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CLINICAL TRIAL MANAGEMENT FOR FOOD SUPPLEMENTS: FROM DESIGN TO MONITORING

PhD Thesis
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Abstract

***Introduction**:* In the European Union, food supplements fall into the macro-category of foods. Directive 2002/46/EC defines food supplements as "*foodstuffs the purpose of which is to supplement the normal diet*…", and establishes harmonized rules for some ingredients, such as vitamins and minerals for the food supplements production. In the past, food supplements were intended only to supplement the deficient diet of some individuals who could not get enough nutrients. During the last twenty years there was an evolution on the role of food supplements. According to Regulation (EC) No 1924/2006, foodstuffs can reduce disease risk factors. The Ministerial guidelines report that the evolution of food Legislation in Europe has led to an increase in the number of efficacy clinical studies on foods to assess health effects.

Methods: Like clinical pharmacological studies, the execution of human clinical studies on food supplements must follow the Good Clinical Practice (GCP), the international ethical and scientific quality standards for designing, recording and reporting trials that involve the participation of human subjects; and must take into account prior and binding opinion of the Ethics Committee. During my Ph.D. course, I designed and monitored clinical trials in accordance with GCP. The first two clinical studies reported in this thesis, concern alpha-lipoic acid (ALA). The first of which, tested the safety and the efficacy of ALA in the reduction of different forms of idiopathic pain, in normoglycemic subjects. The second study evaluated the pharmacokinetic parameters of a liquid formulation of ALA. The third clinical trial investigated the effect of *E. angustifolium* extract (EAE) after the daily intake of hard, gastric-resistant capsules for 6 months in the reduction of the symptoms in subjects with benign prostatic hyperplasia (BPH). The last clinical study was focused on the efficacy of arabinoxylans obtained from barley (AX) to reduce postprandial glycemia, in healthy subjects.

Results. The first clinical study showed that after two months of orally treatment of ALA, none of subjects treated with ALA reported a decreased glycemia or adverse effects. Moreover, the treated subjects showed a significant reduction in two validated questionaries for pain. The second clinical trial on a liquid formulation of R-ALA shows that R-ALA was rapidly absorbed. The Cmax value is higher than the commonly recognized ALA therapeutic effect, and the AUC is higher than a racemic solid formulation of ALA. The clinical study on EAE demonstrated that EAE can be used in subjects with BPH, to improve their quality of life. The last clinical trial demonstrated that AX can reduce post-prandial glucose in a statistically significant way, compared to placebo, producing a lower insulinemic response, consequently.

Conclusions: Clinical studies on food supplements represent a relatively new approach. Therefore, it is important to consider that only following a right methodological approach, it will be possible to put on the market safe and effective food supplements, tested with the highest scientific rigor.

Contents

CHAPTER 1.

1. Introduction

1.1 Food supplements legislative framework

The **Directive 2002/46/EC** of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements (implemented in Italy by **Legislative Decree 21 May 2004, no. 169**), under **article 2, letter a)**, defines **food supplements** as "*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*".

In the European Union, food supplements fall into the **macro-category of food**, presenting some peculiarities regarding their forms of presentation, their consumption units and their pre-set daily supply.

In fact, food supplements are usually presented in small units of consumption such as capsules, tablets, sachets, vials, and are marketed in "dose" form (solid or liquid in measured doses).

As reported in Directive 2002/46/EC (**Whereas 2**) is necessary to adopt Community rules on those products marketed as foodstuffs. "*Those products are regulated in Member States by differing national rules that may block their free movement and create unequal conditions of competition, and thus have a direct impact on the functioning of the internal market*". "*In order to ensure a high level of protection for consumers and facilitate their choice, the products that will be put on to the market must be safe and bear adequate and appropriate labelling*" (Directive 2002/46/EC, **Whereas 5**).

In particular:

- **under article 9, par. 1 of Legislative Decree No 169/2004**, the production and packaging of food supplements has to be carried out in premises authorized by the Health Ministry;
- **under article 6, par. 2 of Directive 2002/46/EC** "*the labelling, presentation and advertising must not attribute to food supplements the property of preventing, treating or curing a human disease, or refer to such properties*", par. 3 lists the mandatory contents of the labels;
- **article 10 of Legislative Decree No 169/2004** provides that the commercialization of food supplements is subject to a prior notification of a model label to the Health Ministry, which verifies its compliance and registers the product giving it a code (which may be shown on the label). The Ministry, in case it deems it necessary in

order to carry out a complete evaluation, may ask for further documentation in respect of effects and safety of the product, and/or may ask for changes in the information contained in the label.

1.2 Italian Ministerial Guidelines on food supplements

Directive 2002/46/EC established **harmonized rules** for some ingredients, such as vitamins and minerals for the food supplements production, instead, there are only national norms for the use of other ingredients and nutrients.

For this purpose, the **"Italian Ministerial Guidelines on Food supplements**" contains rules applicable to food supplements, as provided for by **article 5 of Legislative Decree 169/2004**: "*Intake of vitamins, minerals and other substances"*.

It is structured in the following sections:

- **Vitamins and minerals**, where maximum permitted intake levels are reported.

- **Probiotics and prebiotics**, where specific dispositions for these substances with "physiological" effect are reported.

- **Substances with a nutritional or physiological effect,** in which various dispositions for other nutrients and other substances with a nutritional or physiological effect, other than botanicals, are reported.

European Food Safety Agency (EFSA) stresses that "*a wide range of nutrients and other ingredients might be present in food*

supplements, including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, fibre and various plants and herbal extracts".

It is important to underline that a substance, in order to be used in a food supplement, must have a significant past consumption in the EU as proof of safety. If this condition is not satisfied, the substance is configured as a new ingredient or a new food ("Novel food") according to **Regulation (EC) 2015/2283.** Therefore, any use requires prior authorization at European level.

In addition, if the previous consumption took place with food supplements only, the addition of the substance to food supplement must follow the Regulation (EC) 2015/2283 to re-evaluate the levels of available food sources of the substance in question.

1.2.1 Lists of vitamin and minerals and their forms

For the purposes of **Directive 2002/46/EC,** under article 2, letter b) **nutrients means the following substances**:

(i) vitamins,

(ii) minerals.

Vitamins and minerals and their forms, currently used as ingredients

- in food supplements under Directive 2002/46/EC

- in food as per Regulation (EC) No 1925/2006, **are listed in the Commission Regulation (EC) No 1170/2009 of 30 November 2009 which** amending Directive 2002/46/EC and Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards "*the lists of vitamin and minerals and their forms that can be added to foods, including food supplements*".

  In this regard, Annexes I and Annexes II of Directive 2002/46/EC (implemented by Legislative Decree 21 May 2004, no. 169**)** about food supplements, are replaced respectively in Annex I and Annex II of the Regulation No 1170/2009.

  On the Ministerial Guidelines website, it is possible consult the daily intake of vitamins and minerals allowed in food supplements. The **minimum intake of vitamins and minerals and the daily intake** indicated on the label, must not be less than 15% of the Nutrient Reference Values (NRVs) listed in **Annex XIII, Part A, point 1 of Regulation (EU) 1169/2011**.

  On the label, the weight content of vitamins and minerals daily intake should also be expressed as a percentage of the NRVs.

1.2.2 Substances with a nutritional or physiological effect

On the Italian Ministerial Guidelines website it is possible consult also the list of "**Other nutrients and other substances with a nutritional**

or physiological effect (Ministry of Health, Revision September 2019). The list of substances allowed for use in food supplements is not exhaustive, but somewere, daily intake limits and additional warnings are provided**.**

Examples of these substances are:

- **Essential amino acids;**
- **Lipoic acid;**
- **Arabinoxylane;**
- **Beta glucans;**
- **Citicoline;**
- **Creatine;**
- **Phytosterols;**
- **Flavonoids;**
- **Phospholipids;**
- **Glutathione;**
- **Inositol;**
- **Melatonin;**
- **N-acetylcysteine**

In the section "*Eventual indications*", in some cases, there are examples of properties (which do not constitute health claims) aimed to provide information to guide consumers in their choices.

1.2.3 Botanicals

The use of Botanicals (plant extracts and vegetal preparations) in food supplements is currently regulated by the **Ministerial Decree of August 10, 2018**.

Annex 1 of this DM, containing the **list of permitted plants and their parts**, supplemented by additional dispositions for use, has been amended by the Directorial Decree of July 26, 2019.

Annex 1 provides specific guidance on the documentation to be prepared and provides the procedures to be followed for the use of botanicals in food supplements.

Among the information that the documentation must contain there are:

- **Description of the preparation and processing process of the raw material**.
- **Traditional use and consumption history**.
- **Botanical identification of the plant** (in this regard, it can be referred to the following databases:
    - www.theplantlist.org;
    - www.ars-grin. Gov,
    - www.algaebase.org,
    - www.indexfungorum.org,
    - www.lichens.ie)
- **Finished product information**.
- **Manufacturing process.**

The Food Business Operator (FBO) is responsible about the safety of food supplements placed on the market, in accordance with the evolution of scientific knowledge.

In this regard, FBO is required to communicate to the Ministry of Health (**DGISAN-Office 4**) any new data on side effects of herbal substances and preparations used in his products.

With a **directorial decree of 4 August 2021**, **Annex 1 is subject to a new amendment,** consisting in the introduction of an additional warning, in the light of current scientific evidence, for the labeling of food supplements containing substances, preparations and extracts derived from *Garcinia gummi-gutta sin. Garcinia cambogia* usable in food supplements.

1.2.4 Probiotics and prebiotics

On the Italian Ministerial Guidelines website, it is possible consult also the "**Guidelines on probiotics and prebiotics"**.

These Guidelines provide:

1) Indications for use in food products and food supplements of **probiotic micro-organisms** (bacterial and/or yeasts);
2) Indications for use in food products and food supplements of **prebiotics**.

- The **probiotic micro-organisms** that may be used in food products and food supplements shall comply with the following requirements:

a) "*Having long been used to supplement human intestinal microflora (microbiota);*

b) Being considered safe for use in humans";

c) Being active in the intestines in such a quantity as to be able to multiply there.

The indication for probiotic micro-organisms use is: "*It promotes the intestinal flora balance*".

- The **substances used as prebiotics** shall comply with the following requirements:

- Being safe for human consumption based on a long-standing use that prevents it from being classified as novel food pursuant to Regulation (EU) 2015/2283;

- Being contained in recommended daily consumption amounts in quantities that are plausibly fit to generate a "prebiotic" effect in accordance with the available scientific evidence.

Some examples of constituents that can be used as prebiotics are inulin, fructooligosaccharides (FOS) and galacto-oligosaccharides (GOS).

1.3 Healt claim

In accordance with the Ministerial Guidelines, food supplement can contribute to the general wellness by optimizing the physiological homeostasis or promoting the normal function of the body by providing nutrients or other substances with a nutritional or physiological effect.

During the last twenty years there was an evolution on the role of food supplements. In the past, food supplements were intended only to supplement the deficient diet of some individuals who could not get enough nutrients. In line with reported in Whereas 4 of Directive 2002/46/EC "*Consumers, because of their particular lifestyles or for other reasons, may choose to supplement their intake of some nutrients through food supplements*".

In fact, their name comes from the concept "to supplement the diet".

Over time, there was an evolution of this concept. Today, food supplements are considered like foodstuffs that can exert their action on disease risk reduction, by way of derogation from a preventive effect that foods could not claim. This derogation must be authorized according to **Regulation (EC) No 1924/2006**.

Notwithstanding **Article 2(1)(b) of Directive 2000/13/EC** and **article 7 par. 3 of Regulation (EU) No 1169/2011** "*food information shall not attribute to any food the property of preventing, treating or curing a human disease, nor refer to such properties*", **Regulation (EC) No 1924/2006** introduces the concept of "reduction of disease risk claims".

Hence, according to Regulation (EC) No 1924/2006, foodstuffs can exert disease risk reduction properties, as an exception to the prohibition for foodstuffs (and therefore for food supplements) to claim preventive effects.

In fact, this Regulation harmonizes the legislative dispositions of the Member States concerning nutrition and health claims.

Nutrition claim is defined as "*any claim which states, suggests or implies that a food has particular beneficial nutritional properties"* due to:

- calorific value which provides or not provides;
- the nutrients or other substances it contains or not contains.

The Regulation, in addition to "nutrition claims", define also "*health claim*" and "*reduction of disease risk claim*", which are defined by Article 13 and Article 14 of the same Regulation, respectively.

Specifically, **health claim "***means any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health*" (**Article 13**).

Instead, **reduction of disease risk claim "***means any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease*" (**Article 14**).

The health claim can be reported on the food supplement label only after the authorization by the European Union and the release of a favorable opinion by European Food Safety Authority (EFSA).

Specifically, only the indications contained in the relevant list approved by the European Commission may be used, or in case of new and different indications, a request has to be filed with the Health Ministry, that after the first review, sends the application to the EFSA for its technical-scientific evaluation. The application shall be filed to the European Commission, that heard the Standing Committee on the Food Chain and Animal Health, provided that nothing to the contrary is filed by the European Parliament or by the Council, takes the final decision.

The "*General scientific guidance for stakeholders on health claim applications*," published in 2016 by EFSA, outlines several criteria used by the **Panel on Dietetic Products, Nutrition and Allergies** (**EFSA NDA Panel**) to evaluate health claims.

In assessing each specific food/health relationship which forms the basis of a health claim, the NDA Panel considers the following key questions:

(i) *the food/constituent is **defined** and **characterized**;*

(ii) *the claimed effect is based on the essentiality of a nutrient; OR the claimed effect is **defined** and is a **beneficial physiological effect** for the target population, and can be **measured in vivo in humans***;

(iii) *a **cause-and-effect relationship** is established between the consumption of the food/constituent and the claimed effect (for the target group under the proposed conditions of use)*

Whether or not the alteration of a factor is considered by the NDA Panel to be beneficial in the context of a reduction of a disease risk claim depends on the extent to which it is established that:

a) *the factor is an **independent predictor** of the risk of disease (such a predictor may be established from intervention and/or observational studies)*;

b) *the relationship between the factor and the development of the disease is **biologically plausible***.

Points a) and b) describe two key elements for the approval of a risk reduction claim, by EFSA.

Specifically, a risk factor is considered valid if it is:

- "**well established" risk factor**, i.e., there is evidence, from interventional (with drugs or food supplements) clinical studies, that: 1) reduction of the risk factor generally reduces the incidence of disease and 2) the involvement of the risk factor in the development of the disease is biologically plausible.

In this case, as part of the request for authorization of a risk reduction claim, the evidence that a " food supplement ", with a specific food or one of its components, induces a reduction (or a beneficial alteration)

of a risk factor, is **sufficient to define the scientific basis of the type of claim.**

An example taken from the EFSA guidelines is reported below.

"...It is well established that elevated blood LDL-cholesterol concentration is independently associated with an increased risk of coronary heart disease (CHD), and that reducing blood LDL-cholesterol concentration (by dietary modification or drugs) would generally reduce the risk of development of CHD. It is also well established that elevated (systolic) blood pressure is independently associated with an increased risk of CHD and stroke, and that reducing (systolic) blood pressure (by dietary modification and drugs) would generally reduce the risk of development of CHD and stroke. Reduction in blood LDL-cholesterol concentration, therefore, is considered beneficial in the context of a reduction of disease risk claim for CHD, and reduction in (systolic) blood pressure is considered beneficial in the context of a reduction of disease risk claim for CHD and stroke. It is also well established that falling is a risk factor for bone fractures in the elderly, and that reducing the risk of falling (e.g. by dietary modification, by drugs, by modification of architectonical barriers) reduces the risk of bone fractures…".

The risk factor must be generally accepted by the scientific community. In fact, the reduction of an identified risk factor must be useful to reduce the target disease and to prevent the worsening of the patient's condition. An example is represented by the risk factor: "*high levels of LDL-cholesterol and its first-choice pharmacological treatment represented by statins*".

- **"not well established" risk factor",** i.e., there is no such evidence from intervention studies that a reduction of the risk factor generally reduces the incidence of disease, but 1) there is evidence for an independent association between the proposed risk factor and the incidence of the disease from observational studies and 2) the involvement of the risk factor in the development of the disease is biologically plausible.

In this case, however, evidence that the food supplement with the specific food/constituent induces a reduction (or beneficial alteration) of the risk factor, and a reduced risk of disease, **needs to be provided.**

An example taken from the EFSA guidelines is reported below.

"…There is some evidence that low blood HDL-cholesterol concentration, elevated blood concentration of triglycerides, or elevated blood homocysteine concentration are associated with an increased risk of coronary heart disease (CHD). Reduction in blood concentration of triglycerides, reduction in blood homocysteine concentration, or an increase in blood HDL-cholesterol concentration, have been associated with a decreased incidence of CHD following certain dietary interventions in some human intervention studies. However, changes in any of these factors (by dietary modification or drugs) have not generally been shown to reduce the risk of CHD. ***Therefore, human studies on the risk of CHD are required for the substantiation of these disease risk reduction claims in order to validate the association between these variables and the risk of disease in the context of a particular nutrition intervention****…".*

Under **Article 3, lett. a), Regulation (EC) No 1924/2006** concerning nutritional and health claims used in the labelling, presentation and advertising of foods placed on the market in the Community, the use of nutrition and health claims shall not be false, ambiguous or misleading.

CHAPTER 2.

2. Studies to be conducted on food supplements

2.1 Guidance on studies conducted to evaluate the safety and properties of foodstuffs

As reported in the "“***Linee di indirizzo sugli studi condotti per valutare la sicurezza e le proprietà di prodotti alimentari***” in the food field it is possible to carry out different type of studies on foodstuffs, including food supplements, with different purpose.

In fact, it is possible lead:

1. **SECURITY STUDIES** (a) to assess the safety of use in the case of novel foods; (b) where appropriate, to confirm the safety of use in the case of foods products or ingredients included in list C of Annex 3 of Regulation (EC) 1925/2006 (the addition of vitamins and minerals and of certain other substances to foods);
2. **EFFICACY STUDIES** (a) to assess health effects or reduction of a risk factor disease; (b) to confirm the validity of the indication for use.

These types of studies can be performed both *in vitro* and *in vivo* 1) to support the efficacy of a food, for example by documenting biochemical mechanisms correlated to it; 2) to support the safety of a

food by demonstrating the absence of toxicity under normal exposure conditions.

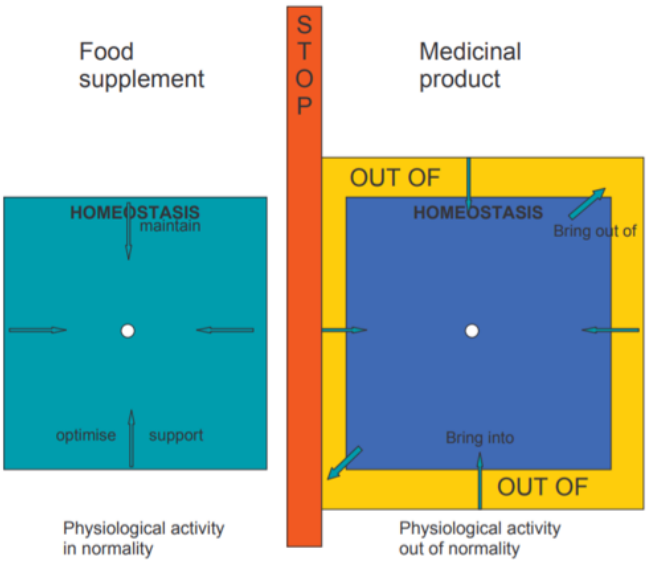
The Ministerial guidelines report that the evolution of food legislation in Europe has led to an **increase in the number of efficacy studies** on foods to assess health effects.

In addition, in order to test the health benefits of a food product, under current regulations two requirements must be satisfied:

1) the product studied must be **effectively a food** (in compliance with all applicable food legislation);

2) the study must be designed to **demonstrate the contribution to the maintenance of the "homeostatic" balance** of a specific function of the organism, which is not deteriorated in a pathological sense, or it must be designed to demonstrate the reduction of a factor of disease risk, according to the model defined by the Council of Europe "*Homeostasis, a model to distinguish between food, including food supplements, and medicinal products*, 07/02/2008".

In the following figure are reported a graphical presentation of the homeostasis model by European council.



Homeostasis as European council definition: «Can be defined as an individual status where the physiological parameters are within normal limits».

Therefore, a **clinical efficacy study** (conducts on humans) involving a food supplement must have a salutary (not therapeutic) purpose. For these reasons, these types of clinical study are generally conducted by general practitioners rather than hospital physicians.

In this regard, **Ministerial Decree of May 10, 2001** define the "*Controlled clinical trials conducted by General Practitioners and Paediatricians*".

In particular:

- **under par. 1.1 of M.D.**, these types of clinical trials refer to diseases that do not require hospitalization (widely spread throughout the territory), which can be conducted completely

or partially outside the hospital with the involvement of General practitioners (GPs) and General Pediatricians (PLSs)".

- **under par. 1.2 of M.D.**, the GPs and PLSs, included in the appropriate registers of the ASL, are authorized by the general director of the same ASL, after the opinion of the Ethics Committee, to conduct the trials referred to in paragraph 1.1.
- **under par. 1.4 of M.D.**, the experimental activities can be conducted at the outpatient setting equipped with the minimum facilities (logistics, equipment, etc..) necessary to conduct the trial in accordance with the study protocol and the principles of ***Good Clinical*** *Practice* (ICH-GCP)".
- **under par. 1.1 of M.D.**, each ASL has the responsibility to verify the existence of the characteristics described in point 1.4.

2.2 Food supplement clinical trials regulation

To date, there is legislative gap on food supplements clinical trials. For the development of these type of clinical studies, we take inspiration from the legislation on drugs.

Like clinical pharmacological studies, the execution of human clinical studies on food supplements must take into account:

- "***Ethical principles that have their origin in the Declaration of Helsinki, and that are consistent with GCP and the applicable***

regulatory requirement(s)". (ICH E6 (R2) Good clinical practice-GCP)

- ***Prior and binding opinion of the Ethics Committee*** (necessary requirement for the development of the clinical study).

The Declaration of Helsinki (DoH) is the World Medical Association's (WMA) best-known policy statement, inspired in part by the revelations of the Nuremberg trials. WMA felt that there was a need to provide the global community of physicians with guidelines for conducting biomedical research involving human participants.
In fact, the Declaration of Helsinki, which was first published in 1964, is considered by many to be the first world standard for biomedical research. Since 1964, The DoH has been amended seven times since, most recently at the General Assembly in October 2013. The current (2013) version is the only official one; all previous versions have been replaced and should not be used or cited except for historical purposes.

As define in the **Directive 2001/20/EC**, Ethics Committee is ": *an independent body in a Member State, consisting of healthcare professionals and non-medical members, whose responsibility it is to protect the rights, safety and wellbeing of human subjects involved in a trial and to provide public assurance of that protection, by, among other things, expressing an opinion on the trial protocol, the suitability of the investigators and the adequacy of facilities, and on the methods*

and documents to be used to inform trial subjects and obtain their informed consent."

In preparing its opinion on clinical trials, the Ethics Committee consider:

- the relevance of the trial and the trial design;
- the protocol;
- the suitability of the investigator (i.e. the person responsible for performing the clinical trials on a site) and his or her supporting staff;
- the quality of the facilities.

The Ethics Committee's opinion must be delivered before the start of the clinical trials.

In this regard, although there is no real legislation on food supplements, in the Ethics Committees recently was introduced a member with expertise in clinical studies conducted on foods.

Both drugs and food supplements follow the same methodology:

- *Clinical trial design*
  - **Research question.**
  - **Development of the experimental design.**
- *Performing a clinical trial*
  - **Execution.**

- **Monitoring.**
- **Analysis.**
- **Conclusions.**

2.3 Clinical trial design

❖ **Research question**

At the beginning of the clinical trial design, a research question must be formulated based on:

- review of scientific literature, clinical practice guidelines and manuals;
- identification of relevant variables;
- formulation of the research questions and hypotheses.

The characteristics of a good research question are summarized by the acronym F-I-N-E-R:

✓ Feasible

Research questions should be answered under objective aspects like time, scope, resources, expertise, or funding.

✓ Interesting

Regardless of your own personal motivation. about a subject, it is important to check if your question corresponds to more practical and broader interests.

✓ **Novel**

Answer to an existing gap in knowledge. Filling one of these gaps is important.

✓ **Ethical**

In empirical research, ethics is an absolute MUST.

