

Analysis of short term and stable DNA damage in patients with differentiated thyroid cancer treated with ^{131}I in hypothyroidism or with rhTSH for remnant ablation

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Running title: DNA damage after ^{131}I therapy

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ABSTRACT

It is well known that ionizing radiations can induce genetic damage and that oxidative stress is a major factor inducing it. Our aim was to investigate whether thyroid remnant ablation with low activities of ^{131}I (1850 MBq), is associated with DNA damage by evaluating Comet assay, micronuclei and chromosome aberrations with multicolor fluorescent in situ hybridization (M-FISH).

Methods: we studied 62 patients prepared with rhTSH or by thyroid hormone withdrawal (THW). In both groups we analyzed stable and unstable genetic alterations before ^{131}I therapy and 1 week and 3 months after ^{131}I administration. We also correlated the genetic damage with several variables, including the degree of radiation-induced oxidative stress, genetic polymorphisms of enzymes involved in DNA-repair and anti-oxidative stress.

Results: we found comparable amount of DNA breaks evaluated by Comet assay and micronuclei test in both groups of patients at different time points, but a significant increase of stable chromosome aberrations evaluated by M-FISH (breaks and translocations) in patients prepared with THW. Overall, high chromosome damage was associated with higher retained body radioactivity and unfavorable gene polymorphism.

A high level of free oxygen radicals and a low level of anti-oxidants was found in all patients at any time point. In particular, patients prepared with THW, at 3 months, had significantly higher levels of free oxygen radicals than those prepared with rhTSH.

Conclusions: an increase of stable chromosome aberrations respect to baseline is detectable after administration of low doses of ^{131}I in patients prepared with THW but not in patients prepared with rhTSH. The clinical significance of these chromosomal alterations remains to be determined.

KEY WORDS

^{131}I -Iodine, Radiation-induced genetic damage, Hypothyroidism, rhTSH, Gene polymorphism, free oxygen radicals, oxygen radical scavengers.

INTRODUCTION

Iodine-131 (¹³¹I) is used in clinical practice for thyroid remnant ablation in patients who undergo thyroidectomy for differentiated thyroid carcinoma (DTC). Radionuclide therapy has been reported to induce harmful effects on cells and tissues (1-7). Indeed, several reports have shown chromosomal damage induced by ¹³¹I, although only chromosomes 1, 2, 4, 8 and 10 have been analyzed (8-12). Despite this is a very controversial issue (3,5,13-19), a significant reduction of ablative treatments has been observed in the last decade.

Therefore, it is important to better elucidate the possible presence of stable genetic damage and of radio-induced oxidative stress following treatment with ¹³¹I. Furthermore, gene polymorphisms that alter the repair of DNA damage should also be investigated (20).

Recombinant human thyroid-stimulating hormone (rhTSH) has effectively been used for exogenous stimulation before ¹³¹I ablation therapy, although it is not yet widely used for preparation of patients receiving high therapeutic amounts of radioiodine (5).

The main aim of this study was to investigate whether the relatively low administered activities of ¹³¹I, used for thyroid remnant ablation, are associated with some stable chromosome damage.

Secondary aims were:

- i) to analyze the level of baseline (i.e., pre- remnant ablation) genetic damage and oxidative stress in patients with DTC;
- ii) to evaluate the role of different DNA-repair and anti-oxidative genes in the occurrence of genetic damage and oxidative stress by analyzing genetic polymorphisms in patients.
- iii) to evaluate whether the yield of damage is comparable in patients prepared with rhTSH or by hypothyroidism.

PATIENTS AND METHODS

Patients

A group of 62 patients to be treated with 1850 MBq (50 mCi) ¹³¹I, were randomly assigned to the two cohorts:

A) 31 patients in hypothyroidism (40 days suspension of L-T4 and replaced with T3 for the first 25 days). These patients were named HYPO 1 to 31.

B) 31 patients in euthyroidism (injected with rhTSH 1mg two days before ¹³¹I and 1mg one day before ¹³¹I). These patients were named rhTSH 1 to 31.

Patients were matched for age, sex, pathologies, life-style habits, non-smokers, no administered drugs; patients with other primary tumors or previously treated with radiotherapy or receiving drugs with an effect on oxidative status or on the immune system were excluded from the study.

All patients were on low-iodine diet for 10 days before therapy.

This study has been approved by local Ethics Committee (n° 736/2014 and 241 SA_2017) and all subjects signed a written informed consent.

Patient recruitment lasted 15 months; study was completed in 2 years.

Measurement of radiation exposure rate was performed at 1 m distance at time of ¹³¹I administration (baseline), after 24h and 48h in all patients, as an indirect measurement of residual body activity. Patients with less than 20 µSv/h at 48h were discharged from hospital. Those with more than 20 µSv/h were counted again after 72h.

Sampling

In all patient cohorts, 10 ml of blood were withdrawn in lithium-heparin:

1. Before treatment with ¹³¹I in euthyroidism, (when sampling for therapeutic purpose is scheduled); blood was immediately processed for micronuclei, translocations, DNA breaks (Comet assay), Thyroglobulin levels (Tg), creatinine and glomerular filtration rate estimated with Epi-CKD formula (Epi-GFR) (21), thyroid stimulating hormone (TSH), plasma Reactive Oxygen Metabolites-derived compounds (d-ROMs), plasma Anti-Reactive Oxygen Metabolites potential (Anti-ROMs) and gene polymorphisms (SNPs).
2. One week after the treatment with ¹³¹I, (when sampling for therapeutic purpose is scheduled); blood was immediately processed for micronuclei, Comet assay, d-ROMs and Anti-ROMs.
3. Three months after ¹³¹I treatment (when sampling for follow-up purpose is scheduled); blood was processed for micronuclei, translocations, Comet assay, Tg, TSH, d-ROMs and Anti-ROMs.

Cell Culture Conditions

A volume of 0.5 ml of blood samples, taken from patients, was diluted with 4.5 ml of complete medium in a culture flask, and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Culture medium was RPMI 1640 (Euroclone, Pero-Milan, Italy) supplemented with 20% heat inactivated fetal bovine serum (Euroclone, Pero-Milan, Italy), 10,000 units/ml penicillin and streptomycin 10 mg/ml (Biological Industries, Beit-Haemek, Israel), 1% L-Glutamine (Euroclone, Pero-Milan, Italy). T-lymphocytes were stimulated to divide for 72 hours using 2% phytohemagglutinin (PHA) (Gibco, Waltham, MA, USA) in the culture medium.

