Blueberry Extract and Turmeric Extract

EO Galliera Hospital of Genoa

(L.833/1978, art. 41; Legislative Decree n.517/1993 art. 4 c. 12)

Complex Structure of Medical Oncology
Tel 010 5634508 - fax 010 57481090
andrea.decensi@galliera.it

PHASE II TRIAL OF BIOMARKER MODULATION IN SUBJECTS WITH COLON ADENOMATOSUS POLYP WITH BLUEBERRY EXTRACT AND TURMERIC EXTRACT

MiRACol Study

ClinicalTrials.gov Identifier : NCT01948661

Sponsor
EO Galliera Hospital

Central Data Management
SC Medical Oncology
Clinical Research Coordination in Oncology
EO Galliera Hospital

Statistical Analysis
Matthew Points
Scientific Coordinator Office
EO Galliera Hospitals

Project Manager:
Andrea De Censi, MD
Director of Medical Oncology
EO Galliera Hospital,
Cappuccine Walls 14, 16128, Genoa, Italy
Tel 010 5634501 - fax 01057481407
andrea.decensi@galliera.it

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1.0 LIST OF COORDINATING CENTER INVESTIGATORS

Andrea De Censi	Project coordinator Director of Medical Oncology Unit, EO Galliera Hospital, Genoa
Gianni Coccia	Sub-Investigator Director of Gastroenterology Unit, EO Galliera Hospital, Genoa
Roberto Bandelloni	Lab Researcher Director, Pathological Anatomy Unit, EO Galliera Hospital, Genoa
Marco Mori	Lab Researcher Director Lab Analysis Unit, EO Galliera Hospital, Genoa
Mariangela Rutigliani	Laboratory Researcher Pathological Anatomy Unit, EO Galliera Hospital, Genoa
Carlotta Defferrari	Clinical Researcher Medical Oncology Unit, EO Galliera Hospital, Genoa
Matteo Puntoni	Biostatistician Office of the Scientific Coordinator, EO Galliera Hospital, Genoa
Marilena Petrera	Study Coordinator Medical Oncology Unit, EO Galliera Hospital, Genoa
Silvia Caviglia	Administrative support and regulatory practices Medical Oncology Unit, EO Galliera Hospital, Genoa

2.0 LIST OF PARTICIPATING CENTERS AND THEIR CO-INVESTIGATORS

Gianni Coccia	Principal Investigator (PI) Director of Gastroenterology Unit, EO Galliera Hospital, Genoa
Bernardo Bonanni	Principal Investigator (PI) Division of Prevention and Oncological Genetics, European Institute of Oncology, Milan
Sebastiano Saccomanno	Principal Investigator (PI) Gastroenterology Unit, Villa Scassi Hospital, ASL3 Genovese
Marina De Mattheis	Principal Investigator (PI) Emergency Gastroenterology and Digestive Endoscopy Unit, Hospital of Lavagna, ASL 4 Chiavari

3.0 COLLABORATIONS

Paolo Morazzoni	Scientific Director, Indena Spa, Milan
Giovanna Petrangolini	Research Scientist Scientific Director, Indena Spa, Milan
Antonella Riva	Senior Research Scientist Scientific Director, Indena Spa, Milan
Massimo Ronchi	Head of the Formulation Development Laboratory Indena Spa, Milan
Elisabetta Frattini	Research Scientist Formulation Development Laboratory, Indena Spa, Milan
Harriet Johansson	Division of Prevention and Oncological Genetics, European Institute of Oncology, Milan
Francesca Mattioli	Di.MI Section of Clinical Pharmacology and Toxicology, University of Genoa

4.0 STUDY SYNOPSIS

STUDY TITLE:

Phase II Study of Biomarker Modulation in Subjects with Colonic Adenomatous Polyp with Bilberry Extract and Turmeric Extract - MiRACol Study

PRINCIPAL INVESTIGATOR: Andrea De Censi

COORDINATION CENTER: EO Galliera Hospital, Medical Oncology Unit, Mura delle Cappuccine, 14 - 16128 Genoa, Italy

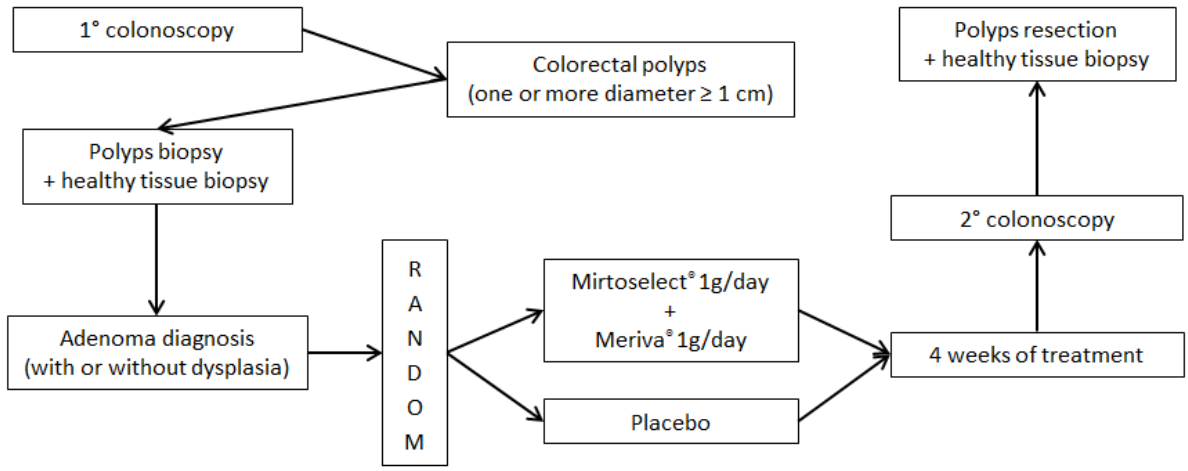
Agents	Formulation	Dose	Regimen	Route
Blueberry extract (Mirtoselect®)	TABLETS from 500 mg	1 g/day (500mgx2/day)	1 tablet 2 times a day at the same time (1 morning tablet + 1 evening tablet)	Oral
Turmeric extract (Meriva®)	TABLETS from 500 mg	1 g/day (500mgx2/day)	1 tablet 2 times a day at the same time (1 morning tablet + 1 evening tablet)	Oral

