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New ways to optimize breast cancer treatment

Schröder, Carolina Pia

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Telomere length in breast cancer patients before and after chemotherapy with or without stem cell transplantation

C.P. Schröder [1], G.B.A. Wisman [2], S. de Jong[3], W.T.A. van der Graaf [1], M.H.J. Ruiters [3], N.H. Mulder [1], L.F.M.H. de Leij [3], A.G.J. van der Zee [2], E.G.E. de Vries [1]

Departments of Medical Oncology [1], Gynecologic Oncology [2], Pathology and Laboratory Medicine [3], University Hospital Groningen, The Netherlands

C.P. Schröder and G.B.A. Wisman contributed equally to this work

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Abstract

High-dose chemotherapy and peripheral blood stem cell transplantation (PBSCT) may accelerate telomere length loss in hematopoietic stem cells. As data including pre- and post-treatment samples are lacking, we studied leukocyte telomere length and telomerase activity before and after treatment in breast cancer patients randomised to receive 5 adjuvant courses FEC (5-FU, epirubicin and cyclophosphamide) (n=17), or 4x FEC followed by high-dose cyclophosphamide, thiotepa, carboplatin and autologous PBSCT (n=16). Haemoglobin, MCV, leukocyte- and platelet numbers were assessed prior to (t_0), 5 months after (t_1) and 9 months after chemotherapy (t_2); these parameters were decreased at t_1 and t_2 compared to t_0 (high-dose: all parameters; standard-dose: leukocytes and platelets), and all parameters were lower after high-dose than standard-dose treatment at t_1. Paired individual leukocyte samples of t_0 and t_1 showed telomere length change (determined by telomere restricted fragment (TRF) assay) ranging from +0.8 to –2.2 kb, with a decreased TRF length in 9 patients of both groups. Telomerase activity (determined by TRAP assay) was below detection limit in leukocyte samples of t_0 and t_1. Thus, standard- and high-dose chemotherapy negatively affect haematological reconstitution in this setting. In individual patients, telomere length can be remarkably changed following haematological proliferative stress after treatment.

Introduction

Human telomeres are regions at the chromosomal ends that play an important role in the structure and function of chromosomes. In normal somatic cells telomeres are shortened with every cell division, and when a critical size is reached, cells lose their proliferative potential (Harley *et al.*, 1990; Hastie *et al.*, 1990; Harley, 1997). Also in purified hematopoietic stem cells telomeric DNA appears to shorten with each cell division and thus with age (Vaziri *et al.*, 1994; Lansdorp, 1995). A number of studies have indicated a possible accelerated shortening of telomere length in hematopoietic stem cells, due to proliferative stress following peripheral blood stem cell transplantation (PBSCT) (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Shapiro *et al.*, 1996; Akiyama *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000). Although low levels of telomerase -a ribonucleoprotein that synthesises telomeric DNA- can be determined in CD34+ hematopoietic stem cells, this appears to be insufficient to compensate increased shortening of telomere length (Notaro *et al.*, 1997). Because of possible negative long-term effects of this shortening, including possible cytogenetic abnormalities (Ball *et al.*, 1998; Ohyashiki *et al.*, 1999), genomic instability preceding myelodysplastic syndromes (Ohyashiki *et al.*, 1994) and reduced response following hematopoietic stress (Rudolph *et al.*, 1999), this is clearly of clinical interest. However, most data so far are obtained from allogeneic transplantation settings (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000), in paediatric patients. Fewer data are available on the effect of autologous PBSCT (Shapiro *et al.*, 1996; Akiyama *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000), while paired data including pre-treatment samples are lacking.

As telomere length of nucleated blood cells was shown to be widely variable between age-matched individuals (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Akiyama *et al.*, 1998; Lee *et al.*, 1999), prospective paired data are essential for determining the impact of autologous PBSCT on this possible ageing process. Therefore, we prospectively studied leukocyte telomere length and telomerase activity in a group of high-risk breast cancer patients randomised to receive either adjuvant standard-dose chemotherapy, or adjuvant high-dose chemotherapy and PBSCT (De Vries *et al.*, 1996). Paired samples before and after these treatments were compared, allowing assessment of the impact of standard and high-dose chemotherapy on telomere length and telomerase activity.

