

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**Genetic susceptibility of the Hungarian general and Roma
populations to harmful alcohol consumption**

by Judit Diószegi, MD

Supervisor: Prof. Róza Ádány, MD, PhD, DSc



UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF HEALTH SCIENCES

DEBRECEN, 2017

Genetic susceptibility of the Hungarian general and Roma populations to harmful alcohol consumption

By Judit Diószegi, MD

Supervisor: Prof. Róza Ádány, MD, PhD, DSc

Doctoral School of Health Sciences, University of Debrecen

Head of the **Examination Committee**: György Paragh, MD, PhD, DSc

Members of the Examination Committee: Edit Paulik, MD, PhD
Zsuzsanna Bereczky, MD, PhD

The Examination takes place at the Bldg A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 14th June, 2017 11 a.m.

Head of the **Defense Committee**: György Paragh, MD, PhD, DSc

Reviewers:

István Kiss, MD, PhD, DSc
Ildikó Seres, PhD

Members of the Defense Committee: Zsuzsanna Bereczky, MD, PhD
Edit Paulik, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 14th June, 2017 13. pm

Introduction

The pathogenesis of alcohol use disorders is multifactorial resulting from both genetic and environmental factors. The heritability of alcohol dependence is relatively high, ranging from 50 to 60 percent, based on twin and adoption studies. A number of polymorphisms in genes involved in alcohol metabolism and neurotransmission systems have been linked to alcohol dependence. Environmental factors also play an important role in the development of harmful alcohol consumption although the extent of their influence can change over relatively short periods of time due to e. g. changes in the wider economy.

Genetic polymorphisms influencing the development of alcohol use disorders

The major pathway of oxidative alcohol metabolism involves two steps. First alcohol dehydrogenase (ADH) converts ethanol to acetaldehyde. Five classes of ADHs have been identified based on their structural and kinetic properties, however under physiological conditions, ADHs from classes I, II, and IV are involved in the human alcohol metabolism. In a second reaction step acetaldehyde is broken down into acetate by aldehyde dehydrogenase (ALDH). Although nine gene families have been identified encoding ALDH in humans only ALDH1 and ALDH2 play an important role in the oxidation of acetaldehyde. Genetic polymorphisms of these ethanol metabolizing enzymes may affect their catalytic activity and determine the levels of blood acetaldehyde. This toxic by-product of hepatic oxidation is responsible for the unpleasant effects of alcohol consumption. Consequently alleles resulting in a more rapid conversion of ethanol to acetaldehyde (more active ADH variants) or slower acetaldehyde oxidation (less active ALDH variants) have a protective effect on the risk of

alcohol dependence. In addition to single nucleotide polymorphisms (SNPs) localized in exonal regions some noncoding SNPs in ADH genes have also been associated with risk for alcohol-related traits, probably by affecting gene expression and thereby influencing alcohol metabolism.

The cytochrome P450 family (including CYP2E1) is responsible for alcohol degradation mainly at higher consumption levels. Under conditions characterized by low to moderate alcohol consumption, CYP2E1 metabolizes only a small fraction of the ingested ethanol accounting for less than 10% of ethanol metabolism. Oxidation by the catalase enzyme, located in the peroxisomes, plays only a minor role in alcohol metabolism, because of the limited availability of hydrogen peroxide. The extent of the non-oxidative degradation of alcohol is considered to be minimal.

A wide range of neurotransmitter systems are affected by alcohol exposure that are implicated in cognition, motivation and emotion. These induced changes in neurotransmission are responsible for the positive reinforcing and behavioural effects of alcohol consumption. Ethanol ingestion alters the balance between inhibitory and excitatory neurotransmitters. It inhibits glutamate transmission and increases serotonin, GABA, glycine, nicotinic acetylcholine activity directly, and dopamine, endocannabinoid opioid activity indirectly. Several studies suggest that the risk for alcoholism is also influenced by gene polymorphisms that affect neurotransmitter responses to alcohol.

Ethnic group disparities in alcohol consumption

Knowledge of the extent to which the genetic and environmental factors are important in the development of alcohol dependence may be useful in situations where ethnically

distinct populations inhabit the same territory. Differences within and among human communities in terms of alcohol consumption and the disproportionate impact of alcohol on some ethnic groups can be attributed to the different genetic background and/or environmental factors, which can be well-targeted by public policy actions. In the USA, certain Native Americans tribes possess elevated rates of alcohol dependence compared to other ethnicities and exhibit the highest alcohol-related deaths of all ethnic groups. Unique pattern of genetic risk may be in part responsible for the higher rates of alcohol dependence and related phenotypes in this group. Previous studies suggest that a higher degree of American Indian ancestry is associated with higher frequencies of potential risk and lower frequencies of protective variants for alcohol dependence phenotypes although there are exceptions.

Roma ethnicity and alcohol consumption

One situation where differences in alcohol consumption across ethnic groups may arise is in Central-Eastern-Southern Europe, where the Roma population represents the largest minority. This minority population suffers from disadvantages in various aspects of life, such as poor living conditions, a high level of unemployment, a low education level, social exclusion and discrimination, throughout Europe. All these factors lead to limited access to health care services, less favourable self-reported health and presumably a higher risk of early mortality compared to the majority population.

