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ARTICLE

Replication of genetic variants from genome-wide association studies with metabolic traits in an island population of the Adriatic coast of Croatia

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Twenty-two single-nucleotide polymorphisms (SNPs) in 10 gene regions previously identified in obesity and type 2 diabetes (T2D) genome-wide association studies (GWAS) were evaluated for association with metabolic traits in a sample from an island population of European descent. We performed a population-based study using 18 anthropometric and biochemical traits considered as continuous variables in a sample of 843 unrelated subjects (360 men and 483 women) aged 18–80 years old from the island of Hvar on the eastern Adriatic coast of Croatia. All eight GWAS SNPs in *FTO* were significantly associated with weight, body mass index, waist circumference and hip circumference; 20 of the 32 nominal *P*-values remained significant after permutation testing for multiple corrections. The strongest associations were found between the two *TCF7L2* GWAS SNPs with fasting plasma glucose and HbA1c levels, all four *P*-values remained significant after permutation tests. Nominally significant associations were found between several SNPs and other metabolic traits; however, the significance did not hold after permutation tests. Although the sample size was modest, our study strongly replicated the association of *FTO* variants with obesity-related measures and *TCF7L2* variants with T2D-related traits. The estimated effect sizes of these variants were larger or comparable to published studies. This is likely attributable to the homogenous genetic background of the relatively isolated study population.
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Keywords: genetic association; obesity; type 2 diabetes; FTO; TCF7L2; isolated population

INTRODUCTION

Recent genome-wide association studies (GWAS) have identified nearly 30 common sequence variants associated with obesity and type 2 diabetes (T2D) related traits in populations primarily of European descent. Genes that have been implicated in T2D harboring these variants include *TCF7L2*, *SLC30A8*, *HHEX/IDE*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *TCF2* and *PPARG*;[1–7] genes that are primarily associated with obesity-related phenotypes are *INSIG2*, *FTO*, *MC4R*, *BDNF* and *SH2B1*.[8–14] Among these genes, *TCF7L2* and *FTO* have emerged as the strongest candidates conferring risks to T2D and obesity, respectively, replicated across studies with relatively large effect sizes.[15,16] The purpose of this study was to replicate a set of the significant GWAS single-nucleotide polymorphisms (SNPs) from the aforementioned studies with several metabolic traits in an island population from the eastern Adriatic coast of Croatia.

Croatian islanders are primarily of Slavic descent, who emigrated from the mainland at successive time periods, the latest during fifteenth and eighteenth century AD following the Turkish invasions.[17,18] Since that time, the populations have remained relatively isolated largely because of their geographic isolation with minimal immigration from the mainland. In spite of sharing a common descent, these populations are distinct from their mainland European ancestors, practicing a traditional life-style pattern based primarily on an agricultural subsistence in a rural setting and living on a typical 'Mediterranean' diet of fish, vegetables, olive oil and red wine.[18] However, previous studies have shown high prevalence of obesity and hypertension,[19–21] which imply factors other than nutritional practices and life-style patterns have influenced variations in metabolic profiles of these populations.

We analyzed 22 significant GWAS SNPs located in and around *INSIG2*, *PPARG*, *CDKAL1*, *SLC30A8*, *CDKN2A*, *CDKN2B*, *HHEX/IDE*, *TCF7L2*, *FTO* and *MC4R* to test for associations with traits that include obesity-related measures of body mass index (BMI), waist circumference (WC), hip circumference (HC), waist–hip ratio (WHR), blood pressure and biochemical measures of fasting plasma glucose (FPG), HbA1c, insulin, HDL, LDL, total cholesterol (TC), triglyceride (TG), fibrinogen, calcium, creatinine and uric acid levels. Among the significant observations, we found strong associations of two SNPs (rs7903146 and rs12255372) in *TCF7L2* with FPG and HbA1c levels; and all eight SNPs in *FTO* were individually associated with weight, BMI, WC and HC.

