Identifikacija genetskih i okolišnih čimbenika uključenih u regulaciju funkcije štitne i doštitne žlijezde

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IDENTIFIKACIJA GENETSKIH I OKOLIŠNIH ČIMBENIKA UKLJUČENIH U REGULACIJU FUNKCIJE ŠTITNE I DOŠTITNE ŽLIJEZDE

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1. SADRŽAJ

2. POPIS OZNAKA I KRATICA

AITD	Autoimune bolesti štitnjače (engl. *autoimmune thyroid disease*)
BMI	Indeks tjelesne mase (engl. *body mass index*)
DLL1	Delta Like Canonical Notch Ligand 1
DPP10	Dipeptidyl Peptidase Like 10
GD	Gravesova bolest (engl. *Graves' disease*)
GRIN3A	Glutamate Ionotropic Receptor NMDA Type Subunit 3A
GWAS	Cjelogenomska analiza povezanosti (engl. *genome-wide association study)*
HDL	Lipoproteini visoke gustoće (engl. *high-density lipoprotein*)
HT	Hashimotov tireoiditis
IL-6	Interleukin 6
LD	Neravnoteža vezanosti gena (engl. *linkage disequlibrium*)
LDL	Lipoproteini niske gustoće (engl. *low-density lipoprotein*)
MANOVA	Multivarijantna analiza varijance
OR	Omjer izgleda (engl. *odds ratio*)
PTH	Paratireoidni hormon
PUFA	Polinezasićene masne kiseline (engl. *polysaturated fatty acids*)
RASGEF1B	RasGEF Domain Family Member 1B
SFA	Zasićene masne kiseline (engl. *saturated fatty acids*)
SLE	Sistemski eritemski lupus (engl. *systemic lupus erythematosus*)
T1D	Dijabetes tipa 1
T3	Trijodtironin
T4	Tiroksin
Tg	Tireoglobulin
Tg-At	Protutijela protiv tireoglobulina
TPO	Štitna peroksidaza
TPO-At	Protutijela protiv štitne peroksidaze

3. PREGLED OBJEDINJENIH RADOVA

Ova doktorska disertacija temelji se na trima objedinjenim znanstvenim radovima:

1. Matana A, Brdar D, Torlak V, Boutin T, Popović M, Gunjača I, Kolčić I, Boraska Perica V, Punda A, Polašek O, Barbalić M, Hayward C, Zemunik T. Genome-wide meta-analysis identifies novel loci associated with parathyroid hormone level. Mol Med. 2018: 24: 15.
   Indeksiran u WoS-u i CC-u, IF (za 2016.): 3,457

2. Matana A, Popović M, Boutin T, Torlak V, Brdar D, Gunjača I, Kolčić I, Boraska Perica V, Punda A, Polašek O, Hayward C, Barbalić M, Zemunik T. Genome-wide meta-analysis identifies novel gender specific loci associated with thyroid antibodies level in Croatians.
   Indeksiran u WoS-u i CC-u, IF (za 2016.): 2,801

3. Matana A, Torlak V, Brdar D, Popović M, Lozić B, Barbalić M, Perica VB, Punda A, Polašek O, Hayward C, Zemunik T. Dietary Factors Associated with Plasma Thyroid Peroxidase and Thyroglobulin Antibodies. Nutrients. 2017: 28;9(11).
   Indeksiran u WoS-u, IF (za 2016.): 3,550

3. 1. UVOD

3.1.1. Paratireoidni hormon (PTH), protutijela protiv štitne peroksidaze (TPO-At) i tireoglobulina (Tg-At)

Hormon doštitne žlijezde (paratireoidni hormon, PTH) je najvažniji čimbenik u regulaciji izvanstanične koncentracije kalcija (1). Balans kalcija u krvi od izuzetne je važnosti za normalno funkcioniranje srca, živčanog sustava, bubrega i kostiju (2). Metabolizam kalcija reguliran je putem tri glavna mehanizma: koštanih promjena, bubrežne reapsorpcije i intestinalne apsorpcije (3). Paratireoidne stanice osjetljive su na promjenu koncentracije kalcija u krvi, te reagiraju na pad koncentracije kalcija pojačanim izlučivanjem PTH i obrnuto (4). PTH stimulira aktivnosti osteoklasta (stanica koje su odgovorne za razgradnju koštane mase u kostima), što dovodi do oslobađanja kalcija u izvanstaničnu tekućinu. Nadalje, potiče reapsorpciju kalcija u završnim distalnim bubrežnim kanalićima i početnim sabirnim cijevima te u manjoj mjeri i u uzlaznom kraku Henleove petlje, što uzrokuje smanjeno izlučivanje kalcija. Također, PTH ima neizravan učinak na apsorpciju kalcija u crijevima tako što povećava crijevnu apsorpciju kalcija induciranjem sinteze aktivnog metabolita vitamina D ($1,25(OH)_2D_3$) u proksimalnim bubrežnim kanalićima, koji regulira apsorpciju kalcija u gastrointestinalnom traktu (5-7).

Tireoidna peroksidaza (TPO) i tireoglobulin (Tg) su glavne sastavnice štitne žlijezde koje igraju ključnu ulogu u sintezi hormona štitne žlijezde, tiroksina (T4) i trijodtironina (T3) (8). Tg jest veliki glikoprotein (660 kDa) koji sadržava aminokiseline tirozina koje se vežu s jodom u stvaranju hormona štitnjače (9). Unutarstanični jod se oksidira uz pomoć enzima TPO, kako bi se mogao izravno vezati na tirozinske ostatke molekule tireoglobulina. Kad je sustav peroksidaze zakočen zaustavlja se stvaranje hormona štitnjače (10).

Autoimune bolesti štitnjače (engl. *autoimmune thyroid disease*, AITD) spadaju u najčešće autoimune bolesti te pogađaju 2-5% generalne populacije (11). Povišena razina protutijela protiv TPO (TPO-At) i/ili Tg (TG-At) može predstavljati ranu fazu u patogenezi AITD (12). Najčešće autoimune bolesti štitne žlijezde su Hashimotov tireoiditis (HT) i Gravesova bolest (engl. *Graves' disease*, GD). HT se manifestira destrukcijom tkiva štitnjače i smanjenom proizvodnjom hormona štitne žlijezde (hipotireoza), dok GD karakterizira prekomjerna proizvodnja hormona štitne žlijezde (hipertireoza) (13, 14). Pozitivni nalaz TG-At prisutan je kod 60-80% osoba oboljelih od HT, te kod 30-60% osoba oboljelih od GD, dok

su povišena TPO-At prisutna kod 90-95% osoba oboljelih od HT i kod 80% osoba oboljelih od GD (15).

Razine hormona doštitne i štitne žlijezde te protutijela protiv štitne žlijezde smatraju se složenim fenotipovima koji su rezultat interakcije između multiplih gena s malim ili umjerenim učinkom i jednako važnih čimbenika okoliša.

3.1.2. Genetski čimbenici

Prema provedenim blizanačkim studijama, procjenjuje se da 60% varijacije u razini PTH određuju genetski čimbenici (16), dok postotak varijacije u razinama protutijela protiv štitne žlijezde koji se može pripisati genetskim čimbenicima ovisi o spolu. Pokazano je da je 39% varijacije u serumskoj razini Tg-At kod muškaraca i 75% kod žena, te 61% u serumskoj razini TPO-At kod muškaraca i 72% kod žena genetički uvjetovano (17). Preostali postotak u varijaciji se može pripisati okolišnim čimbenicima.

Kako bi se definirali genetski čimbenici povezani s određenim svojstvom ili bolesti u novije vrijeme provode se cjelogenomske analize povezanosti (engl. *genome wide association studies*, GWAS). Najvažnija prednost ovog pristupa jest ta što se provodi bez unaprijed postavljene hipoteze o povezanosti određenog gena i istraživanog svojstva, već se istovremeno testira i do nekoliko milijuna genetskih polimorfizama (18). Cjelogenomskim analizama povezanosti otkriveni su brojni genetski polimorfizmi u podlozi različitih bolesti i kvantitativnih obilježja.

Osim naše studije (Rad 1), do sada je publicirana samo jedna cjelogenomska analiza povezanosti za serumsku razinu PTH, u kojoj je identificirano pet genetskih lokusa koji su povezani s razinom PTH (19). Identificirani genetski lokusi smješteni su u blizini gena *CYP24A1, CLDN14, RTDR1* i *CaSR* te unutar gena *RGS14*. Međutim, zajednički učinak navedenih pet genetskih lokusa objašnjava samo 4,2% varijacije u razini PTH, što upućuje na zaključak kako postoje mnogi drugi polimorfizmi koji još uvijek nisu otkriveni, a ukupno značajno doprinose nasljednoj komponenti za vrijednosti PTH. Štoviše, od navedenih pet polimorfizama samo su tri potvrđena u neovisnoj replikacijskoj kohorti (*CYP24A1, RGS14, CLDN14* lokusi) (19).

Do trenutka publiciranja naše studije (Rad 2) nije bila objavljena niti jedna cjelogenomska analiza povezanosti za Tg-At, te su bile publicirane samo dvije cjelogenomske analize povezanosti za TPO-At, od kojih je jedna provedena kod bijelaca (20) a druga kod Azijata (21). Također, publicirana je i studija (22) u kojoj je testirana povezanost 20 genetskih polimorfizama koji su u prije objavljenoj cjelogenomskoj analizi (20) dosegli sugestivnu

razinu značajnosti za povezanost s razinom TPO-At. U navedenim studijama identificirano je devet genetskih polimorfizama povezanih s razinom i/ili pozitivnim nalazom TPO-At, uključujući varijante u blizini gena *TPO, HCP5, HLA-DPB1* te unutar gena *ATXN2, MAG13, KALRN, BACH2, RERE, HLA-DOB*. Zajednički učinak navedenih devet genetskih polimorfizama objašnjava 4,1% varijacije u razini TPO-At (22). Iz navedenog proizlazi nužnost dodatnih genetičkih istraživanja koja će doprinijeti otkrivanju pretpostavljenih čestih varijanti koje utječu na razine PTH, Tg-At i TPO-At.

3.1.3. Okolišni čimbenici

Osim genetskih čimbenika, ističe se važnost utjecaja okolišnih čimbenika na razine hormona doštitne i štitne žlijezde te protutijela protiv štitne žlijezde. Istraživanja su pokazala da žene imaju višu razinu PTH od muškaraca, te da s dobi raste i razina PTH (23). Nadalje, pokazano je da razina PTH varira ovisno i o rasi; naime Afroamerikanci imaju veću razinu hormona od pripadnika bijele rase (23, 24). Također je pokazano da pušači imaju nižu razinu PTH od nepušača, te da osobe s većim indeksom tjelesne mase (engl. *body mass index*, BMI) imaju višu razinu PTH (25). Viša razina PTH povezuje se i s višom serumskom razinom uričnе kiseline, nižom serumskom razinom kalcija, nižom serumskom razinom 25 (OH)D, višim sistoličkim i dijastoličkim krvnim tlakom (23, 26, 27), a vjerojatno postoji povezanost s još velikim brojem nedefiniranih okolišnih čimbenika.

Za nastanak protutijela protiv štitne žlijezde odgovoran je visok unos joda ili suvišak joda u organizmu (28-30). Od ostalih okolišnih čimbenika, pokazano je da pušenje pospješuje nastanak GD, dok nema utjecaja na razvoj HT (28). Prema istraživanjima, umjerena konzumacija alkohola ima dobrotvornu ulogu za razvoj GD i HT (28, 31). Niski unos selena te niska serumska razina vitamina D se povezuju s većim rizikom za razvoj AITD (28, 31). Stres bi također mogao biti rizični faktor za razvoj GD, dok utjecaj stresa na nastanak HT nije dovoljno istražen (28, 31). Unos estrogena djeluje protektivno na nastanak GD (28). Nadalje, pokazano je da virusne infekcije imaju ulogu u nastanku GD i HT, kao i upotreba različitih lijekova (28, 31). Što se tiče prehrambenih navika, provedene su samo dvije studije u kojima je utvrđena protektivna uloga veganske prehrane na razvoj hipotireoze, te protektivna uloga veganske i vegetarijanske prehrane na razvoj hipertireoze (32, 33). Nedostatak navedene dvije studije jest taj što hipotireoza i hipertireoza nisu klinički dijagnosticirane, već su ispitanici samostalno popunjavali upitnik u kojem su naveli boluju li od spomenutih bolesti štitne žlijezde.

3.1.4. Ciljevi istraživanja

Potaknuti prethodno navedenim, osnovni cilj ovog istraživanja bio je cjelogenomskim analizama povezanosti identificirati nove genetske čimbenike povezane s razinama PTH, Tg-At i TPO-At u plazmi. S obzirom da postoje evidentne kliničke razlike u razinama PTH, TPO-At i Tg-At kod muškaraca i kod žena, dodatni cilj ovog istraživanja bio je ispitati postoje li razlike u genetskoj regulaciji ovih fenotipova kod muškaraca i žena.

Drugi cilj istraživanja bio je ispitati povezanost prehrambenih navika, kao okolišnog čimbenika, s povišenim razinama protutijela protiv Tg i TPO.

3.2. PREGLED METODOLOGIJE OBJEDINJENIH RADOVA

3.2.1. Ispitanici

Istraživanje se provelo na ispitanicima koji su uključeni u projekt „10 001 Dalmatinac – Hrvatska biobanka“ (34). U genetičkom dijelu istraživanju inicijalno je sudjelovalo 2 869 ispitanika s područja grada Splita te otoka Korčule i Visa, dok je u istraživanju povezanosti prehrambenih navika s protutijelima Tg-At i TPO-At sudjelovalo 1887 ispitanika s područja grada Splita te otoka Korčule. U istraživanje su bili uključeni punoljetni ispitanici koji su dobrovoljno potpisali informirani pristanak nakon upoznavanja s ciljevima istraživanja.

Kako bi se eliminirao nepoželjni utjecaj određenih faktora na rezultate, primijenjeni su dolje navedeni kriteriji isključenja. U cjelogenomskoj analizi povezanosti za PTH, iz istraživanja su isključeni ispitanici koji su liječeni zbog poremećaja doštitne žlijezde, kao i ispitanici čije su vrijednosti PTH u plazmi manje od 5 pg/ml što je blizu donje granice detekcije razine PTH pomoću korištenog kita (4.3 pg/ml). U cjelogenomskim analizama povezanosti za protutijela Tg-At i TPO-At, iz istraživanja su isključeni ispitanici koji boluju od neke bolesti štitne žlijezde, zatim ispitanici koji su pod terapijom zbog poremećaja štitne žlijezde te ispitanici koji su operirali štitnu žlijezdu. Nakon primjene kriterija isključenja, u cjelogenomskoj analizi povezanosti za PTH sudjelovalo je ukupno 2 596 ispitanika, za Tg-At 2 629 ispitanika, a za TPO-At 2 618 ispitanika. Istraživanje u kojem se ispitivala povezanost prehrambenih navika s pozitivnim protutijelima Tg-At i TPO-At dizajnirano je kao istraživanje slučajeva i kontrola (engl. *case-control study*). Skupinu slučajeva (N=462) činili su oni kod kojih je razina Tg-At i/ili TPO-At bila veća od referentnih vrijednosti, odnosno skupinu kontrola (N=1 425) oni kojima su razine Tg-At i TPO-At bile unutar referentnih vrijednosti. Iz studije su isključene kontrole koje su bile pod terapijom zbog poremećaja štitne žlijezde (N=27) ili su bile podvrgnute operaciji štitne žlijezde (N=10). Na posljetku, u istraživanje je bilo uključeno 462 ispitanika i 1388 kontrola.

3.2.2. Fenotipski podaci

Za svakog od ispitanika bila je dostupna sveobuhvatna baza fenotipskih podataka koja uključuje podatke koji su dobiveni anketnim upitnicima, antropološkim i biokemijskim mjerenjima te fizikalnim i kliničkim pregledima ispitanika.

3.2.2.1. Biokemijska mjerenja

Plazma za analizu dobivena je iz uzorka periferne krvi, te je nakon centrifugiranja i odvajanja krvnih stanica odmah pohranjena na -80°C. Mjerenje koncentracije PTH u plazmi ispitanika obavljeno je ručnom metodom radioimunoesej, a rezultat je dobiven mjerenjem na scintilacijskom brojaču Capintec (Ramsey, New Jersey, USA). Mjerenje koncentracije TPO-At i Tg-At u plazmi ispitanika obavljeno je imunoesej metodom kemiluminscencije korištenjem instrumenta LIAISON (DiaSorin, Saluggia, Italy).

Mjerenja su obavljena na zavodu za Nuklearnu medicinu u Kliničkom bolničkom centru Split. Referentne vrijednosti za razinu PTH u plazmi su 12,26-35,50 pg/ml, za Tg-At <16 IU/mL te za TPO-At 5-100 IU/mL.

3.2.2.2. Upitnik o prehrambenim navikama

Podaci o prehrambenim navikama za svakog ispitanika dobiveni su upitnikom koji je sadržavao pitanja o učestalosti konzumacije 54 namirnice. Na pitanje: „Koliko često konzumirate navedene namirnice?“ ispitanici su mogli izabrati jedan od ponuđenih odgovora: „svaki dan“, „2-3 puta tjedno“, „1 put tjedno“, „povremeno“ ili „nikada“. Upitnik je sadržavao i 4 dodatna pitanja o učestalosti konzumacije raznih vrsta masnoća, a ponuđeni odgovori su bili: „uvijek“, „ponekad“ i „nikad“. Za potrebe statističke analize, kategorijski odgovori o učestalosti konzumacije namirnica su pretvoreni u ekvivalentne tjedne unose na sljedeći način: kategorija „svaki dan“ pretvorena je u 7 puta tjedno, kategorija „2-3 puta tjedno“ u 2,5 puta tjedno, „povremeno“ u 0,5 puta tjedno i „nikada“ u 0 puta tjedno. Odgovori za konzumaciju raznih vrsta masnoća su također pretvoreni u tjedne unose: odgovor „uvijek“ je pretvoren u 7 puta tjedno, „ponekad“ u 2,5 puta tjedno i „nikada“ u 0 puta tjedno.

3.2.3. Genotipski podaci

3.2.3.1. Genotipizacija

Uzorci ispitanika s otoka Korčule kao i 531 ispitanika iz Splita (Split 1) genotipizirani su na genotipizacijskoj platformi HumanCNV370-Duo BeadChip (Illumina, San Diego, California, USA), kojom se dobiju podatci za 350 000 genetskih polimorfizama. Uzorak za preostalih 481 ispitanika iz Splita (Split 2) genotipiziran je na Illumina HumanOmniExpress BeadChip platformi (Illumina, San Diego, California, USA) koja obuhvaća 969 919 genetskih polimorfizama. Genotipizacija uzoraka ispitanika s otoka Visa napravljena je na

HumanHap300-Duo BeadChip platformi (Illumina, San Diego, California, USA) kojom se dobiju podatci za 317 509 polimorfnih varijanti.

Nakon genotipizacije, provedena je kontrola kvalitete genotipiziranih podataka. Iz daljnje analize su isključeni genetski polimorfizmi čija je frekvencija rjeđeg alela manja od 1%, zatim polimorfizmi koji ne zadovoljavaju Hardy-Weinbergovu jednadžbu, te polimorfizmi koji nisu uspješno genotipizirani kod barem 98% ispitanika. Nadalje, iz analize su izbačeni i ispitanici koji nisu uspješno genotipizirani na barem 97% genetskih polimorfizama.

3.2.3.2. Imputacija genotipova

Metoda imputacije genotipova (metoda kojom se s određenom vjerojatnošću predviđa genotip ispitanika za genetske polimorfizme koji nisu direktno genotipizirani) obavljena je na Sveučilištu u Edinburghu. Imputacija za uzroke ispitanika s otoka Korčule i Visa obavljena je korištenjem softvera IMPUTE2 prema bazi „1 000 Genoma“ (prema engl. „1 000 Genomes project“). Uzorci ispitanika sa splitskog područja su prikupljeni i genotipizirani u dva navrata (Split 1 i Split2). Split 2 je imputiran prema bazi „1 000 Genoma“, dok je Split 1 imputiran prema spojenom referentnom panelu „1 000 Genoma“ i Splita 2.

Nakon imputacije, provedena je kontrola kvalitete imputiranih podataka. Iz analize su isključeni genetski polimorfizmi čija je frekvencija rjeđeg alela manja od 1%, zatim polimorfizmi koji ne zadovoljavaju Hardy-Weinbergovu jednadžbu te polimorfizmi čiji je info score (parametar kvalitete imputacije) manji od 0.4.

Konačan broj genetskih polimorfizama uključenih u analizu bio je 9 182 797 za kohortu Korčula, 8 865 173 za kohortu Vis te 8 777 560 za kohortu Split. Broj genetskih polimorfizama koji su se preklapali u sve tri kohorte bio je 7 411 206.

3.2.4. Statistička analiza

3.2.4.1. Cjelogenomske analize povezanosti

Cjelogenomske analize povezanosti provedene su za svaku kohortu zasebno. Potom su meta-analizom objedinjeni rezultati pojedinačnih analiza za područja grada Splita, te otoka Korčule i Visa**.**

Prije provođenja cjelogenomskih analiza povezanosti razine PTH, Tg-At i TPO-At prilagođene su za utjecaj dobi i spola, odrednica za koje je pokazano da utječu na razinu istraživanih fenotipova. Prilagodba se provela metodom linearne regresije, u kojoj je zavisna

varijabla bila razina hormona/protutijela, dok su nezavisne varijable bile dob i spol. Potom su se dobiveni reziduali iz linearne regresije transformirali kako bi se postigla normalna distribucija, te su oni predstavljali novi fenotip koji se dalje analizirao.

Cjelogenomska analiza povezanosti provela se pomoću linearnog mješovitog modela (engl. *linear mixed model*), u kojem su transformirani reziduali zavisna varijabla, a genetski polimorfizmi prestavljaju nezavisnu varijablu, pri čemu se radi prilagodba za srodstvo ispitanika. Analize se provode korištenjem aditivnog genetičkog modela. Kako je već prije naglašeno, ovim pristupom se istovremeno testira nekoliko milijuna polimorfnih varijanti, te je zbog višestrukog testiranja potrebno prilagoditi granicu značajnosti. Zbog činjenice da je milijun genetskih polimorfizama nezavisno, za granicu značajnosti uzima se 5×10^{-8} ($0.05/10^6$). Cjelogenomske analize povezanosti za kohortu Split provele su se pomoću računalnog programa SNPTEST (35), dok su se za kohorte Korčulu i Vis provele pomoću paketa 'GenABEL' (36) i 'VariABEL' (37) programa R.

