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SEVENTH FRAMEWORK PROGRAMME

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1. Introduction

The guidelines presented here have been developed through consultation with PATHWAY-27 project partners to provide practical guidance for designing, implementing and reporting pilot intervention studies using bioactive enriched foods (BEF). These guidelines update and complement previously published guidance documents, but are unique in providing practical information and troubleshooting based on the PATHWAY-27 pilot studies.

The aim of PATHWAY-27 is to evaluate the effectiveness of BEF containing docosahexaenoic acid (DHA), anthocyanins (AC) and beta-glucan (BG) alone or in combination, on improving risk factors of the metabolic syndrome (MetS). These compounds were chosen for their known effects as single compounds, and considered as ingredients of BEF in three different widely consumed food matrices (dairy, bakery and egg products). This approach of using BEF, compared to single pure compounds, will allow a better understanding of possible bioactive synergisms and bioactive-matrix interactions. This knowledge will increase the potential for exploitation of bioactive and BEF commercialization by the food industry.

Previous guidelines by Welch et al., 2011 (1) that describe the designing, conducting and reporting of human intervention studies to evaluate the health benefits of food provide a good introduction to the subject for anyone considering undertaking such a research study. Additionally, there are useful publications describing some of the challenges faced when implementing a human dietary intervention trial. These include articles by Crichton et al., 2012 (2) and Yao et al., 2013 (3) which discuss the difficulties of participant compliance and blinding.

More recently, guidance from the EU-funded project BACCHUS for the design and implementation of human dietary intervention studies testing effects of dietary bioactive peptides and polyphenols on cardiovascular health in humans (4), has provided a useful toolkit to assist substantiation of health claims related to cardiovascular health. Furthermore, the PATHWAY-27 project is developing two guidance papers, for the scientific community and for the food industry, relating to the substantiation of health claims on BEF. These guidance papers, which will be published at the end of the project (deliverables D7.4 and D7.5) after consultation in two stakeholder workshops, will complement earlier guidelines and be particularly useful for small and medium sized enterprises (SMEs) looking for a competitive advantage in the EU market. For a health claim to receive a positive evaluation from the European Food Safety Authority (EFSA) there must be 'generally accepted scientific evidence' to substantiate the claim. EU legislation, Regulation (EC) No. 1924/2006, dictates that the food or nutrient in question must be well characterised and shown to have a beneficial physiological effect (5). The cost of conducting robust scientific research coupled with often limited research and development (R&D) budgets is a barrier for successful applications by SMEs (6).

A pilot trial is a small-scale study that is used to test the physiological effects and participant acceptance of prototype formulations in order to select food products to be tested in a larger scale trial. The pilot is also a great opportunity to test the data collection methods, procedures and logistics that can provide early identification of problems in order to find suitable solutions that can be implemented to improve the quality and efficiency of the main trial.

With examples gathered from the PATHWAY-27 project, this guidance document describes the requirements for the formulation and production of BEF, the design and conductance of a pilot trial, and how the data and information gathered can be used to inform further larger trials and the best practice for public reporting.

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2. Designing a pilot study

2.1. Production of bioactives

To produce BEF it is important to provide ingredients with a high enough concentration of the bioactive compound(s) as well as ensuring the safety of the ingredients.

The ingredients containing the bioactive components of interest must be well characterized. The production process should be described along with the chemical composition and concentration of bioactives. The concentration of bioactives in the ingredients should be consistent; large fluctuations will affect the quantity of the ingredient required to produce BEF. Alteration of one key ingredient may necessitate a reformulation of other ingredients, such as water. Consequently, this may alter the stability, quality and shelf-life of the BEF.

The microbiological criteria and their limits of acceptability for the bioactive ingredients must be established considering microbiological risk assessments associated with the type of product.

The concentration of the bioactive to be administered to volunteers must be set before starting the trial, based on data in the literature on the effectiveness of the bioactive itself on the specific endpoint(s) to be measured. The selection of this concentration drives the choice on the ingredient containing the bioactive.

2.2. Formulation of bioactive-enriched food

2.2.1. BEF formulation

The formulation of foods enriched with bioactive ingredients represents a big challenge for food technologists. Characteristics related to composition, digestibility, bioactive accessibility, shelf-life, sensory properties and consumer acceptance must be optimized. Food formulation is initially conducted on a lab-scale and then increased for production in a manufacturing setting.

As reported above, the concentration of the bioactive to be administered to volunteers is a crucial point. It is usually set based on results in the literature considering the same or similar endpoint(s). Once the concentration is set, the possibility to include it in one portion of the BEF must be checked. Since portion size is different for different food categories, the bioactives concentration per 100 g could be different in different foods. Notably, a high bioactives concentration in the BEF due to a small portion size could negatively affect its sensorial characteristics.

The PATHWAY-27 project represents a useful example on how to manage the formulation and choice of the right products to be used in pilot studies. The PATHWAY-27 pilot studies aimed to test 5 different bioactive enrichment types – docosahexaenoic acid (DHA), beta-glucan (BG) and anthocyanins (AC), individually or in combination of DHA+BG and DHA+AC, within each of 3 different food matrices – bakery, dairy and egg. In the beginning of the project, within each food matrix at least three different foods were considered as possible vehicles to deliver the bioactive ingredients (**Errore. L'origine riferimento non è stata trovata.**). Formulation of the food products was decided using factorial design to simultaneously evaluate the effect of ingredients, stabilizers, salt concentration, pH, and processing parameters such as mixing time/speed, processing time/temperatures, on the production process. Particular attention was devoted to:

- Optimizing the ratio of bioactives, proteins, fats and other ingredients,
- Choosing appropriate ionic strength/pH parameters to control the aggregation of hydrophilic macromolecules to obtain discrete colloidal particles,
- Selecting suitable excipients to direct self-assembly of components to obtain colloidal particles,
- Selecting suitable stabilizers to control 'host-guest' interaction between polysaccharides and AC. Emphasis was placed on studying the dissociation of colloidal structures at varying pH, ionic strength and enzymatic attack to modulate the release of the bioactive in chemically unaltered forms.

Table 1 Food products considered for each matrix in the PATHWAY-27 project

Bakery	Dairy	Egg
Buns	Pudding	Pancake
Bread sticks	Combined dessert	Omelette
Biscuits	Milkshake	Beverage
	UHT milk	Custard
	Melted cheese	

During the design and development of PATHWAY-27 BEF problems occurred. Some were general across all BEF, for example using AC imparted a distinctive purple, sometimes green colour, and some were specific to the food type, for example high viscosity of batter for pancakes containing BG. The challenges and solutions are summarized in **Errore. L'origine riferimento non è stata trovata.**.

Table 2 Problems faced during the design and development of PATHWAY-27 BEF

Goal	Problem	Potential solutions

All products should be stable at room temperature for at least 3 weeks.	- Dairy and egg based foods are typically stored at 4°C to maintain adequate microbiological safety.	- The addition of preservatives and vacuum/modified atmosphere packaging. - Proposal for multiple deliveries from the manufacturer to the recruitment centres (RCs) and from the RCs to participants.
Enrich products with 3 g BG per portion	- BG produced highly viscous solutions when mixed with liquid egg that are difficult to handle in the machines used to produce egg products. - Some products developed unacceptable organoleptic properties. - Less crisp texture of bakery biscuit BEF compared with control due to requirement for extra water.	- Inclusion of more water in the formulation to reduce viscosity. - Use of flavour additives to mask unpleasant flavours. - Longer baking time to produce a drier biscuit.
Enrich products with 320 mg AC per portion.	- AC produced a strong violet colour in all BEF. - Loss of textural quality during storage of bakery buns. - Low density and high staling rate of bakery buns. - Unacceptable taste of bakery buns due to preservatives.	- Microencapsulation of the AC. - Use of coloured ingredients containing no polyphenols to mask the colour. - Recipe modification to change the type of bakery bun. - Reduction of preservatives.
Enrich products with 250 mg DHA per portion.	- DHA imparts a fish-like aroma and taste to the foods.	- Use of flavours and aromas to mask the DHA.

