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Title: Replication confirms association of loci in *FOXE1*, *PDE8B*, *CAPZB* and *PDE10A* with thyroid traits: a GoDARTS study.

Running head: Loci associated with hypothyroidism and serum TSH

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ABSTRACT

Objective: Replication of associations in genome-wide association studies (GWAS) is desirable to ensure that such signals are potentially clinically meaningful. This study aimed to replicate associations of selected single-nucleotide polymorphisms (SNPs) with hypothyroidism and serum thyroid stimulating hormone (TSH) using electronic medical records (EMRs).

Methods: A cross-sectional study was done among patients of European Caucasian ethnicity from the Genetics of Diabetes Audit and Research Tayside (GoDARTS) recruited in Tayside (Scotland, UK). EMRs (biochemistry, prescribing, hospital admissions and demographics) were used to ascertain patients with hypothyroidism and their controls as well as average serum TSH concentration, and linked to genetic biobank data. Genetic tests of association were performed by means of logistic and linear regression models.

Results: We analysed 1,703 cases of hypothyroidism and 9,457 controls. All four SNPs located on chromosome 9 at *FOXE1* were associated with hypothyroidism with similar effect estimates (OR= 0.75- 0.76, $P < 5e-08$). Also loci on chromosomes 1 (PTPN22), 6 (HLA-E/HLA-C) and 12 (SH2B3) were replicated. For serum TSH we confirmed twelve SNPs previously reported at *PDE8B*, *CAPZB*, *PDE10A*, LOC105371356, *NR3C2*, *VEGFA*, *IGFBP5*, *INSR*, *PRDM11*, *NFIA*, *ITPK1*, and *ABO*. Overall, these SNPs accounted for 6.8% of the serum TSH variation ($P < 1e-04$).

Conclusions: EMRs linked to genomic data in large populations allows validation of GWAS discoveries without additional genotyping costs. Our replication confirmed at genome-wide significance the association of loci at *FOXE1* with hypothyroidism, and *PDE8B*, *CAPZB* and *PDE10A* with serum TSH. Twelve SNPs seemed to explain nearly 7% of the serum TSH variation.

Keywords: Single nucleotide polymorphism, thyroid disease/genetics, hypothyroidism, genome-wide association study, thyroid stimulating hormone.

INTRODUCTION

Hypothyroidism is a common thyroid disorder affecting about 3-5% of the general population (1, 2). Treatment includes thyroid hormone replacement therapy, and levothyroxine (thyroxine sodium) is the treatment of choice for maintenance. Patients usually respond well to treatment, and the dose is usually monitored in response to a combination of patients' symptoms and thyroid stimulating hormone (TSH) serum levels (3).

Genome-wide association studies (GWAS) have found association signals with hypothyroidism and serum TSH levels (4-6). However, replication studies are scarce and desirable to help ensure that a genotype-phenotype association observed in a GWAS represents a credible association. Replication of an association requires genotyping the initially discovered genetic variant in a completely independent sample of sufficient size (7).

Recently, electronic medical records (EMR)-derived phenotypes are being linked to genetic biobanks to allow research on the genetic basis of a wide range of traits highly cost effectively (8). Therefore such EMR-linked biobanks might be appropriate to investigate the role that genetics play in thyroid related disorders.

Using the Genetics of Diabetes and Audit Research Tayside Study (GoDARTS) dataset, we aimed to replicate the association of selected GWAS-identified loci with the diagnosis of hypothyroidism and with average serum TSH levels in a Scottish population.

METHODS

A cross-sectional study was done among patients from the GoDARTS population recruited in Tayside, Scotland (UK). All subjects in this population are of white ethnicity and have previously been described elsewhere (9). Each subject has a unique patient identification number (Community Health Index-CHI) which facilitates anonymous data linkage across all available EMRs by the Health Informatics Centre of the University of Dundee (<http://www.dundee.ac.uk/hic>). Records on redeemed drug prescriptions, demography and biochemistry, and several databases were linked to genetic data. Databases included the Scottish Care Information-Diabetes Collaboration- SCIDC, the Scottish Morbidity Records- SMR (hospital admissions and cancer registry), Radioiodine, and the Office of Population Censuses and Surveys Classification of Surgical Operations version 4- OPCS4.

