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Genetic factors affecting susceptibility to alcoholic liver disease in an Indian population

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ABSTRACT

Introduction. Indians are more likely to develop alcoholic cirrhosis compared to Caucasians, though the cause remains obscure. North Indians tend to consume more alcohol than other parts of the country. Genetic factors are likely to play a major role in these observations. This study investigated whether 10 different polymorphisms were associated with alcohol dependence and/or cirrhosis in North Indians. These were in *ADH2*2* (rs1229984), *ADH3*2* (rs698), *CYP2E1*1D, CYP2E1*5* (rs3813867 and rs2031920), *TNF-α* (rs1800629), *TNF-α* (rs361525), *IL-1β* (rs3087258), *CD-14* (rs2569190), *IL-10* (rs1800872) and *PNPLA3* (rs738409). **Material and methods.** Hundred healthy controls and 120 chronic alcoholics (60 alcoholic noncirrhotics and 60 alcoholic cirrhotics) attending various departments of PGIMER, Chandigarh were genotyped using PCR-RFLP methods. **Results.** Alcoholic cirrhotics compared to healthy individuals demonstrated a statistically significant increase in *PNPLA3* (10109G) allele *(p* = 0.037, OR = 2.12, 95% CI 1.29-3.4). Rest of the associations were not significant after correction for multiple testing. **Conclusion.** *PNPLA3* 10109G predisposed North Indian subjects to alcoholic cirrhosis.

Key words. Alcoholic liver disease. Alcohol dependence. Genetic polymorphism. Cytokine. Alcohol metabolizing enzyme. PNPLA3.

INTRODUCTION

Alcoholism remains a major socio-economic and medical problem throughout the world. According to WHO estimates 26% men and 4% women in India drink alcohol which are considerably lower than Europeans (90% men and 81% women). Although alcohol consumption per drinker in India is around 12.9 L/y which is comparable to the rest of the world and alcohol consumption patterns vary considerably within India with North Indians are much more predisposed to alcohol dependence. Alcohol contributes to apparently 4% of total deaths, 20% of hospital admissions, 18% of psychiatric emergencies, 20% of all brain injuries and 60% of all injuries reported in emergency rooms in India. With *per capita* consumption of alcohol rising in India over past two decades alcoholism is likely to cause more problems in future. Though alcohol affects many organs in the body, liver bears the highest brunt as it metabolizes more than 90% of ingested alcohol. There is a spectrum of alcoholic liver disease (ALD) namely steatosis, hepatitis, cirrhosis and hepatocellular carcinoma. However only 6 to 41% of heavy drinkers (> 60 to 80 g/d of alcohol in men and > 20 g/d in women develop cirrhosis.[1] Hence, in addition to alcohol other co-existing factors might influence ALD. Various agents and environmental factors have been implicated in pathogenesis and progression of ALD including type, amount and pattern of drinking, obesity, hyperglycemia, HBV, HCV, HIV infection, diet, toxin and drugs etc. In addition, twin studies have demonstrated that genetic factors are responsible for alcohol dependence and ALD.[2] A number of case-control studies involving single nucleotide polymorphism (SNP) analysis have implicated various genes namely, alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and cytochrome P4502E1, manganese superoxide dismutase (MnSOD), interleukin 1β (*IL-1β)*, interleukin 10

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(*IL-10*), tumor necrosis factor α (TNF-α), matrix metalloproteinase (MMP), tissue inhibitor of matrix metalloproteinase (TIMP) and adiponutrin (PNPLA3) to have causal relationship with alcoholism and/or ALD. But due to heterogeneity in control subjects which constituted of healthy volunteers in some studies whereas alcoholics without liver cirrhosis in others, it is difficult to rule out the confounding effect of alcohol dependence while studying the genetic susceptibility towards ALD. Moreover several cytokines (TNF-α, IL-1β and IL-10) previously thought to be responsible for ALD were found to be responsible for alcohol dependence by modulating serotonin neurotransmission in the brain.[3] Indians may be more susceptible to ALD compared to Caucasians,[4] there have been only few studies in India to comprehend the susceptibility genes towards ALD.[5] But in none of the studies the whole spectrum of susceptibility genes (cytokines and metabolizing enzymes) were studied simultaneously in alcohol dependence and ALD. Hence, a retrospective, observational, case-control study involving the polymorphism in *ADH2*2*, *ADH2*3*, *ADH3*2*, *ALDH2*2*, *CYP2E1*1D, CYP2E1*5*, *TNF-α*, *IL-1β*, *IL-10, CD-14* and *PNPLA3* in healthy controls (HCs) and alcoholics (ALCs) [alcoholic noncirrhotics (ANCs) and alcoholic cirrhotics (ACs)] was undertaken.

