Randomized phase III trial of adjuvant epirubicin followed by cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) versus CMF followed by epirubicin in patients with node-negative or 1-3 node-positive rapidly proliferating breast cancer.

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Abstract

Adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) have proven highly effective in rapidly proliferating breast cancer (RPBC). It has also been seen that sequential administration of doxorubicin and CMF is superior to their alternation, especially in indolent tumors. In a phase III study, we evaluated whether adjuvant epirubicin (E) followed by CMF is superior to the inverse sequence in RPBC. Patients with node-negative or 1–3 node-positive RPBC (Thymidine Labeling Index[3% or histological grade 3 or S-phase[10% or Ki67[20%) were randomized to receive E (100 mg/m2 i.v. d1, q21 days for 4 cycles) followed by CMF (600, 40, 600 mg/m2 i.v. d1 and 8, q28 days for 4 cycles) (E ? CMF) or CMF followed by E (CMF ? E) or CMF for 6 cycles. From November 1997 to December 2004, 1066 patients were enrolled: E ? CMF 440, CMF ? E 438, and CMF 188. At a

median follow-up of 69 months, 5-year OS was 91% (95% CI 88–94) for E ? CMF and 93% (95% CI 90–95) for CMF ? E, with adjusted hazard ratio of 0.88 (95% CI 0.58–1.35), and DFS was 80% in both arms, with adjusted hazard ratio of 0.99 (95% CI 0.73–1.33, Cox model). Adverse events were similar, apart from a higher rate of neutropenia in the CMF ? E arm. No important differences in clinical outcome were observed between the two different sequences, making both a valid option in early breast cancer. Further molecular characterization of the tumors might help to identify subgroups achieving higher benefit from either sequence.

Keywords
Sequential adjuvant chemotherapy strategy _Epirubicin _ CMF _ Randomized phase III study _Rapidly proliferating breast cancer

Introduction

The interplay between tumor cell kinetics and type and sequence of anticancer drugs affects the efficacy of adjuvant chemotherapy. Tumor proliferation is a prognostic marker in breast cancer [1] and affects response to chemotherapy [2]. Antimetabolites act primarily in specific cell cycle phases and are especially active in rapidly proliferating tumors, as shown by the high efficacy of adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) in patients with node-negative, rapidly proliferating breast cancer (RPBC) [3, 4]. Anthracyclines have composite mechanisms of action whose contribution to their activity in vivo is still not fully understood [5]: the inhibition of topoisomerase-2 relies mainly on tumor cell proliferation, whereas other mechanisms are less dependent on proliferation. The superiority of sequential over alternating regimens was predicted by the model of Norton and Simon [6] and confirmed in clinical trials of adjuvant chemotherapy for breast cancer, demonstrating that the sequential administration of 4 courses of doxorubicin followed by 8 courses of CMF yields superior relapse-free and overall survival (OS) rates compared with alternating administration of the same regimens in patients at very high risk of recurrence on the basis of nodal involvement [7]. It is therefore important to establish which regimen should be used first. In a retrospective analysis of the aforementioned trial, the benefit of sequential doxorubicin—CMF was evident mainly in patients with low-intermediate proliferating tumors [8]. We hypothesized that RPBC patients could benefit more from the inverse sequence, receiving CMF first to kill the subpopulation of highly proliferating cells and then the anthracycline to kill the CMF-resistant, probably slowly proliferating subpopulation. To test this hypothesis, we compared the two sequences within a randomized trial.

Patients and methods

Study population Patients were eligible if they had RPBC, as defined, in order of importance, by thymidine labeling index (TLI) [3% or histological grade 3 or S-phase [10% or Ki67/ MIB1 [20%. A cutoff of 3% for TLI has consistently provided prognostic information in large series of patients with early breast cancer [9, 10]. With regard to S-phase, we previously compared different cell kinetic variables in a series of breast cancer specimens, observing that a median S-phase of 10% corresponded to a median TLI of about 3% [11]. Works on Ki67 are based on varying, often arbitrarily chosen cutoffs, and we adopted the threshold of 20% on the basis of literature data and also because it corresponded to the intermediate value between the overall population median and the median for grade 3 tumors in our series [11]. Further eligibility criteria were: females B70 years of age; histological diagnosis of invasive breast carcinoma of any size with 1–3 positive axillary nodes or node-negative tumors [1 cm; radical tumor resection; no evidence of metastatic disease; white blood cell count C3500/ml or neutrophils C1500/ml, platelets C120,000/ml, creatinine B the upper normal limit (UNL), transaminases and bilirubin B 1.5 UNL. Patients gave

their written informed consent and women of child-bearing potential were required to have a negative pregnancy test and to use adequate contraceptive measures. Patients were ineligible if they had a previous history of invasive breast cancer or other previous or concomitant malignancies or concomitant diseases which could interfere with study participation. The study was approved by the institutional review boards of each participating center and has been registered as a National Cancer Institute trial (NCT01031030).

