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DNA damage in blood cells in relation to chemotherapy and nutritional status in colorectal cancer patients – A pilot study

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Highlights

- Genotoxicity was assessed in colorectal cancer patients by means of DNA damage utilizing the comet assay on whole blood samples.
- Colorectal cancer patients on adjuvant chemotherapy had higher levels of DNA damage in blood cells compared to patients not receiving chemotherapy.
- Good nutritional status was associated with less DNA damage indicating a possible protective role of nutritional status against genotoxicity.

Abstract

DNA damage can be considered as a biomarker for toxicity and response to chemotherapy. It is not known whether the chemotherapy-induced genotoxicity is associated with malnutrition. In this pilot study, we assess genotoxicity by means of DNA damage in patients with lymph-node positive colorectal cancer (CRC) and explore associations with chemotherapy treatment...
and nutritional status. DNA damage was compared between patients receiving chemotherapy (n=24) and those not receiving chemotherapy (n=20). DNA damage was measured in frozen whole blood by the comet assay. Associations between DNA damage and various indicators of malnutrition were also explored, including Patient-Generated Subjective Global Assessment (PG-SGA), bioelectrical impedance analysis (BIA) and anthropometric measurements, using multiple linear regression models. Patients on chemotherapy have higher levels of DNA damage in blood cells than patients not receiving chemotherapy (median of 16.9 and 7.9 % tail DNA respectively, p=0.001). The moderately malnourished patients (PG-SGA category B), representing 41 % of the patients, have higher levels of cellular DNA damage than patients with good nutritional status (mean difference of 7.5 % tail DNA, p=0.033). In conclusion, adjuvant chemotherapy and malnutrition are both associated with increased levels of DNA damage in blood cells of CRC patients. Carefully controlled longitudinal studies or randomized controlled trials should be performed to determine whether good nutritional status may protect against chemotherapy-induced genotoxicity and enhance compliance to therapy in CRC patients.

Abbreviations: Q1-Q3: First to third quartile, i.e. interquartile range, N: Number, P: P-value, PG-SGA: Patient-Generated Subjective Global Assessment, BMI: Body mass index, FFM, Fat-free mass, FM: Fat mass, TNM: Tumor, Nodes, and Metastases, 5-FU: Fluorouracil

Keywords: Colorectal cancer; chemotherapy; toxicity; comet assay; DNA damage; nutritional status
1. Introduction

Adjuvant chemotherapy with single-agent fluoropyrimidine-based therapy or fluoropyrimidine and oxaliplatin combination therapy is routinely recommended after resection of TNM/Union Internationale Contre le Cancer (UICC) stage III and high-risk stage II colon cancer (1). Chemotherapy administrated in the adjuvant setting improves survival (1). Although generally well tolerated, side effects and reduced dose or dose-intensity are common (2).

The fluoropyrimidine 5-fluorouracil (5-FU) inhibits cell division, partly by blocking DNA synthesis and partly by forming RNA and DNA with a defect structure (3). 5-FU leads to the formation of both single-stranded breaks and double-stranded breaks in the DNA (4). Oxaliplatin exerts its effects mostly through DNA crosslinking (5), but will also act to enhance the effects of 5-FU. The mechanisms behind this synergism is complex and still unanswered, but oxaliplatin is suggested to reduce the catabolism of 5-FU (6). Administration of 5-FU and oxaliplatin is associated with side-effects in the gastrointestinal, haematological and neurological system (7). For a subset of patients this imposes treatment modifications including dose-reductions, treatment delays and for some also early treatment termination (8,9). This may affect not only quality of life, but also treatment success and hence long-term outcome.

There is a large inter-individual variation in tolerance to chemotherapeutic agents (10). Although some of this variation can be explained by treatment-related factors, growing evidence suggests that intrinsic biological factors such as drug metabolism and DNA repair capacity are important (7,11). In addition, it is likely that exogenous factors such as diet, physical activity and nutritional status affect treatment response and tolerance. In fact, several observational studies have demonstrated associations between various indicators of malnutrition, treatment efficiency and risk of adverse events during therapy (8,9,12-21). As malnutrition is common in patients scheduled to receive chemotherapy (22), research is needed to explore the underlying mechanisms mediating these relations.
DNA damage measured in non-malignant cells (i.e. non-targeted cells) may be a useful biomarker to monitor treatment response and adverse effects accompanying chemotherapy. The comet assay is a standard method to detect DNA damage at cellular levels and has been used for detection of DNA damage in various cancer populations undergoing chemotherapy (23). There are, however, few studies that have investigated the use of DNA damage in blood cells as a marker of genotoxicity in colorectal cancer (CRC) patients.

