beer interventions had a positive impact and reduced low-density lipoprotein cholesterol (LDL-C) levels. In this respect, it is worth mentioning that both AB and NAB groups started with higher mean levels that exceeded the reference limit. Apoliprotein A1 (ApoA1) levels decreased significantly in the NAB compared with the AB group (adjusted difference: −20.9; 95% IC: −36.6, −5.1; *p*-value: 0.010) and almost significantly with regard to the control group (adjusted difference: −16.6; 95% IC: −33.3, 0.29; *p*-value: 0.054). As for the liver profile, gamma-glutamyl

transferase levels were significantly higher after both beer interventions, but final blood concentrations were still below the reference limit (Table 6).

Table 6. Intervention effect on cardiovascular risk factors and hepatic profile at follow-up.

	AB vs. Control		NAB vs. Control		AB vs. NAB	
	Difference Time-Exposure (95% IC)	***p*-Value**	**Difference Time-Exposure (95% IC)**	***p*-Value**	**Difference Time-Exposure (95% IC)**	***p*-Value**
Weight, kg	−0.4 (−3.0, 2.1)	0.742	−6.0 (−16.6, 4.6)	0.267	5.6 (−4.8, 16.0)	0.293
BMI, kg/m^2	−0.4 (−1.4, 0.7)	0.487	−2.8 (−7.1, 1.6)	0.218	2.4 (−1.9, 6.7)	0.275
WC, cm	−0.2 (−3.1, 2.7)	0.887	−5.2 (−12.3, 1.9)	0.150	5.0 (−2.0, 12.3)	0.160
DBP, mmHg	1.4 (−3.6, 6.3)	0.590	−6.3 (−12.9, 0.2)	0.057	7.7 (2.1, 13.3)	0.007
SDP, mmHg	−1.7 (−8.8, 5.4)	0.639	−10.8 (−22.5, 0.9)	0.070	9.1 (−2.2, 20.5)	0.115
Heart rate, bpm	3.6 (−2.3, 9.5)	0.233	−0.4 (−5.9, 5.1)	0.886	4.0 (−1.1, 9.1)	0.125
Glucose, mg/dL	0.7 (−3.3, 4.7)	0.735	3.1 (−5.9, 12.1)	0.496	−2.4 (−11.5, 6.7)	0.601
Total cholesterol, mg/dL	−6.0 (−19.6, 7.6)	0.386	−10.1 (−26.1, 5.8)	0.212	4.1 (−9.7, 17.9)	0.558
LDL-C, mg/dL	−12.8 (−26.4, 0.8)	0.064	−16.1 (−29.2, −3.1)	0.016	3.3 (−9.3, 15.9)	0.600
HDL-C, mg/dL	3.5 (−3.6, 10.6)	0.403	1.3 (−6.0, 8.6)	0.734	2.2 (−6.0, 10.5)	0.518
Triglycerides, mg/dL	7.2 (−11.3, 25.8)	0.446	5.3 (−12.4, 23.0)	0.558	1.9 (−14.5, 18.3)	0.817
ApoA1, mg/dL	4.4 (−13.5, 22.2)	0.633	−16.6 (−33.3, 0.29)	0.054	20.9 (5.1, 36.6)	0.010
ApoB, mg/dL	−2.3 (−12.8, 8.1)	0.663	−3.8 (−17.7, 10.2)	0.598	1.4 (−13.8, 16.7)	0.853
Lpa, mg/dL	18.1 (−6.5, 42.8)	0.149	13.4 (−11.7, 38.4)	0.295	4.8 (−5.6, 14.1)	0.319
AST, U/L	0.4 (−7.8, 8.6)	0.922	−0.9 (−8.6, 6.8)	0.821	1.3 (−5.5, 8.1)	0.706
ALT, U/L	1.2 (−5.0, 7.5)	0.705	−0.7 (−6.6, 5.2)	0.813	1.9 (−3.0, 6.9)	0.445
GGT, U/L	7.2 (0.3, 14.2)	0.042	6.4 (1−1, 11.6)	0.018	0.9 (−6.5, 8.2)	0.817

AB: alcoholic beer; ALT: alanine transaminase; ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; AST: aspartate transaminase; BMI: body mass index; DBP: diastolic blood pressure; GGT: gamma-glutamyl transferase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Lpa: lipoprotein a; NAB: non-alcoholic beer; SDP: systolic blood pressure; WC: waist circumference. Generalized estimating equation (GEE) models were used to estimate the effect (difference) of the intervention among study groups. *p*-value < 0.05.

4. Discussion

4.1. Climacteric Symptoms

Women worldwide usually find that menopausal symptoms negatively affect their quality of life. The results of this study show that a moderate beer consumption (14 g of ethanol a day) significantly reduces several menopause-related symptoms and should therefore improve the quality of life of postmenopausal women. As these improvements were observed after both AB and NAB consumption, they can be attributed to the non-alcoholic fraction of beer, possibly to the phytoestrogenic effect of polyphenols. All the variables controlled in the study have already been described as modifying factors of menopausal symptomatology [38].

The women in the study who were administered beer were consuming a daily dose of 359 ± 17.4 µg and 259 ± 10.3 µg of prenylflavonoids in the AB and NAB groups, respectively. The estrogenic effect of 8-PN, which has a higher affinity for the estrogen receptor α than β, has already been demonstrated. The relative potency of 8-PN is almost equal to that of estrone and is 70 times weaker than that of E2 [39]. In fact, the activity of 8-PN in beer is greater than the effects of phytoestrogens typically found in soya products [20,21]. Three random controlled trials analyzed the effects of 8-PN on vasomotor symptoms and other menopausal discomforts, concluding that a daily dose of 100 µg/day of 8-PN may provide relief for vasomotor symptoms after 4-12 weeks [13,14,18]. A marginal reducing effect on menopausal complaints in the MRS was also found after 8 weeks of standardized hop extract administration [13]. Therefore, as our results indicate, after the isomerization of XN into IX during brewing and subsequent metabolism of 8-PN in the

human body, the effects of a marginal daily dose of phytoestrogens from beer consumption could be clinically significant [12,40].

The observed reduction of psychological symptoms after 6 months of moderate beer consumption in our intervention may also be relevant, considering their notable impact on the quality of life. No significant difference was found between the AB and NAB interventions in terms of effects on these symptoms.

In line with beer's phytoestrogenic effect, other foods have been described as phytoestrogen sources [10,11,41]. Pomegranate seeds are rich in phytosterols [42,43], while legumes (e.g., soy, bean, alfalfa) are rich in isoflavones [10] and flaxseeds in lignans [44,45]. Due to the difference in components, doses, and duration of the interventions, as well as the variability in the metabolism among individuals and a consistent high placebo response rate; additional studies are warranted to further elucidate the association and comparison between phytoestrogen food sources and the relief of climacteric symptoms [41].

4.2. Sex Hormone Profile

The sex hormone profile did not differ significantly between the study groups. Substantial association with lifestyle (e.g., BMI, smoking, diet, physical activity) and physiological factors (e.g., age, time since the onset of menopause) could account for the absence of clear hormonal differences [46–48]. Reporting similar results, Sierksma et al. (2004) did not detect any differences in plasma E2 and T-total in postmenopausal women after a 3-week crossover random controlled trial comparing AB (30 g alcohol/day) and NAB consumption [49]. Other studies have observed lower levels of LH and FSH, and higher levels of SHBG after 4 weeks of beer consumption [50] and a 16.7% decrease in LH concentration (95% IC 0.5, 30.2) 24 h after the administration of a single 750 mg dose of 8-PN [51]. These findings suggest that 8-PN, ingested either in isolation or in beer, may be able to cross the blood–brain barrier and interact with the hypothalamic-pituitary axis [50,51].

Evidence for longitudinal changes in reproductive hormones during natural menopause transitions has been recently reviewed [47]. Menopause is characterized by a reduced synthesis and secretion of E2 by the ovaries, whereas levels of LH and FSH, the products of gonadotropin cells that can be secreted in tandem, increase for up to 5 and 7 years after the onset of menopause, respectively [47]. Postmenopausal estrogens are synthesized from androgens derived from the metabolism of estrone [52], and the release of pituitary-ovarian hormones is controlled by a negative feedback system [3,46,53]. Thus, the inverse correlation found between E2 and FSH at baseline and after the intervention suggests that E2 still affects pituitary FSH output during the postmenopausal state and continues to play an important role in FSH control. As in other studies, the mean levels of FSH in our volunteers were around 2-fold higher than LH, and both hormone values were directly correlated [46,47].

SHBG decreases slightly for about 4 years after the onset of menopause, after which it increases to a small extent [47]. The bioavailability of both E2 and T-total depends on the fractions that are free or transported with albumin in the circulation, as these have rapid access to target tissues, unlike the fraction bound to SHBG [54]. In the present study, changes in SHBG and the bioavailability of E2 and T-total were explored. T-total seemed to decrease after 6 months of NAB consumption due to a reduction in the FTI. Despite this decrease and the reduction in SHBG levels, T-total bioavailability was apparently not affected by the AB intervention and only slightly by the NAB intervention. As the bioavailability of E2 was also stable, the estrogenic effect of beer consumption cannot be explained by lower SHBG levels.

The FSH levels in the AB group decreased significantly more than in the NAB group after the interventions, despite higher baseline values. Our findings are in line with Soares et al. (2020) [47], who conclude that changes in sex hormones do not differ between alcohol drinkers and abstainers, but that females who drink alcohol more often have higher FSH levels from 2 years after menopause and lower SHBG levels throughout the reproductive age than those with a lower alcohol intake frequency [47]. A decrease in

free-E2 and free-T and an increase in SHBG has also been related with a loss of total body fat [4].

4.3. Cardiovascular Risk Factors

The analysis of anthropometric and biochemical parameters revealed the safety of a daily moderate AB and NAB consumption and the plausible role of NAB in the management of the lipid profile and BP in postmenopausal women. Indeed, the effect of moderate alcohol consumption on CVRF in a controlled crossover dietary study was found to be significantly higher in postmenopausal than premenopausal women [55].

4.3.1. Body Weight and Fat

The incidence of most cardiovascular diseases in women increases after menopause when estrogen levels decrease [52]. Evolution in body weight and body fat distribution in either of the interventions did not differ significantly compared to the control group, which does not offer conclusive proof of a beneficial or negative effect of moderate AB and NAB consumption on these health parameters. The results obtained after the 6-month intervention are in line with the available literature, which indicates that beer consumption has an inconsistent effect on adiposity and weight-control outcomes in women [56].

4.3.2. Blood Pressure

No significant effect on BP was observed after AB consumption compared to the control group, but a significant decrease was seen in the women who consumed NAB compared to those in the AB group. This difference between the two drinks may be due to the pressor effects of ethanol, which could counteract the vasodilator properties attributed to polyphenols [57]. The effect of both phytoestrogens and alcohol on BP has been studied [57–62]. Husain et al. (2015) observed no significant change but a reducing trend in SBP or DBP in postmenopausal women after an intervention with soy isoflavones [58]. Another study showed that isoflavone intake reduces SBP and that the consumption of soy foods tends to reduce both SBP and DBP [61]. Moreover, a reduction in SBP was found after NAB intake (990 mL/day) for 4 weeks in 61 $\pm$ 6 year-old men, while DBP remained unchanged. AB (660 mL/day) and gin (30g/day) consumption did not show any effect on BP [57].

4.3.3. Lipid Profile

A decrease in LDL-C levels was observed after NAB intake for 6 months. However, AB did not seem to affect the lipid profile, in contrast with another study that related ethanol consumption to an increase in total cholesterol, HDL-C and ApoA1 [62]. Interestingly, Chiva et al. (2015) observed lower ApoA1 levels (≈0.5%) after NAB intake but higher HDL-C, ApoA1 and adiponectin after moderate gin and AB consumption [57]. Thus, the non-alcoholic and alcohol fractions of beer did not exhibit the same beneficial effects. A similar study on healthy postmenopausal women reported no differential effect on HDL-C and ApoA1 after moderate consumption of AB or NAB after 3 weeks [63].

The impact of alcohol on lipoproteins in postmenopausal women receiving a controlled diet for 8 weeks differed according to the dose: after the intake of 15 g/day of alcohol, LDL-C and triglycerides significantly decreased, while the benefit of an increase in HDL-C and ApoA1 was only significant after 30 g/day of alcohol [55]. In our AB group, women consumed only 14 g of alcohol daily, an insufficient dose to observe an effect on HDL-C.

Although the results of previous studies suggest a leading role of alcohol in the health effects of beer, some of the study designs have not taken into account that NAB has a lower amount of polyphenols than AB. Hence, the overall impact and mechanisms of action of beer polyphenols might not have been well elucidated yet [62].

4.3.4. Hepatic Profile

The liver plays an important role in the enterohepatic recycling of cholesterol and other substances. Higher levels of aspartate transaminase, alanine transaminase, and gamma glutamyl transpetidase (GGT) are related with hepatocyte damage. In this study, both beer interventions increased GGT levels after 6 months. In a previous study, the phytoestrogens tested were not significantly associated with changes in GGT concentration, although a notable negative association between enterolactone, a well-known phytoestrogen metabolite, and GGT levels in urine was reported [64]. The lack of evidence on this relationship hinders the interpretation of the present results.

4.4. Strengths and Limitations

To our knowledge, this is the first human trial specifically conducted to investigate the effect of beer, with or without ethanol, on the menopausal transition in healthy postmenopausal women. The proposed level of phytoestrogen intake was limited in order to comply with the current dietary Spanish guidelines for alcohol intake, which recommend a maximum of 140 g/week of alcohol for women who are habitual drinkers [65]. The NAB intervention was designed to provide a similar amount of total phytoestrogens as regular beer, this being one of the major strengths of this study. However, a dose-response relationship between prenylflavonoids from beer and menopausal symptoms remains undetermined.

An umbrella systematic review and meta-analyses published in 2007 stated that intervention studies with phytoestrogens without specific inclusion criteria might underestimate the clinical efficacy of this therapeutic approach to menopausal symptoms. Thus, the menopausal status (age and time since the onset of menopause), the description of the intervention (type and amount of phytoestrogen), and the baseline intensity of symptoms are key factors in this kind of study. It was concluded that phytoestrogens could be used in early menopausal women (<5 years since menopause) with mild to moderate vasomotor symptoms [8]. In our clinical trial, the participant population was suitable to study the intervention effect on menopausal complaints, as the age range was narrowed to 49–66 years and the mean times since the onset of menopause in the three study arms were 52.1, 36.7 and 46.0 months (3–4.3 years). Moreover, the phytoestrogen intervention was well-characterized, and the severity of the described symptoms was mild to moderate at baseline. As menopausal complaints naturally decrease over longer time periods, 6 months of follow-up seems to be an appropriate timeframe [13]. Additional strengths of the present study are that drinkers were consuming a single type of alcoholic beverage throughout, and good intervention compliance was achieved.

Although our findings indicate that the beer interventions produced significant differences in comparison with the control group, the tentatively positive effect should be interpreted with caution. The greatest weakness of the present trial is the small sample size, which may have insufficient statistical power to identify some of the effects (power AB vs. Control = 65%; power NAB vs. Control = 34%). Nevertheless, statistically significant differences were observed between the two beer interventions and the control group, pointing to a clinically relevant effect. Moreover, participants were not randomized, but reflected real life conditions. Other limitations include the intra-individual variability of the exposure effect, and a probable self-selection bias, as participants were recruited by an advertisement and volunteered to participate in this clinical trial.

5. Conclusions

In conclusion, a daily moderate AB and NAB consumption may provide an alternative approach for postmenopausal women seeking relief from mild to moderate climacteric symptoms. Moreover, NAB was found to have a beneficial effect on LDL-C, ApoA1, and DBP measurements, all known risk factors for cardiovascular disease. However, these results must be considered as preliminary and will require confirmation with larger sample sizes.

The clinical implications of daily moderate AB and NAB consumption have been revealed in this study, but the mechanisms of action and impacts on sex hormones remain unknown. The most effective quantity of beer, with or without alcohol, that can be safely consumed by a postmenopausal woman still needs to be determined, taking into consideration factors such as age, genetics, and ethnicity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13072278/s1, Table S1: Baseline dietary habits of the 3-day food records from all participants in the intervention groups; Table S2: Intragroup analyses of somatic, psychological, and urogenital subscale scores and total MRS score before, during and at the end of the intervention study; Table S3: Intragroup analysis of female sex hormone levels before and after the intervention.

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Publication 7

Effect of moderate beer consumption (with and without ethanol) on osteoporosis in postmenopausal women: results of a pilot parallel clinical trial

Marta Trius-Soler, Anna Tresserra-Rimbau, Juan J. Moreno, Pilar Peris, Ramon Estruch, Rosa M. Lamuela-Raventós

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Supplementary Material available in Annex 6.

Abstract

Aim: The present study aimed to evaluate if a moderate daily intake of beer with (AB) or without alcohol (NAB) could have beneficial effects on bone tissue.

Methods: A total of 31 post-menopausal women were assigned to 3 study groups: 15 were administered AB (330 43 mL/day) and 6, NAB (660 mL/day), whereas the 10 in the control group refrained from consuming alcohol, NAB, and hop-related products. At baseline and subsequent assessment visits, samples of plasma and urine were taken to analyze biochemical parameters, and data on medical history, diet, and exercise were collected. BMD and the trabecular bone score (TBS) were determined by dual-energy X-ray absorptiometry. Markers of bone formation (bone alkaline phosphatase [BAP] and N-propeptide of type I collagen [PINP]) and bone resorption (N-telopeptide of type I collagen [NTX] and C-telopeptide of type I collagen [CTX]) were determined annually.

Results: Bone formation markers had increased in the AB and NAB groups compared to the control after the 2-year intervention. However, the evolution of BMD and TBS did not differ among the three groups throughout the study period.

Conclusions: According to the findings of this pilot study, moderate beer intake does not seem to have a protective effect against bone loss in early post-menopausal women.

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EDITED BY
Panagiotis Zoumpoulakis,
University of West Attica, Greece

REVIEWED BY
Zhenwu Huang,
Chinese Center For Disease Control and Prevention, China
Max Lambert,
Aarhus University, Denmark

*CORRESPONDENCE
Rosa M. Lamuela-Raventós
lamuela@ub.edu

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Effect of moderate beer consumption (with and without ethanol) on osteoporosis in early postmenopausal women: Results of a pilot parallel clinical trial

Marta Trius-Soler[1,2,3], Anna Tresserra-Rimbau[1,2,3], Juan J. Moreno[1,2,3], Pilar Peris[4], Ramon Estruch[3,5] and Rosa M. Lamuela-Raventós[1,2,3]*

[1]Department of Nutrition, Food Sciences and Gastronomy, XIA, School of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain, [2]Nutrition and Food Safety Research Institute (INSA), University of Barcelona, Santa Coloma de Gramanet, Spain, [3]Centro de Investigación Biomédica en Red (CIBER) de Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain, [4]Rheumatology Department, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain, [5]Department of Internal Medicine, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain

Introduction: Osteoporosis is a chronic progressive bone disease characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue, leading to an increase in bone fragility and the risk of fractures. A well-known risk factor for bone loss is postmenopausal status. Beer may have a protective effect against osteoporosis associated with its content of silicon, polyphenols, iso-α-acids and ethanol, and its moderate consumption may therefore help to reduce bone loss in postmenopausal women.

Methods: Accordingly, a 2-year controlled clinical intervention study was conducted to evaluate if a moderate daily intake of beer with (AB) or without alcohol (NAB) could have beneficial effects on bone tissue. A total of 31 postmenopausal women were assigned to three study groups: 15 were administered AB (330 mL/day) and six, NAB (660 mL/day), whereas, the 10 in the control group refrained from consuming alcohol, NAB, and hop-related products. At baseline and subsequent assessment visits, samples of plasma and urine were taken to analyze biochemical parameters, and data on medical history, diet, and exercise were collected. BMD and the trabecular bone score (TBS) were determined by dual-energy X-ray absorptiometry. Markers of bone formation (bone alkaline phosphatase [BAP] and *N*-propeptide of type I collagen [PINP]) and bone resorption (*N*-telopeptide of type I collagen [NTX] and C-telopeptide of type I collagen [CTX]) were determined annually.

Results: Bone formation markers had increased in the AB and NAB groups compared to the control after the 2-year intervention. However, the evolution of BMD and TBS did not differ among the three groups throughout the study period.

Discussion: Therefore, according to the findings of this pilot study, moderate beer intake does not seem to have a protective effect against bone loss in early post-menopausal women.

KEYWORDS

phytoestrogen, polyphenols, alcohol, silicon, bone markers, osteoporosis, menopause

Introduction

Osteoporosis is characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to an increase in bone fragility and risk of bone fractures (1). A major health problem worldwide, this chronic progressive disease constitutes a serious economic burden. The total direct cost of osteoporotic fractures in Europe (excluding the value of quality-adjusted life-years lost) amounted to €56.9 billion in 2019 and 14.8 million women needing osteoporosis treatment were left untreated, generating a treatment gap of 71% (2). The etiology of osteoporosis is multifactorial, and although genetic and hormonal factors strongly influence the rate of bone loss with age, other aspects such as nutrition, lifestyle habits and physical activity also play an important role (1, 3).

Osteoporosis can occur in both sexes but is most frequently observed in postmenopausal women. Estrogen deficiency can increase bone turnover by nearly 90% and the resulting imbalance in bone remodeling leads to a reduction in bone mass and the development of osteoporosis. In women, there are two phases of bone loss: at the onset of menopause, when it can occur at a rapid rate for up to 5 years, and then as a slower aging-related process lasting for 10–20 years, which affects men as well (4). The menopausal transition has also been associated with an accelerated decline in the trabecular bone score (TBS), supporting the thesis that skeletal integrity is particularly at risk at this life stage (5).

Although chronic alcoholism is known to have a negative impact on bone health, beneficial effects on bone tissue have been attributed to a moderate intake of alcohol (3, 6). Thus, bone mineral density (BMD), the gold standard measurement used to diagnose and treat osteoporosis, has been positively associated with alcohol intake in older women in the Framingham Osteoporosis Study (7) and other landmark cohort studies (8). However, only a few studies have compared the effects of different types of alcoholic beverages (e.g., beer, wine, or spirits) on BMD and conflicting results have been obtained (9, 10). In the Framingham Offspring Cohort Study, it was concluded that moderate alcohol intake may be beneficial in postmenopausal women and that beer and wine have a stronger protective effect on BMD compared to spirits, suggesting that beverage constituents other than alcohol may contribute to bone health (11).

The components of beer that may potentiate its protective effects against osteoporosis include silicon, polyphenols, and iso-α-acids. The results of several epidemiological and experimental studies indicate that dietary silicon may increase BMD and reduce bone fragility (12–14). Major sources of silicon in Western diets are cereals/grains and their derivatives, including breakfast cereals, bread, and beer. Other sources are fruits and vegetables (e.g., bananas, raisins, and green beans), as well as unfiltered drinking water. Our exposure to silicon has declined in recent times, due above all to drinking water treatment, cereal processing, and possibly the hydroponic growth of vegetables (15, 16). This would explain why beer is reported to be one of the main sources of dietary silicon in several epidemiological studies, the average content being 6.336 mg/300 mL (14). Moreover, the silicon found in beer is highly bioavailable and most of it is rapidly absorbed and excreted (16–19). Silicon could promote bone formation stimulating cell proliferation and upregulating the expression of osteogenesis gens such as collagen type 1, which is hypothesized to be due to the induction of the extracellular signal-regulated kinases (ERK) pathway. In addition, silicon has been reported to has an influence on both bone remodeling inhibiting the differentiation and activity of osteoclast and early stages of biomineralization (20). Beer is also rich in flavonoids and phytoestrogens (prenylflavonoids) and contains B-vitamins and other minor components (21–23).

Besides the level of bone mass, bone strength is affected by other tissue parameters, such as micro-architecture and the balance and rate of bone remodeling. The TBS evaluates bone texture based on the analysis of lumbar spine dual-energy X-ray absorptiometry (DXA) images and provides information

on bone micro-architecture. On the other hand, biochemical markers of bone turnover (BTMs) are products released during bone formation by osteoblasts and bone resorption by osteoclasts, and monitoring their levels is a non-invasive way of assessing bone health. The acceleration of bone turnover after menopause, in which bone resorption outpaces formation, is reflected by an increase in BTMs (approximate 90% increase in resorption markers and 45% in formation markers). This increase correlates with a higher rate of bone loss, especially 5–10 years after menopause and in the trabecular bone. Therefore, BTMs are useful for the prediction of bone loss, assessment of fracture risk, and particularly to monitor the treatment of postmenopausal osteoporosis (1, 24). In clinical practice, the most recommended markers of bone formation are the bone isoform of alkaline phosphatase (BAP) and fragments of type I procollagen released during the formation of type I collagen (*N*-propeptide of type I collagen, PINP). Resorption markers include the fragments released from the telopeptide region of type I collagen following its enzymatic degradation [including the *N*-telopeptide of type I collagen (NTX) and the C-telopeptide of type I collagen (CTX)]. PINP and CTX have been proposed by the International Osteoporosis Foundation as reference markers and the use of at least two BTMs is recommended in clinical studies (24).

To sum up, postmenopausal status is a well-known risk factor related to BMD loss and the development of osteoporosis. Due to the phenolic, silicon and ethanol content of beer, its moderate consumption may help to maintain BMD in postmenopausal women. However, few long-term controlled clinical trials have been performed to evaluate the impact of beer on bone mass (22). To address this lack, we conducted a 2-year controlled clinical intervention study to assess whether a moderate daily intake of alcoholic beer (AB) or non-alcoholic beer (NAB) could have beneficial effects on bone tissue. With this aim, the impact of beer consumption on BTMs was determined and changes in BMD and TBS were monitored in a cohort of postmenopausal women.

Materials and methods

Experimental design, study population, and recruitment

This study was a long-term three-arm parallel controlled clinical trial investigating the effect of daily moderate beer consumption on bone tissue. Postmenopausal women aged 45–70 years were recruited into the study from April 2017 to June 2019 from the Outpatient Clinic of the Internal Medicine Department of the Hospital Clinic of Barcelona. The recruitment was done through poster boards in different settings and advertisements on the radio.

The postmenopausal status of each participant was validated by the following criteria: (1) absence of menses in the previous 12 months, during early post-menopausal stage; (2) blood levels of follicle-stimulating hormone (FSH) of 23–116 U/L, and (3) blood levels of 17-β-estradiol (E2) < 37 pg/mL. Women using estrogen therapy or taking silicon or polyphenol supplements were excluded, as were those with known diseases affecting bone metabolism (rheumatoid arthritis, hyperthyroidism, hypercortisolism, renal bone disease, chronic liver disease) or using drugs affecting bone metabolism [fluorides, bisphosphonates, teriparatide or parathormone, strontium ranelate, anabolic steroids, chronic glucocorticoids (>3 months), cytostatics, antiandrogens, and antiepileptics].

Participants were allotted to a study group after a run-in period of 15 days (without consumption of alcoholic drinks, NAB or any hop-related products). The AB group consumed 14 g of ethanol a day in the form of AB (330 mL/day); the NAB group were administered NAB (660 mL/day) containing a similar amount of prenylflavonoid compounds as the AB; and the control group were instructed to refrain from consuming alcohol, NAB or any hop-related products. None of the participants were allowed to consume any alcoholic beverages during the study except what was administered.

Considering the long-term nature of the intervention, participants were assigned to the three study groups according to personal preference, taking into account habitual habits of consumption. As the intervention was dietary, it was blinded to the laboratory personnel and technicians but not to the participants or researchers. During the 2-year intervention, the eligible subjects were asked to visit the research center four times for assessment (at baseline, and 6, 12, and 24 months).

Ethical statement

The study was conducted in compliance with the Declaration of Helsinki. All procedures were approved by the Bioethics Commission of the University of Barcelona (Institutional Review Board: IRB 00003099) in March 2017, and the study protocol was registered at ISRCTN (ISRCTN13825020). All participants signed informed consent.

Intervention product characterization and compliance

To standardize the daily consumption of phytoestrogen in each intervention group, the same brand of beer was consumed by all the participants throughout the study. The participants were encouraged to consume beer during meals, which is the recommended dietary practice for alcoholic beverages (25). As NAB has a lower content in polyphenols (26), the NAB

intervention was adapted to provide a similar amount of total phytoestrogens as the AB. NAB has also been reported to have lower levels of silicon than lagers, like the one used for the AB intervention. The silicon average content reported by other researchers in NAB (n = 6) has been 16.3 (6.4–25.7) mg/L, while in lager AB (n = 27) was 23.7 (10.1–56.4) mg/L (27).

Specifically, the women in the study who were administered beer consumed a daily dose of 359 ± 17.4 μg (isoxanthohumol (IX): 302.7 ± 16.8 μg; xanthohumol: 27.9 ± 0.6 μg; 8-prenylnaringenine (8-PN): 5.5 ± 0.4 μg; 6-prenylnaringenine: 22.8 ± 0.3 μg) of prenylflavonoids in the AB (330 mL/day) and 259 ± 10.3 μg [isoxanthohumol (IX): 104.7 ± 3.8 μg; xanthohumol: 81.3 ± 4.0 μg; 8-prenylnaringenine (8-PN): 10.3 ± 0.8 μg; 6-prenylnaringenine: 62.7 ± 2.2 μg] of prenylflavonoids in the NAB (600 mL/day) group. The prenylflavonoid content of the beer was quantified by liquid chromatography coupled to mass spectrometry (LC-MS/MS) in a previous study by Trius-Soler et al. (22, 28), using the methodology of Quifer-Rada et al. with some slight modifications (29).

Intervention compliance was assessed by data obtained from face-to-face interviews, structured dietary questionnaires, and the measurement of IX, a validated biomarker of beer intake. Quantification of IX was carried out in 24-h urine samples collected at baseline, and 6, 12, and 24 months by solid phase extraction LC-MS/MS (30). To facilitate intervention compliance, the participants were supplied with beer every month.

Measurements and outcome assessment

Medical history

Individual information was collected at baseline and updated during each visit by face-to-face interviews. The structured interviews included medical and sociodemographic questions, with special attention given to risk factors for osteoporosis, previous skeletal fractures, menarche and menopause, dietary calcium intake, history of nephrolithiasis, current and past consumption of alcohol and tobacco, and family history of fractures. Sleeping habits, daily life and work stress, time since the onset of menopause, and medication history were also recorded. Participants with serum 25-hydoxyvitamin D (25-OHD) levels < 20 ng/mL were treated with vitamin D supplements, as is usual in clinical practice.

Bone mineral density assessment

We assessed the BMD (g/cm^2) of the lumbar spine, proximal femur (femoral neck and total hip) and whole-body by DXA (GE-LUNAR iDXA Prodigy equipment) at baseline and after 12 and 24 months of intervention. The TBS was calculated using TBS iNsight software (V1.8) (Medimaps Group, Geneva, Switzerland) on the DXA lumbar spine images. Osteoporosis was defined by T-score values ≤−2.5 at the lumbar spine and/or proximal femur according to the WHO criteria and a TBS value < 1.230 indicated degraded micro-architecture (31, 32). BMD assessment was performed following standardized scanning protocols by the CETIR medical group (CETIR Grup Mèdic, Barcelona, Spain).

Anthropometric measurements and body composition

Anthropometric measurements (height, weight, and waist circumference) were obtained at each visit by trained registered staff following anthropometric standardization protocols. Weight was determined using a high-quality calibrated scale, with the participants wearing light clothing and no shoes. Height was measured with a wall-mounted stadiometer. Body mass index (kg/m^2) (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured using an inelastic flexible tape positioned at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest (33).

Total body and regional body composition were estimated using DXA. Lean mass (kg) and fat mass (kg) were both indexed to height to create the fat mass index (kg/m^2) and lean mass index (kg/m^2). Measurements were assessed by the CETIR medical group (CETIR Grup Mèdic, Barcelona, Spain).

Biological samples and biochemical analyses

Overnight fasting blood samples and morning spot urine (between 8–9 a.m., to control circadian cycles) were collected at baseline and 6, 12, and 24 months of intervention. Automated biochemical profiles were measured at the Biomedical Diagnostic Center of the Hospital Clinic. The lower detection limits of plasma E2 was 12 pg/mL. Levels below these limits were defined as 11 pg/mL. 24-h urine samples were also collected at all visits and stored in aliquots at −80°C until analyzed for IX, the biomarker of intervention compliance.

Serum BAP was measured by ELISA (immunodiagnostic Systems, Boldom, UK), and serum CTX and PINP by a Cobas e601 analyzer (Roche Diagnostics, Mannheim, Germany). Urinary NTX was measured by ELISA (Osteomark NTX-I, Alere, Scarborough, ME, USA) and expressed as a ratio to creatinine. Plasma parathyroid hormone (PTH) and serum 25-OHD were determined by Atellica Solution (Siemens Healthineers, Tarrytown, NY, USA) and a Liaison analyzer (DiaSorin, Saluggia, Italy), respectively. A concentration of 25-OHD < 20 ng/mL was considered to be vitamin D deficiency. Blood and urine samples were obtained between 8:00 and 9:00 a.m. after overnight fasting.

Dietary intake and physical activity assessments

Dietary intake over the previous 12 months was assessed by trained staff at baseline, the halfway point (12 months)

and at the end (24 months) of the study using a validated 151-item semi-quantitative food frequency questionnaire (FFQ) (34). Total energy intake (kcal/day) and absolute consumption values of coffee (with caffeine) and tea per day were estimated according to Spanish food composition tables (34). Calcium and vitamin D intake were also estimated by the 151-item FFQ. Total polyphenol intake (mg/day) was estimated by multiplying the polyphenol content in each food item (data obtained from the Phenol-Explorer database) by the daily consumption of the food item according to the FFQ (35). In addition, the 14-point Mediterranean Diet Adherence questionnaire was used as an overall diet quality index to evaluate differences between study groups at baseline (36).

Physical activity was monitored at the four intervention visits. It was measured as the metabolic equivalent of task per day (MET-min/day) using the Minnesota leisure-time questionnaire, previously validated in a population of Spanish women (37).

Sample size calculation

In postmenopausal women, rates of spine and hip bone loss are 0.022 g/cm^2 per year (2.0%) and 0.013 g/cm^2 per year (1.4%), respectively (38). For a parallel design and an analysis of repeated measures, statistical power calculation indicated that to recognize as statistically significant a difference greater than or equal 0.020 g/cm^2 (2.0%) in total hip BMD with a common standard deviation of 0.025 g/cm^2, assuming a maximum loss of 10% of participants, and a correlation coefficient between the initial and final measurements as 0.7; 17 subjects per group will be needed to complete the study ($\alpha = 0.05$; power = 0.8).

Statistical analyses

Continuous variables were expressed as median (Q1–Q3). Categorical variables were expressed as number (*n*) and proportion (%). Differences in the characteristics of volunteers between groups at baseline were tested by the chi-square test for categorical variables and the Kruskal–Wallis test followed by the *post-hoc* Dunn's test for continuous variables.

The effect of the interventions on bone turnover and bone health markers was estimated by performing a generalized estimating equation on gamma regression models for repeated measures (identity link function, autoregressive of order correlation, and robust standard error parameters were specified). Adjusted differences and their corresponding 95% confidence intervals were computed using increasing complexity models. A time-exposure interaction term allowed the evaluation of potential differences between intervention groups in response to changes over time. Spearman's correlations were used to summarize the relationship between the BTMs and the BMD values at baseline and annually.

The % relative changes for bone turnover and bone health markers were calculated. Intergroup differences between baseline, and at 12 and 24 months were analyzed by a non-parametric test for two related samples in each study arm. A Wilcoxon matched-pair signed-rank test for small samples was applied to symmetric variables, and the sign test of matched pairs was used for asymmetric variables. Symmetry was studied by the skewness and kurtosis test for normality (control and AB group) or graphically (NAB group).

Intergroup differences in relevant clinical and anthropometric measurements as well as in dietary patterns between baseline and 24 months were also analyzed by a non-parametric test for two related samples in each study arm. Intragroup differences in dietary patterns were assessed by a Kruskal–Wallis test followed by *post-hoc* Dunn's test in each group.

All statistical analyses were conducted using the Stata statistical software package version 16.0 (StataCorp, College Station, TX, USA). Statistical tests were two-sided and *p*-values below 0.05 were considered significant. Figures were performed using the Prism 9.0.0 software package.

Results

Study subjects, intervention, and compliance

Of the 34 postmenopausal women enrolled at baseline, 31 completed the outcome assessments at 12 and 24 months (**Figure 1**). Of the women that finished the intervention, 15 had chosen to be in the AB group, six in the NAB group, and 10 in the control group. The drop-outs were due to difficulties with continuing the assessment visits or complying with the assigned intervention, as reported by the participant. Otherwise, subject compliance with the intervention was 100% according to dietary self-records and interviews. To confirm intervention adherence, IX concentrations were measured in the 24-h urine provided by the participants at baseline, and 6, 12, and 24 months, thus participants could drink beer at any time of the day but were encouraged to do it with meals. At baseline, IX concentration was below the detection limit (<0.04 ppb) for 71.0% of the urine samples. At follow-up visits (6, 12, and 24 months), IX values confirmed intervention compliance in 96.7, 97.8, and 77.8% of urine samples of the control, AB, and NAB groups, respectively. The concentration of IX was highly variable among samples.

Participant characteristics at baseline

Tables 1, **2** summarize the clinical, anthropometric, densitometric and biochemical parameters of the trial participants. Briefly, the volunteers had a median (Q1, Q3)

age of 55 (53–58) years and a BMI of 26.3 (24.7–29.0) kg/m^2. Most were normo-weight or overweight with an elevated waist circumference (Table 2). Although median baseline values of BTMs were within the reference ranges in all three groups, Q1–Q3 values were in the upper reference range or higher (Table 2; 39). Two participants (one in the control and the other in the AB group) presented densitometric osteoporosis in the lumbar spine at baseline.

