Masarykova univerzita v Brně
Lékařská fakulta
Ústav patologické fyziologie

Úloha vybraných adipokinů a souvisejících molekul a jejich genetické variability v prenatálním/časném postnatálním metabolickém modelování v kontextu komplexních onemocnění později během života

Habilitační práce

MUDr. Julie Bienertová-Vašků, Ph.D.
Brno 2012

Zitušce

Děkuji své dcerce Zitušce za čas, o který jsem ji připravila přípravou této práce. Děkuji svým rodičům za podporu, ať už lidskou nebo odbornou, kterou mi vždy poskytovali. Děkuji všem svým kolegům a spolupracovníkům, ať už současným nebo minulým, a v neposlední řadě děkuji i všem výzkumným subjektům, které se účastnily našich studií a napomohly realizaci předkládaných prací.

Obsah

1. TEORETICKÁ VÝCHODISKA

1.1. TEORIE PRENATÁLNÍHO/POSTNATÁLNÍHO MODELOVÁNÍ

Mnoho epidemiologických důkazů naznačuje, že jak obezita, tak řada dalších tzv. komplexních chorob má kromě nezpochybnitelného genetického pozadí i proměnlivou komponentu environmentální responzivity, jejíž počátek lze vysledovat až do fetálního období.

Suboptimální nutriční podmínky v prenatálním a časném postnatálním období mohou vést k zásadním změnám na úrovni buněčné proliferace, diferenciace a maturace buněk, spojeným s nevratnými strukturálními i funkčními změnami v buňkách, tkáních i orgánových systémech [1]. Tyto změny mohou mít dlouhodobé souvislosti ve smyslu zvýšení individuálního rizika rozvoje komplexních onemocnění včetně hypertenze, obezity, metabolického syndromu i diabetu, a to později v životě [2], jestliže se jedinec ocitne v podmínkách, které rozvoj těchto onemocnění podporují (např. dostatek dostupné potravy nebo nevhodné složení stravy).

Souvislost mezi událostmi v časném postnatálním a perinatálním období (environmentálními faktory, zejména podvýživou) a kardiovaskulárními chorobami později v životě byla zaznamenána již v roce 1934. Tehdy Kermack et al. prokázali, že celková mortalita ve Spojeném Království a Švédsku v letech 1751–1930 poklesla; autoři tuto skutečnost přičítali zlepšení životních podmínek dětí v tomto období [3]. Forsdahl et al. následně publikovali svou práci, ve které upozornili na korelaci mezi různými geografickými regiony v Norsku, výskytem ischemické choroby srdeční v letech 1964–1967 a úrovní kojenecké mortality o 70 let dříve. Autoři této práce formulovali hypotézu, že chudoba vede prostřednictvím nutričního deficitu k dlouhodobé a často celoživotní vnímavosti vůči kardiovaskulárně rizikovému životnímu stylu v dospělosti [4].

V roce 1986 se začali Barker et al. zabývat vlivem různé úrovně mortality na mozkovou mrtvici a kardiovaskulární choroby v různých regionech Anglie a Walesu. Povšimli si přitom,

že geografická distribuce mortality na mozkovou mrtvici a kardiovaskulární choroby v letech 1969–1978 úzce souvisela s novorozeneckou mortalitou v letech 1921–1925. Své zkoumání uzavřeli s tím, že chudoba a špatný zdravotní stav matek v těhotenství jsou u potomstva důležitým rizikovým faktorem výskytu mozkové mrtvice později v životě [5].