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MATERIALS AND METHODS

Study population and phenotypic measurements

The study samples were collected from the island of Hvar, the largest of the middle Dalmatian islands on the Adriatic coast with a population of over 11 500 individuals. Anthropometric measurements of height (Ht), weight (Wt), WC, HC, blood pressure and blood samples were obtained from 843 unrelated individuals (360 men and 483 women) aged 18 to 80 years old from the island of Hvar. Subjects within this age bracket were randomly ascertained without consideration for any known disease status and use of medication. Study protocols were approved by the ethics committee of the Institute for Anthropological Research, Zagreb and the Institutional Review Board of the University of Cincinnati.

Data on Ht, Wt, WC and HC were used to compute the composite scores of BMI (Wt in kg)/(Ht in m^2), and WHR (WC/HC). Blood pressure was measured three times using mercury sphygmomanometer at resting position; the second and the third measurements were used to calculate the mean systolic (SBP) and diastolic (DBP) blood pressure. Blood samples were drawn after a 12-h fast and serum was separated and kept frozen until shipped to the clinical chemistry laboratory in Zagreb, where biochemical tests were performed to measure FPG, HbA1c, insulin, HDL, LDL, TC, TG, fibrinogen, calcium, creatinine and uric acid levels using standard methods.

Genotyping

Twenty-two SNPs (Table 1) significantly associated with anthropometric and biochemical traits in GWAS were genotyped in the total sample of 843 subjects. The five *MC4R* SNPs were genotyped using the TaqMan assay and the remaining 17 SNPs were typed using the SNPlex assay following protocols described previously.[22] Quality control was assured by introducing blind duplicates and negative controls into each batch of samples in the 96-well format. The overall genotype call rate of the 22 SNPs was 96.8% and the genotypic consistency rate based on 8 internal replicates was $>99.5\%$.

Statistical analysis

Descriptive statistics of the phenotypic traits were calculated using SAS v.9. 1 (SAS Institute, Inc., Cary, NC, USA). Allele and genotype frequencies were computed using the HelixTree software (Golden Helix, Inc., Bozeman, MT, USA). Hardy–Weinberg equilibrium (HWE) was assessed by Fisher's exact test. The phenotypic traits were adjusted for the effects of age, gender and their interaction term by linear regression. The association between each SNP and the adjusted traits was performed using HelixTree based on an additive model (1 df test) with *P*-values considered nominally significant at ≤ 0.05. A permutation-based analysis, with 1 00 000 iterations, was performed to account for multiple tests based on the number of markers.

RESULTS

Table 1 shows the description of the examined SNPs with their NCBI dbSNP IDs ('rs' numbers), gene location, chromosomal base positions and minor allele frequencies (MAFs). Genotype proportions at all SNPs conformed to the expectations of HWE with a *P*-value >0.01 (data not shown). All of the SNPs in our population are moderately to highly polymorphic with the MAF ranging between 0.13 and 0.49. In general, the allele frequencies corresponded to those in the European HapMap sample (www.HapMap.org).

Descriptive statistics (mean ± SD) of the phenotypic traits are presented in Table 2. The mean age of the participants at the time of sampling was 57.84 years (59.55 years in men and 56.56 years in women). All of the examined phenotypes were considered as continuous traits for analysis. Of the two classic measures of obesity, mean BMI was within the normal range of variation ($BMI \leq 30\,kg/m^2$) in both genders; however, mean WC was above the normal range as defined either by the ATPIII (Adult Treatment Panel III)[23] or IDF (International Diabetes Federation)[24] definitions in both men and

