Short term fasting, IGF/insulin-axis and therapy outcome in patients with cancer

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Chapter 3

The effects of short-term fasting on tolerance to (neo) adjuvant chemotherapy in HER2-negative breast cancer patients: a randomized pilot study

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Abstract

Background: Preclinical evidence shows that short-term fasting (STF) protects healthy cells against side effects of chemotherapy and makes cancer cells more vulnerable to it. This pilot study examines the feasibility of STF and its effects on tolerance of chemotherapy in a homogeneous patient group with early breast cancer (BC).

Methods: Eligible patients had HER2-negative, stage II/III BC. Women receiving (neo)-adjuvant TAC (docetaxel/doxorubicin/cyclophosphamide) were randomized to fast 24 hours before and after commencing chemotherapy, or to eat according to the guidelines for healthy nutrition. Toxicity in the two groups was compared. Chemotherapy-induced DNA damage in peripheral blood mononuclear cells (PBMCs) was quantified by the level of γ-H2AX analyzed by flow cytometry.

Results: Thirteen patients were included of whom seven were randomized to the STF arm. STF was well tolerated. Mean erythrocyte- and thrombocyte counts 7 days post-chemotherapy were significantly higher (*P=0.007,* 95% CI 0.106-0.638 and *P=0.00007,* 95% CI 38.7-104, respectively) in the STF group compared to the non-STF group. Non-hematological toxicity did not differ between the groups. Levels of γ-H2AX were significantly increased 30 minutes post-chemotherapy in CD45+CD3- cells in non-STF, but not in STF patients.

Conclusions: STF during chemotherapy was well tolerated and reduced hematological toxicity of TAC in HER2-negative BC patients. Moreover, STF may reduce a transient increase in, and/or induce a faster recovery of DNA damage in PBMCs after chemotherapy. Larger studies, investigating a longer fasting period, are required to generate more insight into the possible benefits of STF during chemotherapy.

Background

Chronic reduction of calorie intake without malnutrition reduces spontaneous cancer incidence and delays progression in a variety of tumors in rodents[1-4]. In long-term calorie restricted non-human primates, cancer incidence and mortality are reduced[5], and studies of long-term calorie restricted human subjects have shown a reduction of metabolic and hormonal factors associated with cancer risk[6-8]. Chronic calorie restriction is not practical for clinical use since it causes unacceptable weight loss in cancer patients[9]. However, brief periods of fasting may be feasible in patients and, in mice have been shown to slow cancer growth at least as effectively as chronic calorie restriction without compromising bodyweight[10-12]. Even more importantly, the effects of short-term fasting (STF) on susceptibility to chemotherapy differ between healthy somatic and cancer cells, a phenomenon called differential stress resistance (DSR)[10,11,13,14]. In healthy cells, nutrient deprivation shuts down pathways promoting growth to invest energy in maintenance and repair pathways that contribute to resistance to chemotherapy[15,16]. In contrast, tumor cells are unable to activate this protective response due to uncontrolled activation of growth pathways by oncogenic mutations. Indeed, the persistently increased growth rate of tumor cells requires abundant nutrients, and therefore, STF renders tumor cells more sensitive to chemotherapy[10-12]. Hence, STF is a promising strategy to enhance the efficacy and tolerability of chemotherapy.

In human subjects, STF is safe and well tolerated[17-19]. A case series of 10 patients with various types of cancer demonstrated that fasting in combination with chemotherapy is feasible and might reduce chemotherapy-induced side effects[20]. We conducted a randomized-controlled pilot trial to identify the effects of 48-hours of STF on chemotherapy-induced side effects and hematologic parameters in breast cancer (BC) patients, who received TAC (docetaxel, doxorubicin and cyclophosphamide) chemotherapy. Furthermore, we quantified chemotherapy-induced DNA damage in peripheral blood mononucleated cells (PBMCs) by measuring γ-H2AX accumulation[21]. Upon induction of DNA double strand breaks (DSBs), H2AX is rapidly phosphorylated at the site of DNA damage[22]. γ-H2AX has been widely used to quantify DNA damage after irradiation[23-26], where the expression has been shown to be associated with healthy tissue damage[22,27-30]. However, use of γ-H2AX as a marker for chemotherapy toxicity to healthy cells is relatively unexplored.

