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Immune response to acetaldehyde-human serum albumin adduct among healthy subjects related to alcohol intake

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ABSTRACT:

Acetaldehyde (AA) is the main metabolic product in ethanol metabolism, although it can also derive from sources of airborne pollution. As a typical aldehyde, AA is able to react with a variety of molecular targets, including DNA and protein. This property justifies the hypothesis of a immune reaction against this kind of adduct, to be studied by a seroprevalence screening approach. In this study, the correlation between drinking habits and the amount of circulating AA-Human Serum Albumin adduct (AA-HSA) was evaluated in a group of healthy subjects, non alcohol-addicted. Daily ethanol intake (grams) was inferred for each subject using the information collected through a questionnaire, and AA-HSA antibodies (AA-HSA ab) analyses were performed using the Displacement Assay on whole blood samples. The findings showed a correlation between ethanol intake and immune response to molecular adduct . These results underscore the evaluation of AA-HSA ab amount as a suitable molecular marker for alcohol intake that can be applied in future investigations on a large scale for prevention screening.

KEY WORDS:

Acetaldehyde, ethanol, immune response, molecular adduct.

RUNNING HEAD:

Immune response to AA-HSA adducts

ABBREVIATIONS

AA = acetaldehyde; **HSA** = Human Serum Albumin; **AA-HSA ab =** AA-HSA antibodies

1. INTRODUCTION

Acetaldehyde (AA) is the first and principal product in the metabolism of ethanol. AA forms in the liver through detoxification reactions that are mainly catalysed by alcohol dehydrogenase and aldehyde dehydrogenase (Bosron and Li, 1986). However, AA also occurs in indoor environments as a member of the carbonyl chemical species, and it is an important factor in Indoor Air Quality evaluations (Sarigiannis et al. , 2011). Together with formaldehyde, AA represents one of the most abundant airborne aldehydes, which may be related to traffic emissions, smoking, combustion appliances, cosmetic products, and some hobby supplies such as photographic chemicals and special adhesives (Jurvelin et al. , 2001; Wilke et al. , 2004).

In general, aldehydes exhibit high reactivity, as they have a double bond that renders them a strong electrophile chemical species. Thus, they can easily partake in substitution reactions with aromatic compounds and in addition reaction with alkenes, which is typical for aldehydes. An example of this high reactivity is the formation of molecular adducts with DNA, which may be a critical initiating event in the multistage process of chemical carcinogenesis (Yokoyama and Omori, 2003). Evidence of this type of reactivity is observed in reactions between formaldehyde, which has the simplest structure among aldehydes, and DNA. This reaction forms malondialdehyde-deoxyguanosine adducts, which are found in pathologists who are occupationally exposed to formaldehyde (Bono et al. , 2010). Moreover, AA has been demonstrated to form adducts in reactions involving a number of proteins, such as albumin (Donohue et al. , 1983) and haemoglobin (Stevens et al. , 1981), and also with DNA: the resulting DNA adduct in humans is a Schiff base N^2-ethylidene-2'-deoxyguanosine (N^2-ethylidene-dG) adduct (Vaca et al. , 1995).

Concerning the development of alcoholic liver disease, multiple factors have been suggested. There has been increased interest in focusing on the possible role of immune mechanisms in the pathogenesis and perpetuation of this type of disease. In fact, many features of alcoholic liver disease suggest that immune mechanisms may contribute to liver tissue damage, as evidenced by the detection of circulating auto-antibodies and the presence of CD4+ and CD8+ lymphoid cells in the livers of diseased patients (Cook et al. , 1996; Szabo, 1998). In particular, AA derived from the metabolism of ethanol has been shown to form stable adducts with plasma-circulating proteins, such as albumin, which are recognised by the immune

system as foreign in both animals and in humans. Indeed, studies by Israel *et al.* have shown a type I hypersensitivity reaction yielding IgE antibodies against AA adducts when mice were immunised with plasma proteins binded with AA (Israel et al. , 1992). Human studies showed an increase in anti-AA-albumin IgE antibodies among individuals who consume alcoholic beverages (Israel et al. , 1992).

Humans naturally exhibit basal antibody titres against AA-protein adducts, which is likely due to variation in the amount of alcohol that is typically consumed throughout an individual's lifetime. Some alcoholics present significantly higher levels of anti-AA proteins and adduct antibodies (Hoerner et al. , 1986; Niemela et al. , 1987; Worrall et al. , 1989), which have been proposed as factors that perpetuate alcohol-induced liver injury (Niemela et al. , 1987). Other findings also show the existence in alcoholics of antibodies that specifically react with hepatocytes of animals pre-treated with alcohol (MacSween et al. , 1981; Neuberger et al. , 1984), thus highlighting a cytotoxic and auto-immune effect due to the existence of AA-protein adducts.

