IMPACT OF UBIQUINOL SUPPLEMENTATION ON ENDOTHELIAL FUNCTION IN SUBJECTS AT RISK OF CARDIOVASCULAR DISEASE DEVELOPMENT: A DOUBLE BLIND, RANDOMIZED, PLACEBO – CONTROLLED, PARALLEL GROUPS, SPONTANEOUS CLINICAL STUDY

STUDY CODE: QHHC-FMD-PILOT

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Approval of the Protocol

The study protocol, entitled IMPACT OF UBIQUINOL SUPPLEMENTATION ON ENDOTHELIAL FUNCTION IN SUBJECTS AT RISK OF CARDIOVASCULAR DISEASE DEVELOPMENT: A DOUBLE BLIND, RANDOMIZED, PLACEBO – CONTROLLED, PARALLEL GROUPS, SPONTANEOUS CLINICAL STUDY, final version 08/07/2016, Amendment no. 1 08/11/2016, was written in compliance with the current version of the Declaration of Helsinki, the principles of Good Clinical Practice implemented in the Ministerial Decrees 15/07/1997 and 12/05/2006 and any applicable regulatory requirement(s). The clinical study will be carried on accordingly.

PRINCIPAL INVESTIGATOR

Signature: ..... Date: .....

Dr. Roberto Antonicelli

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Abbreviations

Abbreviation	Term
AE	Adverse event
ALX	Aortic Augmentation Index
ATP	Adenosine triphosphate
BMI	Body Mass Index
BP	Blood Pressure
CRF	Case Report Form
CoQ10	Ubiquinone (Coenzyme Q10)
FMD	Flow Mediated Dilation
GCP	Good Clinical Practice
GTN	Glyceryl trinitrate
ICD	International Classification of Diseases
ICH	International Conference on Harmonization
IEC	Independent Ethics Committee
ITT	Intention to treat
LDL	Low Density Lipoprotein
LVSD	Left Ventricular Systolic Dysfunction
NO	Nitric oxide
OTC	Over-The-Counter
PI	Principal Investigator
PP	Per protocol
PWV	Pulse Wave Velocity
RCT	Randomized Controlled Trial
ROS	Reactive Oxygen Species
SAE	Serious Adverse Event

Synopsis

TITLE	**Impact of ubiquinol supplementation on endothelial function in subjects at risk of cardiovascular disease development: a double blind, randomized, placebo – controlled, parallel groups, spontaneous clinical study**
OBJECTIVES AND ENDPOINTS	To evaluate the impact of a supplementation with ubiquinol (reduced form of ubiquinone or reduced CoQ10) on endothelial function and oxidative stress. <u>Primary Endpoint</u> Change in endothelium-dependent vasodilation (by means of the flow mediated dilation - FMD - technique) at week 8; comparison between both treatment groups and placebo group. <u>Secondary Endpoints</u> • Change in endothelium-dependent vasodilation (by means of the flow mediated dilation - FMD - technique) at week 4; comparison between both treatment groups and placebo group. • Change in red / ox CoQ10 levels at week 4 and 8; comparison between both treatment groups and placebo group. • Evaluation of plasma levels of nitric oxide quantified in terms of its stable reaction product (nitrites and nitrates) through the Griess reaction (Tsikas 2007) at week 4 and 8; comparison both between treatment groups and placebo • Evaluation of plasma levels of peroxinitrite (evaluation of oxidative inactivation of NO to peroxinitrite using fluorescent probes (Rios 2016)) at week 4 and 8; comparison both between treatment groups and placebo. • Evaluation of susceptibility to oxidation of plasma lipoprotein

	quantified as kinetic of conjugate dienes formation following copper exposure (Mazzanti 2015) at week 4 and 8; comparison both between treatment groups and placebo.
METHODOLOGY AND STUDY DESIGN	Double-blind, randomized, placebo – controlled, parallel groups clinical study with 3 intervention groups to be carried out on 51 subjects. Three visits will be envisaged to the test facility: a Screening Visit and 2 Measurement Visits will be performed, each lasting approximately 1 hour. There will be 4 weeks between each visit. At each Visit subjects should have to: •≥12-h overnight fast •Avoid caffeine for 72h •Avoid high-fat food the day before •Avoid tobacco for at least 4-6h before •Abstain from exercise for >12h before the test During Screening Visit the eligibility criteria will be verified and, if fulfilled, each subject will be randomized to one of the 3 study groups after signing the Informed Consent Form. During each visit a short physical examination will be performed and a blood sample will be collected from the vein in the left arm to evaluate CoQ10 levels (red / ox CoQ10 levels) and levels of the markers of oxidative stress. After acclimatisation and rest for ≥ 30 minutes blood pressure (triplicate, first discarded) and FMD (baseline or Week 4 and 8 measurement) will be measured. At Screening Visit and at Week 4, subjects will receive the assigned test product (according to randomization) for consumption at home during the coming 4 weeks and leave the facility.
PRODUCT TO BE TESTED	• GROUP A: Ubiquinol (200 mg daily) • GROUP B: Ubiquinol (100 mg daily) • GROUP C: matched placebo
DURATION OF TREATMENT	Total duration of treatment will be 8 weeks.

SAMPLE SIZE	Fifty-one subjects, considering a 20% dropout rate, are a sample size considered as appropriate for this study (17 for each group).
INCLUSION CRITERIA	This study will include male and post-menopausal female subjects. • Male aged 35 - 65 years (35 and 65 included). • Post-menopausal female (without a period for more than 1 year), until 65 years (65 included). • Subjects enrolled for primary prevention but never treated (only life style modification suggested, according guidelines). • FMD level measured between: 2,5% < FMD baseline value < 6% (at Screening Visit). • BMI: 18.5 - 29.9 kg/m^2. • LDL Cholesterol levels between 130 and 200 mg/dl (based on previous evaluation, performed within 1 month from the Screening Visit).
EXCLUSION CRITERIA	A subject will not be eligible for inclusion in this study if any of the following apply: • Use of any cardiovascular disease related drugs prior to Screening Visit. • Participation in any clinical trial while participating in this trial. • Greater than 5% change in body weight within 1 month of Screening Visit. • Subjects playing competitive physical activity. • Subject taking lipid-altering drug therapy within four weeks prior to Screening Visit. Also excluded are supplements known to have significant lipid altering effects, such as niacin (>100 mg per day), garlic (> 600 mg per day), omega-3 fatty acids (> 1 g omega-3 fatty acids per day), red yeast rice extract, phytostanols / phytosterols (> 0.5 g per day), soluble fiber (>1 g per day), chitosan (> 1 g per day) and conjugated linoleic acid (CLA; > 3 g per day).