S obzirom da postoje razlike u razinama PTH, Tg-At i TPO-At kod muškaraca i kod žena, cjelogenomske analize provedene su i za svaki spol zasebno. Cjelogenomske analize povezanosti po spolu provedene su na isti način kao i primarne cjelogenomske analize, jedina iznimka je što se u ovim analizama fenotip prilagodio samo za godine, a ne i spol.

3.2.4.2. Meta-analize

Meta-analiza cjelogenomskih analiza povezanosti, koje su provedene za svaku od triju kohorti posebno, provela se prema metodi fiksnih učinaka također korištenjem programa R (38).

Rezultati meta-analiza su grafički prikazani pomoću Manhattan (engl. *Manhattan plot*) i kvantil-kvantil grafova (engl. *quantile-quantile plot*, QQ plot) koji su kreirani korištenjem paketa 'qqman' programa R (39). Regionalni grafovi povezanosti za polimorfizme od interesa (engl. *regional association plot*) generirani su korištenjem programa 'Locus Zoom' (40), dok je grafikon raspona pouzdanosti (engl. *forest plot*) generiran korištenjem paketa 'MultiABEL' programa R (41).

3.2.4.3. Bivarijantna analiza

Za Tg-At i TPO-At, svojstva u umjereno visokoj korelaciji (r=0.5-0.7), dodatno je provedena i bivarijantna cjelogenomska analiza u kojoj je ispitana povezanost genetskih polimorfizama s linearnom kombinacijom protutijela protiv Tg-At i TPO-At. Povezanost je

testirana multivarijantnom analizom varijance (MANOVA). Osim na cjelokupnom uzorku, bivarijantna cjelogenomska analiza povezanosti provedena je i na uzorku žena (r=0,6-0,7), dok korelacija između razina Tg-At i TPO-At kod muškaraca nije bila dovoljno visoka da bi se provela ovakva vrsta analize (r=0,3). Bivarijantne cjelogenomske analize provedene su pomoću paketa 'MultiABEL' programa R (41).

3.2.4.4. Prehrambene namirnice povezane s Tg-AT i TPO-AT

Kako bi se ispitala međuovisnost učestalosti konzumacije ukupno 58 namirnica, te kako bi se broj početnih varijabli smanjio na manji broj ključnih faktora, provedena je faktorska analiza. Faktori su rotirani ortogonalnom rotacijom (varimax metoda) kako bi se povećala interpretabilnost rješenja. Pogodnost korištenja faktorske analize na danim podacima testirana je pomoću Kaiserove mjere za adekvatnost uzorkovanja, te pomoću Bartlettovog testa za značajnost broja faktora. Svi faktori sa svojstvenom vrijednošću većom od 1 su zadržani za daljnju analizu.

Povezanost faktora s pozitivnim nalazom protutijela protiv štitne žlijezde testirana je pomoću logističke regresije. Nezavisne varijable koje su bile uključene u logističku regresiju su spol i prehrambeni faktori, dok je zavisna varijabla bila pozitivnost TPO-At i/ili Tg-At. Sve statističke analize provedene su u programu SPSS (prema engl. Statistical Package for the Social Sciences 16,0; Chicago, Illinois, SAD).

3.3. PREGLED REZULTATA OBJEDINJENIH RADOVA

3.3.1. Cjelogenomska analiza povezanosti za razinu PTH

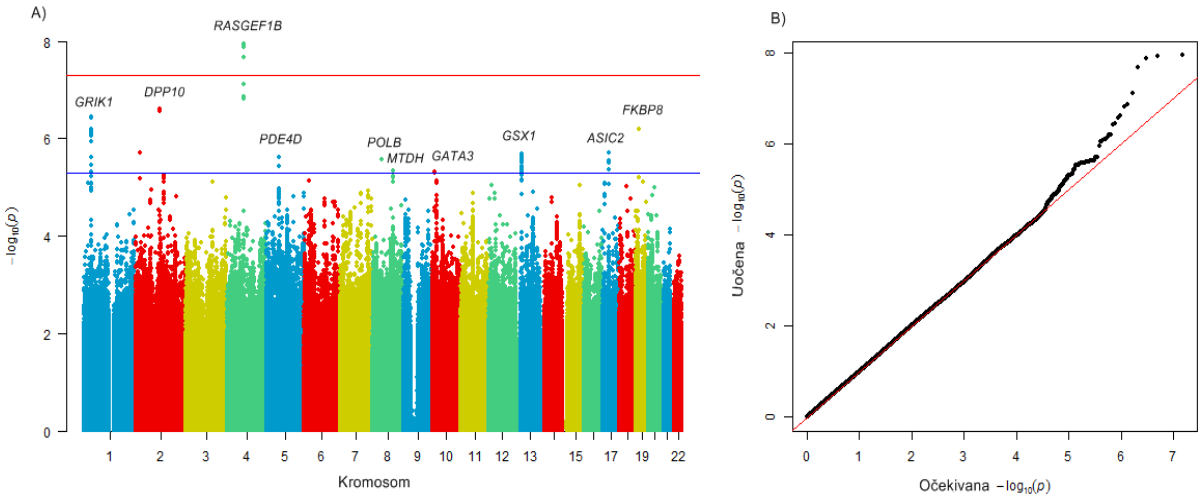
Nakon primjene kriterija isključenja, u istraživanju je sudjelovalo ukupno 2 596 ispitanika čije su karakteristike prikazane u Tablici 1.

Tablica 1. Karakteristike ispitanika uključenih u studiju.

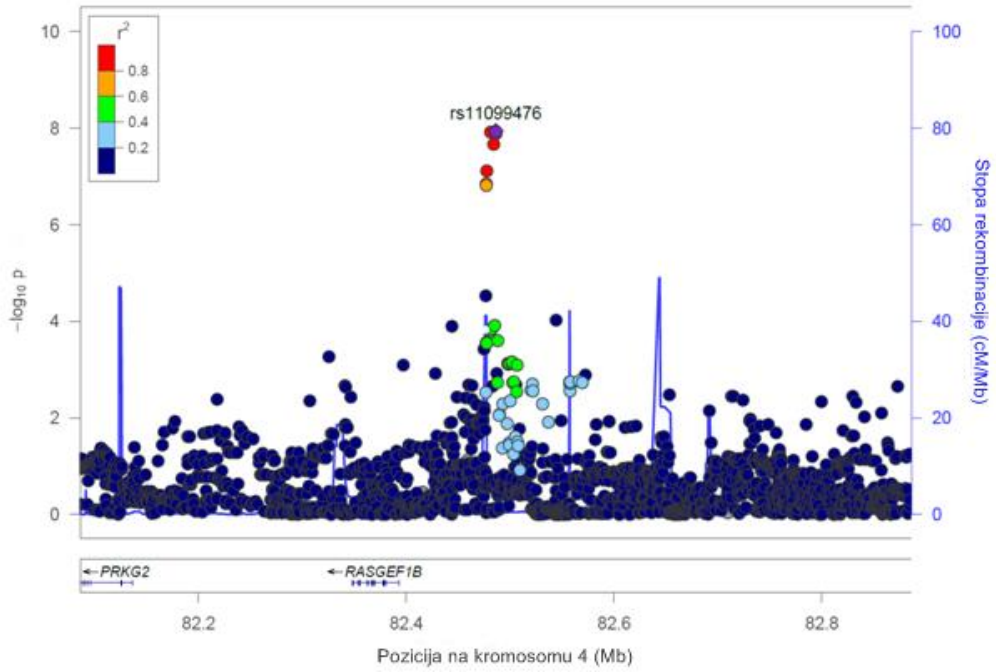
Karakteristike	**Korčula**	**Vis**	**Split**
Broj ispitanika uključenih u analizu	806	831	959
Žene, N (%)	524 (65%)	486 (58%)	586 (61%)
Medijan godine, (q_L,q_U)	57 (47, 67)	57 (45,69)	52 (40, 61)
Medijan PTH, pg/ml (q_L,q_U)	19.9 (13.7, 29.1)	25.9 (18.4, 32.1)	21.6 (17.2, 26.5)

Oznake: q_L- donji kvartil, q_U-gornji kvartil.

Cjelogenomske analize povezanosti su provedene za svaku kohortu zasebno, te su potom rezultati objedinjeni meta-analizom. Rezultati meta-analize grafički su prikazani na Slici 1. Četiri genetska polimorfizma smještena u neposrednoj blizini gena *RASGEF1B* su dosegla cjelogenomsku razinu značajnosti. Najznačajniji polimorfizam rs11099476 ($P = 1{,}15 \times 10^{-8}$) objašnjava 1,14 % varijacije u razini PTH. Pokazano je da je T alel ovog polimorfizma povezan s višom razinom PTH (β = 0.16, SE = 0.03). Regionalni graf povezanosti za identificirani genetski polimorfizam je prikazan na Slici 2.



Slika 1. Rezultati meta-analize cjelogenomskih studija povezanosti za razinu PTH. A) Manhattan graf. B) Kvantil-kvantil graf.



Slika 2. Detaljan prikaz regije na četvrtom kromosomu u kojoj se nalazi identificirani genetski polimorfizam rs11099476.

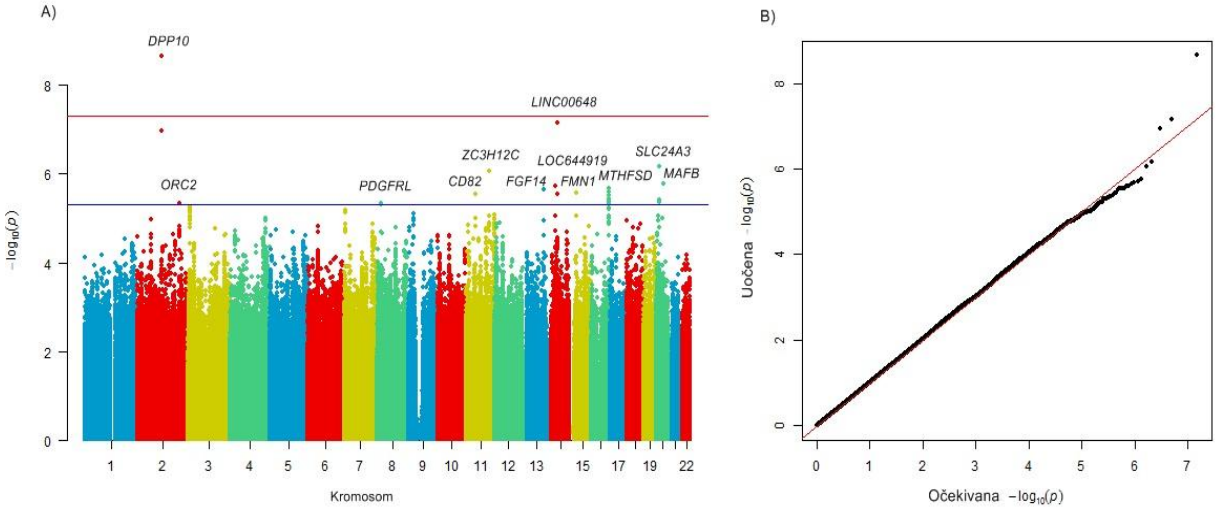
Osim lokusa koji je dosegao cjelogenomsku razinu značajnosti, identificirano je još devet sugestivno značajnih genetskih polimorfizama ($P < 10^{-6}$) čije su karakteristike prikazane u Tablici 2.

Tablica 2. Popis genetskih polimorfizama koji su dosegli sugestivnu granicu značajnosti ($P < 5 \times 10^{-6}$) za povezanost s razinom PTH.

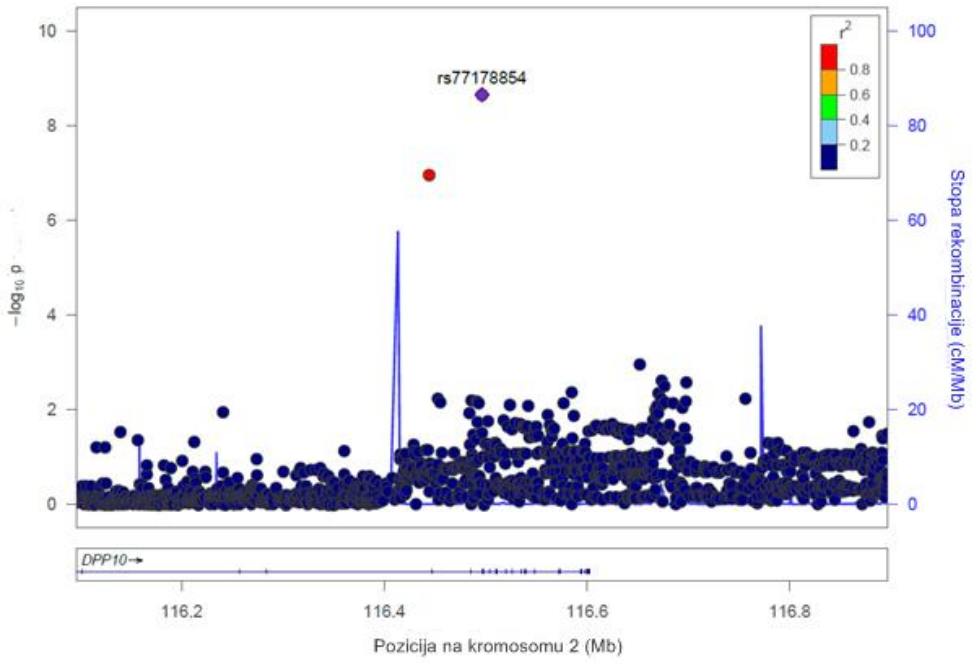
SNP	**Krom.**	**Pozicija**	**Najbliži gen**	**A1**	**A0**	**EAF**	**β**	**SE**	**P**
rs11099476	4	82486056	*RASGEF1B*	T	A	0,55	0,16	0,03	$1{,}15\times10^{-8}$
rs77178854	2	116496539	*DPP10*	C	G	0,98	0,58	0,11	$2{,}46\times10^{-7}$
rs481121	1	37203485	*GRIK1*	A	G	0,57	0,14	0,03	$3{,}58\times10^{-7}$
rs76615278	19	18654588	*FKBP8*	G	A	0,83	0,20	0,04	$6{,}34\times10^{-7}$
rs1875872	17	31795716	*ASIC2*	A	G	0,64	0,14	0,03	$1{,}94\times10^{-6}$
rs9512841	13	28309646	*GSX1*	G	A	0,52	0,13	0,03	$2{,}01\times10^{-6}$
rs191686630	5	58477398	*PDE4D*	A	T	0,16	0,19	0,04	$2{,}36\times10^{-6}$
rs3136797	8	42226805	*POLB*	C	G	0,98	0,57	0,12	$2{,}68\times10^{-6}$
rs499177	8	98472201	*MTDH*	T	C	0,49	0,13	0,03	$4{,}66\times10^{-6}$
rs58726672	10	8407822	*GATA3*	C	T	0,98	0,57	0,13	$4{,}77\times10^{-6}$

Skraćenice: Krom=kromosom, EAF=Frekvencija A1 alela (engl. *effect allele frequency*), β=efekt učinka, SE=standardna pogreška (engl. *standard error*).

Rezultati meta-analize cjelogenomskih studija povezanosti provedenih na pod-uzorku žena prikazani su na Slici 3. Genetski polimorfizam rs77178854 smješten unutar gena *DPP10* je dosegao cjelogenomsku razinu značajnosti (referentni alel C, β = 0.82, SE=0.14, P = $2{,}21 \times 10^{-9}$). Regionalni graf povezanosti za genetski polimorfizam rs77178854 prikazan je na Slici 4.



Slika 3. Rezultati meta-analize cjelogenomskih studija povezanosti za razinu PTH provedene na uzorku žena. A) Manhattan graf. B) Kvantil-kvantil graf.



Slika 4. Detaljan prikaz regije na drugom kromosomu u kojoj se nalazi genetski polimorfizam rs77178854 koji je dosegnuo cjelogenomsku razinu značajnosti u analizi provedenoj na uzorku žena.

Tablica 3 prikazuje sve genetske polimorfizme koji su u meta-analizi provedenoj na uzorku žena dosegli sugestivnu granicu značajnosti. Niti jedan genetski polimorfizam nije dosegao cjelogenomsku razinu značajnosti u meta-analizi provedenoj na uzorku muškaraca (Tablica 4).

Tablica 3. Popis genetskih polimorfizama koji su dosegli sugestivnu granicu značajnosti ($P < 5 \times 10^{-6}$) za povezanost s razinom PTH na uzorku žena.

SNP	**Krom.**	**Pozicija**	**Najbliži gen**	**A1**	**A0**	**EAF**	**β**	**SE**	**P**
rs77178854	2	116496539	*DPP10*	C	G	0,98	0,82	0,14	$2{,}21\times10^{-9}$
rs1890709	14	49101833	*LINC00648*	A	G	0,34	0,20	0,04	$7{,}12\times10^{-8}$
rs16981087	20	19739954	*SLC24A3*	G	C	0,78	0,22	0,04	$6{,}99\times10^{-7}$
rs661171	11	110016519	*ZC3H12C*	G	T	0,72	0,20	0,04	$8{,}94\times10^{-7}$
rs74629672	20	39105870	*MAFB*	T	A	0,94	0,43	0,09	$1{,}68\times10^{-6}$
rs1349573	14	41403160	*LOC644919*	G	A	0,05	0,45	0,10	$1{,}94\times10^{-6}$
rs3866634	16	86567929	*MTHFSD*	G	A	0,93	0,32	0,07	$2{,}14\times10^{-6}$
rs7997888	13	102759325	*FGF14*	A	G	0,04	0,49	0,10	$2{,}19\times10^{-6}$
rs5024438	15	33077401	*FMN1*	G	A	0,74	0,23	0,05	$2{,}76\times10^{-6}$
rs77796218	11	44580581	*CD82*	C	T	0,97	0,49	0,10	$2{,}84\times10^{-6}$
rs13406545	2	201792123	*ORC2*	T	A	0,18	0,21	0,05	$4{,}54\times10^{-6}$
rs2588129	8	17462468	*PDGFRL*	A	G	0,03	0,54	0,12	$4{,}57\times10^{-6}$

Skraćenice: Krom=kromosom, EAF=Frekvencija A1 alela (engl. *effect allele frequency*), β=efekt učinka, SE=standardna pogreška (engl. *standard error*).

Tablica 4. Popis genetskih polimorfizama koji su dosegli sugestivnu granicu značajnosti ($P < 5 \times 10^{-6}$) za povezanost s razinom PTH na uzorku muškaraca.

SNP	**Krom.**	**Pozicija**	**Najbliži gen**	**A1**	**A0**	**EAF**	**β**	**SE**	**P**
rs2024724	1	19237107	*IFFO2*	A	G	0,06	0,55	0,11	$3{,}67\times10^{-7}$
rs75098759	2	184585640	*ACO9369.1*	G	A	0,02	0,70	0,15	$4{,}41\times10^{-6}$
rs9850091	3	7337788	*GRM7*	T	G	0,41	0,22	0,05	$9{,}72\times10^{-6}$
rs143452382	4	185948046	*HELT*	T	A	0,27	0,24	0,05	$5{,}08\times10^{-6}$
rs57117264	5	176075394	*TSPAN17*	T	C	0,29	0,22	0,05	$8{,}17\times10^{-6}$
rs138802122	6	33248372	*B3GALT4*	T	C	0,03	-0,57	0,13	$9{,}34\times10^{-6}$
rs4524627	6	123992030	*TRDN*	G	A	0,14	-0,32	0,07	$2{,}76\times10^{-6}$
rs6964387	7	73727243	*CLIP2*	T	C	0,02	-0,90	0,20	$6{,}38\times10^{-6}$
rs2931353	8	62148882	*CLVS1*	G	A	0,49	0,21	0,04	$1{,}15\times10^{-6}$

Skraćenice: Krom.=kromosom, EAF=Frekvencija A1 alela (engl. *effect allele frequency*), β=efekt učinka, SE=standardna pogreška (engl. *standard error*).

3.3.2. Cjelogenomska analiza povezanosti za razine Tg-At i TPO-At

Nakon primjene kriterija isključenja, u cjelogenomskoj analizi povezanosti za Tg-At sudjelovalo je ukupno 2 629 ispitanika, a za TPO-At ukupno 2 618 ispitanika. Osnovne karakteristike ispitanika uključenih u studiju su prikazane u Tablici 5.

Tablica 5. Karakteristike ispitanika uključenih u studiju.

Tg-At	**Split**	**Korčula**	**Vis**
Ukupan broj ispitanika	942	819	868
Žene, n (%)	587 (62%)	522 (64%)	487 (56%)
Godine, medijan (qL,qU)	52 (40,61)	57 (47,67)	57 (45,69)
Tg-Ab, IU/mL medijan (qL,qU)	6.90 (5.00,15.80)	11.90 (8.10, 32.25)	9.90 (5.10,19.20)
TPO-At	**Split**	**Korčula**	**Vis**
Ukupan broj ispitanika	942	819	857
Žene, n (%)	587 (62%)	522 (64%)	484 (57%)
Godine, medijan (qL,qU)	52 (40,61)	57 (47,67)	57 (45,69)
TPO-Ab, IU/mL medijan (qL,qU)	2.5 (1.3, 7.9)	7.90 (3.85, 18.10)	4.10 (1.80, 11.50)

Oznake: q_L- donji kvartil, q_U-gornji kvartil.

Tablice 6 i 7 prikazuju statistički najznačajnije genetske polimorfizme dobivene u meta-analizi cjelogenomskih analiza povezanosti za razinu Tg-At, odnosno za razinu TPO-At. Niti jedan genetski polimorfizam nije dosegao cjelogenomsku granicu značajnosti u generalnoj populaciji. U ženskoj populaciji graničnu razinu značajnosti pokazao je genetski polimorfizam smješten u blizini gena *DLL1* za povezanost s razinom Tg-At (rs4710782, $P = 6{,}16 \times 10^{-8}$), te genetski polimorfizam smješten pokraj gena *GRIN3*A za povezanost s razinom TPO-At (rs1935377, $P = 8{,}58 \times 10^{-8}$). U analizama provedenima u muškoj populaciji nije identificiran niti jedan statistički značajan genetski polimorfizam (Tablica 6, Tablica 7).

Tablica 6. Popis genetskih polimorfizama koji su dosegli sugestivnu granicu značajnosti ($P < 5 \times 10^{-6}$) za povezanost s razinom Tg-At u cjelokupnom uzorku, te na pod-uzorcima žena i muškaraca.