MATERIAL AND METHODS

The study constituted of 100 HCs and 120 ALCs (60 ACs and 60 ANCs). Selection criteria for ALCs were consumption of at least 80 g alcohol per day for ten or more year. Alcoholic cirrhosis was diagnosed by clinical, radiological and biochemical parameters. All the subjects were residents of North India (Chandigarh, Punjab, Haryana, Delhi, Uttaranchal, Himachal Pradesh and Jammu and Kashmir) for the last three generations, within the age group 25-60 y, were negative for HBsAg and IGM anti HCV and free from any other liver disease. ACs were recruited from the Department of Hepatology and ANCs were recruited from the Department of Psychiatry. HCs were recruited among blood donors. Written consent was taken from all the subjects and the study was approved by the Institute Ethics Committee of PGIMER, Chandigarh. All the subjects were studied for alcohol abuse and dependence by the DSM IV criteria and scored on the basis of CAGE questionnaire. DNA was isolated by the method of Daly *et al.*[6] All mutations were diagnosed by using PCR-RFLP methods available in the literature (Table 1). Three millimetres blood was collected in plain vial and assayed in Random Access Autoanalyzer Modular-P (Hitachi) for bilirubin (total and conjugated), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein and albumin. The variance of biochemical parameters between different groups was calculated by Mann-Whitney and Kruskal-Wallis test (nonparametric ANOVA) and Dunn's multiple comparisons test, using GraphPad StatMate software version 3. Allele frequency was calculated by direct counting. Fisher exact test was used to assess the significance of the differences between allele frequencies (using 2 x 2 contingency table) and genotype distributions (using 3 x 2 contingency table) in different groups. Bonferroni correction was made to the p value to account for multiple testing (10 SNPs). Odds ratio and 95% confidence interval was also calculated to measure the association of the polymorphisms with alcohol dependence or alcoholic cirrhosis. Statistical power of individual analysis was calculated with GraphPad StatMate software version 3. Haplotyping was done for *TNF-α* (-308, -238) and *ADH2*2*, *ADH2*3* and *ADH3*2* alleles. LOD score, D', r^2 and *p* values for the association tests and HWE were calculated with the Haploview version 4.2.

RESULTS

The anthropological and biochemical parameters of the studied subjects are summarized in table 2. The mean age in HCs, ANCs and ACs was 32.89, 37.99 and 49.52 y respectively. The age difference was not statistically significant. None of the subjects in any group demonstrated the presence of *ADH2*3* (rs2066702) or *ALDH2*2* allele (rs671). The data of all the alleles and statistical analysis are presented in table 3. Genotype frequencies of all alleles were in Hardy-Weinberg equilibrium in HCs and ANCs. This study also demonstrated there is no linkage in the TNF-α and ADH gene cluster in North Indians which supports previous findings.[19] The frequency of the mutant alleles ADH2*2 (0.025), CYP2E1*5 (0.005), TNF α -308A (0.04) AND TNF α -238A (0.03) were also rare in healthy North Indians. After Bonferroni correction no other snps excepting PNPLA3 rs738409 were significantly associated with alcoholic cirrhosis or alcoholism. PNPLA3 10109G allele was significantly associated with alcoholic cirrhosis compared to healthy controls (corrected *p* 0.037, OR 2.12, 95% CI 1.29-3.47). However PNPLA3 10109 G/G genotype association

Table 1. PCR-RFLP methods followed.

