Final Report Summary - PATHWAY-27 (PIVOTAL ASSESSMENT OF THE EFFECTS OF BIOACTIVES ON HEALTH AND WELLBEING. FROM HUMAN GENOMA TO FOOD INDUSTRY - PATHWAY)

Executive Summary:
The PATHWAY-27 project started in 2013 with the aim to better understand the physiological effects and mechanism of action of certain bioactive compounds and to develop bioactive-enriched foods (BEFs) with scientifically demonstrated impact on health, especially on metabolic syndrome (MetS).

Three bioactive compounds (oat β-glucans - OBG, anthocyanins - AC and docosahexaenoic acid - DHA) alone or in combinations were used for enriching foods from three different food matrices. In each food
Three bioactive combinations were used for enriching foods from three different food matrices: dairy, bakery, and egg-based products. In total, forty-five BEFs (3 categories * 3 products * 5 bioactive combinations) were developed and produced, which provided satisfactory chemical, physical, microbiological and sensory properties. BEFs were further prioritized based on sensory attributes, results of consumer acceptance tests, microbiological quality, and bioaccessibility and chemical stability.

The selected products (milkshakes, biscuits, pancakes) enriched with five different bioactive combinations were used in three 4-week human pilot studies involving volunteers at risk of MetS. The aim of the pilot studies was to identify the enrichments with the greatest beneficial effect on the two primary clinical endpoints, serum triglycerides and HDL-cholesterol. The selected BEFs were then used in a 3-month multicentric, double-blinded, randomised, placebo-controlled human intervention study run in Italy, France, Germany and the United Kingdom, involving volunteers at risk of or with MetS. The dietary trial allowed not only the evaluation of clinical outcomes; various omics techniques (metabolomics, microbiomics, epigenomics, genotyping, and integrative analyses) were also applied on plasma, urine, stool, blood cells and fat biopsies collected from volunteers.

In parallel, the bioaccessibility and bioavailability of the bioactive compounds were investigated using a pig model, and the effect and mechanisms of action of the bioactive compounds or their primary metabolites (propionate as metabolite of OBG and protocatechuic acid as metabolite of AC) were studied at the cellular and molecular level. Liver and fat cell cultures served as representative models of the main organs involved in regulating certain risk factors of MetS.

Overall, food enrichment with DHA allowed to obtain BEF whose consumption increased blood HDL-cholesterol (“good cholesterol”) in volunteers with low HDL-c at baseline. The positive effect of BEF was already achieved after 6 weeks of consumption, and it lasted over the whole period of treatment. The HDL-c increasing effect was more evident using BEF enriched with DHA+AC. In addition, the group receiving DHA+OBG experienced a clear reduction of LDL-c level (“bad cholesterol”). Consumption of food enriched with DHA was also associated with significant reduction of systolic blood pressure. Notably, the health effects were obtained administering DHA at doses far below the DHA amount (3 g per day) needed to bear the health claim “DHA and EPA contribute to the maintenance of normal blood pressure”. So far, no claim exists on the effect of DHA on HDL-cholesterol. This underlines that the effect of bioactives could be different when they are delivered in combination as ingredients of functional food instead of isolated molecules (or dietary supplements).

The investigation of novel omics technologies and their integration gave new insights into the complex relationships linking genome, metabolism and microbiota, and their relation to nutrition and risk of diseases. Last, the complementary in vitro and in vivo studies generated new and important findings in the field of nutrition research.

Activities performed in PATHWAY-27 enabled the project partners to identify the knowledge gaps associated with (i) the development of BEF and (ii) the clinical trials essential to substantiate the effect of BEFs and eventually the related health claim. These informations were used to develop two sets of Guidelines, the Guidelines for the Food Industry (mainly SMEs) and the Guidelines for the Scientific Community. Both Guidelines are freely available from the PATHWAY-27 project website at www.pathway27.eu.

Project Context and Objectives:
In the global scientific scenario, research on bioactive compounds is very active. Notwithstanding, several
In the global scientific scenario, research on bioactive compounds is very active. Notwithstanding, several questions about the effectiveness of bioactives as preventive agents to reduce the risk of disease, as well as the effectiveness of bioactive-enriched foods (BEF) are still waiting for an answer.

Bioactive compounds (natural components of foods that possess biological activity in addition to their nutritional value) normally occur at very low concentrations in foods, usually far from the optimally effective dose range. To overcome this, the food and beverage industry is developing new products containing elevated concentrations of selected bioactives, so-called “bioactive-enriched food” (BEF).

While formulating a new BEF, the interaction between added bioactives and the food matrix is seldom taken into consideration. Other constituents in a food matrix could aid or hinder the bioaccessibility and bioavailability of the bioactives. The effective dose of the isolated bioactive could change if administered as part of a specific food, since other components already present in the food matrix could have a positive or negative impact on the bioactive final effect. In addition, bioactives can be affected by food processing and storage conditions.

Research on the effect of bioactives is often related more to the theoretical possibility of their impact on health rather than on their real, practical utilization in everyday diets. Most intervention studies administer bioactives as pure compounds, assuming there are no confounding effects related to the food matrix and to food production, and the administration of the bioactive as a standing alone molecule (i.e. as a supplement) or as a BEF has the same effect.

PATHWAY-27 did not consider bioactives as discrete molecules, but as ingredients of BEF that can be part of an everyday diet. BEF within three different matrices (bakery, dairy, and egg-based) were formulated, produced and utilized to perform clinical intervention trials.

The three matrices chosen in PATHWAY-27 were selected because they make major contributions to the diet, and their production is of major economic importance across Europe and relevant to SME and artisan producers. In addition, although they have different compositions, they share the common characteristic of being natural sources of potential bioactives (i.e. fibre for bakery and bioactive peptides for dairy and egg-based products) that could contribute to the final healthy effect of the resulting BEF.

The creation of an effective BEF also depends on a better understanding of the complex interrelationship between the bioactive and the food matrix, and between food structure and performance (Aguilera, JM, 2006). Formulation of a food supplement is much easier than a BEF, and the logical consequence is the extensive marketing and use of supplements delivering the effective bioactive dose. The extensive use of food supplements is in marked contrast to the concept of the Food Based Dietary Guidelines (FBDG), which are centred on foods, rather than nutrients or bioactives. Overcoming the challenges related to BEF formulation can provide opportunities to increase market shares and stimulate economic growth and employment to European food and drink manufacturers. Furthermore, it can enable consumers to a better food selection that together with healthy lifestyle choices is associated with improved health. The preventive nutrition approach based on BEF has gained considerable consumer support because, in general, people prefer consuming tasty BEF rather than no longer eating foods that they enjoy.

The legitimate concerns of many consumers to improve their health and wellbeing in a natural manner led to the adoption of adopted Regulation (EC) 1924/2006 on the use of nutrition and health claims for foods. One of the key objectives of this Regulation is to ensure that any claim made on a food label in the EU is clear and substantiated by scientific evidence. The European Food Safety Authority (EFSA) is responsible for verifying the scientific substantiation of the submitted claims, and human intervention studies are mandatory for health claim approval.

To effectively assess the health-promoting and risk-reducing activities of BEF, their effectiveness must be evaluated against measurable, physiologically relevant endpoints. The lack of strong markers of
evaluated against measurable, physiologically relevant end-points. The lack of strong markers of effectiveness is one of the weak points of clinical studies aimed to substantiate health claim, and it often causes EFSA’s rejection. In PATHWAY-27, we chose to evaluate the effect of BEF administration on the metabolic syndrome (MetS) since there are established criteria for its diagnosis, all of them representing a measurable, physiologically relevant end-point.

