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Polimorfizmi izbranih genov vnetnega procesa kot genetski označevalci napredovanja ateroskleroze vratnih arterij pri bolnikih s sladkorno boleznijo tipa 2

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Polymorphisms of selected genes of inflammatory process as genetic markers of progression of carotid atherosclerosis in patients with type 2 diabetes

Mentor: prof. dr. Danijel Petrovič, dr. med.

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Podpisani Aleš Pleskovič izjavljam, da je doktorsko delo z naslovom Polimorfizmi izbranih genov vnetnega procesa kot genetski označevalci napredovanja ateroskleroze vratnih arterij pri bolnikih s sladkorno boleznijo tipa 2 rezultat lastnega raziskovalnega dela. Laboratorijski del doktorskega dela je bil opravljen v Genetskem laboratoriju na Inštitutu za histologijo in embriologijo MF UL, klinični del pa v kardioloških ambulantah Medicor d.o.o. in MC MEDICOR d.d..

Aleš Pleskovič

Vsebina

Izvleček

Sladkorna bolezen (SB) je kronična presnovna motnja, povezana s kroničnimi zapleti. Eden od kroničnih zapletov je ateroskleroza karotid, ki je multifaktorska bolezen. Zaenkrat ni jasno, kateri so njeni vnetni genetski dejavniki in kako ti vplivajo na nastanek in napredovanje ateroskleroze karotid pri bolnikih s SB tipa 2.

Naše hipoteze so bile: **1**. polimorfizmi testiranih genov vnetnih genov so povezani z debelino intime medije (DIM), debelino plakov in s seštevkom plakov (angl. plaque score) na vratnih arterijah ter z bolj nestabilnimi plaki pri SB tipa 2; **2**. genetska raznolikost testiranih vnetnih genov vpliva na napredovanje aterosklerotičnega procesa v dveletnem obdobju opazovanja pri bolnikih s SB tipa 2; **3.** pri bolnikih s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP ≥ 2 mg/L) poteka aterosklerotični proces hitreje kot pri tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L).

Zasnova raziskave, opis metod, preiskovancev: v prospektivno raziskavo smo vključili 795 preiskovancev; 595 bolnikov s SB tipa 2 in 200 preiskovancev v kontrolni skupini brez SB tipa 2. Izvedli smo genetske analize s pomočjo polimerazne verižne reakcije v realnem času in testirali 17 polimorfizmov v izbranih genih, ki so vpleteni v vnetni odgovor.

Potrdili smo, da poteka pri bolnikih s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP ≥ 2 mg/L) aterosklerotični proces hitreje kot pri tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L). Bolniki s SB tipa 2 z izrazitejšim sistemskim vnetjem so imeli večjo DIM in pogostejše karotidne plake kot bolniki s SB 2 in hs-CRP < 2 mg/L. Primerjava bolnikov s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP ≥ 2 mg/L) in tistih tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L) ni pokazala statistično značilno povezane z UZ markerji napredovanja ateroskleroze karotid (spremembo DIM, porastom števila segmentov s karotidnimi plaki, spremembo seštevka debeline plakov) pri preiskovancih s SB 2. Linearna regresijska analiza je pokazala, da so vrednosti hs-CRP ≥ 2 mg/L statistično značilno povezane s porastom števila segmentov s karotidnimi plaki in spremembo DIM (napredovanje) pri preiskovancih s SB 2. V raziskavi smo ugotovili, da sta bila med 17-imi polimorfizmi dva povezana z DIM, in sicer rs3025058 v *genu za MMP-3* in rs8192673 v genu za koaktivator *PPARγ*.

Ugotovili smo, da je bilo pet polimorfizmov povezanih s pojavom plakov pri preiskovancih s SB 2, in sicer rs1800587 v genu za *IL-1$^{\alpha}$*, rs1143634 v genu za *IL-1β*, rs1801282 v genu za *PPARγ*, rs4754 v genu za *SPP1* in rs2073618 v genu za osteoprotegerin. Ugotovili smo, da je bilo pet polimorfizmov povezanih z atereosklerotičnim procesom pri preiskovancih s SB 2; rs1800587 in rs1143634 v genu za *IL-1$^{\alpha}$* sta bila povezana s seštevkom debeline plakov, rs1143634 v genu za *IL-1β* je bil povezan s številom segmentov s plaki, rs1801282 v genu za *PPARγ* je bil povezan s prisotnostjo plakov, rs4754 v genu za SPP1 s prisotnostjo karotidnih plakov in rs2073618 v genu za osteoprotegerin je bil povezan s številom segmentov s plaki in prisotnostjo karotidnih plakov. Ugotovili smo, da je bil z napredovanjem aterosklerotičnega procesa v 3,5-letnem obdobju opazovanja pri bolnikih s SB tipa 2 povezan rs1143634 v genu za *IL-1β*.

Glede na rezultate naše raziskave lahko zaključimo, da so vnetje in polimorfizmi izbranih vnetnih genov vpleteni v pojav in napredovanje aterosklerotičnega procesa na vratnih arterijah pri bolnikih s SB tipa 2.

Abstract

Background: Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder which is associated with microvascular and macrovascular disorders. One of the most important macrovascular disorders is carotid atherosclerosis (CA). Atherosclerosis is a multifactorial disorder, and several genetic and environmental factors are involved in the development and progression of CA.

Our hypotheses were: Polymorphisms of the inflammatory genes are involved in the development and progression of CA in subjects with T2DM. The progression of CA is faster in subjects with T2DM with more intense inflammation (subjects with hs-CRP ≥ 2 mg/L) in comparison with subjects with a less intense inflammatory response (hs-CRP below 2 mg/L).

Material and methods: 595 subjects with T2DM and 200 subjects without T2DM were enrolled in the prospective study. The DNA of the participants was analyzed with a real-time polymerase chain reaction, and 17 polymorphisms in 12 genes were evaluated. Subclinical markers of carotid atherosclerosis were assessed ultrasonographically (carotid intima media thickness (CIMT), number of affected segments of carotid arteries, and sum of plaque thickness) at the time of enrolment and after a 3.8 ± 0.5-years.

Continuous clinical data were compared using the unpaired Student's *t* test or analysis of variance (ANOVA) when normally distributed, and with the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X^2 test was used to compare discrete variables and to test whether the genotype distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables. A multivariable linear regression analysis was performed to determine the association of the tested polymorphisms with the CIMT/annual progression of CIMT and the change in the number of sites with plaque/total plaque thickness. A multivariate logistic regression analysis was performed to determine the association of the tested polymorphisms with the presence of atherosclerotic plaques on the carotid arteries or the presence of unstable plaques. A two-tailed P value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

Subjects with T2DM with hs-CRP ≥ 2 mg/L had higher carotid intima media thickness (CIMT) in comparison with subjects with T2DM with hs-CRP below 2 mg/L, and a higher incidence of plaques and unstable plaques in comparison with subjects with T2DM with hs-CRP below 2 mg/L. The multivariate logistic regression analysis found an association between the HDL cholesterol level and the presence of plaques, whereas the inflammatory marker hs-CRP was not associated with subclinical markers of progression of carotid atherosclerosis. The multiple linear regression analysis found an association between the hs-CRP level and either the CIMT progression rate or a change in the number of sites with plaques in the 3.8-year follow-up.

Our study demonstrated that among 17 tested polymorphisms, two polymorphisms were associated with CIMT in subjects with T2DM: rs3025058 in the matrix metalloproteinase-3 and rs8192673 in the coactivatory of the *PPARγ*.

Furthermore, our study demonstrated that five polymorphisms were associated with the atherosclerotic process in subjects with T2DM. Namely, rs1800587 and rs1143634 in the interleukin-1α gene were associated with the total plaque score, rs1143634 in the interleukin-1β gene was associated with the number of involved carotid segments, rs1801282 in the *PPARγ* gene was associated with the presence of carotid plaques, rs4754 in the *SPP1* gene was associated with the presence of carotid plaques, and rs2073618 in the osteoprotegerin gene was associated with the presence of carotid plaques and the number of involved carotid segments. Moreover, rs1143634 in the interleukin-1β gene affected the progression of CA in the 3.8-year follow-up in subjects with T2DM.

According to our findings, we may conclude that inflammation and the polymorphisms of the inflammatory genes are associated with the development and progression of CA in subjects with T2DM.

Uvod

Ateroskleroza je kronična vnetna bolezen arterij, ki je vodilni vzrok obolevnosti in umrljivosti v razvitem svetu (Ross, 1999). Ateroskleroza je multifaktorska genetsko pogojena bolezen, kar pomeni, da na njen nastanek vplivajo številni genetski dejavniki in dejavniki okolja. Poznani dejavniki tveganja za aterosklerozo so arterijska hipertenzija, hiperholesterolemija, sladkorna bolezen (SB), spol, starost, prekomerna telesna teža, pozitivna družinska anamneza, povišane vrednosti serumskega kreatinina, povišana serumska vrednost visoko občutljivega CRP in hiperhomocisteinemija (Ridker in sod., 1997; Grundy in sod., 1999; Booth in sod., 2006). Ateroskleroza karotidnih arterij je zelo razširjena bolezen, saj jo ugotovimo pri 9% vseh oseb starejših od 60 let in je s staranjem populacije vedno bolj pogosta (Fuller in sod., 1995; Bots in sod., 1996). Ateroskleroza karotidnih arterij se najbolj pogosto pojavlja na razcepišču skupne karotidne arterije na zunanjo in notranjo karotidno arterijo, verjetno zaradi velikih hemodinamskih sprememb, ki nastanejo po razcepu, kot tudi sprememb strižnih sil (Glagov in sod., 1988). Patomorfološko se kaže z nastajanjem aterosklerotičnega plaka na žilni steni, ki lahko povzroči zožitev lumna in zmanjšanje prožnosti krvne žile (negativna remodelacija) ali nastanek anevrizme (pozitivna remodelacija). Negativna remodelacija je pogostejša in njene posledice so odvisne od stopnje stenoze. Bolniki so lahko brez kliničnih posledic, toda pri stenozi >70% se praviloma pojavijo simptomi, in lahko privede do ishemije možganov (prehodne ali trajne-možganske kapi). Možganska kap je drugi vodilni vzrok smrti po vsem svetu, samo v ZDA zboli za možgansko kapjo vsako leto okoli 700 000 ljudi (Bots in sod., 1996). V 20-25% je vzrok ishemija, ki jo povzroča ateroskleroza karotidnih arterij v njihovem ekstrakranialnem delu.

Med pomembnimi dejavniki tveganja za aterosklerozo je SB (Ross, 1999; Booth in sod., 2006; Ryden in sod., 2007). Za SB je znano, da pospeši potek ateroskleroze arterij, kar se kaže v približno 15 let zgodnejšem pojavu srčnožilnih zapletov (Booth in sod., 2006). Srčnožilne bolezni so hkrati tudi glavni vzrok smrtnosti pri bolnikih s SB (Grundy in sod., 1999). Mehanizmi vpliva SB na hitrejši razvoj ateroskleroze so kompleksni in še niso povsem razjasnjeni (Brownlee, 2001). Dosedanje raziskave kažejo, da naj bi imela pri hitrejšem razvoju ateroskleroze pri bolnikih s SB pomembno vlogo oksidativni stres in vnetje (Brownlee, 2001; Marfella in sod., 2001; Esposito in sod., 2002; Šantl Letonja in sod., 2012). Vnetni proces vpliva na vse faze aterotrombotičnega procesa (Ross, 1999; Libby in sod., 2002). Vnetje je vpleteno pri adheziji celic, rasti plaka, razgradnji matriksa in kolagena, pri razmnoževanju gladkih mišičnih celic, povečani reaktivnosti trombocitov kot tudi pri nastanku tromboze (van der Wal in sod., 1994; Ross, 1999; Libby in Simon, 2001; Libby in sod., 2002). Velik vpliv na uravnavanje vnetnega procesa ima imunski sistem (pridobljena in prirojena imunost) (Hanson in Libby, 2006; Davies in sod., 2012).

Celice, ki sodelujejo v vnetnem procesu pri aterosklerozi vratnih arterij (endotelijske celice, trombociti, limfociti, makrofagi) sproščajo velike količine citokinov in na ta način stimulirajo vnetni proces in aktivirajo gladke mišične celice (van der Wal in sod., 1994; Ross, 1999; Libby in Simon, 2001; Pleskovič in sod., 2011). Poškodba endotelija in vnetje v steni krvne žile povzročijo sprostitev številnih citokinov kot npr. TGF ß, IL-1, IL-12, IL-18, MCSF, INF-γ, MIF (angl. macrofage migration inhibitory factor), PDGF, bFGF, FGF-

2 in FGF-9, ki aktivirajo gladke mišične celice (Ross, 1999; Libby in Simon, 2001). Za proliferacijo gladkih mišičnih celic naj bi bili odgovorni tudi R kadherin in β katenin (Sadie in sod., 2004) ter HGF (angl. hepatocyte growth factor) (Taher in sod., 2002). Rezultati številnih študij kažejo, da se po aktivaciji zniža ekspresija številnih označevalcev na njihovi membrani, kar nakazuje nižjo stopnjo diferenciacije. Začne se njihova proliferacija in migracija proti intimi ter se spremenijo iz kontraktilnih celic v sintetsko aktivne celice, ki ustvarjajo zunajcelični matriks (kolagen, elastin in proteoglikane). Anti- proliferativni učinek je dokazan za IL-19, ki nastaja v samih gladkih mišičnih celicah, ko so te stimulirane s pomočjo citokinov (Tian in sod., 2008). Obstajajo dokazi, da Nogo-B zavira migracijo gladkih mišičnih celic v arterijski steni in odebelitev bazalne membrane, obenem je njegova koncentracija na območju nastajanja plaka bila znižana pri osebah s pomembno zožitvijo arterij (Rodriguez-Feo in sod., 2007).

Interlevkini (IL) so ključni mediatorji sistemskega vnetja pri aterosklerozi. V tem procesu imajo zlasti pomembno vlogo družina IL-1: IL-1α, IL-1β (provnetno delovanje preko vezave na antagonist receporja IL-1) in antagonist receporja IL-1 (IL-1Ra) (Dinarello, 2003; Dinarello, 2009; Davies in sod., 2012). Geni za IL-1α, IL-1β in IL-1Ra so na isti regiji kromosoma: 2q14. IL-1Ra je endogeni inhibitor, ki kompetitivno zavira vezavo IL-1α in β na IL-1 receptor tipa 1. Ravnovesje med provnetnimi in protivnetnimi učinki IL-1 in IL-1Ra pa vpliva tudi IL-1 receptor tipa 2, ki sproži signal transdukcijo signala in tvorbo IL-1β in njegovega neaktivnega prekurzorja - pro-IL-1β (Dinarello, 2003; Dinarello, 2009). Na osnovi številnih dokazov o pomenu interlevkina oziroma zlasti IL-1β je bila nedavno začela multicentrična študija CANTOS, ki bo ugotavljala učinke terapije z zaviralci IL-1β na prognozo bolnikov s stabilno koronarno boleznijo (Ridker in sod., 2011).

Pomembno vlogo pri uravnavanju vnetja v procesu ateroskleroze imajo limfociti T (Robertson in Hansson, 2006). Znana sta dva tipa limfocitov T pomagalk (celice Th). Celice Th1 izločajo IL-2 in IFN-γ ter tako spodbujajo makrofage. Celice Th2 izločajo IL-4 in IL-5 ter s tem zavrejo celični odgovor in spodbujajo humoralni odgovor (Uyemura in sod., 1996). V procesu ateroskleroze se limfociti T spreminjajo predvsem v celice Th1, ki s tvorbo citokinov (IL-1, IL-2, IL-12, IL-18, IFN-γ, TNF-α in limfotoksin-α (LTA)) spodbujajo vnetje (Hanson in Libby, 2006; Robertson in Hansson, 2006). Na vnetni odgovor in na delovanje limfocitov T pa vplivajo različni geni, med njimi tudi glavni histokompatibilni kompleks (MHC) na regiji kromosoma 6p21.3 med regijo HCG27 in HLA-C (Davies in sod., 2012). V meta-analizi 5 raziskav GWAS so ugotovili, da je polimorfizem rs3869109 gena za MHC povezan z razvojem KB (Davies in sod., 2012). V literaturi nismo zasledili podatkov o vlogi tega polimorfizma pri razvoju ateroskleroze pri bolnikih s SB 2.

Dodatni dejavnik, ki vpliva na aktivacijo vnetja pri bolnikih s SB tipa 2, je prekomerna telesna teža (Jacobs in sod., 2011). Raziskovalci so poročali, da je pri prekomerno težkih bolnikih s SB tip 2 povečana aktivacija nevtrofilcev, monocitov in limfocitov T ter provnetnih celic Th 1 (Viardot in sod., 2012). Raziskovalci so poročali, da povišana koncentracija visoko občutljivega CRP povezana s večjim tveganjem srčnožilnih dogodkov (Blaha in sod., 2011). Prav tako so raziskovalci študije JUPITER poročali, da znižanje visoko občutljivega CRP s statini napoveduje natančneje kot znižanje nivoja holesterola LDL zmanjšanje

srčnožilnih dogodkov po uvedbi statinov (Ridker in sod., 2009).

Pri uravnavanju vnetnega odgovora imajo poleg interlevkinov pomembno vlogo tudi osteopontin, osteoprotegerin, SOX6 in receptorji TLR (angl. toll-like receptors) (Hamann in sod., 2005; de las Fuentes, 2008; Dong in sod., 2010; Straface in sod., 2011).

Gena za fosfoprotein 1 (nekoč osteopontin) na kromosomski regiji 4q22.1. Njegov produkt je osteopontin (OPN), ki vpliva na povečano izražanje interferona gama in IL 17 (Chu in sod., 2011). Pri transgenskih miši s povečanim izražanjem fosfoproteina 1 so raziskovalci ugotovili, da imajo povečano debelino intime medije (DIM) (Isoda in sod., 2002). O možni vpletenosti osteopontina pri nastanku atereoskeroze so poročali tudi v raziskavah na ljudeh (de las Fuentes, 2008). De las Fuentes in sodelavci (2008) so poročali, da so imeli posamezniki z genotipom TT polimorfizma T-66G gena za fosfoprotein 1 povečano DIM na karotidah. Raziskovalci sklepajo, da naj bi osteopontin vplival na aterosklerozo karotid preko aktivacije selitve in razmnoževanja gladkih mišičnih celic v arterijah, medtem ko poveča izražanje osteopontina povišana koncentracija glukoze (Takemoto in sod., 2000a; Takemoto in sod., 2000b). Yan in sod. (2010) so prav tako poročali, da je serumski nivo osteopontina neodvisni napovedovalec stopnje diabetične nefropatije (ocenjene preko ocenjene stopnje glomerulne filtracije – eGFR) in napredovalosti KB pri bolnikih s SB tipa 2 (Yan in sod., 2010). V literaturi nismo zasledili podatkov o vlogi teh polimorfizmov pri razvoju ateroskleroze pri bolnikih s SB 2.

Gen receptorja za dejavnik tumorske nekroze 11 b (*TNFRSF11B*) je na kromosomu 8q24. Njegov produkt osteoprotegerin (OPG) je sekretorni glikoprotein, ki sodi v družino dejavnikov tumorske nekroze. Raziskovalci so poročali o korelaciji med povišanim serumskim nivojem OPG in nestabilno angino (Sandberg in sod., 2006) oziroma akutnim miokardnim infarktom (Crisafulli in sod., 2005) v primerjavi s kontrolami s stabilno aterosklerozo compared with controls with stabilno aterosklerozo. Italijanski raziskovalci so nedavno poročali, da je imajo bolniki s simptomatski boleznijo karotid povišan serumski nivo OPG v primerjavi z zdravimi kontrolami (Straface in sod., 2011). Poznane so številne polimorfne variante gena *TNFRSF11B* in nekaj med njimi je funkcionalnih (Straface in sod., 2011). Raziskovalci so poročali, da so polimorfizmi rs 3134069, rs 2073617, and rs 2073618 povezani s serumskim nivojem OPG (Straface in sod., 2011). V literaturi nismo zasledili podatkov o vlogi teh polimorfizmov pri razvoju ateroskleroze pri bolnikih s SB 2.

Med gene, ki sodelujejo pri uravnavanju vnetnega procesa sodi tudi gen za *SOX6* (angl. Sex- determining region Y-box 6). Gen za *SOX6* je na kromosomu 11p15.3. Raziskovalci so v raziskavi GWAS ugotavljali, da je polimorfizem rs16933090 v genu za *SOX6* v regiji poti BMP (angl. bone morphogenic protein) povezan z aterosklerozo karotid v Karibskem otočju (Dong in sod., 2012), medtem ko ni podatkov za kavkaško raso.

Naslednji pomembni gen, ki sodeluje pri uravnavanju vnetnega procesa, je gen za adiponektin. Gen leži na kromosomu 3q27. Raziskovalci so poročali, da je polimorfizem *+45T/G* gena za adiponektin povezan s koronarno boleznijo (Bienertova-Vasku in sod., 2009). Prav tako so poročali, da je bila pri homozigotih TT koncentracija cirkulirajočega adiponektina najnižja, stopnja stenoze pa največja (Bienertova-Vasku in sod., 2009). V literaturi nismo zasledili podatkov o vlogi tega polimorfizma pri razvoju ateroskleroze pri bolnikih s SB 2.

Matriksne metalopeptidaze so skupina encimov, ki se nahajajo na zunanji strani celične membrane, in imajo zelo pomembno vlogo pri razgradnji zunajceličnega matriksa, lipoproteinov, neaktivnih rastnih dejavnikov, kemotaktičnih in celičnih adhezijskih molekulov. Gen za matriksno metalopeptidazo – 3 (*MMP3*) je na lokusu 11q22.3. Dokazano je že, da je polimorfizem 5A/6A v promotorski regiji gena za *MMP3* povezan s količino proteina in aktivnostjo encima. Homozigoti 6A/6A imajo najnižjo aktivnost encima (Medley in sod., 2003). Nekaj študij je bilo narejenih o povezanosti med 5A/6A polimorfizmom v genu za *MMP3* in restenozo koronarnih arterij po vstavitvi žilne opornice. Raziskovalci so poročali, da je bil polimorfizem 6A/6A povezan z nastankom KB oziroma restenoze po vstavitvi opornice v koronarne arterije (Humphries in sod., 2002; Hoppmann in sod., 2004). V literaturi nismo zasledili podatkov o vlogi tega polimorfizma pri razvoju ateroskleroze pri bolnikih s SB 2.

Gen za matriksno metalopeptidazo – 9 (*MMP9*) leži na lokusu 20q12-q13. Polimorfizem -1562 C>T je povezan z večjo količino *MMP9* proteina in večjo aktivnostjo encima MMP9 (Medley in sod., 2004). Dokazana je povezanost tega genotipa in povečane migracije gladkih mišičnih celic kot tudi pospešenega nabiranja makrofagov v aterosklerotičnem plaku (Johnson in sod., 2004). Osebe s tem genotipom imajo večje tveganje za nastanek koronarne ateroskleroze in hude oblike stenoze (Morgan in sod., 2003). V literaturi nismo zasledili podatkov o vlogi tega polimorfizma pri razvoju ateroskleroze pri bolnikih s SB 2.

Pri uravnavanju vnetja imajo poleg interlevkinov, osteopontina pomembno vlogo receptorji TLR (angl. Toll-like receptors). Gen za *TLR-4* je na kromosomu 9q33.1. Nekatere študije so že pokazale povezanost polimorfizma TLR-4 (Asp299Gly) z nižjim tveganjem za nastanek karotidne ateroskleroze (Kiechl in sod., 2002), medtem ko je polimorfizem Arg753Gln bil povezan z višjim tveganjem za nastanek restenoze koronarnih arterij po revaskularizacijskem posegu (Hamann in sod., 2005). V literaturi nismo zasledili podatkov o vlogi tega polimorfizma pri razvoju ateroskleroze pri bolnikih s SB 2.

Hipoteze in namen preiskave

Naše hipoteze so:

- Polimorfizmi testiranih genov vnetnih genov so povezani z DIM, debelino plakov in s seštevkom plakov (angl. plaque score) na vratnih arterijah ter z bolj nestabilnimi plaki pri sladkornih bolnikih tipa 2.
- Genetska raznolikost testiranih vnetnih genov vpliva na napredovanje aterosklerotičnega procesa **v dveletnem obdobju opazovanja** pri bolnikih s SB tipa 2, ki ga bomo kvantificirali z debelino intime-medije, debelino plakov in s seštevkom plakov.
- Pri bolnikih s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP $\geq$ 2 mg/L) poteka aterosklerotični proces hitreje kot pri tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L); napredovanje aterosklerotičnega procesa pri bolnikih s SB tipa 2 bomo kvantificirali s pomočjo primerjave DIM, debeline plakov in s seštevkom plakov v dveletnem obdobju opazovanja.
- Pri bolnikih s SB tipa 2 s povečano telesno težo poteka aterosklerotični proces hitreje kot pri tistih z s normalno telesno težo; napredovanje aterosklerotičnega procesa pri bolnikih s SB tipa 2 bomo kvantificirali s pomočjo primerjave DIM, debeline plakov in s seštevkom plakov v dveletnem obdobju opazovanja.
- Izražanje izbranih genov vnetnega odgovora v vratnih arterijah je večja pri karotidni bolezni pri bolnikih s SB tipa 2 kot v karotidni bolezni pri bolnikih brez SB tipa 2 oziroma v vratnih arterijah preiskovancev brez ateroskleroze karotid.

Namen raziskave - z raziskavo smo želeli:

- preučiti ali raznolikost v izbranih genih vnetnega procesa oziroma njihovi proteinski produkti vplivajo na hitrost napredovanja ateroskleroze karotid pri bolnikih s SB tipa 2
- ugotoviti, ali so polimorfizmi testiranih vnetnih genov povezani z DIM, debelino plakov in s seštevkom plakov (angl. plaque score) na vratnih arterijah ter z bolj nestabilnimi plaki pri sladkornih bolnikih tipa 2
- ugotoviti, ali genetska raznolikost testiranih vnetnih genov vpliva na napredovanje aterosklerotičnega procesa pri sladkornih bolnikih tipa 2, ki ga bomo kvantificirali z debelino intime-medije, debelino plakov in s seštevkom plakov v dveletnem obdobju opazovanja

Bolniki in metode

V prospektivno študijo smo vključili **595 bolnikov s SB tipa 2**. Vsi preiskovanci so bili slovenskega porekla (kavkavška rasa) in si niso bili v sorodu.

Ugotavljali smo trajanje sladkorne bolezni, terapijo sladkorne bolezni, srčnožilne dogodke (event. prebolelo možgansko kap). Preiskovancem smo izmerili krvni tlak in obseg pasu, telesno težo in višino ter izračunali indeks telesne mase (ITM).

Vsem preiskovancem smo **opravili ultrazvočno preiskavo vratnih arterij dvakrat**: prvič ob vključitvi v raziskavo ter drugič ob kontroli 2 leti po vključitvi v raziskavo.

Pri vsakem preiskovancu smo opravili meritev **DIM** en centimeter pod razcepiškem skupne vratne arterije. Ponovljivost meritve DIM v področju skupne vratne arterije je večja kot v področju bulbusa. Ocenili smo prisotnost aterosklerotskih leh na levem in na desnem karotidnem deblu. Levo in desno karotidno deblo smo pregledali najmanj z dveh strani.

Glede na stabilnost plakov smo razdelili lehe v **pet tipov (od I do V)**. Tip I (homogeno hipoehogene lehe). Značilnosti tega tipa so razjede na površini, krvavitve v leho ali visoka vsebnost maščob. Lehe so hipoehogene, do anehogene vsebine. Te lehe so izrazito nestabilne. Tip II (pretežno hipoehogene lehe). Lehe tega tipa vsebujejo več kot 50% neehogenih predelov. Tip III (pretežno hiperehogene lehe). Lehe tega tipa vsebujejo manj kot 50% nestabilnih hipoehogenih elementov. Tip IV (homogene hiperehogene lehe). Lehe lahko vsebujejo drobne kalcinacije, vendar so v glavnem sestavljene iz fibroznega tkiva. Tip V (pretežno kalcinirane lehe). Povzročajo akustično senco, ki onemogoča oceno arterijske stene ali svetline. Lehe tega tipa so nizkega tveganja za razvoj ishemičnega dogodka.

Razširjenost ateroskleroze vratnih arterij smo ocenili s **seštevkom plakov** (angl. plaque score). Ultrazvočno smo ugotavljali prisotnost plakov v skupni vratni arteriji, v razcepišču skupne vratne arterije in notranji vratni arteriji. Prisotnost plakov v eni od arterij nosi eno točko. Preiskava se je vršila na levi in desni strani tako, da je najvišji možni seštevek plakov 6, če plakov ni, je seštevek 0. Bolniki z višjim seštevkom plakov so imeli večjo verjetnost za možgansko kap (Grobbee in Bots, 1994; Lee in sod., 2007).

Biokemijske preiskave

Preiskovancem smo odvzeli 15 ml periferne venske krvi za gensko analizo in za biokemijske preiskave. V krvi smo določili koncentracijo celotnega holesterola, holesterola LDL, holesterola HDL in trigliceridov, nivo glikiranega hemoglobina A ter različne serološke preiskave (visoko občutljivi CRP, osteopontin, osteoprotegerin, adiponektin, metalopeptidaze, interlevkin 1) s pomočjo metode ELISA oziroma CBA.