Study design and treatments

This was a prospective, randomized, multicenter, openlabel phase III trial comparing the efficacy of three treatment arms in patients with RPBC: E for 4 courses followed by CMF for 4 courses (E ? CMF), CMF for 4 courses followed by E for 4 courses (CMF ? E), and CMF alone for 6 courses. The arm with CMF alone was closed after the results of the EBCSG meta-analysis were published in 1998 [12], demonstrating the superiority of anthracyclinebased regimens over CMF alone, and the primary objective remained the comparison of E ? CMF with CMF ? E. The CMF regimen consisted of cyclophosphamide 600 mg/m2 iv, methotrexate 40 mg/m2 iv and 5-fluorouracil 600 mg/m2 iv on days 1 and 8, repeated every 4 weeks. Epirubicin was administered every 3 weeks at 100 mg/m2 iv. Patients with estrogen receptor (ER)-positive tumors received adjuvant tamoxifen for 5 years after the end of chemotherapy. A gonadotropin-releasing hormone (GnRH) agonist could be added in premenopausal patients not achieving amenorrhea after chemotherapy, at the discretion of the participating centers. Patients treated with breast conserving surgery and those submitted to mastectomy for pT3–4 tumors received radiotherapy. Histopathological exams were performed at each participating center and ER and progesterone receptor (PgR) expression was measured by immunohistochemistry in the majority of patients (990 [92.9%] patients for ER, and 985 [92.4%] patients for PgR), and by the charcoal dextran assay [13] in the remaining cases. HER2/neu positivity was determined by immunohistochemistry using Dako Hercep-test or CB11 antibody or by fluorescent in situ hybridization. Tumor proliferation was assessed by TLI in 363 patients (34%), by histological grade in 601 patients (56%) and by Ki67/MIB1 in 102 patients (10%). Grading was considered a surrogate indicator of the proliferative activity based on the strict correlation between the two variables [14]. Baseline workup included medical history, physical examination, laboratory exams, chest X-ray, abdominal ultrasound, bone scan, mammography, ECG, and cardiological consultation. Clinical and laboratory assessments were repeated before each cycle and then at 3-month intervals during years 1 and 2, every 6 months during years 3–5, and yearly thereafter up to the tenth year. Annual chest X-ray, liver ultrasound, and bone scans were carried out for the first 5 years and at the discretion of the investigator thereafter. Mammography was performed yearly. Toxicity was recorded at each clinical examination and scored using World Health Organization (WHO) criteria [15]. Dose modifications, based on common criteria, were outlined in the protocol. Colony-stimulating factors could be used in the event of grade 4 neutropenia.

Statistical considerations

The primary endpoint was OS, defined as the time from randomization to the date of last contact or of death from any cause. Secondary objectives were disease-free survival (DFS) and toxicity. DFS was defined as the time from randomization to the date of locoregional or distant recurrence, second invasive breast carcinoma, second primary cancer, and/or death without evidence of breast cancer. Analysis of outcome according to clinical, pathological, and biological variables was planned in advance, with an explorative intent. The study was performed in accordance with the principles of Good Clinical Practice [16], the ethical standards laid down in the 1964 Declaration of Helsinki [17], and local legal and regulatory requirements. Within 6 weeks of surgery, patients

were randomly assigned to the treatment arms on a 1:1:1 basis by a telephone call to the Biostatistics and Clinical Trials Unit of the coordinating center in Forlı` using computer-generated randomization lists of permutated blocks of varying sizes stratified for participating center, lymph node status (nodenegative vs. node-positive) and ER status (ER-negative vs. ER-positive). The sequences were concealed from the physicians. Sample size was determined assuming a 5-year OS of 75% for patients treated with 6 cycles of CMF and an expected absolute increase of 8% in patients treated with E ? CMF or CMF ? E (5% type I error fixed for a twosided test and power of 80%), planning an accrual of 1200 patients over 3 years. After stopping the CMF arm, the sample size was re-determined assuming a 5-year OS of 78% for patients treated with E ? CMF and an expected absolute increase of 7% in patients treated with CMF ? E (5% type I error fixed for a two-sided test and power of 80%). Continuing a 1:1 randomization, a planned accrual period of 36 months, and a follow-up period of 60 months, it was estimated that 400 patients per arm were necessary. Efficacy analyses were performed according to the intention-to-treat principle. Safety analyses concerned all patients who received at least one dose of study medication. DFS and OS probability and the 95% confidence interval (95% CI) were computed by the Kaplan–Meier product-limit method [18]. The chi-square test or Fisher's exact test were used to compare the incidence and severity of side effects [19]. Estimated hazard ratios (HR) (CMF ? E vs. E ? CMF, CMF ? E and E ? CMF vs. CMF), their 95% CIs and P values were calculated from the Cox proportional hazard regression models [20], adjusted according to center, lymph node status, and ER status. No interim analysis was planned. No correction for multiple testing was performed in subgroup analyses. The relative dose intensity was calculated as the ratio of the delivered dose intensity, i.e., the ratio of the total dose delivered over total time to complete chemotherapy, to the planned dose intensity. All P values were based on twosided testing, and statistical analyses were carried out with SAS Statistical Software (version 9.1, SAS Institute).

Results Study details

Between November 1997 and December 2004, 1066 patients were entered onto the trial by 22 participating centers: 440 were allocated to E ? CMF, 438 to CMF ? E, and 188 to CMF (Fig. 1). Median follow up was 69 months.

Patient characteristics

Patients and tumor characteristics were well balanced in the three treatment arms, as reported in Table 1. Median age was 52 years (range 26–70) and 47% of patients were premenopausal. Most had pT1–2 ductal carcinoma, 47% with nodal involvement. Seventy-nine percent had poorly differentiated tumors and median Ki67/MIB1 was 30%. Sixty-two percent had ER-positive (C10% nuclei immunostained or C10 fmol/mg protein), 50% PgR-positive (C10% nuclei immunostained or C25 fmol/mg protein) and 34% ER/PgR-negative disease. HER2/neu was assessed in about half of the patients and was positive in 44%, reflecting the study selection criteria. Sixty-two percent of the patients had breast conservative surgery and thirty-eight percent had mastectomy, with axillary dissection in all cases. All patients treated with conservative surgery and 1.4% of those who underwent mastectomy received radiotherapy, administered in most cases in concomitance with the CMF regimen. All clinical, pathologic, and biologic characteristics were well balanced in the three treatment arms. About 83% of patients with ER-positive tumors received adjuvant tamoxifen, which was combined with a GnRH agonist in a number of premenopausal patients (43% in the E ? CMF arm, 47% in the CMF ? E arm, and 27% in the CMF alone arm).

