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Effect of aliphatic alcohols and their metabolites on functional state of monocytes and polymorphonuclear leukocytes and population-based assessment of health risk from consumption of spirits

by Mrs. Orsolya Marozsán

Supervisor: Dr. Sándor Szűcs

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By Mrs. Orsolya Marozsán, MSc

Supervisor: Sándor Szűcs, PhD

Doctoral School of Health Sciences, University of Debrecen

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1. Introduction

Alcohol consumption especially heavy drinking is an increasing public health, social and economic concern both in the developed and developing countries. The latest available data from the World Health Organization (WHO), from 2016, showed that the average per capita alcohol consumption was 6.4 liters, which refers to 13.9 grams ethanol/day. In the same year, 43.0 % of the world population (2.35 billion individuals) was regular drinker including those people who have previously consumed alcohol in the previous 12-month period. Among them, the per capita alcohol consumption is considerably higher (15.1 liters of pure alcohol/year or 32.8 grams of pure alcohol/day). Globally, the prevalence of heavy episodic drinking (defined as 40 and 60 or more grams of pure alcohol on at least one occasion at least once per month by women and men, respectively) was 18.2% in 2016. There were also wide variation in total alcohol consumption in the different region of WHO. The highest per capita alcohol consumption was the highest in Europe (10-12.4 liters/year). The alcohol consumption levels were less in the WHO Region of the Americas and the Western Pacific but also in some countries in the African Region. The lowest alcohol consumption, less than 2.5 liters/capita/year, was in the WHO Eastern Mediterranean, African, and South-East Asia Regions.

Although most alcohol consumed worldwide is from commercial sources, with its manufacture and sale subject to regulation by government authorities, a proportion, which in some parts of the world may be considerable, is unregulated and thus unrecorded. The production of recorded alcohols is well controlled. In contrast, unrecorded alcohols are not subjected to quality control.

The term ”unrecorded alcohols”, sometimes termed non-commercial alcohols, comprise a diverse range of beverages. They have a variety of sources, including counterfeit, relabelled, and smuggled alcohols, home-made spirits, and surrogate alcohols not usually considered suitable for human consumption, such as aftershaves, perfumes, and medical tinctures, which often contain very high level of ethanol. The latest available data from the World Health Organization, from 2016, estimated that the worldwide adult per capita alcohol consumption from these unrecorded sources was 1.8 liters, equivalent to 25.5 % of alcohol consumed worldwide, although this varies considerably among countries, with the highest share (47.9 %, 1.42 liter/capita) in low-income countries but comprising a far from negligible share (11.2 %, 1.18 liter/capita) even in the most developed countries. Compared to Western-Europe, the share of unrecorded alcohol drinking especially consumption of home-made fruit spirits is

significantly higher in Central and Eastern-European (CEE) countries. Among 26 European countries, the proportion of unrecorded alcohol consumption was the highest and lowest in The Republic of Moldova (36.8%, 5.6 liter/capita) and Hungary (13.1 %, 1.5 liter/capita), respectively.

Although, the greatest part of exposure from consumption of alcoholic beverages originates from ethanol, alcoholic drinks also contain several toxic and carcinogenic substances that directly or in the form of their metabolites pose a health risk for the consumers. Their concentration in alcoholic beverages depends on raw materials used for production, circumstances of alcoholic fermentation, equipment used during brewing, distillation, bottling and storage.

Hundreds of toxic chemicals have been identified in alcoholic beverages. Some of them such as ethyl-carbamate, nitrate-ions are defined by the International Agency (IARC) as probably carcinogenic to humans (Group 2A). In addition, acetaldehyde alone, that can be produced during alcoholic fermentation, acetaldehyde is probably carcinogenic to humans, while in combination with ethanol is carcinogenic (Group 1) to humans. Previous studies have demonstrated that alcoholic beverages are often contain other aliphatic alcohols (OAAs) including methanol and higher alcohols containing more than two carbon atoms, such as 1- and 2-propanol, 1- and 2-butanol, isobutanol, and isoamyl alcohol. They are usually formed as by-products during alcoholic fermentation of maize, rice, wheat, millet, and, especially, many fruits spirits. Chemical analysis of a range of European alcoholic beverages has found average concentrations of AAs of 85 mg/l and 319 mg/l in beer and wine samples, respectively but in distilled spirits this figure can be as high as 1836 mg/l. Due to different raw materials used for production and methods of production, there can be alterations in the concentration of OAAs in recorded and unrecorded alcoholic beverages. Consequently, it is important to ascertain whether there is any difference between the OAAs in recorded and unrecorded spirits. However, most previous studies from CEE countries were purely descriptive, with no formal comparison of products from different sources using standardized methods. Those studies which did undertake comparisons of recorded and unrecorded alcohols yield inconsistent results. In one study, concentrations of OAAs were significantly higher in home-made spirits than in commercial products but, in another, they were significantly lower in unrecorded than recorded spirits. However, although that study analyzed a large number of samples of recorded spirits, the levels of OAAs in unrecorded products came from previous investigations in which different types of unrecorded beverages from several European countries were analyzed.

Therefore, the comparison in this study could be biased by inclusion of unrecorded alcohols with low concentrations of higher alcohols.

Epidemiological studies have demonstrated that the excessive consumption of recorded and unrecorded alcoholic beverages contributes to global burden of diseases considerably. In 2016, the harmful use of alcohol resulted in some 3 million deaths (5.3% of all deaths) worldwide and 132.6 million disability-adjusted life years (DALYs) – i.e. 5.1% of all DALYs in that year. Among men in 2016, an estimated 2.3 million deaths and 106.5 million DALYs were attributable to the consumption of alcohol. Women experienced 0.7 million deaths and 26.1 million DALYs attributable to alcohol consumption. Epidemiological studies have shown that more than 200 diseases can be associated with harmful alcohol use, ranging from injuries, accidents, digestive diseases, cardiovascular diseases, infectious diseases, cancers and alcohol abuse. In addition to the volume of alcoholic beverages consumed, the health consequences can also be influenced by the type (beer, wine, spirit) and quality of alcoholic drinks.