Patients and methods

Patients

Patients included in this study participated in a national randomised adjuvant breast carcinoma study (De Vries *et al.*, 1996). Chemotherapy naive breast cancer patients with four or more tumour-involved axillary lymph nodes (stage II and III), ≤ 55 years of age with negative chest X-ray, liver ultrasound and bone scan, were randomised to receive 5 courses of standard-dose chemotherapy followed by radiotherapy, or 4 courses of the same combination chemotherapy followed by high-dose chemotherapy, PBSCT and radiotherapy. These groups will be referred to as the standard-dose group, and the high-dose group, respectively. The combination chemotherapy consisted of 5-fluorouracil (500 mg m^{-2}), epirubicin (90 mg m^{-2}) and cyclophosphamide (500 mg m^{-2}), administered intravenously once every 3 weeks. For the high-dose group, PBSC were mobilised following the third or last course of FEC with daily subcutaneous recombinant human granulocyte-colony stimulating growth factor (rhG-CSF, 263 μg), from day 2 of the course. Leucapheresis was performed from day 9 of this course, until $\geq 5.10^6$ CD34+ cells kg^{-1} body weight (as determined by flow cytometric analysis with the fluorescein isothiocyanate-labelled anti-CD34 antibody directed against the HPCA-2 epitope on CD34+ cells, Becton Dickinson, Leiden, the Netherlands) were obtained. High-dose chemotherapy consisted of cyclophosphamide (1500 mg m^{-2}), thiotepa (120 mg m^{-2}) and carboplatin (400 mg m^{-2}) on days -6, -5, -4 and -3, followed by reinfusion of PBSC on day 0. After reinfusion, daily subcutaneous rhG-CSF was administered until the leukocyte count exceeded 3.10^9 l^{-1}. Locoregional radiotherapy (50 Gy in 25 fractions) was administered after completion of the chemotherapy scheme with sufficient bone marrow recovery (defined as platelets $>100.10^9$ l^{-1}). Oral tamoxifen 40 mg daily was administered after platelet recovery for two years, in both groups. The study, and

the collection of blood samples as described, was approved by the Medical Ethical Committee of the University Hospital Groningen. All patients gave informed consent.

Sampling times

Blood samples were collected from all consecutive patients randomised in this study from May 1997 until January 1999. Sampling times were: t_0: directly prior to start of chemotherapy; t_1: 5 months after completion of chemotherapy; t_2: 9 months after completion of chemotherapy.

Telomere length was measured in samples from t_0 and t_1. In a number of these samples it was also possible to measure telomerase activity. Haematological examinations, e.g. haemoglobin, mean corpuscular volume (MCV), leukocytes, and platelets were performed at t_0, t_1 as well as t_2. Haematological parameters were considered normal with haemoglobin ≥ 7.45 mmol l^{-1}, MCV 80- 96 fL, leukocytes ≥ $4.0.10^9$ l^{-1} and platelets ≥ 150.10^9 l^{-1} (Barbui *et al.*, 1996).

Analysis of telomere length

In blood samples from t_0 and t_1, lysis of erythrocytes was performed with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium ethylene diamine tetra acetate, EDTA). The remaining nucleated cell fraction was then washed in phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.4). After centrifugation (150 g, for 10 min) the supernatant was decanted and 1.10^6 cells of the pellet of nucleated cells were transferred onto a slide for assessment of the leukocyte differentiation. The remaining pellet of nucleated cells was stored at -80°C . This erythrocyte lysis procedure was performed in accordance with Wynn *et al.* (1998).

