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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 a randomised controlled trial (RCT) using bioactive-enriched foods (BEF). These guidelines provide an update to, and are intended to complement, previously published guidance documents. They are unique in providing practical information for intervention studies using BEF, based on the PATHWAY-27 project.

The aim of PATHWAY-27 is to evaluate the effectiveness of BEF containing docosahexaenoic acid (DHA), anthocyanins (AC) and oat beta-glucan (OBG) 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 compounds 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 has developed two guidance papers, for the scientific community and for the food industry, relating to the substantiation of health claims on BEF. These guidance papers (submitted as deliverables D7.4 and D7.5) were finalised after consultation in two stakeholder workshops and complement earlier guidelines. They are particularly useful for small and medium sized enterprises (SME) looking for a competitive advantage in the EU market. They are uploaded to the project website and are freely available.

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, states 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 SME [6]. A pilot study can be a cost-effective opportunity to investigate the feasibility of an intervention and to test the methods of recruitment and data and sample collection prior to conducting a larger RCT. PATHWAY-27 submitted a practical guidance document for conducting pilot studies with BEF (project deliverable D5.4).

With examples gathered from the PATHWAY-27 project, this practical guidance document describes the requirements for the formulation and production of BEF, the design and considerations for successful implementation of a RCT, and how to report the data according to good practice.

2. Designing bioactive enriched food

2.1. Bioactive ingredients

To produce BEF it is important to provide bioactive ingredients with an appropriate concentration of the bioactive compound(s) as well as ensuring that the ingredients are safe for human consumption. Ingredients that have not been consumed to a significant degree by humans in the EU prior to May 1997 and have not yet been authorised as novel foods, must be approved for use in compliance with Regulation (EU) 2015/2283 of 25 November 2015. Novel food status will only be approved for use in the EU if the ingredients do not present a risk to public health, are not nutritionally disadvantageous when replacing a similar food and are not misleading to the consumer. Scientific assessment must be carried out prior to authorisation to ensure their safety. The authorisation will determine the conditions for use of the ingredient, their designation as an ingredient and the labelling requirements.

The concentration of the bioactive that will be administered to participants in the BEF must be determined before starting the trial. This should be based on data in the literature or data collected during a pilot study in which the effectiveness of different doses of bioactive compound(s) are tested. The selection of this concentration will influence the choice of the ingredient containing the bioactive compound(s).

Each bioactive ingredient batch must be provided with a product specification providing details of the safety, composition, chemical and physical characteristics of the product.

2.1.1. Safety of bioactive ingredient

The microbiological profile and the limits of acceptability for the bioactive ingredients must be established, and described as part of the ingredient characterisation, taking into account microbiological risk assessments associated with the type of product, for example a dry powder or liquid formulation. Chemical safety must also be ensured by controlling the hazards associated with potential chemical contamination of the product. Such contaminants that may be present include, but are not limited to, mycotoxins, residual solvents from the extraction process and residues of pesticides, heavy metals, and polycyclic aromatic hydrocarbons. Finally, physical safety must ensure the absence of physical particles such as glass or stones. Further detail is provided in the Pathway-27 guidelines for the food industry.

2.1.2. Bioactive composition and stability of the bioactive ingredient

It is important to determine the ingredient composition and concentration of the bioactive component. When working with a mixture of compounds such as a botanical extract in which only some of the compounds are biologically active, characterization of the whole extract must

be provided. This must include characterisation of each compound or component up to 99.9% of the weight of the sample.

The bioactive content of the ingredient may vary between different batches and within the same batch. The target and/or acceptable variability within and between batches, during the ingredient shelf-life, should be defined. Factors affecting the variability include plant species, geographical growing region, season and processing and storage parameters. It is important to limit fluctuations in bioactive concentration since this may affect the quantity of ingredient that is required to produce BEF with a consistent bioactive concentration. This can be difficult to achieve when using bioactive ingredients derived from natural extracts, therefore discussions with ingredient manufacturers must emphasise the need for a reliable and consistent supply of the ingredient for the duration of the study. Appropriate analytical methods should be established, and measurements should be performed by a competent laboratory, with a quality assurance system in place (such as Good Laboratory Practice (GLP) or ISO 17025), that will certify the data. As examples, in PATHWAY-27, anthocyanin content was measured by high performance liquid chromatography (HPLC), DHA by gas chromatography (GC) and OBG by both enzymatic methods and size exclusion chromatography. The methods are described in project deliverable D4.3 (second report on the production of safe BEF with expected shelf life and concentration of bioactive).

2.1.3. Physical and chemical properties of the bioactive ingredient

The physical and chemical properties of the bioactive ingredients and the bioactive compound(s) must be determined. Solid or liquid state, water activity and water content along with physical properties such as powder properties (e.g. particle size, solubility in water), colour, etc. should be described.

The manufacturing process should also be described along with evidence to demonstrate how the process is standardised to produce a consistent product. Any stabilisation or encapsulation process applied should also be described.

2.2. 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.

2.2.1. Chemical stability of bioactive components within BEF

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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.

As discussed in section 3.1.2., reliability and consistency of the bioactive concentration to be administered to participants in the BEF is fundamental to the trial. Administering a bioactive concentration that is lower than the effective dose will bias the intervention study and lead to misinterpretation of the results.

The food matrix in its raw state, after storage and/or during domestic preparation, has an influence on the activity or release of the bioactives. The stability of the bioactive compound(s) must be carefully monitored immediately after BEF production, then at fixed intervals during storage, and the process optimised as much as possible to minimise bioactive losses. Bioactive compounds are often sensitive to conditions encountered during food processing, for example, in PATHWAY-27, yeast fermentation and baking at high temperature decreased the AC content of the bakery BEF during production, which was unavoidable. The amount of bioactive ingredient was increased to compensate for the degradation and to ensure that the effective bioactive dose in the final BEF was correct.

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 different 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 AC in PATHWAY-27 bakery products was different from the extraction method for dairy and egg products.

2.2.2. Bioactive and food matrix interactions

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 was 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.