V roce 1962 Neel et al. zveřejnili hypotézu tzv. „spořivého genotypu“, která si kladla za cíl patofyziologicky vysvětlit souvislosti mezi událostmi v časném postnatálním období a rizikem vzniku komplexních chorob později v životě [6]. Podle Neelovy hypotézy došlo během sběračsko-loveckého období lidského vývoje k selekci „spořivých“ genů, které při snížení dostupnosti potravy zvyšují schopnost vytvářet energetické zásoby. To má nezpochybnitelný evoluční význam pro přežití v podmínkách energetického nedostatku. Druhou stranou mince je fakt, že tyto geny v podmínkách relativního dostatku potravy vedou u predisponovaných jedinců později v životě k vyššímu riziku vzniku např. inzulinové rezistence. Na Neelovu hypotézu navázali v roce 1992 Hales a Barker [7] svou hypotézou „spořivého fenotypu“. Neelovou hypotézou bylo, že spořivé geny byly vyselektovány během evoluce v době nedostatku, což vedlo ke vzniku „rychlé inzulinové spoušti“. Hypotéza spořivého fenotypu naopak říká, že pokud je prostředí, v němž se plod vyvíjí, nějakým způsobem narušeno, dojde k adaptivní negenetické odpovědi, která optimalizuje růst klíčových orgánů těla na úkor jiných. Zároveň dochází ke změnám postnatálního metabolismu, čímž má být dosaženo přežití v podmínkách nárazového dostatku či dlouhodobého nedostatku výživy [7].

Baker a Hales přitom popisují významné souvislosti mezi nízkou porodní hmotností a hypertenzí, ischemickou chorobou srdeční, intolerancí glukózy, inzulinovou rezistencí, hyperlipidémií, hyperkortizolémií, obezitou, chronickou obstrukční plicní chorobou a poruchami reprodukce [8]. Jedním z klíčových produktů definice spořivého genotypu zůstává koncepce vnímavé nebo „kritické periody“ vývoje, během které působení určitých

specifických nutričních vlivů způsobuje dlouhodobé změny ve vývoji, jež mohou případně souviset se zdravotními riziky později v životě [9-11]. Tato koncepce přitom navazuje přímo na myšlenku „metabolického imprintingu“, historicky poprvé zmíněnou Konradem Lorenzem. Ten používal termín „imprinting“ k označení určitého chování zvířat, které vycházelo z časné zkušenosti získané právě v kritické fázi vývoje.

Hypotéza Bakera a Halesa byla v nedávné době podpořena [12] zjištěním, že za vývojem diabetu mellitu 2. typu, metabolického syndromu i obezity stojí s větší pravděpodobností spíše nepříznivé fetální prostředí než genetické determinanty. Tuto hypotézu podporují výsledky studií na dvojčatech udávající vyšší konkordanci výskytu nižší porodní hmotnosti u jednovaječných dvojčat s diabetem mellitem 2. typu oproti geneticky identickým, avšak nediabetickým dvojčatům [13].

Potíže ve smyslu nárustu frekvence komplexních chorob později v dospělosti atd. vznikají v případě výrazného nesouladu environmentálních podmínek mezi fetálním a postnatálním obdobím, např. při vyšší dostupnosti energeticky denzní potravy v postnatálním období [7]. Hypotéza, která říká, že embryonální i fetální adaptivní reakce na suboptimální intrauterinní prostředí vede k trvalým, z dlouhodobého hlediska nepříznivým následkům pro konkrétního jedince, je v souladu s definicí metabolického programování dle Lucase et al. Tu autor publikoval v roce 1991 a metabolické programování je tu definováno jako indukce, ztráta nebo narušení vývoje trvalých somatických struktur nebo „nastavení“ fyziologického systému časným stimulem nebo inzultem působícím během vnímavé periody vývoje [14].

