Synopsis Study Portocol (DigesT trial)	
Study title	**Impact of Dietary Intervention on tumor immunity: the DigesT trial**
Study Sponsor	Fondazione IRCCS Istituto Nazionale dei Tumori di Milano
Coordinating Center	Fondazione IRCCS Istituto Nazionale dei Tumori di Milano
Background	Metabolic reprogramming is a hallmark of cancer. Human cancers are able to use blood metabolites and growth factors to fulfill their energetic and anabolic requirements. In particular, blood glucose stimulates aerobic glycolysis in cancer cells, thus leading to the production of ATP units and precursors of amino acid and nucleotide biosynthesis. Moreover, circulating growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), can induce the activation of the PI3K/AKT/mTOR pathway, which in turn stimulates protein, fatty acid and cholesterol biosynthesis in cancer cells. Not only systemic metabolism can sustain cancer cell growth and proliferation; it also impacts on the proliferation and activation status of circulating immune cells, including protumor and antitumor immune cells. For example, circulating IGF-1 can inhibit the activation and memory functions of T lymphocytes, while the proliferation of myeloid derived suppressive cells (MDSCs) is stimulated by circulating glucose and eicosapentanoic acid. Different metabolic blood profiles could exert stimulatory or inhibitory effects on specific immune cell populations, thus setting a specific balance between protumor and antitumor activities.
Hypotesis/Rationale	Modifying systemic metabolism could affect cancer cell metabolism and the activation of specific immune cell populations. In recent years, a plant-based, calorie-restricted, low-carbohydrate, low-protein diet, also known as the Fasting Mimicking Diet (FMD) has been proposed as a potential anticancer dietary intervention. The FMD is safe when administered cyclically (every 21-28 days) to healthy volunteers, and is capable of significantly reducing the concentration of blood glucose, insulin and IGF-1, while increasing levels of IGFBPs and ketone bodies. The FMD has been shown to inhibit the *in vivo* growth of several tumor models,

	including breast cancer and melanoma mice models. The anticancer effects of the FMD are likely mediated by two concomitant mechanisms: 1) one direct anticancer effect that is mediated by the inhibition of energy production and anabolic pathways, such as protein and fatty acid synthesis, in cancer cells; 2) one indirect effect that is mediated by the activation of antitumor immunity, with the result of enhanced tumor infiltration by cytotoxic T-lymphocytes and reduced infiltration by immunosuppressive populations. Both effects derive from changes in blood metabolites, which can impact on the growth and proliferation of both cancer and immune cells. According to the currently accepted model, the anticancer and immunomodulatory effects of the FMD mostly derive from the reduction of circulating glucose, insulin and IGF-1 levels, and a parallel increase of ketone body and IGF-1 binding protein concentration. However, based on recent discoveries, FMD-mediated changes in many other metabolites, such as specific amino acids (e.g. tryptophan, arginine, methionine, glutamine, serine) or fatty acids (e.g. arachidonic acid, eicosapentanoic acid), could contribute to the cell-autonomous or immune-mediated anticancer effects of the FMD. While the study of the effects of the FMD in combination with standard treatments (e.g. chemotherapy, molecular targeted therapy) in advanced cancers represents the final objective of the ongoing studies, fully uncovering the metabolic and immunological effects of the FMD alone is essential to design future combination studies. From this perspective, the pre- ad post-operative clinical settings in cancer patients who are not candidate to other medical treatments represent an ideal context to assess the effects of the FMD without other confounding factors.
Objetives and corresponding endpoints	<u>Primary objective and corresponding endpoint:</u> To evaluate the short-term and log-term efficacy of the FMD to down-modulate protumor peripheral blood mononuclear cells (PBMCs), including myeloid-derived suppressive cells (MDSCs), and to up-regulate antitumor cells, such as activated T cells and NK cells. Short-term modifications refer to changes assessed the day of FMD discontinuation compared to the day of FMD initiation. Long-term modifications refer to changes detected the day of surgery or 30 days after surgery compared to the day of FMD initiation. <u>Secondary objectives and endpoints:</u> 1. To assess by single-cell “mass cytometry” (CyTOF) phenotypic and functional modifications in PBMCs and tumor-infiltrating lymphocytes during the FMD. 2. To perform transcriptional and miRNA profiling in PBMCs, tumor cells (Cohort A) and immune cell populations inside lymph nodes (Cohort B). 3. To assess changes in the expression of metabolic genes, such as those involved in glycolysis,

