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Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project

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Abstract

The effect of tobacco smoking on the frequency of micronuclei (MN) in human lymphocytes has been the object of many population studies. In most reports, the results were unexpectedly negative, and in many instances smokers had lower frequencies of MN than non-smokers. A pooled re-analysis of 24 databases from the HUMN international collaborative project has been performed with the aim of understanding the impact of smoking habits on MN frequency. The complete database included 5710 subjects, with 3501 non-smokers, 1409 current smokers, and 800 former smokers, among subjects in occupational and environmental surveys. The overall result of the re-analysis confirmed the small decrease of MN frequencies in current smokers (frequency ratio (FR) = 0.97, 95% confidence interval (CI) = 0.93–1.01) and in former smokers (FR = 0.96, 95% CI = 0.91–1.01), when compared to non-smokers. MN frequency was not influenced by the number of cigarettes smoked per day among subjects occupationally exposed to genotoxic agents, whereas a typical U-shaped curve is observed for non-exposed smokers, showing a significant increase of MN frequency in individuals smoking 30 cigarettes or more per day (FR = 1.59, 95% CI = 1.35–1.88). This analysis confirmed that smokers do not experience an overall increase in MN frequency, although when the interaction with occupational exposure is taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the micronucleus assay in lymphocytes. From these results some general recommendations for the design of biomonitoring studies involving smokers can be formulated. Quantitative data about smoking habit should always be collected because, in the absence of such data, the simple comparison

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of smokers versus non-smokers could be misleading. The sub-group of heavy smokers (≥30 cigarettes per day) should be specifically evaluated whenever it is large enough to satisfy statistical requirements. The presence of an interaction between smoking habit and occupational exposure to genotoxic agents should be always tested.

Keywords: Human lymphocytes; Micronuclei; Smoking habit; Pooled analysis

1. Introduction

Lung cancer is the most common malignancy in western countries, and one of the most lethal. The role of tobacco smoking in the etiology of this disease has been well known for many decades, and any approach aimed at expediting the detection of population sub-groups at increased risk should be considered a high priority task. It may be possible to use genotoxicity assays to identify which sub-groups of smokers are more susceptible to the DNA-damaging effect of cigarette smoke and/or which level of smoking produces significant increases in mutation over base-line.

Many of the substances contained in cigarette smoke are genotoxic [1] and therefore cytogenetic damage seems to be an excellent biomarker for determining the effect of exposure to chromosome-damaging agents in smoke. In support of this hypothesis, an increased frequency of chromosome breaks has been recently demonstrated to be an initial event in carcinogenesis, suggesting that these alterations may play a significant role in assessing oncogenic risk [2,3].

Among biomarkers that can be used for this purpose, the measurement of micronuclei (MN) appears to be one of the most suitable. MN originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. They reflect chromosome damage and may thus provide a marker of early-stage carcinogenesis. The measurement of MN in peripheral blood lymphocytes (PBL) is a well-established tool in human biomonitoring [4]. The most commonly used method in human lymphocytes is the cytokinesis-block micronucleus (CBMN) assay. In the CBMN assay, MN are scored after a single cell division using binucleated lymphocytes (accumulated using cytochalasin-B) to eliminate the confounding effect of altered cell division kinetics on the MN index [5].

Despite the well-known presence of carcinogens in the tobacco smoke, results in the scientific literature linking smoking habits to MN frequency are rather controversial. A number of studies have been designed to evaluate the potential influence of background factors such as gender, age, or smoking habit on MN frequency. Subjects in these studies are generally healthy and not occupationally exposed to known genotoxic agents; an increase in MN frequency in smokers has been reported by a number of laboratories [6–10]. Other studies failed to find any effect [11], while the largest of these studies (not specifically designed to assess the effect of smoking, but with a good quality of exposure assessment) showed a reverse association, with smokers presenting a lower frequency of MN [12].

The effect of smoking was evaluated as a potential confounder in a large number of biomonitoring studies of occupationally or environmentally exposed populations and controls. Despite a few reports showing positive results [13–16], the large majority of studies did not find any association between MN and smoking habit [17–49]. The effect of cigarette smoking on the MN frequency was also evaluated in cancer patients and controls; however only weak and occasional associations were demonstrated [50–52]. In dietary intervention studies, no association was found in one study [53], while a significantly higher frequency of MN in smokers (25 cigarettes per day, on average) was reported in another [54].