SNP	Krom.	Pozicija	Najbliži gen	A1	A0	EAF	β	SE	P
Generalna populacija									
rs10889518	1	65544151	*AK4*	A	T	0,14	-0,27	0,05	$1{,}27\times10^{-7}$
rs58150014	10	4249354	*KLF6*	G	A	0,11	0,22	0,05	$2{,}39\times10^{-6}$
rs13253854	8	93372260	*RUNX1T1*	C	A	0,22	0,16	0,03	$3{,}30\times10^{-6}$
Ženska populacija									
rs4710782	6	170582064	*DLL1*	C	G	0,32	0,21	0,04	$6{,}16\times10^{-8}$
rs183893980	7	155106435	*INSIG1*	A	G	0,28	0,23	0,05	$8{,}87\times10^{-7}$
rs1935377	9	104742291	*GRIN3A*	T	C	0,37	-0,18	0,04	$1{,}18\times10^{-6}$
rs12437330	14	98424701	*LINC01550*	A	G	0,19	-0,21	0,04	$1{,}78\times10^{-6}$
rs1889066	10	59776781	*IPMK*	G	A	0,07	0,33	0,07	$2{,}05\times10^{-6}$
rs60767289	22	48875139	*FAM19A5*	G	A	0,23	-0,20	0,04	$2{,}25\times10^{-6}$
rs10889518	1	65544151	*JAK1*	T	A	0,14	-0,32	0,07	$3{,}10\times10^{-6}$
rs2238186	14	72925171	*RGS6*	G	C	0,20	-0,21	0,05	$3{,}31\times10^{-6}$
rs6573038	14	56022792	*KTN1*	T	T	0,28	0,18	0,04	$4{,}37\times10^{-6}$
rs1405966	2	49716853	*FSHR*	A	G	0,20	0,21	0,05	$4{,}58\times10^{-6}$
Muška populacija									
rs73399159	10	134943325	*ADGRA1*	G	A	0,13	-0,33	0,07	$2{,}73\times10^{-7}$
rs323907	7	34736202	*NPSR1*	C	T	0,09	0,37	0,07	$3{,}45\times10^{-7}$
rs9365994	6	158611258	*GTF2H5*	C	A	0,19	0,27	0,05	$6{,}91\times10^{-7}$
rs1288213	5	162212404	*CCNG1*	A	G	0,24	-0,24	0,05	$7{,}21\times10^{-7}$
rs4801635	19	56372705	*NLRP4*	C	T	0,39	0,21	0,04	$9{,}41\times10^{-7}$
rs6097767	20	52717067	*CYP24A1*	A	G	0,07	-0,37	0,08	$2{,}20\times10^{-6}$
rs11952006	5	159022804	*ADRA1B*	A	G	0,16	0,27	0,06	$3{,}14\times10^{-6}$
rs2458778	11	131467380	*NTM*	C	A	0,19	-0,25	0,05	$3{,}60\times10^{-6}$
rs1716169	12	123716930	*MPHOSPH9*	T	A	0,23	-0,23	0,05	$3{,}63\times10^{-6}$
rs9590671	13	42726974	*DGKH*	C	T	0,41	-0,19	0,04	$4{,}51\times10^{-6}$
rs78029223	16	53662833	*RPGRIP1L*	C	T	0,13	0,32	0,07	$4{,}75\times10^{-6}$

Skraćenice: Krom=kromosom, EAF=Frekvencija A1 alela (engl. *effect allele frequency*), β=efekt učinka, SE=standardna pogreška (engl. *standard error*).

Tablica 7. Popis genetskih polimorfizama koji su dosegli sugestivnu granicu značajnosti ($P < 5 \times 10^{-6}$) za povezanost s razinom TPO-At u cjelokupnom uzorku, te na pod-uzorcima žena i muškaraca.

SNP	Krom.	Pozicija	Najbliži gen	A1	A0	EAF	β	SE	P
Generalna populacija									
rs10753297	1	34438957	*CSMD2*	T	A	0,38	-0,14	0,03	$5{,}59\times10^{-7}$
rs7200247	16	74637011	*GLG1*	G	A	0,44	-0,13	0,03	$1{,}53\times10^{-6}$
rs11197050	10	116834272	*ATRNL1*	A	C	0,13	0,22	0,05	$1{,}71\times10^{-6}$
rs12890844	14	92816329	*SLC24A4*	G	A	0,30	0,15	0,03	$2{,}31\times10^{-6}$
rs34926168	14	26550405	*NOVA1*	C	T	0,35	0,14	0,03	$3{,}65\times10^{-6}$
rs1029422	7	117595829	*LSM8*	G	A	0,20	0,16	0,04	$4{,}48\times10^{-6}$
Ženska populacija									
rs1935377	9	104742291	*GRIN3A*	T	C	0,37	-0,20	0,04	$8{,}58\times10^{-8}$
rs10001304	4	154460808	*KIAA0922*	A	G	0,23	-0,20	0,04	$1{,}40\times10^{-6}$
rs13242614	7	132966299	*EXOC4*	T	C	0,06	-0,36	0,08	$2{,}05\times10^{-6}$
rs2088197	4	59978132	*LOC105377247*	G	A	0,36	0,17	0,04	$3{,}35\times10^{-6}$
rs4818857	21	45073775	*HSF2BP*	G	C	0,24	-0,21	0,04	$3{,}73\times10^{-6}$
rs1326248	10	21030854	*NEBL*	C	T	0,40	0,17	0,04	$4{,}16\times10^{-6}$
rs184205168	2	78108992	*LRRTM4*	T	A	0,14	-0,30	0,07	$4{,}95\times10^{-6}$
Muška populacija									
rs2428361	17	31277765	*SPACA3*	C	T	0,27	0,25	0,05	$9{,}16\times10^{-7}$
rs11256909	10	6169140	*PFKFB3*	A	C	0,28	0,23	0,05	$9{,}61\times10^{-7}$
rs1326128	13	50885410	*DLEU1*	G	A	0,46	0,20	0,04	$1{,}28\times10^{-6}$
rs61976526	14	95474079	*DICER1*	G	A	0,29	-0,22	0,05	$2{,}32\times10^{-6}$
rs144436018	2	82236090	*LOC102724542*	G	A	0,22	0,25	0,05	$2{,}86\times10^{-6}$
rs2952635	7	157508424	*PTPRN2*	A	G	0,20	0,25	0,05	$3{,}68\times10^{-6}$
rs111821600	15	24046166	*NDN*	A	G	0,10	-0,40	0,09	$3{,}81\times10^{-6}$

Skraćenice: Krom=kromosom, EAF=Frekvencija A1 alela (engl. *effect allele frequency*), β=efekt učinka, SE=standardna pogreška (engl. *standard error*).

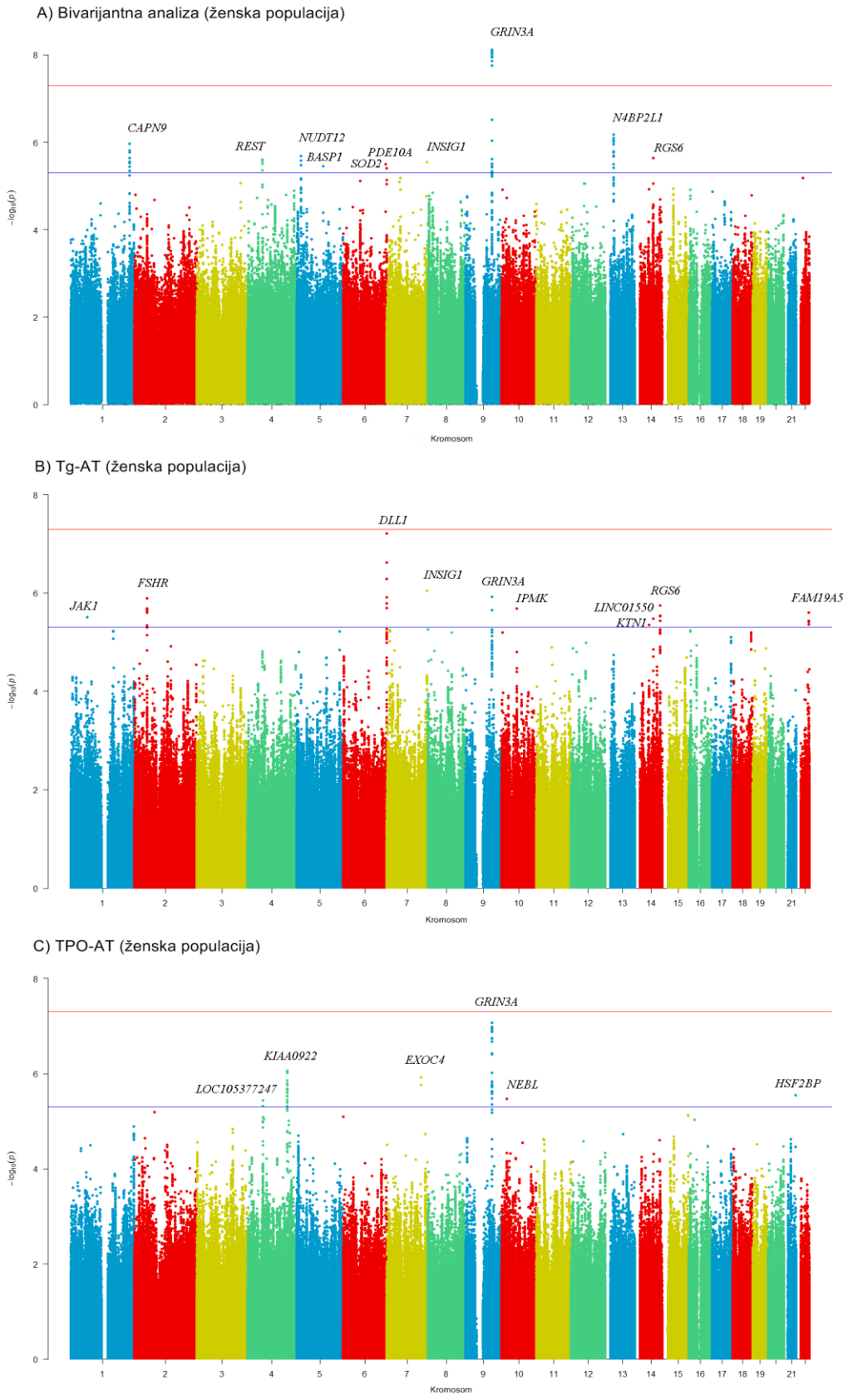
U bivarijantnoj analizi, koja je provedena na cjelokupnom uzorku za Tg-At i TPO-At simultano, niti jedan genetski polimorfizam nije dosegnuo cjelogenomsku razinu značajnosti (Tablica 8). Međutim, u bivarijantnoj analizi koja je provedena u ženskoj populaciji, cjelogenomsku razinu značajnosti dosegao je genetski polimorfizam smješten u blizini gena *GRIN3A* (rs4457391, $P = 7{,}76 \times 10^{-9}$). Slika 5 prikazuje rezultate bivarijantne analize na

pod-uzorku žena, a u Tablici 8 su prikazani genetski polimorfizmi koji su pokazali sugestivnu granicu značajnosti.

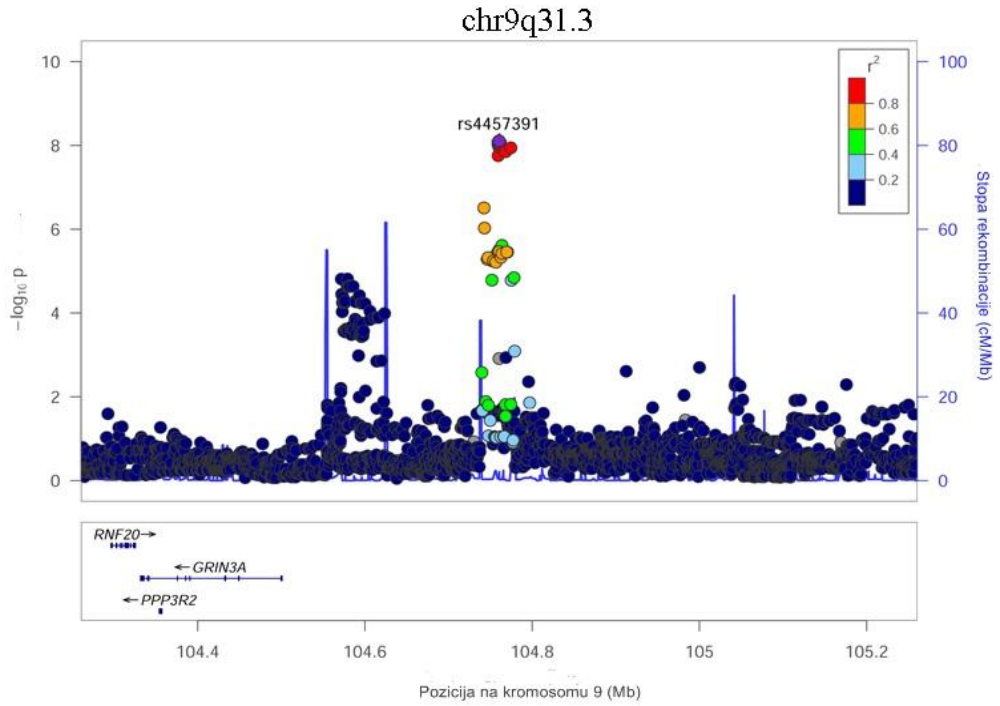
Tablica 8. Popis genetskih polimorfizama koji su dosegli sugestivnu granicu značajnosti ($P < 5 \times 10^{-6}$) u bivarijantnoj analizi provedenoj na cjelokupnom uzorku, te na pod-uzorku žena.

SNP	**Krom.**	**Pozicija**	**Gen**	**A1**	**A0**	**EAF**	**β**	**SE**	**P**
Generalna populacija									
rs8067305	17	1975657	*SMG6*	A	G	0,61	0,01	0,002	$1{,}28\times10^{-6}$
rs4480845	17	1958609	*HIC1*	C	T	0,62	0,01	0,003	$1{,}55\times10^{-6}$
rs611909	6	165924970	*PDE10A*	T	G	0,81	0,01	0,003	$2{,}73\times10^{-6}$
rs56076527	16	74984725	*WDR59*	A	G	0,07	-0,02	0,004	$2{,}76\times10^{-6}$
rs17121639	1	61590306	*NFIA*	T	A	0,06	-0,02	0,004	$2{,}97\times10^{-6}$
rs11012384	10	21198380	*NEBL*	T	G	0,58	0,01	0,002	$3{,}60\times10^{-6}$
rs9999798	4	168977085	*ANXA10*	T	C	0,13	0,01	0,002	$3{,}61\times10^{-6}$
rs147936643	6	159828376	*SOD2*	T	C	0,23	0,01	0,003	$4{,}01\times10^{-6}$
rs76802275	8	51222395	*SNTG1*	A	C	0,09	-0,01	0,002	$4{,}21\times10^{-6}$
Ženska populacija									
rs4457391	9	104760468	*GRIN3A*	G	A	0,59	-0,03	0,005	$7{,}76\times10^{-9}$
rs206327	13	32994393	*N4BP2L1*	C	A	0,20	0,02	0,004	$6{,}6\times10^{-7}$
rs10495300	1	230881762	*CAPN9*	T	C	0,23	0,02	0,004	$1{,}1\times10^{-6}$
rs2238186	14	72925171	*RGS6*	C	G	0,20	0,02	0,004	$2{,}3\times10^{-6}$
rs6554389	4	57708759	*REST*	T	C	0,10	0,02	0,004	$2{,}5\times10^{-6}$
rs183893980	7	155106435	*INSIG1*	G	A	0,72	0,03	0,006	$2{,}9\times10^{-6}$
rs515579	6	165927101	*PDE10A*	C	T	0,46	0,02	0,005	$3{,}2\times10^{-6}$
rs72773267	5	103380718	*NUDT12*	C	T	0,09	0,02	0,005	$3{,}6\times10^{-6}$
rs28882827	21	29123595	*ADAMTS5*	C	T	0,05	0,02	0,004	$3{,}8\times10^{-6}$

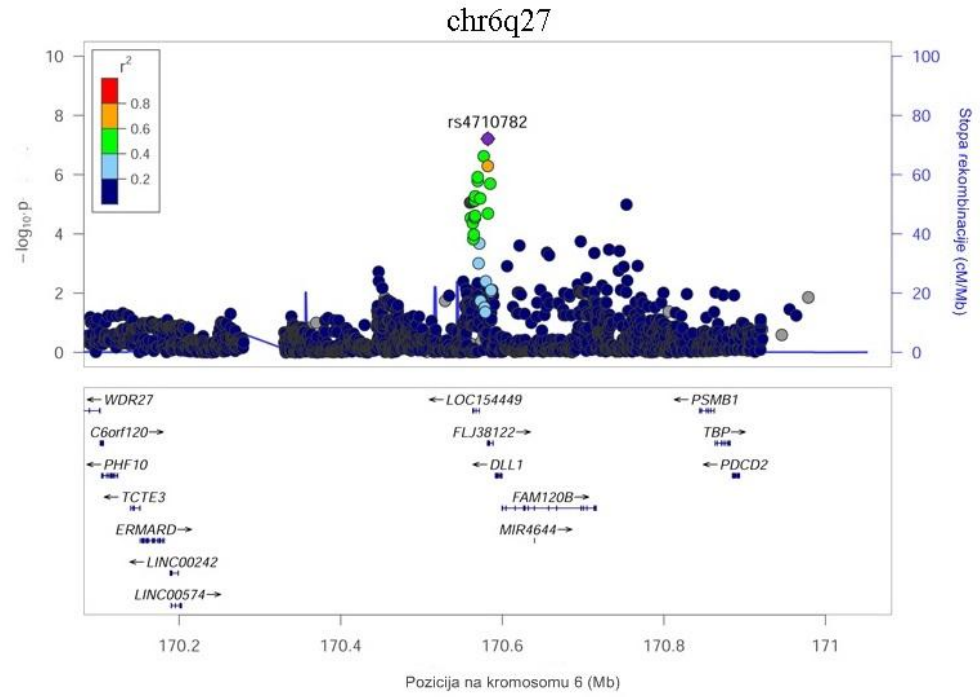
Skraćenice: Krom=kromosom, EAF=Frekvencija A1 alela (engl. *effect allele frequency*), β=efekt učinka, SE=standardna pogreška (engl. *standard error*).



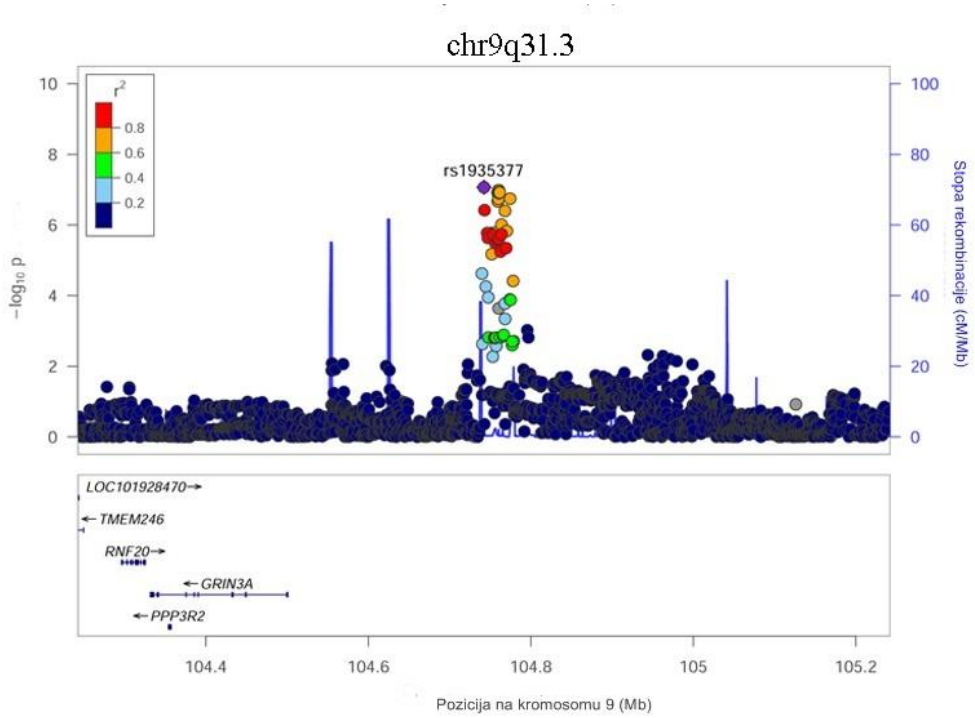
Slika 5. Rezultati meta-analize cjelogenomskih studija povezanosti na pod-uzorku žena za A) bivarijantnu analizu, B) za razinu Tg-At i C) za razinu TPO-At.



Slika 6. Detaljan prikaz regije na devetom kromosomu u kojoj se nalazi genetski polimorfizam rs4457391 koji je dosegnuo cjelogenomsku razinu značajnosti u bivarijantnoj analizi provedenoj na uzorku žena.



Slika 7. Detaljan prikaz regije na šestom kromosomu u kojoj se nalazi genetski polimorfizam rs4710782 koji je bio najznačajniji u cjelogenomskoj meta-analizi za Tg-At provedenoj na ženskoj populaciji.



Slika 8. Detaljan prikaz regije na devetom kromosomu u kojoj se nalazi genetski polimorfizam rs1935377 koji je bio najznačajniji u cjelogenomskoj meta-analizi za TPO-At provedenoj na ženskoj populaciji.

3.3.3. Namirnice povezane s Tg-At i TPO-At

U istraživanju je sudjelovalo ukupno 462 slučajeva i 1388 kontrola. Tablica 9 prikazuje vrijednosti deskriptivne statistike za spol, dob i indeks tjelesne mase (BMI) u uzorku slučajeva i uzorku kontrola. Značajno veći broj žena nego muškaraca imao je pozitivan nalaz za TPO-At i/ili Tg-At, dok nije bilo statistički značajne razlike u godinama i BMI između ispitanika i kontrola (Tablica 9).

Tablica 9. Karakteristike ispitanika uključenih u istraživanje.

	Slučajevi	**Kontrole**	**P vrijednost**
Spol			<0.001[a]
Muško	119 (25.8%)	584 (42%)	
Žensko	343 (74.2%)	804 (58%)	
Dob (godine)	53.66 ± 13.65	52.72 ± 14.93	0.45[b]
BMI	27.78 ± 7.29	27.95 ± 7.79	0.67[b]

Skraćenica: BMI=indeks tjelesne težine. [a] χ^2 test, [b] t-test.