For example, in PATHWAY-27, at the starting storage time different products for each food matrix were comparatively analysed, independently of the bioactive enrichment. Buns were compared with biscuits (bakery matrix), milkshakes with combined desserts (dairy matrix), and pancakes with omelettes (egg matrix), irrespective of DHA, OBG or AC enrichment. To evaluate the contribution of the matrix-related substances on the complete molecular profile,

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only the spectral data points exerting an intensity change below a determined threshold of variation among the five 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, in order 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 PATHWAY-27, 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.

Secondly, a comparative evaluation among the different enrichment types within the same food matrix should be completed to avoid combinations of bioactive ingredients and food matrix which would result in poorly stable food products. In PATHWAY-27, 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+OBG enriched milkshakes had the longest stable molecular profile.

During the design and development of PATHWAY-27 BEF, many technical challenges emerged. Some were general across all BEF matrices, for example adding AC imparted a distinctive purple, sometimes green colour and DHA imparted a fish-like odour, and some were specific to the food type, for example high viscosity of batter for pancakes containing OBG. The challenges and solutions are summarized in table 1.

Table 1 Technical challenges 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 three weeks.	- Dairy and egg-based foods are typically stored at 4°C to maintain adequate microbiological safety. - Unacceptable taste of bakery buns due to preservatives.	- The addition of preservatives and vacuum/modified atmosphere packaging. - Proposal for multiple deliveries from the manufacturer to the recruitment centres (RC) and from the RCs to participants.
Enrich products with OBG	- OBG produced highly viscous solutions when mixed with liquid egg that are difficult to handle in the machines used to produce egg products. - 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. - Longer baking time to produce a drier biscuit.

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Enrich products with AC	- AC produced a strong violet colour in all BEF. In bakery, colour could also be green. - Loss of textural quality during storage of bakery buns. - Low density and high staling rate of bakery buns.	- Microencapsulation of the AC. - Use of coloured ingredients containing no polyphenols to mask the colour. - Recipe modification to change the type of bakery bun. 1.
Enrich products with DHA	- DHA imparts a fish-like aroma and taste to the foods.	- Use of flavours (e.g. anise) 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.

2.2.3. Microbiological safety of BEF

The chemical and microbiological 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.

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 trial, *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 tested in the pilot study. 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 compared with the RC testing the bakery and egg food matrices. Furthermore, since the milkshake BEF and milkshake placebo were to be used at all RC in the subsequent RCT, it was necessary for each RC to confirm approval for the use of the products.

2.2.4. Nutrient Composition

The nutrient profile of BEF must be given consideration in the context of its effect on the overall balance of the consumer's diet. There is the potential for foods bearing a health claim to mislead consumers when trying to make healthy choices [7]. In case the nutrient level, such as saturated fat or sodium, is too high, the food must be reformulated or discarded. Using PATHWAY-27 as an example, the original milkshake formulation required modification to use skimmed milk instead of whole milk in order to reduce the level of saturated fat. This reformulation improved the nutritional profile while retaining acceptable sensory properties.

2.2.5. Digestibility, bioaccessibility and bioavailability of bioactive compounds

Understanding the influence of the food matrix on digestion of BEF and how this affects bioaccessibility and bioavailability of the bioactive compounds will help with selection of the most appropriate food matrix to deliver the effective dose. Bioaccessibility refers to the fraction of the compound that is released from the food matrix and solubilised in the gastrointestinal tract. Bioavailability of the compound refers to the fraction that reaches the systemic circulation. This information can be useful for health claim dossiers to demonstrate that the bioactive is available for absorption.

In PATHWAY-27, research was carried out *in vivo* using a pig model to investigate the effect of the food matrix on DHA bioaccessibility and bioavailability, the results are described in D6.1 (Report describing the effects of assessed food matrices on digestion/availability in the pig model).

2.2.6. Consumer acceptance and sensory analysis of BEF

Consumer acceptance may vary between different European countries and presents a challenge for developing and selecting the most suitable BEF. Understanding consumer acceptance and determining the sensory profile of BEF is important since the use of foods

that are not well-accepted, or foods with poor sensory characteristics, can affect participant recruitment and compliance during the intervention.

In PATHWAY-27, forty five BEF were formulated (three foods per matrix, with five enrichment types per food). All BEF underwent sensory testing as follows:

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 the online survey, focus consumers (not trained in sensory evaluation) 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), leaving two products from each of the three matrices with five enrichment types per food (a total of thirty BEF). These thirty products were subjected to more advanced sensory profiling using trained panellists.

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 trials. Choosing the most optimal product in terms of sensory properties is essential to maximise compliance to the trial.

2.2.7. Consumer perception of BEF

Independent of 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 some of the selected products, biscuits and pancakes, were perceived by consumers as energy-dense. This perception can reduce the number of volunteers willing to enter the study. This was overcome by clearly explaining that the products were low in fat and sugar, and in fact not energy dense.

Based on PATHWAY-27 experience, the evaluation of consumer 'healthiness' perception of the products should be included in the list of determinants for the choice of the BEF to be used in the intervention.

2.2.8. Maintenance of sensory quality during trial duration

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

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trained panel should assess the products at set intervals during the predetermined safe shelf-life period. In PATHWAY-27, this shelf-life exercise was carried out by the trained panellists. Products with a short sensory, or microbial, shelf-life poses greater logistical challenges for RC as the BEF may have to be distributed to participants more frequently in smaller batches.

2.3. BEF selection for the dietary intervention trial

The PATHWAY-27 project represents a useful example on how to manage the formulation and choice of the right products to be investigated. Initially, the PATHWAY-27 project conducted three pilot studies with the aim of selecting three BEF, from a possible fifteen BEF, most effective at improving markers of MetS. Five different bioactive enrichment types – DHA, AC and OBG, individually or in combination of DHA+OBG and DHA+AC, were tested in each of three different food matrices – bakery, dairy and egg. At the beginning of the project, within each food matrix, at least three different foods were considered as possible vehicles to deliver the bioactive ingredients (Table 2).

Table 2 Food products considered for each matrix in the PATHWAY-27 project

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

Integrated analysis of all the above-mentioned parameters facilitated selection of the most suitable food to be used in the RCT. In PATHWAY-27, to select three foods, one in each matrix, to be tested 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: bioaccessibility of bioactive compounds, sensory characteristics, ease of preparation, ease of storage, and food matrix stability). The selection of BEF to be used in the Pathway-27 RCT is described in detail in D2.6 (Report describing the integrated evaluation and selection of PATHWAY-27 BEF). Based on the evaluation decision sieve, the BEF selected were biscuits, milkshake and pancake.