In order to provide a more comprehensive evaluation of the literature, studies measuring MN frequencies in other tissues have been reviewed. Data on buccal, nasal, and urothelial exfoliated cells are most frequently available, but as with the other studies, positive associations [55–57] are counterbalanced by reports of non-association [22,27,35,58,59]. A more homogeneous pattern comes from animal

studies, which report substantial increases in MN in different species and tissues following exposure to tobacco smoke [60–63].

An obvious limitation of most population studies addressing this issue are their small sizes and the consequent lack of statistical power. Furthermore, many of these studies suffer from a poor assessment of exposure, and subjects are often roughly classified as smokers versus non-smokers, without considering the levels of cigarette consumption. The evaluation of smoking cessation is even more difficult, because former smokers sometimes are included in the group of current smokers, and sometimes with non-smokers.

The planning and organization of large studies with high-quality information regarding smoking habit is the best approach to understand the possible use of MN as a marker of exposure/effect in tobacco smokers. However, given the technical difficulties and high costs of designing large studies, the use of existing MN information seems to be a simple, suitable alternative. Experience from clinical trials indicates that a summary of the published studies could be very informative. The most popular approach, *meta-analysis*, combines the individual results published by independent scientists; the advantages and disadvantages of this approach have been described [64]. The results of observational studies can also be efficiently summarized by pooling individual records and re-analyzing the data. This approach has several advantages over the classical meta-analysis of published data, although the cost in time and work of pooling individual data is relatively high [65]. In many cases, this *pooled analysis* is the most informative way to extract information from available studies when the individual studies are too sparse to permit definitive conclusions. Among the other advantages of pooled analysis that seem to be the most valuable is the possibility to analyze variables not specifically evaluated in single studies (e.g. gender, age), to test the effect of different laboratory protocols and methods, to revise individual tests and perform reclassification a posteriori, and to perform prospective studies [66].

The creation of a common database of individual data from the most qualified laboratories working with the CBMN assay in lymphocytes was one of the basic achievements of the Human MicroNucleus (HUMN) project [4,67]. This international collaborative study was launched in 1997 with the purpose of addressing methodological issues related to the MN assay, identifying and controlling the variables in the procedures, providing a network of laboratories from around the world that use this technique, clarifying the long-term significance of MN in human PBL, and assessing the roles of individual characteristics, genetic background, and subject lifestyle on variability in the MN responses. The roles of host factors and methodological parameters on the base-line MN frequency have been addressed in a previous publication [67]. In the present study, another major issue is evaluated: the effect of cigarette smoking. This study has been performed to verify not only the genotoxic effect of cigarette smoking in PBL, but also the effect of smoking intensity and duration, and to evaluate the interaction with exposure to environmental genotoxic agents.

2. Materials and methods

2.1. Subjects and laboratories

The HUMN database has gathered individual data from 24 laboratories in 16 countries. Most of these data come from studies designed for occupational or environmental surveys of people exposed to genotoxic agents. Information about MN frequency, exposure to genotoxic agents, laboratory protocol, scoring criteria, and individual subject characteristics, were collected from participating laboratories through a detailed questionnaire. A more extensive description of the HUMN project, the assembly of the database, and its composition, is available elsewhere [67].

All data included in the HUMN database have been obtained from human lymphocytes using the cytokinesis-block technique [5]. The endpoint used for all statistical analyses is the frequency of micronucleated (MNed) cells per 1000 binucleated cells. There were 16 laboratories that reported the number of micronuclei instead of the frequency of micronucleated cells. In these cases, MN frequencies were estimated using the ratio, *number of micronucleated cells* to *number of micronuclei*, this ratio was calculated from the mean value of laboratories that reported both indexes, which was 1:1.12. This variable was eventually standardized to take into account the heterogeneity among laboratories [67,68].