Acting this way, PATHWAY-27 bridged the gap associated with weak markers of effectiveness. In addition, since one of the promise of omics technologies is to contribute to the process of biomarker development and in recent years their use has led to the discovery of many candidate biomarkers (Quezada H et al, 2017), state-of-the-art omics methodologies (epigenetics, genetics, metabolomics and microbiomics) have been used in the PATHWAY-27 clinical trial to develop biomarkers that may be useful in guiding nutritional therapies, addressing disease risks, and predicting clinical outcomes.

Clinical trials are very often focused on demonstrating the effect of bioactives or BEF on specific end points, without considering the underlying mechanism that is usually studied in vitro. This creates a scientific dichotomy, and the assumption without any demonstration that the in vivo effects are related to the mechanism/s of action observed in vitro represents an enormous, and unscientific, gap in our knowledge.

PATHWAY-27 strategy relied on an integrated in vitro-in vivo approach aimed to reduce the knowledge gap on the mechanisms of action of bioactives, allowing a better understanding of the clinical effect of BEF and its underlying mechanism/s of action. Two different cell models were studied in vitro, adipocytes and hepatocytes, due to their central role in the onset of MetS. Instead of OBG and AC, their metabolites propionic acid (PRO) and protocatechuic acid (PCA) were used for in vitro investigations. Omics technologies (epigenetics, transcriptomics and metabolomics) were used also in cultured cells, and their integration in vitro and in vivo was a powerful and innovative strategy for elucidating the role and mechanism of action of bioactives and BEF.

Contradictory results between in vitro and human studies are often attributed to “bioactive availability”. In BEF, bioactive availability is also dependent on the food matrix, which can modify bioactives digestibility and bioavailability, thus introducing a fundamental bias when translating in vitro data to humans. In PATHWAY-27, the digestibility of BEF and consequent bioavailability of bioactives has been assessed in a pig model, and the bioavailability of bioactives in the PATHWAY-27 BEF has been assessed in volunteers recruited in the pilot intervention studies through direct measuring their concentration in blood, and evaluating the changes in the blood and urine metabolome in volunteers enrolled in pilots and large intervention studies.

Finally, while investigating bioactives and BEF in humans, it is not possible to assume the same effects in all individuals. Several factors (such as lifestyle, diet, genetic variation) can contribute to conflicting results (Guarner-Lans et al, 2011).

In PATHWAY-27 the intervention trial took place in four different countries (IT, FR, UK and DE), thus allowing comparisons of subjects with different dietary habits and lifestyle. In addition, genetic analyses, evaluating the presence of polymorphisms (SNPs) in selected genes has been performed. Since emerging evidence is showing that dysbiosis is involved in various diseases (Yang & Duan, 2018), the effect of the nutritional intervention was also assessed on the gut microflora.

Although the marketing of new, effective BEF represents a great opportunity for the food industry and for the consumers, the number of new products bearing an health claim that is put on the market is still extremely low. One of the most significant barrier to SMEs to perform intervention studies to validate health claims on their product is the high cost. Human intervention studies are mandatory for health claim approval and attempts to reduce their cost often result in poor design and delivery of incomplete or
approval and attempts to reduce their cost often result in poor design and delivery of incomplete or unconvincing data that do not pass scrutiny by EFSA.

To by-pass this gap, PATHWAY-27 developed a strategy involving preliminary pilot studies. The three different pilot studies, each considering a food product coming from one of the selected food matrices and enriched with the selected bioactives alone or in combination, enabled to preliminary evaluate the effects of the different BEF and to select which enrichment was most suitable for achieving measurable results in the larger clinical trial. Furthermore, pilot studies allowed to manage and solve possible problems arising before/during the studies before conducting the large intervention study.

To deliver its strategy, PATHWAY-27 focused on a specific bioactive, docosahexaenoic acid (DHA, C22-6 n-3), examining its effect alone or in combination with two other bioactives: oat beta-glucans (OBG) and anthocyanins (AC). DHA, OBG and AC were used for enriching foods belonging to three different matrices (bakery, dairy, and egg-based products).

The choice of DHA was substantiated by the following reasons: 1. Long chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) improve the blood lipid profile (Binia A et al, 2017), reduce hypertension (Ueshima et al, 2007), inflammation (Jacobo-Cejudo et al, 2017), and may also interact with other dietary factors to modulate the risk factors of MetS (Poudyal et al, 2011); 2. Many intervention studies are focused on eicosapentaenoic acid (EPA) plus DHA, but it is still uncertain which of the two compounds is the most active regarding inflammatory and metabolic diseases; 3. Most intervention studies on n-3 LC-PUFA use fish oil or supplements, and very few have addressed n-3 LC-PUFA-enriched foods; 4. The positive effect of dairy products enriched with n-3 LC PUFA on blood TG and HDL-C, the primary endpoints in PATHWAY-27 intervention studies, has been demonstrated (Dawczynski et al, 2010); 5. DHA is commercially available and, according to EU legislation, may be used in foods. Furthermore, EFSA has approved an Article 13 Health Claim related to DHA effectiveness on one marker of MetS (blood triglycerides in adults), but it refers to DHA and not to food enriched with DHA.

OBG and AC have been chosen since they are considered to act on different or on the same MetS risk factor as DHA via different mechanisms of action (Wey et al, 2011; Qin et al, 2009; Liatis et al. 2002; Juvonen et al. 2009) In addition, food providing >3g OBG per day (also when divided in several servings) can bear a health claim (EFSA, 2010).

In PATHWAY-27 the combined use of DHA + OBG and DHA + AC allowed to give insight of another often forgotten issue that is the possible synergism, or eventually antagonism, between different bioactives. Although PATHWAY-27 used specific bioactives and food matrices for the formulation, design and production of the BEF, the main objective of the project was to develop generic models, best practices and “roadmaps” to be used across a much wider range of bioactives, food matrices and food types. Accordingly, a main PATHWAY-27 output was the development and dissemination of comprehensive guidelines for undertaking studies on BEF, and to support health claims, including investigations on food matrix/bioactive interactions and data analyses related to intervention studies.

References
Project Results:
The PATHWAY-27 project started in 2013 and focused on bioactive compounds, i.e. natural components of foods that possess biological activity in addition to their nutritional value. Bioactives naturally occur at very low concentrations in foods, usually far from the optimally effective dose range. To overcome this, the food and drink industry is developing new products containing high concentrations of selected bioactives, so-called bioactive-enriched foods (BEF). In the global scientific scenario, research on bioactive compounds is very active. Notwithstanding, several questions about the effectiveness of bioactives as preventive agents to reduce the risk of disease, as well as the effectiveness of BEF are still waiting for an answer.

Uniquely, PATHWAY-27 did not consider bioactives as discrete molecules, but as ingredients of bioactive enriched food (BEF) that can be part of an everyday diet.

PATHWAY-27 included 8 work packages (WPs), each one devoted to specific activities but all perfectly integrated and inter-connected to achieve the objective of the project (Figure 1).

WP1 was in charge for the coordination and management, and supervised the research activities between WPs, monitored the overall Project achievements and controlled the scientific quality and timing of the Project results. In addition, WP1 managed contacts with EU Commission and among the consortium; supervised the foreground generated by the project activities monitoring that intellectual property was properly managed, ensured an appropriate overall financial and administrative management and reporting.
properly managed, ensured an appropriate overall financial and administrative management and reporting, and took care of ethics management. Ethical issues were appropriately managed with the help of the internal Ethical Advisory Board (EAB). All protocols for experiments involving animals and human subjects were approved by the PATHWAY-27 EAB before experiments could start, and this process took place prior to submission of protocols to the relevant local research ethics bodies.

Project coordination meetings were organised at the beginning and end of the project, and every 12 months along the project duration. Several on-line meetings were also organized within different WPs, and an additional “in person” meeting for WP5 partners took place in Paris in July 2015.