By chemical analogy, Thrasher *et al*. (Thrasher et al. , 1988; Thrasher et al. , 1987) demonstrated that formaldehyde binds Human Serum Albumin (HSA) covalently, giving rise to a molecular adduct having formaldehyde as hapten. They found antibodies to the formaldehyde-HSA adduct in symptomatic subjects with long-term inhalation exposure to formaldehyde (Thrasher et al. , 1990). Haemoglobin (Hb) and HSA are preferred for monitoring molecules because they are accessible in large amounts, are chemically stable and are not prone to repair mechanisms such as DNA-adducts. Because of the long life span of Hb (approximately 120 days) and the long half-life of HSA (approximately 19 days (Kratz, 2008)), these adducts accumulate in the human body, making their concentrations very sensitive parameters for human biomonitoring (Angerer et al. , 2007). Similar results were found among smoker subjects who inhaled formaldehyde through smoke. In that analysis, the Displacement Assay was confirmed to be a useful method for evaluating formaldehyde exposure (Carraro et al. , 1997).

There were two primary aims of this work. First, the correlation between AA-HSA antibodies (AA-HSA ab) and the amount of alcohol intake was investigated. Second, to investigate a possible role for the AA-HSA adduct as an early indicator of the biological effect of alcohol intake, it was necessary to quantify the immune activity of this adduct in typical alcohol consumers.

2. MATERIALS AND METHODS

2.1 Epidemiological sampling and questionnaire

The sample population was chosen among blood donors of A.V.I.S. Association (Associazione Volontari Italiani Sangue - Torino), without any *a priori* selection concerning drinking habits. Subjects were informed about the objective of the study, and after asking for their voluntary participation, a written consent form was signed and collected. Through this procedure, 76 healthy subjects overall were recruited during a sampling period of four months.

A questionnaire was administered to all 76 of the subjects, who were all healthy and regular alcohol consumers or teetotalers, without any subjects alcohol-addicted. Information about individual and clinical features, smoking habits, and profession were acquired through specific questions about type, quantity and frequency of alcoholic drinks were asked.

The quantity of ethanol assumed varies among different type of alcoholic beverages; specifically, ethanol intake was calculated taking into account its specific weight (= 0.8 gr/cm^3), the percentage in volume (% vvol.) of ethanol quantity of beverages, and the volume ingested. Thus, a number of grams of ethanol was attributed to each subject, taking into account the self-reported average daily alcohol consumption in the questionnaire and the values suggested by the literature reported in **Table 1**.

Table 1: Grams of ethanol in different types and volumes of beverages (Taggi, 2007).

	333 ml (standard can)	**250 ml**	**200 ml** (standard water glass)	**150 ml** (standard wine glass)	**100 ml**	**30 ml**
BEER						
0,05 %vvol.	0.1	0.1	0.1	0.1	-	-
0.5% vvol.	1	1	1	1	-	-
2,5 % vvol.	7	5	4	3	2	1
5% vvol.	13	10	8	6	4	1
8 % vvol.	21	16	13	10	6	2
WINE						
10 % vvol.	27	20	16	12	8	3
11,5% vvol.	31	23	18	14	9	3

12 % vvol.	32	24	19	14	10	3
13 % vvol.	35	26	21	16	10	3
LIQUEURS AND SPIRITS						
Aperitif 18% vvol.	48	36	29	22	14	5
Bitter 27% vvol.	72	54	43	32	22	7
Liqueur 33% vvol.	88	66	53	40	26	8
Strong drink 43% vvol.	115	86	69	52	34	11
Herb infuse 70% vvol.	187	140	112	84	56	18

2.2 Blood sampling and displacement assay

Taking into account possible cumulative effects of the target molecule, subjects had not undergone to any restriction concerning the drinking habits before the biological sampling day.