Micronuclei Assay

To obtain bi-nucleated cells, cytochalasin B (6 µg/ml) (Sigma Aldrich, St. Louis, MO, USA) was added to culture medium 24h before harvesting, as previously described (22). Briefly, cells were pelleted by centrifugation (8 min at 12.000 rpm), resuspended in 0.075 M KCl, incubated for 2 min at 37 °C. The suspension was fixed three times in freshly prepared modified Carnoy solution (5:1 v/v methanol/acetic acid). Bi-nucleated cells were dropped onto slides, air dried and counterstained with 4,6-diamidino-2 phenylindole (DAPI) (Sigma Aldrich, St. Louis, MO, USA) in Vectashield antifade (Vector Laboratories, Burlingame, CA). Micronuclei were identified according to the following criteria: micronuclei was in cytoplasm with a diameter <1/3 of the whole nucleus; the shape of micronuclei was circle or oval and the micronuclei staining and refractivity were in accordance with that of the whole nucleus; structures were similar to the whole nucleus with complete separation and no other fragments and impurities in the vicinity. Images were captured with the Metacyte module of Metafer automated capture software (MetaSystems, Milan, Italy) at 40x magnification using an Axio Imager Z1 microscope (Zeiss, Jena, Germany) equipped with a Cool Cube 1 (CCD) camera (MetaSystems, Altlußheim, Germany). At least 1000 bi-nucleated cells for each patient were analyzed in each experimental condition.

Comet Assay

The Comet assay technique was used to evaluate the frequency of double strand breaks (DSB) and single strand breaks (SSB) induced by ¹³¹I.

The alkaline Comet assay was performed as described by Giovanetti et al. (23). Twenty µl of whole blood were gently re-suspended in 180 µl of 0.7% low-melting-point agarose in phosphate buffer solution (PBS, Ca- and Mg-free) at 38 °C, and immediately pipetted onto a warm frosted glass microscope slide pre-coated with a layer of 1% normal-melting-point agarose, in PBS. Slides were

covered with coverslips, set at 4 °C for solidifying the agarose, then coverslips were removed and slides were incubated in a lysis solution (2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na₂ EDTA, NaOH to pH = 10, 1% Triton X-100, 10% dimethyl sulfoxide) for 45 min; after this step all the operations were performed at 4 °C under dim light. After lysis, slides were rinsed for 10 min with electrophoresis buffer (1 mM Na₂-EDTA, 300 mM NaOH, pH = 13) and placed for 20 min onto a horizontal electrophoresis unit containing the same electrophoresis buffer to allow DNA unwinding. Electrophoresis was conducted with the Sub-Cell GT System (15 Å~ 25 cm) equipped with Power Pack 300 (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 15 min (25 V, 300 mA). Subsequently, slides were gently washed in neutralization buffer solution for 5 min (0.4 M Tris–HCl, pH = 7.5), dehydrated with an ethanol series (70%, 85% and 100%), dried at room temperature and stored. Where not otherwise indicated, all chemicals were purchased from Sigma Aldrich.

For microscopy analysis, slides were stained with ethidium bromide (10 µg/ml) immediately before being analyzed at 400 × magnification by a fluorescent Axiolab Zeiss microscope (Carl Zeiss AG, Oberkochen, Germany).

Slides were analyzed using a fluorescence microscope (Leica) equipped with a camera. On each slide, coded and blindly scored, 200 comets were acquired using "I.A.S." software automatic image analysis system purchased from Delta Sistemi (Rome, Italy).

Collection of Chromosome Spreads and Multicolor Fluorescent in Situ Hybridization (M-FISH)

The M-FISH analysis was used to quantify the stable genomic damage due to 131I. We analyzed in particular:

- exchanges (both simple exchanges, caused by two breaks on two different chromosomes reciprocal and non-reciprocal, and complex exchanges, due to three or more breaks on two or more chromosomes);
- acentric fragments;
- total breaks (as the total number of breakpoints involved in simple and complex exchanges and acentric fragment observed).

Chromosome spreads were obtained after 3h incubation in 5x10⁶ M colchicine (Sigma Aldrich). Metaphase spreads were prepared following standard cytogenetic procedures, consisting of treatment with a hypotonic solution (0.075 M KCl) for 20 min at 37 °C followed by fixation in freshly prepared Carnoy solution (3:1 v/v methanol/acetic acid).

Fixed cells were dropped onto glass slides and hybridised with the 24Xyte Human M-FISH Probe Kit (MetaSystems), as previously reported (24,25). Briefly, the slides were denatured in 0.07 N NaOH and then rinsed in a graded ethanol series. Meanwhile, the probe mix was denatured using a MJ mini personal thermal cycler (Bio-Rad laboratories, Hercules, CA, USA) with the following program: 5 min at 75 °C, 30 s at 10 °C, and 30 min at 37 °C. Probes were added to the slides and a coverslip was added and sealed using rubber cement. Samples were then hybridised in a humidified chamber at 37 °C for 48 h, washed in saline-sodium citrate buffer for 5 min at 75 °C and counterstained with DAPI (Sigma Aldrich) in Vectashield antifade (Vector Laboratories). Finally, images were captured with the M-search module of Metafer software (MetaSystems) at 63x magnification using an Axio Imager Z1 microscope (Zeiss) equipped with a Cool Cube 1 camera (MetaSystems). At least 100 metaphases were analyzed for each patient in each experimental condition. The karyotyping and cytogenetic analysis of each single chromosome was performed using the ISIS software (MetaSystems).

Genotyping (SNPs)

Genomic DNA was isolated from blood samples using the X-tractor Gene system (Corbett life Science, Sydney, Australia).

The SNPs analyzed were: XRCC1 G28152A (rs25487), XRCC3 A4541G (rs1799794), XRCC3 C18067T (rs861539), RAD51 G315C (rs1801320), for enzymes used to repair single strand DNA breaks (DNA1 package); CAT C-262T (rs1001179), OGG1 Ser326Cys (rs1052133), NOS3 Glu298Asp (rs1799983), PON1 A575G (rs662), PON1 C-108T (rs705379), MPO G-463A (rs2333227), for enzymes used to scavenge activity of free oxygen (DNA2 package).

Reference sequences for each gene were obtained from NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/>).

Genotyping of XRCC1 G28152A (rs25487), XRCC3 A4541G (rs1799794), XRCC3 C18067T (rs861539), RAD51 G315C (rs1801320), was performed by pyrosequencing technology, using the PyroMark Q48 Autoprep system (Qiagen, Hilden, Germany) according to manufacturers' directions. Both the amplification and the sequencing primers were obtained by the PSQ Assay Design software (Qiagen, Hilden, Germany).

Sequences of selected primers are reported in supplementary table 1 and 2.