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2.4. Food production

There are three stages to the production of foods for the intervention trial. Firstly, small-scale production is conducted to test the product composition and produce a prototype. At this point ingredient and process specifications are obtained and the shelf-life, stability and packaging can be tested. Consumer acceptance testing follows, and the product formulation or process specification may be modified based on consumer feedback.

The second stage is a pilot-scale production using the modified product formulation and specifications, and specifications for processing and packaging. This will deliver standardisation of the final product, packaging and processing specifications.

The final stage is factory-scale production of the final product. The shelf-life and nutritional profile is verified along with batch testing to ensure that the microbiological safety and bioactive concentration are maintained.

2.4.1. Packaging and labelling

The packaging of food products for an intervention trial needs to ensure that the appropriate level of blinding is maintained, this is discussed in more detail in section 3.1.9 Blinding. The label should provide the following information as a minimum:

- Best before or use by date
- Instruction for preparation/use
- Storage conditions
- Allergen information
- Randomisation number (if available)

Details of the product labels used in PATHWAY-27 are given in project deliverable D4.3 (second report on the production of safe BEF with expected shelf life and concentration of bioactive).

3. Designing a dietary intervention trial

3.1. Writing a research protocol

The description that follows considers the experiences from PATHWAY-27, but it can be used for matrices and bioactive compounds other 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 and a favourable opinion for the trial (refer to section 3.1.15 Ethics and trial registration for more information). 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 provides 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.

3.1.1. 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?
- Are the outcome variable direct measures of the claimed effect?
- Are the assessment methods appropriate?

The most rigorous evaluation of the literature is provided by a systematic review. The PATHWAY-27 Guidelines for the Scientific Community contain valuable information to illustrate the process of performing a systematic review. The BACCHUS Guidance for the Design and Implementation of Human Dietary Intervention Studies for Health Claim Submissions [4] also explains the approaches for conducting a literature search, with useful examples and describes six steps to retrieve relevant literature. 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.

The PATHWAY-27 project identified gaps in current knowledge about the health effects of DHA, OBG and AC that were addressed by the following strategies

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

3.1.2. Study hypothesis, research question and objectives

A research study is based on a clear research question and 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].

3.1.3. Trial design

The most rigorous research study is a double-blind, placebo-controlled, parallel RCT. Random allocation to the treatment prevents systematic differences occurring that can affect the outcomes and a control group is typically used to demonstrate a cause and effect relationship between the treatment group and the outcome [14].

There are two common 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 will be randomly assigned. This type of study can be more cost effective since fewer participants are required to achieve the statistical power [15]. 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 will require a greater number of participants per treatment group. A parallel design was chosen for the PATHWAY-27 pilot studies and RCT to enable a more rapid selection of BEF from the pilot for the subsequent RCT, and then to limit the burden on the participants and prevent carry-over of the treatment effect.

Methods and techniques developed for the assessment of BEF should be founded on the methods used for drug intervention trials assessing similar compounds. However, dietary trials have specific characteristics (table 3) compared to drug intervention trials that may impact on trial design. As an example, a study using BEF must consider whether participants can be expected to consume the same food every day for several months. This may lead to higher drop-out rates or low compliance with food consumption or food restriction. Therefore, a statistician must be included from early stages to advice on different study designs that consider the difference scenarios of completion and compliance. It is also very important to undertake dietary assessment to evaluate the impact of the intervention on general dietary pattern as it may be an important confounding factor.

Table 3 illustrates some of the differences between a food intervention and a drug intervention trial.

Table 3 Comparison of a food intervention study with a drug intervention

Food intervention comparison with a drug intervention study
- Higher drop-out rate
- Compliance to food eaten and to restricted foods drops with time
- More difficult to commit to dietary rules than to taking a given drug
- Statistical methods need to be specific to the study type
- Impact of food introduced on dietary pattern

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Nutritional interventions have a higher impact on food intake than drug trials. Therefore, dietary intake information should be collected from participants before, during and at the end of the trial. In some cases, participants habitual dietary habits may be part of the eligibility criteria (e.g. breakfast consumers, coffee drinkers). In other trials, participants will be asked to exclude or restrict certain foods from their diets for the duration of the trial. For example, in PATHWAY-27, participants who had allergies or intolerance to any of the BEF ingredients, or those consuming supplements containing bioactives, were excluded from entering the trial. During the Pathway-27 trial, participants were asked to restrict their intake of foods containing bioactives to one portion per day. A restriction list was provided for participants.

Furthermore, it is important to collect background dietary information in order to characterise the habitual diet of the participants in terms of food consumption patterns, diet quality, energy content and nutrient composition. The background dietary assessment is usually performed using food frequency questionnaire (FFQ) coupled with 24 hours-recall (24hR) questionnaires, food diary or diet history. Data from the background dietary assessment can also be used to identify possible malnutrition or nutrient imbalance in the cohort.

Diet should also be assessed during and after intervention, in order to detect any potentially confounding changes that may occur as a direct or indirect consequence of the dietary intervention [1]. Notably, dietary assessment is subject to a number of errors including misreporting and measurement errors associated with the reporting method [12]. The tools used for dietary assessment should be carefully considered and adapted to the population under study. Ideally, a combination of previously validated tools should be used, for example FFQ combined with 24 h [12]. The dietary assessment tools should be validated with biomarkers where possible. In PATHWAY-27, an FFQ was used at baseline to evaluate habitual food intake, while 24 h recalls were used at baseline, midpoint and endpoint; and were combined with metabolomics analysis of serum and urine.

In addition, a new FFQ questionnaire was developed as part of PATHWAY-27 to monitor and evaluate the intake of foods naturally containing bioactives. The FFQ focused on food items naturally containing DHA (e.g. fish), AC (fruits, vegetables, cereals) or OBG (cereal products) was prepared and administered at baseline, midpoint and end of the trial. The bioactive FFQ was adapted for each country to contain typical foods.