In this pilot study, we assessed genotoxicity in lymph-node positive CRC patients by measuring DNA damage in blood cells, comparing patients receiving chemotherapy with those not receiving chemotherapy. Furthermore, associations between DNA damage and various indicators of malnutrition were explored.
2. Materials and methods

2.1. Patients and eligibility

Patients included in this study were recruited from the ongoing clinical trial, The “Norwegian Dietary Guidelines and Colorectal Cancer Survival (CRC-NORDIET) study (24). Eligibility criteria for this study were as follows: Men and women aged 50-80 years with a newly diagnosed primary CRC (ICD-10 18-20), histologically confirmed adenocarcinoma and TNM stage I-III. The subgroups included in this cross-sectional investigation consisted of colon cancer patients undergoing adjuvant chemotherapy treatment in the period November 2012 to September 2015, as well as a control group not receiving chemotherapy. The control subjects were selected among patients in the CRC-NORDIET study with the most advanced disease (TNM stage II-III) to match the chemotherapy group with regards to disease severity. There were no inclusion criteria with regards to nutritional status. The control subjects had either not yet started on chemotherapy (n=1) or were not intended for chemotherapy treatment (i.e. due to comorbidity or poor general health condition, low risk stage II colon cancer or rectal cancer). Patients assigned to chemotherapy were given either 5-FU plus folinic acid as monotherapy or 5-FU plus folinic acid and oxaliplatin as combination therapy according to national guidelines (25). Treatment was initiated four to six weeks post-surgery and repeated every 14 days to a maximum of 12 cycles, depending on tolerance.

2.2. Ethics

The CRC-NORDIET study is carried out in accordance with the Helsinki Declaration. The study was approved by the Regional Committees for Medical and Health Research Ethics (REC Protocol Approval 2011/836) and by the data protection officials at Oslo University Hospital and Akershus University Hospital. Informed consent was obtained from all participants. The study is registered on the National Institutes of Health Clinical Trials (www.ClinicalTrials.gov; Identifier: NCT01570010).

2.3. Treatment-specific information
The following information about treatment was collected from patient journals: time of surgery, type of chemotherapeutic drugs initiated and administrated each treatment cycle, duration of chemotherapy before blood sampling (days on chemotherapy, number of treatment cycles completed) and time from last chemotherapy injection to blood sampling. Patients receiving at least one dosage of oxaliplatin before blood sampling were defined as FLOX-users.

2.4. Measurements and sampling

All measurements, including assessment of lifestyle behaviors (2.4.1), nutritional status (2.4.2), and blood sampling (2.4.3) were conducted at the start of the clinical trial (i.e. 2-9 months post CRC-surgery). Hence, for the chemotherapy group, measurements were carried out after initiation of treatment. All, measurements, except self-reported data from questionnaires and the patient-generated historical components of the Patient-Generated Subjective Global Assessment (PG-SGA) were performed by trained personal. Patients were instructed to fast overnight and until all measurements were completed. All measurements were performed in the morning.

2.4.1 Lifestyle behaviors

Information about smoking status, physical activity and comorbidity was based on self-reported registrations. Dietary supplement use and ostomy status (stoma/no-stoma) were recorded by the research staff in connection with measurements. The registration methods are described in detail elsewhere (24).

2.4.2. Nutritional status

Nutritional status was assessed by use of the PG-SGA, anthropometric measurements and bioelectrical impedance analyses (BIA).

2.4.2.1. Patient-Generated Subjective Global Assessment (PG-SGA)

The PG-SGA is a nutritional screening and assessment tool, widely used in clinical practice and academic research as a reference method in cancer patients (26,27). The assessment tool includes
four patient-generated historical components (weight history, food intake, symptoms and activities and function) and three professional components (age and diagnosis, metabolic stress and physical examination). Based on an evaluation of these components, the patients are categorized as either well-nourished (PG-SGA A), moderately malnourished (PG-SGA B) or severely malnourished (PG-SGA C). They also receive an overall global numeric score. Although related, the PG-SGA category score and numerical PG-SGA score are independent assessment and triage systems.

The Norwegian version of the scored PG-SGA (15-004 v10.13.16) was used in the present study, and permission was given by the copyright holder of the instrument. The patient-generated historical components were completed by the patient, while the professional components were carried out by trained registered clinical dietitians. The scoring was controlled by one researcher (H.R).

2.4.2.2. Anthropometric measures

Body weight was measured with light clothes and without shoes by use of a non-slip Marsden M-420 Digital Portable Floor Scale (Marsden, Rotherham, South Yorkshire, United Kingdom) or a digital wireless measuring station for height and weight, Seca 285 (Seca, Birmingham, United Kingdom). Body weight was recorded to the nearest 0.1 kg. To account for clothing, 0.5 kg was subtracted from body weight. Height was measured using either a mechanical height rod (Kern MSF-200) or a digital wireless stadiometer (Seca 285). Height was recorded to the nearest 0.1 cm. BMI (kg/m²) was calculated based on recorded weight and height, and used to categorize patients into BMI groups according to age-specific cut-off values proposed as part of the diagnostic criteria for malnutrition by the European Society for Clinical Nutrition and Metabolism (ESPEN) (28). “Underweight” was defined as BMI < 20 or < 22 kg/m² in patients younger and older than 70 years, respectively, and “normal weight/having increased body weight” was defined as BMI ≥ 20 or 22 kg/m² in patients younger and older than 70 years, respectively. “Weight loss” was defined as any weight loss of ≥ 2% of body weight between the pre-surgery assessment and start of the clinical trial. Waist circumference was
measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest. Waist circumference was recorded to the nearest 0.1 cm.

2.4.2.3. Bioelectrical impedance analyses (BIA)

To measure body composition, a single frequency, whole-body BIA, BIA-101 (SMT Medical, Würzburg, Germany) was used. BIA measures body composition indirectly by measuring the impedance (Resistance (R) and Reactance (Xc)) of a low-voltage current passing through the body.