Chemotherapy administration and safety

Seventy-nine percent of patients in the E ? CMF arm, eighty-one percent in the CMF ? E arm, and eighty-four percent in the CMF arm completed the planned chemotherapy. Three percent in each of the two sequential arms and five percent in the CMF arm stopped treatment in advance due to toxicity, mainly mucositis (11 patients), nausea and vomiting (6), fever-infection (5), and liver toxicity (4). Rarer causes for stopping therapy were neurotoxicity, cardiotoxicity, allergic reactions and myelotoxicity (2 cases each), and actinic dermatitis (1). The remaining patients stopped treatment for other reasons, e.g., treatment refusal, death, progression, lost to follow up. Median relative dose intensity was 0.86 with E ? CMF, 0.88 with CMF ? E, and 0.88 with CMF.

Toxicity

Sequential treatments yielded a higher proportion of grade 3–4 side-effects compared with CMF alone, in particular neutropenia (P = 0.03) and alopecia (P\0.0001) (Table 2). When the two sequential arms were compared, the only difference was a higher incidence in grade 4 neutropenia in the CMF ? E arm (12.0 vs. 7.5%, P = 0.03) with respect to the E ? CMF arm. Other grade 4 toxic events included mucositis, increased AST with chronic C hepatitis, osteoarticular pain, febrile leukopenia, allergic reaction, asthenia with infection, and actinic dermatitis. One treatment-related death due to myelotoxicity was observed in the arm receiving E ? CMF. There were no cases of symptomatic congestive heart failure and the rate of subclinical heart impairment was similar among the three arms; one patient in each sequential arm stopped treatment following grade 2 cardiotoxicity. The rate of chemotherapy-induced amenorrhea was 35% in the E ? CMF arm, 36% in the CMF ? E arm, and 27% in the CMF arm. One patient in the CMF ? E arm was diagnosed with acute myeloid leukemia 27 months after the end of chemotherapy.

Efficacy

Relevant events are reported in Table 3. Five-year OS was 91% (95% CI 88–94) with E ? CMF and 93% (95% CI 90–95) with CMF ? E, with a hazard ratio adjusted for center, nodes (negative or positive) and ER (negative or positive) status of 0.88 (95% CI 0.58–1.35) (Fig. 2a). Fiveyear DFS was 80% in both arms (95% CI 76–85% for E ? CMF and 76–84% for CMF ? E), with an adjusted hazard ratio of 0.99 (95% CI 0.73–1.33) (Fig. 2b). The analyses conducted on the subgroup of patients for whom TLI was available yielded equivalent results, as did those conducted separately in the subgroup of grade 1–2 tumors and in those of grade 3 tumors (data not shown). Likewise, subgroup analyses according to age (\52 vs. C52 years), menopausal status, histology, tumor size, nodal status, and hormone receptor status did not show any differences between the two sequential arms (Fig. 3). An exploratory analysis was conducted to compare the group of patients receiving 6 cycles of CMF with those receiving a sequence schedule (either E ? CMF or CMF ? E) enrolled before the closure of the CMF arm. Five-year OS was 90% (95% CI 87–93) with sequential regimens and 90% with CMF (95% CI 86–94), with an adjusted hazard ratio of 0.91 (95% CI 0.59–1.42). Fiveyear DFS was 77% with sequential regimens (95% CI 73–82%) and 78% with CMF (95% CI 72–84%), with an adjusted hazard ratio of 0.97 (95% CI 0.70–1.35). Subgroup analyses did not show statistically significant differences in outcome between the two treatments (data not shown).

Discussion

Since the publication of the study by Bonadonna and collaborators [21] showing the superiority of 4 courses of doxorubicin followed by 8 courses of CMF over an alternation of the two regimens, sequential schedules have become a common option for the adjuvant therapy of early breast cancer. Although today the most widely used sequence involves an anthracycline-based scheme