In the nucleated leukocyte cell fraction, mean telomere length was determined by the terminal restriction fragment (TRF) assay according to Harley *et al.* (1990), with minor modifications. DNA was isolated using the salt extraction method as described by Miller *et al.* (1988). 5 µg DNA was digested overnight at 37°C using 20 U *Rsa*I and 20 U *Hin*fI (Roche Diagnostics, Almere, The Netherlands). Digested DNA was electrophorised in a 0.6% agarose gel in 0.5x Tris-borate EDTA buffer overnight at 50 V. DNA was depurinated with 0.25 M HCl, denatured in 0.5 M NaOH and 1.5 M NaCl and neutralised in 0.5 M Tris/HCl (pH=7.5) and 1.5 M NaCl, after which the DNA was transferred to a positively charged nylon membrane (Roche Diagnostics, Almere, The Netherlands) using 10x SSC overnight and dried for 2 hours at 80°C. Prehybridisation, hybridisation with 5 ng ml^{-1} probe and washing were performed according to TeloQuant assay (PharMingen, San Diego). Preincubation, incubation with 1:5000 alkaline phosphatase conjugated streptavidine and washing were performed according to biotin luminescence detection kit instructions (Roche Diagnostics, Almere, The Netherlands). Telomeric smears were visualised by incubation of the membrane with the chemiluminescence substrate CPD-Star (1:100), according to the supplied instructions (Tropix, Westburg, Leusden, the Netherlands) and exposure to a film. Films were analysed using a scanner and Diversity One PDI computer software (Pharmacia Biotech, Roosendaal, the Netherlands). The mean TRF lengths were calculated with the formula: mean TRF length= $\sum(OD_i)/\sum(OD_i/L_i)$, in which OD_i is the density output and L_i is the length of the DNA at row i (normally a Gaussian curve was obtained) (Wynn *et al.*, 1998). For standardisation, DNA isolated from leukocytes (one sample) of one healthy donor was included on all gels. The mean TRF length of the leukocytes of the donor was 7.3 kb. TRF lengths from patient samples were normalised to the TRF length of the healthy donor sample, which was set at 7.3 kb for each gel analysed. Paired patient samples were always analysed on the same gel.

The intra-assay variance coefficient in this study was determined to be 1.4% (95% CI) after analysis of 10 aliquots of the control healthy donor sample on one gel, resulting in a mean measurement variance for each sample of ± 100 bp. Therefore, samples of individual patients with a difference in TRF value < 0.2 kb were considered equal. As control, the plasmid pTSK8 (linearized with *Kpn*I; a kind gift from Dr. Royle, Leicester, U.K.) was used, which contains approximately 200 base pairs (bp) of TTAGGG repeats (Royle *et al.*, 1992). TRF length change (Δ TRF length) was defined as the TRF value at t_1 minus the value at t_0.

Telomerase activity (the TRAP assay)

After obtaining the nucleated leukocyte cell fraction as described above, 1.10^6 leukocytes per telomerase activity assay were lysed in 100 µL TRAP lysis buffer (0.5% CHAPS; 10 mM Tris/HCl (pH 7.5); 1 mM $MgCl_2$; 1 mM EGTA; 10% glycerol; 5 mM ß-mercaptoethanol; 0.1 mM PMSF) and incubated on ice for 25 min. After centrifugation at 15,000 g for 20 min at 4°C, the supernatant was quickly frozen in liquid nitrogen and stored at - 80°C until further processing.

The TRAP assay was performed as previously described (Wisman et al., 1998). In short, telomerase activity levels in leukocytes were determined with a fluorescence-based telomeric repeat amplification protocol assay using GLC_4 cells (Zijlstra et al., 1987) as standard in each assay. Peaks representing telomerase activity in GLC_4 cell equivalents were summed, then relatively expressed to telomerase activity of 100 GLC_4 cell equivalents (set at 100%) and normalised to the signal of modified-internal telomerase assay standard (M-ITAS). For the samples (1.10^5 cells and 1.10^4 cells) the peaks representing telomerase activity were also summed and normalised to the signal of M-ITAS, thereafter the relative telomerase activity of the leukocytes was correlated to GLC_4 cell number (relative quantification comparable to 10 GLC_4 cell equivalents = 10 U).

Statistics

Mean TRF length and telomerase activity in blood samples from in individual patients were compared from t_0 and t_1, and statistically analysed with the Wilcoxon signed ranks test for paired samples. Comparisons of haematological parameters and leukocyte differentiations between the standard- and high-dose groups, were performed with the Student's t-test for independent samples, and comparisons between time points in both groups were performed with the t-test for paired samples. Correlations between TRF data, telomerase activity, numbers of CD34+ cells and haematological examinations were examined with the Pearson correlation test. All analyses were performed using the statistical analysis program SPSS. A $p<0.05$ was considered statistically significant.