An overall total of 5710 adult subjects who provided information on their smoking habits were included in the study. There were 3501 non-smokers, 1409 current smokers, and 800 former smokers. Statistical analyses addressing the dose-response relationship between cigarette smoking and MN frequency were based only on those subjects who provided the daily quantity of cigarettes smoked. As a result, 2558 subjects were excluded from the subsequent analyses that considered cigarette consumption, which were then based on 3152 (55.2%) subjects (2364 non-smokers and 788 current smokers) from 21 laboratories. Smoking intensity was categorized into four classes (1–9, 10, 19, 20–29, and ≥30 cigarettes per day). Other variables used in the multivariate analysis were sex, age at blood sampling (categorized in decades), and calendar year of test (≤1989, 1990–1994, ≥1995). As regards exposure to genotoxic agents, all subjects were reclassified as *exposed* or *non-exposed* using the exposure matrix developed by the ESCH study group [3], independently from the categorization assigned in the previous description of the database [67].

2.2. Statistical methods

A negative binomial regression model was applied to analyze the effect of cigarette smoking on MN frequency in each laboratory data set [69]. The use of this model implies a number of advantages. The negative binomial is a mixed model combining Poisson probability density function, i.e. the distribution of MN counts, and the Gamma probability density function. This last distribution takes into account the extra-Poisson variability that is a main cause of overdispersion, thus the resulting negative binomial distribution permits the correct estimate of the standard errors of the parameters. Frequency ratios (FRs) were estimated after adjusting by sex, age at test, year of test, and exposure to known genotoxic agents other than cigarette smoke.

The pooled effect of cigarette smoking on MN frequency was estimated fitting a *random effects* negative binomial regression model to the whole dataset to take into account the clustered nature of the observations (i.e. each laboratory is considered a cluster of data). Statistical analyses were carried out using STATA [70].

3. Results

The main characteristics of the 24 databases evaluated, including the overall frequency of MN and the available indexes of cigarette smoking, are reported in Table 1. The proportion of males is 53.6% and the mean age is 45.2 years (S.D. = 16.5, range = 11–89). The overall proportion of subjects classified as exposed to genotoxic agents was 24.8%. Information on the number of cigarettes smoked daily was available for 1042 subjects (788 current and 254 former smokers), while the dates of smoking beginning and cessation were reported for 259 former smokers. No independent, supporting information on cigarette smoke exposure, e.g. urinary cotinine levels, was available.

The effect of cigarette smoking on MN frequency was calculated for each laboratory before performing the pooled analyses, because the individual laboratory estimates are not affected by the clustered nature of the data or by the inter-laboratory variability. The mean MN FRs in current smokers versus non-smokers, with their 95% confidence intervals (95% CI), were calculated in the 20 databases where this comparison was possible, after adjusting by sex, age, and exposure to genotoxic agents. The result of this analysis is synthesized in Fig. 1.

Most laboratories showed no difference between smokers and non-smokers. A significantly increased frequency of MN in current smokers was found in one laboratory (AM3, FR = 1.82, 95% CI = 1.35–2.46), whereas two laboratories (EU2, AS5) showed significantly lower frequencies of MN in the group of current smokers, FR = 0.82, 95% CI = 0.74–0.90, and FR = 0.94, 95% CI = 0.88–0.99, respectively.

The overall result of the pooled analysis of data is illustrated in Table 2. Pooled FR adjusted by sex, age, laboratory, and calendar year of test showed lower frequencies in current smokers (FR = 0.97, 95% CI = 0.93–1.01) and in former smokers (FR = 0.96, 95% CI = 0.91–1.01), when compared to non-smokers, but the differences were not significant. The decrease in MN frequency was more evident among subjects who were not exposed to genotoxic agents, when compared to the groups of current (FR = 0.95, 95% CI = 0.92–0.99) and former (FR = 0.92, 95% CI = 0.88–0.96) smokers.