After completing the questionnaire, a whole blood sample (10 mL) was collected from each subject to perform the IgG anti-AA-HSA analysis, with the Displacement Assay, considering the analogous reactivity among aldehydes. The Displacement Assay was the analytical method chosen for anti-AA-HSA ab quantification, as it assured high sensitivity for the seroprevalence screening of FA-HSA IgG in an healthy population study (Carraro et al. , 1999; Carraro et al. , 1997); through this method an indirect measurement of AA-HSA adduct can be provided. This assay was applied as described by Gilli *et al.* (Gilli et al. , 2008), with no other relevant modifications. This test is based on a competitive reaction of human IgG antibodies against AA-HSA with rabbit IgG anti-AA-HSA antibodies. Briefly, human serum (1:8 titer, 200 µL) (Thrasher et al. , 1990) obtained from heparinized blood was incubated with the antigens (2 h at 37 ◦C), and then purified rabbit IgG (1:1000 titer, 200 µl) were added (2 h incubation at 37 ◦C). The reliability of the immunological response to AA-HSA was evaluated by comparison with the response against HSA. Positive responses in the human sera were revealed by a decrease of the optical density (OD) compared with the OD value obtained with rabbit IgG (positive control). The subject response was considered positive (presence of AA-HSA adduct antibodies) when the sample OD was lower than the positive control OD (rabbit serum). Negative responses (absence of antibodies to AA-HSA) resulted when the sample OD was greater or equal to that of the positive control. AA exposure marker value was calculated as the difference between the OD of the positive control and the OD of the serum sample: marker of AA exposure = (OD positive control) − (OD sample). The degree of displacement was revealed using a specific swine anti-rabbit

antiserum (1:2500 titre, 200 µl) not showing cross-reactivity with human IgG. Optical densities were read at 492 nm with a micro-plate reader (*Tecan Infinite M200 Pro*, Tecan Trading AG, Switzerland) after addition of the substrate 1,2-*o*-phenylendiamine dihydrochloride.

2.3 Statistical analysis

Student's t-test and one-way analysis of variance (ANOVA) were applied to compare two or more groups of independent samples, respectively, while Pearson's correlation was used to test for possible associations between the variables. In particular, for ANOVA testing, the homogeneity of the variance was firstly assessed through the Levene test, thus the equal variance of Tukey's test was assumed for *post-hoc* multiple comparisons.

Finally, a *P* value of ≤ 0.05 (two-tailed) was considered to be significant for all tests. All of the statistical analyses were performed using SPSS Package, version 17.0.

3. RESULTS

Age and gender were controlled in the sample population (*N*=76), as males (N=41) were not significantly older than females (N=35) (44.1 ± 13.7 *vs.* 39.1 ± 12.8; *p* = 0.101). As tobacco smoke could be considered one of the sources of AA, smoking habits were considered to be possible confounding factors in the subsequent measurements. Smokers comprised 23.7% of the sample population, and there was no appreciable difference in the representation of smokers among males (22%) and females (25.7%) (*p* = 0.706). Additionally, the number of cigarettes smoked *per* day was similar among male and female smokers (males: 3.1 cigarettes/day ± 7.4 *vs.* females: 2.9 cigarettes/day ± 6.2; *p* = 0.878).

Regarding alcohol consumption, subjects were divided into 3 groups: "*nondrinkers*" if they had never consumed an alcoholic drink (e.g. teetotallers), "*slight drinkers*" if they occasionally consumed alcoholic drinks (e.g. if they declared that they rarely consumed alcohol, or if they were former drinkers), and finally "*regular drinkers*" (e.g. if they drink during meals). These groups were used to analyse the correlation between grams of ethanol consumed and the amount of circulating AA-HSA ab. The "*nondrinkers*"

appeared to be the less numerous group, with 16 subjects representing 21.1% of the sample population. By contrast, "*regular drinkers*" represented 43.4% of the sample population with 33 subjects. Finally, 27 subjects (35.5%) belonged to the "*slight drinkers*" group. The majority of "*nondrinkers*" and "*slight drinkers*" were females, with females in these groups representing 40% and 42.9% of all females in the sample population, respectively. Conversely, males comprised the majority of "*regular drinkers*" with 65.9% of all males in the sample population.

Table 2 shows the grams of ethanol daily average intake inferred by the questionnaire and mean values of the AA-HSA ab marker stratified by gender and drinking habits. Alcohol intake in males was statistically higher than in females (males 19.1 ± 13.6 *vs.* female 4.2 ± 5.4; $P \leq 0.0001$); however, no statistically significant differences for alcohol intake were found between males and females within population sub-groups defined by drinking habits.