		- Use DHA as part of a phospholipid or enriched egg powder ingredient

During the product development process, it became apparent that some BEF were not technically feasible. It was therefore decided that UHT milk, melted cheese and custard would not be further considered.

2.2.2. BEF digestion and bioaccessibility of bioactive compounds

Bioaccessibility of a compound or nutrient describes the fraction that is released from its food matrix and solubilized in the gastrointestinal tract so becoming potentially available for intestinal absorption. Bioactive bioaccessibility could be even more relevant than bioactive concentration in the BEF, and it could be dependent on the food matrix. It is therefore mandatory to understand the influence of the food matrix on BEF digestibility and bioactive accessibility. In addition, it could help to select the BEF with the best overall performance for the intervention trial.

A model for static in vitro digestion was recently developed and reported by COST action FA1005, INFOGEST (7). This model comprises oral, gastric and intestinal phases and considers the presence of digestive enzymes, electrolyte composition, temperature, calcium and bile salts, pH and time. It can be applied to various endpoints and can be adapted to accommodate specific requirements.

Based on PATHWAY-27 experience, bioaccessibility of bioactives is influenced by the food matrix and in some cases the simultaneous enrichment with two bioactives. Differences were also observed between two foods within the same matrix. In the PATHWAY-27 project results from digestion and bioaccessibility studies were used to assist the selection of BEF for use in the pilot intervention trial.

2.2.3. Consumer acceptance and sensory analysis of BEF

Understanding consumer acceptance and determining the sensory profile of BEF is an important part of developing BEF for an intervention trial. The use of not well-accepted foods, or foods with bad sensorial characteristics affects participant compliance during the intervention. Accordingly, in the PATHWAY-27 project, 45 BEF were formulated (3 foods per matrix, with 5 enrichment types per food). All BEF were pre-screened and one product from each matrix, with the worst sensory characteristics, was excluded to leave 30 BEF for further sensory and consumer acceptance profiling.

Consumer acceptance was measured using first an online survey followed by tasting sessions. The online survey consisted of four parts: a) to collect socio-demographic data, b) to explore behaviour and attitudes towards health and diet, c) to understand attitudes towards functional foods, and d) to evaluate acceptance of the PATHWAY-27 BEF using photos.

Following on from the online survey, consumers were invited to tasting sessions where they were asked to rate the appearance, aroma, flavour and texture of the BEF using a 9-point hedonic scale. In addition to these main attributes an extended list including specific food colours, aromas, flavours, aftertaste and textures were scored using a 5-point JAR-scale. Consumers were also asked to indicate how much they liked the BEF overall and how likely they were to buy the products, taking into consideration the healthiness of the food. Following this exercise, one food from each matrix was excluded (breadsticks, egg desert and pudding).

A sensory panel was trained in compliance with ISO standards to describe attributes of the foods and rate them on a 0-9 category scale. This is called sensory profiling and provides more specific information that can help technologists improve the products.

The results of consumer acceptance testing and sensory profiling were used to support the selection of BEF for the pilot intervention trial. The selection of the right product is crucial for the intervention. If it is not well-accepted by participants the study compliance will be low. This can result in a high number of dropouts reducing the power of the study, or to a consumption of the food lower than declared by the volunteer. This can represent an important bias in the study outcome.

2.3. Shelf-life evaluation of BEF

The chemical, microbiological and sensorial quality of BEF should be established under the standard storage conditions of the food product. The results will help to characterize the BEF and ensure the provision of safe, stable and acceptable products for the duration of the trial.

2.3.1. Chemical stability

2.3.1.1. Bioactive compounds

It is essential to ensure that participants of the study receive the effective dose of the bioactive compound(s) for the duration of the trial. It is to be expected that the storage conditions (temperature and time), along with the food matrix and bioactive type will affect chemical stability. The bioactive content of BEF should be measured immediately after production and then at set intervals during the storage period under the standard storage conditions.

During the design phase of the study, it is important to establish protocols for the chemical analysis of bioactive compounds in BEF. Different analytical methods are likely to yield varying results so the available methods should be evaluated, tested and selected in advance. It is important to consider that different food types may require different extraction methods, for example the extraction method for anthocyanins in PATHWAY-27 bakery products was different from the extraction method for dairy and egg products.

Bioactive retention is an important parameter of BEF formulation. In PATHWAY-27 AC were found to degrade during food processing and storage of bakery products. To compensate for losses during processing, higher levels of AC were included in the starting formulation. In addition, it was found that bread buns lost more AC compared to biscuits. This factor influenced the choice of biscuits over bread buns in the final selection of BEF for pilot studies. During the PATHWAY-27 studies, bioactive levels were measured for each batch of food produced using standardized analysis protocols. The evaluation of bioactive retention can prevent bias in the intervention study due to administration of bioactive amounts lower than required. Pilot studies are usually quite short, so shelf life and bioactive retention do not represent a big problem, but they must be carefully considered in the light of the further intervention that is usually longer than the pilot.

2.3.1.2. Food matrix

While developing new functional food products containing bioactive compounds, it is current practice to assess the stability of the enrichment ingredients as it may be influenced by their interaction with the matrix, the latter exerting either a protecting or destabilizing action. The other perspective explored by the PATHWAY-27 project is about the effect of the enrichment ingredients on the stability of the matrix, as it is assumed that the bioaccessibility of the other nutrients in the food product remain unaltered. Nuclear Magnetic Resonance (NMR) spectroscopy is a state-of-the-art technique that can be utilized to characterize the food products and assess their molecular profiles. Since high-resolution NMR will only detect molecules that are soluble in the extraction medium it does not directly observe the solid food matrix. Thus, matrix stability is measured by analysing the soluble molecules that are released by the solid phase, that are disappearing by binding to the matrix or are consumed by degradative reactions, during storage.

Firstly, different products for each food category must be comparatively analysed. In the case of PATHWAY-27, two products belonging to bakery, dairy and egg were analysed: buns and biscuits, milkshakes and combined desserts, and pancakes and omelettes. At the starting storage time, all food products with the same food matrix were compared to each other, independently of the enrichment type. To evaluate the contribution of the matrix-related substances on the complete molecular profile, only the spectral data points exerting an intensity change below a determined threshold of variation, among the 5 enrichments of the

same product are considered as descriptors of the matrix. In addition, the subset of selected points must constitute an important fraction of the whole spectral data set, to be a meaningful representative of the soluble fraction of the matrix. These data points are the spectral features that will be evaluated during the shelf-life, by measuring their variance within each product during three storage time-points (for example, T0, T15 and T30 days).

In the PATHWAY-27 study, it was found that milkshakes were the products with highest stability within the dairy category, whilst pancakes were more stable than omelettes among egg products, and biscuits and buns had the same stability.

Additionally, a comparative evaluation among the different enrichment types within the same food matrix should be addressed to avoid combinations of bioactive ingredients and food matrix which result in poorly stable food products. Of the PATHWAY-27 BEF selected for the pilot intervention trial, biscuits were shown to have a relatively stable matrix independent of the specific enrichment type; the most stable pancake enrichment was DHA+AC; and DHA+BG enriched milkshakes had the longest stable molecular profile.

2.3.2. Microbiological stability

The microbiological safety of BEF is one of the most important criteria for selection because it is essential not to cause ill health to participants during the trial. In the PATHWAY-27 project two factors were considered:

1) The growth of intrinsic microorganisms during storage (shelf-life study), and
2) The ability of inoculated risk microorganisms to grow in the stored products (challenge test).

The microbiological criteria that are analysed along with limits of acceptability must consider risk assessments associated with the type of food product. This is based on regulations, standards, review of literature and the manufacturers own experience with similar types of products. A challenge test may not be considered necessary if the food product is unlikely to facilitate the growth of pathogens, for example foods that are frozen immediately at the site of production or provided as a dry powder.