Phenotype definition criteria

All patients with at least one serum TSH recording between 1994 and 2014 were considered for inclusion in this study. A phenotype of all-cause hypothyroidism (i.e. cases) was defined as having been issued at least two prescriptions for thyroid replacement therapy (British National Formulary codes-BNF 6.2.1) during the study period. Controls never received a prescription for thyroid replacement therapy or received just one.

Patients with history of thyroid cancer or probable hyperthyroidism were excluded. Probable hyperthyroidism was considered as having at least one OPCS4 code of treatment with thyroid surgery (OPCS4: B08, B09, B12), radioactive iodine and/or a prescription of anti-thyroid drug use (BNF 6.2.2). At least one International

Classification of Diseases (ICD) 9th-10th edition code (ICD9: 193; ICD10: C73, D093, D440) was required for thyroid cancer.

The TSH (average) was taken as the median of serum TSH measures recorded throughout the study period for each patient. Euthyroid subjects had a normal average TSH serum level (defined as 0.4 to 4.0 mIU/L) and never received thyroid medication.

Genetic data

Genotype data was available from the following platforms: the Human Exome -12 VI_A_chip, the Metabohip (10), Illumina HumanOmni Express -12VI platform (Illumina, San Diego), Affymetrix 6.0 platform (Affymetrix, Santa Clara) and the Illumina Infinium custom GWAS chip (Illumina, San Diego). Imputation was performed against 1000G Phase I V3 reference panel using Impute2 (11) and using the haplotype reference consortium (12); calls made with imputation quality below 90% were discarded. All SNPs were in Hardy-Weinberg equilibrium ($P < 10e-04$).

Selection of genetic loci to be tested

Association signals reported by latest powered GWAS on hypothyroidism (4, 5) and serum TSH levels (6).

Statistical analysis

ANOVA and chi-square tests were used to compare means and frequencies among groups of patients respectively, and nonparametric tests were used where appropriate(13). Single-locus tests of association with hypothyroidism (coded as binary) were performed on cases and controls by logistic regression under the

assumption of an additive genetic model. Odds ratios (OR) from logistic models were adjusted for age at first TSH recording and gender. Linear regression models were used on euthyroid subjects to test the association with average serum TSH levels and to estimate the proportion of variation of TSH explained by the SNPs. Fixed effects meta-analyses were performed to assess the consistency of effects of this study with the previously reported ones, and heterogeneity was quantified using the I-squared measure(14). Statistical analyses were conducted using STATA/SE version 13.1 software (StataCorp, TX, USA) and the statistical significance level set at $P < 5e-0.2$. The significance threshold was further adjusted with Bonferroni correction for the number of independent SNPs simultaneously tested.

Ethical approval

All analyses were performed on anonymised datasets. The study was approved by the Tayside Medical Ethics Committee, and informed consent had been obtained for all participants.

RESULTS

We identified 16,464 individuals as being eligible for the study, of which 2,484 had hypothyroidism (cases) and 13,980 did not (controls), and up to 11,160 (i.e. 1,703 cases and 9,457 controls) had available genomic data in the GoDARTS biobank. Compared to controls, cases were mostly women (73 vs 43.4%, $P < 1e-03$), had a higher average serum TSH level (2.2 vs 1.7 mIU/L, $P < 1e-03$), but there was no difference in age (57 years).

Table 1 shows the adjusted ORs for SNPs associated with hypothyroidism that were identified through published GWAS. All four SNPs located on chromosome 9 at *FOXE1* (rs965513, rs10759944, rs925489 and rs7850258) were associated with hypothyroidism at $P < 5e-08$ with similar effect estimates (OR= 0.75- 0.76). These four SNPs were in strong linkage disequilibrium ($r^2 > 0.99$). An additional fifth polymorphism near *FOXE1* (rs1877432) did not reach statistical significance. We did not find an association for several SNPs located on the same chromosome at *DFNB31* (rs1535971, rs4979402, rs4979397 and rs1408528). In addition to the associations found on chromosome 9 at *FOXE1*, we found significant associations on chromosome 1 at *PTPN22* (rs6679677 and rs2476601; $P < 1.9e-03$).