In terms of absolute analytical values, serum creatinine and calcium concentrations were within the reference ranges, while PTH serum levels were within the reference range or above. Median levels of 25-OHD for each group were above 20 ng/mL (with nine subjects showing values < 20 ng/mL: 2 control, 4 AB, and 3 NAB, respectively) (Table 2). Taking as a reference the results reported for women aged 60 years or more in a cohort study of 5,629 healthy Caucasian men and women (15–98 years), the participants in the present study had similar or higher indices of mean body fat (%) and body fat mass, and a lower lean mass index (40).

Significant differences in baseline characteristics between treatment arms were only observed in family history of fractures, daily life-induced stress/depression score, TBS values (higher in the control group), lean mass index values, FSH levels, aspartate transaminase (AST) and gamma-glutamyl transferase (GGT) (Tables 1, 2). No significant differences were observed in baseline DMD values in any skeletal location or in baseline BTMs between groups. Five out of 6 (83.3%) of the volunteers in the NAB group had a family history of fractures, whereas women in the AB group reported higher levels of stress in their daily life (Table 1). At baseline, median FSH levels of the AB group were significantly higher compared to the NAB group, while the lean mass index was lower in the AB than the control group (Table 2). Moreover, the AB group normally drank alcoholic beverages more often (60% reported a weekly frequency habit) and had significantly higher levels of AST and GGT compared to the control group, but within the reference range (Table 2).

Four women (13%) were taking antihypertensive medication, 2 (6%) antihyperlipidemic medication, 6 (20%) antidepressants/sedative/anxiety pills, and 14 (45%) dietary supplements. No statistical differences in medication use were observed between groups at baseline or at the end of the intervention (Table 1).

Controlled covariates: Anthropometric, clinical, and dietary intake changes during follow-up

For a more in-depth study of the intervention effects on bone tissue, changes in anthropometric and biochemical

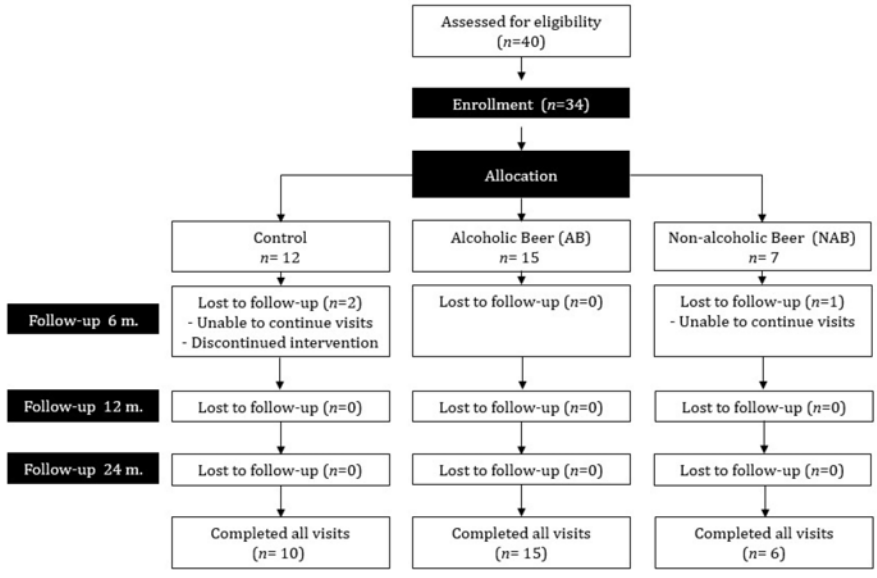

FIGURE 1
Flow diagram of participant recruitment and compliance in each phase of the intervention trial.

TABLE 1 Baseline characteristics, bone turnover markers and dual-energy X-ray absorptiometry (DXA) parameters of the participants according to the intervention group.

	Control (n = 10)	AB (n = 15)	NAB (n = 6)	p-value
Medical history records				
Age, years	55 (53–59)	54 (53–56)	57 (54–59)	0.614
Time since the onset of menopause, months	50.0 (18.0–96.0)	24.0 (15.0–48.0)	22.5 (15.0–50.0)	0.553
Previous fractures (after 45 years), n (%)	2 (20.0)	2 (13.3)	1 (16.7)	0.605
Family history of fractures, n (%)	1 (10.1)	1 (6.7)	5 (83.3)	**<0.001**
Early menopause, n (%)	0 (0.0)	1 (6.3)	1 (16.7)	0.421
Lifestyle habits				
Smoking habit, n (%)				
Current	0 (0.0)	6 (40.0)	2 (33.3)	0.112
Former	3 (30.0)	4 (26.7)	0 (0.0)	
Never	7 (70.0)	5 (33.3)	4 (66.7)	
Sleeping time, hours	6.0 (6.0–7.0)	7.0 (6.5–8.0)	7.3 (6.0–7.5)	0.193
[1]Stress/depression from daily life	2.5 (1.0–3.0)[ab]	3.0 (3.0–4.0)[a]	1.5 (1.0–2.0)[b]	**0.025**
[1]Stress/depression from work	2.5 (2.0–3.0)	3.0 (2.0–4.0)	2.5 (1.0–4.0)	0.905
Physical activity, MET-min/day	840 (480–1,146)	552 (304–807)	460 (396–601)	0.238
DXA parameters				
Lumbar spine				
BMD, g/cm^2	1.05 (0.95–1.17)	1.01 (0.99–1.14)	1.07 (1.00–1.16)	0.861
T-score	−0.95 (−1.90, −0.29)	−1.56 (−1.76, −0.50)	−1.06 (−1.40, −0.35)	0.876
TBS	1.41 (1.35–1.47)[a]	1.33 (1.28–1.35)[b]	1.33 (1.30–1.41)[ab]	0.021
Femoral neck				
BMD, g/cm^2	0.91 (0.86–1.03)	0.86 (0.75–0.90)	0.80 (0.77–0.92)	0.218
T-score	−0.61 (−1.00, 0.42)	−0.98 (−1.93, 0.65)	−1.36 (−1.77, −0.50)	0.226
Total hip				
BMD, g/cm^2	0.99 (0.94–1.07)	0.89 (0.85–1.01)	0.88 (0.81–1.01)	0.099
T-score	−0.06 (−0.48, 0.610)	−0.92 (−1.27, 0.08)	−1.18 (−1.59, 0.06)	0.094
Whole body				
BMD, g/cm^2	1.07 (1.00–1.11)	1.04 (0.98, 1.09)	1.07 (0.99, 1.10)	0.737
T-score	0.20 (−0.50, 0.50)	−0.20 (−0.80, 0.30)	0.20 (−0.10, 0.40)	0.522
Bone turnover markers				
[2]BAP, ng/mL	12.6 (10.1–14.3)	12.2 (10.5–14.8)	11.8 (9.6–15.4)	0.932
[3]PINP, ng/mL	55.5 (43.2–66.4)	55.5 (44.8–64.0)	43.8 (34.7–81.4)	0.724
[4]NTX, nMol/nMol	60.5 (53.0–74.0)	66.0 (45.0–74.0)	49.5 (47.0–55.0)	0.360
[5]CTX, ng/mL	0.54 (0.51–0.75)	0.52 (0.44–0.68)	0.43 (0.31–0.67)	0.320
Medication, n (%)				
Antihypertensive agents	0 (0.0)	3 (20.0)	1 (16.7)	0.328
Lipid-lowering medication	0 (0.0)	2 (13.3)	0 (0.0)	0.320
Antidepressants, sedatives, anxiety pills	2 (20.0)	3 (20.0)	1 (16.7)	0.983
Dietary supplements	4 (40.0)	8 (53.3)	2 (33.3)	0.653

[1]Score from 1–5.
[2]BAP reference values: 6.0–13.8 ng/mL.
[3]PINP reference values: 20.8–60.6 ng/mL.
[4]NTX reference values: 19.3–68.9 nMol/nMol.
[5]CTX reference values: 0.14–0.48 ng/mL. AB, alcoholic beer; BAP, bone alkaline phosphatase; BMD, bone mineral density; CTX, C-telopeptide of type I collagen; NAB, non-alcoholic beer; NTX, N-telopeptide of type I collagen; PINP, N-propeptide of type I collagen; TBS, trabecular bone score.
Categorical variables are expressed as number (n) and proportion (%).
Chi-square test was applied to study differences in categorical variables.
Continuous variables are presented as median values (Q1–Q3).
Kruskal–Wallis test followed by *post-hoc* Dunn's test were applied to study differences in continuous variables.
Medians within the same row carrying different superscripts (a, b) are significantly different.
p-value < 0.05. The bold values represent the p-value < 0.050 is considered statistically significant.

variables that might explain or modify these effects were monitored (**Supplementary Table 1**). At the end of the intervention (24 months), both fat and lean mass indices had significantly increased in the AB group; accordingly, the BMI was also higher, although not significantly. Additionally, median (Q1–Q3) creatinine levels had significantly increased in the control and AB groups, whereas PTH levels increased significantly only in the AB group.

Changes in individual dietary patterns during follow-up were also monitored (**Supplementary Table 2**). Regarding

TABLE 2 Baseline anthropometric measurements, dietary history, and biochemical analyses of the participants according to intervention group.

	Control (n = 10)	AB (n = 15)	NAB (n = 6)	p-value
Anthropometric measures				
BMI, kg/m^2	26.5 (25.3–32.5)	26.5 (23.1–28.6)	25.3 (24.7–29.0)	0.595
WC, cm	90.0 (85.5–100.0)	88.7 (79.5, 96.4)	84.5 (80.3–90.1)	0.588
Body fat mass, %	44.1 (40.2–45.1)	42.7 (39.2–47.5)	40.3 (39.1–48.2)	0.900
Fat mass index, kg/m^2	11.5 (9.6–15.3)	11.2 (8.8–13.0)	10.5 (9.3–12.3)	0.636
Lean mass index, kg/m^2	15.0 (14.5–17.2)^a	14.2 (13.1–14.6)^b	14.6 (14.0–16.5)^{ab}	0.034
Dietary history				
Total energy intake, kcal/day	2,699 (2,556–3,022)	2,599 (2,127–3,138)	2,348 (2,268–2,682)	0.320
Protein intake, % kcal/daily kcal	20.4 (16.3–20.9)	19.2 (17.4–21.8)	18.1 (16.9–20.4)	0.781
Calcium intake, mg/day	1,365 (1,090–15,679)	1,199 (935–1,552)	1,083 (824–1,334)	0.405
Vitamin D intake, µg/day	6.1 (4.0–9.8)	6.4 (4.9–8.3)	6.3 (5.7–7.0)	0.968
Total polyphenol intake, mg/day	1,064 (770–1,419)	753 (487–853)	830 (677–1,450)	0.127
Alcohol drinking habit				
Weekly, n (%)	1 (10.0)	9 (60.0)	1 (16.7)	0.061
Occasionally, n (%)	7 (70.0)	6 (40.0)	4 (66.7)	
Never, n (%)	2 (20.0)	0 (0.00)	1 (16.7)	
Type of alcohol preferred				
Beer, n (%)	3 (30.0)	7 (46.7)	3 (50.0)	0.419
Wine, n (%)	4 (40.0)	7 (46.7)	2 (33.3)	
Spirits, n (%)	0 (0.0)	1 (6.7)	0 (0.0)	
None, n (%)	3 (30.0)	0 (0.0)	1 (16.7)	
MedDiet, 14-item score	9.0 (7.0–9.5)	7.0 (6.0–9.0)	8.5 (7.0–10.0)	0.338
Tea consumption, g/day	14.3 (0.0–21.4)	7.1 (0.0–21.4)	1.7 (0.0– 50)	0.839
Caffeinated coffee consumption, g/day	50 (21–125)	50 (0–125)	88 (0–125)	0.757
Biochemical markers				
Creatinine, mg/dL	0.71 (0.56–0.83)	0.64 (0.59–0.75)	0.68 (0.66–0.69)	0.456
Calcium (serum), ng/dL	9.3 (9.0–9.5)	9.3 (9.0–9.5)	9.3 (9.1–9.5)	0.969
PTH, ng/mL	63.0 (44.0– 80.0)	52.0 (46.0–69.0)	66.5 (46.0–73.0)	0.751
25-hydroxyvitamin D, ng/mL	23.7 (20.6–26.5)	25.4 (18.6–35.7)	24.6 (14.1–38.6)	0.743
FSH, U/L	75.0 (56.3–84.3)^{ab}	88.5 (74.0–105.5)^b	70.4 (37.2–72.2)^a	0.027
E2, pg/mL	23.0 (15.0–31.0)	18.0 (13.5–25.0)	22.0 (21.0–25.0)	0.587
TSH, ng/mL	2.15 (0.94–3.75)	1.82 (1.48–2.96)	2.56 (2.19–2.91)	0.695
FT4, ng/mL	1.17 (1.11–1.32)	1.13 (1.02–1.26)	1.08 (0.98–1.10)	0.159
T3, ng/mL	1.23 (0.98–1.41)	1.14 (1.06–1.25)	1.09 (0.93–1.19)	0.634
AST, U/L	19 (18–20)^a	23 (19–25)^b	20 (17–25)^{ab}	**0.028**
ALT, U/L	16 (14–18)	18 (16–28)	18 (13–22)	0.191
GGT, U/L	13 (10–14)^a	22 (14–26)^b	14 (12–23)^{ab}	**0.044**

AB, alcoholic beer; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; E2, 17-β-estradiol; FSH, follicle-stimulating hormone; FT4, thyroxine; GGT, gamma-glutamyl transferase; MedDiet, mediterranean diet adherence screener 14-item score; NAB, non-alcoholic beer; PTH, parathyroid hormone; T3, triiodothyronine; TSH, thyroid stimulating hormone; WC, waist circumference.

Categorical variables are expressed as number (n) and proportion (%).

Chi-square test was applied to study differences in categorical variables.

Continuous variables are presented as median values (Q1–Q3).

Kruskal–Wallis test followed by *post-hoc* Dunn's test were applied to study differences in continuous variables.

Medians within the same row carrying different superscripts (a, b) are significantly different.

p-value < 0.05. The bold values represent the p-value < 0.050 is considered statistically significant.

the median dietary pattern of the participants, intake was low for carbohydrates (<45–60% kcal/total kcal) and high for sugar (>10% kcal/total kcal), protein (>12–15% kcal/total kcal), fat (>20–35% kcal/total kcal), and saturated fatty acids (<10% kcal/total kcal), according to the reference values of the European Food Safety Authority (41). Fiber intake met the EFSA recommendations and alcohol consumption ranged from low to moderate. Calcium intake also met the recommendation for older people (750 mg/day) or was slightly below, whereas the intake of dietary vitamin D was below the level established for adults (600 IU/day or 15 µg/day) (41).

According to the FFQ data, alcohol consumption at baseline and throughout the study period (due to the intervention) was significantly higher in the AB than in the NAB and control groups. Median (Q1–Q3) percentages of energy provided by carbohydrate and fat intake were significantly higher in the NAB group during follow-up. The percentage of energy provided by simple sugar in the NAB group was also higher than in the AB group at 12 and 24 months. Dietary factors within the NAB

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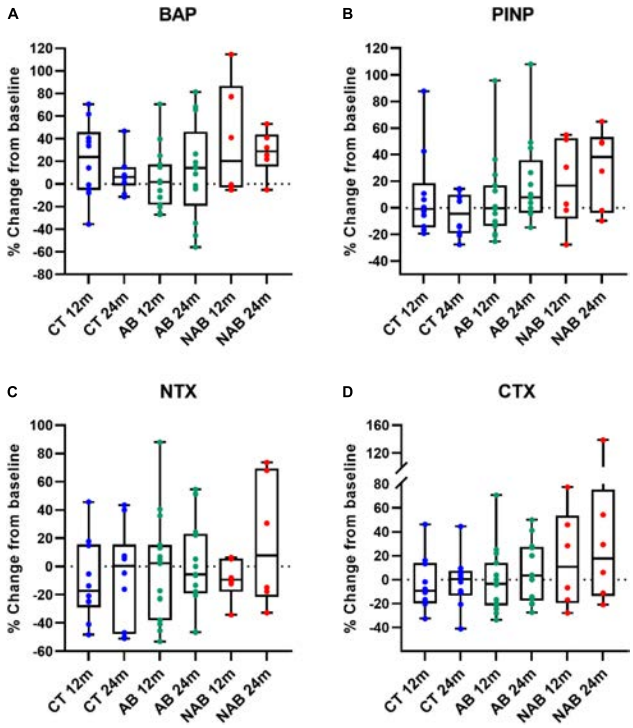

FIGURE 2
Relative change at 12 months (12 m) and 24 months (24 m) in **(A)** bone alkaline phosphatase (BAP); **(B)** *N*-propeptide of type I collagen (PINP); **(C)** *N*-telopeptide of type I collagen (NTX); **(D)** C-telopeptide of type I collagen (CTX) of the control (CT), alcoholic beer (AB) and non-alcoholic beer (NAB) group in comparison to baseline. Median (min, max) values are illustrated. No significant differences were found between baseline and 12 or 24 months in each arm. Matched-pair signed-rank test was used for statistical intragroup comparisons throughout the intervention. Sing-test of matched pairs was used in asymmetric distributed variables.

group did not change significantly during the study, whereas, at 24 months a significant reduction in the percentage of energy intake from carbohydrates was reported by the AB group and from saturated fatty acids by the control group, the latter also reporting a significantly lower intake of calcium.

Changes in bone turnover markers according to beer consumption

Prespecified endpoints were changes in bone formation and bone resorption markers at 12 and 24 months compared to baseline in each group (Figure 2). PINP values in the AB and NAB groups had increased at 24 months but did not change in the control group. All groups displayed a high inter-variability in % changes from baseline.

Table 3 shows the intervention effect on BTMs at follow-up. At 24 months, postmenopausal women consuming AB and NAB exhibited a significantly higher increase in PINP than those in the control group. The linearly measured time-exposure interaction was found to be statistically significant when comparing PINP values of the AB and control groups (*p*-trend: 0.029) and the NAB and control groups (*p*-trend: 0.001); the adjusted differences in PINP levels were expected to increase by 0.39 ng/mL (95% CI: 0.04, 0.74) and 0.76 ng/mL (95% CI: 0.31, 1.21) for every 12 additional months of intervention, respectively. Additionally, the mean difference in BAP values between baseline and 24 months was significantly higher in the NAB than in the control group, with a significant linear time–exposure interaction (adjusted difference: 0.09; 95% CI: 0.01, 0.17; *p*-trend: 0.026). In contrast, no significant changes in the NTX and CTX bone resorption markers were observed in either of the intervention groups compared to the control.

TABLE 3 Intervention effect on bone formation and bone resorption markers at follow-up.

	AB vs. control			NAB vs. control			AB vs. NAB		
	Difference time-exposure (95% CI)	*p*-value	*p*-trend	Difference time-exposure (95% CI)	*p*-value	*p*-trend	Difference time-exposure (95% CI)	*p*-value	*p*-trend
BAP, ng/mL									
Model 1	−0.4 (−3.2, 2.3)	0.748	0.722	2.1 (−0.0, 4.2)	0.053	0.065	−2.5 (−5.7, 0.6)	0.109	0.088
Model 2	−0.4 (−2.8, 1.9)	0.71	0.653	1.9 (0.2, 3.5)	**0.033**	**0.023**	−2.3 (−4.9, 0.3)	0.08	**0.041**
Model 3	−0.9 (−3.1, 1.4)	0.452	0.423	1.8 (0.1, 3.5)	**0.039**	**0.026**	−2.6 (−5.1,−0.1)	**0.038**	**0.019**
PINP, ng/mL									
Model 1	9.9 (1.0, 18.8)	**0.03**	**0.036**	16.7 (3.9, 29.6)	**0.011**	**0.016**	−6.8 (−19.1, 5.5)	0.279	0.315
Model 2	10.6 (2.1, 19.0)	**0.014**	**0.02**	18.2 (7.7, 28.7)	**0.001**	**0.001**	−7.6 (−17.6, 2.4)	0.135	0.146
Model 3	9.5 (1.5, 17.5)	**0.019**	**0.029**	17.9 (7.7, 28.1)	**0.001**	0.001	−8.4 (−18.7, 1.9)	0.111	0.12
NTX, nMol/nMol									
Model 1	4.5 (−14.4, 23.3)	0.641	0.68	12.0 (−10.5, 34.6)	0.327	0.327	−7.6 (−26.6, 11.5)	0.793	0.456
Model 2	5.7 (−11.5, 22.9)	0.516	0.561	9.6 (−11.8, 30.9)	0.381	0.416	−3.9 (−21.7, 14.0)	0.672	0.693
Model 3	3.0 (−13.3, 19.2)	0.721	0.743	8.6 (−12.0, 29.3)	0.413	0.443	−5.7 (−23.5, 12.2)	0.533	0.561
CTX, ng/mL									
Model 1	−0.01 (−0.11, 0.08)	0.92	0.891	0.80 (−0.08, 0.24)	0.327	0.343	−0.08 (−0.24, 0.07)	0.429	0.289
Model 2	0.00 (−0.10, 0.10)	0.977	0.972	0.11 (−0.02, 0.25)	0.111	0.098	−0.11 (−0.24, 0.02)	0.098	0.096
Model 3	0.01 (−0.10, 0.10)	0.983	0.958	0.11 (−0.02, 0.25)	0.104	0.11	−0.11 (−0.25, 0.02)	0.095	0.093

AB, alcoholic beer; BAP, bone alkaline phosphatase; CI, coefficient interval; CTX, C-telopeptide of type I collagen; NAB, non-alcoholic beer; NTX, *N*-telopeptide of type I collagen; PINP, *N*-propeptide of type I collagen.
Generalized estimating equation (GEE) models to estimate the effect (difference group × time 95% CI) on the intervention between the intervention groups and the control group.
Model 1: adjusted by age at baseline; Model 2: adjusted like Model 1 plus time since the onset of menopause, follicle-stimulating hormone concentration, smoking habit, lean mass index at baseline; Model 3: adjusted like Model 2 plus total energy intake, physical activity as MET-min/day, and calcium dietary intake at baseline.
p-value: group × time interaction; *p*-trend: group × time interaction (continuous).
Two participants of the AB group were excluded from the analysis at 24 months due to traumatic fractures during the last year of the intervention.
p-value < 0.05. The bold values represent the *p*-value < 0.050 is considered statistically significant.

The alcoholic fraction derived from AB consumption appeared to have an opposite effect on BAP levels compared to the non-alcoholic fraction of beer at 24 months of intervention (AB vs. NAB group, Table 3).

Among all participants, % changes in PINP were positively correlated with % changes in BAP at 12 (*r*: 0.568; *p*-value: 0.001) and 24 months (*r*: 0.560; *p*-value: 0.002) from baseline. Moreover, % changes in CTX were also correlated with% changes in PINP levels at 12 (*r*: 0.689; *p*-value: < 0.001) and 24 months (*r*: 0.556; *p*-value: 0.002), and BAP levels at 24 months (*r*: 0.381; *p*-value: 0.042). Furthermore, % changes in resorption markers were positively correlated at 24 months from baseline (*r*: 0.375; *p*-value: 0.045).

Changes in bone mass and trabecular bone score according to beer consumption

Prespecified endpoints also included changes in BMD and TBS. Figure 3 shows % changes in lumbar spine, total hip, femoral neck, and whole-body BMD as well as % changes in TBS at 12 and 24 months from baseline values in the three study groups. As shown in the figure, total hip and whole-body BMD significantly decreased in all groups during the 2-year study period. Additionally, a significant decrease in the femoral neck BMD was observed in the control group and in lumbar spine BMD and TBS in the AB group at 24 months.

The intervention effect on BMD and TBS was analyzed considering exposure time interactions (Table 4). Changes in bone health according to DXA measurements were not significantly different when comparing the AB or NAB group with the control; no significant differences were found between the beer interventions either.

When we analyzed the % of subjects with a decrease in BMD > 3% in either lumbar spine, total hip, and femoral neck at 12 and 24 months, no significant differences were observed between the three groups.

Discussion

In this 2-year parallel controlled clinical trial with postmenopausal women, AB and NAB consumption was found to increase bone formation markers (i.e., PINP in both intervention groups and BAP only in the NAB group) in

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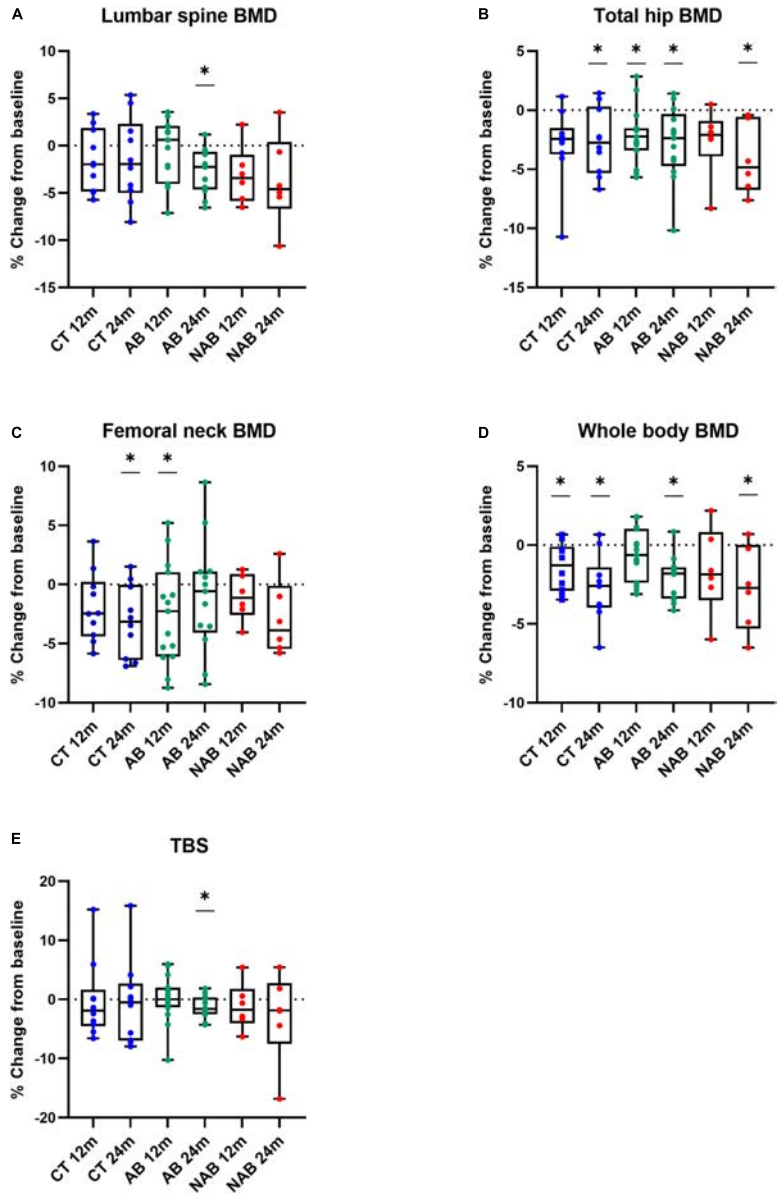

FIGURE 3
Relative change at 12 months (12 m) and 24 months (24 m) in **(A)** Lumbar spine bone mineral density (BMD). **(B)** Total hip BMD. **(C)** Femoral neck BMD. **(D)** Whole body BMD. **(E)** Trabecular bone score (TBS) of the control (CT), alcoholic beer (AB) and non-alcoholic beer (NAB) group in comparison to baseline. Median (min, max) values are illustrated. (*) refers to the difference between baseline and 12 or 24 months in each arm. Matched-pair signed-rank test was used for statistical intragroup comparisons throughout the intervention. Sing-test of matched pairs was used in asymmetric distributed variables.

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TABLE 4 Intervention effect on bone mineral density (BMD) and bone quality at follow-up.

	AB vs. control			NAB vs. control			AB vs. NAB		
	Difference time-exposure (95% CI)	*p*-value	*p*-trend	Difference time-exposure (95% CI)	*p*-value	*p*-trend	Difference time-exposure (95% CI)	*p*-value	*p*-trend
Lumbar spine BMD, g/cm^2									
Model 1	−0.01 (−0.04, 0.02)	0.556	0.599	−0.02 (0.07, 0.02)	0.313	0.324	0.01 (−0.02, 0.05)	0.477	0.464
Model 2	−0.01 (−0.04, 0.02)	0.546	0.586	−0.03 (−0.07, 0.02)	0.258	0.267	0.02 (−0.02, 0.05)	0.393	0.384
Model 3	−0.01 (−0.04, 0.02)	0.484	0.535	−0.03 (−0.07, 0.02)	0.237	0.247	0.02 (−0.02, 0.05)	0.405	0.389
Femoral neck BMD, g/cm^2									
Model 1	0.01 (−0.01, 0.04)	0.337	0.408	0.00 (−0.03, 0.03)	0.87	0.881	0.01 (−0.02, 0.04)	0.47	0.539
Model 2	0.01 (−0.01, 0.04)	0.329	0.386	0.01 (−0.02, 0.03)	0.687	0.687	0.01 (−0.02, 0.04)	0.624	0.693
Model 3	0.01 (−0.02, 0.04)	0.392	0.478	0.01 (−0.02, 0.03)	0.711	0.715	0.01 (−0.02, 0.04)	0.675	0.763
Total hip BMD, g/cm^2									
Model 1	−0.00 (−0.02, 0.02)	0.976	0.904	−0.01 (−0.04, 0.01)	0.378	0.371	0.01 (−0.01, 0.04)	0.387	0.42
Model 2	0.00 (−0.02, 0.02)	0.961	0.893	−0.01 (−0.04, 0.02)	0.49	0.484	0.01 (−0.02, 0.04)	0.501	0.536
Model 3	−0.00 (−0.02, 0.02)	0.945	0.864	−0.01 (−0.04, 0.02)	0.519	0.511	0.01 (−0.02, 0.03)	0.532	0.578
Whole body BMD, g/cm^2									
Model 1	0.00 (−0.01, 0.02)	0.346	0.322	0.00 (−0.02, 0.03)	0.916	0.913	0.01 (−0.02, 0.03)	0.564	0.538
Model 2	0.01 (−0.01, 0.02)	0.406	0.374	−0.00 (−0.03, 0.02)	0.952	0.956	0.01 (−0.01, 0.03)	0.519	0.492
Model 3	0.01 (−0.01, 0.02)	0.446	0.408	−0.00 (−0.03, 0.02)	0.974	0.977	0.01 (−0.02, 0.03)	0.558	0.525
TBS									
Model 1	−0.00 (−0.06, 0.05)	0.952	0.978	−0.03 (−0.12, 0.07)	0.574	0.572	0.03 (−0.05, 0.10)	0.524	0.507
Model 2	−0.00 (−0.06, 0.05)	0.882	0.909	−0.03 (−0.12, 0.07)	0.541	0.54	0.03 (−0.05, 0.10)	0.527	0.509
Model 3	−0.01 (−0.06, 0.05)	0.821	0.847	−0.03 (−0.12, 0.06)	0.546	0.544	0.02 (−0.05, 0.10)	0.574	0.555

AB, alcoholic beer; CI, coefficient interval; NAB, non-alcoholic beer; TBS, trabecular bone score.
Generalized estimating equation (GEE) models to estimate the effect (difference group × time 95% CI) on the intervention between the intervention groups and the control group.
Model 1: adjusted by age at baseline; Model 2: adjusted like Model 1 plus time since the onset of menopause, follicle-stimulating hormone concentration, smoking habit, lean mass index at baseline; Model 3: adjusted like Model 2 plus total energy intake and physical activity as MET-min/day at baseline.
p-value: group × time interaction; *p*-trend: group × time interaction (continuous).
Two participants of the AB group were excluded from the analysis at 24 months due to traumatic fractures during the last year of the intervention.
p-value < 0.05.

comparison with the control group. Nevertheless, DXA scans revealed that neither AB nor NAB interventions attenuated expected postmenopausal BMD and TBS loss, a finding that could be partly attributed to the relatively early postmenopausal stage of the participants (mean age of 55 years), when the menopause-related increase in bone turnover tends to be higher.

The effects of beer or specific beer components on BMD loss have been previously reported (22). Excessive alcohol consumption is associated with a higher risk of osteoporotic fractures (42) and an imbalance in bone remodeling, which becomes skewed toward bone loss (43). Beyond this well-documented association, the effects of moderate alcohol drinking on bone health have also been studied. A recent meta-analysis by Godos et al. (42) found that up to two standard alcoholic drinks/day vs. alcohol abstinence are related with a higher lumbar and femoral neck BMD, while up to one standard drink/day was found to be associated with higher hip BMD (42). In the Framingham Offspring Cohort Study, the relationship between alcohol intake and BMD at three hip sites and the lumbar spine was analyzed in 1,289 postmenopausal and 298 premenopausal women (11). The main conclusion was that moderate alcohol intake may be beneficial for postmenopausal women and that beer and wine have a higher protective effect on BMD compared to spirits, suggesting that beverage constituents other than alcohol may contribute to bone health. The relationship between light to moderate alcohol consumption with higher BMD is supported by observational cross-sectional studies (3, 44, 45), although other researchers have failed to find a significant association (10). However, there is a lack of scientific evidence from long-term intervention studies on beer consumption for comparison with the results of the present study.

Moreover, as pointed out by Godos et al. (42), little evidence is available for the impact of variables such as age, the evaluated skeletal site, duration of exposure to alcohol, and the pattern of drinking (46). Discrepant results between studies on alcohol and bone health could be related to differences in factors such as age and gender. The participants in the present study were in relatively early postmenopause, when bone loss and accelerated bone turnover arising from estrogen deficiency tends to be high (4). The age factor could explain why our results differed from those of the Framingham Offspring Cohort Study, which included older women with a mean age of 62.5 as well as men, who are expected to have lower rates of bone turnover and consequently, bone loss (11).

On the other hand, beer has been described as a rich source of dietary silicon (47). Ingestion of silicon-containing foods stimulates human osteoblasts and osteoblast-like cells to secrete type I collagen, which is involved in bone cell maturation and bone formation and enhances the calcification of the bone matrix. The incorporation of silicon in calcium phosphate bioceramics was also found to improve bone formation (15). In a randomized, placebo-controlled 12-month trial with osteopenic postmenopausal women, supplementation with 6 and 12 mg of choline-stabilized orthosilicic acid (ch-OSA) together with calcium/vitamin D3 resulted in higher PINP levels than the placebo, and a maintenance of lumbar and femoral BMD (48). In the current study, both beer interventions, with and without ethanol, increased the levels of bone formation markers, particularly PINP, which could be explained by the ingestion of silicon, an intrinsic component of beer. The apparent non-effect on bone mass could be attributed to the particularly rapid bone turnover in the early postmenopausal period, when the acceleration of bone resorption renders antiresorptive therapies especially useful. Conversely, in older women or in males, who experience a slower rate of bone loss and bone turnover, a therapeutic agent with moderate effects on bone formation would probably be more effective. We did not observe a decrease in bone resorption related to beer consumption and the slight increase in bone forming markers would be insufficient to prevent the negative imbalance in bone remodeling. In contrast, in previous studies including males and older postmenopausal women, moderate alcohol consumption was found to exert a positive effect on bone mass (6). Clearly, when the effect of moderate beer intake is analyzed, both the age and gender of the consumer need to be considered.

The phytoestrogen content of beer arises from the use of hops (*Humulus lupulus* L.) in its elaboration. Beer is particularly rich in the weakly estrogenic IX, which after ingestion is biotransformed into 8-PN, one of the strongest phytoestrogens known (49–51). In the postmenopausal state, circulation levels of estradiol fall considerably, and estrogen receptors in bones are downregulated. Dietary plant-derived phytoestrogens can induce the expression of these receptors and target specific estrogen receptor actions (52). Although more well-designed randomized clinical trials are still required, three recent meta-analyses restricted to randomized controlled trials concluded that isoflavones can have a positive effect on bone health (53–55). In their review of 63 controlled trials, Sansai et al. (55) found an improvement in BMD in the lumbar spine, femoral neck, and distal radius in postmenopausal women associated with the intake of 54 mg/day of genistein and 600 mg/day of ipriflavone (synthetic isoflavone) (55). In contrast with these findings, and in accordance with the results of Levis et al. (56), who carried out a 2-year, randomized, double-blind clinical trial in which women in early postmenopause consumed 200 mg of soy isoflavones/day (56), we did not observe this beneficial effect of moderate beer consumption in our small cohort during the 2-year intervention. Again, this would suggest that a more potent antiresorptive effect is necessary to prevent bone loss in the early postmenopausal period. The mechanisms of action of the phytoestrogen content of beer and its impact on sex hormones remain unknown.

The impact of silicon on bone health is rendered more complex by the inhibition of its absorption and distribution by sex hormone levels (12). It has been suggested that hormonal factors may overwhelm any favorable effects of dietary silicon on bones in postmenopausal women (13). Conversely, a review published in 2013 found evidence that moderate silicon supplementation enhances bone mineralization and density, independently of other factors (15). Moreover, a single dose intervention study reported that estradiol status had no obvious influence on silicon absorption (57), although the results may have been influenced by the large variation in serum estradiol concentrations among pre-menopause women and young men. More research is needed to determine the synergistic relationship between estrogen and silicon and to better understand the role of silicon in the management of early postmenopausal osteoporosis. Beer constitutes an interesting food matrix in this line of research, as it is rich not only in silicon but also in phenolic compounds with a phytoestrogenic effect.

A wide range of polyphenols are found in beer (23). Known for their antioxidant and anti-inflammatory activity (58), polyphenols can also inhibit osteoclast formation induced by receptor activator of nuclear factor-κB ligand (59, 60). The reported protective effect of wine consumption on bones has been related to its phenolic content, although there is a lack of *in vivo* evidence for the underlying mechanism (61). In a randomized, placebo-controlled trial, postmenopausal women administered capsules containing the wine polyphenol resveratrol (75 mg, twice daily) experienced a slower rate of bone loss in the lumbar spine and femur, and a slight reduction in bone resorption (62).