Allele	Primers	Restriction enzyme	Reference
*ADH2*2*	FP 5'-AATCTTTTCTGAATCTGAACAG-3' RP 5'-GAAGGGGGGTCACCAGGTTGC-3'	*Tsp45*I	[16]Wu, *et al.,* 2005
*ADH2*3*	FP 5'-GGACTCTCACAACAAGCATGTG-3' RP 5'-TTTCTTTGGAAAGCCCCC-3'	*Alw*NI	[17]Frenzer, *et al.,* 2002
*ADH3*2*	FP 5'-TTGTTTATCTGTGATTTTTTTGT-3' RP 5'-CGTTACTGTAGAATACAAAGC-3'	SspI	[18]Yin, et al., 2007
*ALDH2*2*	FP 5'-CAAATTACAGGGTCAACTGCT-3' RP 5'-CCACACTCACAGTTTTCTCTT-3'	MboII	[16]Wu, *et al.*, 2005
*CYP2E1*1D*	FP 5'-TGGTACATTGTGAGACAGTG-3' RP 5'-ATACGGGAACACCTCGTTTG-3'	-	[17]Hu, *et al.,* 1999
*CYP2E1*5* (G-1293C)	FP 5'-CCAGTCGAGTCTACATTGTCA-3' RP 5'-TTCATTCTGTCTTCTAACTGG-3'	*Pst*I	[14]Kato, *et al.,* 1992
*CYP2E1*5* (C-1053T)	FP 5'-CCAGTCGAGTCTACATTGTCA-3' RP 5'-TTCATTCTGTCTTCTAACTGG-3'	*Rsa*I	[14]Kato, *et al.,* 1992
TNF-α (G-308A)	FP 5'-AATAGGTTTTGAGGGCCATG-3' RP 5'-ATCTGGAGGAAGCGGTAGTG-3'	*Nco*I	[17]Grove, *et al.,* 1997
TNF-α (G-238A)	FP 5'-AGAAGACCCCCCTCGGAACC-3' RP 5'-ATCTGGAGGAAGCGGTAGTG-3'	*Msp*I	[17]Grove, *et al.,* 1997
IL-1β (C-511T)	FP 5'-TGGCATTGATCTGGTTCATC-3' RP 5'-TTCACCCTTCTAAGGATTTG-3'	*Ava*I	[18]Takamatsu, *et al.,* 2000
IL-10 (C-627A)	FP 5'-GGTGAGCACTACCTGACTAGC-3' RP 5'-CCTAGGTCACAGTGACGTGG-3'	*Rsa*I	[19]Grove, *et al.,* 2000
CD-14 (C-159T)	FP 5'-ATCATCCTTTTCCCACAC-3' RP 5'-AACTCTTCGGCTGCCTCT-3'	*Hae*III	[20]Shih, *et al.,* 2005
PNPLA3 (C10109G)	FP 5'-TGGGCCTGAAGTCCGAGGGT-3' RP 5'-CCGACACCAGTGCCCTGCAG-3'	*BtsCI*	[21]Dutta, 2011

was borderline significant alcoholic cirrhosis compared to healthy controls (corrected p 0.06). When PNPLA3 10109 C/G and G/G genotypes were clubbed together using a dominant model still this association was not significant between cirrhotics and healthy controls (corrected p 0.08, OR 2.45, 95% CI 1.27-4.72).

DISCUSSION

With fast socioeconomic change India is witnessing a rapid increase in per capita alcohol consumption, especially in younger age group. There is a lacuna in the available literature regarding the national prevalence of alcohol dependence and ALD. However, North Indians tend to consume more alcohol compared to other parts of the country.[7] Moreover there are few reports from United Kingdom suggesting South Asian men are more predisposed to alcohol induced liver cirrhosis. Indian men living in UK tend to develop cirrhosis earlier and after consuming less alcohol compared to Caucasians.[1,4,8] The biochemical and hematological parameters (MCV, AST, ALT, and GGT) also tend to be higher in Indian cirrhotics.[8] It is well known that metabolic syndrome is an important risk factor towards development of liver disease in alcoholics. Thus Indian alcoholics must be more predisposed than Caucasians to develop liver disease because they tend have more abdominal fat content for a given BMI. C to G

Table 2. Comparison of anthropometric and biochemical parameters.

