Micronutrients to reduce levels homocysteine and normalise levels of hormones involved in fertility in women with polycystic ovary syndrome (PCOS)

Protocol code: MED40-2016-01

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LABORATORY INVESTIGATOR:	Not applicable
PHASE OF THE STUDY:	Post-Marketing Study with an approved nutritional supplement (MoH code 80753)
STUDY DESIGN:	Prospective, randomized, controlled, parallel group, open label clinical study

Study synopsis

Study title

Micronutrients to reduce levels homocysteine and normalise levels of hormones involved in fertility in women with polycystic ovary syndrome (PCOS)

Study design

Prospective, randomized, parallel group, controlled vs no-treatment, open label study

Study phase:

Post-marketing study with an approved nutritional supplement

Study objectives:

Primary objective: Reduction of circulating homocysteine

Secondary objectives: Monitoring AMH changes and their correlation with the changes of the FTI.

Study sites

A - Struttura Complessa di Clinica Ostetrica e Ginecologica and B - Struttura Complessa di Medicina Interna e Scienze Endocrine e Metaboliche,

Azienda Ospedaliera Universitaria di Perugia, 06122 Perugia, Italy.

Population

The study population will consist of 56 adult women suffering from PCOS.

Study duration

The enrolment time will be 12 months. The target duration of the treatment/follow-up is 6 months per patient plus 3 months for data collection and analysis and 3 months for the preparation of the study report. Total duration 24 months.

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1. Scientific background

The Polycystic Ovary Syndrome (PCOS) is the most common ovulatory infertility and affects 6%-10% Of women in reproductive age[1]. Hyperandrogenism is a common mark of PCOS from early reproductive life till perimenopausal age and appears to be clinically linked to insulin resistance (IR)[2,3].

IR and/or compensatory hyper-insulinemia play a key role in the physiopathology of PCOS but the cellular mechanism responsible for insulin resistance is not yet clear. Reduction of circulating insulin and/or of IR obtained by weight reduction and/or insulin sensitizing treatments can improve the ovulation rate and the symptoms of hyper-androgenism[4-7]. On the other side, in the ACCORD trial the normalization of blood glucose in type 2 diabetes by using a range of aggressive therapeutic strategies including insulin injections was unsuccessful[8]. One possibility is that overriding IR may have exacerbated intracellular stress by increasing nutrient delivery to an already stressed cell. Indeed, Hoehn et al. (2009) have been able to show in adipocytes, myotubes, and transgenic mice that IR is induced by the excess of food as a compensatory mechanism whose effector is the production of superoxide anions within the mitochondria[9]. According to this model mitochondrial superoxide acts as a metabolic sensor of energy excess that decreases the sensitivity to insulin to protect cells from further oxidative damage. Thus, IR may be viewed as an appropriate response to increased nutrient accumulation as originally suggested by Unger (2003), representing part of the cells attempt to return to an energy neutral situation[10].

Based on this mechanism an oxidative overload may generate non appropriate IR, hyperinsulinemia and IGT in sensitive subjects. The susceptibility may be related to a genetic predisposition or to a specific environmental risk or to absolute/relative dietary shortages or a combination thereof. This mechanism might sustain the clinical onset of many forms of type 2 diabetes.

The same mechanism, when acting in women of reproductive age, will also cause endocrine perturbations that may be clinically evident in the form of PCOS even before or without the occurrence of a clinically evident diabetes. Indeed high insulin causes an increased synthesis of ovarian/adrenal androgens and a reduced hepatic synthesis of Steroid Binding Globulins (SBG). The final effect is an increase of the Free Testosterone Index (FTI). The relative excess of androgens will in turn affect the pituitary feedback with derangements of the release pattern of LH and FSH, which accounts for the disturbances of follicular development in PCOS, leading to ovarian cysts. The excess of androgens will also cause disturbances of lipid metabolism with or without overweight according to the individual setting of the leptin system[11]. Women developing overweight or obesity exert lower androgen and lower LH[12]. Thus, adiposity is one of the possible adaptive mechanisms to the excess of androgens triggered by IR and, in turn, by oxidative stress.