Alcohol-related mortality is significant public health concern in certain CEE countries. Although alcohol-attributable mortality decreased between 2005 and 2016 in the Republic of Moldova (112.52/100 000), Serbia, Hungary (124.53/100 000), the Russian Federation (129.88/100 000), Ukraine (191.22/100 000), Poland (72.29/100 000), and Romania (194.62/100 000), it remained higher than in Western Europe (47.97/100 000 population). One of several reasons can be that compared to European average the share of consumption of alcoholic beverages containing high amount of ethanol is larger in these CEE countries. For example, 16-52 % of all registered alcohol consumed in Hungary, Romania, Slovakia, Lithuania, Belarus, Ukraine, The Russian Federation, and The Republic of Moldova is in the form of spirits. However, the proportion of spirit consumption is probably higher because unrecorded spirits especially home-made fruit spirits are widely consumed in these countries. Compared to ethanol, these aliphatic alcohols have significantly greater hepatotoxic effects. It has been suggested that this may contribute to the very high level of premature mortality from chronic liver diseases and cirrhosis in those CEE countries where these products are widely consumed. However, this alone cannot explain the difference in mortality and attention has focused on two factors, drinking pattern and the role of unrecorded alcohol consumption. Although, cirrhosis related mortality has showed a decreased tendency from 2015, but in the east-European countries 1.9 times (10.05/100 000 capita) more, while in the CEE countries 1.6 times (8.47/100 000 capita) the European average (5.28/100 000 capita) in 2019.

In addition to effects on the liver, gastrointestinal, nervous and cardiovascular systems, acute and chronic alcohol consumption has been found to cause numerous immunotoxic effects including decreased antigen-specific T-lymphocyte activation and proliferation, changes in T-lymphocyte cytokine synthesis and B-lymphocyte immunoglobulin production, and reduced granulocyte chemotactic and phagocytic activities. Consequently, alcohol abuse has been associated with increased susceptibility to bacterial and viral infections and higher morbidity and mortality from a number of infectious diseases including tuberculosis, pneumonia, human immunodeficiency virus-1 and hepatitis B and C infections. (Previous epidemiological studies have showed a strong relationship between heavy drinking and risk of severe bacterial and viral infections such as pneumonia, tuberculosis, Hepatitis B and C.). Monocytes, as essential mononuclear phagocytic cells, are recruited to the site of infections after granulocytes and provide the second line of host defence against bacteria, fungi, viruses, and virally-infected cells. They play a pivotal role in phagocytosis and killing of invading pathogenic microorganisms, moreover monocyte-derived macrophages are the most significant cells responsible for the engulfment and clearance of apoptotic cells. Defects in monocyte functions have been proposed to contribute to the impaired anti-microbial defences noted in alcoholics. It has been reported ethanol can suppress monocyte phagocytosis. We have previously shown that AAs can inhibit superoxide-anion production and phagocytosis by human granulocytes and monocytes. OAAs can also enter the body with the spirits, which had metabolized in the liver by alcohol dehydrogenase (ALD) enzymes, with ethanol, methanol, 1- and 2-propanol, and 1- and 2-butanol giving rise to acetaldehyde, formaldehyde, 1-propanal, acetone, 1-butanal, and 2-butanone, respectively. These metabolites are more hazardous to human health than the original molecules. Although some effects of formaldehyde, acetaldehyde and acetone on tissues are already known, research on human immune cells has been limited. Previous studies have shown that current and heavy drinkers approximately 20-30% suffers from alcoholic liver diseases, including chronic hepatitis and cirrhosis. Alcohol-induced liver damage is a consequence of inflammatory processes and may be associated with increased migration of PMNLs into the liver.

Previous studies have demonstrated that ethanol and peptides can increase the migration activity of PMNLs, so it may contribute to infiltration of PMNLs into the liver parenchyma in alcohol abusers and development of alcoholic hepatitis. Ethanol, the main component of alcoholic beverages, disrupts the physical structure of the cell membrane when incorporated into the phospholipid bilayer, rearranging its compartments in ways that increase its fluidity. This

process leads to disturbances in molecular transport processes, some receptor functions, and enzyme activities. Thus, in addition to its direct hepatotoxic effects, ethanol may contribute further to liver disease by a variety of other mechanisms, including changes to membrane fluidity, a phenomenon whose effects include increased granulocyte migration. Changes in membrane fluidity have been implicated in the aetiology of several chronic inflammatory diseases.

Although, the alcohol consumption poses a significant health risk, the population-based risk assessment of alcohol intake has been performed so far by only one research group. Data on the concentration of OAAs in unrecorded alcohols from several European countries, per capita unrecorded alcohol intake, body weight of consumers, life expectancy at birth, and No Observed Adverse Effect Levels (NOAEL) of OAAs were used in their population-based probabilistic risk assessment using Monte Carlo simulation. However, the concentrations of OAAs, except methanol, in all samples were found to be low so the hazard posed by ethanol and methanol only were taken into consideration in that study. It concluded that the public health risk from consumption of non-commercial alcohols was solely due to their higher concentration of ethanol.

2. Aims of the study

To assess the health risk associated with excessive consumption of spirits, it is important to determine both the concentration of ethanol and OAAs. The composition of recorded and unrecorded spirits, especially spirits may differ. However, those studies which did undertake comparisons of recorded and unrecorded alcohols yield inconsistent results. In one study, concentrations of OAAs were significantly higher in home-made spirits than in commercial products but, in another, they were significantly lower in unrecorded than recorded spirits Although that study analysed a large number of samples of recorded spirits, the levels of OAAs in unrecorded products were measured in different types of non-commercial beverages including wines and beers, home-made spirits, surrogate and medical alcohols, smuggled and relabelled alcoholic beverages. This was a very heterogeneous group of unrecorded alcohols. As a result, the comparison could be biased by inclusion of unrecorded alcohols with low concentrations of OAAs, such as beers, wines, vodka, brandy, smuggled and relabelled alcohols, surrogate and medical alcohols. This limitation can only be overcome by comparing concentrations of OAAs in similar types of recorded and unrecorded alcohols.

1. Therefore, our aim was to ascertain whether there is any difference in the amounts of OAAs in recorded and unrecorded spirits.