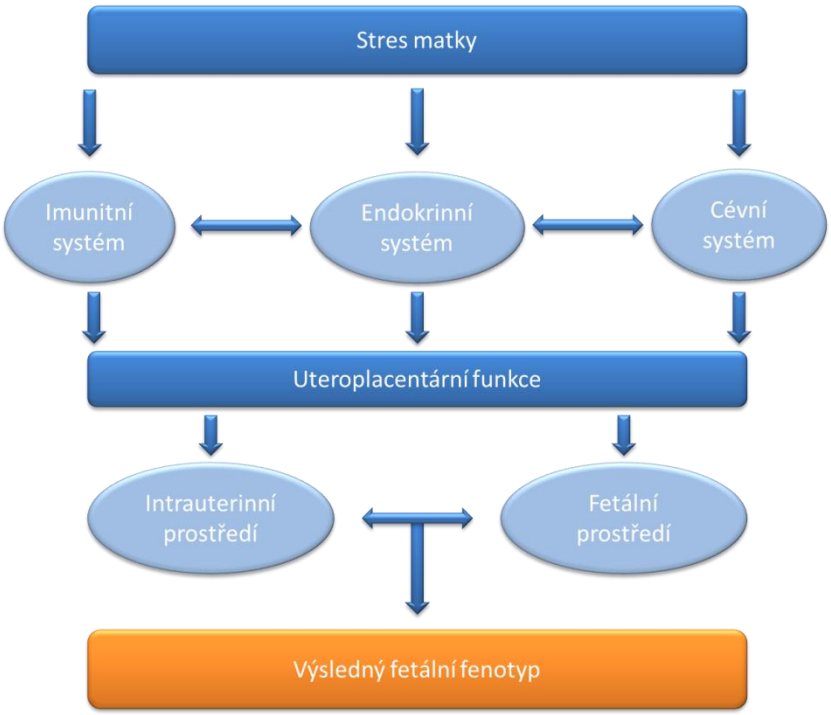
Z výsledků na poli mnoha oblastí výzkumu včetně evoluční ekologie a molekulární biologie vyplývá, že konkrétní genotyp může vést v závislosti na environmentálních podmínkách ke vzniku různých fenotypických projevů [15]. U mnoha živočišných druhů byla pozorována souvislost mezi vlivem okolního prostředí působícího na konkrétní generaci a vývojem a chováním generace následující. Např. u ptáků jsou samičky schopny ovlivnit mnoho aspektů

složení vejce v závislosti na řadě environmentálních faktorů jako dostupnosti potravy, úrovně sourozenecké soutěživosti a kvality partnerů [16]. Tím přímo ovlivňují i strukturální a behaviorální rysy následující generace.

Pozorování skutečnosti, že určité typy událostí v pozdním prenatálním či časném postnatálním životě mohou ovlivňovat riziko určitých událostí později v dospělosti, tzv. paradigma vývojového původu zdraví a nemoci (DOHaD), vedlo k zásadní změně chápání konceptu metabolického programování. Fenomén DOHaD zahrnuje širší řadu procesů vývojové plasticity, pomocí kterých se organismy adaptují na své okolní životní prostředí. Spouštěče těchto procesů plasticity se v typickém případě uplatňují během časného vývoje, mohou postihovat konkrétní tkáň nebo orgán, ale obecně vedou ke vzniku komplexního metabolického fenotypu. To je zprostředkováno zejména nejrůznějšími epigenetickými mechanismy. Základní představa o mechanismech vzniku výsledného fenotypu je uvedena na obrázku 1.

Obrázek 1.

Výsledný fetální fenotyp ve vztahu ke stresu matky, adaptováno dle Wadhwa et al [17].



Z povahy celého procesu logicky vyplývá, že fetální programování, či spíše „metabolická, vývojová či dynamická plasticita“, není samo o sobě manifestací patofyziologického procesu. Jedná se o fyziologickou adaptační reakci plodu pro zajištění optimální adaptace v postnatálním životě s cílem maximalizovat budoucí zdraví a zlepšit přežití [2, 18-20]. Formální definicí vývojové plasticity dle Barkera je přitom schopnost konkrétní genetické varianty vytvořit více alternativních strukturálních forem, mechanismů fyziologického fungování nebo chování v rámci reakce na podmínky okolního prostředí [21].

Plod přizpůsobuje svůj vývoj dodávce nutrientů a kyslíku od matky a z těchto stimulů „odečítá“, do jakého prostředí se chystá narodit. Pokud ovšem dojde později v životě k výrazné změně environmentálních podmínek oproti podmínkám fetálního života, adaptační reakce se může stát výchozím bodem pro maladaptivní metabolické nastavení či chování vedoucí ke zvýšenému riziku vzniku řady onemocnění. Konkrétní genotyp tak může v závislosti na charakteru vnějších podmínek vést k celé řadě fenotypických projevů, z nichž některé jsou adaptivnější a některé naopak méně výhodné.

