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3 Evidence of genotoxicity in lymphocytes of non-smoking alcoholics

4 Alfredo Santovito · Piero Cervella ·
5 Massimiliano Delpero

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8 **Abstract** Alcohol abuse is a significant public health
9 issue. Epidemiological studies conducted on different
10 populations consistently showed that consumption of
11 alcoholic beverages is associated with cytogenetic damages
12 and higher risk for several types of cancer. However, the
13 interpretation of many cytogenetic studies resulted com-
14 plicated because some confounding factors, such as
15 smoking habit, are not always taken into account. In the
16 present study, the frequency of sister chromatid exchanges
17 (SCEs), chromosome aberrations (CAs) and micronuclei
18 (MNs) in cultured human lymphocytes was assessed on 15
19 alcoholic and 15 non-alcoholic control male subjects.
20 Moreover, considering the implication of the Glutathione
21 S-transferases gene polymorphisms in the genetic suscep-
22 tibility to alcoholic liver diseases, we considered an
23 important issue to evaluate the relationship between these
24 gene polymorphisms and the cytogenetic damage. In our
25 sample we exclusively considered individuals that did not
26 smoke nor consume drugs for a period of at least 2 years
27 prior to the analysis. Statistically significant differences
28 were found between alcoholics and controls in the fre-
29 quency of SCEs/cell ($P = 0.001$), RI value ($P = 0.001$),
30 CAs ($P = 0.002$) and CAB ($P = 0.002$). Vice versa, no
31 significant differences were found between alcoholics and
32 controls in terms of MNs frequency and CBPI value. In
33 both samples, no statistically significant association was
34 found between the analysed GSTs gene polymorphisms
35 and the frequencies of MNs, SCEs and CAs. Finally,
36 among alcoholics we found a positive correlation between

SCEs and CAs frequencies and the duration of alcohol 37
abuse. 38

Keywords Micronuclei · Sister chromatid exchanges · 40
Chromosomal aberrations · Genotoxicity · Alcohol 41

42 Introduction

43 Alcohol abuse is a significant social and public health concern. 43
44 In 1988, the International Agency for Research on Cancer 44
45 (IARC) classified alcoholic drinks as group I carcinogens in 45
46 humans for the upper airways, digestive tract and liver [20]. 46
47 Excessive chronic ingestion of ethanol was also associated 47
48 with serious damages to most of the major organ systems such 48
49 as the liver, pancreas, thyroid, pituitary glands, and adrenal 49
50 gland [1, 8, 21, 32, 33]. From a cytogenetic point of view, 50
51 ethanol was shown to induce sister chromatid exchanges 51
52 (SCEs), micronuclei (MNs), and aneuploidy in mouse eggs 52
53 [38]; mis-segregation and/or nondisjunction in *Aspergillus* 53
54 *nidulans* [9, 23], *Drosophila melanogaster* [41], and rodents 54
55 [25–28], as well as MNs in *Zea* and *Tradescantia* [38]. 55

56 In vivo cytogenetic studies among humans showed 56
57 increased frequencies of chromosomal aberrations (CAs) 57
58 [17, 38], SCEs [38], and MNs [19, 32] in peripheral blood 58
59 lymphocytes of alcoholics. Nevertheless, the interpretation 59
60 of some cytogenetic studies could be problematic because 60
61 some confounding factors, such as smoking, were not 61
62 always taken into account. It is known that alcohol 62
63 dependent is usually associated with smoking habit. This 63
64 association could result in further increased levels of CAs 64
65 and MNs [37, 48] and cancer incidence [22, 30, 50]. 65

66 In this study, we analysed the MNs, SCEs and CAs 66
67 frequencies in cultured lymphocytes from a sample of non- 67
68 smoker alcoholic and non-alcoholic control subjects. MN 68

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69 assay detects both clastogenicity (chromosome breakage)
70 and aneugenicity (chromosome lagging due to dysfunction
71 of mitotic apparatus) [12, 14, 39], while the SCE analysis
72 reveals alterations in the chromosome structure only.
73 Moreover, increased levels of CAs, in particular of chro-
74 mosome breaks, were found closely related to cancer
75 development [18].

76 Metabolism of alcohol results in the generation of several
77 classes of DNA-adducts and/or DNA-damaging molecules,
78 including reactive oxygen species (ROS), lipid peroxidation
79 products and acetaldehyde [32]. These compounds were
80 found to affect the DNA-repair systems and can contribute to
81 the hepatocarcinogenesis process [47]. Detoxification cellular
82 systems protect the cells from DNA damage caused by vari-
83 ous reactive substances. Glutathione S-transferases (GSTs)
84 represent one of the major groups of phase II detoxifying
85 enzymes, found in virtually all eukaryotes and evolved pro-
86 viding protection against reactive oxygen metabolites and
87 toxic substances present in the food and the environment [36].
88 In particular, the GST enzymes are able to detoxify harmful
89 ethanol metabolites in the liver by conjugating acetaldehyde
90 and ROS to reduced glutathione [31]. *GSTT1* and *GSTM1*
91 genes are known to be polymorphic in humans for a deletion
92 of a segment of DNA, that results in the absence of protein
93 synthesis and consequent reduced detoxification of xenobi-
94 otics in homozygous individuals [42].

