Reduced levels of genomic damage in young martial artists

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Abstract

The impact of physical activity on the levels of genomic damage is still poorly understood. In this work, we aimed to investigate the influence of a constant martial arts training on the levels of DNA damage. Moreover, we explored the possible association between genomic damage and single-gene polymorphisms on metabolic and DNA repair genes. To do this, we compared the frequency of micronuclei and other nuclear aberrations in the buccal mucosa cells of 35 healthy, young martial artists with 35 healthy, young sedentary controls. Additionally, we genotyped all participants for three metabolic and two DNA repair genes to evaluate the impact of the relative single-gene polymorphisms on DNA damage. Genomic damage was significantly lower in athletes than in sedentary controls, as evidenced by a decrease in both micronuclei and total aberrations. Instead, single-gene polymorphisms did not significantly alter the frequencies of aberrations. These findings suggest that training may have a protective effect against genomic damage, supporting the health benefits provided by physical activity. The influence of other factors should be considered, and further studies focusing on disciplines with different training regimes are necessary to evaluate thoroughly their influence on genomic damage.

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Contributions: AN, sampling, polymorphisms analyses, statistical analyses, manuscript writing, and revision; ML, sampling, statistical analyses, graphs, manuscript writing and revision; AS, study design, polymorphisms analyses, micronuclei count, manuscript writing and revision.

Conflict of interest: the authors declare that they have no competing interests.

Ethics approval and consent to participate: this work has been approved by the local committee responsible for human experimentation (protocol n° 0609375 of 10/28/2021), and all procedures were carried out according to the 2013 Helsinki Declaration. Informed consent was obtained from all individual participants involved in the study.

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Introduction

Sportive activity (both aerobic and anaerobic) is well known to elicit the production of reactive oxygen and nitrogen species (RONS), whose overproduction can disrupt the intracellular redox equilibrium, thus causing oxidative stress.[1] However, RONS overproduction caused by chronically repeated physical exercise is thought to be a signal for the upregulation of the antioxidant system,[1] whose activity has shown considerable increases in athletes.[2-4] The antioxidant response also depends on the training loads: in fact, it was found that while an excessive frequency of training is associated with a constant oxidative insult, a moderate one can stimulate the antioxidant repair systems without implying severe oxidative stress.[3] Both oxidative stress and the unregulated antioxidant system's activity can, in turn, affect the levels of DNA damage.[1,5]

Among the many tests available to assess genotoxicity and study DNA damage, the micronucleus test is one of the most versatile and important ones.[6] Micronuclei (MNi) are small nucleus-like bodies that consist of a membrane containing chromosomes or chromosome fragments that were not included in the main nucleus after a cell division.[7] MNi have been identified as a source of genome rearrangements such as chromothripsis.[7] Moreover, many case-control studies have demonstrated an increase in MNi frequency in most types of cancers.[8,9] High MNi frequencies have also been correlated with many other pathological conditions and/or diseases, such as infertility, diabetes, chronic kidney disease, and cardiovascular and neurological diseases.[8,9]

Oxidative stress can lead to DNA damage and thus to increased levels of MNi.[5,10,11] To date, little literature investigated the correlation between sportive activity and MNi frequency, and

the majority of published studies refer to the variation in MNi frequency in peripheral blood lymphocytes following physical activity.[12] However, studies investigating the role of physical activity in modulating the basal frequency of MNi in buccal mucosa cells remain scant. Besides, the correlation between MNi frequency and training appears to lack a clear and consistent pattern.

