Supplementary Table

Synopsis of the study protocol of the ATHENA trial on anthocyanin supplementation in breast cancer patients undergoing radiotherapy

Study Rationale: Attention has been recently focussed on the development of modalities that can protect healthy organs from the harmful effects of radiation applied during different cancer treatment schedules. As radiation-induced DNA and cellular damage involve oxidative stress, the protective role of antioxidants is evaluated on the toxic condition induced by radiotherapy in breast cancer treatment. Consumption of polyphenol-rich foods, such as fruits and vegetables, and beverages derived from plants, such as cocoa, red wine and tea, may represent a beneficial diet in terms of oxidative protection. Indeed, a retrospective study reports that that moderate wine consumption can reduce the side-effects of radiation therapy in patients with breast cancer. This effect was presumably due to the polyphenol non alcoholic fraction of wine, a finding in agreement with a cross-over intervention study in adult male volunteers, on protection by de-alcoholized red wine from ex vivo radiation-induced DNA damage. However, there are no studies that address the relationship of polyphenol-rich food consumption with the toxic effects of radiations in patients undergoing radiotherapy for breast cancer treatment.

Aim of the study: The main objectives of this study are to evaluate the association between the effect of polyphenol-rich food supplementation and toxicity of radiotherapy for breast cancer.

Study design: Randomized, placebo-controlled double-blind trial with two parallel arms: soluble corn flour at high content in anthocyanins and placebo. Placebo consists of soluble corn flour poor in anthocyanins. The treatment starts one week before starting radiotherapy, and continues during all radiotherapy treatment (of 3 or 5 weeks).

Study Population and recruitment: A total of 300 consecutive breast cancer patients eligible for radiotherapy. Participants are identified at the Department of Radiotherapy of the Fondazione Giovanni Paolo II of Campobasso. Eligible subjects are recruited at their first consultation and subsequently referred to the research investigators. The medical staff in charge of the recruitment operates a pre-screening of participants based on inclusion/exclusion criteria and explains the study at this time. Once signed the informed consent, if the subject is eligible for the study according to all the criteria, she is included into the trial and randomised to receive daily (three times) high-anthocyanin supplement or placebo, using a computer-generated randomization sequence, blinded to the study investigators. To each participant a one-week dietary questionnaire is administered to evaluate the pattern of flavonoids intake, during the week preceding the start of supplementation and during the last week of supplementation.
Subjects start the dietary intervention 7 days before starting the radiotherapy. Then they continue the supplementation for all radiotherapy duration (3 or 5 weeks).
Each patient is asked to answer questionnaires to collect personal information and medical history (social status, previous disease or surgical interventions, risk factors and family history for CVD, drug use). Other relevant clinical data as well as measures of clinical outcomes are collected by medical records. A 1-year food frequency questionnaire is used to investigate dietary habits. Anthropometric measurements are collected using standardized methods. Venous blood (max 30 mL) is collected before starting the supplementation, at the end and one month after the end. Urine (8-hour) are collected for analysis of polyphenols excretion on selected samples.
All patients receive the pharmacological therapy and the lifestyle change advice according to the best clinical practice for the specific disease and clinical intervention.
Inclusion criteria: Female patients, aged 18 years or older; criteria established for the standard radiotherapy access protocol, described below.

Exclusion criteria: According to criteria established for the standard radiotherapy access protocol described below. The clinical trial excludes also people who have already undergone radiotherapy, developed metastasis or present severe problems at connective tissue level.

Conditions for patients eligibility

Women who satisfy all of the following conditions are the only patients eligible for the study.

1. The patient must consent to be in the study and must have signed an approved consent form conforming with institutional guidelines.
2. Patients must be ≥ 18 years old.
3. On histological examination, the tumor must be DCIS or invasive carcinoma of the breast.
4. Surgical treatment of the breast must have been lumpectomy or quadrantectomy. The margins of the resected specimen must be histologically free of tumor (DCIS and invasive). Re-excision of surgical margins is permitted.
5. Patients with *invasive* breast cancer are required to have axillary staging which can include sentinel node biopsy alone (if sentinel node is negative), sentinel node biopsy followed by axillary dissection or sampling with a minimum total of 6 axillary nodes (if sentinel node is positive), or axillary dissection alone (with a minimum of 6 axillary nodes). (Axillary staging is not required for patients with DCIS.)
6. The patient must be randomized within 45 days following the last surgery for breast cancer (lumpectomy, re-excision of margins, or axillary staging procedure) or within 30 days following the last chemotherapy cycle.
7. Patients with a history of *non-breast* malignancies are eligible if they have been disease-free for 5 or more years prior to randomization and are deemed by their physician to be at low risk for recurrence. Patients with the following cancers are eligible if diagnosed and treated within the past 5 years: carcinoma in situ of the cervix, carcinoma in situ of the colon, melanoma in situ, and basal cell and squamous cell carcinoma of the skin.