Concerning the AA-HSA adducts, an interesting statistically significant difference was observed for AA-HSA Ab presence by gender (males 0.07 ± 0.02 *vs.* females 0.05 ± 0.03; $P = 0.03$), but this difference in the distribution was absent in subgroups stratified by drinking habits.

Table 2: Classification of subjects recruited in the seroepidemiological study according to gender, ethanol intake, smoking habits and AA-HSA ab levels.

	Population study (*N*=76)	**Male (*N*=41)**	**Female (*N*=35)**
Ethanol (g.) mean ± SD	12.2 ± 13	19.1 ± 13.6	4.2 ± 5.4
Nondrinkers *N*; mean ± SD	16; 0	2; 0	14; 0
Slightly drinkers *N*; mean ± SD	27; 5.4 ± 4.1	12; 6.9 ± 5.6	15; 4.2 ± 1.8
Regular drinkers *N*; mean ± SD	33; 23.7 ± 11.4	27; 25.9 ± 11.3	6; 13.8 ± 5.3
Anti-AA-HSA-ab mean ± SD	0.06 ±0.02	0.07 ± 0.02	0.05 ± 0.03

Not smokers (*N* = 58)	**1- Nondrinkers *N*; mean** ± SD	12; 0.04 ± 0.01[a, b]	2; 0.05 ± 0.002	10; 0.04 ± 0.01
	2- Slightly drinkers *N*; mean ± SD	20; 0.06 ± 0.02[c]	7; 0.07 ± 0.02	13; 0.05 ± 0.01
	3- Regular drinkers *N*; mean ± SD	26; 0.06 ± 0.02	23; 0.07 ± 0.02	3; 0.05 ± 0.02
Smokers (*N* = 18)	**4- Nondrinkers *N*; mean** ± SD	4; 0.04 ± 0.05[d]	0	4; 0.04 ± 0.05
	5- Slightly drinkers *N*; mean ± SD	7; 0.05 ± 0.02[e]	5; 0.05 ± 0.02	2; 0.04 ± 0.002
	6- Regular drinkers *N*; mean ± SD	7; 0.09 ± 0.02	4; 0.08 ± 0.02	3; 0.1 ± 0.03

[a]Not smokers nondrinkers (*N*=12) *vs* smokers regular drinkers (*N*=7) (*P* <0.0001)

[b]Not smokers nondrinkers (*N*=12) *vs* not smokers regular drinkers (*N*=26) (*P*= 0.01)

[c]Not smokers slightly drinkers (*N*=20) *vs* smokers regular drinkers (*N*=7) (*P* = 0.05)

[d]Smokers nondrinkers (*N*=4) *vs* smokers regular drinkers (*N*=7) (*P* = 0.005)

[e]Smokers slightly drinkers (*N*=7) *vs* smokers regular drinkers (*N*=7) (*P* = 0.03)

Mean values of this biological marker were also compared among the sample population stratified by tobacco smoke, but no statistical difference was found between smokers and not smokers (*N* = 18; 0.06 ± 0.04 and *N* = 58; 0.06 ± 0.02 respectively). However, to better investigate the sensitivity of this biological marker, molecular AA-HSA ab levels were compared among the entire population by taking into account both smoking habits and daily alcohol consumption. Thus, six sub-groups were generated, as shown in **Table 2**, where a globally significant model of data distribution is given ($P \leq 0.0001$).

From this approach, AA-HSA ab mean values show a similar trend when comparing smokers and non-smokers with increased alcohol intakes. In particular, significant comparisons between each of the six resulting groups were detailed in **Table 2** and confirmed by performing Tukey's test *post hoc*. This approach, however, resulted in relatively small sample sizes among groups, especially when the subgroups were furtherly compared by gender (not statistically significant comparisons).

Finally, from the correlation analysis, a significant relationship between values of ethanol daily intake and AA-HSA ab was observed (Pearson's $r = 0.462$; $P \leq 0.0001$) (**Fig.1**), conversely, a not significant relation between AA-HSA ab and smoking habits (measured by n° of cigarettes/day) among smokers was reported (N=18; Pearson's r = 0,064; p = 0,801; data not shown).

4. DISCUSSION

The first aim of this study was to measure AA-HSA adducts values, a molecular marker linked to AA, which exposure is associated with alcohol consumption and airborne pollution. This result is unexpected, as it is already evident among subjects who are in the normal range of drinking habits. Indeed, most studies concerning this topic are focused on stronger drinking habits, often including alcohol abuse, where correlations between the exposure to ethanol and levels of the AA-HSA biological marker are stricter (Neuberger et al. , 1984; Worrall et al. , 1989). With these prior observations, it is important to investigate what values of AA-HSA ab can be expected in a healthy population with a normal range of ethanol intake. This investigation can help determine new conditions for defining very earlier stages of health effects alcohol-related.