The first step of the project was the formulation of the PATHWAY-27 BEF. At the project start 3 foods x 3 matrices were considered, and corresponded to buns, breadsticks, biscuits (bakery), milkshake, milk dessert, pudding (dairy), omelet, egg beverage and pancakes (egg-based). Each food was enriched with 5 bioactive combinations (DHA alone, DHA + AC, DHA + OBG, AC alone and OBG alone). In total, 45 BEF prototypes were developed.

The ingredients containing the different bioactive compounds (AC, OBG and DHA) were produced by the partner SMEs. In particular, ASL produced DHA-enriched egg yolk (2.5% OVO-DHA) and DHA-enriched phospholipids fraction (8% GPL-DHA) from the DHA-enriched egg-yolk; SOF produced OBG-enriched oat bran extracts containing 27-28% OBG; and ABRO produced AC from the skins of red grapes containing 2.5% AC. For the production of BEF, it was important to have the ingredients with high enough concentration of bioactive components as well as ensure the safety of the ingredients. For this purpose, the bioactive content, homogeneity and chemical form were determined for each bioactive formulation by the respective producer SME. The levels of bioactive components were measured and the microbiological safety was analyzed from each production batch. The producer SMEs tested the safety characteristics of bioactive formulations and certified them according to the internal procedures. All ingredients containing the bioactive compounds were in the form of dry powders, so their shelf life was at least one year starting from the production date.

In the 45 prototype BEF, bioactive content was quantified at the end of the manufacturing process, and showed an excellent recovery of DHA in all matrices. Processing led to a decrease in AC concentration in omelet and bakery products, probably due to the cooking step applied during manufacture of these BEF. This loss of AC during manufacture was overcome by increasing the amount of AC in the recipe. Finally, molecular weight of OBG slightly decreased after omelet and bakery products manufacture but the forms present were still bioactive according to the literature.

A pre-screening test to assess the overall sensory quality of the 45 prototypes was organized among partners in order to select only the best two foods in each category. This decreased the number of samples to further analyze from 45 to 30. The 30 selected BEF were in vitro digested to assess the bioaccessibility of the target bioactives. The digestion was done following the oro-gastrointestinal model developed by COST action FA1005, INFOGEST (Minekus et al. 2014). Bioaccessibility (fraction of a compound/nutrient that is released from its matrix and solubilized in the gastrointestinal tract and is potentially available for intestinal absorption) was close to 100% for all the DHA-enriched BEF. All the BEF enriched with AC alone showed higher bioaccessibility than the ones enriched with AC+DHA. OBG bioaccessibility ranged from 57 to 90% for OBG-enriched BEF and from 73 to 119% from DHA+OBG-enriched BEF. Results showed that OBG present after in vitro digestion had a high average molecular weight over the 1000 kDa in most cases. According to the literature OBG molecules higher than 500 kDa should provide similar physiological responses as the native OBG, so we concluded that almost all of the OBG present after in vitro digestion conserved its bioactive properties.

The 30 BEF were extensively studied in order to
The 30 BEF were extensively studied in order to:
- determine the shelf life;
- define the sensory profile;
- understand the consumer acceptance;
- ensure and assess the safety of the samples provided for sensory and consumer tests;
- give inputs for the final selection of BEF to be administered to volunteers in clinical studies;
- set up a questionnaire to be able to measure the preference, overall liking of the developed products during clinical studies.

Comprehensive assessment of food safety risks represented by the experimental samples was carried out. Regarding the microbiological assessment of BEF, challenge test was performed on bakery products only since egg-based products were provided frozen and dairy products as powders. Although preservatives were used to limit bacterial growth during storage of buns at room temperature, this did not avoid the appearance of molds. On the contrary, biscuits, with their low water activity, were highly stable on a microbiological point of view throughout storage.

The shelf life tests were carried out first for the targeted shelf life of 3 weeks, which were changed to 84 days in the second schedule to meet the logistic requirements of the pilot studies and to exploit opportunities provided by the changes of the preservation technology.

The complete sensory characterization of the developed products was done by the trained sensory panel. The main aim of this activity was to have clear picture about the main attributes of the products and to be able to identify the key sensory attributes that drive the consumer liking and preference. In addition, an online questionnaire, which contained questions to explore the consumers’ knowledge on health and healthy diet, bioactive enriched foods, or new and not conventional products, was administered to 120 consumers.

Since the PATHWAY-27 pilot study was evaluating the effectiveness of a single product, enriched with all the five bioactive combinations, within a specific matrix (bakery, dairy and egg-based products), results of consumer acceptance and sensory profile of the five enrichments of the same products were averaged, in order to evidence the product in each matrix having the best mean characteristics considering all enrichments. Results of the pre-screening test were also considered.

Based on results of the consumers’ acceptance test, both bakery products were suitable, and no significant difference was observed between the 2 products during sensory analysis. Among egg-based products, omelet received a slightly higher overall scores than pancake. On the contrary, pancake were slightly more popular than the omelet in the pre-screening. Beside the strange appearance of the AC enriched versions, all pancakes were described with typical pancake aromas and flavours. For evaluating the unusual appearance of the pancakes, a group of children (N: 29) were involved in the study. No significant difference between the pancake and omelet were detected in overall liking scores. Sensory evaluation of both products also appeared satisfactory. Regarding the dairy samples, there were not significant differences between the two products. Almost the same positive and negative comments were mentioned regarding both products. Most of the consumers eat these products at least every month, but the majority of the participants reported to drink milkshake every day. So the difference in the frequencies of the consumption gave priority to milkshake.

Given the importance of the product liking in the clinical studies, a questionnaire for the volunteers involved in the pilot study was developed to identify how they like the appearance, aroma, flavour and texture of the samples and how their opinion changes if they consider the health benefits of the products. The 30 BEF were analyzed by NMR spectroscopy to acquire their molecular profile, and their structural properties were investigated by Confocal Laser Scanning Microscopy.
properties were investigated by Confocal Laser Scanning Microscopy.

Comparison of all attributes between the two food products within each matrix was carried out in order to select the most suitable one considering the characteristics coming from the different five enrichments. The selection method applied was the one of the decision sieve. A decision sieve is based on the thresholds of the quality attributes setting the mesh size; BEF can be considered for selection if they positively pass the mandatory requirements above an acceptable quality threshold. In case both candidate BEF in the same matrix fulfil the selection criteria, the secondary attributes are taken into account.

Mandatory attributes were safety, chemical stability and nutrient profile. Secondary attributes were bioaccessibility of bioactive compounds, sensory characteristics, ease of preparation, ease of storage, food matrix stability.

For the bakery products, biscuits were selected instead of buns mainly for their microbiological stability during storage. The ease of preparation and storage confirmed the better suitability of biscuits over buns. For the dairy products, milkshake was selected over dairy dessert. The nutrient profile of both products was not satisfactory because of level too high in saturated fatty acids (SFA). However, the recipe of the milkshake was optimized to limit the amount of SFA whereas it was not possible to improve the recipe of the dairy dessert commercially available. The pancake was selected for egg-based products over the omelet since it showed a much better nutrient profile (too much salt and SFA in the omelet). In conclusion, the 3 products selected for the pilot study were biscuits, milkshake and pancake.

The 3 selected products enriched with five different bioactive combinations were produced by two partner SMEs at industrial scale to be used in the three 4-week human pilot studies. DPL produced biscuits (bakery product) and ADX milkshake powders (dairy product) and pancakes (egg-based product). Milkshake powder and biscuits were dry products meant to be stored at room temperature, but pancakes needed to be stored and delivered frozen at -18 °C to inhibit the growth of spoilage and pathogenic microbes. The safety characteristics of BEF were determined and certified by the corresponding producer SMEs, according to the internal procedure adopted for new products.