Indeed, for example, it was found no influence of a six-month resistance-training period on the frequency of MNi in buccal cells of institutionalized elderly, while an increase in chromosomal damage was found with the buccal micronucleus cytome assay in professional hockey and baseball-softball players, relatively to sedentary controls.[13,14] This discrepancy may be due to the different age and physical requests of the two training regimes. Other authors compared MNi frequency in lymphocytes of professional road-racing cyclists with non-agonists (subjects with a lower weekly training frequency) and found higher values in the former ones.[3]

This study aimed to evaluate whether physical activity plays a role in the modulation of the MNi frequency levels in buccal mucosa cells of young martial artists. Given the results of previous studies, [3,14] we would expect an increase in MNi frequency in athletes (AT). This increase should be particularly important in individuals who train more, while in less training AT the upregulation of the antioxidant system may compensate for the production of RONS: we will also discuss this possibility.[3]

Moreover, it has been demonstrated that the level of genomic damage also depends on the different individual susceptibility, and single-gene polymorphisms can influence the susceptibility to MNi formation.[15,16] This susceptibility is in large part due to polymorphisms in metabolic genes, such as cytochrome P450 (*CYP*) and glutathione-S-transferase (*GST*s) family genes, or DNA repair genes as *XRCC* and *XPC*.[16-19]

GSTs genes in humans are polymorphic due to a deletion which causes the inability to synthesize the protein; this deletion has been correlated to an increase in DNA damage susceptibility.[20-22]

CYP1A1 gene is fundamental for the metabolism of polycyclic aromatic hydrocarbons, present in cigarette smoke. One of the known polymorphisms at *the CYP1A1* gene consists of a point mutation (A>G) at codon 462 of exon 7, with the subsequent replacement of an isoleucine with a valine.[23]

XRCC1 is a molecular scaffold protein involved in the repair of many DNA single-strand breaks. Among the many *XRCC1* gene polymorphisms, one of the most studied is Arg194Trp on exon 6.[24,25] Regarding this polymorphism, higher levels of MNi have been found in subjects carrying the *XRCC1* 194 Arg/Trp and Trp/Trp genotypes with respect to those carrying the *Arg/Arg* genotype in workers exposed to 1,3-butadiene.[24]

XPC (*i.e.* Xeroderma Pigmentosum group C) gene belongs to the Nucleotide Excision Repair (NER) pathway; this gene encodes a protein binding damaged DNA in apoptosis and the first phases of DNA repair. The Lys939Gln polymorphism of this gene consists of an A>C transition at codon 939 of exon 15.[26-28]

Due to the variation of metabolism and the oxidative stress resulting from physical activity, we expect metabolic and DNA repair genes to be particularly important in athletes.

For these reasons, we also decided to genotype all subjects involved in this study for the following polymorphisms: *GSTT1*, *GSTM1*, *CYP1A1* (Ile/Val), *XRCC1* (Trp194Arg) and *XPC* (Lys939Gln). We analyzed the possible correlation between those polymorphisms and the occurrence frequency of MNi to evaluate their role in predisposing carriers to DNA damage.

Lastly, dietary supplementation is known to be able to reduce the levels of DNA damage.[29,30] Given that 13 of the 35 sampled AT made use of dietary supplements, we compared the levels of cytogenetic damage of the two AT subgroups to exclude a possible role of this factor in reducing aberrations frequencies.

Materials and Methods

Subjects

The sample of this study was composed of 35 healthy agonists practicing martial arts (both striking and wrestling disciplines) and 35 control healthy sedentary subjects. Both the athlete group and the control group were composed of 28 men and 7 women. Athletes (AT) trained from a minimum of 3 to a maximum of 11 times per week (5.657 ± 2.802), while sedentary controls (SC) did not train at all. Each training session lasted at least 90 min; during most of them AT alternated phases of aerobic training, anaerobic training, and rest; few trainings were instead merely based on aerobic (run) or anaerobic (weightlifting) training. The age of the subjects ranged from 18 to 36 years (mean age 22.686 ± 4.276 and 22.857 ± 4.188 for AT and SC, respectively; Table 1).

All participants were informed volunteers and were asked to fill out a questionnaire where data regarding age, sex, training frequency, radiation exposure, supplements, alcohol, diet (meat, fruit, and vegetable consumption), and drug consumption were collected. All subjects were non-smokers, non-alcoholics, were not under any medication, and had not been exposed to X-rays on the cranial region in the last year. Apart from the training activity, all the above-mentioned lifestyle traits of the two sampled groups (AT and SC) were comparable, and all participants lived in the Turin province (thus being subject to the same pollution exposure). This work has been approved by the local committee responsible for human experimentation (protocol n° 0609375 of 10/28/2021), and all procedures were carried out according to the 2013 Helsinki Declaration.