	• Subject taking any concomitant treatment with phosphodiesterase inhibitors (e.g. sildenafil citrate, tadalafil, vardenafil) and donors of NO (nitric oxide), like other long-acting derivatives of GTN (glyceryl trinitrate), such as isosorbide dinitrate and amyl or butyl nitrite. • Excluded concurrent medications are: systemic corticosteroids (nasal and inhaled corticosteroids are permitted), orlistat, bile acid resins, no more than 1 g of prescription omega-3 fatty acids, cyclical or non-continuous hormone therapy (estrogen or testosterone). • No more than 2 alcoholic units per day. Units are defined as 12 grams of ethanol, e.g. a small glass (125 ml) of medium gradation wine, a can of beer (330 ml) of average gradation or 40 ml dose of liquors. • Consumption of flavonoids-enriched products. • Consumption of vitamin C-enriched products or supplements containing vitamin C. • Has a diagnosis of type 1 or type 2 diabetes mellitus, hepatic or renal impairment or diseases, thyroid disorders. • Known cardiovascular disease or stroke, except for conditions that are deemed clinically insignificant by Principle Investigator or Sub-investigator, or study site physician (e.g. clinically insignificant atherosclerotic lesions observed by imaging studies). • History of significant gastrointestinal disease such as severe constipation, diarrhea, malabsorptive disease, inflammatory bowel disease (e.g. Chron's disease, ulcerative colitis). • History of severe psychiatric illness which in the opinion of the investigator would interfere with the optimal participation in the study. • History if cancer within 5 years of Screening Visit (except for successfully treated basal and squamous cell carcinoma of

	the skin). • Known HIV seropositivity. • History of bariatric surgery. • Allergic to the test products or placebo. • Smokers > 10 cigarettes/day. • Individuals who in the opinion of the principal investigator have a risk of non-compliance to the study procedures or who are otherwise not appropriate to include in this clinical trial.

Study flow chart

Visits	V0 Screening visit /Baseline/ Randomization visit	V1	V2 End of Study visit
Week (+/- 2 days)	**0**	**4**	**8**
Informed Consent	x		
Inclusion/Exclusion Criteria	x	x	
Medical history	x		
Demography	x		
Concomitant medication	x	x	x
Physical examination	x	x	x
Randomization	x		
Blood sample	x	x	x
FMD	x	x	x
Study product supply	x	x	
Study product return		x	x
Diary supply	x	x	
Diary return		x	x
AE/SAE evaluation		x	x

1. Background and rationale

1.1 Background Information

Cardiovascular diseases are the leading causes of morbidity and mortality in Europe and all over the world (Ezzati, 2002). Major cardiovascular risk factors, including hypertension and diabetes mellitus mainly contribute to these events causing alterations in cardiovascular structure and function, which occur also in subjects with moderately increased blood pressure and/or hypercholesterolemia, for which a pharmacological treatment is usually not advisable and not cost-effective.

The main international guidelines for cardiovascular disease prevention strongly support the implementation of healthy food choices as a cost-effective preventive tool, in particular for patients with subclinical level of the main cardiovascular risk factors. Nowadays the scientific research is focused to identify preventive dietary strategies to contain the damage induced by oxidative stress, which is a causal factor in the pathogenesis of cardiovascular diseases.

In healthy conditions, the endothelium plays a major role in the maintenance of vascular function and structure through the production of the vasodilating compound nitric oxide (NO), derived via the activity of the NO synthase enzyme. In pathological conditions, endothelium produces vasoconstrictor substances including prostanoids and reactive oxygen species (ROS) (Luscher, 1997). A balance between these vasoconstrictors, which generally also induce cell growth, and NO, which inhibit cell growth, is necessary for the maintenance of normal vascular structure and function. Most of the major cardiovascular risk factors, including hypertension hypercholesterolemia, and diabetes, are characterized by an increased production of ROS, which in turn cause NO breakdown leading to endothelial dysfunction (Taddei, 1998).

Non-invasive assessment of endothelial function by means of flow-mediated dilation (FMD) of the brachial artery technique has increasingly been applied in physiological studies as well as cohort and intervention studies in several labs all over the world (Corretti, 2002; Harris, 2010; Thijssen, 2011; Flammer, 2012). It is evaluated by high-resolution ultrasounds (Ghiadoni, 2008). The endothelial stimulus is represented by the increase in shear stress caused by forearm reactive hyperemia. The latter is obtained by inflating a cuff at supra-systolic levels for 5-minute ischemia. The FMD is the consequent endothelium-dependent vasodilatation in the brachial artery, which is measured following reactive hyperemia. The

endothelium-independent vasodilatation can be obtained by the administration of low-dose sub-lingual glyceryl trinitrate (GTN).

It has been demonstrated that FMD is NO-dependent and its FMD impairment in hypertensive patients is caused by decreased NO-availability (Ghiadoni, 2007). FMD can be negatively modulated by physiological stress (Ghiadoni 2000) and it is reduced in the presence of cardiovascular risk factors (Ghiadoni, 2008). More importantly, a meta-analysis of published reduced showed that FMD is independently associated to poor cardiovascular prognosis.

Reduced FMD in hypertensive patients can be reversed by some antihypertensive drug classes (Ghiadoni, 2003). Moreover, a placebo controlled crossover study showed that combined treatment with antioxidant vitamin C and E ameliorated FMD in newly diagnosed mild hypertensive patients, confirming the central role of oxidative stress in inducing endothelial dysfunction (Plantinga, 2007).

The FMD technique is non-invasive, well tolerated by the patient, and requires relatively simple technical equipment: for these reasons, it is wrongly considered simple to perform. It is clear, however, that minor changes in the methodological approach can critically impact the nature and magnitude of the FMD response, as well as its validity, reproducibility and clinical significance. In the last decade many efforts have been made to standardize the technical approach and set minimum standard requirements for FMD measurement (Corretti, 2002; Harris, 2010; Thijssen, 2011; Flammer, 2012).