Results

Patients

The standard-dose group consisted of 17 patients, and the high-dose group of 16. Mean age at the start of treatment was 44.0 years (range 29-54 years) and 44.6 years (range 37-54 years) in these groups respectively (N.S.). Mean period between blood samples of t_0 and t_1 was 32 weeks in the standard-dose group, and 37 weeks in the high-dose group (N.S.).

Haematological parameters

The analysis of haematological parameters is reflected in figure 1.

Leukocytes: Compared to the standard-dose group, leukocyte counts were lower in the high-dose group at t_1 (standard-dose: mean at t_1 $5.9.10^9$ l^{-1}, high-dose: mean $4.1.10^9$ l^{-1}, $p = 0.008$; leukocytes $< 4.0.10^9$ l^{-1} in 2/17 versus 7/16 patients respectively). At t_2 this difference was not observed.

Compared to t_0, a decreased leukocyte count was shown at t_1 in paired samples after both standard- and high-dose treatment ($p<0.001$); this difference remained present at t_2 in both groups (fig.1A).

Platelets: Compared to the standard-dose group, platelet counts were lower in the high-dose group at t_1 (standard-dose: mean at t_1 220.10^9 l^{-1}, high-dose: mean 137.10^9 l^{-1}, $p < 0.001$; platelets $< 150.10^9$ l^{-1} in 0/17 versus 11/16 patients respectively). At t_2 this difference was not observed.

Compared to t_0, a decreased leukocyte count was shown at t_1 in paired samples after both standard- and high-dose treatment ($p < 0.001$); this difference remained present at t_2 in both groups (fig. 1B).

Haemoglobin: In the standard-dose group, haemoglobin values were not different at t_0, t_1 and t_2. Compared to the standard-dose group, haemoglobin was lower in the high-dose group at t_1 (standard-dose: mean at t_1 8.0 mmol l^{-1}, high-dose: mean 6.7 mmol l^{-1}, $p < 0.001$; haemoglobin < 7.45 mmol l^{-1} in 2/17 versus 14/16 patients respectively) as well as at t_2 ($p=0.003$).

Compared to t_0, a decreased haemoglobin was observed at t_1 in paired samples after high-dose treatment ($p<0.001$); this difference remained present at t_2 (fig. 1C).

MCV: In the standard-dose group, MCV values were not different at t_0, t_1 and t_2. Compared to the standard-dose group, MCV values were increased in the high-dose group at t_1 (standard-dose: mean at t_1 90.9 fL, high-dose: mean 97.9 fL, $p < 0.001$; MCV > 96 fL in 2/17 versus 10/16 patients respectively), but not at t_2.

Compared to t_0, an increased MCV value was observed at t_1 in paired samples after high-dose treatment ($p<0.001$); this difference remained present at t_2 (fig 1D).

CD34+ cell number and haematological parameters

At t_1 in the high-dose group, the number of reinfused CD34+ cells (times 10^6 per kg body weight) correlated positively with the number of leukocytes ($r= 0.63$; $p =0.009$) and platelets ($r= 0.77$; $p <0.001$), and negatively with MCV ($r= -0.6$, $p =0.014$). No relation between haemoglobin and CD34+ cells was found. At t_2, no correlation between CD34+ cells and haematological parameters was observed.

Telomere length and telomerase activity

TRF length (mean of all patients at t_0 8.1 kb, SD 1.4) was in the same range as previously reported in cross-sectional studies (Wynn *et al.*, 1998; Ball *et al.*, 1998; Akiyama *et al.*, 1998; Lee *et al.*, 1999). As shown in figure 2, TRF length decreased

in 9 patients of each group when t_0 and t_1 samples were compared, and 4 patients from the standard- and 5 patients from the high-dose group showed a TRF length increase (mean Δ TRF length of both groups: -0.2 kb, SD 0.6; range Δ TRF length: standard-dose group: +0.4 to -2.2 kb; high-dose group +0.8 to -1.1 kb). Paired analysis of t_0 and t_1 samples showed overall no effect on TRF length of either treatment arm (standard-dose group: p=0.069; high-dose group: p=0.67) or of treatment in general (both groups together: p=0.148). A representative blot is shown in figure 3. No difference in leukocyte differentiation was found when t_0 and t_1 samples were compared of both groups, and no difference between the groups was observed at t_0 or t_1.