4.1.4. Feasibility or pilot study

It may be appropriate to conduct a feasibility or pilot study prior to the main trial in order to test procedures, estimate recruitment rate, compliance and retention, and to determine sample size [13]. The data collected from a pilot trial is not necessarily expected to provide statistically significant results and in cases where the intervention is relatively straight forward, for example if the food matrix is predetermined, the bioactive dose is established and there is just one treatment and one placebo group in a healthy population, a pilot trial may not be necessary.

In PATHWAY-27, pilot studies were conducted to select one BEF in each food matrix that was to be tested in the subsequent randomised controlled trial (RCT). Each pilot study tested five enrichment types in one of three food matrices, thereby considering the interaction of the food matrix and bioactive. Preselection of the BEF in this way greatly reduced the cost of the main RCT, since testing all foods in a long term, multi-centre study would have been extremely costly. However, to perform a single pilot trial to verify the most effective enrichment, and then use it in all food matrices would not have taken into account the food matrix interaction. A similar design can be useful if a SME needs to test different possibilities or if different SME producing different products wish to share the cost of the trial.

Due to the aim of PATHWAY-27 pilot studies, they were performed without including a placebo group. This study design is suggested only if pilots are used for selection of one product among different choices. Particularly as in the case of PATHWAY-27, when the pilot study tested foods prepared by enriching the same matrix with different bioactives. In this case the possible effect of the matrix itself is present in all subgroups, thus reducing the need of a placebo group. In case pilot studies are designed to verify the effectiveness of a single product on a small cohort of volunteers, the inclusion of a placebo group is recommended.

3.1.4. Outcomes

Primary and secondary outcomes must 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 [17]. For a RCT these will include biochemical markers, anthropometric measurements and the data collected from questionnaires. In the case of PATHWAY-27 the target population was at risk of MetS, which is defined by the combination of distinct risk factors. The most modifiable risk factor, possibly identified during a pilot trial, will serve as the primary outcome in the RCT. Any less modifiable outcome variable may be considered as a secondary outcome. Additionally, 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 that are predictive for a disease or function. Any compliance and safety markers may be considered as secondary outcomes. In the case of the PATHWAY-27 RCT, food derived markers (for example, change in serum DHA after consumption of DHA enriched BEF) have been determined and included in the compliance analysis.

3.1.5. Study population

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. Criteria should be broad enough to achieve the required number of participants from the target population within a defined geographical area during the recruitment phase. The criteria used for determining eligibility in the PATHWAY-27 RCT is listed in the following sections.

3.1.5.1. 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 [16]. The cut-off points for each condition associated with MetS are shown below, a diagnosis is made when three out of the five 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)

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

3.1.5.2. Exclusion criteria

Volunteers presenting with all five of the criteria for MetS were excluded since the trial was focused on prevention of MetS and not on its therapy. 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 two 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 dyslipidemia;
- 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 (≥ five hours per week);
- Consumption of nutritional supplements containing DHA, OBG or AC;
- History of allergy or intolerance to any components used in BEFs, celiac disease, lactose intolerance, allergy to milk or egg proteins;

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- 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.

3.1.6. Intervention

The participant activities of the trial should be described in detail. A process flowchart can be useful for illustration (figure 1).

During the PATHWAY-27 RCT, 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, if 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, glucose, thyroid stimulating hormone and free-T4. If the results of the screening examination indicated that the volunteer met the eligibility criteria they were invited to take part in the trial. A minimum of seven 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 and given the study foods for the first three or six weeks, this was dependent on the production schedule for the food as some were produced in smaller batches than others. Physical measurements were performed, and blood and urine were collected. Participants were asked to complete a gastrointestinal symptoms frequency questionnaire, a 24-hour dietary recall, and two food frequency questionnaires (FFQ). One FFQ collected dietary data about the general diet over a 12-month period and one collected data about the usual dietary intake of the PATHWAY-27 bioactive compounds over a 6-week period. A subgroup of participants opted to undertake additional activities that involved providing extra blood for isolation of lymphocytes, a faecal sample and abdominal fat tissue. These participants also underwent a DEXA scan and completed a physical activity questionnaire. On day one of the intervention, after the study foods were consumed for the first time, participants were asked to complete a questionnaire to rate their acceptance of the study foods.

At week six participants attended a midpoint visit at the RC. Physical measurements were performed again along with blood collection. At this time point, participants also completed a gastrointestinal symptoms frequency questionnaire and the 6-week bioactive compounds FFQ. Subgroup participants additionally provided a urine sample, completed a 24-hour dietary recall and physical activity questionnaire. Study foods for the following three to six weeks were supplied to the participants.

At the end of the 12-week trial, participants attended their final appointment at the RC, this was similar to the baseline visit and participants were asked to rate their acceptance of the study foods again.

At weeks three and nine, participants were asked to complete the gastrointestinal symptoms frequency questionnaire and given more study foods if required. At each time point, participants were asked if they had experienced any adverse events so these could be recorded. Participants were also invited to contact the RC at any point during the intervention if they had questions or wanted to report adverse events.

3.1.6.1. Harmonisation of trial procedures

For a multi-centre trial it is essential to harmonise standard operating procedures before recruitment begins and to agree which data and/or samples will be collected and reported, in what format and how they will be processed. Table 4 provides examples of data and samples that will collected during a dietary intervention trial such as the Pathway27 RCT. During Pathway27, all procedures for sample and data collection and processing were harmonised amongst recruitment centres prior to the intervention.

Table 4 Examples of data and samples that could be collected during a dietary intervention trial

Type of measurement/sample	Purpose
Height Weight Waist circumference Blood pressure Dual energy X-ray absorptiometry (DEXA)	Anthropometric characterisation of participants
Biological sampling	
Whole blood	SNPs identification
Serum	Metabolic profile and function (glucose, insulin, glycated haemoglobin, full blood count, lipid profile, kidney and liver function)
Lymphocytes	DNA methylation and gene expression
Urine	Dietary metabolites
Faecal	Microbiome, dietary metabolites
Adipose	DNA methylation and gene expression
Questionnaires and survey	Health and lifestyle screening Gastrointestinal symptoms Dietary assessment (24-hour recall, food frequency for the general diet and bioactive compounds) Physical activity Acceptance of study foods Compliance to the trial