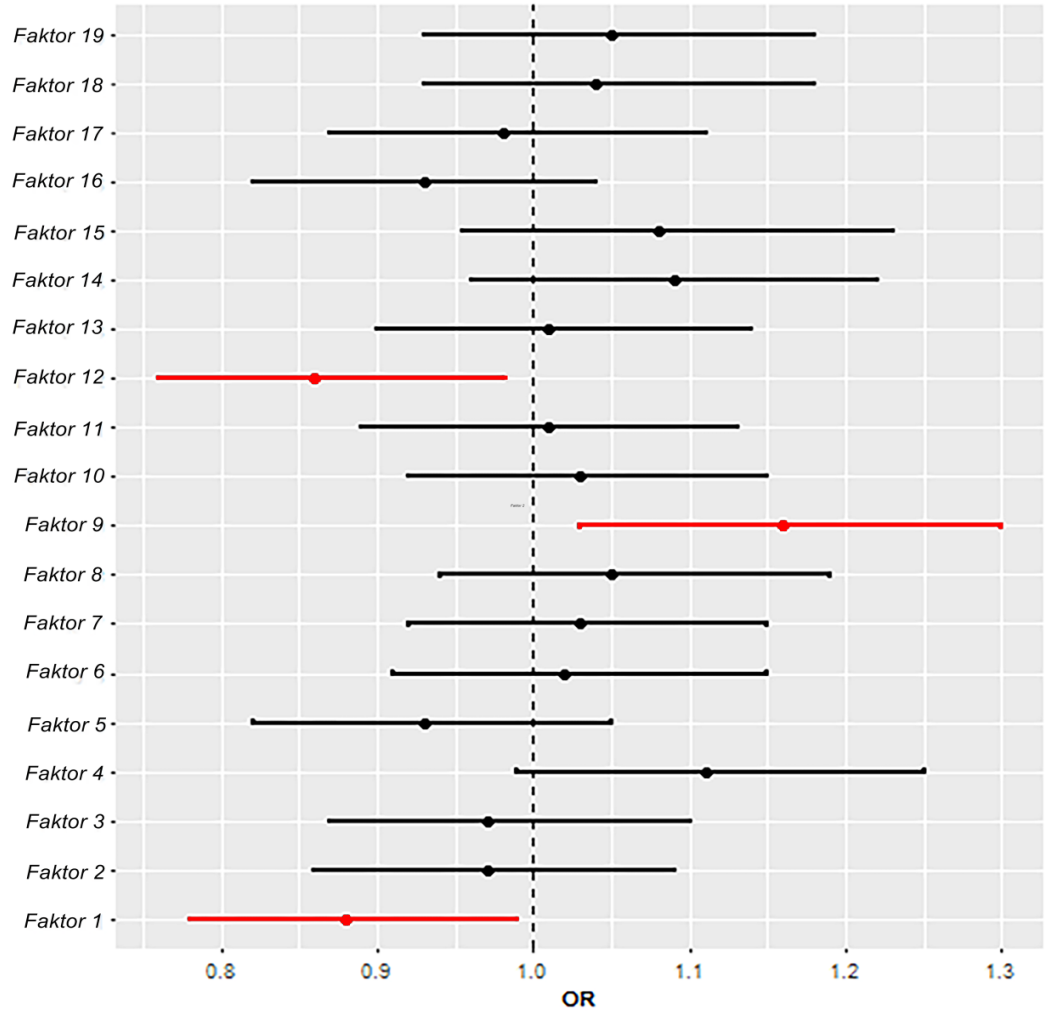
Pogodnost prikupljenih podataka o prehrambenim navikama za korištenje faktorske analize poduprta je Kaiserovom mjerom za adekvatnost uzorkovanja (0.77) i Bartlettovim testom za značajnost broja faktora ($p < 0,001$).

Početni skup od 58 analiziranih namirnica je faktorskom analizom reduciran na 19 ključnih prehrambenih faktora koji zajedno objašnjavaju 54,76% varijance prehrambenih navika ispitanika. Raspodjela namirnica po faktorima s pripadajućim projekcijama na dani faktor je prikazana u Tablici 10.

Tablica 10. Raspodjela namirnica po faktorima s pripadajućim projekcijama na dani faktor. Faktorskom analizom je početni skup od 58 namirnica reduciran na 19 ključnih faktora.

Faktori	**Namirnice (projekcije)**
Faktor 1	Korjenasto povrće (0.79), cvjetasto povrće (0.76), lisnato povrće (0.74), plodasto povrće (0.70), leguminoze (0.54)
Faktor 2	Lignje i hobotnica (0.78), plodovi mora – školjke, rakovi i sl. (0.71), plava riba (0.67), sušena riba i slane srdele (0.56), bijela riba (0.49)
Faktor 3	Čokolada (0.78), keksi (0.76), kolači (0.69), bomboni (0.51)
Faktor 4	Salame (0.69), mesne konzerve (0.62), kobasice (0.57), jaja (0.45), slanina (0.36)
Faktor 5	Cedevita (0.68), voćni sokovi (0.62), osvježavajuća bezalkoholna pića (0.60)
Faktor 6	Integralni kruh (-0.81), bijeli kruh (0.73)
Faktor 7	Svježi sir (0.69), topljeni sir (0.63), tvrdi sir (0.52), vrhnje (0.46)
Faktor 8	Divljač (0.76), riblje prerađevine (0.53)
Faktor 9	Maslac (0.73), životinjska masnoća (0.70)
Faktor 10	Organi i iznutrice (0.73), janjetina (0.52), svinjetina (0.37)
Faktor 11	Gljive (0.64), konzervirano i ukiseljeno povrće (0.61), krumpir (-0.30)
Faktor 12	Muesli (0.70), sušeno voće (0.56), orasi i orašasti proizvodi (0.44)
Faktor 13	Žestoka alkoholna pića (0.68), sokovi od povrća (0.57), industrijske juhe (0.33)
Faktor 14	Čaj (0.64), maslinovo ulje (0.40)
Faktor 15	Mlijeko (0.70), kava (0.51), jogurt (0.49), svježe voće (0.40)
Faktor 16	Piletina (0.68), puretina (0.68)
Faktor 17	Govedina (0.73), teletina (0.34)
Faktor 18	Tjestenina i riža (0.63), džem i marmelada (0.37), voćni kompoti (0.33)
Faktor 19	Biljna ulja (0.77)

Logističkom regresijom je pokazano da je česta konzumacija životinjskih masti i maslaca (Faktor 9) povezana s pozitivnim Tg-At i/ili TPO-At (OR=1,16, p=0,01), dok je česta konzumacija povrća (Faktor 1) (OR=0,88, p=0,048) te sušenog voća, oraha i orašastih proizvoda te mueslia (Faktor12) (OR=0,86, p=0,01) povezana s negativnim nalazima Tg-At i/ili TPO-At. Ostali faktori nisu pokazali statističku značajnu povezanost s Tg-At i/ili TPO-At. Rezultati logističke regresije su grafički prikazani na slici 9.



Slika 9. Rezultati logističke regresije za povezanost prehrambenih faktora s pozitivnim nalazima protutijela Tg-At i/ili TPO-At. Za svaki faktor su prikazini omjeri izgleda (engl. *odds ratio*, OR) i 95%-tni interval pouzdanosti. Statistički značajni faktori su istaknuti crvenom bojom.

3.4. RASPRAVA OBJEDINJENIH RADOVA

Iako genetski faktori znatno utječu na razine PTH te Tg-At i TPO-At, zajednički učinak do sada otkrivenih genetskih polimorfizama objašnjava vrlo mali udio individualne varijabilnosti u razinama ovih fenotipova. Doprinos ovog istraživanja očituje se prvenstveno identifikacijom novih genetskih polimorfizama koji su povezani s razinama PTH, Tg-At i TPO-At u generalnoj populaciji. Također, s obzirom da postoje kliničke razlike u funkciji štitne i doštitne žlijezde kod muškaraca i kod žena, u sklopu ovog istraživanja provedene su po prvi put i cjelogenomske analize povezanosti za svaki spol zasebno. Na taj način su se ispitale razlike po spolu u genetskim odrednicama razine hormona/protutijela doštitne i štitne žlijezde.

U sklopu naše studije (Rad 1) identificiran je novi lokus u neposrednoj blizini gena *RASGEF1B* za povezanost s plazma razinom PTH. *RASGEF1B* je gvanin nukleotidni izmjenjujući faktor sa specifičnošću za Rap2A protein koji je član porodice Ras-sličnih G proteina (42). Na cjelogenomskoj razini značajnosti pokazano je da su varijacije u blizini gena *RASGEF1B* povezane i s visinom (43, 44). Visina pozitivno korelira sa učinkovitošću apsorpcije kalcija, važnom odrednicom ravnoteže kalcija (45). Također, određena razina povezanosti je uočena za varijacije u ovom genu s gustoćom kostiju, opsegom bokova te serumskom razinom cistatina C (46, 47). PTH je značajni negativni prediktor gustoće kostiju u području kukova (48). Serumska razina cistatina C je biomarker bubrežne funkcije i kronične bubrežne bolesti (47). Poremećena funkcija bubrega može utjecati na PTH stimuliranu reapsorpciju kalcija i sintezu $1,25(OH)_2D_3$ (1, 5, 6). Međutim, potrebne su daljnje funkcionalne studije gena *RASGEF1B* kako bi se razumjeli mehanizmi u pozadini uočene povezanosti s razinom PTH.

U okviru iste studije (Rad 1) po prvi put su provedene cjelogenomske analize povezanosti za plazma razine PTH odvojeno po spolu. U navedenim analizama identificiran je dodatni lokus unutar gena *DPP10* kod žena, čime je ukazano na postojanje spolnih specifičnosti u genetskim odrednicama razine PTH. *DPP10* je član serinske obitelji proteaza, eksprimiran u mozgu, gušterači i nadbubrežnoj žlijezdi (44). Može služiti kao prognostički marker za rak debelog crijeva (49). Zanimljivo je napomenuti da su visoke serumske razine PTH povezane s rakom distalnog dijela debelog crijeva kod žena, ali ne i kod muškaraca (50). Prisutnost DPP10 u endokrinim stanicama upućuje na moguću dodatnu ulogu proteina u

regulaciji sekrecije hormona (51), što također podupire naš rezultat. No svakako, potrebne su dodatne funkcionalne studije gena *DPP10* kako bi se razjasnio dobiveni rezultat.

U sklopu naše druge studije (Rad 2), bivarijantnom analizom provedenom na pod-uzorku žena identificiran je novi lokus u blizini gena *GRIN3A* za povezanost s protutijelima Tg-At i TPO-At. Isti lokus pokazao je graničnu povezanost s razinom TPO-At kod žena, dok je lokus smješten pokraj gena *DLL1* pokazao graničnu povezanost s razinom Tg-At, također kod žena.

Gen *GRIN3A* kodira pod-jedinicu N-metil-D-aspartat receptora koji pripadaju super-obitelji ionskih kanala reguliranih glutamatom. Eksprimiran je u mozgu, koštanoj srži, imunološkom sustavu te u muškom i ženskom tkivu (52). Varijacije u ovom genu su povezane s povišenom razinom lipoproteina visoke gustoće (engl. *high-density lipoprotein*, HDL), te sa sniženim razinama lipoproteina niske gustoće (engl. *low-density lipoprotein,* LDL) i triglicerida. Snižene razine hormona štitne žlijezde u jetri imaju utjecaj na smanjenje kolesterola te posljedično i na povećanje razine triglicerida, ukupnog i LDL kolesterola. Pokazano je da su snižene razine LDL-a povezane s hipotireozom (53). Važno je naglasiti da je pronađena povezanost intronske varijante rs9792648 gena *GRIN3A* i hipotireoze ($P=2.7\times10^{-5}$) (54), što ide u prilog našem rezultatu. Međutim, potrebne su funkcionalne studije gena *GRIN3A* kako bi se razjasnila povezanost ovog gena s razinama protutijela protiv štitne žlijezde.

Gen *DLL1* je homolog Notch Delta liganda te je član delta/serrate/jagged obitelji (55). Posreduje u odlukama o staničnoj sudbini tijekom limfopoeze. Notch signalni put sudjeluje u promicanju sazrijevanja CD4 i CD8 T staničnih linija (56). CD4 T stanice induciraju B stanice u proizvodnji protutijela i kod HT i kod GD, dok CD8 T stanice uzrokuju smrt tireocita kod HT (15). Varijacije u blizini ovog gena do sada su povezane s dijabetesom tipa 1 (T1D) te su također sugestivno povezane sa sistemskim eritemskim lupusom (SLE) (57, 58). T1D i SLE se često pojavljuju uz AITD kod istih pojedinaca (59, 60). Kako bi se razjasnila priroda povezanosti gena DLL1 i razine protutijela protiv tireoglobulina, potrebne se daljnje studije.

Kako je već ranije naglašeno, razine protutijela Tg-At i TPO-At osim genetskih čimbenika određuju i razni okolišni čimbenici. Potaknuti nezastupljenošću studija o povezanosti prehrambenih navika s protutijelima protiv štitne žlijezde, u sklopu naše treće studije (Rad 3) po prvi put je analizirana povezanost opsežnog skupa namirnica (58 različitih

namirnica) s pozitivnim nalazom protutijela Tg-At i/ili TPO-At. Rezultati studije sugeriraju da je česta konzumacija životinjskih masti i maslaca povezana s pozitivnim nalazima Tg-At i/ili TPO-At, dok je česta konzumacija povrća te sušenog voća, oraha i orašastih proizvoda te mueslia povezana s negativnim nalazima Tg-At i/ili TPO-At.

Maslac i životinjske masti su bogate zasićenim masnim kiselinama (engl. *saturated fatty acids,* SFA) (61, 62), dok su povrće, žitarice i orašasti proizvodi bogati polinezasićenim masnim kiselinama (engl. *polysaturated fatty acids,* PUFA) (63). Sve namirnice koje su pokazale povezanost s negativnim nalazom Tg-At i/ili TPO-At (povrće, sušeno voće, orasi i orašasti proizvodi, muesli) su bogate omega-6 i omega-3 PUFA.

Omega-6 i omega-3 PUFA su ključne strukturne i funkcijske komponente membranskih fosfolipida te služe kao prekursori za sintezu eikozanoida. Ove dvije vrste PUFA imaju suprotan učinak na upalne reakcije; naime omega-6 PUFA služe kao prekursori upalnih eikozanoida dok omega-3 PUFA generiraju protu-upalne eikozanoide (64). S obzirom da je pretvorba omega-6 PUFA u omega-3 PUFA u ljudskom organizmu manja od 5%, razina protu-upalnih eikozanoida gotovo u cijelosti ovisi o količini n-3 PUFA unesenih prehranom (61). Eikozanoidi, kao što su eikozapentaenoična kiselina i dokosaheksaenoična kiselina, su uključeni u regulaciju proupalnih citokina te djeluju kao protu-upalni medijatori; stoga pogodno djeluju na status autoimunih bolesti kao što je reumatoidni artritis (64).

Studije provedene na eksperimentalnim životinjama su pokazale da prehrana bogata omega-3 PUFA potiskuje upalu koja prati autoimune reakcije (65-68). Rezultati ukazuju da omega-3 PUFA smanjuju diferencijaciju naivnih CD4+ T stanica u Th17 stanice modificirajući regije lipidnih splavi u njihovoj plazma membrani te smanjuju stvaranje IL-6 receptora (65). Poznato je da Th17 stanice pozitivno koreliraju sa serumskom razinom Tg-At i/ili TPO-At (69). Nadalje, nedavno objavljene studije su pokazale da Th17 stanice imaju ključnu ulogu u patogenezi AITD (70, 71).

Povrće, sušeno voće, orasi i orašasti proizvodi te muesli su bogati omega-3 masnim kiselinama te se čini da ove namirnice smanjuju stvaranje protutijela TPO-At i/ili Tg-At. Suprotno tome, izgleda da zasićene masne kiseline životinjskog porijekla imaju štetan efekt za stvaranje TPO-At i/ili Tg-At. Česta konzumacija maslaca i masti životinjskog porijekla može rezultirati u niskom omjeru omega-3 masnih kiselina/omega-6 masnih kiselina, zbog čega dolazi do nedostatka supresije diferencijacije Th17 T stanica, što rezultira stvaranjem protutijela TPO-At i/ili Tg-At (65).

Fitosteroli imaju imunomodulatorna i protu-upalna svojstva, a nalaze se u umjerenim količinama u povrću i u malim količinama u orašastim proizvodima (72, 73). Jedan od

predloženih načina njihove imunomodulatorne aktivnosti je smanjenje plazma razine IL-6. S obzirom da je IL-6 glavni stimulator u diferencijaciji Th17 stanica, moguće obrazloženje protektivnog efekta fitosterola u patogenezi AITD jest redukcija plazma razine IL-6.

Polifenoli imaju protu-upalna, imunomodulatorna i antioksidativna svojstva, a nalaze se u voću i povrću (74, 75). Prisutnost galinske kiseline u sušenom voću i muesliju može biti objašnjenje za njihovu dobrotvornu ulogu za nastanak protutijela Tg-At i/ili TPO-At. Galinska kiselina je komponenta crvenog voća, ali se također nalazi i u kori jabuke te u grejpu (75). Crveno voće se često konzumira sušeno, te je upravo sušeno crveno voće često sadržano u muesliju. Kuppan i sur. su pokazali da kod ljudi tretiranje monocita galinskom kiselinom reducira ekspresiju gena IL-6 (76). Smanjenje razine IL-6 u plazmi može suprimirati diferencijaciju Th17 stanica uključenih u patogenezu AITD.

Novim saznanjima unaprijedile su se dosadašnje spoznaje o genetskim i okolišnim faktorima koji reguliraju PTH, Tg-At i TPO-At, što će pomoći u razumijevanju temeljnih bioloških putova povezanih s funkcijom doštitne i štitne žlijezde te će se pružiti dodatne spoznaje relevantne za kliničku primjenu. Otkrivanje novih genetskih i okolišnih čimbenika uključenih u regulaciju funkcije doštitne i štitne žlijezde može doprinijeti razvoju novih prevencijskih, dijagnostičkih i terapijskih metoda.

3.5. ZAKLJUČCI OBJEDINJENIH RADOVA

U sklopu ove doktorske disertacije koja se temelji na tri objedinjena rada identificirani su novi genetski i okolišni čimbenici koji reguliraju razine PTH, Tg-At i TPO-At. Cjelogenomskom analizom povezanosti za razinu PTH identificiran je gen *RASGEF1B* u generalnoj populaciji, te dodatno gen *DPP10* na pod-uzorku žena. Ovim rezultatima su dopunjene i unaprijeđene postojeće spoznaje o genetskoj regulaciji PTH te je također po prvi put ukazano na postojanje spolnih razlika u genetskim odrednicama PTH. Nadalje, u pod-uzorku žena identificiran je gen *GRIN3A* za povezanost s protutijelima Tg-At i TPO-At. Isti gen dosegao je graničnu značajnost s razinom TPO-At kod žena. Također, identificiran je i lokus u blizini gena *DLL1* koji je pokazao graničnu povezanost s razinom Tg-At kod žena. Ovim rezultatima smo upotpunili dosadašnje znanje o genetskim faktorima koji su povezani s razinom TPO-At i po prvi puta definirali genetske varijante povezane s razinom TgAt te potvrdili postojanje spolnih razlika u genetskoj regulaciji. U trećem radu je pokazano da je česta konzumacija životinjskih masti i maslaca povezana s pozitivnim Tg-At i/ili TPO-At, dok je česta konzumacija povrća te sušenog voća, oraha i orašastih proizvoda te mueslia povezana s negativnim nalazima Tg-At i/ili TPO-At. Rezultati ove studije sugeriraju da je česta konzumacija protu-upalne hrane (hrane bogate polinezasićenim omega-3 masnim kiselinama) povezana s negativnim nalazima Tg-At i TPO-At.

3.6. KRATKI SAŽETAK NA HRVATSKOM JEZIKU

Uvod: Paratireoidni hormon (PTH) je najvažniji čimbenik u regulaciji izvanstanične koncentracije kalcija. Tireoidna peroksidaza (TPO) i tireoglobulin (Tg) su glavne sastavnice štitne žlijezde koje igraju ključnu ulogu u sintezi hormona štitne žlijezde. Povišena razina protutijela protiv TPO (TPO-At) i/ili Tg (Tg-At) može predstavljati ranu fazu u patogenezi autoimunih bolesti štitnjače. Razine PTH, TPO-At i Tg-At rezultat su interakcije između multiplih gena i jednako važnih čimbenika okoliša. Cilj ovog istraživanja bio je identificirati nove genetske čimbenike povezane s razinom PTH, Tg-At i TPO-At te ispitati povezanost prehrambenih navika, kao okolišnog čimbenika, s pozitivnim Tg-AT i/ili TPO-AT.

Metode: Istraživanje se provelo na ispitanicima koji su uključeni u projekt „10 001 Dalmatinac – Hrvatska biobanka“. U genetičkom dijelu istraživanju sudjelovalo je 2 869 ispitanika s područja grada Splita te otoka Korčule i Visa. Cjelogenomske analize povezanosti provedene su korištenjem linearnog mješovitog modela, s prilagodbom za dob, spol i srodstvo ispitanika. U istraživanju povezanosti prehrambenih navika s pozitivnim Tg-At i/ili TPO-At sudjelovalo je ukupno 1887 subjekata (462 ispitanika i 1 425 kontrola). Početni skup od 58 analiziranih namirnica reduciran je faktorskom analizom na 19 ključnih prehrambenih faktora. Povezanost prehrambenih faktora s pozitivnim Tg-At i/ili TPO-At testirana je logističkom regresijom.

Rezultati: Identificiran je lokus u blizini gena *RASGEF1B*, te je dodatno na pod-uzorku žena identificiran i lokus unutar gena *DPP10*, za povezanost s razinom PTH. Nadalje, bivarijantnom analizom na pod-uzorku žena identificiran je lokus u blizini gena *GRIN3A* za povezanost s Tg-At i TPO-At. Isti lokus je pokazao graničnu povezanost s razinom TPO-At na pod-uzorku žena, dok je lokus smješten pokraj gena *DLL1* pokazao graničnu povezanost s razinom Tg-At, također na pod-uzorku žena. U analizi povezanosti namirnica s pozitivnim nalazom Tg-At i/ili TPO-At, pokazano je da je česta konzumacija životinjskih masti i maslaca povezana s pozitivnim Tg-At i/ili TPO-At, dok je česta konzumacija povrća te sušenog voća, oraha i orašastih proizvoda te mueslia povezana s negativnim nalazima Tg-At i/ili TPO-At.

Zaključak: Nova saznanja mogu pomoći u razumijevanju temeljnih bioloških putova povezanih s funkcijom doštitne i štitne žlijezde te pružiti dodatne spoznaje relevantne za kliničku primjenu.

3.7. KRATKI SAŽETAK NA ENGLESKOM JEZIKU (SUMMARY)

Introduction: Parathyroid hormone (PTH) is one of the principal regulators of calcium homeostasis. Thyroglobulin (Tg) and thyroid peroxidise (TPO) are a major components of the thyroid gland, both engaged in the production of thyroid hormones. The presence of autoantibodies with specifity for Tg (TgAb) and TPO (TPOAb) might represent an early stage in autoimmune thyroid diseases. PTH, TgAb and TPOAb levels are complex traits influenced by a combination of genetic and environmental factors. The aim of this study was to identify novel genetic loci associated with PTH, TgAb and TPOAb levels and evaluate the association of dietary factors, as an environmental factor, with positive findings of TPOAb and/or TgAb.