Genetske preiskave

S pomočjo verižne reakcije s polimerazo (RFLP ali PCR v realnem času) smo testirali različne polimorfizme genov vnetnega procesa, ki smo jih izbrali s pomočjo dveh metod (raziskave GWAS in pristop kandidatnih genov):

1. polimorfizma Asp299Gly TLR-4 (angl. toll-like receptor 4) in Arg753Gln gena TLR-2
2. polimorfizmi rs 3134069, rs 2073617, and rs 2073618 gena za receptor dejavnika tumorske nekroze 11b
3. rs16933090 v genu za *SOX6*
4. polimorfizem rs2241766 (+45T/G) gena za adiponektin
5. polimorfizem 5A/6A gena za matriksno metalopeptidazo 3
6. polimorfizem -1562 C>T gena za matriksno metalopeptidazo 9
7. polimorfizem –889 C/T gena za interlevkin-1α
8. polimorfizmi rs1143634 (+3954C/T), +3953, -511C/T gena za interlevkin 1 β
9. polimorfizem introna 2 v genu za antagonist receporja IL-1 (*IL-1Ra*)

Statistična analiza

Numerične vrednosti spremenljivk smo primerjali s Studentovim testom t oziroma z analizo variance. Za analizo ravni proteinskih produktov pri bolnikih s SB smo uporabili Studentov t-test (2 vzorca), po potrebi pa neparametrični Mann-Whitney test in Kruskal-Wallis test. Za analizo parametričnih spremenljivk smo uporabili hi kvadrat test oziroma Fisherjev ekzaktni test.

Za analizo razporeditve genotipov različnih polimorfizmov smo uporabili test hi-kvadrat. Pričakovano število smo izračunali s predpostavko, da so genotipi v Hardy-Weinbergovem ravnovesju. Izračunali smo razmerje obetov (OR) in 95% interval zaupanja. Naredili smo multipla logistična regresijska analiza. Neodvisne spremenljivke so bile tiste, ki so se pokazale statistično pomembne v univariantni analizi ter s testom t.

Pri preizkušanju domnev smo kot statistično pomembno upoštevali vrednost p manjšo od 0,05. Statistično analizo smo naredili s pomočjo računalniškega programa SPSS verzija 20 (SPSS Inc. Illinois).

Raziskava 1

Polymorphisms of the PPAR-γ (rs1801282) and its coactivator (rs8192673) have a minor effect on markers of carotid atherosclerosis in patients with type 2 diabetes mellitus

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Research Article

Polymorphisms of the PPAR-γ (rs1801282) and Its Coactivator (rs8192673) Have a Minor Effect on Markers of Carotid Atherosclerosis in Patients with Type 2 Diabetes Mellitus

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Background. The present study was designed to clarify whether common single nucleotide polymorphisms (SNPs) of the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) gene (rs1801282) and the Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1α) gene (rs8192673) are associated with markers of carotid and coronary atherosclerosis in Caucasians with type 2 diabetes mellitus (T2DM). *Patients and Methods*. 595 T2DM subjects and 200 control subjects were enrolled in the cross-sectional study. Markers of carotid atherosclerosis were assessed ultrasonographically. In 215 out of 595 subjects with T2DM, a coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. Genotyping of either rs1801282 or rs8192673 was performed using KASPar assays. *Results*. In our study, we demonstrated an effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM in univariate and in multivariable linear regression analyses. Finally, we did not demonstrate any association between either rs1801282 or rs8192673 and markers of coronary atherosclerosis. *Conclusions*. In our study, we demonstrated a minor effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM. Moreover, we demonstrated a minor effect of the rs8192673 on CIMT progression in the 3.8-year follow-up in Caucasians with T2DM.

1. Introduction

Patients with diabetes mellitus have an increased risk of premature atherosclerosis [1, 2]. Type 2 diabetes mellitus (T2DM) causes more than a twofold increase in the incidence of myocardial infarction and coronary artery disease-(CAD-) related death [3].

The Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) and its coactivator, the Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1α), are important molecules in atherogenesis because they are associated with metabolic risk factors, such as obesity and diabetes [4, 5]. PPAR-γ regulates insulin sensitivity by transcriptionally activating adipocyte-specific genes involved in insulin signaling, glucose uptake, fatty acid uptake, and lipid-storage [6]. Moreover, PPAR-γ plays an important role in adipogenesis and subcellular metabolism of arterial wall macrophage foam cells [6, 7]. Furthermore, the pharmacological PPAR-γ agonist thiazolidinedione drugs appear to be antiatherogenic at multiple levels, which include a generalized improvement of metabolism reduction of triglyceride accumulation, beneficial effects on vascular wall components (macrophages), and an improvement of the outcome of atherosclerotic disease [8–11].

Genetic polymorphisms of the PPAR-γ and PGC-1α genes have so far been reported to be associated with

metabolic and cardiovascular end points [4, 5, 12–15]. A meta-analysis of 8 case-control studies and 2 family-based studies found that the PPARG A12 allele was associated with a reduced risk of type 2 diabetes [12]. The PPARG A12 allele was also associated with a reduced risk of myocardial infarction [13].

The aim of this study was to clarify whether common single nucleotide polymorphisms (SNPs) of the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) gene (rs1801282) and the Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1α) gene (rs8192673) are associated with markers of carotid atherosclerosis (carotid intima media thickness (CIMT), the number of affected segments of carotid arteries, and the sum of plaques thickness) in subjects with T2DM in the Caucasian population. The second aim of the study was to demonstrate an association between either rs1801282 or rs8192673 and the subclinical markers of CAD in the subset of patients with T2DM.

2. Methods

2.1. Patients. In this cross-sectional study 595 subjects with type 2 diabetes and 200 nondiabetic individuals were enrolled. The Slovene Medical Ethics Committee approved the study protocol. They were selected among patients admitted to the diabetes outpatient clinics of the general hospitals in Murska Sobota and Slovenj Gradec, Slovenia, and from the Cardiology Outpatient Department, MC Medicor, Ljubljana. Patients were classified as having T2DM according to the current report of the American Diabetes Association [16]. Patients were excluded if they had homozygous familial hypercholesterolaemia or a previous cardiovascular event such as myocardial infarction or a cerebral stroke. Clinical data, including smoking habits, duration and treatment of diabetes, arterial hypertension, hyperlipidemia, and consuming any other drugs were obtained from medical records and questionnaires. Patients were asked if they were smokers at the time of recruitment (current smoker).

Two experienced doctors blinded to the participants' diabetes status performed all ultrasound examinations. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured, as described previously [17]. Plaques were defined as a focal intima-media thickening and divided into 5 types according to their echogenic/echolucent characteristics, as described previously [17]. The interobserver reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $p < 0.001$).

Control ultrasound examination was performed on 426 patients with diabetes and 137 healthy controls after 3.8 ± 0.5 years from the first examination. We used the annual CIMT progression rate, the increase in total plaque thickness, and the number of sites with plaques as well as the presence of unstable plaques as markers of carotid atherosclerosis progression.

In 215 out of 595 subjects with T2DM, a coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. In 215 subjects with T2DM, coronary calcium score was measured and the presence of CAD was determined. Four regions (left main (LM), Left anterior descending (LAD) artery, left circumflex (LCX) artery, and right coronary artery (RCA)) were analyzed for the presence of CAD and more than 50% stenotic lesions were looked for in LM, LAD, LCX, RCA regions.

2.2. Biochemical Analyses. Blood samples for biochemical analyses, total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated hemoglobin (HbA1c), hsCRP, and fibrinogen, were collected after a 12-hour fasting period. All the blood biochemical analyses were determined by using standard biochemical methods in the hospital's accredited lab.

2.3. Genotyping. The genomic DNA was extracted from 100 μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagen GmbH, Hilden, Germany). Polymorphisms rs1801282 of the PPAR-γ gene and rs8192673 of the PGC-1α gene were determined with real-time PCR using StepOne™ (48-well) Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA).

3. Statistical Analysis

Continuous variables were expressed as means ± standard deviations, when normally distributed, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Continuous clinical data were compared using an unpaired Student's t-test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson χ^2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables. Due to the high correlation of systolic blood pressure with the diastolic blood pressure ($r = 0.57$, $p < 0.001$) they were not included together in the same statistical model. For the same reason the body mass index (BMI) was not included in the model together with the waist circumference ($r = 0.45$, $p < 0.001$).

Multivariable linear regression analysis was performed to determine the association of the tested polymorphisms with the CIMT/annual progression of CIMT and change in number of sites with plaque/total plaque thickness. To determine the association of the tested polymorphisms with the presence of atherosclerotic plaques on the carotid arteries or the presence of unstable plaques a multivariate logistic regression analysis was performed. All the regression models were adjusted for the presence of well established cardiovascular risk factors: age, gender, hypertension, systolic blood pressure, smoking, plasma levels of LDL and HDL cholesterol, triglycerides, HbA1c, and statin treatment. The results were presented as standardized β coefficients and p values for the linear regression and by odds ratios and

Table 1: Baseline clinical and biochemical characteristics of diabetic patients and controls.

	Diabetic patients $n = 595$	Controls $n = 200$	p
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Male gender (%)	338 (56.8)	92 (46.0)	**0.008**
DM duration (years)	11.25 ± 7.88	—	—
Smoking prevalence (%)	53 (8.91)	34 (17.0)	**0.002**
Statin therapy (%)	375 (63.0)	62 (31.0)	<0.001
Antihypertensive agents (%)	499 (83.8)	58 (29%)	<0.001
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	<**0.001**
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
Systolic blood pressure (mm Hg)	146.98 ± 19.98	143.3 ± 16.6	0.86
Diastolic blood pressure (mm Hg)	85.75 ± 11.62	84.7 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	<**0.001**
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	<**0.001**
Total cholesterol (mmol/L)	4.70 ± 1.19	5.36 ± 1.08	<**0.001**
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	<**0.001**
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	<**0.001**
Triglycerides (mmol/L)	1.9 (1.2–2.7)	1.3 (0.9–1.9)	<**0.001**
hsCRP (mg/L)	2.2 (1.0–4.3)	1.3 (0.8–2.7)	<**0.001**
CIMT (μm)	958 ± 194	890 ± 212	**0.007**

DM: diabetes mellitus; hsCRP: high sensitivity C-reactive protein.

95% CIs for the logistic regression. A two-tailed p value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

4. Results

Patients with T2DM had a greater waist circumference and higher fasting glucose and HbA1c levels compared to controls, whereas there were no differences in BMI or systolic and diastolic blood pressure between patients with T2DM and control subjects (Table 1). Patients with T2DM had lower total, HDL, and LDL cholesterol levels and a higher triglyceride level compared to controls (Table 1). Plasma level of inflammatory marker hsCRP was higher in patients with T2DM compared to controls (Table 1). Additionally, there were a higher percentage of men, statin therapy, and antihypertensive therapy and a lower percentage of smokers in the T2DM group compared to the control group (Table 1).

No statistically significant differences in the rs1801282 and rs8192673 genotype distribution frequencies were observed between T2DM patients and controls (Table 2). The rs1801282 genotype distributions in both patients with DM2 ($\chi^2 = 0.66$; $p = 0.42$) and controls ($\chi^2 = 3.79$; $p = 0.05$) were compatible with Hardy-Weinberg expectations. The rs8192673 genotype distributions in both patients with DM2 ($\chi^2 = 1.52$; $p = 0.22$) and controls ($\chi^2 = 0.50$; $p = 0.48$) were compatible with Hardy-Weinberg expectations (Table 2).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of both polymorphisms (rs1801282, rs8192673) upon enrolment (Tables 3 and 4). In our study, we demonstrated an effect of the rs1801282 on the presence of plaques on subjects with T2DM by univariate and multivariable linear regression analysis (Tables 3 and 5), but we did not demonstrate any association between either the rs1801282 or the rs8192673 and other markers of carotid atherosclerosis CIMT, the sum of plaque thickness, the presence of unstable carotid plaques (Tables 3 and 4).

Finally, we did not demonstrate any association between either rs1801282 or rs8192673 and markers of coronary

Table 2: Genotype distribution and allele frequencies of the polymorphisms rs1801282 and rs8192673 in patients with T2DM and in control subjects.

	Subjects with T2DM $n = 595$	Control subjects $n = 200$	p
rs1801282			
CC genotype	422 (70.9)	137 (68.5)	
GC genotype	155 (26.1)	52 (26.0)	0.27
GG genotype	18 (3.0)	11 (5.5)	
C allele	999 (83.9)	326 (81.5)	0.26
G allele	191 (16.1)	74 (18.5)	
rs8192673			
TT genotype	309 (52.0)	92 (46.0)	0.28
TC genotype	231 (38.8)	84 (42.0)	
CC genotype	55 (9.2)	24 (12.0)	
T allele	849 (71.4)	268 (67.0)	0.10
C allele	341 (28.6)	132 (33.0)	

Table 3: Ultrasonographic markers of carotid atherosclerosis due to rs1801282 genotypes in patients with T2DM at the time of recruitment.

Pro12AlaPPAR	CC	GC + GG	p
CIMT (μm)	1006 ± 210	1026 ± 209	0.39
Number of sites with plaque	2.56 ± 1.57	2.36 ± 1.82	0.31
Total plaque thickness (mm)	7.98 ± 4.47	7.65 ± 4.64	0.58
Presence of plaques			
+	365 (86.5)	133 (76.9)	**0.005**
−	57 (13.5)	40 (23.1)	
Presence of unstable plaques			
+	213 (58.4)	74 (55.6)	0.61
−	152 (41.6)	59 (44.4)	
Coronary calcium score*	250 ± 315	269 ± 367	0.1
Number of coronary arteries with more than 50% stenosis	0.7 ± 0.9	0.9 ± 1.2	0.4
The presence of at least 1 vessel with more than 50% stenosis*	24 (38.9%)	63 (41.1%)	0.2

*Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM.

Table 4: Ultrasonographic markers of carotid atherosclerosis due to rs8192673 genotypes in patients with T2DM at the time of recruitment.

	TT	TC	CC	p
CIMT (μm)	1007 ± 224	1012 ± 191	1012 ± 217	0.94
Number of sites with plaque	2.36 ± 1.60	2.69 ± 1.66	2.72 ± 1.67	0.13
Total plaque thickness (mm)	7.54 ± 4.54	8.06 ± 4.82	8.31 ± 4.35	0.35
Presence of plaques				
+	257 (83.2)	194 (84.0)	47 (85.5)	0.90
−	52 (16.8)	37 (16.0)	8 (14.5)	
Presence of unstable plaques				
+	149 (58.0)	108 (55.7)	29 (61.7)	0.73
−	108 (42.0)	86 (44.3)	18 (38.3)	
Coronary calcium score*	181 ± 170	344 ± 376	200 ± 304	0.2
Number of coronary arteries with more than 50% stenosis*	0.7 ± 1.1	0.9 ± 1.1	0.6 ± 1.3	0.8
The presence of at least 1 vessel with more than 50% stenosis*	7 (31.8%)	33 (39.3%)	48 (44.0%)	0.3

*Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM.

atherosclerosis obtained with CCTA (coronary calcium score, the number of coronary arteries with more than 50% stenosis and the presence of at least one vessel with more than 50% stenosis) in subjects with T2DM (Tables 3 and 4).

In our study, we demonstrated an effect of the rs8192673 on CIMT progression in the 3.8-year follow-up (Table 6). Using the multivariable linear regression analysis we demonstrated an effect of the rs1801282 on the presence of plaques in Caucasians with T2DM (Table 6).

5. Discussion

In the present study we tested the hypothesis that the rs1801282 of the PPAR-γ gene and the rs8192673 of the PGC-1α gene may be genetic markers of subclinical atherosclerosis of carotid and coronary arteries. In our study, we demonstrated an effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM in univariate and in multivariable linear regression analyses. The rs1801282 of the PPAR-γ gene was found to have a protective role against the development of atherosclerosis. Moreover, we demonstrated a minor effect of the rs8192673 on CIMT progression in Caucasians with T2DM in the 3.8-year follow-up.

In our study, we did not demonstrate any association between either the rs1801282 or the rs8192673 and CIMT, despite some previous reports on an association between the rs1801282 and CIMT [18–20]. In few populations (German population, Japanese population, and Canadian Oji-Cree Aborigines), the rs1801282 (Ala12 allele of the PPAR-γ) was reported to be associated with reduced CIMT [18–20]. Contrary to the lack of effect on CIMT, an effect of the rs8192673 on the CIMT progression rate and an effect of the rs1801282 on the presence of plaques in Caucasians with T2DM were demonstrated in univariate and in multivariable linear regression analyses. The rs1801282 of the PPAR-γ gene was found to have protective role against the development of atherosclerosis.

In the present study we pursued the hypothesis that either the rs1801282 of the PPAR-γ gene or the rs8192673 of the PGC-1α gene may be genetic markers of coronary atherosclerosis in subjects with T2DM. In our study, however, we did not demonstrate any association between either the rs1801282 or the rs8192673 and markers of coronary

Table 5: Association of the rs1801282 genotypes with the presence of plaques and presence of unstable plaques in patients with T2DM at the time of recruitment.

	Presence of plaque		Presence of unstable plaque	
	OR (95% CI)	p	OR (95% CI)	p
rs1801282				
Hypertension (0 = no; 1 = yes)	1.71 (0.93–2.58)	**0.04**	1.25 (0.88–2.64)	0.97
Systolic blood pressure (mm Hg)	1.07 (0.92–1.007)	0.17	1.11 (0.86–1.44)	0.32
LDL cholesterol (mmol/L)	1.21 (0.78–1.89)	0.40	1.08 (0.75–1.56)	0.67
HDL cholesterol (mmol/L)	0.18 (0.05–0.63)	**0.008**	0.30 (0.08–1.13)	0.08
Triglycerides (mmol/L)	1.28 (0.63–1.03)	0.09	1.09 (0.66–1.37)	0.34
Hba1c (%)	1.14 (0.64–1.54)	0.28	1.22 (0.74–1.92)	0.42
GC + GG*	0.79 (0.48–1.14)	**0.04**	0.83 (0.34–1.91)	0.65
rs8192673				
Hypertension (0 = no; 1 = yes)	1.35 (1.13–1.93)	**0.04**	1.15 (0.75–2.77)	0.79
Systolic blood pressure (mm Hg)	1.08 (0.96–1.34)	0.16	1.02 (0.97–1.25)	0.31
LDL cholesterol (mmol/L)	1.22 (0.78–1.89)	0.29	1.07 (0.74–1.54)	0.73
HDL cholesterol (mmol/L)	0.19 (0.05–0.72)	0.54	0.29 (0.08–1.05)	0.06
Triglycerides (mmol/L)	1.34 (0.63–1.90)	0.09	1.19 (0.66–1.59)	0.34
Hba1c (%)	1.16 (0.65–2.06)	0.32	1.11 (0.86–1.44)	0.42
TC**	0.97 (0.56–1.38)	0.48	1.16 (0.59–2.70)	0.55
CC**	1.08 (0.38–1.34)	0.63	1.43 (0.43–4.73)	0.56

All the models were adjusted for age, gender, smoking, and statin treatment.
*Reference group were homozygotes for allele C. **Reference group were homozygotes for allele T.

Table 6: Association of the rs1801282 genotypes with ultrasonographic markers of carotid atherosclerosis progression in patients with T2DM.

	CIMT progression rate		Δ Number of sites with plaque		Δ Total plaque thickness	
	β	p	β	p	β	p
rs1801282						
Hypertension (0 = no; 1 = yes)	0.013	0.92	0.020	0.90	0.069	0.26
Systolic blood pressure (mm Hg)	0.022	0.52	0.052	0.69	0.037	0.82
LDL cholesterol (mmol/L)	0.057	0.69	0.051	0.71	0.073	0.49
HDL cholesterol (mmol/L)	−0.211	0.19	−0.230	0.14	−0.189	0.37
Triglycerides (mmol/L)	0.249	0.13	0.343	0.78	0.359	0.44
Hba1c (%)	1.151	0.29	1.097	0.83	1.176	0.41
GC + GG*	0.818	0.93	0.728	0.18	0.684	0.16
rs8192673						
Hypertension (0 = no; 1 = yes)	0.140	0.37	0.062	0.71	0.069	0.29
Systolic blood pressure (mm Hg)	0.186	0.25	0.046	0.88	0.075	0.35
LDL cholesterol (mmol/L)	0.172	0.59	0.143	0.75	0.446	0.35
HDL cholesterol (mmol/L)	−0.203	0.18	−0.232	0.08	−0.192	0.34
Triglycerides (mmol/L)	0.168	0.28	0.117	0.43	0.127	0.21
Hba1c (%)	0.146	0.27	0.143	0.26	0.228	0.16
TC**	0.068	0.63	−0.066	0.64	0.328	0.32
CC**	0.349	**0.01**	−0.115	0.11	0.681	0.06

All the models were adjusted for age, gender, smoking, statin treatment and baseline value of dependent variable.
*Reference group were homozygotes for the allele C; **Reference group were homozygotes for the allele T.

atherosclerosis obtained with CCTA (coronary calcium score, number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis). Our findings are in accordance with the study of Nemoto and coworkers on 91 subjects with T2DM, in which they failed to demonstrate the effect of the variability in the PPAR-γ gene on the coronary calcium score [21]. However, in several studies the effect of polymorphisms of PPARγ2/PGC-1α genes on CAD/MI risk was reported [1, 13, 15, 22–24]. In their case-control study, Galgani and coworkers

demonstrated that homozygosity for the Ala allele at codon 12 of the PPARγ2 (rs1801282) gene was associated with a reduced risk of CAD [22]. Similarly, Ridker and coworkers reported in a prospective study that the rs1801282 of the PPAR-γ (A12 allele) was associated with a 25% reduction in myocardial infarction risk [13]. Ding and coworkers, however, failed to demonstrate a significant effect of the rs1801282 of the PPAR-γ on CAD risk in their meta-analysis (74 studies with 52,998 subjects included) [23]. Cresci and coworkers reported a variant (rs1503298) in a single PPAR pathway gene (i.e., TLL1) that was associated with the extent of CAD in patients with T2DM and CAD [15].

Potential mechanisms of the effect of the variants of both genes (PPAR-γ, PGC-1α) may be speculated to affect serum/tissue levels of both proteins, other risk factors (i.e., obesity and obesity indexes) or other effects (i.e., lipid status).

In our recently published study, we demonstrated that the rs8192673 of the PGC-1α gene and the rs1801282 of the PPAR-γ gene have been associated with waist circumference in subjects with T2DM [4]. Huang and coworkers demonstrated the effect of the rs1801282 of the PPAR-γ gene in the meta-analysis (74 studies with 52,998 subjects) on lipid parameters [25]. They reported that subjects (male) with the AlaAla genotype had lower blood TG than subjects with ProPro genotype in Caucasians [25].

Strength of our study is the community-based sample and the detailed phenotypic characterization of the subjects with regard to ultrasonically determined carotid atherosclerosis, as well as having data of a rather large sample of subjects with T2DM. A limitation is the use of cross-sectional data in the analysis, restricting the possibility of causal inferences from our data and allowing for bias. An additional limitation is that while we assume that the effect of the PPAR-γ/PGC-1α gene variants on plaque is due to their influence on serum/tissue levels of the respective enzymes, we do not have any direct measure to be able to investigate this.

6. Conclusions

To conclude, in our study we demonstrated a minor effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM. Moreover, we demonstrated a minor effect of the rs8192673 on CIMT progression in the 3.8-year follow-up. Our findings suggest that the tested polymorphisms in the PPAR-γ/PGC-1α genes play a minor role in the development of subclinical atherosclerosis in subjects with T2DM.

Conflict of Interests

The authors declare no conflict of interests related to this work.

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Raziskava 2

Polymorphisms rs1800587 and rs1143634 of the interleukin-1α gene are associated with the progression of carotid atherosclerosis in caucasians with type 2 diabetes mellitus

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Polymorphisms rs1800587 and rs1143634 of the Interleukin-1α Gene are associated with the Progression of Carotid Atherosclerosis in Caucasians with Type 2 Diabetes Mellitus

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Abstract

Background: Our study was designed to test the possible association between either polymorphisms T889C (rs1800587) or C3954T (rs1143634) of the interleukin-1 alpha (IL-1α) gene with subclinical markers of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM). Moreover, the effect of both polymorphisms on progression of carotid atherosclerosis in 3.8-year follow-up was studied.

Patients and methods: 595 subjects with T2DM were enrolled in the prospective study. Subclinical markers of carotid atherosclerosis were assessed with ultrasound at the time of recruitment and after 3.8-years. Genotyping of two polymorphisms (rs1800587, rs1143634) was performed with real-time PCR System.

Results: The comparison of atherosclerosis parameters was performed with regard to different genotypes of IL-1α rs1800587 and rs1143634 polymorphisms upon enrolment. Multiple linear regression analysis revealed the association of IL-1α rs1143634 on total plaque thickness progression in a 3.8 year follow up.

Conclusions: An association between either the IL-1α rs1800587 or rs1143634 and total plaque thickness at the time of recruitment was reported. Additionally, we demonstrated the effect of the IL-1α rs1143634 on total plaque thickness progression in the 3.8-year follow-up in patients with T2DM.

Keywords: Carotid atherosclerosis; Genetic polymorphism; Interleukin-1 α; Cross-sectional study

Introduction

Atherosclerosis of carotid arteries is a complex multifactorial disorder that is thought to result from interactions between an individual's genetic background and lifetime exposure to various environmental factors.

It is generally accepted that beside cholesterol accumulation chronic inflammatory is very much involved in the development of atherosclerosis, and inflammation is considered to play a crucial role in the initiation of atherosclerotic process [1-3]. A genetic variability of the inflammatory genes is considered to affect the development of atherosclerosis via interaction with conventional risk factors [4].

Interleukin-1 (IL-1) is an important inflammatory mediator that has been reported to promote the production of inflammatory markers, such as fibrinogen and C-reactive protein, which are involved in the development of atherosclerosis [5-8]. Moreover, high levels of IL-1a mRNA transcripts were demonstrated in atherosclerotic plaques from human patients [9].

The IL-1 gene family exists in two forms, IL-1 and IL-1, which are produced by lymphocytes or monocytes, and one antagonistic cytokine, IL-1 receptor antagonist (IL-1Ra) [10]. The variability of the IL-1α gene has been reported to be associated with either ischemic stroke or coronary heart disease in few studies, but the results are inconsistent [7,8,11-14].

Despite several data demonstrating a relationship between the variability of the IL-1α gene and cardiovascular disorders [7,8,11-14], there is no study evaluating relationship between the tested polymorphisms (rs1800587 and rs1143634) and progression of subclinical markers of carotid atherosclerosis in subjects with T2DM. Therefore, the exact role of rs1800587 and rs1143634 on the development and progression of carotid atherosclerosis remains to be fully elucidated.

Aim of the Study

Our study was designed to test the possible association between either the rs1800587 or the rs1143634 of the IL-1α gene with subclinical markers of carotid atherosclerosis (carotid intima media thickness [CIMT], number of affected segments of carotid arteries, the

sum of plaques thickness, the presence of carotid plaques, and the presence of unstable carotid plaques) in patients with type 2 diabetes mellitus (T2DM).

Moreover, the effect of both polymorphisms on progression of carotid atherosclerosis in 3.8-year follow-up was studied.

Materials and Methods

Patients

In this prospective study approved by the Slovene Medical Ethics Committee, 595 consecutive subjects with T2DM, admitted to the diabetes outpatient clinics of the General Hospitals Murska Sobota, and Slovenj Gradec, Slovenia, and from the outpatient department Medicor, Ljubljana were enrolled.

Patients were classified as having T2DM according to the current report of the American Diabetes Association [15]. Exclusion criteria were: homozygous familial hypercholesterolemia or a history of cardiovascular event (i.e. acute coronary syndrome or a cerebrovascular stroke). Clinical data were obtained from medical records and detailed questionnaires, as previously described [16].

Ultrasonographic analysis

Ultrasonographic analysis was performed by two experienced radiologists according to the same protocol. They were blinded for the participant's clinical status. The CIMT, plaque thickness, were measured, and plaques presence/types were evaluated on both sides, as previously described [16].

Subjects with T2DM were prospectively followed, and after 3.8 ± 0.5 years control ultrasound examination of carotid arteries was performed on 426 out of 595 subjects (71.6%) with T2DM. The annual CIMT progression rate, the increase in total plaque thickness and the number of sites with plaques were evaluated to appreciate the progression of carotid atherosclerosis.

Biochemical analyses and genotyping

Standard biochemical analyses were performed in the hospital's accredited lab after a 12-hour fasting period: lipid status, fasting blood glucose and glycated haemoglobin (HbA1c), and hs-CRP.

After the genomic DNA was extracted with FlexiGene DNA isolation kit (Qiagen GmbH, Hilden, Germany) polymorphisms T889C (rs1800587) of IL-1α gene and C3954T (rs1143634) of IL-1β gene were genotyped with KASP genotyping chemistry in UK (LGC Genomics Ltd, Unit 1-2 Trident Industrial Estate, Hoddesdon, Herts, United Kingdom) according standard protocol.

Thermal cycling conditions for KASP chemistry: hot-start activation 94°C for 15 minutes, 10 cycles: 94°C for 20 seconds, 61-55°C for 60 seconds (dropping 0.6°C per cycle), 26 cycles: 94°C for 20 seconds, 55°C for 60 seconds.

Statistical analysis

Data were expresseticald as frequencies (percentages) or as means ± standard deviation (SD), when normally distributed, and as median (interquartile range) when asymmetrically distributed.