Region covering the SNPs of interest was amplified by PCR. PCR conditions were: 95°C for 3 minutes; 40 cycles with denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds; a final extension step at 72°C for 5 minutes. PCR reactions were performed in a final volume of 25 µl, containing 70 ng of genomic DNA, 10 pmol of each primer, 0.2 mM dNTPs, PCR buffer and 1 U of Taq DNA polymerase (Takara Bio Inc., Otsu, Japan), 1 mM MgCl₂ for XRCC1 G28152A, XRCC3 C18067T and RAD51 G315C, while 1,5 mM MgCl₂ was used for XRCC3 A4541G amplification (26). The other SNPs were genotyped by the Sequenom MassArray iPLEX platform, (Sequenom, San Diego, California, USA). Twenty ng of genomic DNA were standardized for genotyping of each sample. According to the manufacturer's instructions, the DNA samples were amplified by a multiplex PCR reaction and treated with shrimp alkaline phosphatase. The PCR products were then used for locus-specific single-base extension reaction. The resulting products were desalted and transferred to a 96-SpectroCHIP array. The alleles were discriminated by MALDI-ToF mass spectrometry. Data were processed and analyzed by Sequenom MassArray TYPER 4.0 software.

Locus-specific PCR and extension primers were designed by Genotyping Tools and the MassArray Assay Design 4.0 software (Sequenom Inc, San Diego, California, USA).

For each heterozygote mutation we assigned a 0.5 score and for each homozygote mutation we assigned a score of 1. The total mutation score for DNA-1 and DNA-2 enzyme packages were calculated for each patient.

Oxidative Stress and Antioxidant Capacity

d-ROMs and Anti-ROMs have been measured with Diacron kits (Diacron International, Grosseto, Italy). d-ROMs test measures the oxidant ability of a plasma sample towards a particular substance (modified aromatic amine) used as an indicator (chromogen). The change in absorbance per unit time (calculated on the basis of a serum with known titer) is expressed in conventional units (CARR U). The normal range is < 300 CARR U (27).

Anti-ROMs test measures the anti-oxidant capacity of plasma, expressed as iron-reducing activity. The method has been engineered to have 2 phases: the first phase (first minute) provides the value of the so-called "FAST" anti-oxidants (Anti-ROMsF), (i.e. those that act quickly, such as Vitamin C or Vitamin E) and the second phase provides the value of the so-called "SLOW" anti-oxidants (Anti-ROMsS), (such

as the thiolic groups -SH, the uric acid or the polyphenols). The test reference values, in healthy population, are > 200 $\mu\text{Eq/l}$ for fast anti-oxidant and > 1000 $\mu\text{Eq/l}$ for slow anti-oxidant (28).

Statistical Analysis

Sample size was calculated on the basis of the results of M-FISH translocations observed in a previous unpublished pilot study in 10 patients. We hypothesized a difference clinically meaningful $d = \mu_{\text{HYPO}} - \mu_{\text{rhTSH}} = 0.8$, with a standard deviation: $\sigma = 1.1$, and setting $\alpha = 0.05$, with 80% power; the calculated total sample size was of 62 patients, namely 31 subjects per group.

Continuous variables were presented as mean \pm standard deviation (SD) when the normal distribution of the data was verified or median (95%CI – Confidence Interval) otherwise. Categorical variables were expressed as absolute frequencies and percentages – n (%).

The association with group (HYPO vs rhTSH) and gender, pT1, pT3, Papillary and Follicular was tested by χ^2 while with pT2, pT4 and N1 by Fisher exact test because the expected frequencies were less than 5. Shapiro-Wilk test was used to test the normality of the continuous variables and of the residuals; while the homoscedasticity was verified by checking the studentized residuals. For continuous variables, the differences across groups (HYPO vs. rhTSH) were compared by Student's-t or Mann-Whitney's test. A Generalized Linear Mixed Model (GLIMMIX) for repeated measures with distribution=Gaussian and link=identity was used to verify the differences of d-ROMs, Anti-ROMsS and Anti-ROMsF among baseline, 1 week and 3 months in HYPO vs rhTSH groups. Tukey method was used for multiple comparisons to correct the p values.

In order to understand which variables may influence the break or total exchange at 3 month time point in HYPO and rhTSH patients, we performed GLIMMIX with negative binomial function and link = logarithmic, using as independent variables: age, sex, breaks or total exchanges at baseline, radiation exposure rate at 48h, Anti-ROMsF, Anti-ROMsS and d-ROMs at baseline and 3 months; DNA-1, DNA-2 and Epi-GFR at baseline.

To evaluate the differences of micronuclei and Comet assay between the three temporal points (Baseline, 1 week and 3 months) relatively to HYPO vs. rhTSH we applied a GLIMMIX for repeated measures with distribution=Negative Binomial/Gaussian and link=logarithmic/identity (respectively) and Tukey method was used to correct the multiple comparisons.

Finally, a GLIMMIX with distribution = negative binomial and link = logarithmic was used to test the differences between baseline and 1 week of breaks and total exchanges in HYPO vs. rhTSH groups. Tukey method was used to correct the multiple comparisons.

A probability level $p < 0.05$ was considered to be statistically significant. Data were analyzed by SAS v. 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

All patients completed the follow-up period of 1 year. No patient dropout was observed, but in 2 patients we did not have consent to analyze DNA polymorphisms and in 3 patients of rhTSH group we did not measure d-ROMs and Anti-ROMs.

Administration of rhTSH was well tolerated. All patients of both groups had an efficient ablation as evaluated by unstimulated Tg levels at 3 months post-therapy and by rhTSH-stimulated Tg levels at 12 months follow-up.

Mean age of the two groups of patients was not significantly different, as shown in table 1; a significant difference in the levels of TSH, Tg at time of 131I, creatinine and Epi-GFR was observed.

Measurement of radiation exposure rate at 1 m revealed that in HYPO patients was higher at day 1 and day 2 versus rhTSH patients (median 36 vs. 24 $\mu\text{Sv/h}$ at day 1 and 16 vs. 9 $\mu\text{Sv/h}$ at day 2; both $p < 0.0001$; table 1). Most patients were dismissed at day 2 but, 4 patients from HYPO group were dismissed at day 3 and 11 patients from rhTSH group were dismissed at day 1, confirming the lower residual body activity of patients treated with rhTSH as compared to patients in hypothyroidism. The calculated area under the curve (AUC) for the exposure rate through 120 h was 27% lower in rhTSH patients than in HYPO patients, reflecting lower residual body activity, (figure 1).

Micronucleus and Comet Assay

Values of micronuclei showed no differences between HYPO and rhTSH group nor between the same group at 1 week or 3 months after treatment (figure 2).

Analysis of Comet showed no differences between HYPO and rhTSH group. However, all patients showed a statistically significant increase of damage at one-week ($p < 0.0001$) followed by a reduction at three-months ($p < 0.0001$), although not reaching yet the basal values (figure 3).

M-FISH

Results are reported in figure 4 and table 2. In particular, breaks and total exchanges in patients belonging to the HYPO group significantly increased from basal sample to three-month sample (in both $p = 0.004$). By contrast, in patients belonging to rhTSH group no significant increase of breaks or total exchanges was found between basal sample and three-month sample. Overall, 19/32 patients from rhTSH group had no increase or even a reduction in number of chromosome breaks as compared to 8/32 patients from the HYPO group. If we consider the total exchanges, 23/32 patients from rhTSH group had no increase or even a reduction in number of chromosome exchanges as compared to 11/32 patients from the HYPO group.