Although some effects of acetaldehyde on tissues are already known, research on human immune cells has been limited. A previous study reported that acetaldehyde can inhibit PMNL and monocyte phagocytosis. Metabolites of OAAs could also influence phagocytic activity of PMNLs and monocytes, but this has not previously been studied. Therefore, we have sought to investigate granulocyte and monocyte phagocytosis following treatment of cells with some metabolites of OAAs alone and in combination with acetaldehyde. We intend to answer the following questions:

2. How do metabolites of OAAs alone and in combination with acetaldehyde affect phagocytosis by PMNLs and monocytes?
3. Are metabolites of OAAs alone and in combination with acetaldehyde able to influence phagocytosis by PMNLs and monocytes at toxicologically relevant concentrations?
4. Can metabolites of OAAs alone and in combination with acetaldehyde contribute to the ethanol-induced immunosuppression thereby increasing the susceptibility to infectious diseases in alcohol abusers and heavy drinkers?

Ethanol, the main component of alcoholic beverages, disrupts the physical structure of the cell membrane when incorporated into the phospholipid bilayer and increase its fluidity. Ethanol-induced increases in membrane fluidity may be associated with increased migration of PMNLs. OAAs can also affect membrane fluidity and migration. However, their effects on membrane fluidity and migration of PMNLs have not been investigated. Therefore, the other aim of our study was to examine the effects OAAs on these processes. We intend to answer the following question:

5. Can OAAs in spirits alone and in combination with ethanol influence the membrane fluidity and migration of PMNLs?

Epidemiological studies have shown that alcohol-attributable mortality in certain CEE countries remains higher than in their western neighbours. In addition to the volume of per capita alcohol consumption and drinking pattern, the effect of unrecorded alcohol consumption have been suggested as an explanation. The concentration of OAAs in recorded and unrecorded spirits may differ therefore, the health risk from their consumption may also differ. Although the health risk from ingestion of unrecorded alcohols was estimated in a previous study, the differences in the volume of alcohol intake by females and males as well as different types of drinkers were not considered and any risk from exposure to higher levels of OAAs was not investigated. To address these limitations, we intend to answer the following question:

6. Looking separately at groups defined by sex and differences in the volume of alcohol consumption by different types of drinkers, is there any difference between the health risk from ingestion of recorded and unrecorded spirits containing OAAs?

3. Materials and methods

3.1. Collection of spirit samples

The methodology employed in the Alcohol Measures for Public Health Research Alliance project was used to collect of recorded and unrecorded spirit samples Spirits manufactured industrially were categorised as recorded, while spirits produced in small scale distilleries or in private homes were considered to be unrecorded. Recorded spirit samples (n=119) including Hungarian fruit spirits (pálinka, n=34), whiskey (n=25), vodka (n=18), brandy (n=18), rum (n=7), artificially flavoured spirits (n=6), gin (n=5), tequila (n=3), ouzo (n=1), grappa (n=1), and absinth (n=1), produced in 17 European and 4 countries in the Americas, were purchased from Hungarian supermarkets. All commercial spirits were labelled with tax stamps. Unrecorded spirits without tax stamps (n=87) were bought informally in Eastern-Hungary from people who ferment fruits at home and either distil the mash in their own stills or send it to distilled their products in subcontract small local scale distilleries or in private homes. The inclusion criteria were that the unrecorded spirits should be distilled from fruits fermented at private homes and they were not taxed. Following collection, each spirit was labelled with an identification number to prevent mismatches, decanted into glass bottles and kept in the dark at 4°C until gas chromatographic mass spectrometric (GC/MS) analysis could be performed. Information on the origin and raw materials of spirits and date of sampling was recorded. Recorded and unrecorded spirits produced from different raw materials were categorized as follows: fruit spirits distilled from fermented apple, apricot, grape, pear, plum, sour cherry; grain spirits distilled from fermented barley, maize, wheat and other spirits distilled from unknown raw materials; fermented agave plant and molasses.

3.2. Gas chromatographic and mass spectrometric analysis of spirit samples

The spirit samples were analysed on a Hewlett-Packard (Palo Alto, CA, USA) GC/MS system consisting of a HP 5890 gas chromatograph, HP 5973 mass selective detector (MSD) and an Agilent 7683 automatic liquid sampler (Agilent Technologies, Palo Alto, CA, USA). Separations of methanol, ethanol, 2-butanol, 1-propanol, isobutanol, 1-butanol, isoamyl alcohol (target compounds) and internal standard (ISTD) were accomplished using a HP-FFAP (30 m x 0.2 mm internal diameter, 0.33 μm film thickness) cross-linked capillary column (Hewlett-Packard Co.) as described previously with minor modifications. Briefly, the GC/MS parameters were as follows: carrier gas: helium, 3.0 bar, constant pressure; injection: split; inlet

temperature: 200°C. The quantitative analysis of the target compounds was carried out following calibration of the GC/MS system.

3.3. Separation of mononuclear and polymorphonuclear leukocytes

After informed consent and with the approval of the Institutional Ethical Committee at the University of Debrecen, peripheral blood was collected into Vacutainer tubes containing EDTA from healthy volunteers (n = 15, 8 females and 7 males). These were staff of the Department of Preventive Medicine including lecturers, researchers, PhD students and office workers. The subjects were aged 22-46 years [mean = 32.2 ± 10.45 year]. All were non-smokers, not heavy drinkers, had normal dietary habits, and were not taking any alcohol/medications that could influence results of the experiments. PMNLs and mononuclear cells were separated by Ficoll density gradient centrifugation. In brief, blood samples were mixed with an equal volume of Hanks' balanced salt solution (HBSS, pH 7.4) and then layered on the top of a discontinuous Ficoll gradient (1.077 and 1.119 g/ml). The samples were then centrifuged at 400*g* (20°C, 30 min). PMNLs deposited at the interface of Ficoll layers and mononuclear cells from the top of the separation medium were collected. The cells were washed twice with HBSS and their viability determined by trypan blue exclusion test (routinely 96-98%). The purity of PMNLs suspension varied between 95% and 98%, as judged by morphology.