followed by a taxane, the problem of the best sequence has yet to be resolved. Our study addressed the issue of which is the best sequence in two non cross-resistant regimens in patients with RPBC, especially important if the two regimens have different efficacy. Delayed administration of the most effective regimen following a less effective treatment is thought to jeopardize its efficacy [22]. Conversely, computer simulations based on mathematical models for tumor growth and treatment suggest the superiority of the ''worst drug rule'' involving earlier administration of the less effective regimen to rapidly eliminate those cells resistant to the stronger regimen [23]. 5-Fluorouracil and methotrexate are S-phase-specific drugs especially active against highly proliferating cells, while cyclophosphamide is among the alkylating agents with the highest specificity for proliferating cells [24]. Benefit from adjuvant CMF seems, in fact, directly correlated with TLI [3]. Anthracyclines are active during S-phase but also during other phases, including G1, and induce marked cell arrest in G2/M phase [25], suggesting that they may be more active against slowly proliferating tumors when compared with CMF. Response to neoadjuvant doxorubicin plus vincristine appears to be independent of pretreatment TLI [26], and adjuvant treatment of patients with node-negative RPBC comprising fluorouracil, epirubicin, and cyclophosphamide produces a delayed benefit, typical of therapies that are active in slowergrowing tumors, and independent of proliferative activity [27]. A diverse distribution of tumor cells in the different phases of the cell cycle has been observed after neoadjuvant chemotherapy with different drugs: an accumulation of cells in S-phase after CMF and a higher accumulation in G2/M phase after anthracyclines [28, 29]. We hypothesized that the sequence E ? CMF could be highly active in slowly/intermediately proliferating tumors because of the ability of the anthracycline to kill the subpopulation of slowly proliferating cells and to produce a partial synchronization of the remaining, highly proliferating cells sensitive to the S-phase-specific drugs subsequently administered [8]. We also hypothesized that rapidly proliferating tumors could be more effectively treated by administering CMF first to kill the subpopulation of highly proliferating cells and then the anthracycline to kill the CMF-resistant, probably slowly proliferating subpopulation. We did not find important differences between the two sequential treatments in terms of either disease-free or overall survival. It must be emphasized that the only variable tested in our study was the different sequences of two regimens as the overall number of cycles administered and the dose intensity of the drugs used were the same in the two arms. A previous study conducted at the Istituto Nazionale Tumori in Milan did not find any difference between CMF given every 3 weeks for 12 courses and 8 courses of the same CMF followed by 4 courses of doxorubicin in patients with early breast cancer and one to three involved axillary nodes [30]. Although the differing patient populations, drug regimens (CMF every 21 days in the Milan studies and CMF days 1 and 8 every 28 days in our study) and number of cycles prevent direct comparisons from being made between the two trials, our data suggest that the sequence CMF ? anthracycline may be as effective as the more frequently used sequence anthracycline ? CMF in patients with RPBC, supporting the efficacy of CMF, at least with the schedule used in this study, in these tumors. Further molecular characterization of tumor samples is ongoing to ascertain potential differences between the two sequences based on biomolecular profiles. There are probably a number of reasons behind the lack of superiority of CMF ? E over E ? CMF in RPBC. Although anthracyclines have multiple mechanisms of action, inhibition of topoisomerase-II-a is one of the most important. Topoisomerase-II-a expression is associated with cell cycle phases, peaking in G2/M and at its lowest in G0/G1 [31], and is prevalent in highly proliferating cells [32]. Anthracyclines could therefore be as active as antimetabolites in rapidly proliferating cells, in addition to being more active against slowly proliferating ones. On the other hand, cyclophosphamide is also partially active against slowly proliferating cells, contributing to making the two regimens interchangeable. The heterogeneity of proliferation assessment methods in our

study may have diluted the differences among treatment arms. Different methodological problems affect the evaluation of tumor proliferation [33]. Although TLI is reliable and reproducible [10, 34], its complexity has hampered its widespread diffusion. Ki-67/MIB-1, while significantly associated with outcome in patients with early breast cancer, has more limited reproducibility [35]. In conclusion, our study does not show important differences between inverse sequences of two non-crossresistant regimens in early RPBC. Taking into account the heterogeneity of breast cancer, the cell cycle specificity of some agents and the cell cycle-related expression of some targets, e.g., topoisomerase-II-a, it is possible that further molecular characterization of the tumors could identify subgroups that benefit from a specific strategy.

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References

1. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thu¨rlimann B, Senn HJ (2009) Thresholds for therapies: highlights of the St. Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann Oncol 20:1319–1329
2. Amadori D, Volpi A, Maltoni R, Nanni O, Amaducci L, Amadori A, Giunchi DC, Vio A, Saragoni A, Silvestrini R (1997) Cell proliferation as a predictor of response to chemotherapy in metastatic breast cancer: a prospective study. Breast Cancer Res Treat 43:7–14
3. Amadori D, Nanni O, Marangolo M, Pacini P, Ravaioli A, Rossi A, Gambi A, Catalano G, Perroni D, Scarpi E, Giunchi DC, Tienghi A, Becciolini A, Volpi A (2000) Disease-free survival advantage of adjuvant cyclophosphamide, methotrexate, and fluorouracil in patients with node-negative, rapidly proliferating breast cancer: a randomized multicenter study. J Clin Oncol 18:3125–3134
4. Amadori D, Nanni O, Volpi A, Pacini P, Ravaioli A, Rossi AP, Gambi A, Luzi Fedeli S, Perroni D, Scarpi E, Becciolini A, Silvestrini R (2008) Phase III randomized multicenter study on the effects of adjuvant CMF in patients with node-negative, rapidly proliferating breast cancer: twelve-year results and retrospective subgroup analysis. Breast Cancer Res Treat 108: 259–264
5. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L (2009) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 56:185–229
6. Gilewski T, Norton L (1996) Cytokinetics and breast cancer chemotherapy. In: Harris JR, Lippman ME, Morrow M, Hellman S (eds) Diseases of the breast. Lippincott-Raven, Philadelphia
7. Bonadonna G, Zambetti M, Valagussa P (1995) Sequential or alternating doxorubicin and CMF regimens in breast cancer with more than three positive nodes. Ten-year results. JAMA 273: 542–547
8. Silvestrini R, Luisi A, Zambetti M, Cipriani S, Valagussa P, Bonadonna G, Daidone MG (2000) Cell proliferation and outcome following doxorubicin plus CMF regimens in nodepositive breast cancer. Int J Cancer 87:405–411
9. Silvestrini R, Daidone MG, Luisi A, Boracchi P, Mezzetti M, Di Fronzo G, Andreola S, Veronesi U (1995) Biologic and clinicopathologic factors as indicators of specific relapse types in nodenegative breast cancer. J Clin Oncol 13:697–704
10. Silvestrini R, Daidone MG, Luisi A, Mastore M, Leutner M, Salvadori B (1997) Cell proliferation in 3, 800 node-negative breast cancers: consistency over time of biological and clinical information provided by 3H-thymidine labelling index. Int J Cancer 74:122–127
11. Barzanti F, Dal Susino M, Volpi A, Amadori D, Riccobon A, Scarpi E, Medri L, Bernardi L, Naldi S, Aldi M, Gaudio M, Zoli W (2000) Comparison between different cell kinetic variables in human breast cancer. Cell Prolif 33:75–89
12. Early Breast Cancer Trialists' Collaborative Group (1998) Polychemotherapy for early breast cancer: an overview of the randomised trials. Lancet 352:930–942
13. Piffanelli A, Pellizzola D, Giovannini G, Catozzi L, Faggioli L, Giganti M (1989) Characterization of laboratory working standard for quality control of immunometric and radiometric estrogen receptor assays. Clinical evaluation on breast cancer biopsies. Italian Committee for Hormone Receptor Assays Standardization. Tumori 75:550–556
14. Volpi A, Bacci F, Paradiso A, Saragoni L, Scarpi E, Ricci M, Aldi M, Bianchi S, Muretto P, Nuzzo F, Simone G, Mangia A, Schittulli F, Amadori D (2004) Prognostic relevance of histological grade and its components in node-negative breast cancer patients. Mod Pathol 17:1038–1044
15. World Health Organization: World Health Organization handbook for reporting results of cancer treatment (1979). World Health Organization, Geneva

