Evidence of genotoxicity in lymphocytes of non-smoking alcoholics

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Evidence of genotoxicity in lymphocytes of non-smoking alcoholics

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Abstract Alcohol abuse is a significant public health issue. Epidemiological studies conducted on different populations consistently showed that consumption of alcoholic beverages is associated with cytogenetic damages and higher risk for several types of cancer. However, the interpretation of many cytogenetic studies resulted complicated because some confounding factors, such as smoking habit, are not always taken into account. In the present study, the frequency of sister chromatid exchanges (SCEs), chromosome aberrations (CAs) and micronuclei (MNs) in cultured human lymphocytes was assessed on 15 alcoholic and 15 non-alcoholic control male subjects. Moreover, considering the implication of the Glutathione S-transferases gene polymorphisms in the genetic susceptibility to alcoholic liver diseases, we considered an important issue to evaluate the relationship between these gene polymorphisms and the cytogenetic damage. In our sample we exclusively considered individuals that did not smoke nor consume drugs for a period of at least 2 years prior to the analysis. Statistically significant differences were found between alcoholics and controls in the frequency of SCEs/cell ($P = 0.001$), RI value ($P = 0.001$), CAs ($P = 0.002$) and CAB ($P = 0.002$). Vice versa, no significant differences were found between alcoholics and controls in terms of MNs frequency and CBPI value. In both samples, no statistically significant association was found between the analysed GSTs gene polymorphisms and the frequencies of MNs, SCEs and CAs. Finally, among alcoholics we found a positive correlation between SCEs and CAs frequencies and the duration of alcohol abuse.

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

Introduction

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

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

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

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

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

These genes have been also considered as potential candidates for alcoholic liver disease (ALD) susceptibility [34]. Savolainene et al. [46], found a statistically significant association between the occurrence of alcoholic liver cirrhosis and *GSTM1* "null" genotype, suggesting that persons with homozygous deletion of the *GSTM1* gene could be genetically more prone to develop alcoholic liver fibrosis. Nevertheless, no data were found in literature about the possible association of *GSTs* gene polymorphism and cytogenetic damage in samples of alcoholics.

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

Materials and methods

Groups studied

The study was conducted on 15 alcoholics and 15 non-drinker controls, all males. Alcoholic subjects declared to drink regularly both wine and distilled beverages, also during the period while the study was conducted. All participants were extensively interviewed by a specialized physician with a detailed questionnaire in order to collect meaningful information for the study. Age, smoking habits, work-related exposure to hazardous agents, dietary habits, use of therapeutic drugs and alcohol consumption were all recorded.

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] $P = 0.944$

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

All the subjects were volunteers, received information about the study and gave their written informed consent. The procedures followed in this work were in accordance with the ethical standards of the local responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

Blood sample collection

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

Cytokinesis-block micronucleus assay

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

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lymphocytes per subject, following the established criteria for the MN evaluation [15]. The cytokinesis-block proliferation index (CBPI) was calculated according to the formula: $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where N_1–N_4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

Sister chromatid exchanges assay

SCEs assay was performed according to [45]. In order to determine the number of SCE/cell for each subject, we scored 50 well-spread second-division metaphases containing 46 (±1) chromosomes. A total of 100 cells from each donor were scored for the determination of the replication index (RI), calculated according to the formula: $RI = (M_1 + 2M_2 + 3M_3)/N$, 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.

Chromosomal aberrations assay

CAs assay was performed according to [44]. For each subject, a total of 200 well-spread first-division complete metaphases were analyzed for the following categories of CAs: chromatid breaks (B'), chromosome breaks (B''), dicentrics (Dic), acentric fragments (AF), and tri- or tetraradials (RAD). Cells containing any type of chromosomal aberrations were scored as "cells with aberrations" (CAB).

DNA Extraction and Genotyping

Genomic DNA was extracted using the Chelex solution protocol [51]. *GSTM1* and *GSTT1* genotypes were determined 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′-CAACTCATCCACGTTCACC-3′ and 5′-ACACAACT-GTGTTCACTAGC-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