3.4. Assay of phagocytosis by monocytes and polymorphonuclear leukocytes

Phagocytosis of the fluorescein isothiocyanate labeled and opsonized zymosan particles (FITC-OZ) was determined. PMNLs and mononuclear cells in HBSS 5% containing heat-inactivated human AB serum were placed into the wells of chamber slides and the cells allowed to adhere for 30 min at room temperature. Non-adherent cells were removed by washing and the adherent cells and FITC-OZ were incubated in HBSS containing, separately acetaldehyde at concentrations of 0.005 mM, 0.05 mM, 0.5 mM, 5.0 mM, 10.0 mM and formaldehyde, acetone, 1-propanal, 1-butanal, 2-butanone at concentrations of 0.0005 mM, 0.001 mM, 0.005 mM, and 0.05 mM at 37°C in a 5% CO_2 / 95% humidified air chamber for 60 min. Other sets of PMNLs and monocytes were incubated in a mixture containing 0.10 mM acetaldehyde and each of the aliphatic alcohol metabolites at final concentrations of 0.0005 mM, 0.001 mM, 0.005 mM, or 0.05 mM. Untreated cells served as controls. The viability of phagocytic cells was checked using the trypan blue exclusion test after the treatments and was found to be 96-98 %. Following incubation, the fluorescence of non-ingested particles was quenched by addition of 0.2 % trypan blue solution and the cells were then fixed with a 4% paraformaldehyde solution. Monocytes

were identified by an indirect immunofluorescent method. Firstly, the chamber slide cells were incubated at room temperature for 60 min in phosphate-buffered saline (PBS, pH 7.4) containing anti-CD14 monoclonal antibody (1:50 dilution). The cells were then rinsed three times with PBS. Monocytes incubated without anti-CD14 antibody served as negative controls. All monocytes were then stained with anti-mouse IgG conjugated with Dylight 594 fluorescent dye (1:200 dilution) for 45 min. and finally they were washed four times with PBS. The nuclei of granulocytes and monocytes were stained with DAPI and the slide was then removed from the chamber for microscopic evaluation. The number of FITC-OZ/cell was determined with an Axioplan fluorescent microscope by examining 100 phagocytic cells in randomly selected microscopic fields/slide. Each treatment was evaluated in duplicate; each slide was assessed twice/sample. From these values, the phagocytosis index (PI, average number of ingested particles/cell) was calculated.

3.5. Assay of migration by polymorphonuclear leukocytes

Granulocytes were placed into the upper compartment of the migration chamber and incubated in 400 µl aliquots of Hanks' containing, separately, ethanol, methanol, and a mixture of 0.0005 mM, 0.005 mM, 0.05 mM, 0,5 mM OAAs concentration. Untreated cells served as controls. Granulocytes were incubated at 37 ˚C for 4 hours. Following incubation, non-migratory granulocytes were removed by cotton swab from the membrane of the migration chamber. The migrated PMNLs were fixed with a 4% paraformaldehyde solution. Then, the membrane was removed from the chamber and placed on microscopic slide. The nuclei of the PMNLs were stained with DAPI 4',6'-diamidino-2-phenylindole (DAPI)and the slides were covered for microscopic evaluation. The number of migrated PMNLs was determined with an Axioplan fluorescence microscope (Zeiss Oberkochen, Germany. Image analysis was carried out by counting migrated cells in seven randomly selected microscopic fields/slide. The migration index (MI, average number of migrated cells/microscopic field) was calculated from these values.

3.6. Measurement of membrane fluidity of polymorphonuclear leukocytes

PMNLs were incubated in HBSS containing, separately methanol and ethanol at concentrations of 0.0005 mM, 0.005 mM, 0.05 mM, and 0.5 mM at 37°C in a 5% CO_2 / 95% humidified air chamber for 60 min. PMNLs were also treated with a mixture containing methanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutanol, and isoamyl alcohol at final concentrations of 0.0005 mM, 0.005 mM, 0.05 mM, and 0.5 mM. Other sets of PMNLs were incubated in a

mixture containing 10.0 mM ethanol with each of the aliphatic alcohols at final concentrations of 0.0005 mM, 0.005 mM, 0.05 mM, and 0.5 mM. Untreated cells served as controls. Following incubation, the cells were centrifuged at 400 g (20°C, 30 min); the supernatant was removed and PMNLs were washed with HBSS. Fluidity of the cell membrane was measured by labelling PMNLs with 1,6-diphenyl-1,3,5-hexatriene (DPH). Before staining, DPH was dissolved in N,N-dimethylformamide (DMF) at a concentration of 4.3 mM. This stock solution was diluted with HBSS, pH 7.4 to obtain a working solution at a concentration of 0.2 μM. PMNLs were incubated in HBSS with DPH working solution for 20 min. Final concentration of DPH was 0.1 μM. The DPH-labelled cells were then washed three times and re-suspended in HBSS. Steady-state fluorescence anisotropy was measured at room temperature with a spectrofluorometer equipped with L-format polarization. The DPH-labelled cells were excited at 370 nm and fluorescence emission was detected at 430 nm.

3.7. Population-based comparative risk assessment

To compare the health risks associated with the consumption of recorded and unrecorded spirits, a Monte Carlo simulation was used to estimate the range of possible exposures to different alcohols. This uses data on consumption of alcohol per capita, the concentration of different alcohols (e.g. methanol and long chain alcohols) in beverages, and average body weight, here taken as (73.9 ± 14.9 kg) and separately for men (82.0 ± 13.1 kg) and women (67.2 ± 12.8 kg) supposing a normal distribution. Monte Carlo simulations were carried out with @Risk for Excel software, version 7.5 (Palisade Corporation, Ithaca, NY, USA) using 10,000 iterations, Latin Hypercube sampling and Mersenne Twister random number generator. The best fit distributions were selected by Chi-squared statistic with a lower limit fixed at zero.

Three exposure scenarios were employed. The first, to enable comparison with the previous study, was termed “average”, using data on per capita consumption averaged across the entire population aged 15+. The second, termed “regular”, uses data on all drinkers (defined as total population minus abstainers) aged 15+. The third was divided into subcategories termed “chronic heavy drinkers, version A” (defined as consuming 60 g/day and 40 g/day for men and women, respectively) and “chronic heavy drinkers, version B” (defined as consuming 60 g/day by men and 40 g/day by women taking into consideration the share of recorded and unrecorded alcohol consumption). For each, exposure was estimated in mg/kg/day for each substance in recorded and unrecorded spirits and recorded and unrecorded fruit spirits. The next step was to express the results as margin of exposure (MOE) values. MOE is defined as the ratio of

toxicological threshold values [the lower one sided confidence limit of the benchmark dose (BMDL) of ethanol, methanol, 1-butanol, or the "no observed adverse effect level" (NOAEL) of 1-propanol, 2-butanol, isobutanol, and isoamyl alcohol] of particular chemicals and the level to which a human may be exposed. To estimate the cumulative effects of exposure to OAAs (excluding methanol) MOE_c values were also calculated. To allow for uncertainty in assessments and inter-species differences, any value less than 100 is considered to be a potential health concern.