Within the control group there were 9,125 euthyroid patients (i.e. 96% of the controls) aged 57.4 years, 43.4% were women, and had an average serum TSH level of 1.72 mIU/L. For serum TSH concentration in this subgroup (table 2), we confirmed twelve out of twenty statistically significant SNPs previously reported at *PDE8B*, *CAPZB*, *PDE10A*, *LOC105371356*, *NR3C2*, *VEGFA*, *IGFBP5*, *INSR*, *PRDM11*, *NFIA*, *ITPK1*, and *ABO*; the coded allele was associated with increased TSH in five of them (*PDE10A*, *LOC105371356*, *NR3C2*, *IGFBP5*, and *ABO*). Each copy of the coded allele of rs6885099 at *PDE8B* (Phosphodiesterase type 8B) was associated with a decrease of 0.13 mIU/L serum TSH. This SNP accounted for 1.46% of serum TSH variation, followed by signals in the capping protein- actin filament muscle Z-line β (*CAPZB*- rs10799824) and the phosphodiesterase type 10A (*PDE10A*- rs753760) that contributed to 0.74% and 0.71% of variation respectively. Further adjustment of regression models for age and gender did not change the size and direction of the effect estimates. When combined, the twelve significantly associated SNPs accounted

for 6.47% ($n=5,241$, $P<1e-04$) of the TSH variation, that increased to 6.83% after also including age and gender as predictors in the linear model, thus leaving 0.36% (i.e. 6.83- 6.47%) of the variation to age and gender. Male gender was associated with a lower serum TSH ($\beta= -0.052$, $P=1.2e-02$), and each additional year of life conferred an increase of 0.003 mIU/L serum TSH ($P=1.2e-04$).

The consistency of SNPs effects of this study with the previously reported studies was shown in figures 1 and 2. Heterogeneity was quantified using the I-squared measure, and a value of 0% (i.e. no observed heterogeneity) was obtained in almost all meta-analyses. Although no significant heterogeneity was detected, a value close to 50% was found in two of the meta-analyses (figure 1, *PTPN22*-rs2476601 on hypothyroidism; figure 2, *ABO*-rs657152 on average serum TSH).

DISCUSSION

This was a record-linkage study using electronic databases to validate novel genetic loci discovered in GWA studies with hypothyroidism and serum TSH levels in a Scottish Caucasian population from the GoDARTS database.

Phenotype definition is critical in this kind of studies. Patients that received one thyroid replacement therapy prescription were not excluded from the control group because did not differ from the rest of controls. There were 252 of these patients in the control group that had normal TSH values (1.730 mIU/L) similar to controls that never received replacement therapy (1.735 mIU/L). Besides, their inclusion could not result in underestimation of the genetic association effects because they represent less than 3% of the controls. Thus, we found no reason to exclude them.

Four SNPs in linkage disequilibrium at *FOXE1* (thyroid transcription factor 2) were replicated in this study with hypothyroidism, the strongest being rs965513. Loci in *HLA*, *SH2B3*, *PTPN22*, *CTLA4*, *PDE8B* and *VAV3* were also replicated. The association between *FOXE1* and hypothyroidism was first reported by Denny et al. in a GWAS of 1,317 cases and 5,053 controls identified from EMRs, and subsequently by Eriksson et al. in the largest GWAS to date of hypothyroidism of 3,736 cases and 35,546 controls of European ancestry (4, 5). Some of these genes have known immune functions (*HLA*, *SH2B3*, *PTPN22*, *CTLA4*, and *VAV3*) and others in thyroid function (*FOXE1*, *CAPZB* and *PDE8B*). Our associations consistently replicate the effect size and direction of these loci, despite slightly different phenotypes reported by others. Denny et al. searched EMRs for the presence of a thyroid replacement medication for at least three months and at least one ICD code for hypothyroidism. Eriksson et al. used self-administered web-based questionnaires for phenotype identification; cases were those that answered “yes” to hypothyroidism or to elevated TSH levels or to taking medication for hypothyroidism. We did not query ICD codes for phenotype definition as this would have missed the majority of cases with hypothyroidism that did not have a hospital admission.