Table 1 Summary of the studied GWAS SNPs

NCBI SNP ID	*Gene*	*Chromosome*	*Position*[a]	*Study reference*	*Ancestry of reference population*	*Phenotype of reference study*	*Alleles*[b]	*HapMap caucasian MAF*	*MAF*[c]
rs7566605	*INSIG2*	2q14	1.19E+08	Herbert *et al*[8]	Western European African American	Obesity	CG	0.26	0.4
rs1801282	*PPARG*	3p25	12368125	WTCCC[7]	Western European	T2D	CG	0.10	0.16
rs10946398	*CDKAL1*	6p22	20769013	Zeggini *et al*[4]	Western European	T2D	AC	0.34	0.3
rs13266634	*SLC30A8*	8q24	1.18E+08	Sladek *et al*[1]	Western European	T2D	CT	0.24	0.36
rs564398	*CDKN2B*	9p21	22019547	Zeggini *et al*[4]	Western European	T2D	AG	0.43	0.39
rs10811661	*CDKN2A*	9p21	22124094	DGI[3]	Western European	T2D	TC	0.20	0.13
rs1111875	*HHEX-IDE*	10q23	94452862	Sladek *et al*[1]	Western European	T2D	GA	0.42	0.42
rs7903146	*TCF7L2*	10q25	1.15E+08	Grant *et al*[9]	Western European	Obesity	TC	0.28	0.29
rs12255372	*TCF7L2*	10q25	1.15E+08	Grant *et al*[9]	Western European	Obesity	GT	0.25	0.28
rs9939973	*FTO*	16q12	52358069	Hinney *et al*[11]	Western European	Obesity	GA	0.48	0.49
rs1421085	*FTO*	16q12	52358455	Dina *et al*[10]	Western European	Obesity	TC	0.46	0.47
rs1121980	*FTO*	16q12	52366748	Dina *et al*[10]	Western European	Obesity	CT	0.48	0.47
rs17817449	*FTO*	16q12	52370868	Dina *et al*[10]	Western European	Obesity	TG	0.46	0.44
rs8050136	*FTO*	16q12	52373776	Grant *et al*[6]	Western European	Obesity	CA	0.46	0.44
rs3751812	*FTO*	16q12	52375961	Dina *et al*[10]	Western European	Obesity	GT	0.46	0.44
rs9939609	*FTO*	16q12	52378028	Frayling *et al*[9]	Western European	Obesity	TA	0.46	0.43
rs7190492	*FTO*	16q12	52386253	Grant *et al*[6]	Western European	Obesity	GA	0.35	0.33
rs17782313	*MC4R*	18q22	56002077	Loos *et al*[12]	Western European	Obesity	TC	0.26	0.23
rs12970134	*MC4R*	18q22	56035730	Chambers *et al*[13]	Indian Asian, European	T2D-related traits[d]	GA	0.28	0.22
rs477181	*MC4R*	18q22	56047018	Chambers *et al*[13]	Indian Asian, European	T2D-related traits[d]	GT	0.34	0.33
rs502933	*MC4R*	18q22	56047454	Chambers *et al*[13]	Indian Asian, European	T2D-related traits[d]	CA	0.34	0.34
rs4450508	*MC4R*	18q22	56064414	Chambers *et al*[13]	Indian Asian, European	T2D-related traits[d]	GA	0.38	0.35

[a]Chromosomal base position with reference to Human Genome Build 36.3.
[b]Second nucleotide corresponds to the minor allele.
[c]Minor allele frequency (MAF) in this study population.
[d]HOMA-IR, WC, WHR, Wt, BMI, HDL, TG, DBP, T2D and metabolic syndrome.