Roma arrived in the Balkans from North India approximately 1000-1100 years ago and then migrated to Western Europe in three migration waves. Current estimates of the Roma population range between 12-15 million within the World Health Organization's

European Region, of which approximately 10 million are settled in the European Union. The Hungarian Roma population can be divided into three main linguistic, well-described metagroups: Romungros, Vlach and Boyash Gypsies. The Hungarian speaking Romungros (Carpathian Gypsies) came into Hungary through Serbia. This metagroup represents two-thirds of the Roma population of the country. Individuals of the Vlach Roma population, with a proportion of approximately 25 percent of Hungarian Roma, speak the Lovari language and they arrived to Hungary from the territory of Romania. The Boyash metagroup, who migrated from the Central-West Balkans to the Carpathian Basin, represents 8 percent of the Roma living in Hungary. They speak the Boyash language. The geographical distribution of these metagroups is uneven in the country. The highest proportion of Boyash Roma can be found in the Western Transdanubia region of Hungary (up to 30 percent) and the majority of Roma living in Baranya and Somogy counties are members of this metagroup. In other regions of the country their number is very low. Romungros represent the majority of Roma nationwide, except for the Western Transdanubia region. Vlach Roma principally live in the regions of the Southern Great Plain, Danube–Tisza Interfluve, Transdaubia and Szabolcs-Szatmár-Bereg county. Romungros represent 81.4%, Vlach Roma 17.0% and Boyash 1.2% of Roma living in Hajdú-Bihar és Szabolcs-Szatmár-Bereg counties.

Although many Roma people wish to assimilate into majority societies, the most of Roma communities have maintained their cultural identity and traditions, which strongly affect their lifestyle and health-behaviour. Several studies have compared the alcohol drinking habits and levels of consumption between Roma and majority populations in Europe. The results of studies investigating alcohol consumption in Roma populations in different European countries are similar, except the results obtained in a single study when the prevalence of abstainers was significantly higher among Roma compared to the sample representing the general Hungarian adult population. Daily alcohol consumption as well as

drunkenness was found at a higher prevalence among Roma adolescents. A significant association was showed between Roma ethnicity and the lifetime prevalence of alcohol intoxication in a Hungarian study involving Roma and non-Roma adolescents. A study in Moldova showed that expenditure per capita for alcohol and tobacco in Roma families was 3% of the total expenditure compared to 1% in non-Roma families. Logistic regression analysis of potential risk factors for alcoholism resulted in an odds ratio (OR) of 3.2 ($p < 0.05$) for possessing Turkish Gypsy ethnicity compared to other ethnicities investigated. Spanish data noted gender-specific differences in terms of alcohol consumption among Roma: a lower proportion (24.8%) of Roma men are teetotallers compared to male individuals of the general population (31.3%); in the case of women, the results show the opposite situation (62.1% vs 55.9%, respectively). Fundación Secretariado Gitano's research on the health of the Roma indicated that Roma males start drinking alcohol at earlier ages compared to non-Roma and that there are high percentages of households with at least one member possessing an alcohol and/or drug problem, especially in Bulgaria, Greece and Czech Republic.

It is reasonable to suppose that genetic susceptibility underlies the alcohol consumption behaviour of Roma people, since interethnic differences were found regarding other genetic polymorphisms between Roma and majority populations in Europe. A systematic review was carried out to summarize data on the frequency distribution of allelic variants of 9 cytochrome P450 genes in Roma populations and compare them with other populations. Seven variant genotypes showed differences between Roma and Hungarian samples. More than 3-fold difference was observed regarding CYP2C9 *2/*3 and CYP2C19 *2/*2 genotypes. Allele frequencies of rs762551, rs3745274, rs1058930, rs1065852, rs3892097, rs1057910 and rs4244285 polymorphisms differed in Roma population samples. A study involving 189 Valachian Roma and 189 Hungarian Caucasian probands found significant differences in the allele and genotype distributions of the SLC6A3 40 bp VNTR

polymorphism. Review of literature revealed differences in the incidence of MDR1 variants and haplotypes between healthy Roma and Hungarian populations. A statistically significant difference was showed between the Hungarian Roma and non-Roma populations of polymorphisms linked to carcinogenesis: GSTM1, NAT2, TP53, XRCC1 Arg194Trp, Arg280Gln, Arg399Gln (borderline) and pre-miR-146a rs2910164 polymorphisms. Polymorphisms demonstrated to be closely related to smoking behaviour were investigated in DNA samples of Hungarian Roma and general populations. Allele frequencies differed significantly for 16 polymorphisms between the study populations. The susceptible alleles were observed more frequent in the general population and the protective variants were more prevalent among Roma. A study investigating variants of the SLCO1B1 gene when comparing the Hungarian Roma and general populations revealed significant differences in the SLCO1B1 388 AA (24.5 vs 45.5%), GG (33.4 vs 17.9%) genotypes, AG + GG (75.5 vs 54.5 %) carriers and in G allele frequency (0.545 vs 0.362) ($p < 0.001$), respectively. The frequency of SLCO1B1 521 TT was significantly higher in Roma than in Hungarian population samples (67.0 vs 65.2%).

