PURSUING ADVANCES IN CARDIOVASCULAR HEALTH

Phytochemical constituents of certain foods and beverages, in particular a class of compounds called flavanols, have been avidly investigated in recent years. Flavanols, such as epicatechin and catechin, and their oligomers, the procyanidins, represent a major group of secondary, polyphenolic plant metabolites. Accumulating data from epidemiological assessments as well as controlled clinical dietary intervention studies demonstrate an association between flavanol-rich diets and cardiovascular health.

FLAVIOLA Final Publishable Summary

More information about the FLAVIOLA Project can be found at www.flaviola.org

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FLAVIOLA aimed at investigating the absorption and metabolism of flavanols in humans; at establishing the levels of habitual flavanol intake in the EU; at assessing the impact of the dietary intake of flavanols on cardiovascular function, and at developing a cocoa flavanol-containing food product prototype with utility in the context of cardiovascular health.

Flavanols are commonly present in most higher plants, and their high content in certain food plants, such as VITIS VINIFERA (grape wine), CAMELLIA SINENSIS (tea), MALUS DOMESTICA (apples and other pome fruit), as well as THEOBROMA CACAO (cocoa) are especially noteworthy in the context of human nutrition. However, the actual flavanol content of any food ultimately depends on the plant species from which the compounds originate, the specific climate- and agricultural conditions, as well as the methods employed for harvesting and processing the plant material in the context of food manufacture or -preparation. Based on the chemical properties of flavanols, it is not surprising that many commonly employed processes in food preparation [fermentation, heating, pH-modifications] cause a generally dramatic decrease in the flavanol content of foods, as well as the isomerization of a significant quantity of the remaining flavanols. If our knowledge of the importance of stereo-chemical properties on the physiological effects of nutrients are applicable to flavanols, not just the loss of flavanols during food manufacture, but also the transformation of (-)-epicatechin into (-)-catechin (isomerisation), as it occurs during food processing, is an important aspect when considering the potential health benefits of flavanol-containing foods. Despite of this, current epidemiological studies and dietary intake assessments fall short of providing information on this level of granularity. Moreover, a large proportion of currently published dietary intervention studies exhibits significant gaps with regard to: [a] the adequate characterization of the flavanol- and nutritional content of the foods tested; [b] the use of appropriate controls [nutrient-, and calorically-matched, flavanol-free test materials]; and [c] the employment of sufficiently rigorous study designs. Last, but not least, little is currently known about the potential molecular

mechanisms of action (MOAs) that underlie the pharmacodynamic effects observed following flavanol intake. This circumstance is largely due to the fact that almost all previously published papers on this topic do not investigate mammalian flavanol metabolites, i.e. the compounds systemically present in humans following flavanols intake. Thus, such investigations ignore the significant consequences of absorption, distribution, metabolism, and excretion (ADME) on the biological/pharmacological properties of flavanols, and as such the outcomes of the study of native flavanols in cell culture or other in vitro systems is highly unlikely to be very informative with regard to potential mechanisms of action relevant to MOAs in vivo.

It is in this overall context that FLAVIOLA employed a multidisciplinary, translational research approach, aimed at synergistically integrating data and know-how from analytical chemistry, the study of ADME and population-based dietary flavanol intake, clinical dietary intervention studies, MOA assessments in vitro, and food product prototype development and testing. In doing so, the FLAVIOLA research consortium envisioned to proactively address the gaps and challenges detailed above, by employing a state-of-the-art analytical and clinical study infrastructure, and by conducting rigours and well-designed investigations in vivo as well as in vitro. ■

WORKPACKAGE

1

ANALYTICS, FOOD MATRIX & METABOLISM

This WP was primarily concerned with the development of analytical platforms, the assessment of human flavanol absorption and metabolism, and the design and manufacture of cocoa flavanol-containing food product prototypes, which were subsequently tested in the context of FLAVIOLA's clinical dietary intervention studies.