16. International Conference on Harmonisation of Technical Requirement for Requirements for Registration of Pharmaceuticals for Human Use (ICH Harmonised Tripartite Guidelines): Guideline for good clinical practice (GCP) E6 (1996). http:// www.ich.org/LOB/media/MEDIA482.pdf. Accessed 1 February 2009
17. World Medical Association Declaration of Helsinki (1996) Recommendations guiding physician in biomedical research involving human subjects, adopted by the 18th World Medical Assembly Helsinki, Finland, June amended by 48th General Assembly, Somerset West, South Africa. http://www.wma.net/e/ polity/pdf/17cpdf. Accessed 1 February 2009
18. Kaplan El, Meier P (1958) Nonparametric estimation for incomplete observation. J Am Stat Assoc 53:457–481
19. Armitage P, Berry G, Matthews JNS (2000) Statistical methods in medical research, 4th edn. Blackwell Publishing, Oxford
20. Cox DR (1972) Regression models and life tables. J R Stat Soc 34:187–220
21. Buzzoni R, Bonadonna G, Valagussa P, Zambetti M (1991) Adjuvant chemotherapy with doxorubicin plus cyclophosphamide, methotrexate, and fluorouracil in the treatment of resectable breast cancer with more than three positive axillary nodes. J Clin Oncol 9:2134–2140
22. Martin M (2009) Unravelling the mystery of the TACT trial. Lancet 373:1662–1663
23. Day RS (1986) Treatment sequencing, asymmetry, and uncertainty: protocol strategies for combination chemotherapy. Cancer Res 46:3876–3885
24. Valeriote F, van Putten L (1975) Proliferation-dependent cytotoxicity of anticancer agents: a review. Cancer Res 35:2619–2630
25. Ling YH, el-Naggar AK, Priebe W, Perez-Soler R (1996) Cell cycle-dependent cytotoxicity, G2/M phase arrest, and disruption of p34cdc2/cyclin B1 activity induced by doxorubicin in synchronized P388 cells. Mol Pharmacol 49:832–841
26. Silvestrini R, Daidone MG, Valagussa P, Salvadori B, Rovini D, Bonadonna G (1987) Cell kinetics as a prognostic marker in locally advanced breast cancer. Cancer Treat Rep 71:375–379
27. Paradiso A, Schittulli F, Cellamare G, Mangia A, Marzullo F, Lorusso V, De Lena M (2001) Randomized clinical trial of adjuvant fluorouracil, epirubicin, and cyclophosphamide chemotherapy for patients with fast-proliferating, node-negative breast cancer. J Clin Oncol 19:3929–3937
28. Daidone MG, Silvestrini R, Luisi A, Mastore M, Benini E, Veneroni S, Brambilla C, Ferrari L, Greco M, Andreola S (1995) Changes in biological markers after primary chemotherapy for breast cancers. Int J Cancer 61:301–305
29. Remvikos Y, Jouve M, Beuzeboc P, Viehl P, Magdelenat H, Pouillart P (1993) Cell cycle modifications of breast cancers during neoadjuvant chemotherapy: a flow cytometry study on fine needle aspirates. Eur J Cancer 29A:1843–1848
30. Moliterni A, Bonadonna G, Valagussa P, Ferrari L, Zambetti M (1991) Cyclophosphamide, methotrexate, and fluorouracil with and without doxorubicin in the adjuvant treatment of resectable breast cancer with one to three positive axillary nodes. J Clin Oncol 9:1124–1130
31. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH (1991) Proliferation-and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth Differ 2:209–214
32. Negri C, Chiesa R, Cerino A, Bestagno M, Sala C, Zini N, Maraldi NM, Astaldi Ricotti GC (1992) Monoclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerase II: 170- and 180-kDa isozymes. Exp Cell Res 200:452–459
33. Colozza M, Azambuja E, Cardoso F, Sotiriou C, Larsimont D, Piccart MJ (2005) Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now? Ann Oncol 6:1723–1739

34. Silvestrini R (1991) Feasibility and reproducibility of the 3Hthymidine labeling index in breast cancer. The SICCAB Group for Quality Control of Cell Kinetic determination. Cell Prolif 24:437–445
35. de Azambuja E, Cardoso F, de Castro G Jr, Colozza M, Mano MS, Durbecq V, Sotiriou C, Larsimont D, Piccart-Gebhart MJ, Paesmans M (2007) Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12, 155 patients. Br J Cancer 96:1504–1513