Aims

The aim of our present study was to compare the genetic susceptibility of Roma to the Hungarian general population in terms of harmful alcohol consumption by analysing the frequencies of alleles and genotypes demonstrated to be closely linked with alcohol consumption. Unweighted and weighted genetic risk scores (GRS and wGRS) were calculated by summing the total effect of multiple risk-associated genetic variants utilizing effect size estimates from published GWAS studies. Estimating the extent of genetic susceptibility to

harmful alcohol consumption is important for designing and implementing targeted public health intervention programmes among Roma.

Materials and methods

Study design

The present study involved 1273 Hungarian Roma individuals living in segregated colonies (HR) in North-East Hungary and 2967 individuals in the Hungarian general population (HG). Roma, the most vulnerable minority population of the country represent 30% of the population living in North-East Hungary.

Samples

Sample representative of the Hungarian general population

Previously a cross-sectional study was performed to define the prevalence of metabolic syndrome among Hungarians. The population-based disease registry, the General Practitioners' Morbidity Sentinel Stations Programme (GPMSSP), provided the Hungarian reference sample. The Hungarian GPMSSP was established in 1998 by the School of Public Health, University of Debrecen and the National Public Health and Medical Officer Service to monitor the prevalence and incidence of chronic non-communicable diseases (hypertension; diabetes mellitus; liver cirrhosis; ischaemic heart disease; acute myocardial infarction; stroke;

and malignancies of the respiratory tract, colon and rectum, breast, cervix, and prostate) of great public health importance. In the initial phase of the programme, citizens from four counties were enrolled (Hajdú-Bihar, Győr-Moson-Sopron, Szabolcs-Szatmár-Bereg and Zala counties). Subsequently the programme was extended to additional counties (Komárom-Esztergom and Bács-Kiskun from Central Hungary, Baranya from Southern Transdanubia, and Heves from Northern Hungary) of the country.

The source population of this study included all 20-69 year-old Hungarian citizens registered by the 59 participating general practitioners. The study population was randomly selected from the files of residents in the catchment area and represented the Hungarian adult population on the basis of geographic, age and sex distributions. General practitioners recorded the medical history and performed standardised physical examinations (weight, height, waist circumference, blood pressure measurements) and collected blood samples for laboratory tests (serum triglyceride, HDL-cholesterol, glucose levels) and DNA isolation. Within the framework of the above-mentioned study, 2967 DNA samples were obtained, and these samples were used as the reference samples in the present study.

Sample representative of Hungarian Roma living in segregated colonies

Participants were enrolled from counties of Northeast Hungary (Hajdú-Bihar and Szabolcs-Szatmár-Bereg counties), an area where the majority of Roma colonies are found, using a stratified multistep sampling method. Segregated Roma colonies in these counties were identified previously by Roma field workers within the framework of a project of the University of Debrecen and the Hungarian Ministry of Environmental Protection. The ethnicity of the population was assessed by self-declaration. Segregated colonies exceeding

100 inhabitants were identified, resulting in 64 eligible colonies, of which 40 were randomly selected (25 colonies from Hajdú-Bihar county and 15 colonies from Szabolcs-Szatmár-Bereg county). First twenty-five households were randomly chosen in each colony. Then all individuals aged 20 years or older in each household were identified and 1 person was selected by random table in each household. The final sample consisted of 925 people from the practices of 22 GPs (3 GPs refused to participate) in Hajdú-Bihar county (22X25 individuals) and of 15 GPs in Szabolcs-Szatmár-Bereg county (15X25 individuals). As a part of the health examination survey, each participant's medical history was recorded, and a physical examination (weight, height, waist circumference, blood pressure measurements) was carried out. Blood samples were collected for genetic analysis and laboratory investigations (serum triglyceride, HDL-cholesterol, glucose levels). A total of 757 DNA samples were eligible from Roma individuals for genotyping.

The recently launched 'Public Health Focused Model Programme for Organising Primary Care Services Backed by a Virtual Care Service Centre' developed in the framework of the Swiss-Hungarian Cooperation Programme provided additional DNA samples. This ongoing pilot programme targets Hungarian primary health care reform as a means to reduce social inequalities in health. The programme is being implemented in the North Hungarian and the North Great Plain regions, i.e. in the two most-disadvantaged regions of Hungary. In these regions, four general practitioners' (GPs') clusters were established in the territories of Hajdú-Bihar, Borsod-Abaúj-Zemplén, Jász-Nagykun-Szolnok and Heves counties. The enrollment method of the study population was very similar to the one described above. Segregated colonies exceeding 100 inhabitants located in the participating GPs' clusters were identified. The planned sample size was to involve 100 individuals in each cluster. In the initial phase of the programme two additional clusters were involved in the baseline health status assessment survey and in the DNA sample recruitment method, which were not

involved in the final model programme. Five segregated colonies were randomly drawn from each cluster. If there were less than 5 colonies with at least 100 inhabitants, then all colonies exceeding 100 inhabitants were enrolled. Twenty households were randomly selected in each colony. All individuals aged 20 years or older in each household were identified and 1 person was selected by random table in each household. If someone refused to participate, it was not allowed to draw another individual. Within this programme as part of the health status assessment further 516 samples representative of the Roma population living in Northern-East Hungary by age and gender were collected. In total, 1273 Hungarian Roma DNA samples were genotyped in the study.