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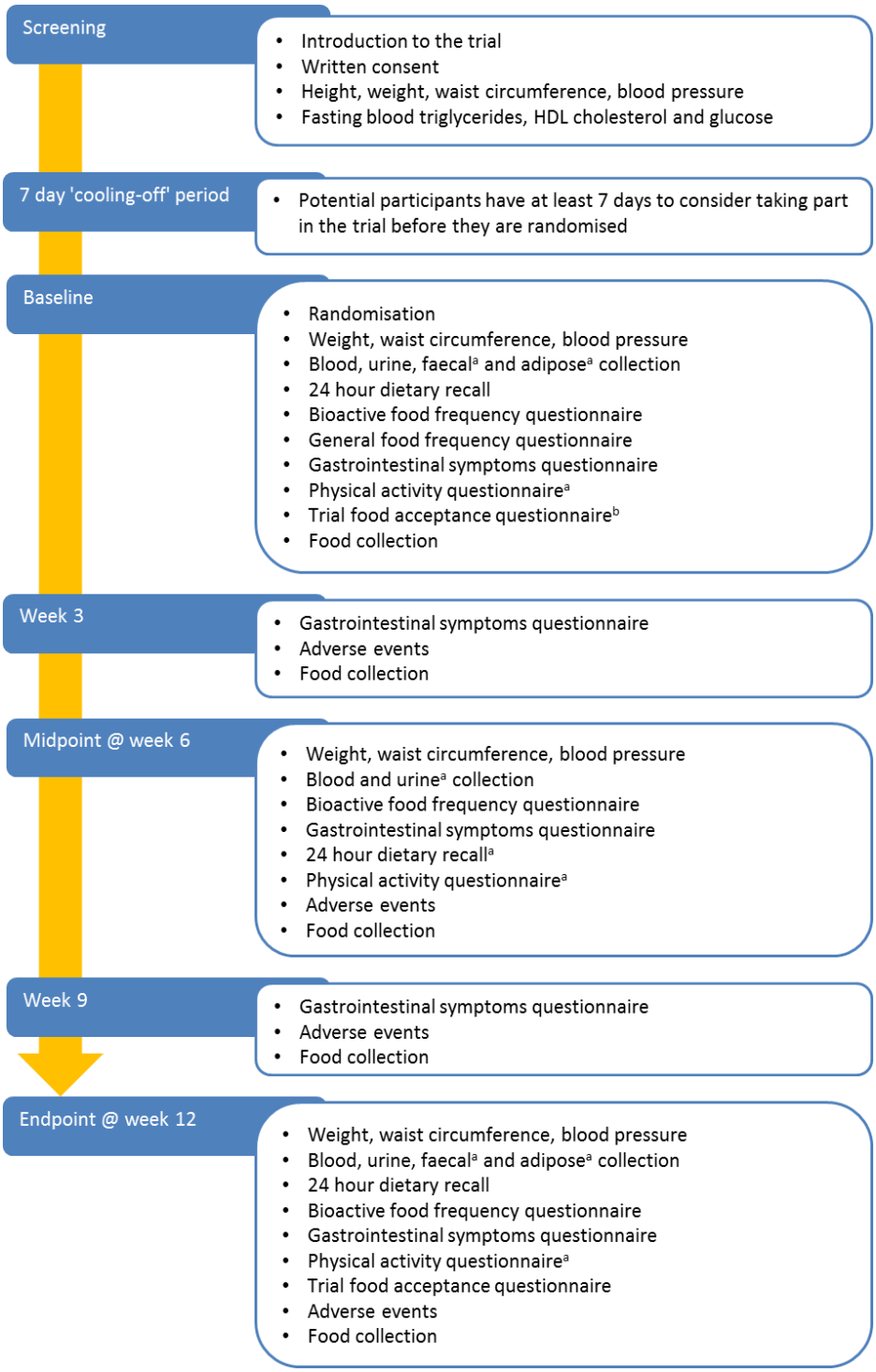

Figure 1 Flowchart of participant activities during the PATHWAY-27 RCT. [a]Only completed by participants enrolled in the subprotocol; [b]Completed on day one of the trial after the participant has consumed the trial foods for the first time.

3.1.7. 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.

In order 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 will be calculated are set, together with specifying the smallest effect size that will be of scientific interest (biologically meaningful). Finally, most of the time, in order 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 trial.

Recalculation of the sample size during the trial using the data that has been gathered to that point may provide a more accurate reflection of the sample required to achieve a statistically significant result as it is based on actual changes in the primary outcome(s) observed in the trial rather than the results obtained from a different study population.

If the study has more than a primary outcome, they all should be considered and the most appropriate selected. During the PATHWAY-27 trial, sample size was recalculated based on the HDL-C results obtained to date, obtaining a lower sample size.

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 [18].

3.1.8. 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 multiples 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.

In order to achieve equal group sizes among the PATHWAY-27 RCT treatment groups the block randomization method was used for each of the RC. The allocation of participants to the different groups was carried out based on predefined randomisation lists created separately for each RC. All three randomisation lists were prepared with a block size of four and with an allocation ratio of 1:1:1:1.

3.1.9. 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 [19]):

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 bioactive enrichment make it impossible to interpret whether the observed health effect is indeed due to the bioactive compound subject of the claim. This type of study design is at high risk of bias therefore considered inappropriate [17].

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 DHA and AC gave the foods distinctive characteristics. DHA tended to impart a fish-like odour to the foods and AC turned the foods purple (Figure 2). It is possible for participants to speculate about the group they have been assigned or, if friends or relatives also take part, to compare their study foods. It is important to ensure blinding to the research team allocating the foods to avoid influencing participants. For this reason, in PATHWAY-27 all food portions given to participants were individually wrapped in opaque packaging that was identical amongst treatment groups (Figure 3).

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

Figure 3 Packaging used in PATHWAY-27. Clockwise from top left: biscuits, milkshake powder, pancakes

3.1.10. 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 and the question being investigated. The most appropriate statistical method(s) cannot be defined *a priori* since it will depend on several specific aspects of the trial (discussed in more detail below). Data will be collected from various sources, for example in PATHWAY-27 biochemical blood analyses, biological metabolomics and dietary assessment provided a large quantity of data that require integration and interpretation.