PURPOSE OF THE RESEARCH :

The study aims to verify the effect of the association of food supplements, blueberry extract and turmeric extract versus placebo, administered for 4 weeks, on some important surrogate biomarkers of colorectal cancer in subjects with colorectal adenomatous polyps candidates for surgical resection.

STUDY DESIGN :

Randomized, double-blind, placebo controlled study



STUDY POPULATION :
Subjects with one or more colorectal adenomatous polyps (with diameter ≥ 1 cm) aged 18 and 85 years, ECOG performance status ≤ 1.
Subjects with hyperplastic polyps and flat and serrated adenomas, with previous colorectal carcinoma, or with carcinomatous tissue in the adenoma will be excluded.

NUMBER OF SUBJECTS : 100 divided into 2 treatment groups.

TREATMENT :
Group 1: Placebo A (Mirtoselect® 0 g) + Placebo B (Meriva® 0 g) / day
Group 2: Mirtoselect® 0.5 g x2/day + Meriva® 0.5 g x2 /day

TREATMENT DURATION : 4 weeks until surgical resection

PRIMARY OBJECTIVE
To evaluate the activity of the association of blueberry extract and turmeric extract on β-catenin expression after 4 weeks of treatment between experimental and placebo arm.

SECONDARY OBJECTIVES

- Evaluate the tolerability of the combination of blueberry extract and turmeric extract and study the plasma and tissue absorption of the active ingredients (anthocyanosides and curcumin);
- Evaluate the effects of treatment in the adjacent adenomatous and healthy tissue (perilesional) by evaluating the IHC expression of Nuclear Factor-Kβ (NFKβ), Ki-67, P53 apoptosis, tissue expression of the EGFRs system; tissue gene expression;
- Evaluate the modulation of some circulating growth factors in the collected serum: IGF system (IGF-I, IGFBP-3 and IGF-I/IGFBP-3), EGFR, inflammation markers (u-PCR).

INCLUSION CRITERIA
1 Age > 18 years and ≤ 85 years
2 Written informed consent
3 Presence of one or more adenomatous polyps in the colon-rectum (with diameter ≥ 1 cm)
4 ECOG performance status ≤ 1.

EXCLUSION CRITERIA
1 Presence of hyperplastic polyps and flat and serrated adenomas;
2 Previous colorectal cancer;
3 Presence of carcinomatous tissue in the adenoma;
4 Taking experimental drugs or dietary supplements containing blueberry or curcumin in the 15 days prior to enrollment;
5 Any other factor that, at the discretion of the investigator, may contraindicate enrollment in the study.

START OF STUDY	DURATION OF ENROLLMENT	END OF STUDY
Date of enrollment of the first subject	**48 months**	**Last follow-up visit date of the last enrolled patient**

5.0 INTRODUCTION AND RATIONALE

In Western countries, the colorectal is the second most common site of tumor occurrence. Colorectal cancer (CRC) is the second most common malignant tumor in terms of incidence and mortality (1) and represents a significant burden in terms of social and economic costs.

CRC is a multifactorial disease with approximately 75% of sporadic cases. The main risk factors are age, family history, the presence of colonic adenomatous polyps (PA), diffuse polyposis and the presence of particular hereditary conditions (HNPCC, FAP).

A growing number of studies indicate that inflammatory activation of the tumor microenvironment is one of the primary causes of CRC progression.

The efficacy of NSAIDs (nonsteroidal anti-inflammatory drugs) in preventing CRC suggests the concept that inflammation may play a crucial role in the development of these tumors (2-3). In fact, following the inflammatory process, several reactive oxygen species (ROS) are produced and it is now known that oxidative stress can significantly contribute to the development of cancer, particularly CRC (4-6).

The colon mucosa represents the body surface most exposed to bacterial antigens, derived from commensal, opportunistic, or frankly pathogenic strains. Consequently, this mucosa presents a particular reactivity that manifests itself with evident inflammatory and immune reactions. In the colon, along with genetic mutations that constitute elements of initiation of carcinogenesis (mutation affecting APC, k-RAS, P53 and others) a continuous promotional effect mediated by inflammatory elements is observed. The limit to the use of aspirin or other NSAIDs (non-steroidal anti-inflammatory drugs) in the prevention of CRC is linked to their possible chronic toxicity especially at the gastrointestinal level.

Adenomatous polyps (AP) are considered high-risk precancerous lesions: colon cancer arises in an adenomatous polyp in approximately 90% of cases, with a sequence that leads from AP to CRC in approximately 10 years (7) and is for these reasons considered a reliable intermediate (surrogate) marker of risk and efficacy for clinical trials of pharmacoprevention (8).

Several case-control studies and clinical trials suggest that AP removal decreases the incidence of CRC; it is therefore logical to think that the regression or elimination of AP through pharmacoprevention strategies could reduce the incidence of CRC (9).