BIA was performed under standardized conditions according to the manufacturer’s protocol. Measurements were performed by placing two adhesive skin electrodes on the right hand and two adhesive electrodes on the right foot on the patient when lying in supine position. The device applies a current of 400 µA at a constant frequency of 50 kHz.

Fat-free mass (FFM) can be calculated by incorporating impedance data into empiric regression equations including information about age, sex, body weight and height. The Schols equation, originally developed in patients with chronic obstructive pulmonary disease, and validated against dual x ray absorptiometry (DXA) in a subgroup of CRC patients included in the CRC-NORDIET study (24,29), was used for calculation of FFM in the current study. Fat mass (FM) was calculated by subtracting FFM from body weight.

FFM values were used to calculate FFM-index (FFM (kg)/height (m)^2). Patients were then categorized with “low FFM-index” (< 15 kg/m^2 for women and < 17 kg/m^2 for men) or “normal FFM-index” (≥ 15 kg/m^2 for women and ≥ 17 kg/m^2 for men) according to cut-off values for FFM-index proposed as part of the diagnostic criteria for malnutrition by ESPEN (28).

2.4.3 Blood samples

2.4.3.1 Chemicals and materials
Lymphoprep was purchased from Axis-Shield, and normal and low melting point (NMP and LMP) agarose as well as SybrGold stain from Invitrogen.

2.4.3.2 Blood collection, processing and storage

All samples used were collected at the start of the clinical trial. Venous whole blood was collected by venipuncture by trained personnel into Vacutainer® tubes containing either ethylenediaminetetraacetic acid (EDTA) or citrate as anticoagulant. Citrate tubes were centrifuged for 10 minutes at 2500 x g. Two buffy coats with a total volume of around 250 μl from each patient were resuspended in 400 μl PBS and PBS added to a final volume of 10 ml. Peripheral blood mononuclear cells (PBMCs) were isolated from the resuspended citrate buffy coats by layering the cell suspension onto 10 ml of Lymphoprep in a 50 ml centrifuge tube, and centrifuged for 30 min at 700× g. The cloudy band above the Lymphoprep containing PBMCs was transferred to a tube containing 9 ml PBS and centrifuged again for 20 min at 700× g. The pelleted PBMCs were resuspended in freezing medium (DMEM with 20 % fetal calf serum, 10 % DMSO) at 10⁶/ml and 1 ml aliquots were frozen slowly to -80°C. The EDTA whole blood was divided into aliquots of 250 μl in 1.5 ml microcentrifuge tubes and frozen directly on dry ice before further storage at – 80°C.

2.4.3.3. Comet assay

To enhance the convenience of the comet assay method for detection of DNA damage in non-targeted cells for the current and future studies, we used a simple sampling procedure based on whole blood rather than customary PBMCs, and include a validation of this approach.

The following samples were prepared for comet assay, using a version of the comet assay with 12 mini-gels on each slide:
1) Frozen EDTA whole blood (“the whole blood method”); Samples were directly embedded in agarose without any further treatment by directly adding 250 μl of thawed whole blood to 400 μl LMP agarose before setting 5 μl aliquots on NMP agarose-precoated slides (Figure 1, right side).

2) Frozen PBMCs from citrate buffy coats (“the PBMC method”); 1 ml of PBS was added to a frozen 1 ml aliquot and as soon as the sample was thawed, the suspended cells were added to 5 ml of PBS in a centrifuge tube and centrifuged for 7 min at 700× g, 4°C. The pellet was suspended in PBS, adjusting the number of cells to 0.25x10⁶/ml. 30 μl of the suspensions was added to 140 μl of 0.8 % LMP agarose at 37°C. Gels of 5 μl were set on NMP agarose-precoated slides (Figure 1, left side).

Slides with mini-gels were immersed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10.0) at 4°C for 1 h. They were then transferred to an electrophoresis tank with 0.3 M NaOH, 1 mM EDTA at 4°C; after 40 min, electrophoresis was carried out for 25 min at 1 Volt/cm across the platform.

Slides were neutralized in PBS, rinsed in water and fixed in 70 % and 100 % ethanol (5 min in each). Slides were stained by immersion in a bath of 25 ml of SybrGold at 10,000× dilution in Tris-EDTA buffer, with agitation, for 30 min at 4°C, in the dark. They were then washed twice with water and left to dry. Comets were scored with Comet Assay IV software (Perceptive Instruments) and results expressed as % tail DNA. Highly damaged cells were not excluded from the analysis.

2.5. Statistical analysis

2.5.1 Main analyses

Continuous variables were tested for normality by the Shapiro-Wilk test and by visual inspection of histograms and Q-Q-plots.

Descriptive statistics are given as median (interquartile range, Q1-Q3) for continuous variables, as most variables violated the assumption of normality. Categorical variables are presented as number
(n) (%). To compare treatment groups with regard to patient characteristics, Mann-Whitney U and Fisher’s exact test was used for continuous and categorical variables, respectively.