During production and microbiological testing of milkshake BEF for the PATHWAY-27 pilot study, Enterococcus spp. was detected in all samples. The levels were acceptable according to regulations in the country of manufacture, however at the time it was unknown whether the levels were acceptable in the country where the milkshake BEF would be trialled. Further microbiological testing was carried out to determine the source of the contamination and expert advice was obtained. Testing showed that the contamination came from the original non-enriched milkshake powder rather than from the bioactive enrichment. Advice from dairy

specialists confirmed that Enterococcus spp. is commonly reported in milk products and that it is difficult to find products with zero-levels. After many discussions, it was agreed that the RC testing the milkshake BEF would be able to use the products, however the start of the pilot trial at this RC was delayed by one month.

2.3.3. Maintenance of sensory quality

BEF must maintain the sensory quality during the shelf-life of the product to maintain a high compliance in participants. Having previously determined the sensory attributes of the BEF, a trained panel should assess the products at set intervals during the predetermined safe shelf-life period.

2.3.4. Nutritional evaluation

BEF must be evaluated from the nutritional point of view, and they must comply with the indication of good nutrient profile provided by EFSA. In case the level of nutrient such as saturated fat or sodium is too high, the food must be reformulated or discarded. Using PATHWAY-27 as experience, we can cite that the original milkshake formulation needed modifications using skimmed milk instead of whole milk to reduce the high level of saturated fat. This reformulation allowed obtaining a good nutritional profile while retaining acceptable sensory properties.

2.3.5. Consumer perception

Independent from their nutritional value BEF must be perceived as healthy by consumers. In PATHWAY-27, BEF were nutritionally evaluated and their nutrient profile was acceptable, but consumer perception was not used to reformulate the study BEF. The selected products were perceived as energy-dense and increasing the possibility of weight gain and this reduced the number of volunteers willing to enter the study. Based on PATHWAY-27 experience the evaluation of consumer perception should be included in the list of determinants for the choice of the BEF to be used in the intervention.

The integrated analysis of all the above-mentioned parameters allows the choice of the most suitable food to be used in the intervention. In PATHWAY-27, to select the three foods, one in each matrix, to be used in the pilot studies, the method of the "decision sieve" was used. Briefly, some parameters were set as mandatory (microbiological safety, chemical stability of bioactive compounds, and nutritional profile), and foods not complying were immediately discarded. Secondary parameters used for BEF selection were: bio-accessibility of bioactive compounds, Sensory characteristics, Ease of preparation, ease of storage, and food matrix stability).

2.4. Writing a research protocol

The description that follows considers the experience built in PATHWAY-27, but it can be used for other matrices and bioactives than those used in the project. It can also be used when a single, specific bioactive should be used to enrich a specific food matrix, as often happens when the producer is a SME. In this case the procedure is easier, but it allows the optimization of the newly formulated food.

A research study protocol is a document that provides detailed information about the background and rationale for the trial and the intervention and activities expected of the participants. It will demonstrate to a Research Ethics Committee (REC) that the study is scientifically valid and has been ethically designed to safeguard the health and wellbeing of the participants. REC members will use the protocol as a basis for providing approval/a favourable opinion for the trial. Researchers involved in the study will use the protocol as an operational handbook.

When writing a research study protocol, it is essential to follow the relevant regulations and good practice guidelines for conducting a research study using human participants. There may be some variation between national guidelines, however, generally in the European Union (EU), these will be based on EU legislation and will adhere to the same governing principles.

The World Health Organization (WHO) have produced a comprehensive Practical Guide for Health Researchers (8) that covers ethics; planning; implementation; analysing, interpreting and communicating results; and writing a scientific manuscript for publication. The document also includes an extensive list of resources to assist a literature search. Regulation (EU) No 536/2014 for clinical trials on medicinal products for human use (9) and the ICH Guidelines for Good Clinical Practice E6(R1) (10) both provide information to ensure the rights, safety and well-being of study participants are maintained. The WHO also provide a simple, user-friendly overview of the recommended format for a research protocol (11).

A research study protocol should follow a standard format. Each of the sections to be included are summarised in this document and, where relevant, links to more detailed resources are provided.

2.4.1. Scientific background and rationale

Performing a review of current literature in the subject area of interest is an essential first step to determine the need for, and feasibility of, the proposed research. Some questions that must be considered include:

- Has research using the pure bioactive been conducted before?
- Has the effective dose of the bioactive been established already?
- What is (are) the main endpoint(s)?
- Who is the target population?
- Have similar BEF been produced before?
  - Have they been studied in intervention trials?
  - Have they been studied in animal models?
- Which food matrix should be selected?
- Is outcome variable(s) direct measures of the claimed effect?
- Are the assessment methods appropriate?

Four main gaps in current knowledge about the health effects of bioactive compounds that were identified will be addressed by the following strategies of the PATHWAY-27 project:

1) Considering bioactives administered as BEF within the everyday diet rather than as discrete compounds there is a need to evaluate:
   a. Possible interactions of bioactives and the food matrix.
   b. Possible effects of the food matrix on the outcome.
2) Bioactive effectiveness will be evaluated against measureable, physiologically-relevant endpoints.
3) *In vitro* and *in vivo* studies will be integrated to better understand the clinical effect of the bioactives and their underlying mechanism(s) of action.
4) The high cost of intervention studies that is often a barrier to SMEs and reduces their ability to develop new products will be shared between SMEs to optimise their cooperation.

The BACCHUS guidance for the design and implementation of human dietary intervention studies for health claim submissions provides detailed information of the approaches for conducting a literature search, with useful examples (4). They describe six steps to retrieve relevant literature and aspects to consider when evaluating human studies. The steps are summarised below:

1) Define the problem and the study question.
   - Use a PICO (participants, intervention, comparison, outcome) search strategy
2) Use online databases to search for papers related to the hypothesis.
   - For example: PubMed, EBSCO, Web of Science, MEDLINE, CAB Abstracts, Scopus, etc.
3) Identify key terms associated with the study question and related terms.
   - For example: anthocyanins, metabolic syndrome.
4) Create a search strategy using the search terms identified.
5) Evaluate the search results.

6) Evaluate the scientific quality of each study.

2.4.2. Study hypothesis/research question and objectives

A research study is based on a clear research question or hypothesis that it is designed to answer. The objectives are statements of the research questions and should be simple, specific and stated in advance (11). There may be an overall research question/hypothesis that initially generates the research study, however a feasibility or pilot study that is conducted prior to a main randomised controlled trial (RCT) could have separate objectives. For example, a pilot study can be used to test procedures, estimate recruitment, compliance and retention, and determine sample size (12). The data collected from a pilot trial are not necessarily expected to provide statistically significant results.

In PATHWAY-27, three pilot studies were used to select the most effective BEF for each food matrix to be tested in a larger intervention trial. This preliminary selection in pilot studies greatly reduced the cost of the larger intervention trial. In fact, each pilot allowed the selection of the most effective enrichment in a specific food matrix, so considering the food matrix/bioactive interaction. In total, 15 BEF were tested in the pilot studies and three of them were selected for the main RCT. To test all foods in a long term, multi-centre study would have been extremely costly. To perform a single pilot to verify the most effective enrichment, and then use it in all matrices would have not considered the food matrix interaction. The design of PATHWAY-27 pilots is therefore a good compromise to reduce cost avoiding the *a priori* assumption that one enrichment fits all matrices. A similar design can be useful when the same food company needs to test different possibilities or when different companies (mainly SMEs) producing different products wish to share the cost of the intervention.

2.4.3. Trial design, randomisation and blinding

2.4.3.1. Trial design

The most rigorous research study is a RCT. A placebo group is typically used to demonstrate a cause and effect relationship between the treatment group and the outcome (13). Random allocation to the treatment prevents systematic differences occurring that can affect the outcomes. Since it is possible that the measured outcomes may be a result of simply consuming the study foods, rather than the bioactive enrichment, the control group acts to validate the results of the treatment group. However, it may not be necessary to use a control group for a pilot trial because the objective of the pilot trial may be to select the most promising formulation, rather than provide statistically conclusive evidence of an effect.