To our knowledge, the present clinical trial is the first to study the impact of daily moderate beer consumption (with and without ethanol) on bone health in a postmenopausal population. Although some positive effects on bone formation markers were found after the two beer interventions, the results should be interpretated with caution. The main weakness of the study is the small sample size, which may lack the statistical power to identify all the effects. Other limitations are the non-randomized design, possible intra-variability of exposure due to phenolic metabolism by gut microbiota and differences in AB and NAB prenylflavonoid profiles, and self-selection bias, as participation was voluntary, based on recruitment through advertisements. Silicon and iso-α-acids content of AB and NAB was not quantified, but the same commercial brand was used, making composition profiles more comparable. Additionally, neither serum silicon nor total silicon intake from the diet was monitored.

In 2001, the NIH Consensus concluded that there is an urgent need for randomized controlled trials of combination therapy, which includes pharmacological, dietary, and lifestyle interventions (including muscle strengthening, balance training, management of multiple drug use, smoking cessation, psychological counseling, and dietary interventions) (1). The present study contributes new insights into the possible benefits of beer consumption for bone health in postmenopausal women and reveals the need for more research in this field.

Conclusion

The effect of beer intake on bone strength depends on the age, sex, and hormonal status of the consumer, as well as the drinking pattern. In this pilot study, daily moderate AB and NAB consumption in early postmenopausal women seemed to increase bone formation markers but had no effect on bone resorption markers, suggesting a positive modulating effect on bone health in this cohort. In contrast, the intervention did not produce changes in BMD and TBS determined at 2-years of treatment. Long-term randomized clinical trials are needed with greater number of participants to evaluate the benefits of moderate beer consumption in an older population of osteopenic post-menopausal women, particularly those aged over 60 years, as well as in males. The effect of both alcoholic and non-alcoholic fractions should also be analyzed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Bioethics Commission of the University of Barcelona (Institutional Review Board: IRB 00003099). The patients/participants provided their written informed consent to participate in this study.

Author contributions

AT-R, PP, RE, and RL-R: conceptualization. MT-S: methodology, formal analysis, investigation, data curation, and writing—original draft preparation. AT-R, JM, PP, RE, and RL-R: validation and writing—review and editing. RE and RL-R: supervision. RL-R: project administration and funding acquisition. All authors had read and agreed to the published version of the manuscript.

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Conflict of interest

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1014140/full#supplementary-material

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Publication 8

Effect of moderate beer consumption (with and without ethanol) and its taste on cardiovascular health in postmenopausal women

Marta Trius-Soler, Pamela Martínez-Carrasco, Anna Tresserra-Rimbau, Juan J. Moreno; Ramon Estruch, Rosa M. Lamuela-Raventós

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Abstract

Aims: The present study aimed to evaluate the long-term effect of moderate daily intake of beer (with and without alcohol) on cardiovascular health in post-menopausal women.

Methods: A 2-years non-randomized parallel controlled clinical trial was conducted, with three study arms: 16 volunteers in the alcoholic beer (AB) group; 6 in the non-alcoholic beer (NAB) and 12 were allocated in the control group. Changes in glucose metabolism, lipid profile, liver enzymes, anthropometric measurements, body composition characteristics and blood pressure were monitored. Data on medical history, diet, and exercise were also collected.

Results: Moderate daily consumption of AB and NAB, seem to have positive effects on biochemical indicators of cardiovascular health in post-menopausal women. First, 660 mL/day of NAB reduced serum levels of low-density lipoprotein cholesterol, while the intake of 330 mL/day of AB increased high-density lipoprotein cholesterol. The evolution of changes on android, gynoid fat percentage and its ratio were significantly different between study groups, being related to interventions or as a consequence of the disparity in the groups in terms of the time elapsed since the onset of menopause. Null effect was observed in terms of glucose control, while gamma-glutamyl transferase levels increase but within a normal range in both beer interventions in comparison to the control group. PTC phenotype was not associated with alcohol drinking frequency.

Conclusions: According to the results of this pilot study, moderate beer consumption might have a preventive effect against cardiometabolic alterations during menopause transition since it presents promising results in terms of improvement of lipid profile.

Effect of moderate beer consumption (with and without ethanol) and its taste on cardiovascular health in postmenopausal women

Marta Trius-Soler [#a,b,c], Pamela Martínez-Carrasco [#a], Anna Tresserra-Rimbau [a,b,c], Juan J. Moreno [a,b,c*], Ramon Estruch [c,d] and Rosa M. Lamuela-Raventós [a,b,c*]

[a] Department of Nutrition, Food Sciences and Gastronomy, XIA, School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain
[b] INSA-UB, Nutrition and Food Safety Research Institute, University of Barcelona, 08921 Santa Coloma de Gramanet, Spain
[c] CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain
[d] Department of Internal Medicine, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, 08036 Barcelona, Spain

Running head: Beer effect on cardiovascular health

* **Correspondence:** Rosa M. Lamuela-Raventós; lamuela@ub.edu; Juan J. Moreno; jjmoreno@ub.edu
# Both authors equally contributed

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Abstract

Aims: The present study aimed to evaluate the long-term effect of moderate daily intake of beer (with and without alcohol) on cardiovascular health in postmenopausal women.

Methods: A 2-years non-randomized parallel controlled clinical trial was conducted, including three study arms: 16 volunteers in the alcoholic beer (AB) group; 6 in the non-alcoholic beer (NAB) group and 12 were allocated in the control group. Changes on glucose metabolism, lipid profile, liver enzymes, anthropometric measurements, body composition and blood pressure variables were monitored. Data on medical history, diet, and exercise were also collected.

Results: Moderate consumption of beer, both AB and NAB, seems to have positive effects on biochemical indicators of cardiovascular health in postmenopausal women. First, 660 mL/day of NAB reduced low-density lipoprotein cholesterol blood levels, while 330 mL/day of AB increased high-density lipoprotein cholesterol. The evolution of changes on android, gynoid fat percentage and its ratio were significantly different between study groups, attributable to either the interventions themselves or to the disparity in the groups regarding the time elapsed since the onset of menopause. PTC phenotype was not associated with alcohol drinking frequency.

Conclusions: Moderate beer consumption might have a preventive effect against cardiometabolic alterations during menopause transition since it presents promising results mainly in the improvement of lipid profile. (Trial registration number: ISRCTN13825020; https://doi.org/10.1186/ISRCTN13825020).

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Keywords: phytoestrogens, polyphenols, alcohol drinking, lipid profile, bitter, PTC phenotype

Introduction

Natural menopause is the permanent cessation of spontaneous menses due to the loss of ovarian follicular activity which occurs normally between 45-55 years of age, causing a consequent drop in steroid and peptide hormones production [1,2]. It is a progressive process that leads to the occurrence of a series of adverse physiological changes and unpleasant symptoms, both in the short and long term, which tend to vary from one person to another and have been consistently described in the literature [3,4].

Bone loss and disturbances in the cardiovascular system are among the most impactful consequences on the health of postmenopausal women [2,5], with cardiovascular disease (CVD) being the main cause of morbidity and mortality in this population group worldwide [6]. The rate of cardiovascular events in postmenopausal women is 2.6 times higher than that in premenopausal women of the same age [7], which is explained by metabolic alterations that occur during the perimenopausal period – between a variable number of years before depending on the person and one year after the event [8]– associated with the circulating oestrogen deficiency [9,10]. Dyslipidaemia is the main health outcome, with a significant increase in serum levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), TC/high-density lipoprotein cholesterol (HDL-c) ratio, and apolipoprotein B (Apo B), which along with the lipid disorders typical of the aging process accelerates the onset of atherosclerosis [11,12]. Subsequently, this situation may lead to an increment in body weight, a tendency to abdominal fat accumulation, insulin resistance, glucose intolerance, high blood pressure (BP), and a consequent increased risk of diabetes, coronary heart disease, heart failure and stroke, among others [13].

On the premise that oestrogens may have a cardioprotective effect, some studies have evaluated the relationship between hormone replacement therapy (HRT) – the most well-known menopausal treatment – with the risk of suffering from CVD in the postmenopausal period. Since the results have been controversial [14,15], no recommendation regarding HRT and prevention of chronic diseases has yet been stablished [16]. For this reason, nowadays, there is a growing number of women who seek other natural treatments to reduce the risk of CVD and symptoms associated with the postmenopausal stage. Therefore, studying alternatives to HRT, its safety and real effectiveness in this matter, has become especially relevant [17,18].

Phytoestrogens are plant compounds similar in structure to the endogenous oestrogens, characteristic that allows them to act as selective modulators of oestrogen-dependent signals [19]. Among the most biologically active phytoestrogens are prenylflavonoids [20], a subclass of flavonoid polyphenols, produced as a secondary metabolite in 37 plant species identified, where hop (*Humulus lupulus* L) is one of them [21]. The prenylflavonoid fraction of hop is

mainly made up of the two prenylated chalcones xanthohumol (XN) and desmethylxanthohumol (DMX), in addition to very small amounts of the prenylated flavanones isoxanthohumol (IX), 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN). The last-mentioned compound is the most potent phytoestrogen discovered to date and, therefore, the one that generates the most interest [22], being 8-PN in beer itself and its conversion from IX by the intestinal microbiota the main source of human exposure to 8-PN [23].

During the beer production process, the female inflorescence of hops is used to provide aroma, bitterness, and preserve it. The perceived bitter taste in beer depends somewhat on the overall level of iso-α-acids - a series of compounds released from hop glands and isomerized during brewing - and their relative proportions [24]. It has also been reported that sensitivity responses to these compounds are mediated by bitter taste receptors (T2Rs), and concretely by T2R1, T2R14 and T2R40 [25]. The applicability of sensory analysis on nutritional and clinical research enlightens new directions towards personalized nutrition, bringing insights on health effects as well as intervention's acceptance and compliance.

Considering all the scientific evidence exposed above, the purpose of this research was to evaluate the effect of moderate daily intake of beer with and without alcohol, for 2-years with intermediate repeated measurements, on cardiovascular (CV) health in postmenopausal women. Parallelly, differences on sensory perception were evaluated to study their influence on beer and alcohol drinking behaviour.

Material and methods

Experimental design

This study was a 2-years non-randomized parallel controlled clinical trial, with three study arms: one whose intervention was the equivalent of 14 g of ethanol per day in the form of alcoholic beer (AB) (300 mL/day); another with non-alcoholic beer (NAB) (660 mL/day); and the last group that could not consume alcoholic beverages, neither NAB nor hop-derived products, acting as a control group. Participants were allocated to a study group after a run-in period of 15 days, where everyone was asked to follow the control group intervention instructions. None of the volunteers were allowed to consume any other alcoholic beverages during the study.

All participants signed informed consent and were required to attend four visits during the entire intervention period (baseline, 6, 12 and 24 months) and later invited to the sensory analysis. The study was conducted in compliance with the Declaration of Helsinki and all procedures were approved by the Bioethics Commission of the University of Barcelona (Institutional Review Board: IRB 00003099) in March 2017 for the main study and in July 2022 for the complementary sensory evaluation.

Study population and recruitment

The eligible participants were women between 45-70 years-old, recruited at the Outpatient Clinic of the Department of Internal Medicine of the Hospital Clinic of Barcelona through informative posters and by radio announcements, between April 2017 and June 2019. To perform the selection, specific inclusion and exclusion criteria were stipulated. The inclusion criteria included those used to validate the menopausal status of potential participants: 1) absence of menses in the previous 12 months; 2) blood levels of follicle-stimulating hormone (FSH) of 23–116 U/L, and 3) blood levels of 17-β-estradiol (E2) <37 pg/mL. Also, since one of the main objectives of the intervention study was originally to assess the effect of beer consumption on bone health, much of the exclusion criteria were related to this issue: 1) use of oestrogen therapy or silicon or polyphenol supplements; 2) known diseases affecting bone metabolism (rheumatoid arthritis, hyperthyroidism, hypercortisolism, renal bone disease, and/or chronic liver disease); and 3) use of drugs affecting bone metabolism (fluorides, bisphosphonates, teriparatide or parathormone, strontium ranelate, anabolic steroids, chronic glucocorticoids (>3 months), cytostatics, antiandrogens, and/or antiepileptics).

A total of 37 women were selected and agreed to be part of the study, but only 34 completed the entire intervention. Two of them, one from the NAB and another from the control group, dropped out due to their incompatibility to continue attending the visits, while the third one did not want to continue with the study after the sixth month, corresponding also to the control group.

Considering that the intervention involved daily moderate alcohol consumption over a 2-year period, volunteers were asked to choose the group based on their preferences and habits, being distributed as follows: 16 participants in the AB group, 6 volunteers in the NAB group, and 12 subjects in the control group. Due to the dietary nature of the intervention, neither the participants nor the principal researchers were blinded. Nonetheless, the information on the study arms remained blinded to the technical and laboratory staff in charge of performing the health analyses seeking to minimize biases associated with this component.

Intervention product characterization and compliance

A specific brand of beer and drinking volumes were selected to standardize the daily dose of phytoestrogens administered within each intervention group (AB and NAB). Moreover, women were encouraged to drink it during meals, according to dietary recommendations based on the Mediterranean diet [26].

The quantification of prenylflavonoids in both types of beers was carried out using the analytical technique of liquid chromatography coupled with mass spectrometry (LC-MS/MS), following the method of Quifer-Rada *et al.* (2013) [27] with some modifications described in Trius-Soler *et al.* (2021) [28]. The contribution of phytoestrogens XN, IX, 8-PN, and 6-PN along

with the alcohol content from beer doses for each of the two intervention arms can be found in *Supplementary Table 1*. The drinking volume was doubled for NAB group compared to the AB group, due to the detrimental impact of non-alcoholic brewing processes on IX content [29].

Products used for intervention were provided in a monthly basis throughout the study, seeking to ensure compliance with the intervention. Nevertheless, the fulfilment level was measured from 7-day dietary record reviewed at each visit, and objectively by the assessment of urinary IX, a beer-specific biomarker of consumption. This analysis was performed from 24-hour urine samples, collected at baseline, 6, 12, and 24-month visits, using solid-phase extraction LC-MS/MS [30].

Measurements and outcome assessment

Medical history

Information related to the medical history was collected at baseline and then updated at each visit. The interviews were standardized and structured using a questionnaire with medical and sociodemographic questions, emphasizing CV health, such as age, baseline diseases, medication history, besides current and past smoking and alcohol drinking habits. Other data, such as the time elapsed since the onset of menopause and sleeping habits were also collected.

Anthropometric measurements and body composition

Anthropometric data such as weight, height, waist circumference (WC), and waist-hip ratio (WHR) were obtained at each visit. Trained technical health personnel performed these measurements following standardized protocols. The weight measurement was carried out with a calibrated scale, with the participants barefoot and wearing light clothing. Height was measured with a wall-mounted stadiometer, while an inextensible tape measure was used for WC in the midpoint between the lower margin of the last floating rib and the upper part of the iliac crest [31]. Body mass index (BMI), expressed as (kg/m^2), was calculated using the mathematical formula of weight divided by height squared.

Diastolic blood pressure (DBP) and systolic blood pressure (SBP) were measured by a validated semi-automatic digital sphygmomanometer (Omron HEM-705CP model) in triplicate. In addition, every participant underwent a 12-lead electrocardiogram in a supine position at baseline, 12, and 24 months between 8-9 am.

Total and regional body composition were estimated using dual-energy X-ray absorptiometry (DXA) (GE-LUNAR iDXA Prodigy equipment). Fat mass index (FMI) (kg/m^2) and lean mass index (LMI) (kg/m^2) were obtained from total fat mass (kg) and total lean mass (kg), respectively, in relation with height. Also, the percentage of android and gynoid distribution of fat was calculated, along with the ratio resulting from the division of both. All these

measurements were evaluated at baseline and annually by the CETIR medical group (CETIR grup Mèdic, Barcelona, Spain).

Biological samples and biochemical analyses

Overnight fasting blood and spot urine samples were collected at each visit (between 8-9 am). The automated biochemical profiles were analysed at the Biomedical Diagnostic Centre of the Hospital Clinic. The lower detection limits of plasma E2 was 12 pg/mL, thus levels below this limit were defined as 11 pg/mL. 24-hour urine samples were also collected and stored in aliquots at 80°C until further analysis. Those samples were used to verify the intervention compliance.

Dietary intake and physical activity assessments

Dietary intake was estimated through a validated semi-quantitative questionnaire of frequency of consumption (FFQ) consisting of 151 items [32], assessed by trained personnel at baseline, 12 and 24 months of follow-up. The total energy intake as well as the macronutrients (protein, carbohydrates, and fats) and alcohol consumption (g/day) were estimated using the Spanish food composition tables. Subsequently, each amount of macronutrient consumed was divided by the absolute value of the total daily energy intake and multiplied by 100, resulting in the proportion (%) of individual daily energy intake attributable to each macronutrient. The estimation of micronutrients in the diet was carried out in the same way, and the absolute values of those that could have some influence on the outcome of CV health were selected to report [33–35]. Under this reasoning, total polyphenol was also calculated by multiplying the daily amount consumed of the foods specified in the 151-item FFQ with the polyphenols content of each of them [36] according to the Phenol-Explorer database [37]. Furthermore, the overall diet quality was evaluated using the 14-point Mediterranean Diet Adherence questionnaire as an index of healthy eating at baseline [38].

Regarding physical activity, it was estimated by the Minnesota leisure-time questionnaire, previously validated in a population of Spanish women, at the four study visits. This data was calculated and reported as the daily metabolic equivalent of task (METs-min/day) [39].

Sensory analysis

Recognition thresholds (RT) were measured using a same-different task approach described elsewhere [40], with some modifications. Sucrose (sweet), monosodium glutamate (umami), sodium chloride (NaCl) (salty), citric acid (sour), phenylthiocarbamide (PTC) (bitter), quinine (bitter), and sinigrin (bitter) were supplied by Sigma Aldrich (St. Louis, MO, USA), while iso-α-acids rich extract (bitter) was supplied by Molina for Brewers (Hopalpha ISO 30%, Molina for Brewers, Spain). Distilled water was used as the solvent to prepare the corresponding solutions and as a blank. Sample sets were administered in ascending concentrations (*Supplementary Table 2*), placing 0.5 mL of each sample at room temperature on the tip of the

tongue. For each pair of samples, participants had to indicate whether the two samples tasted differently or not and to recognize the corresponding basic taste. The assay stopped when the participant correctly recognized the taste that characterize the tasting molecule at a given concentration. RTs were scaled in multiples of 1 standard deviation. Test solutions were randomized and blinded for participants, and they were requested not to smoke, chew gum, or eat/drink any product except for water the 2-hours prior to the test.

Total taste score (TTS), as an overall sensitivity measure, was calculated as the sum of the normalized RT scores divided by the total tastants assessed (n=8). Internal reliability for the Total bitter score (TBS), as an approximation of overall bitter sensitivity, was calculated as the sum of the 4 bitter normalized RT scores divided by 4. Cronbach's α was used to evaluate TTS (α: 0.552) and TBS (α: 0.548). Participants with a PTC RT score of 1 ($\leq$ 0.7 μM) were classified as super-tasters; those with a score of 2 or 3 (3.5-14 μM) were classified as tasters; finally, those with a score higher than 3 (>14 μM) were in the non-taster group [40].

Sweetness, bitterness, and sourness intensity perception of the AB and NAB brand used in the study were recorded through a Likert scale (null, light, moderate, high) with the nose covered. Hedonist perceptions of AB and NAB were also recorded using the same instrument with the nose uncovered. Liking for AB and NAB in general was also asked through a yes/no question.

Statistical analyses

Differences in baseline characteristics and median RTs among groups were assessed using the Kruskal-Wallis test for continuous variables followed by the post-hoc Dunn's multiple comparisons test when significant differences were observed. These variables were tabulated as median with their respective quartiles one and three (Q1 and Q3). For qualitative variables, the chi-square test was performed, being expressed as number (n) and proportion (%). Spearman correlation was applied to study the relationship between sucrose RT and beer sweetness intensity, citric acid RT and beer sourness intensity, and iso-α-acids RT and beer bitterness intensity and the hedonic score perception. Differences between AB and NAB three-dimension intensities were studied with a matched-pair signed-rank.

The existence of possible intragroup differences in dietary intake and physical activity through the intervention (baseline, 12 and 24 months) were analysed with a matched-pair signed-rank test in case of variables distributed symmetrically, while a sing-test of matched pairs was preferred for asymmetric variables. Meanwhile, differences at the intergroup level were performed with the Kruskal-Wallis test followed by Dunn's post-hoc test in case of statistical significance.

The effect of the intervention on biochemical, anthropometric, and BP variables was tested through the generalized estimating equation (GEE) model to compare repeated measurements over time (identity link function, autoregressive of order 1 correlation, and

robust standard error parameters were specified). Three adjustment models of increasing complexity were carried out to avoid influences on outcomes due to other factors, using age as the first variable (Model 1), plus FSH levels (Model 2), plus smoking habit, total energy intake, physical activity, and the cholesterol-lowering (for lipid profile variables) or antihypertensive baseline treatment (for BP variables) in the last model (Model 3). Data were expressed as adjusted mean differences and their 95% confidence interval. A time-exposure interaction term allowed the evaluation of potential differences between intervention groups in response to changes over time. A test for trend was also performed to measure the trend of the extended response over time as a continuous variable.

The evolution of the variables whose changes presented statistically significant differences between study groups at the end of the intervention according to the GEE statistical analysis was graphed. For this, the medians of each study group were calculated with their corresponding Q1 and Q3, at baseline, 12 and 24 months. Additionally, to assess the existence of intra- and inter-group differences for each of these variables, the same steps were followed as for the statistical analysis of the dietary intake and physical activity variables throughout the intervention.

All statistical analyses were performed with STATA software package 16.0 Special Edition (StataCorp LLC, TX, USA). Statistical tests were two-sided and *p*-values below 0.05 were considered significant. Figures were performed using the Prism 9.0.0 software package.

Results

Baseline characteristics of the study participants

Baseline characteristics of each study arm are shown in *Table 1* and *Table 2*. Briefly, the total sample of volunteers had a median (Q1, Q3) age of 55 (53-58) years and a BMI of 26.3 (24.7-29.0) kg/m^2. No significant differences were observed in age, age at menopause and time elapsed since the onset of menopause between the groups. Study arms were also well-balanced in relation to the type of medication used. The only variables with a statistical difference detected were related to alcohol consumption, where the AB group presented a higher intake than the other two groups, being the fermented beverages like beer and wine the preferred ones. Based on the dietary reference values published by the EFSA [41], diet of the study participants was characterized by a being low in carbohydrates but very high in dietary fibre, high in fat especially saturated, and high in sodium *(Supplementary Table 3)*.

Among the anthropometric, body composition or BP variables, no significant differences were observed among groups. The nutritional status of most of the study sample was in the normal or overweight category, with a high WC and a WHR bordering on the limit of abdominal obesity showing an increased risk of metabolic complications [42] *(Table 2)*.

Regarding biochemical markers, significant differences were reported in the liver enzymes aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) at baseline, with higher levels in the AB compared to the control group, but within the reference range. Differences were also found in FSH levels, with the AB group presenting higher levels than the other two study arms. Nevertheless, all the markers were within the normal ranges established by the laboratory, except for TC which was slightly above the reference limit (> 200 mg/dL) in the AB and NAB groups *(Table 2)*.

Study compliance

Of the 34 volunteers who completed the 2-years of intervention, a general level of compliance of 100% was observed according to the dietary self-records and interviews. Nevertheless, based on the evaluation of the presence or absence of biomarker IX in 24-hour urine samples (limit of detection <0.04 ppb), compliance with the intervention was determined at baseline for the run-in period and throughout the study, being respectively 50% and 97.9% for the AB group, 83.3% and 77.8% for the NAB group, and 100% and 97.2% for the control group.

Controlled covariates

Statistical differences were only observed at the dietary level, specifically in carbohydrate intake, being significantly higher in the NAB group at 24 months in contrast to the other two groups. Although the NAB consumption was responsible for an average of 5.2% of the daily energy intake in the form of carbohydrates compared to 1.8% provided by AB, these differences could be a consequence of the carbohydrate low intake in the other study groups, even observing a significant decrease in consumption of this macronutrient in the AB group throughout the intervention. A lower consumption of simple sugars, expressed as % of energy intake was also detected in the AB group compared to both the control and NAB group at 12 months, and only compared to NAB at 24 months. In terms of saturated fatty acids, the control group tended to decline its daily intake over the course of the intervention (*Supplementary Table 3*).

Finally, alcohol consumption was significantly higher in volunteers from the AB group compared to volunteers from the other groups at each of the evaluation times, as expected. However, it should be noted that this intake was also significantly higher in the AB group after than before the intervention.

Intervention effects

The outcomes of the intervention in biochemical markers associated with the risk of CVD are detailed in *Table 3*. It shows the changes in the variables in the middle and at the end of the study in relation to baseline levels, performing contrasts by pairs of groups, while

Supplementary Figure 1 summarizes and illustrates the evolution of biochemical markers whose variations were significantly different after 24 months of intervention between the study groups. Liver enzyme levels were also monitored during the study to assess the risks of the intervention given the alcoholic nature of the product used.

Table 4 presents the outcomes in body composition and BP variables, used as CVD risk factors (CVDRF), during the intervention follow-up. The progression of the variables whose fluctuations evaluated at the end of the intervention were significantly different between the study arms are illustrated in *Supplementary Figure 2*. Additionally, the variable of interest FMI was also graphed to visualize the body composition evolution in each of the study groups throughout the intervention.

Glucose control and lipid profile

No significant differences were observed between the study groups in glycemia, glycosylated haemoglobin, TG, and apolipoprotein A1 (Apo A1) changes during the follow-up of the intervention (Table 3). Apo B levels had a greater decrease in NAB group when compared with control group (adjusted difference: -13.28; 95% CI: -21.46, -5.10; *p*-value: 0.001) and with AB group at 12 months (adjusted difference: 11.20; 95% CI: 1.22, 21.19; *p*-value: 0.028). Nonetheless, these differences between groups lost statistical significance when considering the whole intervention (24-months *p*-value: 0.861 and 0.690, respectively).

One of the relevant findings of the present research is that postmenopausal women who consumed AB presented a significant increase in the levels of HDL-c proved at 24 months of follow-up in contrast to the control group (adjusted difference: 9.01; 95% CI: 2.47, 15.55; *p*-value: 0.007), behaving as a continuous trend over time (*p*-trend: 0.006). Consequently, the TC/HDL-c and LDL-c/HDL-c ratios of the AB group decreased significantly compared to the control group in the same period (adjusted difference: -0.55; 95% CI: -0.92, -0.18; *p*-value: 0.004 and adjusted difference: -0.49; 95% CI: -0.81, -0.17; *p*-value: 0.003), and linearly with time-exposure (*p*-trends: 0.004 and 0.003, respectively).

In addition, another interesting finding was that volunteers consuming NAB presented a significant decrease in their serum LDL-c levels when compared to the control group at 12 and 24 months (adjusted difference: -23.49; 95%CI: -45.65, -1.33; *p*-value: 0.038) and to the AB group only at 12 months (adjusted difference: 23.55; 95%CI: 4.54, 42.57; *p*-value: 0.015). Also, the NAB study arm showed a significant decrease in TC levels at 12 months of study follow-up when compared with participants in both the control and AB group (adjusted difference: -24.78; 95%CI: -46.62, -2.94; *p*-value: 0.026 and adjusted difference: 22.36; 95%CI: 2.49, 42.22; *p*-value: 0.027, respectively). Nevertheless, these differences lost their significance throughout

the intervention. In contrast, no significant differences were found when compared neither LDL-c nor TC changes of the AB group with those of the control group.

Liver enzymes

Both AB and NAB groups presented a significantly greater increase in the GGT enzyme levels after 24 months compared to the control group. No significant difference could be verified between groups in AST and alanine aminotransferase (ALT) enzymes. According to the laboratory ranges, all liver enzymes remained within normal values for the three arms throughout the study.

Anthropometric measurements, body composition and BP

No significant differences were observed between groups regarding the changes in BMI, WHR and FMI *(Table 4)*. Although a greater decrease in WC was observed at 12 months in postmenopausal women who consumed NAB compared to those who consumed AB (adjusted difference: 3.39; 95% CI: 0.19, 6.58; *p*-value: 0.038) and to the control group (adjusted difference: -4.00; 95% CI: -7.25, -0.73; *p*-value: 0.016), this difference was not significant throughout the intervention.

Regarding total fat mass, a significant increase in the AB group compared to the control group was evidenced at 12 months of follow-up. Nonetheless, these differences lost statistical significance when evaluated after 24 months of intervention. On the other hand, the intervention groups showed worse results than the control group in terms of android and gynoid fat mass percentages, as well as in the android-gynoid ratio, with a slight increase observed after 24 months of intervention, but also a marked decrease in the control group. Changes in AB group were not statistically different when compared with the NAB group.

Finally, significant differences were observed in DBP between AB and NAB groups at 24 months of follow-up, with an expected decrease of 0.21 mmHg (95% CI: 0.03, 0.40; *p*-trend: 0.023) for every 12 additional months of consuming 660 mL/day of NAB in comparison with the habit of consuming 330 mL/day of AB during a 2-year period. No significant differences were founded regarding SBP between the study groups.

Sensory analysis

Those participants that decided to drink beer (either AB or NAB) had higher mean TTS (lower sensitivity) than those who decided to be in the control group (mean TTS for beer groups: 0.237 ± 0.142; Control group: 0.137 ± 0.103; *p*-value: 0.050). The beer groups had a higher mean RT for iso-α-acids (iso-α-acids RT score: 4.3 ± 1.8; iso-α-acids RT (μM): 62.8 ± 52.3) and TBS (0.45 ± 0.23) than the control group (iso-α-acids RT score: 3.4 ± 1.5; iso-α-acids RT (μM): 31.89

± 40.5; TBS: 0.28 ± 0.14), but differences were close to be significant (iso-α-acids RT score *p*-value: 0.192; TBS *p*-value: 0.053).

Considering the three arm groups, NAB group had higher iso-α-acids RT in comparison to the control group but not to AB group (mean iso-α-acids RT score for NAB group: 5.6 ± 0.5; Control group: 3.4 ± 1.5; AB group: 3.8 ± 1.8; *p*-value: 0.061). On the contrary, the three study arms were not different in sucrose RT (*p*-value: 0.738), neither in the distribution among PTC phenotypes (*p*-value: 0.713) nor in the TTS (*p*-value: 0.115).

Regarding taste intensity, significant differences between beers were found, being AB bitterer (*p*-value: <0.001) and less sweet (*p*-value: 0.041) than NAB. The mean sourness intensity score for AB was the only dimension recorded that was fairly correlated with the correspondent RT for the molecule that elicits this basic taste (r: 0.391; *p*-value: 0.048). Additionally, iso-α-acids RT was inversely correlated with hedonic liking score from AB (r: -0.506; *p*-value: 0.014), but not with bitterness intensity (r: 0.127; *p*-value: 0.535).

A yes/no question on AB and NAB liking resulted in 70.0% and 80.0% of the control and NAB group, respectively, reporting unliked sensation to AB. In contrast, the totality of the AB group did like it (*p*-values 0.001). On the other hand, 70.0%, 50%, and 20% of the control, AB and NAB groups respectively reported not liking NAB (*p*-value: 0.186). PTC phenotype was not associated with alcohol drinking frequency (*p*-value: 0.650).

Discussion

While in some scientific publications the consumption of beer has been associated with adverse health effects in general population, like weight gain and visceral fat accumulation [43,44], many other authors have reported benefits in the moderate beer intake on lipid profile, cardiovascular risk, and mortality, mainly attributable to the presence of polyphenols [45–47]. Nevertheless, studies that specifically evaluate the relationship between beer and CVD and stratify their conclusions by sex are scarce [46], while previous mind-term results from the present clinical trial showed encouraging results regarding some CV risk factors specifically a decrease in serum levels of LDL-c and a slighter descending trend in DBP after a 6-months of consuming NAB [28].

The population of the present study had an increased cardiovascular risk at baseline, as evidenced by characteristics such as nutritional status and other anthropometric variables related to abdominal obesity. This was an expected situation considering that one of the morphological changes of menopause widely described in the literature is the increase in fat mass and its redistribution at the abdominal level [42]. On the other hand, although significant differences in alcohol consumption were found at baseline between the groups, the intake of the three study arms could be considered as low risk, since the recommendation supported

by scientific evidence on alcohol consumption in women for its cardioprotective effect is 16 grams/day from these fermented beverages [46].

The improvement in HDL-c levels in healthy subjects resulting from moderate and regular alcohol consumption has been repeatedly reported in the literature due to mechanisms that are still not entirely clear [48–50]. In addition, studies reported that this effect has been seen in subjects who consume AB but not in those who consume NAB, which seems to support the hypothesis that this is an exclusive consequence of alcohol consumption [45,51]. A rise in HDL-c represents a protective factor for CVD, since they are molecules that promote the reverse transport of cholesterol from peripheral tissues to the liver for its subsequent excretion, preventing its accumulation in the arterial walls and thus the progression of the atherosclerotic process [52]. As well as HDL-c, the two mentioned ratios TC/HDL-c and LDL-c/HDL-c have been described as cardiovascular risk indicators with greater predictive value than the isolated lipid parameters used independently [53], and therefore their reduction represents a beneficial factor for the health of AB consumers.

Similar to the results obtained in this clinical trial, studies on chronic but moderate beer administration and cardiovascular health in humans show that NAB consumers had significantly lower TC levels when compared to AB consumers, with a homogeneous effect for both men and women [45]. Moreover, the NAB arm of this study also showed a significant long-term decrease on serum LDL-c levels compared to the control group. This explains the greater reductions in TC for the in NAB group compared to the other ones. These outcomes lead to hypothesize that it could be the bioactive fraction of the beer that exerts the beneficial effect on the lipid profile, since studies with phytoestrogen supplementation in postmenopausal women lasting longer than eight weeks state improvements in serum TC and LDL-c as well [54]. Nonetheless, these beneficial effects have been found to be more evident in participants with high baseline cholesterol levels, which is consistent with the characteristics of this study sample. Indeed, a randomized, cross-over, prospective study reported that moderate intake of both NAB and AB brings different pattern of changes in lipid profile on those participants with LDL-c levels > 130 mg/dL than those with lower levels in a mixed cohort [52].

Interventions with AB or NAB did not have an impact over the 2-year duration on glucose-related variables. Similar results were found in an RCT, where the effects of ethanol and phenolic compounds in beer on CV health were evaluated in 33 high-risk men, concluding that neither of the two mentioned beer fractions seemed to generate significant changes in the glucose metabolism parameters [51]. Also, in another RCT where the influence of low to moderate alcohol consumption on some biochemical parameters was evaluated in 51 non-diabetic postmenopausal women, no significant differences were observed in fasting glucose

492 concentrations between the intervention and the control groups [55]. Since the two
493 aforementioned publications reflect short interventions of four and eight weeks respectively,
494 the present study provides relevant information that long-term beer consumption may have
495 neither positive nor negative effects on blood glucose levels and HbA1c in non-diabetic
496 postmenopausal women. From a mechanistic view, it has been hypothesized that phenolic
497 and bitter compounds in beer along its carbohydrate content, could favour glucose
498 metabolism by stimulating GLP-1 secretion through T2Rs activation expressed in the small
499 intestine [56].

500 No significant differences in TG and Apo A1 changes were observed between the study
501 groups at follow-up, and despite Apo B levels in the NAB group had a greater initial decrease
502 compared to the other two study arms, this difference dissipated when considering the whole
503 intervention. A meta-analysis published by Spaggiari *et al.* (2020) analysed 26 RCTs that
504 evaluated the cardiovascular effects of AB consumption, reporting no significant differences
505 in TG levels when compared with groups that consumed NAB as well as placebo or water
506 [45]. In opposition, Wolters *et al.* (2020) published another meta-analysis of RCTs, whose
507 objective was to determine the effects of phytoestrogen supplementation on intermediate CV
508 health in postmenopausal women, with some contradictory evidence. Findings, with a sum
509 of 40 RCTs, a total sample size of 3069 women, and a median intervention duration of 12
510 weeks, indicated that supplementation with phytoestrogens leads to a moderate decrease in
511 TG, whereas 12 RCTs including a total of 985 women showed a reduction of Apo B levels
512 along with an increase in Apo A1 [45,54]. Nevertheless, more than 70% of the studies with
513 such results were classified by the authors as having poor to fair methodological quality, while
514 the differences in outcomes could be explained by the fact that most of the interventions
515 consisted in the evaluation of isoflavones, another phytoestrogenic compound, in its isolated
516 form.

517 Concerning liver enzymes, daily moderate consumption of both AB and NAB was of low
518 hepatic risk in this intervention. Results obtained after regular AB consumption are supported
519 by other studies, which found that AB in moderate doses e.g., one can of beer per day for
520 women, does not modify or only induces small changes on liver enzymes within the reference
521 clinical ranges in plasma [52,57]. The cause of GGT changes after regular NAB remain
522 unknown. Anyway, it is necessary to interpret these findings carefully, since in this case liver
523 enzymes were at normal basal level and participants did not suffer from previously diagnosed
524 hepatic diseases.

525 The anthropometric and body composition parameters reported in the present study are
526 frequently used as CVDRF and associated mortality [65,67–69], thus the fact that no significant
527 long-term differences were found in BMI, FMI, WHR and WC between the intervention

groups and the control group is a relevant health outcome that is supported by other studies [51,52]. For example, Padro *et al.* (2018) observed after eight weeks of consuming AB or NAB no significant changes in body weight, BMI, or WC in adults with overweight or obesity but without other CVDRF [52]. Similarly, Chiva-Blanch *et al.* (2015) in the study mentioned above, found no significant changes before or after the 4 weeks of each intervention or after all three interventions (AB, NAB and gin) in body weight, BMI, or WHR [51]. Despite both studies are of short duration, the results are consistent with those obtained from this clinical trial that did not show a significant impact of moderate alcohol or beer polyphenols long-term consumption on these CVDRF.