The statistical management and integration of omics data and clinical results is important in order to understand the basis of wellness and disease, using global and holistic approach termed systems medicine [20]. The defining feature of systems medicine involves the collection of diverse longitudinal data for each individual that help to better decipher the immense complexity of human biology and disease. The convergence of advances in systems medicine, big data analysis, and individual measurement devices leads to a vision of healthcare that is predictive, preventive, personalized and participatory (P4). Such personal, dense, dynamic data clouds are needed to enable this vision and indeed underlie the essence of what Precision Medicine should be [21].

Several recent studies have illustrated the utility of multi-omic longitudinal data to look for signs of reversible early disease or disease risk factors in single individuals [22-24]. The best way to integrate omics and clinical data has not yet been established, although recent papers give interesting examples [25-26].

The complexity of this integrated approach underlines that inclusion of statistical consultants in the research team from the start of the study is essential, not only to avoid using inappropriate statistical methods but also to exploit results for mining important information. It is important to develop a close collaboration between researchers and statisticians during analysis of the data in order to correctly interpret, and understand the clinical relevance of, the results.

3.1.11. 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 [27], the Declaration of Helsinki [28] and the Council for International Organizations of Medical Sciences (CIOMS) [29]. The following list summarises the main points of each guideline, however it is recommended that the guidelines are 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

3.1.11.1. 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 RCT, 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.

3.1.12. 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 or providing the data. A clinical database that accurately reflects the data collected and is able to 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) [30]. It provides a list of requirements that can be used as a checklist.

Making data findable, accessible, interoperable and reusable (FAIR) is becoming increasingly important, particularly to research funding bodies. Many research councils require a data management plan (DMP) to be prepared within the first six months of the project. A DMP describes how the data will be managed while the project is ongoing and after the end of the project. Research funders may provide a template for a DMP specific to their requirements, the European Commission Horizon 2020 (H2020) Programme guidelines and template [31] is one such example and must include information about:

- The handling of research data during and after the end of the project
- What data will be collected, processed and/or generated
- Which methodology and standards will be applied
- Whether data will be shared/made open access
- How data will be curated and preserved (including after the end of the project)

The AUTOPOST project is part of the H2020 pilot action on open access to research data. Project deliverable 6.3 (Data management plan) provides useful information about what to consider when preparing a DMP [32]. It recommends creating a table that includes, amongst other items, a description of the data, how it will be created and stored, who owns the data and whether it will be destroyed at the end of the project.

Research data collected during PATHWAY-27 was input to an electronic data capture system hosted by project partner AdWare Research Ltd. The system used was Mythos CDMS v2.0, a web application based on Oracle 10gR2 database and accessible through secure Internet connection via a common modern web browser. The system is validated according to GCP and 21 CFR Part 11 regulations to ensure accuracy, reliability, consistent intended performance, and the ability to discern invalid or altered records. Data was transferred to the statistical analysis software using SAS Oracle Data Transfer tool and statistical analysis was performed with SAS 9.2. Database and daily backups were stored on mirrored hard drives at a secure location with access control. A Disaster Recovery Plan, uninterruptible power supply and comprehensive malware protection were also installed.

3.1.13. Quality assurance and quality control

Quality assurance relates to the actions that are established to ensure that the conduct of the trial complies 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 (SOP) should be developed to ensure the study is conducted, and that data is generated, documented and reported, correctly. 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.

The sponsor, an individual or institution takes responsibility for the initiation, management and financing (or arranging financing) of the study. They will ensure provision for management, monitoring and reporting, and must secure access to all trial-related sites, data, documents and reports for monitoring and auditing, and inspection by external regulatory agencies.

3.1.14. 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.

3.1.15. Ethics and trial registration

3.1.15.1. Ethics approvals and amendments

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 REC 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 [33] provides links to relevant EU and national legislation along with details of national research ethics committees.

Table 5 illustrates the ethical approvals required at each PATHWAY-27 RC before recruitment of the first participant.

Table 5 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	UNIBO/AOSP	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-Kommis-sion der Landes-ärtzekammer Baden-Württem-berg)	- Independent ethics committee of the S.Orsola-Malpighi Hospital	- National Health Service (NHS) Research Ethics Committee (REC) - Health Research Authority (HRA)

Writing the ethics application, gathering all the supporting documents and waiting for an approval/favourable opinion can take many weeks so it is important to begin the process as early as possible. However, substantial amendments such as changes to the study protocol or other recruitment documents and procedures will require approval before being applied. Considering this, ideally documents and procedures should be finalised before submitting the initial application, although it is not always possible to avoid the need for an amendment. 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.

3.1.15.2. Trial registration

According to the World Health Organization, registration of intervention trials is a scientific, ethical and moral obligation [34]. The Declaration of Helsinki requires all clinical trials to be registered in a publicly accessible database before recruitment of the first participant.

Trial registration helps to prevent research duplication, improve the transparency of health research by reducing selective reporting and publication bias, and can result in effective collaborations between researchers. It is also a prerequisite for the publication of research articles in many scientific journals. The PATHWAY-27 RCT was registered with ClinicalTrials.gov, registration ID NCT02702713.

3.1.16. 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 Table 6.

Table 6 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, are specific to each country and will be influenced by the recommendations of the REC.

3.1.17. 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 are provided by the Sponsor, which in this case is the RC.

4. Conducting a dietary intervention trial

4.1. Planning

It is essential to have a detailed plan for recruitment and food production. Study food must be produced, dispatched and available at the RC ready for distribution to the participants as required. Any delays in production and/or transport may prevent the participants receiving the food on time, which could result in the intervention treatment being interrupted. This may negatively affect the study outcomes.

If a pilot trial has not been conducted prior to the RCT, it is good practice to test the protocols for data and sample collection, sample processing and analysis. If samples are to be shipped to a centralised analytical laboratory for analysis, it is also helpful to test the shipping methods, such as determining the quantity of dry ice necessary to transport a package of 100 samples from one country to another, to ensure the samples will arrive in good condition at the destination. Similarly, it can be helpful to test that the sample storage containers are suitable to maintain the integrity of the sample. Testing may highlight a problem such as difficulty extracting the sample from the container at the analytical laboratory, in this case an alternative container can be sourced prior to being used in the study.