Continuous clinical data were compared by unpaired Student's t-test or analysis of variance or the Kruskal-Wallis H-test when asymmetrically distributed. Hardy–Weinberg equilibrium was confirmed using the χ^2 test. Moreover, multivariable linear regression analysis was performed, as described previously to determine the association of the tested polymorphisms with the CIMT/annual progression of CIMT and change in number of sites with plaque/total plaque thickness.

A p-value of <0.05 was considered to be statistically significant. Statistical analysis was performed using the SPSS program for Windows 2000 version 19 (SPSS Inc. Chicago, Illinois, USA).

Results

Baseline characteristics (demographic and clinical) of patients with T2DM are demonstrated in Table 1. Almost two third of patients with T2DM were men, and two third of patients were on statin therapy (Table 1). Both polymorphisms (rs1800587 and rs11436348) were demonstrated to be associated with total plaque thickness upon enrolment, whereas they were not associated with either the presence of plaque or presence of unstable plaque (Tables 2 and 3).

The comparison of atherosclerotic markers was performed with regard to different genotypes of IL-1α rs1800587 and rs11436348 upon enrolment and during follow-up (Tables 2-4).

	Subjects with T2DM n=595
Age (years)	62.39 ± 9.61
Male sex (%)	338 (56.8)
Diabetes duration (years)	11.25 ± 7.88
Cigarette smoking (%)	53 (8.91)
Waist circumference (cm)	108.65 ± 12.88
BMI (kg/m^2)	31.00 ± 4.74
SBP (mm Hg)	147.1 ± 19.80
DBP (mm Hg)	85.78 ± 11.60
Fasting glucose (mmol/l)	8.04 ± 2.57
HbA1c (%)	7.89 ± 3.56
Total cholesterol (mmol/l)	4.70 ± 1.18
HDL cholesterol (mmol/l)	1.20 ± 0.35
LDL cholesterol (mmol/l)	2.63 ± 0.94
Triglycerides (mmol/l)	1.9 (1.2-2.7)
hs - CRP (mg/l)	3.5 ± 1.18
CIMT (μm)	1013 ± 208
Number of sites with plaque	2.52 ± 1.63
Total plaque thickness (mm)	7.89 ± 3.51
BMI-Body Mass Index; SBP-Systolic Blood Pressure; DBP-Diastolic Blood Pressure; Hba1c-Glycated Haemoglobin; Hs-CRP-High Sensitivity C-Reactive Protein; CIMT-Carotid Intima Media Thickness.	

Table 1: Baseline characteristics (clinical, ultrasonographical) of subjects with T2DM.

rs1800587		CC	CT	TT	P
CIMT (μm)		1022 ± 197	1010 ± 215	1009 ± 214	0.85
Number of sites with plaque		2.57 ± 1.68	2.43 ± 1.55	2.40 ± 1.39	0.7
Total plaque thickness (mm)		8.56 ± 3.66	7.23 ± 3.12	6.70 ± 3.09	0.04
Presence of plaques	+	278 (85.0)	192 (83.5)	28 (73.7)	0.19
	-	49 (15.0)	38 (16.5)	10 (26.3)	
Presence of unstable plaques	+	163 (58.6)	103 (53.6)	16 (57.1)	0.56
	-	115 (41.4)	89 (46.4)	12 (42.9)	
rs1143634		CC	CT	TT	p
CIMT (μm)		1017 ± 210	1010 ± 205	995 ± 212	0.92
Number of sites with plaque		2.62 ± 1.63	2.30 ± 1.63	2.17 ± 1.51	0.13
Total plaque thickness (mm)		8.40 ± 3.78	7.94 ± 3.29	6.69 ± 2.89	0.02
Presence of plaques	+	315 (84.9)	162 (82.2)	21 (77.8)	0.49
	-	56 (15.1)	35 (17.8)	6 (22.2)	
Presence of unstable plaques	+	186 (59.0)	88 (54.3)	12 (57.1)	0.61
	-	129 (41.0)	74 (45.7)	9 (42.9)	

Table 2: Ultrasonographic markers of carotid atherosclerosis due to rs1800587 and rs1143634 genotypes in patients with T2DM at the time of recruitment.

In our group of patients with T2DM 8.9% of them were cigarette smokers. The genotype distribution in patients with T2DM was in Hardy-Weinberg equilibrium for the IL-1α gene [rs1800587: T2DM genotype frequencies: TT genotype 6.4%, TC genotype 38.7%, CC genotype 54.9%; χ^2=0.08; p=0.77; rs1143634: T2DM genotype frequencies: TT genotype 4.5%, TC genotype 33.1%, CC genotype 62.4%; χ^2=0.02; p=0.89).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of IL-1α rs1800587 and rs11436348 polymorphisms upon enrolment. Multiple linear regression analysis found the association between the rs1143634 TT genotype and total plaque thickness progression in a 3.8 year follow-up (Table 5).

Discussion

In the present study we reported that both polymorphisms of the IL-1α (rs1800587 and rs11436348) were associated with total plaque thickness upon enrolment in subjects with T2DM. Our findings are in accordance with the results of the retrospective association study reported on Chinese population [14].

Zhang and co-workers reported that the TT genotype of the rs1800587 was associated with ischemic stroke due to large artery atherosclerosis in the Han population in Northern China [14]. Contrary to our study, few retrospective studies [7,8,14] did not demonstrate a significant effect of the gene variability of the IL-1α on either ischemic stroke or coronary heart disease. Zee and co-workers failed to demonstrate an important effect of the gene variability of the IL-1α on athero-thrombotic disorders in a prospective cohort of 14916 initially healthy American men (incident myocardial infarction, incident ischemic stroke) [13]. They, however, reported a modest association of rs1143623 of the IL-1β with reduced risk of incident myocardial infarction.

	Presence of plaque		Presence of unstable plaque	
rs1800587	OR (95% CI)	p	OR (95% CI)	P
Hypertension (0=no; 1=yes)	2.36 (0.82-4.67)	0.11	1.25 (0.37-1.95)	0.93
Systolic blood pressure (mmHg)	1.07 (0.97-1.12)	0.34	1.16 (0.79-1.38)	0.41
LDL cholesterol (mmol/L)	1.26 (0.79-1.98)	0.31	1.12 (0.77-1.63)	0.56
HDL cholesterol (mmol/L)	0.14 (0.04-0.52)	0.003	0.27 (0.07-1.06)	0.06
Triglycerides (mmol/L)	1.17 (0.61-1.32)	0.07	1.12 (0.85-1.29)	0.26
Hba1c (%)	1.08 (0.60-1.24)	0.16	1.12 (0.86-1.46)	0.39
CT*	0.89 (0.16-3.22)	0.21	0.93 (0.25-1.34)	0.09
TT*	0.74 (0.20-2.89)	0.89	0.98 (0.43-1.66)	0.58
rs1143634				
Hypertension (0=no; 1=yes)	2.47 (0.91-4.55)	0.07	1.89 (0.74-3.29)	0.84
Systolic blood pressure (mmHg)	1.05 (0.96-1.18)	0.16	1.03 (0.96-1.23)	0.28
LDL cholesterol (mmol/L)	1.26 (0.79-1.97)	0.33	1.34 (0.71-1.89)	0.86
HDL cholesterol (mmol/L)	0.15 (0.04-0.55)	0.004	0.26 (0.07-1.08)	0.06
Triglycerides (mmol/L)	1.37 (0.53-1.96)	0.07	1.60 (0.77-2.42)	0.19
Hba1c (%)	1.38 (0.63-1.86)	0.22	1.09 (0.83-1.44)	0.55
CT**	0.79 (0.22-1.49)	0.64	0.48 (0.19-0.87)	0.22
TT**	0.58 (0.18-1.27)	0.92	0.29 (0.16-0.68)	0.56
All the models were adjusted for age, gender, smoking and statin treatment. *Reference group were homozygotes for the allele C (rs1800587). **Reference group were homozygotes for the allele C (rs1143634).				

Table 3: Association of the presence of plaques/unstable plaques with rs1800587 and rs1143634 genotypes in patients with T2DM at the time of recruitment.

rs1800587	**CC**	**CT**	**TT**	**P**
CIMT progression rate (μm/year)	20.17 (13.90-30.65)	18.68 (14.25-26.08)	16.34 (10.95-22.28)	0.23
Δ number of sites with plaque	2.0 (1.0-3.0)	2.0 (1.0-3.0)	2.0 (1.0-2.25)	0.93
Δ total plaque thickness (mm)	6.30 (3.17-8.50)	5.85 (4.30-9.30)	4.35 (2.80-7.22)	0.38
rs1143634	CC	CT	TT	P
CIMT progression rate (μm/year)	20.67 (13.53-28.25)	20.33 (10.53-25.54)	18.62 (12.34-25.72)	0.77
Δ number of sites with plaque	2.0 (1.0-3.0)	2.0 (0.75-3.0)	1.5 (1.0-2.75)	0.94
Δ total plaque thickness (mm)	5.08 (2.38-7.18)	4.83 (3.30-7.24)	4.64 (3.48-6.88)	0.36
CIMT- carotid intima-media thickness.				

Table 4: Ultrasonographic markers of carotid atherosclerosis progression due to rs1800587 and rs1143634 genotypes in patients with T2DM.

	CIMT progression rate		**Δ Number of sites with plaque**		**Δ Total plaque thickness**	
rs1800587	B	p	β	p	β	P
Hypertension (0=no; 1=yes)	1.03	0.86	1.206	0.71	1.092	0.56
Systolic blood pressure (mmHg)	0.048	0.79	0.051	0.39	0.05	0.76
LDL cholesterol (mmol/L)	0.284	0.56	0.559	0.25	0.45	0.72
HDL cholesterol (mmol/L)	-0.204	0.19	-0.253	0.76	-0.223	0.11
Triglycerides (mmol/L)	0.278	0.09	0.646	0.37	0.118	0.41
Hba1c (%)	1.132	0.32	1.082	0.12	1.175	0.16
CT*	-0.117	0.79	0.337	0.51	-0.144	0.09
TT*	-0.211	0.18	0.332	0.42	-0.225	0.29
rs1143634	B	p	β	p	B	P
Hypertension (0=no; 1=yes)	1.075	0.65	1.26	0.73	1.057	0.7
Systolic blood pressure (mmHg)	0.11	0.51	0.032	0.96	0.044	0.77
LDL cholesterol (mmol/L)	0.255	0.69	0.653	0.29	0.113	0.37
HDL cholesterol (mmol/L)	-0.196	0.24	-0.127	0.91	-0.288	0.06
Triglycerides (mmol/L)	0.237	0.14	0.619	0.41	0.116	0.4
Hba1c (%)	0.14	0.33	0.168	0.28	0.16	0.19
CT**	-0.037	0.84	-0.033	0.61	-0.127	0.69
TT**	-0.06	0.67	-0.064	0.65	-0.229	0.04
All the models were adjusted for age, gender, smoking, statin treatment and baseline value of dependent variable. *Reference group were homozygotes for the allele C (rs1800587). **Reference group were homozygotes for the allele C (rs1143634).						

Table 5: Association of the rs1800587 and rs1143634 genotypes with ultrasonographic markers of carotid atherosclerosis progression in patients with T2DM.

We think that the reported differences in the results of association studies might be due to the fact that different populations and different cohorts of enrolled subjects represent different genetic and environmental background with complex interaction between them. Moreover, multiple linear regression analysis revealed the effect of IL-1α rs1143634 on total plaque thickness progression in a 3.8 year follow up in Caucasians with T2DM.

Our study is the first to report a relationship between the tested polymorphisms (rs1800587 and rs1143634) and progression of subclinical markers of carotid atherosclerosis in subjects in subjects

with T2DM. According to the findings of our study, the exact role of rs1800587 and rs1143634 on the development and progression of carotid atherosclerosis can only be speculated. There are several possible mechanisms of action of IL-1 on the development of atherosclerosis. Gene variability of the IL-1α may exert its affect through the expression (plasma level of inflammatory cytokines) or through other intermediate phenotypes (i.e. obesity and increased body-mass index) [1,10,18,19]. Um and co-workers demonstrated that the polymorphism of rs1800587 affected the transcriptional activity of IL-1a in pre-adipocyte 3T3-L1 cells [10]. Finally, rs1800587 was reported to be associated with few chronic inflammatory diseases including rheumatoid arthritis, Alzheimer disease, and periodontitis [20-22], and chronic inflammation plays a key role in establishing the atherosclerotic lesion [1-3].

The strength of our cross-sectional prospective study is the community-based sample of Caucasians with T2DM, the meticulous evaluation of subclinical markers of carotid atherosclerosis, a rather large cohort of Caucasians with T2DM, and prospective nature of the study. According to calculations the study was appropriately powered to detect differences in subclinical markers of carotid atherosclerosis.

A limitation of the study might be the number of participants involved in the study; however, the study was appropriately powered to detect differences in subclinical markers of carotid atherosclerosis in this cohort of subjects. Another limitation of the study might be the fact that we did not investigate serum levels of IL-1 at the beginning of the study and during follow-up.

To conclude, in our study we reported the effect of both polymorphisms of the IL-1α (rs1800587 and rs11436348) on total plaque thickness upon enrolment in Caucasians with T2DM. Moreover, we demonstrated the effect of IL-1α rs1143634 on total plaque thickness progression in a 3.8 year follow up in Caucasians with T2DM. Our findings indicate that both polymorphisms of the IL-1α gene may have at least a modest effect in the development of carotid atherosclerosis in subjects with T2DM.

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Raziskava 3

SOX6 gene polymorphism (rs16933090) and markers of subclinical atherosclerosis in patients with type 2 diabetes mellitus

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Abstract

Background: The present study was designed to investigate the association between the polymorphism of the *SOX6* gene (rs16933090) and subclinical markers of carotid atherosclerosis, such as carotid intima media thickness (CIMT), the number of affected segments of carotid arteries and the sum of plaque thickness in patients with T2DM. The second aim of the study was to demonstrate an association between the rs16933090 and subclinical markers of coronary artery disease in the same subset of patients with T2DM.

Patients and methods: 595 T2DM subjects were enrolled in the cross-sectional study. Markers of carotid atherosclerosis were assessed ultrasonographically. Additionally, in a subset of subjects with T2DM a coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. Genotyping of *SOX6* gene (rs16933090) was performed using KASPar assays.

Results: In our study we demonstrated the effect of the rs16933090 on coronary calcium score obtained at CCTA, whereas we did not demonstrate any association between the tested polymorphism (rs16933090) and the presence of more than 50% stenotic lesions in coronary arteries, the sum of plaque thickness, the number of involved carotid segments, hsCRP, the presence of carotid plaques, and the presence of unstable carotid plaques.

Conclusions: In our study we demonstrated the effect of the rs16933090 on coronary calcium score obtained at CCTA, whereas we did not demonstrate an important effect of the rs16933090 on either subclinical markers of carotid atherosclerosis or the presence of more than 50% stenotic lesions in coronary arteries in Caucasians with T2DM. We presume that the s16933090 plays a minor role in the development of subclinical atherosclerosis in subjects with T2DM.

Key words: SOX6 gene; genetic polymorphism; association study; carotid intima media thickness; carotid atherosclerosis; coronary computed tomography angiography; coronary calcium score; type 2 diabetes mellitus

Background

Type 2 diabetes mellitus (T2DM) is considered a major epidemic of this century. T2DM is associated with an accelerated progression of atherosclerosis 1. In patients with diabetes, cardiovascular complications are reported about 15 years earlier than in the population without T2DM[1]. Some of the traditional risk factors identified in the general population, such as hypertension, hypercholesterolaemia, smoking and positive family history, also contribute to the high prevalence of cardiovascular disease in patients with diabetes mellitus[2-5]. Moreover, genetic factors have long been known to modulate the risk of atherosclerosis and CVD, and they merit a search for the genes involved in the susceptibility to the atherosclerotic complications of T2DM[6,7].

Dong and co-workers published a GWAS study in which they sought to identify genetic loci influencing carotid plaque in 2 independent samples of 1308 subjects from 100 Dominican families[8]. Carotid plaque was reported to have considerable heritability and might be influenced by loci on chromosomes 11p15, 14q32, and 15q23. After adjustment for age, hypertension, diabetes mellitus, cigarette pack-years, body mass index, and waist-to-hip ratio, significant heritability was detected for plaque presence and plaque area[8]. The authors reported that the *SOX6* gene within the bone morphogenic protein pathway might be a candidate for carotid plaque[8]. Moreover, in the association analysis of the 4 linkage peaks, several single nucleotide polymorphisms in or near *SOX6*, *FSD2*, *AP3S2*, *EFTUD1*, and *MYOD1* were associated with carotid plaque traits in two subsets of Caribbean Hispanics[8].

The present study was thus designed to investigate the association between the polymorphism of the *SOX6* gene (rs16933090) and markers of carotid atherosclerosis, such as carotid intima media thickness (CIMT), the number of affected segments of carotid arteries and the sum of plaque thickness in patients with T2DM. Moreover, the second aim of the study was to demonstrate an association between the rs16933090 and subclinical markers of coronary artery disease - the coronary calcium score and the presence of more than 50% stenotic lesions of coronary arteries in the same subset of patients with T2DM eligible for coronary computed tomography angiography for diagnostic purposes.

Material and methods

This cross-sectional study included 595 consecutive subjects with T2DM, admitted to the diabetes outpatient clinics of the General Hospitals Murska Sobota, and Slovenj Gradec, Slovenia, and from the outpatient department Medicor, Ljubljana[9]. The study protocol was approved by the Slovene Medical Ethics Committee.

Patients were classified as having T2DM according to the current report of the American Diabetes Association[10]. After the informed consent was obtained from the patients, a detailed interview was made concerning smoking habits, the duration and treatment of diabetes, arterial hypertension, and hyperlipidemia. Patients were asked whether they were smokers at the time of recruitment ("current smoker"). Obesity was determined defined as body mass index $\geq$ 30 kg/ m^2 [11]. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the right upper arm of the patients were measured while they were sitting (2 consecutive measurements). Subjects with type 2 diabetes with systolic blood pressure $\geq$ 140 mm Hg or diastolic blood pressure $\geq$ 80 mm Hg and/or subjects who were using antihypertensive drugs were considered to be hypertensive.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured, as described previously[9]. Plaques were defined as a focal intima-media thickening, and divided into 5 types according to their echogenic/echolucent characteristics, as described previously[9]. The inter-observer reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $p < 0.001$).

In 215 out of 595 subjects with T2DM, coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. In 215 subjects with T2DM, coronary calcium score was measured and the presence of CAD was determined. Four regions (LM, LAD, LCX, RCA) were analyzed for the presence of CAD and more than 50% stenotic lesions were looked for in LM, LAD, LCX, RCA.

Blood samples for biochemical analyses were collected. The genomic DNA was extracted from 100 μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). Genotyping of *SOX6* gene (rs16933090) was performed using KASPar assays. Details of the method used can be found on http://www.kbioscience.co.uk/.

Continuous variables are expressed as means ± standard deviations. Continuous clinical data were compared using an unpaired Student's t test or analysis of variance (ANOVA). The Pearson X^2 test was used to compare discrete variables. A two-tailed p-value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 21 (SPSS Inc., Chicago, IL).

Results

Demographic and clinical characteristics in patients with T2DM are demonstrated in Table 1. Almost two third of patients with T2DM were on statin therapy (Table 1). In our cohort of patients with T2DM only 8.9% of them were cigarette smokers. The genotype distribution in patients with T2DM was in Hardy-Weinberg equilibrium for the *SOX6* gene (rs16933090) [T2DM genotype frequencies: TT genotype 67.1%, TC genotype 30.9%, CC genotype 2.0%; $\chi 2 = 3.08$; $p = 0.08$).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of SOX6 gene polymorphism (rs16933090) upon enrolment. In our study, we did not demonstrate an association between the rs16933090 and CIMT, the sum of plaque thickness, the number of involved segments, hsCRP, the presence of carotid plaques, and the presence of unstable carotid plaques (Table 2). We demonstrated the effect of the rs16933090 on the coronary calcium score obtained with CCTA. We did not, however, demonstrate any association between the rs16933090 and other markers of coronary atherosclerosis obtained with CCTA (the number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis) in subjects with T2DM (Tables 2). Additionally, the rs16933090 of the *SOX6* gene was neither associated with other atherosclerosis–associated traits such as increased blood pressure and increased body-mass index) (Table 2).

In a further step, we constructed a logistic regression model to explain the presence of subclinical carotid artery disease in subjects with T2DM according to the rs16933090 that was further adjusted for classic CV risk factors (Table 3). The rs16933090 did not show a significant association before or after adjustment for classic CV risk factors with clinically evident CV disease (Table 3).

Table 1. Baseline characteristics of subjects with T2DM and subjects without T2DM (control group).

	Subjects with T2DM n = 595
Age (years)	62.39 ± 9.61
Male sex (%)	338 (56.8)
Diabetes duration (years)	11.25 ± 7.88
Cigarette smoking (%)	53 (8.91)
Waist circumference (cm)	108.65 ± 12.88
BMI (kg/m2)	31.00 ± 4.74
SBP (mm Hg)	147.1 ± 19.80
DBP (mm Hg)	85.78 ± 11.60
Fasting glucose (mmol/l)	8.04 ± 2.57
HbA1c (%)	7.89 ± 3.56
Total cholesterol (mmol/l)	4.70 ± 1.18
HDL cholesterol (mmol/l)	1.20 ± 0.35
LDL cholesterol (mmol/l)	2.63 ± 0.94
Triglycerides (mmol/l)	1.9 (1.2-2.7)
hs - CRP (mg/l)	3.5 ± 1.18
Statin therapy (%)	375 (63.0)
Antihypertensive agents (%)	499 (83.9)
CIMT (μm)	958 ± 194

BMI – body mass index; SBP - systolic blood pressure; DBP – diastolic blood pressure; HbA1c – glycated haemoglobin; hs-CRP – high sensitivity C-reactive protein; CIMT – carotid intima media thickness

Table 2. Comparison of markers of carotid atherosclerosis in subjects with T2DM at the beginning of the study with regard to the *SOX6* gene (rs16933090) genotypes.

	rs16933090			
	TT	TC	CC	p
Intima media thickness (μm)	1002 ± 190	1008 ± 196	917 ± 210	0.50
Number of involved carotid segments	2.53 ± 1.59	2.48 ± 1.68	2.20 ± 1.80	0.89
Sum of plaque thickness (mm)	7.74 ± 4.40	8.29 ± 4.73	6.73 ± 4.60	0.56
hsCRP (mg/l)	3.26 ± 3.54	4.02 ± 3.59	3.00 ± 3.89	0.16
Presence of carotid plaques n (%)	339 (85.0)	152 (82.6)	9 (75)	0.89
Presence of unstable carotid plaques n (%)	183 (45.9)	95 (51.6)	7 (58.3)	0.63
Coronary calcium score	560 ± 215	429 ± 416	206 ± 282	**0.005**
Number of coronary arteries with more than 50% stenosis**	1.15 ± 1.21	0.57 ± 0.90	0.82 ± 1.06	0.14
The presence of at least one coronary segment with more than 50% stenosis	3 (37.5%)	(24 33.8%)	54 (39.7%)	0.7
BMI (kg/m2)	30.1 ± 2.6	30.8 ± 4.4	31.1 ± 4.9	0.7
SBP (mm Hg)	144.3 ± 24.5	144.5 ± 18.7	144.7 ± 19.2	0.99
DBP (mm Hg)	86.7 ± 7.5	85.4 ± 11.6	85.3 ± 11.6	0.96
HbA1c (%)	7.3 ± 1.2	7.9 ± 1.5	8.1 ± 2.5	0.8

**Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM: **the number of coronary arteries (LM, LAD, LCX, RCA) with more than 50% stenosis*
BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HbA1c – glycated haemoglobin

Table 3. Logistic regression model to explain the presence of carotid atherosclerosis (carotid plaques) according to the rs16933090.

	OR (95% CI)	P value	OR (95% CI)	P value
rs16933090	1.23 (0.82-1.72)	0.6	1.54 (0.95-2.15)*	0.4*

**Analyses adjusted for gender, age, arterial hypertension, dyslipidemia, obesity (BMI ≥ 30 kg/ m2), and cigarette smoking.*

Discussion

In the present study we pursued the hypothesis that the rs16933090 of the *SOX6* gene may be a genetic marker of subclinical atherosclerosis of carotid and coronary arteries. In the study we did not demonstrate an important effect of the rs16933090 on markers of either carotid atherosclerosis or coronary atherosclerosis obtained with CCTA (the number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis) in Caucasians with T2DM, whereas we demonstrated the effect of the rs16933090 on coronary calcium score obtained at CCTA.

In the study we did not demonstrate a statistically significant effect of the rs16933090 on markers of carotid atherosclerosis, such as CIMT, the number of affected segments of carotid arteries and the sum of plaque thickness in carotid arteries in patients with T2DM. Our findings are not in accordance with some previous reports demonstrating that the variability in the *SOX6* gene might be associated with carotid atherosclerosis[8]. Dong and co-workers reported that the rs16933090 was associated with carotid plaque traits (presence of plaque and plaque area) in two subsets of Caribbean Hispanics (the Northern Manhattan Study data set and the Northern Manhattan Study Dominican subset)[8]. We presume that the reported differences of both association studies might be due to the fact that different populations represent different genetic and environmental background with complex interaction between them.

Moreover, variability in the *SOX6* gene was also demonstrated to be associated with other intermediate phenotypes (i.e. obesity, arterial hypertension, hyperinsulinemia, diabetes, cardiovascular calcification) that might influence the development of atherosclerosis[12-17]. In addition, animal models suggested that *SOX6*, located within the bone morphogenic protein pathway, plays an important role in obesity-related insulin resistance[12,13]. Additionally, bone morphogenic proteins were implicated in the pathophysiology of T2DM and cardiovascular calcification[14,15]. Our findings are in accordance with the reported effect of this pathway on cardiovascular calcification, since we demonstrated the effect of the rs16933090 on coronary calcium score obtained at CCTA. In the study, however, we did not demonstrate an important effect of the rs16933090 on other markers of coronary atherosclerosis obtained with CCTA (the number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis) in Caucasians with T2DM. CIMT is considered a separate phenotype from either carotid/coronary plaques or cardiovascular calcification, and we presume that they have distinct genetic determinants. Moreover, they are most probably not regulated via similar genetic/biological mechanisms[18-24]. Additionally, our findings did not demonstrate the variability of *SOX6* gene (rs16933090) to be associated with other atherosclerosis–associated traits such as increased blood pressure and increased body-mass index in subjects with T2DM.

The strength of our cross-sectional study is the community-based sample of Caucasians with T2DM, and the detailed phenotypic characterization of the subjects with regards to ultrasonically determined carotid atherosclerosis, as well as having data about a rather large sample of Caucasians with T2DM. According to calculations the study was appropriately powered to detect differences in CIMT and coronary calcium score.

A limitation is the use of cross-sectional data in the analysis, restricting the possibility of causal inferences from our data.

To conclude, in our study we demonstrated the effect of the rs16933090 on coronary calcium score obtained at coronary computed tomography angiography, whereas we did not demonstrate a significant effect of the rs16933090 on markers of either carotid atherosclerosis or coronary atherosclerosis obtained with CCTA (the number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis) in Caucasians with T2DM. Our findings suggest that the tested polymorphism in the SOX6 gene plays a minor role (if any) in the development of subclinical atherosclerosis in subjects with T2DM.

Conflict of interest

The authors declare no conflict of interest related to this work.

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Raziskava 4

Polymorphisms +45T>G and +276G>T of the adiponectin gene does not affect plasma adiponectin level and carotid intima-media thickness in patients with diabetes mellitus type 2

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Abstract

Background: Despite increasing evidence of adiponectin's anti-inflammatory and antiatherogenic effects, its role in atherogenesis remains uncertain. The aim of the present study is to investigate the association between +45T>G and +276G>T polymorphisms of the adiponectin gene and both plasma adiponectin levels and carotid intima-media thickness in patients with diabetes mellitus type 2.

Methods: 301 diabetic patients, divided into three categories on the basis on BMI were enrolled in the study. Carotid intima-media thickness (CIMT) was assessed ultrasonographically. Plasma adiponectin levels were measured by enzyme-linked immunosorbent assay (ELISA). Genotypes were determined by real-time PCR.

Results: Adiponectin level and prevalence of the G allele of 45T>G polymorphism decreased significantly with increasing BMI category. G allele of +45T>G polymorphism was associated with higher plasma adiponectin level only after adjustment for age, sex and BMI. No statistically significant difference in CIMT and +276T>G genotypes distribution was observed between BMI categories. None of the polymorphisms as well as plasma adiponectin level was associated with CIMT after adjustment for covariates.

Conclusion: The G allele of the +45T>G polymorphism is not independently associated with plasma adiponectin level and is not associated with CIMT. +276G>T polymorphism is not associated with plasma adiponectin levels and CIMT in diabetic patients.

Keywords: adiponectin polymorphism; plasma adiponectin level; carotid atherosclerosis; intima-media thickness; diabetes mellitus

Introduction

Thanks to the advanced insights in its biology, adipose tissue has been recognized not just an inner energy depot but also as an important endocrine organ secreting a variety of proteins that regulate body metabolism, inflammation and immune responses[1]. These actions are mediated by a number of physiologically active molecules secreted by adipocytes, collectively named adipocytokines - such as leptin, adipsin, tumor necrosis factor (TNF)-ά, plasminogen-activator inhibitor type 1 (PAI-1), resistin, and adiponectin[2].