Beyond the traditional estimators of abdominal fat such as WC, with inter- and intra-evaluator variability inherent to the measurement, more sensitive techniques are used to characterize body composition. In fact, significant different changes on android, gynoid fat percentages and its ratio were observed between study groups. Since there are studies that affirm the existence of a directly proportional relation between the consumption of AB and the accumulation of visceral adipose mass [43,44], while others declare the opposite or null effect, the existing evidence regarding this issue is controversial [58,59]. One of the reported explanations is that AB contributes to a higher daily energy intake [60] not only because of its alcohol content, but also because of its carbohydrates and this leads to the accumulation of visceral fat [43]. However, in this study the dietary covariates were controlled, beer encourage to be consumed accompanied by meals and no significant differences in energy or carbohydrates daily consumption were reported between AB group and control group, while paradoxically the AB group significantly decreased its carbohydrate intake in relation to baseline. On the other hand, the pronounced decrease in android and gynoid fat in the control group is not related to alcohol consumption, since it remained at very low levels since the beginning of the study, but could be related to the decrease in saturated fatty acids % of daily energy intake. Other publications have reported that the accelerated increase in fat mass along with changes in its distribution occur during the first four years after menopause and then this process declines [61,62]. Considering that at the beginning of this study the time since the onset of menopause in control group was approximately 3.6 years, while in the AB group it was about 2.3 years and in the NAB group 1.9 years, it is presumably that variations in body composition associated with the menopausal period have decrease for the control group, but not for the other two intervention groups.

Although the present results pointed out a significant decrease in DBP in the NAB group in comparison to the variations in the AB group, this effect was not clinically relevant. Previously, a meta-analysis of controlled clinical trials included eight studies that reported no significant differences in DBP or SBP between the intervention groups with AB and the control groups, whether NAB or placebo [45]. Besides, a systematic review with meta-analysis of cohort

studies found that there was no increased risk of hypertension from consuming one to two servings of alcohol per day in women (RR = 0.94; 0.88 – 1.01) compared with abstainers [63]. The mechanism behind the effect of alcohol on BP is not yet fully elucidated [64], although the effect of light to moderate alcohol consumption is expected to be short-term and reversible [65].

Beer liking was a significant factor in the study arm choice. Furthermore, findings suggest that beer perception could be influenced at least in part, by the iso-α-acids RT. Those in the NAB and control group had lower alcohol intake at baseline, that could indicate that alcohol in addition to or instead of the bitterness in beer is what is not appealing to them, result also reported by Guinard *et al.* (1996) [66]. The iso-α-acids content in lager AB and NAB has been previously reported, ranging approximately from 40-100 μM in lager AB samples and around 35 μM in the analysed NAB sample [67]. Although iso-α-acids content of the study beers was not quantified, mean iso-α-acids RT for the beer groups was similar or above the expected iso-α-acids content in AB and NAB, having this sensory factor a promising biological implication because some beer matrix could not be at suprathreshold level. This result could explain the null correlation between iso-α-acids RT and the bitterness. Hedonic considerations might have also affected how subjects approached the intensity score procedure.

On the other hand, some previous findings have suggested that drinking behaviours and preferences of specific alcoholic beverages might be influenced by genetic variations in taste receptors. In that sense, previous researchers found that taster PTC phenotype predicted fewer average consumption of standard drinks [66,68,69], but we did not. The explored iso-α-acids RT might explain the NAB study arm choice, but further research on large cohorts on individual taste sensations (sensitivity, intensity, and hedonism) might be useful to identify those participants that could get more benefit from moderate beer consumption or those that could have higher acceptance and compliance to a moderate beer intervention.

One of the main strengths of this study is its pioneering design of long extension, which made it possible to evaluate not only the long-term effects of alcohol, but also its bioactive components, in postmenopausal women. In addition, the outcomes obtained allow expanding the limited existing evidence related to cardiovascular health in this population and understanding the properties of beer as a preventive contributor for cardiovascular alterations associated with menopause transition. Limitations of this clinical trial are the small sample size, along with the uneven size between the study arms. Additionally, since the study was initially planned to assess effects on bone health, the exclusion criteria did not consider the existence of a family history of CVD, thus there is the possibility that certain results are biased by this parameter. Moreover, the prenylflavonoid profile was different between the AB and NAB doses, which may have influenced the effects of the intervention beyond the ethanol

content. Due to the difference in components, doses, and duration of the interventions using other phytoestrogens food sources (e.g., soya, flaxseed, red clover), as well as the individual inter-variability in their compound's metabolism, comparison between studies on phytoestrogen food sources are difficult. Finally, sensory evaluation was carried out after the end of the intervention.

In conclusion, the present clinical trial can be used as a pilot study for future research since it presents promising results in terms of beer consumption and improvement of some CVDRF. It remains unclear whether it is polyphenols in general with their antioxidant and anti-inflammatory characteristics that exert positive effects at the metabolic level, or whether it is indeed the phytoestrogenic compounds (that are also phenolics) that act as a replacement of endogenous oestrogens and regulate blood lipids. Nevertheless, these findings could be relevant to boost the healthy properties of beer through food innovation, also considering that 14 g/day of alcohol in a beer form significantly increased serum level of HDL-c.

Further RCT in postmenopausal women about the role of non-alcoholic and alcoholic beer fraction in CVDRF should be performed to confirm these results. In addition, it would be advisable to have more *in vivo* and *in vitro* studies on the mechanisms of action that accurately explain findings in this type of interventions.

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Conflict of interest

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Authors' Contributions

Conceptualization, AT, RE, and RL; methodology, MT, PM; validation, AT, JM, RE, and RL.; formal analysis, MT, PM; data curation, MT, PM; writing—original draft preparation, MT, PM; writing—review and editing, AT, JM, RE, and RL; supervision, RE and RL; project administration, RL; funding acquisition, RL. All authors have read and agreed to the published version of the manuscript.

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Table 1. Participants baseline characteristics on medical history, dietary intake, and medication according to the study group.

	Control group (n = 12)	**AB group (n = 16)**	**NAB group (n = 6)**	***p-value***
Medical history record				
Age, *years*	54.5 (51.5 – 57.5)	54.0 (52.5 – 56.0)	57.0 (54.0 – 59.0)	0.510
Age of menopause, *years*	49.9 (48.1 – 53.9)	51.8 (49.9 – 54.7)	54.1 (49.8 – 55.0)	0.610
Time since the onset of menopause, *months*	43.0 (19.0 – 78.0)	27.0 (18.5 – 51.5)	22.5 (15.0 – 50.0)	0.585
Lifestyle habits				
Physical activity, *METs-min/day*	572 (453 – 1119)	491 (304 – 746)	460 (396 – 601)	0.435
Alcohol consumption				**0.040**
Weekly, *n (%)*	1 (8.3)	9 (56.3)	1 (16.7)	
Occasionally, *n (%)*	8 (66.7)	7 (43.8)	4 (66.7)	
Never, *n (%)*	3 (25.0)	0 (0.0)	1 (16.7)	
Type of alcohol consumed				0.385
Beer, *n (%)*	3 (25.0)	8 (50.0)	3 (50.0)	
Wine, *n (%)*	6 (50.0)	7 (43.8)	2 (33.3)	
Distilled beverages, *n (%)*	0 (0.0)	1 (6.25)	0 (0.0)	
None, *n (%)*	3 (25.0)	0 (0.0)	1 (16.7)	
Smoking status				0.335
Current, *n (%)*	1 (8.3)	6 (37.5)	2 (33.3)	
Former, *n (%)*	3 (25.0)	3 (18.8)	0 (0.0)	
Non-smoker, *n (%)*	8 (66.7)	7 (43.8)	4 (66.7)	
Sleep duration, *hours/d*	6.3 (6.0 – 7.0)	6.8 (6.5 – 8.0)	7.3 (6.0 – 7.5)	0.379
Dietary history				
Med Diet, *14-item score*	9.0 (7.0 – 9.5)	7.0 (6.5 – 8.5)	8.5 (7.0 – 10.0)	0.261
Total energy, k*cal/d*	2699 (2430 – 3042)	2672 (2261 – 3076)	2348 (2268 – 2682)	0.347
Carbohydrates, *% daily kcal*	32.6 (27.6 – 37.2)	33.9 (29.6 – 39.3)	37.4 (34.1 – 40.1)	0.242
Protein, *% daily kcal*	20.4 (16.4 – 20.9)	19.2 (16.7 – 21.5)	18.1 (16.9 – 20.4)	0.694
Fat, *% daily kcal*	47.0 (43.7 – 53.4)	46.9 (38.1 – 50.1)	44.0 (41.3 – 45.3)	0.447
Total polyphenols, *mg/d*	1064 (772 – 1394)	767 (538 – 851)	830 (677 – 1450)	0.107
Alcohol, *g/d*	1.0 (0.3 – 2.0) [a]	7.2 (2.8 – 8.7) [b]	1.9 (0.6 – 6.7) [a]	**<0.001**
Medication, n (%)				
Antihypertensive drugs, *n (%)*	0 (0.0)	3 (18.8)	1 (16.7)	0.288
Cholesterol-lowering agents, *n (%)*	0 (0.0)	2 (12.5)	0 (0.0)	0.303
Sedatives, tranquilizers, or antidepressant treatment, *n (%)*	3 (25.0)	3 (18.8)	1 (16.7)	0.890
Food supplements, *n (%)*	4 (33.3)	8 (50.0)	2 (33.3)	0.615

Abbreviations: AB, Alcoholic beer; NAB, Non-alcoholic beer. For continuous variables, Kruskal–Wallis test followed by post-hoc Dunn's test was performed with data are tabulated as median (Q1 – Q3). Qualitative variables were analysed with the chi-square test and declared as absolute value and percentages. *P*-value < 0.05 is considered statistically significant, highlighted in bold. Different superscripts (a, b) on the same row are significantly different.

Table 2. Participants baseline characteristics on anthropometric measurements, body composition variables, blood pressure and biochemical markers according to the study group.

	Control group (n = 12)	AB group (n = 16)	NAB group (n = 6)	*p-value*
Anthropometric measurements				
BMI, *kg/m²*	26.5 (25.0 – 30.7)	26.4 (22.7 – 28.2)	25.3 (24.7 – 29.0)	0.655
WC, *cm*	89.8 (82.5 – 96.0)	87.9 (78.6 – 95.7)	84.5 (80.3 – 90.1)	0.801
WHR	0.83 (0.79 – 0.87)	0.85 (0.80 – 0.91)	0.83 (0.79 – 0.85)	0.426
LMI, *kg/m²*	14.9 (13.8 – 15.4)	14.2 (12.8 – 14.6)	14.6 (14.0 – 16.5)	0.109
FMI, *kg/m²*	11.5 (9.4 – 12.8)	10.8 (8.7 – 12.9)	10.5 (9.3 – 12.3)	0.879
Total fat mass, *%*	44.1 (39.9 – 44.8)	42.1 (38.7 – 47.3)	40.3 (39.1 – 48.2)	0.953
Body composition variables				
Android fat mass, *%*	44.5 (39.8 – 50.8)	46.3 (40.6 – 52.8)	42.1 (39.1 – 44.6)	0.705
Gynoid fat mass, *%*	48.4 (45.8 – 49.8)	44.3 (42.3 – 50.1)	43.7 (40.4 – 47.0)	0.452
Android-gynoid ratio	0.90 (0.85 – 0.98)	1.00 (0.91 – 1.08)	0.97 (0.83 – 1.05)	0.282
Blood pressure				
DBP, *mmHg*	69 (64 – 84)	73 (68 – 77)	73 (68 – 78)	0.973
SBP, *mmHg*	116 (108 – 135)	117 (108 – 131)	116 (106 – 133)	0.997
Biochemical markers				
Glycemia, *mg/dL*	90 (87 – 94)	94 (87 – 103)	94 (87 – 98)	0.572
HbA1c, *%*	5.5 (4.7 – 5.7)	5.4 (5.3 – 5.7)	5.8 (5.6 – 5.9)	0.232
TG, *mg/dL*	63 (50 – 81)	77 (54 – 98)	61 (54 – 71)	0.637
TC, *mg/dL*	190 (164 – 205)	208 (200 – 221)	203 (192 – 242)	0.135
LDL-c, *mg/dL*	117 (98 – 137)	132 (126 – 151)	144 (134 – 163)	0.105
HDL-c, *mg/dL*	57 (43 – 63)	55 (51 – 64)	57 (51 – 67)	0.905
TC/HDL-c ratio	3.2 (3.0 – 3.8)	3.7 (3.2 – 4.1)	3.6 (3.4 – 4.1)	0.351
LDL-c/HDL-c ratio	1.9 (1.8 – 2.4)	2.4 (1.9 – 2.8)	2.4 (2.2 – 2.8)	0.218
Apo A1, *mg/dL*	150 (136 – 158)	161 (154 – 174)	153 (146 – 185)	0.101
Apo B, *mg/dL*	99 (81 – 104)	109 (96 – 120)	115 (98 – 119)	0.274
AST, *U/L*	19 (18 – 20) [a]	23 (20 – 27) [b]	20 (17 – 25) [ab]	**0.009**
ALT, *U/L*	16 (14 – 18)	19 (16 – 27)	18 (13 – 22)	0.116
GGT, *U/L*	13 (10 – 15) [a]	23 (14 – 27) [b]	14 (12 – 23) [ab]	**0.019**
FSH, *U/L*	74.1 (51.1 – 82.5) [a]	88.6 (74.9 – 112.9) [b]	70.4 (37.2 – 72.2) [a]	**0.015**
17b-estradiol, *pg/mL*	23 (15 – 33)	18 (14 – 25)	22 (21 – 25)	0.538

Abbreviations: AB, Alcoholic beer; NAB, Non-alcoholic beer; BMI, Body mass index; WC, Waist circumference; WHR, Waist-hip ratio; LMI, Lean mass index; FMI, Fat mass index; DBP, Diastolic blood pressure; SPB, Systolic blood pressure; HbA1c, Glycosylated haemoglobin; TG, Triglycerides; TC, Total cholesterol; LDL-c, Low-density lipoprotein cholesterol; HDL-c, High-density lipoprotein cholesterol; Apo A1, Apolipoprotein A1; Apo B, Apolipoprotein B; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; GGT, Gamma-glutamyl transferase; FSH, Follicle-stimulating hormone. Kruskal–Wallis test followed by post-hoc Dunn's test was performed with data are tabulated as median (Q1 – Q3). p-value < 0.05 is considered statistically significant, highlighted in bold. Different superscripts (a, b) on the same row are significantly different.

02 **Table 3.** Effects of intervention on biochemical markers of cardiovascular disease risk and liver enzymes at 12 and 24 months of follow-up.

	AB group vs CG					**NAB group vs CG**					**AB group vs NAB group**				
	Mean difference 12 months (95% CI)	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***
Glycemia, *mg/dL*															
Model 1	1.44 (-3.61, 6.48)	0.576	1.58 (-2.14, 5.31)	0.405	0.408	3.20 (-3.36, 9.75)	0.339	0.09 (-8.21, 8.40)	0.983	0.998	-1.76 (-8.95, 5.43)	0.632	1.49 (-6.86, 9.85)	0.726	0.712
Model 2	1.44 (-3.60, 6.48)	0.576	1.58 (-2.14, 5.31)	0.405	0.410	3.20 (-3.36, 9.75)	0.339	0.09 (-8.21, 8.40)	0.983	0.999	-1.76 (-8.95, 5.44)	0.632	1.50 (-6.86, 9.85)	0.726	0.713
Model 3	1.64 (-3.30, 6.58)	0.515	1.72 (-1.94, 5.38)	0.357	0.363	3.55 (-2.85, 9.95)	0.277	0.25 (-7.71, 8.22)	0.950	0.971	-1.91 (-8.93, 5.11)	0.594	1.46 (-6.54, 9.47)	0.720	0.705
HbA1c, %															
Model 1	-0.16 (-0.37, 0.04)	0.119	-0.06 (-0.31, 0. 18)	0.611	0.598	-0.14 (-0. 37, 0.09)	0.238	-0.14 (-0.45, 0.17)	0.370	0.379	-0.02 (-0.23, 0.18)	0.811	0.08 (-0.15, 0.30)	0.498	0.533
Model 2	-0.16 (-0.37, 0.05)	0.125	-0.06 (-0.31, 0.18)	0.614	0.603	-0.14 (-0.37, -0.09)	0.238	-0.14 (-0.45, 0.17)	0.372	0.381	-0.02 (-0.22, 0.18)	0.836	0.08 (-0.14, 0.30)	0.496	0.529
Model 3	-0.16 (-0.37, 0.04)	0.119	-0.07 (-0.31, 0.17)	0.568	0.558	-0.14 (-0.38, -0.09)	0.227	-0.14 (-0.45, 0.16)	0.356	0.365	-0.02 (-0.22, 0.18)	0.843	0.07 (-0.15, 0.29)	0.514	0.547
TG, *mg/dL*															
Model 1	1.94 (-24.82, 28.69)	0.887	-1.10 (-20.74, 18.53)	0.912	0.919	-9.74 (-31.46, 11.98)	0.379	8.98 (-20.17, 38.13)	0.546	0.669	11.68 (-11.54, 34.90)	0.324	-10.08 (-39.13, 18.97)	0.497	0.620
Model 2	-3.73 (-29.53, 22.06)	0.777	-5.20 (-23.26, 12.88)	0.574	0.537	-11.86 (-36.00, 12.30)	0.336	9.70 (-21.85, 41.26)	0.547	0.683	8.12 (-10.33, 26.57)	0.334	-14.89 (-45.10, 15.31)	0.334	0.397
Model 3	-11.64 (-45.32, 22.04)	0.498	-8.58 (-25.59, 10.43)	0.376	0.362	-18.38 (-52.20, 15.44)	0.287	8.07 (-25.11, 41.23)	0.634	0.815	6.74 (-10.56, 24.05)	0.445	-16.65 (-46.21, 12.92)	0.270	0.330
TC, *mg/dL*															
Model 1	-1.91 (-19.71, 15.89)	0.833	2.94 (-11.68, 17.56)	0.694	0.694	-25.25 (-47.31, -3.19)	**0.025**	-12.97 (-32.04, 6.10)	0.183	0.212	23.34 (3.33, 43.35)	**0.022**	15.91 (-0.24, 32.05)	0.053	0.069
Model 2	-1.89 (-19.72, 15.94)	0.835	2.78 (-11.97, 17.53)	0.712	0.713	-25.19 (-47.30, -3.09)	**0.025**	-12.91 (-32.01, 6.19)	0.185	0.215	23.30 (3.31, 43.30)	**0.022**	15.69 (-0.54, 31.91)	0.058	0.075
Model 3	-2.42 (-20.49, 15.64)	0.792	2.76 (-11.94, 17.46)	0.713	0.715	-24.78 (-46.62, -2.94)	**0.026**	-12.64 (-31.57, 6.28)	0.190	0.220	22.36 (2.49, 42.22)	**0.027**	15.40 (-0.60, 31.40)	0.059	0.076
LDL-c, *mg/dL*															
Model 1	-5.86 (-21.19, 9.46)	0.453	-6.05 (-19.11, 7.00)	0.364	0.361	-30.51 (-49.36, -11.66)	**0.002**	-24.23 (-46.81, -1.66)	**0.035**	0.059	24.65 (5.75, 43.55)	**0.011**	18.18 (-2.65, 39.01)	0.087	0.146
Model 2	-5.87 (-19.30, 9.52)	0.455	-6.19 (-19.30, 6.91)	0.354	0.352	-30.47 (-49.41, -11.53)	**0.002**	-24.20 (-46.60, -1.81)	**0.034**	0.057	24.59 (5.70, 43.48)	**0.011**	18.01 (-2.63, 38.65)	0.087	0.146
Model 3	-6.30 (-22.00, 9.41)	0.432	-6.22 (-19.57, 7.14)	0.361	0.361	-29.85 (-48.79, -10.91)	**0.002**	-23.49 (-45.65, -1.33)	**0.038**	0.067	23.55 (4.54, 42.57)	**0.015**	17.27 (-3.03, 37.57)	0.098	0.159

	AB group vs CG					NAB group vs CG					AB group vs NAB group				
	Mean difference 12 months (95% CI)	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***
HDL-c, *mg/dL*															
Model 1	4.50 (-2.59, 11.59)	0.213	8.92 (2.22, 15.62)	**0.009**	**0.008**	0.80 (-7.51, 9.11)	0.851	2.78 (-6.48, 12.03)	0.556	0.564	3.70 (-3.38, 10.79)	0.306	6.14 (-3.02, 15.30)	0.189	0.184
Model 2	4.37 (-2.76, 11.49)	0.235	8.90 (2.30, 15.50)	**0.008**	**0.007**	0.58 (-7.78, 8.94)	0.892	2.58 (-6.62, 11.77)	0.583	0.589	3.79 (-3.38, 10.96)	0.300	6.32 (-2.67, 15.31)	0.168	0.164
Model 3	3.63 (-3.32, 10.57)	0.306	9.01 (2.47, 15.55)	**0.007**	**0.006**	-0.22 (-8.22, 7.78)	0.956	1.85 (-7.61, 11.32)	0.701	0.702	3.85 (-3.39, 11.09)	0.297	7.16 (-2.21, 16.52)	0.134	0.133
TC/HDL-c ratio															
Model 1	-0.34 (-0.75, 0.07)	0.107	-0.54 (-0.93, -0.14)	**0.008**	**0.007**	-0.48 (-0.98, 0.02)	0.059	-0.35 (-0.93, 0.24)	0.246	0.337	0.14 (-0.36, 0.65)	0.581	-0.19 (-0.76, 0.38)	0.509	0.364
Model 2	-0.35 (-0.76, 0.06)	0.099	-0.55 (-0.94, -0.16)	**0.006**	**0.006**	-0.49 (-0.98, 0.01)	**0.047**	-0.36 (-0.94, 0.23)	0.231	0.317	0.15 (-0.35, 0.65)	0.558	-0.19 (-0.77, 0.39)	0.513	0.378
Model 3	-0.36 (-0.77, 0.04)	0.078	-0.55 (-0.92, -0.18)	**0.004**	**0.004**	-0.52 (-1.02, -0.31)	**0.037**	-0.34 (-1.03, 0.34)	0.325	0.272	0.16 (-0.32, 0.65)	0.515	-0.17 (-0.74, 0.40)	0.554	0.413
LDL-c/HDL-c ratio															
Model 1	-0.30 (-0.67, 0.07)	0.116	-0.48 (-0.82, -0.14)	**0.006**	**0.005**	-0.49 (-0.90, -0.07)	**0.023**	-0.43 (-0.91, 0.04)	0.076	0.120	0.19 (-0.26, 0.63)	0.415	-0.05 (-0.52, 0.42)	0.836	0.572
Model 2	-0.31 (-0.68, 0.07)	0.107	-0.49 (-0.83, -0.15)	**0.005**	**0.004**	-0.50 (-0.91, 0.09)	**0.017**	-0.42 (-0.96, 0.11)	0.123	0.105	0.19 (-0.25, 0.63)	0.391	-0.05 (-0.52, 0.42)	0.843	0.590
Model 3	-0.32 (-0.69, 0.04)	0.084	-0.49 (-0.81, -0.17)	**0.003**	**0.003**	-0.53 (-0.94, -0.12)	**0.011**	-0.47 (-0.92, 0.13)	**0.044**	0.076	0.21 (-0.21, 0.63)	0.329	-0.02 (-0.49, 0.44)	0.928	0.663
Apo A1, *mg/dL*															
Model 1	-2.69 (-15.12, 9.75)	0.672	8.27 (-7.20, 23.75)	0.295	0.293	-13.38 (-30.42, 3.66)	0.124	-0.54 (-19.21, 18.13)	0.955	0.970	10.69 (-5.02, 26.41)	0.182	8.81 (-6.24, 23.86)	0.251	0.274
Model 2	-2.70 (-15.13, 9.74)	0.671	8.27 (-7.21, 23.74)	0.295	0.294	-13.37 (-30.42, 3.68)	0.124	-0.55 (-19.22, 18.11)	0.954	0.972	0.67 (-.505, 26.40)	0.183	8.82 (-6.19, 23.84)	0.249	0.272
Model 3	-2.55 (-15.09, 10.00)	0.691	8.74 (-6.76, 24.25)	0.270	0.270	-13.58 (-30.32, 3.16)	0.112	-0.89 (-19.55, 17.76)	0.925	0.993	11.03 (-4.20, 26.27)	0.156	9.63 (-5.83, 25.09)	0.222	0.241
Apo B, *mg/dL*															
Model 1	-2.29 (-12.00, 7.42)	0.644	1.69 (-6.82, 10.19)	0.698	0.645	-13.34 (-21.64, -5.03)	**0.002**	-1.93 (-17.00, 13.13)	0.801	0.883	11.05 (0.87, 21.23)	**0.033**	3.62 (-11.30, 18.54)	0.634	0.893
Model 2	-1.96 (-11.42, 7.51)	0.685	1.83 (-6.75, 10.41)	0.676	0.628	-13.36 (-21.69, -5.03)	**0.002**	-1.42 (-16.43, 13.58)	0.852	0.844	11.40 (1.48, 21.32)	**0.024**	3.26 (-11.48, 17.99)	0.665	0.918
Model 3	-2.07 (-11.58, 7.43)	0.669	1.62 (-6.76, 9.99)	0.705	0.652	-13.28 (-21.46, -5.10)	**0.001**	-1.30 (-15.84, 13.24)	0.861	0.844	11.20 (1.22, 21.19)	**0.028**	2.92 (-11.40, 17.23)	0.690	0.936
AST, U/L															

	AB group vs CG					NAB group vs CG					AB group vs NAB group				
	Mean difference 12 months (95% CI)	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***
Model 1	-4.40 (-10.65, 1.86)	0.168	-1.17 (-6.94, 4.60)	0.692	0.662	-1.19 (-4.83, 2.45)	0.521	1.23 (-4.61, 7.06)	0.680	0.710	-3.21 (-9.09, 2.68)	0.285	-2.39 (-9.95, 5.16)	0.535	0.534
ALT, U/L															
Model 1	-2.60 (-8.80, 3.60)	0.412	3.00 (-1.44, 7.45)	0.186	0.235	-0.26 (-5.34, 4.82)	0.920	3.23 (-4.14, 10.60)	0.390	0.416	-2.34 (-9.47, 4.80)	0.521	-0.23 (-8.21, 7.74)	0.955	0.922
GGT, U/L															
Model 1	0.13 (-3.95, 4.21)	0.950	4.46 (0.92, 8.01)	**0.013**	**0.011**	0.29 (-2.94, 3.51)	0.861	6.22 (3.77, 8.65)	**<0.001**	**<0.001**	-0.16 (-4.61, 4.30)	0.945	-1.75 (-5.42, 1.92)	0.350	0.412

Abbreviations: AB, Alcoholic beer; NAB, Non-alcoholic beer; CG, Control group; HbA1c, Glycosylated haemoglobin; TG; Triglycerides; TC, Total cholesterol; LDL-c, Low-density lipoprotein cholesterol; HDL-c, High-density lipoprotein cholesterol; Apo A1, Apolipoprotein A1; Apo B, Apolipoprotein B; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; GGT, Gamma-glutamyl transferase.

Generalized estimating equation (GEE) models were used to compare the differences between the study groups throughout the intervention with respect to their own baseline values. Data are expressed as the mean of the differences (95% CI). Model 1: adjusted by age at baseline; Model 2: adjusted like Model 1 plus follicle-stimulating hormone concentration at baseline; Model 3: adjusted like Model 2 plus smoking habit, total energy intake, physical activity as MET-min/day, and hypocholesterolaemia (for lipid profile variables) at baseline. *p*-value: group x time interaction; *p*-trend: group x time interaction (continuous). < 0.05 are statistically significant, highlighted in bold.

27 **Table 4.** Effects of intervention on body composition and blood pressure at 12 and 24 months of follow-up.

	AB group vs CG					NAB group vs CG					AB group vs NAB group				
	Mean difference 12 months (95% CI)	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***
BMI, *kg/m²*															
Model 1	-0.00 (-0.98, 0.97)	0.993	0.58 (-0.36, 1.51)	0.225	0.218	-0.38 (-1.34, 0.59)	0.447	0.17 (-1.06, 1.40)	0.782	0.778	0.37 (-0.77, 1.51)	0.522	0.41 (-0.67, 1.48)	0.462	0.456
Model 2	-0.10 (-1.10, 0.91)	0.853	0.45 (-0.51, 1.42)	0.359	0.358	-0.42 (-1.37, 0.53)	0.359	0.19 (-1.04, 1.41)	0.767	0.763	0.32 (-0.82, 1.47)	0.578	0.27 (-0.84, 1.38)	0.638	0.639
Model 3	-0.01 (-1.02, 0.99)	0.981	0.50 (-0.48, 1.48)	0.318	0.315	-0.39 (-1.31, 0.52)	0.400	0.22 (-1.00, 1.45)	0.719	0.714	0.38 (-0.72, 1.49)	0.498	0.28 (-0.80, 1.36)	0.616	0.619
WC, *cm*															
Model 1	-0.62 (-3.34, 2.10)	0.656	1.05 (-3.37, 5.48)	0.641	0.638	-4.04 (-7.25, -0.84)	**0.013**	-0.96 (-6.81, 4,88)	0.747	0.753	3.42 (0.25, 6.60)	**0.035**	2.02 (-3.94, 7.97)	0.507	0.510
Model 2	-0.68 (-3.47, 2.10)	0.632	0.26 (-4.58, 5.10)	0.918	0.914	-4.02 (-7.34, -0.70)	**0.018**	-0.86 (-6.66, 4.94)	0.771	0.776	3.34 (0.05, 6.62)	**0.046**	1.12 (-5.09, 7.32)	0.724	0.726
Model 3	-0.60 (-3.40, 2.19)	0.672	0.29 (-4.56, 5.13)	0.908	0.903	-4.00 (-7.25, -0.73)	**0.016**	-0.75 (-6.45, 4.96)	0.798	0.801	3.39 (0.19, 6.58)	**0.038**	1.03 (-5.12, 7.18)	0.742	0.742
WHR															
Model 1	0.00 (-0.03, 0.04)	0.770	0.02 (-0.03, 0.04)	0.446	0.440	-0.03 (-0.06, 0.01)	0.116	0.00 (-0.04, 0.05)	0.914	0.914	0.03 (-0.00, 0.07)	0.064	0.02 (-0.05, 0.08)	0.583	0.578
Model 2	0.00 (-0.03, 0.04)	0.774	0.02 (-0.04, 0.07)	0.528	0.522	-0.03 (-0.06, 0.01)	0.125	0.00 (-0.04, 0.05)	0.906	0.906	0.03 (0.00, 0.07)	0.068	0.01 (-0.05, 0.08)	0.662	0.657
Model 3	0.00 (-0.03, 0.04)	0.766	0.02 (-0.04, 0.07)	0.549	0.542	-0.03 (-0.07, 0.01)	0.130	-0.00 (-0.04, 0.05)	0.899	0.899	0.03 (0.00, 0.07)	0.071	0.01 (-0.05, 0.08)	0.688	0.683
FMI, *kg/m²*															
Model 1	0.59 (-0.17, 1.34)	0.128	0.53 (-0.34, 1.34)	0.234	0.229	-0.06 (-1.33, 1.45)	0.932	-0.15 (-1.40, 1.10)	0.817	0.806	0.53 (-0.85, 1.90)	0.453	0.68 (-0.37, 1.72)	0.207	0.198
Model 2	0.45 (-0.33, 1.23)	0.257	0.39 (-0.49, 1.27)	0.384	0.399	-0.03 (-1.46, 1.39)	0.962	-0.18 (-1.47, 1.10)	0.780	0.773	0.48 (-0.95, 1.92)	0.508	0.58 (-0.54, 1.69)	0.310	0.316
Model 3	0.76 (-0.00, 1.53)	0.052	0.38 (-0.61, 1.37)	0.454	0.370	-0.04 (-1.17, 1.24)	0.954	-0.12 (-1.36, 1.15)	0.869	0.838	0.60 (-0.42, 1.62)	0.249	0.37 (-0.80, 1.53)	0.533	0.300
Total fat mass, %															
Model 1	1.85 (-0.06, 3.64)	**0.043**	0.71 (-0.67, 2.09)	0.314	0.313	-0.24 (-4.21, 3.73)	0.904	0.19 (-3.33, 3.71)	0.916	0.919	2.09 (-1.83, 6.02)	0.296	0.52 (-2.92, 3.95)	0.768	0.764
Model 2	1.91 (-0.13, 3.69)	**0.036**	0.74 (-0.62, 2.11)	0.286	0.295	-0.20 (-4.13, 3.74)	0.921	0.20 (-3.29, 3.69)	0.912	0.917	2.11 (-1.77, 5.99)	0.286	0.55 (-2.85, 3.94)	0.752	0.754
Model 3	2.00 (0.20, 3.81)	**0.030**	0.69 (-0.76, 2.13)	0.351	0.347	-0.27 (-4.16, 3.61)	0.890	0.13 (-3.36, 3.62)	0.941	0.943	2.28 (-1.60, 6.15)	0.249	0.56 (-2.82, 3.93)	0.747	0.748

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	AB group vs CG					NAB group vs CG					AB group vs NAB group				
	Mean difference 12 months (95% CI)	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***	**Mean difference 12 months (95% CI)**	***p-value***	**Mean difference 24 months (95% CI)**	***p-value***	***p-trend***
Android fat mass, %															
Model 1	2.82 (-0.03, 5.68)	0.053	4.69 (1.28, 8.10)	**0.007**	**0.006**	1.81 (-1.03, 4.64)	0.211	7.31 (2.84, 11.79)	**0.001**	**0.001**	1.02 (-2.22, 4.25)	0.539	-2.62 (-6.32, 1.08)	0.165	0.170
Model 2	2.86 (0.01, 5.71)	**0.049**	4.72 (1.33, 8.11)	**0.006**	**0.006**	1.82 (-1.01, 4.65)	0.209	7.30 (2.83, 11.77)	**0.001**	**0.001**	1.47 (-2.18, 4.27)	0.525	-2.58 (-6.27, 1.11)	0.170	0.170
Model 3	8.19 (4.69, 11.68)	**<0.001**	3.98 (0.79, 7.17)	**0.014**	**0.008**	1.57 (-1.19, 4.32)	0.265	6.33 (1.64, 11.02)	**0.008**	**0.002**	1.93 (-3.27, 7.12)	0.467	-2.22 (-5.72, 1.27)	0.213	0.180
Gynoid fat mass, %															
Model 1	0.87 (-0.77, 2.52)	0.299	3.76 (0.64, 6.87)	**0.018**	**0.018**	0.44 (-0.97, 1.86)	0.537	4.01 (0.58, 7.46)	**0.022**	**0.021**	0.43 (-1.21, 2.06)	0.610	-0.26 (-2.35, 1.82)	0.805	0.808
Model 2	0.89 (-0.75, 2.53)	0.288	3.76 (0.64, 6.87)	**0.017**	**0.017**	0.45 (-0.97, 1.86)	0.536	4.01 (0.57, 7.44)	**0.022**	**0.021**	0.44 (-1.19, 2.07)	0.596	-0.24 (-2.32, 1.83)	0.819	0.825
Model 3	0.82 (-0.85, 2.49)	0.337	3.73 (0.65, 6.83)	**0.018**	**0.017**	0.36 (-1.06, 1.79)	0.616	4.03 (0.59, 7.46)	**0.022**	**0.021**	0.45 (-1.17, 2.08)	0.585	-0.29 (-2.38, 1.80)	0.785	0.788
Android-gynoid ratio															
Model 1	0.06 (-0.02, 0.12)	**0.043**	0.02 (-0.03, 0.08)	0.407	0.430	0.05 (-0.02, 0.12)	0.152	0.09 (0.01, 0.18)	**0.033**	**0.039**	0.01 (-0.06, 0.08)	0.780	-0.07 (-0.15, 0.01)	0.095	0.102
Model 2	0.06 (-0.03, 0.12)	**0.041**	0.02 (-0.03, 0.08)	0.382	0.412	0.05 (-0.02, 0.12)	0.148	0.09 (-0.01, 0.18)	**0.033**	**0.038**	0.01 (-0.06, 0.08)	0.765	-0.07 (-0.15, 0.01)	0.098	0.104
Model 3	0.09 (0.01, 0.17)	**0.033**	0.02 (-0.03, 0.07)	0.426	0.446	0.05 (-0.02, 0.12)	0.134	0.09 (-0.01, 0.18)	**0.036**	**0.036**	0.04 (-0.06, 0.13)	0.431	-0.07 (-0.15, 0.01)	0.077	0.083
DBP, *mmHg*															
Model 1	-0.54 (-5.40, 4.32)	0.828	-0.13 (-5.93, 5.67)	0.965	0.961	-4.59 (-10.02, 0.85)	0.098	-5.03 (-11.34, 1.27)	0.118	0.109	4.05 (-1.18, 9.28)	0.129	4.90 (0.49, 9.32)	**0.029**	**0.026**
Model 2	-0.56 (-5.42, 4.30)	0.822	-0.15 (-5.96, 5.65)	0.959	0.952	-4.60 (-10.03, 0.83)	0.097	-5.05 (-11.36, 1.26)	0.116	0.108	4.04 (-1.18, 9.27)	0.127	4.90 (0.49, 9.31)	**0.029**	**0.026**
Model 3	-0.26 (-5.18, 4.65)	0.916	0.33 (-5.50, 6.16)	0.912	0.917	-4.63 (-10.03, 0.77)	0.093	-4.98 (-11.26, 1.31)	0.121	0.112	4.36 (-0.56, 9.59)	0.101	5.30 (0.97, 9.64)	**0.017**	**0.023**
SBP *(mmHg)*															
Model 1	-5.16 (-12,94, 2.61)	0.193	-1.65 (-10.46, 7.16)	0.713	0.693	-4.09 (-14.44, 6.25)	0.438	-3.03 (-12.27, 6.21)	0.521	0.503	-1.07 (-11.36, 9.23)	0.839	1.38 (-7.05, 9.80)	0.749	0.748
Model 2	-5.23 (-13.03, 2.57)	0.189	-1.74 (-10.59, 7.12)	0.701	0.678	-4.18 (-14.50, 6.13)	0.427	-3.08 (-12.31, 6.16)	0.514	0.495	-1.05 (-12.32, 9.23)	0.842	1.34 (-7.08, 9.75)	0.755	0.756
Model 3	-5.15 (-12.62, 2.32)	0.177	-1.31 (-10.07, 7.44)	0.769	0.749	-4.39 (-14.41, 5.63)	0.391	-2.34 (-11.62, 6.94)	0.621	0.606	-0.76 (-10.47, 8.95)	0.878	1.02 (-7.61, 9.66)	0.816	0.726

Abbreviations: AB, Alcoholic beer; NAB, Non-alcoholic beer; CG, Control group; BMI, Body mass index; WC, Waist circumference; WHR, Waist-hip ratio; FMI, Fat mass index; DBP, Diastolic blood pressure; SPB, Systolic blood pressure.

