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DNA Damage and Oxidative DNA Damage in Inflammatory Bowel Disease

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Abstract

Background and Aims: Inflammation has long been regarded as a major contributor to cellular oxidative damage and to be involved in the promotion of carcinogenesis.

Methods: We aimed to investigate the oxidative damage in inflammatory bowel disease [IBD] patients through a case–control and prospective study involving 344 IBD patients and 294 healthy controls. DNA damage and oxidative DNA damage were measured by comet assay techniques, and oxidative stress by plasmatic lipid peroxidation, protein carbonyls, and total antioxidant capacity.

Results: Higher DNA damage [$p < 0.001$] was found both in Crohn's disease [CD] (9.7 arbitrary units [AU]; interquartile range [IQR]: 6.2–14.0) and ulcerative colitis [UC] [7.1 AU; IQR: 4.4–11.7], when compared with controls [5.4 AU; IQR: 3.8–6.8], and this was also the case with oxidative DNA damage [$p < 0.001$] [CD: 3.6 AU; IQR: 1.8–6.8; UC: 4.6 AU; IQR: 2.4–8.1], when compared with controls: 2.3 AU; IQR: 1.2–4.2]. Stratifying patients into groups according to therapy (5-aminosalicylic acid [5-ASA], azathioprine, anti-TNF, and combined therapy [azathioprine and anti-TNF]) revealed significant between-group differences in the level of DNA damage, both in CD and UC, with the combined therapy exhibiting the highest DNA damage levels [11.6 AU; IQR: 9.5–14.3, and 12.4 AU; IQR: 10.6–15.0, respectively]. Among CD patients, disease behaviour [B1 and B2], and age at diagnosis over 40 years [A3] stand as risk factors for DNA damage. For UC patients, the risk factors found for DNA damage were disease activity, treatment, age at diagnosis under 40 years [A1 + A2] and disease locations [E2 and E3].

Conclusions: In IBD there is an increase in DNA damage, and treatment, age at diagnosis and inflammatory burden seem to be risk factors.

Key Words: Inflammatory bowel disease; DNA damage; risk factors

1. Introduction

Inflammatory bowel diseases [IBDs] (both Crohn's disease [CD] and ulcerative colitis [UC]) are relapsing inflammatory conditions with still-growing prevalence worldwide.[1] Genetic, immunological and environmental factors are involved in IBD.[2] The chronic inflammation along the gastrointestinal tract that characterizes IBD results from an imbalance of effector lymphocytes and pro-inflammatory cytokines with respect to regulatory lymphocytes and cytokines.[3] Some of the cytokines, as well as the triggered leukocytes and activated macrophages, can produce large amounts of reactive oxygen species [ROS], thus predisposing a patient to oxidative stress disturbances.[4] It has been shown that exposure to activated leukocytes can cause DNA base modifications in human cells, and the oxidative DNA damage is closely related to a lifelong increased risk of cancer development.[5] The activation of inflammation can result in genetic and epigenetic transformations that may promote carcinogenesis,[6] and the increase of mortality and morbidity in IBD is due to increased incidence of colorectal cancer [CRC].[7] Conventional treatments in IBD such as anti-inflammatory drugs [sulfasalazine, mesalazine], corticosteroids [prednisolone, methylprednisolone, budesonide], antibiotics, and immunosuppressants [azathioprine, 6-mercaptopurine] are known to have direct anti-oxidative effects via free radical scavenging properties[8]; on the other hand, biologic therapies (anti-tumour necrosis factor-alpha [TNF-α] monoclonal antibodies), used in the treatment of moderate–severe disease or in cases of refractoriness, have an indirect anti-oxidative effect by decreasing the TNF-α concentrations.[8]

To assess whether IBD may impose oxidatively damaged DNA, we decided to measure peripheral DNA damage [through the alkaline comet assay] and oxidative DNA damage (through the formamidopyrimidine glycosylase [FPG] enzyme version of the comet assay) in isolated lymphocytes, as these are often used as surrogate cells to estimate oxidative stress affecting other tissues. Plasma lipid peroxidation, protein carbonylation content, and total antioxidant capacity were also measured, and clinical data were gathered from patients, in order to determine which factors are more pertinent in contributing to the oxidative burden in IBD, and which may be predictive for DNA damage.

2. Materials and Methods

2.1. Study population

A total of 638 subjects were enrolled in this study. Patients were recruited from the Gastroenterology Unit of Hospital S. João, Porto, Portugal, as they attended their routine IBD specialist medical appointment. All patients were observed by the same physician for several years, which turns the clinical decisions more homogeneous. Of the 638 patients, 344 [221 with CD and 123 with UC] were eligible, and their samples were collected throughout the year of 2012. The control group consisted of healthy blood donors who attend the Blood Bank Unit of Centro Hospitalar S. João, and 294 sex- and age-matched samples were collected. The Ethics Committee of Hospital S. João approved the protocol, and all patients or their legal guardians gave their written informed consent, complying with the principles laid down in the Declaration of Helsinki. A questionnaire concerning each patient's disease characteristics was filled out by the physician, and all data was prospectively compiled in a database [gediibasedados.med.up.pt]. Information on smoking habits, disease score, extra-intestinal manifestations, location and behaviour of disease, therapeutic in use, duration of disease, number of flares, and duration of current therapy was collected. Patients were considered eligible based on their current therapy: 5-aminosalicylic acid [5-ASA], azathioprine, anti-TNFα, and combined therapy of azathioprine and anti-TNFα users were included in the study. Location, behaviour, and age at diagnosis, were classified according to the Montreal Classification.[9] C-reactive protein [C-RP] determination was also requested on sample collection.