There are two types of design that can be used with a RCT, these are crossover or parallel-arm. In a crossover study, each participant will receive both treatment and control, the order in which they receive this can be randomly assigned. This type of study can be more cost effective since fewer participants are required to achieve the statistical power (14). However, a crossover study has the disadvantage that the first intervention effect may carry over to the second intervention and confound the outcomes. Another disadvantage of the crossover is that participants will be required to take part in the study for twice as long which could lead to a higher rate of drop-outs or lower compliance. A crossover study may also affect blinding of the trial if the placebo and treatment foods are noticeably different. A parallel study will compensate for the disadvantages of the crossover design; however, it is likely to require a higher number of participants per treatment group. A parallel design was chosen for the pilot studies to enable a more rapid selection of BEF for the subsequent main RCT.

2.4.3.2. Types of randomisation

Several methods have been proposed for the random assignment of participants to the study groups in clinical trials. These include simple randomization, block randomization, and stratified randomization. Simple randomization is the equivalent of flipping a coin, and the random allocation of subjects into the groups will probably yield balanced group sizes in large trials, but not necessarily in smaller ones. The block randomization method will result in equal sample sizes among groups, as it uses small blocks with sizes that are multiple of the number of treatment groups. The stratified randomization method will also control for the influence of covariates such as gender or (predefined) age groups, etc. Thus, not only will it yield equal group sizes, but also subjects' baseline characteristics will be similar between groups. Practical implementation of this method is very difficult; hence this technique is rarely used in RCTs.
To achieve equal group sizes among the five PATHWAY-27 treatment groups the block randomization method was used for each of the three study centres. The allocation of participants to the different groups was carried out based on predefined randomisation lists created separately for each recruiting centre. All three randomisation lists were prepared with a block size of 5 and with an allocation ratio of 1:1:1:1:1.

2.4.3.3. Blinding

Blinding is the procedure used in a trial to avoid bias by preventing participants and/or researchers being aware of the treatment allocation. There are four categories of blinding [adapted from (15)]:

1) Unblinded: all parties are aware of the treatment allocation.
2) Single-blind: Only the participants are unaware of the treatment allocation.
3) Double-blind: The participants and researchers are unaware of the allocation.
4) Triple-blind: Participant, researchers and data analysts are unaware of the allocation.

When using a food product, it is not always easy to formulate an exact matching placebo, although matching macronutrients and other key nutrients, such as salt, is essential. BEF and placebo that differ in characteristics other than the food or constituent may affect the claimed health effect. Consequently, the study design would be considered not appropriate for the claim and that the study is at high risk of bias so no conclusions can be drawn (16).

The bioactive ingredient used may have a characteristic that is difficult to disguise or provide a non-bioactive corresponding ingredient. For example, the PATHWAY-27 bioactive ingredients of docosahexaenoic acid (DHA) and anthocyanins (AC) give the foods distinctive characteristics. DHA tends to impart a fish-like odour to the foods and AC turns the foods purple (Figure 1). While the participants receiving the treatment, in a parallel-arm trial, are unable to compare their food with the placebo, it is possible for them to speculate about the group they have been assigned. It is important to ensure blinding to the research team allocating the foods to avoid influencing participants. For this reason, in PATHWAY-27 all BEF portions given to participants were individually wrapped in opaque packaging that was identical amongst treatment groups (Figure 2).

Figure 1 BEF and corresponding placebo products used in the PATHWAY-27 pilot studies. 1a = Biscuit BEF, 1b = Biscuit placebo; 2a = Milkshake BEF, 2b = Milkshake placebo; 3a = Pancake BEF, 3b = Pancake placebo.

Figure 2 Packaging used in the PATHWAY-27 project. Clockwise from top left: biscuits, milkshake powder, pancakes

2.4.4. Study population (participants)

This section should describe the eligibility criteria to participation in the trial and depends whether a generally healthy population is required or if a specific health issue is being addressed.

The criteria used for determining eligibility for the PATHWAY-27 pilot trial is listed in the following sections. It was intended that there would be a 1:1 ratio of male and female participants, however this was difficult to achieve in each RC and therefore this criterion was not adopted for the larger trial.

2.4.4.1. Inclusion criteria

MetS is the name given to a cluster of conditions that occur together more often than can be explained by chance. In 2009 a joint statement was published by world-leading authorities on cardiovascular disease and diabetes that harmonised the criteria by which MetS is diagnosed (17). The cut-off points for each condition associated with MetS is shown below, a diagnosis is made when 3 out of the 5 criteria are present.

- Elevated waist circumference (men ≥ 102 cm; women ≥ 88 cm)
- Elevated fasting triglycerides (≥ 150 mg/dL)
- Reduced fasting HDL-cholesterol (men ≤ 40 mg/dL; women ≤ 50 mg/dL)
- Elevated blood pressure (systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg) or hypotensive treatment
- Elevated fasting glucose (≥ 110 mg/dL)

In the PATHWAY-27 pilot trial, volunteers, male or female, age 18 to 80 years, were eligible if they presented with two of the criteria for MetS, with at least one of them being elevated fasting triglycerides or low HDL cholesterol.

After the start of recruitment, the eligibility criteria were amended to include participants with 2, 3 or 4 of the conditions associated with MetS, providing that one of them was elevated fasting triglycerides or low HDL cholesterol. This change was implemented due to many of the volunteers who were screened that typically had altered triglycerides and/or HDL cholesterol plus increased waist circumference and raised blood pressure.

2.4.4.2. Exclusion criteria

Originally participants were excluded if three or more clinical criteria for MetS were met. This was later changed to reflect the amended inclusion criteria so that only volunteers meeting all 5 of the criteria for MetS were excluded. Additionally, major exclusion criteria were:

- Regular drug therapy with impact on serum lipids;
- Diabetes (fasting glucose > 1.26 g/L, or anti-diabetic treatment);
- Recent history of cancer or cancer treatment (less than 2 years);
- Active or recently diagnosed intestinal malabsorption or disorders associated with malabsorption: Crohn's disease, short bowel syndrome, Pancreatic insufficiency, cystic fibrosis, Tropical Sprue, Whipple's disease, chronic pancreatitis, gastrojejunostomy, surgical treatments for obesity, cholestasis, biliary atresia, parasite infections, HIV/AIDS
- Familial dyslipidaemia;
- Use of medication known to cause malabsorption: tetracycline, cholestyramine, thiazide diuretics, aluminium/magnesium hydroxide, colchicine, neomycin, methotrexate, methyldopa, and allopurinol, and laxatives
- Illegal drug use, chronic alcoholism or active smoking;
- Intensive physical exercise (≥ 5 hour/week);
- Consumption of nutritional supplements containing DHA, BG or AC;
- History of allergy or intolerance to any components used in BEFs, celiac disease, lactose intolerance, allergy to milk or egg proteins;

- Institutionalised patients, those who lack autonomy to consent or are unable to meet all examinations;
- Women who are pregnant, lactating or actively trying to conceive;
- Participation in other clinical trials that may impact on outcome;
- Subjects deprived of their liberty by judicial or administrative decision.

2.4.5. Sample size

This is the number of participants required to detect significant differences between primary outcomes in each group. Estimating an adequate sample size has ethical and financial implications. It should be a large enough sample to provide reliable results but not too large that more participants than necessary are included.

To perform a proper sample size calculation, the first and most important step is to formulate the null and the alternative hypothesis. Then the significance level and the power with which the sample size would be calculated will be set, together with specifying the smallest effect size that will be of scientific interest. Finally, most of the time, for the calculation to be performed an estimate of the standard deviation is needed – which can be obtained from historical data – from similar studies conducted in the past, or from a pilot study. After having all the necessary data, the calculation of sample size will be straightforward. There is little published guidance concerning how large a pilot study should be. General guidelines suggest different methods, for example, for using 10% of the PATHWAY-27 sample required for the full study (18).