The elucidation of the mechanisms inducing oxidative stress and their possible involvement in the development and progression of cancer represent an interesting area of study in the pharmacoprevention of CRC.

Pharmacoprevention aims to prevent the onset of cancer by using natural or synthetic drugs that inhibit carcinogenesis.

Approximately 70% of the anti-cancer drugs available today are of plant origin.

Although the main interest is for those that exert frankly antiblastic effects (e.g. vinca alkaloids, camptothecin and taxanes), many others are tested for their anti-inflammatory and/or bacteriostatic properties (e.g. salicylates, curcumin, epigallocatechin-gallate, quercetin, lycopene, resveratrol etc.), and it is precisely among the latter that we find the most interesting candidates for pharmacoprevention strategies.

β-Catenin plays important regulatory roles, in particular it is a key element in the Wnt signaling pathway where it acts as a co-transcriptional activator of target genes in the cell nucleus. The hyperactivation of Wnt signaling essentially influenced by β-catenin functions, is a typical feature of colon cancer and many other tumors; furthermore β-catenin has a crucial role in APC-mediated colon carcinogenesis (10).

The study aims to verify whether the association of 2 food supplements, in the form of standardized botanical extracts, with demonstrated modulatory properties in the process of colon cancer progression, is able to decrease the activation of β-catenin in AP. This is based on the assumption that the hyperactivation of the β-catenin signal is considered one of the earliest events in the sequence of genetic changes that favor the development of colon cancer (11).

6.0 AGENTS IN STUDIO

6.1 Curcumin Extract

Curcumin is the main biologically active component of Indian Saffron (*Curcuma longa L.*) belonging to the ginger family: *Zingiberaceae*. The root and rhizome (part of the underground stem) of the plant *Curcuma longa L.* are crushed and powdered. The powder thus obtained is used throughout the world as an ingredient in curry and curcumin is the polyphenol that characterizes its yellow color (12).

Curcumin was introduced into European medicine by the Dutch, who imported it from their possessions in the East Indies. In its countries of origin, among the populations of India and southern China, it is widely used not only as a condiment and colorant, but also as a remedy for liver disease and as a diuretic.

Curcumin has been tested for its pharmacological properties in thousands of studies, hundreds of which have been published in the last 4 years (13). These studies have confirmed its remarkable anticancer, anti-inflammatory and antioxidant properties. Unlike many other antioxidants, curcumin is able not only to prevent the formation of free radicals but also to neutralize existing free radicals. Therefore, it is considered an effective bio-protector due to this dual activity (13).

As for its antitumor properties, curcumin has been shown to be potentially useful for the treatment of colon, prostate, lung, breast and pancreatic tumors.

In the colon, curcumin has been shown to reduce the occurrence of aberrant foci in a rat model of colorectal carcinogenesis (14).

The mechanisms of action for the antitumor activity of curcumin can be roughly summarized as follows:

a) inhibition of tumor cell proliferation;
b) induction of apoptosis;
c) inhibition of the transformation of cells from normal to tumor;
d) inhibition of invasiveness and metastasis;
e) suppression of inflammation.

Regarding the use of curcumin in clinical trials in oncology, the results, while promising, have shown, however, that the low bioavailability of this natural product remains a serious obstacle to its therapeutic use, as it involves the use of very high dosages with poor patient compliance.

In a phase I trial of 15 patients with advanced colon cancer and refractory to standard therapies (15), curcumin (0.45-3.6 g/die/4 months) reduced patients' plasma PGE2 concentration by 50% at one hour after treatment in the absence of side effects, the authors indicated the 3.6 g dose as the standard for a possible phase II trial. In a previous estimation, doses of 8 g/day (3 months) had been reached without significant toxicity (16).

Curcumin was tested in association with quercetin for the chemoprevention of familial adenomatous polyposis in 5 patients (curcumin 480 mg and quercetin 20 mg/3 times daily/6 months) demonstrating a significant decrease in the number (60.4%) and size (50.9%) of polyps (17).

Curcumin has been tested in a phase II trial against advanced pancreatic cancer (8 g/day/8 weeks) demonstrating once again low toxicity and demonstrating the ability to reduce some inflammatory markers in the PBMC of patients (NF-kB, COX-2, STAT3), 2 out of 21 evaluable patients also demonstrated a clinical response (18).

A very interesting feature of curcumin is its ability to reduce EGFR expression in cells in vitro (19), however no one has yet evaluated whether this also occurs in vivo.

Recently Carroll et al demonstrated a significant reduction in focal aberrant crypts (AFCs) in 44 subjects treated with curcumin at doses of 2 and 4 g/day for 4 weeks (20).

The dose of 4 g/day was found to be more active and plasma levels correlated significantly with the ACF inhibition effect, suggesting in some way that the preventive effect may be systemic and not direct on the rectal mucosa.

For this study, therefore, we propose the use of Meriva®, a supplement based on a standardized extract of curcumin and phospholipid in a 1:2 ratio, with proven efficacy in the treatment of osteoarthritis and anterior uveitis (21) which, due to its pharmacokinetic characteristics and in particular its combination with the phospholipid part, allows us to overcome some of the limitations of the oral curcumin preparations tested to date, such as instability at intestinal pH values, low solubility in water, poor oral bioavailability and rapid conjugation and excretion.

It is also interesting to note that several studies on the conjugation of curcumin with phosphatidylcholine increases its bioavailability by approximately 10 times (21,22).

From the point of view of tolerability, Meriva® has proven to be safe and well tolerated. Oral intake in the studies cited so far has never been associated with significant side effects.