In summary, oxidative damage may be the primary event triggering IR and IGT and may explain the whole clinical picture of PCOS with the severity of overweight and of hyperandrogenism determined by the specific individual substrate. A recent metanalysis confirmed that circulating markers of oxidative stress are abnormal in women with PCOS independent of weight excess and that oxidative stress participates in its pathophysiology[13]. Compared with control women, patients with PCOS presented higher circulating concentrations of homocysteine (23% increase) and malondialdehyde

(47% increase), decreased glutathione levels (50% decrease) and increased superoxide dismutase activity (34% increase).

Among these possible markers of oxidative stress in PCOS, homocysteine (Hcy) is the best suitable in the clinical practice due to the easy access to the test and to the confirmed clinical predictivity. Plasma Hcy is elevated in PCOS proportionally to the IR level and to FTI whereas it is not related to other conditions of hyperandrogenemia like adrenal hyperplasia[14]. Hcy is also high in the follicular fluid of PCOS patients and was shown to predict low oocyte quality, low fertilization rate and low embryo quality in PCOS patients undergoing Assisted Reproductive Technologies (ART)[15]. High Hcy and IR correlate with the rate of recurrent pregnancy loss in PCOS patients and are therefore also related to their obstetric outcomes[16].

Hcy is at the metabolic crossroad of two major cell functions: the one carbon cycle (1CC) responsible for cell growth and differentiation, including DNA methylation, on one side; the transsulfuration pathways leading to glutathione (GSH) synthesis and antioxidant defenses on the other side. The impairment of antioxidant defenses (GSH production) and of the cell growth (1CC) always occur together and generate failure of Hcy recycling and its accumulation. Thus, besides being a marker of oxidative stress in PCOS, Hcy is also likely to play a pathogenic role and may be a relevant target for therapeutic interventions.

B vitamins, that are essential co-factors for the 1CC and that boost the natural antioxidant system, are well known to aid in lowering circulating Hcy and carry a therapeutic potential in PCOS. Folic acid supplementation during 3 months has been shown to decrease plasma Hcy in PCOS patients, whether or not they had IR and high FTI, but the study was not aimed neither powered to show any gain in the metabolic control[17]. Moreover, Hcy lowering may be not enough to correct the IR and FTI and a wider support to the antioxidant system may better work. This means supporting at the same time both the 1CC, to recycle Hcy as a methyl donor, and the trans-sulfurations, to use Hcy as a source of GSH boosting the antioxidant defenses. The critical substances for the 1CC are folic acid and betaine as the methyl donors, Vit.s B2, B3 and B12 as the co-enzymes and zinc to activate several enzymes working by means of zinc fingers. The transulfurations need zinc and Vit. B6 as the essential co-enzyme for CBS, the regulatory enzyme, and can be further boosted by a cysteine donor feeding GSH synthesis downstream to the CBS regulation.

The above substances may be however ineffective in subjects carrying a defective genetic variant of the key metabolic enzymes, which is a common finding. The enzyme MTHFR, which activates dietary folic acid, has two main defective variants whose frequency in the Mediterranean area may be very high. The incidence in Italian newborns was 50.7% for the 677T variant and 32.7% for the 1298C variant[18]. The heterozygous state for the defective variant 66G of MTRR, the enzyme that activates vit. B12 has also been shown to reach 28%[19]. The enzymes BHMT, transferring the methyl group from betaine to Hcy, and CBS, using Hcy for the synthesis of GSH also have genetic variants whose identity and function is far less understood. As a matter of fact genetic mapping is difficult and the outcome from a supplementation remain unpredictable, unless substrates downstream to the possible genetic blockade are used. Noteworthy, the MTHFR variant can be compensated with methylfolate, the MTRR variant with methylcobalamin, whereas the BHMT and CBS defects may be in part compensated by excess of substrates, respectively betaine and cysteine donors.

In summary, there are robust evidences supporting the role of oxidative stress as the trigger of high FTI in PCOS and its possible pathogenic role in the clinical disease. A dietary support to the 1CC and to GSH synthesis by means of methyl donors, B vitamins, zinc and cysteine has the potential to significantly improve the disease given that the genetic variants of the key enzymes are also compensated. The above approach has been recently indicated as capable of improving the reproductive performance of PCOS patients but the involvement of an Improved FTI in these outcome was not investigated[20].

