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Estimation of urinary cotinine cut-off points distinguishing non-smokers, passive and active smokers

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Abstract
An objective assessment of exposure to tobacco smoke may be accomplished by means of examining particular biomarkers in body fluids. The most common biomarker of tobacco smoke exposure is urinary, or serum, cotinine. In order to distinguish non-smokers from passive smokers and passive smokers from active smokers, it is necessary to estimate cotinine cut-off points. The objective of this article was to apply statistical distribution of urinary cotinine concentration to estimate cut-off points distinguishing the three above-mentioned groups. The examined group consisted of 327 volunteers (187 women and 140 men) who were ethnically homogenous inhabitants of the same urban agglomeration (Sosnowiec, Poland). The values which enabled differentiation of the examined population into groups and subgroups were as follows: 50 $\mu g\, l^{-1}$ (differentiation of non-smokers from passive smokers), 170 $\mu g\, l^{-1}$ (to divide the group of passive smokers into two subgroups: minimally and highly exposed to environmental tobacco smoke), 550 $\mu g\ l^{-1}$ (differentiation of passive smokers from active smokers), and 2100 $\mu g\, l^{-1}$ (to divide group of active smokers into two subgroups: minimally and highly exposed to tobacco smoke). The results suggest that statistical distribution of urinary cotinine concentration is useful for estimating urinary cotinine cut-off points and for assessing the smoking status of persons exposed to tobacco smoke.

Keywords: *Cotinine, cut-off point, non-smokers, passive and active smokers*

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Introduction

It is widely known that tobacco smoking causes many diseases and early deaths around the world. Epidemiological studies require differentiation of an examined population into smokers, former smokers and non-smokers. The group of non-smokers can be subdivided into two groups of non-exposed and persons exposed to tobacco smoke (passive smokers). The differentiation is based on self-reports in which examined subjects answer questions concerning their exposure to tobacco smoke. Self-reporting is the cheapest and easiest examination to perform that gives us information about the smoking status of examined persons. Sometimes the differentiation of an examined

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population requires collecting answers to many various questions and analysing the data. The questions have to be unequivocally and thoroughly understood by all examined persons. The results of such self-reports are not completely objective. The reliability and solidity of the answers are key factors that influence the differentiation of an examined population into non-smokers, passive and active smokers. Furthermore, an assessment of personal exposure to tobacco smoke based on self-reports is not completely objective because the amount of toxic constituents in tobacco smoke that is introduced to the body system depends on many factors such as, smoking behaviour and brands and types of cigarettes smoked (Patterson et al. 2003, Hammond et al. 2005). A laboratory quantification of markers and biomarkers of exposure to tobacco smoke is a better way to assess such exposure and objectively identify health hazards associated with active and passive smoking (Scherer 2005).

Among the various potential biomarkers of exposure to tobacco smoke, cotinine, a metabolite of nicotine, is recognized as the most selective and specific (Society for Research on Nicotine and Tobacco 2002). Boffeta et al. (2006) have recently suggested that serum cotinine level is a predictor of lung cancer risk.

A very wide range of determined concentration values in body fluids allows relatively precise separation of smokers from non-smokers but also from persons who are passively exposed to tobacco smoke. A key factor is the establishment of cut-off points that allow differentiation in these three groups. The cotinine cut-off points in published literature are very different (Table I). It should be stated that the majority of authors divided their examined population into only two groups, namely non-smokers and smokers. The third group of passive smokers is very rarely identified.

The Spanish government has recently introduced a complete smoking ban in all public places. This is the fifth country in the European Community that has implemented very restrictive antismoking laws for public places. Unfortunately, in Poland, where the mortality rate due to cardiovascular diseases and lung cancer is very high, legislation is still only under consideration, and the current law is very often disregarded. That is why people living in urban areas in Poland and who visit bars,

Table I. Cut-off points of cotinine urine concentration and corrected cotinine for creatinine concentration cited in the literature, used for differentiation of the examined population into groups of various exposure level to tobacco smoke.