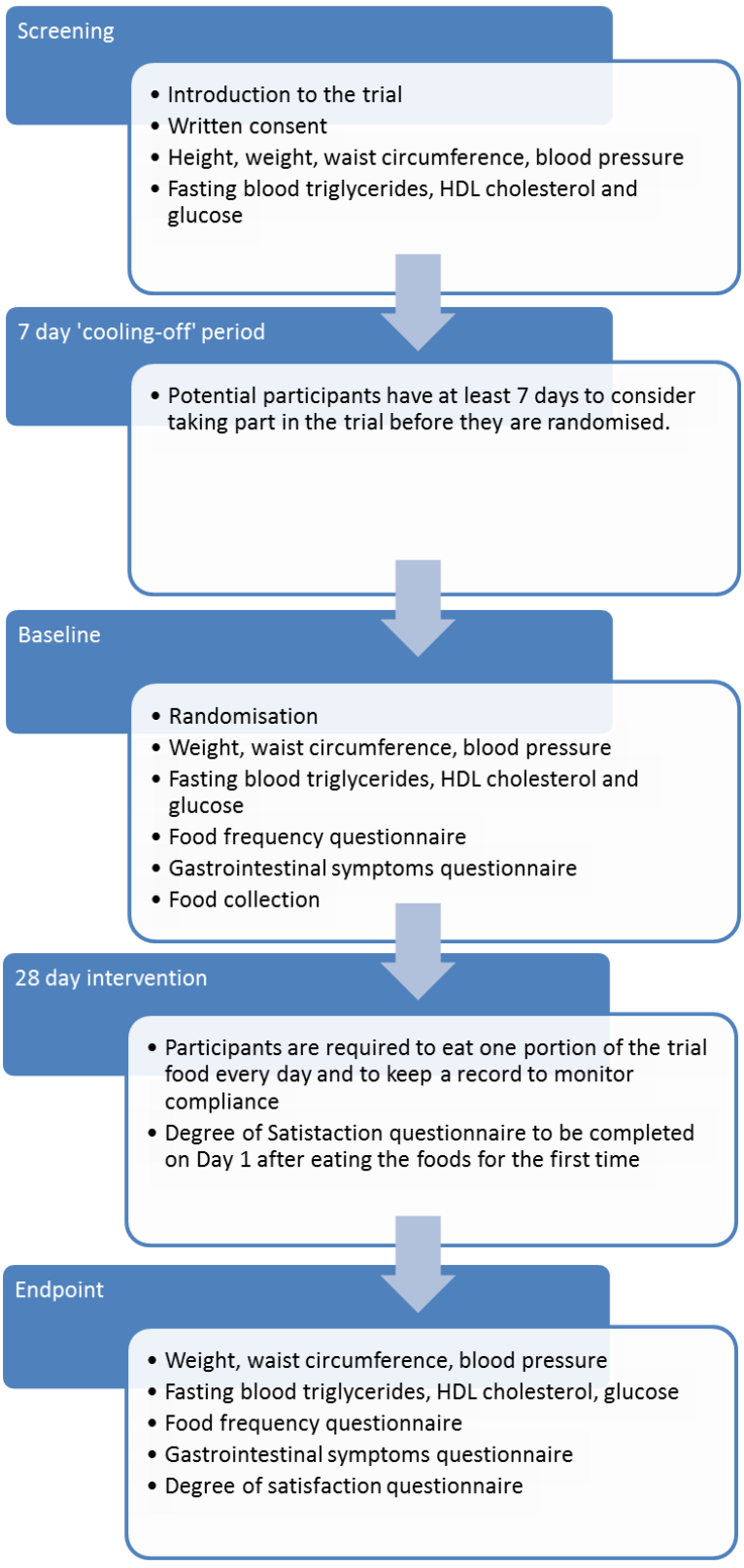
In the PATHWAY-27 main RCT, initial sample size calculation was based on results provided by a reference article, where a difference in the decrease in triglyceride levels of 0.11 mmol/l with standard deviations in the placebo and control groups of 0.34 and 0.31, respectively was obtained. Consequently, for the PATHWAY-27 main RCT a difference in the decrease of triglyceride levels of 0.12 with a common standard deviation of 0.35 was hypothesised. Based on historical data and adjusted values for multiple group comparisons to obtain a power of 80% a sample size of 200 volunteers per group was calculated. According to suggestion from general guidelines, a sample size of 20 volunteers in each treatment group was therefore considered acceptable. Since each pilot focused on five treatments, the total sample size in each pilot was set as 100 volunteers. The sample size was then reduced due to the need of shortening the pilot duration, and set to about 70 in each group to accomplish the “rule of 12”, i.e. at least 12 participants in each group for pilot studies with primary focus of estimating average values and variability for planning larger subsequent studies (19).

The sample size in pilots allowed the selection of the most effective BEF to be used in the main RCT. Significant results were not expected in pilot studies, they needed to be able to discriminate the effectiveness of the different treatments. To do it was quite difficult in the

pilot with the lower sample size. Based on the PATHWAY-27 experience, it is clear that evaluation of sample size is particularly critical for a pilot study. The statement “No sample size justification is needed because of the pilot nature of the proposed study” is not correct. On the contrary, proposals for pilot studies are not exempt from the need to have a clear and well-reasoned rationale for the number of participants to be included. Alignment of the rationale with the scientific aims of the research is critical. Writing a clear and compelling justification for the proposed sample size requires an intellectual analysis of the expected benefits, risks, and costs of the study and the knowledge of which statistical methods are optimal for the study. “Value of information” techniques are becoming more recognized as alternatives to traditional sample size estimation methods that often rely on imprecise parameter inputs (20).

2.4.6. Intervention

The participant activities of the trial should be described in detail. A process flowchart can be useful for illustration (Figure 3).



- Project funded under the Food, Agriculture and Fisheries, and Biotechnology theme (KBBE)

Figure 3 Process flowchart illustrating participant activities during the PATHWAY-27 pilot studies

During the PATHWAY-27 pilot studies volunteers who expressed an interest in taking part in the trial were contacted by the RC, provided with the Participant Information Sheet and asked to complete a screening questionnaire that gathered information about health and lifestyle factors. Subsequently, providing the volunteer did not meet any exclusion criteria, they were invited to a screening appointment. At the screening appointment, the study was explained to the volunteer in detail, they were given the opportunity to ask questions and it was made clear that they had the right to withdraw at any time up until the publication of results. The volunteer gave written consent and had their height, weight, waist circumference and blood pressure measured, and a sample of blood collected to measure fasting triglycerides, HDL cholesterol and glucose. If the results of the screening examination indicated that the volunteer met the inclusion criteria they were invited to take part in the trial. A minimum of 7 days was given prior to baseline to allow potential participants to decide whether they wished to take part in the trial.

At the baseline visit to the RC the volunteer was randomised, had physical measurements and blood sample collection repeated, and were provided with the study foods. They were provided with written study instructions and asked to complete a food frequency questionnaire and gastrointestinal symptoms questionnaire. These activities were repeated at the endpoint visit with an additional questionnaire that measured acceptance of the BEF. During the 28-day intervention period participants were asked to eat one portion of BEF each day and to keep a record of the BEF they consumed particularly noting days where BEF were not consumed or only part consumed. On Day 1 after eating the BEF for the first-time participants were asked to complete the degree of satisfaction questionnaire that measured acceptance of the BEF.