Adiponectin is a specific protein expressed exclusively in differentiated adipocytes[3]. The adiponectin gene (ADIPOQ) consists of three exons and two introns, spanning a 17-kb region on chromosome 3q27[3]. Several single nucleotide polymorphisms (SNPs) in the adiponectin gene have been reported. Because of their high frequencies in all populations the most commonly studied SNPs at the ADIPOQ locus are silent T to G substitution in exon 2 (+45T>G, rs2241766) and G to T substitution in intron 2 (+276G>T, rs1501299). Studies investigating association between these two polymorphisms and plasma adiponectin levels yielded contrasting results. Some studies reported an association between T allele of the SNP +45T>G and lower plasma adiponectin levels[4,5] while others reported no association of the kind[6,7]. The G/G genotype of SNP +276G>T was associated with lower plasma adiponectin levels as compared to the G/T and T/T genotypes[5,8]. However,

some studies failed to report such association[9].

Adiponectin has been recognized to have an important role in the modulation of glucose and lipid metabolism in insulin-sensitive tissues[2]. Plasma adiponectin concentrations are inversely correlated with HOMA score, insulin levels, plasma triglycerides, total and LDL-cholesterol levels[10]. Conversely, adiponectin is directly correlated with VLDL apoB catabolism and HDL-cholesterol levels[10]. Recent studies have indicated that adiponectin has also anti-inflammatory and antiatherogenic effects, supporting a hypothesis that it could take part in atherosclerosis pathophysiology differentially than by affecting insulin resistance, obesity and plasma lipid levels. Adiponectin accumulates in the subendothelial space of an injured human artery[11] and inhibits monocyte adhesion and expression of endothelial adhesion molecules (E-selectin, VCAM-1, ICAM-1) by inhibition of TNF-α induced nuclear factor-κB activation[12,13]. By suppressing expression of macrophage scavenger receptors, adiponectin suppresses lipid accumulation in macrophages[14] and stimulates the production of nitric oxide (NO) in endothelial cells[15] as well, but it could also affect vascular remodelling by inhibiting proliferation and migration of smooth muscle cells[16].

In contrast to the other adipocytokines, whose levels are increased in obesity due to increased total body fat mass, adiponectin levels are paradoxically lower in obese than in lean humans[6,17]. Lower adiponectin plasma levels have also been reported in patients with diabetes, arterial hypertension and coronary artery disease compared to control subjects[12,17-19]. The great majority of patients with diabetes mellitus type 2 (DM2) are obese, hypertensive, hyperlipidemic or have metabolic syndrome. Consequently, it remains unclear whether the decreased adiponectin level is a risk factor for atherogenesis or it is a consequence of the presence of diabetes, obesity or hypertension[20].

Until nowadays, a limited number of studies investigated an association between adiponectin plasma levels and carotid atherosclerosis, especially in diabetic patients. Numerous studies reported that lower plasma adiponectin level is associated with greater carotid intim-media thickness (CIMT)[20,21-25], whereas some reported this association only in males[26]. However, no association between +45T>G and +276G>T polymorphisms and CIMT have not been reported yet[27,28].

The aim of the present study is to investigate association between +45T>G and +276G>T polymorphisms of the adiponectin (ADIPOQ) gene and both plasma adiponectin levels and carotid intima-media thickness in patients with DM2.

Materials and Methods

Subjects

In this cross-sectional study, 301 diabetic patients from the diabetic ambulance of the Murska Sobota General Hospital, Slovenia were observed. They were selected randomly among patients admitted to the diabetic ambulance as satisfying the inclusion criteria. All the subjects enrolled in the study were Slovenian and were

not related. Patients were excluded if they had homozygous familial hypercholesterolemia or previous cardiovascular event such as myocardial infarction or cerebral stroke. The research protocol was approved by the National Medical Ethics Committee. After the informed consent was obtained from the patients, a detailed interview was made concerning smoking habits, duration and treatment of diabetes, arterial hypertension, hyperlipidemia and consuming any other drugs. Patients were asked if they were smokers at the time of recruitment ("current smoker"). Diabetic subjects with systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg and/or subjects using antihypertensive drugs were considered hypertensive. All patients were divided into three categories according to World Health Organization obesity categories (BMI < 25 kg/m^2, 25 ≤ BMI < 30 kg/m^2 and BMI ≥ 30 kg/m^2).

Utrasonographic analysis

Atherosclerotic changes on the carotid arteries were assessed by ultrasonography. High resolution B mode, color Doppler, and pulse Doppler ultrasonography of both carotid arteries were performed with the commercially available ultrasound system Toshiba Aplio SSA-700 (Toshiba Medical. System Corp., Tokyo, Japan) with a multi-frequency linear array transducer. All examinations were performed by a single expert radiologist blinded to the participant's diabetes status. Patients were examined in the supine position with the head tilted backwards. The protocol involved the scanning of the common carotid artery (CCA), carotid bifurcations and origins of internal carotid arteries (ICA). The CIMT was measured at 3 sites along the 10 mm-long segment of the far wall of the CCA free of plaques in agreement with the carotid intima-media thickness consensus[29]. CIMT on the left and on the right were calculated as the mean of three readings, and the mean of the left and right CCA-IMT measurements was used in the analysis.

Biochemical analyses

Blood samples for biochemical analyses were the following: total cholesterol, triglyceride levels, HDL and LDL cholesterol level, fasting blood glucose and C-reactive protein (CRP) were collected after an overnight fasting. All blood biochemical analyses were determined by standard biochemical methods in the hospital's accredited lab. Assays for plasma concentrations of adiponectin were performed in triplicate on microplate reader (Tecan, Männedorf, Switzerland) using human ELISA Kit for adiponectin (BioVendor, Lab. Med. Inc., Brno, Czech Republic). Assay sensitivity was 10 pg/mL for adiponectin.

Genotyping

Genomic DNA was extracted from 100 µL of whole blood using a FlexiGene DNA isolation kit, in accordance to the recommended protocol (Qiagene GmbH, Hilden, Germany). The +45T>G (rs2241766) and +276G>T (rs1501299) were determined by using the KASPar-On-Demand SNP Genotyping Assay (KBioscience Ltd., Hoddesdon, UK) and the StepOne Real-Time PCR System (ABI, Foster City, CA, USA) in accordance to the manufacturer's instructions.

Statistical analysis

Continuous variables were expressed as means ± standard deviations when normally distributed and as medians and interquartile ranges when asymmetrically distributed. Normality was tested using the Kolmogorov–Smirnov test. Continuous clinical data were compared by analysis of variance (ANOVA) when normally distributed and the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X^2 test was used to compare discrete variables. Pearson's correlation was performed to examine the association between independent variables. Because of the high correlation between body mass index (BMI) and waist circumference (Pearson's correlation coefficient $r = 0.45$ and $p < 0.001$) these variables were not included in the same statistical model.

A general linear model analysis was undertaken to test for associations between the +45T>G and +276G>T polymorphisms and both plasma adiponectin level and CIMT after adjusting for confounding variables (age, gender and body mass index - BMI). To determine the association of the +45T>G and +276G>T polymorphisms and plasma adiponectin level with CIMT (using CIMT as continuous dependent variable), a multivariate linear regression analysis was performed. We used an additive model in which common allele homozygotes were coded as 1, heterozygotes as 2 and rare allele homozygotes as 3. Candidate variables to enter the model were the following: age, gender, BMI, hypertension, smoking status, plasma levels of HbA1c, LDL, HDL cholesterol and triglycerides. A two-tailed P value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

Results

Table 1 presents the differences in clinical and biochemical characteristics between diabetic patients divided into three categories, according to World Health Organization obesity criteria. We observed significant trends for increased waist circumference ($p < 0.001$), prevalence of hypertension ($p = 0.007$) and triglyceride level ($p < 0.001$) across the obesity categories, while age, HDL cholesterol level and adiponectin level decreased significantly with increasing BMI category ($p = 0.04$, 0.006 and 0.01, respectively). CIMT increased across BMI categories, but difference was not statistically significant ($p = 0.68$). We observed no stastistically significant difference regarding gender distribution, duration of diabetes, smoking prevalence, systolic and diastolic blood pressure, fasting glucose and HbA1c level, LDL and total cholesterol level between subjects in different obesity categories.

Genotype distribution and allele frequencies of both +45T>G and +276G>T ADIPOQ polymorphisms according to BMI categories were presented in Table 2. The ADIPOQ +45T>G and +276G>T genotypes distributions were compatible with Hardy-Weinberg expectations. Because only six subjects were homozygous for the rare G allele of +45T>G polymorphism, we combined them with the subjects carrying the GT genotype in all statistical analyses. Prevalence of carrying mutant G allele of the +45T>G polymorphism decreased significantly with increasing BMI category ($p = 0.0002$). When comparing +276G>T genotype distribution and allele frequencies between different BMI categories no statistically significant difference was observed ($p = 0.61$).

Table 1. Clinical and biochemical characteristics of study subjects according to World Health Organization obesity categories.

	All subjects n = 301	BMI < 25 kg/m^2 n = 25 (8.3%)	25 ≤ BMI < 30 kg/m^2 n = 101 (33.6%)	BMI ≥ 30 kg/m^2 n = 175 (58.1%)	P*
Age (years)	62.7 ± 9.8	66.5 ± 13.1	63.5 ± 9.5	61.6 ± 9.8	**0.04**
Male sex	139 (46.2)	8 (32.0)	50 (49.5)	83 (47.4)	0.28
Duration of diabetes (years)	9.6 ± 8.2	12.0 ± 12.1	9.3 ± 7.8	9.5 ± 7.8	0.34
Smokers (%)	16 (5.4)	1 (4.0)	7 (6.9)	8 (4.5)	0.67
Waist circumference (cm)	108 (63-155)	88 (82-94)	109 (94-115)	112.5 (88-144)	**<0.001**
Hypertension (%)	262 (88.2)	19 (76.0)	83 (82.1)	162 (92.6)	**0.007**
Systolic blood pressure (mm Hg)	143. ± 19.3	142.0 ± 24.9	140.5 ± 18.3	143.8 ± 18.9	0.61
Diastolic blood pressure (mm Hg)	84.5 ± 10.9	83.5 ± 11.4	82.2 ± 11.9	85.5 ± 10.2	0.23
Fasting glucose (mmol/L)	7.7 (2.5-17.7)	4.1 (3.3-4.9)	8.2 (6.1-15.1)	8.2 (5.5-13.9)	0.09
HbA1c (%)	8.0 ± 1.2	7.3 ± 1.1	8.2 ± 1.4	8.0 ± 1.2	0.32
Total cholesterol (mmol/L)	4.8 (0.8-8.8)	4.8 (4.2-5.4)	4.8 (3.9-5.6)	4.9 (3.3-6.8)	0.87
HDL cholesterol (mmol/L)	1.1 (0.1-2.4)	1.4 (0.9-2.3)	1.3 (1.1-1.4)	1.2 (0.7-2.2)	**0.006**
LDL cholesterol (mmol/L)	2.6 (0.3-5.6)	2.3 (2.0-3.2)	2.5 (1.1-4.4)	2.8 (2.5-3.1)	0.73
Triglycerides (mmol/L)	2.1 (0.6-10.4)	1.6 (1.3-1.9)	2.2 (0.8-6.9)	2.4 (0.8-9.4)	**<0.001**
Plasma adiponectin (µg/mL)	5.2 ± 1.8	6.2 ± 2.0	5.1 ± 1.8	4.9 ± 1.7	**0.01**
CIMT (mm)	1.1 (0.65-1.45)	0.98 (0.80-1.15)	1.10 (0.95-1.20)	1.12 (0.70-1.25)	0.68

Results for continuous variables are reported as means ± SD when normally distributed and median (interquartile range) when asymetrically distributed. Discrete variables were reported as frequency (pecentages).
p values reported for Kruskal-Wallis test for continuous and X^2 test for discrete variables.*
BMI-body mass index; CIMT- carotid intima-media thickness; HDL - high-density lipoprotein; hsCRP- high sensitive C-reactive protein; LDL - low-density lipoprotein;

Table 2. Genotypes and allele frequencies of +45T>G and +276G>T polymorphisms of the adiponectin gene (ADIPOQ) in diabetic patients according to World Health Organization obesity categories.

	BMI < 25 kg/m^2 25 (8.3%)	25 ≤ BMI < 30 kg/m^2 101 (33.6%)	BMI ≥ 30 kg/m^2 175 (58.1%)	p
+45T>G				
TT	141 (80.6)	87 (86.1)	110 (88.0)	**0.0002**
GG+TG	34 (19.4)	14 (13.9)	3 (12.0)	
276G>T				
GG	10 (40.0)	51 (50.5)	95 (54.3)	
TG	11 (44.0)	40 (39.6)	66 (37.7)	0.61
TT	4 (16.0)	10 (9.9)	14 (8.0)	
Alleles				
G	31 (62.0)	142 (70.3)	256 (73.1)	0.25
T	19 (38.0)	60 (29.7)	94 (26.9)	

Results are presented as frequency (percentages). P values calculated using X^2 test.

Relationship between ADIPOQ +45T>G and +276G>T polymorphisms and both plasma adiponectin level and carotid intima-media thickness (CIMT) were presented in Table 3. When compared plasma adiponectin levels between TT homozygotes and carriers of G allele, no statistically significant difference was obseved (p = 0.22) but after adjustment for age, sex and BMI difference between two subgorups became statistically significant (p = 0.03). We observed no statistically significant difference in CIMT in regard to +45T>G genotypes neither before nor after adjustment for confounding variables (p = 0.22 and 0.26, respectively). When comparing plasma adiponectin levels and CIMT in regard to +276T>G genotypes, no statistically significant difference was observed.

Results of multivariate linear regression analysis were presented in Table 4. Both models were adjusted for

the presence of well established risk factor for CIMT: age, sex and BMI, history of hypertension, smoking status, plasma level of HbA1c, HDL, LDL and triglycerides. Multivariate linear regression analysis showed no statistically significant association between plasma adiponectin level and ADIPOQ polymorphisms with CIMT.

Table 3. Relationship between genotypes of the ADIPOQ polymorphisms and both plasma adiponectin level and crotid intima-media thickness (CIMT) in patients with diabetes mellitus type 2.

		Plasma adiponectin (μg/mL)	p	p*	CIMT (mm)	p	p*
+45T>G	TT	5.1 ± 1.7	0.22	**0.03**	1.15 (1.05-1.15)	0.22	0.26
	GG+TG	5.5 ± 2.3			1.10 (1.05-1.15)		
+276G>T	GG	4.9 ± 1.8			1.15 (1.05-1.20)		
	TG	5.3 ± 1.9	0.30	0.10	1.10 (1.05-1.15)	0.24	0.14
	TT	5.4 ± 1.4			1.15 (1.08-1.15)		

Data are presented as mean ± SD when normally distributed and median (interquartile range) when asimetrically distributed.
** general linear model adjusted for age, sex and BMI*
CIMT – carotid intima-media thickness;

Table 4. Multivariate linear regression analysis for association between genotypes of the ADIPOQ polymorphisms and crotid intima-media thickness (CIMT) in patients with diabetes mellitus type 2.

		β	P*
Model 1	Plasma adiponectin (μg/mL)	-0.18	0.31
	+45T>G TG+GG	-0.05	0.72
Model 2	Plasma adiponectin (μg/mL)	-0.19	0.24
	+276G>T**	-0.21	0.11

**BMI-body mass index; CIMT – carotid intima-media thickness; HDL – high-density lipoprotein; hsCRP – high sensitive C-reactive protein; LDL – low-density lipoprotein;*
**Both models adjusted for age, sex and BMI, history of hypertension, smoking status, plasma level of HbA1c, HDL, LDL and triglycerides.*
***common allele homozygotes were coded as 1, heterozygotes as 2 and rare allele homozygotes as 3.*

Discussion

In the present study statistically significant higher adiponectin level in carriers of the G allele of +45T>G polymorphism was observed only after adjustment for confounding variables. No statistically significant difference in adiponectin level was observed in regard to +276G>T genotypes. None of the tested polymorphisms, as well as adiponectin level was associated with CIMT in diabetic patients after adjustment for confounding variables.

The inverse correlation between adiponectin level and BMI has been well documented[6,17]. Consistently to previous reports, our study confirmed statistically significant lower plasma adiponectin levels in diabetics with higher BMI. As it has been reported that plasma adiponectin level could be also affected by age and sex[26], we adjusted general linear model analyses for those three variables.

Numerous studies have already tried to investigate genetic factors affecting plasma adiponectin levels, providing contradictory results. Some studies reported an association between T allele of the SNP +45T>G and lower plasma adiponectin levels[4,5,8]. In the present study, G allele of the +45T>G polymorphism was associated

with higher adiponectin levels only after adjustment for age, sex and BMI. This finding suggests that association is not independent and is probably affected mainly by BMI, as plasma adiponectin levels were statistically significantly different between BMI categories. This finding is consistent to the previous reports suggesting that the +45T>G polymorphism does not affect plasma adiponectin level[6,7,30].

As with the +45T>G polymorphism, the evidence supporting an association between the +276G>T ADIPOQ polymorphism and plasma adiponectin level is inconsistent[5,8]. The G/G genotype of SNP +276G>T has been reported to be associated with both lower[4,5,8] and higher[31] plasma adiponectin level as compared to the G/T and T/T genotypes. In the present study, homozygotes for the G allele had the lowest plasma adiponectin level but the difference was not statistically significant. Our finding is consistent to the previous reports showing that +276G>T genotype does not affect plasma adiponectin level[9,32]. Recent study suggested that observed effects of SNP +45T>G and +276G>T on plasma adiponectin level probably resulted from a linkage disequilibrium with two functional polymorphisms: -1391G>A, and -1377G>C[26] or the other, still unidentified functional variants[8].

Numerous studies reported that lower plasma adiponectin level is associated with greater CIMT[20,21-25], whereas some reported this association only in males[26]. In the present study, the lowest adiponectin level was observed in category with the highest CIMT, but difference in CIMT between categories was not statistically significant. However, multivariate linear regression analysis showed no association between adiponectin level and CIMT in diabetic patients after adjustment for confounding variables.

The great majority of studies of the ADIPOQ polymorphism have been focused on their association with CVD risk, yielded very heterogenous results[8,31]. A limited number of studies focused on association between ADIPOQ polymorphisms and CIMT reported no such association for both +45T>G[27,28] and +276G>T[28] polymorphism. Our study confirmed previous findings, as none of the tested polymorphisms was associated with CIMT, even after adjustment for plasma adiponectin level and other well-known cardiovascular risk factors.

Lower plasma adiponectin level has been reported to be associated with a number of well-known cardiovascular risk factors - such as age, sex, obesity, hyperlipidemia and presence of hypertension or diabetes[12,17-19,26,31]. In the diabetic population plasma level of glycated haemoglobin HbA1c appeared as covariate of considerable importance[26]. It is possible that inconsistency regarding association between adiponectin and atherosclerosis results from its cross-sectional associations with numerous cardiovascular risk factors[33]. Available data provided an evidence that traditional risk factors associated with obesity and the metabolic syndrome accounts for most of the observed inverse relationship between plasma adiponectin level and CIMT[26].

A recent study reported that the ADIPOQ gene is expressed in vascular tissue[34]. This made the authors speculate that adiponectin generated within the vasculature might directly influence endothelial function, without affecting plasma adiponectin levels[35]. Therefore, the further in vitro studies are needed to explore

whether variation in the ADIPOQ gene in vascular tissue affects local adiponectin expression, endothelial function, and ultimately cardiovascular risk.

Our study has possible limitations due to a small size of the sample providing limited power to study moderate genetic effects, cross-sectional design and recruitment of patients from one single centre. Finally, in the present study we did not measure plasma levels of other adipocytokines, such as leptin. The ratio of leptin and adiponectin may be also relevant[36], although the recent data suggest that adiponectin alone is a stronger predictor of atherosclerosis[37].

Conclusion

The present study provided no evidence of association between the +45T>G and the +276G>T polymorphisms of the ADIPOQ gene with both plasma adiponectin level and CIMT in diabetic patients. In the present study plasma adiponectin level did not appear as an independent risk factor for carotid atherosclerosis.

However, the further studies with larger samples in different populations, considering different ADIPOQ polymorphisms are needed in order to get definitely elucidated adiponectin's role in the atherosclerotic process.

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Raziskava 5

C-reactive protein as a marker of progression of carotid atherosclerosis in subjects with type 2 diabetes mellitus

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Abstract

Background: Our prospective study was designed to evaluate the effect of inflammatory markers on the presence and progression of subclinical markers of carotid atherosclerosis in 3.8-year follow-up period in patients with type 2 diabetes mellitus (T2DM).

Patients and methods: 595 subjects with T2DM were enrolled in the prospective study. Subclinical markers of carotid atherosclerosis (carotid intima media thickness, plaque thickness, and plaques presence) were assessed with ultrasound at the time of recruitment and after 3.8-years. Subjects with T2DM were divided according to the plasma high sensitive C-reactive protein (hs-CRP) levels into 2 groups (subjects with hs-CRP≥2 mg/L and subjects with hs-CRP below 2 mg/L).

Results: Subjects with T2DM with hs-CRP≥2 mg/L had higher carotid intima media thickness (CIMT) in comparison with subjects with T2DM with hs-CRP below 2 mg/L, and higher incidence of plaques and unstable plaques in comparison with subjects with T2DM with hs-CRP below 2 mg/L. Multivariate logistic regression analysis found the association between the HDL cholesterol level and presence of plaques, whereas inflammatory marker hs-CRP was not associated with subclinical markers of progression of carotid atherosclerosis. Multiple linear regression analysis found the association between the hs-CRP level and either CIMT progression rate or a change in the number of sites with plaques in a 3.8-year follow-up.

Conclusions: We demonstrated an association between inflammatory marker hs-CRP and either CIMT or incidence of plaques/unstable plaques at the time of recruitment in Caucasians with T2DM. Moreover, we found the association between hs-CRP level and either CIMT progression rate or a change in the number of sites with plaques in a 3.8-year follow-up in subjects with T2DM.

Key words: carotid atherosclerosis**;** inflammation; high sensitive C-reactive protein; prospective study

Introduction

It is generally accepted that beside cholesterol accumulation, chronic inflammation and immune system are involved in the pathogenesis of atherosclerosis, whereas inflammation is considered to play a crucial role in the initiation of atherosclerotic process [1-3]. A genetic variability of the inflammatory genes is presumed to affect the development of atherosclerosis via interaction with environmental factors [4]. Several reports indicate that genetic variability in the inflammatory genes may alter their transcriptional activity and contribute to susceptibility to cardiovascular disease [5-7].

In patients with diabetes mellitus, cardiovascular complications are reported about 15 years earlier than in the population without T2DM [8, 9]. Some of the traditional risk factors identified in the general population, such as arterial hypertension, dyslipidemia, cigarette smoking and parental history of cardiovascular diseases, also contribute to the high prevalence of cardiovascular disease in patients with diabetes mellitus [8, 9].

Ridker and co-workers reported that increasing baseline hs-CRP levels within JUPITER were associated

with increasing vascular risk in analyses treating hs-CRP as a continuous variable, as an ordinal variable, and as a threshold variable [10]. Additionally, the relative risk reduction associated with rosuvastatin was similar in magnitude across the tertile and threshold levels of entry hs-CRP. Ridker and co-workers demonstrated that absolute risk reduction associated with rosuvastatin within JUPITER was greatest among those with the greatest entry hs-CRP levels [10]. Therefore, the benefit of hs-CRP testing appears to rely on its generally consistent association with modestly increased absolute risk, and thus anticipated higher *absolute* benefit from treatment [10]. Secondary analysis of the Heart Protection Study found that statins achieve a similar relative risk reduction at all levels of hs-CRP, including in patients with low hs-CRP [11].

Despite several data demonstrating a relationship between CRP and cardiovascular disorders [10-13, 12], there is no study evaluating between CRP and progression of subclinical markers of carotid atherosclerosis in subjects with T2DM.

Aim of the study: Our prospective study was designed to evaluate the effect of inflammatory markers on the presence and progression of subclinical markers of carotid atherosclerosis in 3.8-year follow-up period in patients with type 2 diabetes mellitus (T2DM).

Materials and methods

Patients

In this prospective study, 595 consecutive subjects with T2DM, admitted to the diabetes outpatient clinics of the General Hospitals Murska Sobota and Slovenj Gradec, Slovenia, and from the outpatient department Medicor, Ljubljana, were enrolled. Patients were classified as having T2DM according to the current report of the American Diabetes Association [14]. Exclusion criteria were: homozygous familial hypercholesterolaemia or a history of cardiovascular event (i.e. acute coronary syndrome or a cerebrovascular stroke). Patients with acute inflammatory disease and acute phase of chronic inflammatory diseases were not included in the study. The research protocol was approved by the National Medical Ethics Committee. Clinical data, including smoking habits, duration and treatment of diabetes, arterial hypertension, hyperlipidemia and consumption of any other drugs, were obtained from medical records and questionnaires. Patients were asked if they were smokers at the time of recruitment (current smoker).

Ultrasonographic analysis

An ultrasound analysis was performed using the portable ultrasound system Toshiba Aplio SSA-700 (transducer 7,5–10 MHz; Toshiba Medical. System Corp., Tokyo, Japan). All examinations were performed by two radiologists, blinded to the participant's diabetes status. The carotid arteries were examined from the supraclavicular fossa to the submandibular angle, including the common carotid artery (CCA), carotid bifurcations and origins of internal carotid arteries (ICA).

The carotid intima media thickness (CIMT), defined as the distance from the leading edge of the lumen-

intima interface to the leading edge of the media-adventitia interface, was measured at 3 sites 10 mm proximal to the flow diverter of the far wall of the CCA free of plaques, in agreement with the carotid intima-media thickness consensus [15]. The CIMT on the left and on the right were calculated as the mean of three readings, and the mean of the left and right CCA-CIMT measurements was used in the analysis, as described previously [16,17]. The interobserver reliability for CIMT measurements was found to be substantial ($\kappa = 0.74$, $p < 0.001$). Plaques were identified on both the near and the far walls in the CCA, bulb and ICA, bilaterally. In the presence of plaque, the CIMT was measured at the segment without plaque. Due to its echogenic/echolucent characteristics we divided all the plaques into 5 types [17].

Subjects with T2DM were prospectively followed, and after 3.8 ± 0.5 years control ultrasound examination of carotid arteries was performed on 426 out of 595 subjects (71.6%) with T2DM. The annual CIMT progression rate, the increase in total plaque thickness and the number of sites with plaques were evaluated to appreciate the progression of carotid atherosclerosis.

Biochemical analyses

Blood samples for standard biochemical analyses, such as total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated haemoglobin (HbA1c), hs-CRP were collected after a 12-hour fasting period, and analysed in the hospitals' accredited lab.

Statistical analysis

Continuous variables were expressed as means ± standard deviations. Continuous clinical data were compared using an unpaired Student's *t* test or analysis of variance (ANOVA). The Pearson X^2 test was used to compare discrete variables. Pearson's correlation was performed to examine the association between independent variables. Due to the high correlation of systolic blood pressure with the diastolic blood pressure ($r = 0.57$, $p < 0.001$) they were not included together in the same statistical model.

Multivariable linear regression analysis was performed to determine the association of the plasma CRP levels with the CIMT/annual progression of CIMT and change in number of sites with plaque/total plaque thickness. A multivariate logistic regression analysis was performed to determine the association of the plasma CRP levels with the presence of atherosclerotic plaques on the carotid arteries or the presence of unstable plaques. All the regression models were adjusted for the presence of well-established cardiovascular risk factors: age, gender, hypertension, systolic blood pressure, smoking, plasma levels of LDL and HDL cholesterol, triglycerides, HbA1c and statin treatment. The results were presented as standardized β coefficients and P-values for the linear regression and by odds ratios and 95% CIs for the logistic regression. A two-tailed P value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

Results

Baseline characteristics (demographic and clinical) of patients with T2DM are demonstrated in Table 1. Baseline characteristics of the 595 subjects with T2DM were analyzed according to hs-CRP status (low [< 2 mg/L] or high [≥ 2 mg/L]). Two hundred and seventy-two subjects with T2DM (45.7%) had low hs-CRP, and 323 subjects with T2DM had high hs-CRP. The comparison of clinical characteristics, biochemical parameters, and atherosclerotic markers was performed with regard to plasma hs-CRP level upon enrolment and during follow-up (Tables 2, 3, and 4). Subjects with T2DM with hs-CRP ≥ 2 mg/L had higher waist circumference, body mass index, and lipid levels (total cholesterol, LDL and triglycerides) at the time of recruitment in comparison with subjects with T2DM with hs-CRP below 2 mg/L (Table 2). Subjects with T2DM with hs-CRP ≥ 2 mg/L had higher CIMT in comparison with subjects with T2DM with hs-CRP below 2 mg/L, and higher incidence of plaques and unstable plaques in comparison with subjects with T2DM with hs-CRP below 2 mg/L (Table 3).

During follow-up, there was a tendency toward faster progression of CIMT thickness and total plaque thickness (Table 4).

Table 1. Baseline clinical and biochemical characteristics of subjects with T2DM.