The present pilot study is aimed at testing the effect of a nutritional supplementation fulfilling the above requirements in PCOS patients with increased FTI to test its efficacy in reverting the FTI and in improving the metabolic control.

2. Test Dietary intervention

Supplement Rationale

An oxidative attack could be neutralized by the endogenous metabolism, where the universal reducing agent is reduced glutathione (GSH). GSH synthesis is largely based on the transsulphuration of homocysteine (Hcy) formed within the so-called one carbon cycle - a ubiquitous biochemical pathway. The rate of Hcy transulfuration to GSH is redox-regulated as the key enzyme, cystathionine beta-synthase (CBS), contains a heme cofactor that functions as a redox sensor: once an oxidative imbalance occurs the heme is oxidized from the ferrous (Fe++) to ferric (Fe+++) state which activates the enzyme [11]. The greater the oxidative load, the greater the activation of GSH synthesis. Conversely, CBS undergoes allosteric activation by the end product of the one carbon cycle, S-adenosyl-methionine (SAMe), increasing its activity by 2.5-5 fold [12]. In summary, GSH synthesis is triggered by the oxidative load but is effective only as long as SAMe is available, i.e. as long as an adequate dietary intake of folates and other Group B vitamins is in place.

These physiologic mechanisms may be hampered by an excess of environmental toxicity and/or bad feeding and/or by a weaker genetic substrate. In particular, two key enzymes of the one carbon cycles, namely MTHFR (responsible for the activation of dietary folic acid) and MTRR (responsible for the activation of dietary vitamin B12), occur at high frequency in the general population and are responsible for defective oxy-redox balance, infertility and resistance to the standard folic acid and vitamin B12 supplementation.

A suitable oral nutritional support intended to feed and activate the one carbon cycle and the transsulphuration pathway should contain a full range of group B vitamins, namely B2, B3, B6, B12, and folic acid mandatory for recycling homocysteine. N-Acetyl-Cysteine and/or L-cystine, the only orally bioavailable precursors for the synthesis of GSH, and Zinc are also important for homocysteine recycling. Zinc is as an essential co-factor for two key enzymes, dihydrofolate reductase and methionine synthase, and does not have any body reservoirs so that the circulating level is largely dependent on daily intake. All these substances are contained in the test product Impryl that also contains the active forms of folic acid (i.e. methyl folate) and of vitamin B12 (i.e. methyl cobalamin) so to overcome the genetic defect if it was in place.

Ingredients

Bulking agent: microcrystalline cellulose; betaine hydrochloride, L-cystine, anticaking agents: mono- and diglycerides of fatty acids, magnesium stearate, silicon dioxide; zinc bisglycinate, stabilizer: cross-linked sodium carboxy methyl cellulose; niacin (nicotinamide), vitamin B6 (pyridoxine hydrochloride), vitamin B2 (riboflavin), folic acid ((6S)5-methyltetrahydrofolic acid, glucosamine salt), vitamin B12 (methylcobalamin).

Nutritional characteristics	Per day (1 tab)	% of NRV
Betaine	200 mg	
L-cystine	200 mg	
Niacin	16 mg	100%
Zinc	10 mg	100%
Vitamine B6	1.4 mg	100%
Riboflavin	1.4 mg	100%
Folic acid (methylfolate)	400 μg	200%
Vitamine B12 (methylcobalamin)	2.5 μg	100%

NRV: nutrient reference value according to Reg. (EU) n.1169/2011

How supplied

Cartoon boxes containing each 2 PVC/aluminium blisters of 15 film coated tablets, for a total of 30 tablets per box. Gluten free, Lactose free.

Instructions for use

The recommended daily dose is 1 tablet per day to be swallowed with a small amount of water, preferentially without food. The assumption of a smaller amount does not guarantee full coverage of the daily need in all subjects. Do not exceed the indicated daily dose.

Impryl is a support to a balanced diet and there is no fixed duration for such support. However, patients with reproductive problems should consider that the maturation process of gametes takes about 4 months to be completed. In addition, those subjects with known issues of homocysteine metabolism should consider that the supportive effects will last only as long as the product is assumed.

Warnings

Keep out of reach of children and do not give to children below 3 years of age.