Table 2 Summary statistics of the phenotypic traits

	Male				*Female*				*Total sample*			
Trait	N	*Mean ± SD*	*Min*	*Max*	N	*Mean ± SD*	*Min*	*Max*	N	*Mean ± SD*	*Min*	*Max*
Age (year)	360	59.55 ± 12.46	19.9	80	483	56.56 ± 13.23	18.1	79.8	843	57.84 ± 12.98	18.1	80
Ht (cm)	360	177.42 ± 7.22	157.2	206	483	164.10 ± 6.78	139	187.2	843	169.78 ± 9.59	139	206
Wt (kg)	360	89.31 ± 12.93	60.2	142.5	483	74.10 ± 12.69	43	123.9	843	80.59 ± 14.84	43	142.5
BMI (kg/m^2)	360	28.32 ± 3.34	19.64	42.23	483	27.54 ± 4.58	16.7	45.79	843	27.87 ± 4.11	16.7	45.79
WC (cm)	359	101.85 ± 8.98	79.3	138.5	482	91.90 ± 12.09	62.3	137.4	841	96.15 ± 11.93	62.3	138.5
HC (cm)	360	104.42 ± 7.29	90.8	132	482	106.15 ± 10.46	75	150	842	105.41 ± 9.27	75	150
WHR	359	0.97 ± 0.05	0.727	1.124	482	0.86 ± 0.06	0.651	1.12	841	0.91 ± 0.08	0.651	1.124
DBP (mm Hg)	360	84.91 ± 9.20	60	120	483	81.49 ± 10.47	55	115	843	82.95 ± 10.08	55	120
SBP (mm Hg)	360	138.59 ± 20.56	100	240	483	132.42 ± 24.46	90	230	843	135.05 ± 23.07	90	240
FPG (mmol/l)	360	6.26 ± 1.40	4.2	16.8	483	5.86 ± 1.37	4.1	14.9	843	6.03 ± 1.39	4.1	16.8
HbA1c (%)	360	5.78 ± 0.84	4.6	12	482	5.77 ± 0.87	4.7	12.4	842	5.77 ± 0.86	4.6	12.4
Insulin (mmol/l)	360	11.15 ± 8.13	1.15	72.26	482	11.383 ± 7.93	1.3	56.26	842	11.28 ± 8.01	1.15	72.26
HDL (mmol/l)	360	1.30 ± 0.34	0.2	3.55	482	1.53 ± 0.39	0.77	4.61	842	1.43 ± 0.38	0.2	4.61
LDL (mmol/l)	358	3.68 ± 0.95	1.1	6.7	481	3.91 ± 1.04	1.2	8	839	3.81 ± 1.01	1.1	8
TC (mmol/l)	360	5.80 ± 1.10	2.4	9.1	482	6.09 ± 1.22	1.5	10.4	842	5.971 ± 1.18	1.5	10.4
TG (mmol/l)	360	1.70 ± 1.03	0.36	6.31	482	1.41 ± 0.75	0.33	5.09	842	1.53 ± 0.89	0.33	6.31
Fibrinogen (mmol/l)	355	3.63 ± 1.25	1.3	8.1	478	4.03 ± 1.38	1.4	9.2	833	3.86 ± 1.34	1.3	9.2
Calcium (mmol/l)	358	2.36 ± 0.13	1.66	3.2	482	2.37 ± 0.11	1.75	2.9	840	2.36 ± 0.12	1.66	3.2
Creatinine (mmol/l)	360	97.68 ± 20.94	54	280	482	78.84 ± 12.99	48	141	842	86.89 ± 19.26	48	280
Uric Acid (mmol/l)	359	365.89 ± 80.58	169	671	482	265.32 ± 73.09	77	632	841	308.25 ± 91.12	77	671

Table 3a *P*-values of single-locus association tests with traits of primary involvement with obesity and T2D