Porcu et al. carried out an analysis of 26,420 subjects pooled from 18 cohorts to identify novel loci related to serum TSH (6). Prior to GWAS they had excluded those with thyroid pathologies, thyroid surgery and/or thyroid medication, as well as those with levels outside the normal TSH range. Overall, their 20 reported loci explained 5.6% of TSH variation in euthyroid subjects. Our study confirmed twelve of these reported associations, which combined accounted for a larger serum TSH variation

(6.5%). As expected, male gender was associated with a lower serum TSH (15). We acknowledge that the higher variance observed from fewer variants could be due to our smaller sample size (as compared to Porcu et al.). However, the allele frequency from our study (see table 2) did not differ from that reported by Porcu et al (6). It is plausible that since the causal variants have still not been confirmed through functional studies, that the Scottish Caucasian population shows a different LD structure between these common SNPs and the causal variants. However, given the lack of differences in TSH levels between the compared populations (i.e. GoDARTS of 1.73mIU/L and Porcu et al. of 1.78 mIU/L), this would be unlikely.

The meta-analyses of the association test results between significant SNPs and hypothyroidism or serum TSH observed across studies indicate that variability between estimated effects from this current study and the previously reported ones can be explained by chance only.

In summary, EMR-linked genomic data allowed replication of discovered genes associated with several traits without additional genotyping costs. Replication confirmed at genome-wide significance the association of loci in *FOXE1* with hypothyroidism, and *PDE8B*, *CAPZB* and *PDE10A* with serum TSH. The GoDARTS database seems to be appropriate to research on genetic associations of thyroid traits, and the identified set of SNPs explains nearly 7% of variation in serum TSH concentration.

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Author contributions

Enrique Soto-Pedre researched/analysed data and wrote the manuscript. Moneeza K. Siddiqui researched data and wrote the manuscript. Alex S. Doney, Colin N. Palmer and Ewan R. Pearson contributed to the discussion and reviewed/edited the manuscript. Graham P. Leese planned the study, researched data, contributed to the discussion and reviewed/edited the manuscript.

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FIGURE LEGENDS

Figure 1 Forest plot representing meta-analysis of the association test results between significant SNPs at Bonferroni threshold and hypothyroidism observed across GoDARTS, Denny et al.[4], and Eriksson et al.[5] studies. Results from logistic regression models are presented; odds ratios are per copy of the coded allele.

Figure 2 Forest plot representing meta-analysis of the association test results between significant SNPs at Bonferroni threshold and average serum TSH concentration observed across GoDARTS and Porcu et al.[6] studies. Results from linear regression models are presented; coefficients are per copy of the coded allele.