Nutritional supplements must be used as part of a healthy lifestyle and should not be used as a substitute of a varied and balanced diet.

Storage

Store in a cool dry place away from light, humidity and direct sources of heat. The best-before date refers to the undamaged and properly stored product.

3. Study Objectives

The primary objective of the study is to demonstrate that the nutritional supplementation of concern (Impryl) is effective, as compared to no-treatment, in decreasing the circulating homocysteine

The secondary objectives of the study are to demonstrate that the nutritional supplementation of concern (Impryl) is effective, as compared to no-treatment, in modulating the circulating homocysteine and Anti-Mullerian Hormone (AMH) and to demonstrate that these changes correlated with the improvements of the FTI.

4. Investigational plan

4.1 Study design

Two centre, randomized, controlled vs no treatment, parallel group, open label study of an approved nutritional supplement.

At visit 1 the patients will be randomised in 2 groups: A - intervention and B - control. Patients randomised in group 1 will assume the nutritional supplementation during 3 months, patients belonging to group 2 will serve as non-treated controls. All patients will be visited again after 3 months (visit 2).

4.2 Study setting

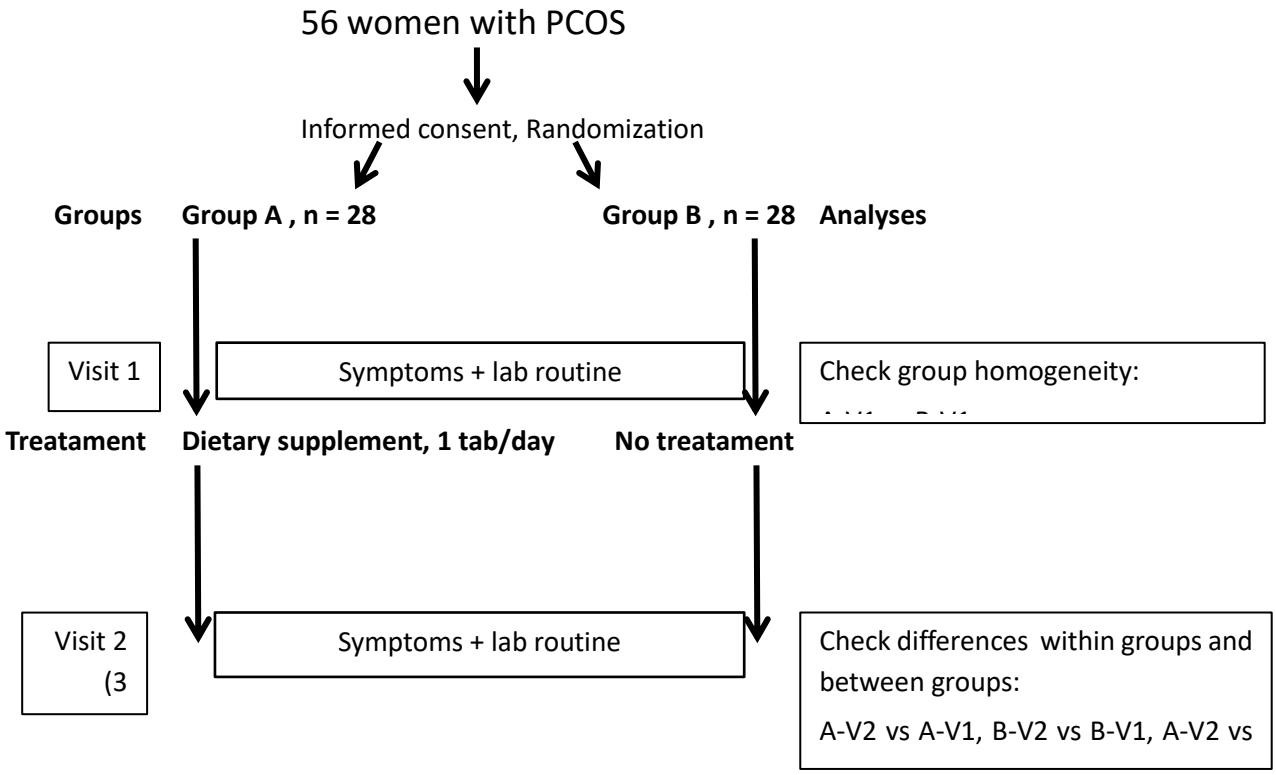
The study will be conducted at Struttura Complessa di Clinica Ostetrica e Ginecologica and at Struttura Complessa di Medicina Interna e Scienze Endocrine e Metaboliche, Azienda Ospedaliera Universitaria di Perugia, 06122 Perugia, Italy. The study will include patients normally referring to the clinics for gynaecological consultations.