Methods: The study was carried-out on samples obtained through the "10,001 Dalmatians" project. The genetic analyses included 2 869 individuals originating from the city of Split and the islands of Korčula and Vis. Genome-wide association analyses were performed using linear mixed model, with adjustments for age, gender and relatedness among participants.
A total of 1887 subjects (462 cases and 1 425 controls) were enrolled to test the association between dietary factors and positive findings of plasma TPOAb and TgAb. Principal component analysis was used to reduce the initial list of 58 food items to 19 key dietary groups (factors). We used logistic regression analysis to examine dietary factors associated with positive TPOAb and/or TgAb.

Results: We identified a novel locus associated with plasma PTH level near *RASGEF1B* gene. We also identified sex-specific association in females in *DPP10* gene. Furthermore, the bivariate analysis of TgAb and TPOAb in females revealed an association for the locus near *GRIN3A*. The same locus showed borderline association with TPOAb levels in females. Also, a novel locus near *DLL1* gene showed borderline significance in association with TgAb levels in females. Frequent consumption of animal fats and butter was associated with positive plasma TPOAb and/or TgAb, while vegetables, dried fruit, nuts and muesli were associated with negative findings of TPOAb and/or TgAb.

Conclusions: New findings can help in understanding the biological pathways involved in thyroid and parathyroid function and provide the additional knowledge relevant for the clinical practice.

3.8. LITERATURA

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4. ŽIVOTOPIS

Osobni podatci

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Obrazovanje

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Mjesto:	Split
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Datum:	2010. – 2012.
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Radno iskustvo

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Ustanova zaposlenja:	Sveučilište u Splitu, Medicinski fakultet, Katedra za medicinsku biologiju
Naziv radnog mjesta:	Asistent
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Datumi (od-do):	2012. - 2015.
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Naziv radnog mjesta:	Stručni suradnik, Vanjski suradnik
Funkcija:	Održavanje nastave

Datumi (od-do):	2014. – 2015.
Ustanova zaposlenja:	Sveučilište u Splitu, Ekonomski fakultet
Naziv radnog mjesta:	Vanjski suradnik
Funkcija:	Održavanje nastave

Datumi (od-do):	2018. - danas
Ustanova zaposlenja:	Sveučilište u Splitu, Kemijsko-tehnološki fakultet
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Usavršavanje/tečajevi

1. Radionica „Introduction to the statistical analysis of genome-wide association studies“, Imperial College London, London, Engleska, 2017.
2. Radionica „Genetic analysis of thyroid and parathyroid function", Medicinski fakultet, Sveučilište u Splitu, Split, Hrvatska, 2017.
3. Radionica “Active learning in STEM education”, Prirodoslovno-matematički fakultet, Sveučilište u Splitu. Split, Hrvatska, 2017.
4. Ljetna škola „First International Summer School on Data Science“, FESB, Sveučilište u Splitu, Split, Hrvatska, 2016.
5. Ljetna škola: „RSSSO2015: Research Summer School in Statistical Omics“, MEDILS, Split, Hrvatska, 2015.
6. Radionica „Bioinformatics Methods in Genomics“, Institut Ruđera Boškovića, Zagreb,

Hrvatska, 2015.

Znanstveno istraživački projekti

2015 - 2018 doktorand na HRZZ projektu "Identification of new genetic loci implicated in regulation of thyroid and parathyroid function".

Znanstveni radovi

1. **Matana A**, Popović M, Boutin T, Torlak V, Brdar D, Gunjača I, et al. Genome-wide meta-analysis identifies novel gender specific loci associated with thyroid antibodies level in Croatians. Genomics. 2018

2. **Matana A**, Brdar D, Torlak V, Boutin T, Popović M, Gunjača I, et al. Genome-wide meta-analysis identifies novel loci associated with parathyroid hormone level. Molecular Medicine. 2018;24(1):15.

3. **Matana A**, Torlak V, Brdar D, Popović M, Lozić B, Barbalić M, Perica VB, Punda A, Polašek O, Hayward C, Zemunik T. Dietary Factors Associated with Plasma Thyroid Peroxidase and Thyroglobulin Antibodies. Nutrients. 2017; 9(11):1186

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2. Tatijana Zemunik, **Antonela Matana**, Marijana Popović, Thibaud Boutin, Vesela Torlak, Dubravka Brdar, Ivana Gunjača, Ivana Kolčić, Vesna Boraska Perica, Ante Punda, Ozren Polašek, Maja Barbalić, Caroline Hayward. Genetic variants in the *ST6GAL1* gene are associated with thyroglobulin plasma levels in healthy individuals. American Society of Human Genetics Annual Meeting, October 16-20 2018, San Diego, California, USA.
3. **Antonela Matana**, Dubravka Brdar, Vesela Torlak, Marijana Popović, Ivana Gunjača, Ozren Polašek, Vesna Boraska Perica, Maja Barbalić, Ante Punda, Caroline Hayward, Tatijana Zemunik. Genome-wide Analysis Identifies Locus associated with Parathyroid Hormone Levels. ICHG 2017: 19th International Conference on Human Genetics, December 18-19 2017, Bangkok, Thailand. **(nagrada za najbolju prezentaciju)**
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5. Dubravka Brdar, **Antonela Boljat**, Vesela Torlak, Ante Punda, Vesna Boraska Perica, Ivana Gunjača, Nikolina Vidan, Bernarda Lozić, Ozren Polašek, Caroline Hayward, Maja Barbalić, Tatijana Zemunik. Identification of novel genetic loci associated with thyroid function. European Congress of Endocrinology, ECE 2016, May 28-31, Munich, Germany.
6. **Antonela Boljat**, Ivana Gunjača, Nikolina Vidan, Vesela Torlak, Dubravka Brdar, Ante Punda, Bernarda Lozić, Vesna Boraska Perica, Ozren Polašek, Caroline Hayward, Maja Barbalić, Tatijana Zemunik. Identification of new genetic loci and environmental factors associated with parathyroid hormone levels. European human genetics conference 2016, May 21-24 2016. Barcelona, Spain.
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5. PRESLIKE OBJEDINJENIH RADOVA

1. Matana A, Brdar D, Torlak V, Boutin T, Popović M, Gunjača I, Kolčić I, Boraska Perica V, Punda A, Polašek O, Barbalić M, Hayward C, Zemunik T. Genome-wide meta-analysis identifies novel loci associated with parathyroid hormone level. Mol Med. 2018: 24: 15.

2. Matana A, Popović M, Boutin T, Torlak V, Brdar D, Gunjača I, Kolčić I, Boraska Perica V, Punda A, Polašek O, Hayward C, Barbalić M, Zemunik T. Genome-wide meta-analysis identifies novel gender specific loci associated with thyroid antibodies level in Croatians.

3. Matana A, Torlak V, Brdar D, Popović M, Lozić B, Barbalić M, Perica VB, Punda A, Polašek O, Hayward C, Zemunik T. Dietary Factors Associated with Plasma Thyroid Peroxidase and Thyroglobulin Antibodies. Nutrients. 2017: 28;9(11).

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RESEARCH ARTICLE Open Access

Genome-wide meta-analysis identifies novel loci associated with parathyroid hormone level

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Abstract

Background: Parathyroid hormone (PTH) is one of the principal regulators of calcium homeostasis. Although serum PTH level is mostly accounted by genetic factors, genetic background underlying PTH level is insufficiently known. Therefore, the aim of this study was to identify novel genetic variants associated with PTH levels.

Methods: We performed GWAS meta-analysis within two genetically isolated Croatian populations followed by replication analysis in a Croatian mainland population and we also combined results across all three analyzed populations. The analyses included 2596 individuals. A total of 7,411,206 variants, imputed using the 1000 Genomes reference panel, were analysed for the association. In addition, a sex-specific GWAS meta-analyses were performed.

Results: Polymorphisms with the lowest *P*-values were located on chromosome 4 approximately 84 kb of the 5′ of *RASGEF1B* gene. The most significant SNP was rs11099476 ($P = 1.15 \times 10^{-8}$). Sex-specific analysis identified genome-wide significant association of the variant rs77178854, located within *DPP10* gene in females only ($P = 2.21 \times 10^{-9}$). There were no genome-wide significant findings in the meta-analysis of males.

Conclusions: We identified two biologically plausible novel loci associated with PTH levels, providing us with further insights into the genetics of this complex trait.

Keywords: Parathyroid hormone, Genome-wide association analysis, Meta-analysis

Background

Parathyroid hormone (PTH) plays a critical role in the regulation of bone mineral metabolism and calcium homeostasis (DeLuca, 1986). PTH regulates serum calcium levels by stimulating osteoclast activity within bone in order to release calcium. Circulating PTH enhances the reabsorption of calcium in distal nephrons and induces the synthesis of the vitamin D active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) within the kidney (Kumar & Thompson, 2011; Kumar et al., 1991; Khundmiri et al., 2016). The $1,25(OH)_2D_3$ stimulates intestinal calcium absorption and moreover, has a synergistic effect with PTH in bone resorption by stimulating proliferation of osteoclasts (Kumar & Thompson, 2011; Kumar et al., 1991; Khundmiri et al., 2016).

Variations in PTH synthesis and secretion are regulated by serum levels of calcium and phosphate, as well as by $1,25(OH)_2D_3$ (Kumar & Thompson, 2011; Gago et al., 2005). Decreases in serum levels of calcium and increases in serum levels of phosphate stimulate the secretion of PTH, while $1,25(OH)_2D_3$ decreases PTH secretion (Silver & Levi, 2005). Regulation of PTH secretion in response to variations in serum calcium is mediated by the calcium-sensing receptors on the membrane of parathyroid cells (Kumar & Thompson, 2011; Brent et al., 1988). $1,25(OH)_2D_3$ associates with the vitamin D receptor and thus represses the transcription of PTH. The secretion of PTH is also indirectly altered by

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$1,25(OH)_2D_3$ and its regulation of calcium-sensing receptor expression (Kumar & Thompson, 2011). Serum phosphate regulates PTH mRNA and serum PTH levels independently of changes in either serum calcium or $1,25(OH)_2D_3$ levels (Kilav et al., 1995).

The most common pathological condition of excessive secretion of parathyroid hormone is hyperparathyroidism. Primary hyperparathyroidism is due to hypersecretion of the parathyroid gland, while secondary hyperparathyroidism can result from conditions that lead to hypocalcemia, especially observed in patients with chronic kidney disease (Fraser, 2009). Hypoparathyroidism, parathyroid hormone deficiency, is an uncommon condition that occurs mostly due to surgical removal of the parathyroid gland (Abate & Clarke, 2017).

Both environmental and genetic factors influence serum PTH levels. It is estimated that 60% of the variation in PTH concentrations is genetically determined. (Hunter et al., 2001). However, the genetic background underlying PTH level is not yet well understood.

Only one high-density genome-wide association study (GWAS) of PTH concentration has been reported to date (Robinson-Cohen et al., 2017). Robinson-Cohen et al. identified five significantly associated loci, including the strongest associated SNP rs6127099 located upstream of *CYP24A1*, a gene that encodes the primary catabolic enzyme for 1.25 $(OH)_2D$ (Robinson-Cohen et al., 2017). The other significantly associated loci were intronic variant rs4074995 within *RGS14* (regulator of G-protein signaling 14), rs219779 adjacent to *CLDN14* (Claudin 14), rs4443100 located near *RTDR1* (RSPH14, radial spoke head 14 homolog) and rs73186030 located near *CASR* (calcium-sensing receptor) gene (Robinson-Cohen et al., 2017). However, only three of these five loci (rs6127099, rs4074995 and rs219779) were replicated within an independent sample. Altogether, the five reported loci explained only 4.2% of the variance in circulating PTH, suggesting that additional genetic variants remain undiscovered.

The aim of our study is identification of novel loci associated with the parathyroid function, by performing a GWAS meta-analysis of plasma PTH levels within two genetically isolated Croatian populations (Korcula and Vis) following by replication analysis in the urban population of Split. To maximize the power of the study, we additionally performed meta-analysis for PTH plasma levels in all three Croatian populations. We also conducted gender-specific GWAS meta-analyses.

Methods

Study cohorts

This study was performed on samples from three Croatian populations: from the Dalmatian islands of Korcula and Vis and the mainland city of Split, within the large-scale project of "10,001 Dalmatians" (Rudan et al., 2009). A detailed description of the cohorts is provided in Table 1. The Korcula population is genetically isolated from Croatian Mainland, while Vis population is genetically isolated from Croatian Mainland and surrounding islands (Vitart et al., 2008). For all study populations, we excluded participants who underwent parathyroid surgery, as well as individuals who had PTH level < 5 pg/ml, which is near the minimum PTH assay detection limit (4.3 pg/ml). After these exclusions, the number of individuals available with PTH level and genotype data was 806 in Korcula, 831 in Vis and 959 in Split. In all three cohorts there were no participants who reported serious renal disease that could affect PTH concentration. The study was approved by the Research Ethics Committees in Croatia and Scotland and all participants provided informed consent. All analyses were in accordance with the relevant guidelines and regulations.

Genotyping and imputation

Additional file 1: Table S1 shows cohort-summary information on genotyping, imputation and quality control procedures. The final numbers of single nucleotide polymorphisms (SNPs) included in analyses were 9,182,797 for the Korcula sample, 8,865,173 for the Vis sample and 8,777,560 for the Split sample. The number of overlapping SNPs present in all three cohorts was 7,411,206.

Table 1 Characteristics of study participants

Variables	Korcula	Vis	Split
N with PTH and GWAS data	863	834	960
N underwent parathyroid surgery	1	0	1
N with PTH level < 5 pg/ml	56	3	3
Sample size used in the analyses	806	831	959
Women, N (%)	524 (65%)	486 (58%)	586 (61%)
Median age, (q_L,q_U)	57 (47, 67)	57 (45,69)	52 (40, 61)
Median PTH, pg/ml (q_L,q_U)	19.9 (13.7, 29.1)	25.9 (18.4, 32.1)	21.6 (17.2, 26.5)

N: number of individuals; q_L: lower quartile, q_u: upper quartile

Measurement of PTH

Plasma PTH levels were determined by radioimmunoassay method (RIA) in the Laboratory of Biochemistry, Department of Nuclear Medicine, University Hospital Split. RIA ran on the Scintillation counter liquid samples, Capintec, and 125I served as a marker. The concentrations of PTH in the plasma were determined using commercial kits (DIAsource hPTH -120 min-IRMA Kit, DIAsource ImmunoAssays S.A, Belgium). The reference range of plasma PTH levels is 12.26–35.50 pg/ml.

Statistical analyses

We performed genome-wide association analysis within each data set and then conducted a meta-analysis of two genetically isolated cohorts (Korcula and Vis) followed by replication analysis in the cohort of the mainland city of Split. To maximize the study power, we also performed a further meta-analysis of all three cohorts.

Genome-wide association analyses

Association analysis for the Split sample was carried out using a combination of R-package GenABEL and SNPTEST software, while for the Korcula and Vis samples analyses were conducted using R-packages GenABEL and VariABEL (Aulchenko et al., 2007; Marchini et al., 2007; Struchalin et al., 2012).

PTH levels were adjusted for age and sex using linear regression analysis and the calculated residuals were inverse-Gaussian transformed to achieve a normal distribution. GWAS was performed on transformed residuals using linear mixed model which accounts for population structure and relatedness. Association statistics for each SNP, including effect size estimates (β-estimates), standard errors and *p*-values were calculated under an additive genetic model.

Prior to performing the meta-analysis we calculated genomic inflation factors (lambdas) in individual data sets. No adjustments were necessary ($\lambda_{Korcula} = 1.026$, $\lambda_{Vis} = 1.001$, $\lambda_{Split} = 0.99$).

Meta-analysis

Meta-analysis was carried out using the R-package MetABEL (R: A Language and Environment for Statistical Computing, 2018). Meta-analysis was conducted using the inverse-variance fixed-effects method on overlapping SNPs based on the β-estimates and standard errors from each study. Meta-analyses showed no significant evidence for inflated statistics (both $\lambda_{Korcula-Vis}$ and $\lambda_{Korcula-Vis-Split}$ were 1.01), thus no genomic correction was applied. To visualize results of the meta-analysis, Manhattan and quantile-quantile (QQ) plots were created using R-package qqman (Turner, 2014). Regional association plots for loci of interest (±400 kb) were produced using Locus Zoom based on hg19 genome build and 1000 genomes EUR population as the linkage disequilibrium (LD) population (Pruim et al., 2010). Forest plots for the most associated SNP were created using R-package MetABEL. To confirm the genotyping quality for the most associated SNPs in the regions, cluster plots were visually inspected using the Illumina GenomeStudio software package. If the SNP of interest was not directly genotyped, but imputed, then cluster plots were examined for directly genotyped SNPs in high LD with the SNP of interest ($r^2 > 0.8$), located on the same chromosome and less than 400 kb apart. A genome-wide significance of association was defined as $p - value \leq 5 \times 10^{-8}$. Power calculations were performed using Quanto version 1.2.4 for quantitative traits (WJ MJ, 2006).

Sex-specific analyses

In order to identify sex-specific effects we performed GWAS analyzing males and females separately in each cohort. We used the same procedures as in the primary analyses with the exception of the gender covariate. Association results were meta-analyzed using the inverse-variance fixed-effects method. The total sample sizes were 1596 in women and 1000 in men.

Results

Meta-analyses

In each population, a separate genome-wide association study of PTH levels was conducted. We meta-analyzed two genetically isolated cohorts, Korcula and Vis (Additional file 1: Figure S1), and then replicated results in the Split population. A total of 1637 individuals were included in the meta-analysis and 959 in the replication analysis (Table 1). The most associated SNP was rs4616742 (reference allele C, $\beta = 0.18$, $SE = 0.04$, $P = 4.42 \times 10^{-7}$). The SNP is located near protein coding gene *RASGEF1B* (RASGEF Domain Family Member 1B). All top SNPs, located on the chromosome 4 near *RASGEF1B* gene with $P < 10^{-6}$ from meta-analysis of 'genetically isolated populations' reached $P < 0.05$ in the Split population.

To maximize the study power, we performed a meta-analysis of all three cohorts. In total, 2596 individuals were included in the meta-analysis (Table 1). The results are shown in Fig. 1a. As seen from the quantile-quantile plot there was no early deviation from expected *P* values (Fig. 1b). Four SNPs, representing one locus, reached genome-wide significance. As in the meta-analysis of two genetically isolated cohorts, SNPs with the lowest *P*-values were located on chromosome 4 near *RASGEF1B*. The most associated SNP was rs11099476 ($P = 1.15 \times 10^{-8}$), which explained 1.14% of the variance in PTH. We found the T allele of the rs11099476 to be associated

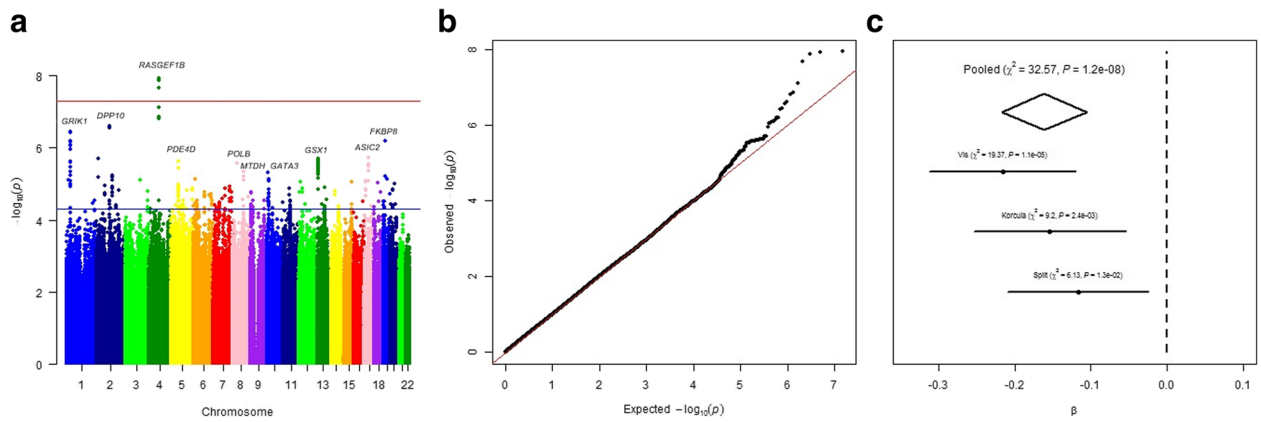

Fig. 1 **a** Manhattan plot of SNPs for PTH levels in the meta-analysis of three cohorts. The *y* axis shows the $-\log_{10}$ *P* values of 7,411,206 SNPs, and the *x* axis shows their chromosomal positions. The blue line indicates the threshold for suggestive hits ($P = 5 \times 10^{-5}$), and the red line represents the threshold for genome-wide significance ($P = 5 \times 10^{-8}$). **b** Quantile-quantile plot in the meta-analysis of three cohorts. **c** Forest plot of rs11099476 effect estimates in individual populations and the combined meta-analysis

with higher PTH level ($\beta = 0.16, SE = 0.03$). Effect sizes were in the same direction across all three cohorts (Fig. 1c). The regional association plot for rs11099476 is given in Fig. 2a. The identified SNP, rs11099476, is in high LD with the top SNP from meta-analysis of 'genetically isolated populations', rs4616742 ($r^2 = 0.9$). These results indicated that associated locus is becoming more significant as the sample size increases and confirmed the consistency of our top finding.

Analysis also revealed several suggestive loci ($P < 5 \times 10^{-6}$), including rs77178854 in the *DPP10* gene ($P = 2.46 \times 10^{-7}$), rs481121 near the *GRIK3* gene ($P = 3.58 \times 10^{-7}$), rs76615278 in the *FKBP8* gene ($P = 6.34 \times 10^{-7}$), rs1875872 near the *ASIC2* gene ($P = 1.94 \times 10^{-6}$), rs9512841 near the *GSX1* gene ($P = 2.01 \times 10^{-6}$), rs191686630 in the *PDE4D* gene, rs3136797 in the *POLB* gene ($P = 2.68 \times 10^{-6}$), rs499177 near the *MTDH* gene and rs58726672 near the *GATA3* gene ($P = 4.77 \times 10^{-6}$) (Table 2).