Parameter	HC (Mean ± SD)	ALC (Mean ± SD)	ANC (Mean ± SD)	AC (Mean ± SD)	HC *vs.* ALC *p*	ANC *vs.* AC *p*
Total nos.	100	120	60	60	-	-
Age (yr)	32.89 ± 9.46	45.16 ± 11.53	37.99 ± 9.85	49.52 ± 10.25	NS	NS
B. wt (kg)	72.84 ± 14.80	69.79 ± 14.82	70.73 ± 15.58	68.40 ± 13.68	NS	NS
T. bil (mg/dL)	0.56 ± 0.33	4.63 ± 8.29	0.69 ± 0.41	7.03 ± 9.77	< 0.001*	< 0.001*
D. bil (mg/dL)	0.06 ± 0.09	2.20 ± 4.46	0.05 ± 0.12	4.38 ± 5.54	NS	< 0.001*
T/D bil	28.69 ± 25.64	24.83 ± 37.19	46.44 ± 42.15	2.96 ± 6.29	< 0.01*	< 0.001*
AST (U/L)	27.12 ± 8.59	80.67 ± 71.06	61.83 ± 39.21	93.18 ± 83.78	< 0.001*	NS
ALT (U/L)	32.22 ±13.23	62.59 ± 59.54	69.21 ± 48.41	58.17 ± 65.79	NS	NS
AST/ALT	0.92 ± 0.44	1.57 ± 0.97	1.07 ± 0.72	1.91 ± 0.97	NS	NS
ALP (U/L)	69.64 ± 34.84	136.44 ± 124.39	54.01 ± 40.28	194.15 ± 130.83	NS	< 0.001*
T. protein (g/dL)	6.88 ± 0.78	7.26 ± 1.15	7.18 ± 0.91	7.33 ± 1.31	NS	NS
Albumin (A) (g/dL)	4.18 ± 0.49	3.74 ± 0.90	4.22 ± 0.50	3.41 ± 0.97	NS	NS
Globulin (G) (g/dL)	2.53 ± 0.78	3.54 ± 1.07	2.96 ± 0.54	3.97 ± 1.16	NS	NS
A/G	1.57 ± 0.21	1.23 ± 0.81	1.48 ± 0.35	1.04 ± 0.99	NS	NS

p values given in the table was calculated by Dunn's multiple comparisons test using GraphPad InStat version 3. *$p < 0.05$ was considered statistically significant.

substitution (rs738409) at genomic position 10109 (cDNA 444) in adiponutrin (patatin-like phospholipase domain containing 3) gene, results in substitution of isoleucine with methionine at 148th position. Meta analysis of the 16 studies reported between 2008 and 2011 has established a strong association of this polymorphism with non-alcoholic fatty liver disease in Hispanics, Asians, African Americans and Caucasians.[9] Mutant homozygotes *(PNPLA3*, G10109G) also demonstrated increased serum alanine transferase activity in different ethnic groups in this meta analysis. This polymorphism is also associated with alcoholic cirrhosis in Mestizos[10] and Germans,[11] steatosis in chronic hepatitis C Italian patients[12] and fibrosis in Germans.[13] Adiponutrin is known to regulate hepatic fat content. *ADH2*3 and ALDH2*2* alleles were not present in North Indians. This finding is consistent with the view that *ADH2*3* is confined to the Africans. While absence of *ALDH2*2* increases the predisposition of Indians towards alcohol dependence, it decreases the predisposition towards oesophageal carcinoma by decreasing the local concentration of acetaldehyde after alcohol consumption.[15] Interestingly according to World Health Organization estimates, Indians have much lower age standardized death rate from esophageal cancer (< 15 per 1,00,000 population) compared to Orientals (> 30 per 1, 00,000 population), who have much higher frequency of *ALDH2*2* allele (0.06-0.27). *CYP2E1*5* allele was rare in North Indians with a frequency of 0.01 among HCs. This observation support previous results that *CYP2E1*5* allele is infrequent among North Indians.[5] This allele was not found to be associated with either alcohol dependence or alcoholic cirrhosis. However, *CYP2E1*5* allele was found to be predisposing to the ALD in North Indians in a prior study[5] carried out in Uttar Pradesh, India. These apparently conflicting findings may be explained by the higher prevalence of tobacco abuse in Uttar Pradesh. Nicotine also acts as an inducer of *CYP2E1*, hence potentiates the harmful effect of alcohol to liver. Frequency of *PNPLA3* 10109G in North Indians (0.235) was slightly higher than Caucasians (0.19)[11] but lower than Hispanics (0.53).[10] *PNPLA3* 10109G is associated with increased hepatic fat content (decreased triglyceride lipase activity) and liver fibrosis.[10,11] This polymorphism (rs738409) was found to be predisposing towards the development of alcoholic cirrhosis (OR = 1.949) in North Indians. Allele frequency of most polymorphisms studied was similar to Caucasians and different from Orientals. In conclusion *PNPLA3* 10109G predisposed North Indians to alcoholic cirrhosis however a much larger sample size is required to rule out the association of other snps with alcoholic liver disease.