6.2 Blueberry extract

Blueberry extract (Mirtoselect®) is derived from the berries of *V. myrtillus* and is rich in anthocyanins, the polyphenols responsible for the blue and/or red pigmentation of many berries. The extract is characterized and standardized to contain 36% anthocyanosides.

A large literature has demonstrated its pharmacological and clinical efficacy. In particular:

a) increased capillary resistance
b) reduction of abnormal vascular permeability
c) antioxidant activity at vascular level
d) induction of arterial vasomotility
e) chemopreventive activity
f) anti-inflammatory activity.

As regards chemopreventive activity, the active component of botanical origin, anthocyanins, has been shown to be effective in some preclinical models of carcinogenesis, especially those of the gastrointestinal tract. Anthocyanins or dry extracts from fruits rich in these compounds have reduced the incidence of focal aberrant crypts and adenocarcinomas in rats treated with different chemical carcinogens (23-25).

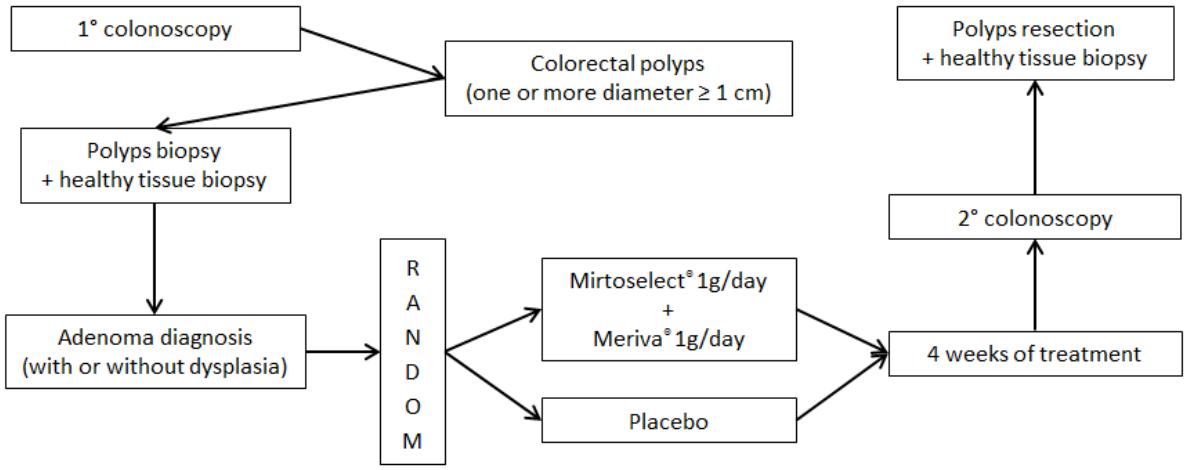
Furthermore, the same compounds were able to reduce the onset of intestinal adenomas in ApcMin mice (26), by reducing the expression of β-catenin.

In a pilot study conducted on 25 patients with colorectal carcinoma (27), the subjects were treated with a dry extract containing anthocyanins for 7 days before surgery (anthocyanin titrated: 0.5-2g/day). Ki67 analysis on pre-operative biopsies and on the resected tumor demonstrated a small but systematic reduction (~7%) of tumor proliferation.

7.0 STUDY DESIGN

Randomized, preoperative, double-blind, placebo-controlled study.

Randomized, double-blind, placebo controlled study



Subjects with polyp of probable colorectal adenomatous nature will undergo biopsy of the polyp and healthy perilesional tissue during colonoscopy. After histological confirmation of AP and eligibility criteria verification, patients will be randomized to one of the two treatment groups. After four weeks of treatment, the second colonoscopy with polyp removal and biopsy of healthy perilesional tissue will be performed. The biopsy of the healthy tissue will be performed in the rectum if there is macroscopic inflammation in the sigma; in the absence of inflammation, it will be performed in the sigma.

The determination of tissue biomarkers to verify the study hypotheses will be performed on adenomatous and normal tissue from adenoma biopsy taken at baseline and on the removed polyp and on the normal tissue biopsy after 4 weeks of treatment. Circulating biomarkers will be measured at baseline and at the end of treatment.

8.0 STUDY POPULATION

A total of 100 subjects with one or more colorectal adenomatous polyps with a diameter ≥ 1 cm will be included in the study.

8.1 Inclusion criteria

1 Age > 18 years and ≤ 85 years;
2 Written informed consent;
3 Presence of one or more adenomatous polyps in the colon-rectum (with diameter ≥ 1 cm);
4 ECOG performance status ≤ 1.

8.2 Exclusion criteria

1 Presence of hyperplastic polyps and serrated and flat adenomas;
2 Previous colorectal cancer;
3 Presence of carcinomatous tissue in the adenoma;
4 Taking experimental drugs or food supplements based on blueberry or curcumin in the 15 days prior to enrollment;
5 Any other factor that, at the discretion of the investigator, may contraindicate enrollment in the study.

9.0 OBJECTIVE

9.1 Primary objective

To evaluate the effect of treatment on the reduction of β-catenin in adenomatous tissue. This biomarker, activator of the Wnt signaling pathway, acts as a transcription factor for some target genes in the nucleus that lead to neoplastic transformation of the cell, and is considered one of the key elements in APC-mediated colon carcinogenesis.

9.2 Secondary objectives

1. Evaluate the effects of treatment on:

- oxidative stress and inflammation through the evaluation of IHC expression changes of Nuclear Factor-Kβ (NFKβ), one of the major transcription factors involved in the regulation of normal and neoplastic cells, closely related to the inflammatory and carcinogenic pathways of colon cancer;
- cell proliferation (Ki-67 labeling index), P53 mutation, tissue gene expression with microarray;
- circulating growth factors of the IGF system (IGF-I, IGFBP-3 and IGF-I/IGFBP-3) and EGFR;
- markers of inflammation (u-PCR).