2.2. Samples

Two blood samples were collected from each subject into heparinized tubes [BD Vacutainer, USA]. Peripheral blood mononuclear cells [PBMCs] were separated from whole blood, by Histopaque-1077™ [Sigma-Aldrich, USA] density gradient centrifugation following the manufacturer's instructions and slowly frozen to –80°C and kept until analysis. Lymphocyte viability was measured by Trypan blue dye exclusion assay. All processed samples were found eligible for comet assay [viability > 90%].[10]

2.3. Comet assay for DNA damage and FPG-sensitive sites determination

The medium-throughput alkaline version[11] of comet assay was performed as described previously.[12] Before proceeding to the scoring step, slides were dyed SYBRGold™ [Invitrogen, Massachusetts, USA] at the dilution recommended by the manufacturer for 15 min. Oxidative DNA damage was assessed using a modified version of the comet assay, as described by Azqueta *et al.*[12] The number of FPG-sensitive sites was obtained by the difference between the % Tail DNA in the buffer-incubated slide and that in the FPG-incubated slide.

2.4. Slides scoring

Blind sample scoring of 100 cells [50 cells per duplicate gel] was carried out using a fluorescence microscope under ×40 objective observation [Eclipse E400, Nikon Instruments, Japan]. The Comet Assay IV [Perceptive Instruments, UK] software was used for DNA measurements, and the parameter used to measure DNA damage was % Tail DNA [Figure 1], here referred as 'arbitrary unit' [AU], and recommended for being the most reliable comet assay measurement.[10,13]

2.5. Lipid peroxidation

Lipid peroxidation was determined by the thiobarbituric acid reactive substances [TBARS] protocol for malondialdehyde [MDA] quantification, following the protocol described by Ohkawa *et al.*[14]

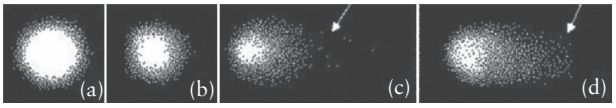

Figure 1. DNA damage measured by comet assay in lymphocytes. [a] Sample from healthy control [S4 – male, 26 years – 3.4% Tail DNA]; [b] Sample from ulcerative colitis [UC] patient [S120 – male, 41 years, 5-ASA – 2.8% Tail DNA]; [c] Sample from Crohn's disease [CD] patient [S136 – female, 35 years, anti-tumour necrosis factor alpha [anti-TNFα] – 9.0% Tail DNA]; [d] Sample from UC patient [S178 – female, 28 years, azathioprine + anti-TNFα – 15.9% Tail DNA] [arrows indicating the tail DNA].

with slight modifications. A calibration curve was prepared with MDA as a standard, and the results were expressed in micromolar MDA. All samples gave results that were within the linear range of the MDA standard curve.

2.6. Plasma protein carbonyls [PPCs]

Detection of carbonyl groups in plasma was performed using the Protein Carbonyl Content Assay Kit [Sigma-Aldrich™, USA] and the Bicinchoninic Acid Kit for Protein Determination [Sigma-Aldrich™, USA], following the manufacturer's instructions. The results obtained were expressed in nanomole carbonyls per milligram of plasma protein.

2.7. Total antioxidant capacity

The total antioxidant capacity [TAC] of plasma was determined using the assay kit 'Antioxidant Assay Kit' [Cayman™, USA] according to manufacturers' instructions, and measuring both aqueous- and lipid-soluble antioxidants. The results obtained were expressed in terms of millimolar of Trolox equivalent per litre.

2.8. Statistics

The study population size was calculated with the 'Open Source Epidemiologic Statistics for Public Health – OpenEPI', available at www.OpenEpi.com, based on the 'Frequency in a Population' tool. For the size of the Portuguese IBD population, estimated at 15 460 patients, studying 344 subjects gave the study greater than a 90% confidence level and, based on the difference in the mean values for DNA damage between cases and controls previously established, a statistical power of 100% by the normal approximation method.