In particular, the development and deployment of various analytical platforms for the analysis of flavanols and their metabolites in biological samples was an important FLAVIOLA-enabling mile stone that was accomplished. Validated by inter-laboratory ring trials, and successfully employed to analyse the large number of samples that emanated from FLAVIOLA activities, the analytical platforms also inspired work ultimately resulting in the 1st AOAC-accredited method for the analysis of cocoa flavanols and procyanidins in food products.

Another important aspect of WP1 was the successful development of an innovative, functional, and nutritionally-responsible cocoa flavanol-containing food product prototype, as well as its zero-flavanol, nutrient-matched control product, which was essential for undertaking controlled, double-masked dietary intervention studies in the context of FLAVIOLA. The development of cocoa flavanol-containing product prototypes in the context of FLAVIOLA required the consideration of 3 key characteristics that such a product should exhibit: (1) The nutritional profile, i.e. the macro-, micro-nutrient content, as well as the energy content [caloric load] of the test product needed to be optimized for applications in the context of cardiovascular health. Thus, the product was envisioned to be low in energy [calories], low in saturated fats, and low in sugars as well as sodium. (2) The test product should also fulfil various consumer-centred criteria that support use and compliance (taste, ease of use, preparation, transport and storage), and the product needed to adhere to all regulations governing food safety; (3) As the product prototypes are initially intended for use in clinical dietary intervention studies, the content of potentially bioactive constituents other than cocoa flavanols needed to be considered.

Consequently, the test products were standardized with regard to their cocoa flavanol content and profile, and with regard to their macro-, micro-nutrient contents. Furthermore, an adequate control product, closely matched to the flavanol-containing test product in terms of appearance, taste, macro-, micro-nutrient content and caloric load, but not containing cocoa flavanols, was also required. Following the development of the test products, a production infrastructure for the manufacture of the larger amounts (approx. 12,000 sachets) required by FLAVIOLA had to be established, and successfully produced the required test products in accordance with FLAVIOLA timelines and requirements.

WP1 was also concerned with establishing basic information with regard to the intra- and inter-individual variations in cocoa flavanol absorption and metabolism. Such information are highly relevant, especially in the context of future dietary recommendations or guidelines, as large differences in absorption, for example, between individual intake occasions or across different segments of the general population would make the establishment of general nutritional recommendations complex or even impossible. Laying the necessary groundwork for future investigations, WP1 assessed the intra- and inter-individual variations present in a relatively homogeneous study population consisting of young healthy Caucasian males. Circulating flavanol metabolites in plasma and urine were analysed by HPLC using the analytical platforms developed. The results demonstrated that the intra- and inter-individual variability in flavanol absorption, excretion, and metabolism was relatively low, and comparable to other nutrients for which dietary guidelines have already been established, thus supporting the notion that future, population-based cocoa flavanols intake recommendations are tenable from this perspective.

As an example for potential nutrient-nutrient interactions, WP1 also studied whether or not such interactions exist between dietary cocoa flavanols and nitrate, and if so, whether these interactions may influence the ADME or efficacy (as measured by arterial dilation [FMD]) of either food constituent, respectively. Employing a randomized, double-masked, cross-over study design, FLAVIOLA investigators conducted a series of dietary intervention studies in this context. Taken together, our data show that both, cocoa flavanols and nitrate, acutely increase endothelial-dependent arterial functions in healthy humans when taken orally together and separately. However, no additive/ synergistic effects on maximally achieved arterial dilation were observed. ■

WORKPACKAGE

2

SUSCEPTIBILITY, AGE & GENDER ASPECTS

The overall goal of WP2 was to establish data on the population-based, habitual, dietary intake of flavanols in the EU, and to investigate specific subsections of the population (i.e. young vs. old persons) with regard to potential differences in CF absorption and efficacy. In addition, using primary human cells in culture, WP2 also aimed at undertaking screenings for potential cytotoxic effects.