1.2. MECHANISMY MODELOVÁNÍ

Mnoho epidemiologických studií ukazuje, že adaptační odpověď na fetální prostředí může mít za následek nežádoucí nepříznivé jevy později v životě jednotlivce. Mechanismy těchto dějů zůstavají dosud z velké části nejasné. Nejnovější data poskytují silné důkazy o tom, že klíčovou roli v regulaci adaptačních odpovědí na nutriční a environmentální faktory během fetálního a časného postnatálního života hrají epigenetické procesy [22]. Epigenetické regulace spolu s genomickým imprintingem představují tedy dvě zásadní regulace, které je nutné zohlednit při uvažování o faktorech ovlivňujících fetální růst, časný postnatální vývoj i metabolické nastavení později v dospělosti [23, 24].

Epigenetické změny v rámci projevů vývojové plasticity postihují velmi široké spektrum genů. V tomto okamžiku nejsme schopni tyto změny výzkumně jednotně obsáhnout a

zejména jednoznačně interpretovat. V zásadě se dá říct, že na vrcholu pomyslné pyramidy celého vývojového modelování stojí konkrétní fenotyp. Ten je podepřený specifickými změnami na úrovni intermediárního metabolismu, za nimiž stojí dlouhá řada událostí na úrovni epigenetických modifikaci cílových genů.

1.3. ZÁKLADNÍ SPECIFIKACE ETAP METABOLICKÉHO MODELOVÁNÍ

Jak bylo zmíněno výše, vývojová plasticita je realizována procesy na subcelulární, celulární či tkáňové úrovni a procesy na úrovni orgánových regulací a regulací v rámci celého organismu. V současnosti nám nejsou známy konkrétní procesy a mechanismy stojící za vznikem daných fenotypů. Na základě jednotlivých asociačních studií ale můžeme usuzovat, že jak na subcelulární úrovni, tak na úrovni intermediárního metabolismu dochází v prenatálním období k řadě adaptačních procesů, kterými plod reaguje úzce na změny v environmentálním prostředí, s nímž je matka v kontaktu.

1.4. RŮST PLODU A JEHO PŘIMĚŘENOST NA GESTAČNÍ VĚK, POJMY AGA, SGA LGA

Suboptimální fetální prostředí se může odrážet v nízké porodní hmotnosti, což ve své podstatě představuje okamžitou adaptační reakci plodu, která mu umožňuje přežít při snížených energetických nárocích až do období porodu. Narušený fetální růst není konstantní proces a souvislost mezi vývojovou plasticitou a rizikem onemocnění je v rozmezí porodních hmotností kontinuální, přičemž maximální nežádoucí účinky mají v daném rozmezí porodních hmotností kontinuální distribuci. Nejvýraznější nežádoucí účinky jsou poměrně logicky pozorovány u obou extrémů porodních hmotností.

Přiměřený vzrůst na gestační věk je označován jako AGA (Appropriate for Gestational Age), anomální fetální růst zahrnuje malý vzrůst na gestační věk (Small for Gestational Age, SGA)

nebo naopak velký růst na gestační věk (Large for Gestational Age, LGA). Zatímco SGA je definován dle American College of Obstetricians and Gynecologists jako porodní hmotnost novorozence pod desátý percentil pro daný gestační věk, LGA je definován jako porodní hmotnost nad devadesátým percentilem spolu s makrosomií (porodní hmotnost minimálně 4 000 gramů) [25, 26]. S extrémy porodní hmotnosti pak souvisí častý výskyt peripartálních komplikací, ať už se jedná o porod mrtvého plodu, neonatální acidózu, záchvaty u SGA nebo zvýšenou fetální a neonatální mortalitu u LGA z důvodu porodních traumat nebo výkyvů glykémie u plodu.