4.1.1. Recruitment plan

The recruitment plan will be developed in conjunction with the RC and food manufacturers taking into account the capacity for recruitment and food production, respectively. The recruitment and food production plans must be developed strategically and closely monitored to maintain concordance with each other. The recruitment plan will probably dictate food production, so it is important that it is realistically achievable to prevent production of food that will be unused. Depending on the food production schedule and duration of the trial, the recruitment plan could be quite broad, for example monthly, or it may need to be quite specific, for example week-by-week. In PATHWAY-27, the recruitment plan was developed on a weekly cycle to coordinate production of the study foods with an achievable target for the RC. Monitoring the recruitment rate on a weekly basis facilitated a process of rapid feedback to the manufacturer to enable them to amend the food production schedule when the recruitment target was not being achieved, thereby reducing the production of study foods and limiting food waste.

4.1.2. Food production plan

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The food production plan must be developed in conjunction with the food manufacturer to take into account availability of production equipment and capacity at the manufacturing site. It is also essential to liaise with raw material suppliers to guarantee continued availability of the ingredients. The storage capacity of the RC, the product shelf life, requirements for quality control batch testing and the length of transportation time must all be taken into account.

In PATHWAY-27, the dehydrated milkshake powders had the longest shelf life and could be manufactured in large batches that were produced on just three occasions. In contrast, biscuits were manufactured every two weeks to guarantee the retention of the appropriate amount of the bioactives.

4.1.3. Food deliveries to RC

Food deliveries must take into consideration weekends and national holidays at the RC. Food packages are usually be shipped on a large pallet so there should be a suitable facility at the RC to unload this type of shipment from the delivery vehicle. The size of the delivery vehicle may also be an issue since some transport companies use large articulated vehicles that may not be able to access the RC. In some cases, where the shipment is small, boxes can be unloaded manually, however it will be impractical to do this with a large number of boxes, particularly in the case of frozen foods that must be transferred to frozen storage as quickly as possible to maintain the cold chain.

4.1.4. Food storage facilities

Ambient foods are easier to store than foods 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 PATHWAY-27 pancakes, are covered by the Quick Frozen Foodstuffs (England) Regulations 2007 [35]. 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 monitoring may be limited to an externally visible thermometer. A break in the cold chain or damage to the packaging may result in the entire shipment being destroyed and participants on the trial will not be able to continue if they do not have the correct foods.

4.1.5. Unused food

In the event that the recruitment target is not fully achieved and food is not used, it should be decided whether foods can be recycled or destroyed. If possible, food should be recycled for other participants in the trial to reduce unnecessary food wastage and additional production

and shipping costs being incurred. If storage space is restricted at the RC, having a contingency plan to recycle or destroy food will help to reduce the burden of storing food that will not be used.

4.1.6. Food distribution to participants

Distributing study food to participants will be influenced by the food storage conditions, shelf-life and storage capacity at the participants' home. Food with a long shelf-life, such as the PATHWAY-27 milkshake powders, that are stored at an ambient temperature and supplied in a small sachet, pose very little problem for distribution and storage. In this instance it may only be necessary for one or two deliveries. Food that has a relatively short shelf-life may require more frequent deliveries. Food that requires cold storage is likely to pose the greatest storage problems.

Short trial duration may not be too problematic whereas a longer trial may require food to be distributed to the participant on multiple occasions. In some cases, it may be necessary to deliver food directly to the home of participants, in addition to distribution during visits to the RC. This may be necessary for various reasons, for example a participant may not be able to return home immediately after visiting the RC so food that requires refrigeration will need to be delivered, or they may not have suitable transport. While this increases the effort of research staff and cost of the study, it can improve compliance to the study protocol. In PATHWAY-27, frozen pancakes were occasionally delivered to the home of participants by research staff. It is important to note that access to a freezer became an additional exclusion criterion for participants.

4.2. Recruitment

4.2.1. 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 for a dietary intervention trial often take an interest in their diet and health and therefore they will typically have a healthy metabolic profile. This was evidenced through the low numbers of eligible volunteers screened at the RC. 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, may be less likely to volunteer to take part in a dietary intervention trial. Finding a successful recruitment strategy is 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. A pilot study can be used to test different advertising methods and identify the most suitable for the target population. An advertising campaign can be far-reaching to a general audience, for example on a local radio station, or it can be targeted to specifically identify potential participants from a clinical patient register, for example at a local doctor's surgery.

The recruitment strategies used in PATHWAY-27 are listed below:

- Selection from databases of interested volunteers and from hospital databases (obese/dysmetabolic/dyslipidemic patients, previous nutritional trials)
- Invitation to pilot study participants
- Collaboration with local GP surgeries to identify potential participants through preliminary screening of the patient register
- Screening of voluntary blood donors
- Interviews/advertisements on local radio stations and in local newspapers
- Online advertisements on newspapers, radios and Universities websites/Facebook pages
- Video reportage at the regional TV news
- Email to the Universities employees
- Posters and flyers in community centres, in the Universities involved, at special event days and in cooperation with health insurance companies
- Word of mouth.

4.2.2. Performance management

Throughout the study it is essential to manage performance of the trial so that problems can be identified and resolved quickly. Difficulty achieving the agreed recruitment rate may indicate an underlying problem that will prevent the overall recruitment target being achieved. Is the problem associated with difficulty recruiting eligible volunteers and/or retaining participants on the trial? It may be that participants in the treatment group (or one of the groups for multiple treatment-arm studies) are experiencing adverse side effects that are resulting in a high number of drop-outs. Providing regular recruitment reports to the management team will allow them to seek advice from experts on techniques that can be implemented to improve recruitment. By reporting and recording performance management data, future studies can use the information to help design and plan recruitment strategies to avoid similar problems.

4.2.3. Participant compliance and retention

One of the biggest challenges when conducting the trial is the compliance and retention 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 and was clearly explained to volunteers 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 drop-out.

The ease with which a participant integrates the study food into their usual diet will influence their motivation. If the study food is 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 ways in which the foods could be consumed were provided to PATHWAY-27 participants, for example suggestions were given on different ways the pancakes could be eaten using 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 food 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.

Incentives may also factor into the motivation for a participant to adhere to the treatment and stay on the trial. Financial incentives such as reimbursement for travel expenses and time may improve participant compliance and retention but this must be carefully calculated since, ethically, financial benefits should be provided as a compensation not as an enticement.