Shelf life of BEF was a key issue for both executing the intervention trial and possible marketing of the products. In fact, a longer shelf life allows better management of production and delivery of BEF during the trial, and consumers consider it as an important positive attribute of food products. Regarding BEF, in order to set a longer shelf life not only food safety and sensory characteristics must be considered, but also the stability of the bioactives. Thus, during the pilot study BEF products were exposed to extended shelf-life studies where microbiological and sensory quality of the products was evaluated and the contents of the bioactive compounds analysed. Taking into account the retention of bioactive concentrations as the key criteria, but sensory and microbiological quality of the BEF products as well, 3 months shelf life was considered for BEF pancakes, 8 months for BEF milkshake and 2 months for BEF biscuits.

In addition, to make sure the bioactive contents in the selected BEF were similar in all production batches, so that similar amounts of bioactives were delivered to all volunteers in pilot studies, bioactive concentration was determined in each batch produced for pilot studies.

Overall, PATHWAY-27 developed a strategy to formulate and select BEF to be used in intervention studies, evaluating food characteristics (acceptability, bioactive accessibility and retention, etc) that are seldom considered although they could interfere with results obtained in human trials.

The three selected products (milkshakes, biscuits, pancakes) enriched with five different bioactive
The three selected products (milkshakes, biscuits, pancakes) enriched with five different bioactive combinations were used in pilot intervention studies. The purpose of pilot studies was to select, for each of the three food matrices, the enrichment delivering the greatest reduction in serum triglycerides (TG) or increase in HDL cholesterol (HDL-c). Three pilot studies were conducted in three clinical research centers (Max Rubner-Institute, Karlsruhe, Germany; University of Leeds, United Kingdom; and Human Nutrition Research Centre of Auvergne, France), each participating center focusing on a specific food matrix. Each pilot study was randomized, double blind, parallel dietary intervention study without a placebo.

In each pilot, participants were divided into five groups, each receiving BEFs enriched with DHA, BG, or AC alone or DHA+BG, or DHA+AC based on bakery, dairy or egg products (Figure 2). Participants were advised to consume the allocated BEF daily for a period of four weeks. The BEF should be consumed in replacement of similar foods and not in addition to the usual diet. Besides BEF consumption, participants were instructed to consume food rich in DHA, OBG and AC only in limited amounts. Therefore they received a list of respective foods.

At baseline and after 4 weeks of intervention, fasting blood samples were collected for further analysis. Additionally, blood pressure and anthropometric data have been determined. A schematic view of the study protocol is given in figure 3.

To standardize and harmonize procedures among the study centers, a set of standard operation procedures (SOPs) were developed for the pilots. Each clinical center obtained the approval from local Ethics Committees and additional regulatory bodies before the start of the trial.

Pilots were designed as exploratory trials aiming to identify the best matrix × enrichment combination that would result in the strongest effect on the designated endpoints. Each pilot explored 5 different BEF within the same matrix, and evaluated the effects compared to baseline.

Pilot studies have also the meaning to solve possible problems arising before/during the intervention.

Considering that MetS is defined as "a group of conditions that put you at risk for heart disease and diabetes" more than a disease itself, criteria for MetS diagnosis (Table 1) were used as inclusion criteria in the study. Subjects presenting two to four of the criteria, at least one of them being alteration of fasting triglycerides or HDL cholesterol were considered eligible to participate. Additionally, major exclusion criteria were selected (among others, regular drug therapy with impact on serum lipids, diabetes, celiac disease, history of allergy or intolerance to any components used in BEFs; recent history of cancer or cancer treatment; familial dyslipidemia; intestinal malabsorption; pregnancy or lactation; etc).

In total, 167 men and women at risk for MetS participated in the pilot study.

The number of volunteers in each intervention subgroup and the duration of the intervention itself did not allow identifying statistically significant changes in the primary endpoints (blood triglycerides - TG and HDL-cholesterol – HDL-c), but some trends were clearly evidenced, allowing the selection of the most promising enrichment within each matrix.

Descriptive statistics data on TG revealed a moderate nominal reduction after 4 weeks of intervention for all matrix groups, more evident for dairy. Considering all matrices, the most prominent effect was for DHA+OBG enrichment. The data indicates that DHA+BG showed for dairy the greatest reduction in blood TG, AC and DHA+AC showed the greatest reduction in blood TG for egg-based and DHA+AC for bakery products. Among matrices, egg-based products had the most prominent effect on HDL-c while DHA+AC was the most effective enrichment.

To further evaluate the impact of BEF intervention we included the following secondary endpoint into the statistical analysis: systolic and diastolic blood pressure, waist circumference and fasting blood glucose. Additionally, the anthropometric measurements of height, body weight and calculated BMI have been.
Additionally, the anthropometric measurements of height, body weight and calculated BMI have been considered. Although no significant effects of BEF intervention on any single secondary endpoint were identified, some interesting trends were observed, particularly on blood pressure.

Sensory acceptance of BEFs was also evaluated, and it evidenced that the volunteers were open to eat such unusual products. The products received relatively high scores for all attributes. Volunteers stated in all three pilot studies that they would eat these products, if proof is provided that the consumption of the products improves their health, or helps to avoid medication. Sensory characteristics and acceptance among enrichments within the same matrix were almost similar, so they did not interfere with the selection. Results obtained in pilot studies were integrated to select the most promising enrichment in each food matrix. Taking the promising effects of DHA+BG on blood TG into account and considering also the positive and stable effects on HDL-cholesterol and blood pressure, the DHA+OBG enrichment was selected for the dairy product (milkshake). For the egg-based product (pancake), considering the positive effect on blood TG and HDL-c, and on blood pressure the enrichment with DHA+AC was selected. The same enrichment (DHA+AC) was selected for the bakery product (biscuits) considering the promising effect in blood TG and the increase of HDL-c (Figure 4).

The three selected BEF were then used in the PATHWAY-27 large intervention study (LIS), a multicentre, randomized, placebo-controlled, parallel-arm dietary intervention study carried out in four European recruiting centres (RC): Human Nutrition Research Centre of Auvergne - CRNH, France; Max Rubner-Institute - MRI, Karlsruhe, Germany; University of Leeds - ULE, United Kingdom; University of Bologna - UNIBO, Italy.

The primary objective of the LIS was the evaluation of the relationship between TG and HDL-c blood levels and the intake of BEF selected in pilot studies in subjects at risk for or affected by MetS. Secondary objectives were to evaluate the effect of the consumption of BEF on other markers of MetS (secondary end-points) and on body composition using total-body Dual Energy X-Ray Absorptiometry (DEXA) in a subgroup of volunteers. In addition, blood, urine, stool and white adipose tissue (WAT) samples were used for omics analyses.

According to the study design, participants were randomly assigned to one of four groups to receive either:
1. Dairy BEF + egg placebo + bakery placebo;
2. Egg BEF + dairy placebo + bakery placebo;
3. Bakery BEF + dairy placebo + egg placebo;
4. Dairy, egg and bakery placebo.

Volunteers were asked to participate to a sub-study, including additional analysis. Participation was on a voluntary basis.

Participants were required to consume all three of the allocated products each day for 12 weeks. Fasting blood and urine samples were collected from all participants at baseline (T0), midpoint (T6 – urine were collected in subgroup participants only) and endpoint (T12). Additionally, blood pressure, body weight (and height) and waist-circumference were collected at baseline, midpoint and endpoint from all participants. At baseline, a dietary assessment was performed for all participants.

As soon as the RCs received the authorization from the respective Ethics Committee, then they actively started contacting and screening the volunteers. Inclusion and exclusion criteria were the same as in the pilot studies.

Although a high number of volunteers was screened during the first 2 months of the trial, the recruitment rate was lower than foreseen, therefore the recruitment phase was extended till the end of July 2017. The LIS concluded in October 2017.