2. Evaluate the tolerability of the treatment, as a combination of 2 food supplements and study the plasma and possibly tissue absorption of the active ingredients present in the botanical extracts (anthocyanosides and curcumin) .

10.0 TREATMENT REGIMEN

10.1 Name of experimental treatment and formulation

Bilberry extract: food supplement formulated in tablets with purple coating of 500 mg each and packaged in blisters.

Composition: Bilberry extract (Mirtoselect ®, 500 mg), microcrystalline cellulose, soy polysaccharides, anhydrous citric acid, croscarmellose sodium, silica dioxide, magnesium stearate, talc FU, purple coating for tablets.

Turmeric extract: food supplement formulated in tablets with yellow coating of 500 mg each and packaged in blisters.

Composition: turmeric extract (Meriva®, 500mg), calcium hydrogen phosphate dihydrate, anhydrous citric acid, soy polysaccharides, sodium croscarmellose, silica dioxide, magnesium stearate, talc FU, yellow coating for tablets.

Placebo A: purple coated tablets, without active ingredients, but with excipients present in blueberry extract tablets.

Placebo B: yellow-coated tablets, without active ingredients, but with excipients present in turmeric extract tablets.

10.2 Dosage groups, duration of exposure and dose selection

Subjects will take blueberry extract (Mirtoselect®) 1 g/daily divided into 2 daily administrations of 500 mg or placebo A, together with turmeric extract (Meriva®) 1 g/daily divided into 2 daily administrations of 500 mg or placebo B for a minimum period of 4 weeks and a maximum period of 6 weeks.

Mirtoselect® 500 mg, Meriva® 500 mg, placebo A or B tablets will be packaged in blisters of 9 tablets each. The intake of the supplement or placebo will be in the order of 2 tablets per day of each supplement or respective placebo to be taken in the morning and evening always at the same time.

The choice of a daily dose of 1 g of blueberry extract is based on a large literature regarding previous studies that ensure its safety even at doses much higher than 1 g. In particular, with blueberry extract the results on efficacy, pharmacokinetics and safety of several clinical studies are available starting from the dose of 160 mg with 2 daily administrations, up to the daily dose of 10.8 g for 7 days of treatment (27-29).

The choice of a daily dose of 1 g of curcumin extract is based on previous studies demonstrating that the complexation of curcumin with phosphatidylcholine is able to increase the bioavailability of the active ingredient (21,22), and this allows to lower the doses compared to previous studies with non-complexed curcumin (20). In fact, the supplement based on curcumin extract and phospholipid in a 1:2 ratio has demonstrated efficacy in the treatment of osteoarthritis and anterior uveitis (30,31) due to its pharmacokinetic characteristics and in particular for its combination with the phospholipid part which allows to overcome some limits of the oral preparations of curcumin, such as instability at intestinal pH values, low solubility in water, poor oral bioavailability and rapid conjugation and excretion. Furthermore, the curcumin extract has proven to be safe and well tolerated. Oral intake in the clinical studies has never been associated with significant side effects.

10.3 Supply, packaging, labelling

The distribution of the supplements (Mirtoselect® and Meriva®) and the respective placebos, packaged in accordance with the regulations for food supplements by the company Indena SpA, is the responsibility of the coordinating center, which will provide the supply to the participating centers.

The blueberry extract and the turmeric extract will be supplied in 2 different packs containing 90 tablets (= 2 tablets per day for 6 weeks + 6 spare tablets, for each supplement). Each patient will receive a “patient kit” with 1 pack of blueberry extract and 1 pack of turmeric extract to cover the entire treatment period. The preparation and packaging will be visually identical to those of the respective placebos. The Indena company guarantees the correct assembly of the compounds (active/placebo) inside the “patient kits”. Indena will deliver the “patient kits” to the EO Ospedali Galliera, c/o the SC Farmacia, which will deliver them to the S.C.Oncologia Medica, for distribution to the enrollment centers involved.

The “patient kit” will have an external label (study name, sponsor and patient ID) with a removable portion that must be applied to the appropriate Case Report Form (CRF) in order to ensure the correct assignment of the product. Inside the kit, the information noted on the boxes are: study name, sponsor, patient ID, expiration date, number of tablets, method of administration, warnings.

10.4 Disposal of residual supplements

Residual, unused, leftover or expired supplements must be stored at the enrolling center until retrieval, which will be done by the monitor, assigned by the coordinating center. This

procedure can be implemented only after accountability verification during the monitoring phase. Unclassified and/or expired supplements cannot be sent independently to the coordinating center.

10.5 Treatment assignment method

All eligible patients after signing the informed consent must be randomized before starting study treatment. Randomization will be centralized at the Medical Oncology Department of the EO Ospedali Galliera through a computerized procedure. The randomization list will be prepared by the biostatistician of the Scientific Coordination Office of the coordinating center independently: permutation blocks of different sizes will be used, stratified for the enrolling sites. Randomization will be performed using a paper form sent by FAX to the Medical Oncology Department at Galliera Hospital. Once it is ascertained that the form has been filled out correctly, a patient ID corresponding to the kit will be associated with the eligible patient.

An operating manual for randomization of study subjects will be provided to centers.

Subjects will be randomized in a strictly sequentially sequenced manner and will remain in their assigned arm for the duration of the study.