Sex-specific analyses

We searched for gender-specific loci by performing sex-specific GWAS meta-analysis, analyzing females and males separately in each cohort. The results for females are shown in Fig. 3. The top hit detected in the meta-analysis of all three cohorts, rs77178854, located within *DPP10* gene, reached genome-wide significance (reference allele C, $\beta = 0.82$, $SE = 0.14$, $P = 2.21 \times 10^{-9}$) in females (Table 3). Effect sizes were in the same direction across all three cohorts (Fig. 3c). Regional association plot of the identified SNP is shown in Fig. 2b. No single locus reaching genome wide significance was identified in males (Additional file 1: Table S2).

Discussion

In this GWAS meta-analysis of three Croatian populations we identified a novel genome-wide significant locus associated with plasma PTH level near gene *RASGEF1B* on chromosome 4. We also identified a sex-specific significant association in females in the *DPP10* gene.

The significance of the identified polymorphism rs11099476 was most influenced by the Vis population, which is isolated from the Croatian Mainland and surrounding islands, then by the Korcula population which is isolated from the Croatian Mainland and the least contributed by the mainland city of Split population (Fig. 2c). However, although the locus significance was most affected by the isolated populations, significance has been amplified in the meta-analysis of all three cohorts compared to meta-analysis of 'genetically isolated populations'.

The identified common variant rs11099476 accounts for 1.14% of population variance in plasma PTH. *RASGEF1B* is the guanine nucleotide exchange factor with specificity for Rap2A, a member of Rap subfamily of Ras-like G proteins (Yaman et al., 2009). Rap2 subfamily contains Rap2A and Rap2B, which share about 90% sequence homology (Paganini et al., 2006). Rap2A protein binds GDP to GTP and exhibits a low intrinsic GTPase activity in the presence of Mg^{2+} (Lerosey et al., 1991), while Rap2B increases intracellular calcium level and phosphorylation level of extracellular signal-related kinase (ERK) 1/2 (Di et al., 2015). Variations near *RASGEF1B* gene have been associated with height (He et al., 2015; Allen et al., 2010). Height is positively correlated with calcium absorption efficiency which is important determinant of calcium balance (Abrams et al., 2005). Some evidence of association was also found for variations in this gene and bone density, hip, and cystatin C

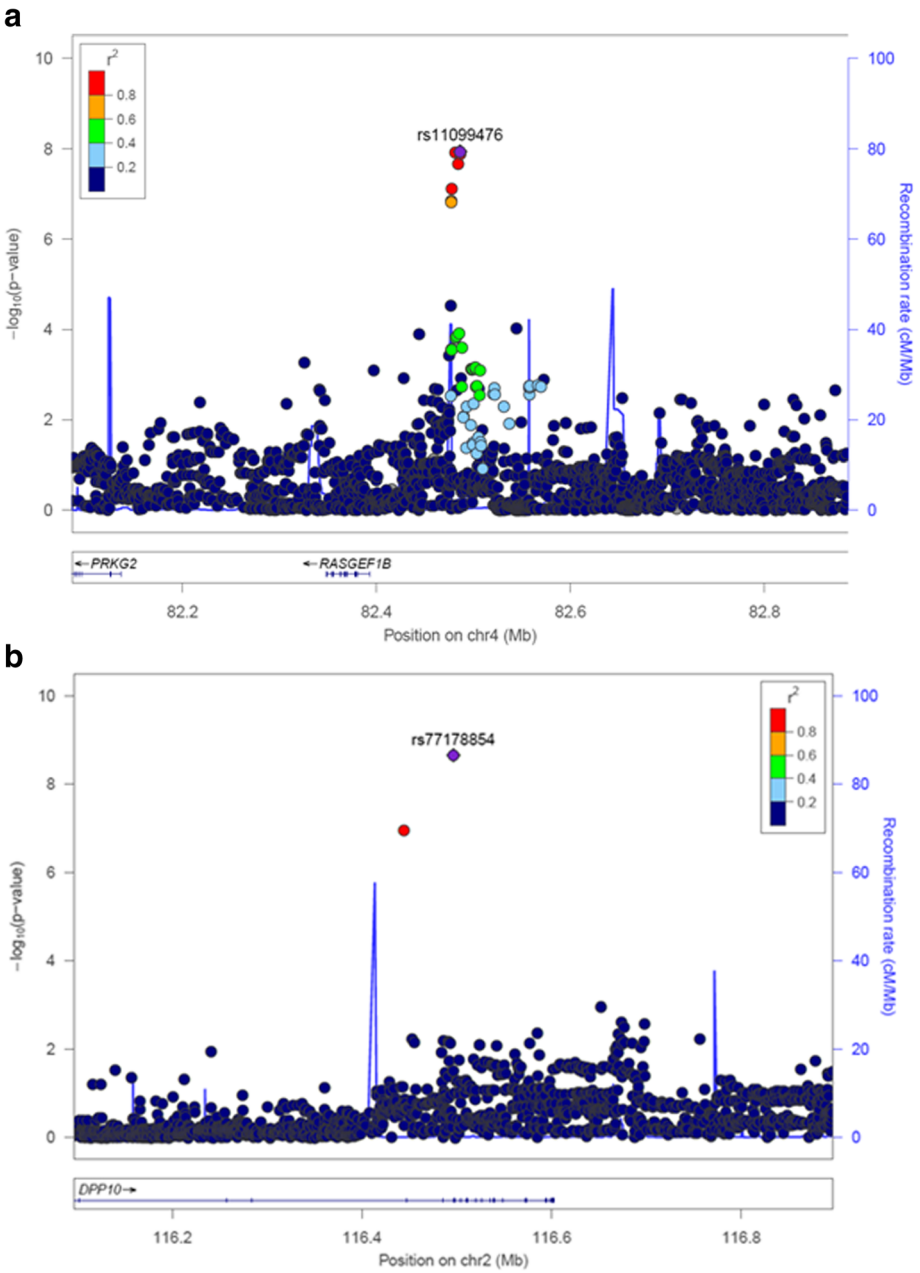

Fig. 2 **a** Regional association plot for the chromosome 4 locus rs11099476 in the meta-analysis of three cohorts. **b** Regional association plot for the chromosome 2 locus rs77178854 in the sex-stratified meta-analysis of three cohorts among females. SNPs are plotted by position against association with PTH ($-\log_{10}$ *P* values). The purple diamond highlights the most significant, whereas the colors of other variant represent LD with most significant SNP

in serum (Kiel et al., 2007; Kottgen et al., 2010). PTH is a significant negative predictor of bone mineral density at the hip (Sneve et al., 2008). Cystatin C in serum is a biomarker of kidney function, and chronic kidney disease (Kottgen et al., 2010). Disturbed kidney function can influence PTH stimulated calcium reabsorption and synthesis of $1,25(OH)_2D_3$ (Kumar & Thompson, 2011; Kumar et al., 1991; Khundmiri et al., 2016). However, to understand the mechanism underlying the observed association further functional studies of *RASGEF1B* will be needed.

Although no signals other than rs11099476 reached genome-wide significance, several candidate loci showed suggestive evidence of association. Particularly interesting is the variant near *GATA3* gene since mutations in this gene are the cause of hypoparathyroidism with sensorineural deafness and renal dysplasia (Van Esch et al., 2000). Of note, in a previous large GWA meta-analysis, variant

Table 2 Associations of top single nucleotide polymorphisms($P < 5 \times 10^{-6}$) with PTH concentrations

SNP	Chr	Position	Nearest Gene	Effect Allele	Other Allele	EAF Korcula	EAF Vis	EAF Split	GnomAD EAF	β	SE	*P* value
rs11099476	4	82,486,056	*RASGEF1B*	T	A	0.57	0.55	0.54	0.59	0.16	0.03	1.15×10^{-8}
rs77178854	2	116,496,539	*DPP10*	C	G	0.97	0.98	0.99	0.98	0.58	0.11	2.46×10^{-7}
rs481121	1	37,203,485	*GRIK1*	A	G	0.56	0.56	0.58	0.49	0.14	0.03	3.58×10^{-7}
rs76615278	19	18,654,588	*FKBP8*	G	A	0.84	0.83	0.82	*	0.20	0.04	6.34×10^{-7}
rs1875872	17	31,795,716	*ASIC2*	A	G	0.62	0.65	0.65	0.65	0.14	0.03	1.94×10^{-6}
rs9512841	13	28,309,646	*GSX1*	G	A	0.51	0.52	0.53	0.58	0.13	0.03	2.01×10^{-6}
rs191686630	5	58,477,398	*PDE4D*	A	T	0.11	0.16	0.21	*	0.19	0.04	2.36×10^{-6}
rs3136797	8	42,226,805	*POLB*	C	G	0.98	0.99	0.98	0.99	0.57	0.12	2.68×10^{-6}
rs499177	8	98,472,201	*MTDH*	T	C	0.46	0.57	0.45	0.44	0.13	0.03	4.66×10^{-6}
rs58726672	10	8,407,822	*GATA3*	C	T	0.98	0.98	0.98	0.98	0.57	0.13	4.77×10^{-6}

Top SNPs were defined as the SNP with lowest P value within a 500 kb window
Chr: chromosome; EAF: effect allele frequency; GnomAD EAF: effect allele frequency from Genome Aggregation Database; β: effect size; SE: standard error
*variants without frequency information in Genome Aggregation Database

near *GATA3* gene was found to be associated with serum calcium (O'Seaghdha et al., 2013).

Given the reported differences in PTH level between males and females, we performed sex-specific analyses (Wei et al., 2015; Serdar et al., 2017). Our study supports the sex-specificity underlying PTH level. Sex-stratified analysis in women identified a novel locus associated with PTH. The identified locus is the intron variant rs77178854, located within *DPP10* gene. *DPP10* encodes a membrane protein that is a member of the serine proteases family, which binds specific voltage-gated potassium channels and alters their expression and biophysical properties. It is highly expressed in brain, pancreas, spinal cord and adrenal gland (Allen et al., 2003), and may serve as a prognostic marker in colorectal cancer (Park et al., 2013). It is interesting to note that Aigner et al. showed that high serum PTH concentrations were associated with distal colorectal cancer in women but not in men (Aigner et al., 2015). The existence of *DPP10* in endocrine cells indicate that the protein might also have an additional role in the regulation of hormone secretion (Bezerra et al., 2015), which also supports our finding. Further studies of *DPP10* will be needed to clarify this result.

The only previously published high-density GWAS for PTH levels did not identify *RASGEF1B* or *DPP10* at a genome-wide significant level, despite having a sample size of over 29,155 participants (22, 653 in discovery stage and 6502 in replication analysis) (Robinson-Cohen et al., 2017). The possible explanation could be an increased relative effect of these loci in our populations due to the reduced genetic and environmental heterogeneity found in two out of three cohorts (i.e., Korcula and Vis) (Rudan et al., 2008) compared to the urban populations used in the

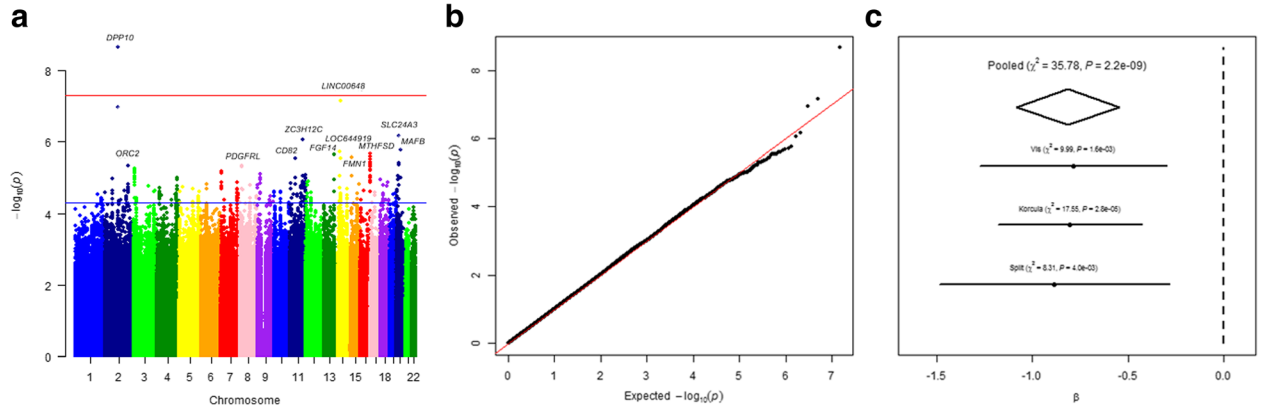

Fig. 3 a Manhattan plot of SNPs for PTH levels in the sex-stratified meta-analysis of three cohorts among females. The *y* axis shows the $-\log_{10}$ *P* values of 7,411,206 SNPs, and the *x* axis shows their chromosomal positions. The blue line indicates threshold for suggestive hits ($P = 5 \times 10^{-5}$), and red line represents the threshold for genome-wide significance ($P = 5 \times 10^{-8}$). **b** Quantile-quantile plot in the sex-stratified meta-analysis of three cohorts among females. **c** Forest plot of rs77178854 effect estimates in individual populations and the combined meta-analysis among females

Table 3 Associations of top single nucleotide polymorphisms($P < 5 \times 10^{-6}$) with PTH concentrations among females

SNP	Chr	Position	Nearest Gene	Effect Allele	Other Allele	EAF Korcula	EAF Vis	EAF Split	GnomAD EAF	β	SE	*P* value
rs77178854	2	116,496,539	*DPP10*	C	G	0.98	0.97	0.99	0.98	0.82	0.14	2.21×10^{-9}
rs1890709	14	49,101,833	*LINC00648*	A	G	0.38	0.30	0.33	0.31	0.20	0.04	7.12×10^{-8}
rs16981087	20	19,739,954	*SLC24A3*	G	C	0.80	0.78	0.77	0.81	0.22	0.04	6.99×10^{-7}
rs661171	11	110,016,519	*ZC3H12C*	G	T	0.74	0.70	0.71	0.70	0.20	0.04	8.94×10^{-7}
rs74629672	20	39,105,870	*MAFB*	T	A	0.95	0.94	0.94	0.96	0.43	0.09	1.68×10^{-6}
rs1349573	14	41,403,160	*LOC644919*	G	A	0.05	0.05	0.06	0.03	0.45	0.10	1.94×10^{-6}
rs3866634	16	86,567,929	*MTHFSD*	G	A	0.93	0.92	0.93	0.91	0.32	0.07	2.14×10^{-6}
rs7997888	13	102,759,325	*FGF14*	A	G	0.04	0.04	0.04	0.15	0.49	0.10	2.19×10^{-6}
rs5024438	15	33,077,401	*FMN1*	G	A	0.72	0.70	0.79	*	0.23	0.05	2.76×10^{-6}
rs77796218	11	44,580,581	*CD82*	C	T	0.97	0.96	0.97	0.98	0.49	0.10	2.84×10^{-6}
rs13406545	2	201,792,123	*ORC2*	T	A	0.15	0.19	0.18	0.23	0.21	0.05	4.54×10^{-6}
rs2588129	8	17,462,468	*PDGFRL*	A	G	0.04	0.02	0.02	0.11	0.54	0.12	4.57×10^{-6}

Top SNPs were defined as the SNP with lowest P value within a 500 kb window
Chr: chromosome; EAF: effect allele frequency; GnomAD EAF: effect allele frequency from Genome Aggregation Database; β: effect size; SE: standard error
*variants without frequency information in Genome Aggregation Database

analysis of Robinson-Cohen et al. *(Robinson-Cohen et al., 2017)*. Previously reported *CYP24A1*, *RGS14* and *CLDN14* variants associated with PTH level (Robinson-Cohen et al., 2017) had the same directions of effect in our study as originally reported but did not show significant associations, probably due to limited sample size of our study or specificity of isolated populations (Additional file 1: Table S3).

The greatest strengths of our study include a comprehensive set of genetic variants examined and ethnically homogeneous sample. We had sufficient data to confidently detect an association for the identified *RASGEF1B* locus, since our meta-analysis had 92% power to detect associated SNP with an effect size of 0.19 and minor allele frequency of 0.45 at the genome-wide level of significance. Furthermore, meta-analysis performed in females only had 86% power to detect *DPP10* locus with an effect size of 0.82 and minor allele frequency of 0.02 at the genome-wide level of significance. Meta-analysis performed in males only had 84% power to detect SNPs with an effect size of 0.35 and minor allele frequency of 0.3 at the genome-wide level of significance. The main limitation of our study is the modest sample size used in the analysis, reducing statistical power for detecting additional associations with smaller effect sizes or minor allele frequencies. Nevertheless, we have identified novel, previously unsuspected and biological plausible associations with PTH variation. Further replication analysis would be required to confirm our findings and to discover additional genetic variants underlying PTH levels in order to explain more of the variability in PTH variations.

Conclusions

In summary, in a GWA meta-analysis of PTH levels we identified a novel significant locus rs11099476 located near a guanine nucleotide exchange factor *RASGEF1B*. The finding appears to be consistent based on analyses of meta-GWAS across all three analyzed cohorts and meta-GWAS across two isolated populations followed by replication analysis in the mainland cohort. Our work also includes the first gender-specific GWAS performed to date and revealed significant association for an intron variant rs77178854 located within the *DPP10* gene in women, indicating the possibility that sex-specificity is underlying PTH level. To conclude, findings from this study improve the current knowledge of the genetic factors regulating PTH levels and their validation in independent populations would be beneficial.

Additional file

Additional file 1: Supporting Information. (XLSX 54 kb)

Abbreviations

GWAS: genome-wide association study; PTH: Parathyroid hormone; RIA: radio-immunoassay method; SNP: single nucleotide polymorphism

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Availability of data and materials
The datasets generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions
TZ conceived the study idea; OP, CH, TZ, VBP, IK and MB formed the biobank "10 001 Dalmatians"; VT, DB and AP performed the measurements of parathyroid hormones and verified the relevance of the results; TB performed imputation of the data; AM, MP, IG researched the data; AM performed the statistical analysis and drafted the manuscript; TZ, CH, MB, MP, IG, VBP and OP contributed to the discussion, review and editing of the manuscript; all authors approved the final version of the manuscript.

Ethics approval and consent to participate
The study was approved by the Research Ethics Committees in Croatia and Scotland and all participants provided informed consent.

Competing interests
The authors declare that they have no competing interests.

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Genome-wide meta-analysis identifies novel gender specific loci associated with thyroid antibodies level in Croatians

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ABSTRACT

Autoimmune thyroid diseases (AITD) are multifactorial endocrine diseases most frequently accompanied by Tg and TPO autoantibodies. Both antibodies have a higher prevalence in females and act under a strong genetic influence.

To identify novel variants underlying thyroid antibody levels, we performed GWAS meta-analysis on the plasma levels of TgAb and TPOAb in three Croatian cohorts, as well as gender specific GWAS and a bivariate analysis.

No significant association was detected with the level of TgAb and TPOAb in the meta-analysis of GWAS or bivariate results for all individuals. The bivariate analysis in females only revealed a genome-wide significant association for the locus near *GRIN3A* (rs4457391, $P = 7.76 \times 10^{-9}$). The same locus had borderline association with TPOAb levels in females (rs1935377, $P = 8.58 \times 10^{-8}$).

In conclusion, we identified a novel gender specific locus associated with TgAb and TPOAb levels. Our findings provide a novel insight into genetic and gender differences associated with thyroid antibodies.

1. Introduction

Thyroglobulin (Tg) and thyroid peroxidase (TPO) are major components of the thyroid gland, both engaged in the production of the thyroid hormones [1]. Autoimmune thyroid diseases (AITD) are one of the most common autoimmune diseases, affecting 2–5% of the general population [2]. The presence of circulating autoantibodies with specificity for Tg and TPO, might represent an early stage in the pathogenesis of AITD [3]. Hashimoto thyroiditis (HT) and Graves' disease (GD) are autoimmune diseases in which the immune system turns against the thyroid gland. HT is characterised by destruction of thyroid gland and underproduction of thyroid hormones (hypothyroidism), whereas antibody stimulation of thyroid gland in GD results in overproduction of thyroid hormones (hyperthyroidism) [4,5]. The prevalence of TgAb and TPOAb positivity in the total and disease-free population is greater in females and increases with age, especially among females [6,7]. The prevalence of TgAb positivity is 60–80% in patients with HT and 30–60% in patients with GD. Positivity of TPOAb is detected in 90–95% patients with HT and 80% patients with GD [8].

Many genetic loci seem to be associated with multiple traits in human complex diseases and have the direct biological influence on more than one phenotypic trait [9]. HT and GD have some unique loci, as well as some common to both diseases, indicating that there is a shared genetic susceptibility to HT and GD [10]. Since various autoimmune diseases often cluster within the same patient, identifying the basis for this shared pathogenesis could be important not only for the fundamental understanding of AITD mechanisms but also in the understanding of other associated diseases [11,12].

Thyroid antibodies are under the strong genetic influence. Autoimmune prevalence and clinical differences in thyroid function are known to be gender-related [6,7,13,14]. According to a twin study, the estimate of genetic influence on serum TgAb concentrations is 39% in males and 75% in females [15]. For serum TPOAb concentrations, the estimates are 61% in males and 72% in females [15].

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Until now, two genome-wide associations studies were performed on TPO antibody in general population, one in Caucasians [16], and the other in an Asian (Korean) population [17]. Also, there is one meta-analysis [18] in which previous GWAS findings [16] were used as a basis for an identification of additional novel genetic variants. Those studies have reported the genome-wide association of several loci with TPOAb level and/or positivity, including variants near *TPO, HCP5, HLA-DPB1* and in *ATXN2, MAGI3, KALRN, BACH2*, *RERE, HLA-DOB* genes [16–18]. Although the heritability of TPOAb accounts for around 70% [15], the identified risk loci for AITD accounts for only 4% of the heritability. The genetic association of TgAb has not been analysed on a genome-wide scale so far.

The aim of this study was to identify novel loci associated with thyroid antibodies. We performed genome-wide meta-analysis for TgAb plasma levels in 2629 individuals from three Croatian cohorts for the first time. Genome-wide meta-analysis for TPOAb plasma levels was also performed in 2618 individuals. In addition, we conducted bivariate analysis for these two correlated traits (i.e., TgAb and TPOAb), gender specific GWAS as well as biological pathway analyses.

2. Methods

The study was carried out on samples from three Croatian populations: the mainland city of Split and the islands of Vis and Korcula. The samples were obtained from the large-scale project of “10,001 Dalmatians” [19]. Cohorts' description is reported in Table 1. We excluded participants with known thyroid pathologies, the ones that underwent thyroid surgery or were treated for thyroid conditions. After these exclusions, 2629 individuals were included in the analyses for TgAb level, and 2618 for TPOAB level. The study was approved by the Research Ethics Committees in Croatia and Scotland, and all participants provided informed consent.