Methods

Patients

All women included in the study had a histologically confirmed diagnosis of HER2-negative stage II and III BC and were receiving (neo) adjuvant TAC-chemotherapy (see below). Eligibility criteria included age ≥ 18 years; BMI ≥19kg/m^2; WHO performance status 0-2; life expectancy of >3 months; adequate bone marrow function (i.e. white blood counts >3.0 x 10^9/L, absolute neutrophil count ≥1.5 x 10^9/l and platelet count ≥ 100 x 10^9/l); adequate liver function (i.e. bilirubin ≤1.5 x upper limit of normal (UNL) range, ALAT and/or ASAT ≤2.5 x UNL, Alkaline Phosphatase ≤5 x UNL); adequate renal function (i.e. calculated creatinine clearance ≥50 mL/min); adequate cardiac function; absence of diabetes mellitus; absence of pregnancy or current lactation; and written informed consent. TNM classification system was used to record stage of disease in accordance with Dutch guidelines of clinical practice (http://www.oncoline.nl).

Study design

Patients were randomized in a 1:1 ratio to fast beginning 24 hours before and lasting until 24 hours after start of chemotherapy ('STF' group) or to eat according to the guidelines for healthy nutrition with a minimum of two pieces of fruit per day ('non-STF' group). STF subjects were only allowed to drink water and coffee or tea without sugar. All patients kept a food diary of the consumption of food and drinks during the 24 hours pre- and post-chemotherapy. All patients gave informed consent in writing. The study (NCT01304251) was conducted in accordance with the Declaration of Helsinki (October 2008) and was approved by the Ethics Committee of the LUMC in agreement with the Dutch law for medical research involving human subjects.

Drugs

On the first day of each 3-weekly cycle (six in total), women received TAC (docetaxel 75mg/m^2 IV for 1 hour, adriamycin 50mg/m^2 IV for 15 minutes and cyclophosphamide 500mg/m^2 IV for 1 hour) with granulocyte-colony stimulating factor (G-CSF; pegfilgrastim 6mg) support the day after chemotherapy administration. Patients received prophylactic dexamethasone (8mg, BID the day before, the day of and the day after chemotherapy administration) in order to prevent fluid retention and hypersensitivity reactions. The anti-emetic agent granisetron (serotonin 5-HT_3 receptor antagonist; 1mg) was administered prior to chemotherapy infusion.

Blood sampling

Venous blood samples were drawn before randomization, at a maximum of 2 weeks prior to treatment (baseline) and directly before each chemotherapy administration (pre-chemotherapy, day 0). Non-fasting blood samples were drawn from subjects in

the non-STF group. The effect of fasting was determined by recording 1) metabolic parameters (insulin, glucose, insulin growth factor 1 (IGF-1), insulin growth factor binding protein 3 (IGFBP3)); 2) endocrine parameters (thyroid-stimulating hormone (TSH), triiodothyronine (T3) and free thyroxine (FT4)); 3) hematologic parameters (erythrocyte-, thrombocytes- and leukocyte count) and 4) inflammatory response (C-Reactive Protein (CRP)). For measurement of metabolic, endocrine and inflammatory parameters , blood was collected in a serum-separating tube and for hematologic parameters, blood was collected in an EDTA tube. In addition, hematologic parameters and CRP were measured on day 7 after each chemotherapy cycle. All samples were analyzed by the accredited clinical laboratory of the LUMC.

To investigate the effect of STF on chemotherapy-induced DNA damage in PBMCs, heparinized venous blood samples (9mL) were collected for both patient groups during each cycle just prior to chemotherapy, for some patients at 30 minutes after completion of chemotherapy, and on day 7 after administration. Samples were stored at room temperature until processing (in most cases directly after withdrawal or at least within 24 hours).

Toxicity

During each cycle, patients were instructed to report the experienced side effects, graded as mild, moderate or severe. Self-reported side effects, side effects documented by the physician and hematological toxicity were graded according to the Common Terminology Criteria for Adverse Events version 4.03 (CTCAE v.4.03)[31].

Isolation of PBMCs and γ-H2AX staining

PBMCs were isolated using Ficoll Paque Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Isolated PBMCs were carefully resuspended in 1 ml of Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 40% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria) and 10% dimethyl sulfoxide (DMSO) and divided over two cryovials. Samples were directly transferred to an isopropanol chamber and incubated at -80°C for a minimum of 24 hours to cryopreserve before they were stored in the vapor phase of liquid nitrogen.