The procedure for assigning treatment to randomized subjects will be kept blind according to the following method: the supplement packages (Mirtoselect® 500 mg, Meriva® 500 mg, placebo A, B) will be prepared in blisters containing 90 identical tablets, supplement and reference placebo, in order to allow for masking. Each pack will contain 10 blisters of 9 tablets for each individual product (supplement or placebo). For treatment, each subject will receive a "patient kit", i.e. a box containing a pack of Mirtoselect® + a pack of Meriva®, or a pack of Placebo A + a pack of Placebo B. Each "patient kit" box will be associated with the subject using the code indicated in the randomization list. The preparation of the supplements will be carried out according to good practices for preparing botanical extracts and will be paid for by Indena SpA. Indena will identify an operator responsible for receiving the open-label (unmasked) randomization lists from the statistician, who will archive the lists for any future checks. The treatment code may be revealed to the clinician and the patient only at the end of the study or if it is necessary following a serious adverse event that could be related to the experimental treatment or in any case in cases where, for medical emergencies, it is necessary to know the treatment associated with the subject in the study.

Unblinding may only be performed by the coordinating center upon specific request of the participating center. An operating procedure for unblinding will be provided to the centers.

11.0 STUDY PROCEDURES

11.1 Duration of study

Subjects will be enrolled over 48 months, so the total study duration is expected to be 64 months, including the determination of SEB (surrogate end-point biomarkers), statistical analysis and drafting of the final report.

The study is considered concluded when the last enrolled subject has completed the follow-up visit one year after the end of the experimental treatment.

11.2 Screening

Within the month preceding randomization, the following must be guaranteed:

- signature of written informed consent;
- eligibility verificaton: evaluation of inclusion/exclusion criteria.
- blood tests and/or instrumental tests;
- colonoscopy with evaluation of the presence of adenomatous polyp ≥ 1 cm and collection of biopsy material necessary for tissue determinations.

11.3 Baseline Visit

- Clinical visit and detection of vital parameters: weight, height, blood pressure (BP), Performance status (PS) according to ECOG;
- Blood sampling for the determination of circulating biomarkers and for the determination of plasma absorption of the active ingredients present in the botanical extracts at time zero (only for patients enrolled at the EO Ospedali Galliera).
- Delivery of the food diary.
- Anamnesis, concomitant pathologies and family history (I and II degree) for CRC/other neoplasms;
- Concomitant therapies;
- Randomization;
- Delivery of supplement/placebo: a package containing sufficient supplement for a six-week administration period + 6 spare tablets (90 tablets) will be delivered. The kit must be returned at the end of the study visit.

11.4 Final Visit (after 4-6 weeks of treatment)

- Clinical visit and detection of vital parameters: weight, BP, PS according to ECOG;

- Blood sampling for the determination of circulating biomarkers.
- Blood sampling to determine the plasma absorption of the active ingredients present in the botanical extracts, before and 1 hour after the last treatment (Only for patients enrolled at the EO Ospedali Galliera);
- Concomitant therapies;
- Adverse events and symptoms;
- Patient kit return and compliance assessment;
- Food diary collection and evaluation;
- Polypectomy and biopsy for tissue determinations, to be carried out after 2-5 hours of taking the last dose.

11.5 Follow-up (1 year after the end of treatment)

The follow-up visit, 1 year after the end of treatment, must be performed one year ± 1 month after the end-of-study visit.

During the visit the following data must be collected:

- Clinical visit and detection of vital parameters: weight, BP, PS according to ECOG;
- Adverse events and symptoms;
- CCR onset (histological evaluation/colonoscopy/polypectomies);
- Relevant clinical notes.

11.6 Compliance

To manage the study supplements accountability, a balance sheet will be drawn up to maintain traceability of the inventory of products, shipping receipts and the kit administration. This register will be updated regularly and, for each shipment, will identify the subject number, the randomization number and the quantity of supplements shipped. The accountability form will be stored/used in all the places where the supplements will be stored for subsequent delivery to patients, the main pharmacy, the pharmacies of the individual centers, the clinical unit and other delivery locations. The form will include, in addition to the delivery register, all other possible operations for the management of the supplements (receipts, transfers, returns, damaged packages). The register form will be provided by the coordinating center to the participating sites.

11.7 Collection and management of biological material

Tissue samples collected during colonoscopies for immunohistochemical determinations will be collected centrally at SC Anatomia Patologica, EO Ospedali Galliera, upon specific request of the coordinating center and at the end of the study (within the month after the last enrolled patient took the last dose of treatment). Transparent labeled envelopes will be provided to the enrolling centers. The labels will contain: name of the study, center number, randomation number and patient registration, date of the exam and time (1=baseline, 2=final).

Blood samples for circulating biomarkers will be collected centrally by the Lab Analysis of the Prevention and Oncology Genetics Division of the IEO in Milan upon specific request and in any case at the end of the study (within the month after the last enrolled patient took the last dose of treatment). In both cases, guides for biological samples collection, preparation, storage and shipment to the appropriate centralized center will be provided.

Blood samples for the determination of plasma absorption of the active ingredients will be performed only on the group of patients enrolled at the Gastroenterology unit of the coordinating center, at EO Ospedali Galliera and will be analyzed by an external laboratory. A guide will be provided for samples collection, preparation, storage times and shipment to the centralized collection center.

11.8 Concomitant treatment

During the study, the intake of Indian foods is not recommended, as is the intake of H_2 blockers (cimetidine, ranitidine, nizatidine, famotidine) and proton pump inhibitors (omeprazole, esomeprazole, rabeprazole, pantoprazole, lansoprazole).

If the subject uses antacids and/or lactic ferments, he/she will be instructed to take them at least 2 hours before or after the experimental treatment.