The studies were approved by the Ethical Committee of the University of Debrecen, Medical Health Sciences Centre (reference No. 2462-2006) and by the Ethical Committee of the Hungarian Scientific Council on Health (Nos. NKFP/1/0003/2005; 8907-O/2011-EKU and 2213-5-2013/EKU). Written informed consent was gained from all participants in each study population.

Selection of SNPs investigated

Systematic literature search (PubMed, HuGE Navigator) was conducted considering susceptible loci for harmful alcohol consumption based on the pathophysiological background of alcohol dependence. The relevant combinations of the following keywords were used for searching: alcoholism, alcohol dependence, alcohol consumption, molecular genetics, genomics, genes, single-nucleotide polymorphism, genetic variants, gene polymorphism, common gene variants, genome-wide association study (GWAS), candidate gene study, case-control study, meta-analysis, review, association, population genetics, Roma, Gypsy. Special

attention was given to publications on meta-analyses and systematic literature reviews. Based on this search altogether 32 SNPs associated with harmful alcohol consumption were identified. These polymorphisms were classified into three priority groups based on their importance during the assay design. The pool created by the service provider was suitable for simultaneous genotyping in iPLEX Gold chemistry in case of 25 SNPs. In the early stage of genotyping process 1 SNP was not recommended for further analysis by the service provider since the assay failed. The reaction format of the applied genotyping methods allowed us to investigate 25 single nucleotide polymorphisms (SNPs) in genes coding for enzymes involved in alcohol metabolism (alcohol dehydrogenase: ADH1B, ADH1C, ADH4, ADH5, ADH7, aldehyde dehydrogenase: ALDH1A1, ALDH2) and neurotransmitters in the dopaminergic (SLC6A3, DDC), GABAergic (GABRA2, GABRG1), serotonergic (HTR1B, MAOA, TPH2), cholinergic (CHRM2), glutamatergic (GRIN2A) and opioidergic (POMC, OPRM1, OPRK1) pathways, as well as one SNP in the gene encoding a protein involved in neural development and dendritic growth neurogenesis (BDNF).

DNA preparation

DNA isolation was performed from 500- μ l aliquots of EDTA-anticoagulated blood samples using the MagNA Pure LC DNA Isolation Kit – Large Volume (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Extracted DNA samples were eluted in 200 μ l MagNA Pure LC DNA Isolation Kit-Large Volume Elution Buffer.

Genotype assessment

Genotyping for rs1693482 in the gene encoding ADH1C was performed by real-time polymerase chain reaction with a LightCycler 1.5 System (Roche Diagnostics, Mannheim, Germany), followed by a melting curve analysis. The probes applied in the melting curve analysis were used in 0.2 $\mu\text{mol/l}$, while the primers in 0.5 $\mu\text{mol/l}$ concentrations. The primers and probes were produced by TIB Molbiol, Berlin, Germany.

Genotyping of the other selected 24 SNPs was performed by the Mutation Analysis Core Facility (MAF) of Clinical Research Center, Karolinska University Hospital (Stockholm, Sweden) using the Mass Array platform with iPLEX Gold Chemistry. This assay is PCR-based followed by MALDI-TOF mass spectrometry, where 28 SNPs can be analysed in a single pool. The multiplexed forward and reverse primers are present together in the multiplexed assay pool. First locus-specific polymerase chain reaction is performed and afterwards a locus-specific PCR extension is carried out (iPLEX assay), where upstream of the polymorphism the oligonucleotide primers are annealed. In the iPLEX assay, the amplified target DNA and the primers are incubated with mass-modified dideoxynucleotide terminators. The mass of the extended primer (with one mass-modified terminator) can be determined by applying the MALDI-TOF mass spectrometry method, whereas the mass of the primer define the sequence. The validation, concordance analysis and quality control were conducted by MAF according to their protocol. Genotyping was successfully performed in case of 4184 (1267 Roma and 2917 reference) DNA samples.

Statistical analyses

The data were analysed using STATA 9.0 Statistical software (StataCorp LP, College Station, TX, USA). To compare the mean age and sex distribution of the two study groups the Mann-Whitney U and χ^2 tests were used. Assessing departure from Hardy-Weinberg equilibrium (HWE) and significant differences in the allele and genotype frequencies between the two populations the χ^2 test was performed. A p-threshold of 0.002 was applied as a result of the Bonferroni correction for multiple comparisons; otherwise the threshold for significance was 0.05. Haploview software version 4.2 was applied to map the linkage between selected markers. D' values were determined and visualized. Pairwise linkage disequilibrium (LD) between the selected single nucleotide polymorphisms was analysed for both study populations, and haplotype blocks were defined.