Table 1. Demographic and training characteristics of the studied groups.

Characteristics Subjects	Athletes 35	Sedentary controls 35
Sex		
Men	28	28
Women	7	7
Age		
Total sample		
Mean years±S.D.	22.69±4.28	22.86±4.19
Range (years)	18-34	18-36
Men		
Mean years±S.D.	23.18±4.48	23.14±4.58
Range (years)	18-34	18-36
Women		
Mean years±S.D.	20.71±2.81	21.71±1.70
Range (years)	18-25	20-25
Trainings per week		
Total sample		
Mean±S.D.	5.66±2.80	/
Range	3-11	/
Men		
Mean±S.D.	5.27±2.57	/
Range	3-11	/
Women		
Mean±S.D.	7.21±3.36	/
Range	3-11	/

S.D., standard deviation.

MNi assay

We made buccal epithelial smears to gather buccal mucosa cells by scraping for 1 minute a toothbrush on the inner side of one cheek of each participant. The tip of the toothbrush was then immediately immersed in a fixative solution composed of methanol/acetic acid 3:1. Test tubes were thus brought to the laboratory, where they were centrifuged and the supernatant was sucked away. The pellet remained in a small amount of fixative, which was aspired with Pasteur pipettes and dripped on slides. Finally, dried slides were stained with 5% Giemsa (pH 6.8) prepared in Sörensen buffer to proceed with the MNi assay.

We performed the microscopic analysis using a light microscope at 1,000x magnification. For each subject we evaluated 1,000 cells with preserved cytoplasm; among these cells, we looked for micronuclei (Figure 1A), nuclear buds (Figure 1B), notched nuclei (indentation, Figure 1C) and broken egg nuclei (Figure 1D). These nuclear anomalies were photographed, and photos were inspected on the computer screen to validate their presence.

DNA extraction and polymorphism analysis

After the toothbrush smear, another smear was performed with a buccal swab. The swab was scraped for 1 minute in the inner side of the cheek of each participant, and then immediately immersed in an eppendorf with 400 µL of lysis solution (10 mM Tris pH 7.6; 10 mM EDTA pH 8; 50 mM NaCl). DNA was extracted according to the following protocol: 7.5 µL of SDS 20% and 15 µL of 10 mg/mL proteinase K were added to the solution, which then was heated at 56°C for 1 hour. Then, 400 µL of chloroform was added, everything was shaken and then centrifuged for 10 min at 640 × g. The supernatant was aspirated and added to a new eppendorf, where an equal volume of isopropanol was added; the eppendorf was gently shaken for 1 min, and then centrifuged at 640 × g for 20 min. The supernatant (isopropanol) was aspired, and 400 µL of 75% ethanol was added. Everything was centrifuged for 15 min, then ethanol was inspired and the pellet was left to completely dry at room temperature. Finally, the pellet was suspended in 50 µL of Tris-EDTA and, thus, it was ready for the PCR analysis.