3.8. Statistical analysis

The levels of OAAs were expressed in mg/liter of pure ethanol to ensure comparability of the measured concentrations in spirit samples with different amounts of ethanol. The concentrations of OAAs were considered to be zero when their levels in spirits were below the limit of detection of the GC/MS analysis. For statistical analyses, the samples were divided into two groups, recorded (n=119) and unrecorded (n=87) spirits. The recorded spirits were divided further into two subgroups comprising recorded fruit (n=53) and recorded grain (n=49) spirits. All unrecorded spirits (n=87) were produced from fruits therefore so were not subcategorised. Recorded and unrecorded spirits distilled from agave (n=3), molasses (n=7) and unknown raw materials (n=7) were not subcategorised due to the small sample size. The normality of data was tested by the Shapiro-Wilk test and found to be non-normally distributed. Consequently, differences between concentrations of ethanol and OAAs in recorded and unrecorded spirits were determined by Mann-Whitney U tests. The same test was used to compare OAA concentrations in spirits in each of the subgroups defined above. Descriptive statistics, including median, minimum and maximum values and interquartile ranges were calculated for each group and presented in bar graphs. Values of $p < 0.05$ were considered to be statistically significant.

Differences in phagocytosis by untreated PMNLs and monocytes and cells treated with metabolites of OAAs were determined by one-way analysis of variance (ANOVA) using a Newman-Keuls *post-hoc* test. The same test was used to compare membrane fluidity and migration activity by untreated PMNLs and cells treated with ethanol and OAAs. Phagocytic activity of cells from female and male donors was compared using an unpaired t-test. P-values < 0.05 were considered to be statistically significant. All results were presented as mean values ± standard deviation (SD) from six independent experiments in line charts.

The distributions of MOE values were compared using the Kolmogorov-Smirnov test. Statistical analyses were carried out with Stata 10.1 for Windows (StataCorp LLC, College Station, TX, USA). Values of $p < 0.05$ were considered to be statistically significant. Median values of MOE, their interquartile ranges, 1^{st} and 99^{th} percentiles were depicted in box plots.

4. Results

4.1. Concentration of ethanol and other aliphatic alcohols in recorded and unrecorded spirits

The concentrations of methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, isoamyl alcohol and the total concentration of OAAs were significantly higher in unrecorded spirits than those in their recorded counterparts. In addition, there was a significant difference between the total OAAs content in recorded and unrecorded fruit spirits. The total concentration of OAAs was significantly lower in recorded grain spirits than in recorded and unrecorded fruit spirits. The levels of ethanol were significantly higher in unrecorded spirits and unrecorded fruit spirits when compared with those of recorded spirits and recorded fruit spirits, respectively.

4.2. Effect of metabolites aliphatic alcohols on phagocytosis by monocytes and polymorphonuclear leukocytes

There was no statistically significant difference in phagocytic activity between untreated PMNLs and monocytes from female and male donors. Both PMNLs and monocytes incubated in HBSS containing 0.005 mM, 0.05 mM, 0.5 mM, and 5.0 mM or 10.0 mM acetaldehyde demonstrated significantly reduced phagocytosis compared to control cells. There was a significant difference between the phagocytic activity of untreated cells and cells exposed to formaldehyde at levels of 0.0005, 0.001 mM, 0.005 mM, and 0.05 mM. PMNLs and monocytes incubated with acetone at concentrations of 0.0005 mM, 0.001 mM, 0.005 mM, 0.05 mM or 1-propanal at levels of 0.0005 mM, 0.001 mM, 0.005 mM, and 0.05 mM exhibited significantly reduced phagocytic activity compared to control cells. Phagocytosis decreased following treatment of the cells with 1-butanal at levels of 0.0005 mM, 0.001 mM, 0.005 mM, and 0.05 mM. Incubation of PMNLs and monocytes with 2-butanon at concentrations of 0.001 mM, 0.005 mM, and 0.05 mM resulted in a concentration dependent inhibition of phagocytosis. As Phagocytosis index was also significantly decreased when the cells were exposed to a mixture containing each metabolite of OAAs at final concentrations of 0.0005 mM, 0.001 mM, 0.005 mM, and 0.05 mM. Compared to the cells treated only with 0.1 mM acetaldehyde, PMNLs and monocytes incubated with a mixture containing 0.1 mM acetaldehyde and OAA metabolites at concentrations of 0.001 mM, 0.005 mM, and 0.05 mM showed significantly decreased phagocytosis.

4.3. Effect of metabolites aliphatic alcohols on migration and membrane fluidity of polymorphonuclear leukocytes

PMNLs treated with 0.0005 mM, 0.005 mM, 0.05 mM, and 0.5 mM ethanol demonstrated significantly reduced fluorescence anisotropy compared to untreated cells. Ethanol increased the migration activity of PMNLs significantly at concentrations of 0,005 mM, 0.05 mM and 0.5 mM compared to untreated controls. There was a significant difference between the fluorescence anisotropy of untreated cells and PMNLs exposed to ethanol at levels of 0.5 mM. Migration activity increased following treatment of PMNLs with methanol at concentrations of 0,0005 mM, 0,005 mM, 0.05 mM and 0.5 mM versus controls. There was a significant difference between the fluorescence anisotropy of untreated cells and PMNLs exposed to methanol at levels of 0.5 mM. The fluorescence anisotropy was also significantly different from values obtained with control cells when PMNLs were exposed to a mixture containing each OAAs at final concentrations of 0.5 mM. Migration activity of PMNLs significantly increased following treatment of the cells with a mixture of OAAs at final levels of 0.0005mM, 0.005 mM, 0.05 mM, and 0.5 mM compared to untreated controls. Compared to the cells treated only with 10.0 mM ethanol, PMNLs incubated with a mixture containing 10.0 mM ethanol and OAAs at final concentrations of 0.05 mM and 0.5 mM showed significantly increased migration activity. There was no statistically significant difference between the fluorescence anisotropy of PMNLs treated only with 10.0 mM ethanol and a mixture containing 10.0 mM ethanol and OAAs.