2.4.7. Outcomes

Primary and secondary outcomes should be well defined. For health claim applications, EFSA will consider whether the outcome describes a beneficial physiological effect, whether the outcome variables are direct measures of the claimed effect and if the assessment methods are appropriate (16). For a main RCT these will include biochemical markers, anthropometric measurements and the data collected from questionnaires. A pilot study will have some of the same outcomes, most likely fewer than the main RCT, however the reason for measuring these outcomes in a pilot will differ. A pilot study will focus on feasibility or testing analytical methods to allow for any necessary improvements to be implemented in the main RCT. In the case of PATHWAY-27 the target population was at risk for MetS, which is defined by the combination of distinct risk factors. Here, a pilot study gives the opportunity to detect the most modifiable risk factor that will serve as primary outcome in the RCT. Any less modifiable outcome variable may be considered as a secondary outcome. Additionally, most promising outcomes related to a specific intervention may be identified by applying novel analytical

(omics) techniques resulting in a single or a set of new markers predictive for a disease or function to be implemented in the future RCT. A pilot study could also have a different scope, i.e. to select the most promising treatments, as in PATHWAY-27. Any compliance and safety markers may be considered as secondary outcomes. In the case of the PATHWAY-27 pilot study food derived markers (for example, change in serum DHA after DHA enriched BEF consumption) have been determined and included in the compliance analysis.

2.4.8. Analytical and statistical methods

The specific analytical methods required will depend on the parameters being assessed. Measurement of biochemical markers will require laboratory analyses. Anthropometric data will be collected by trained research personnel directly from the participant. The equipment used should be of an appropriate standard to provide accurate and precise measurements with a degree of sensitivity that corresponds to the requirements of the study. Validated methods should be used, where possible.

2.4.8.1. Analytical methods

The analytical methods used in the PATHWAY-27 pilot studies are outlined below:

- Blood pressure was measured in participants after being at rest for five minutes in a seated position followed by two additional measurements separated by two minutes each. A calibrated sphygmomanometer (e.g. Boso-Carat professional) was used and the appropriate sized cuff applied after measurement of arm circumference.
- Height was determined using a calibrated stadiometer.
- Body weight was measured using a calibrated balance with the participant barefoot and dressed in underwear.
- Waist circumference was measured in standing position between the iliac crest and the lowest rib using a measuring tape according to the WHO guideline.
- Blood sample analyses were performed by standardised automated methods.
- Food frequency data was captured using the EPIC validated questionnaire.
- Nuclear magnetic resonance (NMR) spectroscopy was used to measure the metabolome of serum.

2.4.8.2. Statistical methods

There are many statistical methods to choose from, with the proper one for a given situation depending both on the type of data to be analysed as well as on the question one wants to find an answer for. Thus, the t-test can be used for estimating a mean, the difference of two means, or to compare two means – either between groups, or within group (repeated measures) or even correlations; χ^2-tests can be used for estimating proportions, the difference

of two proportions, or compare two proportions; the f-test to compare several means, or population variances; or the χ^2-test for the assessment of relationships in two-way tables. Thus, several methods will be applied in most of studies, as various types of data are collected in the trial, and their assessment will require different methods to be applied (**Errore. L'origine riferimento non è stata trovata.**).

Table 3 Examples of statistical methods and comparisons that can be applied to different data types

Data	Methods	Comparisons
Description of continuous variables (such as age, TG levels, BMI, etc.)	- summary statistics including number [n], mean, standard deviation, median, minimum, and maximum	
Description of categorical variables (such as gender, questionnaires, etc.)	- summary statistics giving counts and percentages	
Group comparisons	- 2 normally distributed groups, continuous variables	2-sample t-test
	- 2+ normally distributed groups, continuous variables	one-way ANOVA (f-test)
	- 2 or more groups with categorical data	χ^2-test
	- 2 repeated measures within a group	paired-sample t-test
	- 2+ repeated measures within a group	one-way repeated measures ANOVA (f-test)
	- relationship between two variables	correlation analysis
	- relationship between two variables, with the possibility to adjust for confounding factors	mixed linear model

2.4.9. Safety considerations

Protecting the rights, interests and safety of human participants is fundamental to any research study and is governed by the principles of the Nuremberg Code, the Declaration of Helsinki and the Council for International Organizations of Medical Sciences (CIOMS). The

following list summarises the main points of each guideline, however it is recommended that the guidelines are accessed and read fully to ensure complete understanding.

a) Informed consent and the right to withdraw
b) Risks, burdens and benefits
c) Vulnerable participants
d) Ethical review by a research ethics committee
e) Privacy, confidentiality and anonymity
f) Use of control/placebo
g) Scientific validity
h) Compensation for injury
i) Research registration, publication and dissemination of results

2.4.9.1. Adverse and serious adverse events

An adverse event (AE) is any medical incident affecting a study participant, it does not necessarily have a causal relationship with the study food products. A serious adverse event (SAE) is defined as an occurrence that:

- results in death,
- is life-threatening,
- requires hospitalisation or prolongation of existing hospitalisation,
- results in persistent or significant disability or incapacity,
- consists of a congenital anomaly or birth defect,
- is otherwise considered medically significant by the investigator.

Provision should be made for the reporting of adverse and serious adverse events to the project coordinator. Additionally, events that are considered to be related to the study, or are unexpected, should be notified to the REC.

During the PATHWAY-27 pilot studies, clear instructions were given to participants to contact a doctor if they experienced an AE or SAE and to inform the RC as soon as possible.

2.4.10. Data management

Data management consists of tasks required to capture all study data into electronic form. It includes tasks designed to validate the entered data by means of a variety of edit checks, for example subjecting the data to range checks, valid value checks, cross-checks, and manual review that provide feedback to those entering/providing the data. A clinical database that accurately reflects the data collected and can be used for purposes of analysing study data for regulatory submissions and professional publication should be developed.

The data capturing process and the electronic system that captures clinical data for purposes of analysis and reporting should adhere to Good Clinical Practice (GCP) guidelines for data management systems. The standard requirements for high quality GCP-compliant data management in multinational clinical trials have been developed by the Working Group on Data Centres of the European Clinical Research Infrastructures Network (ECRIN) (21). It provides a list of requirements that can be used as a checklist.

During the PATHWAY-27 pilot trials data were initially captured on paper clinical report forms (CRF) then transferred to an electronic data capture system (EDC) hosted by AdWare Research Ltd.

2.4.11. Quality assurance and quality control

Quality assurance relates to the actions that are established to ensure the trial is conducted in compliance with the ICH GCP guidelines and any other relevant regulatory directives.

Quality control refers to the operational practices, within the quality assurance system, that are employed to safeguard the activities of the research study satisfying the regulatory requirements.

Written Standard Operating Procedures (SOPs) should be developed to ensure the study is conducted and that data is generated, documented and reported appropriately. The ICH GCP guidelines state that quality control should be applied at each stage of data handling to ensure that all data are reliable and have been processed correctly.

It is the responsibility of the sponsor to secure access to all trial-related sites, data, documents and reports for monitoring and auditing, and inspection by external regulatory agencies.

2.4.12. Project management and collaborations

The project management structure should be explained along with links to related projects. This information will be written into the projects' Description of Work to describe the project consortium.

2.4.13. Ethics

Adhering to the ICH GCP guidelines, a research trial should be conducted in compliance with a protocol that has received institutional review board (IRB)/independent ethics committee (IEC) approval/favourable opinion. Each country within the EU will have their own research ethics committees that should be consulted in the early stages of planning a research study

involving human participants. Academic institutions usually have their own ethics committees that are responsible for reviewing research studies that do not involve patients. The European Network of Research Ethics Committees (22) provides links to relevant EU and national legislation along with details of national research ethics committees.

Errore. L'origine riferimento non è stata trovata. illustrates the ethical approvals required at each recruitment centre prior to commencing the PATHWAY-27 pilot studies.

Table 4 Ethical approvals required at the PATHWAY-27 RCs. CRNH: Centre de Recherche en Nutrition Humaine, France; MRI: Max Rubner Institute, Germany; ULE: University of Leeds, UK.