Generalized estimating equation (GEE) models were used to compare the differences between the study groups throughout the intervention with respect to their own baseline values. Data are expressed as the mean of the differences (95% CI). Model 1: adjusted by age at baseline; Model 2: adjusted like Model 1 plus follicle-stimulating hormone concentration at baseline; Model 3: adjusted like Model 2 plus smoking habit, total energy intake, physical activity as MET-min/day, and antihypertensive treatment (for blood pressure variables) at baseline. *p*-value: group x time interaction; p-trend: group x time interaction (continuous). < 0.05 are statistically significant, highlighted in bold

4. GLOBAL DISCUSSION

The present work focuses on the effect of moderate beer consumption on post-menopausal women's health. Concurrently to the conduction of a long-term parallel controlled clinical trial, inter-individual differences in taste sensitivity and their applicability to nutritional research have been evaluated, as well as biomarkers of beer intake, ethanol itself, and other alcoholic beverages discussed. Therefore, this thesis aimed to integrate new promising nutrition research approaches and boost the idea of a personalized nutrition perspective through the study of the benefits and risks of moderate beer intake in a specific population cohort such as post-menopausal women.

Knowledge about the physiological implications of the gustatory function is currently of interest since taste signal transduction have been described as a potential target pathway of specific dietary components with recognized biological effects [129,154]. Indeed, many of the peptide hormones identified in the gut are also expressed in TB cells and are implicated in the modulation of taste functions, although much of the evidence is coming from *in vivo* models [155,156]. In this regard, we worked with the hypothesis that bitter and sweet TR might play an important and complex role in the digestive system and body metabolism. DT and RT are related to ligand affinity to TR, and therefore might be good predictors of individual differences in sensory perception and metabolic responses. More precisely, RTs convey information about the perceptual quality of the nutrients from the very first place of interaction of food with the individual organism [72].

After performing a systematic review and meta-analysis on sweet DT and RT, results pointed out that aging and some pathologies such as type 2 diabetes are factors that significantly increase the sucrose RT. In addition, the higher the BMI, the higher the sucrose DT. Feasible mechanisms underlying changes in the sucrose thresholds

include the modulation of incretin secretion with anorexigenic and glucose-regulatory effects triggered by T1R2/T1R3, a reduction in TB abundance [82,86], or changes in the central taste system [157], among others. Therefore, knowledge about taste genotype/phenotype might be a potential therapeutic e.g., dietetical and pharmacological strategy in the battle against obesity and diabetes.

After this approach, research was focus on understanding individual differences in taste acuity through the influence of some sociodemographic and clinical factors in a cross-sectional study using predictive models of higher or lower RTs for seven molecules representing the five basic tastes. A higher sucrose RT was found in females than in males, while sinusitis and rhinitis were factors in predictive models for higher sensitivity to sucrose and sodium chloride RTs. Although some researchers previously reported some of the studied factors relevant in taste acuity [85,87,158,159], built models did not show a clear pattern. This controversy result could be explained by the short exposure of some studied predicted factors due to the young age and the homogenous characteristics of the studied population cohort.

Finally, the next statistical analysis was on assessing taste sensitivity differences among a gustatory establish classification based on well-known phenotypes corresponding to T2R38 polymorphisms. In this direction, the statistical approach aimed to evaluate if some specific health-related factors were more prevalent in one of the three PTC phenotypes in the same young cohort. BMI was significantly higher in the super-taster PTC group, and lower predicted scores (higher sensitivity) for other RTs molecules were found too. Findings of this study support the PTC taste perception implication on weight homeostasis and body energy balance, previously reported by some other researchers [160–163]. The discovery of extra-oral T2R and T1R in several metabolically active tissues brings new insights into the physiological significance of the gustatory system in human health and nutritional status. As an example, T2R38

was already found to be upregulated in overweight/obese subjects' intestinal tracts [164,165], while an *in vivo* study found a decrease in lipid accumulation after stimulation of T2R38 by its ligand PROP independent of the T2R38 gene variants [166]. Moreover, present findings of higher sucrose RT in PTC non-tasters and the significant correlation between basic taste RTs, add new evidence about individual sensory patterns and nutrient-sensing interactions, as well as the role of taste acuity measurements in glucose homeostasis and food intake. Thus, PTC RT measurement might be an additional approach for the precise diagnosis of the nutritional status.

In that sense, the amount of alcohol intake has been associated with T2R38 genotype [167] and PROP bitterness [168]. Taste sensitivity has also been reported to be different according to alcohol drinking behavior [169,170], having potential applicability as a non-invasive, easy, and non-expensive biomarker of alcohol intake. Nevertheless, more objective approaches must be used to validate intervention compliance or to qualify and quantify dietary exposure [97].

BFIs are useful instruments for accurately assessing food intake, though several challenges to their application as a dietary assessment tool remain (e.g., inter-individual variations, differences in food varieties, and food processing) [99,102]. Indeed, dietary assessment plays a major role in nutrition research, thus it is crucial when exploring the association between specific foods or dietary patterns and their effects on health [171]. To propose promising BFI candidates for a specific food or nutrient such as ethanol, data on food composition or food sources, its metabolism and endogenous production and the related eating behaviors are important nutritional factors, beyond the analytical performance validity [99]. Although low to moderate consumption of alcohol itself and beer have been already related to some health outcomes [149,150]; large-controlled human trials are still needed to validate these effects. Moreover, self-reported alcohol intakes are likely to be influenced by

measurement error, and thus affecting the accuracy and precision of currently established epidemiological associations between ethanol, alcoholic beverage consumption, and health or disease. Thus, the systematic review included in this thesis aimed to list and validate biomarkers of ethanol intake *per se* excluding markers of abuse, and including biomarkers related to common alcoholic beverages such as beer.

The extensive literature review resulted in five main direct markers of alcohol intake: ethanol, EtG, EtS, FAEEs, and PEth. Ethanol is the most obvious biomarker, but EtG and EtS have considerably longer half-lives in plasma [172,173]. At the group level, intake ranges are relatively well studied for EtG, EtS, FAEE and PEth, and all markers can discriminate between low, moderate, and high intakes. At the individual level, there is sometimes an overlap between the ranges due to inter-individual differences in the activities and kinetics of ADH and ALDH enzymes, and in candidate biomarker degradation and excretion. Classification of null intake *versus* low intakes needs further research, which is unfortunate since the major controversy on moderate alcohol intake and health is the effect of abstention *versus* low intakes.

Assessing compliance is also important in clinical trials and demands objective tools too. Factors such as the time lapse since last drink, the frequency of drinking and the type of beverages consumed are important aspects to address. Indeed, there is considerable interest to discriminate between the different alcoholic beverages, because physiological or health effects related to beer intake have recently been reviewed [139,174]. Compounds coming from boiled hops (i.e., iso-α-acids, isoxanthohumol) [175–179] and from barley (i.e., hordenine) [180–182] have been suggested as plausible candidate beer intake biomarkers. Untargeted metabolic approach has been also applied and combined biomarker model proposed [183]. To date, candidates of beer intake cover recent high or moderate intakes reasonably

well, but further research is needed for low-intake doses. IX and iso-α-acids are well qualitative BFIs, while hordenine and its metabolites need further research on dose-response, robustness, and stability criteria.

Considering all the above mentioned, the study on the effect of moderate daily beer consumption (with and without ethanol) on menopausal symptoms, and bone and cardiovascular health was investigated. Intervention compliance was assessed via dietary self-records, interviews and IX as a biomarker of beer intake, while sensory sensitivity of the participants was also evaluated.

A smooth menopause transition is considered a healthy and successful step in aging [10]. HRT effectively reduces menopausal symptoms associated with the decrease of estrogen [184,185]. However, besides these, there is great interest in the use of alternative therapies, such as phytoestrogens intake, to treat menopausal symptoms and menopause-associated risk factors [22,29,34,186]. In recent years some prenylated chalcones, flavonoids present in hops (*Humulus Lupulus* L.), have received much attention for their health effects [43,187–191]. Indeed, the prenylation brings the flavonoids the improvement of bioactivities such as the proestrogenic [188].

After a period of 6-months of moderate daily consumption of AB and NAB, menopause-related symptoms decrease in comparison to the control group in a post-menopausal population suffering from mild-to-moderate symptoms. As these improvements were observed after both AB and NAB consumption, it can be attributed to the non-alcoholic fraction of beer, possibly to the phytoestrogenic effect of polyphenols, although the sex hormone profile did not differ significantly between the study groups. In line with our results and from a clinical perspective, phytoestrogens may be a proper first treatment for women suffering from mild to moderate vasomotor symptoms in early natural post-menopause [23]. This is also in

accordance with the recommendations of the North American Menopause Society [22]. However, only part of these benefits is probably due to the phytoestrogens *per se* because studies with placebo groups have also reported a 20-30% of reduction [192]. A decrease in psychological symptoms was also observed in the AB group (14 g of ethanol/day), but not in the NAB group.

This parallel controlled trial was extended to 2 years of follow-up to study whether the intervention of moderate daily intake of AB and NAB could have beneficial effects on bone tissue. The AB and the NAB consumption were found to increase bone formation markers in comparison to the control group, although dual-energy X-ray absorptiometry (DXA) scans revealed that neither AB nor NAB interventions attenuated expected BMD and TBS loss. The participants in the present study were in relatively early post-menopause, when accelerated bone turnover arising from estrogen deficiency tends to be high, and consequently, bone loss [193]. Beer's beneficial effect on bone tissue has been attributed to its ethanol [194,195], silicon [196], and polyphenol content [197,198]. A wide range of polyphenols with antioxidant and anti-inflammatory activity has been found in beer, which might plain a role in beer's protective effect diminishing bone tissue loss.

On the premise that estrogens may have a cardioprotective effect [199] and the increased rate of cardiovascular events in post-menopausal women [200], the safety and effectiveness of HRT and dietary interventions in reducing the risk of CVD and the associated CVDRF have been studied. Interestingly, 14 g/day of alcohol in a beer form significantly increased high-density lipoprotein cholesterol (HDL-c) serum level compared to the control group, as well as total cholesterol (TC)/HDL-c) and low-density lipoprotein cholesterol (LDL-c)/HDL-c ratios. Previous studies have reported this effect only in subjects who consume AB but not in those consuming NAB, fact

that supports the hypothesis that this is an exclusive consequence of beer's alcohol content [201,202].

Like our results, Spaggiari *et al.* (2020) pointed out that studies on cardiovascular human health show that chronic but moderate NAB consumers had significantly lower TC levels when compared to AB consumers, with a homogeneous effect for both men and women [201]. In addition, the NAB arm of this study showed a significant decrease in serum LDL-c levels compared to the control group. This result explains the greater lowering effects in TC in the NAB group compared to the other study arms. Although it is not clear whether it is polyphenols in general with their antioxidant and anti-inflammatory effects or whether it is indeed the specific phytoestrogenic compounds that regulate blood lipids, it is relevant to boost the healthy properties of the bioactive fraction of beer through food innovation. The baseline TC levels has previously reported as important factor for this associated health effect [203].

Both interventions did not show either positive or negative effects on blood glucose control, triglycerides, and apoliprotein A1 at the follow-up of the intervention. These results are supported by literature [201,202,204]. Changes in body composition did not follow a clear changing pattern, thus effect of low to moderate beer consumption on abdominal fat in post-menopausal women is still in controversy [205]. The effects of NAB intervention in decreasing diastolic blood pressure were significant, but levels were not clinically relevant at 24-months of follow-up. In that sense, the effect of light to moderate alcohol consumption is expected to be short-term and reversible [206]. Although the non-randomization design of this study was one of the exposed limitations, it explained the election of the participants into the study arms. Further research is needed to explore the influence of sucrose and iso-α-acids taste perception, as well as PTC phenotype, on beer health effect and drinking habit. The

applicability of sensory analysis to identify target population that could get more benefit from moderate beer consumption and had a greater adherence to this type of intervention is also a promising.

The main limitation of this dissertation is that sensory analysis among post-menopausal women was carried out after the end of the intervention, which makes its applicability less accurate. On the other hand, IX could be only used as a qualitative biomarker of beer intake, thus the concentration in the analyzed samples was below the limit of quantification and because of the already described limitations of this specific candidate BFIs.

This dissertation has been developed both through college-aged cohort cross-sectional study and a post-menopausal parallel controlled clinical trial. DT and RT measurements provide some information about the sensory function but not the full dynamic range of sensation. In the same line, RT values in the studies presented were estimated by same-different task analysis, that although it is an easy method that allows collection information from large cohorts and with a high number of tastant molecules and solutions, 3-alternative forced choice with reversal steps has been recommended as the more accurate method for it. In the case of the clinical trial, the small sample size was the great weakness of the study. In addition, participants were not randomized, but the allocation reflected real life conditions. Due to the nature of the intervention, blinding was not possible. Finally, the study was performed in a free-living population, a condition that adds higher variability in the background confounder variables.

On the other hand, the main strengths of the sensory analysis study were the low cost and non-invasive methodology applied and the large sample size in comparison with previous studies. In the clinical trial, the study design was the major strength,

thus the phytoestrogen intervention was well-characterized, a biomarker of beer intake for compliance was applied, the population was suitable to the study intervention, as well as the the length of the study was designed as a long-term follow-up.

The results of this dissertation add knowledge regarding the effect of moderate daily beer (with and without ethanol) intake on post-menopausal women's health, and encourage the scientific community to run well-designed clinical trials to prove alcoholic beverage consumption's effect on specific health enpoints and detailed study population.

5. CONCLUSIONS

The results of this dissertation lead to postulate the following conclusions:

1. Regarding the inter-individual variability of taste sensitivity:
    1.1. Aging and type 2 diabetes are factors that increase the sucrose RT, whereas higher BMI increases sucrose DT. Sex and smoking showed no effect, and alcohol consumption or even alcohol abuse is still unknown.
    1.2. In a healthy college-aged Spanish cohort, a higher sucrose RT was found in females than in males, while sinusitis and rhinitis were factors for predictive models for higher sensitivity to sucrose and sodium chloride RTs.
    1.3. The ability to recognize a given taste depends on the ability to recognize another basic taste in a healthy college-aged Spanish cohort, shedding light on the mechanisms underlying taste function and their interactions.
    1.4. PTC super-tasters presented a higher BMI than non-tasters and tasters, and non-taster status was able to predict lower sensitivity in other basic taste RTs and total taste sensitivity. PTC taster status was not associated with liking perception and/or consumption of vegetables with PTC-related bitter taste.

These findings imply the appearance of a new way of optimizing the clinical practice of nutritionists and understanding the complexity of dietary practice and human beings. Future investigations on taste sensitivity should be conducted to evaluate and confirm these associations and analyze the mechanisms involved.

2. Regarding the validation of biomarkers of alcoholic beverages and moderate alcohol intake:
    2.1. Biomarkers of alcohol itself, beer, and wine intake cover recent high or moderate intakes reasonably well, while low intakes may go unnoticed.

2.2. Classification of no intake *versus* low intakes is still only fair at best, which is unfortunate since the major controversy in research on moderate alcohol intake and health is the effects of abstention *versus* low intakes.

BFIs of alcoholic beverages are among the most extensively investigated and several markers of alcohol intake are in common legal use. This work illustrates the usefulness and promise of the area, as well as the caveats and limitations, and hence the need for further development of the theory and technology for this area and for biomarkers in general.

3. In an early post-menopausal population, the moderate daily AB and NAB consumption:
    3.1. Decreases overall mild to moderate climacteric symptoms, while AB also decreases the subscale of psychological symptoms.
    3.2. Seems to increase markers of bone formation. Even so, the intervention did not produce changes in BMD and bone micro-architecture at 2 years of intervention.
    3.3. Appears to have positive effects on biochemical indicators of cardiovascular risk at 2 years of follow-up. NAB could improve blood levels of TC and LDL-c, while moderate intake of AB could generate an increase in HDL-c. No clinical effect was observed in terms of glucose metabolism, while gamma-glutamyl transferase levels increase but within a normal range in both AB and NAB interventions.
    3.4. Did not show a clear effect on body composition within each group throughout the intervention.

Further mechanistic and large well-designed RCT in post-menopausal women about the role of non-alcoholic and alcoholic beer fractions in menopausal discomforts, bone health, and CVDRF should be performed to confirm these results. Overall, this

study could be used as a pilot study for future research that allows the development of new strategies to optimize post-menopausal women's quality of life and minimize the bone and cardiometabolic alterations related to the onset and progression of menopause.

6. FUTURE PERSPECTIVES

Taste acuity measurements are promising tools to study the variability across individuals. In terms of personalized nutrition, taste sensitivity has an open framework of applicability such as: 1) the diagnosis of nutritional status; 2) the identification of susceptible or responsive individuals; and 3) the personalized nutritional therapy or dietary recommendations. However, to improve understanding of its clinical relevance, a standardized methodology for chemical gustatory assessment is lacking in most of the studies.

Regarding BFIs, those obtained from human trials could provide a basis for building up strong associations between AB and NAB and specific health outcomes. In this regard, several challenges to their extensive applicability are still needed. The large inter-individual variability and the potentially sex-dependent variation in excretion kinetics are some of the major issues for the potential applicability of IX as a quantitative biomarker of beer intake, while the main concern of iso-α-acids is its stability. The combined biomarkers approach is a highly promising tool for beer intake but still needs validation in observational studies. Other biospecimens, beyond blood and urine, such as hair or nails could be better samples for estimating chronic exposures.

On the other hand, there is a great interest to increase the knowledge of phytochemicals of foods and update the dietary intake recommendations of it based not only on the macro- and micronutrients but also on the phytochemical content and its health effect. Indeed, some of the identified health benefits of moderate beer consumption could be associated with the fraction of bioactive compounds of beer rather than with the alcohol content by itself. For this reason, it seems a relevant strategy to enhance the effects of its ingredients through food development and innovation. The amount of prenylflavonoids found in beers varies greatly from one

type to another, as it depends on the quality and quantity of hops used, the composition of the wort, and the brewing parameters. In this sense, an alternative proposed has been to boost the beer content of 8-PN by adding lactic acid probiotic strains that could favor the conversions from IX [207]. In this way, a beer with more potential phytoestrogenic effect independent of the consumers' intestinal microbiota could be offered. New insights into this area are coming and might have an impact on the beer industry and the development of new functional food products.

Finally, the popularity of NAB has remarkedly increased over the years, thus the designed intervention comprised in this dissertation would have higher promising acceptance among citizens if performed nowadays. On the other hand, the most effective quantity to get the most significant health benefits from the non-alcoholic fraction from beer consumption remains unknown.

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8. ANNEX

8.1. Supplementary material

Annex 1. Supplementary material of Publication 2

Effect of physiological factors, pathologies and acquired habits on the sweet taste threshold: a systematic review and meta-analysis.

Marta Trius-Soler[1-3#], Dimitri A. Santillán-Alarcón[1, #], Miriam Martínez-Huélamo[1, 2], Rosa M. Lamuela-Raventós[1-3], Juan José Moreno[1-3*]

Supplementary Information

Table S1. PRISMA checklist.

Table S2. Summary of studies included in the systematic review and meta-analysis evaluating the association between age and sucrose thresholds.

Table S3. Summary of studies included in the systematic review and meta-analysis evaluating the association between sex and sucrose thresholds.

Table S4. Summary of studies included in the systematic review and meta-analysis evaluating the association between tobacco smoking and sucrose thresholds.

Table S5. Summary of study included in the systematic review evaluating association between alcohol intake and sucrose thresholds.

Table S6. Summary of studies included in the systematic review and meta-analysis evaluating the association between BMI and sucrose thresholds.

Table S7. Summary of studies included in the systematic review and meta-analysis evaluating the association between T2DM and sucrose thresholds.

Table S8. Summary of meta-analysis results.

Caption supplemental figures

Figure S1. Risk of bias summary: review authors' judgments about each risk of bias item for each included study. Blank space indicates unclear risk of bias.

Figure S2. Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies. Blank space indicates unclear risk of bias.

Table A1. PRISMA checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5-6
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6-7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7-8

Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	8
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	8
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8-9
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	9
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	13-14
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	9-13
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	9-13
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	13-14
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	9-13
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	14-21
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	22-23
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	23-25
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	26

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

Table S2. Summary of studies included in the systematic review and meta-analysis evaluating the association between age and sucrose thresholds.

Author, Year Country	DB	Outcome	Population sample tested	Sample Size	Taste test Data collection	Sucrose concentrations	Key findings
Richter et al, 1939 USA	14	RT	F/M	7-10 y: n=58 19-21 y: n=45 19-50 y: n=10 52-85 y: n=52	Up-down AFC method	21 solutions: 0.01-1.6 %w/w	Oldest subjects and children had higher mean RT than the other groups.
Dye et al, 1981 USA	17	RT	M, non-diabetics, non-alcoholics and normal BMI	40-49 y: n=9 50-59 y: n=9 60-69 y: n=10 70-79 y: n=8 80-89 y: n=6	Randomized carousel sip method	4 solutions: 1-7 mM (in 2.0 mM step increments)	Significant difference between the mean RT of the 40, 50 and 60 y groups and those of the 70 and 80 y groups. No differences among the three younger age groups or between the 70-79 y and 80-89 y groups.
Moore et al, 1982 USA	16	DT	F/M, healthy and non-hospitalized	20-45 y: n=30 60-88 y: n=29	Up-down 2FC method 1h fasting, except water, and refrain from smoking	20 solutions: 0.01-580 mM (in a 1.8 mM dilution factor)	Older subjects had a significantly higher mean DT than younger subjects.
Bales et al, 1986 USA	16	DT	F/M, non-alcoholics, non-diabetics and non-smokers	24.0 (18-30) y: n=30 72.5 y (≥60) y: n=32	Up-down AFC method 1-2h after a meal	11 solutions: 0.03-100 mM	Older subjects had a significantly higher mean DT than younger subjects.
Spitzer, 1988 USA	18	DT	M	21.4 (18-25) y: n=15 73.2 (63-88) y: n=17 74.6 (61-92) y: n=15	Multiple FC ascending method 1h fasting, except water, and refrain from smoking	12-15 solutions: 0.8-377 mM (in a 1.75 mM dilution factor)	No significant differences among groups.
Wayler et al, 1990 USA	16	RT	M, no excessive alcohol consumption, healthy and natural dentition	<65 y: n=25 ≥65 y: n=15	AFC test, staircase method	6 solutions: 1.2-7.2 mM	Older subjects tended to have a higher RT than younger subjects.
Easterby-Smith et al, 1994 UK	13	RT	F/M, 6.25% diabetics and 78% smokers	24 (18-30) y: n=16 73 (60-85) y: n=16	FC ascending method Test between mealtimes after at least 30 min of fasting	8 solutions: 0.29-36 mM	The range of sensitivity was significantly greater in the younger group.
Stevens, 1996 USA	14	DT	F/M	18-29 y: n=109 66-90 y: n=49	Up-down 2AFC test	15 solutions: 0.316-1000 mM (in 0.25 log steps)	Older subjects had a significantly higher mean DT than younger subjects.
James et al, 1997	15	DT	F/M	8.7 y (F): n=28	2AFC test	12 solutions: 0.1-75 mM	Male children had a significantly higher

Australia				22.2 y (F): n=21 8.72 y (M): n=39 22.2 y (M): n=30			DT than male adults. The differences between female children and adults were not significant.
Mojet et al, 2001 Netherlands	17	DT	Healthy, not heavy alcohol users, non-smokers	23.2 y (F): n=11 64.6 y (F): n=11 26.5 y (M): n=11 66.0 y (M): n=10	2AFC test	14 solutions: $4.09x10^{-1}$ - $1.63x10^{2}$ g/L (in 0.2 log steps)	Older men had a higher DT than the younger men.
Yamauchi et al, 2002b Japan	15	DT RT	F/M	10-14 y (M): n=19 15-17 y (M): n=54 18-19 y (M): n=6 20-29 y (M): n=50 30-39 y (M): n=44 40-49 y (M): n=44 50-59 y (M): n=41 60-69 y (M): n=34 70-79 y (M): n=22 10-14 y (F): n=16 15-17 y (F): n=29 18-19 y (F): n=74 20-29 y (F): n=67 30-39 y (F): n=31 40-49 y (F): n=41 50-59 y (F): n=35 60-69 y (F): n=28 70-79 y (F): n=30	Whole mouth ascending method	13 solutions: 0-40 %w/w	Age-related differences were not statistically significant for male or female subjects.
Fukunaga et al, 2005 Japan	15	DT RT	F/M	24.1 (18-29) y: n=30 75.2 (65-85) y: n=30	Filter-paper disk ascending method 2h fasting, except water, and refrain from eating spicy foods within 24h	8 solutions: 5-1000 mM	Older subjects had a significantly higher mean DT and RT than young subjects.
Mojet et al, 2005 Netherlands	17	DT	M/F, not heavy alcohol users, healthy, non-diabetics and non-smokers	19-33 y: n=21 60-75 y: n=21	2AFC-in-a-row method	5 solutions: 8.55-53.95 g/L (in 0.2 log steps)	Older subjects had a higher mean DT than young subjects.
Wardwell et al, 2009	16	DT RT	F/M, no alcohol abuse	21.0 ± 3.0 y: n=48 (F) 66.0 ± 6.3 y: n=50 (F) 25.6 ± 4.0 y: n=50 (M)	3AFC ascending method	7 solutions: 1-13 g/L	Older males had a significantly higher RT than younger males. No other difference between compared groups

USA				76.8±6.5 y: n=43 (M)			and outcomes.
Kennedy, 2010 UK	10	DT RT	F/M	23 (18-33) y: n=36 74.4 (65-85) y: n=48	Ascending method	9 solutions (British Standard ISO 3972:1991)	Significantly higher DT and RT in older adults than in young adults.
Da Silva et al, 2014 Brazil	17	DT	F/M, healthy	18-30 y: n=41 31-45 y: n=16 46-60 y: n=22 61-75 y: n=28 >75 y: n=18	Ascending method Eyes closed during the test	5 solutions: 10-1000 mM	Older subjects (>60 y) had a higher mean DT than younger subjects.
Kalantari et al, 2016 Iran	14	RT	F/M, healthy, non-alcoholics, non-smokers and non-diabetics	22.6 y (F): n=50 59.7 y (F menopausal): n=50 24.4 y (M): n=50 60.4 y (M): n=50	Whole mouth ascending method 8-11:00 a.m. 1h fasting and no chewing gum. No spicy food and perfume since the night before	5 solutions	Menopausal females had a significantly higher RT than young females. No significant differences between old and young males.
Wiriyamattana et al, 2018 Thailand	16	DT RT	F/M, healthy	20-39 y: n=30 40-49 y: n=30 60-85 y: n=30	3AFC test	7 solutions: 3.5-27 g/L	No differences in DT and RT among subjects of different age groups.

(*) Not included in meta-analysis. AFC: alternative forced choice; DB: Downs and Black score; DT: detection threshold; F: females; F/M: females and males; M: males; ns: non-specified; RT: recognition threshold; y: years old.

Table S3. Summary of studies included in the systematic review and meta-analysis evaluating the association between sex and sucrose thresholds.

Author, Year Country	DB	Outcome	Population sample tested	Sample Size	Taste test Data collection	Sucrose concentrations	Key findings
Vreman et al, 1980 USA	15	DT	Healthy	F (50 ± 4 y): n=16 M (65 ± 3 y): n=23	3AFC ascending method Refrain from eating, smoking or using perfumed substances for at least 3h prior to the test	12 solutions: 0.488-1 mM	No significant difference between female and male subjects.
Than et al, 1994 (*) USA	14	RT	18-24 y, non-diabetics and non-smokers. Menstruation, pre-evolution, post-evolution status measurements.	F: n=14 M: n=13	Constant stimuli method	7 solutions: 8-100 mM	Sucrose sensitivity increased in females during pre-ovulation and decreased during post-ovulation.
James et al, 1997 Australia	15	DT		F (8.7 y): n=29 M (8.2 y): n=39 F (22.2 y): n=31 M (22.2 y): n=30	2AFC ascending method	12 solutions: 0.1-75 mM	Difference was not significant between female and male adults. DT of male children was higher compared to female children.
Mojet et al, 2001 Netherlands	17	DT	Healthy, not heavy alcohol users and non-smokers	F (23.2 y): n=11 M (26.5 y): n=11 F (64.6 y): n=11 M (66.0 y): n=10	2AFC ascending method	14 solutions: $4.09x10^{-1}$-$1.63x10^{2}$ g/L (in 0.2 log steps)	Females tended to have a lower sucrose DT. Older men had a significantly higher DT than old and young women.
Yamauchi et al, 2002a	14	DT RT	17-22 y and healthy	F: n=108 M: n=15	Whole mouth ascending method	13 solutions: 0-40 %w/w	Difference in DT and RT between female and male subjects was not significant.
Yamauchi et al, 2002b Japan	15	DT RT	10-79 y and non-smokers	F: n=351 M: n=314	Whole mouth ascending method	13 solutions: 0-40 %w/w	In the 10-15 y group, females had a significantly lower DT and RT than males. Females in the 30 y and onwards group had a lower RT than men. Conversely, in the 18 y group, males had a significantly lower RT than females.
Hong et al, 2005 Korea	16	DT RT	24.5 ± 2.4 y, PCT tasters and non-diabetics	F: n=30 M: n=30	2AFC ascending method 1h fasting, except for water	30 solutions: 0.24-1000 mM (in 0.125 log steps)	Female tasters had a lower RT than male testers, but without a statistically significant difference.

Chang et al, 2006 Korea	16	DT RT	23.9 ± 1.2 y, non-diabetics and non-smokers	F: n=34 M: n=35	2AFC ascending method 1h fasting, except for water	15 solutions: 0.32-1000 mM (in 0.25 log steps)	Although sucrose thresholds were higher in men than in women, the difference was not significant.
Hirokawa et al, 2006 Japan	16	DT RT	19.3 ± 1.3 y, normal weight	F: n=63 M: n=44	Ascending method	9 solutions: 0.1-1.2 %w/w	Masculinity and femininity were not associated with sucrose thresholds.
Wardwell et al, 2009 USA	16	DT RT	No alcohol abuse	F (21 ± 3.0 y): n=48 M (25.6 ± 4.0 y): n=50 F (66 ± 6.3 y): n=46 M (76.8 ± 6.5 y): n=43	3AFC ascending method	7 solutions: 1-13 g/L	Females had a significant higher DT than males. Older males had a significantly higher RT than older females. No difference in the RT between older and younger females and males.
Da Silva et al, 2014 Brazil	17	DT	>18 y, healthy	F: n=65 M: n=61	Ascending method Eyes closed during the test	5 solutions: 10-1000 mM	Female had a significantly lower DT than males in all age groups.
Nagai et al, 2015 (*) Japan	15	RT	20.7 ± 0.3 y, non-smokers	F follicular phase: n=22 F luteal phase: n=17 M: n=40	Ascending method 10-12:30 a.m. Not to skip breakfast and finish it at least 2h before the test	w/v in decrements of 0.1%	Differences among groups were not significant.
Joseph et al, 2016 USA	16	DT	10.4 (7-14) y, BMI percentile >95% (21%)	F: n= 124 M: n= 111	2AFC, paired comparison tracking 1h fasting, except for water. Room illuminated with red light	17 solutions: 0.056-1000 mM (in 0.25 log steps)	Girls had a significantly lower sucrose DT than boys.
Kalantari et al, 2017 Iran	14	RT	Non-alcoholics, healthy, non-smokers and non-diabetics	F (22.6 y): n=50 M (24.4 y): n=50 F menopausal (59.7 y): n=50 M (60.4 y): n=50	Whole mouth ascending method 8-11:00 a.m. 1h fasting and no chewing gum. No spicy food and perfume since the night before	5 solutions	Menopausal females had a significantly lower RT than old males. The difference was not significant between young genders.
Hwang et al, 2018 Korea	18	DT RT	44.96 ± 19.73 y. Patients who had undergone septoplasty and/or rhinoplasty	F: n=107 M: n=190	Whole mouth ascending method 1h fasting and abstain from brushing teeth	6 solutions: 4.80-15.63 g/L	Men had lower sucrose thresholds, than women but the difference was not significant.

Sanematsu et al, 2018 Japan	17	RT	23-67 y. Participants joined a weight-loss program. All subjects were PTC tasters	F (BMI: 34.7 ± 1.9): n=19 M (BMI: 30.8 ± 1.1): n=17	Whole mouth ascending method 8-10:00 a.m. Overnight fasting (after 10:00 p.m.). Abstain from snacks and toothpaste during the day of the test	9 solutions: 1-1000 mM (in 0.25 log steps)	Difference was not significant between female and male subjects.
Fogel et al, 2019 UK	18	DT	7.2 ± 0.13 y. Two pairs of non-twin siblings. The 11% were overweighed and the 11% obese. 14 girls and 11 boys had otitis media history	F: n=47 M: n=48	2AFC ascending method Testing before lunch. 1h fasting, except for water	9 solutions: 0-1.6 %w/v (in 0.2% steps)	Difference was not significant between girls and boys

(*) Not included in meta-analysis. AFC: alternative forced choice; BMI: body mass index; DB: Downs and Black score; DT: detection threshold; F: females; M: males; ns: non-specified; PTC: phenylthiocarbamide; RT: recognition threshold; y: years old.

Table S4. Summary of studies included in the systematic review and meta-analysis evaluating the association between tobacco smoking and sucrose thresholds.

Author, Year Country	DB	Outcome	Population sample tested	Sample Size	Taste test Data collection	Sucrose concentrations	Key findings
Yamauchi et al, 2002b Japan	15	DT RT	M, 20-69 y	Smokers: n= 116 Non-smokers: n= 554	Whole mouth ascending method	13 solutions: 0-40 %w/w	No significant differences between smokers and non-smokers in any age subgroup comparison.
Krut et al, 1961 South Africa	16	RT	F/M	Smokers (25.6 ± 9.5): n=79 Non-smokers (22.0 ± 4.9): n=77	Whole mouth ascending method 9:30-11:00 a.m.	8 solutions: 0.15-20 %w/w (in 0.5% w/w steps)	No significant difference between smoker and non-smokers.
Pepino et al, 2007 USA	18	DT	49 F, 21-40 y. Same phase of menstrual cycle	Current smokers: n=27 Never smokers: n=22	2AFC staircase method 1h fasting. Smokers refrain from smoking 12h prior to test.	17 solutions: 0.056 - 1000 mM (in 0.25 log steps)	DT was higher in smokers (calculated in function of graphic 1, in session 1, between non-smokers and nicotine-smokers trial).
Karatayli-Ozgursoy et al, 2008 Turkey	15	RT	F/M	Smokers n= 20 Non-smokers n= 20	Whole mouth ascending method 2h fasting, except for water	9 solutions: 1.17 - 300 g/L (1:1 dilution)	No significant difference between smokers and non-smokers.
Park et al, 2015 (*) South Korea	14	RT	17 F, 24 M, 20-29 y	Smokers n=14 Nonsmokers: n=25	Electrogustometry and chemical test: different concentrations with a cotton swab 1h fasting, except for water	10 solutions: 50-2000 g/L	No significant difference between smokers and non-smokers.

(*) Not included in meta-analysis. AFC: alternative forced choice; DB: Downs and Black score; DT: detection threshold; F: females; F/M: females and males; M: males; y: years old; RT: recognition threshold; y: years old.

Table S5. Summary of study included in the systematic review evaluating the association between alcohol intake and sucrose thresholds.

Author, Year Country	DB	Outcome	Population sample tested	Sample Size	Taste test Data collection	Sucrose concentrations	Key findings
Park et al, 2015 (*) South Korea	14	RT	17 F, 24 M, 20-29 y	Drinkers: n=31 Non-drinkers: n=8	Electrogustometry and chemical test: different concentrations with a cotton swab 1h fasting, except for water	10 solutions: 50-2000 g/L	No significant differences between groups, or within method.

DB: Downs and Black score; F: females; M: males; ns: non-specified; RT: recognition threshold; y: years old.

Table S6. Summary of studies included in the systematic review and meta-analysis evaluating the association between BMI and sucrose thresholds.

