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Mgr. Dana Dlouhá

Role genu pro FTO v genetické determinaci „civilizačních“ onemocnění.

The role of the *FTO* gene in the genetic determination of common multifactorial diseases.

Disertační práce

Školitel: Ing. Jaroslav Hubáček CSc. DSc.

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Děkuji své rodině a blízkým, bez jejichž pochopení a podpory by tato práce nemohla vzniknout.

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OBSAH

SEZNAM ZKRATEK

5 mC	5 – methylcytosin
BMI	Body Mass Index
cDNA	complementary DeoxyriboNucleotide Acid
CKD	Chronical Kidney Disease
CRC	ColoRectal Carcinom
CVD	CardioVascular Disease
DM2	Diabetes Mellitus druhého typu
DNA	DeoxyriboNucleotide Acid
ESRD	End Stage Renal Disease
GWA	Genome Wide Association
IM	Infarkt Myokardu
KVO/ KVS	Kardiovaskulární onemocnění/ syndrom
mRNA	mediator RiboNucleotide Acid
NCBI	National Center for Biotechonology Information
OD	Optical Density
OMIM	Online Medelian Inheritance in Man
OR	Odds Ratio
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reactin
RFLP	Restriction Fragment Length Polymorphism
RT PCR	Real Time Polymerase Chain Reaction
rTL	relative Telomere Length
SCG	Single Copy Gene
SNPs	Single Nucleotide Polymorphisms
UCL	University College London

ABSTRAKT

Obezita je rizikovým faktorem rozvoje mnoha civilizačních onemocnění. Mezi tato onemocnění s nejvyšší úmrtností patří kardiovaskulární onemocnění (KVO), diabetes mellitus 2. typu (DM2), některá nádorová onemocnění a onemocnění pohybového aparátu. Jedním z genetických rizikových faktorů determinujících tělesnou hmotnost je gen pro FTO („fat mass and obesity associated protein“).

Úkolem této disertační práce bylo zjistit, 1) zda výskyt rizikových alel u vybraných polymorfismů koreluje s BMI u české populace, 2) analyzovat souvislost mezi variantou v 1. intronu v genu pro FTO a rizikem infarktu myokardu/ akutního koronárního syndromu (IM/ AKS), 3) renálním selháním (ESRD) a 4) výskytem kolorektálního karcinomu (CRC). Polymorfismy rs17817449 (1. intron) a rs17818902 (3. intron) jsme analyzovali metodami PCR-RFLP či RT PCR. Potvrdili jsme souvislost varianty v prvním, ale ne ve třetím intronu s BMI u české kontrolní populace. Intervenční studie ukázaly souvislost se změnami BMI (pouze rs178117449) u dětí, ale ne u obézních žen. U pacientů s AKS jsme nalezli korelaci mezi G alelou a zvýšeným rizikem tohoto onemocnění (OR 1,49). U pacientů s ESRD jsme detekovali souvislost mezi výskytem stejné alely a rozvojem onemocnění (OR 1,37). U pacientů s CRC jsme souvislost s výskytem rizikové alely u varianty v 1. intronu nepotvrdili. Jako kontroly byly použity representativně vybrané soubory české populace „MONICA“ a „HAPPIE“.

V rámci analýz potencionálních mechanismů vlivu polymorfismu rs17817449 jsme detekovali souvislost s relativní délkou telomer (rTL) u pre- a postmenopauzálních žen z Prahy (3PMFs).

Přesný mechanizmus účinku FTO není dosud znám, nicméně FTO vykazuje DNA demetylázovou aktivitu. Sledovali jsme, zda existuje rozdíl mezi celkovým metylačním stavem DNA u jedinců kontrolní zdravé populace s odlišným genotypem pro rs17817449.

ABSTRACT

Obesity is a risk factor for development of cardiovascular disease, diabetes type 2 and some cancers. Newly detected genetic risk factor for body weight is the *FTO* gene ("fat mass and obesity associated").

The aim of this thesis was determine 1) whether the presence of risk alleles correlate with BMI in Czech population and to determine 2) whether there is an association between variants in the *FTO* gene and risk of myocardial infarction/ acute coronary syndrome (MI/ ACS), 3) renal failure (ESRD), or 4) incidence of colorectal cancer (CRC). We analyzed polymorphisms rs17817449 (first intron) and rs17818902 (3rd intron) using by PCR-RFLP and then also RT PCR. We found an association of the first intron variant (but not the 3rd one) and BMI in Czech control population. We have detected an association of 1st intron SNP and BMI changes during the intervention study in obese children, but not in obese females. We found a correlation between the risk allele and increased risk of ACS (OR 1.49) in patients with MI. In patients with ESRD was detected association between the risk allele and the risk of disease (OR 1.37). We didn´t confirmed the association between rs17817449 and the development of CRC. Representative selected groups of the Czech populations “MONICA” and “HAPPIE” were used as controls.

One possibility could be the potential impact of polymorphism on the relative telomere length (RTL). We detected in a study of pre- and postmenopausal women from Prague (3PMFs) that carriers of at least one risk allele have shorter rTL.

The exact mechanism of the FTO effect on disease determination is not yet known, however, the FTO exhibit a DNA demethylase activity. The difference between global DNA methylation status of individuals with different genotypes for rs17817449 in healthy control population were analyzed.

ÚVOD

Díky celogenomovým screeningům (GWAs – „genome wide association study“) bylo možné detekovat nové, dosud neznámé asociace fenotypu s DNA variantami bez informací o kauzalitě. V GWAs studiích jsou zkoumány statisíce až miliony jednonukleotidových polymorfizmů („single nucleotide polymorphisms“ - SNPs), víceméně rovnoměrně rozložených podle celého genomu. GWAs hledá asociace mezi jednou či více variantami a fenotypem. Výsledky se obvykle replikují na řadě populací a na řádově tisících jedincích. Často se stává, že kandidátní varianta je lokalizována v genu/oblasti s neznámou funkcí, nebo v genu, jehož dosud známé funkce nejsou spojeny s patofyzioologií sledovaného onemocnění [Hainer a kol. 2008].

GWAs studie odhalily potenciální vliv celé řady nových genů na determinaci BMI. Příkladem mohou být geny pro INSIG2 (insulin-induced gene 2), MC4R (melanocortin-4 receptor gene) nebo dosud nejsilnější známý genetický determinat hodnot BMI, gen pro FTO („fat mass and obesity associated gene“) [Marinou a kol. 2010, Bogardus a kol. 2009].

Vznik nadváhy je ovlivněn celou řadou vnějších faktorů (především nadměrným energetickým příjmem a nedostatkem fyzické aktivity). Index tělesné hmotnosti (body mass index - BMI = hmotnost (kg)/ výška (m^2), hodnoty BMI 25 - 29,9 značí nadváhu, 30 – 34,9 obezitu I. stupně, 35 – 39,9 obezitu II. stupně a hodnoty > 40 obezitu III. stupně) je ovlivněn také genetickými faktory. Odhady o míře dědičnosti obezity se dle typu studie pohybují od 30-ti do 70-ti %. *FTO* varianty v prvním (a v některých populacích i ve třetím) intronu tohoto genu jsou spojeny s hodnotami BMI a přítomnost jedné rizikové alely je spojena s nárůstem tělesné hmotnosti o přibližně 1,5 - 2 kg [Loos a kol. 2008, Dina a kol. 2007, Frayling a kol. 2007, Scuteri a kol. 2007]. Studie sledující možnou kauzalitu (vliv

jednotlivých variant na energetický příjem, bazální metabolizmus, fyzickou aktivitu) nepřinesly konzistentní výsledky.

Gen pro FTO

Experimentální studie

FTO (dříve známý pod názvem Fatso) byl poprvé popsán [Peters a kol. 1999] u myší jako jeden ze šesti genů uvnitř rozsáhlé delece na chromozómu 8, odpovědných za *Ft* (fused toes – „srůstání prstů“) fenotyp. Homozygotní myši s touto delecí umíraly během prenatálního vývoje, vykazovaly vážné malformace hlavy, vývojové defekty centrálního nervového systému, pravo-levou asymetrii, polydaktylii a růstovou retardaci. Heterozygotní myši vykazovaly syndaktylii předních končetin a zvětšení brzlíku. Změny hmotnosti u těchto myší však pozorovány nebyly [Boissel a kol. 2009] a gen na řadu let upadl v zapomnění.

Studie na hlodavcích prokázaly nejvyšší hladiny *Fto* mRNA v mozku a hypotalamu. Hypotalamus hraje klíčovou roli při regulaci energetické rovnováhy a genů odpovědných za funkci monogenní obezity. Exprese *Fto* je regulována stavem výživy (hladovění nebo přejídání) a vykazuje tkáňově specifické odlišnosti. Není však známo, zda jsou tyto rozdíly příčinou nebo důsledkem obezity [Peters a kol. 1999, Loos a kol. 2008, Dina a kol. 2007, Frayling a kol. 2007]. U myší byly popsány dva modely *Fto* nedostatečností: $Fto^{-/-}$ s nulovou mutací, s celkovou absencí exprese Fto proteinu, a druhý model s částečnou ztrátou exprese Fto proteinu, způsobenou bodovou mutací v sekvenci aminokyselin isoleucin → fenylalanin na pozici 367 (I365F). Tato substituce pravděpodobně negativně ovlivňuje funkci Fto tvorbou heterodimerů, což způsobuje změny v energetické rovnováze. U $Fto^{-/-}$ je tělesná hmotnost regulována již od počátku života, u Fto^{I367F} až v dospělosti. $Fto^{-/-}$ myši vykazovaly 30-40% redukci tělesné hmotnosti v porovnání s kontrolami, kdežto Fto^{I367F} vykazovaly pouze 10% pokles. U $Fto^{-/-}$ myší se

vyskytovaly růstové retardace a časná perinatální úmrtnost. Oba modely vykazovaly na vysokotukové dietě snížení tělesné hmotnosti a bílé tukové tkáně v porovnání s kontrolami. Existuje tedy předpoklad, že ztráta funkce *Fto* může působit proti vzniku obezity indukované stravou. V porovnání s kontrolami neexistoval rozdíl v absolutním množství příjmu potravy, přesto byly $Fto^{-/-}$ myši menší a lehčí, pokles tukové hmoty u *Fto* deficitních myší tedy není zprostředkován nižším energetickým příjmem. Pro oba modely je charakteristický také vyšší bazální metabolizmus. Vyšší energetický výdej nesouvisel s fyzickou aktivitou, ale byl ovlivněn zvýšenou aktivitou sympatického nervového systému, který může podporovat lipolýzu a termogenezi tukové tkáně a svalů [Fischer a kol. 2009, Church a kol. 2009, Fawcett a kol. 2010].

Charakteristika lidského genu pro FTO

FTO („fat mass and obesity associated“) obsahuje 9 exonů o celkovém rozpětí více než 400 kb, je lokalizován na lidském chromozómu 16 v pozici 16q12.2. Protein kódovaný tímto genem se skládá z 505 aminokyselin (NCBI Reference Sequence: NP_001073901.1, OMIM ID 610966). Většina funkčně zajímavých polymorfizmů (cluster asi 40ti SNPs) je lokalizovaná na prvním intronu genu a to v oblasti s mezidruhově silně konzervovanou sekvencí [Loos a kol. 2008]. *FTO* je exprimován ve všech lidských embryonálních a dospělých tkáních, nejvyšší hladina exprese byla zjištěna v mozku, hypotalamu a játrech [Boissel a kol. 2009].

Funkce FTO *in vivo* je v detailu neznámá, nicméně bylo zjištěno že FTO sdílí sekvenční motivy s Fe^{2+} a 2 - oxoglutarát - dependentními oxygenázami [Fawcett a kol. 2010]. Rekombinantní protein FTO katalyzuje demetylaci 3-methylthyminu ssDNA a s nižší účinností 3-methyluridinu v ssRNA *in vitro* [Gerken a kol. 2007]. Hlavním substrátem FTO je nukleosid N6-methyladenosine (m6A), který se hojně nachází v mRNA

některých virů a většiny eukaryot včetně savců. Dále bylo zjištěno, že FTO může působit i jako transkripční koaktivátor [Wu a kol. 2010].

Tyto FTO aktivity mohou regulovat expresi genů zapojených do energetického hospodaření, což může vést ke vzniku obezity.

Polymorfizmy v genu pro FTO a jejich role v determinaci BMI

Intron 1

V roce 2007 se objevily, prakticky současně, tři práce [Dina a kol. 2007, Frayling a kol. 2007, Scuteri a kol. 2007], popisující asociaci variant v genu pro FTO a BMI v několika nezávislých bělošských populacích. Primárně byly sledovány tři varianty rs9939609 (T/A), rs1421085 (C/T) a rs17817449 (G/T). Alely A (rs9939609), C (rs1421085) a G (rs17817449) byly silně spjaty s fenotypem obezity. Jak se později ukázalo [Hubacek a kol. 2009], tyto varianty jsou v silné vazebné nerovnováze.

Na evropských populacích bylo dosud provedeno několik desítek studií s různým designem a různým počtem sledovaných jedinců, nicméně s velice konzistentními výsledky. Nejdůležitější studie jsou shrnuty v níže uvedené tabulce. Relativně malá pozornost byla dosud věnována úloze *FTO* u různě definovaných podskupin. Existují studie, které naznačují, že by se vliv *FTO* mohl lišit mezi pohlavími, v několika studiích nebyl potvrzen vztah *FTO* variant k BMI u žen – [Jacobsson a kol. 2008, Song a kol. 2008], či by mohl do určité míry záviset na menopauzálním statusu žen [Hubacek a kol. 2009].

U bělošských populací má alespoň jednu rizikovou alelu přibližně 60 % populace, okolo 20 % je nositelem dvou těchto alel. Vztah *FTO* k tělesné hmotnosti není z klinického hlediska nijak ohromující, ale u bělošských populací byl nalezen velice konzistentní vztah – přítomnost každé rizikové alely je spojena s přibližně 1,5 - 2,0 kg tělesné hmotnosti navíc [Loos a kol. 2008, Dina a kol. 2007, Frayling a kol. 2007, Scuteri a kol. 2007].

Nedlouho po prvních studiích na bělošských populacích byly publikovány výsledky získané na dalších etnických skupinách. Je nezbytné zmínit, že *FTO* vykazuje významné mezi-etnické rozdíly jak ve frekvencích alel, tak v míře nalezené vazebné nerovnováhy. [Bollepalli a kol. 2010, Lopez – Bermejo a kol. 2008, Liu G a kol. 2010, Rendo a kol. 2009, Demerath a kol. 2011, Liu Y a kol. 2010, Cheung a kol. 2010, Adeyemo a kol. 2010].

Vybrané studie sledující vztah variant v prvním intronu genu pro FTO k hodnotám BMI/ parametrům obesity u bělošských populací.

Název studie/populace	**Počet jedinců**	**Varianta**	***FTO* efekt**	**Ref.**
Primární GWA studie/ Evropané	38.759	rs9939609	Spojitost s rozvojem obezity u dospělých i u dětí	[Frayling 2007]
Primární GWA studie/ Evropané	cca 9.000	rs9930506	Souvislost s BMI v řadě populací	[Scuteri 2007]
Primární GWA studie/ Evropané	2.900 obézních, 5.100 kontrol		Spojitost s rozvojem obezity u dospělých a dětí	[Dina 2007]
Studie HAPIEE/ česká populace	3.079 mužů 3.602 žen	rs17817449	Signifikantní vliv na BMI u mužů i u žen	[Hubacek 2011]
/Američané evropského původu	5.607	rs9939609	Souvislost s BMI	[Hunt 2008]
/Švédové	450 obézních, 512 kontrol	rs9939609	Souvislost s obezitou u dívek, ale ne u chlapců	[Jacobsson 2008]
/Australané	3.353 dospělých mužů/dvojčat	rs9939609	Souvislost s BMI	[Cornes 2009]
/Lužičtí Srbové	948 dospělých	rs8050136	Souvislost s BMI	[Tőnjes 2010]
MONICA/ Francie	3.367 dospělých	rs9939609	Souvislost s BMI a s obvodem pasu a boků	[Legry 2009]
	561dětí	rs9939609	Spojitost s rozvojem obezity u dětí	[Bollepalli 2010]
/Američané evropského původu	658 jedinců	rs9939609, rs17820875 rs860713	Vztah k BMI nedetekován v dětství, patrný až v dospělosti	[Mei 2010]
Studie HELENA/ různé evropské populace	752 adolescentů	rs9939609	Souvislost s BMI a se zvýšeným obsahem tuku v těle	[Ruiz 2010]
MONICA+KORA/ německá populace	12.462 dospělých	rs9935401	Souvislost s BMI	[Holzapfel 2010]
Dutch children cohort/ Nizozemsko	101 dětí	rs9939609	Konzistentní vztah *FTO* k BMI krátkodobě narušen během pohlavního dospívání	[Rutters 2011]
The Whitehall II study/ Velká Británie	2.981 mužů a 1.164 žen	rs1421085	Vztah k BMI a obezitě potvrzen během téměř dvacetiletého sledování u mužů, nikoli u žen	[Kivimäki 2011]
The Generation R Study/ Nizozemsko	695 a 216 dětí	rs9939609	Žádný vztah *FTO* k BMI či k tělesnému složení u šestiměsíčních dětí	[Mook–Kanamori 2011]

[Dlouha a kol. 2012]

Intron 3

GWA analýza izolované populace Lužických Srbů detekovala varianty v oblasti intronu 3 jako další a na intronu 1 nezávislé prediktory hodnot BMI. Kombinace alel C a G u SNPs rs10521308 a rs17818902, spojená s nižším průměrným BMI, se vyskytuje přibližně u 18 % populace [Tőnjes a kol. 2010].

Mutace

FTO byl kompletně sekvenován u 1 433 extrémně obézních a u stejného počtu štíhlých Evropanů [Meyre a kol. 2010]. Přestože byly objeveny mutace deaktivující *FTO*, vyskytovaly se jak u obézních, tak u štíhlých jedinců, a je tak jasné, že nemají přímou souvislost se vznikem obezity jako takové. Jedna z mutací, inaktivujících *FTO*, byla zmíněna ve spojitosti s autozomálně recesivní determinací úmrtnosti [Boisel a kol. 2009].

Asociační studie a vybraná onemocnění

Diabetes mellitus 2. typu (DM2).

Primárně byl gen pro FTO detekován pomocí GWAs studie zaměřené na analýzu genetických dispozic k DM2 [Frayling a kol. 2007]. Cluster SNPs na intronu 1 *FTO* vykazoval významnou souvislost s DM2, avšak tento vztah zmizel po adjustaci na BMI. Později se ale objevilo několik studií potvrzujících vztah mezi *FTO* rs9939609 polymorfizmem a rizikem vzniku DM2. Toto riziko bylo vyšší přibližně o 48 % ve francouzské studii MONICA [Legry a kol. 2009], o 50 % u nositelů alely A ve finské studii [Scott a kol. 2007], a přibližně o 25 % ve studii „The Wellcome Trust Case-Control Consortium“ [Burton a kol. 2007]. Souvislost mezi *FTO* a DM2 je částečně, ale ne zcela, vysvětlena vyššími hodnotami BMI.

Vztah *FTO* k DM2 není omezen pouze na bělošské západoevropské etnikum, ale byl prokázán i v rozsáhlé meta-analýze pákistánské populace [Rees a kol. 2011].

Také meta-analýza u skandinávské populace potvrdila souvislost *FTO* s DM2 [Hertel a ko. 2011]. I v japonské populaci byla prokázána významná souvislost *FTO* s diabetem po adjustaci k BMI [Takeuchi a kol. 2011]. V další studii (vzorky vybrány ze studie CURES), provedené na populaci jižní Indie, bylo analyzováno celkem 6 polymorfizmů *FTO* lokalizovaných na třech intronech, přičemž většina z nich souvisela s DM2 [Ramya a kol. 2011].

Doney a kol. 2009 ve své studii zjistili, že větší množství tukové hmoty u nositelů rizikové alely A (rs9939606) nesouvisí pouze s rizikem rozvoje DM2, ale také s vyšším aterogenním lipidovým profilem a rizikem vzniku infarktu myokardu (IM).

FTO a kardiovaskulární onemocnění (KVO).

Další studie poukázaly na možný vztah mezi variantami v intronu 1 *FTO* a KVO.

Lappalainen a kol. [2011] zjistili, že polymorfizmus rs9969609 *FTO* může přispívat k rozvoji KVO u mužů s abnormálním metabolizmem glukózy. Americká studie zaměřená na frekvenci výskytu KVO u zdravých žen bělošského původu zjistila vyšší riziko KVO u žen s rizikovou alelou a současně s nižší pohybovou aktivitou [Ahmad a kol. 2010].

Nádorová onemocnění.

Obezita je známým rizikovým faktorem rozvoje nádorových onemocnění, a proto se přímo nabízí otázka, zda varianty *FTO* spojené s hodnotami BMI ovlivní i výskyt tohoto onemocnění. První publikovaná studie (rs9939609) nalezla zvýšené riziko vzniku nádorových onemocnění v ledvinách především v mladším věku (do 50 let), a zároveň

poněkud překvapivě spojitost mezi přítomností alely zvyšující BMI a nižším rizikem plicních nádorových onemocnění [Brennan a kol. 2009].

Dvě nezávislé studie sledovaly potenciální vliv *FTO* na karcinom endometria. První z nich [Gaudet a kol. 2010] sledovala ženy evropského a australského původu a nepotvrdila souvislost mezi polymorfizmy a rizikem vzniku nádorového onemocnění. Další studie [Lurie a kol. 2011] zjistila zvýšené riziko vzniku rakoviny endometria u nositelek genotypu asociovaného s BMI. I když vztah byl po adjustaci na BMI nesignifikantní, výsledek naznačil, že onemocnění může být zprostředkováno genem způsobujícím nadváhu.

Lewis a kol. [2010] popsal souvislost mezi *FTO* rs9939609 a rizikem vzniku nádorových onemocnění prostaty. Přítomnost rizikové alely A nesouvisela s rizikem vzniku onemocnění, ale jasně souvisela s vyšším stupněm karcinomu prostaty.

Nock a kol. [2011] ve své studii sledovali celkem 5 nejznámějších SNPs *FTO*. U bělošské populace nenalezli souvislost mezi polymorfizmy a rizikem vzniku adenomů, ale u populace afrického původu, byla přítomnost jedné nebo dvou kopií variantních alel u SNPs rs17817449 a rs8050136 spojena se zvýšeným rizikem výskytu kolorektálního adenomu.

Další onemocnění

Některé studie se zabývaly spojitostí mezi variantami v genu pro FTO a syndromem polycystických ovarií (PCOS) [Asunción a kol. 2000, Legro a kol. 2000, Barber a kol. 2008, Attaoua a kol. 2008]. Souvislost mezi hladinou exprese *FTO* v placentě, porodní hmotností a délkou dětí popsal ve své studii Bassols a kol. [2010].

Rozsáhlá, dlouhodobá dánská studie prokázala u mužů signifikantní vztah mezi *FTO* a úmrtností. Vliv genu na celkovou úmrtnost byl podobný jako vliv kouření

[Zimmermann a kol. 2009]. Švédská studie (rs9939609) oproti tomu neprokázala souvislost mezi genotypy *FTO* a celkovou úmrtností u žen, ani u mužů. *FTO* genotyp může ovlivňovat souvislost mezi fyzickou aktivitou a kardiovaskulární mortalitou [Sonestedt a kol. 2011].

SNP na 2. intronu *FTO* byl před několika lety detekován i jako rizikový faktor rozvoje závislosti na tabáku u žen, ale ne u mužů [Bierut a kol. 2007]. Studie sledující souvislost mezi polymorfizmem na 1. intronu a závislostí na alkoholu u polské populace naznačuje protektivní charakter jinak rizikových alel [Sobczyk – Kopciol a kol. 2011].

Nedávné studie prokázaly, že u jedinců s rizikovou alelou běžných *FTO* polymorfismů může ve stáří docházet, jak k redukci objemu frontálního laloku mozku [Ho a kol. 2010], tak k poklesu schopnosti slovní plynulosti [Benedict a kol. 2011]. Švédská studie zjistila, že nositelé alely A u rs9939609 mají zvýšené riziko rozvinutí Alzheimerovy choroby [Keller a kol. 2011].

ANALYZOVANÉ SOUBORY

MONICA – *Multinational monitoring of trends and determinants in cardivascular diseases*, 2559 nepříbuzných jedinců bělošské populace (1 191 mužů, 1 368 žen ve věku 25 – 65 let). Během let 1997-1998 byli zváni jedinci z 9 okresů České republiky a znovu v letech 2000 - 2001 dle WHO protokolu. Tento vzorek představuje representativní vzorek 1 % české populace [Cífková a kol. 2010, Tunstall-Pedoe a kol. 2003].

Czech HAPIEE – *Health, alcohol and psychosocial factors in Eastern Europe*, 6 827 náhodně vybraných jedinců z registru populace ze 7 českých měst (45 % mužů, 45 - 69 let), vzorky sbírány v letech 2002 - 2005. Kontrolní soubor české populace [Peasey a kol. 2006].

107 žen bez anamnézy diabetes mellitus (BMI > 27,5 kg/ m^2, věk 49,2 ± 12,3 let).