To evaluate whether DNA damage was related to treatment characteristics, including treatment modality (chemotherapy/no chemotherapy) and various indicators of treatment status at blood sampling, including days on chemotherapy (continuous), rounds of chemotherapy completed (continuous) and days since last injection (continuous), a Mann-Whitney U Test and multiple linear regression analyses with adjustments for age, sex and TNM status in separate models, were utilized. Multiple linear regression analyses were also used to investigate whether DNA damage was related to various aspects of nutritional status, including overall PG-SGA category (PG-SGA A/PG-SGA B) and numerical score (continuous), patient-generated and professional components of the PG-SGA (dichotomization of variables described in the table legend), low BMI (< 20 or 22 kg/m² for patients younger and older than 70 years, respectively), presence of weight loss (any weight loss ≥ 2 % from pre-surgery assessment), waist circumference (continuous), FFM (continuous), FM (continuous) and low FFM-index (< 17 kg/m² for men and < 15 kg/m² for women). Regression analyses were adjusted for age, sex, TNM status and treatment group in separate models. Results of the regression analyses are presented as crude and treatment group-adjusted estimates.

The frozen whole blood method was validated against the PBMC method in a subgroup of patients (n=27). Values were tested for measurement agreement using Wilcoxon Signed-Rank test, correlation analysis (Spearman’s correlation) and by creating a Bland-Altman plot in which the mean of the two methods (x-axis) is plotted against the difference between the methods (y-axis). As there was an increase in the variability of the differences with increasing measurement values, values were log-transformed as recommended by Bland and Altman (31). The Bland-Altman plot was constructed to explore potential bias, limits of agreements (± 1.96 SD) and to spot for outliers.

All statistical analyses were performed using SPSS 21.0 for Windows (SPSS, Chicago, IL, USA). A significance level of $p<0.05$ was used.
2.5.2 Sample size calculation

Sample size calculation for comparison of treatment groups (chemotherapy group vs no-chemotherapy group) was based on reports from two previous studies investigating the chronic cytotoxicity of chemotherapy by use of the comet assay (32,33). Values assessed pre- and post-administration of chemotherapy were used as an indicator of expected difference between treatment groups. With a power of 80 %, a significance level of 5 % and expected difference between treatment groups with corresponding standard deviations ($SD_{Group1}, SD_{Group2}$) of 1.6 (2.1, 3.0) and 5.2 (3.9, 6.3) % tail DNA, respectively, about 16-41 subjects would be required in each group, to detect a significant difference (34). By May 2015, a total of 49 eligible subjects were included which were within the required number of participants. As we expected the difference in outcome variable to be smaller between groups separated by nutritional status than by treatment, we included all patients, not only the chemotherapy group, in these analyses.

For the method validation between the whole blood method and the PBMC method, the sample size was based on an expected correlation coefficient 0.6, power of 80 % and a significance level of 5 %. Using www.sample-size.net (35) for correlation sample size estimation, a total of 19 samples was necessary to detect a correlation of 0.6. Due to the heterogeneity in different aspects of the population and to be able to perform subgroup analysis we decided to include some extra samples in the validation with a total number of 27 samples.
3. Results

3.1. Patient characteristics

Patient characteristics are shown in Table I. Of the 49 eligible patients, four were excluded due to missing blood samples and one due to concurrent radiotherapy. Of the 44 included patients, 24 patients had received at least one cycle of chemotherapy at time of assessment (referred to as the chemotherapy group). The remaining 20 patients had either not yet started on chemotherapy or were not intended for chemotherapy treatment (referred to as the non-chemotherapy group).

Forty-one patients had colon cancer, two had rectosigmoid cancer and one had rectal cancer. Median time from surgical resection to time of assessment was approximately four months for both treatment groups.

There were no significant differences between treatment groups with regard to clinico-pathological, anthropometric or lifestyle related variables, except for TNM stage, 83% of the patients in the chemotherapy group having TNM stage III compared to 20% in the non-chemotherapy group.

Forty-one percent of patients were classified as moderately malnourished according to the PG-SGA (Table II). Of the patient-generated historical components, 11% reported losing weight the previous month, 51% reported reduced food intake, 49% reported symptoms affecting food consumption and 49% reported reduced level of activity and function. The most commonly reported symptoms affecting food consumption included altered taste (22%), nausea (22%) early satiety (16%) and dry mouth (16%). Eighteen percent of patients had low BMI (BMI < 20 or 22 kg/m\(^2\) for patients younger and older than 70 years, respectively), while 44% had low FFM-index (< 17 kg/m\(^2\) for men and < 15 kg/m\(^2\) for women). Fifty-four percent of the patients had lost weight (any weight loss ≥ 2 % of body weight) from the pre-surgery assessment to start of the clinical trial.