Author, Year Country	DB	Outcome	Population sample tested	Sample Size	Taste test Data collection	Sucrose concentrations	Key findings
Eiber et al, 2002 (*) France	15	RT	F, 25.7 ± 5.6 y	RA (BMI 15.7 ± 1.6): n= 20 AB (BMI 16.1 ± 1.3): n= 20 B (BMI 22.7 ± 2): n= 20	Whole mouth ascending method	10 solutions: 0-40 %w/w	Higher RT in the RA group compared with the other two groups. No difference when BMI was included as a covariate.
Pasquet et al, 2007 France	13	RT	F/M, 11.5-18 y	Obese (BMI 39.5 ± 6): n= 39 Non-obese (BMI 21.0 ± 2.5): n= 48	Staircase-ascending method	10 solutions: 2-1000 mM (in 0.3 log steps)	Massively obese adolescents were more sensitive to sucrose than non-obese adolescents, with significantly lower RTs.
Umabiki et al, 2010b Japan	19	DT	F, 55 ± 7 y, healthy	Beginning (BMI 26.1 ± 1.7): n= 20 After weight reduction: (BMI 24.7 ± 1.7): n= 20	Whole mouth test, with two-down, one-up rule ascending method 10h overnight fasting	10 solutions: 0.0098-5 %w/w	The sucrose taste threshold decreased significantly after weight reduction.
Bueter et al, 2011 UK	15	DT	15 F, 3 M, healthy, non-type 2 diabetics, non-substance abuse, non-smokers	Obese, pre-surgery (BMI 44.8 ± 1.8): n= 9 Obese, post-surgery (BMI 38.4 ± 1.6): n= 9 Normal weight (BMI 22.0 ± 1.0): n=9	2AFC method in random concentration order Overnight fast starting at 11:00 pm	7 solutions: 2.1-100 mM	Increased sweet taste sensitivity in patients after gastric bypass.
Pepino et al, 2014 USA	16	DT	F, non-diabetics, non-smokers LAGB: 46.8 ± 13.9 y RYGB: 42.1 ± 8.4	LAGB, pre-surgery (BMI 48.5 ± 10.5): n=10 LAGB, post-surgery (BMI 39.7 ± 9.5): n=10 RYGB pre-surgery (BMI 46.3 ± 7.7): n=17 RYGB post-surgery (BMI 36.9 ± 5.9): n=17	2AFC staircase procedure 12h overnight fasting	4 solutions: 0-1050 mM	No significant differences between groups.
Green et al, 2015 USA	17	RT	F/M, 44-54 y	Metabolic syndrome patients (BMI 39.26 ± 2.2): n= 16 Healthy patients (BMI 25.25 ±3.3): n=15	2AFC sip and spit ascending method 12h fasting	Beginning with 560 mM (in 0.25 steps)	No significant differences between groups.
Hardikar et al, 2017	13	RT	F/M, healthy	Obese (BMI 33.8 (30.47-38.96)): n= 31	Adaptive Bayesian (QUEST) staircase procedure	12 solutions: 0.1-200 g/L	Higher BMI was associated with higher sensitivity to sucrose taste.

Germany				Lean (BMI 21.881(8.73 - 24.49)): n= 23			
Nance et al, 2017 USA	16	DT	F/M, non-smokers, non-diabetics RYGB: 43.0 ± 9.6 y SG: 36.6 ± 9.9 y	RYGB, pre-surgery (BMI: 46.9 ± 7.5): n= 23 RYGB, post-surgery (BMI: 37.6 ± 6.7): n= 23 SG, pre-surgery (BMI: 53.3 ± 8.7): n= 8 SG, post-surgery (BMI 43.0 ± 7.2): n=8	2AFC staircase procedure 12h overnight fasting	17 solutions: 0.1-1000 mM (in 0.25 log steps)	No significant differences between the two intervention groups.
Abdeen et al, 2018 UK	17	DT	F/M, 12-18 y, non-substance abuse, non-diabetics	LSG, pre-surgery (BMI 49.6 ± 1.6): n= 14 LSG, post-surgery (BMI 39.6 ± 1.5): n= 14 Healthy control (BMI 32 ± 1.6): n=10	Constant stimuli method presented randomly 10h overnight fasting	7 solutions: 2.1-300 mM	No differences were found after LSG or at baseline.
Nishihara et al, 2019 Japan	19	DT	F, 7.9 % smokers Obese/overweight: 51 ± 2.1 y Normal weight: 45 ±1.6 y	Obese/overweight pre-intervention (BMI: 29.8 ± 0.5): n=27 Obese/overweight post-intervention (BMI: 25.1 ± 0.4): n=27 Normal weight (BMI: 20.9 ± 0.3): n=24	2AFC staircase procedure 12h overnight fasting	17 solutions: 0.1-1000 mM (in 0.25 log steps)	No significant difference was found in DT between the two groups before the intervention. No significant difference was detected in the DT after weight loss-intervention in the obese/overweight group.

(*) Not included in meta-analysis. AFC: alternative forced choice; B: bulimic; BA: bulimic anorexia; BMI: body mass index; DB: Downs and Black score; DT: detection threshold; F: females; F/M: females and males; LAGB: laparoscopic adjustable gastric banding; LSG: laparoscopic sleeve gastrectomy; M: males; ns: non-specified; RA: restrictive anorexia; RT: recognition threshold; RYGB: Roux-en-Y gastric bypass; SG: sleeve gastrectomy; y: years old.

Table S7. Summary of studies included in the systematic review and meta-analysis evaluating association between T2DM and sucrose thresholds.

Author, Year Country	DB	Outcome	Population sample tested	Sample Size	Taste test Data collection	Sucrose concentrations	Key findings
Dye et al, 1981 USA	17	RT	M, 40-89 y, non-alcoholics, normal BMI	T2DM: n= 37 Controls: n= 42	Randomized carousel sip method	4 solutions: 1-7 mM (in 2 steps)	No significant differences between diabetic and non-diabetic subjects.
Wasalathanthri et al, 2014 Sri Lanka	19	DT RT	F/M, 20-60 y	T2DM: n= 40 Controls: n= 34	3AFC ascending method Before 11:00 a.m. Overnight fasting, not smoking or drinking alcohol or betal chewing (10-10:30 p.m.). Standard breakfast 1h prior testing	1.25-640 mM (in 0.5 and 0.25 log steps)	Diabetic subjects had a higher DT.
Yazla et al, 2018 Turkey	19	DT RT	F/M, 18-80 y, non-smokers, non-alcoholics	T2DM DPN (60.3 ± 8.5): n= 30 T2DM without DP (55.4 ± 7.8): n=30 Controls (55.5 ± 8.3): n= 30	3AFC ascending method 10h overnight fasting, no teeth brushing	1.25-640 mM (in 0.5 and 0.25 log steps)	Higher sucrose threshold in patients with DPN vs. controls. No significant differences between patients without DPN vs. controls.
De Carli et al, 2018 Italy	20	RT	F/M, non-smokers	T2DM (56.8 ± 6.7): n= 25 Controls (56.2 ± 4.9): n= 25	3AFC ascending method 8-10h fasting and no spicy foods for 24h before	10 solutions: 1.25-640 mM (in a 2 dilution factor)	T2DM patients had a higher RT compared to healthy controls.

AFC: alternative forced choice; DB: Downs and Black score; DPN: diabetic peripheral neuropathy; DT: detection threshold; F/M: females and males; RT: Recognition threshold; T2DM: Type 2 Diabetes Mellitus.

Table S8. Summary of meta-analysis results

Variable	Experimental vs. control group	Outcome	Nº comparisons	SMD (95%CI)	I^2 (%)	Tau^2	95% PI
Age	Young vs. old	DT	17	-0.28 (-0.75, 0.20)	87	0.47	(-1.83, 1.27)
Age	Young vs. old	RT	13	-0.46 (-0.74, -0.19)	73	0.18	(-1,44, 0.52)
Sex	Female vs. male	DT	20	-0.02 (-0.28, 0.24)	88	0.38	(-1.34, 1.30)
Sex	Female vs. male	RT	19	-0.18 (-0.39, 0.02)	75	0.15	(-1.03, 0.67)
Tobacco smoking	Smokers vs. non-smokers	DT	6	0.27 (-0.26, 0.81)	83	0.37	(-1.58, 2.12)
Tobacco smoking	Smokers vs. non-smokers	RT	7	0.10 (-0.12, 0.33)	33	0.03	(-0.44, 0.64)
BMI	Higher BMI vs. lower BMI	DT	10	0.58 (0.35, 0.82)	0	0.00	(0.30, 0.86)
BMI	Higher BMI vs. lower BMI	RT	3	-0.34 (-0.93, 0.25)	70	0.19	(-7.07, 6.39)
T2DM	Diabetics vs. non-diabetics	RT	8	0.30 (0.06, 0.55)	0	0.00	(-0.01, 0.61)

BMI: body mass index; DT: detection threshold; IC: confidence interval; PI: prediction interval; RT: recognition threshold; SMD: standard mean difference; T2DM: Type 2 Diabetes Mellitus.

Figure S1. Risk of bias summary: review authors' judgments about each risk of bias item for each included study. Blank space indicates unclear risk of bias.

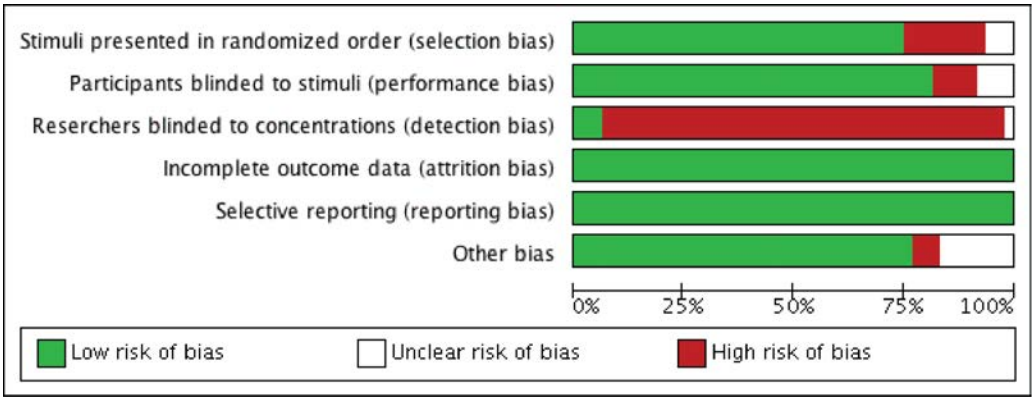

Figure S2. Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies. Blank space indicates unclear risk of bias.

Annex 2. Supplementary material of Publication 3

Supplementary Table 1. Eigenvalues and variance explained by the extracted factors (PCA)

Factor[1]	Eigenvalue	Variance explained (%)	Cumulative variance explained (%)
All participants			
Factor 1	2.226	20.2	20.2
Factor 2	1.560	14.2	34.4
Factor 3	1.387	12.6	47.0
Factor 4	1.143	10.4	57.4
Females			
Factor 1	2.478	24.8	24.8
Factor 2	1.629	16.3	41.1
Factor 3	1.129	11.3	52.4
Factor 4	1.026	10.3	62.6
Males			
Factor 1	2.036	20.4	20.4
Factor 2	1.405	14.1	34.3
Factor 3	1.256	12.6	47.0
Factor 4	1.132	11.3	58.3
Factor 5	1.069	10.7	69.0

[1]Factors reported if the eigenvalue was >1

Supplementary Table 2. Descriptor components of the extracted factors (PCA)

Factor	Components directly correlated (coefficient) [1]	Components inversely correlated (coefficient)[1]
All participants		
Factor 1	Any family antecedents (0.932); family antecedents of hypertension (0.716); family antecedents of diabetes (0.681); family antecedents of overweight/obesity (0.569)	
Factor 2	Rhinitis (0.630); sinusitis (0.664); smoking (0.414); missing teeth (0.404); BMI (0.378)	Female (-0.411)
Factor 3	Female (0.658); sinusitis (0.460); rhinitis (0.455)	BMI (-0.649)
Factor 4	Dental cavities (0.716); missing teeth (0.608)	
Females		
Factor 1	Any family antecedents (0.921); family antecedents of diabetes (0.739); family antecedents of hypertension (0.726); family antecedents of overweight/obesity (0.615); BMI (0.347)	
Factor 2	Rhinitis (0.839); sinusitis (0.833)	
Factor 3	Missing teeth (0.727); dental cavities (0.630)	
Factor 4		Smoking (-0.878)
Males		
Factor 1	Any family antecedents (0.905); family antecedents of hypertension (0.723); family antecedents of diabetes (0.550); family antecedents of overweight/obesity (0.343)	BMI (-0.328)
Factor 2	Sinusitis (0.683); Rhinitis (0.521); smoking (0.502); missing teeth (0.465); dental cavities (0.309)	
Factor 3	BMI (0.699); family antecedents of overweight/obesity (0.660)	Sinusitis (-0.370)
Factor 4	Missing teeth (0.598); rhinitis (0.443)	Smoking (-0.663)
Factor 5	Dental cavities (0.841)	Rhinitis (-0.392); BMI (-0.302)

[1]Components reported if the coefficient was >0.3

Supplementary Table 3. Influence of sex on tastant recognition threshold scores

	Females	**Males**	***p*-value**
Sucrose, mM	5.2 ± 1.2	4.9 ± 1.5	**0.013**
MSG, mM	2.7 ± 0.9	2.7 ± 1.0	0.757
NaCl, mM	4.5 ± 0.9	4.5 ± 1.0	0.501
Citric acid[1], mM	3.4 ± 1.1	3.2 ± 1.3	0.577
PTC, μM	2.7 ± 1.6	2.8 ± 1.7	0.544
Quinine, μM	3.0 ± 1.2	3.1 ± 1.3	0.557
Sinigrin, μM	3.2 ± 1.0	3.0 ± 1.1	0.357
TTS	0.460 ± 0.119	0.455 ± 0.155	0.748

MSG: monosodium glutamate; NaCl: sodium chloride; PTC: phenylthiocarbamide; TTS: total taste score.

Tastant recognition thresholds are expressed as mean ± standard deviation (SD). The Mann-Whitney U test was applied for equal distribution variances. [1] The Median test was used for non-equal distribution variances.

Annex 3. Supplementary material of Publication 4

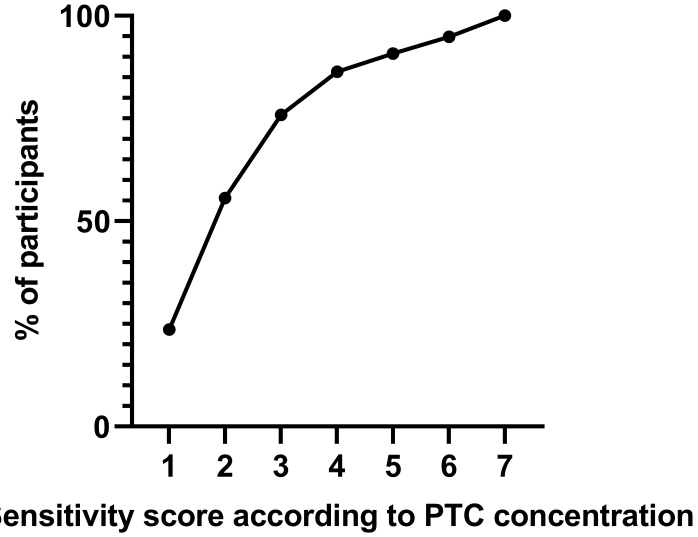

Figure S1. Cumulative frequency curves of the PTC recognition threshold scores.

Annex 4. Supplementary material of Publication 5

Supplementary Table 1. Keyword for the primary literature research

Operator	Database	Field	Keywords
Common keywords			
AND	***Pubmed***	*All Fields*	biomarker OR marker OR metabolite OR biokinetics OR biotransformation
	Web of Science	*Topic*	biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation
	Scopus	*Article Title/ Abstract/ Keywords*	
AND	***Pubmed***	*All Fields*	trial OR experiment OR study OR intervention
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
AND	***Pubmed***	*All Fields*	human OR men OR women OR patient OR volunteer OR participant
	Web of Science	*Topic*	human* OR men OR women OR patient* OR volunteer* OR participant*
	Scopus	*Article Title/ Abstract/ Keywords*	
AND	***Pubmed***	*All Fields*	urine OR plasma OR serum OR blood OR excretion
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
AND	***Pubmed***	*All Fields*	intake OR meal OR diet OR ingestion OR consumption OR drink* OR administration
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
Specific keywords for “alcohol”			
AND	***Pubmed***	*Title/Abstract*	alcohol OR ethanol
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
Specific keywords for “beer”			

AND	***Pubmed***	*Title/Abstract*	beer OR lager
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
NOT	***Pubmed***	*All Fields*	"Beer's Law"
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
Specific keywords for "cider"			
AND	***Pubmed***	*Title/Abstract*	cider OR "apple wine" OR "fermented juice apple" OR "fermented apple*"
	Scopus	*Article Title/ Abstract/ Keywords*	
Specific keywords for "wine"			
AND	***Pubmed***	*Title/Abstract*	wine
	Web of Science	*Topic*	
	Scopus	*Article Title/ Abstract/ Keywords*	
Specific keywords for "sweet wine"			
AND	***Pubmed***	*Title/Abstract All Fields*	"sweet wine" OR "dessert wine" OR "sparkling wine"
	Scopus	*Article Title/ Abstract/ Keywords*	
Specific keywords for "distillates and spirits"			
AND	***Pubmed***	*Title/Abstract*	distillate OR distilled beverage OR spirit OR liquor OR liqueur OR whiskey OR whisky OR wodka OR rum OR brandy OR cognac OR tequila OR gin OR eggnog OR schnapps OR vodka
	Web of Science	*Topic*	

Supplementary Table 2. List of studies reporting candidate biomarkers for alcoholic beverage subgroups and ethanol consumption

Dietary factor	Study design[1]	Study population	Analytical method	Sample type	Discriminating metabolites / Candidate biomarkers	Refs.
Alcohol in general						
All alcoholic beverages	Cross-sectional study	6705 subjects (66% males) (median age 54.5 years)	UPC2-MS/MS Chemical method	Serum Serum	PEth 16.0/18.1 Ethanol	[1]
All alcoholic beverages	Cross-sectional (baseline in a longitudinal cohort study)	5676 Dutch subjects (51.2% males, 53 ± 12 years)	Colorimetric method (DRI dipstick)	24h Urine	EtG	[2]
All alcoholic beverages	Cross-sectional study	4067 pregnant women; 466 were additionally tested later (18-50 years)	UPLC-MS/MS	Blood	PEth 16:0/18:1	[3]
All alcoholic beverages	Cross-sectional study	1875 traffic offenders (87% males) (mean age 40 years, 14-81 years)	Breath analyzer HS-GC-FID	Breath Blood	Ethanol Ethanol	[4]
All alcoholic beverages	Cross-sectional study	1872 subjects (abstinent, low-moderate, moderate, and excessive alcohol consumption) (age n.p.)	GC-MS LC-ESI-MS	Hair Hair	Total FAEEs EtG Combined biomarker: FAEE, EtG	[5]
All alcoholic beverages	Cross-sectional study	849 subjects (57% men, 62.2 ± 16.5 years)	LC-MS	Serum	EtG	[6]
All alcoholic beverages	Cross-sectional study	1369 postmenopausal women (68.3 ± 5.7 years)	LC-MS	Serum	EtG	[7]
All alcoholic beverages	Cross-sectional study	533 females and 506 males at increased CVD risk (55-80 years)	Colorimetric method	Morning spot urine	EtG	[8]
All alcoholic beverages	Cross-sectional study	340 males and 304 females (abstinent, moderate, excessive alcohol consumption or unknown) (age n.p.)	GC-MS	Hair	Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, total FAEEs	[9]

All alcoholic beverages	Cross-sectional study	305 pregnant women in their first trimester coming for first examination, 188 abstainers and 117 with pre-conception alcohol intakes (mean age around 32 years)	HPLC-MS/MS	Blood	Sum of PEth 16:0/16:0, 16:0/18.1 and 18:1/18:1	[10]
All alcoholic beverages	Cross-sectional study	373 females and 231 males (18-26 years)	LC-MS/MS LC-MS/MS	Fingernail Hair	EtG EtG	[11]
All alcoholic beverages	Cross-sectional study	221females and 281males (65 ± 5 years)	UHPLC-MS or GC-MS	Serum	EtG	[12]
All alcoholic beverages	Cross-sectional study	509 subjects referred for evaluation of risky drinking from employment agencies (80% males) (age n.p.)	UPLC-MS/MS UPLC-MS/MS Colorimetric method	Blood Serum Serum	PEth 16:0/18:1 EtG Ethanol	[13]
All alcoholic beverages	Cross-sectional study	418 women (163 pregnant) all with alcohol intake interviews (median (IQR) age 29, 24-35 years)	LC-MS/MS	Dried blood spots	PEth (unspecified)	[14]
All alcoholic beverages	Cross-sectional study	370 legal cases (sex and age n.p.)	Breath analyzer HS-GC-FID	Breath Blood	Ethanol Ethanol	[15]
All alcoholic beverages	Cross-sectional study	300 subjects (abstinence, moderate and excessive alcohol consumption) (50 % males) (mean age around 45 years)	LC-MS/MS	Blood	PEth 16:0/18:1, PEth 16:0/18:2	[16]
All alcoholic beverages	Cross-sectional study	97 hemorrhagic stroke cases and 180 matched referents (39% females, mean age 55 years, 25-74 years)	LC-MS/MS	Blood	PEth 16:0/18:1	[17]

All alcoholic beverages	Cross-sectional study	100 female sex workers and 100 male clients (median age 24.5, 21-29 years)	LC-MS/MS	Dried blood spots	PEth 16:0/18:1	[18]
All alcoholic beverages	Cross-sectional study	139 males, 29 females and 6 with no sex data (0-70 years, 10 with no age data)	HS-SPME-GC-MS LC-ESI-MS-MS n.p.	Hair Hair Blood	Total FAEEs EtG <u>Combined biomarker:</u> Total FAEEs, EtG Ethanol	[19]
<u>Study 1:</u> All alcoholic beverages	Cross-sectional study	100 former abusers (age and sex n.p.)	HPLC-MS/MS	Urine	EtG, ethanol	[20]
<u>Study 2:</u> All alcoholic beverages	Cross-sectional study	12 males (8 based on suspected drinking, 6 from drinking denied, and 4 from high-risk individuals) (36-58 years)	HPLC-MS/MS	Urine	EtG, ethanol	
All alcoholic beverages	Cross-sectional study	92 emergency room patients, 15 chronic alcoholics and 15 episodic heavy drinkers from clinical trials (age n.p.)	HPLC GC	Serum Blood	Ethyl oleate, Ethyl stearate, total FAEEs Ethanol	[21]
All alcoholic beverages	Cross-sectional study	97 subjects providing hair to resolve insurance cases (sex and age n.p.)	LC-MS/MS HS-SPME-GC-MS	Hair Hair	EtG Total FAEEs	[22]
All alcoholic beverages	Cross-sectional study	50 abstainer subjects and 14 in withdrawal treatment (sex and age n.p.)	LC-MS/MS	Hair	EtG	[23]
All alcoholic beverages	Cross-sectional study	17 teetotalers, 20 moderate social drinkers, 47 patients in withdrawal treatment and 171 death cases (sex and age n.p.)	GC-EI-MS GC-MS LC-MS-MS	Hair Hair Hair	Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, total FAEEs EtG EtG <u>Combined biomarker:</u> Total FAEEs, EtG	[24]

All alcoholic beverages	Cross-sectional study	2 abstainers, 20 moderate alcohol consumers, 36 excessive alcohol consumers (sex and age n.p.)	GC-MS GC-MS/MS	Hair Hair	EtG EtG	[25]
All alcoholic beverages	Cross-sectional study	30 healthy males and 15 females (22-66 years) with regular drinking habits	LC-MS/MS	Blood	PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/16:0, PEth 18:1/18:1, total PEth	[26]
All alcoholic beverages	Cross-sectional study	20 men and 24 women abstainer or teetotaler (1-80 years)	HPLC-MS/MS	Hair	EtG	[27]
All alcoholic beverages	Cross-sectional study	29 alcoholics (10 autopsies, 19 in withdrawal treatment, 13 moderate social drinkers and 5 teetotalers) (sex and age n.p.)	HS-SPME-GC-MS	Hair	Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, total FAEEs	[28]
All alcoholic beverages	Cross-sectional study	10 alcoholics in withdrawal treatment, 11 death cases, 4 moderate social drinkers 3 teetotalers (sex and age n.p.)	HS-SPME-GC MS GC-MS	Hair Hair	Total FAEEs EtG	[29]
All alcoholic beverages	Cross-sectional study	13 teetotalers (5 males, 8 females, 6-48 years), 16 social drinkers (7 males, 9 females, 21-77 years) and 10 death cases with known recent alcohol miscues (9 males, 1 female, 35-60 years) and 5 deaths cases without indications of alcohol misuse (5 males, 37-63 years)	HS-SPME-GC-MS	Sebum (skin surface lipids)	Total FAEEs	[30]

All alcoholic beverages	Cross-sectional study	22 alcoholic fatalities, 5 moderate social drinkers, and one teetotaler (26 males, 2 females, 25-65 years)	HS-SPME-GC-MS	Hair	Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, total FAEEs	[31]
All alcoholic beverages	Cross-sectional study	10 non-drinkers and 10 heavy drinkers (> 80 g/d)	GC-MS/MS	Blood plasma proteins	N^{ε}-Ethyl-lysine (acetaldehyde adduct with lysine)	[32]
All alcoholic beverages	Cross-sectional study	18 subjects (sex and age n.p.)	GC-MS Colorimetric method	Serum Serum	Ethyl palmitate, ethyl stearate, ethyl oleate, total FAEEs Ethanol	[33]
All alcoholic beverages	Cross-sectional study	8 heavy drinkers (1 female and 6 males; 44-55 years), 5 social drinkers (1 female and 4 males; 20-59 years); and 7 teetotalers (4 females and 3 males; 26-50 years)	HPLC-MS/MS	Blood	PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/16:0, PEth 18:1/18:1	[34]
All alcoholic beverages	Cross-sectional study	6 ethanol-negative blood samples; 6 ethanol-spiked samples, 6 ethanol-positive blood samples	HS-GC UHPLC-MS/MS	Postmortem blood samples Postmortem blood samples	Ethanol EtG, EtS, total FAEEs, total PEth	[35]
All alcoholic beverages	Cross-sectional study	3 moderate drinkers, 1 teetotaler	CE-MS/MS	Hemoglobin chains	Acetaldehyde	[36]
All alcoholic beverages	Cross-sectional study	n.p.	GC-MS GC-MS	Serum lipoprotein fractions (HDL, LDL, VLDL) Blood	Ethyl oleate, total FAEEs Ethanol	[37]
All alcoholic beverages	Cross-sectional study	213 liver transplant patients (71% previously alcohol dependent (61.3% males, 59.02 ± 10.33 years) and 29% not dependent (72.2%	LC-MS/MS	Dried blood spots	PEth 16.0/18:1	[38]

		males, 59.26 ± 8.09 years)				
All alcoholic beverages	Case-control study	85 pregnant women with substance abuse (42 with self-reported prenatal alcohol exposure and 43 controls) (26.7 ± 4.8 years)	LC-MS/MS LC-MS/MS	Hair Urine	EtG EtG, EtS, PEth (unspecified) Combined biomarkers: hair EtG + urine EtG, hair EtG + urine EtS, hair EtG + PEth	[39]
All alcoholic beverages	Case-control study	8 men and 4 women with acute intoxication (43 ± 3 years) and 15 abstaining men (24 ± 1 years)	GC-MS GC-MS	Pre and postprandial 0-44h serum Pre and postprandial 0-44h lipoprotein fractions (VLDL, LDL, HDL, HDL infranatant)	Ethanol, total FAEEs Total FAEEs	[40]
Study 1: All alcoholic beverages	Case study	1 positive hair pool sample (sex and age n.p.)	n.p.	Hair	Ethyl palmitate, ethyl stearate, ethyl oleate, total FAEEs	[41]
Study 2: All alcoholic beverages	Cross-sectional study	29 children (1-12 years) (sex n.p.)	n.p.	Hair	Ethyl palmitate	
All alcoholic beverages	Cross-sectional study	15 students (sex not n.p.) Providing information on average intakes	LC-MS/MS GC-MS/MS	Hair Hair	EtG FAEEs	[42]
All alcoholic beverages	Longitudinal cohort study with a cross-sectional comparator group	9 male and 3 female withdrawal inpatients (30-58 years) with frequent blood collections over 8-33d and 38 male and 38 female healthy social drinkers (18-75 years)	LC-MS/MS	Blood	PEth 16:0/18:1, PEth 16:0/18:2, PEth 18:0/18:2, PEth 18:0/18:1, PEth 18:1/18:1, PEth 18:0/20:4, PEth 16:0/20:3, PEth 16:0/16:0, PEth 18:0/20:3, PEth 16:1/18:2, PEth 18:1/18:2, PEth 17:0/18:1, PEth 16:0/22:4, PEth 16:0/18:0, total PEth	[43]
Study 1: Ethanol abstention	Cross-sectional study	10 girls and 3 boys (<10 years) and 39 adult abstainers	LC-MS/MS	Urine (first morning void in adults, children spot samples at unknown times)	EtG	[44]
Study 2: Ethanol (as dermal exposure from	5d intervention repeated exposure	6 abstainers males and 3 abstainers females	LC-MS/MS		EtG Ethanol	

61% (w/w) ethanol hand-sanitizer)			Colorimetric method	Pre and postprandial 0-2 to 5d urine and an additional 7d of morning urine		
Study 3: Ethanol (3, 6, 12, 24 g of alcohol as vodka in water or juice)	4 x intervention cross-over dose and time-response single exposure	4 males social drinkers (age n.p.)	LC-MS/MS Colorimetric method		EtG Ethanol	
				Pre and postprandial 0-75h urine		
All alcoholic beverages	5-20w (median of 12 w) longitudinal cohort study	25 men (25-83 years) and 11 women (32-66 years)	HPLC-MS	Blood (1 to 4 times between 5–20d time span)	PEth 16:0/18:1	[45]
All alcoholic beverages	2w longitudinal cohort study	37 men (49.5 (23-72) years) and 12 women (48.7 (30-66) years)	LC-MS/MS	Blood (once daily over 5d and then every second day until 2w) Urine (daily during 2w)	PEth 16.0/18.1, PEth 16.0/18.2, PEth 16.0/24.0	[46]
		heavy drinkers within a detoxification process	LC-MS/MS Breath analyzer	Breath	EtG, EtS Ethanol	
All alcoholic beverages	33 ± 26d (3–74d)) longitudinal cohort study	13 males and 6 females (47 ± 12 years)	LC-MS/MS LC-MS/MS	Urine Dried blood (venous DBS and capillary DBS) and whole blood	EtG, EtS PEth 16.0/18.1, PEth 16.0/18.2	[47]
Study 1: Ethanol (26-32 mM/L alcohol blood concentration as 1:3 ratio vodka with fruit juice)	Intervention time-response after single exposure	4 males and 3 females (21-23 years)	GC-MS	Pre and postprandial 0-24h serum and plasma	Total FAEEs	[48]
			GC	Pre and postprandial 0-24h serum	Ethanol	
	Cross-sectional study	48 samples from anonymous donors (sex and age n.p.)	GC-MS	Blood	Total FAEEs	
Study 2: All alcoholic beverages			GC	Blood	Ethanol	
Study 1: Ethanol (as white wine or beer)	Intervention time-response after single exposure	5 males and 7 females (20-41 years)	LC-MS/MS	Pre and postprandial 0-28.5h urine	EtG, EtS	[49]
			Breath analyzer	Postprandial breath	Ethanol	
			LC-MS/MS	Postprandial 36-132h urine	EtG, EtS	
Study 2: All alcoholic beverages	Cross-sectional study	10 males and 3 females heavy intoxicated by alcohol (30-55 years)	Breath analyzer	Postprandial breath	Ethanol	
All alcoholic beverages	5d longitudinal cohort study after acute withdraw	24 men and 6 women heavy drinkers with acute ethanol intoxication (43 ± 7 years) and 17 healthy subjects who took part	GC GC-MS	Postprandial serum (0-5d twice daily between 8:00-9:00h and between 18:00-19:00h)	Ethanol Total FAEE	[50]

		in a drinking experiment (24 ± 2 years) (sex n.p.)				
Ethanol (4.5 to 6 oz of alcohol as beer, wine, or liquor)	Intervention time-response after single exposure	42 healthy males (21-60 years)	GC-MS Colorimetric method	Postprandial 0.5–2 h blood Postprandial 0.5–2 h saliva	Ethanol Ethanol	[51]
Ethanol (100 mL of sparkling wine)	Intervention time-response after single exposure	18 males and 12 females with Gilbert's syndrome (18-70 years)	LC-MS/MS HS-GC-FID	Pre and postprandial 0-24h urine Postprandial 3h blood	EtG, EtS Ethanol	[52]
Ethanol (64 to 184 g of alcohol as beer, spirits, or wine)	Intervention time-response after single exposure	17 healthy males (22-29 years)	HPLC LC-MS/MS GC GC-MS	Postprandial urine (0-3d, twice daily between 8:00-9:00h and between 17:00-18:00h) Postprandial serum (0-3d, twice daily between 8:00-9:00h and between 17:00-18:00h)	5-HTOL/5-HIAA ratio EtG Ethanol Sum of ethyl palmitate and ethyl stearate	[53]
Ethanol (0.5-0.8 g/kg of blood alcohol concentration as white wine)	Intervention time-response after single exposure	6 healthy females and 7 males (19–42 years)	HPLC-MS/MS HPLC-MS/MS GC-FID and Colorimetric method	Pre and postprandial 0-48h urine Pre and postprandial 0-10h serum Pre and postprandial 0-10h urine and serum	EtG, EtS EtG, EtS Ethanol	[54]
Ethanol (3.6 mL of alcohol/L of water body mass as red wine)	2 x intervention time-response after single exposure	8 men and 4 women (38.6 years)	Breath analyzer	Pre and postprandial 0-4h breath	Ethanol	[55]
Study 1: All alcoholic beverages with orange juice (0.8 g of alcohol/kg of body weight) Study 2: Ethanol (as white wine (0.8 g of alcohol/kg of body weight) or ethanol with orange juice (0.4 g of alcohol/kg of body weight))	Intervention time-response after single exposure Intervention time-response after single exposure	5 women and 5 men (23-39 years) 3 women and 5 men (23-39 years)	GC-MS Colorimetric method HPLC Colorimetric method GC-MS Colorimetric method HPLC Colorimetric method	Pre and postprandial 0-48h urine Pre and postprandial 0-8h blood Pre and postprandial 0-24h urine Pre and postprandial 0-7h blood	5-HTOL Ethanol 5-HIAA Ethanol 5-HTOL Ethanol 5-HIAA Ethanol	[56]

Ethanol (1 mL of alcohol/kg of body weight as red wine and alcohol 40%)	Intervention time-response after single exposure	8 subjects (21-67 years)	GC GC HPLC	Pre and postprandial 0-2h blood Pre and postprandial 0-2h saliva Pre and postprandial 0-2h blood	Ethanol, methanol Methanol Formaldehyde	[57]
Ethanol (0.8 g/kg blood alcohol concentration as vodka mixed with a soft drink)	Intervention time-response after single exposure	6 men and 2 women (19-26 years)	LC-MS/MS HS-GC-FID	Postprandial 100 min-5h dried blood spots Postprandial 100 min-5h serum	Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, total FAEEs Ethanol	[58]
Ethanol (9-18 g of alcohol as sparkling wine)	Intervention time-response after single exposure	4 males and 4 females (21-63 years)	LC-MS/MS	Pre and postprandial 0-44h urine	EtG, EtS	[59]
Ethanol (0.08% blood alcohol concentration as vodka mixed with a soft drink)	Intervention time-response after single exposure	2 females and 6 males (age n.p.)	LC-MS/MS	Pre and postprandial 0-6h blood	PEth 16.0/18.1, PEth 16.0/18.2	[60]
Ethanol (25 g of alcohol as white wine)	Intervention time-response after single exposure	4 healthy females and 3 males (37 ± 5 years)	LC-MS/MS Colorimetric method	Pre and postprandial 0-9h urine Pre and postprandial 0-9h urine	EtG Ethanol	[61]
Study 1: Ethanol (47 g ethanol for males or 32 g ethanol for women (as vodka) and diluted by diet-Coke® up to 500 mL)	Intervention time-response after single exposure	3 men , 2 women (25-47 years) abstaining for 5d prior to the single dose	HPLC-ELSD Breath analyzer	Pre and postprandial 0-5d blood Pre and postprandial breath on 0d	PEth (unspecified) (undetected), ethanol Ethanol	[62]
Study 2: All alcoholic beverages	Intervention dose and time-response after repeated exposure	8 men, 4 women (19-31 years)	HPLC-ELSD	Pre and postprandial 0-2d blood	PEth (unspecified) (detected), ethanol	
Ethanol (47.52 g of alcohol as beer)	Intervention time-response after repeated exposure	17 men and 7 women drinking (24-52 years)	HS-GC-MS LC-MS/MS	Postprandial 30 min blood Pre and postprandial 0-12.5h urine (at 7 time points)	Ethanol EtG, EtS	[63]
Study 1: Ethanol with lemon (placebo, 18g, 30g) Study 2: Ethanol with lemon (placebo, 6, 12 g)	3x intervention cross-over, double-blind, dose and time-response after single exposure	12 males (20-36 years) 6 males (20-36 years) 6 males (20-36 years)	Colorimetric method GC-MS LC-MS	Pre and postprandial 0-6h plasma Pre and postprandial 0-6h plasma Pre and postprandial 0-24h	Ethanol FAEEs EtG	[64]