There is growing evidence that the analysis of arterial stiffness is playing a major role in the clinical assessment of cardiovascular risk, particularly in hypertensive patients, given that it represents the main mechanism responsible for the increase of systolic blood pressure and pulse pressure during ageing (Laurent, 2006). The arterial system generates a sphygmic wave (incidental forward wave) whose characteristics mainly depend on the aortic stiffness. The pulse wave velocity (PWV) is directly proportional to the arterial stiffness and is measured as the ratio between the distance of two sites of the arterial tree and the time needed by the wave to cover this distance. The PWV clinically measured at the carotid-femoral level by applanation tonometry represents a reliable index of the central (aortic) arterial stiffness (Van Bortel, 2012). The forward wave undergoes a reflection in any point of structural and functional discontinuity of the arterial tree, thus generating a reflection wave (reflection backward wave), which depends on the condition of the microcirculation. When the elastic

arteries become rigid, such as with ageing and arterial hypertension, the increase of speed of the forward wave causes the reflection wave is returned to the aorta during the systolic phase, where it is summed with the incidental wave, thus increasing systolic a pulse pressure. The precocity of backward of the reflection wave can be identified with the aortic augmentation index (AIx), as the ratio between the amplification pressure (i.e. the difference between the reflection and the incidental waves) and the central pulse pressure. The aortic pressure wave may be obtained from the peripheral wave (radial artery) by using a transformation function. The AIx represents an integrated index, since it depends by both the site of wave reflection (microcirculation) and by the central and peripheral arterial stiffness. Longitudinal studies have demonstrated the independent predictive value of the arterial stiffness, mainly evaluated as aortic PWV, for cardiovascular morbidity and mortality (Vlachopoulos, 2010). For this reason, recent guidelines have included the aortic PWV in the assessment of organ damage in the hypertensive patient (Mancia, 2007).

Using applanation tonometry for physio-pathological and interventional studies, it has been demonstrated the presence of increased arterial stiffens at very early stages such as in subjects at risk of developing diabetes (Ghiadoni, 2008) or in patients without classical risk factors such as those with rheumatoid arthritis (Bazzichi, 2009). Further studies showed whether the presence of metabolic syndrome (Plantinga, 2008) or secondary form of hypertension (Bernini, 2008) caused a further increase of the indexes of arterial stiffness in hypertensive patients. It has been shown that the increase in arterial stiffness and wave reflection in newly diagnosed hypertensive patients could be reduced by short-term oral antioxidant therapy with vitamin C and E (Plantinga, 2007).

In summary, vascular functional and structural changes are responsible for increased arterial stiffness and peripheral wave reflection, leading to higher central blood pressure (Ghiadoni, 2009). These changes have been recently related to cardiovascular risk, target organ damage and poor prognosis in prospective studies (Lerman, 2005; Vlachopoulos, 2010). Therefore, endothelial dysfunction and arterial stiffness are becoming promising target of interventions (Luscher 2012; Ghiadoni, 2009).

1.2 Rationale for Study

Coenzyme Q10 (CoQ10, also known as ubiquinone -10) is a powerful lipophilic physiological antioxidant; it is part of the mammal mitochondrial electron transport chain and plays an important role in cellular respiration and ATP production. CoQ10 exists in an oxidized form (ubiquinone) that must be converted into the biologically-active, reduced form (ubiquinol) before being utilized. CoQ10 is naturally synthesized by the body and can be obtained in an oxidized or mixed form from the diet in foods such as corn, nuts, soy, broccoli, meat and fish. Oral supplementation of CoQ10 is able to increase CoQ10 levels in plasma, white blood cells and platelets. Supplementation with CoQ10 has been shown to positively affect heart performance in congestive heart failure and ischemic heart disease, and has been shown to have a clinically significant blood pressure lowering effect (Gao et al 2012).

The effect of oral CoQ10 supplementation on endothelial function has been studied in randomized controlled trials focused on the effect of CoQ10 supplementation on endothelial function as measured by FMD of the brachial artery (Gao et al 2012).

In the systematic review and meta-analysis by Gao et al 2012, evidence published from 5 randomized, double-blind, placebo controlled clinical trials that investigated the effect of CoQ10 therapy on endothelial function as measured by FMD, were summarized. It was found that CoQ10 therapy substantially enhances FMD. The absolute increase in FMD of 1.70% after CoQ10 supplementation is of clinically significance, as an absolute improvement in FMD of 1% may already translate into a 10–25% reduction in residual cardiovascular risk for these patients (Neunteufl et al 2000; Shimbo D et al 2007).

In this study the minimum increment of FMD reached with the number of patients provided for each group is to be considered as equal to 1.6% (according to Figure 2 shown in Gao et al, in particular by referring to the study of Watts, which provides a sample size similar to ours).

It was shown that CoQ10 possess the capability of enhancing endothelial functionality by counteracting nitric oxide oxidation (Tiano L et al 2007). Moreover, CoQ10 supplementation is able to improve endothelial dysfunction in statin treated type 2 diabetic patients, possibly by altering local vascular oxidative stress [Hamilton SJ et al 2009] and also to improve endothelial function of conduit arteries of the peripheral circulation in dyslipidemic patients with type 2 diabetes perhaps increasing endothelial release and / or activity of NO due to improvement in vascular oxidative stress (Watts GF et al 2002)

In patients with ischaemic left ventricular systolic dysfunction (LVSD), 8 weeks supplement of CoQ improved mitochondrial function and FMD: in particular the improvement of FMD correlated with the change in mitochondrial function, suggesting that CoQ improved endothelial function via reversal of mitochondrial dysfunction in patients with ischaemic LVSD (Dai YL et al 2011).

Supplementation with either ubiquinol or ubiquinone increases ubiquinol levels in plasma lipoproteins and the heart and thereby enhances resistance of LDL (Low Density Lipoprotein) to free radical oxidation and cardiac ATP (Adenosine triphosphate) production. The higher bioavailability and biological activity of ubiquinol however, increases its beneficial effects (Cohen MM 2015). This was demonstrated in a cross-over trial that involved 4 weeks of supplementation with either 200mg/day of ubiquinol or ubiquinone. While both forms significantly increased total plasma CoQ10 the increase with ubiquinol was nearly twice that produced by ubiquinone. This study also found that plasma ubiquinol/total CoQ10 ratio increased significantly from baseline during ubiquinol supplementation, yet remained unchanged after ubiquinone supplementation [Langsjoen PH and Langsjoen AM 2014]. However, only recently ubiquinol become available as a supplemental nutrient.