Statistical analysis was assessed using the SYSTAT software statistical package (version 10.0, Inc., Chicago, Illinois, USA). The non-parametric Wilcoxon test was used to compare the mean frequencies of SCEs, MNs and CAs between alcoholics and controls. Multiple regression analysis 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 statistical 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)
Alcoholics	15	750	6,831	**9.11 (0.33)**[a]	559	353	288	**1.75 (0.04)**[b]
GSTT1-positive	10	500	4,581	9.16 (0.38)[c]	419	269	212	1.77 (0.04)[f]
GSTT1-null	5	250	2,250	9.00 (0.71)[c]	140	84	76	1.71 (0.06)[f]
GSTM1-positive	10	500	4,528	9.06 (0.48)[d]	416	278	206	1.76 (0.05)[g]
GSTM1-null	5	250	2,303	9.21 (0.36)[d]	143	75	82	1.72 (0.05)[g]
GSTs double positives	8	400	3,709	9.27 (0.47)[e]	374	245	181	1.76 (0.05)[h]
GSTs double nulls	3	150	1,431	9.54 (0.51)[e]	159	81	60	1.67 (0.07)[h]
Controls	15	750	4,019	**5.36 (0.30)**[a]	398	486	316	**1.93 (0.02)**[b]
GSTT1-positive	9	450	2,523	5.61 (0.33)[i]	249	278	173	1.91 (0.02)[n]
GSTT1-null	6	300	1,496	4.99 (0.56)[i]	149	208	143	1.96 (0.04)[n]
GSTM1-positive	8	400	2,118	5.30 (0.44)[*]	207	242	151	1.91 (0.03)[o]
GSTM1-null	7	350	1,901	5.43 (0.42)[*]	191	244	165	1.96 (0.04)[o]
GSTs double positives	7	3590	1,941	5.55 (0.42)[m]	247	280	173	1.89 (0.03)[p]
GSTs 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] $P = 0.068$

[*] $P = 0.612$

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

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

Results

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

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

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

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

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

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

Discussion

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

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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			
Alcoholics	15	15,000	163	26	11	0	248	205	1.65 (0.23)[a]	1.37 (0.20)[b]	1.91 (0.06)[c]
GSTT1-positive	10	10,000	111	20	6	0	169	142	1.69 (0.23)[d]	1.42 (0.16)[g]	1.97 (0.05)[l]
GSTT1-null	5	5,000	52	6	5	0	79	163	1.58 (0.58)[d]	1.26 (0.54)[g]	1.78 (0.14)[l]
GSTM1-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]
GSTM1-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]
GSTs 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]
GSTs 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]
Controls	15	3,000	146	10	14	0	208	170	1.39 (0.28)[a]	1.13 (0.21)[b]	1.95 (0.07)[c]
GSTT1-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]
GSTT1-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]
GSTM1-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]
GSTM1-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]
GSTs 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]
GSTs 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,l] $P = 0.225$; [f,i] $P = 0.593$; [g] $P = 0.786$; [h] $P = 0.343$; [m,q,t] $P = 0.500$; [n] $P = 0.285$, [o] $P = 0.462$; [p] $P = 0.499$; [r,u] $P = 0.600$; [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

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

We found a significantly high frequency of SCEs among alcoholics compared to healthy controls (Table 2). Moreover, the alcohol assumption appeared to influence the lymphocyte replication capacity, as shown by RI values significantly different between alcoholics and controls ($P = 0.001$). These results are concordant with a previously published study of [Butler et al. [7]] who observed an increase of SCEs frequency in alcoholics, that was not related to age, sex, cigarette smoking and duration in years of alcohol abuse. Similarly, [29] and [24] reported an increase in SCEs rates among alcoholics, although in those studies the higher SCEs frequency was associated to smoking habit and age. Considering that SCEs seem to reflect the repair efficiency of DNA lesions by homologous recombination, these results provide further support to the hypothesis that high alcohol consumption could be associated with impaired DNA repair mechanisms, as also suggested by other authors [16, 52].

Accordingly to other published studies [6, 10], among alcoholics we also found a significantly higher frequency of CAs compared to controls (Table 3). This finding could suggest a possible clastogenic effect of ethanol in chronic alcoholics and a higher risk of cancer incidence among them. Indeed, it is known that cancer incidence among healthy individuals of a population increases with increased levels of CAs in their circulating lymphocytes [4, 5].

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

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

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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

[35]. This last compound cause many toxic effects associated with ethanol excess and may be responsible for its mutagenic activity [6].

Ethanol metabolizing process requires different types of enzymes, including GST enzymes, able to detoxify harmful ethanol metabolites in the liver by conjugating acetaldehyde and ROS to reduced glutathione [31]. Some *GST* alleles, such as *GSTM1* null allele, were found associated with liver diseases in alcoholics [34, 46]. However, among heavy and chronic ethanol consumers, no data are present in literature about a possible direct association between *GST* alleles and genomic damage measured in terms of SCEs, CAs and MNs. We investigated this possible association and observed in both groups, no statistically significant association between *GST* genes polymorphisms and the frequencies of SCEs, CAs, and MNs (Tables 2, 3, 4). However, the reduced sample size here considered requires further investigations with a larger number of subjects to provide convincing evidences for the absence of such correlation.

Finally, the increased levels of SCEs and CAs recorded among alcoholics appear to be associated with the duration of alcohol abuse, but not with the age of the subjects (Table 5). An age related increase of spontaneous chromosome instability was showed [2, 3, 13]. The lack of a similar age-related pattern among alcoholics could be probably due to an increased incidence of the chromosomal damage induced by alcohol among younger individuals. In this scenario the effect of alcohol abuse could obscure this relationship among alcoholics.

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