Table 2 shows the results of the GLIMMIX of breaks and total exchanges at three-months in HYPO and in rhTSH patients. In HYPO group, Anti-ROMs at 1 week are negatively associated with breaks at 3 months (with a reduction of 0.3% ($p = 0.046$)); while the exposure rate at 48 hours is positively associated to response variable with an increase of 6.2% ($p = 0.016$). Total chromosome exchanges at 3 months, in HYPO group, are negatively associated with Anti-ROMs at 1 week with a reduction of 0.9% ($p = 0.015$), positively associated with DNA-1 with an increase of 127.5% ($p = 0.027$). Exposure rate at 48 hours is positively associated with response variable with an increase of 0.2% ($p = 0.046$). Finally, in rhTSH group, only exposure rate at 48 hours is positively associated with breaks at 3 months in rhTSH group with an increase of 95.4% ($p = 0.04$).

No association was observed between total exchanges at 3 months in rhTSH group and the analysed covariates.

Genotyping (SNPs)

In the HYPO patient group, 11 patients had a total DNA score (DNA-1 + DNA-2 scores) of 3 or higher, but in the rhTSH patient group, 17 patients had a total DNA mutation score of 3 or higher, indicating a higher frequency of mutations in rhTSH patients. These polymorphisms were predominantly observed in genes of DNA-2 package (for the anti-oxidative stress enzymes) and indeed, positively influenced the number of chromosome breaks at 3 months in rhTSH patients.

Nevertheless, in the HYPO sub-group with DNA mutation score ≥ 3 , chromosome breaks, measured by M-FISH technique, increased from 2.3 ± 2.2 (mean basal sample \pm SD) to 5.5 ± 2.8 (3-month sample), ($p = 0.01$) as compared to rhTSH patients (with DNA mutation score ≥ 3) that increased from 6.6 ± 8.1 (basal sample) to 8.5 ± 8.6 (3-month sample), ($p = \text{ns}$).

Chromosome total exchanges in these HYPO patients, also significantly increased from 1.0 ± 1.1 to 2.1 ± 1.1 (basal vs 3-month sample), ($p=0.03$) as compared to rhTSH patients in whom the frequency of chromosome exchange breaks increased from 2.1 ± 3.0 to 3.3 ± 4.6 (basal vs 3-month sample), ($p=ns$).

Oxidative Stress

Overall, most patients had high levels of d-ROMs and low levels of Anti-ROMs at any time point, with no significant differences between HYPO and rhTSH at baseline and the 1-week-sample but higher values in HYPO at 3 months ($p = 0.03$), (table 3). Only 3 HYPO patients and 4 rhTSH patients had normal d-ROMs values at entry, highlighting the high level of stress induced by cancer, surgery and post-surgical period (including hypothyroidism in HYPO patients) or supra physiologic T4 replacement.

In HYPO patients we observed a reduction of oxidative stress at 1 week with a statistically significant decrease of d-ROMs versus baseline ($p = 0.0001$). However, at 3 months, HYPO patients had a new significant increase of d-ROMs as compared to values at 1 week ($p < 0.0001$).

These differences were not observed in rhTSH patients.

As far as Anti-ROMsF and Anti-ROMsS, we did not observe significant differences between HYPO and rhTSH patients nor significant modifications over time. However, most patients had Anti-ROMs values below the level of normal ranges at any time, presumably, as a result of the high stress level in these patients.

DISCUSSION

Several papers have been published on the potential genetic damage and on the increased risk of secondary cancer in patients treated with ^{131}I , even if with low activities (1-20) although the causative association between ^{131}I therapy and an increased risk of secondary cancers has not yet been definitively established. Indeed, no longitudinal study has been published, indicating that an increase in chromosome breaks or translocations after ^{131}I therapy is associated with an increased risk of malignancies. Nevertheless, there is a worldwide tendency at reducing the ^{131}I ablation treatment in patients with low or intermediate risk after total thyroidectomy for DTC.

Structural aberrations generated by a DNA DSBs can be classified as stable or unstable according to their ability to persist in cellular progeny. Unstable aberrations include deletions, dicentric chromosomes, ring chromosomes, acentric or otherwise asymmetric rearrangements that are normally not tolerated (i.e. lethal) in dividing cells and are, therefore, not transmitted with subsequent cell divisions. Stable aberrations, on the other hand, are generally tolerated by the cells and transmitted to the following cellular generations. It is believed that stable and unstable aberrations are induced with the same frequency, but unstable aberrations seem to be less frequent precisely because they are lost at each cell division.

We investigate, whether 1850 MBq (50 mCi) of ^{131}I for ablation therapy could cause stable genetic damage in patients who underwent surgery for DTC with low and intermediate risk. Moreover, we investigated whether patients prepared with thyroid hormone withdrawal or with rhTSH displayed similar levels of chromosome damage.

We found transient unstable DNA damage in both groups and modest stable DNA damage only in patients treated under hypothyroidism.

Indeed, micronuclei are a sign of early unstable damage, and we expected normal levels at 1 week and 3 months post-therapy. The trend of DNA damage measured by Comet assay after 1 week and 3 months, suggests that the DNA damage observed consists mainly of SSBs, a very sensitive biomarker induced by reactive oxygen species and reversible over time (29). Indeed, cancer, chronic inflammation

and oxidative stress, are closely related and numerous agents, such as ionizing radiations, have been proven to interfere with redox cell signaling pathways (30). Since several studies have shown that unstable DNA damage can also manifest several cellular generations after radiation exposure, generating what is called “delayed damage”, in this study, the unstable damage was assessed also at 3 months after therapy, by Comet assay, but we found no significant difference between the two groups of patients.

Stable aberrations, by contrast, are a marker of radiation exposure, and results obtained by the M-FISH analysis showed a statistically significant increase in the frequency of chromosome breaks between the basal sample and the 3-month sample only in HYPO patients, suggesting that in these patients, not prepared with rhTSH, a genomic instability occurs after treatment and persists with time.

These data could be partially explained by the reduced renal clearance of ¹³¹I (due to reduced GFR), and higher retained total body activity, due to the hypothyroidism induced in HYPO patients by the withdrawal of levothyroxine, but genetic and metabolic factors could also play a role.

The use of rhTSH, by maintaining the condition of euthyroidism and a normal renal clearance (Epi-GFR and creatinine values), reduced by 27% the radiation exposure rate over a period of 120 h, an indirect measurement of the retained body activity, and reduced the genomic instability.