✓ **Relevant**

Relevance can lead to real, visible changes in society.

❖ **Development of the experimental design**

The **CONSORT** (CONsolidated Standards of Reporting Trials) **2010** guideline is intended **to improve the reporting of parallel-group randomized controlled trial (RCT)**, enabling readers to understand the design, the management, the analysis and the final interpretation of a clinical study, and to assess the validity of its results.

See the Appendix 1 for the CONSORT 2010 checklist of information to be included when reporting a randomised clinical trial.

Outcome

All clinical trials evaluate outcomes to compare the results of the involved groups.

As reported in **item 6 of the guidelines CONSORT 2010**:

Most trials have **several outcomes**, some of which are of more interest than others. Where available and appropriate, the use of **previously developed and validated scales or consensus guidelines should be reported**, both to enhance quality of measurement and to assist in comparison with similar studies.

All outcome measures, whether primary or secondary, should be identified and completely defined to allow other researchers to use it.

The **primary outcome measure** is the prespecified outcome and is usually the one used in the sample size calculation.

"Having several primary outcomes incurs the problems of interpretation associated with multiplicity of analyses and is not recommended".

Other outcomes of interest are **secondary outcomes** (additional outcomes). "*There may be several secondary outcomes, which often include unanticipated or unintended effects of the intervention (see item 19), although harms should always be viewed as important whether they are labelled primary or secondary".*

<u>Elegibility criteria</u>

Eligibility criteria are defined in the early steps of study planning to define/identify the **target population.**

The researcher must be careful to not select a population that is too different from those encountered in actual daily clinical practice.
Lack of this requirement removes any value from the results produced by the study.

It must be kept in consideration that much selection increases the internal validity of the study but reduces applicability.
Possible errors include:

- overly restrictive inclusion/exclusion criteria;
- including participants with very low risk of outcome;
- including participants with a high probability to drop out of the study.

Power analysis

Power analysis is a statistical method for calculating sample size.
Sample size calculations are generally performed with three **1-β power values** of **0.80**, **0.95**, and **0.99** and **significance level α =0.05**.
The power of the study, defined as the ability of the trial to find the efficacy of one of the treatments, object of investigation, is generally set at **80%, which corresponds to accept a false-negative error of 20%**. To reduce the probability of a false-negative result to 10%, the power must be increased to 90% with a significant increase in sample size.

Randomization

There are several types of randomizations. For further information see a downloadable scheme from *CONSORT 2010 Guidelines* in the Appendix 2.

The randomization sequence is generally generated by a statistician with a software (e.g., *Stata Statistical Software: Release 16. College Station, TX: StataCorp LL*C). Subjects are randomly and unpredictably assigned to each treatment groups (generally treatment vs control). This procedure minimizes selection bias, i.e. systematic differences between the baseline characteristics of the comparison groups (prognostic and treatment response imbalance).

The CONSORT 2010 Guidelines also provide information about **steps in a typical randomisation process:**

Sequence generation

• *Generate allocation sequence by some random procedure.*

Allocation concealment

• *Develop allocation concealment mechanism (such as numbered, identical bottles or sequentially numbered, sealed, opaque envelopes).*

• *Prepare the allocation concealment mechanism using the allocation sequence from the sequence generation step.*

Implementation

• *Enrol participants:*

- *Assess eligibility*
- *Discuss the trial*
- *Obtain informed consent*

- *Enrol participant in trial.*

• *Ascertain intervention assignment (such as opening next envelope).*

• *Administer intervention.*

Blinding

In case of a **single-blind clinical study**:

- the enrolled subject must be not know which is treatment and which is placebo.

In the case of a **double-blind clinical study**:

- neither the investigating physician nor the enrolled subject must be unaware of which is the treatment, and which is the placebo.

Treatments must be made unrecognizable with respect to:

- packaging
- dosage forms
- color
- flavor

If applicable, it is possible to define:

✓ **Run-in period**

The run-in period (which occurs before the start of treatment, during which time placebo is generally administered) is useful to:

- **remove the effects of prior therapy,**
- **observe the potential compliance to the study**, in order to minimize the drop-out of subjects (increasing the internal validity of the clinical trial).

In addition, at the end of the run-in period:

- false adverse effects may be identified,
- possible therapeutic responses to placebo can be identified,
- the eligibility of the subjects is confirmed and therefore the sample population.

✓ **Cross-over design**

In the cross-over design, **each subject receives more than one treatment in succession**. For each subject the succession of treatments changes, in fact these are randomized to one of two (generally) possible treatment sequences.

The cross-over yields a more efficient comparison of treatments than a parallel design.

Wash-out

At the end of the first treatment, subjects must undergo a wash-out period in which they do not receive any treatment. **This period is used to avoid the carry-over effect.** The carry-over (or residual) effect is defined "as the effect of the treatment from the previous time period on the response at the current time period". The carry-over

effect would produce biases in the statistical analysis that would adversely affect the reliability of the result

.

2.4 *Performing a Clinical trial*

The steps required to perform a clinical trial with food supplements are listed below (the same as drugs):

1. Study protocol design
2. Feasibility study of the center to be involved based on the personnel, equipment, and number of patients that the center can enroll
3. Identification of the Principal Investigator (P.I.)
4. Preparation of documentation to be submitted to the referral Ethics Committee
5. Stipulation of the insurance policy
6. Ethics Committee approval
7. Organization of the investigator meeting
8. Clinical trial execution and monitoring
9. Data collection and statistical analysis

In detail:

1. **Study protocol design**

The experimental protocol on a food supplement, in accordance with good clinical practice, must contain:

- scientific background and rationale;
- outcomes (primary and secondary);
- type of study (monocentric, multicentric, interventional, randomized, double-blind, etc.).
- inclusion and exclusion criteria;
- investigational treatment;
- possible concomitant therapies and/or unauthorized pharmacological treatments;
- exams required by the protocol (diagnostic and follow-up tests);
- run-in (if applicable)
- randomization and masking procedures (if applicable);
- cross-over and wash-out (if applicable)
- study duration (recruitment and total duration).
- sample size/power analysis;
- statistical analysis;
- management of investigational products (labeling, accounting, lot tracking, etc.)
- data ownership and results publication policy;
- study monitoring procedures.

4. Preparation of documentation to be submitted to the Ethics Committee to request clinical trial opinion.

It consists of:

- Letter of intent to request Ethics Committee opinion on the clinical trial.
- Study Protocol.
- Synopsis of Protocol.
- Investigator's Brochure.
- Information sheet and Consent Form to be read and signed by the enrolled patient.
- Information letter for the GP (if applicable).
- Provisions for recruitment and any materials to be given to recruited subjects.
- Insurance policy: in accordance with Ministerial Decree of 14.07.09.
- List of participating centers.
- Case Reporting Form - CRF.
- Center-specific documentation.
- Authorization to participate in the study (experimental/observational) signed by the principal investigator.
- Declaration on the absence of conflicts of interest (signed by the P.I.)
- Curriculum vitae of the principal investigator and curriculum vitae of the site staff.
- Payment to the Ethics Committee for the preliminary investigation phase and examination of the study protocol (in the case of profit studies, the promoter must pay a flat-rate access fee).

More information about the “Information sheet and Consent Form” and the “Case Reporting Form” are provided below.

✓ **Information sheet and Consent Form**

The subjects, before being enrolled in the clinical study, will be informed about the aims of the project. **The information to the patient must be provided with an appropriate and comprehensible form** in order to express an informed written and signed consent. **The investigator, countersigned the form itself, must provide a copy of the Informed consent to the subject and keep the original.**

The subject information sheet and consent form are be evaluated and **by the Ethics Committee** during the examination of the study protocol.

If the **subject chooses to not take part/or to interrupt the study, he can do it freely**, without giving any justification and without changing his health care in the centre.

Similarly, always in the interest of enrolled subject, **clinical study may be interrupted**, if the principal investigator believes that the treatment is not good for him or if there are some undesirable and/or unpredictable effects. In this case, the subject must be immediately informed about his state of health and about possible treatments available to restore it.

The subject must be informed that, there **are any costs** for him from participation to the clinical study, and that the **food supplements is free**.

In the "**Declaration of consent and signature**" (a part of Information sheet and Informed Consent"), the subject must confirm the previous points.

Informed consent must be signed in accordance with Regulation (EU) 2016/679.

✓ **Case Reporting Form – CRF**

The **collection of data is performed through special forms (Case Reporting Form - CRF)** divided into two main sections.

The **first main section** relates to personal data, subject's medical history, the intake of any concomitant medications and it must be completed at the enrollment time.

The **second main section** must be completed during the follow-up visits, and concerns:

- the results of any analyses performed, diagnostic and biochemical investigations, etc.,
- the compliance to the treatment,
- any changes in treatment dosage,
- the results of questionnaires, and any adverse events that occurred during the study period, etc.

5. Stipulation of the insurance policy

In food supplement clinical trials, it is not mandatory to take out insurance, but it is ethical to do it.

Taxes are imposed in accordance with applicable tax regulations. **The policy insures the sponsor/promoter, the monitor, the principal investigator and his collaborators**, and **all study staff involved in the clinical trial**, as a consequence of:

- **injury caused to third parties** in the conduct of the trial,
- **injury** damage the health of enrolled subjects.

Coverage is provided for damaging events that occur during the period of insurance (within 24 months of the end of the clinical trial), as a result of which third parties have submitted a compensation for the first time during the period of insurance, or no later than 36 months after the end of the clinical trial.

6. Ethics Committee approval

After the Ethics Committee's approval of the clinical trial, it is necessary **to sign a contract** between the **Promoter** and the **administrative unit of the Ethical Committee**.

Subsequently, a deliberation shall be published on the institution website.

7. Organization of the investigator meeting

It is important to organize an investigator meeting in the center where the trial is held, to train physicians and staff involved in the study on the experimental protocol.

The investigator meeting, (open to General Practitioners belonging to the selected cooperative or to Physicians of the selected Hospital, depending on the situation,) allows a **high ethical and scientific involvement of the staff**, **on all aspects of the clinical study.**

8. Clinical trial execution and monitoring

At this point, the clinical trial can begin. It is a standard practice notify to the Ethics Committee the start of the study.

The principal investigator must:

- guarantee that the clinical study is conducted according to the protocol,
- coordinate all clinical aspects,
- verify that the clinical trial is conducted in accordance with
    - the **European Directive 2001/20/EC**;
    - the current **Helsinki Declaration on Human** Medical Research;
    - **ICH E6 (R2) Good clinical practice-GCP.**

The European Directive 2001/20/EC legally ensured the implementation of the principles of good clinical practice in clinical trials on medicinal products in Europe. The Directive establishes

specific provisions regarding clinical trials conduction in all Member States. In Italy, the European Directive was **implemented by the Legislative Decree 211/2003**.

In order to ensure compliance with the protocol, with the good clinical practice (GCP) and with the regulatory requirements, it is necessary ensure continuous monitoring, both during and after the study.

In this regard, the safety of the subject must be evaluated end the **suspected adverse events (AEs)**, after ingestion of a food supplement, must be collected within the form used by of the ***Italian National Institute of Health, Ministry of Health*** (www.vigierbe.it).

Confidentiality of data

Clinical data must be processed in accordance with current privacy regulations and, clinical data of individual patients must be processed anonymously. The study data entered into the computer must be stored in accordance with applicable privacy laws as provided in the Regulation (EU) 2016/679.

The clinical data should be report in scientific publications and conference reports. Generally, the data processing and the database are entrusted to a biometrician who produces a statistical report with the data obtained from the clinical study.

It is a standard practice notify to the Ethics Committee the end of the study.

9. Data collection and statistical analysis

In order to demonstrate the **superiority of treatment A (experimental) over treatment B (control)** it is necessary to reject the null hypothesis, according to which, the two treatments have the same efficacy. If the trial demonstrates the superiority of A over B (or vice versa), the null hypothesis is rejected.

The main objective of the statistical analysis is to test whether the observed difference, between the two groups of subjects, is real or is due to chance.

The trial results could be false because the observed difference between the two treatments could be:

- **false positive (α error)** " casualness of observations";
- **false negative (β error)** "insufficient sample size".

Per statistical convention, a **false positivity less than 5% (P value <0.05) is "tolerated".** As the P value decreases, the probability that the observed difference between groups is due to chance, decreases.

Hence, in a research study if the collected data report:

- **a p value < 0.05,** the trial is **statistically significant,** and the results are probably not due to chance (null hypothesis rejected)

- **a p value > 0.05**, the trial is **statistically non-significant**, and the result is probably due to chance (null hypothesis not rejected).

End-of-study report

The research process ends with the interpretation and publication of results.

For clinical studies on food supplements, there is not yet a publication requirement. However, the publication of research:

- **represents a moral obligation,**
- **allows a better transfer of information among researchers.**

Lack of results publications has potential negative consequences for the health care and for the planning of a new research.

The *National Institutes of Health in the United States* **(NIH)** and the *World Health Organization* **(WHO)** have set up many **web registries (**i.e. ClinicalTrials.gov, WHO International Clinical Trials Registry Platform, ISRCTN registry...) in which it is possible to **register clinical trials** and **enter data of clinical study progress, including the results,** in real time.

2.5 *Conclusions*

It is important to consider that only following the methodological approach previously described, **it will be possible to guarantee safe and effectiveness food supplements on the market, tested with the highest scientific rigor, namely thanks to a clinical study**. In fact, according to EFSA and food law, **a food supplement can boast a health claim as long as it is proven in human clinical studies**.

CHAPTER 3.

3. Performed clinical studies

The four clinical studies conducted during my PhD course are reported below. The first and the second ones involve alpha-lipoic acid, the third clinical study was focused on the efficacy of the extract of *Epilobium angustifolium* L. in the reduction of benign prostatic hyperplasia (BPH) symptoms, and the last clinical trial investigated the reduction of the increase of post-prandial glucose induced by arabinoxylans.

In particular, the first clinical study on alpha-lipoic acid evaluated the safety and efficacy of the reduction of different forms of pain by treatment with alpha-lipoic acid, administered orally. The second clinical study on alpha-lipoic acid investigated the bioavailability of a liquid formulation of alpha-lipoic acid "Liponax sol" by evaluating its pharmacokinetic parameters in humans.

The aim of the monocentric, randomized, double-blind, placebo-controlled clinical trial on *E. angustifolium* extract was to evaluate if a daily intake of hard, gastric-resistant capsules induced a significant improvement in symptoms in subjects with BPH.

The aim of the arabinoxylan clinical study was to demonstrate that arabinoxylans obtained from barley are able to reduce postprandial glycemia in healthy subjects.

3.1 Alpha-lipoic acid

The work described in this chapter was also previously published in the article entitled "*Safety and efficacy of alpha-lipoic acid oral supplementation in the reduction of pain with unknown etiology: A monocentric, randomized, double-blind, placebo-controlled clinical trial*" by Cristina Esposito, Emanuele Ugo Garzarella, Cristina Santarcangelo, Alessandro Di Minno, Marco Dacrema, Roberto Sacchi, Gaetano Piccinocchi, Roberto Piccinocchi, Maria Daglia in Biomedicine & Pharmacotherapy 144 (2021) 112308; and in the article entitled "*Pharmacokinetics Parameters of R-α-Lipoic Acid in Healthy Volunteers following the Consumption of Liponax sol a Food Supplement in Liquid Dosage Form*" by Nicola D'Anzi, Eduardo Sommella, Cristina Esposito, Emanuele U. Garzarella, Cristina Santarcangelo, Maria Daglia in Open Acc J of Toxicol. 2021; 5(1):555651.

Alpha-lipoic acid (ALA) is a small amphiphilic organosulfur compound generated by plants, animals, and humans. It is also known as thioctic acid or 1,2-dithiolol-3-pentanoic acid (Busby et al., 199). ALA has a chiral carbon in the C6 position, and it exists in two enantiomeric forms: R-(+) lipoic acid (R-ALA) and S-(-) lipoic acid (S-ALA), with R-ALA being the naturally occurring molecule. Furthermore, R-ALA is a necessary cofactor for mitochondrial enzymes involved in energy production and cellular metabolism (e.g.,

pyruvate dehydrogenase) (Reed et al., 1998). Furthermore, DHLA can be made from ALA (Bast et al., 2003; Biewenga et al., 1997). The ALA/DHLA couple has been dubbed the "universal antioxidant" because of these characteristics. This molecule also has metal-chelating properties (Goraca et al., 2011; Ou et al., 1995) and the ability to scavenge hydroxyl radicals, hypochlorous acid, and single oxygen (Goraca et al., 2011; Ou et al., 1995). (Packer et al., 1995).

3.1.2 Bioavailability

R-ALA is the most active form of ALA, however it is rarely used due to its intrinsic polymerization susceptibility. The racemic combination (which is more stable) is extensively utilized as a medicine or food supplement ingredient, and ALA is mostly synthesized through a chemical process (Brufani et al., 2014; Hornberger et al. 1952).

There are several different ALA formulations on the market, and the most of them contain the racemic combination. However, limited research has been done on the impact of the inactive enantiomer on the pharmacokinetic profile. The S-ALA is known to have an effect on the PK of products due to its influence on the R-polymerization ALA's susceptibility. The potential deleterious consequences of the S-ALA are highlighted by a small number of studies (Brufani et al. 2014; Keith et al. 2012).

When given intravenously, ALA reaches its maximal plasma concentration, which has favorable effects in the treatment of

symptomatic diabetic polyneuropathy and other diabetic-related diseases. In diabetic patients with neuropathy, the ALADIN study (Ziegler et al., 1995) found that intravenous ALA caused a significant reduction in sensations such as burning, paraesthesia, and pain when compared to placebo. ALA, on the other hand, is a food supplement that can only be taken orally and it comes in the form of 600 mg racemic ALA tablets or capsules, usually (Tomassoni et al., 2013). Unlike intravenous dosing, oral administration of ALA tablets has a decreased bioavailability. Previously, the bioavailability of several solid formulations was examined, and a low grade of ALA absorption of around 30% was discovered (Singh et al., 2008). Indeed, ALA's potential is limited by factors such as reduced solubility in an acidic environment and enzymatic breakdown, which characterize its gastric and hepatic passage when taken orally. Furthermore, oral administration involves a number of factors that limit the quantity of ALA absorbed, including solid formulation disintegration, the first pass effect in the liver, and inter-individual variability (Packer et al., 1995; Singh et al., 2008). As a result, a variety of chemical interventions and formulations have been attempted in order to increase ALA plasma bioavailability even after oral administration and provide superior therapeutic benefits (Brufani et al., 2014; Packer et al., 2001; Strickley et al., 2004; Bernkop-Schnurch et al., 2004 a, 2004 b). To far, only one patented oral liquid formulation containing 300 mg of the R-(+) enantiomer stabilized as Na salt in water and co-solvent is available on

the market (Brufani et al., 2013). This formulation improved pharmacokinetic characteristics in experimental animals when given orally at a dose of 50 mg/kg, and it improved nerve conduction velocity, hyperglycemia, and hypertriglyceridemia in a diabetes model induced in Sprague Dawley rats (Brufani et al., 2014).

3.1.3 Efficacy

Over the last two decades, ALA has been ascribed with a variety of biological functions, mostly due to its antioxidant and anti-inflammatory effects (Salehi et al., 2019). Several clinical trials have highlighted the beneficial effects of ALA in subjects suffering from various types of acute and chronic pain (Gao et al. 2007; Kim et al. 2007; Salvemini et al. 2011; Wang et al. 2004) as growing evidence suggests a role for reactive oxygen species and antioxidants in pain modulation (Gao et al. 2007; Kim et al. 2007; Salvemini et al. 2011; Wang et al (Parente et al., 2017). The International Association for the Study of Pain (IASP) defines pain as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*", thus suggesting the need to always consider pain as an important symptom for diagnosis as well as a crucial therapeutic target. In this context, the use of ALA food supplements to alleviate pain has recently attracted researchers' interest, resulting in a substantial body of evidence demonstrating its safety and efficacy, as detailed below. The use of ALA food

supplements is an interesting option, particularly in the case of primary pain of unknown etiology, where no specifically-targeted drug can be chosen and where symptomatic drugs may not always be effective but may be associated with serious side effects if used for an extended period of time. ALA oral supplementation, at doses ranging from 400 to 600 mg/day, has recently been reported to reduce pain in disorders such as migraine, back pain, carpal tunnel syndrome pain, and burning mouth syndrome pain in several clinical trials (Battisti et al., 2013; Cavestro et al., 2018; Monroy Guizar et al., 2018; Femiano et al., 2002). Two other clinical trials, in which the efficacy of ALA was tested against chemotherapy-induced peripheral neuropathy and fibromyalgia, came to opposing conclusions, despite the fact that these studies had several limitations (i.e., small trial size, poor patient compliance) 8 Ametov et al., 2003; Jacob et al., 1999).

Furthermore, many clinical research examining the efficacy of ALA have focused on the alleviation of diabetic polyneuropathy symptoms in diabetic patients, and significant evidence demonstrates that ALA decreases blood glucose levels. ALA helps to regulate insulin levels and reduces insulin resistance through a variety of mechanisms of action (i.e., increase of sugar uptake by the redistribution of glucose transporters (GLUT4) to the plasma membrane, increase in the abundance and intrinsic activity of GLUT4, phosphorylation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1), and activation of intracellular AMP-activated protein kinase (AMPK)

(Rochette et al., 2015). Despite this, there are limited research examining the effects of ALA on glycemia in normoglycemic or prediabetic subjects (Battisti et al., 2013; Farrar et al., 2000; Jensen et al., 2003).

3.1.4 Safety

In terms of ALA's safety, a number of clinical trials have found that it is well tolerated and has no notable side effects when compared to placebo. (Shay et al., 2009; Ametov et al., 2009; Jacob et al., 1999; Reljanovic et al., 1999; Ruhnau et al., 1999; Yadav et al. 2005; Ziegler et al. 1995, 1997, 2006). An observational retrospective study conducted on 610 pregnant women treated for 7 days with a dose of 600 mg/day confirmed the safety of ALA in pregnant women and their babies (Parente et al., 2017). Another study in older subjects (15 people over the age of 65) indicated that ALA was safe at 600 mg/mL, but that it was not totally tolerable. ALA caused gastrointestinal adverse effects and flushing feelings at a dose of 1200 mg/day, but these were minimized with gastrointestinal prophylaxis, enhancing ALA tolerability (Sarezky et al., 2016). ALA can promote the development of insulin autoimmune syndrome (IAS), commonly known as Hirata's disease, in predisposed individuals. This condition is strongly associated with alleles DRB1*04:06, DRB1*04:03 and DRB1*04:07, which are extremely rarely observed in the European population, but are more common in Asian populations. IAS is a rare condition

characterized by hypoglycemic episodes due to the presence of high titres of insulin autoantibodies (IAA). The complex resulting from interactions of insulin with insulin autoantibodies prevents insulin from attaching to its receptor, resulting in a increase in unbound insulin levels and hypoglycemia episodes (Cappellani et al., 2006). IAS induces symptoms that are neuroglycopenic, neurogenic, and cholinergic (Davi et al., 2017; Censi et al., 2018). When the trigger is eliminated, IAS normally disappears within a few months, although some patients may require pharmacological treatment.

i.First clinical study

Safety and efficacy of alpha-lipoic acid oral supplementation in the reduction of pain with unknown etiology: A monocentric, randomized, double-blind, placebo-controlled clinical trial.

The aim of this monocentric, randomised, double-blind placebo-controlled clinical trial, was to evaluate the safety and efficacy of the reduction of different forms of pain by ALA treatment, administered orally for two months at two doses (800 and 400 mg/day), in normoglycemic subjects.

Some subjects, who suffering of different idiopathic pain (such as arthralgia, neuropathic pain, and myalgia in which pain was not secondary to clinically evident tissue damage), cannot or don't want to take pain drugs. Considering that pain is associated with significant functional disability which interfere with quality of life and daily activities, in this study ALA was administered with the purpose to assess if it could be a feasible option respect the common anti-inflammatory drugs.

Regarding ALA safety, limited data on the effect of ALA on glycemia in normoglycemic subjects are present in the literature. Therefore, the effect of ALA on glycemia was evaluated as the primary outcome of this study. Moreover, it was monitored hepatic and renal functions of the subjects, and adverse reactions (ARs), as recommended by the

Italian National Institute of Health, Ministry of Health (www.vigierbe.it).