Study 3: Ethanol with lemon (placebo, 24, 42 g)				urine		
Study 1: Ethanol (0.5, 0.7, 0.7 g) and placebo *vs*. ethanol (0.5, 0.7, 0.7 g) and 4-methylpyrazole (10, 15, 20 g)	6 x intervention cross-over, double-blind, dose and time- response after single exposure	4 males (age n.p.)	GC	Pre and postprandial 0-24h blood	Ethanol	[65]
		4 males (age n.p.)	HPLC-DAD	Pre and postprandial 0-36h urine	Ethanol	
Study 1: Ethanol and 4-methylpyrazole *vs.* placebo and 4-methylpyrazole	3 x intervention cross-over, double-blind, dose and time- response after single exposure					
Ethanol (1, 2 and 3 standard drinks as vodka)	3 x intervention dose and time-response after single exposure	10 women (21-39 years)	LC-MS	Pre and postprandial 0-72h urine	EtG, EtS Combined biomarker: EtG, EtS	[66]
			Breath analyzer	Pre and postprandial breath	Ethanol	
Ethanol (12, 24, 28 g of alcohol)	3 x intervention dose and time-response after single exposure	12 female (22-29 years)	LC-MS/MS	Pre and postprandial 0-48h serum	EtG	[67]
			Immunological method	Pre and postprandial 0-48h serum	Ethanol	
			LC-MS/MS	Pre and postprandial 0-72h urine	EtG	
			Dipstick	Pre and postprandial 0-72h urine	EtG	
Ethanol (0.8 alcohol/kg of body weight) *vs.* banana *vs.* control	3 x intervention partial cross-over, time-response after single exposure	5-9 subjects (21-45 years)	GC-MS	Urine	5-HTOL	[68]
Ethanol (0.5 g alcohol/kg body weight as beer, cachaça, red wine, or whiskey)	4 x intervention cross-over, time-response after single exposure	10 females and 10 males (29.5 ± 3 years)	Colorimetric method	Pre and postprandial 0-6h plasma	Ethanol	[69]
Ethanol ((0.3 g alcohol/kg body weight as beer, white wine, dry sherry, or whiskey)	4 x intervention parallel, time-response after single exposure	11 healthy males (34 ± 3 years)	Breathalyzer	Pre and postprandial 0-4h breath	Ethanol	[70]

Dealcoholized red wine (750 mL, 0.2% ethanol) *vs*. mouthwash *vs*. ethanol (1.8 g of alcohol)	3 x intervention parallel, time-response after single exposure	4 men and 8 women (29-30 years)	UPLC-MS/MS	Pre and postprandial 0-7.5h urine	EtS	[71]
Ethanol (pure, with tonic water) (0.25 or 0.5 g ethanol/kg body weight)	2 x intervention randomized, parallel, time-response after single exposure	14 healthy men and 13 women one-week abstaining social drinkers (28.5 ± 8 years)	HPLC-MS/MS Breath analyzer	Pre and postprandial 0-14d blood Pre and postprandial 0-6d breath	PEth 16:0/18:1, PEth 16:0/18:2, combined PEth Ethanol	[72]
Ethanol (pure, with tonic water) (0.4 or 0.8 g ethanol/kg body weight)	2 x intervention randomized, parallel, dose and time-response after single exposure	27 healthy men and 27 women one-week abstaining social drinkers (27.6 ± 6.32 years)	HPLC-MS/MS Breath analyzer Transdermal ankle monitors	Pre and postprandial 0-14d blood Pre and postprandial 0-6d breath Pre and postprandial 0-22d ancle monitor	PEth 16:0/18:1, PEth 16:0/18:2, combined PEth Ethanol Continuous sweat alcohol monitor	[73]
Ethanol (0.5 g of alcohol/ kg of body weight as beer, vodka/tonic or white wine)	3 x intervention cross-over, time-response after single exposure	15 healthy men (25-65 years)	HS-GC	Pre and postprandial 0-8h blood	Ethanol	[74]
Alcohol (as red wine, 150/300 mL/d females/males) *vs.* control	2 x 3m intervention randomized, parallel, open, controlled, response after repeated exposure	44 (32 females and 12 males; 33.5 ± 9 years)	LC-MS/MS LC-MS/MS	Blood Hair (8-25mg)	PEth 16:0/18:1 EtG	[75] [76]
Study 1: Ethanol (as vodka) (20, 80, 120 mg/dL blood alcohol)	4 x 3w intervention cross-over, dose and time-response after repeated exposure	11 healthy males and 7 females; (21-60 years)	HPLC-MS/MS Breath analyzer HP-GS	Pre and postprandial 0-72h urine Pre and postprandial breath Pre and postprandial blood	EtG, EtS Ethanol Ethanol	[77]
Study 2: All alcoholic beverages (abstinence by mecamylamine treatment *vs*. placebo)	2 x 12d intervention parallel response	42 males and 5 females with problem drinkers (18-60 years)	HPLC-MS/MS	Urine sampling at baseline and at 4w	EtG, EtS	
Study 3: All alcoholic beverages			HPLC-MS/MS	Urine sampling at baseline and at 4w	EtG, EtS	

(on moderation by naltrexone *vs.* placebo)	2 x 12d intervention parallel response	63 males and 20 females with problem drinkers (18-25 years)				
Ethanol (0.03%, 0.05%, 0.07% alcohol blood concentration as vodka)	3 x 3w intervention cross-over, dose and time-response after repeated exposure	5 women and 5 men (22-30 years)	LC-MS/MS Breath analyzer	Pre and postprandial 0-120h oral cavity cells and white blood cells Postprandial breath (30 min)	N2-ethylidene-dG Ethanol	[78]
Ethanol (0.8 g of alcohol/kg body weight as vodka) *vs.* placebo	2 x 15d intervention randomized, cross-over, double-blind, controlled, time-response after repeated exposure	27 ALDH wild type men and 27 heterozygous men (25 ± 3 years)	HS-GC-FID LC-MS/MS	Pre and postprandial 0-6h blood Pre and postprandial 0-6h blood	Ethanol Acetaldehyde	[79]
Ethanol (40 g of alcohol as red wine and gin) *vs*. control (two different standard diets)	4 x intervention cross-over, controlled response after single exposure	8 men (45-55 years)	Colorimetric method	Postprandial 1h blood	Ethanol	[80]
Beer						
Beer (mentioned in case circumstances) *vs.* positive BAC but beer was not mentioned *vs.* neither beer nor alcohol was mentioned	Cross-sectional study	92 male ad 18 female cases with varying causes of death (mean or specific age range n.p.)	UHPLC-MS/MS	Blood (after a body admitted to the mortuary and at autopsy) Serum, vitreous humor, and urine (at autopsy)	IAAs , reduced IAAs, ethanol IAAs , reduced IAAs	[81]
Study 1: Beer (2 L) Study 2: Different kinds of beer, wines, beer plus wine or beer plus digestive (0.4-1.50 g/kg of alcohol blood concentration)	Intervention time-response after single exposure	1 subject (one of the authors) (sex and age n.p.) 10 subjects (sex and age n.p.)	UPLC–MS/MS UPLC–MS/MS	Postprandial 0.5-6h serum Serum	Hordenine, ethanol Hordenine, ethanol	[82]

Beer (330 mL)	Intervention response after single exposure	10 males (21-39 years)	UPLC-MS	Spot urine	Isoxanthohumol	[83]
Beer (762-1000 mL)	Intervention time-response after single exposure	3 males and 1 female (33 ± 13 years)	UHPLC-MS/MS	Pre and postprandial 0-7.5h plasma	Free hordenine, hordenine-Sulf, hordenine-Glc	[84]
			GC-MS	Pre and postprandial 0-7.5h plasma	Ethanol	
			GC-MS	Pre and postprandial 0- 24h urine	Total hordenine	
Beer (0.05% alcohol blood concentration as 2 lagers, 1 low lager)	3 x intervention partially cross-over (1 subject consumed only one of the lagers), time-response after single exposure	2 males and 3 females (25-39 years)	UHPLC–MS/MS	Pre and postprandial 0-6h blood	IAAs, reduced IAAs, ethanol	[85]
			UHPLC–MS/MS	Pre and postprandial 0-6h urine	IAAs, reduced IAAs	
Beer (0.05% alcohol blood concentration as 1 high-hopped beer, 1 low-hopped beer)	2 x intervention time-response after single exposure	5 males (25-44 years)	UPLC-MS/MS	Pre and postprandial 0-6h blood	IAAs, ethanol	[86]
			UPLC-MS/MS	Pre and postprandial 0-6h urine	IAAs	
Beer (2 L) *vs*. control	Intervention response after single exposure	4 subjects (25-50 years)	SPE-LC-MS/MS	Postprandial 1-2h urine	Hordenine	[87]
Beer (0.05% alcohol blood concentration)	Intervention time-response after single exposure	1 subject (sex and age n.p.)	UPLC-MS	Pre and postprandial 0-6h blood	IAAs and reduced IAAs	[88]
Study 1: Beer (300, 660 and 990 mL for males and 330, 495 and 660 mL for females)	3 x intervention cross-over, dose-response after single exposure	20 males and 21 females (28 ± 3 years)	LC-MS/MS	Morning spot urine	Isoxanthohumol	[89]
Study 2: Beer (600 mL/day) *vs.* non-alcoholic beer (990 mL/day) *vs.* gin (92 mL/day)	3 x 4w intervention randomized, cross-over, controlled, response after repeated exposure	33 males (at high cardiovascular risk) (61 ± 7 years)	UHPLC-MS	24h urine	Isoxanthohumol	
			UHPLC-MS	Morning spot urine	Isoxanthohumol	

Study 3: Beer	Cross-sectional study	A subset of 32 males and 14 females (63 ± 5 years)				
Beer (660 mL/day) *vs*. non-alcoholic beer (990 mL/day) *vs*. gin (100 mal/day)	3 x 4w intervention randomized, crossover, open, response after repeated exposure	33 males (55-75 years)	HPLC-LTQ-Orbitrap-MS	24h urine	Humulinone, oxyhumulinic acid, cohumulone, EtS, EtG, 2-phenylethanol-GlcA	[90]
Beer (500 mL/day) *vs*. non-alcoholic beer (500 mL/day)	2 x 14d intervention, cross-over, response after repeated exposure	7 males (30-65 years)	UHPLC-MS UHPLC-MS	Fasting plasma Morning spot urine	Isoxanthohumol Isoxanthohumol	[91]
Study 1: Beer (330 mL as strong lager, 1 regular lager 1 light/non-alcoholic beer) *vs.* soft drink (330 mL)	4 x intervention randomized, cross-over, single-blinded, time-response after single exposure	10 males and 9 females (24-50 years)	UPLC-QTOF UPLC-QTOF	Pre and postprandial 0-3h plasma Pre and postprandial 0-21h urine and 24h pooled urine	Iso-cohumulone, pyro-glutamyl proline Combined biomarker: N-methyl tyramine-Sulf, IAAs, tricyclohumols, pyro-glutamyl proline, 2-ethyl malate	[92]
Study 2: Beer (660 mL as high-hopped beer *vs.* low-hopped beer)	2 x intervention randomized, cross-over, single-blinded, time-response after single exposure	2 males and 2 females (28-60 years)	UPLC-QTOF	Pre and postprandial 0-40h urine	Combined biomarker: N-methyl tyramine-Sulf, IAAs, tricyclohumols, pyro-glutamyl proline, 2-ethyl malate	
Cider						
Cider (500 mL)	Intervention time-response after single exposure	9 healthy subjects (21-42 years) and 5 subjects with an ileostomy (40-54 years)	HPLC-PDA-MSn HPLC-PDA-MSn HPLC-PDA-MSn	Pre and postprandial 0-24h plasma Pre and postprandial 0-24h urine Pre and postprandial 0-24h ileal fluid	Phloretin-2'-O-GlcA Phloretin-2'-O-GlcA, phloretin-O- GlcA, phloretin-O-GlcA-O-Sulf, Total phloretin metabolites Phloretin-2'-(2''-*O*-xylosyl)-glucoside, phloretin-*O*-(*O*-xylosyl)hexoside, phloretin-2'-*O*-GlcA,	[93]

					phloretin-O-GlcA, phloretin-*O*- GlcA-*O*-Sulf, phloretin-*O*-Sulf, total phloretin metabolites	
Cider (1.1 L)	Intervention time-response after single exposure	4 males and 2 females (24-42 years)	HPLC	Pre and postprandial 0-24h urine	Phloretin	[94]
Wine						
Wine and red wine	Cross-sectional study	198 males and 277 females (50-61 years)	LC-MS/MS	24h urine	RV	[95]
Red wine	Cross-sectional study	481 (59% men; 55.3 ± 8.4 years)	UPLC-MS	24h Urine	DHRV-GlcA	[96]
Wine	Cross-sectional study	479 males and 521 females (66.6 ± 6.2 years)	LC-MS/MS	Morning spot urine	TRMs	[97,98]
Red wine	Cross-sectional study	475 subjects (58% females) (33-77 years)	UPLC-MS/MS	24h urine	RV	[99]
All wine, red wine	Cross-sectional study	475 subjects (41.7 men, 53.9 ± 8.5 years)	UPLC-MS/MS	24h urine	RV	[100]
Wine	Cross-sectional study	230 females (66.9 ± 0.4 years)	LC-MS/MS	Urine	Tartaric acid	[101]
Wine	Cross-sectional study	25 subjects (25-55 years)	HPLC-MS/MS	Morning fasting plasma	*Cis*-RV, *trans*-RV	[102]
Red wine (250 mL) *vs.* grape juice (1 L) vs. tablets of red wine extracts (10 tablets)	3 x intervention randomized, cross-over, double-blind, time-response after single exposure	11 males (19-24 years)	GC-MS GC-MS	Pre and postprandial 0-24h plasma Pre and postprandial 0-24h urine	*Trans*-RV, *cis*-RV *Trans*-RV, *cis*-RV, DHRV	[103]
Red wine *vs.* water with sugar-free artificial flavoring *vs.* ethanol (0.09% alcohol blood concentration)	3 x intervention randomized, cross-over, single-blind, time-response after single exposure	7 males and 6 females (mean age 35 (24-47) years)	GC-MS GC-MS Breath analyzer	Pre and postprandial 0-6h plasma Pre and postprandial 0-6h urine Pre and postprandial 0-6h breath	Free-RV Free-RV Ethanol	[104]
Red wine (375 mL) *vs.* grape extract tablets (15 tablets with 400 mL of water)	2 x intervention cross-over, controlled, time-response after single exposure	10 males (24-35 years)	LC-MS/MS	Pre and postprandial 0-24h urine for red wine intervention/ Pre and postprandial 0-48h urine for grape extract tablets intervention	*Trans*-RV-3-GlcA *trans*-RV-4'-GlcA, *trans*-RV-3-Sulf, *trans*-RV-4'-Sulf , *trans*-piceid, *cis*-RV-4'-GlcA, *cis*-RV-3-Sulf , cis-	[105]

				Pre and postprandial 0-24h plasma for red wine intervention/ Pre and postprandial 0-48h plasma for grape extract tablets intervention	RV-4'-Sulf , cis-RV-3-GlcA, *cis*-piceid, piceid-GlcA, piceid-Sulfs, DHRV-GlcAs, DHRV-Sulfs *Trans*-RV-3-GlcA *trans*-RV-4'-GlcA, *trans*-piceid, *cis*-RV-3-GlcA, *cis*-RV-4'-GlcA, *cis*-piceid, DHRV-GlcAs	
Red wine (200 mL)	Intervention response after single exposure	5 males (20-45 years)	LC-MS/MS	Postprandial 10h urine (morning)	Tartaric acid	[106]
Red wine (250 mL)	Intervention response after single exposure	5 males (25-28 years)	HPLC-MS/MS	Pre and postprandial 0-4h urine	*trans*-RV-3-GlcA, *trans*-RV-4'-GlcA, *trans*-RV-3-Sulf , *trans*-RV-4'-Sulf , *cis*-RV-3-GlcA, *cis*-RV-4'-Glc, *cis*-RV-3-Sulf, *cis*-RV-4'-Sulf	[107]
		11 males (18-50 years)	HPLC-MS/MS	Pre and postprandial 0-24h LDL	*Trans*-RV, *trans*-RV-3-GlcA, *trans*-RV-4'-GlcA, *trans*-RV-3-Sulf, *trans*-RV-4'-Sulf , *cis*-RV-3-GlcA, *cis*-RV-3-Sulf, *cis*-RV-4'-Sulf	
Wine	Intervention response after single exposure	2 subjects (sex and age n.p.)	n.p.	Urine	Tartaric acid	[108]
Red wine (150 mL) *vs.* olive oil (25 mL) *vs.* combination of both	3 x intervention randomized, cross-over, response after single exposure	6 males and 6 females (34.0 ± 10.5 years)	HPLC-MS	Pre (2 prior the test-0h) and postprandial 0-6h urine	*Cis*-RV, *trans*-RV, DHRV	[109]
Dealcoholized red wine (100 mL) *vs.* dealcoholized red wine enriched with non-encapsulated (100 mL)	3 x intervention randomized, cross-over, single-blind, controlled, time-response after single exposure	6 females and 6 males (19-50 years)	UPLC-MS/MS	Pre and postprandial 0-6h plasma	RV-Sulf, RV-GlcA	[110]
			UPLC-MS/MS	Pre and postprandial 0-24h urine	RV-Sulf, RV-GlcA	

vs. encapsulated phenolic extract						
Red wine (100, 200, 300 mL)	3 x intervention cross-over, controlled, dose-response after single exposure	21 males (21-50 years)	LC-MS/MS	Morning spot urine	Tartaric acid	[111]
Study 1: Red wine and standard meal (300 mL)	Intervention time-response after single exposure	10 males (25-40 years)	LC-UV-DAD	Pre and postprandial 0-4h serum	*Trans*-RV-3-GlcA, *trans*-RV-4'-GlcA	[112]
Study 2: Red wine fasting (600 mL)	Intervention time-response after single exposure	1 male and 4 females (24-38 years)	HPLC-MS/MS	Pre and postprandial 0-4h serum	Free-*trans*-RV, *trans*-RV-3-GlcA, *trans*-RV-4'-GlcA	
Study 3: red wine and two different meals (differing in the lipid content) (600 mL)	Intervention parallel, time-response after single exposure	3 males and 7 females (24-54 years)	HPLC-MS/MS	Pre and postprandial 0-4h serum	Free-*trans*-RV, *trans*-RV-3-GlcA, *trans*-RV-4'-GlcA	
Study 1: Red wine (272 mL/day) *vs.* dealcoholized red wine (272 mL/day) *vs.* gin (100 mL/day)	3 x 4w intervention randomized, cross-over, controlled, response after repeated exposure	56 subjects (≥ 55 years)	^{1}H-NMR	24h urine	Ethanol, tartaric acid, EtG Combined biomarker: tartaric acid, EtG	[113]
Study 2: Wine	Cross-sectional study	91 (53-79 years)	^{1}H-NMR	Urine	Ethanol, tartaric acid, EtG Combined biomarker: tartaric acid, EtG	
Red wine (272 mL/day) *vs.* dealcoholized red wine (272 mL/day) *vs.* gin (100 mL/day)	3 x 4w intervention randomized, cross-over, open, controlled, response after repeated exposure	67 males (60 ± 8 years)	HPLC-MS/MS LC-MS	24h urine 24h urine	*Cis*-RV, *trans*-RV, Total RVs EtG	[114 – 116]
Red wine (272 mL/day) *vs.* dealcoholized red wine (272 mL/day) *vs.* gin (100 mL/day)	3 x 4w intervention randomized, cross-over, controlled,	61 subjects (≥ 55 years)	^{1}H-NMR	24h urine	Tartaric acid, ethanol	[117]

	response after repeated exposure					
Study 1: Red wine (200 mL/day) *vs.* white wine (200 mL/day)	3 x 4w intervention randomized, cross-over, single-blinded, controlled, response after repeated exposure	10 females (38.1 ± 9.2 years)	LC-MS/MS	Morning spot urine	*Trans*-RV-3-GlcA, *cis*-RV-3-GlcA, TRMs (*trans*-RV-GlcA, *cis*-RV-GlcA)	[118]
Study 2: Wine	Cross-sectional study	30 males and 22 females (55-80 years)	LC-MS/MS	Morning spot urine	*Trans*-RV-3-GlcA, *cis*-RV-3-GlcA, TRMs (*trans*-RV-GlcA, *cis*-RV-GlcA)	
Red wine (272 mL/day) *vs.* dealcoholized red wine (272 mL/day) *vs.* gin (100 mL/day)	3 x 4w cross-over, controlled, RCT	36 males (61 ± 9 years)	UPLC-MS/MS	24h urine	TRMs, total RV microbial metabolites	[119]
Red wine (200 mL/day) *vs.* white wine (200 mL/day)	2 x 4w intervention randomized, cross-over, controlled, response after repeated exposure	35 females (20-50 years)	HPLC	Spot urine	TRMs (*trans*-RV-GlcA, *cis*-RV-GlcA)	[120]
Red wine (272 mL/day) *vs.* dealcoholized red wine (272 mL/day)	2 x 4w intervention randomized, cross-over, controlled, response after repeated exposure	59 subjects (≥ 55 years)	UPLC-MS/MS	24h urine	*Trans*-RV-3-GlcA, *trans*-RV-4-GlcA, *trans*-RV-3-Sulf, *trans*-RV-4-Sulf, *trans*-RV-3,4-diSulf, *cis*-RV-3-GlcA, *cis*-RV-4-GlcA, *cis*-RV-3-GlcA, *cis*-RV-4-Sulf, *cis*-RV-3-Sulf, RV-Sulf-GlcA, piceid-GlcA, piceid-Sulfs, DHRV, DHRV-GlcA, DHRV-Sulf, DHRV-Sulf-GlcA, TRMs, total DHRVs	[121]
Red wine (250 mL/day) *vs.* control	2 x 4w intervention randomized,	22 females and 19 males (36 ± 11 years)	UHPLC-QTOF-MS	24h urine	Tartaric acid, EtS	[122]

	parallel, controlled, response after repeated exposure					
Red wine (270 mL/day)	4w intervention response after repeated exposure	6 males and 4 females (40.4 ± 4.1 years)	UHPLC-MS/MS	Fasting plasma	*Cis*-RV-4-Sulf, DHRV-3-Sulf	[123]
Aged white wine (255 mL/day) *vs.* gin (92 mL/day)	2 x 3w intervention randomized, cross-over, open, controlled, response after repeated exposure	38 males (55-80 years)	LC-ESMS/MS	24h urine	Tartaric acid	[124, 125]
Red wine (272 mL/day) *vs.* dealcoholized red wine (272 mL/day)	3 x 20d intervention randomized, cross-over, controlled, response after repeated exposure	10 males (48 ± 2 years)	HPLC-MS/MS	24h Urine	TRMs, total DHRV metabolites, total metabolites (RV+DHRV metabolites)	[126, 127]
Red wine (300 mL/day) *vs.* white wine (300 mL/day) *vs.* control	15d intervention randomized, parallel, controlled, response after repeated exposure	9 males and 11 females (around 40 years)	HPLC	Fasting plasma	RV	[128, 129]
Spirits and distillates						
Study 1: aniseed spirit (Helenas Ouzu) (120, 200, 360 mL)	Intervention dose and time-response after single exposure	1 male (22 years)	HS-SPME-GC-MS	Pre and postprandial 0-24h serum	Anethole	[130]
Study 2: aniseed spirit (Ouzo, Raki or Küstennebel)	Cross-sectional study	10 females and 40 males (17-57 years) drivers	HS-SPME-GC-MS	Serum	Anethole, ethanol	
Study 1: peppermint liquor (160, 320 and 560 mL)	Intervention dose and time-response after single exposure	1 male (29 years)	HS-SPME-GC-MS	Pre and postprandial 0-24h serum	Menthone, isomenthone, neomenthol, menthol	[131]

Study 2: peppermint liquor	Cross-sectional study	5 females and 95 males (18-66 years)	HS-SPME-GC-MS	Serum	Menthone, isomenthone, neomenthol, menthol, ethanol

Abbreviatures: [1]H-NMR, Proton Nuclear Magnetic Resonance; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HTOL, 5-hydroxytryptophol; DHRV, dihydroresveratrol; d, days; ELSD, Evaporative Light-Scattering Detection; EtG, Ethyl Glucuronide; EtS. Ethyl Sulfate; FAEEs, Free Acids Ethyl Esters; GC-MS, Gas Chromatography–Mass Spectrometry; GlcA, Glucuronide; h; hours; HDL, High Density Lipoprotein; HPLC-MS/MS, High Performance Liquid Chromatography-Tandem Mass Spectrometry; HPLC-PDA-MSn, High Performance Liquid Chromatography-Photodiode Array-Mass Spectrometry; HS-GC, Headspace Gas Chromatography; HS-GC-FID, Headspace Gas Chromatography with Flame Ionization Detection; HS-SPME-GC-MS, Headspace Solid-phase Microextraction Gas Chromatography; IAAs, Iso-α-Acids; LC-ESI-MS/M, Liquid Chromatography- Electrospray Ionization-Tandem Mass Spectrometry LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; LC-UV-DAD, Liquid Chromatography-Ultraviolet-Diode Array Detection; LDL, Low Density Lipoprotein; n.p., not provided; PEths, Phosphatidylethanols; RCT, Randomized Clinical Trial; RV, resveratrol; TRMs, Total Resveratrol Metabolites; Sulf, sulfate; Sulf-GlcA, sulfoglucuronide; UHPLC, Ultra-High-Performance Liquid Chromatography; UHPLC-QTOF- MS, Ultrahigh-Performance Liquid Chromatography−Time-of-Flight Mass Spectrometry; UPC2-MS/MS, Ultra Performance Convergence chromatography-Tandem Mass Spectrometry; VLDL, Very Low-Density Lipoprotein; w, weeks.

[1]Study design listed as the way it was analyzed (not necessarily using the design of the primary study), e.g., prospective studies are typically analyzed cross-sectionally at baseline.

Supplementary table 3. Summary of the excluded candidate BFIs of alcoholic beverages subgroups and ethanol consumption and reasons for exclusion.

Dietary factor	Metabolites	Biofluid locations	Reason for inclusion and exclusion	Reference
Alcohol	5-HTOL and related metabolites	Blood/Urine	Possibly unspecific. Only investigated as a marker of alcohol abuse	[56]
	Propanol	Blood/Urine	Formed from several sources (not robust)	[132]
	GGT, ALT, AST	Blood	Unspecific, possible marker of effect	[133]
	MCV, CDT, SIJ	Blood	Unspecific, possible marker of effect	[134]
	EDAC	Blood	Only validated as a categorical marker for problem drinking	[135]
	HDL and related markers	Blood	Unspecific at the individual level but sensitive above ~1 drink a day at the group level. Possible marker of effect	[136]
Beer	8-Prenylnaringenine	Urine/Plasma	High inter-individual variability	[91,137]
	Sphingomyelin	Serum	Possible biomarker of effect	[138]
	Free tyrosol and hydroxytyrosol	Urine	Not specific as beer intake biomarker (e.g., olive oil and wine)	[139]
	Proline Betaine	Urine	Much lower concentration than citrus fruits	[140]
	16-Hydroxypalmitate	Serum	Possible biomarker of effect	[12]
	5-Hydroxymethylfurfural and 5-hydroxymethylfurfural-2-furoic acid	Urine	Not specific as beer intake biomarker (e.g., coffee, and dried fruits)	[141]
	Mevalonic acid	Urine	Possible biomarker of effect	[142]
	l-Methyl-l,2,3,4-tetrahydro-β-carboline	Urine	Not specific as beer intake biomarker (e.g., wine)	[143]
	l,2,3,4-tetrahydro-β-carboline	Urine	Not specific as beer intake biomarker (e.g., wine, banana)	[143]
Cider	Isorhamnetin (3'-methyl quercetin)	Plasma	Not specific as cider intake biomarker (e.g., grapefruit, orange juice, cranberry juice, almond extract, onion, sea buckthorn, tomato puree)	[94]
	Tamarixetin (4'-methyl quercetin)	Plasma	Not specific as cider intake biomarker (e.g., onion)	[94]
	Caffeic Acid	Plasma	Not specific as cider intake biomarker (e.g., coffee, olive oil, tomato, wine, cocoa, artichoke, berry)	[94]
	Hippuric Acid	Urine	Not specific as cider intake biomarker (e.g., gut microbial fermentation product after consumption of tea, chamomile, wine, coffee, fruit juice)	[94]
Wine				
Tyrosols	Free tyrosol, hydroxytyrosol and derivatives	Urine/Plasma	Not specific as wine intake biomarker (e.g., olive oil and beer)	[96,109,110,144,145]
Anthocyanins	Anthocyanins[1]	Urine/Plasma	Not specific as wine intake biomarker (e.g., berries)	[110,120,146–149]

Flavanols	Catechin and derivatives	Urine/Plasma	Not specific as wine intake biomarker (e.g., apple, apricot, black tea, cocoa)	[99,104,110,119,128,150–155]
	Epicatechin and derivatives	Urine/Plasma	Not specific as wine intake biomarker (e.g., apple, black tea, green tea, cocoa)	[110,119,150,152,156–158]
Flavonols	Isorhamnetin	Urine/Plasma	Not specific as wine intake biomarker (e.g., onion, berries)	[159]
	Kaempferol	Urine	Not specific as wine intake biomarker (e.g., berries, tea, spices)	[159]
	Quercetin	Urine/Plasma	Not specific as wine intake biomarker (e.g., onion, green tea, chocolate)	[159]
Hydroxyphenylacetic acids	Di- and hydroxyphenyl acetic acid and derivatives	Urine/Feces	Not specific as wine intake biomarker (e.g., oat, maize, olive oil)	[99,110,117,119,150,160–162]
Hydroxybenzoic acids	Hippuric acid	Urine	Not specific as wine intake biomarker (e.g., tea, fruit juices, whole grain)	[117,150]
	Syringic acid and derivatives	Urine/Plasma/Feces	Not specific as wine intake biomarker (e.g., walnuts, olive, date)	[96,110,119,150,161–163]
	Gallic acid and derivatives	Urine	Not specific as wine intake biomarker (e.g., cocoa, coffee)	[96,99,110,119,151,164]
	4-O-methylgallic acid	Urine/Plasma	Not specific as wine intake biomarker (e.g., tea, grapes)	[96,151,164–167]
	Hydroxybenzoic acid derivatives	Urine/Feces	Not specific as wine intake biomarker (e.g., berries, grapefruit, date, cereals, beer, coconut)	[119,150,162,163]
Hydroxycinnamic acid	Sinapic acid	Urine	Not specific as wine intake biomarker (e.g., strawberry guava, ryes, cauliflower)	[150]
	Caffeic acid and derivatives	Urine/Plasma	Not specific as wine intake biomarker (e.g., berries, dried fruits, seeds, olive, potato)	[110,119,128,150,151,164,165,168–170]
	m-Coumaric acid and derivatives	Urine	Not specific as wine intake biomarker (e.g., olive, corns, beer, whole grain)	[96,150]
	p-Coumaric acid	Urine/Plasma/Feces	Not specific as wine intake biomarker (e.g., coriander, peanut, date)	[99,119,150,163,168]
	Ferulic acid and derivatives	Urine/Plasma	Not specific as wine intake biomarker (e.g., cocoa, dried fruits, cereal products)	[110,119,150,166,168]
	Caftaric acid	Plasma	Very low or undetectable concentration	[168]
	Fertaric acid	Plasma	undetectable concentration	[168]
Other class of compounds	Malic acid	Urine	Not specific as wine intake biomarker (e.g., apple, apricots,	[171]

			berries, plums, cherries) and produced endogenous	
	Succinic acid	Urine	Endogenous metabolite	[171]
	Scyllo-inositol	Serum	Not specific as wine intake biomarker (e.g., coconut, citrus fruits)	[12]
Sweet wine	*Cis*-resveratrol-3-*O*-glucuronides	Urine	Not specific as sweet or sparkling wine intake biomarker (e.g., all wine)	[118]
	Trans-resveratrol-3-*O*-glucuronides	Urine	Not specific as sweet or sparkling wine intake biomarker (e.g.., all wine)	[118]
Spirits and distillates	TTCA	Urine	Not specific as liquor intake biomarker (e.g., environment, cruciferous vegetables)	[172]

Abbreviations: 5-HTOL, 5-hydroxytryptophol; ALT, ALanine aminoTransferase; AST, ASpartate aminoTransferase; CDT, Carbohydrate Deficient Transferrin; EDAC, Early Detection od Alcohol Cunsumption (a combined marker); GGT, Gamma Glutamyl Transferase; HDL, High Density Lipoprotein (also subfractions and apolipoprotein A1); MCV, Mean Corpuscular Volume of erythrocytes; SIJ, Sialic acid Index of apolipoprotein J; TTCA, 2-thiothiazolidine-4-carboxylic acid.

[1] Cyanidin-3-glucoside, delphinidin-3-glucoside, malvidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, total anthocyanins.

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Annex 5. Supplementary material of Publication 6

Supplementary Material

Table S1. Baseline dietary habits of the 3-day food records from all participants in the intervention groups.

	Control (n= 14)	AB (n= 16)	NAB (n= 7)	p-value
Carbohydrates (% kcal/day)	38.4 ± 5.9	36.6 ± 7.5	42.1 ± 2.9	0.127
Sugar	19.3 ± 6.1	16.8 ± 4.8	21.0 ± 4.3	0.104
Protein (% kcal/day)	19.4 ± 4.0	19.1 ± 2.9	18.0 ± 2.1	0.486
Total fat (% kcal/day)	42.0 ± 7.1	43.5 ± 6.5	39.6 ± 3.6	0.611
SFA	12.1 ± 3.9	11.4 ± 3.0	10.8 ± 3.1	0.904
MUFA	20.0 ± 4.3	21.4 ± 5.0	17.4 ± 2.2	0.109
PUFA	6.6 ± 1.2	6.4 ± 1.5	7.8 ± 1.8	0.276
Fiber (g/day)	25.9 ± 9.0 a	17.0 ± 6.4 b	32.4 ± 14.5 a	**0.008**

AB: alcoholic beer; MUFA: mono-unsaturated fatty acids; NAB: non-alcoholic beer; PUFA: poly-unsaturated fatty acids; SFA: saturated fatty acids.
Kruskal-Wallis with post-hoc Dunn's test analysis was applied to study differences in continuous variables. Means within the same row carrying different superscripts (a,b) are significantly different (p-value <0.05).

Table S2. Intragroup analyses of somatic, psychological, and urogenital subscales scores and total MRS score before, during and at the end of the intervention study.

		Baseline Mean ± SD	1.5 months Changes ± SD	3 months Changes ± SD	6 months Changes ± SD	p-value
Somatic subscale	*Control*	3.9 ± 2.4	-0.2 ± 1.6	-0.5 ± 2.5	-0.6 ± 2.7	0.751
	AB	4.7 ± 2.8	-1.3 ± 1.5	-1.4 ± 1.8	-1.8 ± 1.7	0.277
	NAB	4.6 ± 2.9	-0.3 ± 2.2	-1.6 ± 2.9	-2.0 ± 2.1	0.590
Hot flashes, sweating	*Control*	0.9 ± 1.0	0.1 ± 0.9	0.0 ± 0.8	-0.2 ± 0.8	0.897
	AB	1.1 ± 1.2	0.0 ± 0.5	0.2 ± 0.5	-0.3 ± 0.9	0.957
	NAB	1.1 ± 1.1	0.0 ± 1.0	-0.4 ± 1.0	-0.5 ± 0.8	0.586
Heart discomfort	*Control*	0.5 ± 0.6a	-0.3 ± 0.6a	-0.4 ± 0.8b	-0.5 ± 0.7b	**0.028**
	AB	0.6 ± 0.7	-0.4 ± 0.6	-0.4 ± 0.7	-0.4 ± 0.8	0.071
	NAB	0.6 ± 0.8	0.1 ± 0.4	-0.1 ± 0.4	-0.3 ± 0.8	0.646
Sleep problems	*Control*	1.0 ± 1.1	0.2 ± 0.6	0.2 ± 0.7	0.3 ± 1.1	0.974
	AB	1.6 ± 1.5	-0.5 ± 0.9	-0.5 ± 1.0	-0.8 ± 0.9	0.480
	NAB	1.6 ± 1.3	0.0 ± 0.5	-0.3 ± 1.0	-0.5 ± 0.8	0.896
Joint and muscular discomfort	*Control*	1.4 ± 1.2	-0.3 ± 0.9	-0.4 ± 1.3	-0.3 ± 1.2	0.742
	AB	1.4 ± 1.2	-0.3 ± 0.6	-0.3 ± 0.8	-0.4 ± 1.1	0.788
	NAB	1.3 ± 1.0	-0.4 ± 0.8	-0.7 ± 1.1	-0.7 ± 0.8	0.441
Psychological subscale	*Control*	3.5 ± 2.8	0.0 ± 1.0	0.2 ± 1.9	-0.4 ± 1.4	0.949
	AB	4.1 ± 3.4	-1.4 ± 1.4	-2.2 ± 2.3	-2.7 ± 2.7	0.055
	NAB	3.1 ± 1.9	-0.6 ± 1.0	-1.3 ± 1.3	-1.5 ± 2.1	0.393
Depressive mood	*Control*	0.8 ± 0.7	0.1 ± 0.5	0.1 ± 0.8	-0.3 ± 0.5	0.516
	AB	1.3 ± 1.3	-0.5 ± 0.5	-0.9 ± 0.9	-1.0 ± 1.0	0.075
	NAB	1.1 ± 0.9	-0.3 ± 0.5	-0.7 ± 1.0	-0.8 ± 1.3	0.183
Irritability	*Control*	0.9 ± 1.1	0.1 ± 0.7	0.1 ± 1.0	0.1 ± 0.9	0.940
	AB	0.8 ± 1.0	-0.3 ± 0.6	-0.4 ± 0.7	-0.4 ± 0.8	0.575
	NAB	0.6 ± 0.5	-0.1 ± 0.4	-0.3 ± 0.5	0.0 ± 0.8	0.475
Anxiety	*Control*	0.7 ± 1.0	-0.1 ± 0.4	-0.1 ± 0.5	-0.3 ± 0.5	0.903
	AB	0.7 ± 1.0	-0.3 ± 0.6	-0.4 ± 0.7	-0.6 ± 0.8	0.330
	NAB	0.0 ± 0.0	0.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.414
	Control	1.2 ± 0.9	0.1 ± 0.5	-0.1 ± 0.6	0.0 ± 1.0	0.896
	AB	1.3 ± 1.1	-0.3 ± 0.7	-0.5 ± 0.9	-0.7 ± 1.3	0.262

Physical and mental exhaustion	*NAB*	1.3 ± 1.1	-0.1 ± 0.4	-0.1 ± 0.4	-0.2 ± 0.4	0.959
Urogenital subscale	*Control*	2.8 ± 2.0	0.4 ± 1.2	-0.1 ± 1.0	-0.1 ± 1.1	0.876
	AB	2.6 ± 2.0	-0.1 ± 0.6	-0.4 ± 1.0	-0.8 ± 1.0	0.663
	NAB	2.1 ± 1.9	0.0 ± 1.2	-0.7 ± 1.1	-0.7 ± 1.2	0.893
Sexual problems	*Control*	0.6 ± 1.1	0.1 ± 0.5	0.0 ± 0.4	0.0 ± 0.4	0.876
	AB	1.1 ± 1.3	-0.1 ± 0.3	-0.1 ± 0.4	-0.2 ± 0.5	0.960
	NAB	0.6 ± 1.1	-0.1 ± 0.4	-0.1 ± 0.4	0.0 ± 0.0	0.851
Bladder problems	*Control*	0.9 ± 1.1	0.1 ± 0.7	-0.1 ± 0.9	0.0 ± 0.1	0.889
	AB	0.8 ± 0.9	-0.1 ± 0.5	-0.4 ± 0.6	-0.4 ± 0.7	0.294
	NAB	0.3 ± 0.5	0.0 ± 0.6	-0.3 ± 0.5	0.2 ± 0.9	0.524
Dryness of the vigina	*Control*	1.2 ± 1.2	0.1 ± 0.5	0.0 ± 0.6	0.0 ± 0.6	0.997
	AB	0.8 ± 1.1	0.1 ± 0.5	0.0 ± 0.6	-0.1 ± 0.6	0.909
	NAB	1.3 ± 1.1	0.1 ± 0.7	-0.3 ± 1.1	-0.8 ± 1.0	0.504
Total MRS score	*Control*	10.1 ± 5.8	0.2 ± 2.9	-0.5 ± 4.3	-1.1 ± 4.2	0.915
	AB	11.3 ± 5.6a	-2.8 ± 2.8a	-4.0 ± 3.9a	-5.2 ± 4.4b	**0.014**
	NAB	9.9 ± 5.5	-0.9 ± 3.9	-3.6 ± 4.2	-4.2 ± 3.0	0.398

AB: alcoholic beer; NAB: non-alcoholic beer. Results are presented as mean ± SD and mean changes ± SD compared to baseline visit?. Kruskal Wallis followed by post-hoc Dunn's test was used for statistical intragroup comparisons throughtout the intervention. p-value < 0.05.