n order to assess safe use as well as potential risks that may be associated with the dietary intake of flavanol-containing foods, it is essential to establish a basic understanding of the average habitual dietary intake of flavanols. In this context, a key objective was to determine the flavanol intake in various EU member states and to identify main dietary sources of flavanols using the Comprehensive European Food Consumption Database published by EFSA. Food consumption data for adults [18 to 64 years of age] that originated from 21 individual surveys, and which represent a total of approximately 30,000 individuals are available from 14 EU Member States. In particular, these data sets provide information on food consumption in Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Spain, Sweden, and the UK. In order to assess the dietary intake of flavanols in the EU, we developed a FLAVIOLA Food Composition Database for the Flavanol Content of Foods in Europe. This database contains approximately 3,000 food items, and is based on information provided by the US Department of Agriculture (USDA) as well as by Phenol-Explorer, a comprehensive database on polyphenol content of foods. The habitual dietary intake of flavanols was estimated for 2 major flavanol subgroups, namely [1] flavan-3-ols (catechin, epigallocatechin, epicatechin, epicatechin-3-gallate, epigallocatechin-3-gallate, gallocatechin, catechin-3-gallate), and [2] proanthocyanidins (PA; including flavanol dimers, -trimers, -4-6mers, -7-10mers, polymers with more than monomeric subunits). Our database was expanded and complemented with information from the food database DINER (Data Into Nutrients for Epidemiological Research), which was specifically created for the EPIC (European Prospective Investigation into Cancer and Nutrition) Norfolk cohort study of 25,000 men and women in Norfolk, UK. DINER was used for the calculation of the estimated flavanol content of approximately

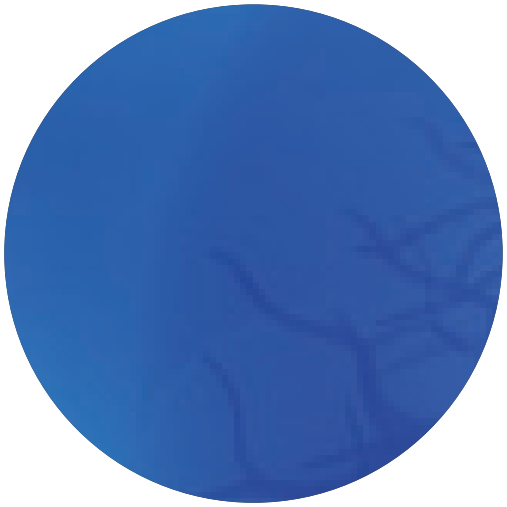
2,500 food items, including approx. 800 food recipes, as well as for allowing for the calculation of the impact of food processing. In summary, the outcomes of this work identified tea, pome fruit (apples, pears, etc.), berries and small fruit, cocoa products, and stone fruit as the main food sources when considering total habitual flavanol intake in the EU. Tea consumption was the major single contributor to flavan-3-ol intake followed by the consumption of pome fruit. Pome fruit were also identified as the most important source for PA. The average habitual intake for total flavanols was found to be 201.9 mg/day, while the average intake of flavan-3-ol monomers and PAs was 78.5 mg/day and 123.4 mg/day, respectively. Considering specific flavanol-3-ol monomers, the main contributors were (-)-epigallocatechin-3-gallate and (-)-epigallocatechin, representing 26.7% and 23.1% of the total flavanol-3-ol intake, respectively.

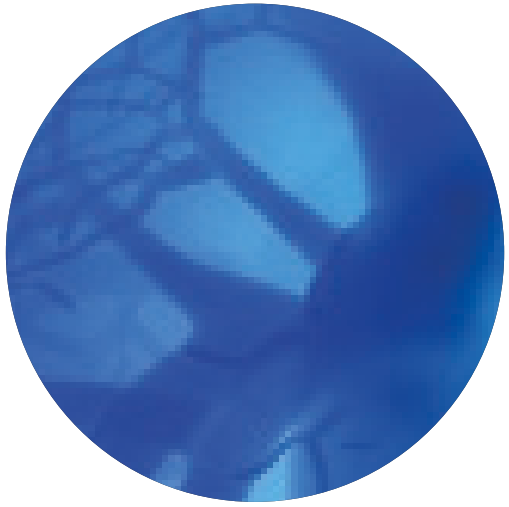
Recent studies in elderly populations suggest that there is an age-dependent decline in the absorption of certain nutrients and drugs. In this context, WP2 assessed whether or not there is an age-dependent change in flavanol absorption, metabolism and excretion following the consumption of the CF-containing FLAVIOLA food product prototype, and also studied whether or not there is an age-dependent decline in the vascular response to flavanols. In order to assess whether the absorption and metabolism of flavanols is age dependent, a randomized, crossover intervention study was performed in 40 healthy, Caucasian men aged 18-35 y (young) or 60-80 y (elderly). The participants received either 5.3 or 10.7 mg of total CF per kg body weight (BW).