95 These genes have been also considered as potential
96 candidates for alcoholic liver disease (ALD) susceptibility
97 [34]. Savolainen et al. [46], found a statistically significant
98 association between the occurrence of alcoholic liver cir-
99 rhosis and *GSTM1* “null” genotype, suggesting that per-
100 sons with homozygous deletion of the *GSTM1* gene could
101 be genetically more prone to develop alcoholic liver
102 fibrosis. Nevertheless, no data were found in literature
103 about the possible association of *GSTs* gene polymorphism
104 and cytogenetic damage in samples of alcoholics.

105 In this study we aimed to perform a cytogenetic analysis on
106 lymphocytes from a selected sample of alcoholic beverage
107 dependent individuals, in order to evaluate possible risks of
108 genomic damage due alcohol genotoxicity. Moreover, con-
109 sidering the implication of the *GSTs* gene polymorphisms in
110 the genetic susceptibility to alcoholic liver diseases and
111 cancer, we considered an important issue to evaluate the
112 possible relationship between these gene polymorphisms and
113 the cytogenetic damage in chronic alcoholics.

114 Materials and methods

115 Groups studied

116 The study was conducted on 15 alcoholics and 15 non-drinker
117 controls, all males. Alcoholic subjects declared to drink

Table 1 Demographic characteristics and alcohol consumption habits of the studied groups

Characteristics	Alcoholics	Controls
Subjects (n)	15	15
Age		
Mean years (SD)	50.73 (8.89) ^a	49.33 (10.83) ^a
Range (years)	36–70	38–70
Alcohol habit		
Years (SD)	7.67 (2.74)	–
Range (years)	4–12	–
Daily assumption		
Mean litres of alcoholic beverages (SD)	3.13 (2.20)	–
Mean grams of ethanol (SD)	284.50 (196.50)	–

SD Standard deviation

^a $P = 0.944$

regularly both wine and distilled beverages, also during the 118
period while the study was conducted. All participants were 119
extensively interviewed by a specialized physician with a 120
detailed questionnaire in order to collect meaningful infor- 121
mation for the study. Age, smoking habits, work-related 122
exposure to hazardous agents, dietary habits, use of thera- 123
peutic drugs and alcohol consumption were all recorded. 124

All subjects were selected avoiding individuals occupa- 125
tionally exposure to known or suspected mutagens/genotoxic 126
agents. In our sample we exclusively considered individuals 127
that have not consumed drugs and have not been subjected to 128
invasive diagnostic examinations for a period of at least 2 years 129
prior to the analysis. Among controls, 9 subjects declared to be 130
teetotalers, and 6 occasional drinkers who declared to drink no 131
more than one glass of wine during week-end. 132

All the subjects were volunteers, received information 133
about the study and gave their written informed consent. 134
The procedures followed in this work were in accordance 135
with the ethical standards of the local responsible com- 136
mittee on human experimentation and with the Helsinki 137
Declaration of 1975, as revised in 1983. 138

Blood sample collection 139

Blood samples were obtained by venipuncture (5–10 mL) 140
and collected into heparinised tubes, for genotoxicity 141
testing. All blood samples were coded, cooled (4 °C), and 142
processed within 2 h after collection. 143

Cytokinesis-block micronucleus assay 144

MNs assay was conducted following the protocol described 145
in [43]. Micronuclei were scored in 1,000 bi-nucleated 146

- 147 lymphocytes per subject, following the established criteria
148 for the MN evaluation [15]. The cytokinesis-block prolifer-
149 ation index (CBPI) was calculated according to the for-
150 mula: $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where
151 N_1 – N_4 represent the number of cells with 1–4 nuclei,
152 respectively, and N is the total number of cells scored.
- 153 Sister chromatid exchanges assay
- 154 SCEs assay was performed according to [45]. In order to
155 determine the number of SCE/cell for each subject, we
156 scored 50 well-spread second-division metaphases con-
157 taining 46 (± 1) chromosomes. A total of 100 cells from
158 each donor were scored for the determination of the rep-
159 lication index (RI), calculated according to the formula:
160 $RI = (M_1 + 2M_2 + 3M_3)/N$, where M_1 , M_2 and M_3 rep-
161 resent the number of cells undergoing first, second, and
162 third mitosis and N is the total number of metaphase
163 scored.
- 164 Chromosomal aberrations assay
- 165 CAs assay was performed according to [44]. For each
166 subject, a total of 200 well-spread first-division complete
167 metaphases were analyzed for the following categories of
168 CAs: chromatid breaks (B'), chromosome breaks (B''),
169 dicentric (Dic), acentric fragments (AF), and tri- or tetra-
radials (RAD). Cells containing any type of chromosomal
aberrations were scored as "cells with aberrations" (CAB).
- DNA Extraction and Genotyping 172
- Genomic DNA was extracted using the Chelex solution
protocol [51]. *GSTMI* and *GSTTI* genotypes were deter-
mined by polymerase chain reaction (PCR) using primers
described in [53] and [40], respectively. In addition, as
internal control, a fragment of the β -globin gene was co-
amplified using the primers 5'-CAACTCATCCACGTT-
CACC-3' and 5'-ACACAAC-TGTGTTCACTAGC-3'. PCR
reactions were carried out following the procedure described
in [44]. Genotypes with homozygous deletion of the *GST*
genes are identified as "GST-null", whereas genotypes
having at least one copy of the gene are "GST-positive".
- Statistical analysis 184
- Statistical analysis was assessed using the SYSTAT soft-
ware statistical package (version 10.0, Inc., Chicago, Illi-
nois, USA). The non-parametric Wilcoxon test was used to
compare the mean frequencies of SCEs, MNs and CAs
between alcoholics and controls. Multiple regression ana-
lysis was used to evaluate the influence of age and years of
employment on SCEs, CAs and MNs frequencies of both
groups. All P values were two tailed and the level of sta-
tistical significance was set at $P < 0.05$ for all tests.