Reference, first author (year)	Differentation	Cut-off points
		(μg cotinine l^{-1} urine)
Cummings (1990)	Non-smokers–smokers	90
Jarvis (1988), Gilbert (1993)	Non-smokers–smokers	50
Apseloff (1994)	Non-smokers–smokers	500
Lee (1995)	Non-smokers–passive smokers–smokers	20–100
Haufroid (1998)	Passive smokers–smokers	100
Holl (1998)	Non-smokers–passive smokers–smokers	100–500
Hobbs (2005)	Non-smokers–passive smokers–smokers	100–500
Savitz (2001)	Non-smokers–passive smokers–smokers	50–500
Agewall (2002)	Non-smokers–smokers	350
Bramer (2003)	Non-smokers–smokers	200
		(μg cotinine g^{-1} creatinine)
Henderson (1989)	Non-smokers–smokers	30
Secker-Walker (1997)	Non-smokers–smokers	550
Greaves (2001)	Non-smokers–smokers	248

clubs, discotheques, restaurants, etc. are highly exposed to environmental tobacco smoke (ETS).

The aim of this work was to establish criteria for differentiation in an examined population living in a highly urbanized area into non-smokers, and passive and active smokers according to determined cut-off points of urine cotinine concentration, and also to compare these results with self-reported smoking status.

Materials and methods

Characteristics of the examined population

Three hundred and twenty-seven volunteers (187 women and 140 men) between the ages of 19 and 60 years who lived in Sosnowiec (Poland) qualified for an examination based on self-reports. The mean age of examined informants was 32 ± 10 years. Using the random-route method, ten start points were selected from the districts of Sosnowiec. Each start point was identified by street address, and the number of start points was proportional to the number of residents living in each district. The volunteers participating in the examination had to answer questions concerning sex, age, current occupational status and facts about cigarette smoking and contact with smokers. Those who identified themselves as cigarette smokers had to declare how many cigarettes they smoked per day (16 ± 7) and the amount of time they had been actively smoking (9 ± 8 years). Passive smokers declared how much time they usually spent during a day with smokers at work or at home (4 ± 3 h). Non-smokers declared that they had never smoked before the examination and excluded possibilities of being in a room with active smokers.

The self-reported data were verified based on statistical analyses of cotinine concentration values in the urine of the examined persons.

All experiments were approved by The Committee on Bioethics at the Medical University of Silesia.

Determination of urine cotinine

Urine samples were collected in the morning, in fasting state (12 h after the last meal), and before smoking the first cigarette (active smokers). In order to extract cotinine from urine samples, the method described by Nakajima et al. (2000) was used. After basic hydrolysis of 10 ml of urine, cotinine was extracted with dichloromethane. The obtained extracts were purified using syringe filters GHP Acrodisc (25 mm, 0.45 μm) (Pall, Ann Arbor, USA). Once the extract was evaporated to dryness it was dissolved in 1 ml of borate buffer (pH 9). The obtained solution was purified using solid-phase extraction on C-18 columns (J.T. Baker, Deventer, the Netherlands). Cotinine was eluted from the column following the evaporation of final eluate to dryness. The dry extract was dissolved in 1 ml of methanol. Then 50 μl of this methanol solution was introduced into a column, LiChrospher 100 RP-18 (250×4, 6 mm, 5 μm) (Merck, Darmstadt), and the cotinine was eluted with a solution as described by Pichini et al. (1992). Separation was performed using high-performance liquid chromatography (Merck-Hitachi) in an isocratic system, in a reversed-phase, with a UV detector (eluate absorbancy was measured at $\lambda = 254$ nm). Cotinine was eluted with water: methanol: sodium acetate (0.1 M): acetonitryl mixture in volume ratios of 63:23:6:8 and pH of 4.3.

The retention time of cotinine was 4.3 min using the conditions described above. This time was confirmed with the chromatographic standard of cotinine. The mean recovery evaluated from five samples was $88.0 \pm 4.9\%$ for concentration values below 500 $\mu g\, l^{-1}$, and $95.4 \pm 3.2\%$ for concentration values higher than 500 $\mu g\, l^{-1}$. The detection limit of urine cotinine was 3 $\mu g\, l^{-1}$. Two ranges of cotinine concentration in urine (3–175 $\mu g\, l^{-1}$ and 200–3000 $\mu g\, l^{-1}$) were applied to validate the method. For the first range, the mean error of estimation, the error of the method and precision were as follows: 0.53, 5.1 and 6.5%, and for the second one: 4.7, 39.1 and 3.1%.