357 obézních nepříbuzných dětí (věk 13,7 ± 4,9 let, BMI 30,8 ± 4,6 kg/ m^2).

1 092 mužů s akutním koronárním onemocněním, vzorky byly kompletovány v letech 2006 - 2009 v rámci studie **GENDEMIP** (*GENetic DEtemination of Myocardial Infarction in Prague)* z celkem pěti spolupracujících koronárních jednotek na území Prahy [Pitha a kol. 2007].

1 014 hemodialyzovaných pacientů (56 % muži, věk 66,9 ± 12,7, hypertenze 63 %, diabetes 39 %, BMI 26,2 ± 5,0 kg/ m^2) z celkem 27 center v ČR.

1 251 pacientů (95 % muži, věk 47,2 ± 13,0), kteří podstoupili transplantaci ledvin, z důvodu CKD nebo renálního selhání v Institutu klinické a experimentální medicíny v letech 1999 - 2007 (prevalence léčených hypertoniků 82 %, diabetiků 35 %, hodnota BMI = 25,5 kg/ m^2).

1 005 dospělých pacientů s kolorektálním carinomem - 583 mužů, 422 žen, věk 61,2 ± 11,0 let [Jiraskova a kol. 2012].

908 žen, účastnících se **3PMFs** (*The Prague Pre and Post Menopausal Females study)* - reprezentativní a náhodně vybraný vzorek (5 %) z 29 440 žen ve věku 45 - 54 let, z registru zdravotních pojišťoven, žijících na území Prahy. Ze vzorku 1 472 žen, 908 dalo souhlas s vyšetřením ke studii. [Cifkova a kol. 2008, Pitha a kol. 2008 a 2013].

201 pacientů s alkoholickou cirhózou jater.

25 vzorků po jaterní resekci.

11 dobrovolníků z řad zaměstnanců CEM.

POUŽITÉ METODY

Izolace DNA

Genomová DNA byla izolována z plné krve mírně modifikovanou metodou dle protokolu Miller a kol. 1988.

FTO genotypizace

FTO polymorfismy byl stanoveny pomocí PCR, varianta rs2302673 pomocí nested PCR, a následnou restrikční analýzou. Fragmenty PCR produktů byly separovány na 10 % polyakrylamidovém gelu a použitím elektroforézy [Hubacek a kol. 2008, 2009].

Sekvence primerů použitých při *FTO* genotypizaci (Fermentas International, Inc. Burlington, Ontario, Canada):

SNP	Forward/ reverse primer	Restrikční enzym
rs17817449	5´ GGT GAA GAG GAG GAG ATT GTG TAA CTG G/ 5´GAA GCC CTG AGA AGT TTA GAG TAA ATT GGG	AlwN I
rs17818902	5´ ATC ATT CTG AAA ACA GAT CTG ACT GG/ 5´ ATG GGT TTA TGA ACC ATA GGA AAG AAT CGA G	Bsa I
rs2302673	5´ ATG TAC TCA TGC CAA CAG GCT ACT TG/ 5´ TTC CAA GTG TCT GAC TTA TGA TGT G Nested 5´ TAG AAA ACT GCT GTT GAG AGC AGC/ 5´ TTA AAT AGA CTT TAA ACA TCG A	Cla I

Exprese *FTO* v jaterní tkáni

Studované vzorky

Pro vlastní izolaci RNA bylo odebráno maximálně 100 mg tkáně, která byla bezprostředně vložena do 1 ml RNA lateru (Qiagen, Germany). Další vzorek tkáně (100 mg) byl použit pro izolaci genomové DNA.

Laboratorní analýza

Celková RNA byla izolována pomocí RNeasy Mini Kit (Qiagen, Germany). RNA byla purifikována pomocí RNase-Free DNase Set (Qiagen, Germany). Integrita všech RNA vzorků byla zkontrolována pomocí elektroforézy (Bio Rad Power Pac 300) na 1 %

agarosovem gelu. Koncentrace a čistota vzorků byla změřena pomocí Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Jednotné množství RNA bylo reversní transkripcí přepsáno do cDNA (Thermo Scientific Verso^{TM} cDNA Synthesis Kit).

Pomocí relativní kvantifikace a $2^{-\Delta\Delta Ct}$ metody byly stanoveny hodnoty mRNA *FTO* měřením na přístroji Rotor Gene (Corbett Research 3000) kvantitativní Real Time PCR detekční systém. Jako endogenní kontrola byl použit housekeeping gen *GAPDH*. Hladiny mRNAs byly kvantifikovány pomocí primerů a duálně značených prob navržených firmou Sigma Aldrich.

Forward/ reverse FTO primer	FTO proba
5′ CAA GGC AAA GAT CTG CTC/ 5′ GTG TGT TTT ATA TTA GAC CCT TTC	5′ [6FAM] CGG TAT CTC GCA TCC TCA TTG G [BHQ1]

Mix pro kvantitativní PCR (25 µl) obsahoval 2 µl cDNA (50 ng), 12,5 µl JumpStart^{TM}*Taq* Ready Mix for Quantitative PCR (Sigma-Aldrich), 4 µl $MgCl_2$ (6 mM), 1 µl probe (250 nM), 1 µl forward primer, 1 µl reverse primer (oba 900 nM), 3,5 µl H_2O. Vzorky byly měřeny v triplikátech [Livak a kol. 2001, Pfaffl a kol. 2001].

Teplotní profil qPCR:

Krok	Teplota	Čas	Cykly
Denaturace	94 °C	2 min	HOLD
Amplifikace	94 °C	15 sec	40 cyklů
	55 °C	60 sec	

Analýza délky telomer

Analýza určení relativní délky telomer (rTL) byla převzata a zavedena s nepatrnými úpravami od kolegů z UCL. Relativní délka telomer byla stanovena pomocí kvantitativní polymerázové reakce (qPCR) dle Cawtona a kol. [2002]. Hodnota rTL je určena jako poměr repetic telomerových sekvencí k počtu kopií single - copy genu (SCG), T/S ratio. Jako SCG slouží při analýze kyselý ribosomální fosfoprotein PO (36B4). Každý vzorek

jsme měřili v triplikátu na přístroji Rotor-Gene 3000 (Corbett Research Ltd). Množství telomerových repetic a SCG kopií jednotlivých vzorků jsme stanovili komparativní kvantifikační analýzou (Rotor-Gene 3000 software, Corbett Research Ltd). Pro všechna měření byl použit identický referenční vzorek DNA. Metoda umožňuje vypočítat relativní množství telomerových repetic a kopií SCG genu na základě hodnoty Take - Off bodu a amplifikace každého vzorku v porovnání s referenčním vzorkem. Pomocí druhé derivace amplifikační křivky je určen vrchol exponenciální amplifikace a Take - Off bod reakce. Take - Off je první bod, který se nachází 80 % pod vrcholem amplifikační křivky.

Sekvence primerů použitých pro stanovení rTL (Fermentas International, Inc. Burlington, Ontario, Canada)

Telomere (135 nM) forward/ (900 nM) reverse	5′ GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T/ 5′ TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA
SCG (300 nM) forward/ (500 nM) reverse	5′ CAG CAA GTG GGA AGG TGT AAT CC/ 5′ CCC ATT CTA TCA TCA ACG GGT ACA A

V každém experimentu jsme měřili 22 vzorků, 1 referenční vzorek a blank. Reakční objem byl 25 µl (12,5 ul SensiMix SYBR No-ROX 2× (Bioline Ltd, UK), 1 ul Fwd/ Rev primery, 30 ng templátu (gDNA) a H_2O. Specifita všech amplifikací byla determinována analýzou melting curve [Cawton 2002, Salpea a kol. 2008].

Teplotní profil qPCR pro stanovení telomerových repetic:

Krok	**Teplota**	**Čas**	**Cykly**
Denaturace	95 °C	10 min	HOLD
Amplifikace	95 °C	15 sec	22 cyklů
	58 °C	120 sec	

Teplotní profil qPCR pro stanovení SCG kopií:

Krok	Teplota	Čas	Cykly
Denaturace	95 °C	10 min	HOLD
Amplifikace	95 °C	15 sec	35 cyklů
	58 °C	60 sec	

Reprodukovatelnost měření rTL byla ověřena intra - a inter - assay testy. Průměrné hodnoty variačního koeficientu intra assaye se pohybovaly mezi 1,9 - 6,9 % a inter assaye mezi 3,4 - 14,8 %.

Detailní popis metod a statistických analýz viz přílohy.

Stanovení celkové DNA metylace

Studované vzorky

Metylaci DNA jsme analyzovali u vzorků vybraných z kontrolního souboru MONICA. První skupinu vzorků tvořilo 21 dvojic kuřáků bez anamnézy diabetes mellitus, se shodným BMI a věkem, které se vzájemně lišily genotypem. Druhou skupinu tvořilo 23 identicky vybraných dvojic - nekuřáků. Další analyzovanou skupinou bylo 13 vzorků gDNA izolované z jaterní tkáně, u kterých jsme měřili expresi *FTO*. Poslední analyzovanou skupinu tvořilo 11 dobrovolníků z pracoviště CEM.

Analýza celkové metylace DNA

Ke stanovení metylace jsme použili MethylFlash^{TM} Methylated DNA Quantification Kit Colorimetric (Epigentek Group Inc., Farmingdale, NY, USA.). DNA se váže s vysokou afinitou k povrchu specificky ošetřených jamek stripů. Metylované úseky DNA jsou detekovány pomocí protilátek a následně kolorimetricky kvantifikovány při 450 nM. Množství metylované DNA (5 - mC) je úměrné naměřené intenzitě OD vzorku. Jako kontrola správnosti měření je v experimentu používána negativní kontrola ME3 (kompletně nemetylovaný polynukleotid obsahující 50 % cytosinu) a pozitivní kontrola

ME4 (metylovaný polynukleotid obsahující 50 % 5 - metylcytosinu). K výpočtu množství 5 - mC je se používá metoda relativní kvantifikace nebo absolutní kvantifikace.

Pozitivní kontrola ME4 slouží k přípravě standardní křivky pro absolutní kvantifikaci.

Zkumavka č.	ME4 (10ng/ µl)	1 x TE pufr (µl)	ME4 (ng/ µl)
1	1.0	19.0	0.5
2	1.0	9.0	1.0
3	1.0	4.0	2.0
4	2.5	2.5	5.0
5	4.0	0.0	10.0

Vstupní množství DNA se dle protokolu může pohybovat v rozmezí 50 ng - 200 ng na reakci. Každý vzorek byl měřen v duplikátu. Množství OD jsme měřili na Spektrofotometru Biotek Synergy 2 (BioTek U.S. - World Headquarters,Winooski, United States)

Množství a počet procent 5 - mC v celkové DNA jsme stanovovali pomocí absolutní kvantifikace dle následujících vzorců.

$$5\text{ - }mC\ (ng) = Sample\ OD\ ME3\ OD / Slope\ x\ 2^{*}$$

$$5\text{ - }mC\% = 5\text{ - }mC\ (ng) / S\ x100\%$$

Slope (OD/ ng) je determinován pomocí funkce lineární regrese

***** 2 - faktor, který normalizuje množství 5 - mC v pozitivní kontrole k 100%

S - množství DNA použité v experimentu (ng)

VÝSLEDKY

Vlastní výsledky práce *in extenso* jsou obsaženy v přiložených pracích.

Příloha 1:

DLOUHA D, ADAMKOVA V, LANSKA V, HUBACEK JA. Lack of association between a new tag SNP in the FTO gene and BMI in Czech-Slavonic population. Eur J Hum Genet. 2010;18(12):1274. IF = 4.38

Příloha 2:

DLOUHÁ D, SUCHÁNEK P, LÁNSKÁ V, HUBÁČEK JA. Body mass index change in females after short-time life style intervention is not dependent on the FTO polymorphisms. Physiol Res. 2011;60(1):199-202. IF = 1.56

Příloha 3:

HUBACEK JA, STANEK V, GEBAUEROVÁ M, PILIPCINCOVÁ A, **DLOUHÁ D**, POLEDNE R, ASCHERMANN M, SKALICKÁ H, MATOUSKOVÁ J, KRUGER A, PENICKA M, HRABÁKOVÁ H, VESELKA J, HÁJEK P, LÁNSKÁ V, ADÁMKOVÁ V, PITHA J. A FTO variant and risk of acute coronary syndrome. Clin Chim Acta. 2010;411(15-16):1069-72. IF = 2.67

Příloha 4:

HUBACEK JA, VIKLICKY O, **DLOUHA D**, BLOUDICKOVA S, KUBINOVA R, PEASEY A, PIKHART H, ADAMKOVA V, BRABCOVA I, POKORNA E, BOBAK M. The FTO gene polymorphism is associated with end-stage renal disease: two large independent case-control studies in a general population. Nephrol Dial Transplant. 2012;27(3):1030-5. IF = 3.37

Příloha 5:

HUBACEK JA, **DLOUHA D**, BOBAK M, JIRASKOVA A, VITEK L. The risk of sporadic colorectal cancer development is not influenced by fat mass and obesity related gene polymorphism in Slavs. Eur J Intern Med. 2012;23(7):e175-6. IF = 2.94

Příloha 6:

DLOUHA D, PITHA J, LANSKA V, HUBACEK JA. Association between FTO 1st intron tagging variant and telomere length in middle aged females. 3PMFs study. Clin Chim Acta. 2012 16;413(15-16):1222-5. IF = 2.54

Příloha 7:

ZLATOHLAVEK L, VRABLIK M, MOTYKOVA E, CESKA R, VASICKOVA L, **DLOUHA D**, HUBACEK JA. FTO and MC4R gene variants determine BMI changes in children after intensive lifestyle intervention. Clin Biochem. 2013;46(4-5):313-6. IF = 2.45

Příloha 8:

HUBACEK JA, ADAMKOVA V, **DLOUHA D**, JIRSA M, ŠPERL J, TÖNJES A, KOVACS P, PIKHART H, PEASEY A, BOBAK M. Fat mass and obesity-associated (FTO) gene and alcohol intake. Addiction. 2012 Jun;107(6):1185-6. IF = 4.75

Příloha 9:

HUBACEK JA, **DLOUHA D**, LANSKA V, ADAMKOVA V. Lack of an association between three tagging SNPs within the FTO gene and smoking behavior. Nicotine Tob Res. 2012 Aug;14(8):998-1002. IF = 2.45

Příloha 10:

HUBACEK JA, **DLOUHA D**, LANSKA V, STAVEK P, PAGACOVA L, KRALOVA-LESNA I, PITHA J. FTO first intron rs1558902 variant and platelets count in white middle-aged women: prague pre- and post-menopausal females (3PMFs) study. J Investig Med. 2013 Feb;61(2):291-3. IF = 1.75

Prohlášení prvního autora:

Prohlašuji, že Mgr. Dana Dlouhá se jako spoluautorka prací významně podílela na designu studií, vlastních genetických analýzách, interpretaci výsledků a na spolupráci při tvorbě textu publikací.

Ing Jaroslav Hubáček CSc., DSc.

KOMENTÁŘ K VÝSLEDKŮM A DISKUZE

I. Prvním cílem předkládané disertační práce bylo zjistit zda *FTO* varianty v 1. a 3. intronu *FTO* genu souvisí s hodnotami BMI u vzorku české populace „MONICA“ a zda korelují se změnami BMI během intervenčních studií u žen a u dětí *(přílohy 1, 2 a 7)*.

V předkládané práci jsme se pokusili replikovat nález Tőnjes a kol., kteří pomocí GWA studie u izolované populace Lužický Srbů nalezl nezávislý *FTO* signál v oblasti intronů 2/3 ve vzdálenosti 40 - 60 kb od primárně potvrzených variant rs9939609, rs1421085 a rs17817449. Nejsilnější vliv na BMI byl zjištěn u varianty rs17818902. Analyzovali jsme skupinu 2 559 nepříbuzných jedinců z projektu „MONICA“ Navíc jsme analýze podrobili 556 studentů VŠ z Českých Budějovic a okolí.

Ačkoliv frekvence jednotlivých genotypů byly v Hardy-Weinbergově rovnováze (P = 0,49) a nelišily se mezi českou a srbskou populací, nepotvrdili jsme souvislost mezi variantou rs17818902 a hodnotami BMI. Odlišné výsledky obou studií nedokážeme dost dobře vysvětlit, jelikož varianty na 1. intronu souvisely s BMI jak u srbské populace [Tőnjes a kol. 2010], tak u české populace [Hubacek a kol. 2008 a 2009]. Je možné, že SNP na 3. intronu představuje novou kausální variantu haplotypu existujícího u srbské populace nebo je vliv varianty rs17818902 na BMI výsledkem interakce s vlivy prostředí nebo s genetickými variantami mimo oblast genu *FTO* specifickými pro isolovanou srbskou populaci. Naše výsledky podporují důležitost detailních analýz interakcí gen-gen nebo gen-prostředí, které by mohly objasnit vliv této varianty na patogenezi obezity různých populací.

V druhé předkládané práci jsme se zaměřili na sledování *FTO* polymorfismu rs17817449 (1. intron) a rs17818902 (3. intron) během krátkodobé intervenční studie u žen bez anamnézy diabetes mellitus. Sledovali jsme, zda varianty genu souvisí se změnou

BMI. Během 10ti týdnů ženy dodržovaly individuálně upravený energetický příjem a cvičební program, který zahrnoval 4krát týdně 60 min aerobního cvičení. Průměrná hodnota BMI před intervencí činila 32,8 ± 4,2 kg/ m^2. Průměrný úbytek váhy byl 4,8 ± 3,5 kg (5,3 ± 3,5 %, maximum - 15,5 kg a minimum + 2,0 kg, $P < 0,01$). Souvislost mezi sledovanými variantami a poklesem BMI jsme však nenalezli.

Souvislost výskytu polymorfismů genu *FTO* (rs17817449) a *MC4R* (rs17782313) s poklesem tělesné hmotnosti jsme sledovali u obézních nepříbuzných dětí během 4týdenní intervenční studie. Sledovaní jedinci měli optimálně dle věku redukovaný energetický příjem a každodenní cvičební program. Průměrný úbytek váhy činil 6,2 ± 2,1 kg ($P < 0,001$). Nalezli jsme statisticky významnou souvislost mezi poklesem BMI dětí a sledovanými polymorfismy obou genů. Nositelé *FTO* varianty GG a/ nebo *MC4R* CC varianty vykazovaly vyšší úbytek váhy než ostatní jedinci ($P < 0,0009$ pro BMI, $P < 0,002$ pro tělesnou hmotnost). Tyto rozdíly zůstaly významné i po adjustaci k pohlaví, věku a vstupním hodnotám ($P = 0,004$ pro BMI a $P = 0,01$ pro tělesnou hmotnost). Naše studie ukázala, že varianty *FTO* a *MC4R* genů ovlivňují výsledný pokles BMI u obézních dětí během intenzivní intervenční studie.

II. Druhým cílem předkládané práce bylo zjistit, zda existuje souvislost mezi *FTO* variantou rs17817449 a rizikem rozvoje vybraných civilizačních chorob v české populaci (*přílohy 3,4,5*).

V naší studii jsme analyzovali variantu rs17817449 u celkem 1 092 mužů s **akutním koronárním syndromem (AKS)** ze studie „GENDEMIP". Kontrolní vzorek představovalo 1 191 mužů z projektu „MONICA". Polymorfismus jsme detekovali pomocí PCR a následnou restrikční analýzou. Pacienti s AKS byli starší, bylo mezi nimi více kuřáků, pacientů s DM a s hypertenzí než v kontrolní skupině. Hodnoty BMI pacientů se nelišily od kontrol. Hladina celkového cholesterolu byl překvapivě nižší u pacientů s AKS i po vyloučení pacientů léčených hypolipemickou terapií. Distribuce jednotlivých genotypů byla v Hardy-Weinbergově rovnováze u obou analyzovaných skupin. Frekvence genotypů v české populaci odpovídala frekvencím v jiných evropských populacích. Analyzovaná varianta významně souvisela s BMI jak u pacientů ($P < 0.01$), tak u kontrolní populace ($P < 0.02$). Nejvyšší hodnoty BMI vykazovali homozygoti pro G alelu a nejnižší TT homozygoti, a to v obou sledovaných skupinách. Frekvence genotypů GG detekované u pacientů s AKS se významně lišily od frekvencí nalezených u kontrolní populace (21,4 % vs. 15,9 %, $P < 0.005$). Nositelé *FTO* varianty GG vykazovali významně vyšší riziko onemocnění AKS v porovnání s TT jedinci. Riziko bylo nezávislé na věku a BMI (OR 1,49, 95 % interval spolehlivosti 1,16 - 1,93). Zvýšené riziko AKS pro GG genotyp zůstalo i po vyloučení diabetiků ze studie (100 kontrol, 339 pacientů AKS) OR 1,32 (95 % CI 1,01 - 1,72).

Naše studie jako první potvrdila souvislost mezi *FTO* polymorfismem a rizikem AKS. Výsledky naší práce potvrdil nález Doney a kol. [2009], kteří nepodpořili hypotézu,

že by souvislost FTO s AKS/ IM byla zprostředkována DM. *FTO* genotyp by tedy mohl být důležitým rizikovým faktorem rozvoje AKS u mužů.

Chronické onemocnění ledvin (CKD) a selhání (ESRD) se, díky vysoké prevalenci, drahé léčbě a výraznému omezení kvality života pacientů, stává celosvětovým zdravotním problémem. Většina chronických onemocnění ledvin úzce souvisí s diabetem a hypertenzí [Levey a kol. 2010]. Dalším rizikovým faktorem renálního selhání je KVO [El Nahas a kol. 2010]. Vedle vnějších faktorů ovlivňují rozvinutí CKD vrozené/ genetické dispozice k onemocnění. Pokusili jsme se rozšířit znalosti o možné korelaci mezi polymorfismem *FTO* genu a onemocněním ledvin [Satko a kol. 2007].

Provedli jsme dvě nezávislé studie na pacientech České republiky, u kterých jsme sledovali variantu rs17817449. V prvním případě jsme analyzovali 1 014 hemodialyzovaných pacientů. Jako kontrolní soubor byl použit soubor 2 559 jedinců z „post - MONICA“, bez CKD či renálního selhání. Ve druhé studii jsme analyzovali 1 251 pacientů, kteří podstoupili transplantaci ledvin, kvůli CKD nebo renálnímu selhání. Jako kontrolní skupinu jsme použili 6 827 jedinců z české studie „HAPIEE“. *FTO* genotyp souvisel u obou kontrolních skupin s BMI. Frekvence *FTO* genotypů se významně lišily mezi pacienty a kontrolní skupinou v obou studiích. Riziko onemocnění (OR) CKD či selhání ledvin se zvyšovalo s výskytem alel G. V prvním případě bylo OR renálního selhání či CKD pro jedince s GG genotypem v porovnání s homozygotem TT 1,56 (95 % CI 1,27 - 1,96) a trend vzrůstajícího OR se zvyšujícím se počtem alel G byl vysoce významný (P = 0,00004). Výsledky druhé studie byly velice podobné, OR renálního selhání či CKD pro GG genotyp vs. TT genotyp bylo 1,27 (95 % CI 1,07 - 1,51, P = 0,006). Frekvence *FTO* genotypů obou skupin pacientů ani kontrolních skupin se významně nelišily (P = 0,60, resp. P = 0,07). Když jsme provedli křížovou analýzu, získali jsme konsistentní výsledky s primární analýzou. Riziko renálního selhání pro GG genotyp

bylo 1,42 (95 % CI 1,16 - 1,72, P = 0,00099) u hemodializovaných pacientů vs. „HAPIEE“, a 1,40 (95 % CI 1,15 - 1,70, P = 0,00036) u pacientů po transplantaci vs. kontrolní skupina „post – MONICA“. Když jsme obě studie spojili, pro CKD/ renální selhání bylo OR pro GG genotyp vs. TT genotyp 1,37 (1,20 – 1,56, P = 0,000011). Po adjustaci k věku a pohlaví se tyto hodnoty téměř nezměnily: OR 1,34 (1,16 – 1,54) resp. OR 1,13 (1,00 – 1,47). Zjistili jsme také, že počátek onemocnění souvisí s genotypem *FTO*. U hemodialyzovaných pacientů s GG genotypem nastupuje onemocnění přibližně o 3 roky dříve. U transplantovaných pacientů docházelo k renálnímu selhání u jedinců s GG genotypem o 2,5 roku dříve než u pacientů s TT genotypem. Tyto dvě nezávislé studie potvrdily silnou souvislost mezi *FTO* rs17817449 a renálním selháním. Výsledky naší studie ukazují, že mezi onemocnění, které mohou být ovlivněny genem *FTO* patří i chronické onemocnění ledvin.