In the high-dose group, no correlation between reinfused CD34+ cells and actual TRF length at t_1, or Δ TRF length could be observed (fig 4).

Also the relation between haematological parameters haemoglobin, MCV, leukocyte- and platelet counts at t_1 and t_2 and TRF length, or Δ TRF length was evaluated. No correlation between these haematological parameters and (Δ) TRF length could be observed.

In 9 patients from each group, paired leukocyte sample size also allowed measurement of telomerase activity at t_0 and t_1. This included the samples with maximum TRF length increase or decrease of both groups. Telomerase activity in all of these patient samples was below the reliable detection limit of 10 U (equivalent to 10 GLC4 cells) per 1.10^5 leukocytes (Wisman *et al.*, 1998), in both groups at t_0 and t_1. This activity level is comparable with telomerase activity found in leukocytes from healthy controls (Wolthers *et al.*, 1999). Therefore, no strong up-regulation of telomerase activity was observed, also not in patients with increased TRF lengths after treatment.

Discussion

The perception that hematopoietic proliferative stress may accelerate the ageing of hematopoietic stem cells has gained interest, in view of the wide spread use of hematopoietic stem cell transplantations for various clinical conditions. Evidence for accelerated telomere shortening after hematopoietic stem cell transplantations was found in a number of studies (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Shapiro *et al.*, 1996; Akiyama *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000). Most data were derived from paediatric patients with haematological malignancies, and frequently mean TRF lengths after therapy were compared to mean TRF lengths of age-matched controls. However, mean TRF length of nucleated blood cells has been shown to be widely variable between these controls (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Akiyama *et al.*, 1998; Lee *et al.*, 1999). Additionally, samples were drawn at a wide range of time after PBSCT, ranging from 1.6 months (Lee *et al.*, 1999) to over 10 years (Akiyama *et al.*, 1998; Wynn *et al.*, 1999). Finally, as TRF dynamics were shown to be different in the various stages of life (Zeichner *et al.*, 1999), predictive value for the adult setting may not automatically be assumed from these paediatric data. Therefore, we studied mean leukocyte TRF length in paired samples before and after treatment, in a group of high-risk breast cancer patients randomised to receive either adjuvant standard-dose chemotherapy, or adjuvant high-dose chemotherapy and PBSCT. These treatment modalities are frequently used for breast cancer (Antman *et al.*, 1997), and their induction of hematopoietic stress and possible consequent effect on individual TRF length could be assessed. TRF length measurement in this study was performed based on the commonly used procedure by Harley *et al.* (1990), and care was taken to standardise measurements. In analogy to the pioneering study by

Wynn *et al*. (1998), we chose unselected leukocytes to measure TRF length and telomerase activity in. In recent studies, it has been suggested that lymphocytes may have a larger TRF length than neutrophils (Wynn *et al*., 1999; Robertson *et al*., 2000). Although TRF length of T lymphocytes and neutrophils was shown to be equally affected by stem cell transplantation (Wynn *et al*., 1999), in case of a change in the relative proportions of these cells, it might be slightly more difficult to draw conclusions from overall leukocyte TRF length. However, in this study no difference in the leukocyte differentiations was found in either group before or after treatment, and TRF length in our leukocyte samples is therefore unlikely to be affected by such a difference. Furthermore, variable differences of TRF length of neutrophils and T lymphocytes have been reported, ranging from approximately 1 kb (Wynn *et al*., 1999) to none (Martens et al., 2000). In light of these data, we consider leukocytes, in line with Wynn *et al*. (1998), sufficient for the purpose of this study.

Hematopoietic proliferative stress to achieve haematological reconstitution after treatment, was analysed by means of haematological parameters in peripheral blood, until 9 months after treatment. A clear negative effect on all haematological parameters was seen after high-dose treatment, and 9 months later still no recovery was made to the pre-treatment level. Even after standard-dose treatment, leukocyte- and platelet counts were significantly affected for at least 9 months. A long-term impact of PBSCT on haematological reconstitution was observed in haematological malignancies (Barbui *et al*., 1996). Our data appear to support this in the solid tumour setting also, but data from longer follow-up periods are needed to confirm this. In line with previous studies (Faucher *et al*., 1996; Bernstein *et al*., 1998), we found that the number of reinfused CD34+ cells correlated with leukocyte- and platelet numbers as well as MCV values, shortly after high-dose treatment.