We genotyped all subjects for *CYP1A1*, *GSTT1*, *GSTM1*,

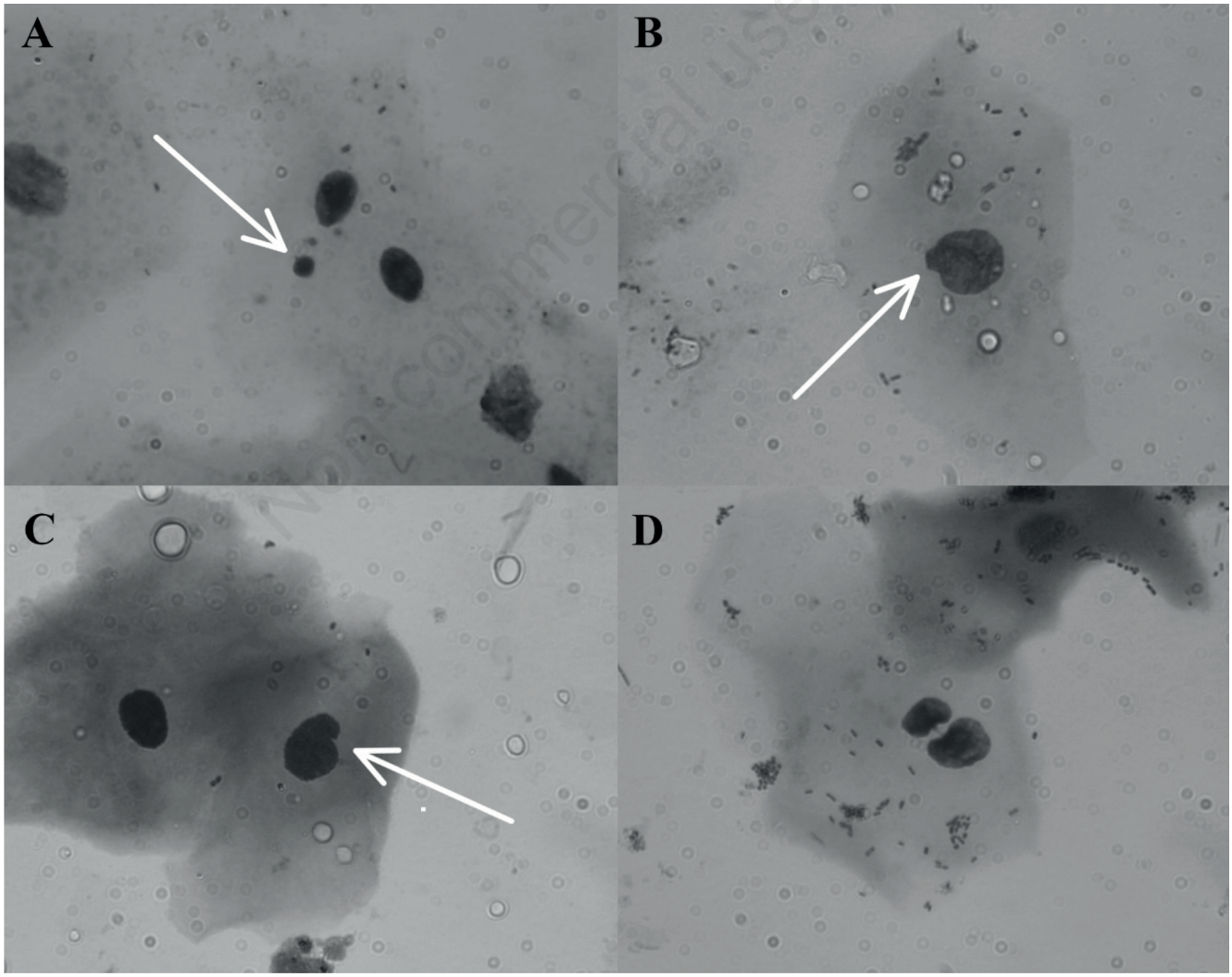

Figure 1. Analyzed nuclear aberrations. A) micronucleated cell; B) cell with a nuclear bud; C) nuclear indentation; D) broken egg nucleus. Pictures were taken at a magnification of 1000x.

XRCC1 Trp194Arg, and *XPC*. Primers and methodologies were described by Scarfò *et al.*[31] (see Supplementary Materials, Table 1 for further details).

For PCR reactions, we used 2 µL of the solution containing DNA. PCR reactions were performed in a final reaction volume of 25 µL, containing 1× reaction buffer, 1.5 mM MgCl2, 5% DMSO, 250 µM dNTPs, 0.5 µM of each primer, 1 U/sample of Taq DNA polymerase (Fischer Scientific, Milan, Italy), and sterile water until reaching the final volume of 25 µL. The PCR cycles were set as follows: 95°C for 5 min, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. The amplification products were electrophoresed using 2% agarose gel and detected by ethidium bromide staining. Negative PCRs were repeated at least once to validate their negativity.