Conditions for patient ineligibility

Men are not eligible for this study. Women with one or more of the following conditions also are ineligible for the study.

1. Axillary nodes with definite evidence of microscopic or macroscopic extracapsular extension.
2. One or more positive *non-axillary* sentinel node(s). (Note that intramammary nodes are staged as axillary nodes.)
3. Palpable or radiographically suspicious ipsilateral or contralateral axillary, supraclavicular, infraclavicular, or internal mammary nodes, unless there is histologic confirmation that these nodes are negative for tumor.
4. *Suspicious* microcalcifications, densities, or palpable abnormalities (in the ipsilateral or contralateral breast) unless biopsied and found to be benign.
5. Non-epithelial breast malignancies such as sarcoma or lymphoma.
6. Proven multicentric carcinoma (invasive cancer or DCIS) in more than one quadrant or separated by 4 or more centimeters.
7. Paget's disease of the nipple.
8. Synchronous bilateral invasive or non-invasive breast cancer.
9. Surgical margins that cannot be microscopically assessed or are positive at pathologic evaluation. (If surgical margins are rendered free of disease by reexcision, the patient is eligible.)
10. Breast implants. (Patients who have had implants removed are eligible.)
11. Prior breast or thoracic RT for any condition.
12. Collagen vascular disease, specifically dermatomyositis with a CPK level above normal or with an active skin rash, systemic lupus erythematosus, or scleroderma.
13. Pregnancy or lactation at the time of proposed randomization. Women of reproductive potential must agree to use an effective non-hormonal method of contraception during therapy.
14. Psychiatric or addictive disorders or other conditions that, in the opinion of the investigator, would preclude the patient from meeting the study requirements.

Radiotherapy: Patients groups and specific treatment criteria

Group A

In Group A, patients at low risk of recurrence are treated with radiotherapy with doses lower than the standard ones (residual breast: 40 Gy in 2.5 Gy/fraction; concomitant boost on the tumor bed: 4 Gy in 0.25 Gy/fraction). The aim of this treatment modality is to significantly reduce the overall treatment time to achieve an organizational advantage. Patients with invasive pT1-3 breast carcinoma undergoing breast-conserving surgery are included. Due to the well-known impact of age and margin status on local control, only post-menopausal (at least 3 years) patients with clear surgical margins (>3 mm) are enrolled. Patients with pT4 pathologic stage, presence of > 3 metastatic axillary's nodes, prescription of nodal irradiation, positive or close resection margins (<3 mm) and presence of distant metastasis are excluded.

Group B

In Group B, patients at moderate-high risk are enrolled and treated with standard doses (residual breast: 50 Gy in 2 Gy/fraction; concomitant boost on tumor bed: 10 Gy in 0.40 Gy/fraction). The aim is to achieve a clinical as well as an organizational advantage. In fact, this schedule of treatment, due to hypofractionation on tumor bed, and considering the low α/β ratio, results in both improved Tumor Control Probability and slightly reduced treatment duration. The following inclusion criteria are used: invasive breast carcinoma undergoing breast-conserving surgery, pT1-4 pathologic stage, pre- or peri-menopausal patients (less than 3 years from last menstruation), > 3 positive axillary's nodes or close resection margins. Patients with positive resection margins or distant metastasis are excluded.