CRNH	MRI	ULE
- CPP Sud Est VI (ethics committee of Clermont-Ferrand) - ANSM (French drugs Agency, Vigilance system to clinical trials)	- Ethics Committee of the State Chamber of Physicians in Baden-Württemberg (Ethik-Kommission der Landesärtzekammer Baden Württemberg)	- University of Leeds Faculty Research Ethics Committee - NHS R&D required for the use of NHS services (for example blood sample analyses)

2.4.13.1. Approvals and amendments

Substantial amendments such as changes to the study protocol or other recruitment documents and procedures will require an ethics amendment before being implemented. Ethics applications and amendments can take many weeks to be considered and given a favourable opinion. Amendments are usually reviewed more quickly although multiple substantial amendments to the study protocol may require submission of a new ethics application and therefore be subject to the full ethics review process.

2.4.14. Budget and funding

Within this section full details of the budget requested should be provided, with justifications for each item, and sources of financial support for the project.

An example summary of costs to be included in the budget proposal is provided in **Errore. L'origine riferimento non è stata trovata.**.

Table 5 Example of costs to be considered for a dietary intervention trial

Cost item	Description

Infrastructure and facilities	Buildings, utilities, insurance etc.
Staff salaries	PI, Co-ordinator, Nurse, Technician, Clerical etc.
Consumables	Blood and urine sample collection, sample processing and storage, sample despatch to analysing laboratories etc.
Participant payments	Commensurate with the time and effort expended by the participant*

*Payments to participants, or compensation for time and effort, is specific to each country and will be influenced by the recommendations of the REC.

2.4.15. Finance and insurance

The agreements for financial support and insurance should be provided. For example, the PATHWAY-27 project is funded by the EU Seventh Framework Programme. Insurance and/or indemnity to meet potential legal liabilities is provided by the Sponsor, which in this case is the recruitment centre.

3. Implementing a pilot study

It is essential to have a detailed plan for food production and recruitment. BEF will typically have a shorter shelf life than a dietary supplement in the form of a pill so the production and recruitment plans must be strategically developed and closely monitored to complement each other. BEF must be produced, dispatched and available at the RC ready for distribution to the participants as necessary.

3.1. Food production plan

The food production plan must be developed in conjunction with the food manufacturer to consider availability of equipment and production capacity at the production facility, as well as the storage capacity of the RC taking into account the product shelf life, requirements for microbiological testing and the transportation time. The food production plan will also be influenced by the study design requirements, such as randomization and gender stratification. For the PATHWAY-27 pilot studies block randomization was stratified by gender. BEF were produced separately for men and women. The randomization by gender complicated the production plan and therefore this type of randomisation was not adopted for the larger trial.

3.1.1. Deliveries to RC

BEF shipments must take into consideration weekends and national holidays at the RC. Deliveries may be made on a large pallet so there should be a suitable facility at the RC to unload this type of shipment from the delivery vehicle. In some cases, where the shipment is small, boxes can be unloaded individually, however it may be impractical to do this with a large number of boxes, particularly in the case of frozen BEF that must be transferred to frozen storage as quickly as possible to maintain the cold chain.

3.1.2. Storage facilities

Ambient BEF are easier to store than BEF that require cold storage. The storage facility should be adequately constructed to maintain the quality of the foods during the shelf-life, this may be to protect against high temperature, light, moisture and pest infestation. Cold storage will be regulated by national legislation, for example in England quick frozen foods, such as the pancakes used in the PATHWAY-27 trial, are covered by the Quick Frozen Foodstuffs (England) Regulations 2007 (23). This regulation states that the temperature on thermal stabilization must be -18°C or colder and that this temperature must be maintained, except for brief periods during transport (including local distribution), where it may reach -15°C, or when in retail display cabinets where it may reach -12°C. Air temperature must be monitored during transport and storage. Where the cold storage facility at the RC is less than 10m^3 this may be limited to an externally visible thermometer.

3.1.3. Unused food

In the event that the recruitment target is not fully achieved and BEF are not used it should be decided whether foods can be recycled or destroyed. If possible BEF should be recycled for other participants in the trial to reduce unnecessary food wastage and additional production/shipping costs being incurred. If storage space is restricted at the RC, having a contingency plan to recycle or destroy foods will help to reduce the burden of storing foods that will not be used.

3.2. Recruitment plan

The recruitment plan will be developed in conjunction with the recruitment centres and food manufacturers considering the capacity for appointments and food production, respectively. The rate of recruitment and randomization to the trial must match the rate of food production and delivery to the RC to ensure the availability of BEF for each participant.

3.2.1. Recruitment strategies

Accessing a target group with a specific health condition and motivating that group to take part in a dietary intervention trial can be challenging. If the health condition manifests through poor dietary and lifestyle choices it can be difficult to motivate such individuals to volunteer to a trial that involves changing firmly established behaviours. Experience from the PATHWAY-27 study indicates that volunteers to a dietary intervention trial are often interested in their diet and health and therefore typically have a healthy metabolic profile. Consequently, they are not eligible to take part in a trial that requires participants with an unhealthy metabolic profile, for example elevated fasting blood triglycerides and/or low HDL cholesterol. Members of the public with an unhealthy metabolic profile, and who are often unaware of it, are less likely to be motivated to volunteer to take part in a dietary intervention trial.

Recruitment strategies are a key component to accessing the target group. Advertising the trial using the most effective media will help to improve the success rate of recruitment. The pilot study can be used to test different advertising methods and identify the most suitable for the target population. Advertising can be broadly focused to a non-specific audience, for example on a local radio station, or directly targeted to identify potential participants from a clinical patient register, for example at a local doctor's surgery.

A radio advert may reach many thousands of listeners and generate a lot of enquiries about the study. Translating those enquiries to participants requires an effective management system since it will not be feasible to speak directly with each advert respondent. A dedicated website or page on the RCs website is a useful first point of contact for enquiries where

information about the trial can be conveyed. A link on the webpage can direct volunteers to an online survey that can be initially used to filter out individuals based on some of the exclusion criteria. For example, if treatment with statins is an exclusion criterion there can be a question on the survey that asks whether the respondent currently takes statins. If the answer is 'yes' the survey can provide an automatic message informing the individual that they are not eligible to participate. At the end of the survey, respondents who are eligible to continue with selection are asked to provide contact details that are collected by the RC. Subsequently potential participants can be contacted and asked to complete a secondary online survey that is used to collect more specific data from volunteers, such as date of birth, height and weight, physical activity, allergies, current medication and health conditions. This data can be used to identify potential participants and invite them to a screening appointment, and to exclude those who meet exclusion criteria.

3.2.2. Food distribution to participants

Distributing BEF to participants will be influenced by the BEF storage conditions, shelf-life and storage capacity at the participants' home. BEF with a long shelf-life, such as the PATHWAY-27 milkshake powders that are stored ambiently and provided in a small sachet, pose very little problem for distribution. In this instance, it may only be necessary for one delivery. BEF that have a limited shelf-life may require more frequent deliveries. The bioactive stability of PATHWAY-27 biscuits dictated the shelf-life of the product. Short study duration, such as the 4-week pilot trial, only required one delivery to each participant. By contrast, a trial with duration longer than the shelf-life of the BEF would require more deliveries. BEF requiring cold storage are likely to pose the greatest storage problems. Firstly, it is important to note that the requirement for a freezer at the participants' home becomes an additional exclusion criterion. Providing that the participant has a freezer, the capacity needs to be considered. Short trial duration may be less problematic whereas a longer trial may necessitate multiple deliveries. In some instances, PATHWAY-27 pancakes had to be delivered to the home of the participants by research staff, since the participant could either not return home immediately after visiting the RC or they did not have suitable transport.

3.2.3. Participant retention and compliance

One of the most important aspects of conducting the trial is the retention and compliance of participants. Motivation for taking part in a trial varies between participants. Predominantly, in the PATHWAY-27 project, volunteers were motivated by potential for weight-loss, even though this was not a primary outcome of the study (this was clearly explained upon recruitment). If the participant does not feel that their personal objective for taking part in the trial is being met, they may lose motivation and withdraw.