In the subset of former smokers (259 subjects overall), no evident effect of time since stopping was

Table 1
Characteristics of the 24 HUMN databases included in the study

Laboratory	Subjects	MN cells (mean (S.D.))	Cells scored (median)	Age (mean)	Males (%)	Subjects exposed (%)	Current smokers		Former smokers		Years since stopping (mean (*N*))
							N (%)	Cigarette per day (mean (*N*))	*N* (%)	Cigarette per day (mean (*N*))	
AM2	30	13.1 (7.5)	1892	49.8	70.0	0	14 (46.7)	1.0 (4)	0	–	–
AM3	59	18.3 (10.0)	1023	39.1	59.3	13.6	25 (42.4)	22.4 (25)	1 (1.7)	–	–
AS1	207	11.7 (9.7)	1000	33.2	31.4	42.0	37 (17.9)	–	0	–	–
AS2	124	10.4 (9.8)	1000	39.2	29.8	0	0	–	0	–	–
AS3	61	9.66 (4.2)	1000	38.4	80.0	0	0	–	0	–	–
AS4	10	16.9 (4.0)	1000	34.7	100.0	0	1 (10.0)	15.0 (1)	0	–	–
AS5	865	52.2 (14.8)	1000	62.3	38.8	100.0	225 (26.0)	17.3 (136)	138 (16.0)	20.0 (63)	12.9 (89)
AU1	118	9.0 (4.2)	2000	35.5	16.1	8.5	5 (4.2)	9.5 (5)	25 (21.2)	9.5 (13)	10.7 (20)
AU2	1019	17.3 (12.7)	1000	45.3	48.6	10.5	98 (9.6)	11.6 (53)	183 (18.0)	13.2 (67)	–
EU1	56	1.7 (1.5)	2000	42.0	64.3	37.5	32 (57.1)	16.6 (32)	0	–	–
EU2	1592	3.5 (3.0)	1000	45.7	48.5	0	419 (26.3)	–	223 (14.0)	–	–
EU3	400	6.0 (3.6)	2000	41.5	79.0	0	113 (28.3)	16.9 (110)	81 (20.3)	18.6 (31)	10.2 (58)
EU4	222	7.1 (4.9)	2000	41.3	82.4	27.9	82 (36.9)	20.0 (79)	52 (23.4)	28.4 (14)	4.6 (12)
EU5	39	7.4 (5.9)	2000	50.8	74.4	0	39 (100.0)	23.7 (39)	0	–	–
EU6	40	9.7 (6.4)	1500	26.1	57.5	0	11 (27.5)	14.2 (10)	3 (7.5)	22.0 (3)	9.0 (3)
EU7	50	8.9 (6.7)	1000	47.5	36.0	0	14 (28.0)	14.0 (7)	2 (4.0)	–	–
EU8	96	9.7 (7.0)	1000	43.1	80.2	0	7 (7.3)	14.3 (7)	26 (27.1)	19.4 (22)	10.5 (24)
EU9	116	9.5 (4.4)	1520	32.3	59.5	0	47 (40.5)	14.8 (47)	1 (0.9)	5.0 (1)	12.0 (1)
EU10	29	16.9 (8.8)	1000	53.0	86.2	0	6 (20.7)	16.3 (4)	9 (31.0)	–	23.6 (8)
EU11	25	9.7 (6.7)	1000	50.7	64.0	0	4 (16.0)	–	4 (16.0)	–	–
EU12	115	39.1 (33.9)	2000	40.2	54.8	49.6	71 (61.7)	16.8 (71)	2 (1.7)	–	5.5 (2)
EU13	212	14.4 (6.9)	1000	36.9	100.0	0	82 (38.7)	15.2 (81)	43 (20.3)	16.9 (40)	7.9 (42)
EU14	72	18.3 (11.3)	500	14.0	66.7	66.7	0	–	0	–	–
EU15	153	53.6 (39.6)	1000	36.7	70.6	100.0	77 (50.3)	21.8 (77)	7 (4.6)	–	–
Total	5710	17.6 (21.2)	1000	45.2	53.6	24.8	1409 (24.6)	17.4 (788)	800 (14.0)	17.4 (254)	10.9 (259)