	trycarboxylic acid cycle, amino acid synthesis, fatty acid and cholesterol *de novo* biosynthesis in PBMCs. 4. To assess FMD-induced changes in blood (glucose, triglycerides, fatty acids, cholesterol, amino acids) and urine (ketone bodies) metabolic parameters. 5. To assess FMD-induced changes in plasmatic growth factors, such as insulin, IGF-1, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), IGF1 binding proteins 1 and 3 (IGFBP-1 and IGFBP-3). 6. To evaluate by immunohistochemistry (IHC) or multiple immunofluorescence (IF) quantitative and qualitative changes in tumor immune cell infiltrate, including myeloid, T and NK cell markers, by comparing the diagnostic biopsy specimen with the tumor specimen after surgical removal of the primary tumor (Cohort A). 7. To assess changes in tumor proliferation index (Ki67), apoptosis (caspase 3 by IHC), autophagy (LC3), glycolysis (Glut1), tumor grade and immunohistochemical expression of estrogen and/progesterone receptor and HER2 oncoprotein in breast cancer patients (Cohort A). 8. To evaluate quantitative and qualitative changes in immune cell suspensions from lymph nodes of melanoma patients undergoing one FMD cycle before lymph node dissection (Cohort B). 9. To evaluate changes in DNA methylation profiles in tumor and lymph node tissues from patients undergoing the FMD before surgery (Cohorts A and B). 10. To assess modifications in gut microbiota community composition, as detected by 16S rRNA gene sequencing and shotgun metagenome sequencing of patient stool samples before and after the FMD. 11. To evaluate short-term and long-term modification of systemic nutritional parameters (serum albumin, transferrin, pre-albumin, retinol-binding protein and lymphocyte counts) after one FMD cycle for patients in cohort A and B and after 4 FMD cycles for patients in Cohort C. 12. To evaluate changes induced by the experimental treatment in the hematologic profile (white blood cell counts, lymphocyte counts, hemoglobin concentration, platelet counts, red blood cell counts). 13. To investigate patient compliance to the diet, as assessed through the analysis of dietary diaries and measured by counting minor and major deviations from the prescribed FMD regimen. 14. To assess the tolerability of the prescribed FMD regimen, including adverse events and serious adverse events occurring during the FMD and after its discontinuation. 15. To correlate FMD-induced changes in serum metabolites with changes in PBMCs, their activation status, as well as with tumor cell and immune infiltrate characteristics.

Study population	***Inclusion criteria*** Patients eligible for inclusion in this study have to meet **all** of the following criteria: 1. Age ≥ 18 and ≤ 75 years. 2. Evidence of a personally signed and dated informed consent document (ICD) indicating that the patient has been informed of all pertinent aspects of the study before enrollment and FMD prescription. 3. Willingness and ability to comply with the FMD protocol, the scheduled visits, treatment plans, laboratory tests and other procedures. 4. Histologically confirmed diagnosis of invasive breast cancer candidate to curative surgery (Cohort A), *or* resected malignant melanoma requiring dissection of the regional lymph node basin for sentinel lymph node involvement (Cohort B), *or* malignant melanoma treated with curative surgery (including, in case, lymph node removal and lymph node dissection) (Cohort C). For breast cancer patients, any biological subgroup (including estrogen receptor-positive, HER2-positive, triple-negative breast cancer) will be admitted; HER2-positive tumors will be defined on the basis of an IHC score of 3, or a score of 2 with ISH evaluation indicative of gene amplification. 5. Availability of archival FFPE tissue blocks of primary breast cancer (Cohort A) or melanoma (Cohort B, Cohort C). 6. Presence of an Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1. 7. Presence of adequate bone marrow and organ function as defined by the following laboratory values: - ANC ≥ 1.5 x 10^9/l - platelets ≥ 100 x 10^9/l - hemoglobin ≥ 9.0 g/dl - calcium (corrected for serum albumin) within normal limits or ≤ grade 1 according to NCI-CTCAE version 4.03 if not clinically significant - potassium within the normal limits, or corrected with supplements - creatinine < 1.5 ULN - blood uric acid < 10 mg/dl - ALT and AST ≤ 2.5 x ULN - total bilirubin < ULN except for patients with Gilbert syndrome who may only be