Statistical analysis

Statistical analysis of the obtained results was performed using Statistica 6.0 (StatSoft Inc. 2001), Origin 4.0 (Microcal Software Inc. 1991–1995).

The homogeneity of each examined group and statistical normal distribution of the results were confirmed using the Shapiro–Wilk test. The results were grouped into classes (k) of the same magnitude (Δx) and then the histograms were generated. The approximate value of Δx was calculated as a quotient of the results range R and the k value (Taylor 1999). The k and Δx values were chosen in order to obtain a statistical normal distribution of the results.

The homogeneity of examined groups variances was evaluated using the Levene test. The analysis of variances and the Scheffe test were used for evaluating the statistical significance of differences between the examined groups if the groups were homogeneous. If they were not homogeneous the Kruskal–Wallis test of the ranks was used.

Results

The cotinine concentration was determined in urine samples taken from 327 volunteers. Of these, 216 declared that they were non-smokers, 114 claimed they were not exposed to tobacco smoke while 102 claimed they were. One hundred and eleven subjects declared active cigarette smoking.

Many authors use values of cotinine level corrected per one gram of creatinine, instead of concentration values. We have determined in this study that the concentration values of cotinine in urine and cotinine: creatinine ratios were closely correlated in the whole examined population ($r = 0.92$, $p < 0.0001$). The correlation presented in the work (cotinine vs. cotinine/creatinine ratio) indicates that both these parameters can be used in the same way in order to divide the examined population into groups and subgroups.

The examined group can be characterized by mean urine cotinine value and its standard deviations only if there is a normal distribution of the values in the whole examined group. The determined values were as follows: 89 ± 90 for non-smokers, 391 ± 451 for passive smokers and 2159 ± 1493 for active smokers. The relatively high values of standard deviation and non-normal distribution of examined values in each group (confirmed by the Shapiro–Wilk test) indicate that all examined groups were heterogeneous. To solve this problem, we decided to analyse the graphic distribution of the frequency of the observed values (histograms). In order to get the Gaussian distribution of those values, the obtained concentrations were arbitrarily divided into classes of the same magnitude. The only criterion of such a manipulation was to

achieve the normal distribution in the histogram. When the Shapiro–Wilk test was applied to the results (whole examined population), we could not confirm the normal distribution for them. Therefore, starting from five classes ($k = 5$), we decreased the magnitude of the class (hence the number of classes increased) as much as it was necessary for the obtaining of the lowest number of curves of the Gaussian type. These curves fitted well the cotinine concentration distribution for the whole population, collected in these classes (Figure 1). We found that three Gaussian curves were sufficient for that procedure (one group of non-smokers and two groups of smokers). An intersection point of two Gaussian curves was taken as a cut-off point for the division of the investigated population into subgroups. We found that the obtained subgroups correlated with the groups generated based on the self-reports. Due to this fact, we proceeded to do further analyses only with the groups of non-smokers and smokers (the self-reports). The first cut-off point that allows for dividing the examined population into smokers and non-smokers was 550 $\mu g\, l^{-1}$ (Figure 1).

The same procedure (fitting of the Gaussian curves) was applied to the subgroup of non-smokers (Figure 2). It was shown that another differentiation of that subgroup was possible. In this case, we succeeded in identifying three subgroups, which included non-smokers and two passive smoker groups.