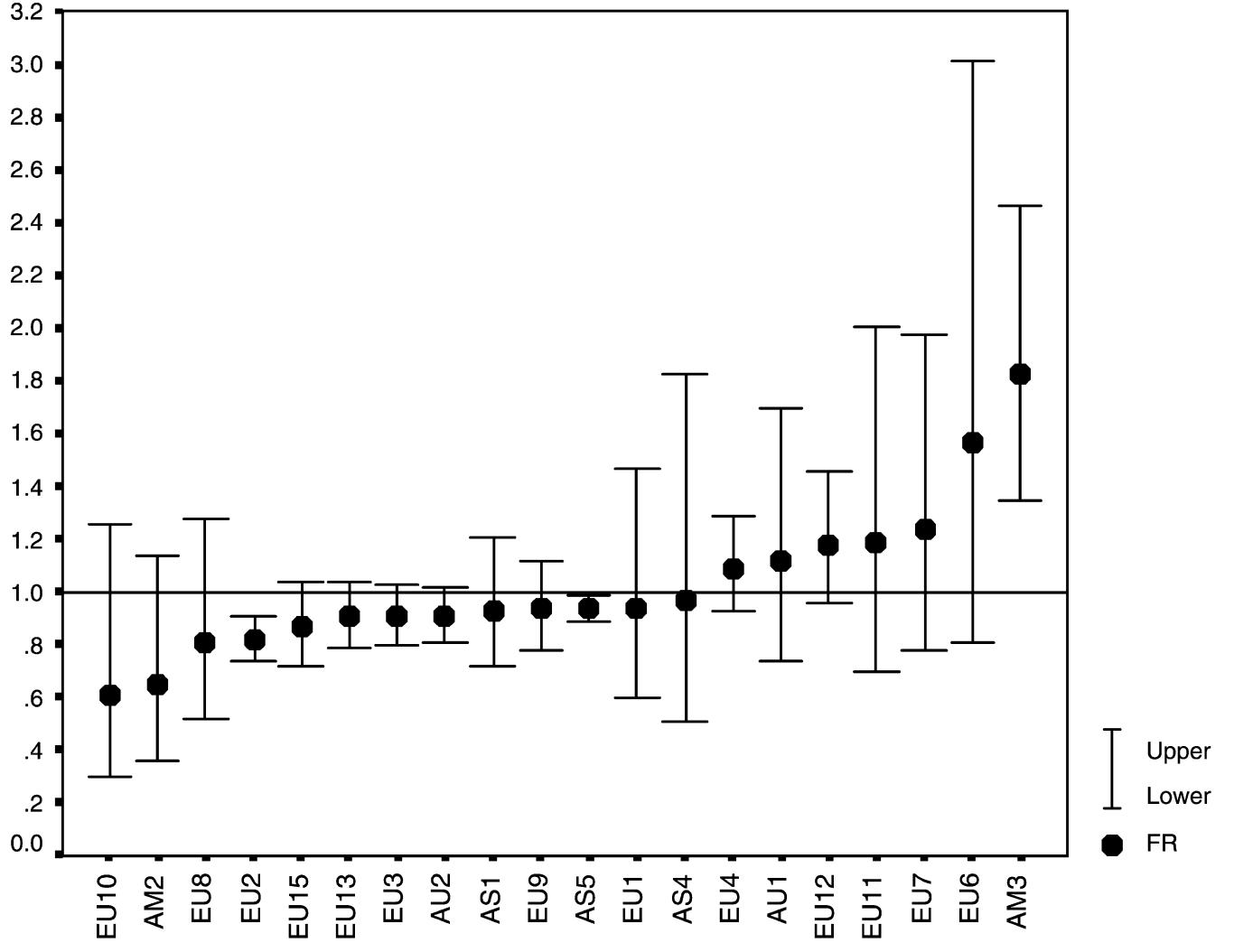

Fig. 1. FRs and 95% CI of MN frequencies in smokers vs. non-smokers in the HUMN databases (adjusted by sex, age, calendar year of test, and exposure to genotoxic agents).

Table 2
FRs according to smoking status and exposure to genotoxic agents (adjusted by sex, age, calendar year of test, and laboratory)

	Non-smokers		Current smokers		Former smokers	
	N	FR (95% CI)	*N*	FR (95% CI)	*N*	FR (95% CI)
Exposed[a]	827	1	413	0.96 (0.88–1.05)	178	0.96 (0.86–1.08)
Non-exposed	2674	1	996	0.95 (0.92–0.99)	622	0.92 (0.88–0.96)
Total[b]	3501	1	1409	0.97 (0.93–1.01)	800	0.96 (0.91–1.01)

[a] According to the ESCH, job-exposure matrix [3].
[b] Adjusted by exposure to genotoxic agents.

found. The FR for subjects who quit smoking more than 5 years before inclusion in the study versus those who ceased since 5 years or less was 0.94 (95% CI = 0.85–1.03).