Table 2 Frequency of SCEs and RI values in metaphases of lymphocytes from alcoholics and controls

Groups	N	NSM	SCEs	SCEs/NSM (SE)	M_1	M_2	M_3	RI (SE)
<i>Alcoholics</i>	15	750	6,831	9.11 (0.33)^a	559	353	288	1.75 (0.04)^b
<i>GSTTI</i> -positive	10	500	4,581	9.16 (0.38) ^c	419	269	212	1.77 (0.04) ^f
<i>GSTTI</i> -null	5	250	2,250	9.00 (0.71) ^c	140	84	76	1.71 (0.06) ^f
<i>GSTMI</i> -positive	10	500	4,528	9.06 (0.48) ^d	416	278	206	1.76 (0.05) ^g
<i>GSTMI</i> -null	5	250	2,303	9.21 (0.36) ^d	143	75	82	1.72 (0.05) ^g
<i>GSTs</i> double positives	8	400	3,709	9.27 (0.47) ^e	374	245	181	1.76 (0.05) ^h
<i>GSTs</i> double nulls	3	150	1,431	9.54 (0.51) ^e	159	81	60	1.67 (0.07) ^h
<i>Controls</i>	15	750	4,019	5.36 (0.30)^a	398	486	316	1.93 (0.02)^b
<i>GSTTI</i> -positive	9	450	2,523	5.61 (0.33) ⁱ	249	278	173	1.91 (0.02) ⁿ
<i>GSTTI</i> -null	6	300	1,496	4.99 (0.56) ⁱ	149	208	143	1.96 (0.04) ⁿ
<i>GSTMI</i> -positive	8	400	2,118	5.30 (0.44) [*]	207	242	151	1.91 (0.03) ^o
<i>GSTMI</i> -null	7	350	1,901	5.43 (0.42) [*]	191	244	165	1.96 (0.04) ^o
<i>GSTs</i> double positives	7	3590	1,941	5.55 (0.42) ^m	247	280	173	1.89 (0.03) ^p
<i>GSTs</i> double nulls	5	250	1,319	5.28 (0.49) ^m	154	201	145	1.98 (0.05) ^p

Superscript letters indicate each comparison (a–p) performed between groups. In bold are highlighted the statistically significant differences (a and b)

SE Standard error; NSM Number of scored metaphases; SCEs Sister chromatid exchanges; RI Replication index = $(M_1 + 2M_2 + 3M_3)/N$, where M_1 , M_2 and M_3 represent the number of cells undergoing first second and third mitosis and N is the total number of metaphase scored

^{a,b} $P = 0.001$; ^c $P = 0.500$; ^d $P = 0.686$; ^{e,h} $P = 1$; ^f $P = 0.500$; ^g $P = 0.892$; ⁱ $P = 0.753$; ^m $P = 0.893$; ⁿ $P = 0.143$; ^o $P = 0.345$; ^p $P = 0.068$

* $P = 0.612$

Table 3 Frequency of chromosomal aberrations in metaphases of lymphocytes from alcoholics and controls

Groups	N	NSM	CAs					Totals CAs	Totals CAB	CAs/NSM %	
			B'	B''	Dic	AF	RAD			Mean (SE)	Mean (SE)
<i>Alcoholics</i>	15	3,000	17	8	1	2	1	29	29	0.97 (0.11)^a	0.97 (0.11)^b
<i>GSTT1</i> -positive	10	2,000	12	4	1	1	1	19	19	0.95 (0.11) ^c	0.95 (0.11) ^f
<i>GSTT1</i> -null	5	1,000	5	4	0	1	0	10	10	1.00 (0.13) ^c	1.00 (0.13) ^f
<i>GSTM1</i> -positive	10	2,000	13	3	1	0	1	18	18	0.90 (0.12) ^d	0.90 (0.12) ^g
<i>GSTM1</i> -null	5	1,000	4	5	0	2	0	11	11	1.10 (0.11) ^d	1.10 (0.11) ^g
<i>GSTs</i> double positives	8	1,600	10	2	1	0	1	14	14	0.88 (0.16) ^e	0.88 (0.16) ^h
<i>GSTs</i> double nulls	3	600	2	3	0	1	0	6	6	1.00 (0.29) ^e	1.00 (0.29) ^h
<i>Controls</i>	15	3,000	0	1	0	2	1	4	4	0.13 (0.08)^a	0.13 (0.08)^b
<i>GSTT1</i> -positive	9	1,800	0	1	0	1	1	3	3	0.17 (0.09) ⁱ	0.17 (0.09) ⁿ
<i>GSTT1</i> -null	6	1,200	0	0	0	1	0	1	1	0.08 (0.05) ⁱ	0.08 (0.05) ⁿ
<i>GSTM1</i> -positive	8	1,600	0	1	0	1	1	3	3	0.19 (0.10) [*]	0.19 (0.10) ^o
<i>GSTM1</i> -null	7	1,400	0	0	0	1	0	1	1	0.07 (0.05) [*]	0.07 (0.05) ^o
<i>GSTs</i> double positives	7	1,400	0	1	0	1	1	3	2	0.21 (0.15) ^m	0.14 (0.09) ^p
<i>GSTs</i> double nulls	5	1,000	0	0	0	1	0	1	1	0.10 (0.10) ^m	0.10 (0.10) ^p