The investigator must detail in the CRF any intake of the above-described substances and any other concomitant drugs or food supplements.

11.9 Food diary

A food diary will be provided to the patient at the beginning of the study and must be filled out daily by the patient. The diary is intended to monitor the intake of foods consumed by the subject in treatment and their possible interaction with the compounds. The diary, like the CRF, must be forwarded to the coordinating center 5 working days after the end-of-study visit.

12.0 FLOW CHART

Exam/Procedure	Screening	Baseline Visit	Final Visit	Follow up
Written Informed Consent	X			
Colonoscopy/identification of polyp >1 cm (histology)	X			
Blood tests[1]	X			
Check eligibility	X			
Randomization		X		
Clinical Visit[2]		X	X	X
Control colonoscopy (according to clinical practice)				X
Anamnesis/clinical history/family history for CRC		X		
Biomarker sampling		X	X [3]	
Pharmacokinetic sampling		X	X [4]	
Polypectomy and Tissue Collection for Biomarker [5]		X	X	
Compliance			X	
Adverse reactions/adverse events			X	X
Concomitant treatment		X	X	X

1 Routine tests required for colonoscopy.
2 Evaluation of vital parameters: weight, height, BP, PS ECOG.
3 Post-dose sampling.
4 To determine the absorption of the active ingredients, a sample will be taken before and 1 hour after taking the last dose of treatment.
5 Polypectomy to be performed within 2-5 hours of taking the last dose of treatment.

13.0 ETHICAL ASPECTS

This protocol complies with the principles established by the 18th World Medical Assembly in Helsinki, 1964 and subsequent amendments/additions and with Good Clinical Practice.

13.1 Informed consent

The investigator must explain to each patient (or legal representative) the nature of the study, its purpose, procedures, expected duration, potential risks and benefits and any adverse events or reactions that may occur. Each patient must be informed that participation in the study is voluntary and that he/she may withdraw from it at any time, withdrawal of consent will not affect his/her subsequent clinical treatment or relationship with the physician. The informed consent will be prepared by the coordinating center by means of a standard written statement, using non-technical language. The patient must demonstrate that he/she has understood the information contained in the consent, by signing and dating it; he/she must also be provided with a copy of the document. If the patient is unable to read or sign the document, the oral and written presentation of the study may be provided to his/her legal representative who can sign and date the document. No patient may be enrolled in the study before his/her informed consent has been obtained. An original signed copy of the patient's written informed consent must be kept in the appropriate section of the Investigator's File of the enrolling center.

13.2 Patient safety

Patient names will not be recorded, but each subject will be identified with a treatment number (randomization number) and a registration number. These numbers will identify the patient and must be included in all CRFs. In order to avoid misidentification, the date of birth must be reported in the CRFs to identify the subjects themselves. The investigators guarantee that all persons involved in this study will respect the confidentiality of any information regarding the subject participating in the trial. All parties involved in this clinical trial will maintain the utmost confidentiality to ensure that neither the person nor the privacy of the patient participant's family is violated; adequate measures will be taken to prevent unauthorized persons from accessing the study data. The processing of personal data of patients participating in the trial, and in particular the related data regarding consent, will be in accordance with local privacy laws:

Legislative Decree 196/03 and subsequent amendments (“Privacy Code”), “Guidelines for the processing of personal data in the context of clinical trials of medicinal products” with particular reference to the profiles relating to the methods of data processing, data requirements, the designation of Managers and Persons in Charge and the custody and security of information.

14.0 DATA COLLECTION AND MONITORING PLAN

14.1 Data collection and monitoring procedures

Study data will be collected via a paper CRF that must be faxed to the coordinating center. CRFs must be sent within 5 working days of the visits. Checks on the receipt of CRFs will be carried out by the Coordinating center on a regular basis during the study.

Monitoring will be carried out according to this plan:

- opening of the center by teleconference (SIV) and concomitant shipment of supplements
- visit to the center after approximately 6 months (± one month) from the inclusion of the first patient
- closing visit of the center at the end of the study.

Periodic telephone monitoring will be carried out to verify the correct entry of data into the CRF.

It is the responsibility of the participating site investigator to prepare and maintain adequate and accurate CRFs for each subject enrolled in the study. All CRFs must be completed to ensure accurate interpretation of the data, a black ballpoint pen must be used to ensure clarity of the data transcription on the CRF. If a correction is necessary, the information that needs to be changed must not be overwritten. The corrected information must be transcribed next to the previous value with the reason for the correction, initialed and dated by the authorized person.

14.2 Quality Assurance

The researchers of the participating centers, will have to guarantee the quality of the data, each information reported on the CRF will be systematically verified for coherence, completeness and correctness by the Coordinating Center that may ask for clarifications in case of doubtful data. Local quality controls will be carried out by the coordinating center as responsible for the monitoring plan.

14.3 Source documents

The source data must be stored and recorded in a hospital medical record; if such records for outpatient subjects are not provided for by the practice of the facility or clinic where the trial is being conducted, they must be prepared for the purposes of the legislation on clinical trials of medicinal products. If paper CRFs are used as source documents, the principal investigator of

the center must declare the methods of archiving such source documents and, after having signed, stamped and dated them, insert the same CRFs into the outpatient record as an integral part of the same.

At the end of the trial, the documents must be kept at the center for a period of 10 years. If the responsible investigator changes workplace, he must forward the name of a new trial manager to the coordinating center.

15.0 END OF STUDY CRITERIA

After 4-6 weeks of treatment, patients will stop study treatment and after all 100 subjects have been randomized, enrollment will be closed. The study will be considered terminated when the 100th patient has made the follow-up visit.