Samples were processed batch wise, so that samples from distinct time points within each cycle were processed simultaneously for each patient. After thawing in RPMI at room temperature, PBMCs were fixed in 1.5% formaldehyde and permealized in ice-cold methanol. Cells were washed 3 times in staining buffer (PBS with 5% bovine serum albumin (BSA, Sigma)) and stained for 30 minutes on ice with anti-CD45-PerCP-Cy5.5 (1:20, BD, clone 2D1), anti-CD3-PE (1:10, BD, clone SK7), anti-CD14-AF700 (1:80, BD, clone M5E2), anti-CD15-PE CF594 (1:100, BD, clone W6D3)

and anti-γ-H2AX-AF488 (1:100, Biolegend, clone 2F3), followed by another washing step. The cell acquisition was performed immediately after the staining procedure (BD LSR Fortessa Flow Cytometer analyzer, BD Bioscience, Breda, The Netherlands) and data was analyzed using BD FACS Diva Software version 6.2. Compensations were set using a mixture of anti-mouse Ig/negative control beads (BD). The CD45+ cells were gated, after which the CD3+ T lymphocytes, CD3- myeloid cells (also harboring B lymphocytes) or CD14+CD15- monocytes were analyzed for the geomean (as measure for the intensity) of γ-H2AX.

Statistical analysis

All parameters were tested for normality using a Kolmogorov-Smirnov test, with Bonferroni adjustment when evaluated in subgroups. Normality distributed parameters, if necessary after log transformation, were summarized as mean (and standard error (SE)) and compared using an independent samples *t*-test for independent groups or paired *t*-test for paired groups. The non-normally distributed parameters were summarized as median (and range) and compared using a Mann-Whitney test for independent groups or Wilcoxon signed rank test for paired groups. Data of different patients and different cycles were combined to test differences between time points and treatment groups. All tests were 2-tailed with a significance level of 0.05. All data were analyzed using IBM SPSS Statistics for Windows (Version 20.0. Armonk, NY: IBM Corp).

Results

Patient characteristics

From May 2011 until December 2012, thirteen women with early BC were included and randomized into the STF (n=7) or non-STF group (n=6). Patient characteristics are summarized in Table 1. In the STF arm, 42.9% of the patients had stage III disease compared to 16.7% of patients in the non-STF arm. Estrogen receptor status was negative for one patient in the STF group (14.3%) and half of the patients in the non-STF group. Three patients had a Bloom-Richardson grade III tumor in the STF group and one in the non-STF group. One patient could not be graded due to the neoadjuvant chemotherapy. None of these patient characteristics was significantly different between the two groups.

Patients were motivated to fast and the STF was well tolerated. Two patients in the STF arm withdrew from fasting after the third chemotherapy cycle: one due to pyrosis and one due to recurrent febrile neutropenia. In both patients, the side effects persisted on a normal diet during cycles 4-6. All patients finished 6 cycles of TAC. There were no significant differences in chemotherapy-related adjustments between the two groups.

Table 1: Patient characteristics.

	STF (n = 7)	Non-STF (n = 6)	*P* Value
Median Age (range), Years	51 (47-64)	52 (44-69)	1.00
Median Body Mass Index (SEM), kg/m²	25.5 (3.3)	23.8 (2.4)	0.53
WHO-status			
Grade 0	6 (85.7%)	6 (100%)	0.34
Grade 1	1 (14.3%)	0 (0.0%)	
Treatment			
Adjuvant	5 (71.4%)	3 (50.0%)	0.43
Neo-adjuvant	2 (28.6%)	3 (50.0%)	
T-classification			
T1	3 (42.9%)	2 (33.3%)	0.94
T2	3 (42.9%)	3 (50.0%)	
T3	1 (14.3%)	1 (16.7%)	
N-classification			
N0	2 (28.6%)	2 (33.3%)	0.85
N+	5 (71.4%)	4 (66.7%)	
Stage			
II	4 (57.2%)	5 (83.3%)	0.31
III	3 (42.9%)	1 (16.7%)	
ER-status			
ER-	1 (14.3%)	3 (50.0%)	0.16
ER+	6 (85.7%)	3 (50.0%)	
PR-status			
PR-	3 (42.9%)	4 (66.7%)	0.39
PR+	4 (57.1%)	2 (33.3%)	
Grade (BR)			
1	1 (14.3%)	1(16.7%)	0.44
2	2 (28.6%)	4(66.7%)	
3	3 (42.9%)	1(16.7%)	
Unknown	1 (14.3%)	0 (0.0%)	
Chemotherapy related adjustment			
No	3 (42.9%)	3 (50.0%)	0.80
Yes	4 (57.1%)	3 (50.0%)	

STF; short-term fasting, SEM; standard error of the mean, ER; estrogen receptor; PR; progesterone receptor, BR; Bloom-Richardson.