4.4. Population-based comparative risk assessment

The results of the population-based comparative risk assessment showed that distributions of MOE values for ethanol were below 20 in all exposure scenarios and were lower for average and regular drinkers consuming recorded spirits and recorded fruit spirits compared to those who drink their unrecorded counterparts. Turning to exposure to methanol from recorded and unrecorded spirits and fruit spirits, the distributions of MOE values reached less than 100 at each consumption level. When consuming 1-propanol, 1-butanol, and isoamyl alcohol from recorded and unrecorded spirits and fruit spirits, the MOE values also reached below 100, but only in chronic heavy drinkers, version A. Consistent with the findings for individual long chain alcohols, the MOE_c values reached below 100 only in chronic heavy drinkers version A. In addition, MOE_c values were also less than 100 for male chronic heavy drinkers, version B when consuming recorded fruit spirits. The median values of MOE were lower at each consumption level for men consuming recorded and unrecorded spirits and fruit spirits than for women. Apart

from chronic heavy drinkers, version A, distributions of MOE values for ethanol differed significantly in all exposure scenarios when consuming recorded and unrecorded spirits and fruit spirits. Distributions of MOE values for methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, isoamyl alcohol, and MOE_c for OAAs except methanol differed significantly at each consumption level from drinking recorded and unrecorded spirits, and fruit spirits. The lowest MOE_c value was obtained for regular drinkers consuming unrecorded fruit spirits.

5. Discussion

Excessive alcohol use is an important public health problem and can contribute to the development of neurological changes, cardiovascular and cerebrovascular diseases, chronic liver diseases and cirrhosis, II-type diabetes and a variety of cancers. In addition, alcohol abusers and chronic heavy drinkers are increasingly susceptible to infectious diseases. Although, the greatest part of exposure from consumption of alcoholic beverages originates from ethanol, alcoholic drinks also contain OAAs which may also contribute to the development of diseases. Both recorded and unrecorded spirits contain OAAs. To assess health risk associated with exposure to OAAs it is important to compare the amount of OAAs in recorded and unrecorded alcohols.

OAAs are present in both recorded and unrecorded alcoholic beverages but their concentrations in different type of alcoholic drinks vary widely. For example, chemical analysis of a large number of European recorded alcoholic beverages has found average levels of OAAs of 98 mg/l and 351 mg/l in beer and red wine samples, respectively. There are also substantial differences in the concentrations of OAAs in a variety of distilled spirits. The mean level of OAAs in grain spirits, including vodka and scotch whiskey, has been found to be 33 mg/l and 844 mg/l, respectively. However, previous studies have shown that the average concentration of OAAs in recorded fruit spirits can be as high as 6356 mg/l. In contrast, surrogate alcohols (aftershaves, perfumes, and medical tinctures), a subgroup of unrecorded alcohols, which usually contain high level of ethanol are typically free of OAAs. Therefore, comparison of different types of recorded and unrecorded alcohols containing different amount of OAAs can be misleading and unless concentrations of OAAs in the same type of recorded and unrecorded alcohols have been compared, it is premature to draw a general conclusion. To avoid the biasing effect of alcoholic beverages with low levels of OAAs, only unrecorded fruit spirits, their recorded counterparts, and recorded grain spirits were included in our comparative study. The results presented here confirm that concentrations of methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, and isoamyl alcohol in unrecorded spirits were significantly higher than those of in their recorded counterparts. When the total amount of OAAs was compared, it was also significantly higher in unrecorded spirits.

A previous study suggested that when the comparator of unrecorded alcohols excluded those recorded alcohols with low levels of OAAs (vodka and whiskey) the difference in the concentrations of OAAs disappear. To test this assumption, first we compared the OAA content

of recorded grain and fruit spirits and unrecorded fruit spirits. Our results demonstrated that levels of OAAs were significantly lower in grain spirits than those of in recorded and unrecorded fruit spirits. Therefore, a separate statistical analysis was performed only comparing the OAA content of recorded and unrecorded fruit spirits. In fact, the difference in concentrations of OAAs did not disappear and the concentrations of OAAs in unrecorded fruit spirits were significantly higher than in their recorded counterparts. These findings reinforce the need to compare concentrations of OAAs in recorded alcoholic beverages with their unrecorded counterparts, something not done in previous studies. One of the possible reasons for the differences we also observed is that different distillation technologies are used to make spirits, as the drinks available from the recorded market are typically prepared under controlled conditions with a qualified large-scale distillation apparatus. In contrast, the conditions for the production of spirits of unrecorded origin can vary considerably from the industry and are often inadequate, and distillation is not always carried out by a specialist. The type of raw materials used in the production of beverages may also contribute to differences in composition. Previous studies have shown that, compared to cereals, the fermentation of fruit produces a much higher proportion of other alcohols in addition to ethanol, for example methanol can be formed from pectin in fruit. Therefore, if the distillation efficiency of the fruit mash is inadequate, OAAs may be present in much higher amounts in the finished distillate. Our results are consistent with these assumptions and demonstrate that the OAAs content of spirits may be affected by both the technology used in distillation and the type of raw materials used to make them, but further studies are needed to further explore the factors determining OAA concentrations in spirits. As spirits from unrecorded sources may increase the burden of alcohol-related diseases due to their higher OAA content, especially in the CEE countries mentioned in the “Introduction” of the thesis, their quality should be controlled regularly.

Previous in vitro studies have demonstrated that OAAs in spirits inhibited phagocytosis of human PMNLs and monocytes at toxicologically relevant concentrations. As these cells may also be exposed to aldehydes formed during the biotransformation of OAAs ingested with spirits, it was reasonable to investigate whether the metabolites of OAAs affect PMNLs and monocytes which play an important role in the host defence against pathogenic microorganisms. Our results showed that metabolites of OAAs inhibited the phagocyte activity of PMNLs and monocytes in a concentration-dependent manner. At combined exposure, a further decrease in phagocytosis of PMNLs and monocytes was observed compared to cells treated with individual metabolites. Therefore, it can be concluded that metabolites of OAAs

could act additively on phagocytosis by PMNLs and monocytes. The Inhibition of phagocytosis cannot be attributed to the cytotoxic effect of the aldehydes studied, as the viability of PMNLs and monocytes did not change after treatment of the cells with the metabolites of OAAs.