Materials and methods

α-Lipoic Acid (ALA) and placebo

The ALA food supplement consisted of tablets containing 400 mg of α-lipoic acid and excipients (dicalcium phosphate, microcrystalline cellulose, polyvinylpolypyrrolidone, croscarmellose sodium, silicon dioxide, vegetable magnesium stearate, hydroxypropyl methylcellulose, polyvinyl pyrrolidone, acetylated monoglycerides, gum lacquer, hydroxypropyl methyl cellulose) while the placebo only contained the inert excipients (dicalcium phosphate, microcrystalline cellulose, silicon dioxide, magnesium stearate, glyceryl dibehenate, talc, calcium carbonate, cross-linked sodium carboxymethylcellulose, hydroxypropyl methylcellulose, polyvinylpolypyrrolidone, gum lacquer, povidone, acetylated mono- and diglyceride esters, stearic acid, titanium dioxide). Both ALA food supplement and placebo were produced by S.I.I.T. (Trezzano sul Naviglio, Italy), in accordance with European specifications for contaminants and microbiologic limits. Both treatments were made unrecognizable in appearance, color, and flavor, and these were packaged in white containers of 60 tablets each. The shipment at the trial center was registered ensuring that the information on the packing slip (inside and outside containers) related to the amount, batch numbers, manufacturing date, expiry date, name

of manufacturer, quantity and storage conditions, had been in line with the protocol. Food supplement accountability logbook were kept and reviewed by the monitor periodically. Both the ALA food supplement and placebo were stored in a locked cabinet in a locked room at environmental temperature, accessible only to essential research personnel.

Clinical trial design

This study was submitted to the Ethics Committee of A.S.L. Napoli 1 Centro. The study was approved by the Ethics Committee (protocol number 532, 19 November 2020) and carried out in accordance with the current Helsinki declaration. This study is listed on the ISRCTN registry (https://www.isrctn.com/ISRCTN89876422).

Once a positive opinion was obtained from the relevant Ethics Committee and the authorising deliberation was published, a monocentric, randomised double-blind placebo-controlled clinical trial was performed by COMEGEN - Società Cooperativa Sociale (Naples, Italy) to evaluate the safety and efficacy of ALA food supplementation on an adult population suffering from idiopathic pain (arthralgia, neuropathic pain, and myalgia with unknown etiology).

The different type of pain was diagnosed through a check-up by physicians, only for those patients which turned to their general practitioner for an alternative pain relief prescription, as they could not

or did not want to take analgesic drugs for the pain relief necessary to increase their quality of life.

The participants received oral and written information concerning the study before they gave their written consent.

The study was double-blind, both for the investigating physician and for the enrolled subjects. At the end of the baseline visits, a randomization sequence was generated by a statistician using STATA 16 software (Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC) and the randomization list was kept hidden. The 210 subjects enrolled in the clinical study, were divided into three groups (70 subjects for each group). In specific, these consisted of subjects assuming the daily dose of 800 mg/day of ALA (two tablets of 400 mg, group 1); subjects assuming 400 mg/day of ALA (one tablet of ALA and one tablet of placebo, group 2); and subjects assuming placebo (two tablets of placebo, group 3).

The subjects were assigned to each of the three treatment groups casually and by simple randomisation (1:1:1 allocation ratio). This procedure minimizes any systematic differences between the characteristics of the studied groups (selection bias). It was not used for stratification or blocking. The concealment of the randomization list protected the allocation sequence until the assignment and was stored at a secured location in the Department of Pharmacy, University of Naples Federico II.

ALA food supplement and placebo were prepacked in white containers of 60 tablets. Each container was consecutively numbered for each participant according to the randomisation list. Each subject was assigned an order number and received the tablets in the corresponding prepacked containers.

The clinical trial duration was 6 months. Participants underwent two visits (baseline = t0 and after 2 months = t1) in an outpatient setting. At the baseline visit (t0) information on the sociodemographic and clinical characteristics of the subjects was collected and reported in the CRF. Numerical rating scale (NRS) and visual analogue scale (VAS) results were reported in the CRF at t0 and after 2 months (t1) for each subject, along with fasting blood glucose assessment, renal and hepatic toxicity assessment by blood test for the evaluation of creatinine (CRE) level, serum glutamic pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase (SGOT). Moreover, at the baseline (t0), each subject was given a form to complete based on that used by Italian Phytovigilance System (IPS), to report the possible ARs after the ingestion of food supplements.

Study population

210 Subjects aged 18-75 and of either sex, with a fasting glycemia below 105 mg/dl at recruitment and with signed informed consent, were enrolled by the general practitioners of Comegen in June 2021. Subjects with the need for pain relief, but who were unable or unwilling

to take analgesic drugs, were considered eligible for the enrolment if they suffered from a mild to moderate primitive pain with no detectable inflammation, no tissue damage or damage to the nervous system, and no identifiable noxious stimulus (i.e., primitive neuropathic pain, arthralgia with unknown aetiology, and idiopathic myalgia). As far as arthralgia and myalgia are concerned, the medical classification list ICD (International Statistical Classification of Diseases and Related Health problems), generated by the World Health Organisation (WHO), was used by the physicians to identify and code the type of pain in the enrolled subjects. ICD-9-CM diagnosis code 719.4 defining "pain in joint" was applied to arthralgia and ICD-9-CM diagnosis code 729.1 was applied to myalgia.

Pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding women, patients with allergies, congenital or acquired immunodeficiency syndrome, fasting glycemia above 105 mg/dl, obesity (BMI>30kg/m^2), undergoing pharmacology therapy for diabetes, cardiovascular diseases, systemic chronic disease, analgesic therapy, anti-inflammatory or food supplements for pain, and those considered unsuitable for participation by the physician were excluded from the study.

Outcomes of the study

In this study the safety and efficacy of ALA oral administration to normoglycemic subjects with primary neuropathic pain, idiopathic myalgia or arthralgia, who needed an alternative treatment to traditional analgesics as they could not or did not want to take pain medications, were evaluated as primary outcomes of the study. Two dosages of ALA were administrated to the subjects. ALA 400 mg/day is used to demonstrate ALA efficacy at the lowest dose, instead ALA 800 mg/day was used to confirm the efficacy and safety of ALA at maximum dose. In particular, as far as ALA safety is concerned, the effect of ALA supplementation on fasting blood glucose was determined in the recruited normoglycemic or mild dysglycemic subjects. The evaluation of the efficacy of pain reduction from ALA oral supplementation after two months of treatment, was evaluated using validated questionnaires, such as the Numerical Rating Scale (NRS) and the Visual Analogue Scale (VAS). The NRS is a unidimensional measure of pain intensity in which the subject indicates the intensity of the pain by drawing a circle on the number that best describes it. The instrument is represented by a horizontal line on which a scale of values between 0 and 10 are indicated, corresponding to "no pain" and "worst pain imaginable," respectively (Breivik, et al., 2008). Higher scores indicate greater pain intensity. The minimal perceptible clinical improvement (MPCI) is a 2 point or a 30% reduction on the pain NRS scores (Farrar et al., 2000; Jensen et al., 2003).

The VAS is another pain assessment tool, used for a variety of purposes and in the assessment of general pain. It consists of a 100 cm paper strip, with the two end points of "no pain" and "worst pain I can imagine" at either end. The subject must mark the level of perceived pain on the strip. The following cut-off points are recommended for the pain VAS: no pain (0–4 cm), mild pain (5–44 cm), moderate pain (45–74 cm), and severe pain (75– 100 cm) (Jensen et al., 2003).

The secondary outcome was the evaluation of possible ARs occurred after the intake of ALA, during the study. ARs were registered by filling in a form, specifically prepared according to IPS standards, for the reporting of suspected ARs that may occur after the intake of food supplements. This serves to assess the severity of the ARs, and the causal relationship between the oral administration of ALA and/or any concomitant therapy, and the ARs.

Moreover, renal and hepatic functions were monitored for two months following the oral administration of ALA, through the determination of blood tests to evaluate CRE, SGPT, and SGOT, performed at t0 and t1.

Statistical analysis

Sample size calculation was conducted using three 1-β power values (0.80, 0.95 and 0.99), a significance threshold value of α equal to 0.05, and three effect size values (Cohen's f = 0.10, 0.14 and 0.25, respectively). Sample size was determined to be 210 participants (70 each group).

The effect of the treatments on the response variables (NRS, VAS, glycemia, SGOT, SGPT and creatinine), was assessed through a Random Intercept Linear Mixed Model (LMM), where the treatment groups (G1, G2 and G3), the measurement times (t0 and t1), and the age and sex of subjects entered the model as fixed effects. The interaction group × treatment was also added to the fixed effects in order to account for differential patterns of responses of groups to measurements. Finally, subject identity was entered into the model as a random effect, to account for repeated measures within subjects.

In a second analysis, we searched for possible differences in NRS and VAS scores among treatment groups due to pain source (arthralgia or neuropathic pain). To do so, we were able to collect data for a subsample of 198 out of 210 subjects enrolled in the study (G1: 66, G2: 67, G3: 64). On this subsample we ran the same LMM we used for the previous analysis but updated to evaluate the three-way interaction group × treatment × pain, accounting for differential responses between treatment groups due to the combination of measurement time (t0 and t1) and nature of pain (arthralgia or neuropathic pain).

Analyses were performed using the lme4 (D. Bates, M. Maechler, B. Bolker, S. Walker, Fitting Linear Mixed-Effects Models Using lme4, J. Stat. Soft. 67 (2015) 1–48) and MuMIn (Barton, K. 2020. 'MuMIn': Multi-Model Inference. R Package Version 1.43.17. Available online: https://CRAN.R-project.org (accessed on 2 August 2021) packages in

R ver. 4.0.1 (R core Team 2021), and unless otherwise stated, data are reported as means ± standard errors.

Results

Clinical trial

The study flow chart is reported in figure 1 according to the CONSORT PRO reporting guideline (Calvert et al., 2013).

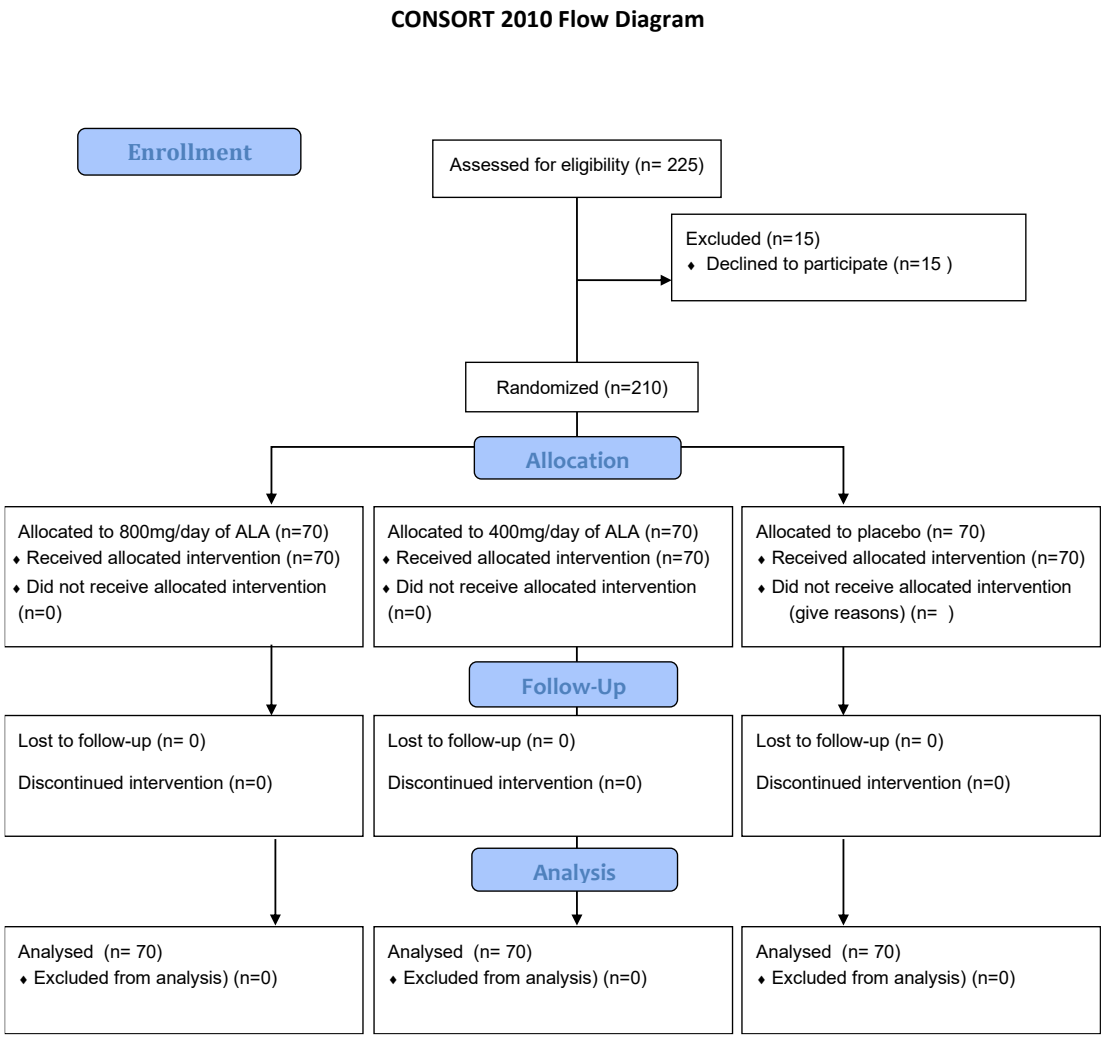


Figure 1. CONSORT Flow diagram.

The two ALA treated groups consisted of 140 subjects: 26 male (corresponding to 37 %) and 44 female (63 %) treated with the ALA dose of 800 mg/day (two tablets of 400 mg of ALA), and 29 (41 %) male and 41 female (59 %) treated with the ALA dose of 400 mg/day (one tablet of 400 mg of ALA and one tablet of placebo). The untreated group consisted of 70 subjects, 25 male (36 %) and 45 female (64 %), treated with two tablets of placebo. The participants in the three groups had similar sociodemographic characteristics and clinical data with no significant differences. The baseline characteristics of the subjects for each group are summarized in table 1.

Characteristic/ Treated Untreated Clinical data	**Group 1** (n=70) **Treated with ALA (dose 800 mg/day)**	**Group 2** (n=70) **Treated with ALA (dose 400 mg/day)**	**Group 3** (n=70) **Untreated (placebo)**
Age	Male= 53.5 ± 7.98 Female= 54.2 ± 7.25	Male= 54.6 ± 7.47 Female= 52.7 ± 7.85	Male= 55.7 ± 5.17 Female= 5.8 ± 6.58
Gender	Male= 26 Female= 44	Male= 29 Female= 41	Male= 25 Female= 45
Type of pain (n° subjects):			
arthralgia	50	45	46
neuropathic pain	16	22	19
idiopathic myalgia	4	3	5
NRS	6.2±2.2 (3-10)	6.2±2.4 (3-10)	6.0±2.1 (3-10)
VAS	57.7±24.7 (1-100)	59.9±23.7 (1-100)	62.4±26.2 (1-100)
Glycemia	82.3±11.5 (70-104)	86±11.1 (70-105)	87.5±10.9 (70-104)
SGOT	37±8 (24-50)	37±8 (24-50)	37±8 (24-50)
SGPT	37±8 (24-50)	37±6 (24-49)	37±6 (24-49)
Creatinine	0.95±0.10 (0.80-1.10)	0.93±0.08 (0.80-1.09)	0.96±0.09 (0.81-1.10)

Table 1.— Characteristics of the study population: demographic and clinical data at baseline.

In table 2 the data regarding the primary and secondary outcomes at baseline and t1 are reported. The study revealed that the two response variables (NRS and VAS) changed among the ALA food supplement groups and the placebo group between the beginning (t0) and the end (t1) of the clinical trial.

Variable	t0	t1
NRS		
G1	6.2±2.2 (3-10)	1.7±0.8 (0-4)
G2	6.2±2.4 (3-10)	1.9±1.0 (0-5)
G3	6.0±2.1 (3-10)	6.0±2.1 (1-10)
VAS		
G1	57.7±24.7 (10-100)	6.6±6.8 (0-30)
G2	59.9±23.7 (10-100)	24.5±15.2 (0-80)
G3	62.4±26.2 (10-100)	63.4±26.9 (10-100)
Glycemia		
G1	82.3±11.5 (70-104)	83.4±10.8 (70-105)
G2	86±11.1 (70-105)	85.7±10.4 (70-108)
G3	87.5±10.9 (70-104)	86.7±10.2 (70-105)
SGOT		
G1	37±8 (24-50)	37±8 (24-50)
G2	37±8 (25-50)	37±8 (24-50)
G3	37±8 (25-50)	36±8 (25-50)
SGPT		

G1	36±8 (25-50)	36±8 (25-50)
G2	38±8 (25-50)	38±7 (25-50)
G3	37±6 (25-49)	37±6 (25-50)
Creatinine		
G1	0.95±0.10 (0.80-1.10)	0.94±0.09 (0.78-1.10)
G2	0.93±0.08 (0.80-1.09)	0.92±0.080 (0.80-1.10)
G3	0.96±0.09 (0.81-1.10)	0.96±0.09 (0.81-1.10)

Table 2.— Primary and secondary outcomes at baseline (t0) and t1

Indeed, the results from the LMM applied to the NRS and VAS scale (Table 3) highlighted significant effects over time for the group, time of measurement and group-measurement time interaction, but not for the age and sex of subjects, suggesting that age and gender do not influence these variables.

Model	**F**	**df**	**P**
NRS			
Measurement	499.22	1,207	**<0.001**
Group	35.03	2,205	**<0.001**
Gender	1.96	1,205	0.16
Age	1.09	1,205	0.30
Measurement × Group	114.33	2,207	**<0.001**
VAS			
Measurement	528.80	1,207	**<0.001**
Group	41.77	2,205	**<0.001**
Gender	1.01	1,205	0.31
Age	1.98	1,205	0.16
Measurement × Group	155.01	2,207	**<0.001**
Glycemia			
Measurement	0.02	1,207	0.89
Group	2.87	2,205	0.06
Gender	1.04	1,205	0.31

Age	0.03	1,205	0.87
Measurement × Group	11.39	2,207	**<0.001**

Table 3.— Results for the LMM models for the analysis related to the primary outcome of the study.

In each experimental group, there was a significant difference in NRS pain scale values between t0 and t1. In particular, the NRS values in the G1 group significantly decreased from t0 to t1 (-4.55±0.24, t_{207}=19.34, P<0.001, Fig.2) and the same occurred in the G2 group (-4.25±0.24, t_{207}=18.03, P<0.001, Fig.2). On the contrary, in the G3 group there were no significant differences in the NRS values between t0 and t1 (-0.04±0.24, t_{207}=0.18, P=0.86, Fig.2). The random effect between subjects was highly significant in both models (LRχ^2=45.27, df=1, P<0.001).

The VAS pain scale yielded similar results as the NRS scale. Indeed, the model showed significant effects between the groups, times of measurement and their interaction, but not for age and sex of subjects (Table 2). In particular, the values on the VAS scale in G1 significantly decreased from t0 to t1 (-51.07±2.15, t_{207}=23.80, P<0.001, Fig. 2) passing from moderate to mild pain (Table 1). The same thing occurred in group G2 (-35.37±2.15, t_{207}=16.49, P<0.001, Fig.2), passing through moderate to mild (Table V). As in the previous analysis, for G3 there was no significant difference in the intensity of pain VAS values from t0 to t1 (+1.00±0.24, t_{207}=0.47, P=0.64, Fig.2) which remained

moderate until the end of trial. The random effect between subjects was still highly significant ($LR\chi^2$=118.43, df=1, P<0.001).

One of the modes of action of ALA, as explained previously, is the reduction of glycemia via insulin metabolic pathways, glucose uptake, and glycogen synthesis. Only the interaction group measurement had a significant influence on fasting blood glucose levels, while no significant effect was discovered for the measures or the age and sex of the subjects (Table 3). However, the variations in glycemia identified by the LMM between t0 and t1 were only in the order of one or two points (Fig.2): variations were +1.13±0.29 (t_{207}=3.83, P<0.001) in G1 and -0.80±0.29 (t_{207}=2.72, P=0.0072) in G3, while in G2 the variation was not significant (-0.26±0.29, t_{207}=0.87, P=0.40). The random effect was found to be highly significant ($LR\chi^2$=615.18, df=1, P<0.001).

The LMMs did not identify any significant effect on hepatic and renal functions (Table 8, Fig.2). However, the random effect between subjects was highly significant in all cases ($LR\chi^2$>363.97, df=1, P<0.001).

Model	F	df	P
SGOT			
Measurement	0.19	1,207	0.66
Group	0.07	2,205	0.93
Gender	<0.01	1,205	0.99
Age	0.11	1,205	0.74
Measurement × Group	1.12	2,207	0.33
SGPT			
Measurement	0.61	1,207	0.44
Group	0.93	2,205	0.39
Gender	0.44	1,205	0.51

Age	0.76	1,205	0.38
Measurement × Group	<0.01	2,207	0.99
Creatinine			
Measurement	1.74	1,207	0.19
Group	2.73	2,205	0.07
Gender	0.33	1,205	0.57
Age	<0.01	1,205	0.92
Measurement × Group	1.30	2,207	0.27

Table 3.— Results of the LMM models for the hepatic and renal toxicity analysis.

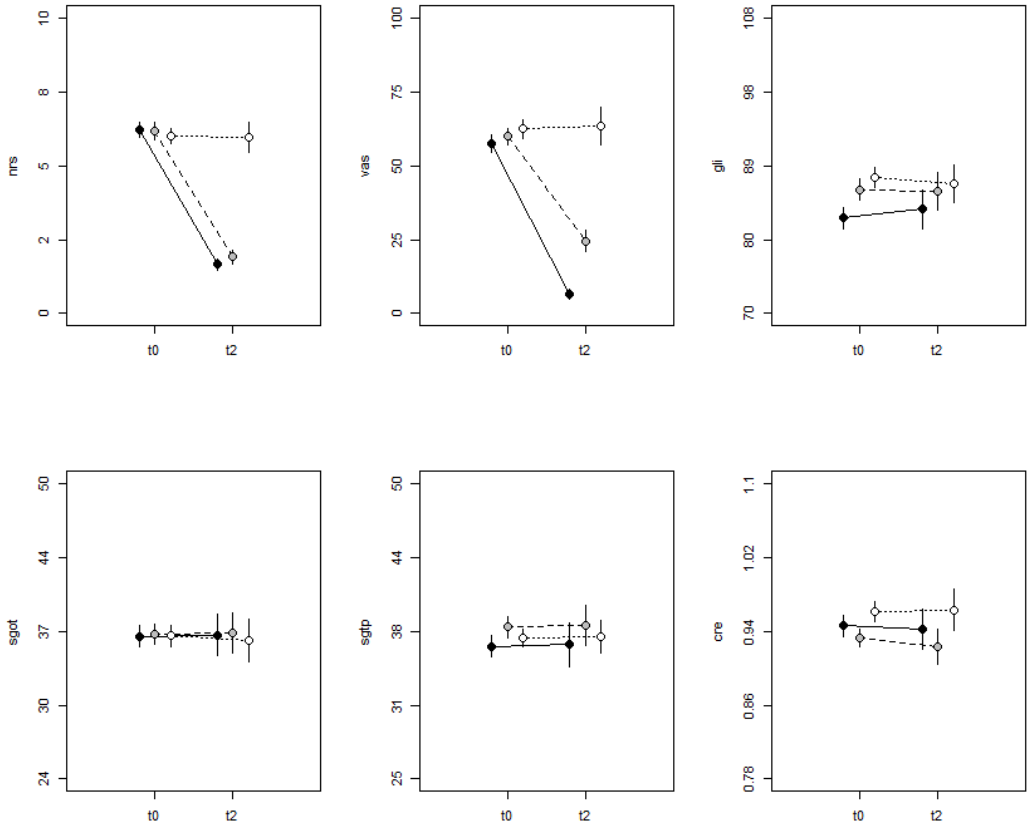


Figure 2. Variations of the six response variables evaluated at t0 and t1 in the three experimental groups (black and continuous line: G1; grey and dashed line: G2; blank and speckled line: G3).

No individuals reported ARs related to the administration of ALA at either dose during the two-month treatment period, including the absence of allergies, and the principal investigator concluded that the use of ALA tablets was well tolerated.