Toxicity

The most frequently observed side effects, were grade I/II and the percentage of occurrence of each side effect is recorded in Table 2. No significant differences were observed between the two patient groups. The total incidence of grade III/IV side effects that occurred in both groups is given in Table 2. The observed grade III/IV side effects were neutropenic fever, fatigue and infection (pneumonia and neutropenic enterocolitis (typhlitis)). There was no significant difference in incidence of grade III/IV side effects between the STF and non-STF group. No grade V toxicity occurred during the chemotherapy in either group.

Table 2: Grade I/II and grade III/IV toxicity during 6 cycles of TAC in both groups.

	Grade I/II	
	STF	**Non-STF**
Fatigue	5 (71%)	6 (100%)
Infection	3 (43%)	1 (17%)
Mucositis	4 (57%)	4 (67%)
Neuropathy	5 (71%)	3 (50%)
Diarrhea	5 (71%)	2 (33%)
Dizziness	3 (43%)	3 (50%)
Nausea	7 (100%)	4 (67%)
Eye complaints	4 (57%)	2 (33%)
Constipation	4 (57%)	2 (33%)
	Grade III/IV	
Total	6	3
Neutropenic fever	2 (29%)	2 (33%)
Fatigue	2 (29%)	0 (0%)
Infection	2 (29%)	1 (17%)

All side effects were scored according CTCAE4.03. Each side effect was scored maximal once per patient during the course (the highest grade of occurrence was scored).
STF; short-term fasting.

Metabolic, endocrine and inflammatory parameters

Metabolic and endocrine parameters at randomization (maximum 2 weeks before first chemotherapy cycle) and the mean or median (depending on distribution) of the day 0 values (immediately before chemotherapy infusion, when patients in the STF group had fasted for 24 hours) were compared (Table 3). As no baseline values were available for three patients, no paired *t*-test could be performed, hence the deviating N values. In the STF and non-STF groups, median blood glucose values were significantly increased between the two time points (*P=0.042* and *P=0.043,* respectively). There was no significant difference in median insulin level between the two time points in the STF group, but in the non-STF group, the insulin level was significantly increased (*P=0.043).* Mean IGF-1 levels were significantly decreased (*P=0.012*) in the STF group; no change was observed in the non-STF group. IGF-BP3 levels did not change in either group. TSH was significantly reduced (*P=0.034*) in the non-STF group, but not in the STF group. The FT4 did not change significantly over time in patients in either group.

Table 3: Metabolic and endocrine parameters at baseline (before randomization) and day 0 (immediately before chemotherapy infusion during the use of prophylactic dexamethasone).

Parameter	N	Baseline Median (range)	Day 0 (with DEX) Median (range)	In/decrease	*P* value
Glucose	STF (n = 5)	5.2 (4.3-5.5)	6.8 (5.6-9.0)	↑	**0.042**
(3.1-6.4mmol/L)	Non-STF (n = 5)	4.8 (4.7-6.7)	7.0 (6.1-8.8)	↑	**0.043**
Insulin	STF (n = 5)	14.0 (2.0-40.0)	13.0 (6.0-36.0)	=	0.500
(0-20mU/L)	Non-STF (n = 5)	2.0 (2.0-9.0)	16.0 (9.0-63.0)	↑	**0.043**
Parameter	**N**	**Baseline Mean (SE)**	**Day 0 (with DEX) Mean (SE)**	**In/decrease**	***P* value**
IGF-1	STF (n = 4)	23.7 (2.9)	19.6 (3.3)	↓	**0.012**
(5.4-24.3nmol/L)	Non-STF (n = 5)	17.5 (3.5)	16.8 (2.8)	=	0.634
IGF-BP3	STF (n = 4)	5.0 (0.5)	4.2 (0.3)	=	0.212
(2.2-5.8mg/L)	Non-STF (n = 5)	4.5 (0.2)	3.9 (0.3)	=	0.122
TSH	STF (n = 3)	1.38 (0.26)	0.61 (0.08)	=	0.065
(0.3-4.8mU/L	Non-STF(n = 5)	1.49 (0.14)	0.42 (0.06)	↓	**0.034**
FT4	STF (n = 3)	15.4 (0.92)	13.9 (0.94)	=	0.117
(12-22pmol/L)	Non-STF (n = 5)	15.0 (0.54)	14.0 (0.34)	=	0.149

Bold value indicates that p<0.05. Abbreviations: DEX; dexamethasone, IGF-1; Insulin-like growth factor 1, IGF-BP3; insulin- like growth factor binding protein 3, TSH; thyroid-stimulating hormone; FT4;,free thyroxine; STF; short-term fasting, SE; standard error.