Figure 1

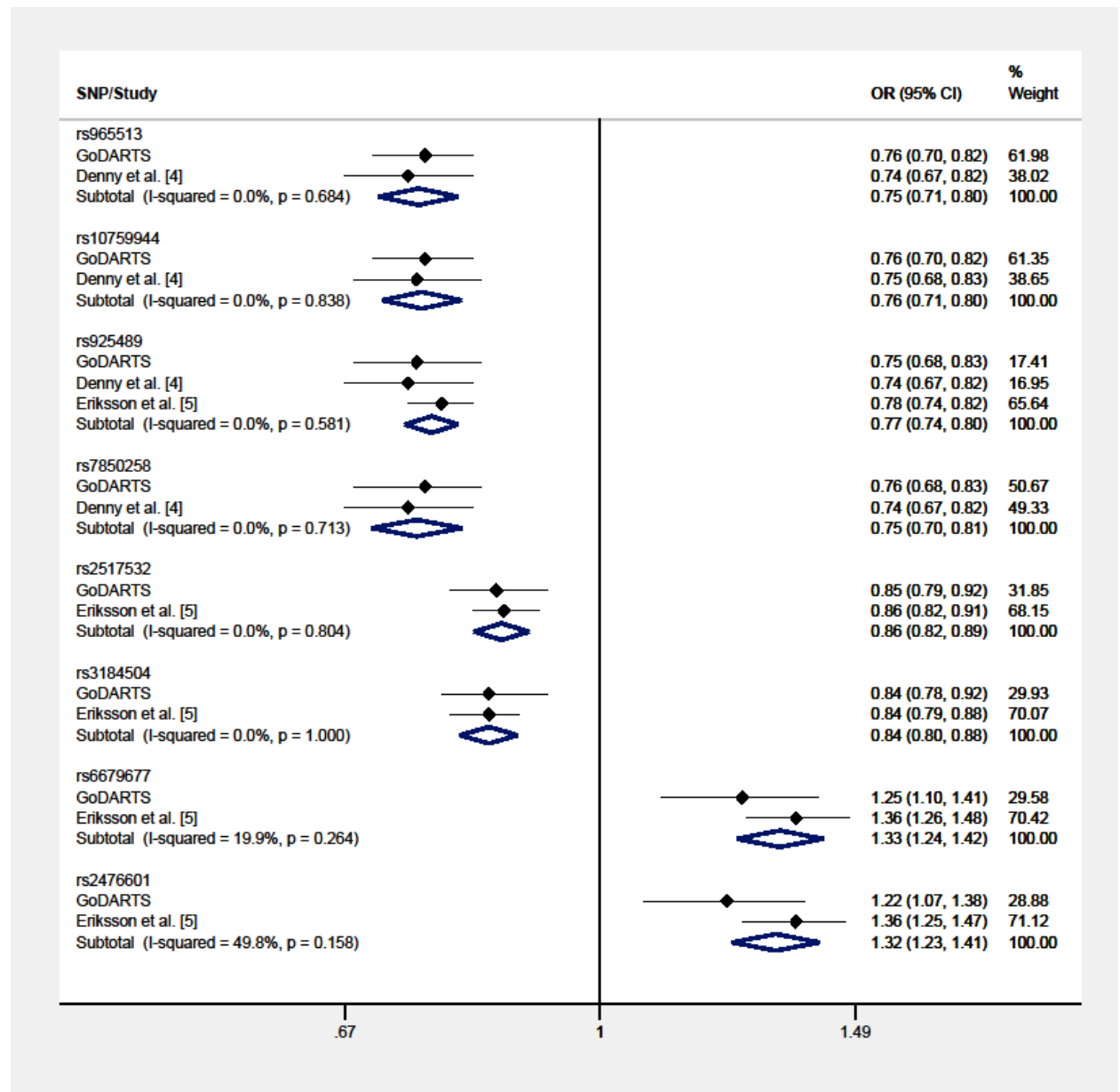


Figure 2