The distribution of the results from the combined group has two maximum values and can be subdivided into two separate ranges ($ab_{(-)}$) and ($b_{(+)}$). However, the results of 15 samples were out of these ranges (Figure 2). The range ($ab_{(-)}$) had a non-normal distribution of the analysed results (Shapiro–Wilk test). Moreover, a

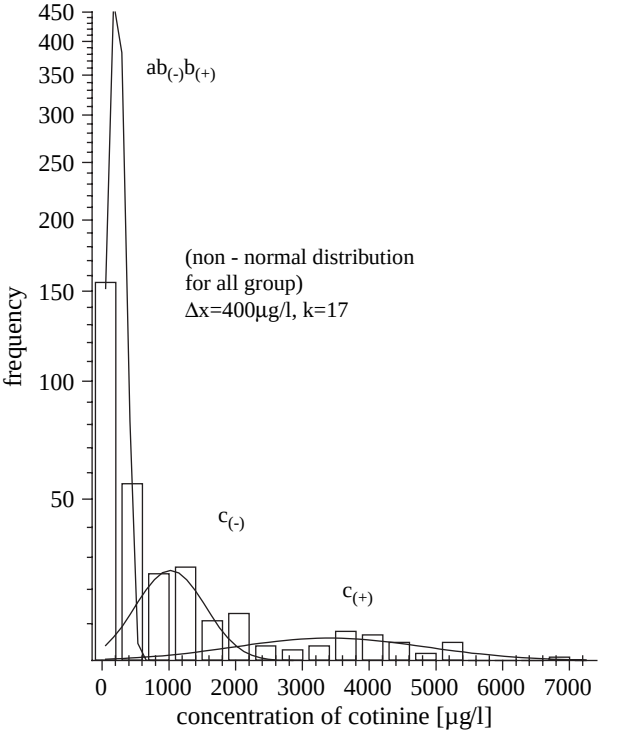

Figure 1. Statistical distribution of cotinine concentration in urine for the whole examined population. Δx, class range; k, class number.

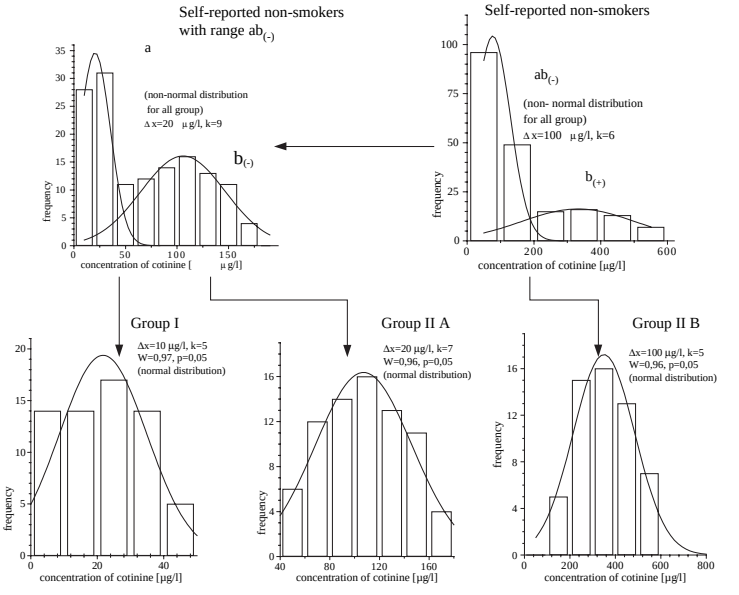

Figure 2. Statistical distribution of cotinine concentration in urine of self-reported non-smokers. Δx, class range; k, class number; W and p, results of Shapiro–Wilk test.

theoretical mean value calculated for that range had a high standard deviation (higher than the mean value): 34 ± 111 μg l^{-1}. The above-mentioned facts indicated that there were further differences within the range ($ab_{(-)}$). This fact was confirmed during the next analysis after decreasing the Δx value from 100 to 20 μg cotinine l^{-1} (Figure 2). As a result, the group was subdivided into two ranges with a normal distribution: (a) with lower and ($b_{(-)}$) with higher urine cotinine values. This means that the number of non-exposed non-smokers was lower than the one declared in self-reports and that this group corresponds to the results from range (a). The number of non-smokers actually exposed to second-hand tobacco smoke was higher than the one declared in self-reports and corresponds to the results from ranges ($b_{(-)}$) and ($b_{(+)}$). The scheme of the above-discussed differentiation is presented in Figure 3.