Categorical variables were described as absolute frequencies [*n*] and relative frequencies [%]. Descriptive statistics (median and interquartile range [IQR], and percentages for continuous and categorical variables, respectively) were calculated for all variables. Missing data were excluded from analyses; on average, <2% of data were missing. When testing a hypothesis about continuous variables, non-parametric tests [Mann–Whitney or Kruskall–Wallis] were used, as appropriate, taking into account normality assumptions and the number of groups compared. When testing a hypothesis about categorical variables, a chi-square test and Fisher's exact test were used, as appropriate. All the reported *p* values were two-sided, and *p* values < 0.05 were considered statistically significant. All data were arranged, processed and analysed with SPSS© v. 20.0 data [Statistical Package for Social Sciences, IBM]. The predictors for DNA damage and oxidative DNA damage were assessed by univariate and multivariate analysis linear regression, and 95% confidence intervals were considered to make inferences with the regression coefficients; the tested independent variables were age, sex, C-reactive protein [C-RP], therapy, disease location, disease behaviour [for CD], age at diagnosis, years of follow-up, number of flares per year of follow-up, and smoking habits.

3. Results

3.1. Baseline characteristics

Relevant demographic and clinical characteristics of the enrolled subjects are described in Table 1. In total, 344 cases and 294 controls were included. Although a sex- and age-matched control population was recruited, it significantly differed from the IBD population in age distribution [$p = 0.0151$]. Also, CD and UC patients significantly

Table 1. Demographic and clinical characteristics of enrolled subjects {median (interquartile range [IQR] 25–75)}.

	Controls	IBD	CD	UC	*p1*	*p2*	*p3*
	[*n* = 294]	[*n* = 344]	[*n* = 221]	[*n* = 123]			
Sex [% men/% women]	55.1/44.9	50.4/49.6	55.5/44.5	41.5/58.5	0.982*	**0.011***†	**0.015***†
Age (median [IQR 25–75])	43 [33–50]	39 [30–49]	36 [27–47]	43 [35–55]	**<0.001**†	0.169	**<0.001**†
Smoking [% yes/% no]	NA	20.1/79.9	26.1/73.9	7.8/92.2	–	–	**<0.001***†
C-RP [mg/mL] (median [IQR 25–75])	–	2.1 [0.9–6.6]	2.60 [1.1–7.90]	1.8 [0.8–4.0]	–	–	**0.022**†
C-RP <3 mg/mL *n* [%]	–	–	92 [46%]	70 [63%]	–	–	–
C-RP ≥3mg/mL *n* [%]	–	–	109 [54%]	42 [37%]	–	–	–
Disease location [%]:	–	–	–	–			
CD [L1/L2/L3]			46.4/13.6/40.0	–	–	–	–
UC [E1/E2/E3]	–		–	33.6/27.1/39.3	–	–	–
Behaviour [%] [B1/B2/B3]	–	–	47.7/22.0/30.3	–	–	–	–
Therapeutics %		–					
5-ASA	–	–	15.9	42.6	–	–	–
Azathioprine	–	–	37.0	33.9	–	–	–
Anti-TNFα	–	–	24.5	12.2	–	–	–
Azathioprine + anti-TNFα	–	–	22.6	11.3	–	–	–
Years of follow-up (median [IQR 25–75])	–	–	8 [4–12]	9 [5–13]	–	–	0.534
Number of flares/years of disease (median [IQR 25–75])	–	–	0.2 [0.1–0.3]	0.16 [0.08–0.27]	–	–	0.551
Time on 5-ASA [months] (median [IQR 25–75])	–	–	139.6 [52.8–217.5]	80.9 [35.4–143.2]	–	–	0.055
Time on azathioprine [months] (median [IQR 25–75])	–	–	40.4 [13.3–82.1]	43.6 [19.4–98.8]	–	–	0.518
Time on anti-TNFα [months] (median [IQR 25–75])	–	–	38.6 [17.0–67.5]	40.4 [13.3–58.6]	–	–	0.599
Time on azathioprine + anti-TNFα [months] (median [IQR 25–75])	–	–	46.2 [24.0–70.8]	17.7 [10.1–43.6]	–	–	0.100

differed in gender distribution [$p = 0.015$], median age [$p < 0.001$], and smoking habits [$p < 0.001$]. Univariate analysis was made to account for confounders, age, and sex, and significant differences [$p < 0.001$] were found for DNA damage in CD (β-value [95% CI]: 5.3 [4.4–6.1]) and UC (β-value [95% CI]: 3.2 [0.2–4.2]) in reference to the control group, and for oxidative DNA damage in CD (β-value [95% CI]: 1.4 [0.6–2.1]) and UC (β-value [95% CI]: 2.6 [1.7–3.6]) in reference to the control group. Disease activity was measured by C-RP levels [cut-off: >3 mg/mL] and number of flares per year of follow-up. The two groups of patients significantly differed for C-RP [$p = 0.022$], with CD exhibiting a higher level and also a larger fraction of subjects with C-RP above the cut-off; no significant differences were found for number of flares per year or years of follow-up [Table 1].

3.2. Oxidative damage markers among IBD population

Oxidative damage markers were measured in all subjects. The IBD population exhibited a statistically significant increase in DNA damage [9.0 AU; IQR: 5.4–13.4] [$p < 0.001$] and a statistically significant increase in oxidative DNA damage [4.0 AU; IQR: 2.1–7.5] [$p < 0.001$], when compared with controls [Figures 2 and 3]. The differences observed for lipid peroxidation and protein carbonylation were not statistically significant, but the TAC was significantly higher in IBD patients than in controls [$p < 0.001$] [Supplementary material – Table 1].