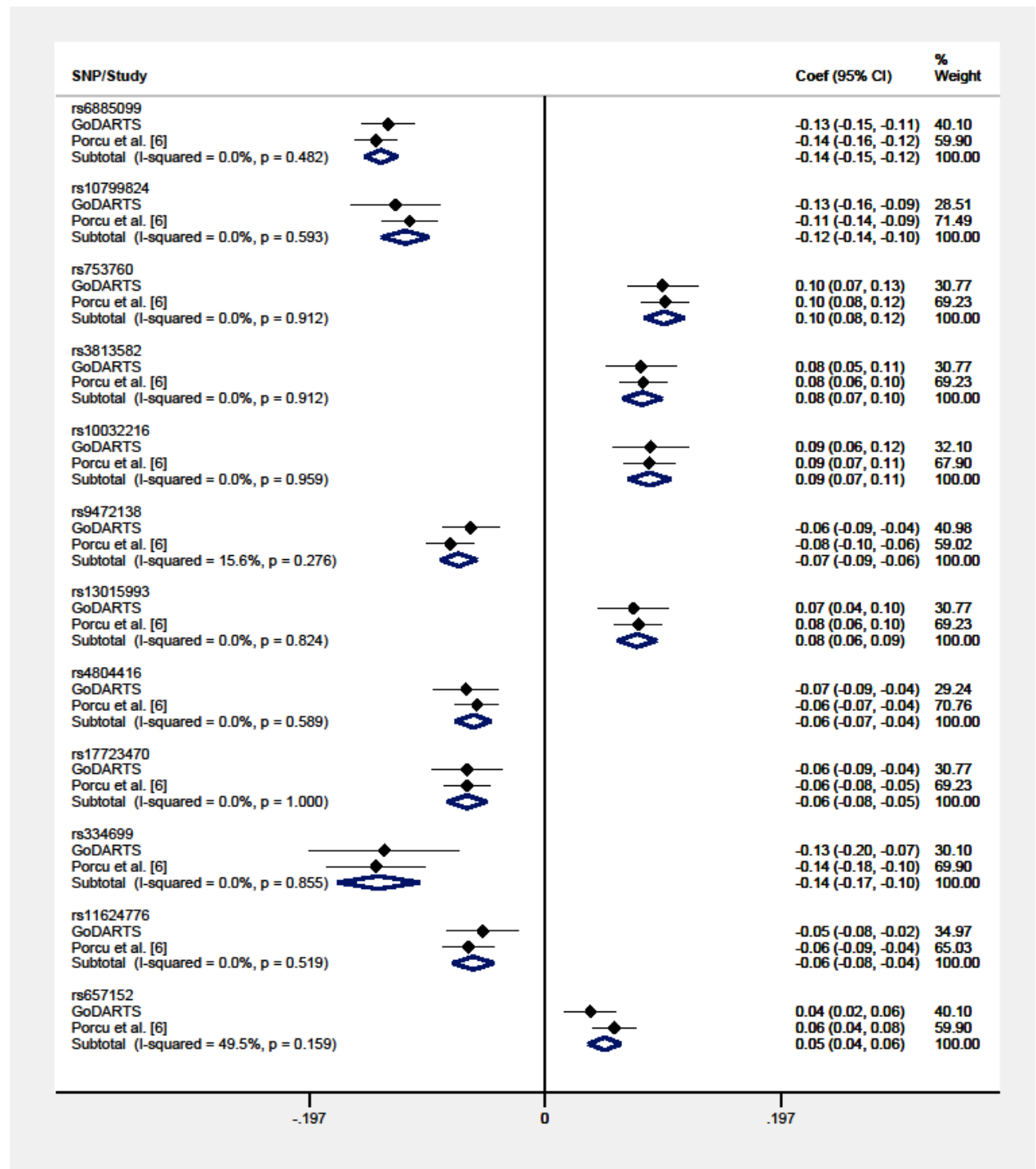


TABLE 1. SNPs associated with hypothyroidism.