2.1. Genotyping and imputation

Genotyping platforms and quality control procedures are summarized in Table 2. SHAPEIT2 was used for genotypes pre-phasing, along with duoHMM for refine phasing [20,21]. Samples from Split cohort were collected and genotyped in two rounds (Split1 and Split2) with two different genotyping platforms (Table 2). Cohorts of Vis, Korcula, and Split2 were directly imputed using 1000 Genomes project phase I version 3, whereas for the imputation of Split1 a merged reference panel of 1000 Genomes and Split2 was used. For imputation, we used IMPUTE2 [22,23]. Variants with minor allele frequency > 5%, no significant deviation from HWE ($p > 10^{-7}$), and imputation info score > 0.4 were kept for further analysis. The final number of overlapping SNPs was 5,527,232.

Table 1
Characteristics of study participants.

Variables for Tg-Ab	Split	Korčula	Vis
Overall sample size	942	819	868
Women, n (%)	587 (62%)	522 (64%)	487 (56%)
Median age, (qL,qU)	52 (40,61)	57 (47,67)	57 (45,69)
Median Tg-Ab, IU/mL (qL,qU)	6.90 (5.00,15.80)	11.90 (8.10, 32.25)	9.90 (5.10,19.20)

Variables for TPO-Ab	Split	Korčula	Vis
Overall sample size	942	819	857
Women, n (%)	587 (62%)	522 (64%)	484 (57%)
Median age, (qL,qU)	52 (40,61)	57 (47,67)	57 (45,69)
Median TPO-Ab, IU/mL (qL,qU)	2.5 (1.3, 7.9)	7.90 (3.85, 18.10)	4.10 (1.80, 11.50)

N: number of individuals; q_L: lower quartile, q_u: upper quartile.

2.2. Measurement of Tg and TPO antibodies

Plasma TgAb and TPOAb were determined by a sandwich chemiluminescence immunoassay method in the Laboratory of Biochemistry, Department of Nuclear Medicine, University Hospital Split. The immunoassay was conducted in a fully automated instrument “Liaison” Biomedica Chemiluminescence Analyzer, using LIAISON®Anti-Tg and LIAISON®Anti-TPO in vitro assays for the quantitative determination of TgAb and TPOAb in the plasma. The reference range of TgAb is 5–100 IU/mL, and for the TPOAb is 1–16 IU/mL.

2.3. Genome-wide association analyses

TgAb and TPOAb levels were adjusted for age and sex under a linear regression model. Derived residuals were inverse-normal transformed and included in the linear mixed model, which accounts for population structure and relatedness. Association analysis was performed assuming an additive genetic model to test for association between each SNP and adjusted TgAb and TPOAb levels. For the Split sample analysis was carried out using a combination of R-package GenABEL and SNPTEST software, while for the Korčula and Vis samples association analyses were conducted using R-packages GenABEL and VariABEL [24–26]. Genomic inflation factors (lambdas) were calculated in each data set prior performing meta-analysis. There was no need for adjustments ($\lambda_{TgAb\_Korčula} = 1.00$, $\lambda_{TgAb\_Split} = 0.93$, $\lambda_{TgAb\_Vis} = 1.01$; $\lambda_{TPOAb\_Korčula} = 0.99$, $\lambda_{TPOAb\_Split} = 1.00$, $\lambda_{TPOAb\_Vis} = 1.02$).

2.4. Meta-analyses

We combined evidence of associations from single GWAS using inverse-variance fixed-effect meta-analysis. Meta-analyses showed no significant evidence for inflated statistics ($\lambda_{TgAb} = 0.97$, $\lambda_{TPOAb} = 1.01$) hence no genomic correction was applied. Manhattan and quantile-quantile (QQ) plots were generated using the qqman R package [27]. Regional association plots for loci of interest (400 kb) were created using Locus Zoom based on 1000 genomes EUR population [28]. Illumina GenomeStudio software package was used to create cluster plots for confirmation of genotyping quality for associated SNPs. In cases where the SNP of interest was imputed, and not directly genotyped, cluster plots were created for directly genotyped SNPs that were in high LD with the SNP of interest ($r^2 > 0.8$). The genome-wide significance of association was defined as $p - value \leq 5 \times 10^{-8}$. Meta-analyses were performed with the R-package MetABEL [29].

2.5. Gender specific analyses

The prevalence of positive TPOAb and positive TgAb in the general population is higher in females than males [6]. To identify gender specific effects we performed GWAS for each gender separately in each cohort. We used the same procedures as in the primary analyses except for the gender covariate. Association results were meta-analysed using the inverse-variance fixed-effects method. The total sample sizes for TgAb were 1596 for women and 1033 for men, while 1593 for women and 1025 for men for the TPOAb.

2.6. Bivariate analysis

We applied a multiple-trait analysis method to test the association between each SNP and the two correlated traits TgAb and TPOAb simultaneously, since joint analysis of correlated traits may increase power for identification of novel loci [30]. The association was tested using multivariate analysis of variance (MANOVA). Multi-trait association test statistic was calculated on the basis of the summary statistics from single univariate GWAS. We have also performed bivariate analysis of TgAb and TPOAb for females. Correlation among antibodies in males was not sufficient for the performance of bivariate analysis.

Table 2
Genotyping methods and quality control procedures.

	Cohorts	Split1 (first 531 individuals from Split sample)	Split2 (other 481 individuals from Split sample)	Korcula	Vis
Genome-wide genotyping	N individuals	531	481	897	960
	Genotyping platform and SNP panel	Illumina HumanHap 370CNV QUAD Phase 1	Illumina HumanOmni ExpressExome8v1-2_A	Illumina HumanHap 370CNV DUO Phase 1	Illumina Human Hap300v1 BeadChip
	N SNPs	351514	969919	346034	317509
	Genotype-calling algorithm	Illumina BeadStudio V3	Illumina BeadStudio V3	Illumina BeadStudio V3	Illumina BeadStudio V3
SNP QC (prior to imputation)	Call rate	≥98% per SNP	≥98% per SNP	≥98% per SNP	≥98% per SNP
	MAF	≥1%	≥1%	≥1%	≥1%
	HWE	$p < 10^{-7}$	$p < 10^{-7}$	$p < 10^{-7}$	$p < 10^{-7}$
Sample QC (prior to imputation)	Call rate	≥97%	≥97%	> 97%	> 95%

MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium.

2.7. Pathway analysis

Pathway analysis was performed with ConsensusPathDB (http://cpdb.molgen.mpg.de) [31,32]. For each of the loci with $P < 5x10^{-6}$, downstream genes in ± 500 kb window were extracted and used as the input for analysis. The significance of results was defined as a $P < 0.01$. The same analysis was performed separately for males and females.

3. Results

3.1. Meta-analyses

Suggestive associations from genome-wide meta-analyses of TgAb and TPOAb levels are shown in Supplementary Tables 1 and 2. We did not find any significant association in the meta-analysis for TgAb or TPOAb level. The Manhattan and Quantile-quantile plots for both traits are shown in Supplementary Figs. 1 and 2.

Genome-wide meta-analysis of TgAb levels in females revealed a borderline significant association with rs4710782 genetic variant. SNP is located near protein coding gene *DLL1* (Table 3, Figs. 1 and 2).

The rs1935377 locus had borderline significance in females for association with TPOAb (reference allele C, $P = 8.58 \times 10^{-8}$) (Table 3, Figs. 1 and 2). The SNP is located near protein coding gene *GRIN3A*.

Top findings from bivariate genome-wide meta-analyses are shown in Supplementary Fig. 3 and Supplementary Table 3. The bivariate analysis in females revealed a genome-wide significant rs4457391 locus near *GRIN3A* gene (reference allele G, $P = 7.76 \times 10^{-9}$) (Table 3, Figs. 1 and 2).

3.2. Pathway analysis

No enrichment was obtained in general population for neither TgAb nor TPOAb. Results of pathway analyses performed for each gender separately are shown in the Supplementary Tables 4 and 5.

For females, there were ten significantly enriched pathways at the $P < 0.01$ for TgAb level, while only one pathway was enriched for the level of TPOAb. For males, the enrichment for TgAb level was obtained for twenty-nine significant pathways at the $P < 0.01$, while two pathways were enriched for the level of TPOAb (Supplementary Tables 4 and 5).

3.3. Replication of previous GWA findings

We investigated variants that were previously associated with the level or/and positivity of TPOAb [16,18]. Nine loci were reported in previous studies (*TPO, ATXN2, MAGI3, KALRN, BACH2, RERE, HCP5, HLA-DOB, HLA-DPB1*), however locus *HLA-DOB* could not be tested since the SNPs or any surrogate ($r^2 > 0.5$) were not available in our data. From eight reported variants that were available for the analysis in our data set, two were nominally replicated with $P < 0.05$, SNP rs11675434 for *TPO* gene ($P = 0.009$) and SNP rs653178 in *ATXN2* gene ($P = 0.035$). All other variants with available data for effect sizes were similar in size and direction as in our study (Supplementary Table 6).

4. Discussion

Our study confirms the gender specificity of genetic influences on serum thyroid antibody level. In females, a novel significantly associated locus near *GRIN3A* gene was identified in the bivariate analysis of TgAb and TPOAb, and the same locus showed borderline significance in association with TPOAb levels. Furthermore, we detected marginally significant locus associated with variation in TgAb levels in females (*DLL1*). Genetic variants affecting the TgAb level at a genome-wide scale are analysed for the first time in this study.

The levels of TPOAb and TgAb are correlated and participate in the onset and diagnosis of AITD [33]. In our study, an intermediate

Table 3
Associations between genetic variants and TgAb and TPOAb level.

	SNP	Chr.	Position GRCh37.p13	Gene	Region of the gene	Minor allele	MAF	β	SE	*P* value
Bivariate analysis Female	rs4457391	9	104760468	*GRIN3A*	260 kb downstream	T	0.4	−0.027	0.005	$7{,}76 \times 10^{-9}$
Tg-Ab levels Female	rs4710782	6	170582064	*DLL1*	9 kb upstream	C	0.32	0.210	0.039	$6{,}16 \times 10^{-8}$
TPO-Ab levels Female	rs1935377	9	104742291	*GRIN3A*	241 kb downstream	T	0.37	−0.200	0.037	$8{,}58 \times 10^{-8}$

SNP - single nucleotide polymorphism.Chr. - chromosome.MAF - minor allele frequency.β - effect size.SE - standard error.

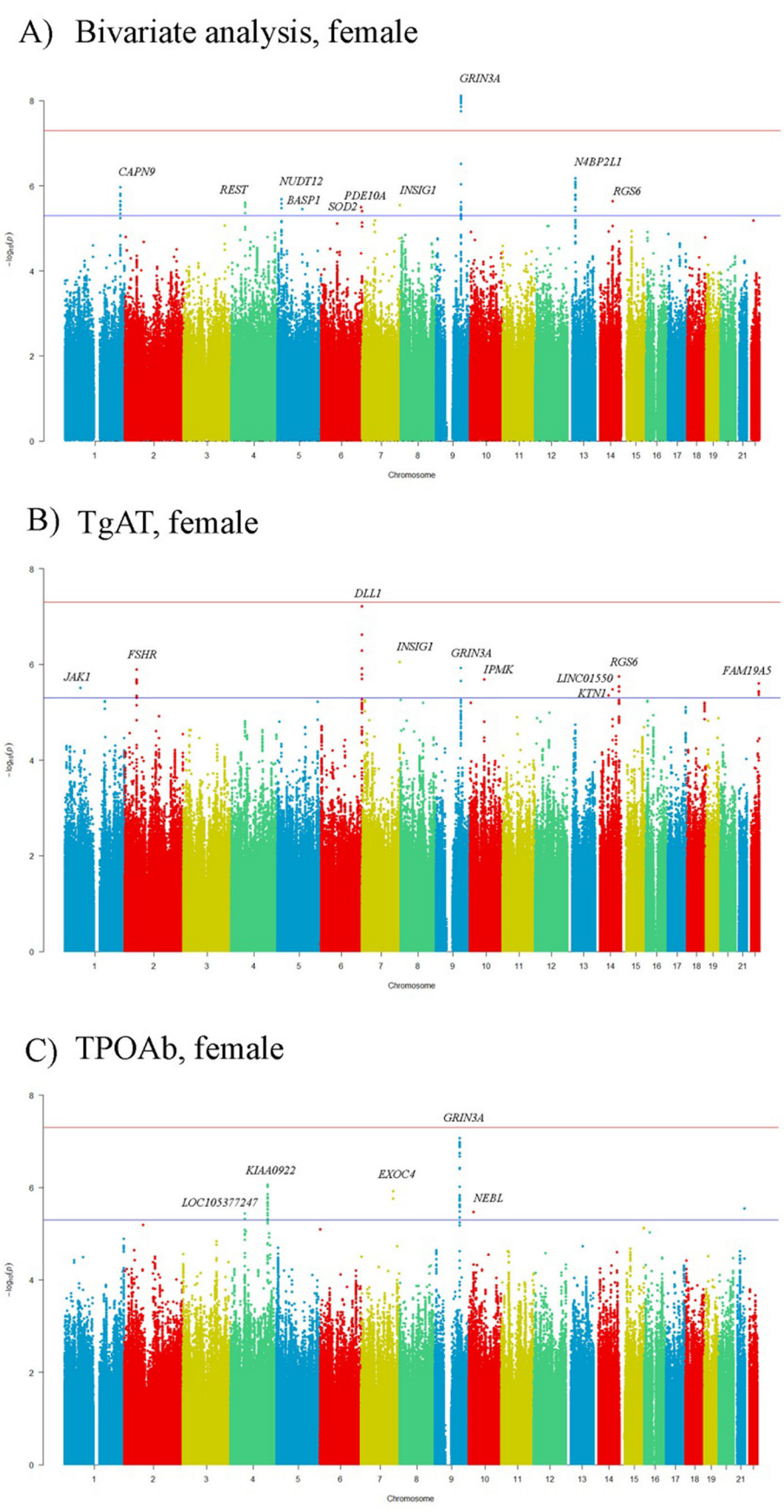

Fig. 1. A) Manhattan plot of SNPs for the bivariate and meta-analysis of females in three cohorts. The *y*-axis shows the $-\log_{10}P$ values of 5527232 SNPs, and the *x*-axis shows their chromosomal positions. The red line indicates the threshold for significant hits ($P = 5 \times 10^{-8}$) while the blue line indicates the threshold for suggestive hits ($P = 5 \times 10^{-6}$). Gene labels are provided for suggestive hits ($P = 5 \times 10^{-6}$) only B) Manhattan plot of SNPs for TgAb levels in the meta-analysis of females in three cohorts C) Manhattan plot of SNPs for TPOAb levels in the meta-analysis of females in three cohorts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

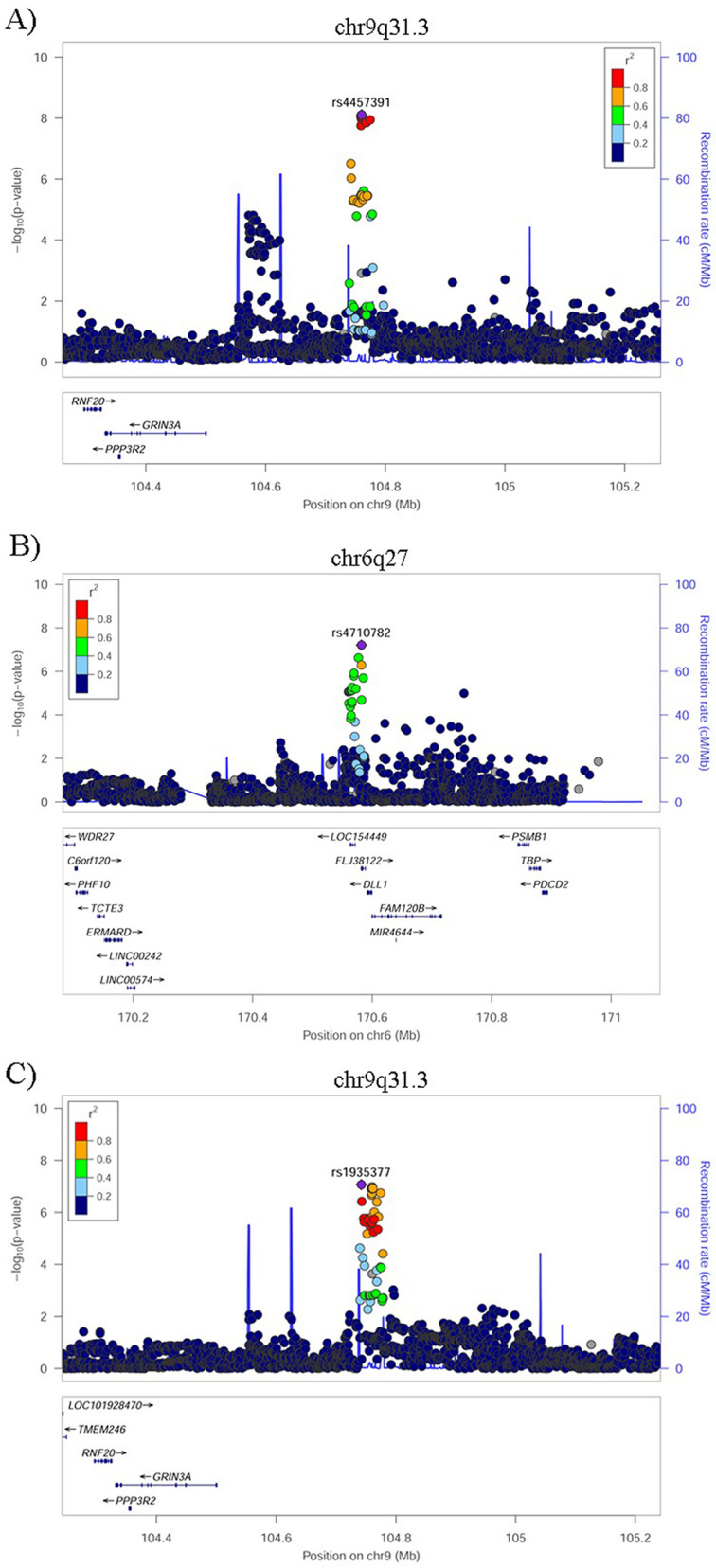

Fig. 2. A) Regional association plot for bivariate and meta-analysis of females in three cohorts for the locus rs4457391 on chromosome 9. SNPs are plotted by position against association with two correlated traits TgAb and TPOAb simultaneously ($-\log_{10}P$ values). The purple diamond highlights the most significant SNP in the meta-analysis, whereas the colours of other variant represent LD with most significant SNP. B) Regional association plot for TgAb level in the meta-analysis of females in three cohorts for the locus rs4710782 on chromosome 6. SNPs are plotted by position against association with TgAb (−log10 P values). C) Regional association plot for TPOAb level in the meta-analysis of females in three cohorts for the locus rs1935377 on chromosome 9. SNPs are plotted by position against association with TPOAb (−log10 P values). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlation between both antibodies was found in our 3 general populations (r = 0.5–0.7) as well as in females only (r = 0.6–0.7), which enabled us to perform the bivariate analysis. The bivariate analysis revealed a significant association for a locus near the *GRIN3A* gene in females, with rs4457391 as a leading SNP ($P = 7.76 \times 10^{-9}$). The rs4457391 SNP showed evidence of association for the level of TPOAb and TgAb in females ($P = 1.04 \times 10^{-7}$ and $P = 1.17 \times 10^{-5}$, respectively). The same locus with rs1935377 as leading SNP was marginally associated with TPOAb levels ($P = 8.58 \times 10^{-8}$) and suggestively associated at the bivariate analysis ($P = 3.09 \times 10^{-7}$). The rs1935377 is

in moderate LD with rs4457391 ($r^2 = 0.72$, 1000Genomes phase3). Both these polymorphisms showed no association in men with p values < 0.05, however effect sizes were in the same direction as in females.

GRIN3A gene encodes a subunit of the N-methyl-D-aspartate (NMDA) receptors, which belongs to the superfamily of glutamate-regulated ion channels. *GRIN3A* gene is expressed in several tissues, mostly in brain, bone marrow, immune system, female and male tissue [34]. The *GRIN3A* gene is associated with the high-density lipoprotein (HDL) cholesterol levels and suggestively associated with the low-density lipoprotein (LDL) cholesterol and triglyceride levels [35]. Decreased levels of thyroid hormones in the liver have an effect on the breakdown of circulating cholesterol, consequently, the higher level of triglycerides, total and LDL cholesterol and lower level of HDL cholesterol are associated with hypothyroidism [36]. It is important to emphasize that Eriksson et al. found evidence of association of intronic variant (rs9792648, 2.7×10^{-5}) in *GRIN3A* gene with the hypothyroidism [37]. A recent study by Joehanes et al. showed that SNPs associated with *GRIN3A* have the trans-eQTL effect, thus may act on phenotypes by affecting the expression of distant genes [38]. For distant affected genes (*ZNF782*, *LCE2B*, *TEKT5* and *MORF4L2*) from the study [38], polymorphisms with evidence of association with the level of hypothyroidism, LDL, HDL and total cholesterol, as well as with adiponectin level were found [37,39–42]. For polymorphisms in *ZNF782* gene evidence of genome-wide association with hypothyroidism ($P = 10^{-5}$), as well as with LDL and total cholesterol level ($P = 10^{-2}$–10^{-3}) were detected.

Genes underlying AITD can be divided into thyroid-specific genes and immunoregulatory genes [14]. GD and HT, although clinically antithetical, share number of immunological features including thyroid lymphocytic infiltration and autoreactivity against the key thyroid autoantigens [43]. Different genes and mechanisms seem to be implicated in autoimmune prevalence in men and woman [13,14].

Gender specific GWAS meta-analysis identified a novel locus associated with TgAb levels in females. Identified variant rs4710782 ($P = 6 \times 10^{-8}$) is located on chromosome 6, 9 kb upstream of the protein coding gene *DLL1*. *DLL1* is a human homolog of the Notch Delta ligand and a member of the delta/serrate/jagged family [44]. It plays a role in mediating cell fate decisions during lymphopoiesis. Notch ligand Delta-1 inhibits the differentiation of human hematopoietic progenitors into the B cell lineage, while promotes the emergence of cells with a phenotype of T cell/natural killer (NK) precursor [45]. Notch1 signaling plays a role in promoting maturation into both the CD4 and CD8 T cell lineages [46]. CD4 T cells induce B cells in antibody production both in HD and GD, while CD8 T cells cause the death of thyrocytes in HT [8]. Variations near *DLL1* gene are associated with type 1 diabetes (T1D) [47] and suggestively associated with systemic lupus erythematosus (SLE) [48]. T1D, as well as SLE, frequently occur with AITD within the same individuals [49,50].