CD patients exhibited a statistically significant increase in DNA damage [9.7 AU; IQR: 6.2–14.0] when compared with the UC population [7.1 AU; IQR: 4.6–11.7] [$p < 0.001$], but significantly lower oxidative DNA damage [3.6 AU; IQR: 1.8–3.8 vs. 4.6 AU; IQR: 2.4–8.1] [$p = 0.048$] [Figures 2 and 3]. No significant differences were found between CD and UC patients for lipid peroxidation, protein carbonylation, or TAC [Supplementary material – Table 1].

Each group of patients was subgrouped according to age [grouped as <20, 21–30, 31–40, 41–50, >50 years] [Supplementary material – Tables 2 and 3], smoking habits [smokers, former smokers, and never smokers] [Supplementary material – Table 4], and therapeutic [5-ASA, azathioprine, anti-TNFα, and combined therapy] [Tables 2 and 3] to look for significant differences.

3.3. CD population

3.3.1. Age

Between the defined age groups, DNA damage was significantly different [$p = 0.002$]; the highest level was found in patients between 21 and 30 years old and the lowest in patients under 20 years of age [Supplementary material – Table 2]. Under linear regression analysis, DNA damage showed a significant negative correlation with age [$r_s = -0.179$, $p = 0.009$]. Number of flares per year of follow-up was found to be significantly different for different age groups [$p < 0.001$], with the group under 20 years exhibiting the highest level and the group over 50 years the lowest [Supplementary material – Table 2].

3.3.2. Disease activity and disease phenotype

Between the C-RP < 3 mg/mL and C-RP≥3 mg/mL groups, DNA damage [9.5 AU; IQR: 6.1–13.9, and 10.7 AU; IQR: 6.8–14.5, respectively; $p = 0.4$] and oxidative DNA damage [4.4 AU; IQR: 2.3–8.5, and 4.2 AU; IQR: 2.2–6.9, respectively; $p = 0.520$] were not statistically different. C-RP levels were not statistically different between age groups, smoking habits, or therapeutic. DNA damage was not found to be significantly different for 'number of flares per year of follow-up' distribution [Supplementary material – Figure 3]. Disease behaviour subgrouping did not reveal a significant difference for DNA damage [B1: 11.1 AU, IQR: 6.3–16.9; B2: 9.6 AU, IQR: 6.8–13.5; B3: 8.9 AU, IQR: 5.9–12.1, $p = 0.056$], nor did location [L1: 9.1 AU, IQR: 5.9–13.9; L2: 9.4 AU, IQR: 6.6–13.4; L3: 10.5, IQR: 6.7–14.9, $p = 0.459$]. Oxidative DNA damage difference was also found to be non-significant for disease behaviour subgrouping [B1: 4.2 AU, IQR: 2.1–6.8; B2: 3.3 AU, IQR: 1.4–5.8; B3: 3.1 AU, IQR: 1.7–5.6, $p = 0.512$] and disease location [L1: 4.0 AU, IQR: 1.5–7.4; L2: 3.1 AU, IQR: 1.7–6.9; L3: 3.2, IQR: 2.1–5.0, $p = 0.840$]. Total antioxidant status was significantly different for

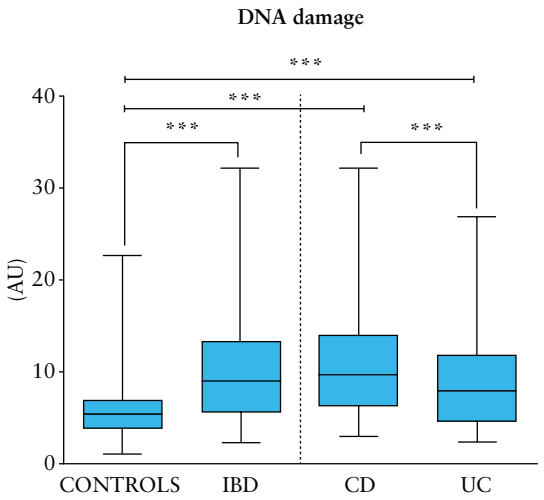

Figure 2. DNA damage measured in controls (5.4 arbitrary units [AU]; interquartile range [IQR]: 3.8–6.8), irritable bowel disease [IBD] population [9.0 AU; IQR: 5.4–13.4], Crohn's disease [CD] [9.7 AU; IQR: 6.2–14.0] and ulcerative colitis [UC] patients [7.1 AU; IQR: 4.6–11.7] [box-plot: medians, IQRs 25–75, max–min] [Mann–Whitney *U* test; ***$p < 0.001$].