15.1 Premature discontinuation

Subjects who, for various reasons, do not complete the treatment period will be classified as having dropped out of the study early.

Some possible reasons for early exit include:

- Inappropriate recruitment;
- Adverse event;
- Personal reasons: a patient may withdraw from the study at any time and for any reason.

16.0 TOXICITY MONITORING AND ADVERSE EVENT MANAGEMENT

16.1 Adverse Event Definitions

Adverse event (AE): is any undesirable medical event in a patient or subject included in a clinical trial and which does not necessarily have a causal relationship with the treatment.

Serious adverse event (SAE) or serious adverse reaction (SAR) or suspected unexpected serious adverse reaction (SUSAR): any adverse event or adverse reaction that, regardless of dose, results in death or is life-threatening, requires hospitalisation or prolongation of hospitalisation, or results in severe or prolonged disability or incapacity, or involves a congenital anomaly or birth defect.

16.2 Adverse Event (AE) and Serious Adverse Events (SAE) Reporting

All AEs must be recorded in the appropriate section of the CRF, regardless of whether or not they are directly related to the study treatment.

All SAEs and SUSARs must be reported on a specific form provided by the coordinating center and processed in the following manner:

SAE and SUSAR. Each investigator will ensure that all information relating to SAE is recorded on the appropriate phytovigilance form and notified to the Coordinating Centre within 24 hours of the first knowledge of the SAE by the responsible investigator (tel: 010-563.4580; fax: 01057481407), and that subsequent relevant information is communicated within eight days of

the first report. The Coordinating Centre will be responsible for sending reports of suspected reactions to the study treatment to the Istituto Superiore di Sanità.

Once a year, the principal investigator will provide the Ethics Committees with a list of all SAEs and SUSARs observed throughout the entire period.

The occurrence of CCR will not be considered as a SAE.

17.0 STATISTICAL METHODS

17.1 Statistical design and sample calculation

The primary endpoint of the study is the difference in β-catenin values [post-treatment value - pre-treatment value].

Data from our previous trial on the same study population (APAC Study) allow us to make some assumptions: we assume a baseline mean of β-catenin expression in adenomatous tissue equal to 25% (standard deviation=35) and a correlation between baseline and final β-catenin values equal to approximately 0.9. Given these assumptions, using an ANCOVA model (therefore adjusting the analysis for baseline β-catenin values), with 50 subjects per arm, for a total of 100 subjects, we will have an 85% power to observe a mean difference between the two arms equal to 10%, in the levels of β-catenin expression in adenomatous tissue. The sample calculation takes into account 10% of subjects lost to follow-up and a 2-tailed alpha error equal to 5%.

17.2 Interim analysis

At approximately halfway through the study, which will approximately coincide with 50% of enrolled patients completing treatment, the study monitoring committee, composed of the principal investigators from each center, the biostatistician, and the study coordinator, will perform an interim analysis to assess any adverse and/or toxic effect of the active treatment compared to placebo. This analysis will therefore consider 2 endpoints in the comparison arms: 1) number of adverse events 2) size of the adenoma.

With a total of n=50 subjects, there will be sufficient power to detect an absolute difference of 40% in terms of frequency of adverse events and in terms of change in size of the adenoma between the active arm and placebo at the end of treatment (assuming an ANCOVA model corrected for baseline size and a correlation coefficient of 0.7 between mean baseline and final size). In case of a significantly greater adverse and/or toxic effect in the active arm, the monitoring committee will consider the possibility of prematurely stopping the study.

17.3 Statistical analysis methods

All subjects enrolled in the study will be considered in the “Intention To Treat” (ITT) population, and the statistical analysis will be performed in accordance with this principle, therefore considering the randomized subjects regardless of their actual adherence to the experimental treatment.

The descriptive statistics used for continuous variables will be: mean, standard deviation, median, quartiles, minimum and maximum; for discrete variables they will be: absolute frequency and relative frequency.

In agreement with previous results, the difference in post- and pre-treatment values of β-catenin [post-treatment value - pre-treatment value] is a normally distributed variable. However, the normality of the distribution will be tested and, if necessary, the necessary transformations will be applied to obtain normality.

Fisher's exact test and chi-square test will be used to compare proportions in the two treatment arms (toxicity: number of adverse events), while parametric (t-test) and non-parametric (Mann-Whitney U) tests will be used to compare continuous variables.

Parametric statistics will be used for the primary objective. Hypotheses will be tested using the general linear model: the null hypothesis of no treatment effect will be tested using standard analysis of variance (ANOVA) and covariance (ANCOVA) methods, adjusting for baseline β-catenin and the effect of other possible confounding factors such as age, sex, body mass index (BMI), family history of colon cancer, smoking habits, alcohol consumption and other important factors (demographic and/or biological) collected.

Acceptance of the study protocol / Responsibilities of the investigator

I read the study protocol with the following title:

" A PHASE II STUDY OF BIOMARKER MODULATION IN SUBJECTS WITH COLON ADENOMATOSUS POLYP WITH BLUEBERRY EXTRACT AND TURMERIC EXTRACT
"MIRACLE STUDIO"

and I agree to conduct the study as specified in the protocol and to observe the Good Clinical Practice (GCP) standards, I accept periodic visits by the monitor and all other data control and verification procedures.

Name of the Center:

_____

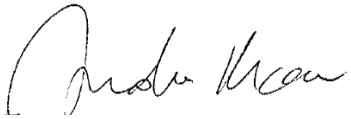
Name of Responsible Investigator:

_____

Signature Date

_____ _____

Signature of Project Manager Date



_____ 01 October 2016

Andrea De Censi

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