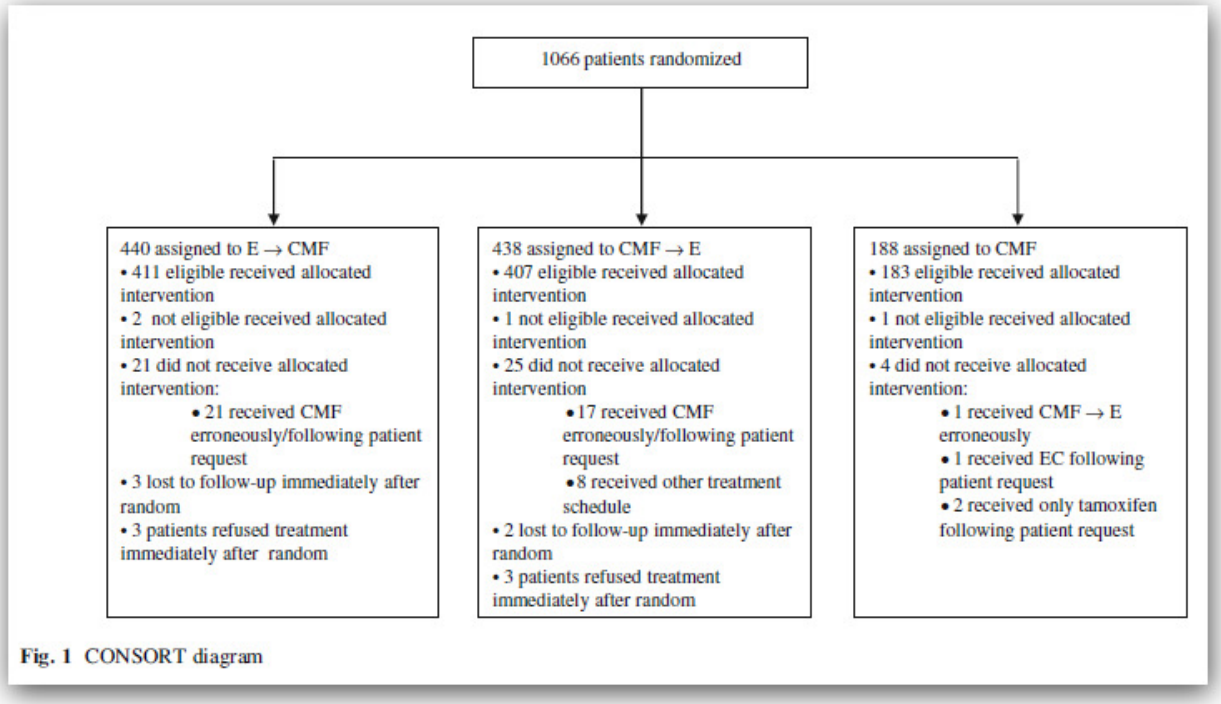

Fig. 1 CONSORT diagram

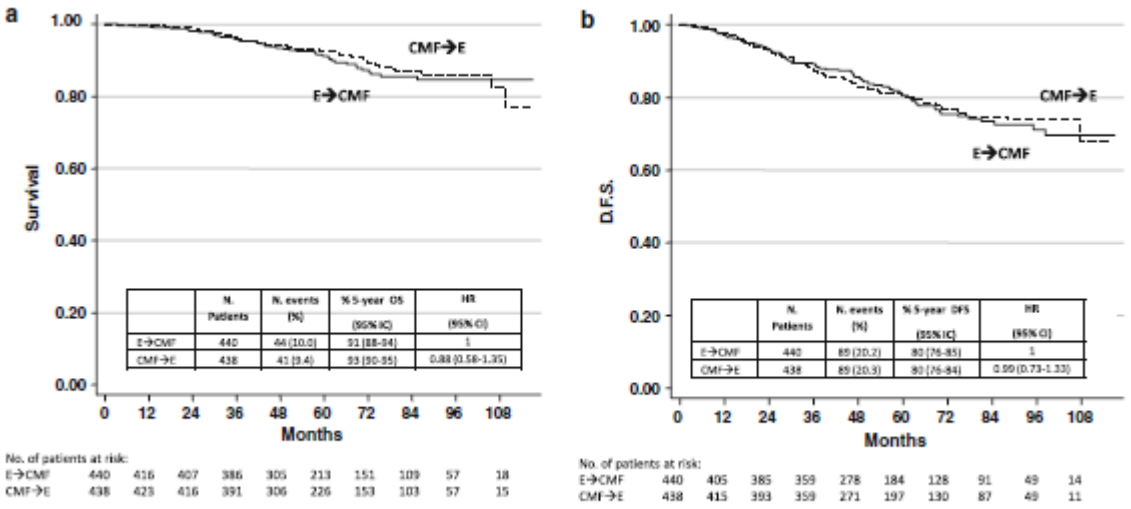

Fig. 2 Overall (**a**) and disease-free (**b**) survival

Table 1 Characteristics of randomized patients

Characteristic	No. of patients (%)			
	Total ($n = 1066$)	E → CMF ($n = 440$)	CMF → E ($n = 438$)	CMF ($n = 188$)
Age [years; median (range)]	52 (26–70)	52 (26–70)	52 (27–70)	50 (27–70)
Age group (years)				
<40	116 (10.9)	48 (10.9)	47 (10.7)	21 (11.2)
40-49	338 (31.7)	136 (30.9)	134 (30.6)	68 (36.2)
50-59	321 (30.1)	137 (31.1)	134 (30.6)	50 (26.6)
≥60	291 (27.3)	119 (27.1)	123 (28.1)	49 (26.0)
Menopausal status				
Pre-menopause	499 (46.8)	201 (45.7)	207 (47.3)	91 (48.4)
Post-menopause	567 (53.2)	239 (54.3)	231 (52.7)	97 (51.6)
Histological type				
Ductal	954 (89.5)	395 (89.8)	394 (90.0)	165 (87.8)
Lobular	59 (5.5)	21 (4.8)	21 (4.8)	17 (9.0)
Other	53 (5.0)	24 (5.4)	23 (5.2)	6 (3.2)
pT				
T1	498 (49.0)	204 (49.0)	205 (48.6)	89 (49.7)
T2	468 (46.0)	196 (47.1)	192 (45.5)	80 (44.7)
T3	29 (2.8)	9 (2.2)	16 (3.8)	4 (2.2)
T4	22 (2.2)	7 (1.7)	9 (2.1)	6 (3.4)
Missing	49	24	16	9
Lymph node status				
Negative	567 (53.2)	234 (53.2)	231 (52.7)	102 (54.3)
Positive	499 (46.8)	206 (46.8)	207 (47.3)	86 (45.7)
1	248 (23.3)	101 (23.0)	97 (22.1)	50 (26.6)
2	140 (13.1)	56 (12.7)	65 (14.8)	19 (10.1)
3	111 (10.4)	49 (11.1)	45 (10.3)	17 (9.0)
No. of lymph nodes examined				
Median (range)	19 (10–47)	19 (10–43)	19 (10-47)	18 (10–47)
Receptor status				
ER-negative[a]	404 (37.9)	167 (37.9)	164 (37.4)	73 (38.8)
ER-positive	662 (62.1)	273 (62.1)	274 (62.6)	115 (61.2)
PgR-negative[b]	534 (50.1)	210 (47.7)	224 (51.1)	100 (53.2)
PgR-positive	532 (49.9)	230 (52.3)	214 (48.9)	88 (46.8)
Local treatment				
Mastectomy alone	394 (37.0)	167 (38.0)	151 (34.5)	76 (40.5)
Mastectomy + radiotherapy	15 (1.4)	4 (0.9)	10 (2.3)	1 (0.5)
Conservative + radiotherapy	657 (61.6)	269 (61.1)	277 (63.2)	111 (59.0)
Systemic treatment				
Hormonotherapy (ER+)	550 (51.6)	232 (52.7)	220 (50.2)	98 (52.1)
Hormonotherapy (ER−)	60 (5.6)	27 (6.1)	28 (6.4)	5 (2.7)