		Subjects with T2DM n = 595
Age (years)		61.38 ± 9.65
Male gender (%)		338 (56.8)
DM duration (years)		11.25 ± 7.88
Smoking prevalence (%)		53 (8.91)
Waist circumference (cm) – males		106.76 ± 14.26
Waist circumference (cm) – females		110.78 ± 10.76
BMI (kg/m2)		30.96 ± 4.74
Systolic blood pressure (mm Hg)		146.98 ± 19.98
Diastolic blood pressure (mm Hg)		85.75 ± 11.62
Fasting glucose (mmol/L)		8.04 ± 2.57
HbA1c (%)		7.89 ± 3.56
Total cholesterol (mmol/L)		4.70 ± 1.19
HDL cholesterol (mmol/L)		1.19 ± 0.35
LDL cholesterol (mmol/L)		2.63 ± 0.94
Triglycerides (mmol/L)		1.9 (1.2-2.7)
Non –HDL-cholesterol (mmol/L)		3.52 ± 1.19
hs-CRP (mg/L)	< 2	272 (45.7)
	≥ 2	323 (54.3)

DM - diabetes mellitus; hs-CRP - high sensitivity C-reactive protein; BMI – body mass index

Table 2. Baseline clinical and biochemical characteristics of subjects with T2DM according to plasma CRP levels at the enrollment.

	Subjects with hs-CRP < 2 n = 272	Subjects with hs-CRP ≥ 2 n = 323	P
Age (years)	62.45 ± 9.52	62.38 ± 9.79	0.94
Male gender (%)	161 (59.2)	162 (50.2)	**0.03**
DM duration (years)	11.75 ± 8.24	10.85 ± 7.57	0.21
Smoking prevalence (%)	20 (7.4)	22 (6.8)	0.79
Waist circumference (cm)	105.35 ± 12.31	110.71 ± 12.89	**<0.001**
Body mass index (kg/m2)	29.79 ± 4.07	31.98 ± 5.02	**<0.001**
Arterial hypertension (%)	218 (80.1)	284 (87.9)	**0.009**
Systolic blood pressure (mm Hg)	146.78 ± 20.11	147.35 ± 19.90	0.79
Diastolic blood pressure (mm Hg)	86.30 ± 10.58	85.28 ± 12.56	0.40
Fasting glucose (mmol/L)	7.71 ± 2.43	8.28 ± 2.63	0.06
HbA1c (%)	7.61 ± 1.46	8.17 ± 4.79	0.11
Total cholesterol (mmol/L)	4.51 ± 1.15	4.86 ± 1.19	**0.001**
HDL cholesterol (mmol/L)	1.24 ± 0.36	1.16 ± 0.34	**0.01**
LDL cholesterol (mmol/L)	2.51 ± 0.94	2.74 ± 0.93	**0.009**
Triglycerides (mmol/L)	1.99 ± 1.31	2.55 ± 2.08	**<0.001**
Non-HDL cholesterol (mmol/L)	3.27 ± 1.15	3.70 ± 1.19	**<0.001**
Statin therapy (%)	178 (65.4)	204 (63.2)	0.56

Table 3. Baseline ultrasonographic markers of carotid atherosclerosis in subjects with T2DM at the enrollment.

	Subjects with hs- CRP < 2 n = 272	Subjects with hs-CRP ≥ 2 n = 323	P
CIMT (μm)	980 ± 226	1037 ± 192	**0.01**
Number of sites with plaque	2.48 ± 1.72	2.55 ± 1.57	0.70
Total plaque thickness (mm)	8.36 ± 4.71	7.58 ± 4.37	0.13
Presence of plaques +	215 (79.0)	280 (86.7)	**0.03**
–	57 (21.0)	43 (13.3)	
Presence of unstable plaques	105 (38.6)	169 (52.3)	
Presence of stable plaques	110 (40.4)	111 (34.4)	
Absence of plaques	57 (21.0)	43 (13.3)	

CIMT- carotid intima-media thickness

Table 4. Ultrasonographic markers of carotid atherosclerosis progression in subjects with T2DM regarding plasma CRP levels.

	hs- CRP < 2 n = 272	hs-CRP ≥ 2 n = 323	P
CIMT progression rate (μm/year)	14.28 (0-20.34)	23.74 (18.19-35.65)	0.07
Δ number of sites with plaque	1.0 (0.75-2.25)	2.0 (1.0-3.0)	0.52
Δ total plaque thickness (mm)	3.90 (1.30-7.82)	7.64 (4.40-8.92)	0.08

CIMT- carotid intima-media thickness

Multivariate logistic regression analysis found the association between the HDL cholesterol level and presence of plaques, whereas inflammatory marker hs-CRP was not associated with subclinical markers of progression of carotid atherosclerosis (Table 5).

Table 5. Association of the plasma hs-CRP levels with the presence of plaques and presence of unstable plaques in in subjects with T2DM at the time of recrutment.

	Presence of plaque		Presence of unstable plaque	
	OR (95% CI)	P value	OR (95% CI)	P value
Hypertension (0 = no; 1 = yes)	2.66 (0.99-4.12)	0.05	1.35 (0.93-2.58)	0.92
Systolic blood pressure (mmHg)	1.06 (0.96-1.07)	0.17	1.15 (0.97-1.36)	0.27
LDL cholesterol (mmol/L)	1.23 (0.79-1.91)	0.36	1.05 (0.73-1.51)	0.79
HDL cholesterol (mmol/L)	0.18 (0.09-0.66)	**0.009**	0.32 (0.08-1.21)	0.09
Triglycerides (mmol/L)	1.09 (0.69-1.54)	0.08	1.20 (0.96-1.48)	0.28
HbA1c (%)	1.58 (0.64-2.48)	0.28	1.09 (0.84-1.41)	0.50
hs-CRP≥ 2	1.16 (0.51-2.63)	0.73	1.54 (0.76-3.18)	0.23

All the models were adjusted for age, gender, smoking and statin treatment.
** Reference group were diabetic patients with hs-CRP < 2 mg/L.*

Multiple linear regression analysis found the association between the hs-CRP level and either CIMT progression rate or a change in the number of sites with plaques in a 3.8-year follow-up (Table 6).

Table 6. Association of the plasma hs-CRP levels with ultrasonographic markers of carotid atherosclerosis progression in subjects with T2DM.

	CIMT progression rate		Δ Number of sites with plaque		Δ Total plaque thickness	
	ß coefficient	P value	β	P value	ß coefficient	P value
Hypertension (0 = no; 1 = yes)	1.104	0.54	1.084	0.57	1.271	0.55
Systolic blood pressure (mmHg)	0.099	0.78	0.096	0.54	0.212	0.62
LDL cholesterol (mmol/L)	0.059	0.83	0.037	0.77	0.507	0.27
HDL cholesterol (mmol/L)	-0.137	0.40	-0.287	**0.04**	-0.895	0.37
Triglycerides (mmol/L)	0.206	0.18	0.120	0.40	0.255	0.63
HbA1c (%)	1.168	0.23	1.154	0.21	1.259	0.46
hs-CRP ≥ 2	1.363	**0.03**	1.355	**0.04**	1.538	0.21

All the models were adjusted for age, gender, smoking, statin treatment and baseline value of dependent variable.
** Reference group were diabetic patients with hs-CRP<2 mg/L.*

Discussion

In our study we report an association between inflammatory marker hs-CRP and either CIMT or incidence of plaques/unstable plaques in Caucasians with T2DM. Moreover, we found the association between the hs-CRP level and either CIMT progression rate or a change in the number of sites with plaques in a 3.8-year follow-up.

Our findings demonstrating the relationship between CRP and progression of subclinical atherosclerosis are in accordance with some larger studies demonstrating a relationship between CRP and cardiovascular disorders [10, 11, 13]. Additionally, our findings demonstrating a connection between inflammatory marker hs-CRP and subclinical carotid atherosclerosis (CIMT, incidence of plaques/unstable plaques, CIMT progression rate) in Caucasians with T2DM are in accordance with our recently published data demonstrating an association between polymorphisms of few inflammatory genes (i.e. ICAM-1, PPAR-γ) and progression of subclinical atherosclerosis [7, 17]. Despite several reports demonstrating a relationship between CRP and cardiovascular disorders (coronary artery disease and ischemic stroke), a causal relationship between CRP and CAD was not demonstrated with Mendelian randomization [11-13, 18]. Persistent low grade inflammation is presumed to affect the pathogenesis of cardiovascular diseases as well as mortality (vascular and non-vascular) via different mechanisms [13, 19-24]; however, the exact mechanism is not known. Obesity, which often accompanies T2DM, is characterized as a state of chronic low-grade inflammation caused by overnutrition,

and is a major cause of decreased insulin sensitivity, which makes obesity a major risk factor for insulin resistance and development of T2DM [25]. CRP is speculated to contribute to vascular inflammation by activating complement proteins and increasing the production of thrombogenic components bound to the membranes of injured vascular cells, which contributes to the development of insulin resistance [26, 27]. However, there is no apparent causality between serum CRP, insulin resistance, and T2DM, which suggests that CRP is more likely to be a downstream marker rather than an upstream effector that links inflammation to insulin resistance [26].

In our study, we wanted to emphasize two kinds of connective: first, between hs-CRP and CIMT, and second, between hs-CRP and typical atherosclerotic phenotype (incidence of plaques/ unstable plaques, a change in the number of sites with plaques). This fact is important, since CIMT is biologically distinct from atherosclerotic plaques, not really atherosclerosis, but it represents an indicator for cardiovascular risk. In contrast, carotid plaques are a characteristic phenotype of atherosclerosis, not a simple continuum of CIMT progression, and predict the cardiovascular disease better than CIMT [28, 29].

The strengths of our prospective study are the community-based sample of Caucasians with T2DM, the exact evaluation of subclinical markers of carotid atherosclerosis, a rather large cohort of subjects with T2DM without evident CAD, and the prospective nature of the study. A limitation of the study might be the number of participants involved in the study; however, the study was appropriately powered to detect differences in subclinical markers of carotid atherosclerosis in this cohort of subjects. Another limitation of the study might be the fact that we had the data about hs-CRP at the beginning of the study.

To conclude, we demonstrated an association between an inflammatory marker hs-CRP and either CIMT or incidence of plaques/unstable plaques at the time of recruitment in Caucasians with T2DM. Moreover, we found the association between the hs-CRP level and either CIMT progression rate or a change in the number of sites with plaques in a 3.8-year follow-up in subjects with T2DM.

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Raziskava 6

Matrix metalloproteinase-3 gene polymorphism (rs3025058) affects markers of subclinical atherosclerosis in patients with type 2 diabetes mellitus

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Abstract

Background: The current study was designed to test the possible association between either polymorphisms of the matrix metalloproteinase-9 (*MMP-9*) gene (rs17576, rs39182420) or the *MMP-3* 5A/6A gene polymorphism (rs3025058) with markers of carotid atherosclerosis (carotid intima media thickness [CIMT], number of affected segments of carotid arteries, the sum of plaques thickness, the presence of carotid plaques, and the presence of unstable carotid plaques) in patients with type 2 diabetes mellitus (T2DM). The second aim of the study was to demonstrate an association between either the rs17576, or rs39182420, or rs3025058, and subclinical markers of coronary artery disease in a subset of patients with T2DM.

Patients and methods: 595 subjects with T2DM and 200 subjects without T2DM (control group) were enrolled in the prospective study. Subclinical markers of carotid atherosclerosis were assessed ultrasonographically. Additionally, in a subset of subjects with T2DM a coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. Genotyping of all three polymorphisms (rs17576, rs3918242; rs3025058) was performed with real-time PCR Systems.

Results: The comparison of atherosclerosis parameters was performed with regard to different genotypes of *MMP-9* rs17576, rs3918242, and *MMP-3* rs3025058 polymorphisms upon enrolment and during follow-up. In our study we found an association between the *MMP-3* rs3025058 and CIMT at the time of recruitment. Multiple linear regression analysis revealed the association of either the A- allele or the A- genotypes of the rs3025058 (*MMP-3*) with carotid intima media thickness (CIMT) progression in a 3.8 year follow up. We did not find any association between either the *MMP-9* rs17576 or rs3918242 and subclinical markers of carotid atherosclerosis. We demonstrated the effect of the rs3025058 on subclinical markers of coronary atherosclerosis (coronary calcium score, number of coronary arteries with more than 50% stenosis, and presence of at least one vessel with more than 50% stenosis).

Conclusions: In our study, we found an association between the *MMP-3* rs3025058 and subclinical markers of carotid (CIMT) and coronary atherosclerosis at the time of recruitment. Moreover, we demonstrated the effect of the *MMP-3* rs3025058 on CIMT progression in the 3.8-year follow-up in patients with T2DM.

Key words: carotid atherosclerosis**;** genetic polymorphism; cross-sectional study; matrix metallo-proteinase 3 gene; matrix metalloproteinase 9 gene; rs17576, rs3918242; rs3025058

Introduction

Carotid intima media thickness (CIMT) was reported to have a predictive role for coronary artery disease (CAD) [1, 2]. A recent report (including a meta-analysis), however, raised doubts regarding the clinical utility of CIMT measurement, and concluded that the ultrasound assessment of carotid plaque, compared with that of CIMT, had a higher diagnostic accuracy for the prediction of future CAD events [3, 4].

The pathogenesis of atherosclerosis is very complex, and several genes and their products have so far been implicated in the pathogenesis of atherosclerosis [5-8]. A very interesting candidate gene system is the matrix metalloproteinase (MMP) family. The MMPs are proteolytic enzymes that degrade the extracellular matrix, leading to connective tissue remodelling during normal and pathological vascular remodelling [5-8]. It is generally accepted that the deregulation of the MMP system has a crucial role in vascular remodelling in the atherosclerotic process [5].

MMP-3 is an important member of the MMP family. The expression of the *MMP3* is primarily regulated at the transcriptional level [9]. A common variant in the promoter region of the *MMP-3* gene (rs3025058) was reported [9]. Two alleles may be present; one allele has six adenosines (6A) and the other allele has five (5A) [9]. In vitro studies have shown that the 5A allele expressed higher MMP-3 levels than the 6A allele in both cultured fibroblasts and vascular smooth muscle cells [9].

MMP-9 is another important member of the MMP family. MMP-9 is characterized by several substrates, such as gelatin, type IV collagen, and elastin, and it has proteolytic activity against type IV collagen, a component of the carotid artery basement membrane lying under the endothelium [10].

Several matrix metalloproteinases (MMPs) have been reported to be expressed in atherosclerotic lesions; however, the exact role of tested polymorphisms of both genes (*MMP-3* and *MMP-9*) on subclinical markers of carotid atherosclerosis remains to be fully elucidated [11].

Aim of the study

The current study was designed to test the possible association between either polymorphisms of the *MMP-9* gene (rs17576, rs39182420) or the *MMP-3* gene polymorphism (rs3025058) with markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries, sum of plaques thickness, presence of carotid plaques, and presence of unstable carotid plaques) in patients with T2DM. The second aim of the study was to demonstrate an association between either rs17576, rs39182420 or rs3025058 and subclinical markers of coronary artery disease in the same subset of patients with type 2 diabetes mellitus (T2DM).

Material and methods

The study protocol was approved by the Slovene Medical Ethics Committee in 2010 and 2012 (114/07/2010 and 116/06/2012). After an informed consent for the participation in the study had been obtained, a detailed interview was made. In this cross-sectional study 595 subjects with T2DM aged 40 years or more were enrolled. They were selected among patients admitted to the diabetes outpatient departments of two general hospitals from Slovenia (Murska Sobota and Slovenj Gradec), and patients from the outpatient cardiology department of the International Center for Cardiovascular Diseases Medicor, Ljubljana. Subjects with T2DM and control subjects were excluded if they had a previous cardiovascular event (myocardial infarction or cerebrovascular stroke) or homozygous familial hypercholesterolaemia at the time of enrollment.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured at 3 sites 10 mm proximal to the flow diverter of the far wall of the common carotid artery free of plaques, in agreement with the carotid intima-media thickness consensus [12, 13]. Plaques were defined as a focal intima-media thickening, and divided into 5 types according to their plaque characteristics [12]. Unstable plaques are plaques types from 1 to 3, while stable plaques are types 4 and 5 [12]. Type 1 plaque is de ned as dominantly echolucent with a thin echogenic cap, type 2 plaque as predominant echolucent with small areas of echogenicity, type 3 plaque as dominant echogenic with small areas of echolucency (less than 25%), type 4 plaque as uniformly echogenic (equivalent to homogenous), and type 5 as predominantly calcified plaque. Plaque types 1, 2 and 3 were considered stable, while types 4 and 5 were considered unstable [20]. Additionally, the length of scanning of carotid arteries regarding carotid plaques was defined: 20 mm of the distal CCA and 20 mm of the proximal ICA. The inter-observer and intra-observer reliability for carotid plaque characterization was found to be substantial (CIMT: inter-observer variability: $\kappa = 0.74$, $p < 0.001$; intra-observer variability – examiner 1: $\kappa = 0.76$, $p < 0.001$; intra-observer variability – examiner 2: $\kappa = 0.73$, $p < 0.001$; plaque type characterization: inter-observer variability: $\kappa = 0.74$, $p < 0.001$; intra-observer variability – examiner 1: $\kappa = 0.72$, $p < 0.001$; intra-observer variability – examiner 2: $\kappa = 0.77$, $p < 0.001$; sum of plaque thickness: inter-observer variability: $\kappa = 0.68$, $p < 0.001$; intra-observer variability – examiner 1: $\kappa = 0.68$, $p < 0.001$; intra-observer variability – examiner 2: $\kappa = 0.70$, $p < 0.001$).

After several years, patients were re-assessed and markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries, and sum of plaques thickness) were checked again.

Subjects with T2DM were prospectively followed, and after 3.8 ± 0.5 years control ultrasound examination of carotid arteries was performed on 426 out of 595 subjects (71.6%) with T2DM. The annual CIMT progression rate, the increase in total plaque thickness and the number of sites with plaques were evaluated to appreciate the progression of carotid atherosclerosis.

In 215 out of 595 subjects with T2DM, coronary computed tomography angiography (CCTA) was performed for diagnostic purposes (angina or dyspnea during exercise) at the beginning of the study. In 215 subjects with T2DM, coronary arteries (left main, left anterior descendent coronary artery, left circumflex coronary artery, and right coronary artery) were evaluated for the presence/degree of stenosis. Moreover, a semi-automated method was used for determination of the coronary calcium score.

Biochemical analyses

Blood samples for biochemical analyses: total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated haemoglobin (HbA1c), and hsCRP were collected at the beginning of the study. All the biochemical analyses were determined in the hospital's accredited laboratory.

Genotyping

Polymorphisms 5A/6A (rs3025058) of the gene for the *MMP-3* and R279Q (rs17576) and C1562T (rs3918242) of the gene for the *MMP-9* were determined in LGC – Genomics Laboratories (Registered office: LGC, Queens Road, Teddington, Middlesex, TW11 0LY, UK). PCR included primers as follows:

5A/6A (rs3025058): TGATGGGGGGAAAAA**[A/-]**CCATGTCTTGTCCT
R279Q (rs17576): CAGGACTCTACACCC**[A/G]**GGACGGCAATGCTG
C1562T (rs3918242): AGGCGTGGTGGCGCA**[C/T]**GCCTATAATACCAG

Statistical analysis

Continuous variables were expressed as means ± standard deviations, when normally distributed (unpaired Student's t test or analysis of variance (ANOVA)), and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Continuous clinical data were compared using an unpaired Student's t test or ANOVA when normally distributed and the Mann-Whitney U-test when asymmetrically distributed. The Pearson chi-square test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables.

A multivariate linear regression analysis was performed to determine the association of *MMP-9* rs17576, rs39182420, and MMP3 rs3025058 with CIMT (Table 4), and a multivariate linear regression analysis was performed to determine the association of the *MMP-9* rs17576, rs39182420, and *MMP-3* rs3025058 polymorphisms with the progression of carotid atherosclerosis (CIMT progression rate, changes in the number of sites with plaques, changes in total plaque thickness),. The regression models were adjusted for the presence of well-established cardiovascular risk factors. The results were presented as β coefficients and P-values for the linear regression. A two-tailed P value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 21 (SPSS Inc., Chicago, IL).

Results

Characteristics in patients with T2DM and in subjects without DM (control group) at the enrolment are demonstrated in Table 1. In our cohort of patients with T2DM only 8.9% of them were cigarette smokers, whereas the percentage was higher in the control group (Table 1). Patients with T2DM had higher waist circumference, fasting glucose, HbA1c, hs-CRP and lower levels of total and LDL cholesterol in comparison with the control group (Table 1).

Table 1. Baseline clinical and biochemical characteristics in patients with T2DM.

	Patients with T2DM N = 595
Age (years)	61.38 ± 9.65
Male gender (%)	338 (56.8)
DM duration (years)	11.25 ± 7.88
Smoking prevalence (%)	53 (8.91)
Waist circumference (cm)	108.65 ± 12.88
BMI (kg/m2)	30.96 ± 4.74
Systolic blood pressure (mm Hg)	146.98 ± 19.98
Diastolic blood pressure (mm Hg)	85.75 ± 11.62
Fasting glucose (mmol/L)	8.04 ± 2.57
HbA1c (%)	7.89 ± 3.56
Total cholesterol (mmol/L)	4.70 ± 1.19
HDL cholesterol (mmol/L)	1.19 ± 0.35
LDL cholesterol (mmol/L)	2.63 ± 0.94
Triglycerides (mmol/L)	1.9 (1.2-2.7)
hs-CRP (mg/L)	2.2 (1.0-4.3)

The genotype distribution in patients with T2DM and in the control group were in Hardy-Weinberg equilibrium for the *MMP-9* (rs17576, rs3918242), and *MMP-3* rs3025058 polymorphisms (data not shown).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of *MMP-9* rs17576, rs3918242, and *MMP-3* rs3025058 polymorphisms upon enrolment and during follow-up (Tables 2 and 3). In our study we found an association between *MMP-3* rs3025058 and CIMT at the time of recruitment, whereas we did not find any association between *MMP-3* rs3025058 and other subclinical markers of carotid atherosclerosis (sum of plaque thickness, number of involved segments, presence of carotid plaques, and presence of unstable carotid plaques at the time of recruitment (Table 2)). Moreover, we did not find any association between either *MMP-9* rs17576 or rs3918242 and subclinical markers of carotid atherosclerosis (CIMT, sum of plaque thickness, number of involved segments, presence of carotid plaques, and presence of unstable carotid plaques) at the time of recruitment (Table 2).

We demonstrated the effect of the rs3025058 on subclinical markers of coronary atherosclerosis (coronary calcium score, number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis). We failed, however, to demonstrate an association between either the rs17576 or the rs3918242, and any marker of coronary atherosclerosis obtained with CCTA (the number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis) in the subset of subjects with T2DM (Tables 3).

Table 2. Ultrasonographic markers of carotid atherosclerosis due to MMP9 rs17576, rs39182420, and MMP3 rs3025058 genotypes in patients with T2DM at the time of recruitment (unpaired Student's t test, analysis of variance, and the Pearson chi-square).

		Genoptype			
rs3025058 (MMP 3 5A/6A)		- genotype 144 subjects	A- genotype 297 subjects	AA genotype 154 subjects	P
CIMT (μm)		1008 ± 206	1010 ± 206	1078 ± 165	**0.02**
Number of sites with plaque		2.49 ± 1.62	2.54 ± 1.63	2.80 ± 1.72	0.35
Total plaque thickness (mm)		7.68 ± 4.53	8.14 ± 4.69	8.72 ± 4.28	0.28
Presence of plaques	+	121 (84.0)	245 (82.5)	132 (85.7)	0.67
	-	23 (16.0)	52 (17.5)	22 (14.3)	
Presence of unstable plaques	+	55 (45.5)	128 (52.2)	60 (45.5)	0.32
	-	66 (54.5)	117 (47.8)	72 (54.5)	
Coronary calcium score		148 ± 105	253 ± 146	336 ± 181	**0.009**
rs17576 (MMP 9 R279Q)		AA genotype 240 subjects	AG genotype 274 subjects	GG genotype 81 subjects	P
CIMT (μm)		1008 ± 217	1025 ± 194	1062 ± 168	0.26
Number of sites with plaque		2.51 ± 1.62	2.54 ± 1.69	2.74 ± 1.51	0.69
Total plaque thickness (mm)		8.06 ± 4.42	7.95 ± 4.54	7.94 ± 4.50	0.94
Presence of plaques	+	198 (82.5)	226 (82.5)	74 (91.4)	0.15
	–	42 (17.5)	48 (17.5)	7 (8.6)	
Presence of unstable plaques	+	92 (46.5)	108 (47.8)	43 (58.1)	0.23
	–	106 (53.5)	118 (52.2)	31 (41.9)	
rs3918242 (MMP 9 C1562T)		CC genotype 436 subjects	TT+CT genotypes 159 subjects		P
CIMT (μm)		1004 ± 190	1014 ± 170		0.58
Number of sites with plaque		2.36 ± 1.67	2.47 ± 1.74		0.54
Total plaque thickness (mm)		7.81 ± 4.55	7.69 ± 4.68		0.82
Presence of plaques	+	361 (82.8)	137 (86.2)		0.38
	–	75 (17.2)	22 (13.8)		
Presence of unstable plaques	+	173 (47.9)	71 (51.4)		0.42
	–	188 (52.1)	67 (48.6)		

Table 3. Comparison of subclinical markers of coronary atherosclerosis in subjects with T2DM at the beginning of the study with regard to the MMP9 rs17576, rs39182420, and MMP3 rs3025058 genotypes (unpaired Student's t test, analysis of variance, and the Pearson chi-square).

	Genoptype			
rs3025058 (MMP 3 5A/6A)	- genotype 51 subjects	A- genotype 108 subjects	AA genotype 56 subjects	P
Coronary calcium score	148 ± 105	253 ± 146	336 ± 181	**0.009**
Number of coronary arteries with more than 50% stenosis**	0.47 ± 0.91	0.67 ± 0.93	1.16 ± 1.06	**0.02**
The presence of at least one coronary segment with more than 50% stenosis	16 (31.5%)	36 (33.3%)	25 (44.6%)	**0.01**
rs17576 (MMP 9 R279Q)	AA genotype 85 subjects	AG genotype 100 subjects	GG genotype 30 subjects	P
Coronary calcium score	307 ± 290	219 ± 270	259 ± 305	0.75
Number of coronary arteries with more than 50% stenosis**	0.94 ± 1.16	0.69 ± 0.92	0.52 ± 1.08	0.74
The presence of at least one coronary segment with more than 50% stenosis	32 (37.6%)	42 (42%)	13 (43.3%)	0.2
rs3918242 (MMP 9 C1562T)	CC genotype 158 subjects	TT+CT genotypes 57 subjects		P
Coronary calcium score	264 ± 250	295 ± 249		0.78
Number of coronary arteries with more than 50% stenosis**	0.85 ± 1.07	0.62 ± 1.04		0.54
The presence of at least one coronary segment with more than 50% stenosis	53 (33.5%)	21 (36.8%)		0.30

**Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM: **the number of coronary arteries (LM, LAD, LCX, RCA) with more than 50% stenosis*

As shown by multiple linear regression analysis, the association of either the A- allele or the A- genotypes of the rs3025058 (*MMP-3* 5A/6A) with CIMT progression was statistically significant after adjustment for confounding variables (Table 4).

Table 4. Association of the MMP9 rs17576, rs39182420, and MMP3 rs3025058 genotypes with ultrasonographic markers of carotid atherosclerosis progression in subjects with T2DM (multivariate linear regression analysis) in 3.8 ± 0.5 years follow-up.

	CIMT progression		Δ Number of sites with plaque		Δ Total plaque thickness	
	B	p	β	p	β	p
rs3025058 (MMP 3 5A/6A)*						
Hypertension (0 = no; 1 = yes)	0.033	0.82	0.020	0.89	0.109	0.59
Systolic blood pressure (mmHg)	0.046	0.76	0.037	0.82	0.182	0.35
LDL cholesterol (mmol/L)	0.102	0.41	0.085	0.52	0.156	0.34
HDL cholesterol (mmol/L)	-0.184	0.17	-0.299	**0.04**	-0.276	0.132
Triglycerides (mmol/L)	0.178	0.18	0.089	0.54	0.160	0.38
Hba1c (%)	1.137	0.32	0.138	0.28	0.257	0.12
A-	0.190	**0.04**	0.032	0.84	0.092	0.52
AA	0.283	**0.03**	0.072	0.64	0.132	0.62
rs17576 (MMP 9 R279Q) **	B	p	β	p	β	P
Hypertension (0 = no; 1 = yes)	0.086	0.61	0.027	0.86	0.085	0.66
Systolic blood pressure (mmHg)	0.111	0.52	0.015	0.26	0.137	0.48
LDL cholesterol (mmol/L)	0.087	0.55	0.042	0.75	0.135	0.40
HDL cholesterol (mmol/L)	-0.205	0.42	-0.281	0.06	-0.292	0.12
Triglycerides (mmol/L)	0.260	0.13	0.054	0.74	0.101	0.60
Hba1c (%)	0.149	0.31	0.118	0.38	0.255	0.14
AG	0.048	0.75	0.027	0.17	0.102	0.58
GG	0.082	0.49	0.050	0.73	0.160	0.34
rs3918242 (*MMP 9 C1562T)****	B	p	β	p	β	P
Hypertension (0 = no; 1 = yes)	0.084	0.61	0.017	0.17	0.054	0.78
Systolic blood pressure (mmHg)	0.080	0.64	0.019	0.89	0.128	0.51
LDL cholesterol (mmol/L)	0.088	0.54	0.087	0.52	0.104	0.52
HDL cholesterol (mmol/L)	-0.215	0.18	-0.284	0.06	-0.237	0.22
Triglycerides (mmol/L)	0.241	0.12	0.094	0.52	0.110	0.53
Hba1c (%)	0.135	0.34	0.133	0.32	0.238	0.17
TT+CT	0.096	0.79	0.045	0.73	0.042	0.38

All the models were adjusted for age, gender, smoking, statin treatment and baseline value of dependent variable.
** Reference group were homozygotes for the allele -.*
All the models were adjusted for age, gender, smoking and statin treatment.
*** Reference group were homozygotes for the allele A.*
**** Reference group were homozygotes for the allele C.*

Discussion

In the present study we tested the hypothesis that rs17576 and rs3918242 of the *MMP-9* gene and the rs3025058 of *MMP-3* gene may be genetic markers of subclinical carotid and coronary atherosclerosis in patients with T2DM. In the study we found an association between *MMP-3* rs3025058 and CIMT at the time of recruitment. Additionally, the effect of *MMP-3* rs3025058 (heterozygosity, homozygosity) on CIMT progression was demonstrated by multiple linear regression analysis. The effect of *MMP-3* rs3025058 on CIMT and CIMT progression is presumed to be an effect of rs3025058 on gene expression, since higher MMP-3 levels were reported in 5A allele carriers in comparison with the 6A allele carriers [9]. Our findings are in accordance with some previous reports demonstrating that the variability in the *MMP-3* gene

(rs3025058) might be associated with CIMT in the general population, whereas in 762 Cyprus community dwellers (general population) no association between rs3025058 and CIMT was found [12-17]. We presume that the reported differences of reported association studies might be due to the fact that different populations represent different genetic and environmental background with complex interaction between them. Moreover, since sample size of our study and Cyprus study are of similar size, we hypothesize that differences in the effect of rs3025058 on CIMT might be due to different enrolment criteria (our study enrolled subjects with T2DM, whereas the Cyprus study enrolled general populations).