The analysis (Figure 2) leads to differentiation of the examined population into group I of non-exposed non-smokers (range a, from 0–50 μg l^{-1}) and group II of passive smokers (ranges $b_{(-)}$ and $b_{(+)}$, from 50 to 550 μg l^{-1}). The analysis leads also to the differentiation of group II into two subgroups, IIA and IIB, of different exposure levels. The cut-off point for these two group was 170 μg l^{-1}. The cut-off points that corresponds to crossing points of Gaussian curves are presented in Figure 3.

The results of active smokers were analysed separately (Figure 4). The analogous analysis of active smokers also leads to the differentiation of this group into two ranges with normal distribution ($c_{(-)}$) and ($c_{(+)}$) (Figure 4). This means that the group of active smokers can also be subdivided into two subgroups: IIIA (range $c_{(-)}$) and IIIB (range $c_{(+)}$) of lower and higher urine cotinine values, respectively. The groups (IIIA and IIIB) also contained results of 21 non-smokers with higher urine cotinine concentration that were outside the range ($b_{(+)}$). The cut-off point (Figure 3) that corrects group IIIA from IIIB is 2100 μg l^{-1}.

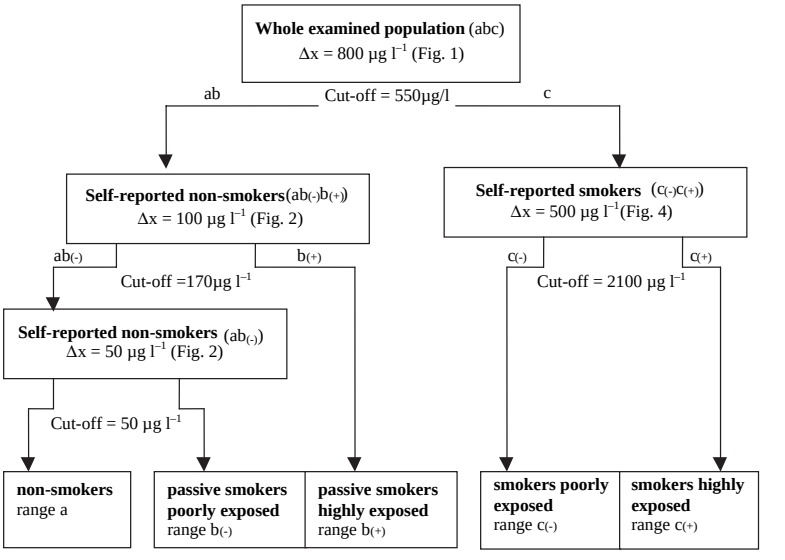

Figure 3. Scheme of differentiation of population into subgroups.

The presented cut-off points enable a more objective differentiation (relative to self-reporting) into groups of various exposure levels to tobacco smoke. The method presented in this work enables the separation of non-smokers and so-called passive smokers, as well as the further differentiation of the latter group – due to exposure level – in the same way that active smokers can be subdivided. This approach also leads to a normal distribution of separated ranges (Figures 1, 2 and 4). The characteristics of each group after verification of corresponding self-reports are presented in Table II.

The next step of the work was verification of the self-reported exposure status against the obtained cut-off points (Figure 5). Among the 114 examined persons who declared no exposure to tobacco smoke, only 53 were classified in group I of non-exposed non-smokers and 61 in group II of passive smokers (44 in group IIA and 17 in group IIB). Probably those persons were unknowingly exposed to environmental tobacco smoke. This phenomenon influenced the error of self-reports differentiation of those persons, which was 54%.

Among the 102 subjects who declared passive smoking, 72 were classified in group II, but 11 with lower cotinine concentration were classified in group I, and 21 were classified in group III (19 in group IIIA and two in group IIIB). The error of self-report differentiation of those persons was 32%.

All 111 of the volunteers who declared active smoking were classified in group III of active smokers. Fourteen smokers had urine cotinine concentration in the group IIB range. However, these persons declared smoking a few cigarettes per day or occasionally smoking. So, in this case these persons were classified as being members of group III according to their self-report declaration.

The proposed method can be used for any other examined population in order to create such subgroups.