In addition, in related experiments the volunteers ingested 1 g of acetaminophen to study age-related differences in phase II drug metabolism and to enable benchmarking against the CF data obtained. Blood and urine samples were collected over 24 hours post-consumption with 1 week washout in between interventions. Plasma and urine levels of flavanol-, acetaminophen- and methylxanthine metabolites were analysed by HPLC with UV-Visible, fluorescence, and electrochemical detection. The area under the curve of the plasma concentration over time (AUC) of total plasma flavanols was not significantly different between young and elderly subjects. However, the AUC of individual metabolites, in particular (-)-epicatechin-3'-ß-D-glucuronide was found to be significantly greater in elderly individuals as compared to the group of the young. On the contrary, the AUCs of 3'-O-methyl-(-)-epicatechin-5-sulfate and 3'-O-methyl-(-)-epicatechin-7-sulfate were lower in elderly as compared to young men. Although no age-related differences were found in the urinary excretion of total flavanol metabolites, the urinary excretion of □-valerolactone (□-VL) metabolites was lower in the elderly men as compared to young men. The plasma AUC of total acetaminophen and its glucuronide

was significantly higher in older participants, while no differences were observed for methylxanthines. Whereas our data suggest that the absorption of flavanols is not age-dependent, differences in xenobiotic metabolism are potentially important, and should be studied in more detail in future. In terms of age-dependent differences regarding the vascular responses to dietary CF, our data demonstrated that both, young and elderly, participants exhibited measurable and statistically significant improvements in endothelium-dependent arterial dilation (FMD), diastolic blood pressure, vasodilator capacity of resistance arteries, perfusion of the microcirculation and red blood cells deformability. In line with the ADME data described above, no age-dependent differences in the magnitude of the response with regard to the above parameters were noted. These outcomes demonstrate that dietary CF interventions are able to improve cardiovascular function in healthy young and elderly subjects. However, we noted statistically significant differences with regard to CF-intake-related changes in systolic blood pressure, a feature physiologically linked to vascular stiffening related to aging. In this context, our data suggest that CF intake decreases systolic blood pressure only in elderly subjects by decreasing arterial stiffness. Greater detail is provided below [WP3].

In the context of the WP2-related studies on cultured human cells that aimed at screening for potential adverse effects we conducted various experiments by testing a wide range of concentrations, the highest of which included supraphysiological levels that would practically not occur in humans in the context of dietary intake. Our data show that exposure of human umbilical vein endothelial cells (HUVECs) to (+)-catechin, (-)-epicatechin, and their metabolites at concentrations ranging from (0.4 µM to 100 µM) did not result in the observation of relevant cytotoxicity. ■





WORKPACKAGE

3

ORGAN FUNCTIONALITY AND ORGANISM VITALITY

This WP was aimed at undertaking clinical dietary intervention studies to investigate the effects of CF intake on cardiovascular functions, to provide a Proof-Of-Efficacy for the CF-containing food product prototype developed in the context of FLAVIOLA, and to study the longer-term effects of CF intake in a larger cohort of a healthy European population. In addition, and to potentially gain further insights into the MOAs, which are causally related to the CF effects seen in humans, WP3 also studied potential CF-mediated effects on cardiovascular morphology and function in vivo, as well as the potential impact of CF on nitric oxide (NO)-dependent processes, involving both, NOS-catalysed NO synthesis, as well as interactions between CF and dietary nitrate and nitrite.