Rakovina tlustého střeva, kolorektální karcinom (CRC), je celosvětově třetí nejvyšší příčinou úmrtí na onemocnění rakovinou. Riziko onemocnění je ovlivněno jak různými faktory prostředí (výživa, nízká fyzická aktivita, alkohol, obezita a kouření), tak genetickými faktory. Dědičnost CRC je přibližně 35 %, s pouze malými efekty jednotlivých genů. Většina kauzálních variant zůstává neznámá [Lichtenstein a kol. 2000]. Některé studie potvrdili vztah mezi variantami genu *FTO* a určitými typy rakoviny. *FTO* gen je lokalizován na chromozomu 16, nedaleko lokusu detekovaného využitím GWA studie v souvislosti s CRC [Houlston a kol. 2008]. Nedávná analýza naznačila, že varianty *FTO* genu korelují s rozdílným stupněm metylace DNA [Almen a kol. 2012]. Je známo, že hypermetylace regulačních oblastí tumor supresorových genů je společným rysem všech typů rakovin.

Rozhodli jsme se zjistit, zda výše sledovaná varianta rs17817449 genu *FTO* koreluje s výskytem CRC v české populaci. Je známo, že Česká republika se vyznačuje celosvětově nejvyšším výskytem CRC. Analyzovali jsme rozsáhlou skupinu dospělých 1 005 pacientů. Jako kontrolní populaci jsme použili 6 827 jedinců ze studie „HAPIEE". Frekvence genotypů mezi pacienty s CRC a zdravými kontrolami se významně nelišily pro dominantní (P = 0,31), kodominantní (P = 0,78) ani recesivní (P = 0,85) model. Naše studie, která je zatím nejrozsáhlejší na skupině pacientů s kolorektálním onemocněním, nenalezla souvislost mezi variantou rs17817449 a rizikem CRC onemocnění. Tyto výsledky posléze podpořily další studie [Tarabra a kol. 2012, Nock a kol. 2011, Li a kol. 2012]. Přestože varianta rs17817449 na prvním intronu genu *FTO* souvisí minimálně s jedním rizikovým faktorem CRC a potencionálně také se stavem metylace DNA, nepotvrdili jsme hypotézu, že tato varianta koreluje s kolorektálním karcinomem.

III. Třetí část této práce jsme zaměřili na sledování souvislosti mezi *FTO* polymorfismem rs17817449 a relativní délkou telomer měřenou v leukocytech periferní krve *(Příloha 6)*.

Epidemiologické a jiné studie jasně ukazují, že délka telomer měřená v leukocytech periferní krve je ukazatelem biologického stárnutí a mohla by být i ukazatelem kardiovaskulárního stárnutí [De Meyer a kol. 2011, Brouilette a kol. 2008].

Rozhodli jsme se analyzovat potencionální souvislost mezi *FTO* polymorfismem, u kterého jsme nalezli souvislost s rizikem AKS, a délkou telomer u skupiny 908 žen středního věku (3PMFs). Délku telomer jsme analyzovali pomocí qPCR metody. Relativní délka telomer byla určena jako podíl telomerových repetic a kopií single copy genu (T/S). Statistická analýza byla provedena analýzou ANOVA a ANCOVA. Frekvence genotypů byly v Hardy-Weinbergově rovnováze (P = 0,95) a nelišily se od frekvencí bělošské populace. Zjistili jsme ale, že nositelky alespoň jedné rizikové alely G mají významně kratší relativní délku telomer v porovnání s TT homozygoty (0,85 ± 0,39 vs. 0,93 ± 0,48; P = 0,016) a to i po adjustaci na BMI, podkožním tuku, obvodu pasu a věku (P = 0,009).

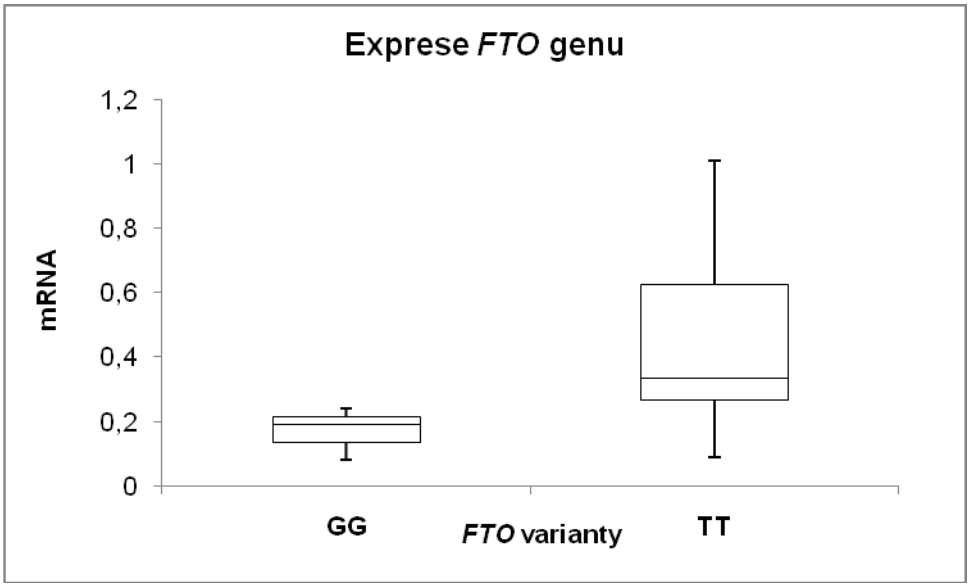
Přesný mechanismus působení FTO není doposud znám. Předpokládá se, že patří mezi regulační proteiny, protože vykazuje nízkou DNA demetylázovou aktivitu a pravděpodobně působí jako transkripční kofaktor. Nicméně právě buněčné/ biologické stárnutí organismu by mohlo být jedním z mechanismů jak varianty *FTO* ovlivňují výskyt a průběh zdánlivě nesouvisejících onemocnění.

IV. Čtvrtým úkolem disertační práce bylo zjistit, zda odlišný genotyp varianty rs17817449 *FTO* genu souvisí s hladinou mRNA *FTO* v jaterní tkáni a analyzovat celkový stav metylace DNA u vybraných skupin jedinců s odlišným genotypem této varianty.

Na základě doposud známých vlastností o *FTO* genu, jsme se rozhodli analyzovat expresi tohoto genu v lidské jaterní tkáni. Vzorky pro analýzu byly odborně odebrány z oblasti nezasažené tumorem během jaterní resekce u pacientů bez předešlé chemoterapie.

RNA a DNA jsme izolovali celkem z 25 vzorků. Restrikční analýzou bylo detekováno 13 homozygotních jedinců pro alelu TT a 3 pro alelu GG. Distribuce genotypů odpovídala Hardy-Weinbergově rovnováze a byla testována chí-kvadrát testem (P = 0,23). Z 13 TT nositelů byla RNA použitelná pouze u 10. V experimentu jsme porovnávali expresi mRNA mezi homozygotními nositeli alely G a homozygoty pro alelu T. U homozygotních jedinců s rizikovou alelou G byla hladina mRNA přibližně 2,6x nižší než u homozygotů TT (Obr. 1 a Tab. 1). Pomocí Mann-Whitney testu jsme zjistili, že rozdíl v expresi FTO mezi uvedenými genotypy je statisticky významný P = 0,043. Tento výsledek je však nutné potvrdit na větším počtu jedinců.

Obr. 1: Porovnání hladin mRNA *FTO* genu v jaterní tkáni u GG a TT homozygotů.



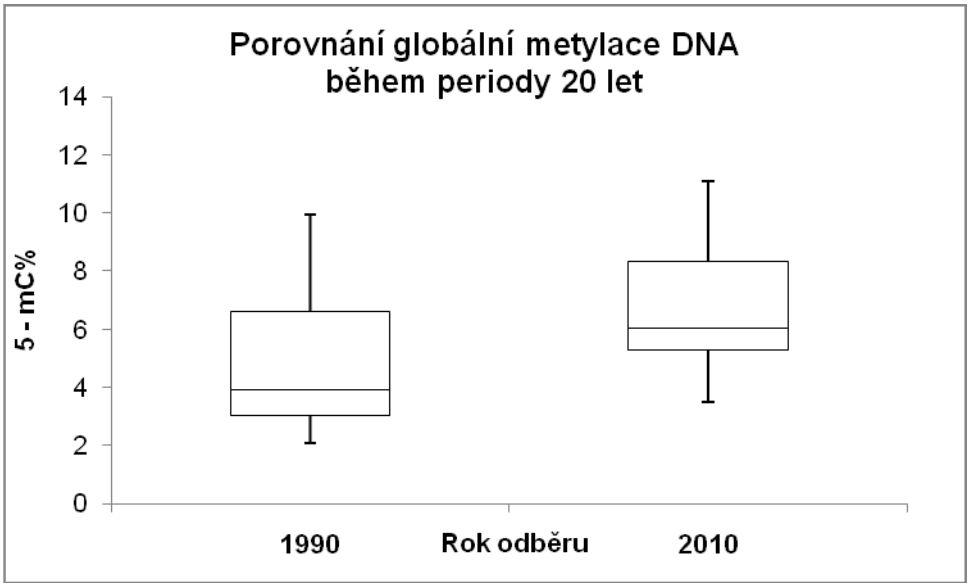
Tab. 1: Porovnání hladin mRNA *FTO* genu v jaterní tkáni u GG a TT homozygotů.

N = 13	rs17817449		
	GG (n = 3)	TT (n = 10)	
FTO mRNA*	0,17 ± 0,08	0,44 ± 0,29	**P = 0,043**

Hladiny mRNA jsou vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

Metylace DNA je jedním z klíčových mechanizmů epigenetických procesů u savců. Hraje důležitou roli při genomovém imprintingu, při globálních změnách metylomu v embryogenezi a působí protektivně před nežádoucí aktivitou transponovatelných elementů. Studie srovnávající stupeň metylace DNA a acetylace histonů H3 a H4 u jednovaječných dvojčat ukázaly, že se vzrůstajícím věkem stoupá rozdílnost epigenetických modifikací mezi dvojčaty. Obecně platí, že s věkem nastává globální pokles DNA metylací a soudí se, že procesy (de)metylace vedou ke stárnutí. Celkovou metylaci jsme stanovovali kolorimetricky použitím MethylFlash kitu. Analýzou 11 vzorků gDNA izolované z leukocytů dobrovolníků z řad zaměstnanců CEM, jsme u 9 jedinců detekovali pokles celkové metylace DNA průměrně o 1,81 % 5 - mC za 20 let. Rozdíl metylace byl dle Wilcoxnova testu statisticky významný P = 0,014. Zároveň jsme u všech 11 jedinců detekovali pokles relativní délky telomer, P = 0,004 (Obr. 2, 3 a Tab. 2, 3).

Obr. 2: Rozdíl v celkové metylaci DNA sledovaný ve vzorcích odebraných v letech 1990 a 2010 u totožných jedinců.

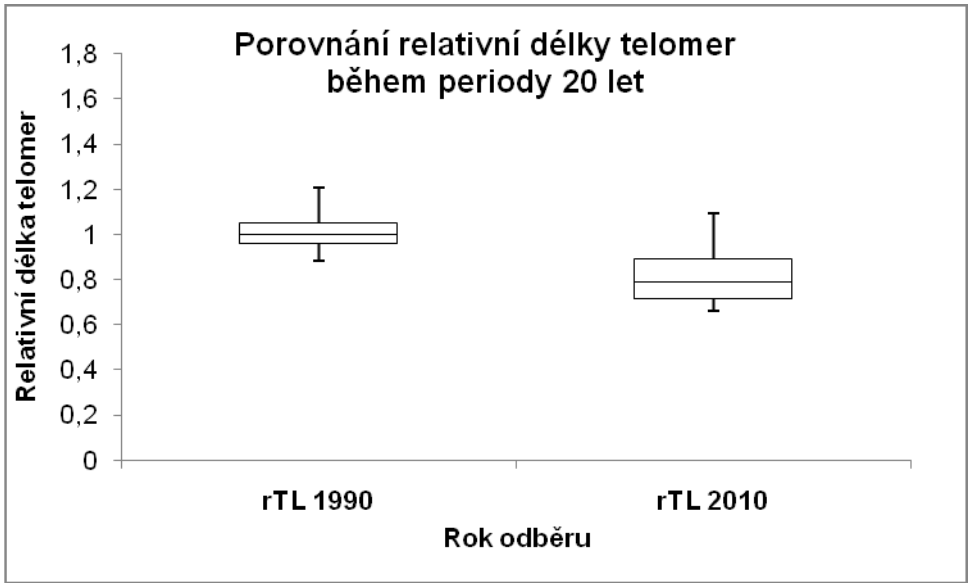


Tab. 2: Rozdíl v celkové metylaci DNA sledovaný ve vzorcích odebraných v letech 1990 a 2010 u totožných jedinců.

Rok	1990	2010	N = 13
5 – mC%	5,07 ± 2,66	6,88 ± 2,41	**P =0,014**

V tabulce jsou hodnoty vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

Obr. 3: Sledování poklesu rTL s věkem jedinců.



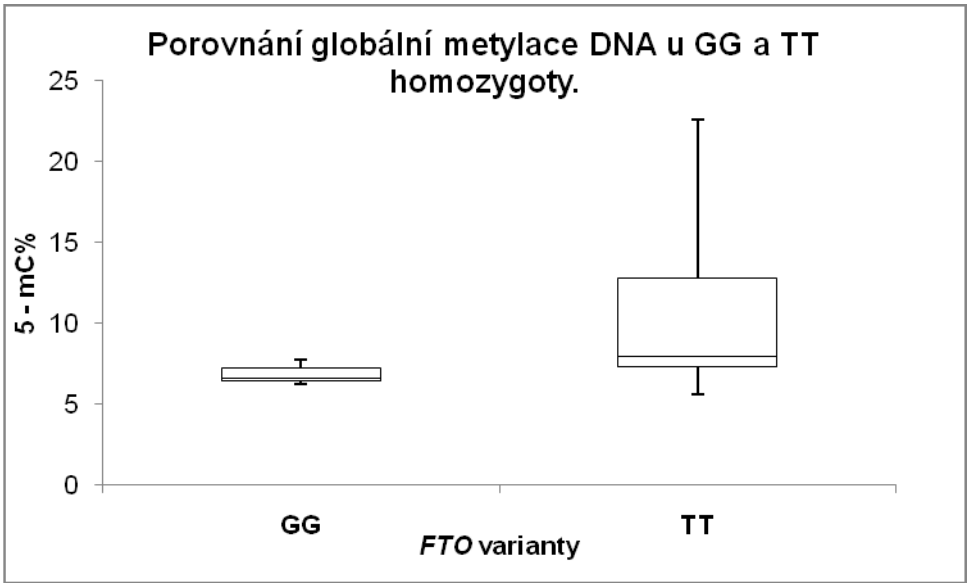
Tab. 3: Sledování poklesu rTL s věkem jedinců.

Rok	1990	2010	N = 9
rTL	1,01 ± 0,09	0,83 ± 0,14	**P = 0,004**

V tabulce jsou hodnoty vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

FTO vykazuje mírnou DNA demetylázovou aktivitu. Zajímalo nás, zda exprese *FTO* genu v jaterní tkáni bude korelovat se stavem celkové metylace DNA. K této analýze jsme použili gDNA jedinců, u kterých byla sledována exprese *FTO*. Hladiny celkové metylace DNA ve skupině jedinců s GG genotypem byly nižší než u homozygotů TT. Mann-Whitney testem jsme však zjistili, že tento rozdíl je statisticky nevýznamný P = 0,128 (Obr. 4, Tab. 4). Tento výsledek je nutné ověřit na větším počtu jedinců.

Obr. 4: Rozdíl v množství 5 – mC% mezi homozygoty GG a TT.



Tab. 4: Rozdíl v množství 5 – mC% mezi homozygoty GG a TT.

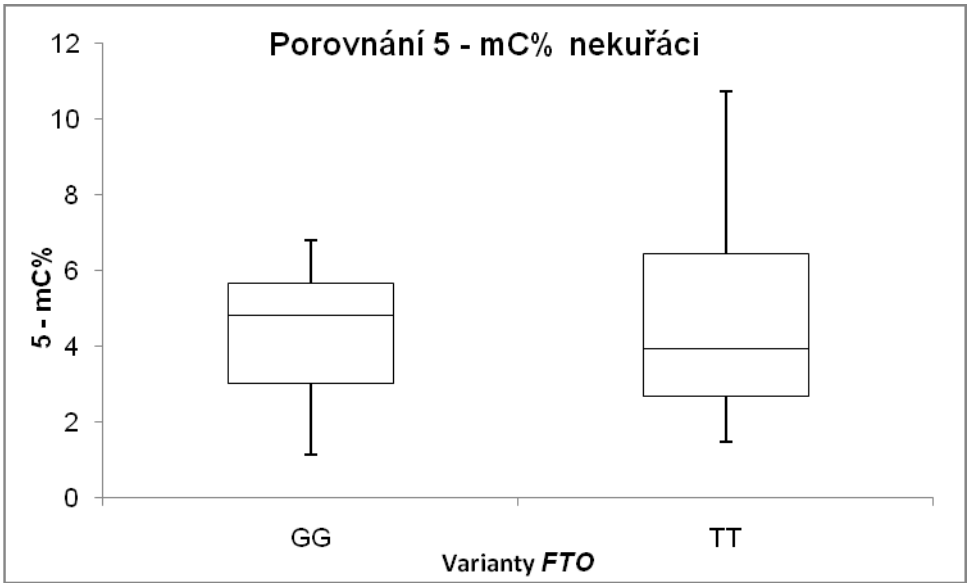
	rs17817449		
	GG (n = 3)	TT (n = 10)	
5 mC %	6,86 ± 0,82	10,68 ± 5,78	**P = 0,128**

V tabulce jsou hodnoty vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

Enviromentální faktory mohou významně ovlivňovat celkový stav DNA. Sledovali jsme globální metylaci DNA ve skupině kuřáků a nekuřáků vybraných z kontrolní populace „MONICA“. U skupiny nekuřáků, s věkovým průměrem 43,7 let, jsme detekovali nižší metylaci u homozygotů GG. Rozdíl v množství 5 – mC% byl mezi GG a TT genotypem v této skupině statisticky nevýznamný P = 0,942 (Obr. 5, Tab. 5). U kuřáků, věkový průměr 38,5 let, byla globální metylace DNA také nižší u jedinců s GG genotypem, P = 0,869 (Obr. 6, Tab. 6). Globální metylace DNA u jedinců s genotypem GG resp. TT byla ve skupině kuřáků nevýznamně vyšší než ve skupině nekuřáků, P = 0,122 resp. P = 0,229. Když jsme však porovnali obě skupiny vůči sobě navzájem, bez ohledu na *FTO* genotyp, nalezli jsme významně vyšší hladinu globální metylace DNA u kuřáků, P = 0,049, po adjustaci k věku, byl rodíl mezi oběma analyzovanými skupinami ještě významnější, P = 0,016 (Obr. 7, Tab. 7).

V našem pilotním experimentu jsme nepotvrdili souvislost mezi hladinou globální metylace DNA a rozdílným *FTO* genotypem. Hladina 5 – mC% nesouvisela ani s věkem testovaných jedinců. Nalezli jsme však signifikantní korelaci mezi hladinou celkové metylace DNA a statusem kuřáctví.

Obr. 5: Porovnání hladin globální metylace DNA ve skupině nekuřáků.

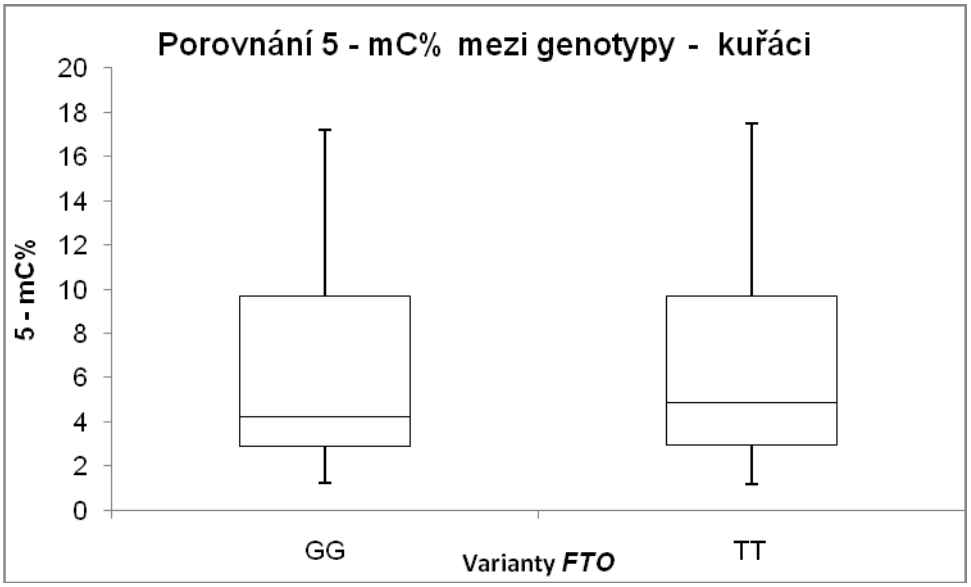


Tab 5: Porovnání hladin globální metylace DNA ve skupině nekuřáků.

	rs17817449		
	GG (n = 23)	TT (n = 23)	
5 mC %	4,45 ± 1,71	4,67 ± 2,53	**P = 0,942**

V tabulce jsou hodnoty vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

Obr. 6: Porovnání hladin globální metylace DNA ve skupině kuřáků.

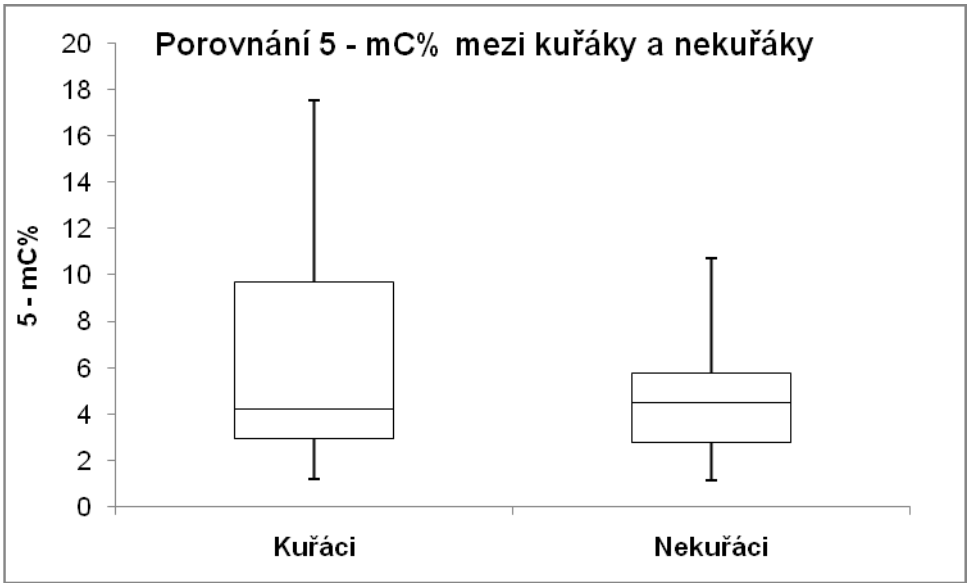


Tab 6: Porovnání hladin globální metylace DNA ve skupině kuřáků.

	rs17817449		
	GG (n = 21)	TT (n = 21)	
5 mC %	6,33 ± 4,75	6,44 ± 4,51	**P = 0,869**

V tabulce jsou hodnoty vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

Obr. 7: Rozdíl hladin globální metylace DNA mezi kuřáky a nekuřáky.



Tab. 7: Rozdíl hladin globální metylace DNA mezi kuřáky a nekuřáky.

	Kuřáci (n = 42)	Nekuřáci (n = 46)	
5 mC %	6,39 ± 4,62	4,56 ± 2,14	**P = 0,049**
Věk	38,57 ± 6,99	43,70 ± 11,28	**P = 0,016**

V tabulce jsou hodnoty vyjádřeny jako aritmetické průměry ± směrodatná odchylka.

V. Poslední část disertační práce je věnována konfirmační studii vybraných polymorfismů *FTO* (rs17817449, rs2302673, rs17818902) s rizikem vzniku závislosti na tabáku či alkoholu u české populace. Souvislost mezi variantou rs1558902 a počtem krevních destiček jsme analyzovali u žen z 3PMFs studie *(přílohy 8-10)*.

SNP na intronu 2 *FTO* byl před několika lety detekován i jako rizikový faktor rozvoje závislosti na tabáku [Bierut a kol. 2007]. Asociace mezi *FTO* polymorfizmem a kuřáckým statutem byla prokázána u žen, ale ne u mužů. Nebyl nalezen vztah k počtu vykouřených cigaret. Genotyp na intronu 1, predisponující k vyšším hodnotám BMI, je spojen i s mírně zvýšeným počtem vykouřených cigaret u kuřáků v době jejich nejintenzivnějšího kouření s nižším věkem počátku kouření, ale zdá se být protektivní proti vzniku závislosti na alkoholu v polské populaci [Sobczyk-Kopciol a kol. 2011].

V české studii „post-MONICA“ (1 191 mužů, z toho 32,1% kuřáků, 1 368 žen, z toho 22,5% kuřaček) nebyl nalezen vztah mezi kuřáckým statutem či počtem vykouřených cigaret pro *FTO* varianty v intronech 1, 2 a 3.