Finally, the LMMs were used to see if there were any variations in NRS and VAS scores across treatment groups depending on the source of pain (i.e., arthralgia or neuropathic pain), did not reveal any significant effect, as the three-way interaction group × measurement × pain was not significant for both models (NRS: $F_{2,192}=0.217$, $P=0.81$; VAS: $F_{2,192}=1.109$, $P=0.33$). The two-way interaction group × pain was also not significant in both models (NRS: $F_{2,190}=1.861$, $P=0.16$; VAS: $F_{2,190}=0.133$, $P=0.86$).

Discussion

The main purpose of this clinical study was to investigate the safety and the efficacy in the reduction of mild or moderate pain (arthralgia, neuropathic pain, and fibromyalgia, with unknown etiology) of an ALA food supplement, administered orally for two months at doses of 400 mg/day or 800 mg/day. We enrolled 210 normoglycemic subjects who ask to their general practitioner an alternative to analgesic drugs commonly used to treat pain. The common analgesic drugs are defined by WHO analgesic ladder in to two analgesic steps, the first one includes for instance aspirin, paracetamol and the non-steroidal anti-inflammatory drugs, and the

second ones include e.g. weak opioids such as codeine (Hylands-White et al., 2017).

ALA treatment was found to be able to significantly reduce pain intensity as measured by the two most commonly used unidimensional pain intensity scales, NRS and VAS, at both doses, with the higher dose being more effective than the lower one. Although VAS highlighted a greater difference in pain relief between the two ALA doses, these scales showed good correlation in agreement with the conclusions reported by systematic reviews on comparison of pain scales (Karcioglu et al., 2018; Williamson et al., 2005). The results of this investigation are all the more significant considering the need for novel pain treatments alternative to commonly used drugs, which often fail in the achievement of an adequate pain management. In this regard, a recent study of Breivik et al., showed that 64% of subjects taking prescription drugs found that their pain medication was inadequate and, of the 48% of chronic pain suffering subjects not taking pain medication, 14% had stopped due to side effects (Breivik et al., 2006)].

In this clinical trial 57 subjects were affected by neuropathic pain, 141 subjects by arthralgia, and only 12 subjects by myalgia.

The ALA food supplement shows its effectiveness independently from the type of pain (arthralgia, neuropathic pain, and myalgia with unknown etiology).

About neuropathic pain, the obtained results are in line with those present in the literature on the efficacy and safety of ALA in the

treatment of neuropathy caused by diabetes. This fact extends the use of ALA to idiopathic neuropathy. Neuropathic pain, defined by the International Association for the Study of Pain (IASP) as: "*pain that arises as a direct consequence of a lesion or diseases affecting the somatosensory nervous system*" (Scholz et al., 2019), affects about 7-10 % of the general population, being more frequent in subjects with age >50 years. Neuropathic pain has a complex etiopathogenesis, with diabetes being known as the most common cause of neuropathic pain, which affects about one third of diabetic patients. Although many causes are considered responsible for neuropathic pain (i.e., mechanical-compressive, traumatic, viral and inflammatory causes), in many cases (about 20-30 %) the etiology of neuropathy remains idiopathic (Farhad et al., 2016). Especially in its idiopathic forms, the management of neuropathic pain is a real challenge for physicians as they cannot treat the causes underlying this symptom, and they can only relieve the pain with symptomatic drugs. In fact, the most common interventions used to treat diabetic neuropathy (lifestyle improvement, intervention on glycemic control, and pathogenesis-oriented pharmacotherapy, which exert effects on the processes by which hyperglycemia leads to cell damage) cannot be used, and only symptomatic pain relief can be prescribed (Ziegler et al., 2021). The drugs used to relieve neuropathic pain include tricyclic antidepressants, serotonin–norepinephrine reuptake inhibitors (SNRIs) and calcium-channel anticonvulsants and opioids, which are limited in their

effectiveness and have considerable side effects (Binder et al., 2016). In the Riediger et. al meta-analysis on the adverse effects of antidepressant drugs used in pain relief is shown that amitryptiline, a tryciclic antidepressant, induces adverse effects in a percentage ranging from 52 to 100% of treated patients (Riediger et. al, 2017). The adverse effects that may occur include dry mouth, drowsiness, urinary difficulty, constipation, sweating, headache, irritability, palpitations, diarrhea, blurred vision, dizziness, edema, gastritis, thirst, tachycardia, weight gain, and nausea. In particular, the adverse effects (i.e., nausea, vomiting, dizziness, and somnolence) of venlafaxine (SNRI) showing that the percentage of subjects suffering from adverse effects ranges from 14 to 100% of the treated patients. As far as calcium-channel anticonvulsants are concerned, the most common adverse effects of anticonvulsants are sedation and cerebellar symptoms (nystagmus, tremor and incoordination), occurring quite frequently (Jensen et al., 2002).

Regarding the Arthralgia, it causes a pain in one or multiple joints (i.e., hands, knees, hips and spine). It is estimated to be the second of the ten most common reasons for a visit to a physician maybe be due to either inflammatory or non-inflammatory forms of arthritis. About 50% of subjects with arthralgia or poly-arthralgia have an unclassifiable condition, not accompanied by typical signs of inflammation and extra-articular symptoms. In this study, 141 subjects suffering from arthralgia with unknown etiology were recruited. At the end of this study, subjects

with arthralgia treated with an ALA food supplement had an improvement in pain respect to the placebo group, without any clinically significant changes in glycemia and without any indication of adverse effects. To our knowledge, this clinical trial is the first that demonstrates an effect of ALA food supplements in non-inflammatory forms of arthralgia. The unclassifiable forms of arthralgia are generally transient, of little clinical significance and may not require a pharmacologic treatment, going into remission within a year. Notwithstanding, this type of pain interferes with the daily activities and induces a significant emotional distress. For this condition, the most common drug used to relieve pain is acetaminophen (N-acetyl para-aminophenol or paracetamol), which is the most widely used as an over-the-counter non-opioid analgesic agent used to treat mild to moderate pain. Although the common perception is that acetaminophen is an extremely safe drug, it can however cause serious adverse effects and is responsible for 56,000 emergency department visits, 2,600 hospitalizations, and 500 deaths per year in the United States (with fifty percent of these being due to unintentional overdoses) and is the second most common cause of liver transplantation worldwide (Agrawal et al., 2021). The most common adverse effects are skin rash, hypersensitivity reactions, nephrotoxicity, hepatotoxicity (increased aminotransferase activity at therapeutic doses, hepatic failure in the case of overuse, enhanced previous liver damage caused by alcohol consumption), hematological (i.e. anemia, leukopenia, neutropenia, pancytopenia),

metabolic (hyperglycemia, increased bilirubin and alkaline phosphatase) and electrolyte (i.e. decreased serum bicarbonate, decreased concentrations of sodium and calcium, hyperammonemia, and hyperchloremia) disorders (Kennon-McGill et al., 2018), and liver injury. When acetaminophen fails to relieve pain, non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs, although these exert many adverse effects (i.e., gastrointestinal bleeding, renal toxicity, and hypertension), being responsible for about 30 % of hospitalizations due to adverse drug reactions. It is estimated that 5,000–16,500 annual deaths in the United States and 400–1,000 deaths in the United Kingdom are directly related to upper gastro-intestinal ulceration and bleeding caused by NSAIDs (McEvoy et al., 2021).

Ac concerns fibromyalgia, this condition affects between 2 and 4% of the general population, mainly consisting of women, and it is characterized by generalized chronic pain in the absence of clinically evident structural abnormalities explaining said pain. In this study, 12 subjects with a further type of pain lacking an organic basis, such as fibromyalgia, were recruited. The low number of recruited subjects suffering from idiopathic fibromyalgia made it impossible to perform an appropriate statistical analysis, although pain improvement in ALA treated subjects was registered. In this regard, a recent clinical study on the effect of ALA on pain intensity shows contradicting results. The NRS was measured in 27 subjects (5 males and 22 females; age range:

25-74 yrs) suffering from fibromyalgia, treated for 4 weeks with ALA at increasing doses ranging from 300 mg/day in the first week to 1800 mg/day in the fourth week. No statistically significant differences were found between placebo and ALA groups, nevertheless, the *post hoc* exploratory subgroup analysis showed a significant difference between male and female, probably due to gendered differences in the pharmacokinetics of ALA. Drugs (i.e anti-epileptic drugs, tricyclic anti-depressants, selective serotonin reuptake inhibitors, and serotonin-norepinephrine reuptake inhibitors) used to treat fibromyalgia present serious adverse effects and limited efficacy, it is necessary to carry out large-scale population clinical trials to define the actual effects of ALA in the reduction of pain due to fibromyalgia.

Another important assessment of this study regard ALA safety. Subjects enrolled did not show any clinically significant changes in glycemia or any indication of adverse effects. Our results are in agreement with those obtained by Gosselin et al. who studied the effect of ALA oral supplementation (600 mg/mL for 30 days) on plasmatic glucose levels in 12 pre-diabetic subjects showing a glucose level of 102.1 ± 5 mg/dL at baseline, in a randomized, double-blinded, placebo controlled cross-over clinical trial. Any statistically evidence regard the decrease in serum glucose, after the ingestion of ALA was revelated. In contrast, statistically significant decrease in insulin and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index was evaluated. These results suggesting that ALA also exerts its properties

in healthy subjects with mild dysglycemia (Gosselin et al., 2019). Moreover, no variation on serum glucose was noted after 6 months of ALA oral supplementation (400 mg/day) in a clinical trial on the improvement of migraine in patients with insulin resistance (Cavestro et al). In 2020, Derosa et al., in a retrospective, observational study enrolling 322 patients treated with different dosages of ALA (i.e. 400, 600, 800 and 1200 mg/day), concluded that the chronic use (4 years) of ALA is well tolerated at all dosages, with an improvement in glycemic status only at high dosages in disglycemic subjects (Derosa et al., 2020). Recently, in 2021, Gatti et al. conducted the first real world assessment of the safety profile of ALA-containing products by analysing spontaneous reports of suspected adverse reactions (ARs) collected from March 2002 to February 2020 by the IPS, coordinated by the Italian Institute of Health. Of the 2147 total reports found, 116 reports (about 5.4 % of the total number of collected reports) regarded the ARs to ALA-containing products, and of these 15 reports (about 0.7 % of the total number of collected reports) showed a definite causality assessment. In accordance with WHO-VigiBase data, this study showed that the ARs consist of cutaneous, gastrointestinal, nervous and immune disorders with varying degrees of seriousness. Skin (44.9%) and gastrointestinal disorders (10.8%) were the most frequently represented ARs. Skin and subcutaneous tissue disorders were found to be significantly predominant in non-serious events (52.5% vs. 30.9%; p=0.004). Overall, 45 (38.8%) cases were classified as serious, but no

fatal cases were reported by the IPS. In particular, ten cases of IAS, mostly represented as serious cases, were registered (about 0.5 % of the total number of collected reports), among which only one case was considered confirmed (about 0.05 % of the total number of collected reports) according to the WHO system for standardized case causality assessment. In the letter to the Editor of the Clinical Nutrition Journal published in 2020 some concerns about the interpretation of these data were reported, concluding that warning for ALA should be cautious as the high ALA safety profile is reported in large meta-analyses (Salehi et al., 2019. The result of Fogacci et.al study showed that ALA was not associated with an increased risk of any treatment-emergent adverse event ($p > 0.05$). All these evidence show that ALA supplementation is safe in different populations groups such as smokers, pregnant women, children/adolescents, diabetics, heart patients (Fogacci et al., 2020).

In 2021 the European Food Safety Authority (EFSA) Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked by the European Commission to deliver an opinion on the relationship between the intake of ALA and the risk of IAS. In the opinion, EFSA reported data published by Yamada et al. in 2020, indicating that the incidence of IAS in the general Japanese population was 0.017 cases per 100.000 inhabitants in the years 2017-2018, while the incidence in the Caucasian population was lower than that found in the Japanese population, likely due to the lower presence of the Human Leukocyte Antigen HLA-DR4, and in particular the alleles DRB1*04:03

(responsible for most Caucasian cases) ranging from 0.4 to 3.9% in European Countries and from 1.6 to 12.3 in Japan and South Korea. The results from the comprehensive literature search performed by EFSA on the published case reports in the English language yielded 49 cases of IAS linked to ALA intake worldwide. Of these 49 cases, 20 were observed in Europe. 22 cases out of the 49 did not report the symptoms involved, and in 12 cases the symptoms were serious but not lethal. The EFSA NDA panel concluded that "*Based on the limited data available and the low prevalence of IAS in Europe the risk associated with the development of IAS following consumption of ALA cannot be quantified precisely neither for the general population overall nor for sub-groups or individuals with genetic susceptibility*." (Turck et al., 2021).

Definitively, even if analgesic drugs are considered the gold standard for the treatment of pain, the extent and incidence of the adverse effects of these types of drugs, led to understand that ALA taken orally through food supplements could be a feasible option compared to them.

This work present limitations and strengths. First, a follow up was not performed, making it impossible to learn about any longer-term effects of ALA supplementation on pain relief. Moreover, the VAS and NRS methods used to estimate the severity of pain and estimate the extent of pain relief, take into account only the intensity of pain and not the complexity of the pain experience. The third limitation regards the impossibility to assess the therapeutic effect on idiopathic myalgia owing the low number of subjects suffering from this pain.

In contrast, the major strength of this clinical trial is the robustness of the experimental design in the assessment of safety of ALA supplementation, as the effect of ALA on glycemia for a statistically significant number of normoglycemic subjects.

Conclusion

In conclusion, bearing in mind the safety of ALA supplementation in comparison with that of commonly-used analgesic drugs and its efficacy in pain treatment, the use of ALA food supplements, which according to current legislation is not intended to treat or prevent diseases in humans and is addressed towards the general population, could be considered as a feasible option in pain treatment.

The management of idiopathic pain is a real challenge for physicians, as they cannot treat the causes underlying this symptom but can only relieve the pain with the use of symptomatic drugs which generally have a good and rapid efficacy, although in some cases possess limited efficacy and considerable side effects. Thus, in the absence of a diagnosis of pain-causing disease.

In the future, further larger-scale studies will be necessary to consolidate the promising findings of the present study.

ii.Second clinical study

Pharmacokinetics Parameters of R-α-Lipoic Acid in Healthy Volunteers following the Consumption of Liponax sol a Food Supplement in Liquid Dosage Form.

With the aim to improve the bioavailability of food supplements containing ALA, an improved liquid formula, Liponax sol, was formulated. Its biological activity, tolerability and safety were already tested in different clinical studies. In a group of 38 patients with peripheral neuropathy treated for 4 weeks with R-ALA liquid formulation, a significant reduction in the total pain value by the *Italian Neuropathic Pain Scale* (NPS*)* was registered (Maglione et al., 2015). Even if this study is performed on a limited number of patients, it confirms that the liquid solution of R-ALA provides relief from the pain symptoms of peripheral neuropathy. The results obtained may be attributed also to the improved bioavailability of the new formulation. For this purpose, the aim of the study is to confirm the high bioavailability of the liquid formulation Liponax sol, administered in fasting healthy adult at 300 mg dose of R-ALA, evaluating its pharmacokinetics parameters in human.

Materials and methods

R-ALA stock solution (1mg/mL) was prepared in methanol and stored at 4 °C. The calibration curve was obtained in a concentration range of

0.05 – 5.00 µg/mL with six concentration levels and performing triplicate analysis for each level. Naproxen (NA) was selected as internal standard at the concentration of 5 µg/mL. All the solutions were stored at 4 °C until the beginning of the analysis and there was no change in stability after 30 days (data not shown).

UHPLC-MS analyses were performed on an Acquity *I class*, equipped with a QDa single quadrupole mass detector (Waters, Milford (MA), U.S) system. The separation was performed on a Kinetex® Biphenyl 100 Å column with geometry (L × I.D) 10 cm × 2.1 mm, 2.6 µm (Phenomenex®, Bologna, Italy) employing as mobile phases: A) 0.1% CH_3COOH in H_2O and B) ACN, with the following gradient: 0 min, 45% B, isocratic for 2.50 min, 2.51 min, 99 % B, isocratic for 1 min. Returning to 45 % in 1.50 min. The flow rate was set to 0.4 mL/min. Column oven was set to 40 °C, 5 µL of extract were injected.

The ESI was operated in negative mode. Source temperature was 600°C, Probe voltage -3.5 kV. Nitrogen was used as nebulizer gas (10 L/min). MS analysis were conducted in selected ion recording (SIR) mode, employing 205.0 m/z for R-LA and 229.0 m/z for NA.

Pharmacokinetic parameters

The pharmacokinetics parameters calculated for each subject included: maximum plasma concentration (Cmax) of R-ALA after oral administration of Liponax sol (10 mL), time to reach maximum concentration (Tmax), area under the concentration–time curve (AUC)

equivalent to the total amount of R-ALA that reaches the systemic circulation unmodified, and elimination half-life ($t_{1/2}$).

Clinical trial

This study was submitted to the Ethics Committee of A.S.L. Napoli 1 Centro. The study was approved by the Ethics Committee (protocol number 122) and carried out in accordance with the current Helsinki declaration.

Once a positive opinion was obtained from the relevant Ethics Committee and the authorising deliberation was published, a monocentric, randomised double-blind placebo-controlled clinical trial was performed by COMEGEN - Società Cooperativa Sociale (Naples, Italy) to evaluate pharmacokinetic parameters (Tmax, Cmax, AUC and $T_{1/2}$) on a healthy adult population. Blood samples were collected after the consumption of an oral dose of a food supplement (Liponax sol) containing 300 mg of R-LA after an overnight fast of 12 h. The subjects received oral and written information concerning the study before they gave their written consent.

Study population

Subjects of both sexes, aged 18-65 years, were considered eligible for enrolment if they are in healthy status with a BMI < 25 kg/m^2. Pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding, subjects with R-ALA allergy,

smokers, subjects exposed to a high risk of cardiovascular events, subjects suffering from endocrine disorder (e.g. hypothyroidism, Cushing's syndrome, polycystic ovary syndrome or PCOS), subjects taking corticosteroids, subjects who have taken food supplements in the two weeks before recruiting, subjects suffering from liver disease, and not self-sufficient were excluded from the study.

Blood samples (2.5 mL) for the determination of R-ALA plasma concentration were collected at the time of 0, 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, and 180 min after oral administration as reported in Figure 3.

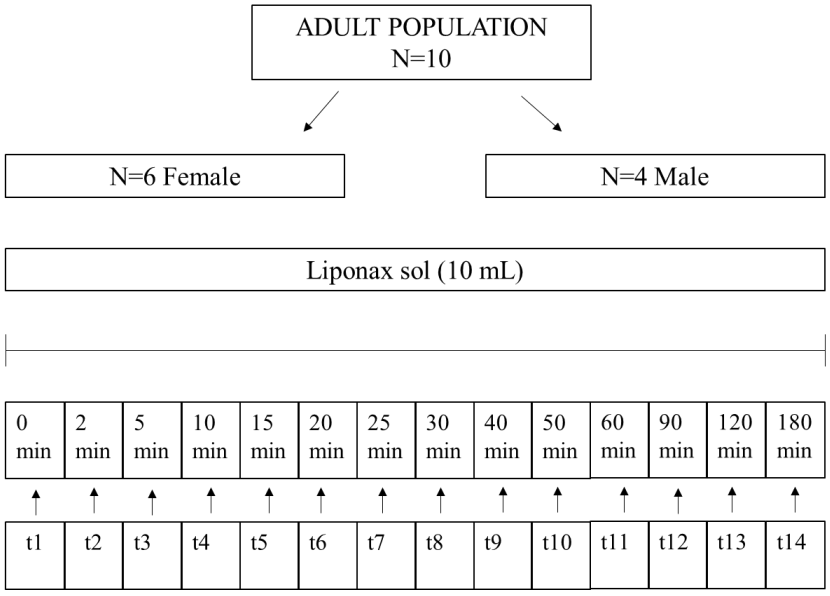


Figure 3. Study design.

Results

An ultra-high performance liquid chromatographic coupled with mass spectrometer (UHPLC-MS) method useful to determine the concentration of R-ALA in human plasma after the oral consumption of Liponax sol in healthy subjects, was developed as the first aim of the study. Figure 4 shows Single Ion Recording (SIR) chromatograms of R-ALA and Naproxen obtained from the analysis of the standard compound in methanol (A), spiked plasma sample (B) and plasma from a healthy subject who consumed Liponax sol (C). R-ALA and NA peaks resulted to be well separated under the used experimental conditions. R-ALA and NA were identified using the standard compound retention times (1.12 and 1.33 min, respectively) and *m/z* values obtained from parent ions (for R-LA *m/z* $[M-H]^-$ = 205.0, for NA *m/z* $[M-H]^-$ = 229.0).

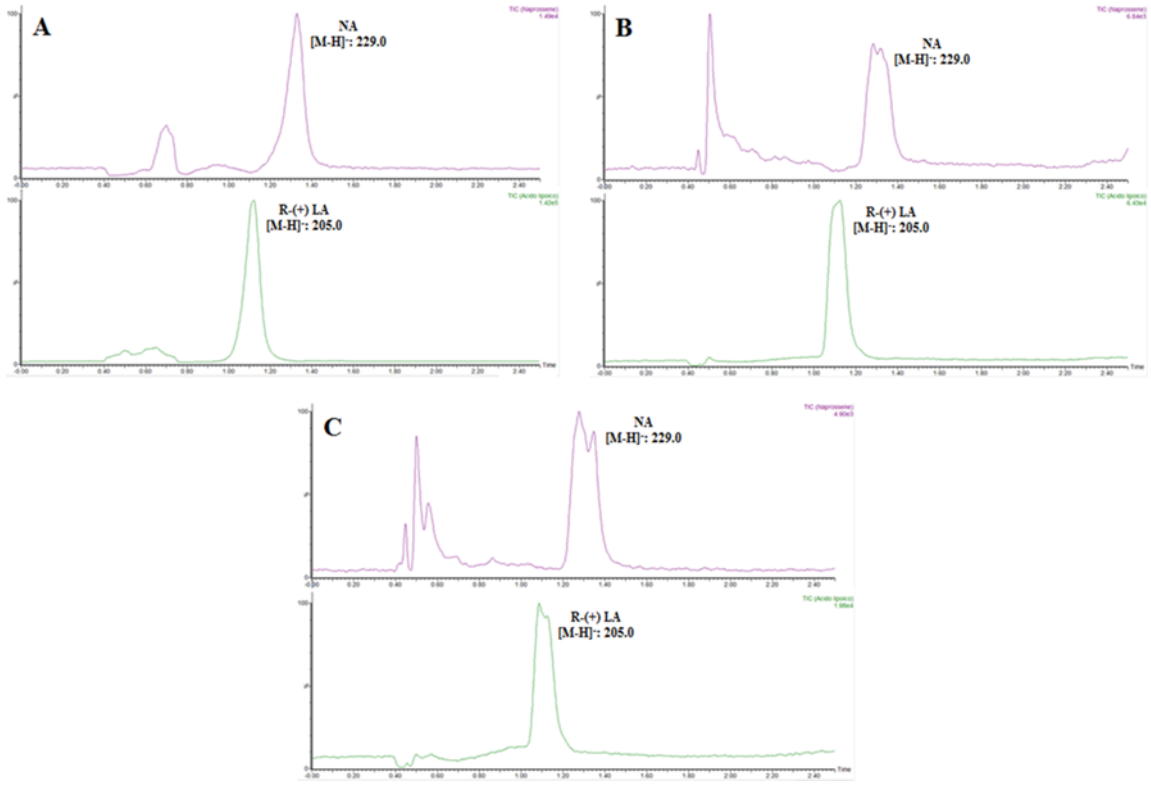


Figure 4. Single Ion Recording (SIR) chromatograms of R-(+) ALA and NA in methanol (A), spiked plasma (B) and plasma from a healthy subject who consumed Liponax sol.

To determine R-LA plasma concentration, a calibration curve was obtained in a concentration range of 0.05 – 5 µg/mL with six concentration levels and performing triplicate analysis for each level. Naproxen was selected as internal standard in the concentration of 5 µg/mL. Linear regression analysis showed a high correlation coefficient (R^2 of 0.9997; y = 0.1257x - 0.0033), proving a good linearity of the method in the selected concentration range.

The values obtained from intra- and inter-day precision and accuracy showed good repeatability in terms of retention time and concentration, as reported in Table 5. Intra-day RSD % value relative to R-ALA spiked in human plasma, ranges from 1.174 to 1.880 and inter-day RSD % value ranges from 1.694 to 3.914, respectively (Table 6).