Gene	SNP	CHR	Position	Coded allele	GoDARTS			OR [‡] (95%CI)	P	PRIOR ASSOCIATION	
					MAF	Cases [†]	Controls [†]			OR (95%CI)	P
<i>FOXE1</i>	rs965513	9	100556109	A	0.34	1,670	9,250	0.76 (0.70- 0.82)	7.6e-11*	0.74 (0.67- 0.82) ¹	4.1e-09
<i>FOXE1</i>	rs10759944	9	100556972	A	0.34	1,669	9,260	0.76 (0.70- 0.82)	1.0e-10*	0.75 (0.68- 0.83) ¹	8.1e-09
<i>FOXE1</i>	rs925489	9	100546600	C	0.34	1,212	5,937	0.75 (0.68- 0.83)	3.8e-08*	0.74 (0.67- 0.82) ¹	4.6e-09
										0.78 (0.74- 0.82) ²	2.4e-19
<i>FOXE1</i>	rs7850258	9	100549013	A	0.34	1,212	5,937	0.76 (0.68- 0.83)	4.7e-08*	0.74 (0.67- 0.82) ¹	3.9e-09
<i>HLA-E/HLA-C</i>	rs2517532	6	31018407	A	0.42	1,703	9,457	0.85 (0.79- 0.92)	7.5e-05*	0.86 (0.82- 0.91) ²	1.3e-08
<i>SH2B3</i>	rs3184504	12	111884608	C	0.49	1,410	7,573	0.84 (0.78- 0.92)	9.4e-05*	0.84 (0.79- 0.88) ²	2.6e-12
<i>PTPN22</i>	rs6679677	1	114303808	A	0.09	1,695	9,403	1.25 (1.10- 1.41)	3.0e-04*	1.36 (1.26- 1.48) ²	2.8e-13
<i>PTPN22</i>	rs2476601	1	114377568	A	0.09	1,662	9,281	1.22 (1.07- 1.38)	1.9e-03*	1.36 (1.25- 1.47) ²	3.9e-13
<i>CTLA4</i>	rs231779	2	204734487	T	0.40	1,208	5,908	1.15 (1.05- 1.26)	2.2e-03	1.12 (1.07- 1.19) ²	7.0e-06
<i>PDE8B</i>	rs4704397	5	76518442	A	0.37	1,212	5,937	1.14 (1.04- 1.25)	5.0e-03	1.17 (1.10- 1.26) ²	2.1e-06
<i>VAV3</i>	rs4915077	1	108366016	C	0.08	1,208	5,908	1.22 (1.05- 1.43)	9.9e-03	1.30 (1.20- 1.42) ²	7.5e-10
<i>near FOXE1</i>	rs1877432	9	100543880	A	0.39	1,212	5,937	1.08 (0.98- 1.18)	8.4e-02	1.25 (1.14- 1.37) ¹	1.9e-06
<i>PVTI</i>	rs4733792	8	128840276	T	0.41	1,212	5,937	0.95 (0.87- 1.05)	3.8e-01	1.18 (1.08- 1.30) ¹	2.3e-04
<i>PVTI</i>	rs4733789	8	128834403	C	0.41	1,212	5,937	0.95 (0.87- 1.05)	3.6e-01	1.17 (1.07- 1.28) ¹	5.5e-04
<i>CAPZB</i>	rs1472565	1	19755030	C	0.49	1,198	5,876	1.03 (0.94- 1.12)	5.2e-01	1.11 (1.05- 1.16) ²	6.3e-05
<i>DFNB31</i>	rs1535971	9	117229400	T	0.06	1,200	5,894	1.03 (0.92- 1.14)	5.5e-01	1.27 (1.15- 1.40) ¹	3.6e-06
<i>DFNB31</i>	rs4979402	9	117222675	G	0.24	1,202	5,904	1.02 (0.91- 1.13)	7.1e-01	1.29 (1.16- 1.42) ¹	1.2e-06
<i>closest to KIT</i>	rs17827152	4	55478686	A	0.23	1,171	5,742	0.97 (0.87- 1.09)	7.1e-01	1.28 (1.15- 1.42) ¹	3.2e-06
<i>HLA-DRB1</i>	rs2516049	6	32570400	C	0.32	1,018	5,099	1.01 (0.91- 1.12)	7.9e-01	1.15 (1.09- 1.21) ²	6.0e-07
<i>DFNB31</i>	rs4979397	9	117219888	T	0.24	1,203	5,891	1.01 (0.91- 1.12)	8.1e-01	1.28 (1.16- 1.42) ¹	1.9e-06
<i>DFNB31</i>	rs1408528	9	117220773	C	0.24	1,203	5,891	1.01 (0.91- 1.12)	8.1e-01	1.28 (1.16- 1.42) ¹	2.0e-06
<i>SHQ1</i>	rs17043990	3	72880470	C	0.01	1,212	5,937	1.12 (0.31- 4.03)	8.6e-01	3.71 (2.08- 6.60) ¹	8.3e-06
<i>CCBE1</i>	rs1791303	18	57455226	T	0.40	1,167	5,690	1.00 (0.91- 1.10)	9.6e-01	0.83 (0.75- 0.91) ¹	6.7e-04
<i>CCBE1</i>	rs4940904	18	57455061	T	0.40	1,212	5,937	1.00 (0.91- 1.09)	9.9e-01	0.83 (0.76- 0.91) ¹	1.1e-04
<i>TBLIX</i>	rs17280788	X	--	T	--	(Not available)		--	--	1.14 (1.02- 1.28) ¹	1.9e-02

CHR= chromosome. MAF= Minor allele frequency. SNP= single-nucleotide polymorphism. (φ) Logistic regression models adjusted for age and gender. (†) Number of subjects used for the association test. (*) SNPs reaching the Bonferroni significance threshold at $\alpha=0.05/24= 2.1e-03$.

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(2) Eriksson N, Tung JY, Kiefer A, et al. PLoS One 2012; 7(4): e34442.

TABLE 2. SNPs associated with average TSH levels in euthyroid subjects.