In chronic heavy drinkers, acetaldehyde can also affect the function of the immune system. Of the metabolites of OAAs, only the effect of acetaldehyde on phagocytosis of monocytes has been investigated in previous in vitro experiments. Acetaldehyde at concentrations of 0.0625 mM, 0.125 mM, and 0.5 mM was found to inhibit cell phagocyte activity. At similar concentrations of 0.05 mM and 0.5 mM we also observed a decrease in phagocyte function. However, our results showed that acetaldehyde was able to inhibit phagocytosis by PMNLs and monocytes at a concentration less than 0.0625 mM (0.005 mM).

Given the concentrations observed in our in vitro experiments, the question arose as to whether the concentration of aldehydes in the blood could be reached at the level at which phagocytosis could be inhibited in vivo when consuming spirits containing OAAs. There are few data in the scientific literature on plasma concentrations of metabolites of OAAs. In the toxicological studies so far, the concentrations of acetaldehyde and acetone in the blood were measured in addition to ethanol after alcohol poisoning. The results of one such study showed that, following ethanol intoxication, the blood of the affected subjects contained, on average, 0.014 mM acetaldehyde at a concentration of 21.0 mM to 54 mM ethanol. In another study, also in ethanol poisoning, shortly after alcohol consumption, the mean concentration of ethanol was 68 mM, while that of acetaldehyde and acetone was 0.136 mM and 0.172 mM, respectively. Concentrations of 8 mM ethanol and 11.0 mM acetone were measured in the blood of a man transported to hospital for detoxification. The lowest effective concentrations that inhibited phagocytosis of PMNLs and monocytes in our experimental system were 0.005 mM for acetaldehyde and 0.0005 mM for acetone. Comparing these data with the concentrations of acetaldehyde (0.014 mM, 0.136 mM) and acetone (0.172 mM, 11.0 mM) measured in the blood of alcohol abusers, it can be concluded that these in vivo values are higher than the lowest concentrations determined in our research. Therefore, we hypothesize that not only OAAs but also their metabolites may inhibit the phagocytosis by PMNL and monocytes in alcohol abusers and chronic heavy drinkers, thereby contributing to the development of susceptibility to infectious diseases. However, further research is needed to address this assumption.

Several studies suggest that chronic alcohol consumption may contribute to the accumulation of PMNLs in the liver which may play a role in the development and progression of alcoholic hepatitis. The mechanism of this is described in detail in the “Introduction” of my dissertation.

Animal studies have also shown that PMNLs isolated from the blood of rats consuming ethanol show an enhanced migration ability. It is hypothesized that ethanol-induced increase in migration is associated with increased membrane fluidity of PMNLs. By consuming spirits, OAAs enter the gastrointestinal tract and can accumulate in the liver, we investigated whether OAAs in addition to ethanol, can affect the migration and membrane fluidity of PMNLs, thereby increase the risk of alcoholic hepatitis. Our results showed that, ethanol and methanol increased the migration of PMNLs in a concentration dependent manner and ethanol was able to increase the migration capacity of the cells at a concentration of 0.005 mM while methanol at a level of 0.0005 mM. The same alcohols caused a significant increase in the membrane fluidity of PMNLs at higher concentrations of 0.5 mM. We also showed that the migration of PMNLs was also increased when cells were treated with a 0.0005 mM mixture of OAAs. Previous studies have found that after consuming spirits the concentrations of ethanol and OAAs in the blood of alcohol abusers ranged from 10.0 mM and 0.01 to 0.10 mM, respectively. These values are higher than the concentrations at which migration of PMNLs increased during our experiments. In addition, a 0.05 mM mixture of OAAs in the presence of 10.0 mM ethanol caused a further increase in the migration activity of PMNLs compared to cells treated with 10.0 mM ethanol alone. Therefore, it can be concluded that the migration-enhancing effect of OAAs can be observed even at relatively high ethanol concentrations. Although fluorescence anisotropy was further reduced when cells were treated with a mixture of OAAs and 10.0 mM ethanol, there was no statistically significant difference between PMNLs exposed to 10.0 mM ethanol alone and a mixture containing OAAs. This result suggests that at higher ethanol concentrations, the effect of OAAs on the membrane fluidity of PMNLs cannot be detected.

Previous in vitro investigations have shown that ethanol, 1-propanol, 1-butanol and 1-pentanol increased the membrane fluidity and FMLP-induced migration of human PMNLs at higher concentrations (9.1-27.3 mM) than observed in our experiments. One factor to be considered is the different experimental conditions. In those studies, PMNLs were pre-incubated with OAAs for 15-30 minutes. In contrast, we treated PMNLs with OAAs for 60 minutes and 4 hours before determination of fluorescence anisotropy and migration activity, respectively. This suggests that, with even longer incubation periods, OAAs could enhance the membrane fluidity and migration of PMNLs at lower concentrations than seen in our experiments. The relevance of this point is that the biological half-life of OAAs, in the presence of ethanol, varies between 1.71-8.36 hours. Consequently, it is reasonable to assume that PMNLs of some alcohol abusers could be exposed to OAAs for long periods, increasing membrane fluidity and migration

activity of the cells. Our findings suggest that OAAS may exacerbate membrane fluidity and migration of granulocytes at concentrations observed in alcohol abusers and in this manner further increase the risk of alcoholic hepatitis. However, further in vitro and in vivo studies are needed to address this question.

The function of the cell membrane, which separates the cytosol from its extracellular environment, is susceptible to exposure to a number of substances associated with diet, smoking, but, especially, alcohol consumption. The vulnerability of cell membranes stems from their complex dynamic structure, with multiple roles including transport of ions and molecules essential for cell metabolism, ligand-receptor recognition, antigen presentation, signal transduction, and cell activation. These functions depend upon the continuing ability of the membrane to reorganise plasma membrane phospholipids and proteins, thereby sustaining the integrity of the cells. Ethanol, the main component of alcoholic beverages, disrupts the physical structure of the cell membrane when incorporated into the phospholipid bilayer, rearranging its compartments in ways that increase its fluidity. This process leads to disturbances in molecular transport processes, some receptor functions, and enzyme activities. Our experiments showed that not only ethanol but also methanol and mixture of OAAs could increase the membrane fluidity of the PMNLs but at higher concentration (0.5 mM) than that of increased the migration (0.0005 mM) of the cells. Therefore, further studies are needed to examine whether OAAs at the levels measured in the blood of alcohol abusers may contribute to the development of alcoholic hepatitis.