Considering food production, the pilot studies were valuable source of experience for the LIS, both for the
Considering food production, the pilot studies were valuable source of experience for the LIS, both for the manufacturing SMEs and the research centers. The difficulties in BEF distribution and storage encountered during the pilot studies were solved, allowing successful production, delivery and storage of the required amount of BEF during the LIS. Therefore, to conduct pilot studies represented a good strategy to reduce the cost of the large intervention not only because they allowed selecting the most promising BEF, but also solving problems related to food production and delivery to recruiting centres. Microbiological quality of BEF pancakes and milkshakes was analysed right after production, and the food was sent to research centers only after the analysis results were ready and the batch thus declared as safe. Biscuits have very low water activity (<0.6) and the data obtained from a microbiological challenge test was enough to assure microbiological safety and stability.

For the LIS, 25 000 active and 73 000 placebo pancake portions were produced by ADX. For milkshakes the corresponding numbers were 27 000 and 70 000 (produced by ADX), and for biscuits 19 000 and 60 000 (produced by DPL). To ensure research centers an un-stoppable supply of fresh BEFs and placebos, the food productions were arranged based on pre-planned schedules where different shelf lives of the products were taken into account and the number production batches minimized.

Reproducibility of tested food is one of the basic requirements of EFSA for the substantiation of the health claims. In order to establish the cause and effect relationship between consumption of the food constituent and the claimed effect, a standardised concentration of the constituent must be verified by checking the variation of the initial data between different batches. Thus during the LIS, for each lot produced, the concentration of bioactives in the BEF was analysed. The results from the analyses showed that bioactive concentration was quite constant in all production batches of the LIS. The foodomics approach was applied to assess the effect of the incorporation of a functional ingredient on the reproducibility and/or stability of three different food matrices. Foodomics has the advantage of capturing a holistic view of the molecular complexity of the food extracts, and allowed a simplified appreciation, granted by 1H-NMR spectroscopy, of the molecular homogeneity among the production batches of the 3 different food matrices before storage and the subsequent stability performances of several products (enrichment, storage time) produced and consumed during the LIS.

Due to the difficulties in recruitments and the LIS extension, food production lasted longer than foreseen. To minimise food waste coming from low recruitment and the SMEs’ efforts, an excel-based planning protocol was developed and taken in use. It was based on a continuous flux of information from RC to producing SMEs and on re-labeling of placebo servings that were not used earlier, but still had long enough shelf-life. To ensure the blindness in the study, re-labelling was performed by contact persons (one in each RC), otherwise not involved in the study.

The SMEs were able to continue the productions also after the original ending date of the study and provide BEFs and placebos for the research centres for the whole duration of the LIS, altogether 1.5 years.

The most promising BEF were selected in pilot studies and used in the large intervention trial. All problems related to the production of BEF to be used in human intervention trials were successfully solved. Overall, PATHWAY-27 activities paved the road for the industrial exploitation of PATHWAY-27 BEF.

During the LIS, 5401 putative volunteers were contacted by the 4 clinical centres, of whom 1361 accepted to carry out the screening visit and/or blood sampling for lipid profile and exclusion criteria: 420 resulted eligible and 325 accepted to enter the study and were randomized. Among them, 109 accepted to carry out additional analysis included in the sub-study. During the trial, 89 subjects dropped out while 236...
during the trial, 89 subjects dropped out while 236 volunteers (82 in the sub-study) completed the 12-week trial and were considered in the Intention to Treat (ITT) statistical analysis. Subjects with available TG and HDL-c value at all 3 main time points and at least 70% compliance to consumption for each administered food were included in the per protocol (PP) statistical analysis. Four subjects with TG$>400$ mg/dl (possibly indicating familial hypertriglyceridemia) were excluded from the analysis. In total, 195 volunteers were included in the PP analysis. Restrictive inclusion/exclusion criteria allowed us to select a homogeneous cohort of high metabolic-risk subjects who potentially could have benefited from BEF consumption.

In volunteers with low HDL-c ($\leq50$ mg/dl for females, $\leq40$ mg/dl for males) at baseline, all BEF significantly increase HDL-c. HDL-c is the well-behaved “good” cholesterol. High HDL levels reduce the risk for heart disease but low levels increase the risk. HDL cholesterol scavenges and removes LDL-c (“bad” cholesterol) by transporting it to the liver where it can be reprocessed. The positive effect of BEF on HDL-c was already achieved after 6 weeks of consumption, and it lasted over the whole period of treatment. Since HDL-c is biologically stable, the HDL-c improvement may be long standing with potential clinical benefits.

The HDL-c increasing effect was more evident using BEF enriched DHA+AC (pancake and biscuits). The nutritional evaluation of volunteers evidenced that consumption of these BEF did not induce a significant increase in AC intake while DHA intake was significantly higher than at baseline. So, we could speculate that DHA is the more active compound, AC having a facilitating activities.

The group receiving DHA+OBG experienced a clear reduction of LDL-c level (mean changes T0-T12 = -6.844; % differences of T0= -3.69%). It is worth noting that DHA+OBG treatment was the only one reducing LDL-c, other treatments having an increasing effect. A modest increase of LDL-c level after DHA administration has been already reported in clinical trials, although the clinical relevance of such finding is uncertain (Mozaffarian D, 2012). Anyway, the association of OBG to DHA could have antagonized the LDL-c increasing effect. A decrease in LDL-c level was also observed in the placebo group. This could reflect changes in the dietary habits of volunteers, who possibly turned to a healthier diet during the trial. Consumption of all food enriched with DHA was associated to significant reduction of systolic blood pressure (SBP) compared to placebo, both at T6 and T12. The effect appeared mainly ascribable to administration of DHA+OBG and DHA+AC embedded in pancakes. Consumption of DHA+AC enriched biscuits had no effect on SBP.

Importantly, the observed metabolic effect seems to be independent by changes in BMI and waist-circumference.

Samples from volunteers enrolled in the LIS were analysed with state-of-the-art omics techniques. An intensive literature search led to the selection of 39 single nucleotide polymorphisms (SNPs) to be investigated in all recruited volunteers to highlight the possible correlation of the genetic profile to the risk of MetS. Among selected SNPs, one (ADIPOQ_ rs17300539) was found associated with the risk of MetS in all national sub-cohorts, and the presence of the polymorph allele A (in homo- and heterozygosis) increased the risk.

An NMR-based metabolomics approach applied to serum and urine evidenced that samples collected at the beginning and the end of the study could discriminate with high accuracy, so indicating a clear modification of the metabolome (i.e. the complete set of small-molecule chemicals found within a biological sample) due to treatment. From the combined analysis of the serum and urine samples in the different BEF
Due to treatment, it appeared that the enrichment with DHA+AC was more effective than DHA+OBG in introducing effects in the metabolomic profiles of the volunteers, confirming the clinical observation on HDL-c. In particular, in serum we highlighted significant changes in the lipoprotein profiles of the volunteers receiving DHA+AC embedded in the bakery product, but not in volunteers receiving the same bioactives embedded in the egg-based product.

The effect of BEF intervention on unbalances in gut microbiota and intestinal metabolites that impact on human physiology was monitored in a subgroup of volunteers (n=82). At the bacterial species level, some components of gut microbial population were apparently positively associated to changes in the lifestyle of volunteers due to participation in the study and consumption of any BEF. Other shifts were more related to the effect of one or two BEF. Interestingly, increase in some microbial groups seemed to exclude the increase of pathogenic members of the group, suggesting an ecological barrier function induced by BEF consumption.

Single omics datasets were merged into an integrated multi-omics collection to test correlations between pairs of variables and perform multivariate analysis. The most important finding obtained was a weak tendency to separate subjects in the multi-omics space consequent to the consumption of BEF, associated to two genetic variants of ADIPOQ and FTO genes, together with LDL-c, HDL-c and total cholesterol, as well as a couple of microbial species and the lipids region of the NMR spectra.