Calculation of GRS and wGRS values

Unweighted and weighted genetic scores (GRS and wGRS) were calculated. Subjects with any missing SNP genotypes were excluded. Each person was assigned a summary score based simply on the number of risk alleles carried while calculating unweighted genetic risk scores. Individuals carrying two risk alleles (risk allele homozygotes) were coded by genotype “2”, risk allele heterozygotes by genotypes “1”, while “0” indicated the absence of risk allele. When the effect allele was reported as protective “0” stood for effect allele homozygotes and “2” for other allele homozygotes. The equation (1) below describes the calculation of the unweighted genetic risk score (GRS) in which G_i represents the number of

the risk alleles for the i -th SNP. This model assumes that all alleles in the score make additive contribution to the phenotype and sums up all risk alleles over all loci as a summary score.

$$GRS = \sum_{i=1}^I G_i \quad (1)$$

To collect the effect size estimates of the SNPs included in the study a review of literature on large scale alcohol dependence genomic association studies and meta-analyses was carried out. As a result 11 SNPs were selected in 8 candidate genes (POMC gene: rs1866146, rs6713532; BDNF gene: rs6265; GABRA2 gene: rs567926, rs279871, rs279858; ALDH1A1 gene: rs610529; ADH1B gene: rs1229984; ADH7 gene: rs1154458; CHRM2 gene: rs324650 and OPRM1 gene: rs1799971).

The weighted score takes into account that effect sizes among the polymorphisms vary. While calculating weighted genetic risk scores described by equations (2) and (3), weights (w_{OR_i}) were derived from the risk coefficient for each allele based on odds ratios reported in previous association studies. The log per-allele odds ratio from meta-analysis or from other independent data is considered generally as a reasonable weight to apply to each SNP. These weights (w_{OR_i}) were multiplied by 0, 1, or 2 according to the number of effect alleles (X_i) carried by each individual.

$$wGRS = \sum_{i=1}^I w_{OR_i} X_i \quad (2)$$

$$w_{OR_i} = \log(OR_i) \quad (3)$$

Where effect estimates were reported for carriage of either one or two copies of each risk allele as a single group (OPRM1), the risk coefficients were multiplied by a score of 0 or

1. Two sample Student's t-test and Mann-Whitney U test were applied to compare the distribution of GRS and wGRS, respectively.

Considering the effects of gender and age as confounders on differences between study populations in terms of GRS, analysis of variance models were constructed. For wGRS the study samples were stratified by the potential confounding variables (groups of age: 20-29, 30-39, 40-49, 50-59, 60-X).

Results

Allele and genotype comparisons between the study groups

Altogether, 25 SNPs were tested using samples from 1267 Roma and 2917 participants representing the Hungarian general population. The Mann-Whitney U test was applied to compare the mean age of the two study groups, since the age distribution of both study samples did not follow the normal distribution (Shapiro-Wilk test, $p < 0.0001$) and a significant difference in the two variances was observed. The mean age of the two study groups differed significantly according to the Mann-Whitney U test ($p < 0.001$). The mean age of the Roma population was 40.67 ± 12.44 and 45.43 ± 14.5 of the Hungarian general population. The proportion of male individuals in Roma samples was significantly lower (HR: 0.42 vs HG: 0.49, $p < 0.01$) compared to the Hungarian reference sample.

All SNPs were tested for Hardy–Weinberg equilibrium. One SNP was not in HWE (TPH2 rs1386496) in both populations (presumably due to genotyping error), so this polymorphism was excluded from further analysis. MAOA rs979606 was in a deviation from

the Hardy-Weinberg equilibrium within the Roma study group and DDC rs3779084 within the Hungarian reference sample. Since these deviations are not necessarily due to genotyping error, they can be attributed to genetic factors, non-random mating, population stratification and selection effect of the SNPs, these polymorphisms were retained for subsequent analyses.

The allele frequencies of the two study populations were calculated from genotypic frequencies. Two polymorphisms, ALDH2 rs671 and SLC6A3 rs6530, were found to be monomorphic in both groups. After applying the Bonferroni correction, the frequency of alleles, with 17 SNPs, remained significantly different ($p < 0.002$) between the two groups. Regarding the susceptibility variants, rs7694646 (ADH4), rs1154400 (ADH5), rs279858 and rs567926 (GABRA2) and rs6985606 (OPRK1) were more frequent among the participants representing the general Hungarian population (30.77% vs 26.68%, 34.25% vs 29.31%, 38.65% vs 27.17%, 39.77% vs 28.54% and 47.6% vs 33.99%, respectively), whereas higher allele frequencies were found in the Roma population for rs1799971 (OPRM1), rs1866146 and rs6713532 (POMC) (19.90% vs 13.04%, 52.35% vs 39.37% and 40.17% vs 26.15%, respectively). Three protective variants (rs1229984 in ADH1B, rs130058 in HTR1B and rs979606 in MAOA) were observed more frequent among Roma (12.87% vs 8.0%, 35.59% vs 27.88% and 41.93% vs 31.66%, respectively), and six others (rs1800759 in ADH4B, rs610529 in ALDH1A1, rs6265 in BDNF, rs279871 in GABRA2, rs2221020 in GABRG1 and rs2072450 in GRIN2A) were more frequent in the Hungarian study sample (40.79% vs 35.7%, 45.05% vs 31.51%, 19.88% vs 11.34%, 38.67% vs 27.19%, 48.23% vs 36.33% and 13.72% vs 9.04%, respectively).