Polymorphisms (SNPs) represent different variants of the same gene, present in the population. The type and combination of polymorphisms of genes involved in DNA repair and for enzymes acting as free oxygen radical scavenger, present in each individual, influenced the amount of DNA damage observed. Thus, analysis of gene polymorphism allowed us to identify a sub-group of HYPO and rhTSH patients more susceptible to chromosome damage induced by ¹³¹I because of mutations in enzymes deputed to DNA repair (DNA-1) or enzymes involved in scavenging of free oxygen radicals (DNA-2). It was interesting to note that amongst these patients, those in hypothyroidism showed a higher increase in chromosome damage after ¹³¹I, than patients prepared with rhTSH. Indeed, polymorphism of gene regulating the redox status has been involved in several other malignancies, mainly breast cancer (31). Overall, in HYPO patients the number of chromosome breaks at 3 months was associated to the level of breaks at baseline, residual body activity at 48 h and levels of Anti-ROMs (table 2). As far as the Anti-ROMs are concerned, we found a significant positive association with Anti-ROMsF at 1 week and a negative association with Anti-ROMsS at 1 week. This could be explained considering that a high level of d-ROMs (as observed in these patients) induces an increase of Anti-ROMs, and amongst these, the Fast Anti-ROMs rise quickly and result positively associated with an increase of chromosome damage, whereas the Slow Anti-ROMs may require more time to rise and results negatively associated with an increase of chromosome damage.

Patients prepared with rhTSH showed no significant increase of both breaks and total exchanges after ¹³¹I, suggesting that patients prepared with rhTSH have less radiation-induced chromosome damage, even in presence of mutations in several enzymes. Interestingly, the number of chromosome breaks at 3 months in rhTSH patients was positively associated with polymorphisms of genes for DNA-repairing enzymes (DNA-2 group).

These data confirm previously published findings that ¹³¹I therapy can induce stable DNA damage, but for the first time we are able to demonstrate that rhTSH may significantly reduce this damage, particularly in patients with unfavorable polymorphism of genes involved in DNA repair. Whether chromosome damages found in peripheral lymphocytes can be related to an increased risk of secondary malignancies, remains a matter of debate. In addition, data obtained by the measurement of d-ROMs and Anti-ROMs demonstrate a crucial role of the oxidative stress. To this regard, patients with high levels of d-ROMs and low levels of Anti-ROMs, or with an unfavorable genotype for DNA-repairing

enzymes or for free oxygen radical scavengers, might be more susceptible to radiation-induced DNA damage.

CONCLUSIONS

In summary, administered activity, DNA polymorphisms, GFR value, oxidant/antioxidant homeostasis, are all parameters that may influence DNA damage in patients treated with 131I. Our study also highlights the importance of rhTSH in preventing radiation-induced stable chromosome damage, even if an unfavorable genetic background is present.

Even if stable DNA damage are considered of particular importance for their involvement in tumor cell clonal evolution, in our study we do not provide any evidence that stable DNA damage has clinical consequences or induce secondary tumors. To evaluate these specific end points, larger longitudinal studies should be warranted.

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CONFLICT OF INTEREST

Authors have no conflict of interest. Genzyme-Sanofi supports research in thyroid cancer in our institution but did not sponsor this specific study. rhTSH was regularly purchased for all patients.

KEY POINTS

QUESTION: Is there transient DNA damage after 131I ablation therapy?

FINDINGS: We found transient DNA damage after 131I ablation therapy in most patients treated with 1850 MBq, that disappeared after 3 months.

IMPLICATIONS: Transient chromosome damage is not relevant in patients treated with 1850 MBq of 131I.

QUESTION: Is there a detectable permanent chromosome damage induced by 131I?

FINDINGS: We found some chromosome breaks and exchanges after 131I therapy particularly in patients prepared with hypothyroidism as compared to those prepared with rhTSH. This may be due to the higher body retained activity in hypothyroid patients and/or high oxidative stress.

IMPLICATIONS: Patients candidate for 131I ablation therapy should be prepared with rhTSH, avoiding discomfort and symptoms of hypothyroidism and chromosome damage.

QUESTION: To what extent permanent chromosome damage is due to 131I dose, or to oxidative stress of the patient and gene polymorphism?

FINDINGS: We found that the majority of patients had a reduction of anti-free oxygen radical scavengers and an increase of free oxygen radicals. An unfavorable genotype, furthermore, exposes patients to higher radiation-induced damages. When matching patients for severity of gene mutations, hypothyroid patients had higher levels of chromosome damage.

IMPLICATIONS: Genes responsible for the control of oxidative stress are possible factors that influence the induction of DNA damage in patients treated with 131I, although larger studies are needed to confirm our preliminary results.

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Table 1
Differences of the clinical and biochemical variables between HYPO and rhTSH group

| Variable | HYPO Median (95%CI) | rhTSH Median (95%CI) | p |
|---|---------------------------------|----------------------------------|-------------------|
| Age* (years) | 47.64 ± 11.18; (43.54 to 51.75) | 48.64 ± 11.53; (44.42 to 52.87) | 0.73 |
| Gender** (M/F) | 4 (12.90) / 27 (87.10) | 7 (22.58) / 24 (77.42) | 0.32 |
| pT1** | 18 (58.06) | 20 (64.52) | 0.60 |
| pT2** | 5 (16.13) | 1 (3.23) | 0.19 |
| pT3** | 7 (22.58) | 10 (32.26) | 0.39 |
| pT4** | 1 (3.23) | 0 (0.00) | 1.00 |
| Papillary** | 20 (64.52) | 19 (61.29) | 0.79 |
| Follicular** | 11 (35.48) | 12 (38.71) | 0.79 |
| N1** | 2 (6.45) | 4 (12.90) | 0.67 |
| TSH at ¹³¹ I (μU/ml) | 87.37 (76.62 to 99.72) | 112.80 (98.84 to 135.80) | 0.01 |
| Thyroglobulin at ¹³¹ I (ng/ml) | 0.79 (0.25 to 2.77) | 0.25 (0.20 to 0.34) | 0.008 |
| Thyroglobulin at 3 months (ng/ml) | 0.10 (0.10 to 0.10) | 0.10 (0.10 to 0.10) | 0.53 |
| Exposure rate at 24h (mSv/h) | 33 (28 to 37) | 24 (20 to 28) | <0.0001 |
| Exposure rate at 48h (mSv/h) | 16 (12 to 18) | 9 (8 to 9) | <0.0001 |
| Creatinine (mg/ml) | 0.98 (0.85 to 1.05) | 0.76 (0.72 to 0.80) | <0.0001 |
| Epi-GFR* (ml/min) | 74.77 ± 17.50; (68.36 to 81.19) | 97.61 ± 12.50; (93.03 to 102.20) | <0.0001 |

*data presented as mean±SD; (95%Confidence Interval); **data presented as n (%)

Table 2**Parameter estimates by Generalized Linear Mixed Model of chromosome breaks and exchanges at 3 months in HYPO and rhTSH group****Chromosome breaks at 3 months in HYPO patients**

| Variable | Mean ± SE | (95% CI) | Exp (mean) | % chance | p |
|---------------------------|------------------|----------------------|-------------------|-----------------|--------------|
| Anti-ROMsS at 1 week | -0.003±0.001 | (-0.005 to -0.00005) | 1.00 | -0.3 | 0.046 |
| Exposure rate at 48 hours | 0.06±0.02 | (0.01 to 0.10) | 1.06 | 6.2 | 0.016 |