Of the 24 patients receiving chemotherapy, 71% were scheduled for FLOX combination therapy (5-FU and oxaliplatin), while 29% were scheduled for FLV monotherapy (5-FU). Median (Q1-Q3) time on
chemotherapy at blood sampling was 58 (44-79) days, corresponding to approximately 4 (3-6) cycles of the intended twelve treatment cycles.
3.2. DNA damage in relation to chemotherapy treatment

To investigate genotoxicity, we compared DNA damage (strand breaks and alkali-labile sites) in frozen whole blood, measured as % tail DNA, between patients receiving and not receiving chemotherapy. The level of DNA damage was more than twice as high in patients receiving chemotherapy compared to those not receiving chemotherapy (median values of 16.9 and 7.9 % tail DNA respectively, \( p<0.001 \)) (Figure 2). As patients receiving chemotherapy in general were characterized by a more advanced disease, indicated by a higher proportion of patients with TNM stage III (83 % compared to 20 % in the non-chemotherapy group), regression analysis with adjustment for TNM stage was conducted to examine whether the observed difference could be driven by the underlying disease. However, the difference in DNA damage levels remained significant after including TNM stage in the model (mean difference in % tail DNA: 9.4 (95 % CI: 2.3, 16.5), \( p=0.011 \)). Moreover, the difference remained significant after adjustment for age and sex and when excluding the participant with rectum cancer from the analysis (data not shown). There was a large inter-individual variation in the level of DNA damage. This was particularly evident among chemotherapy patients with values ranging from 3.6 to 46.6 % tail DNA.

To explore whether treatment received at blood sampling was related to DNA damage in the chemotherapy group (n=24), associations between various indicators of treatment status, including type of chemotherapy initiated (FLOX/FLV), duration of chemotherapy before blood sampling (days on chemotherapy, number of treatment cycles completed) and time since last injection, and DNA damage levels were evaluated. No significant associations were found between DNA damage in whole blood and any of these treatment status measures explored (Supplemental Table 1 and Figure 1A-D). Despite the lack of associations, firm conclusions should not be drawn from these subgroup analyses given the small sample size and limited power.

3.3. DNA damage in relation to nutritional status
To evaluate whether DNA damage was related to nutritional status, associations between this biomarker and data collected from PG-SGA, BIA and anthropometric measurements were explored (Table II). To ensure statistical power, data from all subjects, both patients receiving chemotherapy and patients not receiving chemotherapy, were included in the subsequent analysis.

DNA damage was significantly higher in patients categorized as moderately malnourished (PG-SGA B) compared to patients with good nutritional status (PG-SGA A) with a mean difference of 7.5% tail DNA ($p=0.033$). The difference remained significant after separate adjustment for treatment group (Table II), age, sex and TNM status (data not shown). Looking at the individual components contributing to the overall PG-SGA categorization, both the patient-generated components “nutrition impact symptoms” and “activities and function” and the professional component “physical examination” were related to DNA damage. Patients having nutrition impact symptoms had significantly higher levels of DNA damage than patients without any symptoms with a mean difference of 9.6% tail DNA ($p=0.004$). The difference remained significant after separate adjustment for treatment group (Table II), age, sex and TNM status (data not shown). Patients having reduced levels of activity and function had significantly higher DNA damage levels compared to patients reporting normal activity and function with a mean difference of 7.2% tail DNA ($p=0.037$). The difference was however no longer significant after adjustment for treatment group (Table II). Also, patients with moderate to severe muscle and fat depletion had significantly higher DNA damage levels than patients with no depletion with a mean difference of 7.3% tail DNA ($p=0.036$). However, the difference was no longer significant after adjustment for treatment group (Table II).

The other indicators of malnutrition, including low BMI, weight loss and low FFM-index were not related to level of DNA damage. Furthermore, neither waist circumference, nor FFM or FM, indicators of abdominal obesity and body composition, respectively, were linearly related to level of DNA damage.
3.4. Method validation

In order to assess DNA damage in vivo, PBMCs are usually the preferred cell type. However, as PBMC isolation is time consuming, this limits its applicability in large clinical trials. To increase the applicability of this method, we performed a method validation in a subgroup of our patients to evaluate if the more readily available sample source; frozen whole blood, could be used to measure DNA damage. DNA damage measured in whole blood correlated well with DNA damage measured in isolated PBMCs, with a correlation coefficient of $r=0.68$ ($p<0.001$). However, the absolute level of DNA damage was significantly higher in whole blood compared to PBMCs with median values of 11.3 and 3.9 % tail DNA, respectively ($p<0.001$). This was also demonstrated in the Bland-Altman plot of the log-transformed values, showing consistently higher values for the whole blood method compared to the PBMC method (Figure 3). However, no outliers or proportional bias (i.e. change in vertical spread with increasing values of DNA damage) were detected, indicating good concordance between the methods.

Using the subgroup of PBMC samples we confirmed the results using the whole blood method i.e. significant differences in DNA damage between treatment groups (median values of 4.4 and 3.2 % tail DNA, respectively, $p=0.051$, data not shown).

4. Discussion

4.1. DNA damage in relation to chemotherapy treatment

To the best of our knowledge, our study is the first to apply the comet assay for assessment of genotoxicity in patients undergoing first-line treatment for CRC. We observe that patients receiving chemotherapy has more than twice as high levels of DNA damage compared to patients not receiving chemotherapy. The difference in DNA damage levels observed, may be due to a DNA-damaging effect of chemotherapy, or alternatively, there may be differences in treatment groups with regards to factors influencing DNA damage levels. In support of a DNA-damaging effect of chemotherapy,
several studies across various cancer populations have observed an increase in DNA damage levels in response to chemotherapy injection (32,33,37-41). In line with our result, Almeida et al (42), using a modified version of the comet assay, found that 5-FU- and oxaliplatin-based treatment led to DNA crosslinks in PBMCs of five CRC patients with metastatic cancers. Evidence of crosslinking was observed in all patients immediately and 24 hours after injection. Similar to our findings, high inter-individual variability was observed. This has also been reported in other studies, both before (32,37,38,43) and after chemotherapy administration (37,38,43,44). The variability in response may have implications for treatment tolerance and compliance, and hence treatment success. In contrast to our results, irinotecan-based treatment, mostly combined with 5-FU did not result in detectable DNA damage either immediately or 24 hours post treatment (45). The contradictory findings might be due the presence of distant spread, masking a potential effect of chemotherapy.