To evaluate food digestion and bioactive availability, considered as a possible variable of BEF effectiveness, in vitro and in vivo studies were performed. In vivo, experiments were performed in a pig model since it is considered as the most physiologically-relevant model to mimic the human digestive system. Each pig was fed the three BEF selected after the pilot study and used in the large intervention study. Effluents and plasma were collected continuously during 7h after the meal and the resulting digested material was analyzed. The kinetics of DHA concentration in plasma appeared to be affected by the type of BEF ingested. After pancake ingestion, the peak of DHA concentration appeared earlier but it was of lower magnitude than for milkshake and biscuits. DHA concentration peaked at later time when biscuits were administered, but it decreased slower than using pancake and milkshake.

Within the PATHWAY-27 project, in vitro studies were also performed to understand the protective role and mechanism of action of the three bioactive compounds (DHA, OBG, and AC) used to enrich food tested in clinical studies. Two main cell model systems were used, adipocytes and hepatocytes, due to their pivotal role in development of the MetS.
Despite the wide use of cell cultures for the determination of bioactive effectiveness and mechanism of action, many studies reporting a protective effect of bioactives have been performed supplementing cells with putative active compounds in the form they are present in the food. In vivo, foods are digested and some bioactives are extensively metabolized, so that the effective molecules are very different from parent compounds. In PATHWAY-27, we took it into account, and we used for cell supplementation protocatechuic acid (PCA), the major metabolite of most anthocyanins (Vitaglione et al. 2007; Czank et al. 2013), and propionic acid (PRO), one of the short fatty acid produced in the colon upon OBG fermentation by the microbiota. PRO produced in the colon is absorbed, and around 90% is metabolized by the liver (Al-Lahham et al. 2010). Since DHA is absorbed by intestinal cells and it is delivered to peripheral cells in its parent form, cells were supplemented with DHA.

An extensive literature search was performed to identify the physiological range of concentrations of the studied bioactives. In addition, since cell cultures represent a close system, and their direct and continuous exposure to bioactives could alter the cell response, inducing cytotoxicity due to the lack of the continuous detoxification and clearance of compounds occurring in the whole body, the possible cytotoxicity of supplemented compounds was assessed prior to further experiments.

A systemic analysis of the action of bioactive compounds, alone and in combination, on lipogenesis, lipolysis, adipokine secretion (i.e. inflammatory status) and adipogenesis of human in vitro differentiated adipocytes was made. As model, primary human pre-adipocytes from subjects between 26-65 years of age with a body mass index (BMI) ranging from 21-28 kg/m2 (mean 25) were isolated from subcutaneous white adipose tissue (WAT) obtained as a waste product from cosmetic liposuction. No cytotoxic effect was observed up to 6 days of treatment with bioactives at the tested concentrations (DHA 0.5 µM; PRO 100 µM and PCA 13 µM). As well, no effects of the selected compounds on lipid accumulation nor on gene expression of selected marker genes (PPARG, CEBPA, PLIN1, LIPE, UCP1 and ADIPOQ) were observed after 6 days of treatment. To investigate the effect of the bioactives on the function of adipose cells, a panel of the most important pro- and anti-inflammatory adipokines, were measured, as well as the effect on lipolysis and insulin-stimulated lipogenesis. All bioactives decreased basal lipolytic activity and secretion of inflammatory markers in human fat cells, which might explain some of the reported health benefits induced by bioactive compound-containing diets in humans, including reduced insulin resistance, hypertension and dyslipidemia.

In preliminary studies on hepatocytes, possible bioactive cytotoxicity was assessed by means of different methods and the highest concentrations of bioactives to be used in further experiments were set as follow: DHA 50 µM; PRO 70 µM; PCA 20 µM; DHA 50 µM + PRO 70µM; DHA 50 µM + PCA 20 µM (Di Nunzio et al, 2017). Then it was evidenced that DHA supplemented alone or in combination with PRO or PCA is significantly taken up by cells in a time-dependent manner, and modified cell fatty acid composition. A significant decrease in intracellular cholesterol content was detected in DHA supplemented cells compared to controls. This effect was due to an increased cholesterol secretion but mainly to the reduced expression of genes involved in cholesterol metabolism. Results showed that in normal condition (no insulin resistance) 24h supplementation with the bioactives increase glycogen storage and alter the expression of genes of the insulin pathway (AKT, GYS, GLUT2).

The overall effect of bioactive supplementation on hepatocyte lipidome and metabolome was investigated using NMR combined with traditional techniques. DHA supplementation, alone or in combination with PCA or PRO, strongly altered the cell lipid profile, and cells receiving DHA (alone or in combination) were perfectly discriminated from other cells. This “DHA signature” was identified not only in the cell lipidome, but also in the metabolome (Ghini et al. 2017).
but also in the metabolome (Ghini et al., 2017).

We also compared metabolic changes occurring in vitro in human hepatocytes to changes observed in serum and urine of volunteers enrolled in the intervention trial. Overall, we provide evidence that the NMR-based metabolomics approach can predict modifications in the metabolome consequent to the treatment with bioactives in vitro or administration of BEF in vivo.

At molecular level, the analysis of the hepatocytes transcriptome showed a significant modulation of genes involved in cholesterol and fatty acid synthesis following DHA supplementation. PRO appeared to potentiate the effects of DHA whereas there was little effect of PCA co-supplementation. Supplementation with PCA or PRO alone had almost no effect on the transcriptome.

To study the epigenetic effect of bioactives in hepatocytes, two approaches were used: acute treatment (24h) and chronic treatment (4 weeks). In the short treatment, bioactives induced chromatin changes on a global and on a genome wide level. The changes were mainly induced by DHA, alone or in combination. The observed chromatin changes potentially regulate genes that were identified as differential expressed by RNA-seq. All genes are referred to mechanisms which are involved in the pathogenesis of metabolic syndrome, more precisely fatty acid metabolism and chronic inflammatory processes, and were downregulated after the short-term treatment with DHA (alone and in combination).

After the long-term exposure, we observed a clear separation between DHA-treated and not-treated cells at the transcriptomic level, with additional distinction of DHA and the combination treatments. In general, fatty acid-related metabolic processes were downregulated by DHA. Methylation change was mostly very small, so we suppose that DNA methylation is not a major mechanism that contributes to the regulation of expression of the considered genes in the selected cell model (HepG2 cells), but eventually reinforces a gene regulatory effect.

Other molecular studies were performed in the human pre-adipocyte cell system Simpson-Golabi-Behmel syndrome (SGBS) cells. In these cells, an extremely useful new strategy for administration of fatty acids to human adipocytes was developed. This strategy is based on differentiation under serum free conditions followed by adaptation of adipocytes to serum prior to administration of DHA. As in hepatocytes, DHA appeared by far the bioactive with the greatest impact on the transcriptome and promoted subtle but consistent activation of a subset of known adipocyte genes and suppression of proinflammatory genes, which is suggested to collectively promote fatty acid storage and turnover. Bioinformatic analyses of the data indicated that these effects of DHA in adipocytes are primarily mediated by activation of PPARγ. Depending on the subgroup of genes, PRO and PCA displayed positive or negative cross-talk with DHA. This gene group dependent cross-talk between the different bioactives in human adipocytes is novel and uncovering the mechanisms for this cross-talk will be important for the interpretation of the physiological results.

Long term treatment of SGBS preadipocytes with DHA and combinations induced strong changes in DNA methylation, mainly hypomethylation, while PCA and PRO had little effects on this epigenetic mark. Differentially methylated CpG sites identified both in vitro and in vivo (WAT from LIS volunteers) partly overlapped with stable and dynamic enhancer regions of adipocyte differentiation.