SNP	*Gene*	*Wt*	*BMI*	*WC*	*HC*	*WHR*	*FPG*	*HbA1c*	*Insulin*
rs7566605	*INSIG2*	0.8241	0.6250	0.6983	0.8846	0.4685	0.7412	0.3214	0.3683
rs1801282	*PPARG*	0.9015	0.9478	0.7123	0.2166	0.4526	**0.0463**	**0.0402**	0.8157
rs10946398	*CDKAL1*	0.4687	0.7888	0.8522	0.8098	0.6234	0.5921	0.0380	0.0809
rs13266634	*SLC30A8*	0.3859	0.8006	0.5610	0.9760	0.3627	0.7612	0.3965	0.3110
rs564398	*CDKN2B*	0.2659	0.5976	0.3366	0.3005	0.6022	0.7524	0.8627	0.2888
rs10811661	*CDKN2A*	0.7389	0.6424	0.3315	0.8007	0.3216	**0.0426**	**0.0336**	0.6773
rs1111875	*HHEX/IDE*	**0.0289**	**0.0413**	0.1624	0.2168	0.5880	**0.0164**	**0.0163**	0.4383
rs7903146	*TCF7L2*	0.1785	0.2602	0.3611	0.5749	0.4348	**1.29E-07**	**1.14E-09**	0.4202
rs12255372	*TCF7L2*	0.1930	0.0950	0.2746	0.3026	0.5428	**9.62E-08**	**6.37E-09**	0.1310
rs9939973	*FTO*	**0.0024**	**0.0047**	**0.0009**	**0.0139**	**0.0305**	0.9428	0.3743	**0.0167**
rs1421085	*FTO*	**0.0013**	**0.0010**	**0.0004**	**0.0010**	0.1277	0.9686	0.4779	0.0664
rs1121980	*FTO*	**0.0010**	**0.0013**	**0.0006**	**0.0022**	0.1169	0.8934	0.5187	**0.0457**
rs17817449	*FTO*	**0.0009**	**0.0025**	**0.0009**	**0.0021**	0.1462	0.5949	0.8865	0.1571
rs8050136	*FTO*	**0.0051**	**0.0179**	**0.0026**	**0.0078**	0.1524	0.6895	0.7871	0.1714
rs3751812	*FTO*	**0.0029**	**0.0064**	**0.0020**	**0.0029**	0.2310	0.5585	0.9416	0.1086
rs9939609	*FTO*	**0.0018**	**0.0043**	**0.0016**	**0.0063**	0.1027	0.5013	0.7326	0.2418
rs7190492	*FTO*	**0.0054**	**0.0130**	**0.0009**	**0.0110**	**0.0229**	0.2070	0.4397	0.0768
rs17782313	*MC4R*	0.0817	0.3312	0.1987	0.2315	0.5412	0.8683	0.1626	0.5135
rs12970134	*MC4R*	0.0540	0.1401	0.1414	0.1822	0.4766	0.8727	0.4638	0.9882
rs477181	*MC4R*	0.2032	0.4211	0.6506	0.1405	0.3210	0.9218	0.4512	0.5074
rs502933	*MC4R*	0.0961	0.2539	0.3708	0.0711	0.4804	0.9472	0.4479	0.9060
rs4450508	*MC4R*	0.1356	0.2424	0.3561	0.0918	0.5768	0.8037	0.6887	0.9428

Empirical *P*-values (<0.05) are shown in bold; *P*-values that maintain significance after permutation tests are underlined.

women (men=101.9 cm, women=91.9 cm). Among the biochemical traits, mean FPG in men (6.3 mmol/l), TC in men (5.8 mmol/l) and women (6.1 mmol/l), and fibrinogen (3.6 g/l in men and 4.0 g/l in women) were slightly above the normal cutoff values. With an expected correlation of these measures with age, a likely explanation for this observation is the study group represents a relatively older population as indicated by the mean age of the participants.

The results of genetic association of the 22 SNPs with the 18 traits are presented in Tables 3a and b. In Table 3a, we show the *P*-values of single-locus association tests with traits that are primarily associated with measures of obesity (Wt, BMI, WC, HC and WHR) and T2D (FPG, HbA1c and insulin). The most notable observations were with the SNPs in *FTO* and *TCF7L2*. All 8 *FTO* SNPs were individually associated with Wt, BMI, WC and HC; 20 of the 32 nominal *P*-values

Table 3b *P*-values of single-locus association test with metabolic traits of secondary involvement with obesity and T2D