Varianty rs17817449 resp. rs9939609 jsme sledovali u třech nezávislých kontrolních skupin (2 559 jedinců české „post-MONICA“, 6681 jedinců „HAPIEE“, izolovaná skupina 948 Lužických Srbů) a také 201 pacientů s alkoholickou cirhózou jater. Souvislost mezi variantou na 1. intronu *FTO* a rizikem alkoholismu jsme v naší rozsáhlé studii nepotvrdili.

Nedávno byla nalezena souvislost mezi *FTO* polymorfizmem na 1. intronu rs1558902 a počtem krevních destiček u japonské populace (Kotani a spol. 2012). Tato studie naznačuje, že počet krevních destiček by mohl ovlivňovat hemokoagulační stav, jako možný faktor přispívající k rozvoji KVO. V naší studii jsme analýze podrobili 669 žen ze studie „3PMFs“. Frekvence genotypů *FTO* byly podobné a počet krevních destiček nekoreloval s variantou rs1558902.

ZÁVĚR

V předkládané dizertační práci jsou shrnuty výsledky studií provedených v Laboratoři pro výzkum aterosklerózy, která je součástí Centra experimentální medicíny Institutu klinické a experimentální medicíny v Praze. Cílem všech studií bylo objasnit, do jaké míry může genotyp *FTO* a jeho varianty sloužit jako ukazatel rizika daného onemocnění. Podařilo se nám prokázat že:

1. Varianta rs17817449 v 1. intronu koreluje s BMI, naproti tomu varianta rs17818902 na 3. intronu *FTO* nesouvisí s hodnotami BMI u české populace. Oba sledované polymorfizmy nesouvisí s poklesem BMI během krátkodobé intervenční studie u žen. Varianta na 1. intronu však ovlivnila pokles BMI u obézních dětí během intenzivní intervenční studie.

2. Zjistili jsme souvislost mezi *FTO* polymorfismem a zvýšeným rizikem akutního koronárního syndromu u mužů. Riziko onemocnění bylo nezávislé na věku a BMI a zůstalo zvýšené i po vyloučení diabetiků.

Varianta rs17817449 souvisí významně jak s rizikem renálního selhání u pacientů s ESRD a CKD, tak s nástupem tohoto onemocnění. Hemodialyzovaní pacienti s rizikovou alelou onemocněli v průměru o 3 roky a transplantovaní o 2,5 roku dříve.

U skupiny pacientů s CRC jsme souvislost s variantou v 1. intronu *FTO* genu neprokázali.

3. Kratší relativní délku telomer jsme detekovali u nositelek alespoň jedné rizikové alely *FTO* rs17817449 genotypu a to i po adjustaci k věku, BMI, obvodu pasu a podkožnímu tuku.

4. Hladina mRNA *FTO* v jaterní tkáni je rozdílná mezi homozygoty GG a TT. Celková metylace DNA nesouvisí s rozdílným *FTO* genotypem. Globální metylace DNA koreluje se statusem kuřáctví.

5. Nepotvrdili jsme souvislost mezi variantami v 1., 2. ani 3. intronu *FTO* a rizikem vzniku závislosti na tabáku či alkoholu u české populace ani mezi variantou rs1558902 a počtem krevních destiček u žen.

Závěrem lze konstatovat, že tato studie přispívá k identifikaci nového genetického determinantu nejen některých civilizačních onemocnění, ale pravděpodobně i biologického stárnutí organismu.

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PŘÍLOHA 1

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LETTERS

Lack of association between a new tag SNP in the *FTO* gene and BMI in Czech–Slavonic population

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We read with great interest the recent article by Tönjes *et al*[1] published in the European Journal of Human Genetics. In the last 2 years, the association between variants within the first intron of the *FTO* ('fat mass and obesity related') gene and obesity was recognized and widely replicated.[2] Despite recent data suggesting that FTO has a DNA demethylase activity[3] and non/heme dioxygenase activity,[4] the exact role of *FTO* genetic variants in BMI determination are yet to be identified. Tönjes *et al*[1] performed a genome-wide association study in the Sorbian population (isolate of Slavonic origin resident in eastern part of Germany) and detected an independent *FTO* signal, mapping to a region in introns 2/3 and about 40–60 kb away from the originally reported SNPs (rs9939609, rs1421085, and rs17817449). There, the strongest effect on BMI values was associated with rs17818902. As the signal has not so far been confirmed in independent populations, we have genotyped this *FTO* SNP in a group of 2559 unrelated Caucasians (1191 males and 1368 females, aged 25–65 years), a 3-year cohort of the selected 1% Czech–Slavonic population sample from nine districts. The individuals were recruited in 1997–1998 and reinvited in 2000–2001, according to the WHO protocol (*Multinational monitoring of trends and determinants in cardiovascular diseases: 'MONICA Project'.* Manual of operations WHO/MNC 82.2, Nov. 1983). Further, 556 university students (both males and females) with known birth length and birth weight were genotyped.

The frequencies of the individual genotypes were in Hardy–Weinberg equilibrium (P=0.49) and did not differ between the Czechs (TT – 63.8%; TG – 31.7%; GG – 4.5%) and the Sorbs (TT – 66.4%; TG – 30.6%; GG – 2.9%). All statistical analyses were performed separately for both the measurements obtained at the 1997/8 and 2000/1 surveys and for the means of these analyses. The results were not significant for either survey. Namely, we did not confirm the original association between *FTO* rs17818902 SNP and BMI, despite the fact that the slight similar trend (like in Sorbs) in BMI was detected at least in females (data from 2000/01 survey: males, TT – 28.2 ± 3.9 kg/m^2; TG – 28.3 ± 4.1 kg/m^2; GG – 28.1 ± 4.0 kg/m^2; females, TT – 27.6 ± 5.7 kg/m^2; TG – 27.5 ± 5.4 kg/m^2; GG – 28.0 ± 5.4 kg/m^2). Further, neither waist–hip ratio (males, TT – 0.93 ± 0.07; TG – 0.93 ± 0.07; GG – 0.93 ± 0.06; females, TT – 0.81 ± 0.07; TG – 0.81 ± 0.07; GG – 0.80 ± 0.06) nor plasma levels of total cholesterol (males, TT – 5.76 ± 1.03 mmol/l; TG – 5.75 ± 1.08 mmol/l; GG – 5.78 ± 1.14 mmol/l; females, TT – 5.78 ± 1.14 mmol/l; TG – 5.82 ± 1.12 mmol/l; GG – 5.49 ± 1.32 mmol/l), LDL and HDL cholesterol, triglycerides, glycaemia, nor hsCRP levels were associated with the newly detected *FTO* rs17818902 variant in Czechs. Also birth length and weight were independent in *FTO* rs17818902 variant. Discrepancies between the studies could not be easily explained. First, both in Sorbs[1] and in Czechs,[5,6] SNPs in intron 1 are associated with BMI and we also don't suppose some major differences in general genetic background between the two neighboring middle-European Slavonic populations,[7] despite the fact that recent neighbors could have different historical ancestry.[8] Also, the common cause of non-replication in genetic association studies – different definitions of the analyzed populations – is most probably not the cause as both analyzed groups are based on general populations. However, it is still possible that the SNP in intron 3 tagged a causal novel variant on an existing haplotype in the Sorbian population, or the BMI effect of rs17818902 is the result of interaction with environmental factors and/or genetic variant(s) outside the *FTO* region which is/are specific to the Sorbian isolate.[1] Our results underline the importance of detailed analyses of the gene–gene and/or gene–environmental interactions, which could clarify the exact role of this variant in pathogenesis of obesity in different populations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Dana Dlouha[1,2], Vera Adamkova[1], Vera Lanska[1] and Jaroslav A Hubacek[1,2]

[1]*Department of Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czech Republic;*

[2]*Centre for Cardiovascular Research, Prague, Czech Republic*

E-mail: jahb@ikem.cz

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PŘÍLOHA 2

Physiol. Res. 60: 199-202, 2011

SHORT COMMUNICATION

Body Mass Index Change in Females After Short-Time Life Style Intervention Is Not Dependent on the *FTO* Polymorphisms

D. DLOUHÁ[1], P. SUCHÁNEK[1,2], V. LÁNSKÁ[1], J. A. HUBÁČEK[1,2,3]

[1]Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [2]Centre for Cardiovascular Research, Prague, Czech Republic, [3]South Bohemia University, Faculty for Public Health and Social Studies, Ceske Budejovice, Czech Republic

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Summary

Variants within the *FTO* gene are important determinants of body mass index (BMI), but their role in determination of BMI changes after combined dietary/physical activity intervention is unclear. We have analyzed 107 unrelated overweight non-diabetic Czech females (BMI over 27.5 kg/m^2, age 49.2±12.3 years). *FTO* variants rs17817449 (first intron) and rs17818902 (third intron) were genotyped. The life style modification program (10 weeks) consisted of an age-matched reduction of energy intake and exercise program (aerobic exercise 4 times a week, 60 min each). The mean BMI before intervention was 32.8±4.2 kg/m^2 and the mean achieved weight loss was 4.8±3.5 kg (5.3±3.5 %, max. –15.5 kg, min. +2.0 kg, $p<0.01$). No significant association between BMI decrease and *FTO* variants was found. Also waist-to-hip ratio, body composition (body fat, water, active tissue), lipid parameters (total, LDL and HDL cholesterol, triglycerides) glucose and hsCRP changes were independent on *FTO* variants. *FTO* variants rs17817449 and rs17818902 are not associated with BMI changes after combined short time dietary/physical activity intervention in overweight females.

Key words

Intervention • Diet • Exercise • *FTO* • Polymorphism • Lipid • Glycaemia

Corresponding author

Jaroslav A. Hubacek, IKEM-DEM-LMG, Videnska 1958/9, 140 21 Prague 4, Czech Republic. Fax: +420 241 721 666. E-mail: jahb@ikem.cz

Obesity is a serious health problem worldwide and is associated with increased mortality and morbidity. In combination with unfavorable genetic background, abundant energy intake and low physical activity are major causes of obesity on a population level, but, for example, sleeping habits, non-exercise activity thermogenesis and more stable inside room temperatures could also contribute to the recent increase of obesity prevalence (Hubacek 2009, Adamkova *et al.* 2009).

Independent genome wide association studies detected variants in the first (rs17817449) (Dina *et al.* 2007, Scuteri *et al.* 2007) and third (rs17818902) (Tönjes *et al.* 2010) intron of the *FTO* ("fat mass and obesity associated") gene to be (independently) responsible for the BMI determination. The exact function of FTO is unknown so far and the protein has no known similarity to other proteins that could help to predict its function. How *FTO* gene could affect BMI is not clear – possible role in determination of the energy intake or energy expenditure were suggested, but the achieved results are inconsistent (Cecil *et al.* 2008, Haupt *et al.* 2009, Liu *et al.* 2010, Hasselbalch *et al.* 2010).

The decrease of the BMI could be in most individuals achieved through restriction of energy intake and enhanced physical activity (both exercise and non-exercise). Nevertheless, the individual responses to lifestyle modification vary (Hainer *et al.* 2008) and it is clear, that it is partially genetically determined.

We have investigated the influence of *FTO* rs17817449 and rs17818902 variants on a response to a

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life style changes in 107 overweight (BMI over 27.5 kg/m^2, age 49.2±12.3 years), but healthy (without diabetes, thyroid gland disease, any other endocrine disorders, autoimmune diseases, any chronic inflammation, or neoplastic disease) Czech Caucasian females.

The ten weeks life style modification program consisted of equilibrium to the recommended dietary energy intake for the appropriate age and controlled physical activity (for more details see Suchanek *et al.* 2008, Suchanek *et al.* 2009).

Dietary intervention was aimed at lowering energy intake (to recommended values), together with decrease in animal fat intake and increase of fruits and vegetables intake. Volunteers participated 3 times weekly in a supervised 1-hour training session at a fitness center, and 3 more sessions per week (cycling, jogging, or brisk walking) were recommended (at least one session was performed by all individual). All these activities included an aerobic exercise component – the participants were supervised (and advised) to sustain heart rate of 115 to 145 beats (according to age) per minute within 60 min of exercise.

The probands have their anthropometrical parameters and blood pressure determined at baseline, after each 2 weeks of intervention and on the end of the study.

Body weight (measured with electronic weight), height (measured with a stadiometer), waist and hip circumferences, were measured by trained staff according the standard protocols. The waist-to-hip ratio (WHR) and BMI were calculated from obtained measurements.

DNA was isolated from frozen EDTA blood by standard method (Miller *et al.* 1988). *FTO* variants were analyzed by PCR and restriction analysis. Briefly oligonucleotides 5` GGT GAA GAG GAG GAG ATT GTG TAA CTG G and 5` GAA GCC CTG AGA AGT TTA GAG TAA ATT GGG with restriction enzyme AlwNI were used for the genotyping of rs17817449 variant (Hubacek *et al.* 2008, Hubacek *et al.* 2009) and oligonucleotides 5` ATC ATT CTG AAA ACA GAT CTG ACT GG and 5` ATG GGT TTA TGA ACC ATA GGA AAG AAT CGA G with restriction enzyme BsaI were used for the genotyping of rs17818902 variant.

The ethics committee of the Institute approved the study and all participants signed their informed consent. ANOVA for repeated measures was used in order to evaluate the statistical significance of the differences between before and after study tests. Because of the multiple testing, P value less than 0.01 was considered to be significant. All data are presented as means ± SD.

There was no evidence for deviation of genotype frequencies from Hardy–Weinberg equilibrium. In comparison to the general populations (Hubacek *et al.* 2008, Dlouha *et al.* 2010) between obese females were nonsignificantly more carriers of the obesity associated GG genotypes (22.4 % vs. 18.8 %, for rs17817449 and 5.8 % vs. 4.5 % for rs17818902).

All subjects completed the follow-up during ten weeks, with the mean weight loss of 4.8±3.5 kg (5.3±3.5 %), with minimum "loss" of +2 kg, and maximum loss of –15.5 kg.

No significant association between BMI changes and *FTO* rs17817449 and rs17818902 genotypes was found (Figure 1).

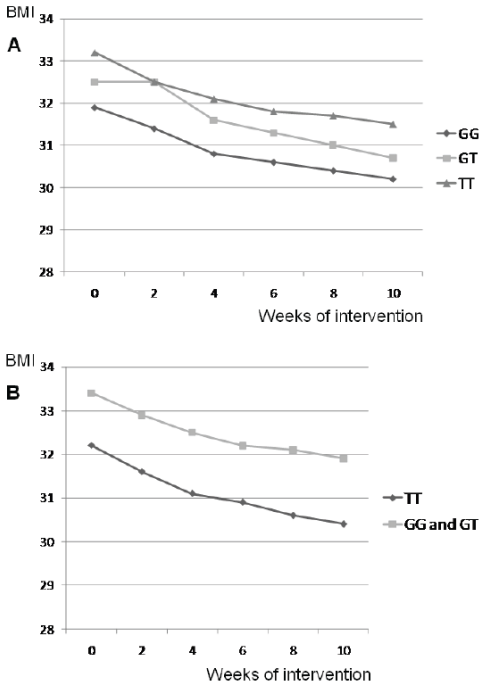

Fig. 1. BMI decrease according the *FTO* genotypes (**A** for rs17817449 and **B** for rs1788902). (In the figure just means are given, SD were, according the weeks of intervention ±3.9; ±3.8; ±3.7; ±3.7; ±3.7; ±3.7; for TT and ±4.6; ±4.5; ±4.5; ±4.3; ±4.4; ±4.3; for G allele carriers of the rs1788902 SNP and for rs17817449 than for TT ±3.2; ±3.0; ±2.9; ±2.9; ±3.1; ±3.0; for TG ±3.8; ±3.8; ±3.6; ±3.7; ±3.7; ±3.8; and finally for GG ±5.3; ±5.2; ±5.1; ±4.9; ±5.0; ±4.7).

FTO gene variants had also no influence on waist, hip, body weight, waist to hip ratio, body composition (body fat, water, active tissue), lipid parameters (total, LDL and HDL cholesterol, triglycerides) glucose and hsCRP values development through the intervention (data not shown in details).

After ten weeks of lifestyle modification, consisting of at least 4 units of exercise per week and changing of dietary habits, we have observed significant decrease in BMI and body weight in all females. Further, our results confirmed a large interindividual variability in response to the applied lifestyle modification. Most importantly the difference between the maximal and minimal body weight change was 17.5 kg. As the exercise training was three times a week performed under supervision at fitness centre, we suppose, that the differences likely reflect the different genetic predisposition than failure in the adherence to the dietary regimen and physical activity program.

So far, just interventions in children (Rendo *et al.* 2009), leads to the significant decrease of BMI, which was modulated by the *FTO* genotype and carriers of the obesity related genotype profit more from the intervention. In contrast, some another recent studies on differently defined individuals [children and adults (Müller *et al.* 2008) and diabetic patients (Lappalainen *et al.* 2009), the exact protocols used there for the duration and style of intervention differ from our study] also failed to report significant effect of the *FTO* variant on intervention dependent BMI changes. All these studies have analyzed first intron *FTO* variants (rs17817449 is in almost complete linkage disequilibrium with all of them) only.

Our study have at first time analyzed the role of *FTO* rs17818902 variation in third intron on life style modification associated changes on anthropometric and biochemical parameters in females, however, also with negative results.

In conclusion, in overweight healthy females, *FTO* gene variants in first (rs17817449) and third (rs17818902) intron have no effect on BMI, body composition and lipid parameters development over time of short lifestyle intervention. Most likely, despite the important role in obesity development per se, *FTO* variants have no effect on life style induced profitable changes in anthropometric and biochemical parameters.

Conflict of Interest

There is no conflict of interest.

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PŘÍLOHA 3

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A *FTO* variant and risk of acute coronary syndrome

Jaroslav A. Hubacek [a,b,c,*], Vladimír Staněk [a], Marie Gebauerová [a], Alexandra Pilipčincová [a], Dana Dlouhá [a,b], Rudolf Poledne [a,b], Michal Aschermann [c], Hana Skalická [c], Jana Matoušková [d], Andreas Kruger [e], Martin Pěnička [f], Hana Hrabáková [f], Josef Veselka [f], Petr Hájek [g], Věra Lánská [a], Věra Adámková [a,c], Jan Pit'ha [a,b]

[a] *Institute for Clinical and Experimental Medicine, Prague, Czech Republic*
[b] *Centre for Cardiovascular Research, Prague, Czech Republic*
[c] *South Bohemia University, Faculty for Public Health and Social Studies, Ceske Budejovice, Czech Republic*
[d] *2nd Department of Internal Medicine, General Teaching Hospital, Prague, Czech Republic*
[e] *Department of Cardiology, Homolka Hospital, Prague, Czech Republic*
[f] *Cardiocenter, Department of Cardiology, University Hospital Královské Vinohrady, Prague, Czech Republic*
[g] *Department of Cardiology, Teaching Hospital Motol, Prague, Czech Republic*

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ABSTRACT

Background: The *FTO* gene plays an important role in the determination of body weight and BMI and it has been suspected of being associated with all-case mortality.
Methods: We have analyzed the *FTO* rs17817449 variant in consecutive 1092 male patients with acute coronary syndrome (ACS) and in 1191 randomly selected Caucasian individuals (population controls).
Results: The *FTO* variant was significantly associated with BMI both in controls ($P<0.02$) and ACS patients ($P<0.01$). In both groups, BMI was highest in GG homozygotes and lowest in TT homozygotes. There was a significant difference between the ACS patients and controls in the frequency of the *FTO* genotype GG (21.4% vs. 15.9%, $P<0.005$). *FTO* GG homozygotes had a significantly increased risk of ACS, compared with TT homozygotes which was independent of age and BMI (odds ratio 1.49, 95% confidence interval 1.16–1.93). The odds ratio of ACS patients for the GG genotype remained significant even after the exclusion of diabetics (100 controls and 339 ACS patients), with OR 1.32 (95% CI 1.01–1.72).
Conclusions: This study provides an evidence of an association between the *FTO* variant and risk of ACS in Caucasian males.

In recent years, a major progress has been achieved in establishing the genetic basis of several common multifactorial diseases. Many new candidate genes associated with myocardial infarction, diabetes and obesity have been identified using genome wide association studies [1].

Among the newly detected genes, particular interest has been focused on the *FTO* gene. The *FTO* ("fat mass and obesity related", gene ID 79068, OMIM accession number 610966) gene (and its three SNPs — rs9939609, rs1421085 and rs17817449 — which are in almost complete linkage disequilibrium) was simultaneously detected by several research groups as a significant predictor of body mass index (BMI) [2,3]; further studies suggested the *FTO* importance also in other human pathologies [4–6]. *FTO* is expressed in almost all tissue; despite the fact that the exact FTO function remains unknown, the FTO has been described as a 2-oxoglutarate-dependent nucleic acid demethylase [7]. *FTO* variants are associated with BMI in a wide range of populations (mainly in Caucasians, the effect in other ethnic groups is less homogenous and still uncertain) [2,3,8–14], but it remains unclear if this effect is mediated through the increased energy intake or decreased lipolysis [15,16] or through another mechanism. It has been shown that *Fto* knock-out mice develop postnatal growth retardation with a significant reduction of adipose tissue. This extreme leanness of *Fto* knock-out mice was a consequence of increased energy expenditure (despite the decreased activity of the animals and increased energy intake) [17]. Increased energy intake (but independent on body weight) was also found in children with the *FTO* risky allele [15].

Recently, an association between the *FTO* SNP and overall mortality has been described in a large cohort of Danish men [18]. Interestingly, this effect was similar in obese and lean individuals and comparable with the risk associated with smoking; at the same time it was not predominantly associated with a particular cause of death.

Based on the results and size of the Danish study mentioned above [18], we expected that, in a study with sufficient power, a direct

Abbreviations: ACS, acute coronary syndrome; BMI, body mass index; CR, call rate; FTO, fat mass and obesity related; MAF, minor allele frequency; SNP, single nucleotide polymorphisms; WHR, waist hip ratio.

* Corresponding author. IKEM-DEM-LMG, Videnska 1958/9, 140 21, Prague 4, Czech Republic. Tel.: +420 261 363 367; fax: +420 241 721 666.
E-mail address: jahb@ikem.cz (J.A. Hubacek).

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association between *FTO* SNP and acute coronary syndrome (ACS) could also be demonstrated. We have examined this hypothesis in a case–control study, and we report here for the first time the association between the *FTO* and ACS.

2. Material and methods

2.1. Subjects

All consecutive males younger than 65 years ($n = 1092$), hospitalized in five participating coronary units (covering almost all complete ACS capacity in Prague) for ACS (STEMI and non-STEMI acute myocardial infarction, minimal myocardial lesion and unstable angina pectoris) between April 2006 and April 2009 were included in the study [19] (GENDEMIP study — GENetic DEtermination of Myocardial Infarction in Prague). The only exclusion criteria were age and refusal to participate in the study. The 1191 control males (response rate of 84%) represent a 1% Czech (Caucasian) random population sample. The individuals were recruited in 9 districts of the Czech Republic in 1997/1998 and re-invited in 2000/2001 according to the WHO protocol (*"MONICA Project"*. Manual WHO/MNC 82.2, Nov-1983.). A standard questionnaire focused on the presence of traditional cardiovascular risk factors, including family and personal history. Written informed consent was obtained from all study participants, and the study was approved by the local ethics committee.

2.2. Genetic and biochemical analysis

DNA was extracted using a salting out method [20], and *FTO* SNP rs17817449 was genotyped using PCR–RFLP as described elsewhere [8]. To ensure the accuracy of genotyping, one plate (containing 94 DNA samples) was analyzed twice within one week with 100% conformity. Call rate for genotyping was 96% in controls and 95.1% in patients. The lipoprotein parameters were measured by the WHO Regional Lipid Reference Centre, IKEM, Prague on a Roche COBAS MIRA autoanalyser, using reagents from Boehringer Mannheim Diagnostics (Mannheim, Germany) and Hoffmann-La Roche (Basel, Switzerland). Body height and weight were measured according to standardized WHO MONICA Project protocol.

2.3. Statistical analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were analyzed using the chi-square test (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls). ANOVA was used for the analysis of the potential effect of *FTO* variants on individual risk factors for ACS development. Values are given as means ± standard deviations. The power of our study was >80% to detect, with 95% confidence level, an odds ratio of 1.4 for the GG vs. TT genotype. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for each genotype, with the TT genotype as the reference category.

3. Results

3.1. Study subjects

The basic characteristics of the groups under study are summarized in Table 1. As expected, the patients were older and they have higher prevalence of smoking, diabetes mellitus (DM) and hypertension. These differences remain significant after the adjustment for age. On the other hand, BMI of patients was similar to the BMI of controls, and plasma cholesterol level was lower in ACS patients. This is almost certainly explained by the fact that many more patients (20%) than controls (8%) were on lipid lowering drugs at the time of ACS.

Table 1
Basic characteristics of the analyzed individuals.