Superscript letters indicate each comparison (a–p) performed between groups. In bold are highlighted the statistically significant differences (a and b)

^{a,b} $P = 0.002$; ^c $P = 1$; ^{d,g} $P = 0.257$; ^{e,h} $P = 0.157$; ^f $P = 1$; ⁱ $P = 0.317$; ^{*,o} $P = 0.414$, ^{m,n,p} $P = 0.317$

N Number of tested individuals; *NSM* Number of scored metaphases; *CAs* Chromosome aberrations; *B'* Chromatid breaks; *B''* Chromosome breaks; *Dic* Dicentric chromosome; *AF* Acenatric fragments, *RAD* Tri- or tetra-radials; *CAB* Cells with aberrations; *AB.C %* percentage of cells with aberrations

194 Results

195 Table 1 summarizes the main characteristics of the two studied
196 groups. Alcoholic subjects had a mean age of $50.73 \pm$
197 8.89 years (range: 36–70 years) and an average duration of
198 alcohol abuse of 7.67 ± 2.74 years (range: 4–12 years). The
199 mean age of controls was 49.33 ± 10.83 years (range: 38–70).
200 No significant differences were found between groups in terms
201 of mean age ($P = 0.944$).

202 Table 2 reports the SCEs frequencies recorded in
203 peripheral blood lymphocytes of alcoholics and control
204 subjects. A total of 750 metaphases for each group were
205 analysed. Statistically significant differences were found
206 between alcoholics and controls in both SCEs/cell fre-
207 quency ($P = 0.001$) and RI value ($P = 0.001$).

208 Table 3 shows the CAs frequencies in the two subject
209 groups. A total of 3000 metaphases for each group were
210 analysed. Statistically significant differences were found
211 between alcoholics and controls in terms of CAs and CAB
212 frequencies ($P = 0.002$).

213 Table 4 shows the frequencies of MNs and cells with
214 MNs in the studied groups. A total of 15,000 bi-nucleated
215 cells for each group were scored. No statistical significant
216 differences were found between alcoholics and controls in
217 the frequency of MNs ($P = 0.509$), cells with MNs
218 ($P = 0.394$) and CBPI values ($P = 0.691$).

219 Among both the alcoholics and control groups, we did
220 not find statistically significant associations between any
221 *GST* genes polymorphisms and the frequencies of SCEs
222 (Table 2), CAs (Table 3) or MNs (Table 4).

223 Results of multiple regression analysis are summarized
224 in Table 5. The duration of alcohol exposure (expressed as
225 years of alcohol abuse) was seen to have effects on the
226 frequencies of SCEs ($P = 0.015$), CAs ($P = 0.45$), but not
227 on MNs frequency ($P = 0.396$). Finally, in both groups, no
228 significant correlations were found between the age of
229 subjects and the frequencies of the analysed cytogenetic
230 biomarkers.

231 Discussion

232 Epidemiological studies conducted on different human
233 populations consistently showed that consumption of
234 alcoholic beverages is associated with a higher risk for
235 several types of cancer, such as oral, pharyngeal, colorectal
236 and liver cancers [21]. Nevertheless, the interpretation of
237 some cytogenetic studies is complicated by factors, such as
238 smoking or other potential confounders, not always taken
239 into account. Specifically, the effects of smoking and
240 alcohol consumption appear to be multiplicative. It seems
241 plausible that the synergism between tobacco and alcohol

Table 4 Frequencies of MNs, Cells with MNs and CBPI values in bi-nucleated lymphocytes from alcoholics and controls