SNP	*Gene*	*DBP*	*SBP*	*HDL*	*LDL*	*TC*	*TG*	*Fibrinogen*	*Calcium*	*Creatinine*	*Uric acid*
rs7566605	*INSIG2*	0.2291	0.7331	0.6855	0.8362	0.6363	0.7372	0.1060	0.1316	0.8093	0.9117
rs1801282	*PPARG*	0.1928	0.5681	0.2944	0.4522	0.3466	0.1923	0.5983	0.2750	0.3073	0.5802
rs10946398	*CDKAL1*	0.5244	0.2873	0.1381	0.9542	0.6557	0.7126	**0.0085**	0.7534	0.2626	0.4152
rs13266634	*SLC30A8*	0.7711	0.4559	0.6692	0.0740	**0.0473**	0.7669	0.6007	0.1229	0.5746	**0.0160**
rs564398	*CDKN2B*	0.6574	0.2279	0.2422	0.3836	0.3882	0.1684	0.5774	0.3860	0.7380	0.6599
rs10811661	*CDKN2A*	0.8407	0.1204	0.7581	0.2645	0.3898	0.7571	0.9392	0.4871	0.5388	0.9719
rs1111875	*HHEX/IDE*	0.9582	0.4485	0.1979	0.4477	0.2921	0.3319	0.4013	0.7220	0.3647	0.5799
rs7903146	*TCF7L2*	0.7694	0.8916	0.6502	0.5222	0.6610	0.9198	0.5471	0.1897	0.2615	0.9639
rs12255372	*TCF7L2*	0.9222	0.6293	0.5218	0.6823	0.7637	0.6648	0.2400	0.4415	0.1634	0.9961
rs9939973	*FTO*	0.1408	0.0927	**0.0202**	0.3841	0.4224	0.1599	**0.0369**	0.2461	0.3442	0.2037
rs1421085	*FTO*	0.1986	0.3867	**0.0228**	0.3840	0.4475	0.1721	0.0620	0.2077	0.5951	0.2503
rs1121980	*FTO*	0.2001	0.3761	**0.0425**	0.2936	0.4192	0.1627	0.1273	0.2662	0.6252	0.2009
rs17817449	*FTO*	0.2621	0.4452	**0.0482**	0.6007	0.7767	0.1453	0.0583	0.2830	0.6856	0.3106
rs8050136	*FTO*	0.1485	0.3353	**0.0379**	0.6076	0.8816	0.0583	0.0511	0.3911	0.7973	0.3517
rs3751812	*FTO*	0.2783	0.3822	0.0649	0.4587	0.6521	0.1799	0.0599	0.2399	0.7485	0.4303
rs9939609	*FTO*	0.3198	0.6684	**0.0303**	0.4098	0.7927	**0.0421**	0.0833	0.0530	0.5885	0.2899
rs7190492	*FTO*	0.0761	**0.0358**	0.0983	0.3679	0.2185	0.7813	0.0610	0.9017	0.3096	0.2108
rs17782313	*MC4R*	0.4253	0.3599	0.3851	0.4106	0.5881	0.4449	0.6862	0.7231	**0.0237**	0.1261
rs12970134	*MC4R*	0.7060	0.1162	0.3348	0.9171	0.9739	0.1056	0.5220	0.7346	0.1317	0.2670
rs477181	*MC4R*	0.9406	0.8286	0.8359	0.9309	0.9146	0.9021	0.3465	0.6542	0.5027	0.1809
rs502933	*MC4R*	0.9499	0.8887	0.6123	0.9957	0.9446	0.8991	0.2029	0.7798	0.4066	0.2404
rs4450508	*MC4R*	0.8625	0.7548	0.6526	0.8450	0.8617	0.9218	0.3814	0.7657	0.6040	0.5451

Empirical *P*-values (<0.05) are shown in bold.

remained significant after permutation testing. Two *FTO* SNPs (rs9939973 and rs1121980) were also nominally associated with insulin. The strongest associations were found between the two *TCF7L2* SNPs (rs7903146 and rs12255372) and FPG and HbA1c, with *P*-values (all $\leq 1\times10^{-5}$) remaining significant after the permutation tests. In addition, rs1801282 (*PPARG*), rs10811661 (*CDKN2A/B*) and rs1111875 (*HHEX/IDE*) were marginally associated with both FPG and HbA1c levels. The *HHEX/IDE* variant was also nominally associated with Wt and BMI.

Table 3b shows the results of single SNP associations with traits that are involved in the larger milieu of metabolic abnormalities and secondarily associated with obesity and T2D. Although the *P*-values are nominal, six *FTO* SNPs (rs9939973, rs1421085, rs1121980, rs17817449, rs8050136 and rs9939609) were associated with HDL; rs7190492 in *FTO* was associated with SBP; rs132266634 in *SLC30A8* was associated with TC; rs9939609 in *FTO* was associated with TG; rs10946398 in *CDKAL1* and rs9939973 in *FTO* were associated with fibrinogen; rs17782313 in *MC4R* was associated with creatinine; and rs13266634 in *SLC30A8* was associated with uric acid.