Statistical analysis

Statistical analyses were performed with the R Studio interface for R software (version 4.0.2), and the significance level was set as $p<0.05$. We performed the Shapiro test to verify the normality of the distribution of nuclear aberrations in the two groups. Since the distribution was never found to be normal, we carried on the Wilcoxon-Mann-Whitney test (using the wilcox_test function in the "coin" package) to search for differences in the frequencies of the above mentioned aberrations between AT and SC. We applied the Wilcoxon-Mann-Whitney test also to evaluate whether genetic polymorphisms could influence the frequency of nuclear damage and to exclude, within the AT group, a possible influence of dietary supplements.

Spearman test was performed to investigate a correlation between nuclear aberrations and age or weekly training sessions.

Results

Sports influence on nuclear aberrations

We analyzed 35,000 buccal cells for each studied category, for a total of 70,000 cells observed. Frequencies of the analyzed aberrations in the two groups are shown in Figure 2. MNi were found to be significantly higher in the SC group with respect to the AT group ($p <0.001$); however, the frequency of the other 3 analyzed nuclear aberrations (nuclear buds, notched nuclei, and broken egg nuclei) was not found to be statistically different between the two groups. The total number of aberrations was significantly higher in the SC group ($p = 0.01$, see Figure 2). We report in Supplementary Materials (Table 2) the frequencies of aberrations in the two groups and the men/women subgroups.

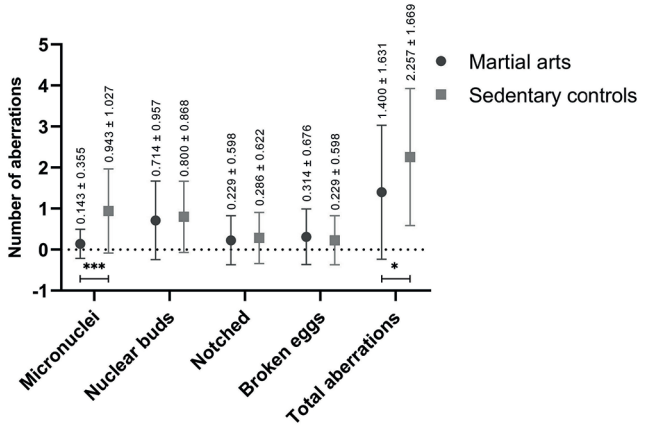

Figure 2. Frequency of nuclear aberrations in the two study groups. *p<0.05; ***p<0.001.

Genetic polymorphisms and nuclear aberrations

Figure 3 shows correlations between the studied polymorphisms and the incidence of cytogenetic damage. Overall, the genetic polymorphisms object of this study did not induce any significant difference in the aberration frequencies (see Figure 3A-3C, 3E), except for *XRCC1* and broken egg nuclei: subjects carrying the mutated allele of this gene showed a slightly but significantly higher frequency of broken egg nuclei ($p = 0.045$, see Figure 3D). Supplementary materials (Table 3) report p-values for each polymorphism and each aberration.

Age, sex, weight, training sessions per week, and dietary supplements

Spearman test did not reveal any significant correlation between the levels of cytogenetic damage and age, sex, or weight. We didn't find any correlation between the number of training per week and the frequency of analyzed aberrations (Supplementary Materials, Table 4). Dietary supplementations did not imply a reduction in the levels of aberrations; conversely, AT undertaking dietary supplements showed a slight but significant increase in notched nuclei relative to the other, non-supplementing, AT subgroup ($p = 0.040$, see Figure 3F).

Discussion

It is well known that physical activity can lead to many benefits for human health; these benefits include the prevention of obesity and cardiovascular and tumor diseases.[32] However, the molecular relationship between training and the subsequently gained health benefits is not fully understood.[33] In this context, the production of RONS has ambivalent effects: it is important for signaling pathways and the upregulation of the antioxidant system,[1,34] but on the other hand, overproduction can lead to protein, lipid, and DNA damage.[35] It follows that although the activity of the antioxidant system can be enhanced in subjects practicing sports,[2-4] an excessive training load can bring unbearable levels of RONS and subsequent genomic damage.[2,10,11] In this scenario, to date it is yet not fully understood how physical activity can influence the levels of antioxidant response, and the correlation between training and genomic damage remains univocal. Our results are (at least partially) in disagreement with the ones obtained by other authors.[3,14] Sharma *et al.* found higher levels of cytogenetic aberrations in hockey and baseball-soft ball players relative to sedentary controls;[14] however, these authors carried out the test on professional players who trained 4-6 hours a day, while athletes in our AT group didn't maintain such a high training frequency. Hence, it is probable that sampling martial artists with a higher training frequency could lead to different results. On the other hand, Pittaluga *et al.* did not include a sedentary control group in their study but found an increase in the basal levels of MNi in professional athletes with respect to non-agonists;[3] instead, we were not able to find a significant relationship between training frequency and cytogenetic damage within the AT group at the Spearman test. Nevertheless, the relationship