4.3 Study duration

The enrolment time will be 12 months. The target duration of the treatment/follow-up is 6 months per patient. per patient plus 3 months for data collection and analysis and 3 months for the preparation of the study report.

The total study duration will be 24 months.

4.4 Study scheme



5. Study population

5.1 Type of patients

The study population will be constituted of women referring for gynaecological problems and diagnosed as affected by PCOS.

The referring women will be checked for their adherence to the inclusion and exclusion criteria here below listed. All the consecutively referring women will be screened up to the enrolment of a total of 56 patients.

5.2 Inclusion criteria

- Female gender, aged at least 18 years or older;
- Clincal diagnosis of PCOS according to the Rotterdam criteria[21];
- Written informed consent

5.3 Exclusion criteria

Considering the clinical practice-oriented nature of this post-marketing study there are no strict exclusion criteria set up. However, the following criteria should be checked at the time of study entry:

- Ongoing pregnancy;
- Ongoing pharmacological treatment: oral antidiabetic drugs, insulin, antihypertensives, any hormone;
- Ongoing systemic or endocrine diseases including hypertension and tyroid diseases;

- Assumption of any other dietary supplement during 1 week before enrolment or expected during the study period;
- Known or suspected hypersensitivity to any ingredient of the intended nutritional support;
- Any other condition that, in the opinion of investigator, could preclude evaluation of the response;
- Any condition that may hamper the ability of the enrolled subject to release the consent to participate to the study.

5.4 Participant withdrawal

The possible reasons for the withdrawal of a participant from the study are the following:

- Judgement by the investigator at any point during the study that the participant's safety was jeopardized or potentially compromised;
- Withdrawal of participant consent for any reasons

The participant will be clearly informed that the withdrawal of the consent to the study may occur at any time even without explaining the reason and that such withdrawal will not decrease by any means the level of clinical assistance received.

6. Treatment

6.1 Randomisation

A randomization list will be prepared using a pseudo-random number generator and will contain 28 randomisation positions for each of the two study enrolment sites. The randomization list will be divided in balanced blocks size 4, each including 2 positions for active intervention and 2 positions for no treatment for a total of 6 blocks per study enrolment site. The randomization code will be made by a letter indicating the enrolment site (A or B), a number from 01 to 10 indicating the block, and a letter A to D, indicating the intra-block position. I.e., the first position of centre A will be A01A, and so on.

At time of enrolment the first available position from the list will be assigned to the patient and the randomisation code will be duly noted on the relevant Case Record Form (CRF).

6.2 Treatment modalities

At time of enrolment each patient will be delivered a treatment set (Impryl, 3 boxes of 30 tablets) instructed on how to assume the treatment. The prescribed dose will be 1 tablet of Impryl per day. The tablets will be swallowed with the help of some water between meals at any preferred day time. Skipped doses will not be compensated.

Participants will take the first treatment dose on the day of enrolment and will continue for 3 months. Patients whose treatment will last less than 2 months will be excluded from the per-protocol analysis.

6.3 Treatment compliance

Participants will also be instructed on how to keep records of their actual compliance to the treatment by taking note of doses skipped as well as of the total amount of Impryl boxes and tablets

consumed. To do so, they will be provided a Patient Diary. The patients will be also asked to report about major changes of their lifestyle (e.g. occupation, diet, smoking) occurring during the study period.

6.4 Discontinuation of treatment

The treatment may be discontinued due to patient's decision or to medical reasons at investigator's judgement. Medical reasons for treatment discontinuation include, but are not limited to, worsening of symptoms and severe, serious adverse events. Patients who discontinued the treatment will be excluded from the per-protocol analysis.

6.5 Adverse events

An Adverse Event (AE) is defined as any event occurred during a clinical trial, including modifications of the concomitant illnesses, or any accident, which could impair the health status of the subject.