This study will be conducted to understand the effects of a supplementation of ubiquinol (200 mg / 100 mg daily) on FMD in male and female subjects at risk of cardiovascular disease development.

Nitric oxide (NO) plays a major role as a regulator of endothelial vasomotor tone. NO, released as a gas by the arterial endothelium, stimulates guanylyl cyclase, thus increasing cGMP concentrations. cGMP biological effects vary depending on biological site. Specifically, in vascular smooth cells, increased cGMP activates GMP-dependent kinases that decrease intracellular calcium, producing relaxation.

Considered alone, NO is an important anti-atherosclerotic biofactor, with anti-aggregatory effects on platelets, antioxidant, anti-inflammatory, anti-proliferative, and vasodilatory effects on vasculature, while in combination with proinflammatory oxidants (e.g., superoxide and hydrogen peroxide) it forms proatherogenic mediators, such as peroxynitrite, that modify lipids and proteins (Cannon, 1998). Reactive oxygen and nitrogen species oxidatively damage LDL trapped in the arterial intima forming oxidized LDL, which in turn initiates

numerous events facilitating the development of atherosclerotic lesions.

Oxidation of lipoproteins involves the peroxidation of their polyunsaturated fatty acids (PUFA) and yields large amounts of lipid peroxidation products such as conjugated diene hydroperoxides. Cleavage of these products generates aldehydes, such as malondialdehyde, which act as toxic messengers in the processes of atherosclerotic lesion formation (Viigimaa, 2010).

Peroxynitrite contributes to the development of early vascular lesions and as the lesion develops, other reactive nitrogen species derived from the reaction of nitrite with proteins, such as myeloperoxidase, in inflammatory cells, are also thought to contribute to nitrosative stress. It is well known that peroxynitrite is able to modify proteins and lipids in LDL and HDL even in the presence of endogenous lipophilic antioxidants and regulates signaling pathways in the endothelial and vascular smooth muscle, thereby modulating the vascular response to atherogenetic stimuli (Rubbo, 2009).

Several pathophysiological states are characterized by a variable degree of endothelial dysfunction, mainly due to a decrease in endothelium-dependent vasodilation. A comprehensive but not exhaustive list of these conditions includes hypercholesterolemia, atherosclerosis, diabetes mellitus and heart failure. Specifically, an impairment in flow mediated endothelium-dependent vascular dilation was observed in hypercholesterolemia. This may be due to increased levels of intracellular superoxide inactivating NO more rapidly (Loffredo, 2012).

Since NO bioavailability is reduced in the presence of reactive oxygen species (ROS), with subsequent production of other compounds including peroxynitrite, quantification of NO and peroxynitrite levels is currently proposed as a marker of oxidative stress.

In order to monitor the beneficial antioxidant effects of ubiquinol in this respect we will evaluate

1) Plasma levels of nitric oxide quantified in terms of its stable reaction product (nitrites and nitrates) through the Griess reaction (Tsikas 2007)
2) Oxidative inactivation of NO to peroxinitrite using fluorescent probes (Rios 2016).
3) Susceptibility to oxidation of plasma lipoprotein quantified as kinetic of conjugate

dienes formation following copper exposure (Mazzanti 2015)

2. Study Objectives and Endpoints

To evaluate the impact of a supplementation with ubiquinol (reduced form of ubiquinone or reduced CoQ10) on endothelial function and oxidative stress.

Primary Endpoint

Change in endothelium-dependent vasodilation (by means of the flow mediated dilation - FMD - technique) at week 8; comparison between both treatment groups and placebo.

Secondary Endpoints

- Change in endothelium-dependent vasodilation (by means of the flow mediated dilation - FMD - technique) at week 4; comparison between both treatment groups and placebo.
- Change in red / ox CoQ10 levels at week 4 and 8; comparison both between treatment groups and placebo
- Evaluation of plasma levels of nitric oxide quantified in terms of its stable reaction product (nitrites and nitrates) through the Griess reaction (Tsikas 2007) at week 4 and 8; comparison both between treatment groups and placebo
- Evaluation of plasma levels of peroxinitrite (evaluation of oxidative inactivation of NO to peroxinitrite using fluorescent probes (Rios 2016)) at week 4 and 8; comparison both between treatment groups and placebo.
- Evaluation of susceptibility to oxidation of plasma lipoprotein quantified as kinetic of conjugate dienes formation following copper exposure (Mazzanti 2015) at week 4 and 8; comparison both between treatment groups and placebo.

3. Study Design

3.1 Study Outline

Double-blind, randomized, placebo – controlled, parallel groups clinical study with 3 intervention groups to be carried out on 51 subjects.

Three visits will be envisaged to the test facility: a Screening / Baseline / Randomization Visit and 2 Measurement Visits will be performed, each lasting approximately 1 hour. There will be 4 weeks between each visit.

At each Visits subjects should have to:

•≥12-h overnight fast

•Avoid caffeine for 72h

•Avoid high-fat food the day before

•Avoid tobacco for at least 4-6h before

•Abstain from exercise for >12h before the test

During Screening Visit the eligibility criteria will be verified and, if fulfilled, each subject will be randomized to one of the 3 study groups after signing the Informed Consent Form.

During each visit a short physical examination will be performed and a blood sample (approximately 10 ml) will be collected from the vein in the left arm to evaluate CoQ10 levels. After acclimatisation and rest for ≥ 30 minutes blood pressure (triplicate, first discarded) and FMD (baseline or Week 4 and 8 measurement) will be measured.

At Screening Visit and at Week 4, subjects will receive the assigned test product, according to randomization, for consumption at home during the coming 4 weeks and leave the facility.

The study products include test treatment (ubiquinol, two dosages, 100 and 200 mg daily) and placebo; details on these study products are given in the study products dossiers. Each product (ubiquinol or placebo), have to be taken daily at breakfast and dinner (total two pills: 1 before breakfast and 1 before dinner).

FMD measurements will be taken as follows:

Measurement 1: baseline measurement on Day 0 (Visit 0 - Screening / Baseline / Randomization Visit)

Measurement 2: second measurement on Week 4 (Visit 1)

Measurement 3: third measurement on Week 8 (Visit 2 – End of Study Visit)

3.2 Regulatory status of the study product

The active study products are already marketed as Kaneka Ubiquinol™ (Kaneka QH™ active antioxidant form of coenzyme Q10).