Figure 1 shows the mean, log transformation of the mean or the median (dependent of the distribution) of day 0 metabolic, endocrine and inflammatory parameters of all cycles compared between STF and non-STF subjects. The FT4 levels were significantly higher (*P=0.034,* 95% CI 0.08-1.91) in the STF group compared to the non-STF group. Glucose and insulin levels appeared to be lower in the STF group compared to the non-STF group, but the difference was not statistically significant. IGF-1, IGF-BP3, TSH and T3 showed similar levels in STF and non-STF patients.

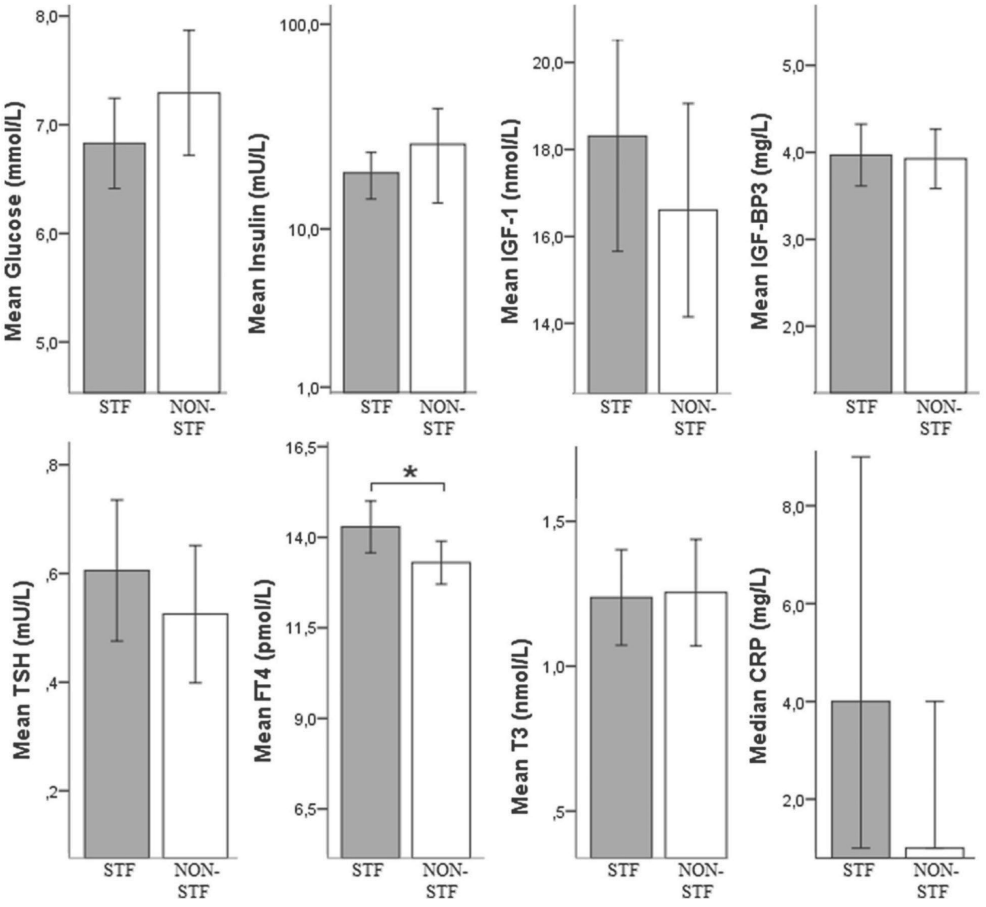

Figure 1: Metabolic, endocrine and inflammatory parameters on day 0 compared between STF and non-STF subjects. Values are measured on day 0 immediately before chemotherapy infusion (during the use of prophylactic dexamethasone). Mean values of different patients of different cycles(1-6) are combined to test differences between both treatment groups. * P value <0.05. Reference values: glucose 3.1-6.4mmol/L; insulin 0-20mU/L; IGF-1 5.4-24.3nmol/L; IGF-BP3 2.2-5.8mg/L; TSH 0.3-4.8mU/L; FT412-22pmol/L, T31.1-3.1nmol/L; CRP 0.0-5.0mg/L;. IGF-1; Abbreviations: STF: short-term fasting, IGF-1:Insulin-like growth factor 1, IGF-BP3: insulin- like growth factor binding protein 3, TSH: thyroid-stimulating hormone; FT4:,free thyroxine; T3: CRP; C-reactive protein.