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Table 3. Genotypes and allele frequencies of studied polymorphisms in HCs, ALCs, ANCs and ACs.

Frequency	HCs N (%)	ALCs N (%)	ANCs N (%)	ACs	ACs *vs.* ANCs	Frequency N (%)	HCs N (%)	ALCs N (%)	ANCs N (%)	Acs	ACs *vs.* ANCs
*ADH2*2* genotype						*ADH2*2* allele					
*1/*1	95 (95)	120 (100)	60 (100)	60 (100)		*1	195 (0.975)	240 (1)	120 (1)	120 (1)	
*1/*2	5 (5)	0	0	0		*2	5 (0.025)	0	0	0	
*2/*2	0	0	0	0		*P*	-	0.19*	1.61	1.60	
P	-	0.18*	1.60*	1.60*		OR/95% CI	-	0.07*	0.15*	0.15*	
								(0.004-1.35)	(0.008-2.69)	(0.008-2.69)	
*ADH3*2* genotype						*ADH3*2* allele					
*1/*1	44 (44)	49 (40.83)	25 (41.67)	24 (40.00)		*1	136 (0.68)	156 (0.65)	77 (0.64)	79 (0.66)	
*1/*2	48 (48)	58 (48.33)	27 (45.00)	31 (51.67)		*2	64 (0.32)	84 (0.35)	43 (0.36)	41 (0.34)	
*2/*2	8 (8)	13 (10.83)	8 (13.33)	5 (8.33)		*P*	-	5.44*	5.41*	7.13*	8.92**
P	-	7.40*	5.50*	8.80*	6.10**	OR/95% CI	-	1.14*	1.19*	1.103*	0.93**
								(0.77-1.70)	(0.74-1.91)	(0.68-1.78)	(0.55-1.58)
*CYP2E1*1D* genotype						*CYP2E1*1D* allele					
*1/*1	74 (74)	82 (68.33)	44 (73.33)	38 (63.33)		*1	169 (0.845)	195 (0.8125)	103 (0.858)	92 (0.77)	
*1/*1D	21 (21)	31 (25.83)	15 (25)	16 (26.67)		*1D	31 (0.155)	45 (0.1875)	17 (0.142)	28 (0.23)	
*1/*1D	5 (5)	7 (5.83)	1 (1.67)	6 (10)		*P*		3.79*	8.72*	1.01*	0.97**
P	-	6.50*	5.00*	2.90*	1.30**	OR/95% CI	-	1.26*	0.90*	1.66*	1.84**
								(0.76-2.08)	(0.47-1.71)	(0.94-2.94)	(0.95-3.59)
*CYP2E1*5* genotype						*CYP2E1*5* allele					
*1/*1	99 (99)	117 (97.50)	57 (95)	60 (100)		*1	199 (0.995)	237 (0.9875)	117 (0.975)	120 (1.00)	
*1/*5	1 (1)	3 (2.50)	3 (5)	0		*5	1 (0.005)	3 (0.0125)	3 (0.025)	0	
*5/*5	0	0	0	0		*P*	-	6.30*	1.50*	10*	2.50**
P	-	6.30*	11.50*	10*	1.40**	OR/95% CI	-	2.52*	5.10*	0.55*	0.14**
								(0.26-24.42)	(0.52-49.65)	(0.02-13.67)	(0.007-2.73)
TNF-α (G-308A) genotype						*TNF-α (G-308A)* allele					
G/G	93 (93)	94 (78.33)	47 (78.33)	47 (78.33)		G	192 (0.96)	212 (0.88)	105 (0.875)	107 (0.89)	
G/A	6 (6)	24 (20)	11 (18.33)	13 (21.67)		A	8 (0.04)	28 (0.12)	15 (0.125)	13 (0.11)	
A/A	1 (1)	2 (1.67)	2 (3.33)	0		*P*	-	0.05*	0 .07*	0.20*	8.40**
P	-	0.09*	0.20*	0.10*	3.40**	OR/95% CI	-	3.17*	3.43*	2.91*	0.85**
TNF-α (G238A) genotype						*TNF-α (G238A)* allele					
G/G	94 (94)	109 (90.83)	57 (95)	52 (86.67)		G	194 (0.97)	229 (0.95)	117 (0.975)	112 (0.93)	
G/A	6 (6)	11 (9.17)	3 (5)	8 (13.33)		A	6 (0.03)	11 (0.05)	3 (0.025)	8 (0.07)	
A/A	0	0	0	0		*P*	-	4.60*	10*	1.60*	2.20**
P	-	4.50*	10*	1.50*	2.00**	OR/ 95% CI	-	1.55*	0.83*	2.31*	2.79**
								(0.56-4.28)	(0.20-3.38)	(0.78-6.83)	(0.72-10.77)
IL-1β (C-511T) genotype						*IL-1β (C-511T)* allele					
C/C	12 (12)	17 (14.17)	11 (18.33)	6 (10)		C	84 (0.42)	88 (0.37)	49 (0.41)	39 (0.325)	
C/T	60 (60)	54 (45)	27 (45)	27 (45)		T	116 (0.58)	152 (0.63)	71 (0.59)	81 (0.675)	
T/T	28 (28)	49 (40.83)	22 (36.67)	27 (45)		*P*	-	2.81*	9.10*	1.00*	2.30**
P	-	0.80*	1.70*	0.90*	3.70**	OR/95% CI	-	1.25*	1.05*	1.50*	1.43**
								(0.85-1.84)	(0.66-1.66)	(0.94-2.42)	(0.85-2.43)
IL-10 (C-627A) genotype						*IL-10 (C-627A)* allele					
C/C	36 (36)	57 (47.50)	27 (45)	30 (50)		C	116 (0.58)	168 (0.70)	82 (0.68)	86 (0.72)	
C/A	44 (44)	54 (55)	28 (46.67)	26 (43.33)		A	84 (0.42)	72 (0.30)	38 (0.32)	34 (0.28)	
A/A	20 (20)	9 (7.50)	5 (8.33)	4 (6.67)		*P*	-	0.09*	0.70*	0.20*	6.70**
P	-	0.20*	1.30*	0.40*	8.40**	OR/95% CI	-	0.59*	0.64*	0.55*	0.85**
								(0.40-0.88)	(0.40-1.03)	(0.34-0.88)	(0.49-1.48)