Building upon the achievements of WP1 and WP2, especially with regard to analytical platforms and the successful development of a standardized CF-containing food product prototype and its matched zero-CF control, WP3 employed a state-of-the-art cardiovascular research infrastructure to undertake high-quality clinical dietary intervention studies. WP3 was able to address some of the shortfalls common in nutritional research, by studying well characterized CF test products, by using an adequate CF-free control, which allowed for establishing causality and for double-masking, and by investigating accredited primary- and secondary endpoints and biomarkers, which allowed for meaningful outcomes assessment in the context of cardiovascular health and primary- and secondary prevention. Moreover, as any future dietary recommendations or guidelines would require data from healthy populations, and as such data from well-controlled, longer-term flavanol-based dietary intervention studies are currently scarce, WP3 prospectively addressed this need by conducting studies in healthy populations.

One of the two main studies in the context of FLAVIOLA investigated the effect of

dietary CF intake on circulatory function in healthy young and elderly persons. The primary endpoint was endothelium-dependent arterial dilation as measured by flow-mediated vasodilation [FMD], an EFSA-accredited endpoint. Secondary endpoints tested the changes in cardiac output, conductance of conduit- and resistance arteries, and the perfusion in the microcirculation. Using a randomized controlled, double-masked, parallel-group study design, we recruited young (<35 y) and elderly (50-80 y) male participants. Adhering to the CONSORT statement, we comprehensively characterized the study cohorts, and established that all participants were healthy, non-smoking persons, who were not taking medication and who had no history or symptoms of vascular disease. Participants were asked to consume the CF-containing food product prototype or its nutrient-matched zero-CF control twice a day over 14 days. Both, young and elderly participants, who were part of the group receiving the CF-containing product, exhibited significant improvements in endothelial function with the effect size being independent of age. However, elderly participants showed significantly lower FMD values at the beginning of the study, which was expected as FMD/endothelial function generally decreases with age, even in healthy people. Importantly, the group consuming the zero-CF control product did not exhibit any changes or improvements with regard to the primary and secondary endpoints investigated. Taken together, our data demonstrate that CF intake was efficacious to improve endothelial function in healthy young and elderly persons. The secondary endpoint analysis revealed several novel insights into the effects of flavanols on physiological functions of the circulatory system. Our data show that cardiac function was not affected by CF intake, suggesting that the observed CF-related effects were not mediated by modulating cardiac function per se, but rather by improving arterial function and the vascular system. Along with improvements of endothelial function, beneficial decreases in pulse wave velocity as well as total peripheral resistance were also observed. These changes were associated with a significant decrease in diastolic blood pressure in both, healthy young and elderly participants. Notably, systolic blood pressure physiologically increases with age due to progressive stiffening of the large, so called conductance, arteries, a phenomenon known for more than 100 years as blood pressure augmentation. Our data show for the first time a CF-intake-related decrease in systolic blood pressure in healthy elderly men, and we were able to link this improvement to a significant decrease in arterial stiffness as indicated by statistically significant decreases in pulse wave velocity and aortic augmentation index.
In conclusion, the intake of the CF-containing food prototype, but not that of the zero-CF control product, improves circulatory function in healthy individuals, and reverses age-related increases in blood pressure and vascular stiffness. Our findings provide tenable insights that scientifically underpin the potential of CF-based dietary interventions to counteract vascular dysfunction related to aging.