	Male controls	ACS patients	*P*	OR (95% CI)
N	1191	1092		–
Age (years)	49.0 ± 10.8	55.2 ± 7.5	0.001	–
Cholesterol (mmol/L)	5.76 ± 1.06	5.22 ± 1.15	0.001	–
Triglycerides (mmol/L)	1.97 ± 1.28	2.05 ± 1.46	n.s.	–
BMI (kg/m^2)	28.2 ± 4.0	28.5 ± 4.3	n.s.	–
Lipid lowering drugs (%)	8.1	19.9	0.001	2.9 (2.3–3.7)
Smoking prevalence (%)	32.7	60.1	0.001	3.0 (2.4–4.0)
Diabetes prevalence (%)	8.9	34.7	0.001	4.9 (4.0–6.1)
Hypertension prevalence (%)	40.7	52.8	0.001	1.5 (1.3–1.9)

Values are expressed as mean (SD). Prevalence of diabetes and hypertension is based on self-reported data and medication. OR is age adjusted.

3.2. FTO genotypes in controls and ACS patients

In both analyzed groups, distributions of individual genotypes are within Hardy–Weinberg equilibrium and frequencies detected in the Czech Slavonic population were similar to the European samples reported previously [2,3,10].

The frequencies of the *FTO* genotypes differed significantly between ACS patients and healthy controls. In patients, there were more GG homozygotes (21.2% vs. 15.9%; $P<0.005$; OR 1.49; 95% CI 1.16–1.93) than in the controls (Table 2).

These differences remained significant ($P<0.01$) after the adjustment for age and BMI. In fully adjusted model, which included further adjustments for DM and lipid parameters, the effect of *FTO* remained virtually unchanged (OR 1.45, 95% CI 0.98–2.15); the loss of statistical significance is due to the loss of statistical power in multivariate analysis, rather than to confounding.

Because of the previously reported association between FTO and diabetes and the fact, that also in our study there is a higher frequency of suspected diabetics, we have conducted further analyses after the exclusion of diabetic persons from both controls ($N = 100$) and ACS patients ($N = 339$). Despite the smaller number of individuals in this analysis, the frequency of the GG homozygotes remained significantly higher in ACS patients than in controls (18.7% vs. 14.9%, $P<0.05$; OR 1.32, 95% CI 1.01–1.72).

3.3. Association between FTO SNP and other risk factors

Associations between FTO genotype and other risk factors are shown in Table 3. In agreement with previous findings, *FTO* SNP rs17817449 was associated with BMI; the mean BMI values in controls [8] were as follows: 28.7 ± 3.7 kg/m^2 in GG homozygotes; 28.3 ± 4.1 kg/m^2 in GT heterozygotes and 27.8 ± 3.9 kg/m^2 in TT homozygotes ($P<0.02$). In ACS patients, the effect of *FTO* variant on BMI levels was similar (GG — 29.2 ± 4.1 kg/m^2; GT — 28.3 ± 4.5 kg/m^2; TT — 28.2 ± 3.8 kg/m^2; $P<0.01$). Other analyzed MI/CAD risk factors (total cholesterol, triglycerides, self-reported diabetes and self-reported hypertension) were not associated with the *FTO* genotypes in controls

Table 2
Frequencies of the FTO rs17817449 genotypes and alleles between healthy controls and ACS patients.

	Controls		ACS patients		OR (95% CI)	OR (95% CI)
	N	%	*N*	%	Crude	Adjusted[a]
T/T	374	32.7	303	29.2	1.0	1.0
G/T	587	51.4	515	49.6	1.08 (0.89–1.32)	1.12 (0.82–1.42)
G/G	182	15.9	220	21.2	1.49 (1.16–1.93)	1.54 (1.17–2.02)

P for genotypes<0.005.
ACS — acute coronary syndrome.
[a] Adjusted for age and BMI.

Table 3
Association between FTO rs17817449 genotypes and individual characteristics.

Variable	Controls			*P*	ACS patients			*P*
	GG	GT	TT		GG	GT	TT	
N	182	587	374		220	515	303	
Age	49.1 ± 10.9	49.1 ± 11.0	49.0 ± 10.6	0.99	55.1 ± 7.8	55.2 ± 7.5	55.4 ± 7.3	0.94
Total cholesterol	5.69 ± 0.96	5.74 ± 1.09	5.81 ± 1.05	0.38	5.15 ± 1.06	5.22 ± 1.24	5.28 ± ± 1.04	0.71
Triglycerides	2.02 ± 1.24	1.94 ± 1.23	2.05 ± 1.43	0.44	2.15 ± 1.47	2.05 ± 1.50	2.02 ± 1.49	0.63
BMI	28.7 ± 3.7	28.3 ± 4.1	27.8 ± 3.9	0.02	29.2 ± 4.1	28.3 ± 4.5	28.2 ± 3.8	0.01
DM prevalence (%)	11.5	8.7	7.6	0.28	42.9	33.3	33.5	0.04
Hypertension (%)	46.3	41.1	37.7	0.16	55.7	53.4	48.8	0.28
Smoking (%)	30.5	31.7	35.2	0.42	60.0	58.4	62.4	0.54
Hypolipidemic treatment (%)	9.6	7.7	8.5	0.70	19.7	21.7	18.9	0.61

[8]. In ACS patients, only the prevalence of self-reported DM was slightly higher in GG vs. TT carriers ($P = 0.04$) but this difference disappeared after the adjustment for age and BMI.

4. Discussion

This study provides the first evidence for an association between the *FTO* gene variant and ACS in males. Carriers of the GG genotype (rs17817449) were at higher risk of ACS in comparison with carriers of the T allele. The effect of *FTO* was apparent even after the adjustment for BMI and the exclusion of diabetic subjects. Our results extend the finding of Doney et al. [21] and they do not support the hypothesis that the association between ACS/MI is mediated by diabetes.

It is known that both genetic and environmental factors contribute on ACS risk and both are of high importance [22]. Despite the known rare mutations, the vast majority of ACS risk is under polygenic control, with many variants in a couple of genes contributing in the total effect. The estimated effect of each individual SNP is estimated to be small but significant [23].

The *FTO* gene variants were primarily described to be associated with BMI levels in Caucasian studies [2,3,9,10], with potentially sex-specific effects [8,24]. It is possible that the part of the *FTO* risk associated with ACS development is caused by elevated BMI and/or higher DM prevalence. However, in our study, the mean BMI values of controls and ACS patients were almost identical (Table 1), and adjustment for BMI did not reduce the odds ratio. In addition, the association between FTO and ACS remained significant after the exclusion of all diabetic subjects. It is difficult, however, to determine if such adjustment (the fully adjusted model including lipids in the case of the high prescription of lipid lowering drugs in the ACS group) is appropriate for the assessment of real causality. For example, controlling for plasma lipids, in the case that a substantial number of individuals are on statins and in primary prevention care may obscure potentially relevant associations — total cholesterol was here recognized like a protective factor of ACS development. Nevertheless, the effect of FTO could not be explained by the other risk factors.

The gene for the *FTO* codes for the enzyme that does not seem to have a direct effect on energy management. Data about the possible association of the *FTO* gene variants with the preference of energy dense food, higher fat intake or markers of physical activity [15,25–29] are inconsistent, and the exact mechanism of the effect of FTO on obesity remains elusive.

We believe that *FTO* variants could enhance the risk of ACS through another mechanism, namely through its possible effect on DNA methylation (i.e. the epigenetic status of the organism). Animal experiments show clearly that different nutritional and lifestyle factors affect the methylation (epigenetic) status of many genes, thus most likely also genes influencing ACS development, and methylation therefore play a significant regulatory role in the wide spectrum of human diseases [30,31]. *FTO*, coding a 2-oxoglutarate-dependent nucleic acid demethylase [7] could thus present an important link between unhealthy lifestyle and real outbreak of the disease.

This hypothesis is supported by the finding that the genomic DNA methylation in patients with cardiovascular disease *per se* is significantly higher than in healthy controls [32]. To our knowledge, however, there are no data available about the total DNA methylation status and *FTO* genotype, either from experimental or clinical studies.

To what degree will *FTO* variants interact with unhealthy lifestyle factors (such as dietary habits, smoking or lack of physical activity) which may affect the epigenetic status [33], remains to be answered in future studies. The *FTO* gene variants could interact with an unhealthy lifestyle and with the presence of the general disease susceptible alleles. This could contribute not just to the risk of acute coronary syndrome/myocardial infarction development, but also to other diseases where an unhealthy lifestyle also plays an important role — in diabetes [5], metabolic syndrome [6] and cancer [4] as suggested by others.

The detection of risky ACS alleles may, in the future, lead to really in time and early detection of individuals under risk. At the moment, with reliance on the classical risk factors (anthropometrical and/or biochemical), it may be already too late for primary prevention when classical risk factors are identified.

In conclusion, our study suggests that the *FTO* genotype is an important risk factor for ACS in males. The *FTO* gene may be, besides the apolipoprotein E [23] and A5 [34] genes (major genetic determinants of plasma lipids), the next candidate gene to be used in the future in personalized preventive medicine.

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The *FTO* gene polymorphism is associated with end-stage renal disease: two large independent case–control studies in a general population

Jaroslav A. Hubacek[1,2], Ondrej Viklicky[1], Dana Dlouha[1,2], Silvie Bloudickova[1], Ruzena Kubinova[3], Anne Peasey[4], Hynek Pikhart[4], Vera Adamkova[1], Irena Brabcova[1], Eva Pokorna[1] and Martin Bobak[4]

[1]Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [2]Centre for Cardiovascular Research, Prague, Czech Republic, [3]Environmental Health Centre, National Institute of Public Health, Prague, Czech Republic and [4]Department of Epidemiology and Public Health, University College London, London, UK

Correspondence and offprint requests to: Martin Bobak; E-mail: m.bobak@ucl.ac.uk

Abstract

Background. Genome-wide association studies identified the *FTO* (fat mass and obesity gene) gene as an important determinant of body weight. More recently, the *FTO* gene was reported to be associated with other outcomes, including major risk factors for chronic kidney disease (CKD). We investigated the role of this gene in the risk of end-stage renal disease (ESRD) caused by CKD.

Methods. We conducted two large population-based case–control studies of ESRD. Study 1 compared 984 haemodialysed patients with ESRD with 2501 participants in the Czech post-MONICA study; Study 2 compared 1188 patients included in a kidney transplantation programme for ESRD with 6681 participants in the Czech HAPIEE study. The frequencies of the *FTO* rs17817449 single nucleotide polymorphism genotype were compared between cases and controls.

Results. The *FTO* rs17817449 genotype was significantly associated with CKD in both studies (P-values 0.00004 and 0.006, respectively). In the pooled data, the odds ratios of CKD for GG and GT, versus TT genotype, were 1.37 (95% confidence interval 1.20–1.56) and 1.17 (1.05–1.31), respectively (P for trend <0.0001). Among haemodialysed and kidney transplant patients, the onset of ESRD in GG homozygotes was 3.3 (P = 0.012) and 2.5 (P = 0.032) years, respectively, earlier than in TT homozygotes.

Conclusions. These two large independent case–control studies in the general population found robust associations between the *FTO* rs17817449 polymorphism and the ESRD. The results suggest that the morbidities associated with the *FTO* gene include CKD.

Keywords: chronic kidney disease; end-stage renal disease; FTO; genetic epidemiology

Introduction

Genome-wide association studies identified variations in the first intron of the *FTO* (fat mass and obesity associated) gene as genetic risk factors for obesity in a wide range of populations [1–4]. The *FTO* gene (ID 79068, OMIM acc. N. 610966) codes a protein, the exact role of which remains unknown, but the *FTO* gene is widely expressed in human tissues, and some experiments suggest that *FTO* gene products exhibit slight and different enzymatic activities, namely 2-oxoglutarate-

dependent nucleic acid demethylase activity [5] and non-haeme dioxygenase activity [6]. The research on *FTO* has primarily concentrated on its association with obesity and body mass index (BMI) values, food intake and energy expenditure [7].

More recently, a large cohort of Danish men reported an association between the *FTO* single nucleotide polymorphism and all-cause mortality [8]. The effect of the *FTO* gene appeared similar in obese and lean individuals, its magnitude was comparable with that of smoking, and the gene was not associated with a particular cause of death. Other recent reports suggest that the *FTO* variants are associated with a range of conditions, such as cancer [9], diabetes [10], hypertension [11], polycystic ovaria [12], acute coronary syndrome [13], metabolic syndrome [14], Alzheimer disease [15] and reduced brain volume [16]. Taken together, these recent reports imply a pleiotropic effect of the *FTO* gene (i.e. that its effects are not limited to body weight or obesity).

Chronic kidney disease (CKD) has become a major public health problem due to its high prevalence, enormous cost and reduction in patients' life expectancy and quality of life [17]. The majority of CKD, leading to end-stage renal disease (ESRD), is closely linked to diabetes and hypertension; virtually, all patients with ESRD have diabetes, hypertension or both [17, 18]. Other risk factors for ESRD include history of cardiovascular disease (CVD), which has led to a proposition that CKD primarily reflects vascular disease [19]. Besides environmental/external factors, inherited/genetic dispositions have been suggested to influence the progression of CKD and ESRD development. Previous studies detected a strong genetic component of ESRD, with heritability ranging from 0.30 to 0.44 [20]. Indeed, several genetic polymorphisms affecting CKD/ESRD development have already been detected [21], but the current knowledge about the genetic determinants of CKD/ESRD is far from complete.

Given the fact that majority of ESRD is caused by diabetes and hypertension, which in turn is related to conditions linked with the *FTO* gene, an association between ESRD and *FTO* is plausible. To expand our understanding of the morbidities associated with the *FTO* gene, we have investigated whether there is a relationship between CKD/ESRD and the *FTO* polymorphism and whether the *FTO* genotype was associated with the age of onset of ESRD in patients with CKD.

Materials and methods

We conducted two independent case–control studies in the Czech Republic, using two different groups of patients with CKD/ESRD and two different sets of population-based controls. The local Ethical Committees of the Institute for Clinical and Experimental Medicine in Prague, Czech National Institute of Public Health and University College London approved the protocol of these studies. All subjects included in the study were Caucasian, and all gave their informed consent to participate in the study.

Study 1

In the first study, there were 1014 cases (56% males, mean age 66.9 ± 12.7 years) from 27 haemodialysis centres in the Czech Republic. The prevalence of drug-treated hypertension was 63%, prevalence of diabetes was 39% and the mean BMI at the time of the inclusion into the study was 26.2 (±5.0) kg/m^2 (BMI was available for 64.2% of patients). To be included in the study, the patients had to be on dialysis therapy for at least 3 months, and they had to give consent to participate in the study. Patients with generalized cancer, history of poisoning or another known exogenous cause of ESRD were excluded from the study.

The controls in Study 1 were 2559 individuals without CKD/ESRD (49% males, mean age 49.0 ± 10.7 years) who participated in the post-MONICA study [22]. The post-MONICA study, using the WHO MONICA Project protocol [23], examined a 1% random population sample of men and women aged 25–64 years in nine Czech districts in 1997/1998; the subjects were re-examined in 2000/2001, when blood samples used for DNA extraction were taken.

Study 2

In the second study, there were 1251 cases (65% males, mean age 47.2 ± 13.0 years) who underwent kidney transplantation for CKD/ESRD at the Institute of Clinical and Experimental Medicine in Prague in 1999–2007. Among those cases, prevalence of drug-treated hypertension was 82%, prevalence of diabetes 35% and mean BMI at inclusion to study 25.5 kg/m^2 (BMI was available for 50.6% of the patients). Patients were referred for kidney transplantation by the same dialysis centres that provided cases for the first study. The blood samples for DNA extraction were drawn during hospitalization for kidney transplantation.

The control group consisted of participants from the baseline survey of the Czech HAPIEE study conducted 2002–05 [24]. The participants, men and women aged 45–69 years, were randomly selected from population registers of seven Czech towns. DNA samples are available for 6827 (45% males, mean age 58.2 ± 7.1 years) individuals.

Genotyping

The *FTO* gene variant rs17817449 (which is in almost complete linkage disequilibrium with other often analysed variants rs9939609 and rs1421085) was analysed by polymerase chain reaction (PCR) and restriction analysis [25, 26]. Briefly, genotyping of the rs17817449 variant was performed by PCR using oligonucleotides 5′ ggt gaa gag gag gag att gtg taa ctg g 3′ and 5′ gaa gcc ctg aga agt tta gag taa att ggg 3′ followed by treatment with restriction enzyme AlwNI (uncut PCR product 198 bp represents allele G, restriction fragments of 99 and 99 bp allele T). The genotype call rates were 97.0% in haemodialysed cases and 96.4% in post-MONICA-based controls in Study 1 and 96.9% in kidney transplantation cases and 97.9% in the HAPIEE study-based controls in Study 2. To ensure the accuracy of the method, one plate (94 samples) was genotyped twice within 1 week; the agreement between the two analyses was 100%. The reliability of the genotyping results is further supported by the fact that the allele frequencies were very similar to those previously reported in western European populations [1–3, 8].

Statistical analyses

The statistical analyses are based on subjects with valid *FTO* genotype data (and reflecting the call rates described above); there were 984 cases and 2501 controls in Study 1 and 1188 cases and 6681 controls in Study 2.

The Hardy–Weinberg test was applied to confirm independent segregation of the *FTO* alleles. There was a borderline significant deviation of the *FTO* genotype frequencies from the Hardy–Weinberg equilibrium (HWE) for the controls in Study 2 from the HAPIEE study ($P = 0.03$).

The differences in the genotype frequencies between cases and controls were assessed by chi-square and likelihood ratio tests. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were estimated using the STATA software. In the primary analyses, the association between the *FTO* genotype and ESRD was assessed in each case–control study separately; after that, we also analysed the pooled data (i.e. both groups of cases combined versus both control groups combined). The relationship between the age of onset of CKD and the *FTO* genotype was analysed using analysis of variance and linear regression. The pooled dataset provided a statistical power of >95% to detect an OR of ≥1.25, for GG versus TT genotype at 95% confidence level.

Results

In both sets of healthy controls, the *FTO* genotype was associated with BMI in the expected direction; i.e. the carriers of the GG genotype had significantly higher BMI than carriers of GT and TT alleles [25, 26]. Among cases, data

Table 1. Frequencies of the *FTO* rs17817449 genotypes in cases and controls and ORs (95% CIs) for ESRD by *FTO* genotype

	Study 1			Study 2			Pooled data		
	Cases, *N* (%)	Controls, *N* (%)	OR (95% CI)	Cases, *N* (%)	Controls, *N* (%)	OR (95% CI)	Cases, *N* (%)	Controls, *N* (%)	OR (95% CI)
TT	273 (28%)	829 (33%)	1.0	350 (29%)	2204 (33%)	1.0	623 (29%)	3033 (33%)	1.0
GT	487 (49%)	1240 (50%)	1.19 (1.00–1.42)	578 (49%)	3188 (48%)	1.14 (0.99–1.32)	1065 (49%)	4428 (48%)	1.17 (1.05–1.31)
GG	224 (23%)	432 (17%)	1.56 (1.27–1.96)	260 (22%)	1289 (19%)	1.27 (1.07–1.51)	484 (22%)	1721 (19%)	1.37 (1.20–1.56)
P (chi-square)			0.0002			0.024			<0.0001
P for trend			<0.0001			0.006			<0.0001

on BMI before the onset of CKD were not available. However, BMI was available for a subset of cases at the time of the inclusion into the haemodialysis/transplantation programme. Similar to the controls, the *FTO* G allele was positively associated with BMI both in haemodialysed and transplant patients.

The main results are shown in Table 1. There are several notable findings. Firstly, the frequencies of the *FTO* genotypes differ significantly between cases and controls in both studies, and in both studies, the OR of ESRD/CKD increases with the number of G alleles. In Study 1, the OR of ESRD/CKD for GG homozygotes compared with TT homozygotes was 1.56 (95% CI 1.27–1.96), and trends of increasing OR with increasing number of G alleles were highly significant (P = 0.00004). In Study 2, the results were similar, although the association was weaker than in Study 1; the OR of ESRD for GG versus TT genotype was 1.27 (95% CI 1.07–1.51), and the P-value for trends was 0.006.

Secondly, there were no significant differences in the frequencies of the *FTO* genotypes between the two sets of cases (P = 0.60) and between the two sets of controls (P = 0.07).

Finally, cross analyses produced results consistent with those found in the primary analysis; the ORs of ESRD for GG versus TT genotype were 1.42 (95% CI 1.16–1.72, P for trend 0.00099) for comparing cases from Study 1 with controls in Study 2 and 1.40 (95% CI 1.15–1.70, P for trend 0.00036) for comparing cases from Study 2 with controls in Study 1. Given these results, it is not surprising that there was a highly significant association between the *FTO* genotype and ESRD/CKD in data pooled from both studies (2196 cases and 9048 controls). The OR for GG versus TT genotype was 1.37 (1.20–1.56), and the trend for increasing OR with increasing number of G alleles was highly significant (P = 0.000011). Further adjustment for age and sex did not materially change these estimates; the ORs for GG and GT versus TT genotypes were 1.34 (1.16–1.54) and 1.13 (1.00–1.47), respectively.

In addition to the case–control comparisons, we have also examined whether the age of onset of ESRD differs by *FTO* genotype in a subset of 753 haemodialysed and 1095 kidney transplantation patients of whom the age of onset was available (Table 2). We found that in haemodialysed patients (cases from Study 1), the age at onset of ESRD was significantly associated with *FTO* genotype; the onset of the disease in carriers of the G allele was ~3 years earlier than in TT homozygotes. The association in kidney transplant patients (cases from Study 2) was similar: GG homozygotes had onset of ESRD ~2.5 years earlier than TT homozygotes. Additional adjustment for presence of diabetes and sex did not change these results significantly (not shown in table).

Table 2. Mean age (SD) at onset of ESRD in cases from Study 1 and Study 2 by *FTO* genotype[a]

	Haemodialysed patients (cases from Study 1), *N* = 753	Kidney transplantation patients (cases from Study 2), *N* = 1095
TT	63.5 (13.3)	47.9 (13.3)
GT	59.9 (13.9)	47.3 (12.8)
GG	60.1 (14.6)	45.4 (13.2)
P-value (ANOVA)	0.008	0.071
P-value (trend)	0.012	0.032

[a]ANOVA, analysis of variance.

Discussion

It is known that both genetic and environmental factors contribute to CKD/ESRD risk and both are of significant importance [20, 21]. As in other diseases, the vast majority of CKD/ESRD risk is under polygenic control, with many variants in a number of genes contributing to the total effect, each with a small, but detectable effect, in studies with sufficient power. The genes previously studied in association with CKD/ESRD and its complications include apolipoprotein E, methylentetrahydropholate reductase, interleukins and tumour necrosis factor alpha [27]. This is the first report that examines the possible role of the *FTO* gene. Our results, based on two large independent sets of cases and controls selected from general central European Slavonic population, comprising in total >2000 cases and almost 10 000 controls, suggest a robust association between the *FTO* genotype and the risk of CKD/ESRD. We also found a suggestion of an earlier onset of ESRD in carriers of the G allele.

To our knowledge, this is the first study explicitly investigating this topic, although there are some indirect indications from other studies (see below). This study has several important strengths. Firstly, the large sample size means that the study had a large enough statistical power to detect modest effects. Secondly, we analysed two different sets of cases and controls and effectively replicated the main

finding in an independent investigation. This greatly reduces the possibility that the results are due to chance (false positive).

Thirdly, this study was population based. Although the cases came from medical centres, the nature of ESRD (requiring referral for haemodialysis or transplantation) means that the cases were population based rather than drawn from specific sub-populations. Controls were random population samples. While, strictly speaking, controls and cases were not recruited from identical geographical areas, the fact that the controls were not selected on the basis of their health status reduces the scope for selection bias.

There are several limitations of this study. Firstly, we did not have more details about the cases. To recruit large numbers of cases, we had to compromise on the amount of data about them e.g. details about their health behaviours, other risk factors and clinical and laboratory data. Some of this information was available for subsets of both sets of cases, but because it was not collected in a comparable fashion with controls, these data do not allow detailed statistical analyses. Moreover, even if such data were available, they would reflect the patients' status at a very late stage of CKD; this would be insufficient to estimate the role of, for example, diabetes and hypertension, in the development of the disease.

Secondly, ~50% of the cases did not undergo renal biopsy in the phase of ESRD; therefore, exclusion of potential exogenous causes of the CKD may not be reliable. However, it has been estimated that at least 75% of CKD cases are due to diabetes and hypertension [17], while other causes, such as cancer or poisoning, are relatively uncommon (and patients suffering from cancer or poisoning were not included in our study). While we could not reliably exclude all such secondary CKD from our study, their presence would probably lead to underestimation of the association between *FTO* and ESRD (assuming that the secondary CKD is not associated with the *FTO* gene). Our estimates of the effect of the *FTO* genotype are therefore likely to be realistic.

Finally, the distribution of the genotypes among controls in Study 2 was not in HWE. This can be partly explained by chance and high statistical power (even modest differences between expected and observed frequencies are statistically significant with sufficiently large numbers of subjects, and the P-value in our study was of a borderline significance). The literature suggests that the most common reason of Hardy-Weinberg disequilibrium in genetic association studies is genotyping error [28]. Our quality control did not suggest any problem with genotyping, and the relationship between *FTO* and BMI was similar in both sets of controls. The main difference between the observed and HWE expected genotype frequencies among the controls in Study 2 was the proportion of GT heterozygotes (47.7% in observed data versus 49.1% in expected data), while the observed and expected proportions of GG and TT homozygotes were similar to the expected. Moreover, the OR of ESRD based on the expected frequencies among controls in Study 2 was 1.29, which is slightly higher than the observed OR of 1.27. These sensitivity calculations suggest that the association between *FTO* and ESRD was not overestimated in Study 2.