Gene	SNP	CHR	Position	Coded allele	GoDARTS					PRIOR ASSOCIATION	
					Freq.	N [†]	β (SE) ^φ	P	% Var.	β (SE) ¹	P
<i>PDE8B</i>	rs6885099	5	76530349	A	0.61	8,896	-0.131 (0.011)	2.3e-30*	1.46	-0.141 (0.009)	1.9e-56
<i>CAPZB</i>	rs10799824	1	19841174	A	0.15	5,710	-0.125 (0.019)	6.6e-11*	0.74	-0.113 (0.012)	3.6e-21
<i>PDE10A</i>	rs753760	6	166046483	C	0.69	5,637	0.098 (0.015)	2.1e-10*	0.71	0.100 (0.010)	1.2e-24
<i>LOC105371356</i>	rs3813582	16	79749353	T	0.69	5,711	0.080 (0.015)	1.5e-07*	0.48	0.082 (0.010)	8.4e-18
<i>NR3C2</i>	rs10032216	4	149669506	T	0.79	5,714	0.088 (0.016)	2.1e-07*	0.47	0.087 (0.011)	9.2e-16
<i>VEGFA</i>	rs9472138	6	43811762	T	0.30	9,125	-0.062 (0.012)	2.2e-07*	0.29	-0.079 (0.010)	6.7e-16
<i>IGFBP5</i>	rs13015993	2	217625523	A	0.73	5,715	0.074 (0.015)	2.0e-06*	0.39	0.078 (0.010)	3.2e-15
<i>INSR</i>	rs4804416	19	7223848	T	0.57	5,715	-0.066 (0.014)	3.5e-06*	0.38	-0.057 (0.009)	3.1e-10
<i>PRDM11</i>	rs17723470	11	45227567	T	0.30	5,467	-0.065 (0.015)	2.7e-05*	0.32	-0.065 (0.010)	8.8e-11
<i>NFIA</i>	rs334699	1	61620496	A	0.05	5,704	-0.134 (0.032)	3.2e-05*	0.30	-0.141 (0.021)	5.4e-12
<i>ITPK1</i>	rs11624776	14	93595591	A	0.68	5,597	-0.052 (0.015)	5.6e-04*	0.21	-0.064 (0.011)	1.7e-09
<i>ABO</i>	rs657152	9	136139265	A	0.31	9,125	0.038 (0.011)	1.3e-03*	0.13	0.058 (0.009)	4.1e-10
<i>NRG1</i>	rs7825175	8	32416274	A	0.20	5,715	-0.052 (0.017)	2.9e-03	0.16	-0.066 (0.011)	2.9e-09
<i>MIR1179</i>	rs17776563	15	89119104	A	0.32	5,639	-0.037 (0.015)	1.3e-02	0.11	-0.060 (0.010)	2.8e-10
<i>VEGFA</i>	rs11755845	6	43904780	T	0.24	5,715	-0.039 (0.016)	1.7e-02	0.10	-0.065 (0.010)	1.6e-10
<i>GLIS3</i>	rs1571583	9	4267209	A	0.21	8,880	0.023 (0.013)	9.2e-02	0.03	0.057 (0.010)	2.5e-08
<i>SOX9</i>	rs9915657	17	70127536	T	0.51	5,528	-0.016 (0.011)	2.7e-01	0.02	-0.064 (0.009)	7.5e-13
<i>SASH1</i>	rs9497965	6	148521292	T	0.40	5,243	0.011 (0.010)	4.5e-01	0.01	0.051 (0.009)	2.2e-08
<i>FGF7</i>	rs10519227	15	49746364	A	0.24	5,619	-0.008 (0.012)	5.7e-01	0.01	-0.072 (0.011)	1.0e-11
<i>MBIP</i>	rs1537424	14	36574018	T	0.59	5,715	-0.005 (0.014)	7.4e-01	0.01	-0.052 (0.009)	1.1e-08

CHR= chromosome. Freq= Coded allele frequency. SE= standard error. SNP= single-nucleotide polymorphism. TSH= thyroid stimulating hormone. %Var= Percentage of variation within the phenotype explained by the polymorphism. (φ) Univariate linear regression models; β=effect size (mIU/L). (†) Number of subjects used for the association test. (*) SNPs reaching the Bonferroni significance threshold at $\alpha=0.05/20= 2.5e-03$

(1) Porcu E, Medici M, Pistis G, et al. PLoS Genetics 2013; 9(2): e1003266.