In contrast to most previous investigators measuring DNA damage prior to and immediately after chemotherapy administration, we explored the chronic genotoxicity of chemotherapy by comparing patients receiving chemotherapy to those not receiving chemotherapy. The control subjects were selected in attempt to match the severity of the disease between treatment groups. However, more patients in the chemotherapy group (83 %) had TNM stage III compared to the control group (20 %). Although we controlled for this in our analyses, we had a multi-collinearity problem, treatment group and TNM stage being highly correlated. Hence, we cannot rule out the possibility that disease severity contributed to the difference in DNA damage observed. Although studies have been unable to confirm an association between DNA damage and disease severity (38,43), there are reports that cancer patients have higher DNA damage levels in blood cells compared to healthy controls (33,38,40,41). Thus, the disease itself may be accompanied by an increase in DNA damage. However, the relationship between these two is complex, genomic instability being an enabling characteristic of the underlying hallmarks of cancer (46). Hence, these patients may be characterized by increased DNA damage levels which may or may not be causally related to disease severity.
4.2. DNA damage in relation to nutritional status

We demonstrate that moderately malnourished patients, as classified by the PG-SGA, have higher levels of DNA damage than well-nourished patients. Furthermore, patients with nutrition impact symptoms have higher levels of DNA damage than patients without symptoms. The other indicators of malnutrition, i.e. weight loss, low BMI and low FFM-index, were however not related to DNA damage.

To our knowledge, our results are novel in indicating a link between nutritional status as measured by the PG-SGA and protection from DNA damage. The PG-SGA was designed in the context of anabolic competence and is regarded internationally as a reference method for assessment of nutritional status in cancer patients (26). Hence, it may capture aspects of nutritional status of particular importance for the oncologic patient which may be missed by the use of more crude indicators.

A good nutritional status may exert cytoprotective effects, making the cells less vulnerable to external toxic exposures such as chemotherapy. On the other hand, it is also possible that increased levels of cellular toxicity adversely affect nutritional status.

Malnutrition has been associated with chemotherapy toxicity in several studies (8,9,12,14-21). However, none of these has assessed DNA damage in whole blood as an indicator of chemotherapy toxicity, and the mechanisms mediating the malnutrition-toxicity association remains poorly understood. Suggested mechanisms include altered pharmacokinetic properties, reduced glomerular filtration rate and altered cellular immune response (47,48). Malnourished patients may also receive higher dosages of chemotherapy drugs per distribution volume, as most chemotherapeutic agents are administrated based on body surface area rather than FFM (49). All these pathways and mechanisms may result in increased level of cellular toxicity.

An increased level of cellular DNA damage could also make patients more prone to develop malnutrition. Chemotherapy typically affects fast-proliferating tissues such as the bone marrow, oral cavity and gastrointestinal tract. If DNA damage in blood cells reflects the level of DNA damage in fast-
proliferating cells, such as the epithelial cells lining the gastrointestinal tract, this may result in a variety of clinical manifestations of importance to nutritional status such as altered taste, stomatitis, nausea, vomiting and diarrhea. This may adversely affect nutritional intake and hence energy balance. Furthermore, DNA damage may induce inflammation, an important contributor to cancer cachexia characterized by anorexia and muscle depletion (50).

Future studies should explore whether it is possible to improve tolerance to 5-FU-based chemotherapy in non-metastatic CRC patients by modulating nutritional status, and whether the effect is mediated through decreased DNA damage levels in non-targeted cells. If proven beneficial, targeting nutritional status may be an easy and cost-effective strategy to improve tolerance and achieve maximum benefit from treatment.

4.3. Method validation

We observed high relative agreement between the PBMC and the whole blood method for detection of DNA damage. Similar to our results, good agreement has been observed in a rat and human sample (51). A human study also demonstrated that the whole-blood method was suitable for direct use with alkaline and enzyme-modified comet assays (52). However, we find that the whole blood method produced consistently higher values than the PBMC method. This could be due to differences in sampling and freezing procedures, for example damaged PBMCs might be lost during the cell isolation protocol leading to under-estimation of DNA damage. Alternatively, there may be actual differences in the samples, the two methods representing different cell populations.

4.4. Limitations

Despite great promise with use of the comet assay in clinical practice, some issues should be addressed. DNA damage can be influenced by a variety of external factors including diet, smoking and air pollution among others. This results in a wide range of intra- and inter-individual variation,
complicating the interpretation of the results (23). Furthermore, there are challenges with experimental validation, standardization and data interpretation, which limits its current use as a predictive test in clinical practice (23). Also, the comet assay capture only one aspect of cytotoxicity and other aspects are probably also important.

A limitation with our study is the cross-sectional design which makes it impossible to make causal inferences both with regards to effects of chemotherapy on DNA damage and implications of nutritional status. Carefully controlled longitudinal studies or randomized controlled trials should be performed to determine whether such causal relationships exist.