Groups	N	NSCs	Distribution of BNCs according to the number of MNs					% MNs/NSCs (SE)	% CMNs/NSCs (SE)	CBPI (SE)	
			1	2	3	4	Total MNs				Total CMNs
<i>Alcoholics</i>	15	15,000	163	26	11	0	248	205	1.65 (0.23) ^a	1.37 (0.20) ^b	1.91 (0.06) ^c
<i>GSTT1</i> -positive	10	10,000	111	20	6	0	169	142	1.69 (0.23) ^d	1.42 (0.16) ^e	1.97 (0.05) ^f
<i>GSTT1</i> -null	5	5,000	52	6	5	0	79	163	1.58 (0.58) ^d	1.26 (0.54) ^e	1.78 (0.14) ^f
<i>GSTM1</i> -positive	10	10,000	93	17	5	0	142	120	1.42 (0.24) ^e	1.20 (0.19) ^h	1.97 (0.07) ^m
<i>GSTM1</i> -null	5	5,000	70	9	6	0	106	85	2.12 (0.48) ^e	1.70 (0.44) ^h	1.80 (0.25) ^m
<i>GSTs</i> double positives	8	8,000	85	16	4	0	129	110	1.61 (0.25) ^f	1.38 (0.19) ⁱ	1.99 (0.07) ⁿ
<i>GSTs</i> double nulls	3	3,000	44	5	4	0	66	53	2.20 (0.78) ^f	1.77 (0.44) ⁱ	1.73 (0.19) ⁿ
<i>Controls</i>	15	3,000	146	10	14	0	208	170	1.39 (0.28) ^a	1.13 (0.21) ^b	1.95 (0.07) ^c
<i>GSTT1</i> -positive	9	9,000	99	8	12	0	151	119	1.68 (0.43) ^o	1.32 (0.32) ^r	1.96 (0.10) ^u
<i>GSTT1</i> -null	6	6,000	47	2	2	0	57	51	0.95 (0.23) ^o	0.85 (0.18) ^r	1.94 (0.08) ^u
<i>GSTM1</i> -positive	8	8,000	75	8	7	0	112	90	1.40 (0.43) ^p	1.13 (0.31) ^s	1.85 (0.08) ^v
<i>GSTM1</i> -null	7	7,000	71	2	7	0	96	80	1.37 (0.40) ^p	1.14 (0.31) ^s	2.07 (0.10) ^v
<i>GSTs</i> double positives	7	7,000	66	7	7	0	101	80	1.44 (0.49) ^q	1.14 (0.36) ^t	1.86 (0.10) ^w
<i>GSTs</i> double nulls	5	5,000	38	1	2	0	46	41	0.92 (0.27) ^q	0.82 (0.22) ^t	1.96 (0.09) ^w

Superscript letters indicate each comparison (a–w) performed between groups

^a $P = 0.509$; ^b $P = 0.394$; ^c $P = 0.691$; ^d $P = 0.686$; ^e $P = 0.225$; ^f $P = 0.593$; ^g $P = 0.786$; ^h $P = 0.343$; ^m $P = 0.500$; ⁿ $P = 0.285$, ^o $P = 0.462$; ^p $P = 0.499$; ^r $P = 0.600$; ^s $P = 0.553$; ^v $P = 0.173$; ^w $P = 0.498$

NSCs Number of scored metaphases; BNCs Bi-nucleated cells; MNs Micronuclei; CMNs Cells with micronuclei; SE Standard error; CBPI (Cytokinesis-block proliferation index) = $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where N_1 – N_4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored

242 in the causation of cancer is due to the enhancement of the 267
 243 effects of tobacco carcinogens by ethanol [11]. While 268
 244 several previous studies have addressed this topic, this is 269
 245 the first study about non-smoker alcoholics. 270

246 We found a significantly high frequency of SCEs among 271
 247 alcoholics compared to healthy controls (Table 2). More- 272
 248 over, the alcohol assumption appeared to influence the 273
 249 lymphocyte replication capacity, as shown by RI values 274
 250 significantly different between alcoholics and controls 275
 251 ($P = 0.001$). These results are concordant with a previ- 276
 252 ously published study of [Butler et al. [7]] who observed an 277
 253 increase of SCEs frequency in alcoholics, that was not 278
 254 related to age, sex, cigarette smoking and duration in years 279
 255 of alcohol abuse. Similarly, [29] and [24] reported an 280
 256 increase in SCEs rates among alcoholics, although in those 281
 257 studies the higher SCEs frequency was associated to 282
 258 smoking habit and age. Considering that SCEs seem to 283
 259 reflect the repair efficiency of DNA lesions by homologous 284
 260 recombination, these results provide further support to the 285
 261 hypothesis that high alcohol consumption could be associ- 286
 262 ated with impaired DNA repair mechanisms, as also 287
 263 suggested by other authors [16, 52]. 288

264 Accordingly to other published studies [6, 10], among 289
 265 alcoholics we also found a significantly higher frequency 290
 266 of CAs compared to controls (Table 3). This finding could 291

267 suggest a possible clastogenic effect of ethanol in chronic 268
 269 alcoholics and a higher risk of cancer incidence among 269
 270 healthy individuals of a population increases with 270
 271 increased levels of CAs in their circulating lymphocytes [4, 271
 272 5]. 272

273 Although a possible aneugenic effect of ethanol has 273
 274 been evidenced by different authors [8, 19, 32, 49], in our 274
 275 study the MNs test did not reveal any significant difference 275
 276 between alcoholics and controls in terms of MNs frequency 276
 277 formation, as well as the number of cells with MNs and 277
 278 CBPI value (Table 4). Nevertheless, results reported in the 278
 279 above studies are referred to subjects that were both alco- 279
 280 holics and smokers, and thus the effects of smoking as a 280
 281 confounder factor should be taken into account. Vice versa, 281
 282 our selected sample included only non-smoking alcoholics, 282
 283 and thus the observed cytogenetic damage appeared to be 283
 284 exclusively due to the effects of alcohol consumption. 284

285 Overall, our data suggest that alcoholism may cause 285
 286 chromosome damage in humans, in terms of increased 286
 287 levels of SCEs and CAs. However, it cannot be concluded 287
 288 that the direct action of ethanol on chromosomes is 288
 289 responsible for these effects. Indeed, ethanol is quickly 289
 290 metabolized and significant levels of ROS and acetalde- 290
 291 hyde accumulate in the blood during ethanol oxidation 291