The finding that, among haemodialysis patients, the GG homozygotes had lower age of CKD/ESRD onset than the TT homozygotes is consistent with the results of our two case–control studies. This was replicated in transplant patients, although in this group the difference in age of onset was somewhat smaller. This may be due to the fact that transplant patients are generally younger and have lower rates of diabetes, CVD and other comorbidities than patients with ESRD not selected for transplantation. This was also the case in our study; transplant patients represent a healthier pre-selected group of CKD/ESRD patients, and this may explain the weaker association between the *FTO* polymorphism and the age of onset of ESRD.

It is difficult to assess the consistency of our findings with previous studies, because the existing literature on *FTO* and CKD/ESRD is sparse. We identified only two sets of reports in the literature, and they are only partly relevant. Firstly, using two Korean studies analysing the *FTO* variant rs8050136, 629 healthy controls from a study by Cho *et al.* [29] were compared with 583 kidney transplantation patients from a separate independent study [30]. In this comparison, kidney transplant patients had a frequency of the less common allele of 25%, which is slightly higher than the 22.7% among healthy non-diabetic subjects; however, the OR 1.14 is not statistically significant, mainly because of the relatively small numbers of individuals in both groups. However, these Korean data are based on a population with a different ethnic background and using different *FTO* gene polymorphisms with different allelic frequencies and cannot be directly compared with our study. The second study investigated the role of the *FTO* gene variant (rs9939609) in the development of diabetic nephropathy in diabetic patients [31]. While the *FTO* genotype was associated with BMI, it was not related to the risk of nephropathy. This study, however, did not focus on the relation between ESRD and the *FTO* gene.

While the association between the *FTO* genotype and ESRD in our data is statistically robust, we can only speculate about the biological basis for this link. It is known that *FTO* is associated with obesity and diabetes mellitus and both these conditions are risk factors of CKD/ESRD development. It is possible that the *FTO* effect on CKD/ESRD is at least partly mediated through these conditions. However, pathways independent from diabetes and obesity are also likely, in analogy to earlier studies that showed that the *FTO* genotype was related to the risk of acute coronary syndrome after controlling for BMI and diabetes [13] or with increased mortality independently of BMI [8].

The gene translation product of *FTO* exhibits more enzymatic activities, but the real function of FTO remains largely unknown and is a matter of speculation. As *FTO* was originally identified as a determinant of obesity, functional studies have focused on the possible associations of the *FTO* gene variants with the preference of energy-dense food, with higher fat intake or with markers of physical activity. While the association of *FTO* with higher energy intake has been replicated in children and adolescents, it seems unlikely that the *FTO* gene affects the development of CVD or ESRD through this mechanism.

Alternatively, the *FTO* polymorphisms may enhance the risk of ESRD through its possible effect on DNA methylation (i.e. the epigenetic status). There is strong experimental evidence that the *FTO* gene could be involved in demethylation of the 1-methyladenine and 3-methylcitosine [32]. In addition, animal experiments suggest that nutrition and other life style factors significantly affect the methylation (epigenetic) status of many genes, which play a significant regulatory role in wide spectrum of human diseases [33, 34] and which may include genes influencing ESRD. Since the *FTO* gene codes protein with slight 2-oxoglutarate-dependent nucleic acid demethylase activity [5], it could provide the link between life style and risk of chronic diseases. Finally, at least in some conditions (newborns with low weight for gestational age; other individuals were so far not studied), it was proven that *FTO* variants within the first intron are associated with different methylation status of the peroxisome proliferator-activated receptor gamma gene promoter [35].

Despite the uncertainty about biological mechanisms, there is strong evidence that the *FTO* genotype is associated with obesity and there is emerging evidence that the *FTO* gene is related to several other health outcomes. This well-powered population-based case–control study suggests that the *FTO* polymorphism is also associated with CKD. Further studies are required to replicate this association in other populations and to clarify the molecular and pathophysiological mechanisms.

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Conflict of interest statement. None declared.

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Letter to the Editor

The risk of sporadic colorectal cancer development is not influenced by fat mass and obesity related gene polymorphism in Slavs

Colorectal cancer (CRC) is the third leading cause of cancer related mortality worldwide. Both environmental factors (e.g. nutrition, low physical activity, alcohol intake, obesity or smoking) and genetic factors contribute to the individual risk. The inheritance of CRC is estimated to be about 35% [1], with only small single gene effects (usual risk ratios are between 1.1 and 1.2); the majority of the causal variants remain unknown.

Variants within the gene for FTO ("fat mass and obesity related", OMIM acc. No. 610966), primarily detected as "obesity-associated" gene, have been reported to be linked with certain types of cancer in some, but not in all, studies. Furthermore, the *FTO* gene is located on chromosome 16 not far away from a locus found to be associated with CRC in a GWAs study [2]. A recently performed analysis of the DNA methylation profile suggests that common variants within the *FTO* gene are associated with different DNA methylation level [3], and abundant methylation of regulatory parts of tumor suppressor genes is a common feature of all human cancers.

In light of the several strands of evidence summarise above, we analysed a large cohort of sporadic CRC patients originating from the Czech Republic, a country with the incidence of CRC among the highest in the world, to investigate whether tagging rs17817449 variant at the first intron of the *FTO* gene is associated with elevated risk of CRC.

Using the PCR-RFLP method described previously [4], we have genotyped the first intron tagging variant (rs17817449, G>C) at the *FTO* gene in 1005 adult patients with sporadic CRC (583 males and 422 females aged between 26 and 89 years, mean age 61.2 ± 11.0 years); further details on patient selection have been reported by Jiraskova et al. [5]. Controls were 6,827 healthy adults (3079 males and 3602 females aged 45–69 years, mean age 58.3 ± 7.1 years) participating in the Czech branch of the HAPIEE study [6]. In 1451 controls, the genotyping results were verified by KASPER method (KBioScience, London, UK) with 99.2% conformity. All participants signed an informed consent and the study was approved by the institutional Ethics committees and conducted according to the Good Clinical Practice guidelines.

Chi-square tests and odds ratio (95% CI) of CRC by the *FTO* genotype were calculated. *P*-values less than 0.05 were considered to be significant.

Genotype call rate was 91.3% in CRC patients and 97.0% in healthy controls and the minor allele frequencies in controls (0.43) were similar to the other Caucasian populations [7].

We found no differences between the CRC patients and healthy controls in the genotype frequencies, regardless on the model of the analysis performed; either in dominant, ($P=0.31$) co-dominant ($P=0.78$) or recessive model ($P=0.85$) (for more details, see Table 1). Finally, the numbers of alleles were identical ($P=0.66$) in both groups (43.2% in controls and 43.7% in CRC patients for the minor G allele). In the subset of cases with relevant data, neither the age at diagnosis nor mortality, were associated with the *FTO* genotypes.

Table 1
Frequencies of the rs17817449 genotypes between healthy controls and sporadic CRC patients.

	Controls		CRC patients		OR (95%CI)	*P*
	N	%	*N*	%	Crude	
GG	1290	19.3	190	20.7	1.06 (0.88–1.29)	0.25
GT	3190	47.8	422	46.0	0.95 (0.82–1.12)	0.56
TT	2201	32.9	305	33.3	1.0	

Our study, the largest on this topic so far, produced negative results. This is consistent with other studies. The recently published study of Tarabra et al. [8] did not detect any significant role of the rs9939609 variant (particular allele of rs9939609 and rs17817449 corresponding allele co-segregate together in almost 100% linkage disequilibrium [4]) with risk of colorectal adenoma or cancer in Italian population. Another multi-ethnic study [9] also failed to detect an association between *FTO* tagging SNPs (among others also rs9939609 and rs17817449) and risk of colorectal adenoma in Caucasians, but were associated with higher risk of colorectal adenomas in African-Americans. Finally, a large meta-analysis involving 13 studies focusing on *FTO* polymorphism and cancer risk [10] suggested that rs9939609 is not significantly associated with the increased risk of cancer, with exception valid for the slightly elevated risk for pancreatic cancer.

In conclusion, we found no evidence for the hypothesis that the 1st intron tagging *FTO* SNP rs17817449 is associated with increased risk of sporadic CRC, although the *FTO* genotype is associated with at least one risk factor of sporadic CRC (obesity) and potentially also with DNA methylation status.

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Jaroslav A. Hubacek*
Dana Dlouha
Institute for Clinical and Experimental Medicine, Prague, Czech Republic
**Corresponding author at:* IKEM-DEM-LMG, Videnska 1958/9, 140 21 Prague 4, Czech Republic. Tel.: +420 261 363 379; fax: +420 241 721 666.
E-mail address: jahb@ikem.cz (J.A. Hubacek).

Martin Bobak
Department of Epidemiology and Public Health, University College London, London, United Kingdom

Alena Jiraskova
Institute of Medical Chemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Libor Vitek
Institute of Medical Chemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic
4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

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Association between *FTO* 1st intron tagging variant and telomere length in middle aged females. 3PMFs study

D. Dlouha *, J. Pitha, V. Lanska, J.A. Hubacek

Institute for Clinical and Experimental Medicine, Videnska 1958/9, Prague, 14021, Czech Republic

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ABSTRACT

The *FTO* gene plays an important role in the determination of body weight and BMI and it has been suspected of being associated with all-cause mortality, cardiovascular disease, cancer and end stage renal disease, but the causal mechanism of these effects is still unknown. One of the possibilities is the potential association with telomere length. Telomeres are repetitive DNA-sequences located at the ends of eukaryotic chromosomes' length of which is reduced in all somatic cells during ageing. Out of the 908 females (3PMFs study), in 783 females both *FTO* 1st intron tagging polymorphism (G>T, rs17817449) and the relative telomere length were successfully analysed. The relative telomere length was calculated as the ratio of telomere repeats to single-copy gene copies. The frequencies of the *FTO* genotypes were similar to other populations (GG = 18.3%, GT = 49.1% and TT = 32.6%). We have detected, that the relative telomere length was significantly shorter ($P<0.02$, $P<0.01$ after adjustment for age, BMI, waist and subcutaneous fat), in carriers of at least one *FTO* risky (G) allele (0.85 ± 0.39) in comparison to the carriers of the protective TT genotype (0.93 ± 0.48). We have demonstrated that the *FTO* variant could be associated with the relative telomere length. Whether this represents a causality of association between the *FTO* variant and the non-communicable diseases needs to be further analysed.

1. Introduction

Cardiovascular diseases (CVD) are the most common cause of mortality and morbidity in developed countries. Genetic plays substantial role in the development of CVD, but particular knowledge is far from the entire estimate of genetic predisposition of CVD, which should be about 50%. Nearby the "classical" candidate genes, like for example apolipoprotein E [1,2] or apolipoprotein A5 [3], high throughput methods (especially chips used in genome wide association studies) lead to the detection of the new genes of interest, mainly, however, without clear link between the gene and the disease.

Among these genes belongs also the gene for FTO (fat mass and obesity associated protein, OMIM ID: 610966, gene ID: 79068), primarily recognised like body mass index (BMI) associated gene (for review see [4]). In Caucasian, a cluster of SNPs located within the 1st intron of the *FTO* gene was recognised as sufficient for the detection of the risky *FTO* variant. This variant could be detected by the analysis of one of the three tagging SNPs (rs9939609, rs1421085 and rs17817449). Later the importance of the variants within this gene in the determination of non-communicable diseases, especially, but not only, of CVD was detected [5,6]. Despite the intensive research the mechanism linking the *FTO* gene to the obesity or CVD development remains unclear. However, it is clear that the association between *FTO* polymorphism and CVD is at least partially independent of obesity or diabetes mellitus [6].

Epidemiological and other studies show clearly that the length of telomeres measured in peripheral blood leucocytes is the marker for biological ageing and so could be also the biomarker in monitoring cardiovascular ageing [7].

Telomeres are composed of nucleotides and are located at the end of chromosomes in eukaryotic cells. They cap the termination of the double strands of DNA and preserve the integrity and stability of the genome during replication [8]. Telomeres are made up of a repetitive sequence of six bases (TTAGGG). This sequence is repeated over several thousand base pairs at the 3′ end of DNA (4 to 15 kilo bases in humans). In most human somatic cells, the length of the telomeres decreases by 20 to 200 base pairs with each cell division [9]. In normal somatic cells, telomeres shorten due to the inability of DNA-polymerase to fully replicate the chromosomal ends, up to a critical length which induces the loss of the complex nucleoprotein structure, thereby triggering replicative senescence [10]. The telomere hypothesis of cellular ageing suggests that when telomere shortening reaches a critical level, the chromosome may become unstable and

Abbreviations: CVD, cardiovascular diseases; FTO, fat mass and obesity associated; BMI, body mass index; 3PMFs, the Prague Pre and Post Menopausal Females study; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SCG, single-copy gene; T/S, telomere repeats/single-copy gene copies ratio; OMIM, Online Mendelian Inheritance in Man.

* Corresponding author at: Institute for Clinical and Experimental Medicine, PEM, Videnska 1958/9, Prague 4, 14021, Czech Republic. Tel.: +420 261 362 251; fax: +420 241 721 574.
E-mail address: dadl@ikem.cz (D. Dlouha).

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the cells stop dividing [11,12]. This hypothesis has prevailed as one of the most feasible hypotheses for explaining the cause of not only cellular ageing but also ageing of individuals [13].

Among the CVD patients there exists considerable variability in sensibility, age of onset and extent of progression of disease. Too short telomeres lead to a decrease of functional cells which contributes to overall tissue and organ dysfunction. Reduction of length of telomeres may provide the explanation for highly variable symptoms of heart failure [14]. As cell ageing based on telomere shortening is one of the mentioned risk factors of the CVD development, we have analysed the potential association between the *FTO* polymorphism and the length of telomeres in middle aged females.

2. Material and methods

2.1. Subjects

Individuals included within the Prague Pre and Post Menopausal Females study (3PMFs) were analysed. Briefly, 5% of the representative random sample of the population consisting of 29,440 women aged 45–54 years living in Prague was selected from the registries of health insurance companies. From a random sample of 1472 women, 908 give their informed consent to participate in the study and were primarily examined. The protocol of the study was approved by an institutional ethics committee. Clinical characteristics of the analysed women are presented in Table 1.

2.2. DNA and SNP analyses

Genomic DNA was extracted from EDTA blood by a standard method [15].

Tagging variant within the first intron of the *FTO* gene (G>T; rs17817449) was analysed using the PCR–RFLP based method, as described in details before [16].

2.3. Measurement of telomere length

Telomere length was measured using a quantitative polymerase chain reaction (qPCR)-based method [17]. The relative telomere length was calculated as the ratio of telomere repeats to single-copy gene (SCG) copies (T/S ratio). For each sample the quantity of telomere repeats and the quantity of SCG copies were determined in comparison to a reference sample. The identical reference DNA was used in all runs to allow comparison of the results in different runs. All PCRs were performed in duplicate on the Rotor-Gene 3000 (Corbett Research Ltd). The raw data from each PCR were analysed using the comparative quantification analysis (Rotor-Gene 3000 software, Corbett Research Ltd). The second derivative of the amplification curve was considered in order to identify the peak of the exponential amplification and determine the Take-Off of the reaction. The Take-Off was estimated by finding the first point to be 80% below the peak level. Based on the Take-Off point and the amplification, the method calculated the relative quantity of telomere repeats or SCG copies in each sample compared to the reference sample.

The acidic ribosomal phosphoprotein PO (36B4) gene was selected as SCG. The primers used for the telomere and the SCG amplification were [17,18] as follows — telomere forward: 5′ GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T, telomere reverse: 5′ TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA, SCG forward: 5′ CAG CAA GTG GGA AGG TGT AAT CC and SCG reverse: 5′ CCC ATT CTA TCA TCA ACG GGT ACA A.

In the telomere PCR, primer concentrations were (forward/reverse) 135/900 nM and in the SCG PCR, primer concentrations were (forward/reverse) 300/500 nM. In each run of 32 samples, a reference sample and a no-template control, all in duplicate were included.

The cycling profile for telomere PCR was: 95 °C incubation for 10 min, followed by 22 cycles of 95 °C for 15 s and 58 °C for 120 s. For SCG PCR the cycling profile was: 95 °C incubation for 10 min, followed by 35 cycles of 95 °C for 15 s and 58 °C for 60 s. For both the telomere and the SCG PCR the final reaction volume was 25 μl consisting of SensiMix SYBR No-ROX (2×) (Bioline Ltd, UK), 100 ng of template and the respective primer concentrations.

The specificity of all amplifications was determined by melting curve analysis.

To examine the measurement stability of the telomere length by qPCR analysis both the intra-assay and the inter-assay reproducibility were evaluated.

In the intra-assay test, we performed T/S measurement for 8 genomic DNA samples. The samples were simultaneously assayed in heptaplicate repeats in two runs. The intra-assay mean value ranges of the coefficient of variation from the results of the different DNA sample assays were 1.9–6.9%.

In the inter-assay test, we repeated the T/S measurements for the same 8 samples in heptaplicate repeats on the 2nd and 3rd days. The inter-assay mean value ranges of the coefficient of variation from the results of the different DNA samples assays were 3.4–14.8%.

2.4. Statistical analysis

Data are presented as percentages for categorical variables and means for continuous variables. Between-group comparison of continuous variables was performed using analysis of variance (ANOVA) and analysis of covariance (ANCOVA) was performed to exclude potential confounding by age, body mass index, waist circumference and subcutaneous fat measured by ultrasound. To reach Gaussian distribution in the final model, the square root of telomere length was used.

Table 1
Clinical characteristics and lipid profiles of women in 3PMFs.

Clinical characteristic	3PMFs
N	783
Age (years)[a]	50.1 ± 3.3
BMI (kg/m^2)[a]	26.0 ± 4.9
WHR [a]	0.84 ± 0.10
Diabetes mellitus (N/%)	22/2.4
Hypertension (N/%)	271/29.9
Smoking prevalence (N/%)	267/29.4
Relative telomere length[a]	0.88 ± 0.43
Cholesterol (mmol/L)[a]	5.58 ± 0.90
Triglycerides (mmol/L)[a]	1.35 ± 0.79
HDL-cholesterol (mmol/L)[a]	1.63 ± 0.40

[a] Result presented as median, BMI — body mass index, WHR — waist–hip ratio, HDL— high density lipoprotein.

3. Results

Frequencies of the genotypes in our study (GG = 18.3%, GT = 49.1% and TT = 32.6%) are similar to the other Caucasian populations analysed so far and are within the Hardy–Weinberg equilibrium (P = 0.95).

Out of the 908 individuals included in 3PMFs, both the *FTO* genotype and the valid relative telomere length data were available in 783 individuals (86.2%).

Neither *FTO* variant rs17817449 (data not shown in details) nor telomere length (Table 2) was significantly associated with BMI, waist circumference, subcutaneous fat, plasma lipids or prevalences of smoking, type 2 diabetes or hypertension.

There was a borderline significant difference (P = 0.056, using square root P = 0.087) if the relative telomere length was analysed in co-dominant manner (GG vs. GT vs. TT genotypes). More detailed analysis, however reveals the dominant effect of the *FTO* variability at the first intron on the relative telomere length. Namely, the carriers of at least one risky G allele have a significantly shorter relative telomere

Table 2
The relative telomere length in individuals with different smoking, diabetes and hypertension status; values expressed as mean (SD).

Groups under study	Telomere length	P value
GG + GT variants vs	0.85 (0.39)	0.016
TT variant	0.92 (0.48)	
Smokers vs	0.83 (0.36)	0.08
non smokers	0.89 (0.45)	
Diabetics vs	0.83 (0.39)	0.14
non diabetics	0.88 (0.44)	
Hypertensive vs	0.89 (0.48)	0.87
non hypertensive	0.86 (0.41)	

length in comparison to the TT homozygotes (0.85 ± 0.39 vs. 0.93 ± 0.48; P = 0.016, square root P = 0.027). After standardization for age, BMI, waist circumference and subcutaneous fat this significance even increased (P = 0.009, square root P = 0.017, Table 3).

4. Discussion

The major finding of our study is the so far undescribed association between the *FTO* genotypes and the telomere length. Carriers of at least one risky G allele have a significantly shorter relative telomere length in comparison to the TT homozygotes, independently on body mass index and/or other markers for obesity. The detected association could at least partially explain the mechanism of how *FTO* variants could affect the risk of the development of a couple of non-communicable diseases. Variants within the first intron of the *FTO* gene were primarily recognised as a risk factor of obesity development [19–21]. Short time after, *FTO* variants were described to be, independently on BMI, associated also with cardiovascular disease [6,22], cancer [23,24], diabetes mellitus type 2 [25,26], end stage renal disease [27] and total mortality [28,29]. Despite an intensive research, the underlying mechanism remains unclear. First experiments were focused on the possible effect on dietary habits (revised by [30]), but recent publications suggest that the FTO is more likely a regulatory protein, exhibiting low DNA demethylase activity [31] and functioning like a possible transcriptional co-factor [32].

Another possibility on how to influence independently and simultaneously a wide spectrum of diseases is a potential effect on cell/biology ageing. This could be estimated by the analysis of the telomere length.

Shorter telomeres in white blood cells were detected, in comparison with the healthy controls, in patients with cardiovascular disease [33,34], and the role of telomere shortening in the development of further diseases (for example cancer, kidney failure or diabetes mellitus type 2) is discussed [35–38].

The positive findings summarised in the above mentioned studies could be discussed within the recently widely used approach of Mendelian randomisation. This approach was used recently to access a causality of plasma C-reactive protein (CRP) levels and cardiovascular disease [39]. On almost 200,000 participants, a clear link between CRP SNPs and plasma CRP levels on one side and CRP levels and CVD on the second side was detected. However, the risk ratios for CVD per additional copy of an allele associated with raised C reactive protein were almost identical, and thus, the "third line of the Mendelian randomisation triangle" was missing. This result indicates that CRP concentration itself is unlikely to be a causal factor in CVD [39].

Table 3
The relative telomere length grouped by *FTO* variant rs17817449.

	GG	GT	TT	P
Co-dominant model				
N(%)	139 (17.7)	380 (48.5)	264 (33.7)	
Mean	0.85 ± 0.39	0.85 ± 0.39	0.92 ± 0.48	0.055
M.delog	0.90 ± 0.22	0.90 ± 0.22	0.93 ± 0.24	0.086
	GG + GT		TT	P
Dominant model without adjustment				
Mean	0.85 ± 0.39		0.92 ± 0.48	0.016
M.delog	0.90 ± 0.22		0.93 ± 0.23	0.027
Dominant model, adjustment for BMI, age, waist circumference and subcutaneous fat				
Mean	0.84 ± 0.019		0.93 ± 0.026	0.009
M.delog	0.89 ± 0.010		0.93 ± 0.013	0.017

The independent findings that *FTO* variants and telomere length are associated with a variety of non-communicable diseases, do not necessarily mean that there is a functional causality among them. But, our results link the *FTO* genetic variability, the relative telomere length and the disease risks in expected directions within the Mendelian randomisation triangle.

Further experiments are necessary in order to determine, the mechanism of the causality between the *FTO* variant and the relative telomere length.

In summary, we have identified an association between the *FTO* 1st intron tagging polymorphism and the relative telomere length in a group of adult females in menopause. Whether this effect represents the causality between the *FTO* and the enhanced risk of non-communicable diseases, needs to be further examined.

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FTO and *MC4R* gene variants determine BMI changes in children after intensive lifestyle intervention

L. Zlatohlavek [a], M. Vrablik [a,*], E. Motykova [a], R. Ceska [a], L. Vasickova [b], D. Dlouha [c], J.A. Hubacek [c]

[a] 3rd Department of Medicine, 1st Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic
[b] Dr. Filip's Institute for Children, Poděbrady, Czech Republic
[c] Institute for Clinical and Experimental Medicine, Prague, Czech Republic

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ABSTRACT

Objectives: This study aimed to determine whether there is a relationship between common *FTO* (rs17817449) and *MC4R* (rs17782313) gene variants and body mass reduction or weight loss after a one-month lifestyle intervention in overweight/obese children.

Design and methods: We genotyped 357 unrelated non-diabetic Czech children (age 13.7 ± 4.9 years, average BMI at baseline 30.8 ± 4.6 kg/m^2). Biochemical and anthropometrical measurements were performed before and after 4 weeks of lifestyle interventions (comprising a reduction in energy intake to the age-matched optimum and a supervised exercise program consisting of 5 exercise units per day, 50 min each).

Results: The mean weight loss achieved was 6.2 ± 2.1 kg ($P<0.001$). Significant associations were found between a BMI decrease and the *FTO* and *MC4R* variants. Carriers of the *FTO* GG genotype and/or *MC4R* CC genotype lost significantly more body weight compared to noncarriers ($P<0.0009$ for BMI and $P<0.002$ for body weight). These differences remained significant following adjustment for sex, age and baseline values ($P=0.004$ for BMI and $P=0.01$ for body weight).