Radiotherapy treatment planning

In all patients, a CT-planning is used. At the time of CT scanning, the patient are placed in the treatment position (supine position with one arm raised). An alpha-cradle is used to ensure setup reproducibility. Contiguous 5-mm CT axial images are obtained extending from the larynx to the upper abdomen, including the entire breasts and lungs bilateral. Treatment is performed with the tangential technique and slight beam (not opposed) angulation to reduce the dose to the organs at risk (OARs). In patients also undergoing supraclavicular irradiation, the technique with a single isocenter is performed. Caudal to the isocenter the breast volume is irradiated with the tangential technique. Cranial to the isocenter the supraclavicular volume is irradiated with 2 opposed beams. Their angulations are optimized to prevent spinal cord irradiation. In all patients, dose reference is performed according to the ICRU report 62, even though the intrinsic difficulties in achieving a homogeneous dose in the breast tissue does not allow to comply with the minimum (95%) and maximum (107%) dose limit to the PTV in all patients. CTV1 and CTV2 is defined. The CTV1 is defined as tumor bed. Contouring is performed based on preoperative mammography, type of surgery, position of surgical clips and identification on CT-simulation scans of areas of surgical breast rearrangement. The CTV2 is defined as the whole breast excluding the most external cutaneous-subcutaneous 5 mm (except for pT4 for cutaneous infiltration). PTV1 and PTV2 are obtained by adding 8-mm margin to the corresponding CTVs, directed in cranial, caudal, medial, and lateral directions. A forward planned IMRT technique is used for treatment planning optimization. In all patients for PTV2 irradiation, the dose to each of the two tangential beams is divided into two different segments. One segment is designed to include the whole breast without filters (6 MV photons). This configuration, in the absence of filters results in a volume of under dosage in the thickest region of the breast. A second segment is directed to this area of under dosage to compensate for dose loss (15 MV photons). The weight of the two segments is determined by an iterative process repeated by the operator to the attainment of the optimal result. The PTV1 is treated with two conformed tangential photon beams with standard MLC and wedge filters at the same time of PTV2. Photon beam dose calculation is performed by Plato Sunrise treatment planning system (Nucletron B.VC., Veenendaal, The Netherlands) with a three-dimensional pencil beam algorithm, described by Bortfeld. It comprises a convolution-based approach, where the energy fluence distribution is convolved with a dose pencil beam. Inhomogeneity correction is applied by the equivalent tissue air ratio (ETAR) method.

Radiotherapy

In Group A, patients are treated with forward planned IMRT. The PTV2 (residual breast) will receive 40 Gy in 2.5 Gy fractions. The PTV1 receives a concomitant boost of 4 Gy in 0.25 Gy/fraction, delivered with 3D technique, photon beams and wedge filters. In Group B, patients are treated with IMRT. The PTV2 (residual breast) receives 50 Gy in 2.0 Gy/fraction). The PTV1 (tumor bed) receives a concomitant 3D boost of 10 Gy in 0.4 Gy/fraction delivered with 3D technique, photon beams and wedge filters. In all patients undergoing adjuvant chemotherapy, radiotherapy starts at least 3 weeks after systemic treatment. In all patients daily portal images in the first phase of irradiation (5–10 MU) are acquired on both beams. Deviations larger than 5 mm in the isocenter position are immediately corrected. Toxicity is scored prospectively in all patients groups, using the same timing and scoring system. All patients are evaluated at least once a week with clinical examination. Supportive therapy is similar in all groups of patients: Biafin is applied daily on the irradiated skin. In patients with grade 1–2 toxicity, steroids are administered topically. In patients with grade 3 toxicity, treatment is discontinued until grade 2 toxicity is resumed. Acute skin toxicity is differentiated based on the site (breast or supraclavicular region). Overall treatment time is of 16 fractions for Group A (3.2 weeks) and 25 fractions for Group B (5 weeks).

Dosimetry

The following dosimetric parameters are recorded and evaluated: D_{max}, D_{min}, $V_{95\%}$, and $V_{107\%}$ of PTV, D_{mean} of the lung and D_{mean} of the heart.

Evaluation criteria: The primary end point is the acute toxicity of radiotherapy treatment, which is evaluated by clinical analysis and with the objective skin analysis performed with a cutometer. Secondary end-points are cellular and plasma markers of oxidative stress and inflammation, as well as long term toxicity. Bioavailability of administered anthocyanins is evaluated in urine from a subsample of patients by analytical techniques LC-MS techniques, as described in WP3.

Power calculation: Preliminary data indicate an incidence of radiotherapy-induced toxicity of 33.6% in patients undergoing radiotherapy after surgery for breast cancer (Morganti et al, 2012). With the hypothesis of an OR=0.50 of the toxicity studied, 138 patients have to be treated, matched 1:1 with a placebo group, in order to rule out the null hypothesis of a relative risk=1 (power=90% and α=0.05). As previous data from the observational study showed a drop out of almost 8%, the total number of patients to be recruited is 300.

Statistical analysis: All analyses are performed using statistical analysis software SAS (SAS, 9.1.3 for Windows, Cary, NC: SAS Institute Inc.1989). All individuals enrolled are included in the analysis. Descriptive summaries are presented for all the individuals, and for subgroups of individuals. Statistical tests are carried out for exploratory purposes, as appropriate. The descriptive analysis of nominal/ordinal data comprises tabulation of frequency and percentages. Chi-square test (or Fisher exact test in the presence of small frequencies) are used to compare categories. The descriptive analysis of continuous data comprises the mean, standard deviation, median, extreme values, and 95% confidence interval.