3.3 Selection of Subjects

3.3.1 Planned number of subjects

Sample size: 51 (17 per group), healthy, adult males and females.

In females, FMD varies with the hormonal cycle. Therefore, to avoid such complications, the study will be conducted in post-menopausal female subjects only.

Subjects will be recruited from subjects referred as outpatients to U.O.C. Laboratorio Analisi – POR I.N.R.C.A. Ancona, Italy. Selection of suitable subjects will be made according to the inclusion and exclusion criteria described in the following sections.

3.3.2 Inclusion Criteria

- Male aged 35 - 65 years (35 and 65 included).
- Post-menopausal female (without a period for more than 1 year), until 65 years (65 included).
- Subjects enrolled for primary prevention but never treated (only life style modification suggested, according guidelines).
- FMD level measured between:
  2,5% < FMD baseline value < 6% (at Screening Visit).
- BMI: 18.5 - 29.9 kg/m^2.
- LDL Cholesterol levels between 130 and 200 mg/dl (based on previous evaluation, performed within 1 month from the Screening Visit).

3.3.3 Exclusion Criteria

- Use of any cardiovascular disease related drugs prior to Screening Visit.
- Participation in any clinical trial while participating in this trial.
- Greater than 5% change in body weight within 1 month of Screening Visit.
- Subjects playing competitive physical activity.
- Subject taking lipid-altering drug therapy within four weeks prior to Screening Visit. Also excluded are supplements known to have significant lipid altering effects, such

as niacin (>100 mg per day), garlic (> 600 mg per day), omega-3 fatty acids (> 1 g omega-3 fatty acids per day), red yeast rice extract, phytostanols / phytosterols (> 0.5 g per day), soluble fiber (>1 g per day), chitosan (> 1 g per day) and conjugated linoleic acid (CLA; > 3 g per day).

- Subject taking any concomitant treatment with phosphodiesterase inhibitors (e.g. sildenafil citrate, tadalafil, vardenafil) and donors of NO (nitric oxide), like other long-acting derivatives of GTN (glyceryl trinitrate), such as isosorbide dinitrate and amyl or butyl nitrite.
- Excluded concurrent medications are: systemic corticosteroids (nasal and inhaled corticosteroids are permitted), orlistat, bile acid resins, no more than 1 g of prescription omega-3 fatty acids, cyclical or non-continuous hormone therapy (estrogen or testosterone).
- No more than 2 alcoholic units per day. Units are defined as 12 grams of ethanol, e.g. a small glass (125 ml) of medium gradation wine, a can of beer (330 ml) of average gradation or 40 ml dose of liquors.
- Consumption of flavonoids-enriched products.
- Consumption of vitamin C-enriched products or supplements containing vitamin C.
- Has a diagnosis of type 1 or type 2 diabetes mellitus, hepatic or renal impairment or diseases, thyroid disorders.
- Known cardiovascular disease or stroke, except for conditions that are deemed clinically insignificant by Principle Investigator or Sub-investigator, or study site physician (e.g. clinically insignificant atherosclerotic lesions observed by imaging studies).
- History of significant gastrointestinal disease such as severe constipation, diarrhea, malabsorptive disease, inflammatory bowel disease (e.g. Chron's disease, ulcerative colitis).
- History of severe psychiatric illness which in the opinion of the investigator would interfere with the optimal participation in the study.
- History if cancer within 5 years of Screening Visit (except for successfully treated basal and squamous cell carcinoma of the skin).
- Known HIV seropositivity.
- History of bariatric surgery.

- Allergic to the test products or placebo.
- Smokers > 10 cigarettes/day.
- Individuals who in the opinion of the principal investigator have a risk of non-compliance to the study procedures or who are otherwise not appropriate to include in this clinical trial.

3.3.4 Dietary Restrictions

- Subjects will be asked not to consume supplements known to have significant lipid altering effects, from the start to the end of the study, such as niacin (>100 mg per day), garlic (> 600 mg per day), omega-3 fatty acids (> 1 g omega-3 fatty acids per day), red yeast rice extract, phytostanols / phytosterols (> 0.5 g per day), soluble fiber (>1 g per day), chitosan (> 1 g per day) and conjugated linoleic acid (CLA; > 3 g per day) or flavonoids-enriched products.
- Subjects will be allowed to drink no more than 2 alcoholic units per day from the start to the end of the study. Units are defined as 12 grams of ethanol, e.g. a small glass (125 ml) of medium gradation wine, a can of beer (330 ml) of average gradation or 40 ml dose of liquors.
- Subjects are not allowed to consume any other ubiquinol / ubiquinone supplement from the start to the end of the study.
- Subjects are not allowed to consume vitamin C supplements from the start to the end of the study.

3.4 Randomization Procedure

A simple randomization technique, i.e. a randomization based on a single sequence of random assignments, will be used. A computer-generated random numbers list will be used and subjects will be assigned a number according to their order of inclusion in the study.

According to the technique above outlined, 51 subjects will be randomly allocated to one of the 3 study groups; treatments will be randomised to subject numbers (1-51).
Randomized subjects who are withdrawn from the study after receiving at least one dose of study product will not be replaced.

3.5 Blinding

This will be a double blind study; the assessors will be blinded. The placebo capsules will be equal in colour, shape and weight to study products capsules (ubiquinol, provided as softgel capsules) but without active ingredients.

3.6 Withdrawal of Subjects.

Subjects may discontinue from the study at any time. In addition, the Principal Investigator (PI) or his/her designee has the right to withdraw a subject for any reason that is in the best interests of the subject. Whenever a subject is withdrawn from a study, an early withdrawal assessment must be completed stating the reason for withdrawal, assessing any eventual AE and taking other necessary assessments if needed. Withdrawals due to non-attendance must be followed up to attempt to obtain the reason that the subject is "lost to follow up".

3.7 Protocol deviations and violations

A protocol violation or deviation is any failure to comply with the protocol. All the protocol violations / deviations are required to be reported in the CRF by the investigator.