Linkage analysis

The pairwise analysis in the Hungarian study population showed complete linkage disequilibrium for GABRA2 markers (rs279871–rs279858). Polymorphisms in genes of ADH5 rs1154400–ADH4 rs7694646, ADH4 rs7694646–rs1800759, ADH5 rs1154400–ADH4 rs1800759, GABRA2 (rs567926–rs279871, rs567926–rs279858) and POMC gene (rs1866146 –rs6713532) were in strong linkage disequilibrium. Three haplotype blocks were identified. Block 1 spanned the variants of POMC (rs1866146, rs6713532). Block 2 covered GABRA2 markers (rs567926, rs279871, rs279858) and block 3 spanned the regions of ADH5-ADH4 genes (rs1154400, rs7694646, rs1800759).

In the group representing the Roma, rs279871 (GABRA2) and rs279858 (GABRA2) were in complete LD. Markers for GABRA2 (rs567926 and rs279871, rs567926 and rs279858), ADH (ADH5 rs1154400–ADH4 rs7694646, ADH5 rs1154400–ADH4 rs1800759, ADH5 rs1154400–ADH1B rs1229984, ADH4 rs7694646–rs1800759, ADH4 rs7694646–ADH1B rs1229984, ADH4 rs1800759–ADH1B rs1229984, ADH1B rs1229984–ADH7 rs1154458) and POMC genes (rs1866146 – rs6713532) showed strong LD. Markers of the POMC (rs1866146, rs6713532), the GABRA2 (rs567926, rs279871, rs279858), and the region of ADH5, ADH4 and ADH1B genes (rs1154400, rs7694646, rs1800759, rs1229984) belonged to three haplotype blocks.

Comparison of genetic risk scores

The GRS for the HR population, combining all SNPs included in the study, was constructed for 1090 individuals and ranged from 13 to 28 risk alleles. The GRS for HG

subjects, based on all SNPs, was calculated for 2607 individuals and ranged from 12 to 29 risk alleles. The GRS followed a normal distribution in both groups according to the Shapiro-Wilk test (Hungarian general population: $p=0.69117$, Hungarian Roma living in segregated colonies: $p=0.99925$). The mean risk allele count score was 20.73 (SD 2.44) in people of the HR group and 21.02 (SD 2.49) in those in the HG group. Distribution of gene count scores differed significantly between study groups applying the two sample Student's t-test ($p=0.0013$).

Thirty per cent of the Hungarian general population were in the bottom fifth (GRS ≤ 19) of the risk score vs 27.1 % of the Hungarian Roma population. Thirteen per cent of people in HR group were in the top fifth (GRS ≥ 24) of the GRS compared with 16.9% of those in HG population ($p=0.017$), i.e. the distribution of unweighted genetic risk scores was found to be left-shifted in the HR population compared to the HG population.

There were no available effect size estimates for SNPs of ADH4 and ADH5 genes so those were not included in the wGRS function. The wGRS was derived from 8 SNPs, since the polymorphisms of POMC and GABRA2 genes were in strong linkage disequilibrium only rs1866146 of POMC and rs567926 of GABRA2 (systematically the first SNPs in the haplotype blocks with available effect size estimates) were chosen to enter the wGRS.

The median wGRS of HR vs HG were 0.53 (interquartile range (IQR): 0.33- 0.76) vs 0.66 (IQR: 0.45-0.85). The lower HR median suggests that the HR population has lower weighted genetic risk scores on average ($p=3.33 \times 10^{-27}$). Data in wGRS were non-normally distributed with skewness of -0.18 (SE=0.07) and kurtosis of -0.13 (SE=0.14) in HR population and with skewness of -0.12 (SE=0.05) and kurtosis of -0.17 (SE=0.1) in HG population.

Adjustment for confounders was done where records were available without missing data. The pattern of GRS ($p=0.0236$) was not changed after adjusting for gender and age. The results are consistent across the strata since the stratum-specific effect estimates of wGRS (median and IQR) show that the Roma population has lower weighted genetic risk scores on average, independent of age and gender.

Discussion

The European Roma people migrated from India to the Balkans 1000-1100 years ago and are today spread throughout Europe. In the majority of Central, Eastern and Southern European countries, this group represents over 5% of the population. Their health status is significantly worse than that of the majority population, which is strongly related to their unfavourable health behaviour characteristics. On the basis of results (i) demonstrating a high prevalence of daily alcohol consumption as well as drunkenness, even among Roma adolescents, (ii) the increasing amount of data accumulating in the literature on the genetic background of alcohol consumption and (iii) the high consanguinity rate known to be characteristic for the Roma population, it is reasonable to suppose that genetic susceptibility underlies the alcohol consumption behaviour of Roma people.