The effects of age, sex, and number of cigarettes smoked were also examined. The results of multiple regression analysis are presented in Table III. The results show that

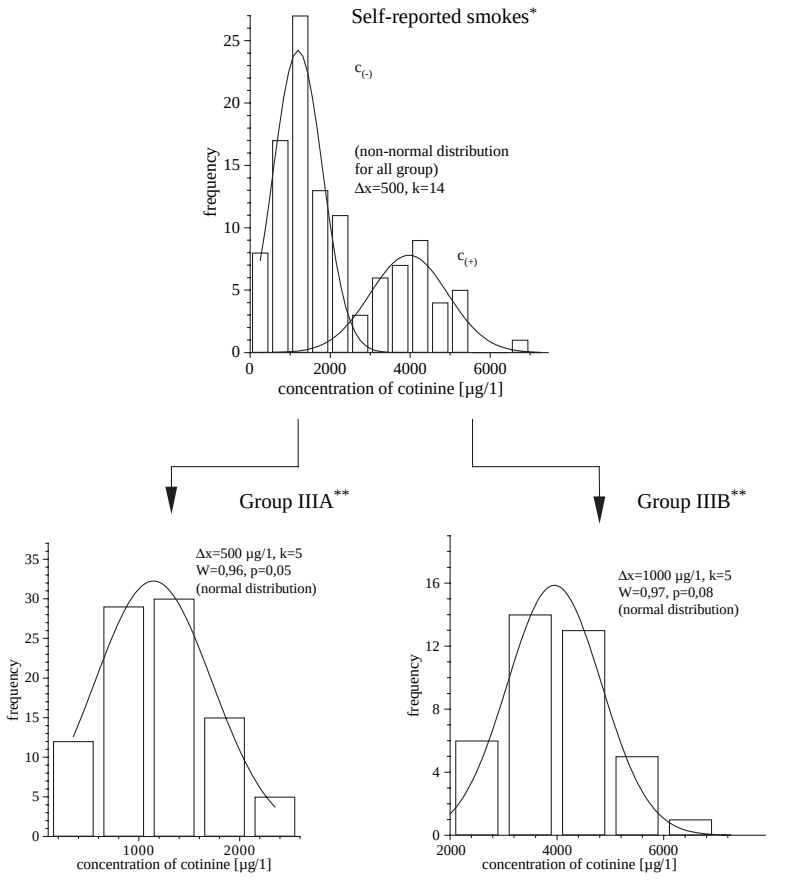

Figure 4. Statistical distribution of cotinine concentration in urine of self-reported active smokers. *Number of smokers consistent with questionnaire; **number of smokers extension about 21 persons self-reported passive smokers (19 persons included in the group IIIA and 2 person to the group IIIB). Δx, class range; k, class number; W and p, results of Shapiro–Wilk test.

age and number of cigarettes smoked have a significant effect on urine levels of cotinine ($p < 0.0001$). The age factor was observed for the whole examined population in 26%; however, the mean age of each obtained group was similar (Table II). The effect of number of cigarettes smoked was observed for the whole examined population in 67%. Moreover, the effect of the number of smoked cigarettes was observed among all self-reported smokers in 66%.

Table II. Characteristics of groups I, IIA, IIB, IIIA and IIIB.

Group (range)	I (a)	IIA ($b_{(-)}$)	IIB ($b_{(+)}$)	IIIA ($c_{(-)}$) +19	IIIB ($c_{(+)}$) +2
n	64	76	55	93	39
$\bar{x} \pm$ SD (μg l^{-1})	22 ± 13	107 ± 33	350 ± 120	1073 ± 470	3729 ± 1070
min ÷ max (μg l^{-1})	0.0 ÷ 48.4	51.0 ÷ 169.3	172.8 ÷ 550.0	185.0 ÷ 2100.0	2151.0 ÷ 6859.0
age ± SD (years)	34 ± 13	32 ± 13	33 ± 13	30 ± 10	32 ± 15