In our study, however, we did not find any association between *MMP-3* rs3025058 and other subclinical markers of carotid atherosclerosis (number of affected segments of carotid arteries, sum of plaques thickness, presence of carotid plaques, and presence of unstable carotid plaques). Our findings are in accordance with the findings of the Cyprus study, which failed to demonstrate the effect of rs3025058 on either carotid or femoral atherosclerosis [17]. Similarly, Koch and co-workers failed to demonstrate in a case-control association study and a meta-analysis the association between the *MMP-3* rs3025058 and CAD [18]. Our findings (association with CIMT and lack of association with other carotid plaque traits) provide further support to the fact that CIMT and atherosclerotic plaque most probably have a different pathophysiological background.

In the study we did not demonstrate a statistically significant effect of the *MMP-9* rs17576, rs3918242 on subclinical markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries and sum of plaque thickness in carotid arteries, presence of carotid plaques, and presence of unstable carotid plaques) in patients with T2DM. Our findings are in accordance with the report on the Cyprus study and German population, in which rs17576 was not associated with CIMT [17, 19]. Moreover, serum MMP-9 levels were not reported to be associated with CIMT in the Framingham Offspring study [20]. Moreover, in the Cyprus study rs17576 was not associated with subclinical markers of either carotid or femoral atherosclerosis, whereas MMP-9 serum levels were consistently associated with markers of carotid atherosclerosis and lesion vulnerability [17]. In some other reports, an association between *MMP-9* rs3918242 and other cardiovascular disorders (CAD, myocardial infarction) and other disorders (breast cancer, metabolic syndrome, primary open-angle glaucoma, idiopathic recurrent spontaneous abortion) was reported [21-26].

We demonstrated the effect of rs3025058 on subclinical markers of coronary atherosclerosis (coronary calcium score, number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis). The effect of the rs3025058 on subclinical markers of coronary atherosclerosis in our study is in accordance with the effect of the rs3025058 on subclinical markers of carotid atherosclerosis. We failed, however, to demonstrate an association between either the rs17576 or rs3918242 and any marker of coronary atherosclerosis obtained with CCTA in subjects with T2DM.

A limitation of the study was the sample size and the lack of MMP-3 and MMP-9 serum levels, since the effect of the *MMP-3 and MMP-9* polymorphisms on atherosclerosis progression is speculated to be due to their influence on serum and tissue levels of both enzymes. The strength of our prospective study is the community-based sample of subjects with T2DM and without T2DM, and the precisely defined and ultrasonically determined subclinical markers of carotid atherosclerosis in subjects with/without T2DM. Moreover, due to its prospective nature the study enables the possibility of causal inferences from our findings.

Conclusions

In our study, we found an association between the *MMP-3* rs3025058 and subclinical markers of carotid (CIMT) and coronary atherosclerosis at the time of recruitment. Moreover, we demonstrated the effect of the *MMP-3* rs3025058 on CIMT progression in the 3.8-year follow-up in patients with T2DM.

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Conflicts of interest

There are no conflicts of interest.

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Raziskava 7

Phosphoprotein 1 (osteopontin) gene (RS4754) affects markers of subclinical atherosclerosis in patients with type 2 diabetes mellitus

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Abstract

Aim: Our study was designed to test a possible association between polymorphisms of the SPP1 gene (rs4754, rs28357094) and markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries, sum of plaque thickness, presence of carotid plaques, and presence of unstable carotid plaques) in subjects with T2DM. The second aim was to test the possible association between polymorphisms of the SPP1 gene (rs4754, rs28357094) and the progression of carotid atherosclerosis (CIMT progression, change in total plaque thickness, change in the number of sites with plaques) in subjects with T2DM.

Patients and methods: 595 T2DM subjects were enrolled in the cross-sectional study. Markers of carotid atherosclerosis were assessed ultrasonographically. rs4754 and rs28357094 polymorphisms of the phosphoprotein 1 (SPP1) gene were determined with real-time PCR.

Results: In our study we found an association between *SPP1* rs4754 and the presence of plaques at the time of recruitment, whereas we did not find any association between *SPP1* rs28357094 and subclinical markers of carotid atherosclerosis at the time of recruitment.

Moreover, we did not find any statistically significant effect of either rs4754 or rs28357094 on subclinical markers of carotid atherosclerosis progression (CIMT progression, change in total plaque thickness, change in the number of sites with plaques). As shown by the multiple linear regression analysis, genotypes of either rs4754 or rs28357094 did not have a statistically significant effect on the progression of subclinical markers of carotid atherosclerosis (CIMT progression, change in total plaque thickness, change in the number of sites with plaques) after the adjustment for confounding variables.

Conclusion: We demonstrated an important effect of the *SPP1* rs4754 on subclinical markers of carotid atherosclerosis in subjects with T2DM, however, as demonstrated by the multiple linear regression analysis, neither rs4754 nor rs28357094 had an important impact on the progression of subclinical markers of carotid atherosclerosis in subjects with T2DM.

Introduction

Atherosclerosis of carotid arteries is a complex multifactorial disorder that is thought to result from interactions between an individual's genetic background and lifetime exposure to various environmental factors[1-5]. Inflammation is considered to play a crucial role in the initiation and progression of the atherosclerotic process[6]. Moreover, inflammatory genes and genes regulating inflammatory response affect the development of atherosclerosis via interaction with conventional risk factors[7-9].

Several reports indicate that genetic variability in the inflammatory genes may alter their transcriptional activity and contribute to susceptibility to cardiovascular disease[10-12].

The gene for phosphoprotein 1 (Spp1; formerly osteopontin gene), composed of 7 exons, is located on chromosome region 4q22.1[13]. Its protein product is osteopontin (OPN) that binds to several integrin

receptors, and affects cell adhesion, migration, and survival[14]. OPN is expressed in a range of immune cells and reported to act as an immune modulator, which promotes cell recruitment to inflammatory sites[14-16]. Stimulation of OPN expression leads to an increase in cell proinflammatory cytokine levels, although the regulatory pathways are not yet known[14-15]. Evidence from several genetic mouse models and basic studies suggests that the Spp1 gene affects the atherosclerotic process[9,15-18]. In accordance with these reports, Spp1 (OPN) gene polymorphisms were demonstrated to affect its transcriptional activity[19]. Moreover, in case-control studies for MI (ECTIM: 990 cases, 900 controls) and brain infarction (BI) (GENIC: 466 cases, 444 controls) it was demonstrated that a portion of the Spp1 gene polymorphism is likely to be associated with cardiovascular disease-related phenotypes[10].

Due to the involvement of the Spp1 gene in the inflammatory process, we designed our study to test a possible association between polymorphisms of the SPP1 gene (rs4754, rs28357094) and markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries, sum of plaques thickness, presence of carotid plaques, and presence of unstable carotid plaques) in subjects with T2DM. The second aim was to test the possible association between polymorphisms of the SPP1 gene (rs4754, rs28357094) and the progression of carotid atherosclerosis CIMT progression, change in total plaque thickness, change in the number of sites with plaques in subjects with T2DM.

Material and methods

The study protocol was approved by the Slovene Medical Ethics Committee in 2010 and 2012 (114/07/2010 and 116/06/2012). A detailed interview was conducted after an informed consent for the participation in the study had been obtained. 595 subjects with T2DM aged 40 or more were enrolled in this cross-sectional study. They were selected among patients admitted to the diabetes outpatient departments of two general hospitals in Slovenia (Murska Sobota and Slovenj Gradec), and patients from the outpatient cardiology department of the International Center for Cardiovascular Diseases Medicor, Ljubljana. Subjects with T2DM and control subjects were excluded if they had a previous cardiovascular event (myocardial infarction or cerebrovascular stroke) or homozygous familial hypercholesterolaemia at the time of enrolment.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured at 3 sites 10 mm proximal to the flow diverter of the far wall of the common carotid artery free of plaques, in agreement with the carotid intima-media thickness consensus[20-21]. Plaques were defined as a focal intima-media thickening, and divided into 5 types according to their plaque characteristics[20]. Unstable plaques are plaque types from 1 to 3, while stable plaques are types 4 and 5[20]. Type 1 plaque is defined as dominantly echolucent with a thin echogenic cap, type 2 plaque as predominantly echolucent with small areas of echogenicity, type 3 plaque as dominantly echogenic with small areas of echolucency (less than 25%), type 4 plaque as uniformly echogenic (equivalent to homogenous), and type 5 as predominantly calcified plaque. Plaque types 1, 2 and

3 were considered as stable, while types 4 and 5 were considered as unstable[11]. Additionally, the length of scanning of carotid arteries in terms of carotid plaques was defined: 20 mm of the distal CCA and 20 mm of the proximal ICA. The inter-observer and intra-observer reliability for carotid plaque characterization was found to be substantial (CIMT: inter-observer variability: $\kappa = 0.74$, $p < 0.001$; intra-observer variability – examiner 1: $\kappa = 0.76$, $p < 0.001$; intra-observer variability – examiner 2: $\kappa = 0.73$, $p < 0.001$; plaque type characterization: inter-observer variability: $\kappa = 0.74$, $p < 0.001$; intra-observer variability – examiner 1: $\kappa = 0.72$, $p < 0.001$; intra-observer variability – examiner 2: $\kappa = 0.77$, $p < 0.001$; sum of plaque thickness: inter-observer variability: $\kappa = 0.68$, $p < 0.001$; intra-observer variability – examiner 1: $\kappa = 0.68$, $p < 0.001$; intra-observer variability – examiner 2: $\kappa = 0.70$, $p < 0.001$).

Subjects with T2DM were prospectively followed and after a 3.8 ± 0.5-year control, an ultrasound examination of carotid arteries (CIMT, number of affected segments of carotid arteries, and sum of plaques thickness) was performed on 426 out of 595 subjects (71.6%) with T2DM. The annual CIMT progression rate, the increase in total plaque thickness and the number of sites with plaques were evaluated to assess the progression of carotid atherosclerosis.

Biochemical analyses

Blood samples for biochemical analyses: total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated haemoglobin (HbA1c), and hsCRP were collected at the beginning of the study. All biochemical analyses were determined in the hospital's accredited laboratory.

Genotyping

The genomic DNA was extracted from 100μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). rs4754 and rs28357094 polymorphisms of the phosphoprotein 1 (SPP1) gene were determined with real-time PCR using StepOne™ (48-well) Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Continuous variables were expressed as means ± standard deviations when normally distributed, and as median (interquartile range) when asymmetrically distributed. The normality of the continuous variables was examined by the Kolmogorov–Smirnov test. Continuous clinical data were compared using an unpaired Student's *t* test or analysis of variance (ANOVA) when normally distributed, and with the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X^2 test was used to compare discrete variables and to test whether the genotype distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables. Due to the high correlation of systolic blood pressure with the diastolic blood pressure ($r = 0.57$, $p < 0.001$) they were not included together in the same statistical model. For the same reason, body mass index (BMI) was not included in the model together with the waist circumference ($r = 0.45$, $p < 0.001$).

A multivariable linear regression analysis was performed to determine the association of the tested polymorphisms with the CIMT/annual progression of CIMT and the change in the number of sites with plaque/total plaque thickness. A multivariate logistic regression analysis was performed to determine the association of the tested polymorphisms with the presence of atherosclerotic plaques on the carotid arteries or the presence of unstable plaques. All the regression models were adjusted for the presence of well-established cardiovascular risk factors: age, gender, hypertension, systolic blood pressure, smoking, plasma levels of LDL and HDL cholesterol, triglycerides, HbA1c and statin treatment. The results were presented as standardized β coefficients and P-values for the linear regression and by odds ratios and 95% CIs for the logistic regression. A two-tailed P value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

Results

Characteristics in patients with T2DM and in subjects without DM (control group) at enrolment are demonstrated in Table 1. In our cohort of patients with T2DM, only 8.9% of them were cigarette smokers, whereas the percentage was higher in the control group (Table 1). Patients with T2DM had a higher waist circumference, fasting glucose, HbA1c, hs-CRP and lower levels of total and LDL cholesterol in comparison with the control group (Table 1).

Table 1. Baseline clinical and biochemical characteristics of diabetic patients and controls.

	Diabetic patients n = 595	Controls n = 200	p
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Male gender (%)	338 (56.8)	92 (46.0)	**0.008**
DM duration (years)	11.25 ± 7.88	-	-
Smoking prevalence (%)	53 (8.9)	34 (17.0)	**0.002**
Waist circumference (cm)	108.6 ± 12.9	93.3 ± 13.2	**<0.001**
ITM (kg/m2)	31.0 ± 4.7	27.9 ± 4.4	0.16
Systolic blood pressure (mm Hg)	147.0 ± 20.0	143.3 ± 16.6	0.86
Diastolic blood pressure (mm Hg)	85.7 ± 11.6	84.7 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	**<0.001**
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	**<0.001**
Total cholesterol (mmol/L)	4.70 ± 1.19	5.36 ± 1.08	**<0.001**
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	**<0.001**
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	**<0.001**
Triglycerides (mmol/L)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	**<0.001**
hs-CRP (mg/L)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	**<0.001**
CIMT (μm)	1013 ± 208	979 ± 141	**0.03**

DM- diabetes mellitus; hs-CRP - high sensitivity C-reactive protein

The genotype distribution in patients with T2DM and in the control group were in the Hardy-Weinberg equilibrium for SPP1 (rs4754, rs28357094) polymorphisms.

Table 2. Genotype distribution and allele frequencies of the polymorphism rs4754/rs28357094 in patients with diabetes and healthy controls.

rs4754	Subjects with T2DM n = 595	Controls n = 200	p
TT genotype	323 (54.3)	96 (48.0)	0.30
TC genotype	233 (39.2)	88 (44.0)	
CC genotype	39 (6.5)	16 (8.0)	
T allele	879 (73.9)	280 (70.0)	0.13
C allele	311 (26.1)	120 (30.0)	
rs28357094			
TT genotype	367 (61.7)	133 (66.5)	0.30
TG genotype	204 (34.3)	59 (29.5)	
GG genotype	24 (4.0)	8 (4.0)	
T allele	938 (78.8)	325 (81.3)	0.30
G allele	252 (21.2)	75 (18.7)	

The genotype distributions (rs4754) in both patients with DM2 ($\chi2 = 0.12$; $p = 0.73$) and controls ($\chi2 = 0.45$; $p = 0.50$) were compatible with Hardy-Weinberg expectations. The genotype distributions (rs28357094) in both patients with DM2 ($\chi2 = 0.43$; $p = 0.51$) and controls ($\chi2 = 0.20$; $p = 0.65$) were compatible with Hardy-Weinberg expectations.

The comparison of atherosclerosis parameters was performed with regard to different genotypes of SPP1 (rs4754, rs28357094) polymorphisms upon enrolment (Table 3). In our study, we found an association between *SPP1* rs4754 and the presence of plaques at the time of recruitment, whereas we did not find any association between *SPP1* rs28357094 and subclinical markers of carotid atherosclerosis (sum of plaque thickness, number of involved segments, presence of carotid plaques, and presence of unstable carotid plaques) at the time of recruitment (Table 3).

Table 3. Ultrasonographic markers of carotid atherosclerosis due to rs4754/rs28357094 genotypes in patients with T2DM at the time of recruitment.

rs4754		TT	TC	CC	p
CIMT (μm)		999 ± 216	1030 ± 199	1045 ± 181	0.29
Number of sites with plaque		2.35 ± 1.65	2.61 ± 1.58	3.19 ± 1.49	**0.03**
Total plaque thickness (mm)		7.58 ± 3.61	8.19 ± 3.42	8.41 ± 3.19	0.44
Presence of plaques	+	261 (80.8)	198 (85.0)	39 (100)	**0.007**
	-	62 (19.2)	35 (15.0)	0 (0.0)	
Presence of unstable	+	153 (58.6)	111 (56.1)	23 (59.0)	0.85
plaques	-	108 (41.4)	87 (43.9)	16 (41.0)	
rs28357094		TT	TG	GG	p
CIMT (μm)		1013 ± 209	1011 ± 213	1006 ± 186	0.99
Number of sites with plaque		2.48 ± 1.59	2.62 ± 1.71	2.64 ± 1.56	0.39
Total plaque thickness (mm)		8.26 ± 3.12	7.69 ± 3.66	7.91 ± 3.09	0.58
Presence of plaques	+	312 (85.0)	168 (82.4)	18 (75.0)	0.36
	-	55 (15.0)	36 (17.6)	6 (25.0)	
Presence of unstable	+	178 (57.1)	101 (60.1)	8 (44.4)	0.42
plaques	-	134 (42.9)	67 (39.9)	10 (55.6)	

Moreover, we did not find any statistically significant effect of either rs4754 or rs28357094 on subclinical markers of carotid atherosclerosis progression (CIMT progression, change in total plaque thickness, change in the number of sites with plaques) (Table 4).

Table 4. Ultrasonographic markers of carotid atherosclerosis progression in T2DM patients due to rs4754/ rs28357094 genotypes.

rs4754	TT	TC	CC	P
CIMT progression rate (μm/year)	16.28 (10.71-21.05)	21.56 (10.71-27.81)	23.68 (14.42- 30.38)	0.29
Δ number of sites with plaque	1.5 (1.0-2.75)	1.5 (1.0-3.0)	2.0 (1.0-3.00)	0.54
Δ total plaque thickness (mm)	4.9 (2.47-7.85)	5.62 (2.63-7.38)	7.60 (4.30-11.4)	0.64
rs28357094	TT	TG	GG	p
CIMT progression rate (μm/year)	22.12 (10.54-30.28)	20.69 (20.00-22.43)	14.29 (7.89-31.81)	0.63
Δ number of sites with plaque	2.0 (1.0-3.0)	2.0 (1.0-3.00)	2.5 (0.5-3.00)	0.62
Δ total plaque thickness (mm)	6.46 (2.28-9.34)	5.85 (1.75-8.60)	4.30 (1.50-8.26)	0.12

CIMT – carotid intima-media thickness.

A multivariate logistic regression analysis demonstrated that rs4754 was associated with the presence of plaques after adjustment for age, gender, smoking and statin treatment (Table 5).

Table 5. Association of the rs4754/ rs28357094 genotypes with the presence of plaques and presence of unstable plaques in T2DM patients at the time of recruitment.

	Presence of plaque		Presence of unstable plaque	
rs4754	OR (95% CI)	p	OR (95% CI)	p
Hypertension (0 = no; 1 = yes)	1.41 (1.06-2.51)	0.13	1.22 (1.03-1.65)	0.58
Systolic blood pressure (mmHg)	1.04 (0.96-1.36)	0.92	1.08 (0.97-1.24)	0.74
LDL cholesterol (mmol/L)	2.04 (0.93-3.46)	0.07	1.14 (0.95-1.58)	0.89
HDL cholesterol (mmol/L)	0.15 (0.04-0.38)	**0.01**	0.36 (0.05-0.88)	0.20
Triglycerides (mmol/L)	1.24 (0.85-1.82)	0.36	1.08 (0.62-1.47)	0.52
Hba1c (%)	1.42 (1.04-1.82)	**0.01**	1.45 (0.97-2.15)	0.07
TC	1.74 (1.14-2.46)	**0.03**	1.34 (0.88-1.92)	0.28
CC	1.92 (1.28-3.62)	**0.04**	1.48 (1.04-2.26)	0.36
rs28357094				
Hypertension (0 = no; 1 = yes)	1.42 (1.08-2.62)	0.10	1.25 (1.05-2.24)	0.74
Systolic blood pressure (mmHg)	1.02 (0.97-1.38)	0.97	1.06 (0.97-1.21)	0.71
LDL cholesterol (mmol/L)	1.78 (0.97-2.36)	0.16	1.22 (0.86-1.73)	0.44
HDL cholesterol (mmol/L)	0.21 (0.04-0.42)	**0.01**	0.12 (0.04-0.39)	**0.04**
Triglycerides (mmol/L)	1.16 (0.86-1.38)	0.51	1.06 (0.64-1.39)	0.78
Hba1c (%)	1.48 (0.96-2.24)	**0.02**	1.63 (1.06-2.50)	**0.02**
TG	0.96 (0.39-1.48)	0.53	1.53 (1.07-2.08)	0.08
GG	0.92 (0.27-1.84)	0.76	1.88 (1.36-3.92)	0.14

All models were adjusted for age, gender, smoking and statin treatment.
**Reference group were homozygotes for the allele T (rs4754, rs28357094).*

As shown by the multiple linear regression analysis, genotypes of either rs4754 or rs28357094 did not have a statistically significant effect on the progression of subclinical markers of carotid atherosclerosis (CIMT progression, change in total plaque thickness, change in the number of sites with plaques) after adjustment for confounding variables (Table 6).

Table 6. Association of the rs4754/ rs28357094 genotypes with ultrasonographic markers of carotid atherosclerosis progression in T2DM patients.

	CIMT progression rate		Δ Number of sites with plaque		Δ Total plaque thickness	
rs4754	β	p	β	p	β	p
Hypertension (0 = no; 1 = yes)	1.089	0.91	1.148	0.68	1.067	0.27
Systolic blood pressure (mmHg)	0.044	0.78	0.048	0.64	0.012	0.85
LDL cholesterol (mmol/L)	0.278	0.64	0.059	0.60	0.604	0.46
HDL cholesterol (mmol/L)	-0.965	0.31	-0.789	**0.04**	-0.797	0.45
Triglycerides (mmol/L)	0.258	0.14	0.295	0.67	0.109	0.33
Hba1c (%)	1.146	0.18	1.161	**0.02**	1.134	0.85
TC	1.269	0.18	0.369	0.06	1.157	0.16
CC	1.417	0.12	0.413	0.11	1.389	0.22
rs28357094						
Hypertension (0 = no; 1 = yes)	1.072	0.66	1.328	0.35	1.037	0.66
Systolic blood pressure (mmHg)	0.102	0.53	0.054	0.49	0.080	0.81
LDL cholesterol (mmol/L)	0.488	0.39	0.016	0.89	0.248	0.53
HDL cholesterol (mmol/L)	-0.632	0.31	-0.479	**0.04**	-0.397	0.56
Triglycerides (mmol/L)	0.612	0.14	0.401	0.54	0.182	0.70
Hba1c (%)	1.057	0.27	1.089	**0.04**	1.157	0.96
TG	-0.286	0.12	0.544	0.33	-0.228	0.22
GG	-0.763	0.51	0.623	0.25	-0.324	0.56

All models were adjusted for age, gender, smoking, statin treatment and baseline value of dependent variable.
**The reference group were homozygotes for the allele T (rs4754, rs28357094).*

Discussion

In our study, we demonstrated an important effect of the *SPP1* rs4754 on subclinical markers of carotid atherosclerosis in subjects with T2DM, whereas rs28357094 did not have a major impact on subclinical markers of carotid atherosclerosis.

In our study, we found an association between *SPP1* rs4754 and the presence of plaques at the time of recruitment in subjects with T2DM. Moreover, a multivariate logistic regression analysis demonstrated that rs4754 was associated with the presence of plaques after adjustment for age, gender, smoking and statin treatment. To our knowledge, this is the first report on the effect of *SPP1* rs4754 on subclinical carotid atherosclerosis. The role of the Spp1 gene is incompletely understood. Liu et al. have recently demonstrated that Spp1 might mediate cell activation and cytokine production[22]. The Spp-1 (OPN) gene was reported to be connected with coronary artery disease in a large GWAS study[1]. Moreover, Spp-1 (OPN) gene variability was demonstrated to affect CIMT which is not in accordance with the findings of our study, since we did not find a significant effect of the tested polymorphisms on CIMT thickness[23,24]. Interestingly, an increased expression of OPN was demonstrated in smooth muscle-derived foam cells from human atherosclerotic lesions of the aorta[15]. OPN is presumed to influence the inflammatory response, namely it acts as a macrophage chemotactic factor and plays an important role in mast cell migration[14,16,25,26]. Stimulation of Spp1 expression resulted in cytokine expression changes and may regulate c-Fos, PKCα and p-ERK/ERK pathways in vitro[22,27]. The Spp-1 gene has just recently been demonstrated to be the most up-regulated gene in atherosclerotic plaques in comparison to healthy control arteries[9]. Additionally, OPN also correlated with the plaques' stability as the amount and subtype of macrophages correlate with plaque stability[25].

The first limitation of the study is the number of participants involved in the study, as this should be higher, however, the study was powered to detect differences in subclinical markers of carotid atherosclerosis in our group of patients with T2DM. The second limitation of the study might be the fact that the CIMT was detected manually. The strengths of our prospective study, on the other hand, were the fact that this is a community-based real-world sample of Caucasians with T2DM, and the prospective nature of the study.

To conclude, we demonstrated an important effect of the *SPP1* rs4754 on the subclinical markers of carotid atherosclerosis in subjects with T2DM, however, as demonstrated by the multiple linear regression analysis, neither rs4754 nor rs28357094 had an important impact on the progression of subclinical markers of carotid atherosclerosis in subjects with T2DM.

Conflict of interest

The authors declare no conflict of interest related to this work.

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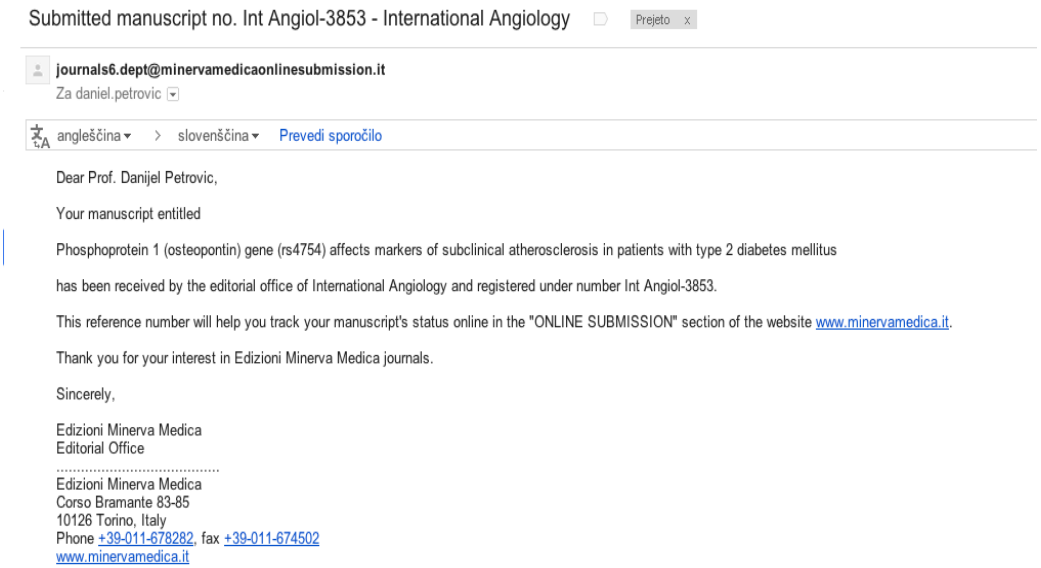

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Raziskava 8

Major histocompatibility complex polymorphism rs3869109 and ultrasound markers of carotid plaque stability in patients with type 2 diabetes mellitus

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Abstract

Background: The aim of our study was to investigate if polymorphism rs3869109 within the major histocompatibility complex (MHC) locus is associated with ultrasound markers of carotid atherosclerosis in subjects with type 2 diabetes (T2DM) of Slovenian origin. The MHC genes regulate inflammation and T cell responses that play an important role in initiation and progression of atherosclerosis.

Patients and methods: The association of polymorphism rs3869109 was tested in a case-control cross-sectional study including 330 subjects with T2DM. A high resolution B mode ultrasound analysis of carotid arteries was performed with the assessment of carotid intima-media thickness (CIMT) and plaque characteristics (presence and structure). Polymorphism rs3869109 of the MHC gene complex was determined with real-time PCR.