In general population, bivariate analysis revealed some interesting, suggestive associations with *PDE10A* gene (rs611909, $P = 2.37 \times 10^{-6}$), which was previously associated with thyroid stimulating hormone (TSH) levels, as well as with the hypothyroidism [43]. Likewise, *NFIA* gene (rs17121639, $P = 2.97 \times 10^{-6}$), previously associated with the level of TSH [43], had the suggestive association in the general population. These findings imply on the possibility of shared genetic susceptibility for thyroid function and autoimmunity.

While there were no functionally enrichment pathways for general populations, interesting findings were obtained for both antibodies in gender specific manner. Most of the enrichments were related with different immune and inflammatory responses. The most interesting pathways enriched for TgAb levels in females were Proteasome Degradation (Wikipathways), Notch, Hedgehog and GPCR signaling-G alpha i (INOH). For TPOAb levels in females, the pathway for Neutrophil degranulation (Reactome) was enriched. The most interesting pathway enriched for TgAb levels in males were Alpha9 beta1 integrin signaling events (PID) and Vitamin D Receptor Pathway (Wikipathways), while for the TPOAb Inflammatory mediator regulation of TRP channels (KEGG) and Vitamin D Receptor Pathway (Wikipathways).

Our study has helped in additional clarification of genetic variants associated with the TgAb and the TPOAb level. Thyroid autoimmunity is a consequence of the complex interaction of multiple genes and pathways, and possibly has different ethology depending on the gender. More GWA studies will be needed in the further enlightenment of this complex trait.

There are several limitations in our study. We had a modest number of participants for genome-wide association analyses, a larger study should be performed in order to replicate our findings and discover novel associated loci. Also, our analysis was restricted to participants of European ancestry, thus further GWAS on populations of different ancestry will be required. We did not perform additional functional studies for identified variants to clarify biological mechanism behind our findings.

5. Conclusion

We identified gender specific genetic factors associated with thyroid autoimmunity. We detected significantly associated locus (*GRIN3A)* in females with bivariate analysis, and likewise, the same locus was marginally associated in females with variation in TPOAb levels. Furthermore, we found a novel locus (*DLL1)* marginally associated with TgAb levels in females. Overall, our findings add to the knowledge of shared genetic susceptibility affecting thyroid antibodies, as well as of genetic factors that differently affect thyroid autoimmunity in males and females.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2018.04.012.

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Dietary Factors Associated with Plasma Thyroid Peroxidase and Thyroglobulin Antibodies

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Abstract: The knowledge about dietary habits and their influence in the development of autoimmune thyroid disease is insufficient. The aim of this study was to analyse the association of dietary factors and plasma thyroid peroxidase antibodies (TPO-Ab) and/or thyroglobulin antibodies (Tg-Ab). The study enrolled 1887 participants originating from the South Croatia. Participants with elevated plasma TPO-Ab and/or Tg-Ab were defined as cases (n = 462) and those with TPO-Ab and/or Tg-Ab within referent values were defined as controls (n = 1425). Dietary intake was evaluated according to a food frequency questionnaire containing 58 food items. Principal component analysis was used to group food items into dietary groups. We used logistic regression analysis to examine dietary groups associated with positive plasma TPO-Ab and/or Tg-Ab. The results indicate that the dietary group with frequent consumption of animal fats and butter is associated with positive plasma TPO-Ab and/or Tg-Ab (p = 0.01). The dietary group with frequent consumption of vegetables as well as the dietary group with high consumption of dried fruit, nuts, and muesli are associated with negative findings of TPO-Ab and/or Tg-Ab (p = 0.048 and p = 0.02, respectively). We showed that the anti-inflammatory dietary groups are associated with the negative findings of plasma TPO-Ab and/or Tg-Ab.

Keywords: autoimmune thyroid diseases; thyroid peroxidase antibodies; thyroglobulin antibodies; dietary habits

1. Introduction

Thyroid disorders are, beside diabetes mellitus, the most frequent disorders affecting endocrine systems. Autoimmune thyroid diseases (AITD) such as Hashimoto disease and Graves disease are characterised by an autoimmune reaction against thyroid autoantigens [1]. One of the first findings of AITD is positive serum thyroid peroxidase antibody (TPO-Ab) [2]. Genetic background significantly contributes to the development of autoimmune thyroid diseases (70 to 80%), but their occurrences are also associated with different environmental factors (20 to 30%) [1].

It has been shown that increased occurrence of thyroid autoantibodies is the result of iodine sufficiency or excessive iodine intake [3–5].

Among other environmental factors, smoking was associated with increased risk for Graves disease (GD) but not with Hashimoto thyroiditis (HT), while moderate alcohol consumption had a protective role in the development of GD and HT [1,3]. Low selenium intake and low serum vitamin D levels were shown to be associated with higher risk of AITD, but the data is still inconclusive [1,3]. Stress could be a risk factor for GD, but has not been studied enough for HT [1,3]. A lower incidence of GD was linked to estrogen intake [1]. Infection with Yersinia enterocolitica was shown to be associated with GD, while enterovirus infection was associated with HT [1,3]. Molecular mimicry is a possible mechanism for pathogen association with infections and immune response. Recent studies based on bioinformatics data support the triggering role of several bacterial and viruses in the onset of AITD [6]. The intake of different drugs was related with GD and HT [1,3].

However, there are no comprehensive studies investigating dietary habits and their influence on AITD to date. Only two studies analysed the prevalence of self-reported hypothyroidism and hyperthyroidism in relation to dietary habits, and found a protective effect of vegan diet from hypothyroidism, and both vegan and vegetarian diets from hyperthyroidism [7,8].

In the current study, we evaluated the association of dietary factors and plasma thyroid antibodies. The goal of the study was to identify groups of food items that are associated with positive or negative findings of plasma TPO-Ab and/or Tg-Ab.

2. Materials and Methods

This case-control study was performed using the data collected through the "10,001 Dalmatians" project [9,10]. The project provided comprehensive data about the dietary habits of participants, as well as stored plasma samples that were used for biochemical measurements in this study. We included 1887 participants originating from the Dalmatian region of South Croatia (921 from the island of Korcula and 966 from the city of Split). The participants were adult volunteers from the general population (over 18 years of age). A written informed consent was obtained from participants and the study protocol was approved by the Ethical board of the University of Split, School of Medicine (No: 2181-198-03-04-14-0031).

Participants were allocated in either group based on thyroid antibodies status; subjects were considered as cases if their level of TPO-Ab and/or TgAb were higher than referent values (TPO-Ab > 16 IU/mL and Tg-Ab > 100 IU/mL), while those with negative findings (within referent values) of TPO-Ab and Tg-Ab were determined as controls. Controls who were taking thyroid medication (n = 27) or who had undergone thyroid surgery (n = 10) were excluded from the analysis. Finally, a total of 462 cases and 1388 controls were included in the study. Out of 462 cases, five had other autoimmune diseases: four had psoriasis, and one had psoriatic arthritis. In the control group, one out of 1388 participants had systemic lupus erythematosus.

Plasma TPO-Ab and Tg-Ab were determined by the sandwich chemiluminescence immunoassay method in the Laboratory of Biochemistry, Department of Nuclear Medicine, University Hospital Split (Split, Croatia). The immunoassay was conducted in a fully automated instrument "Liaison" Biomedica Chemiluminescence Analyser (DiaSorin, Saluggia, Italy) using in vitro assays for the quantitative determination of TPO-Ab and Tg-Ab in the plasma.

Dietary intake was assessed with a food frequency questionnaire (FFQ) that consisted of 54 foods and beverages. The frequency of food intake was measured using five categories: every day, 2–3 times a week, once a week, occasionally, and never. Additionally, there were four questions regarding the frequency of fat consumption with three possible answers (always, sometimes, never). For analysis, frequency categories for each food item were converted into an equivalent weekly intake as follows: every day (converted to 7 times a week), 2–3 times a week (2.5), once a week (1), occasionally (0.5—once in two weeks), and never (0). Responses on the frequency of fat consumption were also converted to an equivalent weekly intake as follows: always (7 times a week), sometimes (2.5 times a week), and never (0 times a week).

The data are presented as the mean ± standard deviation (SD) for continuous variables and as frequencies (percentages) for categorical variables in Table 1. The χ^2 test was used to assess the differences between groups for categorical variables, and the t-test was used for numerical variables.

Principal Component Analysis (PCA) was used to identify underlying patterns of food consumption to reduce the list of 58 food items to key dietary groups (factors), such that the foods in each dietary group tend to be consumed equally often. The factors were rotated by orthogonal transformation (varimax rotation) to obtain a more interpretable structure. Propriety of using factor analysis was tested by the Kaiser-Meyer-Olkin measure of sampling adequacy and Bartlett's test of sphericity. Factors with an eigenvalue greater than 1.0 were retained. A food item was considered to load on a given factor if the absolute factor loading value was >0.3 for that factor and ≤0.3 for all other factors. The factor loading of a food item increases as the contribution to the corresponding dietary group increases. Each factor contains a distinct set of food items. Factor-specific scores were calculated using the regression method and assigned to each participant. Odds ratio (OR) and 95% confidence intervals (CI) were calculated by multiple logistic regression to examine factors associated with positive plasma TPO-Ab and/or Tg-Ab, i.e., between cases and controls. The logistic regression model included gender and dietary factors. *p*-values of less than 0.05 were considered as statistically significant. Statistical analysis was conducted using Statistical Package Software for Social Science, version 16 (SPSS Inc., Chicago, IL, USA).

3. Results

A total of 462 cases and 1388 healthy individuals were enrolled in this study. The characteristics of study participants are shown in Table 1. A significantly higher number of women had positive TPO-Ab and/or Tg-Ab, while there was no statistically significant difference in age and body mass index (BMI) between cases and controls (Table 1).

Table 1. Differences between cases and controls in sociodemographic characteristics.

Variable	Cases	Controls	*p* Value
Gender			<0.001 [a]
Males	119 (25.8%)	584 (42%)	
Females	343 (74.2%)	804 (58%)	
Age (year)	53.66 ± 13.65	52.72 ± 14.93	0.45 [b]
BMI	27.78 ± 7.29	27.95 ± 7.79	0.67 [b]

BMI: Body mass index. [a] χ^2 test, [b] *t*-test.

Suitability of the respondent data for factor analysis was supported by the Kaiser-Meyer-Olkin measure of sampling adequacy (0.77) and Bartlett's test of sphericity ($p < 0.001$). Factor analysis revealed 19 dietary factors, which explained 54.76% of the total variance in food intake. The factors were generally in accordance with conventional dietary groups. Loading values for factors are presented in Table 2.

Logistic regression analysis revealed that the dietary group with high loadings for root vegetables, flower vegetables, leafy vegetables, fruity vegetables, and legumes was negatively associated with the plasma TPO-Ab and/or Tg-Ab (OR = 0.88, 95% CI 0.78–0.99, $p = 0.048$). The dietary group with high loadings for dried fruit, nuts, and muesli was also negatively associated (OR = 0.86, 95% CI 0.76–0.98, $p = 0.02$), while the dietary group with high loadings for butter and animal fats was positively associated with plasma TPO-Ab and/or Tg-Ab (OR = 1.16, 95% CI 1.03–1.30, $p = 0.01$). Other dietary groups showed no association with plasma TPO-Ab and/or Tg-Ab. Results from the logistic regression analysis are shown in Figure 1.

Table 2. List of food items and factor loadings for 19 dietary groups (factors) identified using principal component analysis.

Factors	Food Items (Factor Loadings)
Factor 1	Root vegetables (0.79), flower vegetables (0.76), leafy vegetables (0.74), fruity vegetables (0.70), legumes (0.54)
Factor 2	Squid and octopus (0.78), sea-food (shells, crab) (0.71), blue fish (0.67), dried fish and salted sardines (0.56), white fish (0.49)
Factor 3	Chocolate (0.78), cookies (0.76), cakes (0.69), bonbons (0.51)
Factor 4	Salami (0.69), canned meat derivates (0.62), sausages (0.57), eggs (0.45), bacon (0.36)
Factor 5	Cedevita (powder based vitamin juice) (0.68), fruits juices (0.62), refreshing non-alcoholic drinks (0.60)
Factor 6	Bran bread (−0.81), white bread (0.73)
Factor 7	Full-fat cheese (0.69), cottage cheese (0.63), hard cheese (0.52), sour cream (0.46)
Factor 8	Venison (0.76), fish derivates (0.53)
Factor 9	Butter (0.73), animal fats (0.70)
Factor 10	Internal organs (0.73), lamb (0.52), pork (0.37)
Factor 11	Mushrooms (0.64), canned and pickled vegetables (0.61), potato (−0.30)
Factor 12	Muesli (0.70), dried fruit (0.56), nuts (0.44)
Factor 13	Hard liquor (0.68), vegetables juices (0.57), powder soups (0.33)
Factor 14	Tea (0.64), olive oil (0.40)
Factor 15	Milk (0.70), coffee (0.51), yoghurt (0.49), fresh fruits (0.40)
Factor 16	Chicken (0.68), turkey (0.68)
Factor 17	Beef (0.73), veal (0.34)
Factor 18	Macaroni or rice (0.63), jam and marmalade (0.37), fruit compote (0.33)
Factor 19	Plant oil (0.77)

Absolute values ≤ 0.3 were excluded for simplicity.

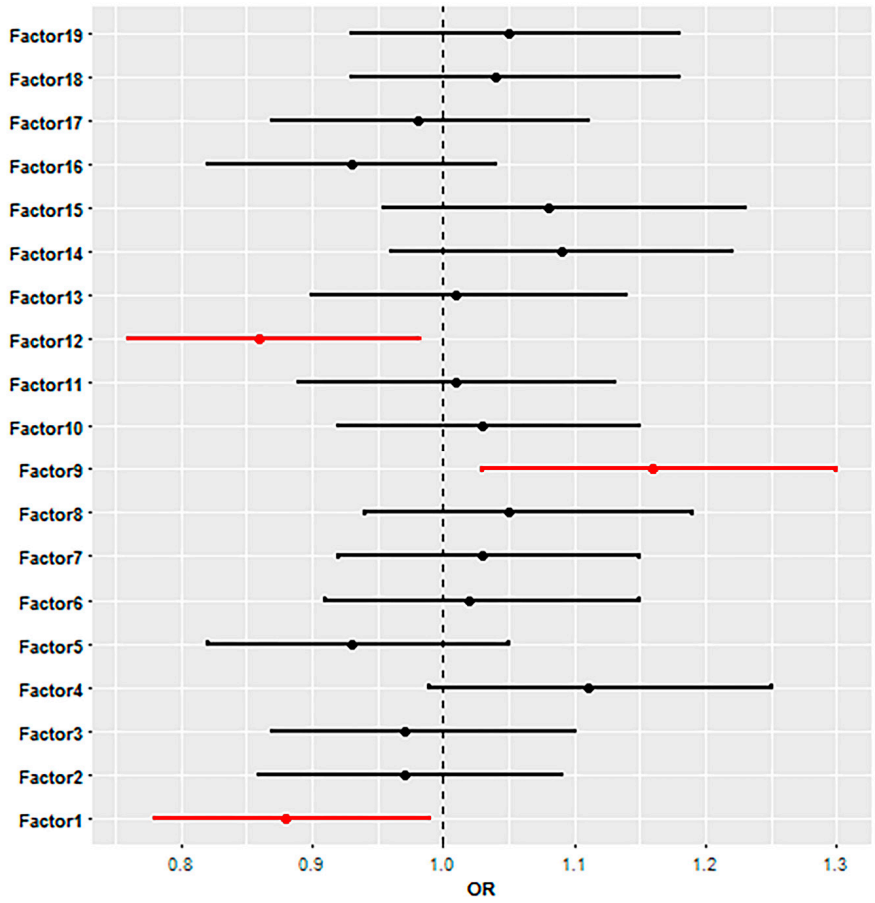

Figure 1. Odds ratios (OR) and 95% confidence intervals obtained from the logistic regression analysis for the association of dietary groups (factors) with plasma TPO-Ab and/or Tg-Ab. Significant dietary groups are coloured in red, while not-significant dietary groups are coloured in black. If the OR and the lower limit of the 95% confidence interval are above 1, the dietary group is positively associated with plasma TPO-Ab and/or Tg-Ab, whereas if the OR and the upper limit of

the 95% confidence interval are below 1, the dietary group is negatively associated with plasma TPO-Ab and/or Tg-Ab.

4. Discussion

This study has shown that the dietary group (factor 9) with frequent consumption of animal fats and butter was associated with positive plasma TPO-Ab and/or Tg-Ab, while the dietary groups (factors 1 and 12) with frequent consumption of different sorts of vegetables, dried fruit, nuts, and muesli were associated with negative findings of TPO-Ab and/or Tg-Ab.

Furthermore, the study showed that a higher number of women had positive TPO-Ab and/or Tg-Ab, which is in accordance with previously published studies [1,3,11]. Age and body mass index did not show association with positive TPO-Ab and/or Tg-Ab. The influence of body mass index is controversial in the literature, while the prevalence of positive thyroid antibodies increases with age [11–13].

Butter and animal fats are rich in saturated fatty acids (SFA) [14,15]. Vegetables, nuts, and cereals are rich in polyunsaturated fatty acids (PUFA) [16]. All the food items from dietary groups 1 and 12 of this study are rich in *n*-6 and *n*-3 PUFAs. Dietary *n*-6 and *n*-3 PUFAs are imbedded into the cell plasma membranes, where they serve as precursors in the synthesis of eicosanoids. These two types of PUFAs have opposite effects on inflammatory responses, whereas *n*-6 PUFAs serve as the precursors of inflammatory eicosanoids while *n*-3 generates anti-inflammatory eicosanoids [17]. The rate of *n*-6 conversion into *n*-3 in humans is below 5% and, consequently, the levels of anti-inflammatory eicosanoids are almost completely linked to the amount of dietary *n*-3 consumption [14]. Eicosanoids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are involved in the regulation of pro-inflammatory cytokines and act as anti-inflammatory mediators, and therefore have benefits in autoimmune diseases such as rheumatoid arthritis [17].

Studies performed in experimental animals showed that a diet enriched with *n*-3 fatty acids suppresses the inflammation that accompanies autoimmune reactions [18–21]. The results indicate that *n*-3 PUFAs reduce the differentiation of Th17 cells from naive CD4+ T cells by modifying the lipid rafts regions in their plasma membrane and decreasing the formation of IL-6 receptors [18]. It is known that Th17 cells positively correlate with serum TPO-Ab and Tg-Ab [22]. Recently published studies showed that Th17 cells play the main role in the pathogenesis of AITD [23,24].

Food items included in dietary groups 1 and 12 are rich in *n*-3 fatty acids and seem to suppress the production of plasma TPO-Ab and Tg-Ab. On the contrary, saturated fatty acids of animal origin (dietary group 9) seem to have harmful influence on TPO-Ab and/or Tg-Ab production. Frequent consumption of animal fats and butter could result in a low ratio of *n*-3 fatty acids (anti-inflammatory)/*n*-6 fatty acids (inflammatory) in the diet and thus a lack of suppression of Th17 T cells differentiation, resulting in the promote TPO-Ab and/or Tg-Ab production [18,22].

Phytosterols are present in moderate and small amounts in nuts and vegetables, respectively, and have shown immunomodulatory and anti-inflammatory properties [25,26]. They are contained in dietary groups 1 and 12, and are associated with negative findings of plasma TPO-Ab and/or Tg-Ab. One of the proposed ways of their immunomodulation activity is the reduction of IL-6 plasma levels [25,26]. Since IL-6 is the main stimulator in Th17 cells differentiation, the reduction of IL-6 could be the reason behind the protective effect of phytosterols in the pathogenesis of AITD.

Polyphenols, abundant micronutrients in the diet, are components of fruits and vegetables. They are known for their anti-inflammatory, immunomodulatory, and antioxidative effect in the body [27,28]. Gallic acid could have a major role in the explanation of the beneficial effect of dried fruit and muesli (dietary group 12) on positive plasma TPO-Ab and/or Tg-Ab observed in this study. Gallic acid is a component of red fruit, but it can also be found in apple peels and grapes [28]. Red fruits are often consumed as dried fruit, and some of them are common components of muesli. Kuppan et al. found that treatment of human monocytes with gallic acid reduces the expression of IL-6 gene [29]. Hence, the reduction of IL-6 plasma levels could have a suppression effect in differentiation of Th17 cells involved in the pathogenesis of AITD.

To date, two studies have been published regarding diet and hypothyroidism/hyperthyroidism. The authors reported an association of vegan diet and lower risk of hypothyroidism [7]. The same group of authors also showed that vegan and vegetarian diets are associated with lower risk of hyperthyroidism [8]. The authors suggest a possible protective effect of polyphenols (such as flavonoids) against autoimmune processes, but a detailed explanation on the molecular level is not provided. They also discuss the possible effect of environmental toxins from food on the microbiome and their possible stimulation of autoimmune disease [7,8].

Benvenga et al. showed an association of lower serum thyroid autoantibodies and oily fish consumption and hypothesised about the protective effect of *n*-3 fatty acids [30]. In our study, dietary group 2, composed of different seafoods, did not reach significance in the association. The possible reason for this could be the presence of squids, octopus, shells, crabs, and white fish in that group, which are not as rich in *n*-3 fatty acids as blue (oily) fish [31]. Milerova et al. showed a positive correlation of phytoestrogen genistein with TgAb level in the sera of school children screened for iodine deficiency. However, relatively low iodine intake might be related with this finding [32].

Saturated fatty acids of animal origin, mostly presented in pro-inflammatory Western diets, showed a negative effect, while anti-inflammatory vegetarian and especially vegan diets showed a beneficial effect in the pathogenesis of other autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus [33–36].

The present study has several limitations. This is a cross-sectional study and therefore only associations, and not causations, were inferred. Furthermore, all variables regarding food consumption were self-reported. Food frequency questionnaire had limitations for quantitative assessment of food intake; however, it provided relevant dietary information. Although we controlled for sociodemographic variables, unmeasured confounding factors could be present, including inflammatory parameters that may influence associations.

5. Conclusions

In summary, the present study demonstrated the association of animal fats and butter consumption with the positive plasma TPO-Ab and/or Tg-Ab. Vegetables, dried fruit, nuts, and muesli consumption was associated with the negative findings of TPO-Ab and/or Tg-Ab. In light of recently published studies, we discussed the possible protective effect of *n*-3 fatty acids from vegetables and nuts on the IL-6 receptors of the CD4+ T cells plasma membranes, and the suppression of their differentiation in Th17 T cells involved in the pathogenesis of AITD. Likewise, we discussed the suppressive effect of phytosterols and polyphenols found in vegetables, nuts, dried fruit, and muesli on IL-6 secretion. It seems that the frequent consumption of anti-inflammatory food items (vegetables, nuts, dried fruit, and muesli, as presented in dietary groups 1 and 12) is associated with the negative findings of plasma TPO-Ab and/or Tg-Ab.

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