	included in the total bilirubin is < 3.0 x ULN or direct bilirubin < 1.5 x ULN - Albumin > 3 g/dL 8. Fasting glucose ≤ 200 mg/dl. 9. Total Cholesterol ≤ 300 mg/dl. 10. Triglycerides ≤ 300 mg/dl. 11. Female patients of childbearing potential must agree to sexual abstinence or to use two highly effective method of contraception throughout the study and for at least 30 days after the end of the FMD. Abstinence is only acceptable if it is in line with the preferred and usual lifestyle of the patient. Examples of contraceptive methods with a failure rate of < 1% per year include tubal ligation, male sterilization, hormonal implants, established, proper use of combined oral or injected hormonal contraceptives, and certain intrauterine devices. Alternatively, two methods (e.g., two barrier methods such as a condom and a cervical cap) may be combined to achieve a failure rate of < 1% per year. Barrier methods must always be supplemented with the use of a spermicide. A patient is of childbearing potential if, in the opinion of the Investigator, she is biologically capable of having children and is sexually active. Female patients are not of childbearing potential if they meet at least one of the following criteria: • Have undergone a documented hysterectomy and/or bilateral oophorectomy • Have medically confirmed ovarian failure • Achieved post-menopausal status, defined as: (≥ 12 months of non-therapy-induced amenorrhea) or surgically sterile (absence of ovaries) and have a serum FSH level within the laboratory's reference range for postmenopausal females. ***<u>5.3 Exclusion criteria</u>*** Patients eligible for this study must not meet **any** of the following criteria: 1. Prior systemic treatment for breast cancer or melanoma. 2. Diagnosis of a concurrent malignancy other than breast cancer or melanoma, or malignancy other than breast cancer or melanoma diagnosed within 5 years of treatment enrollment, with the exception of adequately treated, basal or squamous cell carcinoma, non-melanomatous skin cancer or curatively resected cervical cancer. 3. Body Mass Index (BMI) < 20 Kg/m^2.

	4. Anamnesis of alcohol abuse. 5. Unintentional weight loss ≥ 5% in the last three months, unless the patient has a BMI > 25 Kg/m^2 at study enrollment. Intentional weight loss is permitted if < 10% in the last three months and patient BMI is > 22 kg/m^2. 6. Severe heart, liver, pulmonary, kidney comorbidities. 7. Current status of pregnancy or lactation, where pregnancy is defined as the state of a female after conception and until the termination of gestation, confirmed by a positive hCG laboratory test (> 5 mIU/mL). 8. Active HBV or HCV infection. 9. Severe infections within 4 weeks prior to FMD initiation, including, but not limited to, hospitalization for complications of infection, bacteremia, or severe pneumonia. 10. Active autoimmune diseases that require systemic treatment (i.e. with use of disease modifying agents, corticosteroids or immunosuppressive drugs). 11. History of recent diagnosis of hypothyroidism for which replacement therapy (eg., thyroxine) and blood endocrine profile are not stabilized yet. 12. Established diagnosis of diabetes mellitus type I or diabetes mellitus type II that requires pharmacological treatment (including, but not limited to, insulin, insulin secretagogues and metformin). 13. Severe impairment of the gastrointestinal (GI) function or GI disease that may alter the digestion and absorption of nutrients during the re-feeding phase (e.g. active ulcerative diseases of the stomach or intestine, uncontrolled nausea, vomiting, diarrhea, malabsorption syndrome, or small bowel resection). 14. Known history of Human Immunodeficiency Virus (HIV) infection. 15. Clinically significant heart disease and/or recent cardiac events including: a) history of angina pectoris, coronary artery bypass graft (CABG), symptomatic pericarditis, or myocardial infarction within 12 months prior to the start of study treatment; b) history of documented congestive heart failure (NYHA III-IV); c) documented cardiomyopathy. 16. History of cardiac arythmias, (e.g. ventricular tachycardia, chronic atrial fibrillation), complete left bundle branch block, high grade AV block (e.g. bifascicular block, Mobitz type II and third degree AV block), supraventricular, nodal arrhythmias, or conduction abnormality in the previous 12 months. 17. Uncontrolled hypertension defined by a Systolic Blood Pressure (SBP) ≥ 160 mmHg and/or Diastolic Blood Pressure (DBP) ≥ 100 mmHg, with or without anti-hypertensive medication. 18. Known reduction of left-ventricular ejection fraction (LVEF) to less than 50%, as assessed by multigated radionuclide scintigraphic scan (MUGA) or echocardiography. 19. Previous episodes of symptomatic hypotension causing unconsciousness.