Covariate: Age, sex, breaks at baseline, d-ROMs at baseline and one week, Anti-ROMsF at baseline and one week, Anti-ROMsS at baseline, DNA-1, DNA-2 and Epi-GFR

Chromosome exchanges at 3 months in HYPO patients

| Variable | Mean ± SE | (95% CI) | Exp (mean) | % chance | p |
|---------------------------|------------------|-------------------|-------------------|-----------------|--------------|
| Anti-ROMsS at 1 week | -0.009±0.003 | (-0.02 to -0.002) | 0.99 | -0.9 | 0.015 |
| DNA-1 | 0.82±0.34 | (0.10 to 1.54) | 2.28 | 127.5 | 0.027 |
| Exposure rate at 48 hours | 0.06±0.03 | (0.002 to 0.12) | 1.06 | 6.2 | 0.046 |

Covariate: Age, sex, exchanges at baseline, d-ROMs at baseline and one week, Anti-ROMsF at baseline and one week, Anti-ROMsS at baseline, DNA-2 and Epi-GFR

Chromosome breaks at 3 months in rhTSH patients

| Variable | Mean ± SE | (95% CI) | Exp (mean) | % chance | p |
|-----------------|------------------|-----------------|-------------------|-----------------|-------------|
| DNA-2 | 0.67±0.25 | (0.03 to 1.31) | 1.95 | 95.4 | 0.04 |

Covariate: Age, sex, breaks at baseline, d-ROMs at baseline and one week, Anti-ROMsF at baseline and one week, Anti-ROMsS at baseline and 1 week, DNA-1, dosimetry at 48 hours and Epi-GFR

Table 3
Differences in longitudinal data of d-ROMs and Anti-ROMsS/F between HYPO and rhTSH group

| Variable | HYPO | | | | rhTSH | | | |
|------------|-------------------------------------|-------------------------------------|-------------------------------------|---------|-------------------------------------|------------------------------------|-------------------------------------|------|
| | Baseline Mean±SD (95%CI) | 1 week Mean±SD (95%CI) | 3 months Mean±SD (95%CI) | p | Baseline Mean±SD (95%CI) | 1 week Mean±SD (95%CI) | 3 months Mean±SD (95%CI) | p |
| d-ROMs | 399.75±86.51 (368.02 to 431.48) | 333.46±71.07 (307.39 to 359.53) | 402.93±72.60* (375.82 to 430.04) | <0.0001 | 374.22±75.56 (345.72 to 402.72) | 355.20±93.50 (319.93 to 390.47) | 363.62±66.84* (338.20 to 389.04) | 0.29 |
| Anti-ROMsF | 209.15±50.53 (190.61 to 227.68) | 215.13±47.25 (197.80 to 232.46) | 212.13±52.46 (192.89 to 231.37) | 0.83 | 211.49±35.75 (197.63 to 225.36) | 219.53±36.46 (205.39 to 233.67) | 202.81±30.95 (191.46 to 214.16) | 0.07 |
| Anti-ROMsS | 727.15±121.06 (682.75 to 771.55) | 731.30±172.46 (668.04 to 794.56) | 727.31±134.85 (677.85 to 776.77) | 0.99 | 681.79±116.81 (636.50 to 727.09) | 720.57±96.55 (683.13 to 758.01) | 665.52±105.12 (626.96 to 704.08) | 0.09 |

Post-hoc analysis: HYPO

d-ROMs – p(Baseline vs. 1 Week)=**0.0001**; p(1 week vs. 3 months)<**0.0001**

***HYPO vs. rhTSH:**

d-ROMs – p(3 months vs. 3 months)=**0.03**

Normal values for d-ROMs are < 300 CARR U; for Anti-ROMsF > 200 µEq/l; for Anti-ROMsS > 1000 µEq/l.