The integrated analysis of all results indicated that DHA, alone or in combination, is the main modulator at molecular and cellular level although the effects in adipocytes and hepatocytes are different, as well as the cross-talk between DHA and PRO or PCA. Overall, studies performed in PATHWAY-27 suggest that beneficial effects of DHA in relation to the MetS is mediated in part by the ability to reduce cholesterol synthesis in hepatocytes and adipocytes, and to promote lipid storage and adipokine secretion and to reduce the inflammatory tone in adipocytes.
One of the main aims of PATHWAY-27 was the preparation, publication and implementation of guidance documents that could inform and assist the food industry sector, especially SMEs, to produce bioactive-enriched foods (BEF) with supportive health claims according to the EU legislation, thereby supporting implementation of the legislation claim framework and enhancing cooperation between complementary scientific disciplines, public-private partnership in the food sector, stakeholder interactions, and ultimately the health of consumers.

Two sets of guidelines were produced:

➢ Guidelines for the scientific community

This set of Guidelines is addressed to scientists from both academia and the food industry. The guidance helps to understand and apply the relevant steps of the health claim substantiation process. These steps include:

1. Thoroughly reviewing the published evidence concerning any putative beneficial physiological effect(s) of the food or food constituent of interest (e.g., a bioactive compound); and
2. Correctly designing, conducting, interpreting, and reporting any necessary human dietary interventions.

The guidance focuses on randomised controlled trials as they are the most rigorous type of interventions to investigate the claimed health effect of a specific food. In this regard, the critical aspects of randomisation, blinding and control are discussed, with hands-on examples from the PATHWAY-27 project of issues that can be encountered when dealing with bioactive compounds and how to solve them. Furthermore, it addresses sample size and data analysis, emphasising the need to involve an experienced (bio)statistician from the outset. Study duration and (non-)compliance are also discussed. As recruitment of study participants can often be a major bottleneck, various recruitment strategies are described. This guidance document also refers to successfully submitted dossiers as well as failed applications and other publically available relevant resources which will help with the appropriate scientific substantiation of health claims.

➢ Guidelines for the food industry

The first step of the work was to conduct a survey amongst relevant stakeholders to collect and identify industry/SME needs and difficulties in establishing and submitting health-claims for food products enriched with health-promoting bioactives. The results of the survey were used to ensure that both industry and scientific guidelines address the issues mentioned by the industry/SMEs (Hegyi et al., 2015). The proposed integrated Guidelines for food industry/SMEs thus offer a structured product development approach addressing all aspects that SMEs and their suppliers of material, knowledge and related services should consider when designing products with health claims in Europe (Figure 5):

- Description of the targeted cause-effect relationship to obtain the claimed effect;
- Composition of the product including the characterization of the bioactive constituent;
- Measures to ensure the requested minimum quantity of the active constituent within a batch, between different batches, lots and through the whole shelf-life of the product;
- Selection and verification of health claims to prepare scientific substantiation;
- Food safety assessments such as determination of shelf-life;
- Sensory analysis methods for product acceptability testing;
- Manufacturing ability;
- Financial feasibility;
- Marketing strategy and market launch of products with health claims.
Finalisation of both Scientific and Industry Guidelines was based on interaction with key stakeholders. The Guidance Paper Workshops held in Brussels in September 2017 allowed reviewing the Guidelines before publication (Figure 6).

During this two-day event, workshops in a combination of presentations and working groups sessions were organized to give the opportunity to the end users representatives (industry/SMEs and academic representative, EFSA staff, etc.) of the guidelines to provide feedback on how to improve and strengthen the usefulness and readiness of both documents. The workshop on the Scientific Guidelines was attended by 64 stakeholders and the one on the Industry Guidelines was attended by 50 stakeholders. The feedback received during these events was critical to finalize these Guidelines.

The final version of the PATHWAY-27 guidelines is downloadable free of charge on the PATHWAY-27 website.

An additional set of information supporting the implementation of the European legislation frameworks on health claims was prepared and uploaded on PATHWAY-27 website. The information provided refers to the current Regulation on nutrition and health claim made on foods, a series of workshops organized by EFSA and related Guidance documents that brings key information on how to address health claims related to different health functions (immune system, cognition, etc.) but also reference to peer-reviewed articles showing the importance of how the selection of relevant makers to show an action of the bioactive compound(s) is key when applying for a claim in Europe.

An interactive diagram was produced to ease the understanding of the regulatory framework and to facilitate the finding of the relevant information (Figure 7).

PATHWAY-27 provided two sets of guidelines based on PATHWAY-27 experience. The PATHWAY-27 guidelines are downloadable free of charge on the PATHWAY-27 website.

References

- Guidelines for the Scientific Comunity
- Guidelines for the food industry.
• Guidelines for the food industry

Potential Impact:
Many naturally-occurring compounds in dietary plants and animal products possess a variety of physiological functions which could promote human health and wellbeing and contribute to reduced risk of diet-related-disease (DRD). These compounds are known collectively as bioactives, i.e. natural components of foods that possess biological activity in addition to their nutritional value. In the global scientific scenario, research on bioactive compounds is very active. Identifying bioactives, establishing their mechanisms of actions and health effects are all active areas of scientific inquiry and, through industrial exploitation, potential societal benefit.

Bioactives normally occur at very low concentrations in foods. The agro-food industry has adopted different approaches to provide foods with higher concentrations of bioactives (the so-called functional foods). Until now, enrichment of food matrices with selected bioactives, leading to bioactive-enriched foods (BEF), represents the most common technique.

The scientific community considers that bioactives and related BEF have a high potential for reducing health risks and improving health quality and eating habits. However, notwithstanding a very substantial increase in knowledge on certain bioactives, the impact of this research in delivering novel food products has been limited. This is due to inefficient knowledge transfer to food industry (which is dominated by SMEs) and to difficulties in convincing EFSA of the validity of the requested health claim.

PATHWAY-27, involving a pan-European interdisciplinary team of life- and social scientists, and SME, uniquely focused on the role and mechanisms of action of 3 bioactives considering them as ingredients for the enrichment of 3 different food matrices.

PATHWAY-27 used three model compounds (docosahexaenoic acid -DHA; oat beta glucan -OBG and anthocyanin -AC) and three model food matrices (dairy, bakery and egg products). However, the conclusions derived from PATHWAY-27 could be widely applicable and allow the exploitation of other bioactive compounds as components of foods that, within the common diet, could significantly benefit human health and wellbeing.

Scientific impact.
A key scientific objective of modern nutrition is to determine the role of bioactives and BEF in metabolic regulation, in health maintenance and in the reduction in risk of diseases.

Opportunities for a bioactive food or component begin with identifying a link between a BEF and health or to a disease. Although scientific evidence is accumulated, reliable, science-based information is lacking for health benefits of BEF. It is difficult to demonstrate causality for BEF and health. The highest quality of evidence is the randomized controlled trial (RCT). An RCT is less susceptible to bias than other study designs for assessing therapeutic interventions; notwithstanding, RCTs are vulnerable to bias throughout their entire life span.
PATHWAY-27 run three pilot intervention studies and one large intervention study. Beside the specific knowledge on BEF effectiveness, these clinical studies were the bases for the PATHWAY-27 Guidelines to the scientific community. The guidelines are based on real experience (and failures) and are aimed to prevent mistakes in the different steps of an RCT, starting from its design, through to implementation and reporting.

In multicenter RCT, bias could be related to different operating procedures in the involved recruiting centres. In PATHWAY-27, a complete set of standard operating procedures (SOPs) have been developed and adopted.