Results: We demonstrated an association between rs3869109 genotypes and plaque morphology (unstable vs. stable plaques) in carotid arteries (P = 0.041). Multivariate logistic regression analysis showed that subjects with AA and AG genotypes have a higher likelihood of unstable carotid plaques compared to subjects with GG genotype (odds ratio [OR] 2.59, 95% CI: 1.00-6.69, P = 0.049; OR = 1.97, 95% CI: 1.02-3.82, P = 0.044; respectively). We did not, however, demonstrate any association between the tested polymorphism and either CIMT, the sum of plaque thickness or the number of involved segments with plaques.

Conclusions: In the present study, we demonstrated an association between the rs3869109 polymorphism and the ultrasound markers of plaque stability in carotid arteries of T2DM Caucasian subjects.

Key words: Type 2 diabetes mellitus, carotid atherosclerosis, ultrasound, single nucleotide polymorphism, major histocompatibility complex,

Introduction

Type 2 diabetes mellitus (T2DM) is a serious and growing public health problem with significant mortality, morbidity, and health-system costs in the world [1]. Since 1980, the number of adults with T2DM worldwide has increased from 108 million to 422 million in 2014 [1]. T2DM is associated with diffuse and accelerated progression of atherosclerosis, the major cause of increased morbidity and cardiovascular complications [2].

Chronic inflammation and immune activation play a central role in the pathogenesis of both T2DM and atherosclerosis [3]. Atherosclerotic plaques of subjects with T2DM are characterized by larger necrotic cores and significantly greater inflammation containing mainly macrophages and T lymphocytes relative to subjects without T2DM [2]. Enhanced plaque instability in T2DM is associated with increased incidence and severity of clinical events [4].

Ultrasonography of carotid arteries is widely used to monitor atherosclerotic disease progression in T2DM [5]. Moreover, carotid intima - media thickness (CIMT) and ultrasonographic markers of carotid plaques have been reported as important surrogate markers for the prediction of future coronary artery

disease and cerebral ischemic events in the general population [6,7]. Sonographic plaque characteristics, such as plaque echogenicity, provide a clue to differentiate unstable from stable atherosclerotic lesions [8]. It has been demonstrated that T2DM patients have more unstable echolucent plaques compared with nondiabetic subjects [9]. Unstable echolucent plaques are associated with a higher risk of ischemic cerebrovascular events in comparison with stable echogenic carotid plaques [10].

The results of genetic studies support the hypothesis that proinflammatory pathways, involving both innate and adaptive immunity, play a causal role in atherosclerosis-associated clinical manifestations [11]. The major histocompatibility complex (MHC) on the short arm of chromosome 6 is the most important region in the human genome with respect to infection and autoimmunity, and is essential in adaptive and innate immunity [12]. The complex spans about 4 Mb and covers more than 120 expressed genes, thus representing one of the most gene-dense and complex regions of the human genome with extreme levels of polymorphism and linkage disequilibrium [13]. 40% of the expressed loci encode proteins with functions related to immune defense, including the highly polymorphic class I and class II human leukocyte antigen (HLA) membrane glycoproteins that present peptides for recognition by T lymphocytes [13]. Emphasizing its biomedical importance, the MHC is associated with susceptibility to more common diseases than any other region of the human genome, including almost all autoimmune disorders [14]. Since MHC genes regulate inflammation and T-cell responses, they could contribute substantially to the initiation and propagation of atherosclerosis [15].

In a recent genome wide association study, Davies et al. identified a novel coronary artery disease risk allele at 6p21.3 within the major histocompatibility locus [16]. The observed signal at rs3869109 lies in an intergenic region between HCG27 (HLA complex group 27) and HLA-C [16]. Similarly, the rs3869109 polymorphism was associated with premature coronary artery disease in a Chinese Han population [17].

As immune-mediated inflammation plays an important role in the pathogenesis of diabetes-accelerated atherosclerosis, we investigated whether the single nucleotide polymorphism rs3869109 is associated with ultrasound markers of carotid atherosclerosis in subjects with type 2 DM of Caucasian origin.

Materials and methods

Patients

In this cross-sectional study 330 subjects with T2DM were enrolled. They were selected among patients admitted to the diabetes outpatient clinics of the General Hospitals Murska Sobota and Slovenj Gradec, Slovenia. Patients were excluded if they had homozygous familial hypercholesterolemia or a previous cardiovascular event such as myocardial infarction or a cerebral stroke. The research protocol was approved by the National Medical Ethics Committee. Clinical data, including smoking habits, duration and treatment of diabetes, arterial hypertension, hyperlipidemia, and consuming any other drugs were obtained from

medical records and questionnaires. Patients were asked if they were smokers at the time of recruitment (current smoker).

Ultrasonographic analysis

A high resolution B mode ultrasound analysis was performed using a portable ultrasound system, Toshiba Aplio SSA-700 (Toshiba Medical. System Corp., Tokyo, Japan) connected to a multi-frequency (7.5-10 MHz) linear array transducer. All examinations were performed by two radiologists, blinded to the participant's diabetes status. Patients were examined in the supine position with the head tilted backwards. The carotid arteries were examined from the supraclavicular fossa to the submandibular angle, including the common carotid artery (CCA), carotid bifurcations and origins of internal carotid arteries (ICA).

The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured, as described previously [18]. Plaques were defined as a focal intima-media thickening and divided into 5 types according to their echogenic/echolucent characteristics, as described previously [18]. The interobserver reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $p < 0.001$).

Biochemical analyses

Blood samples for biochemical analyses: total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated haemoglobin (HbA1c), hs-CRP were collected after a 12-hour fasting period. All the blood biochemical analyses were determined by using standard biochemical methods in the hospital's accredited lab.

Genotyping

The genomic DNA was extracted from 100μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). Polymorphism rs3869109 of the HLA gene were determined with real-time PCR using StepOne™ (48-well) Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Continuous variables were expressed as means ± standard deviations, when normally distributed, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov–Smirnov test. Continuous clinical data were compared using an unpaired Student's t test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables.

To determine the association of the tested polymorphism with the presence of unstable plaques a

multivariate logistic regression analysis was performed. All the regression models were adjusted for the presence of well-established cardiovascular risk factors: age, gender, duration of DM, duration of hypertension, plasma levels of total and HDL cholesterol.

The results were presented by odds ratios and 95% CIs for the logistic regression. A two-tailed P value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

Results

We divided T2DM subjects according to sonographically determined carotid plaque stability into two groups (Table 1). T2DM subjects with unstable plaques had ultrasonographic carotid plaque types 1, 2, and 3, while T2DM subjects with stable plaques had ultrasonographic plaque types 4 and 5.

T2DM subjects with unstable plaques had a smaller waist circumference and shorter duration of diabetes compared to T2DM subjects with stable plaques in carotid arteries (Table 1). The two groupus did not differ significantly in age, gender distribution, cigarette smoking, history of hypertension and its duration, body mass index (BMI), systolic and diastolic blood pressure, fasting glucose, HbA1c, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, hs-CRP, as well as traetment with statins, ACE inhibitors, beta-blockers, and calcium channel blockers (Table 1).

Table 1. Demographic, biochemical, risk factors and treatment profile of the Slovenian T2DM subjects according to the plaque type.

		Subjects with T2DM with unstable plaques N = 190	Subjects with T2DM with stable plaques N = 140	P-value
Age (years)		63.42±9.42	64.94±8.83	0.132
Male sex, *N* (%)		96 (50.3)	66 (47.2)	0.582
Cigarette smoking, *N* (%)		11 (5.7)	10 (7)	0.64
History of hipertension, *N* (%)		170 (89.7)	124 (88.3)	0.702
Duration of diabetes (years)		8 (3; 14)	11 (5.5; 21)	**0.003**
Duration of hypertension (years)		7 (4; 16.25)	10 (5; 15)	0.169
Waist circumference (cm)		107 (100.75; 115)	110 (102; 118.50)	**0.03**
BMI (kg/m^2)		30.06 (27.71; 33.31)	31.07 (27.59; 33.96)	0.292
SBP (mm H g)		140 (130; 160)	145 (132; 160)	0.263
DBP (mm Hg)		85 (80; 90)	85 (80; 90)	0.651
Fasting glucose (mmol/L)		7.6 (6.4; 9.2)	7.85 (6.3; 9.7)	0.884
HbA1c (%)		7.6 (6.9; 8.8)	7.6 (6.64; 8.4)	0.170
Total cholesterol (mmol/L)		4.6 (4; 5.5)	4.6 (3.9; 5.5)	0.483
HDL cholesterol (mmol/L)		1.1 (1; 1.3)	1.1 (1; 1.4)	0.309
LDL cholesterol (mmol/L)		2.6 (2.05; 3.2)	2.5 (1.89; 3.2)	0.344
Triglycerides (mmol/L)		2 (1.4; 2.85)	2 (1.2; 2.9)	0.453
hs-CRP		2.65 (1.3; 5.3)	2.3 (1.1; 4.8)	0.347
Treatment:	Statins, *N* (%)	140 (73.8)	99 (70.5)	0.658
	ACEi, *N* (%)	97 (51.1)	78 (56)	0.376
	BBs, *N* (%)	32 (17)	27 (19.5)	0.667
	CCBs, *N* (%)	19 (10)	21 (14.9)	0.232

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure, HbA1c: glycated haemoglobin; hs-CRP: high sensitivity C reactive protein; ACEi: angiotensin converting enzyme inhibitor; BB: beta blocker; CCB: calcium chanell blocker

The genotype distribution and allele frequencies of rs3869109 polymorphism in T2DM subjects with stable and unstable plaques are presented in Table 2. Subjects with unstable plaques had a higher frequency of allele A and a lower frequency of allel G in comparison with subjects with stable carotid plaques (p = 0.006). The frequency of genotype GG was lower, while the frequencies of genotypes AA and AG were higher in T2DM subjects with unstable plaques compared to T2DM subjects with stable plaques (p = 0.041) (Table 2). The genotype distributions in both subjects with unstable plaques and subjects with stable plaques were in Hardy-Weinberg equilibrium (Table 2).

Table 2. Frequency distribution of genotypes and alleles of the rs3869109 polymorphism.

rs3869109	Subjects with T2DM with unstable plaques (N = 190)	Subjects with T2DM with stable plaques (N = 140)	P-value
Genotype			
AA, N (%)	30 (15.6)	17 (12.2)	
AG, N (%)	94 (49.4)	55 (38.9)	0.041
GG, N (%)	66 (35)	68 (48.9)	
Allele			
A, N (%)	154 (40.5)	89 (32.1)	0.006
G, N (%)	226 (59.5)	191 (67.9)	
HWE P-value	0.72	0.27	

On the contrary, we found no association between the rs3869109 polymorphism and either CIMT, sum of plaque thickness, or the number of segments with plaques (Table 3).

Table 3. Ultrasound markers of atherosclerosis in carotid artery.

rs3869109	AA	AG	GG	P-value
CIMT (mm)	1.1 (1; 1.15)	1.1 (0.9; 1.15)	1.1 (0.91; 1.15)	0.875
Sum of plaque thickness (mm)	7.8 (4.94; 10.3)	7.95 (4.73; 11.2)	7.2 (3.8; 11.5)	0.427
Number of segments with plaques (%)	3 (2; 4)	3 (1; 4)	3 (1; 4)	0.664

Multiple logistic regression analysis adjusted for age, sex, duration of DM, duration of hypertension, total and HDL cholesterol revealed that T2DM subjects carrying the AA genotype or the AG genotype had higher prevalence of unstable carotid plaques compared with the carriers of the GG genotype (OR 2.59, 95% CI 1.00-6.69, p = 0.049; OR 1.97, 95% CI 1.02-3.82, p = 0.044) (Table 4). In addition, in the recessive model, the prevalence of unstable carotide plaques was significanly higher in T2DM subjects carrying the AA + AG genotype in comparison with those carrying the GG genotype (OR 2.11, 95% CI 1.14-3.93, p = 0.018).

Table 4. The association between the rs3869109 polymorphism and unstable plaques by logistic regression analysis adjusted for age, sex, duration of DM, duration of hypertension, total and HDL cholesterol.

Genetic model			OR (95 % CI)	P-value
Dominant :	AA+AG	GG (Ref.)	2.11 (1.14-3.93)	**0.018**
Co-dominant:	AA		2.59 (1.00-6.69)	**0.049**
	AG	GG (Ref.)	1.97 (1.02-3.82)	**0.044**
Recessive:	AA	AG+GG (Ref.)	1.83 (0.76-4.43)	0.181

Discussion

In the present study, we demonstrated an association between the rs3869109 polymorphism within the MHC locus and carotid plaque stability in subjects with T2DM. T2DM subjects carrying the AA and GG genotypes had a higher prevalence of unstable plaque morphology than GG genotype carriers.

To the best of our knowledge, this is the first study to investigate the association between the rs3869309 polymorphism and different ultrasound phenotypes of carotid atherosclerosis in subjects with T2DM. In a genome wide association study, rs3869109 was associated with coronary artery disease in patients of European descent (risk allele G, OR 1.14, $p = 1.12x10^{-9}$) [16]. Similarly, the rs3869109 polymorphism was associated with premature coronary artery disease in a Chinese Han population [17]. Multivariate logistic regression showed that carriers with AG and GG genotypes had a higher risk of premature coronary artery disease than carriers of AA genotype (OR 1.997, 95% CI 1.166-3.419, p = 0.012; OR 1.695, 95% CI: 1.044-2.752, p = 0.033; respectively). In addition, the variant was also associated with the severity of premature coronary artery disease (p = 0.038) [17]. Contrary to these findings, in our study subjects with AA and AG genotypes had a higher likelihood of unstable carotid plaques compared to subjects with GG genotype (OR 2.59, 95% CI 1.00-6.69, p = 0.049; OR 1.97, 95% CI 1.02-3.82, p = 0.044; respectively).

We did not, however, demonstrate any association between the tested polymorphism and either CIMT, the sum of plaque thickness or the number of involved segments with plaques. It is well appreciated that carotid ultrasound phenotypes, such as CIMT and carotid plaque, reflect biologically and genetically different features of the atherosclerotic process [19]. While CIMT represents mainly hypertensive medial hypertrophy, and is more predictive of stroke than of myocardial infarction, carotid plaque area is more strongly associated with traditional risk factors, and is more predictive of myocardial infarction than of stroke [19]. From a genetic viewpoint, different ultrasound phenotypes will thus have different genetic associations [20]. For this reason, ultrasound phenotypes of atherosclerosis are a useful and important tool for genetic research [19].

It is well established that both atherosclerosis and T2DM are associated with a systemic inflammatory state. Immune-mediated inflammation of the macrovasculature is implicated in the pathogenesis of diabetes-accelerated atherosclerosis and its complications [3]. Innate and adaptive immune responses to lipoprotein deposition and oxidation in the arterial wall play a significant role in the development and progression of atherosclerosis [21]. As MHC genes regulate immunity and T-cell responses, they could contribute substantially to the inflammatory process and influence plaque vulnerability [15]. Consistent with enhanced inflammatory response, carotid plaques from diabetics contain a larger lipid-rich necrotic core and have more macrophages, T lymphocytes, and human leukocyte antigen-DR-positive cells in comparison to nondiabetic plaques [2, 4]. Consequently, carotid plaques in T2DM subjects are more vulnerable and appear more echolucent on B-mode ultrasound images [9]. In comparison, stable athero-sclerotic plaques are characterized by fibrosis and calcification and thus appear more echogenic on ultrasound [22]. Ultrasound

carotid plaque echolucency is a predictive risk factor for ipsilateral stroke in patients across a wide range of carotid stenosis severity [10], and is associated with higher degrees of systemic inflammation [23].

The association between genetic markers of inflammation and carotid plaque phenotypes was previously reported. Gardner et al. observed significant associations between genetic variants in 7 genes related to inflammation (TNF, NOS2A, IL6R, TNFSF4, PPARA, IL1A, TLR4) and carotid plaque phenotypes in people of Dominican descent [24]. Similar to our study, none of the inflammatory genes associated with plaque phenotypes were associated with carotid intima-media thickness in the same population [24]. In an Italian study, variations in genes encoding for typical inflammatory molecules were significantly and independently associated with internal carotid artery stenosis and histologically determined plaque stability [25].

The rs3869109 polymorphism is located in an intergenic region between HCG27 (long noncoding RNA) and HLA-C [16]. The signal spans a large area containing numerous genes in addition to HLA-B and HLA-C, many of which have known functions in immune mediated processes [15]. Newly, rs3130683, another GWAS variant in the MHC locus approximately 700 kb away from rs3869109 variant, has been reported to be associated with coronary artery disease in subjects with Western-European ancestry [26]. The rs3130683 polymorphism lies in the HLA complex in intron 1 of C2, which encodes the complement C2 protein. However, the CAD signal spans a region of approximately 300 kb including more than 20 genes [26]. Interestingly, rs3130683 was also significantly associated with T2DM ($p = 2.7x10^{-5}$) [26]. In addition, smaller candidate gene studies have reported associations between coronary artery disease and MHC2TA encoding the MHC class II transactivator [27], as well as genes implicated in leukotriene synthesis [28]. There is also inconsistent evidence regarding the association of a MHC class III lymphotoxin A gene with myocardial infarction [29].

The location of the rs3869109 polymorphism in the major histocompatibility locus at 6p21.3 would strongly indicate that it acts through the innate immune system to enhance the vascular inflammatory response. However, the exact biological mechanism of this polymorphic variant is not clear. Similar to the majority of GWAS-identified variants, the observed signal at rs3869109 lies in a non-coding region between HCG27 and HLA-C [16]. The non-coding genomic regions include non-coding RNAs that may influence gene transcription through multiple mechanisms, active promoters and enhancers, regions affecting histone acetylation and deacetylation and chromatin remodeling, susceptibility to DNA methylation, and micro-RNAs and micro-RNA binding sites [30]. Newly, McManus et al. found that MHC class I (*MR1*) is the top gene target of miR-505, a micro-RNA associated with cardiometabolic traits (body mass index, blood pressure, and triglycerides) [31] and endothelial function [32]. Notably, Davies et al. found that no single common HLA type contributed significantly or fully explained the observed association [16]. Last but not least, the polymorphisms identified in GWAS are rarely causal themselves but rather in linkage disequilibrium with a neighboring or even distal causal polymorphism. The disease-associated loci identified by GWASs may span several kilobases, and some may encompass multiple causative variants in more than

1 gene [30].

Our study has certain limitations related to its cross-sectional design and relatively small sample size. However, all the participants were recruited from an ethnically homogenous population and all underwent detailed ultrasound evaluation of carotid atherosclerosis phenotypes.

To conclude, the MHC complex polymorphism rs3869109 may be a genetic marker of atherosclerotic carotid plaque stability in T2DM subjects of Caucasian descent. Further studies are warranted to unravel the underlying molecular mechanisms of this association and find the causative gene(s).

Conflict of interest

The authors declare no conflict of interest related to this work.

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Raziskava 9

Polymorphism rs2073618 of the osteoprotegerin gene as a potential marker of subclinical carotid atherosclerosis in caucasians with type 2 diabetes mellitus

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Abstract

Background: The OPG/RANKL/RANK (osteoprotegerin/receptor-activator of nuclear factor κB ligand/ receptor-activator of nuclear factor κB) axis has been recently linked to the development of atherosclerosis and plaque destabilization. We have investigated whether polymorphism rs2073618 of the OPG gene is associated with subclinical markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus (T2DM).

Methods: 595 subjects with T2DM were enrolled in the cross-sectional study. Subclinical markers of carotid atherosclerosis (carotid intima media thickness, plaque thickness, and plaques presence) were assessed with ultrasound at the time of recruitment. Genotyping for rs2073618 (a missense variant located in exon I of the *OPG* gene) was performed, and OPG serum levels were determined by ELISA.

Results: Compared to the GG genotype, the CC genotype of the rs2073618 polymorphism had a significantly increased risk for the presence of carotid plaque (OR = 2.54, 95% CI = 1.22-5.28, $p = 0.01$). No statistically significant difference could be detected ($p = 0.68$) upon comparing median values of serum OPG levels among studied genotype groups in subjects with T2DM. Multivariable linear regression analyses in T2DM subjects demonstrated that GC and CC genotypes ($p = 0.03$ and $p = 0.003$), together with statin therapy ($p = 0.009$), were independent predictors of the number of carotid segments with plaques.

Conclusions: Despite the fact that *OPG* rs2073618 genotypes failed to predict the serum OPG levels as there was no statistical difference among compared genotypes, our results demonstrate that the rs2073618 polymorphism could be a possible genetic marker for the prediction of increased risk for carotid plaque burden as a measure of advanced subclinical atherosclerosis in T2DM subjects.

Key words: subclinical carotid atherosclerosis; genetic polymorphism; osteoprotegerin (OPG), type 2 diabetes mellitus (T2DM)

Introduction

Atherosclerotic disease in both its subclinical and clinically established phases is widely prevalent throughout the world. Disease progression can eventually lead to the occurrence of acute cardiovascular events (CVE), such as myocardial infarction, unstable angina pectoris and sudden cardiac death [1]. The processes of accelerated and premature atherosclerosis in subjects with type 2 diabetes mellitus (T2DM) lead to an increased risk of macrovascular complications, including an increased risk of cardiovascular events [2]. Subjects with T2DM have an increased risk of cardiovascular diseases (CVD), a higher cardiovascular morbidity and mortality compared to non-diabetic subjects [3]. Various traditional and non-traditional risk factors are involved in the pathogenesis of atherosclerosis in subjects with T2DM [4]. Atherosclerosis is triggered by active processes involving metabolic factors, inflammatory cytokines and other signals, or by disordered calcium/phosphate homeostasis [5-8]. An important regulator of mineral metabolism, namely osteoprotegrin (OPG), a member of the tumour necrosis factor (TNF) receptor

superfamily, is a soluble decoy receptor for the osteoclast differentiation factor receptor-activator of nuclear factor κB ligand (RANKL) that inhibits the interaction between RANKL and its membrane-bound receptor RANK [9]. The OPG/RANKL/RANK axis has been shown to regulate bone remodelling [10,11], however, it has more recently been linked to the development of atherosclerosis and plaque destabilization [12,13]. In bones, OPG inhibits bone resorption, whereas RANKL promotes bone resorption in contrast to their action in the vasculature, where RANKL promotes calcification and OPG has a protective effect [14]. Data suggest that OPG is induced by atherosclerosis and may be upregulated as an incomplete compensatory response to the vessel insult, possibly thereby limiting vascular calcification [15]. Although it has been demonstrated that increased carotid intima-media thickness (CIMT) and the presence of carotid plaques are correlated with plasma OPG levels in healthy individuals [16,17], only recently relevant data has been published concerning subjects with T2DM [18].

Despite the widespread use of non-invasive imaging in early detection of subclinical atherosclerosis as measured by CT and/or carotid ultrasound, novel protein biomarkers would have utility in predicting CVE risk. In the Second Manifestations of arterial disease (SMART) study, 23 novel biomarkers were evaluated for their potential to enhance predictive performance in patients with type 2 diabetes [19]. In particular, their research was focused mainly on other bone-related proteins (osteocalcin, osteonectin and osteopontin), not on OPG. However, sparse data are available on the relationship between plasma OPG levels and markers of subclinical atherosclerosis in subjects with T2DM. Furthermore, increased plasma OPG concentration is associated with carotid and peripheral arterial disease in T2DM [20].

More importantly, there is growing evidence that genetic polymorphisms related to the alteration in the expression of OPG could contribute significantly to the atherosclerotic processes in subjects with T2DM. Subsequently, we evaluated whether polymorphism rs2073618 in the exon I of the OPG gene affects plasma OPG level, and whether this polymorphism is associated with markers of subclinical carotid atherosclerosis in Slovenian subjects with T2DM.

Materials and Methods

Subjects

This cross-sectional study enrolled 595 consecutive subjects with T2DM which were admitted to the diabetes outpatient clinics of the General Hospitals Murska Sobota and Slovenj Gradec, Slovenia, and from the outpatient department Medicor, Ljubljana. Patients were classified as having T2DM according to the current report of the American Diabetes Association [21]. Exclusion criteria were: homozygous familial hypercholesterolaemia or a history of cardiovascular event (i.e. acute coronary syndrome or a cerebrovascular stroke). The research protocol was approved by the National Medical Ethics Committee. Clinical data, including smoking habits, duration and treatment of diabetes, arterial hypertension, hyperlipidemia and consumption of any other drugs, were obtained from medical records and questionnaires. Patients were asked if they were smokers at the time of recruitment (current smoker).

Clinical and laboratory assessment

Clinical and anthropometric data, including smoking habits, the duration and treatment of diabetes, the presence of arterial hypertension, hyperlipidaemia and data on the consumption of any other drugs were collected from medical records and questionnaires at the time of enrolment. Subjects with systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 85 mmHg and/or subjects using antihypertensive drugs were considered hypertensive. Body mass index (BMI) was calculated as total body mass (kg) divided by height (m) squared.

A 12-hour overnight fasting blood sample was drawn to determine the serum levels of total, HDL and LDL-cholesterol, triglycerides and glycated haemoglobin (HbA1c), and OPG. Serum non-HDL-cholesterol levels were calculated by subtracting HDL-cholesterol from the total cholesterol. Plasma was used for fasting glucose, hs-CRP concentration measurements.

HbA1c was measured by high-performance liquid chromatography and had a non-diabetic range of 3.8-5.3%.

The Human Osteoprotegerin Instant ELISA (Enzyme-Linked-Immunosorbent Assay, eBioscience, Bender MedSystems GmbH, Austria) kit was used for the quantitative determination of OPG in serum samples, according to the manufacturer's instructions. The assay employs a polyclonal antibody adsorbed onto microwells, specific for human OPG. A biotin-conjugated polyclonal anti-human OPG antibody was used as a capture antibody. Streptavidin-HRP binds to the biotin conjugated anti-human OPG and generates coloured precipitate. The absorbance was measured at 450 nm using a spectrophotometer (Bio-rad, USA), and the concentration (pg/ml) was determined by comparison with the standard curve.

Genetic analyses

Genomic DNA was extracted from 200 µl of peripheral venous blood from each subject using a FlexiGene isolation kit according to the recommended protocol (Qiagene, Germany). Genotyping for rs2073618 (a missense variant located in exon I of the *OPG* gene, NM_002546.3:c.9C>G, NP_002537.3:p.Asn3Lys) was outsourced to KBioscience which uses a mixture of competitive allele specific PCR (KASPar) (KBioscience, Hoddesdon, United Kingdom). Genotypes were assigned using the Klustercaller software (KBioscience, Hoddesdon, United Kingdom).

Carotid artery evaluation

An ultrasound analysis was performed using the portable ultrasound system Toshiba Aplio SSA-700 (Toshiba Medical. System Corp., Tokyo, Japan). All examinations were performed by three radiologists, blinded to the participants diabetes status. The carotid arteries were examined from the supraclavicular fossa to the submandibular angle, including the common carotid artery (CCA), carotid bifurcations and origins of internal carotid arteries (ICA).

The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading

edge of the media-adventitia interface, was measured at 3 sites along the 10mm-long segment of the far wall of the CCA free of plaques, in agreement with the carotid intima-media thickness consensus [22]. The CIMT on the left and on the right were calculated as the mean of three readings, and the mean of the left and right CCA-CIMT measurements was used in the analysis [22]. Plaques were identified on both the near and the far walls in the CCA, bulb and ICA, bilaterally. In the presence of plaque, the IMT was measured at the segment without plaque. Due to their chogenic/echolucent characteristics, we divided all the plaques into 5 types [22].

Statistics

Statistical analyses were performed using the Statistical Package of Social Sciences (SPSS) software, version 20 (SPSS Inc., Illinois). Normal distribution of data was analysed by the Shapiro-Wilk normality test. Continuous variables were summarized as mean ± standard deviation when normally distributed, and as median values (first and third quartile) when asymmetrically distributed. Continuous clinical, anthropometric and biochemical data were compared by one-way analysis of variance (ANOVA) when normally distributed, and by the Kruskall-Wallis H-test when asymmetrically distributed. Post hoc tests were run to confirm the statistical significance of the differences between rs2073618 genotypes. Multiple comparisons were made using pairwise comparisons, and a Bonferroni correction was applied. The Pearson χ^2 test was used to compare discrete variables. The associations between risk genotypes and ultrasonographic markers of atherosclerosis, namely, CIMT, the sum of plaque thickness and the number of segments with plaques were tested by a linear regression model adjusted for age, sex, smoking status, systolic blood pressure, non-HDL-cholesterol, HDL-cholesterol and the use of statins in Model 1. In Model 2 age, sex, smoking status, duration of diabetes and history of hypertension were included in linear regression analyses as adjusting variables. Further, a multivariate binary logistic regression was carried out in order to estimate the association between genotypes and the presence of plaques or the presence of unstable plaques. The presence and the type of plaque were coded as bimodal variables (presence of plaques = 1, without plaques = 0, and unstable plaques = 1 and stable plaques = 0, respectively). The co-dominant genetic model with the GG genotype being the reference was employed. Adjusted odd ratios (OR) with 95% confidence intervals (95% CI) were reported. Both models (Model 1 and Model 2) were adjusted for the following potentially confounding variables: age, sex, smoking status, systolic blood pressure, non-HDL-cholesterol, HDL-cholesterol, the use of statins, history of hypertension, duration of diabetes and hypertension. A *p* value less than 0.05 was considered statistically significant.