The R-ALA absolutely recovery from plasma was 120.00 %, 105.21 % and 102.23 % at the concentrations of 0.5, 1.0 and 5.0 µg/mL, respectively. The matrix effect calculated, as reported in materials and method, resulted to be on average 21%.

The limits of detection (LOD) and the limit of quantification (LOQ) were 0.012 and 0.038 µg/mL, respectively.

Compound		Intra-day Rt (min) (*n*=5)	Inter-day Rt(min) (3 days; n=5)
R-(+) Lipoic acid	Mean ± S.D	1.124 ± 0.001	1.29 ± 0.005
	R.S.D. %	0.01 %	0.487 %
Naproxen	Mean ± S.D	1.331 ± 0.005	1.333 ± 0.006
	R.S.D. %	0.263 %	0.487 %

Table 5.— Intra-and inter-day retention time repeatability of ALA and NA.

Sample Concentration (µg/mL)		Intra-day µg/mL (*n*=5)	Inter-day µg/mL (3 days; n=5)	Recovery %	Matrix effect %
0.50	Mean ± S.D	0.608 ± 0.01	0.590 ± 0.01	120.00 %	11.66 %
	R.S.D. %	1.236 %	1.694 %		
1	Mean ± S.D	1.419 ± 0.03	1.388 ± 0.03	105.21 %	8.09 %
	R.S.D. %	1.880 %	2.302 %		
5	Mean ± S.D	5.168 ± 0.06	5.379 ± 0.21	102.23 %	43.11 %
	R.S.D. %	1.174 %	3.914 %		

Table 6.— Intra- and inter-day precision and accuracy, recovery and matrix effect of ALA spiked in human plasma.

Pharmacokinetic study

The 10 healthy subjects (6 female and 4 male) involved in the pharmacokinetic study, were recruited one a day for 10 days in fasted state. Then blood samples were collected at the time of 0, 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90 and 180 min after the oral administration of

the food supplement (10 ml of Liponax sol) in an outpatient setting. The mean plasma concentration versus time profile of R-ALA following a single 300 mg oral dose in 10 subjects were shown in Figure 3. As reported in the Table 7, R-ALA is rapidly absorbed as highlighted by the Tmax value of 11 min. Moreover, R-ALA reaches high plasma concentrations with a mean Cmax of 8 µ/mL. R-ALA distribution in various tissues and its clearance confirm the plasma pharmacokinetic profile shown in Figure 5.

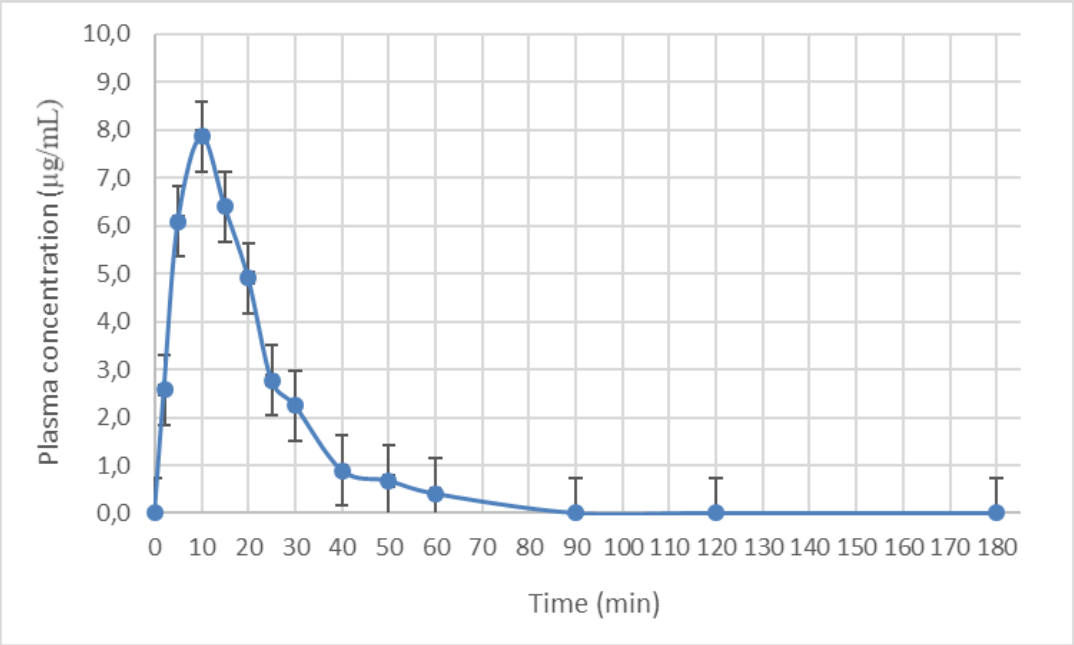


Figure 3. Plasma concentration-time profile of ALA in healthy subjects

	Cmax (µg/mL)	Tmax (min)	$T_{1/2}$ (min)	AUC_{0-t} (min x µg/mL)
Mean	8 ± 4.7	11 ± 4	6.1	178.778± 106.432
S.D.	4.7	4	3	106.432

Table 7.— Pharmacokinetic parameters of R-ALA in 10 healthy volunteers after the consumption of Liponax sol (300 mg of R-ALA).

Discussion

ALA, as a strong antioxidant compound, can exert its activity in a wide range of oxidative stress-related clinical conditions (e.g. diabetic complications, mechanical compression neuropathies, neurodegenerative and cardiovascular pathologies, physical and mental impairment, obesity, etc) (Packer et al., 1995; Packer et al., 2001; Maglione et al., 2015; Kim et al., 2004; Carbonelli et al., 2010; Anderson et al., 2011; Brufani et al., 2014). Despite its beneficial properties, standard solid LA formulations for oral use have pharmacokinetic limitations such as high Tmax and low bioavailability (Table 8).

Reference	ALA dose	AUC (min*µg/mL)	Cmax (µg/mL)
Teichert et al. (1998)	200 mg racemic ALA tablets	46.82 ± 21.46	0.66 ± 0.33
	600 mg racemic ALA tablets	157.83 ± 35.82	2.85 ± 1.49

	200 mg racemic ALA intravenous	157.97 ± 35.05	8.32 ± 2.35
Breithaupt-Grogler et al. (1999)	600 mg racemic ALA tablets	R: 140.8824	R: 1.81232
		S: 74.5944	S: 0.9782
Mignini et al. (2007)	Thioctacid 600 mg racemic ALA tablets	210.654 ± 65.316	1.3386 ± 0.7518
	Tiocil N 600 mg racemic ALA tablets	213.81 ± 82.446	1.2158 ± 0.5605
Amenta et al. (2008)	Thioctacid 600 mg racemic ALA tablets	196.254 ± 22.368	1.2662 ± 0.2377
	Tiocronal 600 mg racemic ALA tablets	175.512 ± 26.904	2.2905 ± 0.2866
	Biodynoral 600 mg racemic ALA tablets	188.538 ± 19.89	2.2628 ± 0.3928
	Tiobec retard 600 mg racemic ALA tablets	191.244 ± 9.168	1.3977 ± 0.0704
Mignini et al. 2011	ALA600 600 mg racemic ALA, vitamins of the B complex	339 ± 47.4	6.86 ± 1.29
Hermann et al. (2014)	Thioctacid HR 600 mg racemic ALA tablets	R: 145.308	R: 2.8225
		S: 69.384	S: 1.4059
	Thioctacid 600 mg racemic liquid for intravenous somminatrion, taken orally	R: 226.062	R: 7.8438
		S: 102.834	S: 3.7859

Table 8.— Pharmacokinetic parameters of α-lipoic acid different formulation. Area under the concentration–time curve (AUC) values are converted and reported in min x µg/mL. Maximum plasmatic concentration (Cmax) values are converted and reported in µg/mL.

The present study evaluated the pharmacokinetic profile of the patented oral liquid formulation containing 300 mg of the active enantiomer R-ALA, (Liponax sol), in healthy humans. The results obtained, in line with pharmacokinetic data obtained with a liquid formulation of R-

ALA in Sprague-Dawley rats (Brufani et al., 2014) and in line from the study on LA saline solution for injection (Hermann et al., 1996), confirm both the efficacy and the improved PK parameters of the Liponax sol (R-ALA liquid formula). Similar results, about PK results in humans, are obtained by Carlson et al. using freshly dissolved R-ALA Na salt in water that is not suitable as commercial formula due to taste and stability issue (Carlson et al., 2008). It maybe is due to the fact that at low concentrations, ALA is actively transported by intestinal protein carriers, such as the monocarboxylate transporter usually involved for medium-chain fatty acids absorption (Maglione et al., 2015). Instead, at high concentration the passive diffusion mechanism is favored for absorption (Takaishi et al., 2007). The innovative and patented formulation contained in Liponax sol is able to overcome this difficulty because is completely dissolved in solution, stable over the time and in the gastric environment (Brufani et al., 2013). The immediate availability of R-ALA in Liponax sol liquid formulation allows the transient saturation of the first-pass metabolism in the liver showing an high Cmax, unlike the traditional tablets which must first disaggregate, dissolve and then be metabolized. (Hermann et al., 1996). Additionally, the new formulation stabilizes and makes readily available the only active enantiomer R-ALA, without any possible impact on PK value by the S-ALA (Brufani et al., 2014; Keith et al. 2012). In the Table IV are summarized the pharmacokinetic parameters of several studies with healthy human volunteers. Many of

them studied the pharmacokinetic of tablets containing 600 mg of racemic mixture (R-S), whereas the liquid formula tested here contained only 300 mg of the form R-ALA. Mignini's et al. study demonstrated that a tablet consisting of 600 mg racemic of ALA and B complex vitamins, manufactured with a patented technology, containing surfactants, superdisintegrating, maltodextrins and lecithin, showed better results than traditional ALA tablets but a Cmax lower than that found for Liponax sol.

Therefore, the comparison with previous studies on ALA show that Liponax sol have a good pharmacokinetic parameter, the new technologies aimed to increase the absorption of ALA are important to obtain better pharmacokinetic parameters and consequently to improve the clinical efficacy, and also that Liponax sol as a strong antioxidant can be used for numerous diseases related to oxidative stress.

Conclusions

Our results demonstrated that the liquid formula for oral use containing 300 mg of R-ALA tested in human healthy voluntaries, has better pharmacokinetic parameters with a rapid absorption, high bioavailability, and optimized half-life. Instead, data on pharmacokinetic parameters of traditional ALA tablets shows that, in our experimental conditions, this liquid oral formulation demonstrated to overcome the limitations ascribed to oral lipoic acid

supplementations. For this purpose, Liponax sol could be a great alternative to tablets with better patient. Anyway, further clinical trials are needed to show the beneficial effects for the consumption of the liquid R-ALA formulation.

3.2 Epilobium angustifolium L.

The work described in this chapter was also previously published in the article entitled "*Epilobium angustifolium* L. *extract with high content in oenothein B on benign prostatic hyperplasia: a monocentric, randomized, double-blind, placebo-controlled clinical trial*" by Cristina Esposito, Cristina Santarcangelo, Raffaello Masselli, Giuseppe Buonomo, Giovanna Nicotra, Violetta Insolia, Maria D'Avino, Giuseppe Caruso, Antonio Riccardo Buonomo, Roberto Sacchi, Eduardo Sommella, Pietro Campiglia, Gian Carlo Tenore, Maria Daglia in Biomedicine & Pharmacotherapy 138 (2021) 111414.

The *Epilobium* genus belongs to the *Onagracee* family and includes over 200 species spread across temperate and cold regions (Raven, et al.,1976), of these *E. angustifolium* L. (erect stem herbaceous plant), is used for wounds and skin diseases in traditional medicine (Soukand et al., 2020). In Europe, preparations based on the aerial parts of *E. angustifolium* are used in the treatment of prostatic disorders. Recent research suggests that *E. angustifolium* yields positive effects on the inflammation of urethra and prostate, as well as micturition problems (Committee on Herbal Medicinal Products, 2015). In the monograph on *E. augustifolium* published by the European Medicinal Agency (EMA), many European Union countries were found to have used it for over 30 years, thus meeting the requirements for "traditional use" with the following indications: "*Relief of lower urinary tract symptoms related*

to benign prostatic hyperplasia, after serious conditions have been excluded by a medical doctor" (Ducrey et al., 1997).

The main classes of polyphenols occurring in *E. angustifolium* are phenolic acids, flavonoids and ellagitannins (Close et al., 2012), and the beneficial effects of *E. angustifolium* have been ascribed to these (Ramstead et al., 2015). The main ellagitannin in *E. angustifolium* is oenothein B, which has shown *in vitro* and *in vivo* biological activities such as antioxidant, anti-inflammatory, inhibitory enzyme activity, antitumor, antimicrobial, and immunomodulatory activities (Deng et al., 20019). In particular, the ellagitannin oenothein B exert beneficial effects on prostate health. In 2004, Kiss et al. demonstrated the activity of *E. angustifolium* in the inhibition of metalloproteinases which is correlatedwith the development of BPH (Kiss et al., 2004, 2006). In 2013, the results of a randomized, double-blind, placebo-controlled, phase II trial were published, showing the efficacy and safety of a traditional herbal medicinal product based on *E. angustifolium*, *S. lycopersicum*, *P. africanum* and *S. repens*, on BPH symptoms (by means of *International Prostate Specific Score,* IPSS) and reduction of night-time urinary frequency (Coulson et al., 2013).

i.Clinical study

Epilobium angustifolium L. extract with high content in oenothein B on benign prostatic hyperplasia: a monocentric, randomized, double-blind, placebo-controlled clinical trial

The main pharmacological treatments for BPH are α-blockers and 5-α-reductase inhibitors. The former act as 1-α-adrenergic receptor antagonists, which relax bladder neck muscles and prostate muscle fibres making urination easier. The latter target 5-α-reductase, increasing DHT affinity for androgen receptors. The clinical use of these drugs in the treatment of BPH may put patients at high risk of adverse events including erectile dysfunction, orthostatic hypotension, dizziness and tachycardia (Deng et al., 2919). Due to the adverse effects of drug therapies currently used in BPH, which particularly affect the subject's quality of life, the aim of this monocentric, randomized, double-blind, placebo-controlled clinical trial is to evaluate if a daily intake of a food supplement based on *E. angustifolium* extracts (EAE) for a period of 6 months may allow a significant improvement in symptoms and urinary flow in subjects with BPH.

Material and methods

Food supplement based on E. angustifolium extract and placebo

The food supplement and placebo consisted of hard, gastric-resistant capsules containing EAE (500 mg) and magnesium stearate (5 mg) as a sliding agent, and microcrystalline cellulose (350 mg) and magnesium stearate (5 mg), respectively. EAE, standardized to contain ≥ 15 % oenothein B, is a commercial food supplement ingredient (ENOTprost®) produced by EPO S.r.l. (Milan, Italy) in according to the manufacturer, EAE complies with European specifications for contaminants and microbiological limits. Both EAE food supplement and placebo capsules, produced by Sorgente del Benessere S.r.l. (Fiuggi, Italy), were made unrecognizable in appearance, color, and flavor, and these were packaged in white containers of 60 tablets each. Regarding capsules wight, it was managed by means of Metrostat statistical software, in agreement with Italian law (Legge 25 ottobre 1978 n. 690) and standard UNI ISO 2859.

Clinical trial design

This clinical study was approved by the Committee (protocol number 10534 of 24/01/2020) and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000). Moreover, this study is listed on the ISRCTN registry (www.isrctn.com) with ID ISRCTN18705154 (doi.org/10.1186/ISRCTN18705154).

Once a positive opinion was obtained from the relevant Ethics Committee and the authorising deliberation was published, a monocentric, randomised double-blind placebo-controlled clinical trial was performed by Samnium Medical Cooperative (Benevento, Italy) to

evaluate the effects of the EAE food supplement on an adult male population (mean age ± SD, treated group: 67 ± 10, placebo group: 64 ± 10), suffering from BPH diagnosed through prostate ultrasound, blood tests and IPSS score.

The participants received oral and written information concerning the study before they gave their written consent.

The study was double-blind, both for the investigating physician and for the enrolled subjects. At the end of the baseline visits, the randomization sequence was generated using STATA 16 software (Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC) and the randomization list was kept hidden. The 128 male adults enrolled in the clinical study, were divided into two groups (70 in the treated group and 58 in the placebo group) by simple randomisation (about 1:1 allocation ratio). In specific, group 1 consisted of subjects assuming the daily dose of EAE food supplement, or placebo (group 2). The EAE food supplement group was provided one hard gastro-resistant capsule per day for 6 months; it contained 500 mg of EAE, corresponding to 2 g of aerial parts of *E. angustifolium,* according to the indications of the Assessment Report on *E. angustifolium* L. and/or *Epilobium parviflorum* Schreb., herba. The placebo group received one hard gastro-resistant capsule per day containing 350 mg of microcrystalline cellulose for six months. Hard gastric-resistant capsules containing EAE or microcrystalline cellulose were made

unrecognizable by identical colour, shape and taste. Both treatments were prepacked in white containers of 60 tablets.

The clinical trial duration was 7 months. Participants underwent four visits (baseline = t0, after 15 days = t1, after 2 months = t2, and after 6 months = t3) in an outpatient setting. For the baseline visit (t0) information on the sociodemographic, clinical and symptomatologic characteristics of the subjects were collected and reported in the case report form (CRF). In particular, bladder post-void residual volume (PVR) and prostate volume (PV) were obtained by prostate ultrasound; prostate-specific antigen (PSA), and neutrofile/lymphocyte ratio (N/L) were derived from blood tests analysed by Unisannio Lab (San Giorgio del Sannio, BN, Italy); number of urinations during the night before the clinical visits, and IPSS score were registered by the physicians.

Clinical visits were carried out at t1 (after 15 days of treatment) to monitor the possibility of kidney and liver toxicity, t2 (after 2 months of treatment), and t3 (after 6 months of treatment). After each clinical visit, all data were compiled in the CRF by the physicians.

In particular, the specific analyses carried out are shown in Table 9.

Clinical visits	Analyses
t0 (baseline)	PV, PVR, PSA, N/L, CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GFR
t1 (15 days)	CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GRF
t2 (2 months)	PSA, N/R, CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GFR
t3 (6 months)	PV, PVR, PSA, N/R, CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GFR

Table 9.—Analyses planned at t0, t1, t2, and t3. Prostate volume (PV), post-void residual volume (PVR), prostate-specific antigen (PSA), neutrofile/lymphocyte ratio (N/L), creatinine (CRE), bilirubin (BR direct/indirect/total), prothrombin, aspartate transaminase (AST), alanine transaminase (ALT), cholinesterase (CHE).

Outcome of the study

The primary endpoint was to investigate the efficacy of a 6 month-daily dose of EAE food supplement to reduce PVR and PV in subjects with BPH, assessed at baseline (t0) and after 6 months (t3). A BPH diagnosis was made by the physician based on prostatic ultrasound, PSA assay and IPSS score.

Study population

The subjects were recruited following these inclusion criteria: no clinically significant deviation in laboratory tests; history of BPH for at least one year, IPSS score ≤ 25, prostate volume ranging from 25 cc to 200 cc, no medication intake for BPH prior to baseline assessment and during the study, PVR ≤300 ± 2 mL and serum total PSA lower than 4 ng/ml. Subjects with the following criteria were excluded from the study: acute or chronic disease that could interfere with the study or endanger the subject; use of any of the following concomitant drugs: immunosuppressants, anticoagulants, α-blockers, 5α-reductase inhibitors, antipsychotics, chemotherapy drugs, drugs for dementia, male hormone replacement therapy and drugs for overactive bladder,

atonic and/or neurogenic bladder; bladder neck contracture; acute prostatitis; bladder calculosis; urinary tract infection more than once in the last 12 months; prostate or bladder cancer; history of pelvic trauma or surgery; clinically significant kidney or hepatic insufficiency; microscopic hematuria that was not evaluated by a urologist and not attributed to BPH; any condition that might interfere with the subject's ability to give informed consent, to comply with study instructions, to provide an objective evaluation of his or her symptoms, or that might confuse the interpretation of study results; those considered unsuitable for participation by the physician.

Statistical analysis

Sample size calculation was conducted using three 1-β power values equal to 0.95 and a significance level $\alpha = 0.05$. The sample size was determined to be 130 participants, allowing for a 15 % drop out rate.

The effect of the treatment with EAE food supplements on the response variables for the primary and secondary outcomes of the study (i.e., PV, PVR, urinations during night, and IPCC score) was assessed by generalized linear mixed models (GLMM) including treatment (EAE food supplement vs placebo), measure (t0 vs t3), and age of the subject (standardized) as explanatory variables. We also added the treatment × measure interaction to account for differential effects of the treatment between t0 and t3. The subjects entered the model at a random intercept to account for unexplained variation at the individual level (σ^2_{ind}) after

we controlled for the explanatory variables. We ran an independent model for each response variable. The PV (after log transformation) and IPSS were normally distributed and consequently the distribution error of the two corresponding GLMMs was set as Gaussian. By contrast, both PVR and nightly urinations followed a Poissonian distribution, and consequently the distribution error of the two corresponding GLMMs was set as Poisson.

Biochemical variables were analyzed using a GLMM with the same predictors as for the four main response variables. All variables were normally distributed or achieved normality after log transformation (i.e., PSA, N/L, AST, ALT, and BR total). Analyses were performed using the lme4 (Bates et al. 2015) and MuMIn (Barton 2016) packages in R ver. 3.2.4 (R core Team 2016), and unless otherwise stated, data are reported as means ± standard error.

Results

Clinical trial

The "*study flow chart*", in accordance with the *CONSORT PRO reporting guidelines* (Calvert et al., 2013) is shown in figure 6.

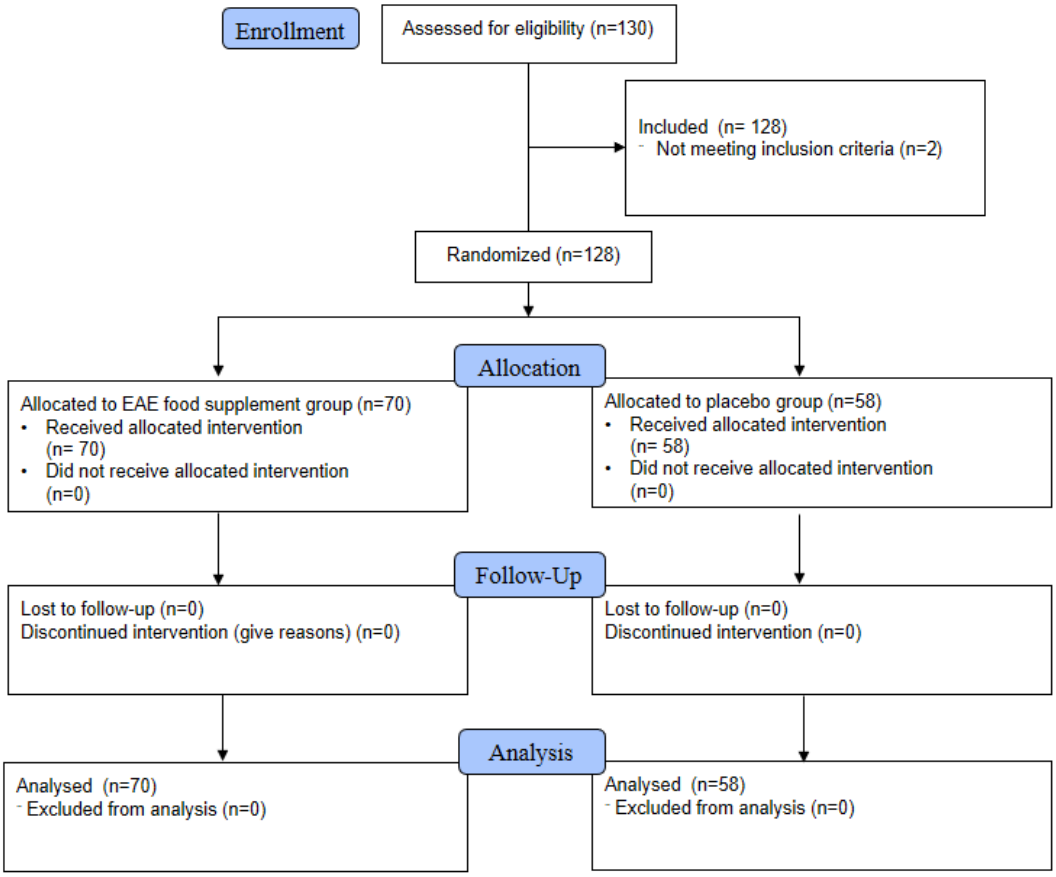


Figure 6. CONSORT flow diagram.