A causal relationship with the study treatment does not have to be necessarily implied. Any AE will be duly reported on the relevant CRF.

AEs will be classified and recorded according to seriousness, intensity and causality as here below described.

Seriousness

- Definition of serious Adverse Events (SAE)

A “Serious Adverse Event” is defined as an adverse experience that fulfils at least one of the following criteria:results in death, is life threatening, requires inpatient hospitalisation or prolongation of existing hospitalisation, results in persistent or significant disability/incapacity, is a congenital anomaly/birth defect, overdose, cancer.

- Definition of Non-Serious Adverse Events

Any adverse events, which do not fall into the above-described categories, are defined as Non Serious. The evaluation of the AE as serious or not-serious will be made up independently of any attribution of causality.

Intensity

Any AE must be judged for intensity and will be graded on the following:

- MILD: causing no limitation of usual activities; the subject may experience mild discomfort;
- MODERATE: causing some limitation of usual activities; the subject may experience annoying discomfort;
- SEVERE: causing inability to carry out usual activities; the subject may experience intolerable discomfort or pain.

Causality

Any AE must be judged for causality. The relationship of an AE to the treatment will be graded on the following:

- RELATED: an AE is to be considered as definitely related to the test treatment if the following criteria are met:

1. The AE follows a reasonable temporal sequence from administration of the drug;

2. The AE follows a known response pattern to the suspected drug;

3. The AE is confirmed by improving on stopping the drug (dechallenge) and reappearance of the reaction on repeated exposure (rechallenge).

- POSSIBLE: an AE is to be considered as possible test treatment-related if the following two conditions are met:

1. There is a reasonable temporal relationship between the administration of the medical device and the AE; but

2. None of the conditions of RELATED is met or an alternative explanation for the AE is more likely. An AE will also be classified as possible test treatment-related if there is sufficient information to know that an AE occurs during test treatment but the temporal relationship is unknown.

- UNRELATED: An AE is to be considered as unrelated if any of the following tests are met:

1. There is no reasonable temporal relationship, or

2. A causal relationship between the test treatment and the AE is biologically implausible (e.g. a subject in a clinical trial is injured as a passenger in a car accident), or

3. A clearly more likely alternative explanation is present.

- UNCLEAR: a reported AE is to be classified as not clear if the available information does not permit the assessment of a causal relationship in one of the above categories.

Immediately reportable adverse events

During the study, if any serious or unexpected AE occurs, the Investigator(s) will immediately inform the competent health authorities. In addition, the Investigator(s) will perform appropriate diagnostic and therapeutic measures, and will interrupt the study treatment, if appropriate.

7. Study procedures

7.1 Summary of study procedures

Women referring due to gynecological problems and diagnosed PCOS according to the Rotterdam criteria[21] will be offered to participate in the study. After a preliminary check of the inclusion/exclusion criteria an informed consent will be obtained and a randomisation position will be assigned. It is noted that the dosage of testosterone and SHBG necessary for the calculation of the FTI are already included in the standard lab routine of the concerned study centres for the concerned patients, therefore these data will be available for all the potential study subjects before enrolment.

Those patients randomised for the active intervention will receive instructions on how to assume the treatment and how to report on treatment compliance. All patients will be instructed on how to report on major lifestyle changes during the study period. Patients will be planned for a post-treatment visit about 3 months later. Patients with an interval between visit 1 and 2 shorter than 2 months will be excluded from the per-protocol analysis.

7.2 Visit 1: screening, enrolment and treatment allocation

Women referring for to gynaecological problems and diagnosed PCOS will undergo a preliminary screening to verify the compliance with the inclusion/exclusion criteria. The patients fitting all the criteria will be asked an informed consent for the study and, if obtained, will be assigned a randomization position.

All patients, independently of their group assignment, will undergo an interview to record age, height and weight and to collect information on their menstrual cycles (age of menarche, date of last menstruation, occurring of menstruation and ovulatory symptoms during the latest 6 months). It is noted that the dosage of testosterone and SHBG necessary for the calculation of the FTI and the dosage of fasting homocysteine and of AMH are already included in the standard lab routine of the concerned study centres for the concerned patients and therefore already available at time of visit 1. Accordingly no further blood sampling will be required.