Conclusions: *FTO* and *MC4R* gene variants modify the impact of an intensive lifestyle intervention on BMI decrease in overweight/obese children. Carriers of the *FTO* GG genotype and *MC4R* CC genotype benefit significantly more from the lifestyle intervention.

Introduction

Over the past twenty years we have witnessed a steeply increasing prevalence of obesity that occurs not just in adults but also in children. The latest surveys estimate that approximately 20% of children are overweight in developed countries and some 10% are overweight worldwide [1]. Similarly, in the Czech Republic, the prevalence of obese and overweight children is approximately 15% [2].

The reasons are widely discussed. In addition to a lack of physical activity (increased time watching TV, playing computer games, etc.), abundant energy intake (particularly a high proportion of processed foods and sweetened beverages) is to be blamed for this negative trend. However, there are also other factors, e.g., shorter sleeping times, higher indoor temperatures and/or the in-building environment, that play important roles [3,4]. Moreover, twin and family studies have suggested a surprisingly high heritability of obesity, ranging from 40 to 80% [5].

Many studies have shown that individuals do not respond uniformly to overfeeding or dietary/physical activity interventions. It is assumed that these differences have a significant genetic background, but the number of nutrigenetic and actigenetic studies that have been performed, particularly on children, is limited.

Variants within the *FTO* ("fat mass and obesity related gene", OMIM acc. No. 610966) and *MC4R* ("melanocortin 4 receptor", OMIM acc. No. 155541) genes, identified using the genome-wide association approach, are the most important genetic determinants of body mass index (BMI) recognised thus far.

The association between *FTO* polymorphisms within the first intron and BMI has been unambiguously reproduced in Caucasians [6]. Interestingly, variants within this gene are, independent of BMI, also associated with overall mortality [7], the risk of acute coronary syndrome [8], end-stage renal disease [9] and cancer [10].

Abbreviations: APO A, apolipoprotein A; APO B, apolipoprotein B; BMI, body mass index; *FTO*, fat mass and obesity associated protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *MC4R*, melanocortin receptor 4; OMIM, online Mendelian inheritance in man; TGs, triglycerides.

* Corresponding author at: 3rd Department of Medicine, 1st Faculty of Medicine, Charles University and General Teaching Hospital, U Nemocnice 1, Prague 128 00, Czech Republic. Fax: +420 22496 6677.
E-mail addresses: l.zlato@tiscali.cz (L. Zlatohlavek), vrablikm@seznam.cz (M. Vrablik), e.motykova@gmail.com (E. Motykova), richard.ceska@vfn.cz (R. Ceska), L.Vasickova@seznam.cz (L. Vasickova), dadl@ikem.cz (D. Dlouha), jahb@ikem.cz (J.A. Hubacek).

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The *MC4R* gene encodes a widely expressed G-protein-coupled receptor that binds the α-melanocyte stimulating hormone. It has been demonstrated that *MC4R* is an important regulator of energy homeostasis with the potential to influence both food intake and energy expenditure through melanocortin neuronal pathways [11]. The rs17782313 polymorphism within the *MC4R* gene has been recognised as an important determinant of BMI in humans [12].

Based on the summarised results, both *FTO* and *MC4R* genes may modify the magnitude of BMI changes after dietary/exercise intervention in adults. However, studies on the association between genetic background and lifestyle intervention are prone to confounding factors (e.g., due to difficulties in diet and physical activity standardisation, medication use, concomitant diseases). Therefore, we decided to test the effects of common variants within the *FTO* and *MC4R* genes on anthropometric and biochemical parameters in a large group of obese children who underwent a standardised, inpatient, four-week lifestyle modification program aimed at weight loss.

Patients and methods

Patients

Between June 2009 and March 2011, 357 individuals agreed to participate in the study. For 343 (96.1%) individuals, a complete data set and DNA samples were available. Overweight/obese children between 8 and 15 years of age were considered eligible for the study. Overweight was defined as a BMI at or above the 85th percentile and lower than the 95th percentile for children of the same age and sex. Obesity was defined as a BMI at or above the 95th percentile for children of the same age and sex.

Exclusion criteria comprised known diabetes mellitus, arterial hypertension, smoking, use of any medications and contraindication of prescribed physical activity. All participants were of Caucasian ethnicity, and written informed consent was given by the parents/guardians of all individuals. The study was approved by the Institutional Ethics Committee and conducted according to Good Clinical Practice guidelines.

Intervention

The participants were enrolled in an intensive, one-month, inpatient weight reduction program. The program comprised individualised dietary changes made after a thorough nutritional assessment by an experienced dietician to achieve a caloric intake of 5000 kJ for the age category of 8 to 10 years old and 7000 kJ for those 11 to 15 years old. All participants were assessed by an exercise specialist, and five units (50 min each) of daily, supervised physical activity (endurance training with a heart rate of 65–75% of the maximum measured at the beginning of the intervention program) were prescribed [13]. The exercise program comprised aerobic and resistance training complemented with ball games, swimming, dancing and fast walking. All children participated in all sessions, and the proportion of each activity was identical regardless of initial BMI, and age or sex of the participants.

Anthropometric measurements

All participants underwent a thorough physical examination. Body weight was measured with a horizontally placed and calibrated electronic weight scale (scaled to the nearest 100 g). Height was measured to the nearest 0.5 cm. Waist (defined as the narrowest diameter between the xiphoid process and iliac crest) and hip (defined as the widest diameter over the greater trochanters) circumferences were also measured with an accuracy of 0.5 cm. BMI (kg per m^2) was calculated from obtained measurements. Diastolic and systolic blood pressures were measured after 10 min in a sitting position using the average of 3 readings on the right arm with an automated blood pressure unit (Automated sphygmomanometer BP-203 NA, Nippon Colin co., Ltd). All measurements were performed by the same experienced nurse to eliminate inter-individual variability.

The total body fat was determined by impedance analysis using a Bodystat analyser (1500 MDD, Bodystat, Isle of Man. UK). Skinfold thickness was measured by calliper BEST II K-501. This measurement was performed in three areas: subscapular area (in the projection of the angle of left scapula), triceps area (in the middle between the acromion and the elbow) and abdominal area (1/3 of the distance between the navel and the left anterior superior iliac spine).

Biochemical analyses

Venous blood was collected after 12 h of fasting, and plasma lipid levels together with glycaemia were assessed by enzymatic methods using automated analysers (Hitachi, Japan). LDL-cholesterol level was calculated by the Friedewald equation [LDL-C = TC − (HDL-C) − TG/2.2].

Genetic analyses

DNA was isolated using the standard salting-out method [14]. Genotyping of the *MC4R* rs17782313 variant was performed by PCR using oligonucleotides 5‘ AAG TTC TAC CTA CCA TGT TCT TGG and 5‘ TTC CCC CTG AAG CTT TTC TTG TCA TTT TGA T followed by the treatment with the restriction enzyme BclI (an uncut PCR product of 137 bp represents allele C, while restriction fragments of 30 bp and 107 bp represent allele T). For PCR-RFLP analysis of the rs17817449 variant within the gene for *FTO*, the oligonucleotides 5‘ GGT GAA GAG GAG GAG ATT GTG TAA CTG G and 5‘ GAA GCC CTG AGA AGT TTA GAG TAA ATT GGG were used followed by the treatment with the restriction enzyme AlwNI (an uncut PCR product of 198 bp represents allele G, while restriction fragments of 99 bp and 99 bp represent allele T). To ensure the accuracy of these methods for genotyping, one plate containing 94 samples was genotyped twice within one week with 100% conformity.

Statistical analyses

The chi-square test, ANOVA and ANCOVA (adjusted for age, sex and baseline values for each variable) were used for statistical analysis. All tests were two-tailed, and the significance level 2 alpha = 0.05 was considered significant.

Results

Anthropometric and biochemical measurements

As expected, there was a significant positive change in all anthropometrical parameters of interest after the intervention. Not only the average BMI decreased significantly, but also the waist circumference, suggesting a reduction in visceral fat mass. This was accompanied by a significant reduction in total and LDL-cholesterol and triglycerides and a significant increase in HDL-cholesterol (Table 1). All of these changes were more pronounced in girls than in boys, but all changes were significant when girls and boys were analysed separately or after the adjustment for sex, age and baseline values.

DNA analysis

In the subgroup with a complete dataset, the call rates of *FTO* rs17817449 and *MC4R* rs17782313 variants were 96.8% and 95.6% respectively. The Hardy–Weinberg test confirmed the independent segregation of individual genotypes (P = 0.38 for *FTO* and P = 0.37 for *MC4R*). No gender differences in genotype distribution were observed.

In the entire population, the allelic frequencies of individual polymorphisms were comparable with previously published frequencies obtained in other Caucasian populations (Table 2).

Table 1
Characteristics of the study participants before and after intervention (mean ± SD).

(n = 357)	Basal	After 4 weeks	Crude P	Adjusted P*
Age, years	13.1 ± 1.9			
Boys/girls	139/210			
BMI	30.8 ± 4.4	28.4 ± 4.3	0.001	0.015
Weight, kg	80.7 ± 19.9	74.5 ± 18.4	0.001	0.001
Waist, cm	89.9 ± 11.7	84.1 ± 11.2	0.001	0.046
Hip, cm	103.4 ± 11.4	97.7 ± 11.3	0.001	0.001
Total body fat, %	31.3 ± 12.7	28.7 ± 12.0	0.001	0.007
Glycaemia, mmol/l	4.97 ± 0.42	4.95 ± 0.37	0.727	0.092
Total cholesterol, mmol/l	4.56 ± 0.95	3.62 ± 0.79	0.001	0.001
TGs, mmol/l	1.06 ± 0.52	0.82 ± 0.32	0.001	0.013
HDL-C mmol/l	1.29 ± 0.28	1.13 ± 0.24	0.001	0.891
LDL-C mmo/l	2.79 ± 0.79	2.11 ± 0.68	0.001	0.001
APOB, mmol/l	0.89 ± 0.25	0.72 ± 0.22	0.001	0.071
APOA mmol/l	1.31 ± 0.18	1.13 ± 0.18	0.001	0.739
Insulin	15.5 ± 8.0	11.8 ± 6.9	0.001	0.945
Sub. fat—subscapular area	2.28 ± 0.88	2.12 ± 0.76	0.001	0.375
Sub. fat—triceps area	2.51 ± 0.87	2.03 ± 0.65	0.001	0.366
Sub. fat—abdominal area	3.90 ± 1.01	3.25 ± 0.71	0.001	0.665

P* is a P value for differences between boys and girls adjusted for age and baseline level of the individual parameter.

It should be mentioned that the allelic frequencies were not compared to the appropriate control group of age-matched healthy lean children. However, when compared with the representatively selected group of Czech adults (post-MONICA study, 1191 males and 1368 females, aged 25–64 years, mean age 49.0 ± 10.7 years) a higher frequency of the *FTO* GG genotype was detected (24.9% vs. 17.3%, P = 0.0005) [15]. The distribution of the *MC4R* genotypes (Hubacek, unpublished data) was not different between the child and adult populations (P = 0.06).

At baseline, neither polymorphism exhibited a significant effect on obesity-related parameters (BMI, body weight, WHR), but as expected, elevated initial BMI values were detected in carriers of the *FTO* GG genotype and *MC4R* CC genotype (in the case of *MC4R*, the observed difference between homozygotes was almost 2 BMI units). This lack of effect is easily explained; probands represent a relatively narrow part of the population (obese and overweight individuals only) and extreme BMI values could mask the real effect of both polymorphisms, which is estimated to be, in absolute numbers, 1.5–2.0 kg of body weight in adults per one risky allele.

The *FTO* variant was a significant determinant for the spa intervention. Carriers of the GG genotype lost more (P = 0.02 for BMI and P = 0.04 for body weight, respectively, P = 0.05 for BMI and P = n.s. for body weight after adjustment) body weight (6.7 ± 2.3 kg, 2.52 ± 0.75 BMI units) than carriers of at least one T allele (6.0 ± 2.1 kg, 2.25 ± 0.69 BMI units).

In absolute numbers, even stronger effects on the BMI were observed if the *MC4R* variant was analysed. Here, carriers of the CC genotype lost more (P = 0.004, P = 0.03 after adjustments) BMI units (2.8 ± 0.9 BMI units) than carriers of at least one T allele (2.3 ± 0.7 BMI units).

Table 2
Distribution of the individual genotypes and their combinations in analysed children.

			MC4R					
			CC		CT		TT	
		N (%)	N	%	N	%	N	%
			21	6.0	144	41.3	184	52.7
			21	6.0	144	41.3	184	52.7
FTO	GG	87 (24.9)	4	1.2	34	9.7	49	14.0
	GT	182 (52.1)	11	3.2	79	22.6	92	26.4
	TT	80 (22.9)	6	1.7	31	8.9	43	12.3

Table 3
Effect of the *FTO* and *MC4R* polymorphisms on BMI and body weight loss. Unadjusted P values are given.

	Δ kg	Δ BMI	N	P kg	P BMI
FTO GG	6.7 ± 2.3	2.5 ± 0.8	87	0.04	0.02
FTO GT + TT	6.0 ± 2.1	2.2 ± 0.7	162		
MC4R CC	4.2 ± 3.6	2.8 ± 0.9	21	0.03	0.0004
MC4R CT + TT	3.1 ± 2.6	2.3 ± 0.7	328		
FTO GG/*MCR4* CC	7.4 ± 2.3	3.9 ± 1.3	4	n.s.	n.s.
FTO TT/*MC4R* TT	5.7 ± 2.1	2.1 ± 0.6	43		
FTO GG and/or *MCR4* CC	6.7 ± 2.2	2.5 ± 0.7	98	0.002	0.0009
FTO GT + TT and/or *MCR4* CT + TT	5.9 ± 2.1	2.2 ± 0.7	231		

In the case of *MC4R*, the effect on BMI is further supported by the finding that carriers of this genotype showed the largest decrease in total fat as estimated by Bodystat measurements (4.2 ± 3.6 kg vs. 3.1 ± 2.6 kg, P = 0.03).

The changes in other analysed parameters (plasma lipids, CRP etc.) were not associated with either the *FTO* or *MC4R* polymorphisms (details not shown).

FTO–MC4R interactions

If all nine possible combinations of *FTO* and *MC4R* genotypes were analysed (and all nine were also detected), the largest decrease in BMI values was obtained in carriers of the *FTO* GG/*MC4R* CC combination (Δ−3.9 ± 1.3 BMI units), whereas the *FTO* TT/*MC4R* TT combination exhibited the lowest BMI decrease (Δ−2.1 ± 0.6 BMI units). Unfortunately, there were only 4 carriers of the advantageous combination compared to 43 individuals with the "resistant to intervention" combination and the difference remained insignificant.

Extended analysis of genotype combinations revealed that carriers of at least one obesity-associated genotype (N = 98) either in *FTO* (GG) or *MC4R* (CC) genes lowered their body weight (Δ−6.7 ± 2.2 kg) and BMI (Δ−2.5 ± 0.7 units) more efficiently (P = 0.002 for body weight and P = 0.0009 for BMI, after adjustment P = 0.01 for both body weight and BMI) than non-carriers (N = 231; body weight Δ−5.9 ± 2.1 kg; BMI Δ−2.2 ± 0.7 units) (Table 3).

Discussion

For the first time, we report an important role for the common *FTO* and *MC4R* gene variants in determining body weight loss after an intensive lifestyle intervention in children/adolescents. After four weeks of focused lifestyle modification in children under close supervision within an inpatient facility (consisting of five units of exercise per day and a change in dietary habits to the age- and sex-matched optimums), we observed a significant decrease in most of the analysed anthropometric and biochemical parameters. Interestingly, a rather large interindividual variability in response to the interventions has been documented: the highest body weight decrease observed was 14.4 kg in contrast to the lowest decrease, which was only 1.8 kg. Although the study was designed to minimise heterogeneity in exposure to the intervention, the observed differences do not necessarily and solely reflect genetic predisposition but could be a result of variable adherence to the intervention program.

Conversely, it is unlikely that the observed differences between the carriers of different *FTO* and/or *MC4R* genotypes could be fully explained by this confounding result.

Previous interventional studies on *FTO* rs1717817449 and *MC4R* rs17782313 polymorphisms have been focused mainly on adults. In the case of the *FTO* variant, the results are far from consistent (reviewed by Dlouha et al. [16]), and most studies have failed to detect an important role for *FTO* variants. Some of the studies performed in children were summarised by Rendo et al. [17]. Overall, these

studies have also confirmed the importance of variants within the first intron of the *FTO* gene on BMI in children. Over 40 variants have been found in this region in varying degrees of linkage disequilibrium, although rs1781744 has been shown to be among the most significantly associated with BMI and is putatively functional [18]. However, no effect of the *FTO* variant on body weight/BMI change was observed after interventions. Thus, our study is the first to detect an important role for *FTO* in determining BMI changes after lifestyle intervention. This difference is most likely explained by the different protocols used. In our study, the children were exposed to high-intensity training under the well-defined conditions of an inpatient facility. Moreover, our study included three hundred and fifty examined individuals and therefore ranks among the largest paediatric studies ever performed on the topic.

Previous studies have shown that the impact of *FTO* variants on BMI is similar regardless of the baseline BMI values — each "obesity-associated" allele was associated with a 1.5 to 2 kg increase in body weight. Differences in energy intake [19], level of physical activity [20] or basal metabolic rate [21] in individuals with distinct *FTO* genotypes have been suggested (but not unambiguously proven) to mediate the effect of *FTO* on BMI. The fact that carriers of obesity-associated alleles in our study group showed better responsiveness to lifestyle intervention suggests that the effect of *FTO* on BMI is not mediated by the determination of energy intake or physical activity level.

Even fewer studies have followed the effects of *MC4R* gene polymorphisms on body weight changes after intervention. In German adults, no impact of the rs17782313 variant on changes in body weight or fat distribution was detected [22]. To date, no study has been performed on children/adolescents.

Our results suggest that the rs17817449 variant within the *FTO* gene and the rs17782313 variant within the *MC4R* gene have the potential to significantly influence the response to regular physical activity in children, assuming an optimised energy intake.

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Addiction

Letters to the Editor

The journal publishes both invited and unsolicited letters.

FAT MASS AND OBESITY-ASSOCIATED (FTO) GENE AND ALCOHOL INTAKE

In a recent issue of *Addiction*, Sobczyk-Kopciol *et al.* [1] reported an association between the first intron tagging variant rs9939609 in the obesity-predisposing fat mass and obesity-associated (*FTO*) gene (OMIM no. 610966) and both alcohol consumption and alcohol dependence. Interestingly, the genotype generally associated with elevated risk of obesity [2], acute coronary syndrome [3], some types of cancer [4], end-stage renal disease [5] or overall mortality [6] [in all cases at least partially independently on body mass index (BMI)] was associated with lower ethanol consumption, and was less common in alcohol-dependent individuals compared to healthy controls.

Despite the large number of individuals included (6584), an important limitation of the original study [1] is the lack of the confirmatory study. Even large studies are prone to type I error (false positive), and their results need to be replicated [7,8].

To confirm the results by Sobczyk-Kopciol *et al.* [1], we analysed self-reported alcohol intake (recorded in a standardized interview) and the first intron tagging single nucleotide polymorphisms (SNPs) (rs17817449 or rs9939609; both occur in almost 100% linkage disequilibrium [9]) in three large independent cohorts. We used the Czech post-MONItoring of trends and determinants in Cardiovascular disease (MONICA) study (2559 individuals, examined twice within 3 years) [9], the Czech part of the Health, Alcohol and Psychosocial factors In Eastern Europe (HAPIEE) study (6681 individuals) [10], and a study of the self-contained population of 948 Sorbs in Germany [11]. Finally, 201 Czech patients with alcoholic liver cirrhosis were also genotyped. Data were analysed using logistic regression with the additive mode of inheritance and adjustment for age and gender.

The *FTO* genotype and allele frequencies did not differ significantly between our three cohorts (0.42–0.43 of the minor allele) and the study by Sobczyk-Kopciol *et al.* [1] (0.45). We detected no significant effect of rs9939609 or rs17817449 on alcohol intake in any of the studies; the *P*-values were 0.47 (0.51 in the second survey) in the Czech post-MONICA study, 0.37 in the Czech HAPIEE study and 0.82 in the Sorbs study. For example, in the Czech HAPIEE study, the largest of our replication samples, the mean (± standard deviation) alcohol intakes (in grams) in the last week in CC, CT and TT carriers were 174 (±201), 187 (±214) and 177 (±184), respectively ($P = 0.92$), in males and 38 (±70), 41 (±72) and 43 (±77), respectively ($P = 0.12$), in females. There were no statistically significant differences in the frequencies of the individual *FTO* genotypes in pooled data of the Czech cohorts ($n = 9148$, CC = 18.7%, CT = 48.3%, TT = 33.0%) and in patients with alcoholic cirrhosis ($n = 201$, CC = 13.9%, CT = 53.7%, TT = 32.3%), $P = 0.16$, although the pattern of results was similar to the results by Sobczyk-Kopciol *et al.* [1].

In summary, we were unable to replicate the association between the *FTO* 1st intron tagging SNPs and alcohol consumption in any of the confirmatory samples. We conclude that 1st intron *FTO* tagging SNPs are unlikely to be major and general genetic determinants of total alcohol intake/alcohol consumption.

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Declarations of interest

None.

JAROSLAV A. HUBACEK[1], VERA ADAMKOVA[1], DANA DLOUHA[1], MILAN JIRSA[1], JAN ŠPERL[1], ANKE TÖNJES[2], PETER KOVACS[2], HYNEK PIKHART[3], ANNE PEASEY[3] & MARTIN BOBAK[3]

Institute for Clinical and Experimental Medicine, Videnska 1958/9, Prague 4, 14021, Czech Republic[1], *Medical University of Leipzig, Leipzig, Germany*[2] *and Department of Epidemiology and Public Health, University College London, London, UK.*[3]

E-mail: jahb@ikem.cz

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Brief Report

Lack of an Association Between Three Tagging SNPs Within the *FTO* Gene and Smoking Behavior

Jaroslav A. Hubacek,[1,2,3] *Dana Dlouha,*[2,3] *Vera Lanska,*[4] *& Vera Adamkova*[3,5]

[1] *Department of Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czech Republic*
[2] *Centre for Cardiovascular Research, Prague, Czech Republic*
[3] *Faculty of Health and Social Studies, University of South Bohemia, Ceske Budejovice, Czech Republic*
[4] *Statistical Unit, Institute for Clinical and Experimental Medicine, Prague, Czech Republic*
[5] *Department of Preventive Cardiology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic*

Corresponding Author: Jaroslav A. Hubacek, *Institute for Clinical and Experimental Medicine, Department of Medicine, Videnska 1958/9, 14021 Prague 4, Czech Republic. Telephone: +420-261-363-367; Fax: +420-241-721-574; E-mail: jahb@ikem.cz*

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Abstract

Introduction: Using genome-wide screening, a polymorphism within the second intron of the *FTO* gene (rs2302673) was found to be associated with smoking habits in females. In a population-based, cross-sectional study, we analyzed three tagging *FTO* single-nucleotide polymorphisms (SNPs) for their association with smoking behavior.

Methods: Subjects from the Czech post-MONICA study, including 1,191 adult males (32.1% smokers) and 1,368 adult females (22.5% smokers) were included in this study. Smoking habits were obtained through questionnaire data analysis, and three *FTO* tagging SNPs were genotyped (rs17817449: intron 1, rs2302673: intron 2, and rs17818902: intron 3).

Results: We detected slightly lower frequencies ($p = .043$) of the GG genotype of the rs17818902 SNP in males who quit smoking compared with others. However, the significance disappeared after adjusting for multiple testing. Within the entire population, or in either males or females alone, we failed to detect a significant difference between other *FTO* genotypes and smoking status. Also, the number of cigarettes smoked per day was independent of individual *FTO* genotypes in both genders.

Conclusions: We did not find an association between the *FTO* gene tagging variants and smoking status. *FTO* is unlikely to be a major genetic determinant of smoking status.

Introduction

Cigarette smoking is the most common form of tobacco use and is a major preventable cause of total mortality around the world (Bergen & Caporaso, 1999). The environmental influences on tobacco smoking are known, and twin studies provide evidence that smoking has a significant hereditary component (Li, 2006; Sellman, 2010). The analysis of genetic determination of nicotine dependence is extremely difficult; indeed, the genetic components differ according to the tests/characteristics used (e.g., Fagerström Test for Nicotine Dependence, Heaviness of Smoking Index, Diagnostic Nicotine Dependence) and because there are significant gender differences for smoking initiation, persistence, and ability to quit smoking (Munafò & Johnson, 2008).

Recent genome-wide association studies have identified novel genes and particular single-nucleotide polymorphisms (SNPs) associated with smoking phenotypes/nicotine dependence. Among them, the *FTO* gene ("fat mass and obesity related gene"; OMIM accession number: 610966), which has been suggested to function as a transcriptional cofactor (Wu, Saunders, Szkudlarek-Mikho, Serna Ide, & Chin, 2010) having DNA demethylase activity (Gerken et al., 2007), was identified as being among the top 14 candidate genes whose SNPs are responsible for nicotine dependence (Bierut et al., 2007).