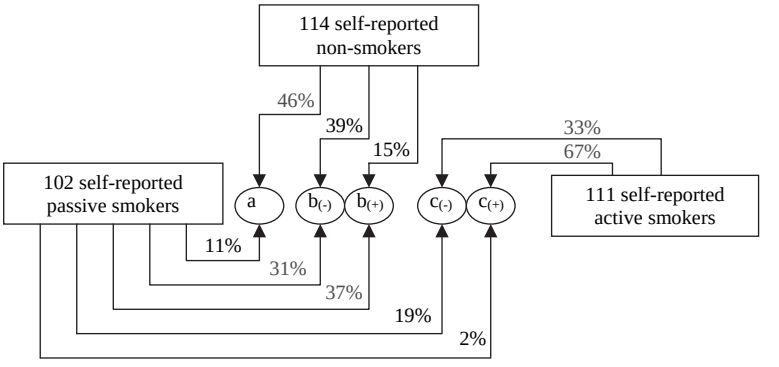

Figure 5. Verification of the self-reported exposure status.

Discussion

In other published work, groups of passive smokers and non-smokers are very often taken together (Apseloff et al. 1994, Secker-Walker et al. 1997, Greaves et al. 2001, Agewall et al. 2002). However, the separation of these groups is a very important factor because of the health effects observed in the group of passive smokers (Barnoya & Glantz 2005).

In order to separate the group of passive smokers from the group of non-exposed non-smokers, different cut-off points varied from 20 to 100 μg cotinine l^{-1} (Table I). Exposure to second-hand smoke in public places has been a particular public health concern for more than two decades. Studies using biomarkers confirm that second-hand smoke exposure in public places continues to affect non-smokers. Using NHANES III data, several investigators have shown that people with home or workplace exposures still had detectable levels of cotinine in their serum (US Department of Health and Human Services 2006). This finding suggests that many people are exposed to second-hand smoke in other locations. Restaurants, cafeterias and bars are worksites as well as public places where smoking is frequently unrestricted or restricted in a manner that does not effectively decrease exposure. There were some difficulties with urine cotinine of non-exposed persons who eat vegetarian food containing nicotine (and also smokeless tobacco). Cotinine concentration in the urine samples taken from those persons varied from 1.5 to 6.0 μg l^{-1} (Benowitz 1996), sometimes much higher, but always lower than the cut-off point of 50 μg l^{-1} (Jarvis et al. 1988, Gilbert 1993, Society for Research on Nicotine and Tobacco 2002). The same cut-off point of 50 μg l^{-1} was the result of our analysis of

Table III. Multiple regression between the cotinine concentration and age, gender, number of cigarettes smoked, for all 327 volunteers.

	B	SE B	ß	SE ß	*p*-values
Intercept			**961.2**	**155.2**	**0.000001**
Age	**−0.26**	**0.04**	**−24.4**	**3.9**	**0.000001**
Gender	0.032	0.04	74.5	95.2	0.43
Number of cigarettes smoked	**0.67**	**0.06**	**99.4**	**8.9**	**0.000001**

Significant factors are marked. SE, standard error.

the examined population. By applying this cut-off point in our study, the mean cotinine concentration in group I (non-smokers) was 22 ± 13 μg l^{-1}. This mean value was similar to ones cited by other authors: 17 μg l^{-1} (Vine et al. 1993, Chambers et al. 2001) and 23.2 μg l^{-1} (Agewall et al. 2002). However, Upper Silesia, where the examined population came from, is the most highly industrialized and urbanized region of Poland (and Europe). Although the smoking restrictions in public places are obligatory, there are still many places, like bars, pubs and restaurants, where smoking is commonly tolerated. This might be the reason people who declared not being exposed to tobacco smoke are indeed exposed to ETS.

The cut-off point used to separate groups of passive and active smokers cited in literature was 100 (Lee 1995), and in the other studies even as high as 500 μg cotinine l^{-1} (Holl et al. 1998, Savitz et al. 2001). However, sometimes the concentration of cotinine in urine samples taken from passive smokers exposed to ETS with high intensity can be very similar to that measured in samples taken from active smokers who smoke only a few cigarettes a day or smoke only occasionally. The cut-off point for separating groups of passive and active smokers in our study was 550 μg cotinine l^{-1}. On the other hand, by using the next cut-off point of 170 μg l^{-1} it was possible to divide group II, passive smokers, into two subgroups, IIA and IIB, with different intensities of exposure to ETS. The mean urine cotinine concentration in group IIA of passive smokers minimally exposed to ETS was 107 ± 33 μg l^{-1}, and in group IIB of passive smokers highly exposed to ETS was 350 ± 120 μg l^{-1}. The mean concentration in the whole group II was 229 ± 77 μg l^{-1}. It is difficult to discuss the results because in the literature there are only mean values for homogenous groups of passive smokers. They are as follows: from 198.6 (Cok & Öztürk 2000), 260 (Suter et al. 1995) to 372 μg l^{-1} (Piekoszewski et al. 2002).