between weekly training sessions of the AT group and MNi frequency, albeit not significant, was negative (rho = -0.295 and p = 0.086, see Supplementary Materials, Table 4). These differences could be explained by the training regime required by the nature of each discipline, as even the nature of the sport (aerobic/anaerobic/mixed) and the training duration may influence the results. Different levels of oxidative stress biomarkers have been found in athletes practicing different disciplines.[36,37] Moreover, the level of oxidative stress also depends on the training load.[2] The variable biochemical and cellular responses to the different sport types are thus anything but univocal and could explain our results.

Besides, our results could be exacerbated by the fact that sampling occurred in the first two years of the Covid-19 pandemic, during which lockdowns sometimes led people to adopt a series of unhealthy habits (as reducing the hours of sleep),[38] which are typically correlated to an increase in genomic damage,[39,40] but not easily detectable by a questionnaire. In this context, practicing sportive activity could have represented an outlet for people who were authorized to keep on doing it, and may have prevented them from such unhealthy behaviors. Albeit these possible explanations, the relationship between sportive activity and genomic damage is far from being clear, and more studies are needed to better understand the cellular benefits and damages induced by a certain training regime.

According to available literature, MNi frequency increases in women with respect to men with aging.[41] However, the relationship between MNi frequency and sex is not univocal, especially with younger individuals.[42,43] Our sample deals with young subjects, for which the above-mentioned increment for women shouldn't be already evident, and the limited number of involved subjects (7