Although identified over a decade ago, *FTO* has attracted more recent attention due to its recognition as an obesity predisposing gene (Frayling et al., 2007). Its association with body mass index (BMI) was widely reproduced; however, variants within this gene, independent of BMI, were also associated with overall mortality (Zimmermann et al., 2009), acute coronary syndrome (ACS; Hubacek et al., 2010), or cancer (Brennan et al., 2009). The functional link between these associations remains unknown. A possible *FTO* effect on smoking behavior, as suggested in a study by Bierut et al. (2007), could be one of the plausible explanations. In this study, the rs2302673 SNP within the *FTO* gene was associated with smoking dependence, and this association was more pronounced in females than in males.

To extend the original data, a population-based study was performed. First, we assessed the genotype frequencies of the *FTO* rs2302673 (intron 2) variant within the Czech population. Second, we assessed the prevalence of this and another two tagging *FTO* SNPs, rs17817449 (intron 1; Frayling et al., 2007) and rs17818902 (intron 3; Tönjes et al., 2010), in groups defined

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according to smoking behavior (i.e., smokers, never-smokers, past smokers, and number of cigarettes smoked per week in smokers) to analyze their potential in determination of smoking status.

Methods

We have analyzed the three *FTO* tagging SNPs in two groups of representatively selected individuals, including 1,191 males and 1,368 females, aged 25–64 years (mean: 49.0 ± 10.7 years) at the first examination. All individuals took part in the post-MONICA study (Cífková et al., 2010). The WHO MONICA Project protocol (Tunstall-Pedoe, Kuulasmaa, Tolonen, Davidson, & Mendis, 2003) was created to examine risk factors of cardiovascular disease development, including smoking. The subjects, representing 9 Czech districts, were examined in 1997/1998 and re-examined in 2000/2001. Data on smoking status and number of cigarettes consumed per day were collected by self-completed questionnaires and were available from both examinations. Never-smokers were self-identified individuals who smoked less than five lifetime packages. Written informed consent was given by all individuals. The study was approved by Institutional Ethics Committee.

Genomic DNA was extracted from peripheral blood white cells (Miller, Dykes, & Polesky, 1988). *FTO* SNPs, rs17817449 and rs17818902, were analyzed as previously described (Dlouha, Adamkova, Lanska, & Hubacek, 2010; Hubacek, Pitha, Adamkova, Lanska, & Poledne, 2009). Rs2302673 was genotyped using the nested polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis. All PCR chemicals and restriction enzymes were purchased from Fermentas International, Inc. (Burlington, Ontario, Canada). PCR reactions were performed using the MJ Research DYAD Disciple thermal cycler. Briefly, DNA was amplified in a total volume of 25 µl using the oligonucleotide primers 5′ATGTACTCATGCCAACAGGCTACTTG and 5′TTCCAAGTGTCTGACTTATGATGTG. One microliter of diluted PCR product (diluted with sterile water in a ratio of 1:19) was used for the nested PCR reaction (primers: 5′TAGAAAACTGCTGTTGAGAGCAGC and 5′TTAAATAGACTTTAAACATCGA) in a total volume of 25 µl. The final PCR product (93 basepairs [bp]) was cut using 5 units of *Cla*I restriction enzyme, and restriction fragments were separated on a 10% polyacrylamide gel by microtiter array diagonal gel electrophoresis (Day & Humphries, 1994). Allele A was represented by restriction fragments of 76 and 17 bp, while the presence of uncut product represented allele G.

Deviations from Hardy–Weinberg (HW) equilibrium were tested using the online HW calculator (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls). Differences in the genotype frequencies between the groups were assessed by the chi-square test in dominant, codominant, and recessive models for both years of examination. In Table 1, the *p* values for all three unadjusted models at first examination are given. The number of cigarettes smoked per day, in association with individual genotypes, was analyzed by ANOVA in dominant, codominant, and recessive models for both years of examination. In Table 2, numbers and *p* values for the codominant model at first examination are given.

Results

There were 32.1% current and 27.6% past smokers in the male cohort and 22.5% current and 13.8% past smokers in the female cohort at the 1998/1999 examination. The mean number of cigarettes smoked per day was 15.7 ± 8.7 for males and 11.3 ± 6.4 for females. Tobacco pipe smoking and tobacco chewing were not documented in our sample. These data were almost identical at the time of the second survey (data not shown).

Table 1. Smoking Habits and *FTO* Variants

	Total population						Males						Females					
	Smokers		Past smokers		Never-smokers		Smokers		Past smokers		Never-smokers		Smokers		Past smokers		Never-smokers	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Rs17817449																		
GG	107	16.0	100	20.1	220	16.8	54	14.8	57	18.4	66	14.9	53	17.5	43	22.8	154	17.8
GT	335	50.3	238	47.8	654	50.0	182	49.9	157	50.6	235	53.2	153	50.7	81	43.1	419	48.4
TT	225	33.7	160	32.1	434	33.2	129	35.3	96	31.0	141	31.9	96	31.8	64	34.1	293	33.8
p	.162		.454		.845		.353		.498		.426		.239		.399		.794	
Rs17818902																		
TT	444	67.3	337	67.5	875	66.5	251	68.8	207	65.7	314	69.3	193	65.4	130	70.7	561	65.0
TG	183	27.7	149	29.9	388	29.5	97	26.6	104	33.0	123	27.2	86	29.4	45	24.5	265	30.7
GG	33	5.0	13	2.6	53	4.0	17	4.6	4	1.3	16	3.5	16	5.4	9	4.9	37	4.3
p	.890		.314		.119		.547		.050		.043		.335		.490		.712	
Rs 2302673																		
AA	522	77.3	380	76.0	993	75.4	287	76.3	239	75.6	347	76.3	236	78.3	141	76.6	646	75.2
AG	139	20.6	112	22.4	296	22.5	78	20.7	71	22.5	98	21.5	61	20.3	41	22.3	198	23.1
GG	14	2.1	8	1.6	25	1.9	11	3.0	6	1.9	10	2.2	3	1.0	2	1.1	15	1.7
p	.681		.855		.839		.973		.895		.648		.475		.707		.583	

Note. Data are from the survey performed during 1998/1999. The uncorrected *p* values for genotype differences between the subgroups are displayed in the order of dominant/codominant/recessive models. After Bonferroni correction, no significant differences were observed.

Table 2. Number of Cigarettes Smoked per Day According to the *FTO* Genotype

	Population		Males		Females	
	N	No. cigarettes	*N*	No. cigarettes	*N*	No. cigarettes
Rs17817449						
GG	107	14.2 ± 8.2	54	16.0 ± 8.8	53	12.3 ± 7.2
GT	335	13.8 ± 8.6	182	16.0 ± 9.4	153	11.2 ± 6.6
TT	215	13.7 ± 7.4	129	15.4 ± 7.9	96	11.2 ± 5.8
p		.869		.844		.563
Rs17818902						
TT	444	13.9 ± 8.2	251	16.1 ± 8.8	193	11.2 ± 6.4
TG	183	13.7 ± 7.9	97	15.2 ± 8.6	86	11.9 ± 6.4
GG	33	14.8 ± 8.1	17	17.5 ± 7.9	16	11.6 ± 7.4
p		.708		.502		.727
Rs2302673						
AA	523	13.8 ± 8.1	287	15.8 ± 8.6	236	11.5 ± 6.6
AG	139	13.4 ± 8.5	78	15.6 ± 9.8	61	10.9 ± 5.7
GG	14	16.5 ± 8.6	11	16.4 ± 9.1	3	16.7 ± 7.6
p		.351		.948		.296

Note. Data are from the survey performed during 1998/1999. The uncorrected *p* values for genotype differences between the subgroups are displayed (all *p* values are not significant).

The genotype call rates were 96.4% for the rs17817449 SNP, 97.1% for the rs17818902 SNP, and 97.7% for the rs2302673 SNP. Genotype distributions of all three analyzed SNPs ($p = .37$, $p = .30$, and $p = .09$) were in Hardy–Weinberg equilibrium in the entire population.

Allelic and genotypic distributions (Table 1) of the individual SNPs were similar to previously published results, and the SNPs were not in linkage disequilibrium.

To determine whether the *FTO* SNPs are associated with smoking behavior, we compared the *FTO* genotype frequencies between current smokers, past smokers, and never-smokers (Table 1). We have not confirmed the previously described association between the rs2302673 SNP and smoking behavior. Furthermore, the genotype distributions of another two *FTO* tagging SNPs, rs17817449 and rs17818902, were similar in both past smokers and never-smokers. This was valid for both the male and female populations, with one exception.

We have detected a significantly lower frequency ($p = .043$) for the GG genotype (rs17818902) carriers in male past smokers when compared with current and never-smokers. This suggests that the carriers of this genotype could have certain problems quitting smoking. However, the difference is low, especially because of the very low frequency of this genotype in the population and, most importantly, significance disappeared after the adjustment (Bonferroni correction) for multiple testing.

Additionally, we pooled (a) current smokers and past smokers and compared them with never-smokers and (b) compared current and former smokers. These analyses did not reveal any significant differences between individual genotypes (data not shown).

As displayed in Table 2, in current smokers, the number of cigarettes smoked per day was also independent of different *FTO* genotypes, regardless of which polymorphism was analyzed. These negative results were observed in both surveys. Furthermore, the negative results were obtained regardless of the survey or type of analysis used (within the tables, data from the first survey [1997/1998] are presented, whereas data for the second survey [2000/2001] are not shown in detail, as they are almost identical).

Discussion

The high prevalence of smoking in the eastern and central European region has been well known for many years. The environmental causes of this phenomenon are a matter of debate, and information about the possible genetic causes is very sparse (Hubacek, Adamkova, Skodova, Lanska, & Poledne, 2004; Santos et al., 2008, Sieminska et al., 2009).

The *FTO* gene (namely the intron 2, rs2302673 variant) was recognized through a genome-wide association analysis to be a candidate gene for smoking dependence in the United States and Australian Caucasian populations (Bierut et al., 2007); however, the original finding was not confirmed or excluded by further studies. The most likely reason for this is that more attention was paid to *FTO* as a candidate gene for predisposition to obesity (Frayling et al., 2007) and other noncommunicable diseases (Brennan et al., 2009; Hubacek et al., 2010). Also, there were many more suitable candidate genes having a clear role in the pathophysiology of smoking/nicotine dependence described at this time. This includes, for example, the genes for nicotinic acetylcholine receptor subunits, nicotinic cholinergic receptors β3 and β6, nicotine-metabolizing enzymes CYP2A6, D2 dopamine receptor, opioid receptor, brain-derived neurothropic factor, and others (Thorgeirsson et al., 2010; Tobacco and Genetics Consortium, 2010). Even so, the individual differences in smoking habits could be based on differences in hundreds of genes, and only those with the most computational strength and importance have been detected thus far.

The fact that there is no clear link between the *FTO* gene and smoking behavior does not necessarily mean that this gene deserves less attention than others. Notably, the associations with DNA variants detected through the genome-wide association approach are often located within genes that lack a physiological link between the monitored parameter or pathology. *FTO* itself is the best example. The exact mechanism leading to the higher BMI/mortality/ACS prevalence associated with *FTO* genotypes remains unknown, despite data that appear in recent publications suggesting the role of FTO as a regulatory protein (Gerken et al., 2007; Wu et al., 2010).

We were not able to reproduce the original finding, suggesting that the *FTO* rs2302673 SNP is not implicated in the prevalence of smoking in a Slavonic population. Furthermore, we did not find any significant association between the other two tagging SNPs within introns 1 and 3 and self-reported smoking status or number of cigarettes smoked. The discrepancy in findings observed between the current study and that of Bierut et al. (2007) may be attributable to differences in the smoking-related phenotypes studied. Bierut et al. (2007) focused on nicotine dependence, while we examined smoking status and smoking quantity. Whereas daily cigarette consumption may be considered as a proxy for nicotine dependence, the association between the *FTO* variants and nicotine dependence

was not explicitly examined in this study; therefore, comparison with the Bierut et al. (2007) study should only be made tentatively. Quite recently (Sobczyk-Kopciol et al., 2011), an association was identified between the first intron *FTO* SNP (the second and third intron SNPs were not analyzed in this study) and the number of cigarettes smoked per day during the year of most intense smoking and age of smoking initiation.

Sample size and ethnic differences are likely not reasons for the different finding. Our study and the original study were performed on roughly the same number of individuals of the same ethnicity; however, we cannot exclude that there could be some differences in the effects of the gene variant in individuals of the same ethnicity but of different nationalities. We have already observed a similar pattern, for example, when analyzing the SNP within the third *FTO* intron (Dlouha et al., 2010) and BMI.

The relatively low power of our study could also be a reason why we were not able to detect some subtle effects of the *FTO* variants on smoking habits. On the other hand, we were not able to detect any similar trends in our results. Finally, it should be mentioned that in post-communist countries, the environment affecting smoking behavior does not discourage smoking enough and is likely the major reason why smoking prevalence is higher in this population. This fact could also influence the results significantly. Indeed, it could be hypothesized that due to a higher prevalence of smoking within the population, environmental causes (e.g., socioeconomic status, family history of smoking, and others) could contribute significantly more than the genetic predispositions.

In conclusion, we find that the association between the *FTO* genotypes and smoking may be definition dependent and that smoking does not likely represent a link between *FTO* genotypes and ACS or cancer.

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Declaration of Interests

There are no competing interests.

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PŘÍLOHA 10

BRIEF REPORT

FTO First Intron rs1558902 Variant and Platelets Count in White Middle-aged Women: Prague Pre- and Post-Menopausal Females (3PMFs) Study

Jaroslav A. Hubacek, PhD, DSc, Dana Dlouha, Vera Lanska, PhD, Petr Stavek, PhD, Libuse Pagacova, MD, PhD, Ivana Kralova-Lesna, MD, and Jan Pitha, MD, PhD

Abstract: The polymorphisms within the *FTO* gene play an important role in the genetic determination of body weight and body mass index and have been associated with cardiovascular disease, but the causal mechanism is still a matter of debate. The possible effect on the platelet count as a marker of hemocoagulation status as a possible cardiovascular risk factor was suggested in Japanese population. We have analyzed both rs1558902 *FTO* polymorphism (T > A) and platelet counts in the Prague Pre and Post Menopausal Females (3PMFs) study, including those of 669 women (mean age, 55.7 ± 2.7 years). The frequencies of the *FTO* genotypes were similar to other populations (TT, 30.4%; TA, 48.1%; and AA, 21.5%). We have not detected a significant association between the *FTO* rs1558902 variant and platelet counts in white women (TT, 242 ± 55 × 10^9; TA, 246 ± 67 × 10^9; and AA, 247 ± 55 × 10^9; F[2.642] = 0.30, P = 0.75). At least in white persons, platelet count seems not to be a link between the *FTO* variation and risk of cardiovascular disease.

Key Words: *FTO* gene, polymorphism, platelets, white persons

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Cardiovascular diseases (CVDs) are the most common cause of mortality and morbidity in developed countries. Genetics plays a substantial role in development of CVD, but particular knowledge is far from the entire estimate of genetic predisposition of CVD, which should be approximately 50%. Near the "classical" candidate genes, for example, genes for apolipoprotein E[1,2] or apolipoprotein A5[3] (where the association is based on the known physiological effects on plasma lipids), high throughput methods (especially chips used in genomewide association studies) lead to the detection of the new genes of interest, mainly, however, without clear biochemical or pathological links between the gene and disease.

Among these genes belongs also the gene for FTO (fat mass and obesity–associated protein, OMIM ID, 610966; gene ID, 79068), primarily recognized as body mass index (BMI)-associated gene (for review, see Fawcett and Barroso[4]). A cluster of single-nucleotide polymorphisms (SNPs) located within the first intron of the *FTO* gene was recognized as sufficient for the detection of the obesity-associated *FTO* variant. This variant could be detected by the analysis of 1 of the 3 tagging SNPs (rs9939609, rs1421085, and rs17817449; these SNPs are in almost complete linkage disequilibrium).

Later, the importance of the first intron variants within the *FTO* gene in determination of noncommunicable diseases, especially, but not only, of CVD, was detected.[5–8] Interestingly, the association between *FTO* polymorphism and CVD is at least partially independent on common risk factors such as obesity, diabetes mellitus, or smoking.[5,6] Despite the intensive research and the fact that some regulatory functions of the FTO are supposed,[9,10] the mechanism linking the *FTO* gene to the CVD development remains unclear.

Recently, an association between the rs1558902 (also located within the first *FTO* intron) and platelet counts was detected in Japanese individuals.[11] Obesity-associated A allele was associated also with the approximately 10% increase of platelet count in contrast to the values observed in the "normal" homozygotes.

Especially because the relative low number of included individuals (209), an important limitation of the original finding[1] is the lack of the confirmatory study. It was described that even large studies are prone to be false positive (type I error), and their results need to be replicated.[12]

To confirm the original study, we have analyzed the potential association between the rs1558902 *FTO* polymorphism and (not only) platelet counts in a group of middle-aged white women.

MATERIALS AND METHODS

Individuals included within the Prague Pre and Post Menopausal Females study (3PMFs) were analyzed.[13] Briefly, 5% representative random sample (individuals with severe kidney or liver disease were not included in the study) of the population consisting of 29,440 women aged 45 to 54 years living in Prague was selected from the registers of health insurance companies. From a random sample of 1472 women, 908 gave their informed consent to participate in the study and were primarily examined. In 2010/2011, 669 women (73.7%) participated in the second survey of the study.

Anthropometrical parameters and blood pressure were measured according the standardized protocols. Plasma lipids were analyzed in fasting plasma by the WHO Regional Lipid Reference Centre, IKEM, Prague, on a Roche COBAS-MIRA autoanalyzer (Hoffmann-LaRoche, Basel, Switzerland), using reagents from Boehringer Mannheim Diagnostics (Mannheim, Germany), and Hoffmann-La Roche. For the hematological analysis, vials containing tripotassium ethylenediaminetetraacetic acid (EDTA) were used. Parameters were automatically determined by an impedance method using COULTER LH-750 analyzer (Beckman Coulter, Brea, CA).

From the Institute for Clinical and Experimental Medicine, Videnska, Prague, Czech Republic.
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Reprints: Jaroslav A. Hubacek, PhD, DSc, Institute for Clinical and Experimental Medicine, DEM, Videnska 1958/9, Prague 4, 14021, Czech Republic. E-mail: jahb@ikem.cz.
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Genomic DNA was extracted from EDTA blood by a standard method.[14] Rs1558902 variant within the first intron of the *FTO* gene (T > A) was analyzed using the polymerase chain reaction restriction fragment length polymorphism method. Briefly, oligonucleotides 5′-TAG CAA CTG CGA TAC AAG TGT TAG ATA TC and 5′-TAG GGT ACG TTG CAG CAA TAA CCT ACC CGA were used for amplification of 181-base pair (bp) DNA fragment at annealing temperature of 64°C. Treatment with restriction enzyme *Mbo*I distinguished between the common T allele (uncut polymerase chain reaction product of 181 bp) and minor A allele (restriction fragments of 150 and 31 bp). To ensure the accuracy of the methods for genotyping, one plate, obtaining 94 samples, was genotyped twice within 1 week with 100% conformity.

Data are presented as percentages for categorical variables and means ± SD for continuous variables. Between-group comparison of continuous variables was performed using analysis of variance, and analysis of covariance was performed to exclude potential confounding by age. Discrete variables were tested by χ^2 statistics.

The protocol of the study was approved by institutional ethic committee. Clinical characteristics of the analyzed women are presented in Table 1.

RESULTS

Frequencies of the rs1558902 genotypes in our study (TT, 30.4%; AT, 48.1%; and AA, 21.5%) are within the expected range and are in agreement with the Hardy-Weinberg equilibrium ($P = 0.43$).

Of the 669 individuals included in the second survey of the 3PMFs study, both the rs1558902 *FTO* genotype and valid platelet counts were available in 646 individuals (96.4%).

The *FTO* variant rs1558902 (Table 1) was not significantly associated with BMI, waist circumference, subcutaneous fat, or plasma lipids.

Furthermore, we did not detect any association between the *FTO* rs1558902 polymorphism and platelet counts (TT, 242 ± 55 × 10^9; TA, 246 ± 67 × 10^9; and AA, 247 ± 55 × 10^9; F[2.642] = 0.30, $P = 0.74$). Of the other parameters analyzed, AA homozygotes have slightly lower plasma levels of total cholesterol (F[2.643] = 3.19, $P = 0.043$) and hemoglobin (F[2.643] = 3.12, $P = 0.045$) than others, but the significance disappeared after adjustment for multiple testing. Other anthropometric, lipid and hematological parameters were independent of the *FTO* rs1558902 genotypes (for more details, see Table 1).

DISCUSSION

In our study, we did not confirm the original finding of Kotani et al.[11] who have detected an increased platelet counts in carriers of the obesity-associated AA (rs1558902) *FTO* genotype.

There are a couple of plausible explanations for the observed difference. At first, the original study has included only slightly more than 200 individuals, and such studies are prone to false-positive results, especially if any correction for multiple testing was performed. Furthermore, age and sex differences between the studies could cause the observed differences, as we have included only women of narrow age range.

In contrast, it is not very likely that the ethnical differences (whites vs Japanese) could be the reason for the observed differences, as the *FTO* variants have the similar effects on BMI values across the different ethnics. In the case of the rs1558902 variant, no association between the SNP and BMI was observed both in our study and in the Japanese study.

Based on our results, it seems not likely that an effect of the *FTO* variant on platelet count could at least partially explain the mechanism, how *FTO* variants affect the risk of development of a couple of noncommunicable diseases.

TABLE 1. Clinical Characteristics of the Analyzed Women in 3PMFs

Clinical Characteristic				
***FTO* rs1558902**	**TT**	**TA**	**AA**	***P***
n (%)	196 (30.3)	311 (48.1)	139 (21.5)	
Age, yrs*	55.5 ± 2.7	55.7 ± 2.7	55.9 ± 2.8	0.402
BMI, kg/m^2*	26.8 ± 4.8	26.5 ± 4.7	27.07 ± 5.3	0.548
WHR*	0.856 ± 0.079	0.841 ± 0.069	0.845 ± 0.075	0.077
Diabetes, n (%)	8 (4.1)	7 (2.3)	7 (5.0)	0.265
Hypertension, n (%)	99 (51.0)	143 (46.4)	67 (48.9)	0.597
Smoking prevalence, n (%)	39 (19.9)	87 (28.0)	34 (24.5)	0.121
Total cholesterol, mmol/L*	5.71 ± 1.02	5.59 ± 0.93	5.36 ± 0.93	0.043†
Triglycerides, mmol/L*	1.46 ± 0.81	1.32 ± 0.64	1.34 ± 0.68	0.112
HDL cholesterol, mmol/L*	1.66 ± 0.39	1.67 ± 0.37	1.63 ± 0.42	0.444
Glycemia*	5.47 ± 0.93	5.37 ± 0.60	5.49 ± 0.91	0.201
Insulin*	7.17 ± 3.95	6.95 ± 4.29	7.66 ± 5.71	0.314
Hemoglobin, g/L*	139.3 ± 8.3	138.9 ± 8.6	136.9 ± 10.4	0.045†
Platelets, ×10^9/L*	242.2 ± 55.3	246.0 ± 64.7	246.6 ± 54.9	0.737
Leukocytes, ×10^9/L*	6.17 ± 1.64	6.16 ± 1.84	6.31 ± 1.68	0.680
Neutrophils, ×10^9/L*	3.47 ± 1.20	3.47 ± 1.46	3.67 ± 1.26	0.309
Lymphocytes, ×10^9/L*	1.97 ± 0.60	1.97 ± 0.55	1.93 ± 0.59	0.741
Erythrocytes, ×10^{12}/L*	4.41 ± 0.30	4.41 ± 0.31	4.40 ± 0.31	0.915

*Results are presented as mean ± SD.

†Not significant after Bonferroni correction for multiple testing.

HDL indicates high-density lipoprotein; WHR, waist-hip ratio.

Variants within the first intron of the *FTO* gene (especially the rs1421085, rs17817449, and rs9939609 variants) are between the exciting pleiotropic genetic variants. They were primarily recognized as a risk factor of obesity development.[15–17] A short time after, *FTO* variants were described to be (at least partially), independently of BMI, associated also with CVD,[5,6] diabetes mellitus type 2,[7,18] end-stage renal disease,[19] some but not all types of cancer,[8,20,21] and total mortality.[22] Despite an intensive research during the past years, the underlying mechanism remains unclear. The first experiments were focused on the possible effect on dietary habits, physical activity (reviewed by Dlouha et al.[23]), or basal metabolic rate[24] and led to inconsistent results. Recent publications suggest that the FTO is more likely a regulatory protein, exhibiting low DNA demethylase activity[9] functioning like a possible transcriptional cofactor[10] and potentially influencing also the telomere length[25] and, thus, the biological aging.

In summary, we have not confirmed a potential association between the first intron SNP rs1558902 and platelet counts in white middle-aged women. The underlying mechanism connecting the *FTO* first intron tagging polymorphisms and enhanced risk of noncommunicable diseases needs to be examined in future studies.

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PREZENTACE NA KONGERSECH A SYMPOZIÍCH

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