Table 5 Multiple regression analysis of confounding factors on SCEs and CAs frequencies in peripheral lymphocytes of the study groups

CF	SCEs frequency			CAs frequency			MNs frequency		
	β -co	P value	95 % CI Lower-upper	β -co	P value	95 % CI Lower-upper	β -co	P value	95 % CI Lower-upper
Alcoholics									
Age	0.33	0.15	-0.94-5.70	0.21	0.40	-0.11-0.26	0.21	0.45	-0.39-0.82
YA	0.60	0.02	4.16-30.99	0.53	0.05	0.020-1.52	0.24	0.40	-1.46-3.44
Controls									
Age	0.37	0.18	-1.01-4.87	-0.05	0.87	-0.040-0.03	0.23	0.40	-0.35-0.83

CF Confounding factor; β -co β -coefficient; YA Years of alcohol abuse

292 [35]. This last compound cause many toxic effects asso- 330
 293 ciated with ethanol excess and may be responsible for its 331
 294 mutagenic activity [6]. 332

295 Ethanol metabolizing process requires different types of 333
 296 enzymes, including GST enzymes, able to detoxify harmful 334
 297 ethanol metabolites in the liver by conjugating acetalde- 335
 298 hyde and ROS to reduced glutathione [31]. Some GST 336
 299 alleles, such as *GSTM1* null allele, were found associated 337
 300 with liver diseases in alcoholics [34, 46]. However, among 338
 301 heavy and chronic ethanol consumers, no data are present 339
 302 in literature about a possible direct association between 340
 303 GST alleles and genomic damage measured in terms of 341
 304 SCEs, CAs and MNs. We investigated this possible asso- 342
 305 ciation and observed in both groups, no statistically sig- 343
 306 nificant association between GST genes polymorphisms 344
 307 and the frequencies of SCEs, CAs, and MNs (Tables 2, 3, 345
 308 4). However, the reduced sample size here considered 346
 309 requires further investigations with a larger number of 347
 310 subjects to provide convincing evidences for the absence of 348
 311 such correlation. 349

312 Finally, the increased levels of SCEs and CAs recorded 350
 313 among alcoholics appear to be associated with the duration 351
 314 of alcohol abuse, but not with the age of the subjects 352
 315 (Table 5). An age related increase of spontaneous chro- 353
 316 mosome instability was showed [2, 3, 13]. The lack of a 354
 317 similar age-related pattern among alcoholics could be 355
 318 probably due to an increased incidence of the chromosomal 356
 319 damage induced by alcohol among younger individuals. In 357
 320 this scenario the effect of alcohol abuse could obscure this 358
 321 relationship among alcoholics. 359

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326 References

327 1. Adler RA (1992) Clinical review 33: clinically important effects 370
 328 of alcohol on endocrine function. J Clin Endocrinol Metab 371
 329 74:957-960 372

2. Barnett YA, King CM (1995) An investigation of antioxidants 330
 status, DNA repair capacity and mutation as a function of age in 331
 humans. Mutat Res 338:115-128 332
 3. Bolognesi C, Abbondandolo A, Barale R et al (1997) Age-related 333
 increase of baseline frequencies of sister chromatid exchanges, 334
 chromosome aberrations, and micronuclei in human lympho- 335
 cytes. Cancer Epidemiol Biomark Prev 6(4):249-256 336
 4. Bonassi S, Hagmar L, Strömberg U et al (2000) Chromosomal 337
 aberrations in lymphocytes predict human cancer independently 338
 of exposure to carcinogens. Cancer Res 60:1619-1625 339
 5. Bonassi S, Znaor A, Norppa H, Hagmar L (2004) Chromosomal 340
 aberrations and risk of cancer in humans: an epidemiologic per- 341
 spective. Cytogenet Genome Res 104:376-382 342
 6. Burim RV, Canalle R, Takahashi CS et al (2004) Clastogenic 343
 effect of ethanol in chronic and abstinent alcoholics. Mutat Res 344
 560(2):187-198 345
 7. Butler MG, Sanger WG, Veonett GE (1981) Increased frequency 346
 of sister-chromatid exchanges in alcoholics. Mutat Res 347
 85(2):71-76 348
 8. Castelli E, Hrelia P, Maffei F, Fimognari C, Foschi FG, Caputo F, 349
 Cantelli-Forti G, Stefanini GF, Gasbarrini G (1999) Indicators of 350
 genetic damage in alcoholics: reversibility after alcohol abstinence. 351
 Hepatogastroenterology 46(27):1664-1668 352
 9. Crebelli R, Conti G, Conti L, Carere A (1989) A comparative 353
 study on ethanol and acetaldehyde as inducers of chromosome 354
 malsegregation in *Aspergillus nidulans*. Mutat Res 215:187-195 355
 10. Demirhan O, Tastemir D (2008) Cytogenetic effects of ethanol on 356
 chronic alcohol users. Alcohol Alcohol 43(2):127-136 357
 11. Du X, Squier CA, Kremer MJ, Wertz PW (2000) Penetration of 358
 N-nitrosornicotine (NNN) across oral mucosa in the presence 359
 of ethanol and nicotine. J Oral Pathol Med 29:80-85 360
 12. Elhajouji A, Cunha M, Kirsch-Volders M (1998) Spindle poisons can 361
 induce polyploidy by mitotic slippage and micronucleate mononu- 362
 cleates in the cytokinesis-block assay. Mutagenesis 13:193-198 363
 13. Fenech M (1998) Important variables that influence base-line 364
 micronucleus frequency in cytokinesis-blocked lymphocytes as 365
 biomarker for DNA damage in human populations. Mutat Res 366
 404:155-165 367
 14. Fenech M (2000) The in vitro micronucleus technique. Mutat Res 368
 455:81-95 369
 15. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, 370
 Zeiger E (2003) Human Micronucleus project (HUMN pro- 371
 ject): detailed description of the scoring criteria for the cyto- 372
 kinesis-block micronucleus assay using isolated human lymphocyte 373
 cultures. Mutat Res 534(1-2):65-75 374
 16. Garcia-Sagredo JM (2008) Fifty years of cytogenetics: a parallel 375
 view of the evolution of cytogenetics and genotoxicology. Bio- 376
 chim Biophys Acta 1779(6-7):363-375 377
 17. Gattás GJ, Saldanha PH (1997) Chromosomal aberrations in 378
 peripheral lymphocytes of abstinent alcoholics. Alcohol Clin Exp 379
 Res 21:238-243 380