Hematologic parameters

Hematologic parameters measured on day 0 (i.e., immediately before chemotherapy infusion, when the STF group had fasted for 24 hours), were similar in the two groups. Erythrocyte counts were significantly higher in the STF group during chemotherapy treatment at day 7 (*P=0.007,* 95% CI 0.106-0.638) and at day 21 (*P=0.002,* 95% CI 0.121-0.506) compared to the control group (Figure 2). Thrombocyte counts were only significantly higher at day 7 (*P=0.00007,* 95% CI 38.7-104) in the STF arm compared to the non-STF arm. For leukocytes and neutrophils, no significant difference in counts was observed, both at day 7 and day 21 between STF and non-STF patients (not shown).

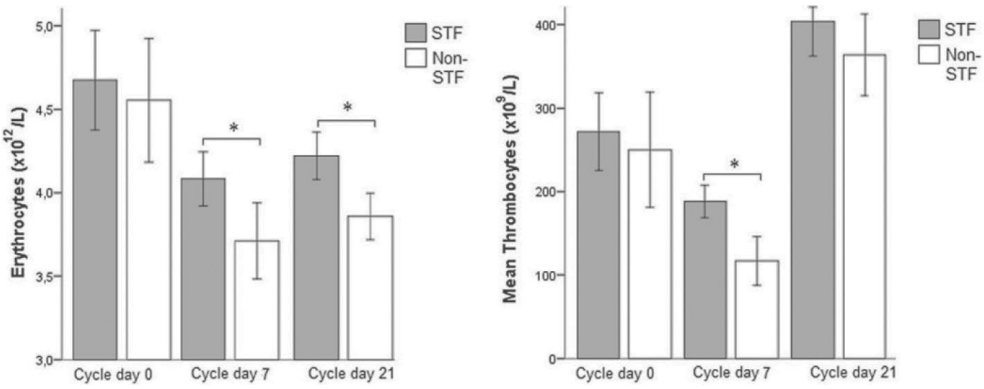

Figure 2: Hematologic parameters compared between both groups. Values are measured on day 0 of cycle 1 immediately before the chemotherapy infusion, on day 7 of cycle 1-5 combined and day 21 of cycle 1-5 combined. * *P* value <0.05. STF; short-term fasting, Reference values: erythrocytes 4-5*10^{12}/L; thrombocytes 150-400*10^{9}/L

DNA damage in PBMCs

No cumulative effect on DNA damage of chemotherapy was seen during the 6 cycles of TAC in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3- myeloid as no significant differences in γ-H2AX intensity were seen throughout 6 cycles, (see Supplemantary table 1). Therefore, the measured γ-H2AX intensity from each cycle at the same time point (before chemotherapy, after 30 minutes, and after 7 days) was combined for analysis. The level of γ-H2AX intensity (given as geomean) measured by flow cytometry in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3- myeloid are given in Table 4. γ-H2AX intensity was increased after chemotherapy infusion in the CD45+CD3+ lymphocytes 30 minutes after chemotherapy infusion in both groups and in the non-STF group after 7 days as well. In the CD45+CD14+CD15- monocytes no difference in γ-H2AX intensity was seen after 30 minutes, but after 7 days, a significant increase was seen in both groups. In the CD45+CD3- myeloid cells, a significantly increase was seen in γ-H2AX intensity at 30 minutes post-chemotherapy only in the non-STF group. γ-H2AX intensity was consistently higher in CD45+CD14+CD15- monocytes than in CD45+CD3+ lymphocytes and CD45+CD3- myeloid cells.

Table 4: γ-H2AX intensity in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3-myeloid cells.