Following the evident hematopoietic stress induced by both treatment arms (and PBSCT in particular), TRF length was clearly changed in individual patients. The majority of patients (n=9 in both arms) showed a TRF length decrease at t_1, but also remarkable TRF length increases were observed; no significant decrease due to either treatment was found in paired samples. The high-dose treatment scheme used in this study is classically combined with stem cell support in view of its profound myolotoxicity, causing prolonged life threatening marrow aplasia (Ayash *et al.*, 1993; Antman *et al.*, 1994). It is possible that in individual patients the lack of TRF length decrease due to treatment may be interpreted as a sign of insufficient treatment toxicity, as stem cells remaining in the patient after high-dose treatment will have an impact on the requirements to divide for haematopoietic reconstitution. In line with the presumed ablative nature of the treatment regimen in our study however, its profound impact on hematological parameters is clear. The maximum myelosuppression at t_1 and the (partial) hematological recovery at t_2, indicate hematopoietic proliferative stress at the time-point at which TRF length was measured (at t_1). Full recovery of hematological parameters after this high-dose treatment may actually take years (Nieboer *et al.*, 2000), and the impact of this lengthy process on TRF length changes at later time-points than t_1 is currently being studied.

The detection of a distinct increase of TRF length in some patients was surprising. We hypothesised that up-regulation of telomerase activity in response to replicative stress might be responsible for this, in agreement with in vitro studies with purified CD34+ cells (Engelhardt *et al.*, 1997; Yui *et al.*, 1999). However, in our samples telomerase activity remained undetectable after treatment. In drawing conclusions from this, it should be considered that telomerase activity is a much more dynamic parameter than TRF length. Possibly, telomerase activity changes took place at other time-points than were measured in this study. Furthermore, in

contrast to the comparable TRF length of leukocytes and CD34+ cells (Kronenwett *et al.*, 1996), telomerase activity in purified CD34+ cells is likely higher than in terminally differentiated cells such as leukocytes (Engelhardt *et al.*, 1997).

CD34+ cell numbers in our study were not related to (Δ) TRF length. Previously, it was assumed that if small numbers of CD34+ cells are reinfused, these cells may have to undergo more cell divisions than larger numbers, for a similar net hematopoietic effect (Notaro *et al.*, 1997). However, no relationship was found between the degree of TRF length shortening and the number of reinfused CD34+ cells in recent studies (Lee *et al.*, 1999; Wynn *et al.*, 1999) and our data support this. Possibly, in vitro culturing of CD34+ cells may provide more insight into the balance between cell proliferation and the ability to upregulate telomerase activity in individuals, leading to (change of) telomere length in vivo. Disturbances in this balance may be related to haematological malignancies (Engelhardt *et al.*, 2000). In this respect, the ability to measure TRF length in individual chromosomes or cells by means of flow cytometry (Rufer *et al.*, 1998) or in situ hybridisation (Martens *et al.*, 2000) may be of interest. It remains conceivable that a rapid TRF decrease, predisposes for long-term effects such as secondary malignancies in individual patients. This has to be evaluated after a longer period of follow-up.

In conclusion, in this study we found that standard- and high-dose chemotherapy (in particular) negatively affect haematological reconstitution. Leukocyte TRF length was remarkably changed in individual patients after treatment, showing both decrease (in the majority of patients), as well as increase. Therefore, although no accelerated telomere loss was observed in general, TRF length was clearly affected following proliferative stress in this setting.