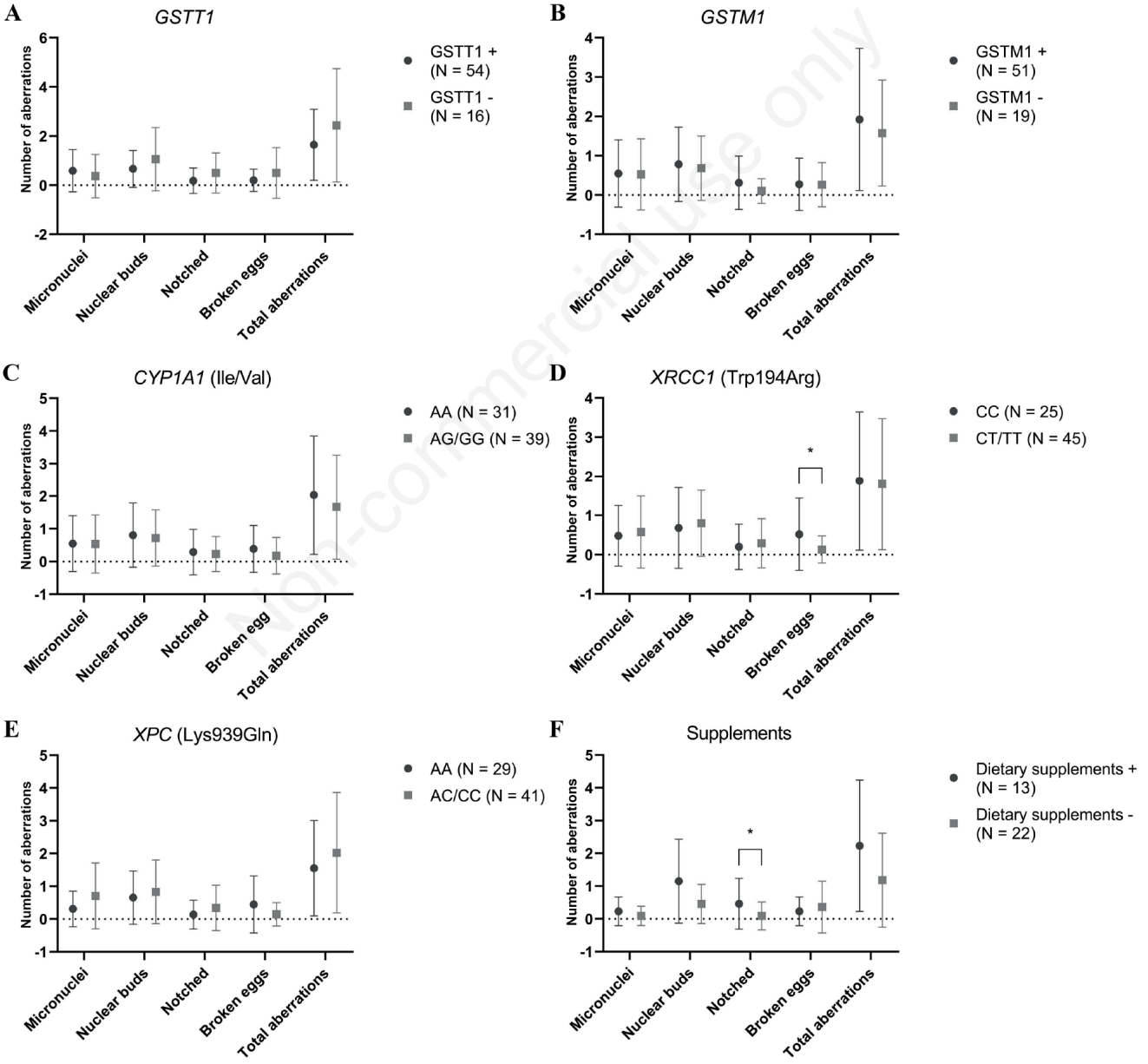

Figure 3. A-E): frequency of nuclear aberrations in subjects carrying the different single-gene polymorphisms; F): frequency of nuclear aberrations compared in the two AT subgroups: subjects undertaking dietary supplements or not. *p<0.05.

women and 28 men) prevents us from defining a neat correlation with sex. The outcome of our analyses indicates a minor mean number of micronuclei for women with respect to men.

Regarding polymorphism analysis, despite the analyzed polymorphisms are well known to influence the levels of genomic damage,[16,19] their influence in our sample was not relevant. The absence of any correlations (except for *XRCC1* and broken eggs, see Figure 3 D) between a particular allele and an increase/decrease of aberrations helps to better understand the great magnitude of influence of the training status on the observed differences in genomic damage between SC and AT. In fact, despite our sample may have been too small to detect specific allele-mediated differences in the levels of genomic damage, the differences in MNi and total aberrations between SC and AT were significant.

Conclusions

We compared the levels of nuclear aberrations between a group of 35 sedentary controls and one of 35 martial artists. We suggest that practicing martial arts can lead to a reduction of genomic damage, at least in terms of MNi and total aberrations; this reduction was particularly relevant in our sample, given that (predictable) influences of single-gene polymorphisms on nuclear aberrations were instead not found. Our results are partially in disagreement with the ones obtained by other authors,[3,14] who evaluated the frequencies of MNi in athletes of other disciplines. These differences may be due to the different training frequencies of the sampled subjects, as well as to the variable training regime required by each sport and to the positive effects that sport may have provided to athletes' habits and behaviors during Covid-19 lockdowns. However, other studies on subjects practicing different disciplines are necessary to draw general conclusions about the influence of training on the levels of genomic damage.

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Online supplementary material:

Table 1. Gene polymorphisms, reference sequence (rs) numbers, Primers, annealing temperature and genotypig methodologies for each of the analysed polymorphisms.

Table 2. Frequencies of aberrations in the two groups and in the males/females subgroups.

Table 3. Correlation between polymorphisms/supplements intake and the frequency of analyzed aberrations.

Table 4. Correlation between the number of trainings per week and the frequency of analyzed aberrations.