	20. Baseline fasting plasma glucose ≤ 65 mg/dl. 21. Ongoing therapy with systemic corticosteroids, or systemic corticosteroid therapy ≤ 2 weeks before study enrollment, or who have not recovered from side effects of such treatment. The following uses of corticosteroids are permitted: topical applications (e.g. for rash), inhaled sprays (e.g. for obstructive airways diseases), eye drops. 22. Any serious medical or psychiatric illness that in the assessment of the investigator renders the patient not suitable for participation in this clinical study.
Study Design and Treatment	Single-arm, monocentric, prospective study designed to estimate the immunomodulatory and metabolic effects induced by the FMD in breast cancer and melanoma patients. Three different patient cohorts will be enrolled: 1) Cohort A: 40 breast cancer patients candidate to curative surgery without pre-operative systemic treatment. Patients with clinical stages T1N0M0, or those with T1N1M0-T2N0M0 tumors not requiring pre-operative systemic treatment, will be enrolled. Patients with any breast cancer biology, including hormone receptor-positive, HER2-overexpressing and triple-negative cancers, will be included in the study; 2) Cohort B: 40 melanoma patients candidate to lymph node basin dissection after the histological documentation of sentinel lymph node tumor involvement; 3) Cohort C: 20 patients with surgically resected malignant melanoma not candidate to any adjuvant treatment. All patients will be prescribed 5-day low-protein, low-carbohydrate FMD, consisting in a list of allowed foods and quantities to be consumed during each of the five days. Foods or beverages not explicitly included in the prescribed scheme are not permitted. On day 1, the total calorie content of the FMD is of about 700 KCal; on days 2-5, the caloric content is 300 KCal. Patients in Cohort A and B (i.e. those patients receiving the FMD in the pre-operative setting) will receive only one FMD cycle, to be initiated within 15 days from surgery, and to be completed at least 7 days before surgery. Blood, urine and stool samples will be collected just before FMD initiation and at diet completion, to assess FMD-induced effects on systemic immunity and metabolism, as well as the modulatory effects on gut microbiota composition and stool metabolites. Moreover, FMD-induced modifications in the intratumor immune infiltrate and tumor biology (e.g. proliferation index, ER, PgR and Her2 status) will be assessed by comparing diagnostic biopsies with surgical tumor specimens (Cohort A); in Cohort B, the composition of immune cell populations in sentinel lymph node(s) (before the FMD) and in lymph nodes resected at the moment of basin dissection (after the FMD) will be compared as well. Moreover, immune cell suspensions from surgically removed lymph nodes will be obtained from fresh lymph node samples after the FMD to quantify different population subsets, and compared with matched controls of patients with similar characteristics, who were not eligible for enrollment in the trial, or directly refused to be enrolled.

	Patients in Cohort C will receive four consecutive FMD cycles every 28 days. Blood, urine and stool samples will be collected before and after FMD cycles n.1 and n.4, as well as 30 days after completion of 4 FMD cycles, to assess short- and long-term changes in systemic metabolism and immunity.
Duration of the treatment	Enrolled patients will undergo the experimental intervention (namely the FMD) for one cycle (Cohorts A, B) or 4 consecutive cycles (Cohort C). The intervention will be interrupted in the case of unacceptable toxicities, consent withdrawal, patient death or for other reasons at the discretion of the Investigators.
Planned sample size	100 patients (40 in cohort A; 40 in cohort B; 20 in cohort C)
Study period	30 months of enrollment and 6 months of follow up

Statistical methodology	The main goal of this project is to find systemic, tumor or lymph node immunologic modulation patterns that are significantly modified by the FMD. This assessment will be made on the basis of many indicators belonging to distinct biological systems, while a synthetic criterion for judging treatment effectiveness on this ground is lacking. This situation precludes the possibility to estimate upfront the statistical properties of our study. Nevertheless, the planned sample size in the three patient Cohorts was chosen in the attempt to guarantee study feasibility and patient enrollment within two years from study initiation, while contemporarily providing a sufficient patient number to describe the eventual variability in FMD-induced immunological, metabolic and gut microbiota modifications. The analysis of study results will be based on calculation of descriptive statistics suitable for continuous (mean, median, standard deviation, interquartile range) or categorical (absolute/ relative frequencies) variables. Statistical testing will be preferably based on non-parametric location tests for bivariate comparisons like the Wilcoxon-Mann-Whitney test or the Wilcoxon Signed-Rank Test in case of paired data. Generalized linear models will also be used for a comprehensive analysis of longitudinal data. Finally, robustness of statistical results focusing on single variables will be checked using suitable adjustments for multiple testing, like the Hommel's procedure (1988) for controlling the family wise error rate (FWER). The conventional 5% threshold of statistical significance for two-sided tests will be used throughout.