The baseline characteristics of the subjects for each group are summarized in Table 10.

Characteristic/ Clinical data	**Treated (n=70)**	**Untreated (n=58)**
Age	67 ± 10	64 ± 10
Ethnic origin:		
All Europeans	70	58
PV (cc)	45.2 ± 2.4	44.1 ± 3.1
PVR (ml)	40.4 ± 7.3	28.0 ± 5.4
Urinations during night	1.2 ± 0.1	1.1 ± 0.1
IPSS	13.4 ± 0.7	13.0 ± 0.8
PSA (ng/ml)	1.7 ± 0.2	2.6 ± 0.8

N/L (%)	2.2 ± 0.1	1.9 ± 0.1
AST (U/L)	22.1 ± 0.7	22.1 ± 0.9
ALT (U/L)	21.7 ± 1.0	21.6 ± 1.3
BR (direct/indirect) (mg/dl)	0.24 ± 0.03	0.35 ± 0.05
BR total (mg/dl)	0.88 ± 0.05	0.84 ± 0.05
Protrombine (Inr)	1.01 ± 0.01	1.01 ± 0.01
CHE (U/L)	7983 ± 175	8150 ± 163
GFR (ml/min/1,73mq)	62.3 ± 1.4	69.8 ± 2.2
CRE (mg/dl)	1.19 ± 0.02	1.10 ± 0.03

Table 10. — Characteristics of the study population: demographic and clinical data at baseline.

Between the beginning (t0) and the end (t3) of the clinical trial, the three response variables (IPSS, PVR, and the number of night urinations) changed between the EAE food supplement group and the placebo group (Table 11). The treatment x measure interaction was statistically significant for IPSS, PVR, and the number of urinations throughout the night, but not for PV, according to GLMM analysis (Table 12). PV values did not differ significantly between t0 and t3 or between the EAE food supplement and placebo groups (Table 12, Fig. 7A), but were dependent on the age of the subjects. In fact, as expected, older individual in both groups had higher PV ($\beta=0.16 \pm 0.03$, $t_{125}=4.611$, $P<0.001$, Fig. 7B).

Instead, IPSS score decreased significantly between t0 and t3 in the EAE food supplement group ($\beta=-1.9 \pm 0.2$, $t_{126}=7.89$ $P<0.001$ Fig. 7C), while it slightly increased in the placebo group ($\beta=+0.6 \pm 0.2$, $t_{126}=2.36$ $P=0.02$, Fig. 7C). Similarly to PV, the IPSS score significantly

increased with increasing subjects' age, irrespective of the treatment (β=1.43 ± 0.49, t_{125}=2.888, P=0.005, Fig. 7D).

The Poisson GLMM for the PVR value showed that the number of subjects with a low residual urine volume in the bladder increased significantly in the EAE food supplement group, whereas the proportion of subjects with residual urine volumes greater than 100 µl decreased (β=-0.17 ± 0.03, Z=5.792, P<0.001, Fig. 8). The opposite pattern was instead observed in the placebo group (β=0.12 ± 0.03, Z=3.419, P<0.001, Fig. 8).

The number of urinations during the night (Fig. 9) showed a similar pattern: in the EAE food supplement group, the frequency of individuals without urination increased while the number of subjects urinating more than once per night decreased (=-0.41 0.17, Z=2.408, P=0.016). In the placebo group, there was no significant difference between t0 and t3. The frequency of urination increased significantly with the subject's age, regardless of the group (=0.19 0.07, Z=2.561, P=0.010). The number of participants not urinating overnight in the treatment group increased by 21.7%, while it decreased by 10.2% in the placebo group. Means, standard errors and ranges for each biochemical variable of EAE food supplement and placebo groups are reported in Table 13. The GLMM analyses did not find any significant effects of the experimental treatment on any of the biochemical variables (statistics not shown), with the exception of N/L. In detail, the N/L was significantly higher in the placebo group in comparison with the EAE

food supplement group ($F_{1,125}$=5.893, P=0.017), but did not change between t0 and t3 ($F_{1,126}$=1.191, P=0.27).

All participants were included in the analysis of the groups to which they were originally assigned at the end of the treatment (intention-to-treat analysis). During the 6 months of treatment, no individuals reported any adverse effects (AEs) due to the oral intake of *E. angustifolium* food supplement. Furthermore, the results in Table 13 reveal that *E. angustifolium* food supplement has neither hepatic or renal toxicity. The principal investigator concluded that the treatment with *E. angustifolium* capsules was well tolerated in this regard.

Response variable	Placebo group (n=58)		EAE food supplement group (n=70)	
	t0	t3	t0	t3
PV	44.1 ± 3.1 (17-143)	50.4 ± 4.6 (15-200)	45.2 ± 2.4 (13-100)	47.2 ± 2.8 (10-118)
IPSS	13.0 ± 0.8 (2-25)	13.6 ± 0.7 (2-25)	13.4 ± 0.7 (3-25)	11.5 ± 0.6 (1-25)
PVR	28 ± 5.4 (0-200)	31.4 ± 5.2 (0-210)	40.4 ± 7.3 (0-296)	39.5 ± 8.2 (0-360)
Urinations during the night before clinical visits	1.1 ± 0.1 (0-4)	1.3 ± 0.1 (0-4)	1.2 ± 0.1 (0-5)	0.8 ± 0.1 (0-3)

Table 11.— Descriptive statistics (mean ± SE, range) for the four response variables measured at t0 and t3 in the two experimental groups

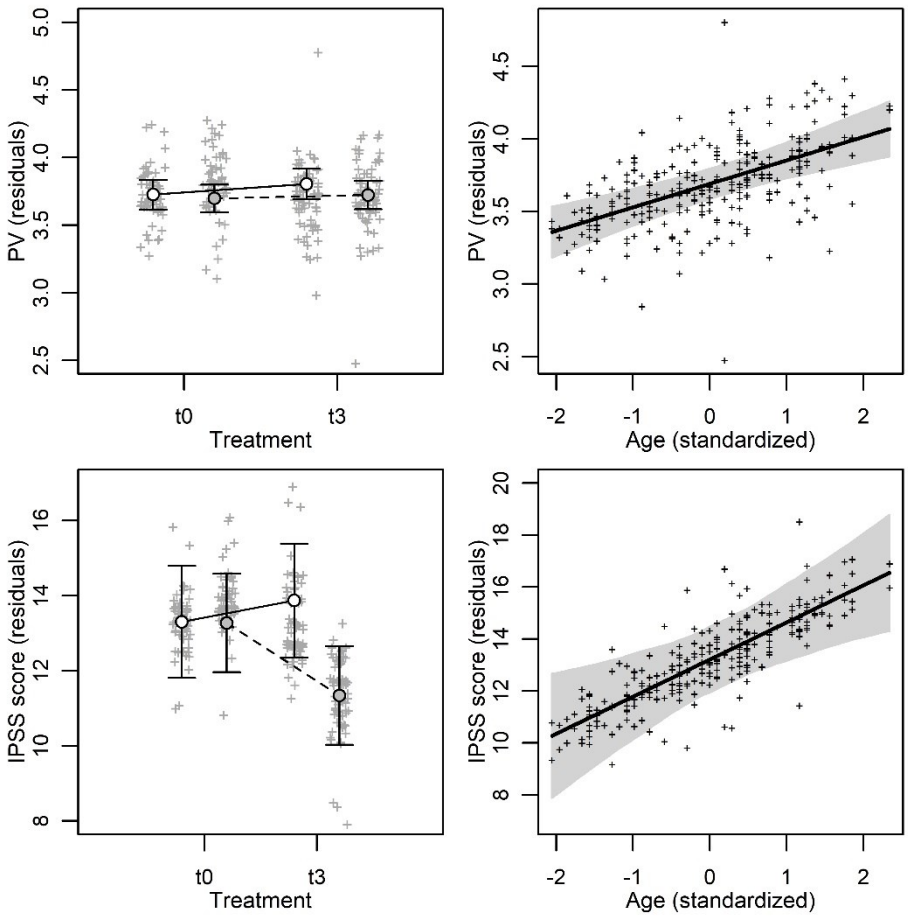


Figure 7. Effects of the treatment (white circles: placebo group, gray circles: EAE food supplement group) and age of subjects on the PV and IPSS score predicted by GLMMs. Bars and grey areas are for 95% confidence intervals of the estimate coefficients.

Effect	F*/χ^{2}**	Df	P
PV*			
Treatment	0.612	1,125	0.43
Measure	1.921	1,126	0.17
Age	21.26	1,125	<0.001
Treat. × Meas.***	0.482	1,126	0.49
IPSS*			
Treatment	1.666	1,125	0.20
Measure	17.39	1,126	<0.001

Age	8.341	1,125	0.004
Treat. × Meas.	58.67	1,126	<0.001
VR**			
Treatment	0.211	1	0.64
Measure	4.399	1	0.036
Age	1.820	1	0.18
Treat. × Mis.	40.84	1	<0.001
Number of Urinations**			
Treatment	2.253	1	0.13
Measure	0.855	1	0.35
Age	6.559	1	0.010
Treat. × Meas.	6.174	1	0.013

Table 12. — Fixed effects of the GLMMs used for the four response variables. *Models with Gaussian error distribution, ** models with Poissonian error distribution.***Treatment x Measures; *** Poissonian distribution

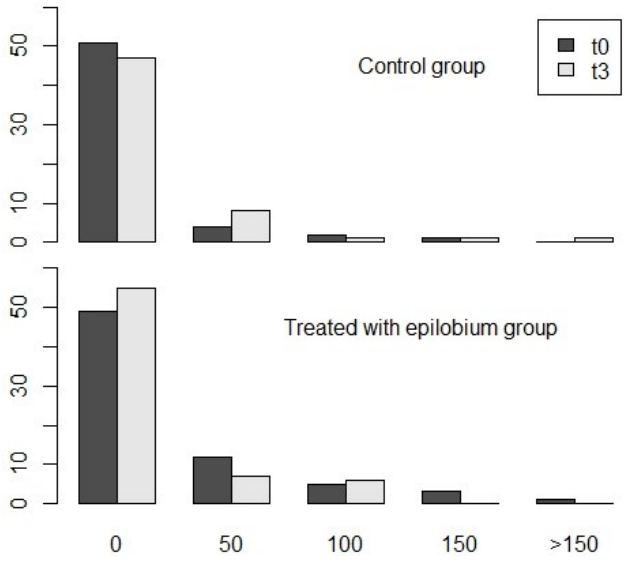


Figure 8. Frequency distributions of the PVR in the two experimental groups at t0 and t3 as predicted by the GLMM.

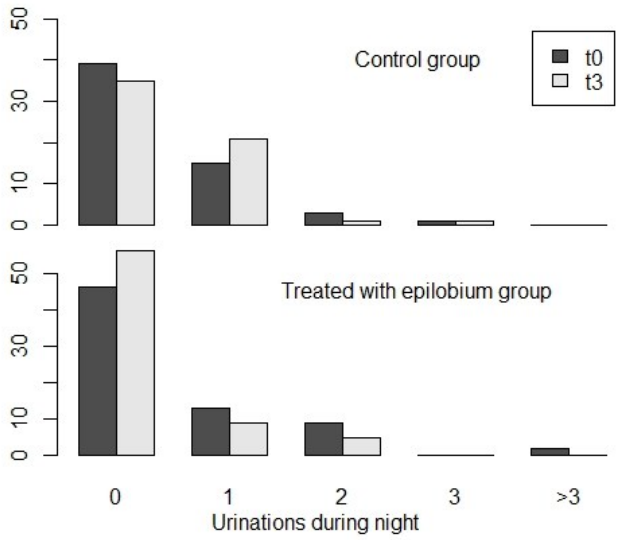


Figure 9. Frequency distributions of urinations during night in the two experimental groups measured at t0 and t3 as predicted by GLMM.

	Placebo group (n=58)		EAE food supplement group (n=70)	
	t0	t3	t0	t3
PSA	2.6 ± 0.8 (0.3-35.6)	3.2 ± 1.3 (0.2-53.3)	1.7 ± 0.2 (0.1-9.3)	1.9 ± 0.2 (0.1-11.6)
N/L	1.9 ± 0.1 (0.8-3.5)	2.0 ± 0.1 (0.9-3.5)	2.2 ± 0.1 (0.9-5)	2.3 ± 0.1 (1-5.7)
AST	22.1 ± 0.9 (10-49)	21.2 ± 0.7 (10-38)	22.1 ± 0.7 (13-40)	20.8 ± 0.7 (11-44)
ALT	21.6 ± 1.3 (8-50)	23.4 ± 1.4 (2-53)	21.7 ± 1 (9-52)	24.4 ± 1.3 (6-56)
BR (direct/indirect)	0.35 ± 0.05 (0.14-2.45)	0.3 ± 0.04 (0.09-1.33)	0.24 ± 0.03 (0.09-1.92)	0.21 ± 0.01 (0.12-0.33)
BR total	0.84 ± 0.05 (0.14-1.76)	0.80 ± 0.04 (0.37-1.8)	0.88 ± 0.05 (0.3-3.04)	0.84 ± 0.04 (0.33-1.93)
Protrombine	1.01 ± 0.01 (0.80-1.51)	1.02 ± 0.01 (0.80-1.41)	1.01 ± 0.01 (0.85-1.44)	1.04 ± 0.02 (0.85-1.69)
CHE	8150 ± 163 (4625-10921)	9058 ± 179 (5092-12434)	7983 ± 175 (4335-11765)	8928 ± 193 (4646-13030)
GFR	69.8 ± 2.2	69 ± 1.7	62.3 ± 1.4	68.1 ± 1.4

	(42-124.1)	(43-97)	(35-100)	(42-102)
CRE	1.10 ± 0.03 (0.49-1.60)	1.11 ± 0.02 (0.8-1.55)	1.19 ± 0.02 (0.79-1.86)	1.11 ± 0.02 (0.78-1.61)

Table 13. — Descriptive statistics (mean ± SE, range) for the four response variables measured at t0 and t3 in the two experimental groups.

Discussion

BPH is a condition mainly characterized by a proliferation of both stromal and epithelial cells of the prostate with an alteration of periurethral area responsible for symptoms that can strongly affect the quality of life (Paweł Miotła et al., 2017). In fact, the symptoms of BPH are often very mild at first, but they become more serious if PBH is not treated. In the literature, data on the efficacy of *E. angustifolium* extract alone against BPH are limited. With this purpose, a monocentric, double-blind, placebo controlled clinical trial was conducted to demonstrate the effects of *E. angustifolium*, with a high content of oenothein B, in subjects with BPH. The results of this clinical study clearly show that a daily intake of *E. angustifolium* for a period of 6 months may allow a significant improvement in symptoms, and the improvement of urinary flow. In fact, in the treated group the frequency of subjects without urinations during night increased and the subjects urinating more than once per night significantly decreased. On the other hand, the placebo group did not follow the same trend. The same result was achieved regarding PVR value. In fact, in EAE food supplement group the frequency of subjects with a low residual urine volume in the

bladder significantly increased while there was a decreasing frequency of subjects with residual urine volume higher than 100 µl. The most important aspect regarding the improvement in quality of life of the treated subjects. In fact, IPSS score significantly decreased while in the placebo group it slightly increased.

This work has limitations and strengths. The main limitation is imputable to the fact that it was not possible to follow up the subject after the 6 months of treatment being impossible to learn about longer term effects after EAE food supplement treatment.

Furthermore, the major strength of this study is that to the best of our knowledge, it was the first double blind, controlled interventional study on the effects of *E. angustifolium* extract alone on PBH suggesting a significant reduction of nocturia. This result is very remarkable for the impact on wellness and health, as nocturia is a serious therapeutic problem with serious impact on the quality of sleep leading to sleep disorders, decreased quality of life and depression (Varilla et al. 2011). Another important strength of our study concerns the use of International Prostate Symptom Score (IPSS), a validated questionnaire to assess BHP symptoms in men with urinary complaints. Our results show that, the IPSS score significantly decreased by nearly 2 points between t0 and t3 in the treated group and slightly increased (0.6 points) in the placebo group leading to an improvement in the quality of life of the subjects treated with EAE food supplement and highlighting the protective effect of this supplementation. In addition, the continuous

monitoring of potential AEs allowed us to confirm the safety of *E. angustifolium* food supplement. It was well tolerated, and it not showed hepatic and renal toxicity.

In conclusion, a daily intake of *E. angustifolium* food supplement may allow a significant improvement in symptoms, and the improvement of urinary flow in the early stage of BPH, in terms of post-void residual volume reduction and consequently nocturia.

3.3 Arabinoxylans obtained from barley

Wheat (*Triticum aestivum* L.) is one of the main cereals belonging to the *Gramineae* family. In terms of production and consumption, barley (*Hordeum vulgare* L.) is one of the most important cereal crops in the world following wheat, maize, and rice. It is cultivated both in highly productive agricultural systems and at the subsistence level in marginal environments (Newton et al., 2011).

The caryopsis of cereals includes three main fractions: 85% is composed of endosperm, the starchy inner part from which flour is produced; 13% by bran, outer integument of the caryopsis; and 2% by the germ, portion containing the energy reserves, from which, in the case of wheat, oil is obtained. In addition to the starchy polysaccharide portion, mostly represented in the endosperm, the bran and the endosperm of several cereals (i.e., wheat, barley, rice, oats, and sorghum) contain a portion of non-starchy polysaccharides, such as arabinoxylans, β-glucans, pectins, arabinogalactans, xylans, xyloglucans, galactomannans, cellulose and lignin. Among these, arabinoxylans and β-glucans, which, together with pectins and arabinogalactans, are part of the soluble fiber, have gained the interest of the medical-scientific community for some beneficial activities, demonstrated by a large body of evidence occurring in the literature, such as:

1) increased food bolus viscosity, as soluble fibers create a gel in aqueous environment (Salunier et al., 1995). The highly viscous food

bolus incorporates glucose and cholesterol into the gel and removes them from absorption;

2) the reduction in gastric emptying time and the consequent increase in the sense of satiety also due to the gelling and swelling properties of soluble fibres (Hartvigsen et al., 2014);

3) the beneficial effect on the trophism of the intestinal mucosa, due to the metabolism of these compounds by the microbiota, which releases short chain fatty acids *(*SCFAs), such as acetate, propionate and butyrate, into the intestinal lumen (Kjolbaek et al., 2020).

The main sources of arabinoxylans are wheat, barley, rice, rye, oats and sorghum. However, these compounds are also present, in different proportions, in non-cereal species such as psyllium seeds (*Plantago indica* L.) and bamboo shoots (*Bambusa* spp.).

The molecular structure of arabinoxylans includes a central linear carbonaceous skeleton consisting of β-1,4-linked xylose monomers (D-xylopyranose) and, as substituents, arabinose (L-arabinofuranose) side chains, which may have ferulic acid on the fifth carbon (see Figure 10). The covalent bond between the ferulic acid residues is the primarily responsible for gel formation.

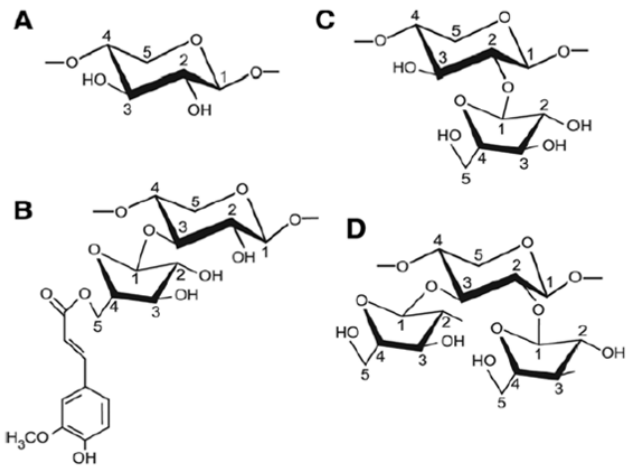


Figure 10. The structure of arabinoxylans. **A**. Arabinoxylan molecule without substituents. **B**. Single-substituted arabinoxylan molecule. **C**. Arabinoxylan molecule monosubstituted with arabinose and ferulic acid. **D**. Arabinoxylan molecule with two substituents.

Furthermore, it has been shown that arabinoxylans may also contain other sugar residues, such as glucuronic acid, 4-O-methyl glucuronic acid or small oligomers comprising L-arabinose, D-xylose, D-galactose, D- glucose and/or uronic acids, short oligomers consisting of L-arabinose, D- xylose, which bind to the oxygen hydrogen atom at position 2 of the xylose, composing the central skeleton (Chen et al., 2019).

Despite the characteristic structure, arabinoxylans are characterized by morphological heterogeneity, depending on several factors, including:

- the botanical species, cultivar,
- the type of tissue in the caryopsis (bran, endosperm),
- the external environment.

The morphological differences result in different physicochemical properties, which can significantly affect the solubility of arabinoxylans, and, consequently, the compound's relative viscosity and gelling properties.

The arabinoxylans, on the basis of structural and conformational properties, are classified into two categories:

1. water extractable arabinoxylans;
2. non-water extractable arabinoxylans.

The ratio between the two types of compounds varies depending on the species, cultivar, type of caryopsis tissue (bran, endosperm), external environment and processing or extraction process (Ain et al., 2018).

Arabinoxylans once introduced through the diet, undergo certain structural changes due essentially to: 1) passage through the gastric tract, due to the acidic environment and the presence of digestive enzymes, and 2) metabolism by the gut microbiota.

In recent studies, water-soluble arabinoxylans were found to be effective in modulating glucose metabolism. The mechanisms of action that contribute to reducing glucose absorption and attenuating the postprandial glycemic response, are:

- delaying gastric emptying time,

- slowing intestinal transit,
- reducing the rate of glucose diffusion in the intestinal lumen,
- lowering the availability and inhibiting the activity of digestive enzymes in the intestinal lumen,
- the bifidogenic effect.

In addition, the gelling properties of arabinoxylans delay the degradation and, consequently, the absorption of proteins by attenuating the insulin response due to insulinogenic amino acids (Christensen et al., 2013).

Also in this case, functional evidence were observed to occur depending on the molecular structure of the employed arabinoxylans. In particular, the degree of polymerization, molecular weight, water solubility and the presence of ferulic acid, have a significant impact in determining the biological activity of arabinoxylans in glucose metabolism.

3.3.1 *Health claim on arabinoxylan*

Wheat endosperm arabinoxylans have been granted a *health claim* for reducing postprandial blood glucose.

The approved claim falls under Article 13.1 of Regulation (EC) 1924/2006.

CLAIM SUBJECT: *Arabinoxylan produced from wheat endosperm*.

HEALTH INDICATION: *A cause and effect relationship has been established between the consumption of arabinoxylan produced from wheat endosperm and reduction of post-prandial glycaemic responses.*

CONDITIONS OF USE: *In order to obtain the claimed effect, 8 g of arabinoxylan-rich fibre produced from wheat endosperm (at least 60 % arabinoxylan by weight) per 100 g of available carbohydrates should be consumed. The target population is individuals who wish to reduce their postprandial glycaemic responses*.

This claim was approved by the European Commission following the positive opinion by EFSA: "*Scientific Opinion on the substantiation of health claims related to arabinoxylan produced from wheat endosperm and reduction of post-prandial glycaemic responses (ID 830) pursuant to Article 13(1) of Regulation (EC) 1924/2006"* (EFSA Journal 2011; 9(6):2205).

In EFSA opinion the following claimed effect is reported *"carbohydrate metabolism and insulin sensitivity". The target population is assumed to be individuals who wish to reduce their post-prandial glycaemic responses. A reduction of post-prandial glycaemic responses (as long as post-prandial insulinaemic responses are not disproportionally increased) may be a beneficial physiological effect.*

Three intervention studies investigated the effects of AX consumption on post-prandial blood glucose and insulin responses were considerated useful by the Panel (Lu et al., 2000; Lu et al., 2004; Mohlig et al., 2005).

In the study of Möhlig et al., 15 enrolled subjects (9 female) first consumed a control breakfast without AX. During the second visit, a breakfast with 6 g of AX-enriched fibre was administered. (Möhlig et al., 2005). *The Panel considers that no conclusions can be drawn from this uncontrolled study for the scientific substantiation of the claim*.