PATHWAY-27 went behind the statement that “one treatment (BEF) fits all” considering that dietary, lifestyle and genetic characteristics could have an impact on effectiveness. The aim of this approach was not “personalised nutrition” or “personalised BEF” (i.e. unique nutrition guidelines or BEF for each individual) but to develop effective approaches based on the combination of individual characteristics (precision nutrition). While running human intervention trials, PATHWAY-27 exploited novel omics technologies and by integrating them gave new insights in the investigation of the complex relationships linking genome and metabolism and their regulation, nutrition, and risk of diseases. The gut microbiome was also considered, since a further complication in understanding the benefits of bioactive components on health may be that their activity arises from the metabolites produced by the host or the gut microbiome rather than from their presence in the food. While evaluating BEF effectiveness it is important to identify the factors that contribute to normal physiological variation, so that normal metabolomic fluctuations are not confused with biomarkers representing a metabolic change due to nutritional intervention. PATHWAY-27 faced such challenge while exploring the multi-omics dataset delivered during the project. The dominant background of the large intervention study was the tetra-centric distribution of the recruitment. This was the strength of the experimental design because it guarantees the generalised validity of the results, but it also rises a challenge because it introduces a strong source of variation (genetics, environment, lifestyle, dietary habits) that may cover other wanted weak changes linked to the effect of BEF consumption on the clinical and molecular end-points. Once every single omics dataset was thoroughly explored, they all have been merged in an integrated multi-omics collection. This poses the basis for the development of ad hoc algorithms able to correlate all parameters to possibly predict how genetic, environmental and/or behavioural ‘lifestyle’ variance within each person may provide a more effective basis for administration of specific BEF.

Another weak point in the design of RCT administering BEF, and not drug, is the bioavailability of the administered bioactives. In BEF, bioactive availability is also dependent on the food matrix and technological processing, which can modify bioactives digestibility. In PATHWAY-27, the digestibility of BEF has been assessed in vitro, and consequent bioavailability in a pig model and in volunteers recruited in the pilot intervention studies through direct measuring the bioactive concentration in blood. The assessment of BEF digestibility and bioactive availability has not only a scientific impact but can also support the food industry in the development of new, improved BEF.

Last, the complementary in vitro and in vivo studies performed within the project verified whether the mechanisms by which PATHWAY-27 bioactives modulate cell functions are also effective in selected target organs, generating new and important findings in the field of nutrition research.

Impact on the European Food Industry.

PATHWAY-27 will increase the European food industry’s competitiveness not only with the new BEF that have been delivered by the project, but also through provision of protocols and guidelines drafted specifically for SMEs for the production of other effective BEF. PATHWAY-27 did not simply evaluate the
specifically for SMEs for the production of other effective BEF. PATHWAY-27 did not simply evaluate the effectiveness of specific BEF to reduce the risk of metabolic syndrome, but also provided generic principles that will drive development of new BEF utilizing other bioactives and other food matrices. In the field of food technology, PATHWAY-27 enabled science-based selection of different food matrices suitable for enrichment with bioactives. Technological methodologies (formulation, processing, etc.) were generated by partner SME to produce specific BEF possessing high concentrations of available bioactives with optimal sensory properties. Kinetic studies during storage gave information on the stability of quality parameters, on retention of bioactives within the food matrix and on the objective determination of the shelf-life of the designed BEF.

Bioactives with EFSA-substantiated health benefits can be used as supplements, but the possibility to administer them as ingredient of common foods within a common diet, maintaining their bioavailability and protective activity, is one of the most important and challenging areas of concern and investigation in the inter-disciplinary food/nutritional sciences.

By overcoming these challenges, PATHWAY-27 provides opportunities to increase market shares and stimulate economic growth and employment to European food and drink manufacturers, enabling consumers to a better food selection (together with healthy lifestyle choices) associated with improved health, and contributing to a reduction in spiraling health and social costs associated with an ageing population.

Impact on consumers.

Although the relationship between diet, nutrition and health has long been established, many consumers find it difficult to follow nutritional rules in everyday life. Although a correct diet and lifestyle remain the basis for a healthy life, BEF could be helpful for consumers to reduce the risk of some diseases without having to completely modify their dietary habits. Consumer acceptance and the meeting of consumer demand were both focal points of PATHWAY-27. The project included the development of traditional foods in its overall strategy, to allow consumers to preserve their cultural identity while enhancing their health and wellbeing. The focus on affordability was also considered since all consumers should benefit from BEF.

Notably, in the PATHWAY-27 intervention adult volunteers were considered regardless of the age and gender. The result of an RCT is only applicable to everyday practice if the volunteers included in the trial were similar to those who would be treated in practice. Thus, if a treatment has only been tested in men aged under 65, it is generally impossible to know whether it will benefit a 95-year old woman. The inclusion of a broad population in the intervention study makes results more generalisable.

Impact on Public Health.

Nutrition is a key priority for EU public health policy, and bioactives could contribute to health improvement and reduction of the risks of diet-related diseases. However, if bioactives are consumed as part of BEF and not as supplements, the importance of determining how bioactive/food matrix interactions might alter the nature and extent of biological activities becomes of very great significance. PATHWAY-27 contributes to a greater understanding of the beneficial effects of bioactives as ingredients of BEF. In this way, PATHWAY-27 address and support specific European public health and social concerns, priorities and policies. This is not limited to the developed BEF and their effectiveness in the counteracting MetS, since the two sets of PATHWAY-27 Guidelines will allow to apply the project strategy to other bioactives, BEF, and disease risk.

A national and international dialog is underway on whether public health recommendations can be made on bioactive foods and ingredients that have health benefits. Recent publications evaluated the readiness of bioactive components for public health recommendations and set the stage for an evidence-based
of bioactive components for public health recommendations and set the stage for an evidence-based process.

Existing processes for setting requirements for essential nutrients may not be practical for bioactive components, for which this process is yet to be defined. Such a process is needed to be able to set a framework for research and the evidence base needed before consensus messaging can be given to health care professionals.

Currently, bioactive foods and ingredients have almost no role in public policy. We believe that PATHWAY-27 results can give a significant contribution to the process that will ultimately lead to public health message, whose ultimate beneficiary is the consumer.

The results of the PATHWAY-27 project were extensively communicated, disseminated and transferred to the main stakeholders, to the industry, SMEs, policy makers and regulators, food safety and food control authorities, retailers, intermediaries, NGO and consumers, etc.

The project corporate identity and communication tools included:

- The project web site (www.pathway27.eu) with 3 different levels of access (public, intranet and blog surface). The website was regularly updated with events, news and the available results of the project.
- The project logo (Figure 8), templates for presentations, posters, deliverable reports and research summary sheets,
- The core presentation, which was updated with the results,
- The project brochure,
- 2 press releases that were translated to 6 languages (Italian, Spanish, German, Finnish, Turkish and Hungarian),
- 3 project articles,
- Research Summary Sheets for all research work packages,
- 3 project leaflets that were translated to 6 languages (Italian, Spanish, German, Finnish, Turkish and Hungarian, French),
- So far, 14 publications in international journals,
- 3 social media sites: Facebook page, LinkedIn Group and a Twitter account,
- Template for collecting the expected/achieved results.

Training activities were also performed:

2-phased “Train the trainers” workshops with an aim to train the project partners to transfer the results of the project to the industry and to other stakeholders at national level were performed.

Three on-line demonstration workshops targeting the industry.

A local training on the 30 January 2018 in Budapest.

During the project, 5 researchers participated in the staff exchange programme between the PATHWAY-27 partner institutions, and 7 mobility conferences were organized to share the latest research results of the project partners.

The developed Scientific and Industry Guidelines were presented at 6 Conferences, at 7 Meetings, 8 workshops, 4 Open Days, and 17 presentations on the guidelines were held on different events.

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workshops, 4 Open Days (Campden BRI UK, 2016 and 2017 and Hungary Open Days, 2016 and 2017), and 17 presentations on the guidelines were held on different events.

The project results and achievements were presented at several national and international events:

- dissemination of PATHWAY-27 at 32 Conferences,
- presentation of PATHWAY-27 at 31 Meetings,
- 64 presentations of the PATHWAY-27 project within 37 different events,
- 16 workshops,
- 10 Open Days.

Finally, an exploitation plan of the PATHWAY-27 project results has been developed, and several results have been identified, which can be exploited for one or more purposes.

List of Websites:
Project website: www.pathway27.eu

Related documents

final1-pathway-attachment-final-report.pdf

Last update: 3 September 2018
Record number: 239136