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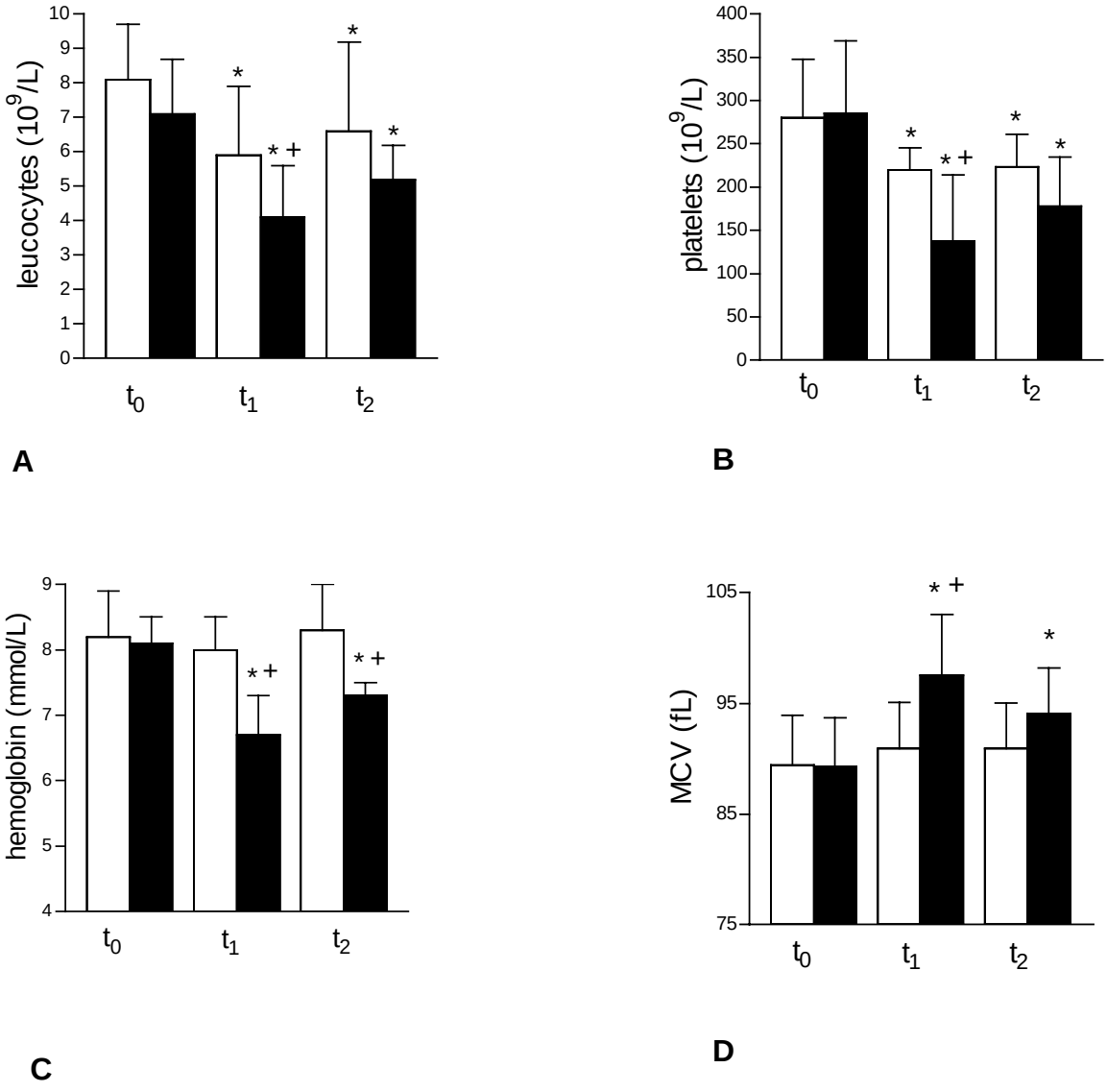
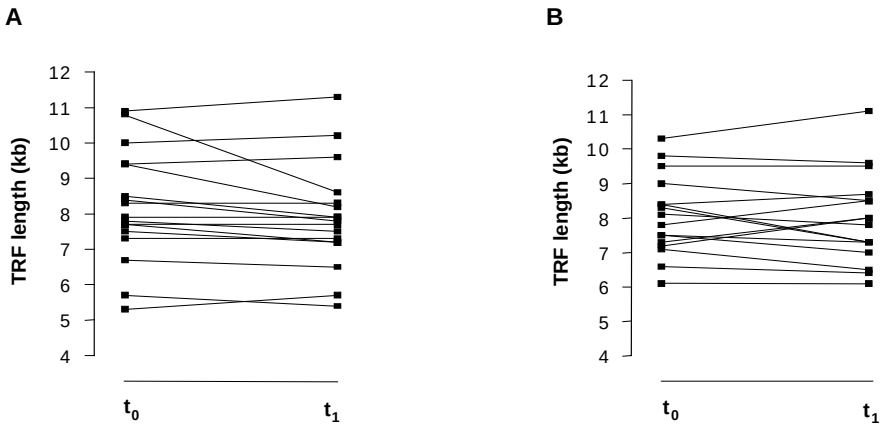