4.2.3.1. Communication

Regular communication with each participant is important to develop and maintain a good relationship. Participants who feel valued and well-informed will be more motivated to comply with the study protocol. 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.

Using modern media for communication such as text messaging allows researchers to communicate quickly and easily with participants, although it is important to note that this will depend on the acceptability of the method with the target group. The use of an online survey platform may improve the response rate for completing questionnaires. Many people now use smart mobile devices so a well-designed survey that works equally well on a mobile device or desktop will be a useful tool. Capturing data electronically may also assist researchers since the data can be easily exported in a tabular format to the analytical software. Paper-based questionnaires require the data to be manually input to the analytical software, this is time-consuming and a source of potential error when copying the data. By contrast, electronic data will typically require less manipulation and therefore will be less prone to typographical errors.

4.3. Sample collection, processing and analysis

4.3.1. Collection

For a human study investigating the effect of BEF on physiologically-relevant endpoints biological samples will be required for analyses. The type of sample will depend on the selected endpoints but as a minimum requirement blood and 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.

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, can be useful for screening volunteers as the results are available within a few minutes. At baseline and control visits 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.

4.3.2. Processing

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 so as to minimise discomfort to the participant and ethically it will be important to justify the volume required. As a contingency it is good practice to factor in collecting extra blood to ensure a sufficient reserve in case some samples are lost or destroyed, for example in transport to the analytical laboratory. 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.

Multiple tube collections must adhere to recommendations for the order of blood 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 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 storage containers that are adequate for the storage conditions and to label the samples correctly to ensure traceability. This is why standard operation procedures must be established during trail design.

4.3.3. Analysis

The protocols for sample analyses should be determined prior to the start of the trial and ideally, they should have been tested in advance. 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 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, was stored at -80°C and had to maintain its frozen state until it was required for analysis. By contrast, whole blood used to analyse single nucleotide polymorphisms (SNPs) was stored at -20°C at the RC but could be shipped at ambient temperature.

For a multi-centre trial as the PATHWAY-27 RCT, the analyses should, where practicable, be performed by a centralised laboratory to limit sources of random error. 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. In this case the sample would not be stored and shipped to a centralised laboratory, it would be analysed by a local laboratory. In the PATHWAY-27 pilot studies, it was acceptable for sample analyses to be conducted at different laboratories since the data was not collated for statistical analysis. However, the analytical methods, including the type of sample used – for example serum or plasma, were the same.

5. Reporting a dietary intervention trial

Transparent reporting of the pre-specified outcomes of the trial is important to prevent bias and misinterpretation of the results that may occur if reporting is selective [36]. A summary of the trial protocol should be readily available during the trial, for example through the trial register (discussed in section 3.1.15 Ethics and trial registration) and the results made available after completion of the trial [37].

5.1. End of trial declaration

The 'End of Trial' is usually defined as the last visit of the last participant although it may refer to a later time point as described in the study protocol. The trial sponsor is responsible for declaring the 'End of Trial' to relevant regulatory bodies, such as the REC, within a specific timeframe, for example 90 days after the last visit of the last participant.

5.2. Summary report

A summary report of the trial must be submitted within a specific timeframe after the end of the trial. This is twelve months for studies involving adults and six months for studies involving children [38]. As a minimum the report should state whether the study achieved its objective, the main findings and arrangements for publication or dissemination of the research, including any feedback to participants [39]. The ClinicalTrials.gov registry requires information about participant flow, participant baseline characteristics, outcome measures and statistical analyses, and adverse events [40].

5.3. Dissemination of results

The dissemination plan will have been prepared before the start of the trial as it is typically a requirement of the ethics application. Where this is the case, changes to the plan must be notified to the REC by submitting an amendment so this should ideally be carried out before the end of trial declaration is submitted.

The trial results will be disseminated through publication in peer-reviewed scientific journals and the anonymised dataset should be submitted to an appropriate open-access repository (see section 3.1.12 Data management for discussion about the principles of FAIR data). The findings of the research should also be disseminated to participants and the public generally in a format that is accessible to the lay person.

Guidelines exist for the reporting of different types of studies. The EQUATOR Network is a collaboration of researchers, medical journal editors, peer reviewers, developers of reporting guidelines, research funding bodies, and others, with the objective of improving the quality of research publications. They provide a comprehensive list of guidelines for different study types and a flowchart to help researchers choose the most appropriate guidelines to follow [41].

5.3.1. Guidelines for reporting randomised trials

The International Committee of Medical Journal Editors (ICJME) have developed recommendations for best practice in the conduct and reporting of research to ensure accurate, clear, reproducible and unbiased reporting [42]. A manuscript should follow the general structure of Introduction, Methods, Results and Discussion (IMRAD).

The current recommendation for reporting randomised trials is to follow the Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines [43]. These guidelines provide a minimum requirement for reporting of trials. Increasingly however, it is appreciated that following CONSORT may not be sufficient in some cases. A discussion of what else could be reported in relation to dietary trials is provided in the PATHWAY-27 Guidelines for the Scientific Community. The SPIRIT 2013 guidelines will also apply when reporting the study protocol.

5.3.1.1. CONSORT 2010

The CONSORT statement provides a minimum set of recommendations for reporting a RCT with a series of extension statements that are applicable for variations on the standard trial methodology. For example, the CONSORT statement for pilot and feasibility trials [44] provides guidance for reporting randomised trials in which a prospective RCT, or part of it, is conducted on a smaller scale.

The CONSORT 2010 statement comprises a checklist of items that should be included in the report. The main points of the checklist are summarised below and it is recommended that researchers familiarise themselves with the statement and its supporting documentation.

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
- Statistical 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

6. Conclusion

Valuable experiences gathered from the PATHWAY-27 pilot studies and multi-centre RCT have been collated and presented here with the aim of providing a practical guidance document for health researchers. Dietary intervention trials are complex and often involve asking participants to consume foods that they would not usually chose 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.

Good design and careful planning are fundamental to the success of a dietary intervention trial using BEF. Close collaboration between all relevant partners (ingredient suppliers, food manufacturers, researchers, and coordinators) is central to designing and implementing a study that will generate useful and exploitable outcomes.

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SEVENTH FRAMEWORK PROGRAMME

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8. Abbreviations not provided in the text

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