GnRH agonist	98 (38.8)	45 (42.9)	42 (46.7)	11 (26.8)
Grade[c]				
1	11 (1.1)	7 (1.7)	2 (0.5)	2 (1.2)
2	200 (19.9)	79 (19.0)	86 (20.8)	35 (20.1)
3	793 (79.0)	330 (79.3)	326 (78.7)	137 (78.7)
TLI (%), median (range)	5.6 (3.1–24.5)	5.2 (3.1–24.5)	5.6 (3.1–16.7)	5.9 (3.1–20.6)
Missing	703	306	286	111

Leukopenia	103 (24.9)	56 (13.6)	7 (1.7)	89 (21.8)	63 (15.4)	15 (3.7)	46 (25.0)	20 (10.9)	3 (1.6)
Neutropenia	72 (17.4)	82 (19.9)	31 (7.5)	69 (16.9)	67 (16.4)	49 (12.0)	31 (16.8)	28 (15.2)	9 (4.9)
Thrombocytopenia	10 (2.4)	3 (0.7)	1 (0.2)	9 (2.2)	7 (1.7)	0	4 (2.2)	3 (1.6)	0
Anemia	36 (8.7)	1 (0.2)	0	30 (7.4)	4 (1.0)	0	10 (5.4)	1 (0.5)	0
Alopecia	58 (14.0)	219 (53.0)	0	58 (14.2)	185 (45.3)	0	25 (13.6)	11 (6.0)	0
Nausea/vomiting	148 (35.8)	37 (9.0)	0	145 (35.5)	30 (7.4)	3 (0.7)	42 (22.8)	11 (6.0)	1 (0.5)
Diarrhea	10 (2.4)	4 (1.0)	0	17 (4.2)	6 (1.5)	0	4 (2.2)	0	0
Mucositis	48 (11.6)	10 (2.4)	2 (0.5)	29 (7.1)	10 (2.5)	1 (0.2)	12 (6.5)	7 (3.8)	0
Cardiotoxicity	2 (0.5)	0	0	4 (1.0)	0	0	1 (0.5)	0	0
Other	89 (21.5)	19 (4.6)	2 (0.5)	76 (18.6)	19 (4.7)	4 (1.0)	26 (14.1)	8 (4.3)	0

Table 2 Toxicity

Toxicity	E → CMF No. (%)			CMF → E No. (%)			CMF No. (%)		
	Grade 2	Grade 3	Grade 4	Grade 2	Grade 3	Grade 4	Grade 2	Grade 3	Grade 4
Leukopenia	103 (24.9)	56 (13.6)	7 (1.7)	89 (21.8)	63 (15.4)	15 (3.7)	46 (25.0)	20 (10.9)	3 (1.6)
Neutropenia	72 (17.4)	82 (19.9)	31 (7.5)	69 (16.9)	67 (16.4)	49 (12.0)	31 (16.8)	28 (15.2)	9 (4.9)
Thrombocytopenia	10 (2.4)	3 (0.7)	1 (0.2)	9 (2.2)	7 (1.7)	0	4 (2.2)	3 (1.6)	0
Anemia	36 (8.7)	1 (0.2)	0	30 (7.4)	4 (1.0)	0	10 (5.4)	1 (0.5)	0
Alopecia	58 (14.0)	219 (53.0)	0	58 (14.2)	185 (45.3)	0	25 (13.6)	11 (6.0)	0
Nausea/vomiting	148 (35.8)	37 (9.0)	0	145 (35.5)	30 (7.4)	3 (0.7)	42 (22.8)	11 (6.0)	1 (0.5)
Diarrhea	10 (2.4)	4 (1.0)	0	17 (4.2)	6 (1.5)	0	4 (2.2)	0	0
Mucositis	48 (11.6)	10 (2.4)	2 (0.5)	29 (7.1)	10 (2.5)	1 (0.2)	12 (6.5)	7 (3.8)	0
Cardiotoxicity	2 (0.5)	0	0	4 (1.0)	0	0	1 (0.5)	0	0
Other	89 (21.5)	19 (4.6)	2 (0.5)	76 (18.6)	19 (4.7)	4 (1.0)	26 (14.1)	8 (4.3)	0