<u>Protocol deviations</u>

- minor

- misuse of study product not affecting compliance (e.g. missed more than three times in total; study product not taken at proper time);
- 1 day of product missed;
- a visit is delayed or anticipated for 1 day from the initially scheduled visit (if study product consumption is not affected);
- no return of unused study product;
- minor lack of compliance with restricted diet to be followed (e.g. consumed more than 2 units of ethanol per day once or twice).

- major

- no fasting state at the arrival to the study center;
- major lack of compliance with restricted diet to be followed (consumed ethanol several times (> 2) in significant quantities);
- three consecutive times and four or more non-consecutive times of product missed;

- a visit is delayed or anticipated for more than 1 day from the initially scheduled visit (even if study product consumption is not affected);
- not complying with instructions given by the study personnel.

Protocol violations (minor/major according to Sponsor's decision on a case-by-case basis)

- informed consent obtaining process not adequately performed;
- violation of inclusion / exclusion criteria;
- use of prohibited concomitant medications (i.e. taking prescribed drugs or OTCs or dietary supplement that can influence FMD);
- required tests and sampling procedures incorrectly performed or not performed at all (e.g. samples not collected);
- any other GCP non compliance.

Both minor and major protocol deviations / violations will be recorded in the Case Report Form (CRF) and will be addressed in the blind review performed before the de-blinding and statistical analysis. In case of the violation or the deviation may affect the study results (this decision will be taken according to Sponsor's decision) the subject will be withdrawn from the Per Protocol statistical analysis.

3.8 Endpoints

3.8.1 Assessment of Effects

Measurement will be taken by sonographers who have been trained to measure FMD. Subjects will be invited to study site as per randomisation schedule. After collection of the blood sample from the left arm, they will be allowed to get acclimatized at study site (temp 20-22 °C) for 30 minutes. During this time, they will be given instructions about the measurement and their blood pressure will be measured (3 individual measurements, the first discarded). After at least 30 minutes rest, they will be taken to the bed and will be asked to rest while the arm support will be adjusted and the probe and occlusion cuff will be fitted to their arm.

Each FMD measurement cycle will have the following steps and will take approximately 30 minutes:

- Subjects rests comfortably on the equipment side bed

- Right arm of the subject is partially immobilized and brachial artery is focussed
- Operator waits for 1 minute after the image on screen gets stabilised
- FMD measurement including the following steps:
    - 1-minute base scan to measure the baseline diameter of artery (Resting stage)
    - 5 minutes of forearm occlusion at 300±30 mmHg (cuff occlusion stage), just below the elbow (2-5 cm from antecubital crease)
    - 4 minutes FMD scan, which will start immediately after release of occlusion (reactive hyperaemia stage)
    - When the artery has returned to baseline a second 1-minute scan is taken
- FMD will calculated as maximal percent increase in diameter above baseline (mean value of measures obtained during 1 minute before cuff inflation). In case of an unusable baseline diameter, the expert assessing the scans can use alternative options to determine the baseline diameter (recovery diameter) as rescue option.

In the blood sample triglycerides and glucose in blood will be used to detect subjects who were not fasted. Highly sensitive C-reactive protein will be measured to assess inflammatory status. Inflammation and food intake may affect FMD but it is not anticipated that the intervention will affect these laboratory values.
Red / ox CoQ10 levels will be measured.

3.8.2 Blood sample collection

To collect blood and to perform the analysis standard methodologies will be used. On collected blood sample will not be performed genetic tests, but they will be collected only as regards what described in the study protocol and they will be destroyed at the end of the study.

3.8.3 Assessment of Safety

Safety and tolerability will be assessed by monitoring adverse events (AEs) during the whole study period. Adverse events are any untoward medical occurrence, whether or not related to study product or study procedures. AEs will be coded using ICD-9.

Ubiquinol is usually well tolerated, and cases of overdose are not known. Rarely, it may cause mild gastrointestinal upset, decrease in appetite, nausea, vomiting, diarrhoea or constipation and rash. CoQ10 may be contra-indicated for individuals taking blood thinning medication.

3.9 Concomitant medication

Any concomitant medication, including prophylactic treatments, OTC medication and all food supplements, should be reported in the subject diaries and will be entered in the case report form (CRF).

3.10 Excluded medication

Subjects on prescribed medication (including prophylactic treatments) will not be selected for the study. Any subject that will start taking prescribed medication during the study, which, in the Investigator opinion may interfere in the study, will be excluded.

Regular intake of OTC (Over-The-Counter) medication and consumption of nutritional supplement is allowed if an effect on vascular function is not expected as judged by the PI or his/her designee at screening.

Occasional intake of drugs or supplements will be evaluated during a blind review at the end of the study and may lead to exclusion from the per protocol analyses.

3.11 Assessments at Each Visit

Visit 0 – Day 0 - Screening / Baseline / Randomization Visit

Before any screening procedures take place, potential subjects will be provided with written and oral information about the study and the procedures involved. They will be fully informed about their responsibilities and of all the procedures involved in the study, the possible risks with the study product and their rights while participating in the study. They will have the opportunity to ask questions and time to consider participation. If the subjects wish to participate in the study, they will be asked to sign and date an Informed Consent Form. The form must be signed and dated by the subjects as well as by the person who conducted the informed consent procedure before any study-related activities are performed.

Subjects will be warned to come to study centre (for all the Visits: V0, V1 and V2):

• ≥ 12 hours overnight fast

• Avoid caffeine for 72h before

• Avoid high-fat food the day before

• Avoid tobacco for at least 4-6h before

• Abstain from exercise for >12h before

All subjects will have a screening evaluation that includes the following:

- Signature on Informed Consent Form
- Check eligibility criteria
- Medical history and demography
- Concomitant medication monitoring and recording
- Brief physical examination
- Measuring weight and height (BMI calculated)

If they will be deemed eligible the randomization process will take place, i.e. each subject will be assigned a number according to the order of inclusion in the study; the following procedures will take place:

- Explanation of dietary restrictions (to be followed for the whole study)
- Randomisation: subjects will be assigned a number according to order of inclusion in the study
- Blood sample collection for laboratory parameters (triglycerides, glucose, C-reactive protein – CRP, LDL Cholesterol - to be checked for inclusion criteria), red / ox CoQ10 levels evaluation and assessment of the levels of the markers of oxidative stress
- Acclimatisation
- Vital signs (blood pressure)
- FMD measurement
- Supply products (according to randomization) for consumption at home
- Supply of daily diary

Until the following visit the subjects will be instructed to consume the study product at home and to complete the daily diary. Diary and unopened and empty study products packs will be returned as an assessment of compliance. The following visit will be scheduled after 4 weeks.