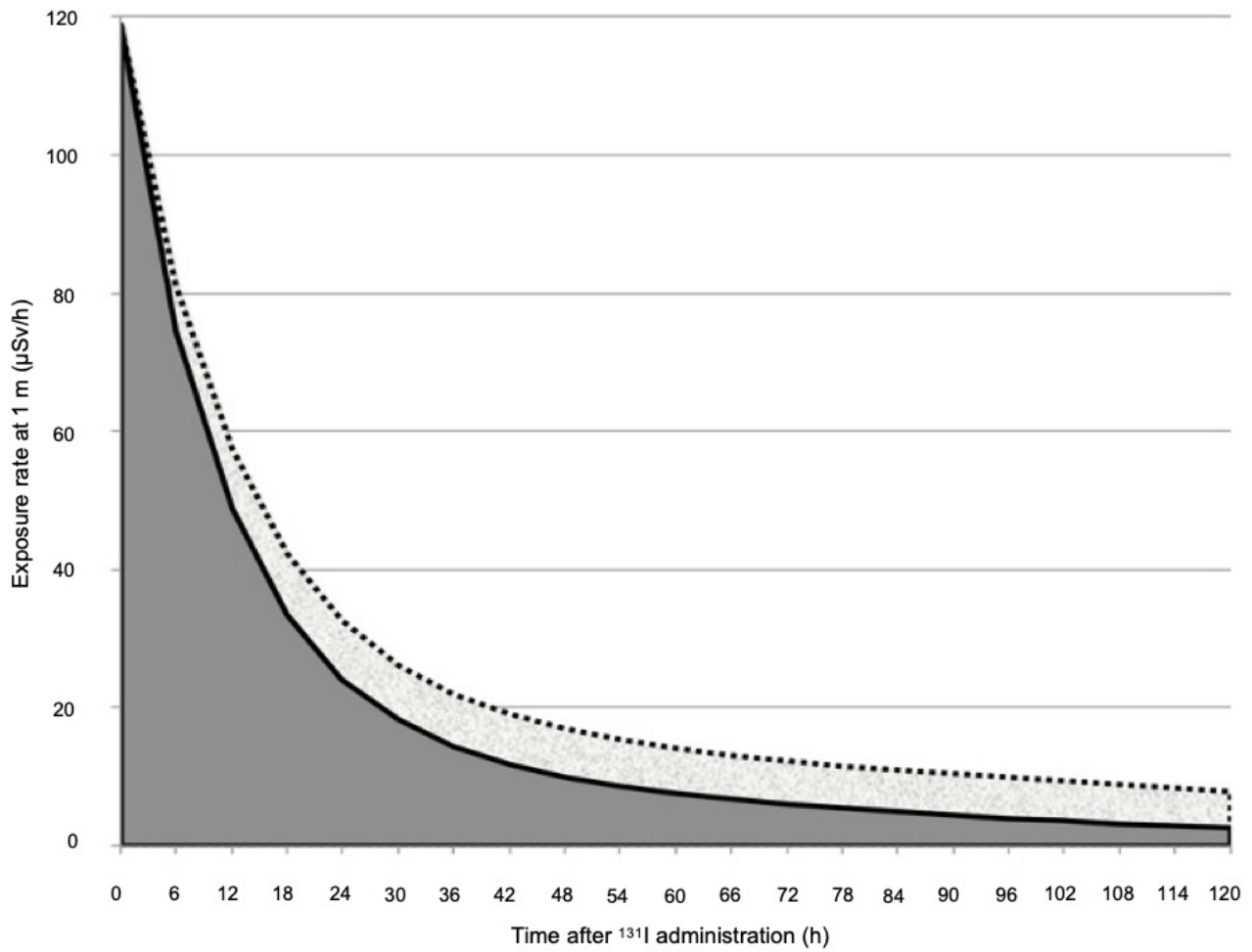


Figure 1. Results of fitted radiation exposure rate in patients, up to 120 h, measured at 1 m distance. Dotted line refers to HYPO patients, continuous line refers to rhTSH patients. In HYPO patients there is a higher retained body activity respect to rhTSH patients with 27% higher exposure rate, calculated comparing the two areas under the curves.

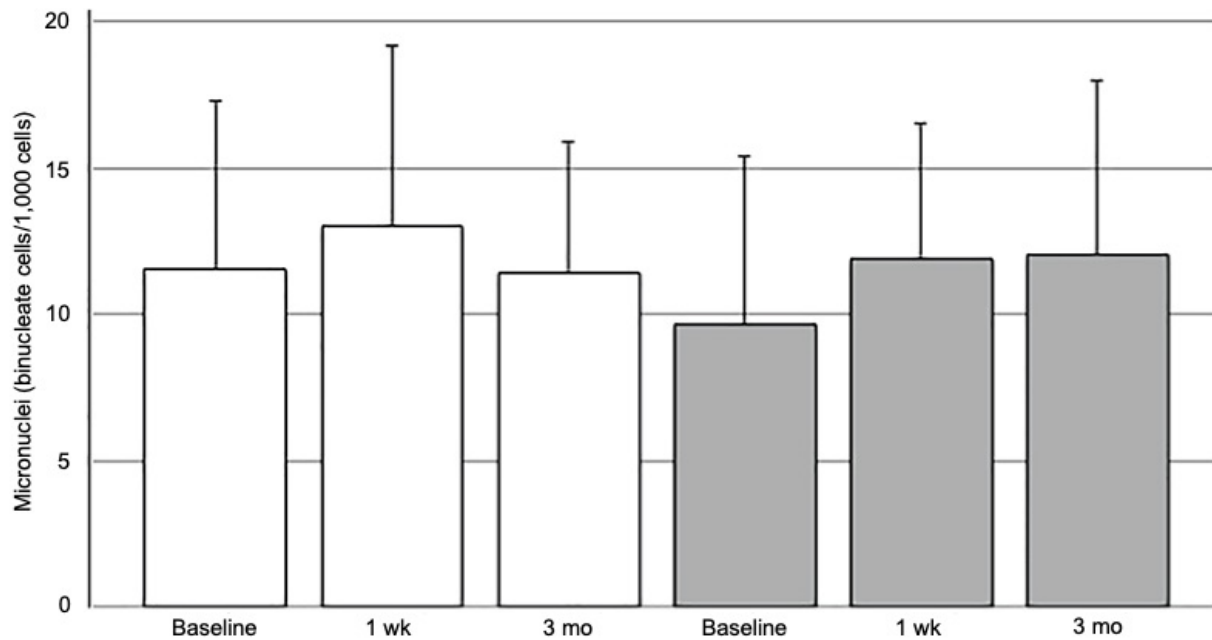


Figure 2. Results of unstable damage measured by micronuclei (empty columns HYP0, dark columns rhTSH). No differences between and within groups. Tukey method was used to correct the multiple comparisons.

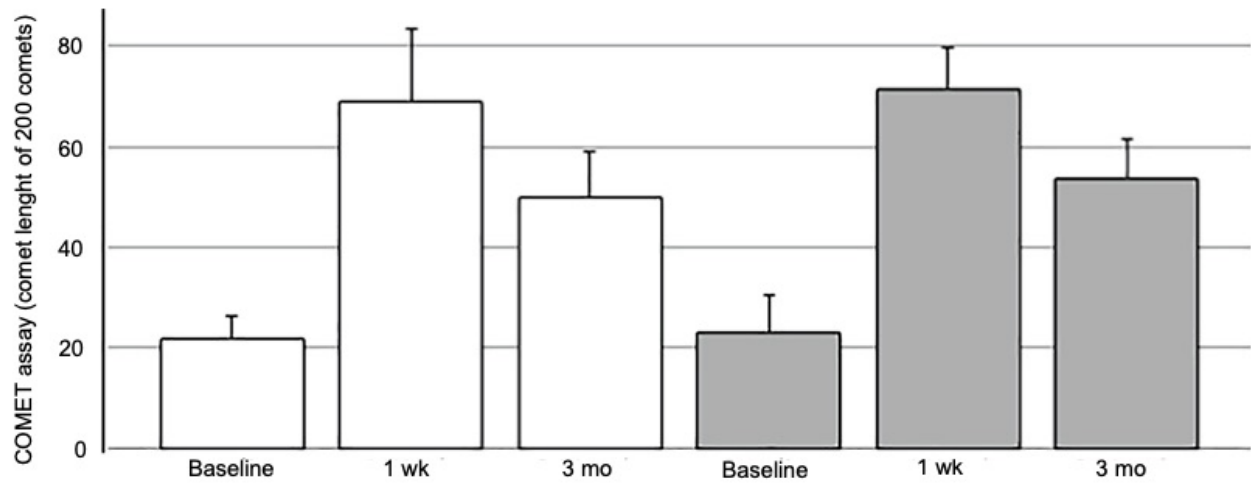


Figure 3. Results of Comet assay (empty columns HYPO, dark columns rhTSH). Significant differences were found between baseline and 1 week values, between 1 week and 3 months values and between baseline and 3 months values in both groups (all $p < 0.0001$), as described in the results session. No differences were found between HYPO and rhTSH patients at any time point. Tukey method was used to correct the multiple comparisons.