Table 3 Events contributing to overall and disease-free survival analysis

Events	No. of patients (%)			
	Total ($n = 1066$)	E → CMF ($n = 440$)	CMF → E ($n = 438$)	CMF ($n = 188$)
All deaths	115 (10.8)	44 (10.0)	41 (9.4)	30 (16.0)
Cancer	105 (9.8)	40 (9.1)	38 (8.7)	27 (14.4)
Toxicity	1 (0.1)	1 (0.2)	0	0
Other	9 (0.8)	3 (0.7)	3 (0.7)	3 (1.6)
All events contributing to DFS	231 (21.7)	89 (20.2)	89 (20.3)	53 (28.2)
Locoregional relapse only	31 (2.9)	14 (3.2)	12 (2.7)	5 (2.7)
Distant relapse only	141 (13.2)	51 (11.6)	59 (13.5)	31 (16.5)
Bone	39 (3.7)	15 (3.4)	15 (3.4)	9 (4.8)
Liver	22 (2.1)	8 (1.8)	8 (1.8)	6 (3.2)
Lung	10 (0.9)	2 (0.4)	5 (1.1)	3 (1.6)
Supraclavicular lymph nodes	6 (0.6)	3 (0.7)	2 (0.5)	1 (0.5)
Other	22 (2.1)	8 (1.8)	11 (2.5)	3 (1.6)
Multiple sites	42 (3.9)	15 (3.4)	18 (4.1)	9 (4.8)
Second malignancies only	49 (4.6)	20 (4.5)	16 (3.7)	13 (6.9)
Contralateral breast cancers	20 (1.9)	12 (2.7)	4 (0.9)	4 (2.1)
Other sites	26 (2.5)	8 (1.8)	10 (2.3)	8 (4.3)
Endometrium	6 (0.6)	1 (0.2)	2 (0.5)	3 (1.6)
Thyroid	3 (0.3)	1 (0.2)	2 (0.5)	–
Lung	3 (0.3)	1 (0.2)	1 (0.2)	1 (0.5)
Colon	3 (0.3)	1 (0.2)	1 (0.2)	1 (0.5)
Uterus	2 (0.2)	1 (0.2)	–	1 (0.5)
Other	12 (1.1)	3 (0.7)	6 (1.4)	3 (1.6)
Deaths without breast cancer	10 (0.9)	4 (0.9)	2 (0.5)	4 (2.1)

DFS (disease-free survival) events: locoregional + distant metastases + second malignancies + deaths without breast cancer

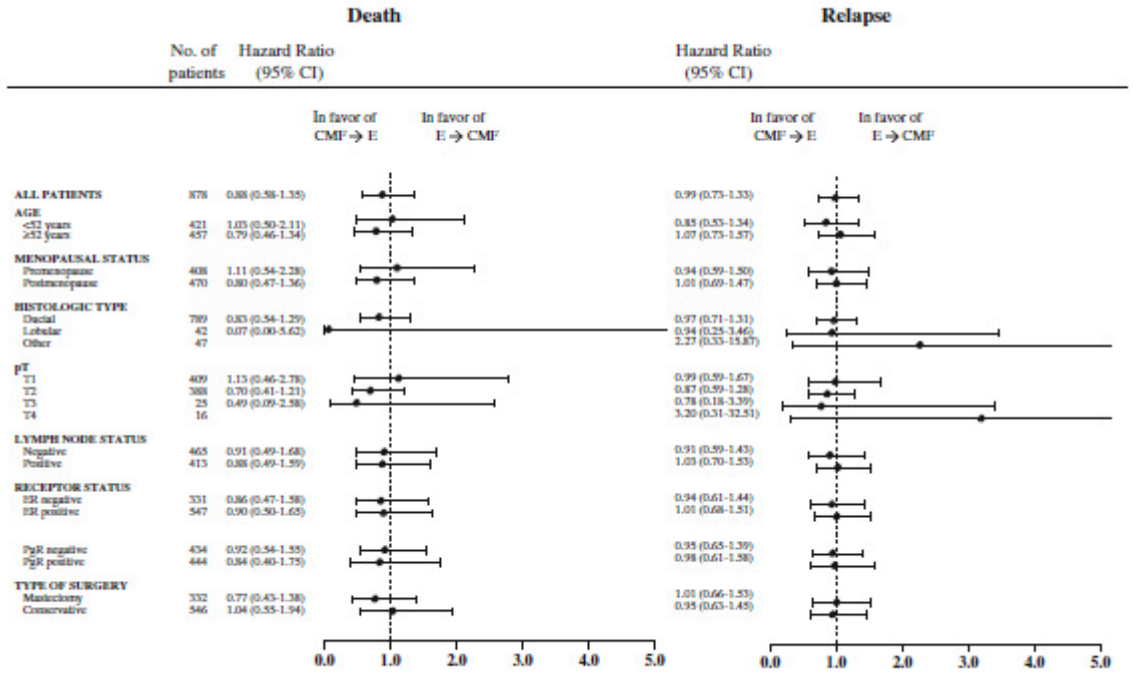

Fig. 3 Cox proportional adjusted hazards model of OS and DFS, E → CMF vs. CMF → E