Results

The basic clinical and biochemical features in relation to rs2073618 genotypes of the participants with T2DM are provided in Table 1.

The median durations of diabetes for C allele carriers were 8.5 (2-15) and 10 (5-16) years, for CC and

CG carriers, respectively. Specifically, carriers of the C allele retained statistical significance for diabetes duration after post-hoc test analysis (for both pairwise comparisons CC versus GG, and GC versus GG, the p value was 0.02, respectively).

Except for the therapy with calcium channel blockers (CCBs) (p = 0.04), no statistically significant differences were observed among analysed genotype groups with respect to antihypertensive drugs and statin use (Table 1). Further, the distribution of subjects receiving insulin therapy and subjects with a history of hypertension were significantly different among genotype groups (p = 0.04).

In further analysis, we evaluated if serum OPG could serve as a biomarker for subclinical atherosclerosis. In randomly selected subgroup of subjects with T2DM (178 subjects), OPG serum levels were determined, and they were divided according to their rs2073618 genotype into three groups (Figure 1). Median values of serum OPG levels were significantly higher in subjects with T2DM than in controls (108 subjects).

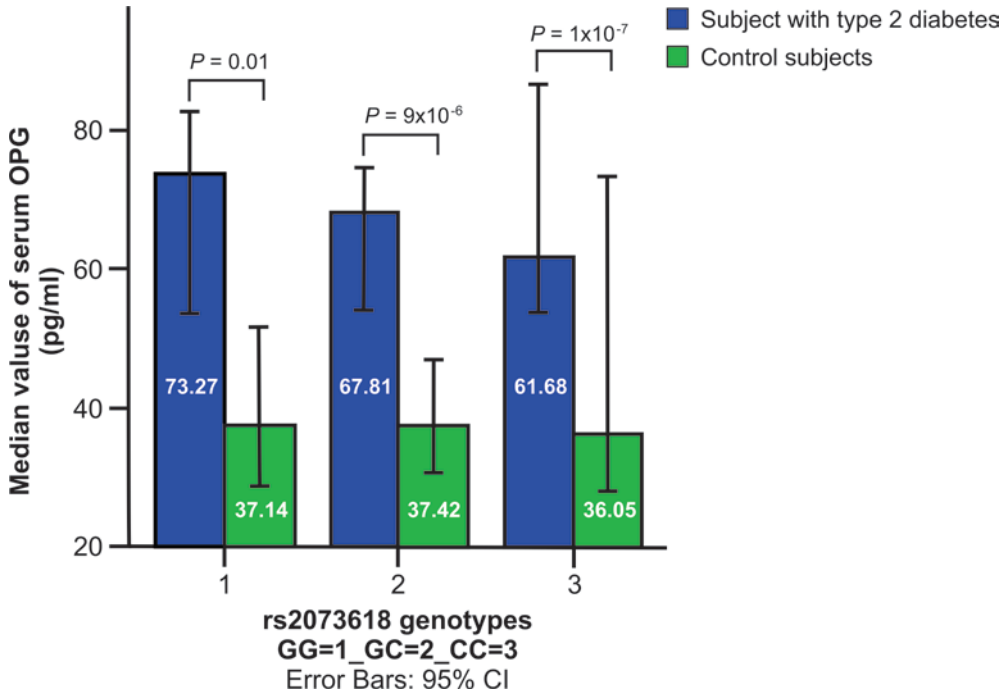

Figure 1. Median values of serum OPG levels with 95% confidence intervals stratified by rs2073618 genotypes. P-values were obtained by the Mann-Whitney test.

However, no statistically significant difference could be detected (p = 0.68) upon comparing median values of serum OPG levels among studied genotype groups when only subjects with T2DM were included in the analysis (Table 1). The GG genotype had a median level of 73.26 (47.03-96) pg/ml, the GC genotype had a median level of 67.56 (44.26-90.43), and CC genotype had a median level of 61.6 (47.63-137.89) pg/ml (Table 1).

Table 1. Clinical and biochemical characteristics in relation to rs2073618 genotypes in subjects with type 2 diabetes mellitus (*N = 595*).

Characteristics	1181GG	1181GC	1181CC	*P value*
Genotypes, n (%)	205 (34.5)	252 (42.3)	138 (23.2)	
Age (years)	62.52 ± 9.14	63.24 ± 9.75	60.79 ± 9.84	0.09
Male sex, n (%)	115 (56.4)	134 (52.5)	72 (51.8)	0.68
Smokers, n (%)	13 (6.4)	18 (7.2)	10 (6.9)	0.96
BMI (kg/m^2)	30.48 (25.92-33.44)	30.40 (27.45-33.71)	30.96 (28.08-33.39)	0.84
Waist circumference (cm)	108 (103-115.5)	108.5 (101-116)	107 (99-117)	0.69
Duration of diabetes (years)	11 (5-15)	10 (5-16)	8.5 (2-15)	**0.04**
Patients on insulin therapy, n (%)	101 (49)	91 (36)	65 (46.7)	**0.04**
History of hypertension, n (%)	161 (78.6)	223 (88.6)	119 (85.9)	**0.04**
Duration of hypertension (years)	10 (4-15)	9 (4-15.5)	7.5 (3-17.25)	0.81
Systolic blood pressure (mm Hg)	140 (130-158)	146 (137-161)	144 (130-160)	0.14
Diastolic blood pressure (mm Hg)	84 (77-90)	86.5 (80-93)	85 (80-90)	0.32
Fasting plasma glucose (mmol/l)	8.1 (6.5-9.9)	7.7 (6.1-9.7)	7.3 (6.2-8.6)	0.09
Serum OPG (pg/ml)	73.3 (47.0-96.0)	67.6 (44.3-90.4)	61.6 (47.6-137.9)	0.65
Log-OPG	1.9 (1. 7-2.0)	1.8 (1.6-2.0)	1.8 (1.7-2.14)	0.65
HbA1c (%)	7.5 (6.8-8.6)	7.6 (6.7-8.4)	7.5 (6.9-8.6)	0.84
hs-CRP (mg/l)	2.5 (1.2-5.0)	2.3 (1-4.5)	2 (1.1-3.3)	0.27
Plasma fibrinogen (g/l)	4.2 (3.5-5.4)	4.3 (3.6-5.2)	4.3 (3.7-5)	0.94
Total cholesterol (mmol/l)	4.5 (3.9-5.4)	4.4 (3.9-5.5)	4.7 (3.9-5.6)	0.67
HDL-cholesterol (mmol/l)	1.1 (0.9-1.4)	1.2 (1-1.4)	1.1 (1-1.4)	0.49
LDL-cholesterol (mmol/l)	2.5 (2-3.2)	2.5 (1.9-3.1)	2.6 (1.9-3.4)	0.78
Triglycerides (mmol/l)	2 (1.3-3.1)	1.9 (1.3-2.5)	1.9 (1.2-2.8)	0.57
Non HDL-cholesterol (mmol/l)	3.3 (2.6-4.3)	3.3 (2.7-4.2)	3.6 (2.7-4.5)	0.63
Treatment				
ACEi, n (%)	110 (53.4)	148 (58.6)	79 (57.3)	0.59
ARBs, n (%)	40 (19.6)	56 (22.2)	20 (14.5)	0.27
CCBs, n (%)	29 (14.1)	45 (17.7)	10 (7.3)	**0.04**
Statins, n (%)	125 (60.9)	161 (63.8)	99 (71.6)	0.31

Continuous variables were expressed as median (quartile 1, quartile 3) when asymmetrically distributed, except for age where data were provided as means ± standard deviations. Categorical variables were expressed as frequency (number (n) and percentage (%)). BMI: body mass index; OPG: osteoprotegerin; Log-OPG: logarithmic transformation of serum OPG levels; HbA1c: the average value for glycated haemoglobin; hs-CRP: high-sensitivity C-reactive protein; HDL-cholesterol: high density lipoprotein cholesterol; LDL-cholesterol: low density lipoprotein cholesterol; ACEi: angiotensin-converting enzyme (ACE) inhibitors; ARBs: angiotensin II receptor blockers; CCBs: calcium channel blocker

In addition, to assess whether there is any difference in median values of serum OPG levels among genotypes, subjects with T2DM were divided into four groups according to dichotomous variables (history of hypertension, statin therapy, presence of carotid plaques and type of carotid plaque) (Table 2). A significant difference of median OPG values among genotypes was observed only in the subgroup of subjects with stable plaques ($p = 0.01$).

Table 2. The comparisons of median values of serum OPG levels (pg/ml) among rs2073618 genotypes according to dichotomous independent variables in subjects with type 2 diabetes mellitus.

Parameters		rs2073618 genotypes			*P value*[a]
		1181GG	1181GC	1181CC	
History of hypertension	Y	60.1 (45.9-94.1)	71.0 (46.8-100.0)	60.8 (49.2-91.6)	0.97
	N	73.11 (38.5-84.5)	72.37 (47.4)	49.4 (41.3-228.1)	0.87
Statin therapy	Y	62 (39-89.2)	66.62 (51.41-94.34)	61.6 (49.3-97.6)	0.82
	N	51.39 (36.0-185.1)	65.17 (43.3-85.8)	72.6 (49.7-321.0)	0.4
Presence of carotid plaques	Y	73.01 (47.0-95.2)	68.05 (44.3-95.8)	74.18 (53.2-141.6)	0.43
	N	78.66 (38.2-103.6)	54.01 (41.4-77.4)	50.51 (30.9-90.1)	0.36
Type of carotid plaque	U	73.11 (43.6-90.4)	72.37 (51.7-95.8)	61.68 (47.3-110.3)	0.92
	S	73.17 (44.5-111.9)	48.9 (40.1-87.9)	116.19 (57.6-159.0)	**0.01**

Statistical analysis was performed by Kruskall-Wallis test. Y: yes; N: no; U: unstable; S: stable.

The results of the Spearman test (Table 3) showed that serum OPG levels significantly positively correlated with age ($\rho = 0.195$, $p = 0.009$) and negatively with BMI ($\rho = -0.148$, $p = 0.05$), whereas none of the ultrasonographic markers of carotid atherosclerosis nor other clinical variables did so.

Table 3. Spearman correlation coefficients between the osteoprotegerin levels and clinical variables or sonographic markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus

Variables	Spearman coefficient of correlation	*P* value
Age (years)	0.195	**0.009**
BMI (kg/m^2)	-0.148	**0.05**
Waist circumference (cm)	-0.055	0.47
Duration of diabetes (years)	-0.03	0.67
Duration of hypertension (years)	0.00	0.99
Systolic blood pressure (mm Hg)	0.003	0.98
Diastolic blood pressure (mm Hg)	0.064	0.56
Fasting plasma glucose (mmol/l)	0.014	0.86
HbA1c(%)	-0.087	0.35
hs-CRP (mg/l)	0.09	0.24
Plasma fibrinogen (g/l)	0.02	0.82
HDL-cholesterol (mmol/l)	0.07	0.36
Non HDL-cholesterol (mmol/l)	0.1	0.19
CIMT (μm)	0.06	0.44
Sum of plaque thickness (mm)	0.09	0.28
Number of segments with plaques	0.135	0.07

Next, we investigated the interplay between rs2073618 genotypes and ultrasonographic markers of carotid atherosclerosis in Slovenian subjects with T2DM. Except for the number of involved carotid segments with plaques ($p = 0.01$) and the occurrence of carotid plaques ($p = 0.02$), there were no differences in other carotid ultrasound findings among genotypes, as summarized in Table 4. In terms of the number of involved carotid segments, the post-hoc analysis thus showed that subjects carrying the GC and CC genotypes had more vessel segments with plaques than GG subjects (GC versus GG: $p = 0.03$ and CC versus GG: $p = 0.006$, respectively). Finally, the distribution of plaque occurrence among three genotypes was statistically significant ($p = 0.02$). The prevalence of carotid plaques was higher in both GC and CC carriers (GC (69.2%) versus GG (58.9%): $p = 0.03$ and CC (71.8%) versus GG (58.9%): $p = 0.03$, respectively) (Table 4).

Table 4. Ultrasonographic markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus with regard to rs2073618 genotypes at the time of recruitment.

rs2073618 of the *OPG* gene	1181GG	1181GC	1181CC	*P* value
CIMT (μm)	1075 (885-1150)	1100 (911.2-1150)	1100 (1000-1150)	0.45
Sum of plaque thickness (mm)	6.9 (3.2-10.4)	7.7 (4.6-10.9)	7.75 (4.7-12.0)	0.16
Number of segments with plaques	2 (1-3)	3 (2-4)	3 (2-4)	**0.01**
Presence of carotid plaques, n (%)	121 (58.9)	174 (69.2)	99 (71.8)	**0.02**
Absence of carotid plaques, n (%)	84 (41.1)	78 (30.8)	39 (28.2)	
Presence of unstable carotid plaques, n (%)	105 (51)	144 (57.2)	90 (64.9)	0.18
Presence of stable carotid plaques, n (%)	100 (49)	108 (42.8)	48 (35.1)	

To evaluate if the presence of carotid plaques in subjects with T2DM could be associated with rs2073618 genotypes, we performed logistic regression analyses (Table 5).

Table 5. The association between rs2073618 polymorphism and the carotid artery plaques in subjects with type 2 diabetes mellitus at the time of recruitment.

	Presence of carotid plaque		Presence of unstable carotid plaque	
	OR (95 % CI)	*P* value	OR (95 % CI)	*P* value
Model 1				
Age	1.04 (0.99-1.1)	0.06	1.01 (0.46-2.23)	0.94
Sex (0 = man; 1 = women)	1.34 (0.68-2.63)	0.39	0.69 (0.34-1.41)	0.31
Smoking status (0 = no; 1 = yes)	5.11 (0.93-7)	0.06	0.5 (0.12-2.12)	0.35
Systolic blood pressure (mm Hg)	0.99 (0.97-1.01)	0.27	0.99 (0.97-1.01)	0.15
Non-HDL cholesterol (mmol/l)	1.62 (1.18-2.22)	**0.003**	1.13 (0.85-1.5)	0.42
HDL cholesterol (mmol/l)	0.52 (0.19-1.47)	0.22	0.43 (0.13-1.39)	0.16
Statin therapy (0 = no; 1 = yes)	2.12 (1.05-4.29)	**0.04**	1.42 (0.64-3.11)	0.39
1181CC*	2.54 (1.22-5.28)	**0.01**	2.08 (0.7-6.24)	0.19
1181GC*	1.89 (0.72-4.91)	0.19	1.01 (0.46-2.23)	0.98
Hosmer and Lemeshow Test (χ2; *P* value)	(12.99; 0.11)		(14.8; 0.06)	
Model 2				
Age	1.05 (1.01-1.08)	**0.01**	0.99 (0.96-1.03)	0.85
Sex (0 = man; 1 = women)	1.24 (0.67-2.31)	0.5	0.94 (0.49-1.90)	0.86
Smoking status (0 = no; 1 = yes)	0.76 (0.33-1.7)	0.88	0.81 (0.26-2.88)	0.81
Duration of diabetes (years)	0.99 (0.95-1.04)	0.69	0.99 (0.95-1.04)	0.86
History of hypertension (0 = no; 1 = yes)	1.77 (0.29-10.63)	0.54	0.68 (0.05-8.68)	0.77
Duration of hypertension (years)	1.04 (0.99-1.08)	0.07	0.98 (0.94-1.02)	0.34
1181CC*	2.98 (1.27-6.92)	**0.01**	2.82 (1.09-7.28)	**0.03**
1181GC*	2.6 (1.26-5.37)	**0.01**	1.32 (0.62-2.82)	0.48
Hosmer and Lemeshow Test (χ2; *P* value)	(6.64; 0.58)		(5.61; 0.69)	

**The reference group were homozygotes for the G allele.*

In Model 1 with age, sex, smoking status, systolic blood pressure, non-HDL-cholesterol, HDL-cholesterol, statin therapy, GC and CC genotypes as covariates, a significant association was found between the presence of carotid plaque and the CC genotype. Compared to the GG genotype, the CC genotype of the rs2073618 polymorphism had a significantly increased risk for the presence of carotid plaque (OR = 2.54, 95% CI = 1.22-5.28, p = 0.01). Moreover, users of statins had a 2-fold increased risk for the presence of carotid plaques (OR = 2.12, 95% CI = 1.05-4.29, p = 0.04). Likewise, the non-HDL-cholesterol was associated with the presence of carotid plaque (OR = 1.62, 95% CI = 1.18-2.22, p = 0.003). Additionally, in Model 2, subjects with either CC or GC genotypes had a more than 2-fold increased risk for the occurrence of carotid plaques (OR = 2.98, 95% CI = 1.27-6.92, p = 0.01 and OR = 2.6, 95% CI = 1.26-5.37, p = 0.01, respectively). Among other variables (age, sex, smoking status, duration of T2DM, history and

duration of hypertension) that were included in the multivariable logistic regression Model 2, only age (OR = 1.05, 95% CI = 1.01-1.08, $p = 0.01$) was a risk predictor of the carotid plaque presence (Table 5). Further, after the adjustment for the most relevant clinical variables (age, sex, smoking status, duration of T2DM, history and duration of hypertension) in the logistic regression Model 2, only carriers of the CC genotype showed an increased risk (OR = 2.82, 95% CI = 1.09-7.28, $p = 0.03$) of having unstable plaques when compared to individuals carrying the GG genotype (Table 5).

Lastly, to clarify whether the rs2073618 polymorphism is independently associated with CIMT levels, the sum of plaque thickness and the number of carotid segments with plaques, we performed multivariable linear regression analyses in all T2DM subjects (Table 6).

Table 6. Multivariable linear regression analyses of factors that affect CIMT, plaque thickness and number of segments with plaques.

	CIMT (μm)		Sum of plaque thickness (mm)		Number of segments with plaques	
	ß value	*P* value	ß value	*P* value	ß value	*P* value
Model 1						
Age	0.183	**0.02**	0.175	**0.04**	0.221	**0.003**
Sex (0 = man; 1 = women)	-0.019	0.81	0.229	**0.01**	0.108	0.14
Smoking status (0 = no; 1 = yes)	0.093	0.23	0.154	0.06	0.092	0.197
Systolic blood pressure (mm Hg)	0.018	0.82	-0.035	0.68	-0.056	0.45
Non-HDL cholesterol (mmol/l)	0.16	**0.05**	0.315	**<0.0001**	0.192	**0.009**
HDL cholesterol (mmol/l)	-0.093	0.25	-0.027	0.75	-0.051	0.49
Statin therapy (0 = no; 1 = yes)	-0.077	0.32	-0.121	0.14	-0.189	**0.009**
1181CC*	0.11	0.2	0.137	0.14	0.177	**0.003**
1181GC*	0.076	0.38	0.122	0.19	0.235	**0.03**
Model summary (R square; *P* value)	(0.086; 0.1)		(0.211; **<0.0001**)		(0.161; **<0.0001**)	
Model 2						
Age	0.176	**0.01**	0.148	**0.04**	0.289	**<0.0001**
Sex (0 = man; 1 = women)	-0.002	0.97	0.234	**0.002**	0.102	0.12
Smoking status (0 = no; 1 = yes)	0.062	0.35	0.104	0.14	0.109	0.09
Duration of diabetes (years)	-0.134	0.05	0.043	0.56	-0.065	0.32
Duration of hypertension (years)	0.202	**0.004**	0.243	**0.002**	0.089	0.18
1181CC*	0.134	0.09	0.177	**0.04**	0.213	**0.005**
1181GC*	0.156	0.05	0.07	0.42	0.205	**0.007**
Model summary (R square; *P* value)	(0.107; **0.001**)		(0.143; **<0.0001**)		(0.152; **<0.0001**)	

**The reference group were homozygotes for the G allele.*

Both linear regression models (Model 1 and Model 2) included age, sex, smoking status, GC and CC genotypes, with the GG genotype being the reference group. First, there was an obvious effect of the age and non-HDL-cholesterol on all three quantitative ultrasonographic markers for atherosclerosis in Model 1. In Model 2, age and duration of hypertension were independently linked to CIMT ($p = 0.01$ and $p = 0.004$, respectively) and plaque thickness ($p = 0.04$ and $p = 0.002$). Moreover, women tended to have significantly higher sum of plaque thickness compared with man in both Model 1 ($p = 0.01$) and Model 2 ($p = 0.002$). Similarly, the CC genotype ($p = 0.04$) in Model 2 was independently associated with plaque thickness. Furthermore, in Model 1, the GC and CC genotypes ($p = 0.03$ and $p = 0.003$), together with statin therapy ($p = 0.009$), were independent predictors of the number of carotid segments with plaques. Finally, two genotypes retained their significance as independent factors of carotid plaque number in Model 2 (GC genotype: $p = 0.007$ and CC genotype: $p = 0.005$, respectively).

Discussion

The main finding of this study is a clear evidence of the genetic impact of the rs2073618 CC and GC genotypes on subclinical atherosclerosis. Among early ultrasound-based markers of carotid atherosclerosis, rs2073618 genotypes differed in the number of segments with plaques and in plaque presence, whilst there was no overall difference in CIMT, sum of plaque thickness or type of plaque. An interesting fact is that plaques were more prevalent in both GC and CC carriers; moreover, carries of both genotypes had more carotid segments with plaques compared to GG genotype carriers.

The higher prevalence of the observed CC and GC genotypes in Slovenian subjects with T2DM highlights their potential impact on atherosclerosis. Indeed, more than a 2-fold increased risk for carotid artery plaque formation was seen in carriers of the CC or GC genotypes when compared to T2DM subjects with the GG genotype. Age- and gender-adjusted multivariable linear logistic regression not only confirmed the positive association between the CC and GC genotypes with the number of segments with plaques, but also revealed that the CC genotype was a statistically significant predictor of plaque thickness. Despite the well-established association between the CC genotype and the development of unstable plaques, we did not observe any significant differences among rs2073618 genotypes when median serum OPG levels in the group of 101 T2DM subjects with unstable plaques was assessed. In particular, subjects carrying the high-risk CC genotype showed a lower median OPG level compared to GC or GG genotypes, although the difference was not statistically significant ($p = 0.92$). On the other hand, and perhaps more importantly, the median serum OPG level was statistically higher ($p = 0.004$) in the CC genotype group compared to those carrying the GC genotype when 57 T2DM subjects with stable plaques were analysed. This finding is consistent with the recent clinical observation from Davaine et al. [23] who proposed a new concept of OPG plaque stability modulation. They observed that circulating OPG levels strictly mirrored intraplaque OPG presence and correlated positively with carotid plaque stability. Moreover, plasma OPG should be seriously considered as an interesting potential marker for carotid plaque stability [24]. Golledge et al. [17] also observed elevated OPG levels in symptomatic carotid lesions, but no histological data regarding plaque vulnerability were provided [24]. Similarly, Straface et al. [24] suggested that high serum OPG levels might be associated with plaque instability. In their study, patients with the CC variant genotype of the G1181C polymorphism with unstable plaques showed a statistically higher median OPG concentration than the subjects with stable plaques. On the contrary, we did not observe any significant variation in the serum OPG levels between the Slovenian groups of T2DM subjects regarding the two types of plaques (data not shown).

When serum OPG levels of all T2DM subjects were taken into account, no difference among rs2073618 genotypes were found. Although statistically not significant, our data indicate that the CC carriers had the lowest median serum OPG level. This might be explained at least partially by the fact that almost 47% of the CC carriers required insulin treatment and more than 70% subjects with the same genotype were receiving statins. Several treatments are known to affect OPG concentrations, such as insulin, glitazones,

and statins [25]. It has also been reported that an increased OPG concentration is associated with carotid and peripheral arterial disease in type 2 DM [20, 26]. Our data could, however, not demonstrate the existence of a meaningful linear relationship between any of the ultrasonographic markers of subclinical atherosclerosis and serum OPG level in T2DM subjects. Furthermore, our results are in accordance with another study [26], which confirmed that OPG levels were significantly higher in T2DM subjects compared to healthy controls.

Up to now, very little studies have assessed the relationship between polymorphisms of the *OPG* gene and macrovasular diseases in T2DM subjects. Of note, the study of Guo et al. [27] provided evidence that the C allele of the T950C (rs2073617) polymorphism was associated with an increased risk of cardiovascular disease in diabetic patients. Furthermore, in some recent studies performed in Italian diabetic patients, the SNPs, T245G (rs3134069), T950C (rs2073617) and G1181C (rs2073618), respectively, were significantly associated with the risk of stroke [28]. On the other hand, Soysal-Atiel et al. [29] did not observe any significant association of the A163G (rs3102735) polymorphism in the promoter region of OPG with either micro- or macrovascular complications in a cohort of 116 Turkish T2DM patients.

Some limitations of the study should be addressed. As our study sample size was relatively small, it could produce false-positive results, or it overestimated the magnitude of the association [30]. Our findings need to be evaluated in larger groups of different ethnicities. In an attempt to clarify the potential role of the OPG/RANKL/RANK system in atherogenesis, it would have been rational to measure not only OPG, but also RANKL and RANK serum levels. Interactions between genes of the OPG/RANKL/RANK axis and the development of carotid atherosclerosis remain to be investigated.

Conclusion

To conclude, logistic regression analyses on Slovenian T2DM subjects showed that carriers of the CC genotype had a more than 2.5-times increased risk for the presence of carotid plaque compared to those with GG the genotype. Moreover, linear regression revealed that CC and GC genotypes of the rs2073618 polymorphism were significantly positively associated with the total number of plaques. To summarize, despite the fact that *OPG* rs2073618 genotypes failed to predict the serum OPG levels as there was no statistical difference among the compared genotypes, our results demonstrate that the rs2073618 polymorphism could be a possible genetic marker for the prediction of an increased risk for carotid plaque burden as a measure of advanced subclinical atherosclerosis in T2DM subjects.

Conflicts of interest

The authors declare no conflict of interest.

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Zaključki

V naši prospektivni raziskavi, ki je zajela 595 bolnikov s SB 2 in 200 preiskovancev brez SB (kontrolna skupina), smo genetsko testirali 17 polimorfizmov v 12 izbranih genih, ki so vpleteni v vnetni odgovor, smo testirali štiri hipoteze.

Zanimalo nas je ali poteka aterosklerotični proces hitreje pri bolnikih s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP ≥ 2 mg/L) kot pri tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L).

- Potrdili smo, da poteka pri bolnikih s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP ≥ 2 mg/L) aterosklerotični proces hitreje kot pri tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L).
- Bolniki s SB tipa 2 z izrazitejšim sistemskim vnetjem so imeli večjo DIM in pogostejše karotidne plake kot bolniki s SB tipa 2 in hs-CRP < 2 mg/L.
- Primerjava bolnikiov s SB tipa 2 z izrazitejšim sistemskim vnetjem (visoko občutljivi CRP ≥ 2 mg/L) in tistih z manj intenzivnim sistemskim vnetjem (visoko občutljivi CRP < 2 mg/L) ni pokazala statistično značilne povezave z UZ markerji in napredovanja ateroskleroze karotid (spremembo DIM, porastom števila segmentov s karotidnimi plaki, spremembo seštevka debeline plakov) pri preiskovancih s SB tipa 2.
- Linearna regresijska analiza je pokazala, da so vrednosti hs-CRP ≥ 2 mg/L statistično značilno povezane s porastom števila segmentov s karotidnimi plaki in spremembo DIM (napredovanje) pri preiskovancih s SB tipa 2.

Zanimalo nas je ali so polimorfizmi testiranih vnetnih genov so povezani z DIM, debelino plakov in s seštevkom plakov (angl. plaque score) na vratnih arterijah ter z bolj nestabilnimi plaki pri preiskovancih s SB tipa 2.

- Ugotovili smo, da sta bila med 17 polimorfizmi dva povezana z DIM, in sicer rs3025058 v genu za MMP-3 in rs8192673 v genu za koaktivator PPARγ.
- Ugotovili smo, da je bilo pet polimorfizmov povezanih s pojavom plakov pri preiskovancih s SB tipa 2, in sicer rs1800587 v genu za IL-1α, rs1143634 v genu za IL-1β, rs1801282 v genu za PPARγ, rs4754 v genu za SPP1 in rs2073618 v genu za osteoprotegerin. Ugotovili smo, da je bilo pet polimorfizmov povezanih z atereosklerotičnim procesom pri preiskovancih s SB 2. Natančneje, rs1800587 in rs1143634 v genu za IL-1α sta bila povezana s seštevkom debeline plakov, rs1143634 v genu za IL-1β je bil povezan s številom segmentov s plaki, rs1801282 v genu za PPARγ je bil povezan s prisotnostjo plakov, rs4754 v genu za SPP1 in prisotnostjo karotidnih plakov in rs2073618 v genu za osteoprotegerin je bil povezan s številom segmentov s plaki in prisotnostjo karotidnih plakov.

Zanimalo nas je ali genetska raznolikost testiranih vnetnih genov vpliva na napredovanje aterosklerotičnega procesa v več kot dveletnem obdobju opazovanja pri bolnikih s SB tipa 2.

- Ugotovili smo, da je bil z napredovanjem aterosklerotičnega procesa v 3,5-letnem obdobju opazovanja pri bolnikih s SB tipa 2 povezan rs1143634 v genu za IL-1β.

Zaključimo lahko, da so vnetje in polimorfizmi izbranih vnetnih genov vpleteni v pojav in napredovanje aterosklerotičnega procesa pri bolnikih s SB tipa 2.

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