Table S3. Intragroup analysis of female sex hormone levels before and after intervention.

		Baseline Mean ± SD	6 months Changes ± SD	p-value
LH	*Control*	29.3 ± 10.4	2.2 ± 4.0	0.129
(15.9-54.0 U/L)[1]	*AB*	41.6 ± 15.3	-2.2 ± 6.5	0.175
	NAB	36.9 ± 12.6	1.6 ± 10.3	0.688
FSH	*Control*	62.9 ± 21.3	2.3 ± 14.4	0.151
(23-116 U/L)[1]	*AB*	96.8 ± 42.5	-6.5 ± 10.9	0.039
	NAB	59.9 ± 20.2	3.5 ± 9.6	0.438
E2	*Control*	51.9 ± 13.0	4.1 ± 23.7	0.685
(>37 pg/mL)[1]	*AB*	35.8 ± 6.6	3.1 ± 20.3	0.815
	NAB	53.0 ± 35.5	-3.0 ± 10.3	0.438
Progesterone	*Control*	0.39 ± 0.27	-0.02 ± 0.29	0.549*
(ng/mL)	*AB*	0.32 ± 0.13	0.02 ± 0.08	0.515
	NAB	0.29 ± 0.05	-0.01 ± 0.09	1.000
T-Total	*Control*	18.7 ± 10.9	1.6 ± 8.2	0.519
(10-50 ng/dL)	*AB*	14.4 ± 8.4	0.4 ± 4.6	0.901
	NAB	15.3 ± 9.4	-2.8 ± 4.8	0.219*
SHBG	*Control*	53.5 ± 24.0	0.8 ± 12.6	0.470
(25.0-96.0 nmol/L)	*AB*	59.1 ± 24.3	-7.4 ± 16.5	0.386
	NAB	63.0 ± 32.6	-8.6 ± 17.8	0.219
TFI	*Control*	1.76 ± 2.32	0.13 ± 0.46	0.470
(0.43-8.10)	*AB*	1.01 ± 0.80	0.07 ± 0.56	0.561
	NAB	1.06 ± 0.79	-0.08 ± 0.30	0.688*
FEI	*Control*	0.46 ± 0.32	0.03 ± 0.21	0.850
(nmol/L)	*AB*	0.27 ± 0.14	0.04 ± 0.11	0.231
	NAB	0.55 ± 0.78	-0.01 ± 0.10	1.000*

[1]Postmenopausal reference values. AB: alcoholic beer; FEI: free estradiol index; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; NAB: non-alcoholic beer; SHBG: sex hormone-binding globulin; TFI: Free testosterone index; T-Total: Total testosterone. Wilcoxon matched-pair signed-rank test was was used for statistical intragroup comparisons throughtout the intervention. Sing test of matched-pairs was used in asymmetric distributed variables (*).

Annex 6. Supplementary material of Publication 7

Supplementary Material

Supplementary Table 1. Intragroup and intergroup analysis of anthropometric and clinical measurements during the intervention study

	Control (n = 10)	**AB (n = 15)**	**NAB (n = 6)**	***p*-value[1]**
BMI, kg/m^2				
Baseline	26.5 (25.3-32.5)	26.5 (23.1-28.6)	25.3 (24.7-29.0)	0.595
12 months	28.4 (25.5-33.2)	28.1 (21.6-28.8)	24.9 (24.8-28.2)	0.291
24 months	27.5 (25.1-32.0)	27.3 (21.4-28.8)	25.2 (24.4-28.8)	0.472
p-value[2]	0.557	0.121	0.688	
WC, cm				
Baseline	90.0 (85.5-100.0)	88.7 (79.5-96.4)	84.5 80.3-90.1)	0.588
12 months	91.1 (84.0-103.0)	89.0 (82.0-96.4)	82.3 (78.0-83.2)	0.208
24 months	94.0 (82.0-98.5)	94.0 (83.0-99.0)	85.3 (77.0-92.0)	0.641
p-value[2]	0.723	0.302	0.625	
Body fat mass, %				
Baseline	44.1 (40.2-45.1)	42.7 (39.2-47.5)	40.3 (39.1-48.2)	0.900
12 months	43.3 (39.3-44.6)	44.0 (39.2-47.9)	39.6 (36.4-41.5)	0.409
24 months	43.1 (38.0-46.1)	43.1 (38.0-47.0)	40.7 (37.6-42.0)	0.671
p-value[2]	0.922	0.417	1.000	
Fat mass index, kg/m^2				
Baseline	11.5 (9.6-15.3)	11.2 (8.8-13.0)	10.5 (9.3-12.3)	0.636
12 months	11.8 (9.6-13.8)	12.1 (8.2-13.1)	9.6 (8.5-11.0)	0.354
24 months	12.2 (9.7-13.4)	11.6 (8.1-13.4)	9.7 (8.7-11.7)	0.592
p-value[2]	0.695	**0.030**	0.688	
Lean mass index, kg/m^2				
Baseline	15.0 (14.5-17.2)	14.2 (13.1-14.6)	14.6 (14.0-16.5)	**0.034**
12 months	15.2 (14.6-17.1)	14.2 (13.5-14.4)	15.6 (14.4-16.4)	**0.008**
24 months	15.8 (14.8-17.7)	14.9 (13.3-15.1)	15.2 (14.5-16.3)	**0.043**
p-value[2]	0.432	**0.013**	1.000	
Physical activity, METS-min/day				
Baseline	840 (480-1146)	552 (304-807)	460 (396-601)	0.238
12 months	635 (517-1105)	477 (266-731)	748 (313-1124)	0.273
24 months	673 (535-1326)	471 (207-709)	764 (385-997)	0.284
p-value[2]	0.922	0.525	0.156	
Creatinine, mg/dL				
Baseline	0.71 (0.56-0.83)	0.64 (0.59-0.75)	0.68 (0.66-0.69)	0.456
12 months	0.73 (0.60-0.78)	0.64 (0.61-0.74)	0.72 (0.69-0.75)	0.407
24 months	0.79 (0.66-0.85)	0.73 (0.64-0.87)	0.86 (0.79-0.88)	0.223
p-value2	0.014	0.001	0.094	
Calcium (serum), mg/dL				
Baseline	9.3 (9.0-9.5)	9.3 (9.0-9.5)	9.3 (9.3-9.5)	0.969
12 months	9.3 (9.1-9.4)	9.3 (9.1-9.5)	9.0 (8.8-9.3)	0.314
24 months	9.4 (9.0-9.7)	9.2 (8.9-9.4)	9.2 (8.9-9.4)	0.862
p-value[2]	0.984	0.751	1.000	
PTH, ng/mL				

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Baseline	63.0 (44.0-80.0)	52.0 (46.0-69.0)	66.5 (46.0-73.0)	0.751
12 months	65.0 (57.0-80.0)	60.0 (52.0-68.0)	70.0 (57.0-71.0)	0.747
24 months	66.0 (53.0-91.0)	71.0 (64.0-94.0)	66.5 (42.0-78.0)	0.659
p-value[2]	0.053	**0.004**	0.375	
25-hydroxy-vitamin D, ng/mL				
Baseline	23.7 (20.6-26.5)	25.4 (18.6-35.7)	24.6 (14.1-38.6)	0.743
12 months	23.2 (21.6-24.4)	24.8 (13.6-31.6)	21.2 (13.6-26.5)	0.710
24 months	22.6 (20.2-23.7)	25.2 (18.0-26.8)	25.4 (22.4-28.2)	0.620
p-value[2]	0.846	0.241	0.675	

AB: alcoholic beer; BMI: body mass index; NAB: non-alcoholic beer; PTH: parathyroid hormone; WC: waist circumference. Data are expressed as median values (Q1-Q3).

p-value[1] refers to the intergroup comparisons by the Kruskal–Wallis test followed by Dunn's test. $p < 0.050$ are statistically significant.

p-value[2] refers to the difference between baseline and 24 months in each study arm. Matched-pair signed-rank test was used for statistical intragroup comparisons throughout the intervention. Sing-test of matched pairs was used for asymmetrically distributed variables.

Supplementary Table 2. Intragroup and intergroup analysis of dietary habits from food frequency questionnaire during the intervention study

	Control (n = 10)	**AB (n = 15)**	**NAB (n = 6)**	***p*-value[1]**
Energy, kcal/day				
Baseline	2699 (2556-3022)	2599 (2127-3138)	2348 (2268,-682)	0.320
12 months	2682 (2384-2757)	2478 (1946-3668)	2583 (2297-3075)	0.928
24 months	2439 (2242-2636)	2775 (1909-3712)	2354 (2169-2781)	0.611
p-value[2]	0.160	0.600	1.000	
Carbohydrates, % kcal/day				
Baseline	31.4 (24.7-33.7)	33.5 (29.2-38.9)	37.4 (34.1-40.1)	0.076
12 months	33.6 (28.3-37.7)	30.2 (26.6-36.9)	37.9 (34.9-42.5)	0.085
24 months	34.1 (31.8-35.4) [a]	28.9 (24.8-33.5) [a]	40.9 (38.0-43.1) [b]	**<0.001**
p-value[2]	0.193	**0.008**	0.063	
Sugar, % kcal/day				
Baseline	17.1 (13.9-18.9)	14.6 (11.3-16.9)	19.7 (11.8-26.5)	0.330
12 months	18.0 (15.5-21.2) ab	14.8 (10.7-18.0) b	20.3 (16.9-24.2) a	0.034
24 months	16.5 (11.9-20.0) ab	14.2 (10.9-16.9) b	24.8 (16.1-25.9) a	0.012
p-value[2]	0.625	0.208	0.219	
Fiber, g/day				
Baseline	37.6 (33.2-44.3)	36.6 (29.7-41.0)	37.4 (33.7-37.8)	0.829
12 months	40.3 (33.1-44.5)	33.5 (22.5-45.6)	40.3 (39.2-41.1)	0.589
24 months	39.4 (34.9-44.5)	29.6 (21.9-41.6)	37.6 (34.1-38.6)	0.246
p-value[2]	0.492	0.169	0.679	
Protein, % kcal/day				
Baseline	20.4 (16.3, 20.9)	19.2 (17.4, 21.8)	18.1 (16.9, 20.4)	0.781
12 months	18.0 (16.5, 20.8)	18.2 (15.0, 20.5)	16.7 (16.4, 19.4)	0.736
24 months	17.3 (16.6, 20.6)	17.7 (15.9, 20.0)	17.2 (14.8, 18.4)	0.691
p-value[2]	0.492	0.302	0.313	
Total fat, % kcal/day				
Baseline	49.7 (44.6, 54.0)	47.3 (37.4, 50.2)	44.0 (41.3, 45.3)	0.153
12 months	46.0 (43.9, 54.1)	47.2 (41.5, 51.9)	41.9 (38.6, 49.4)	0.424
24 months	48.1 (46.5, 51.8) a	51.5 (45.3, 53.8) a	41.2 (37.6, 46.6) b	**0.037**
p-value[2]	0.695	0.073	0.313	
SFA, % kcal/day				
Baseline	13.8 (13.2, 15.0)	12.7 (10.9, 14.5)	12.5 (11.8, 14.1)	0.176
12 months	12.8 (11.4, 14.7)	12.1 (11.3, 13.1)	11.9 (10.1, 12.7)	0.660
24 months	12.6 (11.2, 13.3)	12.8 (11.7, 13.9)	10.6 (9.6, 11.6)	0.134
p-value[2]	0.006	0.169	0.156	
MUFAs, % kcal/day				
Baseline	21.8 (20.3, 27.6)	23.3 (16.5, 26.8)	20.3 (18.4, 24.0)	0.469
12 months	21.8 (20.4, 28.7)	23.9 (19.2, 27.9)	19.7 (16.8, 25.0)	0.335
24 months	24.3 (23.4, 26.1)	24.5 (22.7, 28.9)	19.8 (18.2, 21.4)	0.079
p-value[2]	0.770	0.107	0.844	
PUFAs, % kcal/day				
Baseline	7.6 (6.9, 8.2)	6.6 (6.0, 8.4)	6.7 (5.6, 8.8)	0.440
12 months	8.0 (7.3, 9.4)	7.2 (5.9, 7.9)	6.5 (6.0, 8.5)	0.163
24 months	7.8 (7.4, 8.9)	7.3 (6.1, 8.2)	6.8 (6.5, 7.7)	0.091

p-value[2]	0.557	0.359	1.000	
Alcohol, g/day				
Baseline	1.0 (0.0, 1.7) a	7.7 (2.9, 9.2) b	1.9 (0.6, 3.1) a	**<0.001**
12 months	0.6 (0.0, 0.8) a	12.4 (11.6, 13.7) b	3.9 (3.3, 4.6) a	**<0.001**
24 months	0.0 (0.0, 1.3) a	12.4 (11.2, 12.7) b	3.9 (3.3, 4.6) a	**<0.001**
p-value[2]	0.297	**<0.001**	0.313	
Calcium, mg/day				
Baseline	1365 (1090, 1679)	1199 (935, 1552)	1083 (824, 1334)	0.405
12 months	1122 (810, 1543)	1108 (810, 1543)	1108 (645, 1244)	0.904
24 months	915 (837, 1188)	1251 (827, 1766)	872 (638, 895)	0.206
p-value[2]	0.010	0.934	0.219	
Vitamin D, µg/day				
Baseline	6.1 (4.0, 9.8)	6.4 (4.9, 8.3)	6.3 (5.7, 7.0)	0.995
12 months	6.4 (5.1, 7.8)	5.8 (5.3, 7.4)	9.7 (5.8, 11.9)	0.253
24 months	6.4 (4.0, 8.0)	8.4 (5.3, 10.8)	5.9 (3.6, 7.1)	0.143
p-value[2]	1.000	0.107	0.438	
Total polyphenols, mg/day				
Baseline	1064 (770, 1419)	753 (487, 853)	830 (677, 1450)	0.127
12 months	1243 (810, 1562)	844 (681, 973)	1175 (1100, 1295)	0.224
24 months	1006 (589, 1455)	742 (487, 958)	1126 (1107, 1638)	0.173
p-value[2]	0.846	0.107	0.210	

AB: alcoholic beer; NAB: non-alcoholic beer; SFA: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids. Data are expressed as median values (Q1-Q3).
p-value[1] refers to the intergroup comparisons by the Kruskal–Wallis test followed by Dunn's test. $p < 0.050$ are statistically significant.
p-value[2] refers to the difference between baseline and 24 months in each study arm. Matched-pair signed-rank test was used for statistical intragroup comparisons throughout the intervention. Sing-test of matched pairs was used for asymmetrically distributed variables.

Annex 7. Supplementary material of Publication 8

Supplementary Table 1. Phytoestrogen and alcohol content for daily dose of alcoholic (AB) and non-alcoholic beer (NAB) administered in the intervention.

Intervention group	IX µg/day	XN µg/day	8-PN µg/day	6-PN µg/day	Total amount µg/day	Alcohol g/day
AB (330 mL)	302.7 ± 16.8	27.9 ± 0.6	5.5 ± 0.4	22.8 ± 0.3	358.9 ± 17.4	14.0
NAB (660 mL)	104.7 ± 3.8	81.3 ± 4.0	10.3 ± 0.8	62.7 ± 2.2	259.0 ± 10.3	0.0

Abbreviations: 6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; IX, isoxanthohumol; XN, xanthohumol. Values are means of triplicate analyses ± standard deviation (SD).

Supplementary Table 2. Concentrations of taste test solutions.

	Sweet	Umami	Salty	Sour	Bitter			
Score	Sucrose (mM)	MSG (mM)	NaCl (mM)	Citric acid (mM)	PTC (μM)	Quinine (μM)	Sinigrin (μM)	iso-α-acids (μM)
1	1.2	3.0	3.9	1.2	0.7	9.4	100	0.8
2	2.3	7.5	7.8	2.3	3.5	18.7	300	1.6
3	4.7	15.0	15.6	4.7	14	37.5	600	15.6
4	9.4	30.0	31.3	9.4	56.2	75	>600	31.3
5	18.8	60.0	62.5	18.7	112.5	150	-	62.5
6	37.5	120.0	125.0	37.5	225	300	-	125
7	75.0	-	250.0	75.0	900	-	-	250
8	150.0	-	500.0	-	-	-	-	-

Abbreviations: MSG, monosodium glutamate; NaCl, sodium chloride; PTC, phenylthiocarbamide.

Supplementary Table 3. Intragroup and intergroup analysis of dietary habits from food frequency questionnaire during the intervention study

	Control group (n = 12)	AB group (n = 16)	NAB group (n = 6)	*p*-value[2]
Physical activity, *METS-min/day*				
Baseline	572 (453 – 1118)	491 (304 – 746)	460 (396 – 601)	0.435
12 months	635 (497 – 1033)	448 (285 – 645)	748 (313 – 1124)	0.241
24 months	673 (433 – 1168)	461 (231 – 685)	764 (385 – 997)	0.234
***p*-value[1]**	0.850	0.525	0.156	
Energy, *kcal/day*				
Baseline	2699 (2430 – 3042)	2672 (2261 – 3076)	2348 (2268 – 2682)	0.347
12 months	2644 (2302 – 2741)	2533 (2061 – 3525)	2583 (2297 – 3075)	0.948
24 months	2439 (2222 – 2722)	2732 (1921 – 3520)	2354 (2169 – 2781)	0.610
***p*-value[1]**	0.204	0.821	1.000	
Protein, *% daily kcal*				
Baseline	20.4 (16.4 – 20.9)	19.2 (16.7 – 21.5)	18.1 (16.9 – 20.4)	0.694
12 months	19.2 (16.6 – 20.7)	17.8 (145.0 – 20.0)	16.7 (16.4 – 19.4)	0.488
24 months	18.4 (16.8 – 20.3)	17.6 (15.5 – 19.8)	17.2 (14.8 – 18.4)	0.582
***p*-value[1]**	0.424	0.962	0.313	
Carbohydrates, *% daily kcal*				
Baseline	32.6 (27.6 – 37.2)	33.9 (29.6 – 39.3)	37.4 (34.1 – 40.1)	0.242
12 months	33.6 (30.7 – 37.8)	30.6 (26.7 – 37.9)	37.9 (34.9 – 42.5)	0.121
24 months	34.1 (32.0 – 35.8)[a]	29.1 (25.1 – 34.4)[a]	40.9 (38.0 – 43.1)[b]	**0.002**
***p*-value[1]**	0.470	**0.004**	0.063	
Simple sugars, *% daily kcal*				
Baseline	17.1 (13.8 – 20.2)	15.3 (12.2 – 16.9)	19.7 (11.8 – 26.5)	0.347
12 months	18.0 (15.5 – 21.9)[b]	15.0 (12.2 – 17.8)[a]	20.3 (16.9 – 24.2)[b]	**0.029**
24 months	16.5 (13.5 – 20.3)[ab]	14.5 (11.4 – 17.1)[a]	24.8 (16.1 – 25.9)[b]	**0.017**
***p*-value[1]**	0.622	0.274	0.219	
Fibre, *g/day*				
Baseline	40.2 (34.6 – 46.4)	37.9 (30.5 – 41.5)	37.4 (33.7 – 37.8)	0.600
12 months	40.3 (32.6 – 47.3)	35.2 (24.3 – 46.8)	40.3 (39.2 – 41.1)	0.723
24 months	40.1 (35.6 – 46.8)	31.6 (22.0 – 42.7)	37.6 (34.1 – 38.6)	0.319
***p*-value[1]**	0.677	0.175	0.679	
Fat, *% daily kcal*				
Baseline	47.0 (43.7 – 53.4)	46.9 (38.1 – 50.1)	44.0 (41.3 – 45.3)	0.447
12 months	46.0 (43.3 – 51.5)	46.9 (43.2 – 51.1)	41.9 (38.6 – 49.4)	0.520
24 months	48.1 (45.8 – 50.1)	50.8 (45.1 – 53.8)	41.2 (37.6 – 46.6)	0.051
***p*-value[1]**	0.910	0.051	0.313	
SFA, *% daily kcal*				
Baseline	13.7 (13.0 – 14.6)	12.8 (11.0 – 14.0)	12.5 (11.8 – 14.1)	0.317
12 months	12.7 (11.1 – 14.6)	12.2 (11.3 – 13.1)	11.9 (10.1 – 12.7)	0.840
24 months	12.6 (10.4 – 14.4)	12.8 (11.7 – 13.9)	10.6 (9.6 – 11.6)	0.159
***p*-value[1]**	**0.043**	0.231	0.156	
MUFAs, *% daily kcal*				
Baseline	21.3 (19.7 – 27.3)	22.2 (16.4 – 25.8)	20.3 (18.4 – 24.0)	0.737
12 months	21.8 (20.1 – 26.3)	23.8 (19.4 – 27.6)	19.7 (16.8 – 25.0)	0.339
24 months	23.7 (21.3 – 25.5)	24.3 (22.3 – 28.4)	19.8 (18.2 – 21.4)	0.094
***p*-value[1]**	0.519	0.058	0.844	
PUFAs, *% daily kcal*				
Baseline	7.6 (6.8 – 8.0)	6.7 (6.1 – 8.4)	6.7 (5.6 – 8.8)	0.574
12 months	7.7 (7.0 – 9.0)	7.2 (6.2 – 7.8)	6.5 (6.0 – 8.5)	0.220
24 months	7.7 (7.3 – 8.6)	7.3 (6.3 – 8.1)	6.8 (6.5 – 7.7)	0.115
***p*-value[1]**	0.301	0.570	1.000	
Sodium, *mg/day*				
Baseline	3693 (3240 – 5576)	4455 (3547 – 5517)	3451 (2636 – 5621)	0.552
12 months	3499 (2965 – 4876)	3969 (3485 – 4860)	3993 (3501 – 5914)	0.475

24 months	3853 (3144 – 5367)	4540 (3112 – 6221)	3355 (3320 – 3985)	0.534
***p*-value[1]**	0.151	0.804	1.000	
Iron, *mg/day*				
Baseline	18.0 (17.5 – 20.8)	18.3 (17.0 – 21.2)	17.3 (16.5 – 18.6)	0.542
12 months	19.0 (16.7 – 20.6)	17.9 (13.6 – 21.0)	17.8 (16.4 – 18.4)	0.760
24 months	18.6 (16.3 – 20.0)	17.7 (12.7 – 22.2)	16.5 (14.6 – 17.8)	0.354
***p*-value[1]**	0.519	0.323	0.219	
Zinc, *mg/day*				
Baseline	15.2 (14.1 – 16.6)	14.6 (11.7 – 16.3)	13.8 (13.2 – 14.3)	0.406
12 months	14.5 (13.1 – 15.4)	12.7 (10.6 – 15.5)	14.2 (12.9 – 14.9)	0.665
24 months	13.8 (11.9 – 15.2)	14.2 (9.1 – 18.2)	12.4 (11.5 – 12.7)	0.583
***p*-value[1]**	0.052	0.274	0.219	
Total carotenoids, *µg/day*				
Baseline	7030 (4289 – 7972)	5399 (3948 – 8562)	5906 (3121 – 11250)	0.917
12 months	7283 (3543 – 8353)	5085 (3304 – 7320)	4253 (3488 – 4958)	0.581
24 months	6662 (4769 – 7683)	5462 (3389 – 8601)	4952 (3305 – 5902)	0.650
***p*-value[1]**	0.850	0.464	1.000	
Vitamin C, *mg/day*				
Baseline	309 (213 – 423)	234 (197 – 278)	186 (186 – 334)	0.267
12 months	340 (151 – 414)	212 (165 – 254)	217 (169 – 324)	0.388
24 months	289 (247 – 347)	196 (128 – 279)	198 (160 – 379)	0.119
***p*-value[1]**	0.569	0.083	0.688	
Vitamin E, *mg/day*				
Baseline	20.6 (17.8 – 24.5)	17.5 (15.3 – 22.2)	17.8 (14.7 – 19.2)	0.195
12 months	19.9 (18.2 – 22.6)	19.5 (15.0 – 22.7)	20.3 (14.7 – 23.6)	0.994
24 months	19.6 (17.7 – 25.2)	17.4 (14.6 – 27.1)	17.7 (14.7 – 20.8)	0.591
***p*-value[1]**	0.850	0.376	0.312	
Total polyphenols, *mg/day*				
Baseline	1064 (772 – 1394)	767 (538 – 851)	830 (677 – 1450)	0.107
12 months	1092 (775 – 1520)	870 (681 – 1056)	1175 (1100 – 1295)	0.320
24 months	1135 (627 – 1493)	788 (536 – 951)	1126 (1107 – 1638)	0.115
***p*-value[1]**	0.733	0.058	0.210	
Alcohol, *g/day*				
Baseline	1.0 (0.3 – 2.0)[a]	7.2 (2.8 – 8.7)[b]	1.9 (0.6 – 6.7)[a]	**<0.001**
12 months	0.5 (0.2 – 0.8)[a]	12.4 (11.4 – 13.4)[b]	3.9 (3.3 – 4.6)[a]	**<0.001**
24 months	0.3 (0.0 – 1.0)[a]	12.4 (11.2 – 12.6)[b]	3.9 (3.3 – 4.6)[a]	**<0.001**
***p*-value[1]**	0.164	**<0.001**	0.313	

Abbreviations: AB, Alcoholic beer; NAB, Non-alcoholic beer; SFA, Saturated fatty acids; MUFAs, Monounsaturated fatty acids; PUFAs, Polyunsaturated fatty acids. Data are expressed as median (Q1 – Q3).

p-value[1] refers to the intragroup statistical comparison between baseline and 24-month follow-up of each study arm, performed through the matched-pair signed-rank test for variables with symmetric distribution or sing-test of matched pairs test in case of asymmetrically distributed variables.

p-value[2] refers to the intergroup comparisons made with Kruskal–Wallis test followed by Dunn's test.

p-value < 0.050 is considered statistically significant, highlighted in bold.

Different superscripts (a, b) on the same row are significantly different.

Figure legends

Supplementary Figure 1. Evolution of biochemical markers for cardiovascular disease risk that showed significantly different variations between the study groups at the end of the intervention

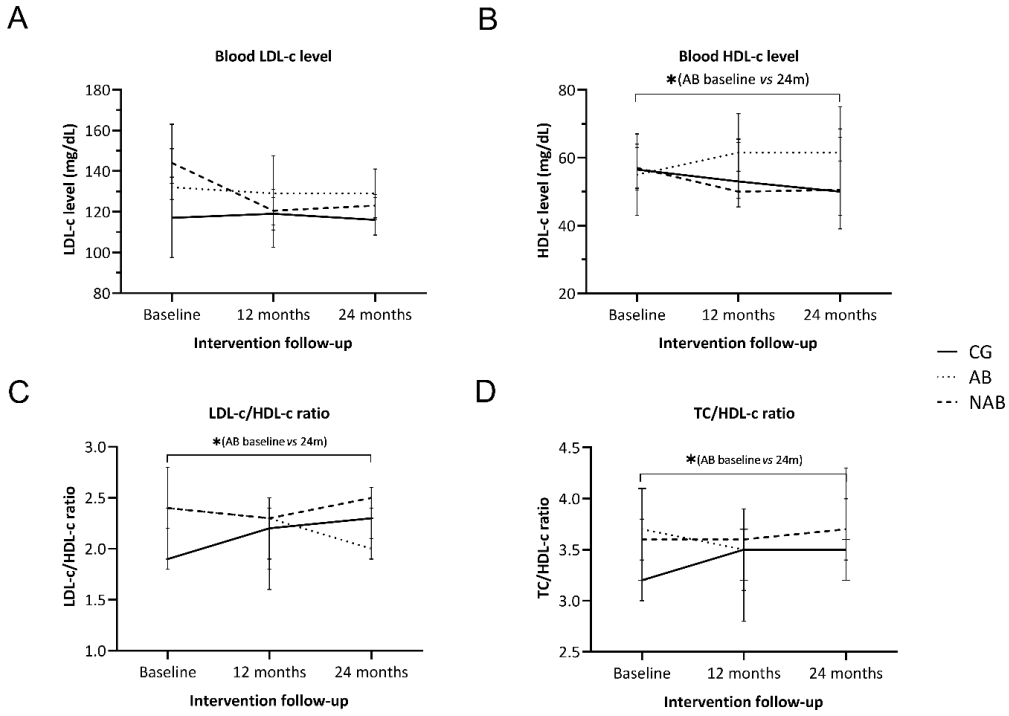
Abbreviations: CG, Control group; AB, Alcoholic beer group; NAB, Non-alcoholic beer group; TC; LDL-c, Low-density lipoprotein cholesterol; HDL-c, High-density lipoprotein cholesterol.
Intragroup statistical comparison between baseline and 24-month follow-up of each study arm was performed through the matched-pair signed-rank test.
Intergroup comparisons were made with Kruskal–Wallis test.
Declared data correspond to medians and their respective quartiles (Q1 and Q3).
*Existence of significant differences intra or intergroup, as specified in each graph. p-value < 0.050 is considered statistically significant.

Supplementary Figure 2. Evolution of the cardiovascular disease risk variables of interest related to body composition and blood pressure or that showed significantly different variations between the study groups at the end of the intervention

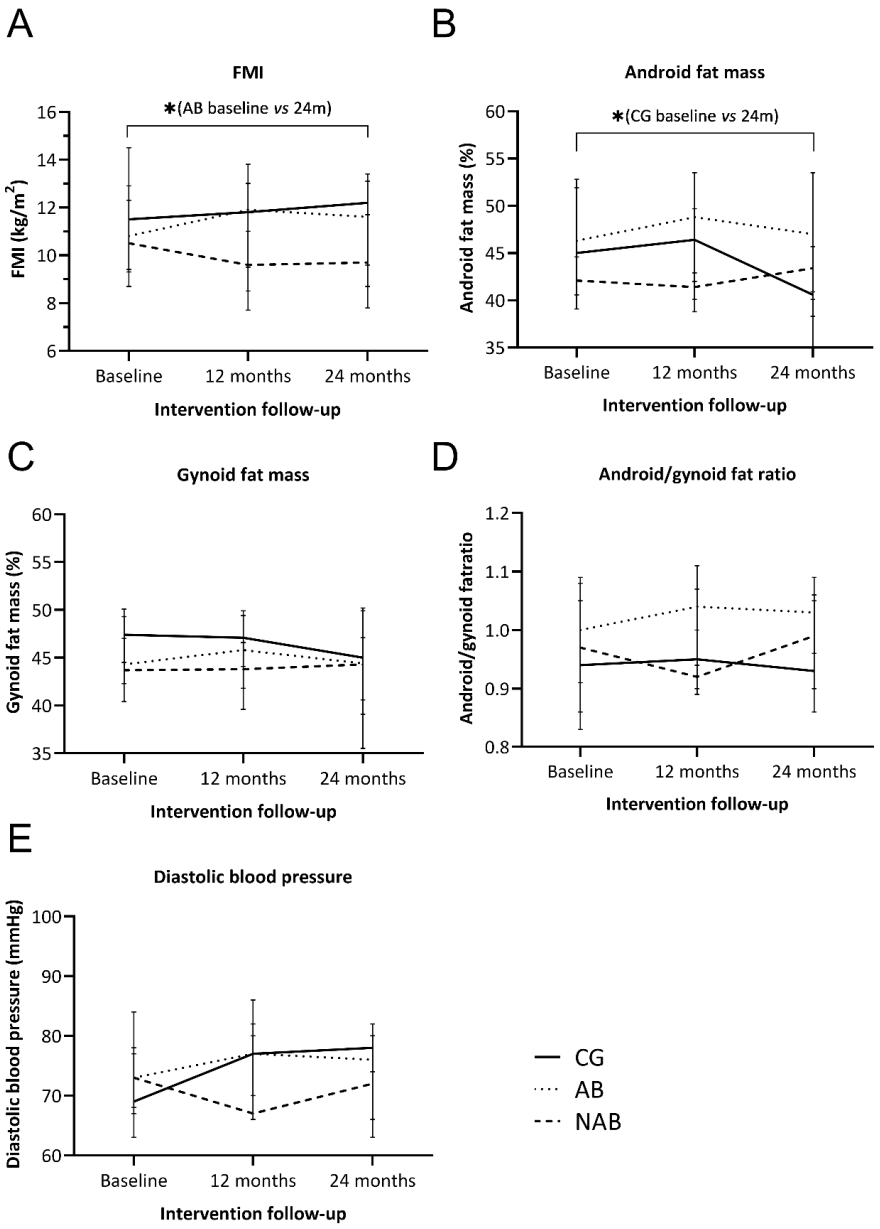
Abbreviations: CG, Control group; AB, Alcoholic beer group; NAB, Non-alcoholic beer group; FMI, Fat mass index.
Intragroup statistical comparison between baseline and 24-month follow-up of each study arm was performed through the matched-pair signed-rank test.
Intergroup comparisons were made with Kruskal–Wallis test.
Declared data correspond to medians and their respective quartiles (Q1 and Q3).
*Existence of significant differences intra or intergroup, as specified in each graph. p-value < 0.050 is considered statistically significant.

Supplementary Figures

Supplementary Figure 1



Supplementary Figure 2



8.2. Other publications

- Parilli-Moser I, Domínguez-López I, **Trius-Soler M**, Castellví M, Bosch B, Castro-Barquero S, Estruch R, Hurtado-Barroso S, Lamuela-Raventós RM (2021). Consumption of peanut products improves memory and stress response in healthy adults from the ARISTOTLE study: A 6-month randomized controlled trial. Clinical Nutrition 40:5556–5567

- **Trius-Soler M*,** Huarte E*, Domínguez-Fernández M, de Peña MP, Cid C (2022). (Poly)phenol characterisation in white and red cardoon stalks: could the sous-vide technique improve their bioaccessibility? International Journal of Food Sciences and Nutrition 73:184–194

- **Trius-Soler M***, Hurtado-Barroso S*, Lamuela-Raventós RM, Zamora-Ros R (2020). Vegetable and Fruit Consumption and Prognosis among Cancer Survivors: A Systematic Review and Meta-Analysis of Cohort Studies. Advances in Nutrition 11:1569–1582

- Ginex T, **Trius M**, Luque FJ (2018). Computational Study of the Aza-Michael Addition of the Flavonoid (+)-Taxifolin in the Inhibition of β-Amyloid Fibril Aggregation. Chemistry - A European Journal 24:5813–5824

*Equally contributing authors

8.3. Communications

- **Trius-Soler M,** Tresserra-Rimbau A, Moreno JJ, Peris P, Estruch R, Lamuela-Raventós RM. Effect of moderate consumption of beer (with and without ethanol) on bone turnover and bone mass in postmenopausal women. In the annual VI Workshop INSA- UB. February 9th, 2022, Barcelona, Spain

- **Trius-Soler M**. Effect of moderate beer consumption on postmenopausal women health". In the Scientific Antipasti before FBHC, ECS Pitch Session. June 21st, 2022, Parma, Italy.

8.4. Awards

- Beer and Health Publication Award 2021, for the publication: **Trius-Soler M**, Marhuenda-Muñoz M, Laveriano-Santos EP, Martínez-Huélamo M, Sasot G, Storniolo CE, Estruch R, Lamuela-Raventós RM, Tresserra-Rimbau A (2021). Moderate consumption of beer (with and without ethanol) and menopausal symptoms: Results from a parallel clinical trial in postmenopausal women. Nutrients. Beer and Health initiative.

- Best poster presentation award, for the poster: **Trius-Soler M**, Laveriano Santos EP, Moreno JJ (2022). Taste perception in a college-aged cohort: the relationship among the five basic tastes. In the 3rd International Conference on Food Bioactives and Health. June 21-24, 2022, Parma, Italy.