It was also possible to divide group III, active smokers, into to subgroups IIIA and IIIB by using the cut-off point 2100 μg l^{-1}. The mean concentrations of urine cotinine were 1073 ± 470 μg l^{-1} and 3730 ± 1070 μg l^{-1} for smokers minimally and highly exposed to tobacco smoke, respectively. The mean concentration for the whole group III was 2200 ± 800 μg l^{-1}. The obtained results are in a range of formerly published values of 580–7870 μg l^{-1} (Suter et al. 1995, Holl et al. 1998, Chambers et al. 2001, Agewall et al. 2002, Becker et al. 2003). The obtained mean values are similar to those published by Cok & Öztürk (2000), but two times lower than those published by Vine et al. (1993). However, the differentiation of active smokers into minimally and highly exposed persons, cited in both of the above-mentioned works, was performed only according to their self-reported number of cigarettes smoked per day.

In only one case does the verification of the differentiation methodology for an examined population – according to their tobacco smoke exposure status – require an arbitrary decision. It concerns smokers who smoke only a few cigarettes a day or who only smoke occasionally. Although the cotinine concentration in urine samples taken from these persons is relatively low, they were classified in group III of active smokers according to their self-reports.

The effect of many factors, like type of smoked cigarettes, smoking behaviour and topography, number of cigarettes smoked, time of exposure to indoor air polluted with ETS, room volume and air exchange, diet, sex, race, metabolisms and many other individuals factors, must be taken into consideration when such differentiation is performed. On the other hand, environmental pollution, geographic and climate conditions must also be analysed. It should also be stated that urine cotinine might

be influenced by the polymorphism of the *CYP2A6* gene because of its role in nicotine metabolism (Yoshida et al. 2002). However, a frequency of CYP2A6*2, CYP2A6*4 and CYP 2A6*5 alleles that cause isoenzyme activity depletion is low in Caucasian populations (represented by examined persons) and equals 0–3%. So, it can be concluded that the polymorphism of the *CYP2A6* gene might only slightly influence the obtained results (Malaiyandi et al. 2005, 2006).

The characteristics of the examined population determine which of the above-mentioned factors might be omitted during its differentiation, e.g. for people who live in the same small area or are of the same sex. It must be assumed that the number of factors that influence the cotinine results of the same group is unknown, even though it is sTable I of the differentiation process leads to many subgroups, it means that there will be some such factors. Such factors for urine cotinine levels are number of cigarettes smoked per day and exposure level to environmental tobacco smoke.

The cut-off points estimated in the study might only differ among different populations living in various areas of world (and even in Poland) just slightly. The main aim of the paper is the methodology of the differentiation procedure, which seems to be very useful for any examined population (from various regions). Attention should be given only to the special factors that might influence cotinine urine levels (or any other biomarker), because these factors, in special situations, might lead to further differentiation of the examined population.

Conclusions

It can be concluded that under controlled study conditions: (i) analysis of obtained results and determined cut-off points of cotinine urine concentration presented in the work allow for more objective differentiation of an examined population into groups of non-smokers, and passive and active smokers; (ii) the presented methodology allows for differentiation of selected groups into subgroups – a group of passive and active smokers can be divided into subgroups of 'minimally' exposed and 'highly' exposed persons; (iii) although the cut-off points presented in the work are not universal, the proposed statistical analysis of the results seems to be adequate for determining the limiting values that allow differentiation of every examined population into the discussed groups and subgroups; (iv) the presented results might be used in campaigns for smoke-free environments in Poland.

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