Those patients randomised for the nutritional intervention will receive a treatment set (3 boxes of Impryl, 30 tablets each) detailed instructions on how to assume the treatment and on how to report on the actual compliance to the treatment. All patients, both treated and controls, will be asked to refrain from assuming other nutritional supplements during the study period and, should they assume any, to report on the type and amount assumed. Finally, all patients will receive instructions on how to report on the occurrence of the menstrual cycle and of ovulation symptoms during the study period. All of these information will have to be noted on a duly prepared patient's diary that will be delivered to the patients with the due instructions.

The date for next visit (end of treatment) will be scheduled 3 months later trying to fit with day 3-5 of the follicular cycle. The occurrence of menses before the planned visit will be confirmed by a telephone call and if needed the appointment will be re-scheduled to fit with the appropriate day of the cycle.

7.3 Visit 2: Treatment response and compliance

Visit 2 will occur 3 months after visit one at day 3-5 of the follicular cycle.

All subjects will undergo a new blood sampling. Patients belonging to the active intervention group will also be interviewed to check the compliance to the treatment and about possible adverse events. All patients will be questioned on the possible assumption of other nutritional products and about major changes of the lifestyle (diet, smoking, occupation) during the study period. Finally, all patients will report on their menstrual activity during the study interval. The information on compliance, adverse events, other supplements and menstrual cycle will be collected from the patient diary and integrated with the interview.

7.4 End of the study

The end of the study is defined as the day of the last assessment of the latest complete patient.

However, patients will be informed that they are free to withdraw from the study at any time and for any reason without affecting their subsequent treatment. In addition, a subject may be removed from the study if, in the investigators' opinion, it is not in the best medical interest of the subject to continue. The date the subject is withdrawn from the study and the reason for withdrawal will be recorded. Withdrawn patients will not be replaced.

8. Criteria of evaluation

8.1 Primary efficacy endpoint

The primary efficacy end-point is the improvement of the FTI after 3 months of dietary supplementation in comparison to the control group not receiving any supplementation during the same period of time. The outcomes from group A at visit 2 vs Group B at visit 2 and of both groups at visit 2 vs baseline will be used for this purpose.

8.2 Secondary and ancillary efficacy endpoints

The secondary efficacy endpoint will be the following:

- Decrease of fasting blood homocysteine and of AMH
- Correlation between homocysteine and AMH changes and the improvement of FTI

9. Data management

9.1 Source Data

All clinical records, laboratory reports as reported into the study centre archive and diaries as delivered by the patients are considered source data. Upon request of the competent authorities, the investigator/institution will permit inspection providing direct access to source documents.

9.2 Retention of Documentation

All study related documentation shall be retained for a period of minimal 5 years after the study completion or longer if deemed necessary. It is the investigator's responsibility to ensure these are filed in a secure place.

9.3 Data collection and processing

Clinical data will be copied from the source records and entered into a duly formatted Case Record Form (CRF). The data transcription will occur in real time, i.e. as soon as possible. At the end of the study the data from the CRFs will be entered into a database suitable for statistical analyses.

10. Statistics

10.1 Sample size

This is a pilot study and no historical data are available to formulate a statistical hypothesis on the sample size. Therefore, it is established that a target number of 56 patients, 28 per group, will constitute a clinically significant sample allowing the detection of a possible trend of efficacy. Should such a trend occur, it will be available for a sample size calculation in future studies on the same objective.

10.2 Statistical methods

Basic descriptive statistics (mean, range and standard deviation as appropriate) and statistical analysis will be performed using IBM SPSS statistics on a personal computer.

For continuous variables the Wilcoxon test or the Mann-Whitney test will used. These two tests have greater efficiency than the t-test on non-normal distributions and they are nearly as efficient as the t-test on normal distributions. The Wilcoxon test will be used to compare two continuous variables in a same group of patients. The Mann-Whitney test will be used to compare continuous variables in two independent groups. In both cases, the null hypothesis is that the values are the same and the alternative hypothesis is that the values are different. A p value < 0.05 will be assumed as statistically significant.