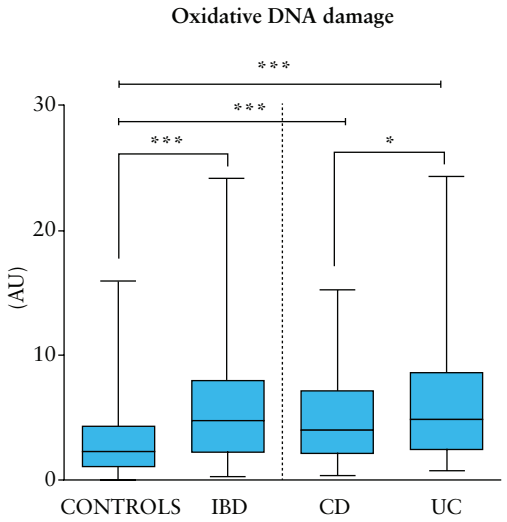

Figure 3. Oxidative DNA damage [median] measured in controls (2.3 arbitrary units [AU]; interquartile range [IQR]: 1.2–4.2), irritable bowel disease [IBD] population [4.0 AU; IQR: 2.1–7.5], CD [3.6 AU; IQR: 1.8–3.8] and UC patients [4.6 AU; IQR: 2.4–8.1] [box-plot: medians, IQRs 25–75, max–min] [Mann–Whitney *U* test; *$p < 0.05$; ***$p < 0.001$].

disease behaviour, with B1 exhibiting the lowest level [1.0 mM; IQR: 0.8–1.2] and B3 the highest [1.1 mM; IQR: 1.0–1.3] [$p = 0.022$].

3.3.3. Smoking habits

The measured oxidative DNA damage in smokers was significantly higher when compared with former-smokers [$p = 0.006$] and never-smokers [$p = 0.018$]. No significant differences were found for DNA damage, lipid peroxidation, protein carbonyls or total antioxidant status [Supplementary material – Table 4].

3.3.4. Therapeutic subgroups

For CD patients, DNA damage was significantly different between therapeutic groups [$p = 0.010$], with azathioprine exhibiting the lowest value, and the combined therapy [azathioprine + anti-TNFα] the highest [Table 2] [Supplementary material – Figure 1]. Significant differences were also found for lipid peroxidation [$p = 0.002$] and total antioxidant status [$p < 0.001$]. Regarding oxidative DNA damage, no statistically significant differences were observed between groups [Table 2]. CD patients on 5-ASA exhibited the highest levels of lipid peroxidation and total antioxidant status. The azathioprine group showed the lowest values for lipid peroxidation. In the anti-TNFα group, we found the lowest total antioxidant capacity [Table 2]. Number of flares per year of follow-up was significantly different between the therapeutic groups [$p = 0.004$], being highest in the combined therapy group and lowest in the 5-ASA group [Table 2] [Figure 4].

3.4. Univariate and multivariate analysis

Univariate analysis of DNA damage predictors [Supplementary material – Table 5] found a significant negative correlation with age

Table 2. Results for oxidative damage markers {DNA damage, oxidative DNA damage, lipid peroxidation (thiobarbituric acid reactive substances [TBARS], protein carbonylation [PCC])}, total antioxidant capacity [TAC], C-reactive protein [C-RP], and number of flares/year of follow-up for CD population stratified for therapy – 5-ASA, azathioprine, anti-TNFα, and combined therapy of azathioprine and anti-TNFα [AU: arbitrary units] {median (interquartile range [IQR] 25–75)}.

Crohn's disease					
N = 208					
	5-ASA	Azathioprine	Anti-TNFα	Azathioprine + anti-TNFα	*p*
	[*n* = 33]	[*n* = 77]	[*n* = 51]	[*n* = 47]	
DNA damage [AU] (median [IQR 25–75])	10.5 [4.8–14.9]	8.7 [6.2–11.8]	10.1 [6.1–18.5]	11.6 [9.5–14.3]	**0.010**†a
Oxidative DNA damage [AU] (median [IQR 25–75])	4.4 [1.4–5.9]	4.0 [2.0–6.8]	3.6 [2.0–8.9]	3.2 [1.6–4.3]	0.613
TBARS [μM] (median [IQR 25–75])	4.8 [4.2–5.4]	4.1 [3.3–5.0]	4.2 [3.6–5.3]	4.6 [4.0–5.7]	**0.002**†b
PCC [nmol/mg prot] (median [IQR 25–75])	0.9 [0.8–0.9]	0.8 [0.8–0.9]	0.8 [0.7–0.9]	0.8 [0.7–0.9]	0.445
TAC [mM] (median [IQR 25–75])	1.2 [0.9–1.3]	1.1 [1.0–1.3]	0.9 [0.8–1.1]	1.0 [0.8–1.3]	**<0.001**†c
C-RP [mg/L] (median [IQR 25–75])	1.3 [0.4–6.0]	1.9 [1.1–7.2]	4.5 [1.3–10.5]	3.1 [1.3–5.5]	0.063
No. flares/years of follow-up (median [IQR 25–75])	0.1 [0.0–0.2]	0.1 [0.1–0.1]	0.2 [0.1–0.3]	0.2 [0.1–0.3]	**0.004**†d

p-values refer to Kruskall–Wallis test. †Statistically significant [$P < 0.05$];
p-values for multiple comparisons with Bonferroni *post hoc*:
†a – Azathioprine vs. azathioprine + anti-TNFα [$p < 0.001$];
†b – 5-ASA vs. azathioprine [$p = 0.048$]; azathioprine vs. azathioprine + anti-TNFα [$p < 0.001$];
†c – 5-ASA vs. anti-TNFα [$p = 0.018$]; azathioprine vs. Anti-TNFα [$p < 0.001$];
†d – 5-ASA vs. azathioprine [$p = 0.012$]; 5-ASA vs. anti-TNFα [$p = 0.03$]; 5-ASA *vs* azathioprine + anti-TNFα [$p < 0.006$].