Since the concentrations of OAAs were significantly higher in unrecorded spirits, it was reasonable to examine whether there is any difference in exposure to potentially hazardous levels in consumers of recorded and unrecorded spirits. However, it is also important to look at this within different groups in the population, defined by sex and alcohol consumption. Our population-based comparative risk assessment demonstrated that average and regular drinkers were more likely to be exposed to potentially hazardous levels of ethanol from recorded spirits and fruit spirits than those who drink their unrecorded counterparts. In contrast, there was not significant difference between the health risk from consumption of ethanol by chronic heavy drinkers which we can attribute to our assumption that this group consumes similar amounts of ethanol from recorded and unrecorded spirits and fruit spirits. In addition, exposure to methanol from each type of spirit was also potentially hazardous at each consumption level, but significantly more so when drinking unrecorded fruit spirits. When combined exposure to OAAs was assessed, only chronic heavy drinkers, supposing the A version of the third scenario

were at risk from consumption of both recorded and unrecorded spirits and fruit spirits, with a significantly higher potential health risk associated with drinking unrecorded fruit spirits. In contrast, assuming the B version of the third scenario in chronic heavy drinkers, the risk associated with combined exposure to OAAs was not higher in chronic heavy drinkers consuming unrecorded fruit spirits. However, they typically have a low socioeconomic status, with limited income, so, given the lower prices of illegal alcohols, their relative consumption of unrecorded alcohol is probably higher than in the general population. Therefore, the health risk derived from our use of a 15% figure for unrecorded alcohol consumption is probably an underestimate. Our comparative risk assessment thus suggests that the view that the only health risk associated with consumption of unrecorded alcohols is due to their higher concentration of ethanol is only partially correct. While this may be true when looking at aggregate data, we find that a more disaggregated analysis identifies a group that is exposed to potential risk. In addition, our risk assessment using data from previous studies also suggests that unrecorded fruit spirits may pose a greater higher health risk than compared to the other types of unrecorded spirits. However, further studies are needed to confirm this finding.

One strength of our study is that this is the first large study to compare comprehensively the concentrations and frequency of OAAs found in the same types of recorded and unrecorded spirits. Although our unrecorded fruit spirit samples were not representative of all types of unrecorded spirits (vodka, whiskey, cognac), they were truly representative of unrecorded fruit spirits consumed in CEE countries including Hungary. The other strength of this study is that potential health risks were compared at different consumption levels, considering both sexes, men and women separately. Our research also has some limitations. First, to ensure the comparability of recorded and unrecorded spirits, the samples were purchased only in Hungary which can make the extrapolation of our results to other CEE countries more difficult. Second, our health risk assessments were based on a few assumptions. All of the alcohol consumed by chronic heavy drinkers was assumed to be drunk in the form of recorded and unrecorded spirits and fruit spirits which could result in an overestimation of OAAs intake. Third, BMDL values were available only for ethanol, methanol and 1-butanol thus, instead of them NOAEL values of other higher alcohols were used in our health risk assessments. In contrast to BMDLs, the dose that corresponds to a specific change in an adverse effect, NOAELs do not provide information on dose-response relationship. Therefore, when NOAELs were used to calculate MOE values, the health risks associated with consumption of recorded or unrecorded spirits and fruit spirits could be underestimated. Further toxicological studies are needed to determine

the BMDL value of each higher alcohol enabling the conduction of more precise health risk assessments.

6. Summary of the results and conclusions

Our aim was to ascertain whether there is any difference in the amounts of OAAs in recorded and unrecorded spirits.

1. We demonstrated that the concentrations of OAAs in unrecorded spirits were significantly higher than those in their recorded counterparts. The spirits from unrecorded sources may increase the burden of alcohol-related diseases due to their higher OAA content, especially in the CEE countries. Therefore, the levels of OAAs in alcoholic beverages especially in unrecorded spirits should be controlled regularly.
2. Our results revealed that metabolites of OAAs inhibited phagocytosis by human PMNLs and monocytes in a concentration-dependent manner and when combined with acetaldehyde, they caused a further decrease in phagocytic activity.
3. We found that in combination with acetaldehyde, metabolites of OAAs may inhibit phagocytosis by PMNLs and monocytes at toxicologically relevant concentrations in alcohol abusers and chronic heavy drinkers.
4. Metabolites OAAs alone and in combination with acetaldehyde may contribute to ethanol-induced immunosuppression in alcohol abusers and chronic heavy drinkers, thereby contributing their increased susceptibility to infectious diseases.
5. Our findings showed that OAAs increased membrane fluidity and migration activity of PMNLs in a concentration-dependent manner and when combined with ethanol they caused a further increase in membrane fluidity and migration activity of the cells. It is possible that OAAs consumed with spirits may further enhance migration of PMNLs in alcohol abusers and chronic heavy drinkers and contribute to ethanol-induced liver damage.
6. Our population-based comparative risk assessment demonstrated that average and regular drinkers were more likely to be exposed to potentially hazardous levels of ethanol from recorded spirits and fruit spirits than those who drink their unrecorded counterparts. The findings of our comparative risk assessment supported that not only ethanol but also methanol posed a higher health risk for average consumption on a population level, regular and chronic heavy drinkers and the health risk associated with combined exposure to OAAs except methanol was higher only in chronic heavy drinkers consuming unrecorded spirits. The highest health risk was associated with consumption

of unrecorded fruit spirits. Therefore, further studies are required to assess whether there is any association between the increased potential health risk from consumption of unrecorded spirits and alcohol-related mortality in CEE countries.

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List of publications related to the dissertation

1. **Bujdosó, O.**, Pál, L., Nagy, A. C., Árnyas, E., Ádány, R., Sándor, J., McKee, M., Szűcs, S.: Is there any difference between the health risk from consumption of recorded and unrecorded spirits containing alcohols other than ethanol? A population-based comparative risk assessment.
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2. Pál, L., **Bujdosó, O.**, Szűcs, S., Baranyi, G., Borbásné Sebestyén, V., Vámosi, G., Rácz, G., Ádány, R., McKee, M., Árnyas, E.: How do methanol and higher alcohols found in alcoholic beverages affect membrane fluidity and migration of granulocytes?
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List of other publications

4. Pál, L., Muhollari, T., **Bujdosó, O.**, Fehérné Baranyai, E., Nagy, A. C., Árnyas, E., Ádány, R., Sándor, J., McKee, M., Szűcs, S.: Heavy metal contamination in recorded and unrecorded spirits. Should we worry?
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