5. Conclusion

CRC patients on adjuvant 5-FU based chemotherapy had higher levels of DNA damage in blood cells compared to CRC patients not receiving chemotherapy. Good nutritional status was associated with less DNA damage indicating a possible protective role of nutritional status against genotoxicity. Carefully controlled longitudinal studies or randomized controlled trials are needed to get a broader understanding of the complex relations between genotoxicity, treatment tolerance and nutritional status.

6. List of abbreviations

5-FU: 5-fluorouracil; BIA: Bioelectrical impedance analysis; BMI: Body mass index; CRC: Colorectal cancer; DXA: Dual x ray absorptiometry; FFM: Fat-free mass; ICD: International classification of diseases and related health problems; LMP: Low melting point; N: Number; NMP: Normal melting point; PBMCs: Peripheral blood mononuclear cells; PG-SGA: Patient-Generated subjective global assessment; SD: Standard deviation; SGA: Subjective Global Assessment, TNM: Tumor node metastasis; UICC: Union for International Cancer Control

7. Funding
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Conflict of Interest Statement: Rune Blomhoff is a shareholder of Vitas, Oslo, Norway. The remaining authors declare that there are no conflicts of interest.
8. Acknowledgements

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9. Statement of authorship

ASK and SKB had the main responsibility for data analysis and writing of the manuscript. ASK, SKB, JM, NEY, CH, HR, IP, HBH, GW, SS, RB and AC contributed to the conception and the design of the study, analysis and interpretation of the data and drafting of the manuscript. ASK, SKB, JM, NEY, HR, CH, IP and HBH contributed to acquisition of data. All authors contributed to the writing and final approval of the manuscript.

10. References


The PBMC method

1) 

2) 

3) 

4) 

The whole blood method

Figr-1
Figure 2

DNA damage in blood cells, % tail DNA

Chemotherapy

No chemotherapy

Treatment group
Table I. Characteristics of the study population by treatment group (chemotherapy/non-chemotherapy). Numbers are medians (Q1-Q3) if not otherwise stated.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Chemotherapy (n=24)</th>
<th>Non-chemotherapy (n=20)</th>
<th>P^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63.6 (61.0, 71.3)</td>
<td>67.5 (60.5, 72.5)</td>
<td>0.377</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>9 (37.5)</td>
<td>11 (55.0)</td>
<td>0.363</td>
</tr>
<tr>
<td>Women</td>
<td>15 (62.5)</td>
<td>9 (45.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Lifestyle behaviors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>2 (8.3)</td>
<td>2 (10.0)</td>
<td>1.000</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>22 (91.7)</td>
<td>18 (90.0)</td>
<td></td>
</tr>
<tr>
<td>Dietary supplementsb</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Users</td>
<td>14 (60.9)</td>
<td>16 (80.0)</td>
<td>0.203</td>
</tr>
<tr>
<td>Non-users</td>
<td>9 (39.1)</td>
<td>4 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Inactivity, hours/day</td>
<td>7.0 (6.0, 10.0)</td>
<td>6.0 (4.0, 8.0)</td>
<td>0.183</td>
</tr>
<tr>
<td>Number of comorbidities, n (%)</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9 (37.5)</td>
<td>3 (15.0)</td>
<td>0.207</td>
</tr>
<tr>
<td>1</td>
<td>9 (37.5)</td>
<td>12 (60.0)</td>
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<tr>
<td>≥ 2</td>
<td>6 (25.0)</td>
<td>5 (25.0)</td>
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<tr>
<td><strong>Clinicopathological information</strong></td>
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<tr>
<td>Cancer localization, n (%)</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon (C18)</td>
<td>22 (91.7)</td>
<td>19 (95.0)</td>
<td>0.340</td>
</tr>
<tr>
<td>Rectosigmoideum (C19)</td>
<td>2 (8.3)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Rectum (C20)</td>
<td>0 (0.0)</td>
<td>1 (5.0)</td>
<td></td>
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<tr>
<td>TNM stadium, n (%)</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4 (16.7)</td>
<td>16 (80.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>III</td>
<td>20 (83.3)</td>
<td>4 (20.0)</td>
<td></td>
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<tr>
<td><strong>Treatment-specific information</strong></td>
<td></td>
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<tr>
<td>Ostomy, n (%)</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (16.7)</td>
<td>1 (5.3)</td>
<td>0.363</td>
</tr>
<tr>
<td>No</td>
<td>20 (83.3)</td>
<td>18 (94.7)</td>
<td></td>
</tr>
</tbody>
</table>
Days since surgery, n | 44 | 107 (90, 111) | 109 (87, 124) | 0.673
Days on chemotherapy, n | 24 | 58 (44, 79) | - | -
Treatment rounds completed, n | 24 | 4 (3, 6) | - | -
Days since last injection, n | 24 | 8 (6, 12) | - | -
Type of chemotherapy initiated, n (%) | 24 |
| FLOX (5-FU, Oxaliplatin) | 17 (70.8) | - | - |
| FLV (5-FU) | 7 (29.2) | - | - |