- 381 18. Hagmar L, Bonassi S, Stromberg U, Brogger A, Knudsen LE, 445
 382 Norppa H, Reuterwall C (1998) Chromosomal aberrations in 446
 383 lymphocytes predict human cancer: a report from the European 447
 384 study group on cytogenetic biomarkers and health (ESCH). 448
 385 *Cancer Res* 58:4117–4121
 386 19. Iarmarcovai G, Bonassi S, Sari-Minodier I, Baciuchka-Palmaro 449
 387 M, Botta A, Orsiere T (2007) Exposure to genotoxic agents, host 450
 388 factors, and lifestyle influence the number of centromeric signals 451
 389 in micronuclei: a pooled re-analysis. *Mutat Res* 615:18–27 452
 390 20. IARC (1988) Alcohol drinking. international agency for research 453
 391 on cancer. Monographs on the evaluation of carcinogenic risk to 454
 392 human, vol 54. International Agency for Research on Cancer, 455
 393 Lyon, pp 251–321
 394 21. IARC (2010) Alcohol consumption and Ethyl Carbamate. 456
 395 Monographs on the evaluation of carcinogenic risk to human, vol 457
 396 96. International Agency for Research on Cancer, Lyon, 458
 397 pp 173–1001
 398 22. Inoue M, Tajima K, Hirose K, Kuroishi T, Gao CM, Kitoh T 459
 399 (1994) Life-style and subsite of gastric cancer-joint effect of 460
 400 smoking and drinking habits. *Int J Cancer* 56:494–499
 401 23. Kafer E (1984) Disruptive effects of ethyl alcohol on mitotic 461
 402 chromosomes segregation in diploid and haploid strains of 462
 403 *Aspergillus nidulans*. *Mutat Res* 135:53–75
 404 24. Karaoguz MY, Coşar B, Arıkan Z, Başaran F, Menevşe A, 463
 405 Menevşe S (2005) Increased frequency of sister chromatid 464
 406 exchanges in peripheral lymphocytes of alcoholics and cigarette 465
 407 smokers. *Cell Biol Int* 29(2):165–168
 408 25. Kaufman MH (1983) Ethanol-induced chromosomal abnormali- 466
 409 ties at conception. *Nature* 302:258–260
 410 26. Kaufman MH (1985) An hypothesis regarding the origin of 467
 411 aneuploidy in man: indirect evidence from an experimental 468
 412 model. *J Med Genet* 22:171–178
 413 27. Kaufman MH (1997) The teratogenic effects of alcohol following 469
 414 exposure during pregnancy, and its influence on the chromosome 470
 415 constitution of the pre-ovulatory egg. *Alcohol Alcohol* 471
 416 32:113–128
 417 28. Kaufman MH, Bain IM (1984) Influence of ethanol on chromo- 472
 418 some segregation during the first and second meiotic divisions in 473
 419 the mouse egg. *J Exp Zool* 230:315–320
 420 29. Lazutka JR, Dedonate V, Lekevicius RK (1992) Sister chromatid 474
 421 exchanges in lymphocytes of normal and alcoholic subjects. 475
 422 *Experientia* 48(5):508–512
 423 30. Lee KW, Kuo WR, Tsai SM et al (2005) Different impact from 476
 424 betel quid, alcohol and cigarette: risk factors for pharyngeal and 477
 425 laryngeal cancer. *Int J Cancer* 117:831–836
 426 31. Lieber CS (1994) Alcohol and the liver: update. *Gastroenterology* 478
 427 106:1085–1105
 428 32. Maffei F, Fimognari C, Castelli E, Stefanini GF, Forti GC, Hrelia 479
 429 P (2000) Increased cytogenetic damage detected by FISH analy- 480
 430 sis on micronuclei in peripheral lymphocytes from alcoholics. 481
 431 *Mutagenesis* 15(6):517–523
 432 33. Mandayam S, Jamal MM, Morgan TR (2004) Epidemiology of 482
 433 alcoholic liver disease. *Semin Liver Dis* 24:217–232
 434 34. Marcos M, Pastor I, Chamorro AJ, Ciria-Abad S, Gonzalez- 483
 435 Sarmiento R, Laso FJ (2011) Meta-analysis: glutathione-S- 484
 436 transferase allelic variants are associated with alcoholic liver 485
 437 disease. *Aliment Pharmacol Ther* 34:1159–1172
 438 35. Nakamura K, Iwalashi K, Itoh M, Ameno K, Ijiri I, Takeuchi Y, 486
 439 Suwaki H (2000) Immunohistochemical study on acetaldehyde 487
 440 adducts in alcohol-fed mice. *Alcohol Clin Exp Res* 24(4):93S– 488
 441 96S