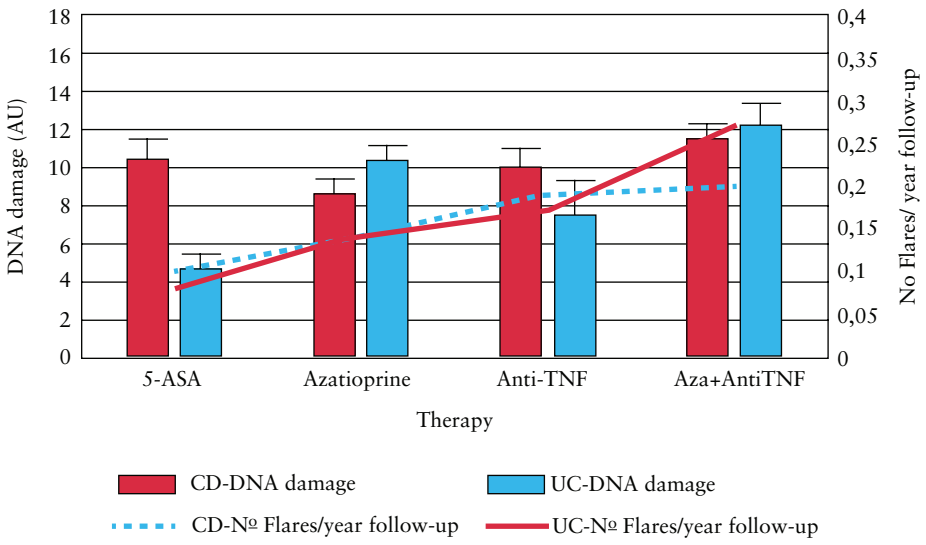

Figure 4. Distribution of DNA damage [median + SEM] and Disease Activity [No. of flares/year of follow-up] for Crohn's disease [CD] and ulcerative colitis [UC] patients.

and significant association with disease behaviours B1 [inflammatory] and B2 [structuring] [$p < 0.001$ and $p = 0.003$, respectively]. Under univariate analysis of oxidative DNA damage [Supplementary material – Table 6], we found positive significant correlation for A3 [over 40 years old] group of 'age at diagnosis' [$p = 0.015$], for smokers, and for sex. On multivariate analysis [Supplementary material – Table 5], no significant associations were found for DNA damage; however, for oxidative DNA damage [Supplementary material – Table 6], a significant association was found with the A3 group of 'age at diagnosis' [over 40 years old] [$p = 0.025$], and sex.

3.5. UC population

3.5.1. Age

Between the defined age groups, no significant differences were observed for the studied oxidative damage markers [Supplementary material – Table 3]. Under linear regression analysis, DNA damage and lipid peroxidation showed a significant negative correlation with age [$r_s = -0.211$, $p = 0.020$ and $r_s = -0.190$, $p = 0.039$, respectively]. Number of flares per year of follow up was found to be significantly different for the various age groups distribution [$p = 0.005$], with the 21–30 years age group exhibiting the highest level and the group over 50 years old exhibiting the lowest [Supplementary material – Table 3].

3.5.2. Disease activity and disease phenotype

Between the C-RP < 3 mg/mL and C-RP ≥ 3 mg/mL groups, DNA damage [7.7 AU; IQR: 4.6–11.8, and 7.7 AU; IQR: 4.2–11.0, respectively; $p = 0.707$] and oxidative DNA damage [4.4 AU; IQR: 2.5–8.3, and 5.3 AU; IQR: 2.6–9.3, respectively; $p = 0.512$] were not statistically different. C-RP levels were not statistically different between age groups, smoking habits, or therapeutic group. DNA damage was found significantly different for 'number of flares per year of follow-up' distribution [$p = 0.012$] [Supplementary material – Figure 4] and for disease location with E1 exhibiting the lowest level [4.2 AU; IQR: 3.3–5.0] and E2 the highest [8.3 AU; IQR: 4.7–12.4] [E3: 8.0 AU; IQR: 4.6–12.1] [$p = 0.005$].

3.5.3. Smoking habits

We found no significant differences for any of the oxidative damage markers or TAC between these groups. It is noteworthy that in the UC group, the number of smokers is quite small [7.8%], thus possibly misleading [Supplementary material – Table 4].