The aim of our study was to determine whether a genetic susceptibility does indeed contribute to the higher prevalence of harmful alcohol consumption in the Roma population by comparing differences in the genotype and allele frequencies of several alcohol consumption-related polymorphisms between the Hungarian Roma and general populations, since interethnic differences were found regarding other polymorphisms between Roma and majority populations in Europe. This is the first study to investigate several susceptible loci

for harmful alcohol consumption among Roma living in segregated colonies and to compare them with data for the majority population. The SNPs in the current study are located in genes encoding proteins involved in alcohol metabolism and in several neurotransmission systems linked to alcohol consumption.

Twenty-five polymorphisms associated with alcohol drinking habits were genotyped, and differences in 17 polymorphisms remained significant after multiple test correction when the two groups were compared. The susceptibility alleles of rs7694646, rs1154400, rs279858, rs567926 and rs6985606 were more frequent among participants representing the general Hungarian population, whereas rs1799971, rs1866146 and rs6713532 were more frequent in the Roma sample. Among the protective variants, the rs1800759, rs610529, rs6265, rs279871, rs2221020 and rs2072450 polymorphisms were observed more frequent Hungarian study sample; with rs1229984, rs130058 and rs979606, higher allele frequencies were observed in the Roma sample.

Although the frequency of 17 genetic alterations differed significantly between the Roma and Hungarian study populations, the allele frequency of the ALDH2 polymorphism was concordant, that is, the allele was found to be monomorphic within both study populations. This result is in accordance with dbSNP data for the Central European populations, i.e. the frequency of this protective allele in the HapMap-CEU population is 0%. The rs671 polymorphism of the aldehyde dehydrogenase 2 gene has a strong protective effect against alcohol dependence and other alcohol-induced diseases in Asians, leading to alcohol sensitivity as a result of an enzyme with low – if any – catalytic activity, with consequently strongly delayed acetaldehyde oxidation and raised acetaldehyde levels.

Although the allele frequencies of ALDH2 rs671 and SLC6A3 rs6530 were identical (monomorphic) in the two study samples, our results show differences in allele frequencies in the vast majority of the selected polymorphisms between the general Hungarian and Roma

populations. Out of the polymorphisms investigated by a meta-analysis an association with harmful alcohol consumption was confirmed for 5 SNPs. In case of the variant of ADH1B (rs1229984) results of a meta-analysis of association studies published in the previous 21 years showed a strong association with alcohol use disorders (dependence and abuse), with an allelic OR of 2.06 (95% CI:1.84-2.31). The association of BDNF rs6265 with different categories of mental disorders (eating disorders, substance-related disorders, mood disorders and schizophrenia) was tested in a meta-analysis of individual case-control studies, and in the case of substance-related disorders, a 21% protective effect was estimated. Concerning alcoholism, a generalized odds ratio (ORG) of 1.27 (95% CI: 1.01-1.60) for GABRA2 rs279858 and an allele-specific OR of 1.24 (95% CI: 1.06-1.46) for GABRA2 rs567926 were estimated. Ethnicity-specific meta-analyses of the OPRM1 variant (rs1799971) revealed a significant association with alcohol dependence in Asians (OR: 1.73) but not in Caucasians.

Pairwise linkage disequilibrium analysis showed differences in LD among the selected SNPs between the two populations. The extent of linkage disequilibrium, block boundaries and specific haplotypes have been reported to be variable among different loci and populations, and Roma and individuals of the Hungarian general population have different ancestries, with different population admixture, which contribute to LD differences.

In addition, our study introduced the concept of GRS, means to combine information across variants into a quantitative measurement as a single score. Our results indicate that both GRS and wGRS were significantly lower in the HR population even after controlling for the effects of possible confounding factors. Although a relatively small set of SNPs was included in wGRS, the results imply that it is possible to derive a genetic score that has at least some discriminative ability. Since at the present time the number of genome-wide association studies related to alcohol dependence are limited, the effect size estimates used to calculate wGRS are derived from heterogenous studies. Accordingly the results of the wGRS

calculation must be interpreted with caution due to these uncertainty factors. Our results of the GRS calculation can be used with certainty to sum the total effect of multiple risk-associated genetic variants. However, since the literature lacks a sufficient number of genome-wide association studies in respect of alcohol dependence, the weighted genetic risk score model applied in our recent study rather provides methodological fundamentals for future research.

The results strongly suggest that no genetic susceptibility underlies the higher prevalence of harmful alcohol consumption in the Roma population compared to the majority population. On the basis of this finding it is reasonable to suppose that environmental, including socio-economic and cultural factors that explain differences in drinking behaviour.