The ease with which a participant integrates the BEF into their usual diet will influence their motivation. If the BEF are not well accepted then compliance to the study protocol will be impaired and may even lead to participants choosing to withdraw from the trial. Examples of how the foods could be consumed was provided (e.g. pancakes can be eaten on their own, or with sweet or savoury fillings). The time and effort that is required from a participant will also influence compliance and retention. A short trial that requires a participant to consume one BEF per day with relatively few questionnaires and visits to the RC will be more accepted than a longer trial with multiple foods and/or multiple questionnaires and visits to the RC.

Regular communication with each participant is essential to develop and maintain a good relationship. Participants who feel valued and well-informed will be more motivated to consume the BEF, complete the questionnaires and come to the RC when required. There are exceptions when participants are simply not well motivated, however in these cases it is unlikely that the research team will be able to improve compliance or prevent withdrawal from the trial.

3.2.4. Sample collection

For a human study investigating the effect of BEF on physiologically-relevant endpoints it is likely that biological samples will be required for analyses. The type of sample will depend on the selected endpoints but as a minimum requirement blood and possibly urine will be collected. It is common to collect a fasted sample from participants, particularly when measuring analytes that are influenced by consumption of food, such as glucose and triglycerides that will increase after a meal. To measure baseline values a fasting period of 10-12 hours may be necessary.

In some cases, it might be sufficient to use a point-of-care device such as the Cardiochek Professional Analyser test system that can measure the concentration of single or multiple analytes, using analyte-specific test strips, in a small volume of capillary blood (15-40 µL) collected from a fingertip. This can be useful for screening volunteers as the results are available within a few minutes. In most cases it is more appropriate to collect larger volumes of blood to perform a wider range of analyses. In this case a trained phlebotomist will be required to perform venepuncture, typically on the antecubital fossa region.

It is essential to determine the biological markers prior to the start of the trial to ensure the most suitable blood collection tubes are used. The number and type of analytes to be measured will influence the tube volume and additive type. The volume of blood drawn should not be excessive to minimise discomfort to the participant. Ethically it will be important to justify the volume required. The correct tube type will depend on the analyte being measured and the analytical method. For example, to measure lipids it is necessary to collect blood into a tube containing a coagulant that causes the blood to clot. The tube is typically left for 30

minutes at room temperature following blood collection to allow the clot to form. The tube can then be centrifuged to separate the sample and extract serum. Multiple tube collections must adhere to recommendations for the order of draw to prevent potential cross-contamination of additives that may affect the accuracy of sample analyses. Current practice for the order of draw is:

1) Blood cultures
2) Citrate
3) Serum
4) Heparin
5) EDTA
6) Fluoride

Some analytical methods will require the sample to be refrigerated immediately after collection to prevent sample/analyte degradation, in other cases it may not be necessary. In all cases it is essential to know the correct storage conditions for the sample, use suitable storage containers that are appropriate for the storage conditions and to label the samples adequately to guarantee traceability.

3.2.5. Sample analysis

The protocols for sample analyses should be determined prior to the start of the trial. Changes to the analytical protocols, including changing equipment or laboratories, during the trial should be avoided as this could introduce a source of error into the results. Failure to manage the samples correctly may affect the accuracy of the analyses. If there are special conditions that are required for the processing and storage of samples they must be communicated to the research team involved with collection and processing of samples. For samples that are to be stored prior to being shipped to the analytical laboratory, it is essential that the storage conditions are maintained during transport, where necessary. Repeated freeze-thaw cycles may reduce the integrity of the sample and affect the results. For example, serum obtained for the analysis of lipids, glucose, thyroid, kidney and liver function in the PATHWAY-27 project, is stored at -80°C and must maintain its frozen state until it is required for analysis. By contrast, whole blood used to analyse single nucleotide polymorphisms (SNPs) is stored at -20°C at the RC but can be shipped at ambient temperature.

Ideally all samples will be analysed by one laboratory to limit sources of random error. For multi monocentric studies, such as the PATHWAY-27 pilot studies, it is acceptable for sample analyses to be conducted at different laboratories however the analytical method, including the type of sample used – for example serum or plasma, should be the same. For a multi-centre trial (PATHWAY-27 larger trial), the analyses should, where practicable, be done by a centralised laboratory. In some cases, it is not feasible to use one analytical laboratory due to

the type of analysis that is to be performed. For example, a full blood count requires relatively fast analysis of whole blood to prevent artefacts from the EDTA anticoagulant that affect the results.

- Project funded under the Food, Agriculture and Fisheries, and Biotechnology theme (KBBE)

SEVENTH FRAMEWORK PROGRAMME

4. Reporting a pilot study

4.1. Pilot study outcomes

As discussed previously, the aim of a pilot study is to test the study protocol as well as addressing whether consumption of a particular BEF causes a beneficial physiological effect. The pilot study outcomes will be used to inform decisions for a subsequent RCT. Using the PATHWAY-27 pilot study as an example, the following outcomes were achieved:

- Primary outcomes measured at baseline and endpoint
  - Fasting blood triglycerides
  - HDL cholesterol
- Secondary outcomes measured at baseline and endpoint
  - Fasting blood glucose
  - Body height, weight and BMI
  - Waist circumference
  - Blood pressure
- Acceptance of BEF
- Participant compliance with the study protocol
- Participant recruitment strategies
- Testing of data collection methods
- Logistics of food delivery and storage
- BEF selection for main RCT

Collating the pilot study outcomes and using them to learn what works well and what doesn't, then implementing any necessary changes will help to ensure the subsequent RCT has a good quality, robust design that will provide substantial evidence to support the claim of a beneficial health effect.

4.2. CONSORT 2010 Statement: extension to randomised pilot and feasibility trials

One of the main outputs of a research study is the dissemination of results. The Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines (24) were developed to improve reporting of RCTs. Transparency is the core objective.

The CONSORT statement provides a minimum set of recommendations for reporting RCTs with a series of extension statements to include variations of the standard trial methodology. For example, the CONSORT statement for pilot and feasibility trials (25) provides guidance for reporting randomised trials in which a prospective RCT, or part of it, is conducted on a smaller scale.

The CONSORT statement: extension to randomised pilot and feasibility trials comprises a checklist of items that should be included in the report. The main points of the checklist are summarised below:

a) Title and abstract
b) Introduction
   - Background and objectives
c) Methods
   - Trial design
   - Participants
   - Intervention
   - Outcomes
   - Sample size
   - Randomisation
   - Sequence generation
   - Allocation concealment mechanism
   - Implementation
   - Blinding
   - Analytical methods
d) Results
   - Participant flow
   - Recruitment
   - Baseline data
   - Numbers analysed
   - Outcomes and estimations
   - Ancillary analyses
   - Harms
e) Discussion
   - Limitations
   - Generalisability
   - Interpretation
f) Other information
   - Registration
   - Protocol
   - Funding

5. Conclusion

Valuable experiences gathered from the PATHWAY-27 pilot studies have been collated and presented here with the aim of providing a useful guidance document for health researchers. A well-designed pilot trial can improve the quality and robustness of a larger RCT. Dietary intervention trials are complex and often involve asking participants to consume foods that they would not usually choose to eat and to change behaviours that have developed over many years. BEF add an additional level of complexity since the food product is novel and experimental and therefore unfamiliar to most consumers. For SMEs to invest time and money in R&D there has to be an incentive, such as gaining market competitiveness. This may be achieved through successful health claim applications that allow the SME to market and promote their food as beneficial to health. Achieving a successful health claim application for a functional food requires formulation of a BEF that is well characterised and can be shown to have a beneficial physiologically-relevant effect on health. Strategic planning and close collaboration between all relevant partners (production companies, researchers, co-ordinators) is central to designing and implementing a pilot study and subsequent larger RCT that will generate exploitable outcomes.

6. References

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7. Abbreviations:

ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use