3.5.4. Therapeutic subgroups

In UC patients, DNA damage was found to be significantly different [$p < 0.001$] between the various therapeutic options [Supplementary material – Figure 2] [Table 3]; the lowest value was obtained for the 5-ASA group and the highest for the combined therapy [azathioprine + anti-TNFα]. Differences in oxidative DNA damage, lipid peroxidation, protein carbonylation, and TAC were non-significant [Table 3]. The number of flares per year of follow-up was found to be significantly different between the therapeutic groups [$p < 0.001$], being highest for the combined therapy group and lowest for the 5-ASA group [Table 3] [Figure 4].

3.6. Univariate and multivariate analysis

Associations with DNA damage were found under univariate analysis for age, therapeutic groups, disease locations [E2 and E3], 'age at diagnosis' [A3 group], and disease activity measured by number of flares per year of follow-up [Supplementary material – Table 7]. Under univariate analysis, association with oxidative DNA damage was found for smokers, yet due to the small number of included subjects [$n = 9$], these results should be considered cautiously [Supplementary material – Table 8].

Multivariate analysis for factors associated with DNA damage [Supplementary material – Table 7] found significant β-values for therapeutic groups and for 'sex'. No significant associations were found for oxidative DNA damage under multivariate analysis [Supplementary material – Table 8].

4. Discussion

To our best knowledge, this is the largest study performed over a representative IBD population assessing both peripheral and oxidative DNA damage and involving a stratified approach to analysing the

Table 3. Results for oxidative damage markers {DNA damage, oxidative DNA damage, lipid peroxidation (thiobarbituric acid reactive substances [TBARS]), Protein carbonylation [PCC]}, total antioxidant caspacity [TAC], C-reactive protein [C-RP], and number of flares/year of follow-up for UC population stratified for therapy – 5-ASA, azathioprine, anti-TNFα, and combined therapy of azathioprine and anti-TNFα [AU: arbitrary units] {median (interquartile range [IQR] 25–75)}.

Ulcerative colitis					
N = 115					
	5-ASA	Azathioprine	Anti-TNFα	Azathioprine + anti-TNFα	*p*
	N = 49	*N* = 39	*N* = 14	*N* = 13	
DNA damage [AU] (median [IQR 25–75])	4.7 [3.6–7.1]	10.4 [7.6–13.6]	7.5 [3.1–17.0]	12.4 [10.6–15.0]	**<0.001**†a
Oxidative DNA damage [AU] (median [IQR 25–75])	4.4 [2.2–9.1]	5.9 [2.3–8.1]	4.2 [1.3–8.5]	4.4 [2.5–6.6]	0.945
TBARS [μM] (median [IQR 25–75])	4.6 [3.6–5.4]	4.6 [3.4–5.2]	5.4 [5.1–6.3]	4.5 [3.8–5.1]	0.05
PCC [nmol/mg prot] (median [IQR 25–75])	0.8 [0.8–0.9]	0.8 [0.7–1.0]	0.9 [0.7–1.0]	0.8 [0.7–1.0]	0.957
TAC [mM] (median [IQR 25–75])	1.0 [0.9–1.2]	1.1 [0.9–1.3]	1.0 [0.9–1.1]	1.0 [0.8–1.3]	0.553
C-RP [mg/L] (median [IQR 25–75])	2.2 [0.8–4.0]	1.9 [0.8–3.9]	1.8 [0.7–8.0]	1.1 [0.4–2.1]	0.244
No. flares/years of follow-up (median [IQR 25–75])	0.1 [0.0–0.2]	0.1 [0.1–0.4]	0.2 [0.1–0.3]	0.3 [0.2–0.5]	**<0.001**†b

p-values refer to Kruskall–Wallis test. †Statistically significant [$p < 0.05$];
p-values for multiple comparisons with Bonferroni *post hoc*:
†a – 5-ASA vs. azathioprine [$p < 0.001$]; 5-ASA vs. azathioprine + anti-TNFα [$p < 0.001$];
†b – 5-ASA vs. azathioprine [$p < 0.001$]; 5-ASA vs. azathioprine + anti-TNFα [$p = 0.006$].

results. Our findings confirmed that IBD was related to higher levels of peripheral and oxidative DNA damage, when compared with a healthy population. These increases differed between CD and UC patients. Peripheral DNA damage was significantly higher in CD than in UC, yet oxidative DNA damage was significantly higher in UC patients. The observed differences between CD and UC may indicate that the systemic involvement of CD results in higher steady-state levels of DNA damage. Moreover, the higher oxidative DNA damage observed in UC patients might indicate some downregulation of DNA repair systems, as studies have shown that defects in the ability to repair 8-oxo-7,8-dihydro-2'-deoxyguanosine [8-oxo-dG] result in the accumulation of endogenously produced oxidized DNA bases, and increased susceptibility to tumour development.[15,16] Oxidative DNA damage levels in IBD patients has already been reported in small studies measuring 8-hydroxy-2'-deoxyguanosine [8-OHdG] either in CD or UC patients' colon biopsies or plasma, all of them observing higher levels in patients compared with controls and/or higher levels in inflamed tissues compared with non-inflamed tissues.[17–21] Risques *et al.*[22] found significantly higher levels of DNA damage in the colonocytes of UC patients between 30 and 59 years when compared with controls, but failed to relate this increase to duration, age of disease onset, disease activity, or medication. Unlike other studies,[20,23–29] in our work no significant differences for lipid or protein oxidation products between patients and controls were observed. However, some inconsistency is found in the literature as others[30,31] also report no significant differences for lipid peroxidation levels.