The obvious limitation of the current study is that the Roma study population was not representative of the overall Roma population living in Hungary. Roma who have, to various degrees, assimilated with the general Hungarian population were not included in the analysis due to the nature of the study design. Many people are reluctant to self-define their ethnicity as Roma and the representative sample of the general Hungarian population included some people who are Roma as well. It is possible that the inclusion of Roma in the general Hungarian population resulted in a slight underestimation of the differences between the populations.

The genotyping technology applied in the current study is a high-throughput SNP genotyping method, but it does not allow the analysis of variable number tandem repeat (VNTR) polymorphisms. However it would be advantageous to extend our study towards investigation of VNTR polymorphisms, since convincing findings in the literature show that some VNTR polymorphisms may affect alcohol consumption patterns.

There are no genetic association studies available in the literature related to harmful alcohol consumption among Roma people. There is only one single effect size estimate (A1A1+A1A2, OR: 1.74) analysis study of TaqIA site of ANKK1 gene among Meitei communities of Manipur in India (considering the Indian origins of Roma populations) that can be compared to the results of the metaanalysis of European studies investigating the same polymorphism (A1A1+A1A2, OR: 1.23). These estimated odds ratios of the A1A1+A1A2 genotype in respect of alcohol dependence do not differ greatly. Our recent study assumes that the effect size of SNPs applied in the current study was similar in our study populations than it was found in the populations previously studied and the effect size described for. On the basis of data showing concordance on different populations there is no reason to suppose that the effect sizes would substantially differ in the populations we studied. However due to the lack of study data we cannot rule out the possibility that the effect size estimates – to a certain extent - may show differences in some cases. Based on the similar distribution patterns of genetic risk scores weighted and unweighted the possibility of significant distortions due to varying effect size estimates can be excluded. However a further study may answer the question whether there is any - even minor - difference in the degree of correlation between unweighted and weighted genetic risk scores and alcohol consumption (prevalence of harmful alcohol consumption and amount of alcohol consumed) in the Hungarian general and Roma populations.

The unfavourable health behaviour of Roma, with smoking and harmful alcohol drinking habits even among adolescents, may be linked to the permissiveness of Roma families towards alcohol and tobacco consumption. Roma people are not aware of the harmful effects of active and passive smoking, and it is acceptable even among children. Regarding alcohol consumption, Roma women believe that having alcoholic spouses is inherent in normal family life, most likely due to a lack of knowledge on the harmful effects of alcohol.

The differences in alcohol consumption behaviour between the two populations do not appear to be linked to genetic constitution but may occur as a result of different environmental exposure and cultural values. Highlighting the role of environmental and social factors has great importance in the planning of hopefully effective public health interventions among the Roma population; regardless, it is still important to emphasise that further genetic research on minority populations is needed.

Summary

Several studies suggest that harmful alcohol drinking habits, even among Roma children and adolescents, are more common than in the majority population. The aim of the study was to evaluate the genetic susceptibility of Roma to uncontrolled alcohol consumption compared to the Hungarian general population.

A total of 1273 samples from the population of segregated Hungarian Roma colonies and 2967 samples from the Hungarian general population were genotyped for 25 polymorphisms. Differences in genotype and allele distributions were investigated. Genetic risk scores (GRS) were generated to estimate the joint effect of multiple SNPs. After unweighted and weighted GRS were calculated the distribution of scores in study populations was compared.

The allele frequencies differed significantly between the study populations for 17 SNPs ($p < 0.002$). The distribution of unweighted GRS in Roma population was left shifted compared to general population ($p = 0.0013$). The median weighted genetic risk score was lower among the subjects of Roma population compared to the individuals of general population (0.53 vs 0.65, $p = 3.33 \times 10^{-27}$) even after adjustment for confounding factors.

Differences in alcohol consumption patterns between the Hungarian Roma and Hungarian general populations do not appear to be linked to genetic constitution, but they may occur as a result of different cultural values and environmental exposures. Preventive interventions focusing on cultural and environmental factors are preferred for controlling harmful alcohol consumption in the Roma population.



Registry number: DEENK/11/2017.PL
Subject: PhD Publikációs Lista

Candidate: Judit Diószegi
Neptun ID: L118BW
Doctoral School: Doctoral School of Health Sciences

List of publications related to the dissertation

1. **Diószegi, J.**, Fiatal, S., Tóth, R., Moravcsik-Kornyicki, Á., Kósa, Z., Sándor, J., McKee, M., Ádány, R.: Distribution Characteristics and Combined Effect of Polymorphisms Affecting Alcohol Consumption Behaviour in the Hungarian General and Roma Populations.
Alcohol Alcohol. 52 (1), 104-111, 2017.
DOI: <http://dx.doi.org/10.1093/alcalc/agw052>
IF: 2.724 (2015)
2. Kósa, Z., Moravcsik-Kornyicki, Á., **Diószegi, J.**, Roberts, B., Szabó, Z., Sándor, J., Ádány, R.: Prevalence of metabolic syndrome among Roma: a comparative health examination survey in Hungary.
Eur. J. Public Health. 25 (2), 299-304, 2015.
IF: 2.751

Total IF of journals (all publications): 5,475

Total IF of journals (publications related to the dissertation): 5,475

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

24 January, 2017