In the randomised, controlled, cross-over clinical trial by Lu et al., the effects of three breads containing 0, 6 or 12 g of AX-rich fibre on induced post-prandial glycaemic and insulinaemic responses were investigated in 14 subjects (9 female) with normal glucose tolerance. The three breakfasts were administered in the three different groups, interspersed with a wash-out period of three days or more, before the treatment cross-over. Blood glucose and insulin concentrations were measured at baseline and 15, 30, 45, 60, 75, 90 and 120 min after each breakfast (Lu et al., 2000).

The Panel notes that this study shows a dose-dependant effect of AX-rich fibre on the reduction of post-prandial glycaemic responses without disproportionally increasing post-prandial insulinaemic responses.

Subsequently, the same research group conducted a randomised, single-blind, controlled, cross-over clinical trial to study the effect of the same type of fibre, used in the 2000 reference, on the control of post-prandial glycaemia in 15 subjects suffering from type 2 diabetes (Lu et al., 2004).

Participants underwent three consecutive treatments (in five weeks), without a wash-out period. It consisted of muffins enriched with arabinoxylan-rich fibre, unenriched muffins, and unenriched bread. The AX-rich foods provided 14-17 g of NSPs (of which 90 % was AX) daily compared to the control foods. Plasma glucose and insulin were assessed in fasting and at 2 hours after a 75 g oral glucose tolerance test (OGTT), both at baseline and after each study phase. The results showed that the sequence in which the diets were administered had no effect on the variables tested.

In evaluating the evidence provided by the applicant, the NDA Panel took into account that a well-designed interventional study was conducted in healthy subjects, the results of which showed a dose-response relationship between the intake of arabinoxylans produced from wheat endosperm and a reduction in postprandial glycaemic and insulinemic responses. *The Panel notes that although post-prandial glycaemic and insulinaemic responses following AX consumption were not measured directly in this study, the results are consistent with an effect of AX on the reduction of post-prandial glycaemic responses, asobserved in the study by Lu et al. (2000).*

i.Clinical study

Efficacy study of a food supplement based on arabinoxylans obtained from barley on the reduction of postprandial glycemic response in healthy subjects: a monocentric, randomized, cross-over, double-blind, placebo-controlled clinical trial.

Considering that the claim on arabinoxylans from wheat endosperm cannot be ascribed to arabinoxylans obtained from barley, as the latter are derived from another plant species, the aim of this study was to demonstrate that arabinoxylans obtained from barley exhibit the same property of reducing post-prandial blood glucose in healthy subjects.

Materials and methods

Food supplement containing arabinoxylans obtained from barley and placebo

The subjects recruited in the present clinical study consumed a bakery product (breadsticks) characterized by a high content of available carbohydrates, previously portioned and packaged in such a quantity as to provide 50 g of available carbohydrates, characterized in terms of nutrients and caloric value. These bakery products included in the addition, in one case, a soluble granulate in stick-pack containing the bioactive compound (STANDARD MEAL + arabinoxylans - AX obtained from barley) to be dissolved in a glass of water, and in the other case, a soluble granulate in stick-pack containing

microcrystalline cellulose to be dissolved in a glass of water (STD MEAL + placebo).

Specifically, first the subject consumed the food supplement containing arabinoxylans or microcrystalline cellulose and immediately thereafter the subject consumed the baked goods.

It should be noted that, in order to mimic the conditions of real-life use of the food supplement, the subjects presented themselves fasting in the late morning for the examination, as they consumed breakfast no less than three hours before the start of the intake of the treatments.

The composition and technical characteristics of the above products are reported below. The treatments were provided by the company HEALLO s.r.l. - C.so Europa 13, 20122 Milan, as Promoter of the present clinical study.

Bakery product (STD MEAL)

Based on the information on the nutrition table provided (Table 14), it was estimated that the single portions of bakery product should have a net weight of 65 g each.

INGREDIENTS: soft wheat flour type "00", brewer's yeast, salt, flour and barley malt extract. Coated with soft wheat flour.

AVERAGE NUTRITIONAL VALUES	100 g of product

Energy	1563 kJ - 369 kcal
Fats of which saturated fatty acids	1,6 g 0,4 g
Carbohydrates of which sugars	76,5 g 6,8 g
Dietary fibres	1,9 g
Protein	11,5 g
Halls	2,8 g

Table 14.— Breadsticks nutritional table.

Treatment - food supplement containing AX obtained from barley

Treatment in the form of soluble granules in stick-packs of 2.5 g.

COMPOSITION OF ACTIVE INGREDIENTS	2,00 g/stick-pack
Soluble fibre-arabinoxylans	

COMPOSITION OF EXCIPIENTS	0.50 g/stick-pack
Inulin (20% of the finished product of 2.50 g)	

TECHNICAL DATA

Pharmaceutical form	Soluble granules in stick-pack sachet
Warnings	Do not exceed the recommended dose. Keep out of reach of children under three years of age.
Storage mode	The expiry date refers to the product correctly stored, in unopened package. Store in a cool and dry place away from light and localized heat sources, sunlight and avoid contact with water.
How to use	One sachet to dissolve in a glass of water.

Table 15.—Ingredients, excipients and technical data related to food supplement containing arabinoxylans from barley.

Placebo -containing microcrystalline cellulose

Placebo in the form of soluble granules in stick-packs of 2.5 g.

COMPOSITION OF EXCIPIENTS 2,50 g/stick-pack
Microcrystalline cellulose Inulin 20% (0.5g)

TECHNICAL DATA

Dosage form	Soluble granules in stick-pack sachet
Warnings	Do not exceed the recommended dose. Keep out of reach of children under three years of age. Excessive consumption may have laxative effects.
Storage mode	The expiry date refers to the product correctly stored, in unopened package. Store in a cool and dry place away from light and localized heat sources, sunlight and avoid contact with water.
How to use	One sachet to dissolve in a glass of water.

Table 16.— Excipients and technical data for placebo.

Clinical trial design

This monocentric randomized, placebo-controlled, double-blind cross-over clinical trial was approved by the Ethics Committee of ASL Napoli1CENTRO on April 12, 2021 (Prot n° 222) and was conducted at the cooperative of General Practitioners Comegen located in Via Maria Bakunin, 41 80126 Naples.

All enrolled subjects remained unaware of the type of treatment to which they were subjected, and assignment to treatment groups was not made known to the investigator, sponsor, or any other person involved in the clinical study. In addition, both the food supplement containing

arabinoxylans obtained from barley (AX) and the placebo, were made unrecognizable in shape, weight, color, and, as far as possible in taste.

Subjects, before being enrolled in the clinical study, were informed about the purpose of the clinical study by a special informed sheet written in an understandable form. After that, the enrolled subject filled in with their data and signed the Consent Form as required by Regulation (EU) 2016/679. This form was subsequently countersigned by the investigator.

Throughout the study, the principal investigator ensured that the trial was conducted according to the *European Union's Standards of Good Clinical Practice* in accordance with the current *Declaration of Helsinki* concerning medical research on humans.

The study design included two experimental groups, specifically:

- subjects belonging to group A who had to take the food supplement containing AX obtained from barley,
- subjects belonging to group B who had to take the placebo.

Subjects were assigned to each of the two groups in a random and unpredictable way by means of a simple randomization (allocation ratio 1:1).

During the first visit (t0), recruited subjects:

1. initially they underwent a fasting blood draw,
2. subsequently they consumed the STD MEAL + AX or the STD MEAL + placebo,

3. 15 minutes after ingestion of the bakery product, they underwent blood sample to measure glycemia and insulinemia [at 15 (t1), 30 (t2), 60 (t3), 90 (t4), and 120 (t5) minutes after intake of the treatment and breadsticks].

This step was followed by five days of *wash-out* period (in which subjects took no treatment), prior to cross-over of treatments.

After the five-days of *wash-out period* (t6) each subject in the two groups underwent blood sample again (at the times indicated for t0) for measurement of fasting blood glucose and insulin, subsequently, after ingestion of STD MEAL+ placebo or STD MEAL + AX (according to a cross-over design).

Participants were asked to reduce their fiber intake from two weeks before the start of the study until the end, and not to significantly change their eating habits for the entire duration of the study.

Blood samples was carried out by health personnel qualified, sent by Laboratorio Basile (Naples). Blood tests, on the other hand, were carried out at the Laboratorio Basile (head office Viale Michelangelo, 13, 80129 Naples).

Study population

The subjects recruited by the cooperative of general practitioners Comegen (40 subjects: 20 in the AX treated group and 20 in the placebo group) presented the following inclusion criteria: healthy subjects,

according to the clinical history; aged between 18 and 65 years; non-smokers; subjects able to understand and to sign the Informed Consent. Instead, subjects with the following characteristics were excluded from the study: subjects with type 1 or 2 diabetes; subjects with fasting blood glucose > 110 mg/dl; subjects with blood pressure values > 160/100 mm Hg; subjects with metabolic disorders or eating disorders; subjects with endocrine, cardiovascular, pulmonary, renal or gastrointestinal diseases, which may interfere with the results of the study; subjects with sensitivity, intolerance or allergy to products used in the clinical trial; pregnant or lactating women; blood donors in the three months prior to recruitment; subjects under pharmacological treatment, with drugs that could interfere with the study, such as alpha-glucosidase inhibitors (acarbose, etc.), insulin-sensitive drugs (metformin, etc..), sulfanilureee, cholesterol medications, and any other medications that the physician does not deem compatible with the study; subjects who were taking food supplements that could interfere with the study, such as products high in vitamins and minerals (> 200% VNR), B vitamins, C vitamin, calcium, zinc, copper, chromium, iodine, iron, magnesium, manganese, phosphorus, essential fatty acid products, botanicals, and any other products that the physician does not deem compatible with the study.

Outcome of the study

Data collection was performed by means of a CRF divided into two main sections. A first section concerning personal data, subject's medical history, intake of any concomitant drugs, and the treatment group, was filled at the time of enrollment. Instead, the second section was filled with the results of the analyses performed on the blood samples taken.

The main objective of the present clinical study was to evaluate the contribution of AX obtained from barley, as part of a standard meal, in promoting the reduction of postprandial blood glucose increase in healthy subjects.

On the other hand, the secondary objective was to evaluate the impact of the food supplement based on arabinoxylans obtained from barley on the postprandial insulinemic response.

Power analysis

In order to calculate the sample size, the EFSA opinions relating to the claim on the reduction of post-prandial blood glucose were analyzed. Following the analysis of the efficacy studies that were submitted in support of the *health claim* request and, following the analysis of the comments made by the EFSA on the correct sample size for these types of studies, the number of subjects to be enrolled is at least 30 (15 subjects per arm). In support of this choice, based on the EFSA indications, a power analysis was also conducted to verify the validity of the sample size identified for this specific study.

The analysis was conducted with the following assumptions:

- Balanced design (a necessary condition for a cross-over study).
- For the estimation of the effect size, values between 0.20 and 0.30 were used, which correspond to small to medium effects according to Cohen (1969, *Statistical power analysis for the behavioural sciences*. New York: Academic Press).
- Within-subject correlation set at r = 0.5.
- Power (1-β) values of 0.80, 0.95 and 0.99;
- Significance level (α) was chosen to be 0.05.

Based on these parameters, the sample size for the different power and effect size assumptions is shown in Table 17.

Effect size	**(1-β) = 0,99 α =0.05**	**(1-β) = 0.,95 α =0.05**	**(1-β) = 0,80 α =0.05**
f=0.20	118	84	52
f=0.25	76	54	34
f=0.30	54	**40**	24

Table 17.— Sample size of the population to be co-investigated in the clinical trial**.**

Therefore, considering the number of subjects accepted by EFSA (at least 30 subjects) and the results of the power analysis reported above, it was considered appropriate to enroll 40 subjects (20 subjects per group).

Safety

Arabinoxylans obtained from barley are an approved ingredient for food supplements.

Although no adverse events related to the intake of the food supplement were expected, the enrolled subjects were continuously monitored. Instead, subjects with sensitivity, intolerance or allergy to gluten or barley were categorically excluded from the study.

Results

Clinical study

The "*study flow chart*", in accordance with the *CONSORT PRO reporting guidelines* (Calvert et al., 2013) is shown in figure 11. Initially, 42 subjects were screened in the clinical study, however, two subjects did not present all the inclusion criteria required by the protocol, therefore, the final number of subjects enrolled was 40 (sample size in accordance with that indicated by the power analysis).

The group of subjects who initially took the AX food supplement (group 1) consisted of 13 women and 7 men (mean age ± SD: 53 ± 5). In contrast, the group of subjects who initially took the placebo (group 2) consisted of 12 women and 8 men (mean age ± SD: 57 ± 7).

Furthermore, although the cross-over design of the study would have nullified any significant sociodemographic differences, subjects in the two experimental groups were appropriately randomized, and, they had similar sociodemographic characteristics with non-significant

differences, at t0 (baseline). The characteristics at baseline of each group are shown in Table 18.

Features	Treated (n=20)	Not treated (n=20)
Age	53 ± 5	57 ± 7
Ethnicity: All European	2	20
	0	

Table 18.— Demographic characteristics of the *study population*

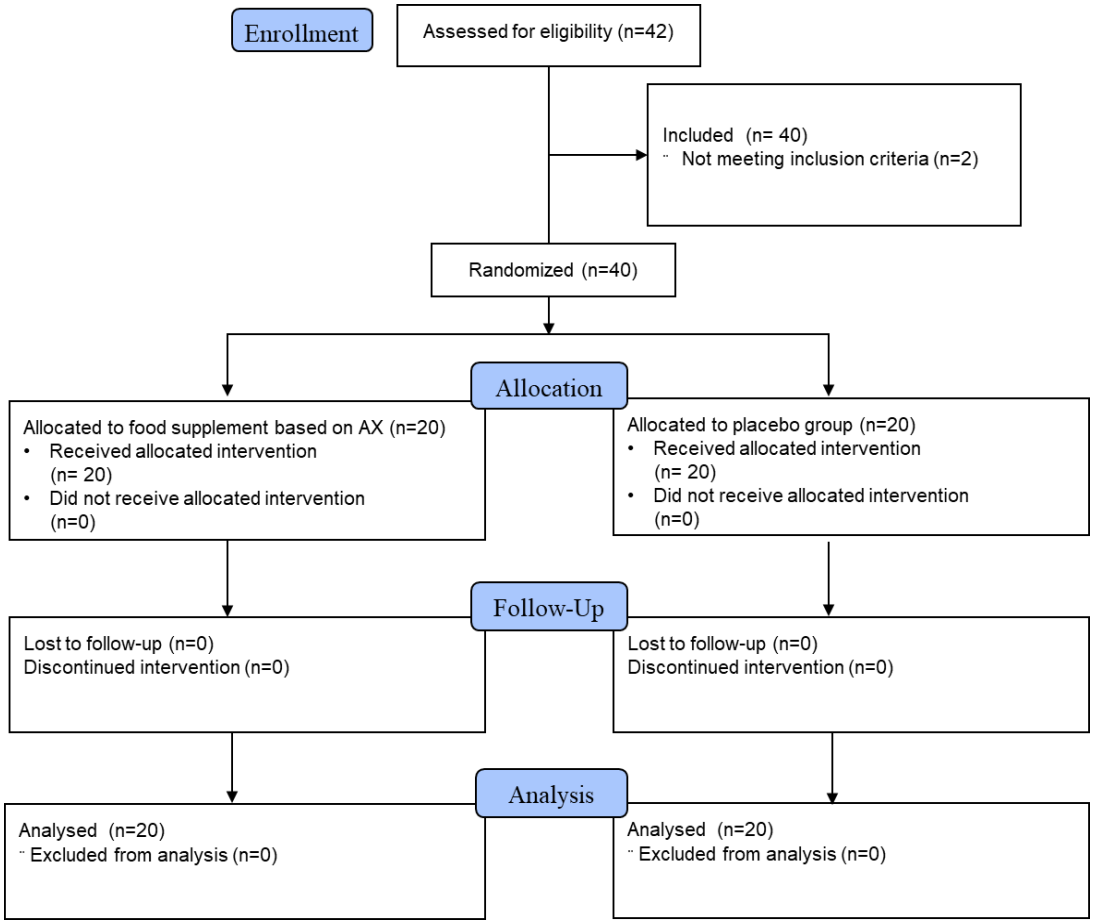


Figure 11. CONSORT flow diagram.

The primary objective of the study was to evaluate the contribution of arabinoxylans in promoting the reduction of postprandial blood glucose in healthy subjects.

Tables 19 and 20 show, respectively, the blood glucose values, expressed in mg/dl, of the 20 subjects in group 1 who initially took the AX food supplement and who, after the wash-out period, took the placebo. Tables 21 and 22 show the blood glucose values of the 20 subjects in group 2 who initially took the placebo and who, after the wash-out period, took the AX food supplement.

As shown in the tables, all the 40 subjects met the inclusion criterion of being normoglycemic (normal fasting blood glucose values: 70 and 100mg/dl):

- Group 1
  - presenting fasting glycemia equal to 78±5 mg/dl, before taking the glycemic load and the food supplement,
  - presenting fasting glycemia equal to 78±6 mg/dl, before taking the glycemic load and the placebo;
- Group 2
  - presenting fasting glycemia equal to 82±7 mg/dl before taking the glycemic load and the placebo,
  - presenting fasting glycemia equal to 79±6 mg/dl before taking the glycemic load and the food supplement.

BLOOD GLUCOSE					
0 min	**15 min**	**30min**	**60min**	**90min**	**120min**
80	85	90	95	90	83
76	80	85	90	85	80
78	82	87	92	90	88
74	79	84	89	86	83
76	81	86	91	89	85
83	88	92	97	99	95
78	80	85	90	88	85
76	82	87	92	89	83
82	86	91	96	93	85
91	96	100	103	98	93
70	75	80	90	85	78
76	80	85	90	86	80
79	83	87	92	97	99
72	78	84	90	85	78
71	77	82	87	85	80
86	90	95	99	94	87
73	78	85	90	92	91
83	85	89	93	95	95
85	90	95	98	95	92
72	78	84	90	87	79

Table 19.—Blood glucose of the 20 subjects in group 1 who initially took the AX food supplement.

BLOOD GLUCOSE					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
76	80	85	95	95	90
82	86	91	96	93	85
91	96	100	103	98	93
85	90	95	98	95	92
72	80	85	90	95	93
81	86	96	100	98	97
70	77	83	89	95	96
73	78	86	90	96	95
83	88	92	98	100	98
84	90	95	98	95	90
76	82	87	92	98	96

74	79	84	89	93	92
78	85	90	95	93	91
74	80	89	96	94	90
70	75	83	87	93	91
77	80	85	90	87	85
76	82	85	90	96	94
80	85	90	98	95	93
78	80	85	88	84	80
76	81	86	91	95	90

Table 20.—Blood glucose of the 20 subjects in group 1 who took placebo after the washout period.

BLOOD GLUCOSE					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
76	81	87	98	94	91
85	81	86	95	93	90
72	77	92	99	97	94
83	88	93	100	95	90
82	87	91	98	94	91
72	78	84	95	93	90
83	88	92	99	96	92
76	82	87	93	91	90
90	94	99	104	100	97
87	92	96	99	97	95
93	97	102	107	105	104
81	87	92	99	97	92
86	90	95	100	97	94
84	88	93	98	95	93
74	79	85	95	90	85
85	92	97	101	99	95
77	82	87	92	90	89
82	88	92	99	97	90
92	96	100	103	100	99
71	77	82	87	95	91

Table 21.— Blood glucose of the 20 subjects in group 2 who initially took placebo.

BLOOD GLUCOSE					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
84	89	94	99	95	85
83	88	93	99	94	90
70	75	80	90	85	80
76	81	86	92	89	80
71	76	81	90	80	75
70	75	80	95	90	82
72	77	82	92	87	80
84	89	94	100	95	90
77	82	87	97	90	82
79	83	88	95	98	99
74	79	84	96	88	76
77	82	87	93	89	85
75	79	84	93	84	78
82	88	93	99	94	90
83	88	92	100	98	88
84	89	94	98	96	95
86	91	96	101	98	93
82	87	92	97	93	85
92	94	99	102	96	93
80	85	90	95	90	83

Table 22.— Blood glucose of the 20 subjects in group 2 who took the AX food supplement after washout.

For each subject, the area under the glycemic curve following the intake of the AX food supplement and the placebo, was calculated.

Hence, the percentage difference between the area under the glycemic curve determined following the intake of the placebo (set equal to 100%), and the area under the glycemic curve determined following the intake of the AX food supplement, were calculated (Tables 23 and 24).

AREA UNDER GLYCEMIC CURVE		**% Decrease in area under the AX curve compared with placebo curve**
Food supplement AX	Placebo	
10695,0	10732,5	**0,35**
10132,5	10897,5	7,00
10552,5	11797,5	10,55
10125,0	11295,0	10,35
10395,0	10597,5	1,91
11317,5	11452,5	1,18
10312,5	10507,5	1,86
10432,5	10657,5	2,11
10897,5	11422,5	4,60
11797,5	11257,5	**-4,80**
9870,0	10897,5	9,43
10162,5	10470,0	2,94
10950,0	10890,0	**-0,55**
10020,0	10807,5	7,30
9892,5	10282,5	3,80
11227,5	10275,0	**-9,27**
10455,0	10702,5	2,31
10965,0	11085,0	1,09
11295,0	10057,5	**-12,3**
10095,0	10650,0	5,21

Table 23.— Area under the glycemic curve and percentage difference in area under the AX curve compared with placebo curve referred to group 1.

AREA UNDER GLYCEMIC CURVE		% Decrease in area under the AX curve compared with placebo curve
Placebo	Food supplement AX	
10867,5	11175,0	**-2,83**
10777,5	11175,0	**-3,69**
11055,0	9900,0	10,45
11235,0	10350,0	7,88
11092,5	9720,0	12,37
10590,0	10230,0	3,4
11242,5	10110,0	10,07
10627,5	11280,0	**-6,14**
11887,5	10605,0	10,79
11497,5	11092,5	3,52
12367,5	10290,0	16,6
11242,5	10500,0	6,6
11452,5	10117,5	11,656
11227,5	11167,5	**0,53**
10477,5	11272,5	**-7,59**
11625,0	11325,0	2,58
10560,0	11535.0	**-9,23**
11235,0	10965.0	2,4
11955,0	11662,5	2,45
10357,5	10695,0	**-3,26**

Table 24.— Area under the glycemic curve and percentage difference in area under the AX curve compared with placebo curve referred to group 2.

In group 1 and group 2 there are, respectively, 5 and 7 subjects presenting a % decrease in the area under the curve (AX compared to the placebo) close to the 0 or negative value. It indicates that 12.5% and 17.5% of the subjects, respectively, do not respond to the treatment with the AX food supplement.

Hence, the mean value and standard deviation of the % decrease in this area under the curve, were then calculated for the 28 subjects who responded positively to treatment with the AX food supplement. The value obtained is 6.15 ± 4.21 % (1.09 < % >12.37).

Comparison of the data obtained in the present clinical trial with those obtained by Lu et al., indicate that the reduction in area under the glycaemic curve reported in this study is modest compared with that reported by Lu et al. which decreased by 20.2 and 41.4% following intake of 6 and 12 g of arabinoxylan-rich fiber, respectively.

The secondary objective was to evaluate the impact of the AX food supplement on the postprandial insulinemic response.

Tables 25 and 26 show, respectively, the insulinemia values, expressed in µU/ml, of the 20 subjects in group 1 who initially took the AX food supplement and who, after the wash-out period, took the placebo.

Tables 27 and 28 show the insulinemia values of the 20 subjects in group 2 who initially took the placebo and who, after the wash-out period, took the AX food supplement.

Moreover, is possible to note that in Table 26 and Table 2, among the 40 normoglycaemic subjects who took placebo, 24 subjects had an

insulinemia at 120 min (written in bold) higher than normal values (generally between 18 and 58 μU/ml). It indicates a potential slight insulin resistance which cannot be confirmed to date, but which requires further data on these subjects (lipid profile, BMI, abdominal circumference, blood pressure).

In addition to the abovementioned, Tables 25 and 28 show that, among the 40 normoglycemic subjects who took the AX food supplement, all the subjects present a normal insulinemia at 120 min (15 <insulinemia at 120 min> 50 μU/ml). It indicates that, taking the supplement at the same time as the glycemic load, allows even the 24 subjects with potential insulin resistance (i.e., at 120 min had moderately high insulinemia after taking the glycemic load) to restore normal insulinemia values. This unexpected result is certainly interesting and deserves further investigation.