The independence of two categorical variables will be tested by the independence Chi2 test, also known as the test of homogeneity. The null hypothesis is that the two variables are independent, the alternative hypothesis corresponds to the variables having an association or relationship. A p value < 0.05 will be assumed as statistically significant.

11. Ethics

11.1 Declaration of Helsinki

The study will be performed according to the revised Declaration of Helsinki for biomedical research involving human subjects (Declaration of Helsinki, Seoul, South Korea amendment of 2008).

11.2 Independent Ethics Committee Review and Approval

The relevant Ethical Committee will be notified in accordance with local regulations and the due approval will be obtained before implementing any study related activities.

Any major amendment of this protocol that seems appropriate, as the study progresses, will be reported.

11.3 Informed Consent

All patients who are eligible for inclusion will be informed of the aims and nature of the study. They will be informed of the fact that all clinical data concerning them will be treated confidentially, but that their medical records may be reviewed by authorized persons other than their treating physician for study purposes. All patients will be informed that participation is voluntary and that the patient is allowed to refuse participation at any time, without consequences for his or her further treatment. Subjects will sign informed consent forms at baseline after reading a subject information sheet and discussing the study with the participating physician. The physician will describe the purpose of the study and timing. Consent will include permission to contact any treating physician to follow up on specific safety outcomes. Subjects will be informed that the physician will contact them during the follow-up phase to ask a predefined set of safety related questions or to update alternative contact information. Subjects will be asked to provide personal contact information (e.g., telephone number, home and e-mail address) and information regarding alternative contacts (e.g., relative, friend, general practitioner) in case they cannot be reached. In the event that a subject cannot be reached during the follow-up phase, physician will attempt to reach an alternative contact to re-establish contact with the subject, or, in the event of a subject's death, to confirm the cause of death. Subjects may be contacted between two follow-up points to confirm that their personal contact information is correct.

Subjects retain the right to withdraw their consent at any time during the study. If they wish to withdraw consent during the follow-up phase, they may inform the investigator and no further direct contact will be made.

Documented informed consent must be obtained for all patients before they are registered. The informed consent procedure must conform to the ICH guidelines on Good Clinical Practice and must be in accordance with national and local regulatory requirements.

11.4 Data Protection

All personnel involved in the project will observe or work within the confines of the European Data Protection Directive as interpreted by the national law.

11.5 Risk-benefit and ethical consideration

The patients included in the present study, i.e. symptomatic PCOS ladies, do not benefit of pharmacologic treatments unless other complications are in place and dietary advices are the most common prescription from the physician. The adherence to dietary regimens is however modest, especially in the long run. These ladies are also frequently assuming dietary supplements, often by self-prescription and based on oral antioxidants that may interfere with their metabolism.

The dietary supplementation tested in this study is totally free of antioxidants and contains a series of micronutrients that are known to be part of the Mediterranean diet and to positively influence the metabolism of subjects assuming a defective diet[19]. These consist in B vitamins, aminoacids and zinc, all of them widely tested and used as dietary supplements. Accordingly, these substances constitute a mere integration to the standard diet and are not expect to create any imbalance. In summary, the proposed intervention is not expected to create any harm to the treated subjects.

It is also to be noted that the study procedures will not create any further burden of medicalization for these patients if not the need to undergo to a second visit at 3 months, which is however often indicated independently of the study participation.

On the other side, those subjects assuming the dietary integration, besides the opportunity to improve their PCOS symptoms and signs, which is the study question, are expected to benefit in their general health and well being from a period of better complete feeding. In addition, the data resulting from the study may help in individuating supplementations and/or feeding habits with a positive effect on their disease with a potential long term benefit for all those suffering from the same condition.

Finally, the data resulting from the study will be published on a peer-reviewed journal and will contribute to the scientific knowledge in the field.

Based on the above considerations the present study may be assumed as carrying a positive risk to benefit ratio.

12. Final report and publication

A final integrated report containing clinical, laboratory and statistical data will be prepared. This report and relevant report sections will be reviewed and signed by the Investigators. It will be regarded as confidential.

The study report will be used as the source of data for editing a scientific publication on a peer-reviewed journal, likely within 12 months from the conclusion of the study. The study itself will be duly announced on an international clinical trial directory and the data will be published independently of the final, positive or negative, study outcomes.

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