The pooled evaluation of FR according to cigarette use shows a discrepancy between the group of subjects occupationally exposed to genotoxic agents and the unexposed controls (Table 3). The FR appeared to not be influenced by the number of cigarettes smoked per day among the exposed subjects, whereas a typical U-shaped curve is observed for non-exposed smokers. In this latter group, subjects smoking fewer than 20 cigarettes per day show slightly reduced MN frequencies in comparison with non-smokers; a limited increase is present in those smoking 20–29 cigarettes per day (FR = 1.05, 95% CI = 0.95–1.16); and a significant increase is evident in those smoking 30 cigarettes or more per day (FR = 1.59, 95% CI = 1.35–1.88). The presence of an increasing trend at high cigarette use is also confirmed by findings in the sub-group of 11 subjects smoking 40 cigarettes per day or more, who experienced an even higher FR of 2.47 (95% CI = 1.79–3.42).

The effect of smoking duration was evaluated in 206 current smokers. Subjects smoking longer than 20 years showed a FR of 1.11 (95% CI = 0.88–1.39) when compared with those smoking 20 years or less, after adjusting for main confounders, cigarettes smoked per day, and age at starting.

4. Discussion

The main results of this analysis can be summarized in two major statements.

(i) Smokers do not show an overall increase in MN frequency when compared to non-smokers.
(ii) A significant increase in MN frequency is evident in heavy smokers in the group not occupationally exposed to genotoxic agents.

These statements are qualified by the observation that occupational exposure to genotoxic agents is likely to mask the effect of smoking, and by the finding of a reduced frequency of MN in smokers of fewer than 20 cigarettes per day with respect to non-smokers.

These results provide a useful tool to interpret findings reported in the literature, which generally do not describe any effect of smoking status on MN

Table 3
FRs by daily consumption of cigarettes in those databases that reported the number of cigarettes smoked per day (adjusted by sex, age, calendar year of test, exposure to genotoxic agents, and laboratory); current smokers only

	Non-smoker		1–9 cigarettes per day		10–19 cigarettes per day		20–29 cigarettes per day		≥30 cigarettes per day	
	N	FR (95% CI)	*N*	FR (95% CI)	*N*	FR (95% CI)	*N*	FR (95% CI)	*N*	FR (95% CI)
Exposed[a]	757	1	41	0.90 (0.73–1.11)	85	0.97 (0.84–1.14)	130	1.03 (0.89–1.18)	50	0.98 (0.79–1.19)
Non-exposed	1607	1	73	0.91 (0.79–1.04)	178	0.98 (0.89–1.08)	182	1.05 (0.95–1.16)	49	1.59 (1.35–1.88)
Total[b]	2364	1	114	0.90 (0.79–1.03)	263	0.96 (0.87–1.05)	312	1.09 (0.99–1.19)	99	1.11 (0.96–1.29)

[a] According to the ESCH, job-exposure matrix [3].
[b] Adjusted by exposure to genotoxic agents.

frequency [71]. A Medline search of studies performed in subjects environmentally or professionally exposed to genotoxins (and their controls) that also analyzed the role of cigarette smoking, identified 33 publications out of the 37 evaluated (89.2%) that did not find an association between MN frequency and smoking habits [13–49]. In most of these studies, when the information about cigarette consumption was reported, heavy smokers were under-represented in the study groups, which could explain the apparent null effect.

The studies that we evaluated in the pooled analysis were not specifically designed to study the effect of smoking, and therefore misclassification may have occurred; such misclassification is likely to induce a towards-the-null bias. This interpretation is plausible, but is hardly the only reason for our findings, since it is rather unlikely that the same bias occurred in all laboratories.

Among the most plausible interpretations for this lack of effect are the low sensitivity of the MN assay for monitoring exposure to environmental genotoxic agents, or the lower effective concentration of cigarette smoke chemicals in the blood than in other organs, such as the lung. However, alternative hypotheses regarding individual susceptibility have been proposed. Only 10–15% of smokers develop lung cancer, and a smaller percentage develop lymphoid cancers [72]. Therefore, if the MN assay perfectly reflects the carcinogenic effect of smoking, then only a small percentage of smokers would be expected to have an increased frequency of MN caused by smoking. A further plausible explanation is the possible interaction between the numbers of cigarettes smoked and diet, e.g. heavy smokers tend to have a lower intake of folate and Vitamin B12, both of which are important contributors the frequency of MN in humans [53,54].