Parameter	N	Before CT Day 0 Mean (SE)	30 minutes after CT Day 0 Mean (SE)	Increase	*P* value
CD45+CD3+ lymphocytes	STF (n = 14)	75.5 (4.7)	89.5 (6.5)	↑	**0.020**
	Non-STF (n = 6)	78.8 (5.6)	95.7 (5.9)	↑	**0.001**
CD45+CD14+CD15- monocytes	STF (n = 12)	162.2 (11.9)	192.5 (14.3)	=	0.055
	Non-STF (n = 6)	180.8 (15.6)	206.2 (20.8)	=	0.051
CD45+CD13- myeloid cells	STF (n =14)	104.0 (7.0)	109.5 (8.4)	=	0.594
	Non-STF (n = 6)	109.0 (7.8)	123.0 (6.7)	↑	**0.009**
Parameter	**N**	**Before CT Day 0 Median (range)**	**7 days after CT Median (range)**	**Increase**	***P* value**
CD45+CD3+ lymphocytes	STF (n =16)	75.5 (49-157)	83.0 (64-141)	=	0.109
	Non-STF (n = 9)	78.0 (47-102)	90.0 (71-114)	↑	**0.015**
CD45+CD14+CD15- monocytes	STF (n = 14)	157.0(114-231)	186.5 (132-295)	↑	**0.035**
	Non-STF (n = 8)	203.5 (116-273)	258.5 (183-319)	↑	**0.021**
CD45+CD13- myeloid cells	STF (n = 16)	106.0 (71-173)	84.0 (65-145)	=	0.379
	Non-STF (n = 9)	88.0 (49-137)	88.0 (74-119)	=	0.477

Paired comparison between pre- and post- chemotherapy (30 minutes and 7 days; median of 6 cycles of TAC) for the different cell types. γ-H2AX intensity is given as mean and median depending on the distribution.
Bold value indicates that p<0.05. 95% CI; 95% confidence interval. P values are given for differences of intensity of γ-H2AX between pre- and post-chemotherapy

Discussion

This is the first randomized pilot study to explore the effects of 48 hours STF on the side effects of chemotherapy in early BC patients. Only one study to date[20] has examined the effects of fasting on chemotherapy-induced side effects in cancer patients, but therein the patients served as their own controls and had various tumor types and treatment protocols. The main findings of our study were that STF was well-tolerated, safe and had beneficial effects on hematologic toxicity and possibly on DNA damage in healthy cells (lymphocytes and myeloid cells). Additionally, we found that dexamethasone, when administered during the fasting period, causes an increase in metabolic values.

Although STF was well tolerated, two patients withdrew from STF after 3 cycles of chemotherapy after experiencing a side effect (pyrosis and recurrent febrile neutropenia, respectively). Since these side effects persisted in both patients during the subsequent 3 cycles of chemotherapy without STF, they may not be related to STF. All patients finished their treatment schedule of 6 cycles of TAC and no significant difference in occurrence of chemotherapy-related adjustments were found between the two groups. The side effect profile of the TAC protocol seen in this study was consistent with the existing literature[32-34]. STF had no beneficial effect on patient-reported side effects in this study. This may be explained by the large variability of side effects between patients, which may be attributable to occurrence of symptom clusters and pharmacogenomics[35,36]. This

may have masked any beneficial effects of STF. Additionally, the relatively short period of fasting (48 hours) may explain the lack of benefit in terms of side effects: previous studies have shown that a longer fasting period is required to cause major changes in IGF-1 levels[20,37]. Reduction of plasma IGF-1 levels is a critical mediator of differential stress resistance in response to nutrient restriction (see below).

γ-H2AX phosphorylation indicates the presence of double-strand DNA breaks and could serve as a marker for chemotherapy toxicity in healthy cells, as seen in a phase I/II trial with patients treated with chemotherapy and belinostat[38]. We measured the induction of chemotherapy-induced DNA damage in PBMCs by phosphorylation of H2AX (i.e. γ-H2AX). The level of γ-H2AX in CD45+CD3+ lymphocytes was increased after 30 minutes in both groups. After 7 days, γ-H2AX accumulation remained increased in the non-STF group only, suggesting that STF promotes the recovery of chemotherapy-induced DNA damage in these cells. In CD45+CD3- myeloid cells, the level of γ-H2AX was increased after 30 minutes in the non-STF group, but not in the STF group, suggesting STF protected these cells against the induction of DNA damage by chemotherapy. As these myeloid cells may harbor the antigen-presenting cells required for induction of an effective anti-tumor immune response, this result warrants further study[39]. Moreover, the relation of this finding with the clinical benefit of STF still needs to be established.