Other kind of assay have been considered as top test for the evaluation of drinking status, in particular the CDT quantification (= Carbon Deficient Transferrin) is widely diffuse; however the accuracy for this marker is generally poorer among drinkers with a low level of alcohol consumption, younger and women (Gronbaek et al. , 1995), conversely it seems to be suitable in distinguish chronic and heavy drinkers subjects (Sillanaukee et al. , 2001).

Also Ethyl Glucuronide quantification in hair (HEtG) has recently gained great attention, because of the high sensitivity and specificity in the diagnosis of chronic heavy drinking (Hoiseth et al. , 2009; Morini et al. , 2009; Morini et al. , 2010), and it has been recently adopted by the Society of Hair Testing for the chronic excessive alcohol consumption assessment (Soht, 2009). In general the quantification of a marker measured in hair provide an indication about possible cumulative and time-dependent effects of the substance, since the hair growth is estimated about 1 cm/month; on the other hand this concern affects the sensitivity of the technique, especially when subjects are in the regular range of alcohol use.

Conversely, HSA exhibits a shorter average life-time (Kratz, 2008) and represents the main target of AA, on which an immune reaction takes place; in our view such type of reaction is better defined through a biological and dynamic assay, especially when low levels of ethanol could be expected as in the general healthy population.

It's also important to note that certain common non-alcoholic beverages, including sodas and fruit juices, contain small amount of ethanol (Logan and Distefano, 1998) in which it is present as result from fermentation processes of sugar or sugarcane, as well as baked foods, in which ethanol is used as antimicrobial agent. All these foods are widely consumed, thus this could explain the amount of AA-HSA ab among non and slightly drinkers subjects.

Further AA exposure was also investigated, taking into account exposure to tobacco smoke as possible source of air pollution. Far from our hypothesised results and expectations based on the literature, AA-HSA adduct levels do not show any appreciable differences between populations stratified by tobacco smoke exposure. In fact, this study indicates that AA-HSA ab formation does not seem to depend on tobacco smoke intake. Moreover, these findings were reinforced by the results shown in **Table 2**, in which both smoking habits and alcohol intake were considered together as potential contributors in AA-HSA ab induction. Although non-smokers/nondrinkers have AA-HSA ab levels that are significantly lower than those of smokers/regular drinkers ($p \leq 0.0001$), globally not-smokers have AA-HSA ab levels comparable to those of smokers. We suppose that this distribution could be due to differing sample sizes when comparing across smokers and not smokers (N= 18 *vs.* N=58, respectively) and to the low number of cigarettes smoked *per* day by smokers. Thus, the results shown in **Table 2** seem to be better explained by differing levels of alcohol intake, where similar trends were observed within each drink category, rather than by smoking habits.

Gender seems to be involved in the formation of AA-HSA ab, as values for the molecular adduct are significantly different between males and females. This result is most likely due to the little number of drinkers among females than those among males (females *N*=6 *vs.* males *N*= 27); on the other hand also differences in alcohol metabolism genetically pre-determined should be taken into account, even if literature provide quite controversial findings concerning gender and alcohol metabolism (Ramchandani et al. , 2001). However, when considering the sample population stratified by different alcohol consumption and smoking habits, differences between gender are missing.

In conclusion, even considering the limited number of subjects analysed in this seroprevalence investigation, the results show that ethanol intake causes a clear antibody response, as highlighted by the

formation of AA-HSA ab. The biological importance of these findings is linked to the possibility for useful applications of this molecular adduct as a biologically effective dose marker for ethanol exposure. Indeed, the need to promote healthy lifestyles among human populations, especially among younger demographics, arises from an awareness of alcohol abuse, smoking, and the consumption of drugs and unhealthy food as risk factors for poor health in the general population, as they can cause illness and mortality. The abuse of such substances causes high health care costs; consequently, their effects have a variety of consequences in healthcare administration, social contexts, and the workplace.

For the above-mentioned reasons, the evaluation of the presence of AA-HSA ab can represent a useful approach for screening activities related to preventive actions.

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FIGURE CAPTIONS

Figure 1: Bivariate correlation between quantity of assumed ethanol (g.) and AA-HSA ab values (OD).