CD-14(C-159T) genotype						*CD-14 (C-159T)* allele					
C/C	6 (6)	12 (10)	11 (18.33)	1 (1.67)		C	72 (0.36)	80 (0.33)	48 (0.40)	32 (0.27)	
C/T	60 (60)	56 (46.67)	26 (43.33)	30 (50)		T	128 (0.64)	160 (0.67)	72 (0.60)	88 (0.73)	
T/T	34 (34)	52 (43.33)	23 (38.33)	29 (48.33)		*P*	-	6.10*	4.80*	1.10*	0.40**
P	-	1.30*	0.20*	1.20*	0.09**	OR/95% CI	-	1.12*	0.84*	1.55*	1.83**
								(0.76-1.67)	(0.53-1.34)	(0.94-2.54)	(1.06-3.16)
PNPLA3 (C10109G) genotype						*PNPLA3 (C10109G)* allele					
C/C	62 (62)	58 (48)	34 (57)	24 (40)		C	135 (0.765)	156 (0.65)	87 (0.725)	69 (0.575)	
C/G	29 (29)	40 (33)	19 (32)	21 (35)		G	47 (0.235)	84 (0.35)	33 (0.275)	51 (0.425)	
G/G	9 (9)	22 (18)	7 (11)	15 (25)		*P*	-	0.45*	7.94*	0.037*	0.21**
P	-	0.60*	7.60*	0.06*	0.90**	OR/95% CI	-	1.55*	1.09*	2.12*	1.949**
								(1.01-2.37)	(0.65-1.83)	(1.29-3.47)	(1.13-3.34)

Fisher's exact test was performed. *p: p* value after Bonferroni correction for multiple testing (10), $p < 0.05$ was considered statistically significant. **p* or OR – ALCs, ANCs and ACs *vs.* HCs. ***p* or OR - ACs *vs.* ANCs, 95% CI- 95% confidence interval.

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