The significantly higher erythrocyte and thrombocyte counts observed after chemotherapy in the STF group could be explained by decreased breakdown of circulating cells and/or less severe bone marrow suppression. This supports the hypothesis that STF may protect against chemotherapy-associated hematological toxicity. No significant difference in leukocyte and neutrophil counts was seen. This could be explained by the use of pegfilgrastim, which acts to increase the production of white blood cells in bone marrow and may therefore prevent a decrease in leukocyte counts in response to chemotherapy.

Plasma glucose levels increased and insulin levels remained constant in response to STF. The use of dexamethasone may explain this phenomenon[40-42]. Dexamethasone was administered for anti-emesis, reduction of fluid retention and dampening of hypersensitivity reactions in response to docetaxel[43]. However, the metabolic effects of dexamethasone may have attenuated the benefits of STF. In the absence of dexamethasone, STF reduces circulating glucose, insulin and IGF-1 levels[19,44]. A decrease in IGF-1 affects other factors (e.g. Akt, Ras and mammalian target of rapamycin (mTOR)) to down-regulate cell growth and proliferation[45-47]. Reduction of IGF-1 is one of the key mediators of the protective effects of STF in healthy cells[44]. Although fasting modestly reduced plasma IGF-1 concentrations in the current trial, the concomitant use of dexamethasone probably attenuated the decline and thereby probably counteracted the

beneficial impact of the dietary intervention.

Our study has some limitations. The most obvious limitation of our study is the small sample size, which may have limited the power of the study and precludes firm statistical conclusions. Moreover, as high dose dexamethasone induces insulin resistance, compensatory hyperinsulinemia and hyperglycemia, its prophylactic use may have counteracted the beneficial effects of STF. Therefore the use of this drug warrants further study for future clinical trials with STF. Finally, as DNA damage is repaired rapidly[48], our protocol may not be rapid enough to obtain a reliable quantification. Therefore, a consistent and rapid protocol for the isolation and fixation of PBMCs immediately after blood withdrawal should be applied in future studies to allow for reliable quantification of damage induced by chemotherapy.

Larger randomized trials such as the DIRECT study (NCT02126449) are now ongoing to evaluate the impact of STF on tolerance to and efficacy of neoadjuvant chemotherapy in women with stage II or III BC. Because it is likely that the positive effects of STF will be enhanced if the period of fasting is prolonged[37,49], a very low calorie, low protein fasting mimicking diet (FMD) is used to ease the burden of prolonged fasting[50]. Prophylactic dexamethasone will be omitted in the FMD arm during the first 4 chemotherapy cycles to reduce its potentially counteractive metabolic effects. Moreover, blood will be processed immediately after sampling to prevent potential recovery of DNA damage.

Conclusions

We demonstrate for the first time that STF is feasible for a period of 48 hours during chemotherapy in a homogeneous group of patients with early breast cancer. This study provides evidence that STF attenuates bone marrow toxicity in these patients and reduces chemotherapy-induced DNA damage in PBMCs and/or accelerate its recovery. A larger trial with a longer fasting period is ongoing to investigate the possible benefits of STF during chemotherapy.

List of abbreviations

BC:	breast cancer
CRP :	C-Reactive Protein
DSBs:	double-strand breaks
DSR:	differential stress resistance
FT4:	free thyroxine
G-CSF:	granulocyte-colony stimulating factor
IGF-1:	insulin growth factor 1
IGF-BP3:	insulin growth factor binding protein 3
PBMCs:	peripheral blood mononuclear cells
STF:	short-term fasting
T3:	triiodothyronine
TAC:	docetaxel, doxorubicin and cyclophosphamide
TSH:	thyroid-stimulating hormone
UNL:	upper limit of normal

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Supplementary material

Supplementary Table 1: Median of γ-H2AX geomean intensity in CD45+CD3+ lymphocytes, CD45+CD14+CD15- monocytes and CD45+CD3- myeloid cells among the six cycles tested with the median test, testing for differences of γ-H2AX between cycles.

	CD45+CD3+ lymphocytes		CD45+CD14+ CD15- monocytes		CD45+CD3- myeloid cells	
Time point	**Median γ-H2AX intensity**	***P* value***	**Median H2AX intensity**	***P* value***	**Median γ-H2AX intensity**	***P* value***
before CT Day 0	78.0	0.265	156.5	0.147	106.0	0.953
30 minutes after CT Day 0	97.0	0.931	177.5	0.502	123.0	0.931
7 days after CT	86.0	0.440	181.5	0.602	84.0	0.514

P values* given for differences of intensity of γ-H2AX tested among the six cycles.