Figure 1:

Haematological recovery

The open bar indicates the standard-dose group, the black bar the high-dose group. A (*) indicates a significant difference in paired samples compared to t_0; a (+) indicates a significant difference between groups at that time point. On the X-axis blood sampling times t_0 (prior to chemotherapy), t_1 (5 months after chemotherapy) and t_2 (9 months after chemotherapy) are indicated. On the Y-axis, the following values are indicated with mean + SD: **A**: leukocytes (10^9 l^{-1}); **B**: platelets (10^9 l^{-1}; **C**: haemoglobin (mmol l^{-1}) and **D**: MCV (fL).

Figure 2:



Paired TRF samples

A: X-axis: standard-dose group samples, at t_0 and t_1; Y-axis: TRF length (kilobase, kb). **B**: X-axis: high-dose group samples, at t_0 and t_1; Y-axis: TRF length (kb).

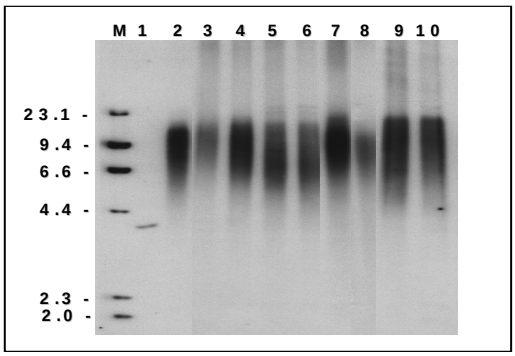


Figure 3:

Representative example of blot to measure TRF length

M: marker; lane 1: plasmid control; lane 2: leukocyte control healthy volunteer; lanes 3-10: paired patient samples; lanes 3 and 4: t_0 and t_1 sample, standard-dose treatment (Δ TRF length -0.7 kb); lanes 5 and 6: t_0 and t_1 sample, standard-dose treatment (Δ TRF length 0 kb); lanes 7 and 8: t_0 and t_1 sample, high-dose treatment (Δ TRF length -1.1 kb); lanes 9 and 10: t_0 and t_1 sample, high-dose treatment (Δ TRF length + 0.3 kb).

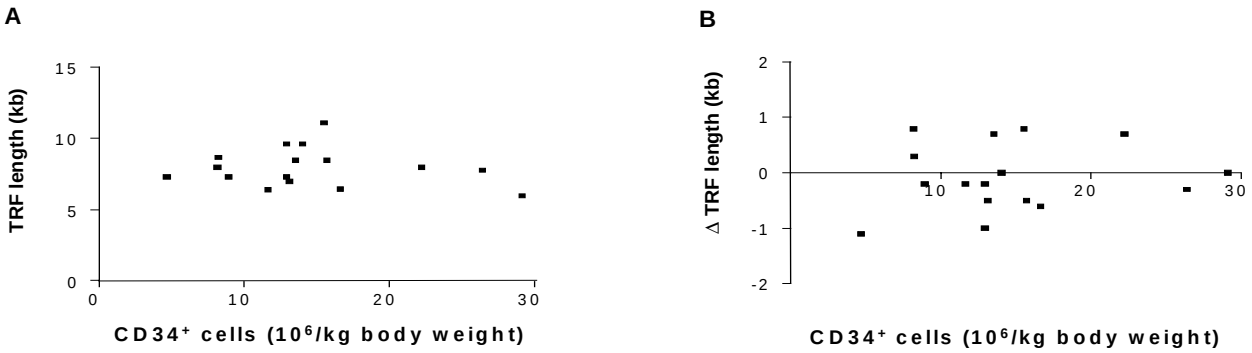


Figure 4:

TRF length and CD34+ cell numbers

On the X-axis, the number of reinfused CD34+ cells (10^6per kg body weight) is indicated; on the Y-axis the following values are indicated: **A**: TRF length (kb): measured value at t_1 after high-dose treatment, **B**: Δ TRF length (kb): calculated difference between TRF values at t_0 and t_1 of high-dose treatment.

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