**Nutritional status**

PG-SGA global score, n (%) | 37 |
| PG-SGA A | 11 (55.0) | 11 (64.7) | 0.738 |
| PG-SGA B | 9 (45.0) | 6 (35.3) |
PG-SGA, numeric score | 37 |
| 5.5 (3.0, 8.5) | 5.0 (3.0, 6.0) | 0.257 |
BMI, kg m² | 44 |
| 25.4 (23.1, 26.3) | 25.2 (20.9, 31.6) | 0.981 |
Presence of weight loss, n (%) | 37 |
| Yes | 12 (60.0) | 8 (47.1) | 0.517 |
| No | 8 (40.0) | 9 (52.9) |
Waist circumference, cm | 44 |
| 91.7 (87.7, 98.7) | 90.9 (78.1, 110.5) | 0.878 |
FFM, kg | 43 |
| 44.0 (41.8, 50.9) | 52.6 (40.8, 59.4) | 0.284 |
FM, kg | 43 |
| 28.2 (23.1, 30.6) | 26.0 (17.1, 32.2) | 0.543 |
FFM-index, kg/m² | 43 |
| 15.3 (14.6, 17.5) | 16.3 (14.8, 20.6) | 0.189 |
Table II. Association between DNA damage level (% tail DNA) and nutritional status (n=44).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Crude estimates</th>
<th>Adjusted estimates</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>(95 % CI)(^a)</td>
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<tr>
<td><strong>PG-SGA</strong></td>
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<tr>
<td>Overall scoring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-nourished (PG-SGA A)</td>
<td>22</td>
<td>7.5 (0.7, 14.4)</td>
</tr>
<tr>
<td>Moderately malnourished (PG-SGA B)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>PG-SGA score, numeric scoring</td>
<td>37</td>
<td>0.8 (-0.1, 1.8)</td>
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<tr>
<td><strong>Individual components</strong></td>
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<tr>
<td><em>Patient-generated historical components</em></td>
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<tr>
<td>Weight stable/anabolic</td>
<td>33</td>
<td>3.8 (-7.7, 15.3)</td>
</tr>
<tr>
<td>Catabolic(^1)</td>
<td>4</td>
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<tr>
<td>Normal/increased food intake</td>
<td>18</td>
<td>1.6 (-5.6, 8.8)</td>
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<tr>
<td>Reduced food intake(^2)</td>
<td>19</td>
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<tr>
<td>Nutrition impact symptoms</td>
<td>18</td>
<td>-9.6 (-16.0, -3.2)</td>
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<tr>
<td>No nutrition impact symptoms(^3)</td>
<td>19</td>
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### Symptom score, numeric scoring

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|---|---|---|---|
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### Normal activity and function

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### Reduced activity and function

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**Professional components**

### Low disease score

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### Elevated disease score

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### Low metabolic demands

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### Increased metabolic demands

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### No depletion

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### Moderate to severe depletion

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**Anthropometric measures**

### Normal to increased body weight (BMI ≥ 20 or 22 kg/m²)

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### Underweight (BMI < 20 or 22 kg/m²)

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### Presence of weight loss

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33
<table>
<thead>
<tr>
<th>No weight loss</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference, cm</td>
<td>44</td>
</tr>
<tr>
<td><strong>BIA</strong></td>
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</tr>
<tr>
<td>FFM, kg</td>
<td>43</td>
</tr>
<tr>
<td>FM, kg</td>
<td>43</td>
</tr>
<tr>
<td>Normal FFM-index</td>
<td>24</td>
</tr>
<tr>
<td>Low FFM-index&lt;sup&gt;10&lt;/sup&gt;</td>
<td>19</td>
</tr>
</tbody>
</table>

Linear regression analysis with listwise comparisons

<sup>a</sup>Crude estimates, <sup>b</sup>Adjusted for treatment group (chemotherapy/no-chemotherapy). Adjustments for age, sex and TNM status in separate models were also performed, but is not presented in the table as the adjustments did not alter the interpretation of the results.

<sup>c</sup>Significance level \( p < 0.05 \)

<sup>1</sup>Patients who records that they have lost \( \geq 2 \) kg last month or \( \geq 2 \) kg last 6 months (if data from last month is missing) or are weight losing at recording (indicated by weight loss last two weeks).

<sup>2</sup>Patients who record eating less than usual.

<sup>3</sup>Patients who records they have no symptoms affecting food intake.

<sup>4</sup>Patients who record that their level of activity and function have been normal with no limitations the past month.

<sup>5</sup>Patients are scored based on age (>65 years) and presence of selected chronic diseases (36).

<sup>6</sup>Score for metabolic demands is based on presence of fever and use of corticosteroids.
7Indicate loss of muscle or adipose tissue stores or presence of oedema.

8Underweight: BMI < 20 or 22 kg/m² for patients younger and older than 70 years, respectively, normal to increased body weight: ≥ 20 or 22 kg/m² for patients younger and older than 70 years, respectively.

9Patients are characterized with weight loss if they have lost ≥ 2 % of body weight last 2-9 months (from pre-surgery assessment to start of the clinical trial).

10Low FFM-Index is defined as < 17 kg/m² for men and < 15 kg/m² for women.

Abbreviations: N: Number, P: P-value, CI; Confidence intervals, PG-SGA: Patient-Generated Subjective Global Assessment, BMI; Body mass index, FFM: Fat-free mass, FM: Fat mass