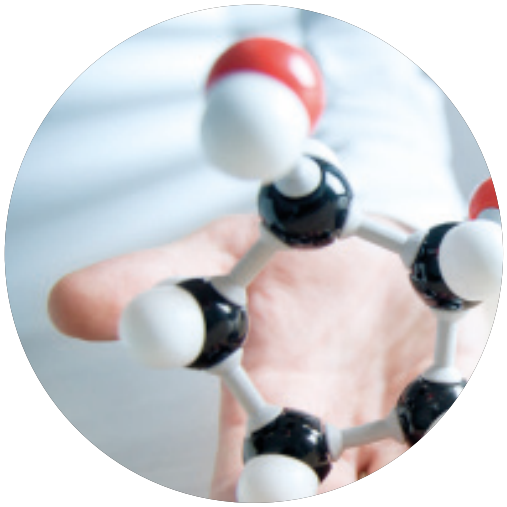
Building on the insights generated by the study described above, and to broaden our data set, FLAVIOLA also studied the longer-term effects of CF intake in a larger cohort (n=100) of a healthy Europeans. In this context, we performed a randomized, double-masked, controlled clinical dietary intervention in healthy middle-aged males and females (n=50 male, n=50 female). Again, the participants were asked to consume either the CF-containing food product prototype or the zero-CF control twice daily over 30 days, a time-frame considered by EFSA as being relevant in the food-claims regulatory framework related to cardiovascular health benefits [endothelial function and blood pressure]. Endothelial function as measured by FMD [an EFSA-accredited endpoint] was chosen as the primary endpoint. In addition, and to assess the impact of CF intake on vascular health in this healthy population, we also chose prognostically validated clinical determinants of atherosclerosis development and progression, including blood pressure, cholesterol, and glucose. At the present time, the study has been concluded with regard to the cohort of middle-aged male participants, but is still ongoing in the female study cohort. A preliminary data analysis of outcomes from the male cohort shows significant CF-intake-related improvements in endothelial function and blood pressure, effects similar to those observed in the elderly participants discussed above. However, a final outcomes assessment cannot be undertaken before the final completion of study, including the female study cohort, which is expected for the end of May, 2013. ■

FLAVIOLA COMPRISES 8 PARTNERS FROM 7 EUROPEAN COUNTRIES, INCLUDING UNIVERSITIES, INDUSTRY PARTNERS, SMES AND RESEARCH INSTITUTES

WORKPACKAGE

4

CELLULAR TARGETS AND MECHANISMS



WP4 aimed at discovering the molecular mechanisms of action (MOAs) that dietary flavanols exert in the human vasculature. For this purpose, standardized cell culture models of human vascular- and inflammatory cells were used to study flavanol-specific effects on cell-cell-interactions, redox-sensitive processes, protein signalling and gene expression pathways as well as epigenetic mechanisms. In addition, the effects on blood flow and new blood vessel formation were investigated on an organism level

Based on the use of flavanols, as well as their main mammalian metabolites, as systemically present following CF intake in vivo, our data demonstrate that flavanols and some of their metabolites are able to effectively attenuate inflammatory processes in vitro by affecting the adherence of inflammatory cells to the vascular endothelium and by the modulation of the expression of key proteins and genes involved in inflammation. Along the same line, we found that flavanols and their metabolites trigger DNA methylation changes in vascular endothelial cells and blood-born cells that indicate the modulation of gene expression in the context of cell adhesion. Moreover, flavanols and their metabolites effectively protect vascular cells from extra- and intracellular oxidative damage, maintaining the anti-inflammatory action of glucocorticoids in the presence of oxidative stress. Flavanols positively affect the restoration of blood flow and new blood vessel formation in vivo. Taken together, flavanols and some of their metabolites attenuate various cellular- and subcellular processes that are believed to be causally related to the development of cardiovascular diseases. The novel insights emerging from WP4 provide mechanistic insights for the explanation of the cardiovascular health effects observed following CF intake in humans, and will help to pave new avenues for novel therapeutic strategies and dietary guidelines in this field. ■

OVERALL SUMMARY OF FLAVIOLA OUTCOMES

TAKEN TOGETHER, FLAVIOLA RESULTED IN THE FOLLOWING KEY OUTCOMES

- ***Development of optimized CF-containing food product prototype;***
- ***Creation of dedicated analytical platforms;***
- ***Elucidation of flavanol ADME;***
- ***Assessment of habitual dietary intake of CF and procyanidins in EU;***
- ***Demonstration of CF-mediated cardiovascular health benefits in clinical dietary intervention studies:***
  - ***Longer-term effect in healthy individuals***
  - ***Assessment of age-dependent CF efficacy***
  - ***Evaluation of applicability to general population***
- ***Novel insights into potential mechanisms of action;***