Visit 1 - Week 4

During this visit the following activities will have to be performed:

- Confirmation of eligibility criteria
- Compliance check (fasted, dietary restriction and diary check)
- Concomitant medication / adverse events monitoring and recording
- Blood sample collection for laboratory parameters (triglycerides, glucose, C-reactive protein – CRP, LDL Cholesterol - to be checked for inclusion criteria), red / ox CoQ10 levels evaluation and assessment of the levels of the markers of oxidative stress
- Brief physical examination
- Acclimatisation
- Vital signs (blood pressure)
- FMD measurement
- Supply products (according to randomization) for consumption at home
- Supply of a new daily diary
- Collection of the completed daily diary and unopened and empty product packs

Diary and unopened and empty study products packs will be returned as an assessment of compliance. The following visit will be scheduled after 4 weeks.

Visit 2 – Week 8 – End of Study Visit

During this visit the following activities should be performed:

- Compliance check (fasted, dietary restriction and diary check)
- Concomitant medication / adverse events monitoring and recording
- Blood sample collection for laboratory parameters (triglycerides, glucose, C-reactive protein – CRP, LDL Cholesterol - to be checked for inclusion criteria), red / ox CoQ10 levels evaluation and assessment of the levels of the markers of oxidative stress
- Brief physical examination
- Acclimatisation
- Vital signs (blood pressure)
- FMD measurement
- Collection of the completed daily diary and unopened and empty product packs

Table 1. Study flow chart

Visits	V0 Screening visit /Baseline/ Randomization visit	V1	V2 End of Study visit
Week (+/- 2 days)	**0**	**4**	**8**
Informed Consent	x		
Inclusion/Exclusion Criteria	x	x	
Medical history	x		
Demography	x		
Concomitant medication	x	x	x
Physical examination	x	x	x
Randomization	x		
Blood sample	x	x	x
FMD	x	x	x
Study product supply	x	x	
Study product return		x	x
Diary supply	x	x	
Diary return		x	x
AE/SAE evaluation		x	x

4. Study Products

The active study products, Ubiquinol-QH 100 mg, will be provided in the form of softgel capsules.

Each active softgel capsule contains ubiquinol (Kaneka QH™ active antioxidant form of coenzyme Q10) 100 mg.

The product contains also soy and other ingredients: medium chain triglycerides, gelatin, glycerin, ascorbyl palmitate, purified water, beeswax, soy lecithin, annatto extract.

Placebo will consist of softgel capsules too, matching the active study products but without any active principle.

The subjects will consume 2 softgel capsules according to the following scheme:

	Number of soft capsules/day	
	Ubiquinol-QH 100 mg	Placebo
Group A: Ubiquinol 200mg/day	2	0
Group B Ubiquinol 100mg/day	1	1
Group C: Matched placebo	0	2

4.1 Presentation and Administration

The study products, provided as softgel capsules, will be consumed at home by the subjects in the morning and in the evening, with a meal.

4.2 Storage

The study products will be stored in the pharmacy of I.N.R.C.A., Ancona; dose packets will be handed over at the study site by the PI or his/her designee.

4.3 Accountability

The accountability of the study products will be regularly checked and a person from the medical staff will maintain the accounts of study products in the study product log.

4.4 Breaking the Code in an Emergency

Subjects and site personnel will be blinded with regard to the identification of specific treatments.

A randomization schedule will be generated. The Pharmacy custodian / designate independent of the study will conduct the dispensing procedure.

The link between the treatment code and the treatment will not be available to the personnel involved in the collection, revision, or evaluation of adverse events, to the clinical laboratory, or to personnel who could have an impact on the outcome of the study, until after the end of the study (when all adverse events have been finalized) and analytical phases.

After the study, blind review meeting will be organized with soft data between PI and Statistician; decision on breaking of code will be taken only after the blind review team decides to hard lock the data.

5. Safety Monitoring

5.1 Adverse Event

An adverse event (AE) is any untoward medical occurrence in a subject, whether or not related to study product or study procedures. Adverse events include any occurrence that is new in onset, an exacerbation of a pre-existing condition and clinically significant laboratory values.

5.1.2 Study Specific Expected Adverse Event

While trained and experienced nurses/phlebotomists will draw the blood with utmost care, there is a possibility that some of the subjects may experience haematoma (bruise or collection of blood under the skin with minor swelling in the area, which may last for few days), fainting or injury to the nerves after venipuncture. Moreover, temporary occlusion of arm during FMD measurement may cause transient hematoma, discomfort or paresthesia. These symptoms will not be recorded as adverse events.

Rarely, ubiquinol may cause mild gastrointestinal upset, decrease in appetite, nausea, vomiting, diarrhea or constipation and rash.

5.2 Serious Adverse Event

A serious adverse event (SAE) is an AE that results in any of the following outcomes: death; a life-threatening event; in-patient hospitalization; prolongation of existing hospitalization; a persistent or significant disability/incapacity; a congenital anomaly or birth defect. Any other important medical event may be considered a SAE when the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed previously. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, or convulsions that do not result in in-patient hospitalization. Just as a stable pre-existing condition is not an AE, hospitalisation for elective treatment (e.g. cosmetic or dental procedure) of a pre-existing condition that did not worsen from baseline is not an SAE.

5.3 Severity and Relatedness of Adverse Events

An AE will be recorded only once, with the most extreme severity. Severities are defined as:

Mild	Awareness of symptoms which require minimal or no treatment and do not interfere with daily activity
Moderate	Discomfort or low level of interference which is enough to interfere with but not prevent daily activity
Severe	Interrupted or unable to perform usual daily activity and usually requires treatment.