Surprisingly, the association between increased MN frequency and heavy cigarette smoking was found only in subjects who were not exposed to occupational carcinogens or mutagens. One explanation for this finding is that occupational exposure to genotoxins may have stimulated the expression of DNA repair genes or detoxification mechanisms that are also important in attenuating the genotoxic effects of chemicals in cigarette smoke. Also, the presence of a residual confounding due to differences in the genotoxic effect of various exposures may have been responsible for this result, i.e. subjects were classified as exposed regardless of whether it was to weak or potent mutagens/carcinogens. As regards the role of other indexes that have been recognized in the epidemiological literature to confound the carcinogenic effect of smoking, i.e. duration of smoking, and age at start, the analyses performed using our database were inconclusive, largely because of the limited number of subjects for which such information was available.

The lower frequency of MN observed in the PBL of light-medium smokers when compared with non-smokers, was definitely the most challenging result. This finding has been previously reported by other studies, and the adaptive response was often mentioned among the possible reasons. A few cigarettes per day may stimulate an adaptive (cell-protective) response, causing an apparent lowering in the MN frequency, and a continued exposure to mutagens/carcinogens may induce resistance to further DNA damage [58,73,74].

On the other hand, tobacco smoking may induce damage to lymphocytes, and the damaged cells may not survive the culture period in the CBMN assay, or may not divide. If they do not divide, they will not form binucleated cells and will not be scored for MN. The inverse relationship found between daily consumption of tobacco and frequency of BN cells may suggest that the genotoxic activity of tobacco smoke is expressed not only as direct damage to DNA in the form of chromosomal aberrations, but also as an inhibition of the proliferation induced in vitro by phytohemagglutinin [7]. The other possibilities are that: (a) exposure to cigarette smoke may induce in vivo expression of MN, but cells already containing MN may not divide in culture and are therefore not included as BN cells in the CBMN assay; and (b) cells damaged by cigarette smoke do not complete nuclear division in culture because they are more likely to die of necrosis or apoptosis. Kirsch-Volders and Fenech [75] proposed that the CBMN assay should be applied in its comprehensive mode by scoring MN not only in BN cells, but also in non-divided mononucleated cells, necrotic cells, and apoptotic cells. The inclusion of these parameters in the scoring process may result in a CBMN assay that is more suitable for biomonitoring purposes.

A possible effect of cigarette smoking on the lymphocyte proliferation rate can generate false negative results if damaged cells are delayed in their cell cycle [12,32]. This effect of tobacco could vary in different

cellular sub-populations, e.g. T-lymphocytes seem to be the most sensitive cells, and more evident effects of cigarette smoking measured in these cells have been reported [76]. The subset of T8-lymphocytes is the most sensitive to the effect of smoking, while a lack of effect is observed in the numerically larger population of T4 cells [77].

In conclusion, the re-evaluation of data from the large HUMN database that was assembled from laboratories in many countries has provided some interesting results concerning the effect of cigarette smoking on chromosome stability (measured as MN in PBL) in human populations. The data obtained suggest that only heavy smoking produces observable increases in the micronucleus frequency of lymphocytes however, the absence of an increase in moderate smokers does not imply that genotoxic effects are not being caused in the lung or other tissues. The extent to which lymphocytes can act as a surrogate for DNA damage in lung cells from inhaled toxins should be further investigated.

From these results, some general recommendations for the design of biomonitoring studies involving smokers can be formulated. Quantitative data about smoking habit should be always collected, because the simple comparison of smokers versus non-smokers could be misleading. The sub-group of heavy smokers ($\geq$30 cigarettes per day) should be specifically evaluated whenever it is large enough to satisfy statistical requirements. Finally, the presence of an interaction between smoking habit and occupational exposure to genotoxic agents should be always tested. Future studies should also consider implementing the CBMN assay in its comprehensive mode to include, apart from the frequency of MN in binucleated cells, measures of MN during necrosis, apoptosis, and cytostasis, as well as frequency of MN in mononucleated (non-divided) cells.

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