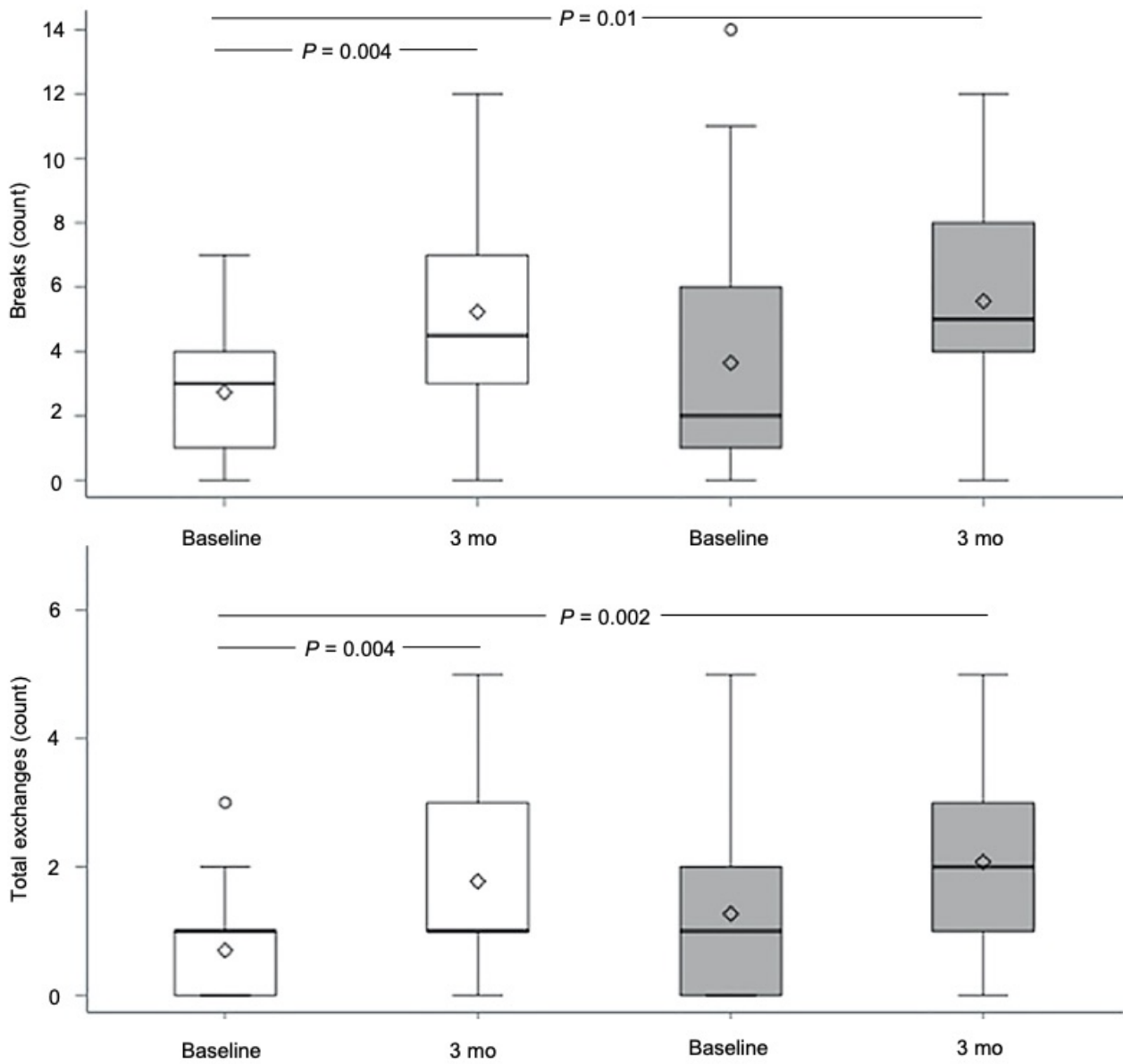


Figure 4. Stable chromosome damage (chromosome breaks and total exchanges) in patients before and 3 months after ^{131}I therapy (empty columns HYPO, dark columns rhTSH). Tukey method was used to correct the multiple comparisons.

Supplementary Table 1
Primers for PCR amplification and pyrosequencing for DNA-1 package

| Gene name | Polymorphism | | Oligonucleotides |
|--------------|---------------------|---|---------------------------------|
| <i>XRCC1</i> | G28152A rs25487 | F | ^a AGTACAGCCAGGTCCTAG |
| | | R | CGCTCCTCTCAGTAGTCT |
| | | S | CGTGTGAGGCCTTACC |
| <i>XRCC3</i> | A4541G rs1799794 | F | GCCTGTAAACCAAGTTC |
| | | R | ^a TGACGGATAACAGACTCA |
| | | S | TTAAACCAAGTTCTCAGC |
| <i>XRCC3</i> | C18067T rs861539 | F | ^a ATAAGAAGGTCCCGTAC |
| | | R | CTAAAAATACGAGCTCAGG |
| | | S | CTGCTCAGCTCACGC |
| <i>RAD51</i> | G315C rs1801320 | F | GAGAAGTGGAGCGTAAGCC |
| | | R | ^a CGCCTCACACTCACCT |
| | | S | GAAGTGGAGCGTAAGC |

Abbreviations – F: Forward, R: Reverse, E: Extension; ^abiotin molecular attached

Supplementary Table 2
Amplification and extension oligonucleotides used in the multiplex genotyping assay for DNA-2 package

| Gene name | Polymorphism | | Oligonucleotides |
|-------------|------------------------|---|---------------------------------|
| <i>SOD2</i> | V16A rs4880 | F | ACGTTGGATGTTCTGCCTGGAGCCAGATA |
| | | R | ACGTTGGATGCTGTGCTTTCTCGTCTTCAG |
| | | E | GAGCCCAGATACCCCAA |
| <i>CAT</i> | C-262T rs1001179 | F | ACGTTGGATGAGGATGCTGATAACCGGGAG |
| | | R | ACGTTGGATGAGCAATTGGAGAGCCTCGC |
| | | E | AGCCCCGCCCTGGGTTCCGGCTAT |
| <i>NOS3</i> | Glu298Asp rs1799983 | F | ACGTTGGATGTGCTGCCCTGCTGCTGC |
| | | R | ACGTTGGATGACCTCAAGGACCAGCTCGG |
| | | E | GCTGCAGGCCCCAGATGAT |
| <i>PON1</i> | A575G rs662 | F | ACGTTGGATGGATCACTATTTTCTTGACCC |
| | | R | ACGTTGGATGTAGACAACATACGACCACGC |
| | | E | TTTCTTGACCCCTACTTAC |
| <i>PON1</i> | C-108T rs705379 | F | ACGTTGGATGTGCTGGGGCAGCGCCGATT |
| | | R | ACGTTGGATGCTTCTGTGCACCTGGTCGG |
| | | E | CGCCGATTGGCCCGCCCC |
| <i>OGG1</i> | Ser326Cys rs1052133 | F | ACGTTGGATGTCCTACAGGTGCTGTTTCAGT |
| | | R | ACGTTGGATGGGAACCCTTTCTGCGCTTTG |
| | | E | TGCCGACCTGCGCCAAT |
| <i>MPO</i> | G-463A rs2333227 | F | ACGTTGGATGGGCTGGTAGTGCTAAATTC |
| | | R | ACGTTGGATGCTCTAGCCACATCATCAAT |
| | | E | CTTTGGGAGGCTGAGGC |

Abbreviations – F: Forward, R: Reverse, E: Extension