INSULIN					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
6	20	30	40	35	20
10	30	45	50	45	15
26	35	48	53	49	30
25	30	42	47	41	32
9	20	34	38	32	20
17	30	38	42	44	40
23	33	40	48	43	28
20	38	45	55	50	48
8	20	34	44	39	15
21	35	42	52	47	30
22	38	44	49	40	28
25	34	46	57	50	32
26	40	48	55	50	38
10	20	34	40	34	28

7	18	25	35	30	17
14	26	34	44	43	44
26	35	45	56	45	32
6	15	25	35	36	37
10	25	35	46	40	29
19	28	39	48	40	21

Table 25.— Insulinemia of the 20 subjects in group 1 who initially took the AX food supplement.

INSULIN					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
6	30	40	100	90	**85**
10	35	55	83	88	**90**
12	22	35	55	75	**70**
14	28	39	45	65	**60**
22	30	40	55	70	**65**
8	18	28	48	56	50
17	30	42	95	90	**85**
10	33	40	50	40	30
9	20	32	45	68	**65**
23	33	45	64	72	**70**
27	37	48	68	77	**75**
23	33	42	54	67	**70**
25	39	50	61	78	**75**
10	15	30	45	60	**65**
27	37	48	64	76	**80**
13	26	36	54	68	**69**
20	25	35	58	70	**71**
24	34	47	60	79	**77**
8	20	34	42	57	**59**
17	25	35	44	56	**60**

Table 26.— Insulinemia of the 20 subjects in group 1 who took placebo after the washout period.

INSULIN					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
18	30	42	95	90	**85**
11	26	36	54	70	**69**
7	17	27	44	52	50
19	29	39	56	58	56
17	25	32	47	59	55
19	27	37	44	50	51
29	37	50	55	60	58
13	26	36	46	56	54
17	27	37	47	59	55
22	35	50	58	55	52
6	30	40	100	85	**80**
30	40	50	70	65	**60**
8	18	28	48	43	40
14	34	44	65	60	58
25	40	55	66	62	**60**
15	35	48	67	65	**61**
10	30	40	55	50	48
8	20	30	50	45	41
22	38	48	60	55	52
10	25	35	52	49	47

Table 27.— Insulinemia of the 20 subjects in group 2 who initially took placebo.

INSULIN					
0min	**15min**	**30min**	**60min**	**90min**	**120min**
20	30	40	55	42	29
29	38	45	55	48	32
28	38	47	60	50	34
19	29	39	48	43	28
12	22	34	50	39	21
7	17	27	47	35	18
9	18	27	36	25	15
17	27	37	47	30	22
30	40	50	68	57	35
11	21	32	45	38	21

25	35	45	59	55	50
6	12	23	45	32	28
18	27	36	54	44	22
25	35	45	56	38	29
23	33	43	53	49	31
6	20	30	40	35	30
17	28	37	48	38	30
7	17	27	47	39	24
29	39	49	59	40	30
23	33	43	55	50	30

Table 28.— Insulinemia of the 20 subjects in group 2 who took the food supplement after the washout period.

For each subject, the area under the insulinemic curve following intake of the food supplement and the placebo was calculated, and then the percentage difference between the area under the insulinemic curve determined following intake of the placebo, set equal to 100%, and the area under the insulinemic curve determined following intake of the AX food supplement was calculated (Tables 29 and 30).

AREA UNDER INSULINEMIC CURVE		**% Decrease in area under the AX curve compared to placebo curve**
Placebo	Food supplement AX	
3570	8370	57,4
4612,5	8317,5	44,6
5310	6157,5	13,8
4702,5	5602,5	16,1
3532,5	6240	43,4
4612,5	4830	4,5
4717,5	8347,5	43,5

5602,5	4620	-21,3
3840	5452,5	29,6
5047,5	6810	25,9
4815	7312,5	34,2
5422,5	6292,5	13,8
5595	7192,5	22,2
3780	5100	25,9
3090	7237,5	57,3
4530	5992,5	24,4
5242,5	6217,5	15,7
3517,5	7072,5	50,3
4252,5	4980	14,6
4395	5190	15,3

Table 29.— Area under the insulinemic curve and percentage difference in area under the AX curve compared with placebo curve referred to group 1.

AREA UNDER INSULINEMIC CURVE		**% Decrease in area under the AX curve compared to placebo curve**
Placebo	Food supplement AX	
8355,0	4845,0	42,0
6037,5	5370,0	11,1
4545,0	5647,5	-24,3
5715,0	4605,0	19,4
5227,5	4170,0	20,2
4965,0	3645,0	26,6
6217,5	3000,0	51,8
5167,5	4005,0	22,5

5370,0	6225,0	-15,9
5985,0	3922,5	34,5
8145,0	5895,0	27,6
6900,0	3472,5	49,7
4290,0	4620,0	-7,7
6225,0	4980,0	20,0
6765,0	5160,0	23,7
6592,5	3720,0	43,6
5295,0	4410,0	16,7
4500,0	3855,0	14,3
6045,0	5325,0	11,9
4972,5	5235,0	-5,3

Table 30.— Area under the insulinemic curve and percentage difference in area under the AX curve compared with placebo curve referred to group 1.

The mean values recorded for groups 1 and 2 referring to the percentage decrease in the area under the insulinemic curve of the AX compared with placebo were 26.5 and 19.1 %, respectively. These data are in line with those obtained by Lu et al, equal to 17 and 32 % following the intake of 6 and 12 g of fiber rich in arabinoxylans, respectively.

Statistical Analysis.

Following are reported the independent variables considered:

- **Measurement**: 6-level factor corresponding to the measurement of the parameter of interest (blood glucose, insulin) immediately after the meal (t0) and after 15 min (t1), 30 min (t2), 60 min (t3), 90 min (t4) , and 120 min (t5);

- **Treatment**: two-level factor A and B corresponding to treatment with AX and placebo, respectively. Levels are indicated A and B as the analysis is blinded, i.e., without knowing the identity of the two experimental groups;
- **Treatment order**: a two-level factor (AB and BA) corresponding to the order in which the experimental treatments were carried out;
- **Sex** of recruited subjects (two-level factor, male: n=15, female: n=25)
- **Age of** subjects (Continuous variable, men: 54.1 ± 5.7; women: 55.5 ± 7.1).

The aim of the statistical analysis of this clinical study was to compare the response curve (glycaemic and insulinemic) in the two experimental groups (A and B) taking into account the effects of the order of treatment and the characteristics of the subjects recruited (sex and age). The most suitable statistical analysis for this type of data is Linear Mixed Model (LMM). Blood glucose and insulin value represent (in two separate models) the dependent variable, while, measurement, treatment, treatment order, sex and age of recruited subjects represent the independent variables. The "measurement x treatment" and "measurement x treatment order" interactions were included among the independent variables. The "measure x treatment" interaction is the key

variable for the primary endpoint as it allows to test whether the trend over the course of the measurements differs in the two treatments.

About the interaction “measurement x treatment order”, it is used to check whether the trends during the measurement were different according to the order of administration of the treatments (before or after wash-out). The identity of the subject constitutes the random effect which consent to control differences of the enrolled subjects.

Table 31 shows the descriptive statistics (mean, standard deviation and range) for the blood glucose and insulin values of men and women at the different measurements, in the two experimental treatments.

Variable		t0	t1	t2	t3	t4	t5
Glcemia							
Women	A	79.4 ± 6.3	84.1 ± 5.9	89 ± 5.6	94.7 ± 4.6	91.7 ± 5.4	87 ± 7.3
		(70 - 92)	(75 - 96)	(80 - 100)	(87 - 103)	(80 - 100)	(75 - 99)
	B	80.5 ± 6.1	85.3 ± 5.9	91.1 ± 5.3	96.7 ± 4.5	95.3 ± 3.4	92.3 ± 3.9
		(72 - 93)	(77 - 97)	(84 - 102)	(89 - 107)	(87 - 105)	(85 - 104)
Men	A	77.4 ± 5.6	82.1 ± 5.6	87.3 ± 5.5	94 ± 4.3	90.4 ± 4.1	84.5 ± 4.9
		(70 - 86)	(75 - 91)	(80 - 96)	(87 - 101)	(85 - 98)	(78 - 93)
	B	78.2 ± 5.5	83.3 ± 5.1	88.5 ± 4.5	94.7 ± 5	94.3 ± 4.1	91.4 ± 4.7
		(70 - 90)	(75 - 94)	(83 - 99)	(87 - 104)	(84 - 100)	(80 - 98)
Insulin							
Women	A	18.2 ± 7.9	29.3 ± 7.7	39.4 ± 7.1	50 ± 7.4	43.7 ± 6.7	32.3 ± 9.5
		(6 - 29)	(15 - 40)	(25 - 49)	(35 - 60)	(32 - 55)	(20 - 52)
	B	14.8 ± 6.6	28.1 ± 7.2	39 ± 7.9	58 ± 15.1	64 ± 12.8	61.4 ± 13.4
		(6 - 25)	(15 - 40)	(27 - 55)	(44 - 100)	(40 - 90)	(30 - 85)
Men	A	15.4 ± 7.8	26.7 ± 8.2	36.5 ± 8.1	48.1 ± 7.8	38.9 ± 7.9	24.6 ± 6.9

	(6 - 30)	(12 - 40)	(23 - 50)	(35 - 68)	(25 - 57)	(15 - 35)
B	18.2 ± 7.4	30 ± 6.2	41.7 ± 7	61.5 ± 17.7	67.6 ± 13.8	66.1 ± 13.8
	(8 - 30)	(20 - 40)	(32 - 55)	(42 - 95)	(50 - 90)	(48 - 90)

Table 31.— Descriptive statistics (mean ± SD, minimum and maximum) for the two biochemical variables blood glucose and insulin measured in the two groups at t0, t1, t2, t3, t4 and t5.

In line with *intention to treat*, all subjects enrolled in the study were included in the final analysis.

The LMM model for blood glucose (Table 32) identified a statistically significant effect for measurement (P<0.001), treatment (P<0.001) and also for the measurement x treatment interaction (P<0.001).

No significant effect was found for sex (P=0.15) and age (P=0.69) of the recruited patients.

However, the effect of treatment order was significant (P=0.014), but the measurement x treatment order interaction was not (P=0.97).

Model	**F**	**Gdl**	**P**
Blood Sugar			
Measuring	164.14	5,422	**<0.001**
Treatment	56.341	1,427	**<0.001**
Sex	2.169	1,37	0.15
Age	0.158	1,37	0.69
Treatment order	6.076	1,423	**0.014**
Measurement x Treatment	4.325	5,423	**<0.001**
Measurement x Order of treat.	0.183	5,423	0.97
Insulin			
Measuring	257.49	5,422	**<0.001**
Treatment	233.07	1,427	**<0.001**
Sex	0.065	1,37	0.80
Age	0.264	1,37	0.61

Treatment order	4.137	1,423	**0.043**
Measurement x Treatment	57.878	5,423	**<0.001**
Measurement x Order of treat.	1.237	5,423	0.29

Table 32.— Results of LMM models for blood glucose and insulin response curves.

These results indicate that there is a difference in the postprandial blood glucose curve in patients when they assumed treatment A compared to treatment B (Figure 12). Blood glucose did not differ between treatments at the initial measurement (dB-A= 1.37 ± 0.93, t424=1.480, P=0.14) and at 15 minutes (dB-A= 1.56 ± 0.93, t424=1.683, P=0.093), but starting from 30 minutes (dB-A= 2.15 ± 0.93, t424=2.328, P=0.020). The blood glucose value in treatment B was significantly higher than the corresponding value in treatment A. At 60 minutes the difference was significant (dB-A= 1.88 ± 0.93, t424=2.030, P=0.042), but reached the maximum values after 90 minutes (dB-A= 4.14 ± 0.93, t424=4.465, P<0.001) and 120 minutes (dB-A= 6.24 ± 0.93, t424=6.737, P<0.001). The effect of the order indicates that the blood glucose value after the wash-out was significantly lower than that observed before the washout (-0.92 ± 0.37, t423=2.465, P=0.014), regardless of the experimental treatment. The blood glucose curve trend, however, was not different between before and after the wash-out period, as attested by the fact that the “measurement x treatment order” interaction was not significant (Table XXVII). The random

effect of the subject was statistically significant (LRχ^2=143.32, P<0.001).

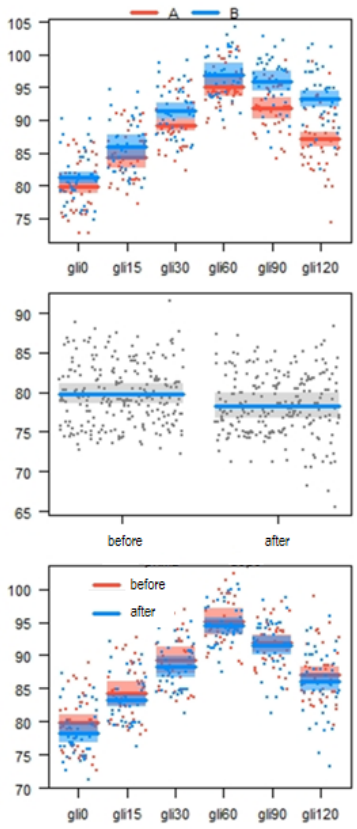


Figure 12. Post-prandial blood glucose variation in the two experimental treatments. Top: blood glucose variation in the two experimental treatments A and B; middle: blood glucose values before and after washout; bottom: blood glucose variation before and after washout regardless of experimental treatment.

The LMM model for insulin (Table XXVII) provided similar results to that for blood glucose. The effects of measurement (P<0.001), treatment (P<0.001) and "measurement x treatment" interaction (P<0.001) were statistically significant. No significant effect emerged for sex (P=0.80) and age (P=0.61) of the recruited patients. The effect

of treatment order was again statistically significant (P=0.043), but the "measurement x treatment order" interaction was not statistically significant (P=0.29).

These results indicate that there is a difference in the postprandial insulinemic curve in patients when they undergo treatment A compared to when they undergo treatment B (Fig. 13). The insulin value did not differ between treatments at the initial measurement (dB-A= -0.59 ± 1.86, t424=0.318, P=0.75), at 15 minutes (dB-A= 1.04 ± 1.86, t424=0.556, P=0.58) and at 30 minutes (dB-A= 2.24 ± 1.86, t424=1.200, P=0.23). Starting at 60 minutes, the insulin value in treatment B was significantly higher than the corresponding value in treatment A (dB-A= 10.47 ± 1.86, t424=5.612, P<0.001) and the difference increases at 90 minutes (dB-A= 23.76 ± 1.86, t424=12.733, P<0.001) to be maximal in the measurement at 120 minutes (dB-A= 34.04 ± 1.86, t424=18.248, P<0.001). The order effect indicates that the insulin value after washout is significantly higher than that observed before washout (+1.54 ± 0.76, t423=2.304, P=0.043), regardless of experimental treatment. As for glycaemia, also for insulin the trend of the curve was not different between before and after the wash-out period, as attested by the fact that the interaction "measurement x treatment order" was not significant. The random effect of the subject was statistically significant (LRχ^2=78.87, P<0.001).

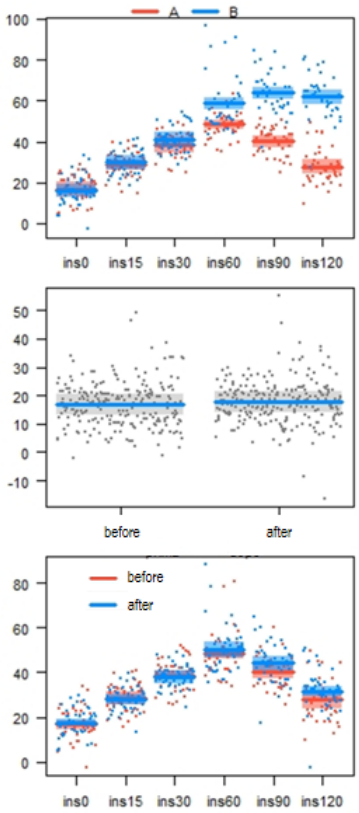


Figure 13. Variation of post-prandial insulin values in the two experimental treatments. Top: change in insulin concentration in the two experimental treatments A and B; middle: insulin concentration values before and after washout; bottom: change in insulin concentration before and after washout regardless of experimental treatment.

Safety

Regarding the safety of the AX food supplement, no subjects reported adverse events (AEs) after taking the supplement containing AX obtained from barley.

Conclusions

This monocentric, placebo-controlled, randomized, cross-over clinical trial showed that the food supplement based on arabinoxylans from barley, can reduce post-prandial glucose in a statistically significant way, compared to placebo, producing a lower insulinemic response, consequently.

Regarding the clinical relevance of the results, a decrease in the area under the glycemic curve (following the intake of the AX food supplement compared to placebo) of about 6 %, is modest when it is compared to the data obtained from arabinoxylans from wheat endosperm. Further studies with an increase in the dose of arabinoxylans from barley are needed to evaluate if higher concentrations of these compounds allow an increase in the desired effect.

In relation to the insulinemic response of the subjects, it can be concluded that the decrease in the area under the insulinemic curve following the intake of the AX food supplement compared to placebo, is in line with the data obtained from arabinoxylans from wheat endosperm.

The inclusion of normoglycemic subjects who, after the insulinemic curve test, showed a potential insulin resistance, allowed to highlight an unexpected and interesting effect of insulin normalization after the intake of the glycemic load and the AX food supplement.

CONCLUSIONS

As previously discussed, foods are defined as: finished products, ingredients and substances for food use that include both nutrients and substances with a nutritional and physiological effect. Therefore, in this context, the food supplement that contains food-type ingredients and any excipients allowed in the food industry, is defined as a foodstuff.

The foodstuff identified and well characterized, must be evaluated by the Food Business Operator (FBO) for its safety and its admissibility in the food market. Food safety is guaranteed by its presence on the market prior to May 15, 1997. If this requirement is not met, preclinical data are needed to prove safety and following EFSA opinion on their safety the European Union can authorize their presence on the market.

As far as the healthy properties of foods, to demonstrate the food efficacy in maintaining health of reduce a disease risk factor, human clinical studies are needed. In any case, manufacturing companies must ensure that the foodstuff is not contaminated by pathogenic microorganisms, chemical contaminants or foreign bodies. It must contain ingredients defined in terms of quantity, and it must comply with all standard quality criteria defined by the competent Regulatory Institutions. In particular, this aspect is essential when the foodstuff contains plant-derived products characterized by a wide qualiquantitative variability.

Fortunately, thanks to the evolution of food legislation, there was a greater focus on these types of controls, to promote both safety and efficacy of foodstuffs.

Moreover, as described in the second part of the thesis, the most critical aspect is the legislation gap and the crucial aspects to perform clinical studies on food supplements concern:

- the use of a representative sample of the target population that must be healthy;
- appropriate outcome measures to validate the designated effect;
- the opinion of the relevant Ethics Committee as a prerequisite;
- the compliance with GCP, as far as applicable.

With regard to the management of clinical trials on food supplements, in the last part of my thesis, I reported the results of four different types of clinical studies on food supplements conducted during my PhD course, that include 1) the study of the pharmacokinetic properties of a food supplement ingredient (i.e. lipoic acid), 2) the evaluation of safety and efficacy of a food supplement ingredient (i.e. lipoic acid), 3) the evaluation of the capacity to reduce BPH symptoms and 4) finally the study of a healthy properties (i.e. reducing the increase of post-prandial blood glucose levels)

In more detail, since the management of idiopathic pain is challenging for physicians, the safety and efficacy study on ALA reported above, allowed us to ensure that the use of food supplements containing ALA

can be a feasible option compared with the commonly-used analgesic drugs.

On the other hand, the pharmacokinetics study conducted on 10 healthy volunteers permitted us to understand that the poor bioavailability of ALA was improved with a liquid formulation.

E. angustifolium extract food supplements can be used in subjects with BPH, to improve their quality of life by reducing post-void residual volume and consequently nocturia and general renal function without hepatic or renal toxicity.

Instead, the last clinical study on AX showed that AX can reduce post-prandial glucose in a statistically significant way, compared to placebo, producing a lower insulinemic response, consequently.

The scientific literature is increasingly interested in the definition of the biological role of foodstuffs and its most relevant components in health and disease conditions. Consequently, the consumers are increasingly more informed about it, and unlike to the past, FBOs are increasingly more interested to test their food supplements by clinical studies, before marketing.

Nevertheless, it has been just over three years since Guidelines on food supplements were published in 2018 by the Ministry of Health and this means that clinical studies on food supplements represented a relatively new approach. To date, the harmonization process by the Regulatory Institutions is still ongoing. The road ahead is still long, but only through this methodological approach, so closely similar to

clinical studies on drugs, it will be possible to put on the market high quality food supplements proved to be safe and effective in humans both in in health and disease conditions.

APPENDIX 1. CONSORT 2010 checklist of information to include when reporting a randomized trial.

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts [21,31])	
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	
	2b	Specific objectives or hypotheses	
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	
Participants	4a	Eligibility criteria for participants	
	4b	Settings and locations where the data were collected	
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	
	6b	Any changes to trial outcomes after the trial commenced, with reasons	
Sample size	7a	How sample size was determined	
	7b	When applicable, explanation of any interim analyses and stopping guidelines	
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	
	13b	For each group, losses and exclusions after randomisation, together with reasons	
Recruitment	14a	Dates defining the periods of recruitment and follow-up	
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms [28])	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	
Other information			
Registration	23	Registration number and name of trial registry	
Protocol	24	Where the full trial protocol can be accessed, if available	
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration [13] for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials [11], non-inferiority and equivalence trials [12], non-pharmacological treatments [32], herbal interventions [33], and pragmatic trials [34]. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see http://www.consort-statement.org.

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APPENDIX 2. CONSORT 2010 randomisation and minimisation

Must be not know

- **Simple randomisation**—Pure randomisation based on a single allocation ratio is known as simple randomisation. Simple randomisation with a 1:1 allocation ratio is analogous to a coin toss, although we do not advocate coin tossing for randomisation in an RCT. "Simple" is somewhat of a misnomer. While other randomisation schemes sound complex and more sophisticated, in reality, simple randomisation is elegantly sophisticated in that it is more unpredictable and surpasses the bias prevention levels of all other alternatives.
- **Restricted randomisation**—Any randomised approach that is not simple randomisation. Blocked randomisation is the most common form. Other means of restricted randomisation include replacement, biased coin, and urn randomisation, although these are used much less frequently.[141]
- **Blocked randomisation**—Blocking is used to ensure that comparison groups will be generated according to a predetermined ratio, usually 1:1 or groups of approximately the same size. Blocking can be used to ensure close balance of the numbers in each group at any time during the trial. For every block of eight participants, for example, four would be allocated to each arm of the trial.[142] Improved balance comes at the cost of reducing the unpredictability of the sequence. Although the order of interventions varies randomly within each block, a person running the trial could deduce some of the next treatment allocations if he or she knew the block size.[143] Blinding the interventions, using larger block sizes, and randomly varying the block size can ameliorate this problem.
- **Stratified randomisation**—Stratification is used to ensure good balance of participant characteristics in each group. By chance, particularly in small trials, study groups may not be well matched for baseline characteristics, such as age and stage of disease. This weakens the trial's credibility.[144] Such imbalances can be avoided without sacrificing the advantages of randomisation. Stratification ensures that the numbers of participants receiving each intervention are closely balanced within each stratum. Stratified randomisation is achieved by performing a separate randomisation procedure within each of two or more subsets of participants (for example, those defining each study centre, age, or disease severity). Stratification by centre is common in multicentre trials. Stratification requires some form of restriction (such as blocking within strata). Stratification without blocking is ineffective.
- **Minimisation**—Minimisation ensures balance between intervention groups for several selected patient factors (such as age).[22 60] The first patient is truly randomly allocated; for each subsequent participant, the treatment allocation that minimises the imbalance on the selected factors between groups at that time is identified. That allocation may then be used, or a choice may be made at random with a heavy weighting in favour of the intervention that would minimise imbalance (for example, with a probability of 0.8). The use of a random component is generally preferable. Minimisation has the advantage of making small groups closely similar in terms of participant characteristics at all stages of the trial. Minimisation offers the only acceptable alternative to randomisation, and some have argued that it is superior.[145] On the other hand, minimisation lacks the theoretical basis for eliminating bias on all known and unknown factors. Nevertheless, in general, trials that use minimisation are considered methodologically equivalent to randomised trials, even when a random element is not incorporated.

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