Regarding 'age at diagnosis', univariate analysis showed it to be an individual risk for oxidative DNA damage in CD patients and for DNA damage in UC patients, but with contrasting effects—for CD A3 [over 40 years] is the risk group, whereas in UC A3 is protective against DNA damage. Some cancers have been associated with young age of disease onset for IBD, e.g. cervical dysplasia and non-Hodgkin lymphoma.[32] Nevertheless, Fries *et al.*[33] report that IBD patients diagnosed over the age of 40 years more frequently develop tumours [$p < 0.000$], and Baars *et al.*,[34] in a large retrospective study with 251 IBD patients with diagnosed IBD-related colorectal cancer [CRC], found older age at onset of IBD to be a risk factor for developing CRC. Taken all together, these facts indicate that age at diagnosis might be a useful predictor for oxidative DNA damage burden through disease course, and as seen before, through DNA repair failure. Our results also revealed that in CD, disease behavior B1 [inflammatory] and B2 [structuring], and in UC, disease locations E2 and E3, and disease burden, namely number of flares/year of follow-up, constitute risk factors for DNA damage, indicating that inflammation is a major contributor to DNA damage in IBD patients. Although a clear definition for aggressive UC has not yet been determined, it is known that in UC the severity of the disease is proportional to the amount of inflamed colon involved, and some studies show that E2 and E3 phenotypes are associated with more complicated disease.[35] Moreover, regarding IBD, the extent of inflammatory lesions, the duration of the disease, and the presence of active chronic inflammation have all been pointed out as risk factors for CRC.[36] Furthermore, we found significant differences in peripheral DNA damage between the therapeutic groups, both in CD and UC. The combined therapy with azathioprine and anti-TNFα exerted the highest levels of peripheral DNA damage, both in CD and UC. The lowest DNA damage levels were found for the azathioprine group in CD patients and for the 5-ASA group in UC patients. UC patients on 5-ASA, besides having the lowest number of flares per year of follow-up [i.e. less active disease], exhibited the lowest level of DNA damage; the value found was even lower than the DNA damage found in controls, and this could be attributed to the antioxidant and free radicals scavenging action of 5-ASA. Concerns for therapeutic-related carcinogenicity have reported in the literature. Azathioprine, anti-TNF, and the combination of both drugs have been associated with increased risk of lymphomas.[37] In IBD patients, high risk for non-melanoma skin cancer[38] and lymphomas[39,40] have been related to exposure to azathioprine. In addition to lymphomas, anti-TNF has been associated with increased risk for melanoma[37]; combined therapy of azathioprine and anti-TNFα has been associated with a specifically increased risk for hepatosplenic T-cell lymphoma.[41] Our results showed that CD patients had an increased level of DNA damage when undergoing anti-TNFα therapy, compared with azathioprine, and the highest DNA damage level for the combined therapy, both in CD and UC; despite this, oxidative DNA damage levels did not differ between all groups.

5. Conclusion

The strengths of this study lie in the well-established cohort, the prospective nature of the study, and the fact that it is one of the largest studies performed measuring oxidative damage markers in IBD patients. Nevertheless, the small number of UC patients in some of the subgroups, namely smokers and patients under 16 years of age, constitutes a weakness that could only be circumvented by including patients from other referral centres. In view of our results, we concluded that DNA damage was evident among IBD patients, and that age at diagnosis, inflammatory burden, and therapeutic options might be associated with DNA damage levels. The predictors found for DNA damage and oxidative DNA damage might be useful in the assessment of individual susceptibilities to IBD-related cancers. Moreover, the oxidative DNA damage levels in UC patients may indicate some impairment of the DNA repair systems in these patients, and this should be thoroughly assessed in future research. Although establishing the occurrence of oxidative damage is an important step in determining the pathophysiology of the disease, it does not determine whether oxidative stress is a cause or a consequence of the disease. Yet as a first step, it might open ways to explore these pathways and promote research studies targeting the benefits of and the potential for developing pharmacological antioxidant agents, as well as alerting physicians to potential implications concerning the disease phenotype.

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Conflict of Interest

Professor Magro has received fees for speaking engagements from ScheringPlough/MSD, Abbvie, Lab Vitória, and Dr Falk Pharma Portugal, and fees for consultancies and honoraria from MSD.

The other authors state no conflicts of interest.

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Author Contributions

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Carla Costa, João Paulo Teixeira and Guilherme Macedo: Critical revision of the manuscript for important intellectual content.

Fernando Magro: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; study supervision; critical revision of the manuscript for important intellectual content.

Supplementary Material

Supplementary data to this article can be found at *ECCO-JCC* Online.

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