DISCUSSION

We sought to replicate associations of 22 GWAS variants in 10 gene regions with a series of metabolic traits in an isolated population of European descent. The published studies have provided both direct and indirect explanations of biological plausibility of these genes/regions (*INSIG2*, *PPARG*, *HHEX/IDE*, *CDKAL1*, *SLC30A8*, *CDKN2A*, *CDKN2B*, *TCF7L2*, *FTO* and *MC4R*) in the pathophysiology of carbohydrate intolerance and the maintenance of energy balance. Our study replicated the associations of *FTO* and *TCF7L2* GWAS SNPs with obesity and T2D-related traits, respectively. The *FTO* gene variants have shown consistent associations with obesity across several studies.[9–11,14,25–27] These studies primarily considered BMI as the measure of obesity. We extended the list of the phenotypic traits influencing obesity and found without exception, *FTO* variants were associated with each of the four obesity-related traits (Wt, BMI, WC and HC, with *P*-values ranging from 0.018 to 0.0004). We found clear replication of previously reported associations of two SNPs, rs3751821 and rs1421085, with Wt and BMI, respectively.[14,28] Overall, the consistent associations between the extended measures of obesity and all eight SNPs provide further evidence that *FTO* variants influence body fatness. In a recent study, we evaluated association of 29 SNPs on *FTO*, including the eight variants reported here, with a larger number of obesity-related phenotypic traits.[29] In addition to the body fatness measures (BMI, Wt, WC and HC), this study showed that *FTO* variants influence lean mass (eg, bicondilar upper arm width), suggesting pleiotropic effects of the FTO gene. Also, we found that the body fatness measures were highly correlated (all $r^2 > 0.8$),[29] which at least partially explain the consistent association of the obesity-related traits with *FTO* SNPs. However, precise relationships between *FTO* SNPs and each of the obesity-related traits are difficult to assert from these observations, as the genetic and environmental contributions to their correlations are unknown.

With respect to the risk of T2D, the strongest evidence for association has been found with variants on the *TCF7L2* gene with odds ratios >1.5 in multiple studies.[1,2,30–32] We genotyped two GWAS SNPs, each showing very strong associations with FPG (rs7903146, $P=1.29\times10^{-7}$; rs12255372, $P=9.62\times10^{-8}$) and HbA1c (rs7903146, $P=1.14\times10^{-9}$; rs12255372, $P=6.37\times10^{-9}$). We also performed a secondary analysis of all SNPs taking into consideration FPG and HbA1c as dichotomous traits being surrogates for T2D state (all data not shown). In the total sample of 843 individuals, 113 had FPG $\geq$126 mg per 100 ml and 100 subjects had HbA1c $\geq$6.5%, which are cut points for T2D according to the World Health Organization, which are also used as standards for the Croatian populations. We found highly significant associations of the *TCF7L2* SNPs with T2D status based on both FPG (rs7903146, $P=5.8\times10^{-5}$; rs12255372, $P=0.0001$) and HbA1c (rs7903146, $P=9.93\times10^{-6}$; rs12255372,

$P=3.33\times10^{-5}$), all *P*-values remained significant after corrections for multiple tests. In spite of the relatively smaller sample size of our study, these results reiterate the importance of genetic variation in *FTO* and *TCF7L2* influencing the risk of obesity and T2D in our study population.