 442 36. Nebert DW, McKinnon RA, Puga A (1996) Human drug meta- 489
 443 bolising enzyme polymorphisms effects on risk of toxicity and 490
 444 cancer DNA. *Cell Biol* 15:273–280
 37. Nersesyan A, Muradyan R, Kundi M, Knasmueller S (2011) 491
 Impact of smoking on the frequencies of micronuclei and other 492
 nuclear abnormalities in exfoliated oral cells: a comparative study 493
 with different cigarette types. *Mutagenesis* 26(2):295–301
 38. Obe G, Anderson D (1987) International commission for pro- 494
 tection against environmental mutagens and carcinogens. IC- 495
 PEMC Working Paper No. 15/1. Genetic effects of ethanol. 496
Mutat Res 186(3):177–200
 39. Parry EM, Parry JM, Corso C et al (2002) Detection and char- 497
 acterization of mechanisms of action of aneugenic chemicals. 498
Mutagenesis 17(6):509–521
 40. Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt 499
 HM, Ketterer B, Taylor JB (1994) Human glutathione S-trans- 500
 ferase theta (GSTT1): cDNA cloning and the characterization of a 501
 genetic polymorphism. *Biochem J* 300:271–276
 41. Rey M, Palermo AM, Muñoz ER (1992) Nondisjunction induced 502
 by ethanol in *Drosophila melanogaster* females. *Mutat Res* 503
 268(1):95–104
 42. Roy B, Majumder PP, Dey B, Chakraborty M, Banerjee S, Roy 504
 M, Mukherjee N, Sil SK (2001) Ethnic differences in distribu- 505
 tions of GSTM1 and GSTT1 homozygous “Null” genotypes in 506
 India. *Hum Biol* 73(3):443–450
 43. Santovito S, Cervella P, DelPero M (2011) In vitro aneugenic 507
 effects of the fungicide thiabendazole evaluated in human lym- 508
 phocytes by the micronucleus assay. *Arch Toxicol* 85:689–693
 44. Santovito A, Schilirò T, Castellano S, Cervella P, Bigatti MP, 509
 Gilli G, Bono R, DelPero M (2011) Combined analysis of 510
 chromosomal aberrations and glutathione S-transferase M1 and 511
 T1 polymorphisms in pathologists occupationally exposed to 512
 formaldehyde. *Arch Toxicol* 85:1295–1302
 45. Santovito S, Cervella P, DelPero M (2014) Increased frequency 513
 of chromosomal aberrations and sister chromatid exchanges in 514
 peripheral lymphocytes of radiology technicians chronically 515
 exposed to low levels of ionizing radiations. *Environ Toxicol* 516
Phar 37:396–403
 46. Savolainen VT, Pajarinen J, Perola M, Penttila A, Karhunen PJ 517
 (1996) Glutathione-S-Transferase GST M1 “Null” genotype and 518
 the risk of alcoholic liver disease. *Alcohol Clin Exp Res* 519
 20(8):1340–1345
 47. Seitz HK, Stickel F (2006) Risk factors and mechanisms of hep- 520
 atocarcinogenesis with special emphasis on alcohol and oxida- 521
 tive stress. *Biol Chem* 387(4):349–360
 48. Sierra-Torres MS, Arboleda-Moreno YY, Hoyos LS, Sierra- 522
 Torres CH (2004) Chromosome aberrations among cigarette 523
 smokers in Colombia. *Mutat Res* 562(1–2):67–75
 49. Stich HF, Rosin MP (1983) Quantitating the synergistic effect of 524
 smoking and alcohol consumption with the micronucleus test on 525
 human buccal mucosa cells. *Int J Cancer* 31(3):305–308
 50. Vioque J, Barber X, Bolumar F, Porta M, Santibáñez M, de la 526
 Hera MG, Moreno-Osset E, PANESOES Study Group (2008) 527
 Oesophageal cancer risk by type of alcohol drinking and smok- 528
 ing: a case-control study in Spain. *BMC Cancer* 8(1):221
 51. Walsh P, Metzger DA, Higuchi R (1991) Chelex 100 as a 529
 Medium for simple extraction of DNA for PCR-based typing 530
 from forensic material. *Biotechniques* 10:506
 52. Wilson DM 3rd, Thompson LH (2007) Molecular mechanisms of 531
 sister-chromatid exchange. *Mutat Res* 616(1–2):11–23
 53. Zhong S, Wyllie AH, Barnes D, Wolf CR, Spurr NK (1993) 532
 Relationship between GSTM1 genetic polymorphism and sus- 533
 ceptibility to bladder, breast and colon cancer. *Carcinogenesis* 534
 14:1821–1824 535