The likelihood that the AE was related to the study product or study procedure is defined as;

Not Related	The AE is clearly due to an alternative cause, even if this cannot be definitely identified. Alternative causes include disease or environmental factors.
Unlikely	A connection between the AE and the study product or procedure is unlikely. • The AE has a relationship in time to the study product or procedure • An alternative cause (e.g. disease or environmental factors) is the most likely explanation, even if this cannot be identified.
Possibly	A connection between the AE and the study product or procedure cannot be ruled out with certainty. • The AE has a relationship in time to the study product or procedure

	• An alternative cause (e.g. disease or environmental factors) seems likely or possible or there is significant uncertainty about the cause of the AE.
Probably	There is a high degree of certainty that the AE is related to the study product or procedure. • The AE has a relationship in time to the study product or procedure • A possible alternative cause may be present. • AE disappears or decreases on withdrawal or reduction of study product or procedure (if performed).
Definitely	The AE is clearly related to the study product or procedure. • There is a strong relationship in time • An alternative cause is unlikely • AE disappears or decreases on withdrawal or reduction of study product or procedure (if performed)

5.4 Reporting of Adverse Events

All AEs will be recorded in the CRF. All AEs will be coded using ICD-9 and reported according to local regulations.

It should also be remembered that, referring to the legislation currently in force, doctors are obliged to notify any suspected adverse reaction of which they become aware in their clinical practice, by completing the appropriate specific reporting form.

Once completed, this form will be sent to the Pharmacovigilance team responsible for the relevant health institution the Investigator belongs to.

5.5 Follow-up of Adverse Events

The PI or designee will ensure that adequate medical care (if required) is provided to a subject for any study related AE. If an AE is ongoing at the end of the study, follow-up visits will be performed until the AE has resolved or stabilised or the PI determines that no further follow up is necessary. Follow-up visits may take the form of subject visits, a referral to another specialist, site telephone calls to the subject or letters from the treating physician.

The PI or designee must comply with the specific reporting requirement(s) of the ethics committee.

6. Statistical Considerations

6.1 Sample Size Calculation

Sample size calculation is carried out with use of G*power Software.

The parameters used to compute sample size for this study were taken from the reference article by Gao at al 2012, a meta-analysis of 5 randomized controlled trials.

Mean and SD taken from the article by Watts GF et al 2002, one of the RCT mentioned in reference article (see fig. 2 in the article by Gao et al), were considered as appropriate values to be taken into account: considering group one mean and SD as 1.6 (1.16) and second group mean and SD as -0.4 (2.24), with 80% power and 5% two-sided significance level, 14 subjects are required for one group.

In this study, considering a 20% dropout rate, a total of 52.5 subjects should have been enrolled; a sample size equal to 51 subjects was deemed as appropriate.

It is to be noted that in this study the minimum increment of FMD reached with the number of patients provided for each group is to be considered as equal to 1.6% (according to Figure 2 shown in Gao et al, in particular by referring to the study of Watts, which provides a sample size similar to ours).

6.2 Definition of Analysis Population

All subjects receiving at least one study product will be used for safety analyses. All subjects with at least 1 FMD measurements will be part of the intention-to-treat population. All subjects with no major deviation from the protocol will form the per-protocol population. All FMD measurements with acceptable quality will be used for analyses. Decision on the per-protocol population and FMD measurement quality will be made during blind review and documented.

6.3 Statistical Methods

FMD measurements will be taken as follows:

- Measurement 1: Baseline measurement on Day 0 (Visit 0 - Screening / Baseline / Randomization Visit)
- Measurement 2: second measurement on Week 4 (Visit 1)

- Measurement 3: third measurement on Week 8 (Visit 2 – End of Study Visit)

In order to evaluate a potential effect size among groups according to Primary and Secondary Endpoints the comparison will be made comparing:

- Measurement 3 vs Measurement 1
- Measurement 2 vs Measurement 1
- Measurement 3 vs Measurement 2

Parametric or non-parametric tests, ANOVA and Kruskal-Wallis tests, will be performed basing on the normality of the data.

6.4 Statistical data quality control

Both an Intention To Treat (ITT) and a Per Protocol (PP) analysis will be performed.

Descriptive statistics will be provided for baseline variables including demographic and anthropometric values. The number and percentage of AEs will be tabulated by verbatim term and for categories via ICD-9. Adverse events will be defined as pre-treatment or treatment emergent if pre-treatment AEs are reported for that study.

7. Data Handling and Record Keeping

Clinical data (including AEs and Concomitant Medications) will be captured into a paper CRF. It will be the responsibility of the PI and monitor to ensure the completeness and accuracy of the data.

All videos from the FMD scans will be evaluated by experts (Quipu srl, via Moruzzi,1, 56124 Pisa, Italy) in dedicated software (FMD studio). The expert will also provide comments on the quality of the scans made by the sonographers and note when adjustments of the baseline diameter were made. It is to be noted that all FMD assessment will be made by a fully blinded expert assessor.

All site staff must ensure that the subject's anonymity will be maintained. On all documents that are to be submitted to external laboratory, subjects must be identified only by an identification code and not by their names. The PI or designee must keep a separate confidential enrolment log that matches identifying codes with the subject's names and addresses. The PI or designee must maintain these documents at the site.

It is the responsibility of the PI or designee to maintain adequate study records. All documents must be archived in a secure place and treated as confidential material.
All the patients' data will be treated according to Data Protection Code - Legislative Decree no. 196/2003.

8. Quality Standards

It is the responsibility of the PI to ensure that the study is conducted in accordance with the principles of Good Clinical Practice, the 2008 version of the Declaration of Helsinki and according to applicable local laws and regulations concerning studies conducted on human subjects, which are outside of the definition of a medicinal product or medical device.
Quality assurance audits may be performed by any ethics committee or regulatory authority during the course of the study or at study completion.

9. Ethics and Informed Consent

The PI or designee must submit a copy of the protocol, subject information sheet and consent form to an Independent Ethics Committee or Institutional Review Board who must provide written approval before study specific procedures commence. The IEC/IRB must also approve any other information that is given to subjects such as advertisements and may require other documents such as study product documentation.
Any modification to the agreed protocol must be approved in writing by the IEC/IRB. Written approval must be obtained from the IEC/IRB before any amendment is implemented, unless immediate change is required to eliminate hazards to the subjects or when the change(s) involves only logistical or administrative aspects of the study (e.g., change of telephone number(s)).
The PI or designee must obtain informed consent from each subject participating in the study, after explanation of the aims, methods, benefits and potential hazards of the study. The consent must be obtained before any study-specific procedures are performed. It must be made completely and unambiguously clear to each subject that they are free to refuse to participate in the study, or that they can withdraw their consent at any time and for any reason, without incurring any penalty or withholding of treatment. The subject must be given their own copy of the information sheet and signed consent form. The original signed informed consent must be kept on file by the PI or designee.

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