We found effect size estimates of variants in *FTO* on measures of body fatness were comparable to those reported in previous GWAS, which reported various *FTO* SNP effect sizes between 0.33 and 1.45 kg/m^2 per risk allele.[9,14,25,28] For example, each copy of the C allele of the most significant *FTO* SNP rs1421085 was associated with an elevation of age and gender adjusted values of Wt (1.98 kg), HC (1.42 cm), WC (1.78 cm) and BMI (0.64 kg/m^2). Under the additive model, the variance explained by rs1421085 was 1.22% (Wt), 1.27% (HC), 1.49% (WC) and 1.29% (BMI). An example of this is presented graphically in Supplementary Figure 1 illustrating the comparability of effect sizes (in BMI units) between this study and previously reported GWAS based on rs1421085 and SNPs that are in strong linkage disequilibrium (LD) with this SNP in our population. The effect sizes obtained by Dina *et al*[10] are somewhat larger, which is likely because of their use of morbidly obese individuals in effect size calculations. Importantly, the most significant SNP in our study, rs1421085, overlaps the 95% confidence intervals of SNPs in high LD in the reported GWAS. Similarly, in *TCF7L2*, each copy of the T allele of rs12255372 corresponded with an elevation of age and gender adjusted values of FPG (0.39 mmol/l) and HbA1c (0.27%). The variance explained by the effect of rs12255372 was 4.4% (FPG) and 4.3% (HbA1c). As noted above, in GWAS *FTO* and *TCF7L2* have emerged as the most important genes influencing the risks for obesity and T2D and have been replicated in populations of diverse origins. Our results of association not only confirmed these findings, but were further augmented by higher effect sizes of the variants, which is likely attributable to a homogenous genetic background of the study population. It should also be noted that the eight *FTO* SNPs in our study population were in high LD, with average pairwise $r^2>0.8$ and the two *TCF7L2* SNPs were in near perfect LD ($r^2=0.92$).

The modest sample size of our study limited the power to detect signals of association of variants of small effect sizes or to provide confirmation of previously reported findings. On the basis of power calculations for 22 SNPs, however, we have sufficient power to detect SNPs with moderate effect size. For example, our study has 94.8% power to detect SNPs, which explain 1.5% variance at the nominal significance level ($\alpha=0.05$). Even using the multiple-testing adjusted significance level ($\alpha=0.05/14$, 14 is the estimated number of equivalent independent tests of the 22 SNPs based on permutation), the power is 74.8%. We found nominal replications of the SNPs on *PPARG* (rs1801282), *CDKN2A/B* (rs10811661) and *HHEX/IDE* (rs111875) with both FPG and HbA1c levels. Each of these three SNPs was previously associated with T2D risk in GWAS;[4,7] direction of associations in our study was consistent with these studies. Of note is the *PPARG* SNP rs1801282, which is the Pro12Ala coding variant and was found to be associated with decreased T2D risk.[33] We found the association of this SNP was in the same direction as was found by Altshuler *et al*;[33] the minor allele was protective against T2D when defined by HbA1c $\geq$6.5%. We did not find association of the variants in the two previously implicated T2D loci, *CDKAL1* and *SLC30A8*, with FPG, HbA1c or insulin levels. Our study failed to confirm the association between variants in *MC4R* and measures of body fatness. The *MC4R* SNPs were found to be associated with fat mass, Wt, WC, insulin resistance in previous GWAS.[12,13] We also did not find association of the *INSIG2* SNP, rs7566605, with any of the phenotypic traits. This was the first variant implicated in obesity in a GWAS.[8] However, its association remains largely inconclusive.[34] Failure to replicate these findings, whose effect sizes are small, could have stemmed from insufficient sample size. It should also be noted that the GWAS SNPs may not be the true trait or disease-specific variants, rather these could be in LD with the causal variants. Varied patterns of LD across populations owing to genetic diversity could mask the association of an index SNP with the trait in different populations. We report nominal associations of the GWAS variants with several other metabolic traits, which have not yet been explored in other studies. Therefore, these results need further verification in larger samples of varied ancestries.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

The study was conceived and designed by RD, PR and RC. Sample and data collection was conducted by NJ, DH-A, SM, NSN, ZD and PR. Genotyping was performed by RK, SRI and GS. Statistical analysis was performed by RK, GZ, WN and RC. The draft paper was prepared by RK, GZ, ZD, NSN, RC, PR and RD. All authors read and approved the final draft.

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