Ve velké studii zkoumající 123 383 porodů v Milwaukee byl výskyt SGA o 57 % vyšší než výskyt LGA (11 % oproti 7 %). Úroveň novorozenecké mortality v této studii byla u AGA 5,3/1000, u SGA 11,0/1000 a u LGA 2,7/1000 a tato úroveň se udržovala po celých 12 let pokračování studie. Dále bylo zjištěno, že SGA je rizikovým faktorem časné novorozenecké mortality (0–6 dní po porodu), ale nikoli postneonatální mortality (28–364 dní po porodu) [27], což by naznačovalo, že pokud dítě přežije rizikové období porodu, jeho šance na přežití se blíží obecné populaci.

Je známo, že podvýživa matky nebo narušená placentární vaskularizace z nejrůznějších příčin navozují intrauterinní růstovou retardaci (IUGR) a rezistenci vůči růstovému hormonu v časném dětství. Převážná většina novorozenců malých na svůj gestační věk však v prvních letech života růstovou retardaci z fetálního období vyrovná [15]. SGA novorozenci, u kterých k tomuto růstovému vyrovnání dochází, mají často v porovnání s běžnou populací vyšší body mass index, větší množství tukové tkáně, inzulinovou rezistenci a vyšší systolický krevní tlak během dětství i adolescence [28-30]. V současné době nejsou známy mechanismy tohoto růstového vyrovnání, ani není jasné, jak postupovat v otázce časné výživy a behaviorálních intervencí v časném postnatálním období u SGA novorozenců a kojenců. SGA fenotyp plodu v době porodu může být odrazem epizody malnutrice ve třetím trimestru těhotenství, naopak

malý obvod hlavičky plodu (který je korelován s krevním tlakem silněji než porodní hmotnost) odráží zpomalení růstu během celého těhotenství. Dá se tedy očekávat, že k patofyziologickému „inzultu“ došlo v ranější fázi gestace. Klíčovým obdobím pro vývoj dlouhodobých zdravotních konsekvencí ve smyslu komplexních onemocnění později v dospělosti je i časné postnatální období. Bylo pozorováno, že tkáně, které byly v průběhu fetálního života vystaveny nízkým koncentracím inzulínu a inzulínu podobného růstového faktoru 1 (IGF-1), mohou v rámci metabolické obrany proti hypoglykémii při expozici vyšším hladinám těchto hormonů vyvinout v poporodním období inzulínovou rezistenci. Zdá se tedy, že události ve fetálním období dostávají svůj konečný metabolický význam až v kontextu časného postnatálního období [15].

1.5. ADIPOKINY V INTERAKCI MATKA DÍTĚ

Těhotenství představuje v životě ženy specifické období, kdy dochází k reprodukčně zásadně významné adaptaci intermediárního metabolismu matky na rostoucí metabolické nároky plodu. Zároveň však matka zprostředkovává i neustálý tok informací o charakteru vnějšího prostředí směrem k samotnému plodu, čímž dochází k unikátnímu a neopakovatelnému modelování celého metabolického nastavení plodu ještě *in utero*.

Tuková tkáň matky představuje metabolicky nesmírně aktivní orgán, který během těhotenství hraje specifickou úlohu ve smyslu zajištění energetických rezerv pro zdárný průběh těhotenství a zejména navazující laktace ve smyslu regulace vývojové plasticity. Během těhotenství dochází fyziologicky k redistribuci mateřské tukové tkáně. Přitom se ukazuje, že množství podkožního tuku se zvyšuje již od prvního trimestru a je pravděpodobné, že adipokiny, do značné míry secernované proporcionálně s celkovou masou tukové tkáně, zvyšují inzulinovou rezistenci s pokračujícím těhotenstvím. Tím umožňují organismu matky uvolnit dostatečnou funkční rezervu glukózy pro saturaci rostoucích nároků plodu [31]. Tento komplexní proces zvyšování inzulinové rezistence, který je markantně znatelný ve druhém a

třetím trimestru, je zprostředkován působením celé řady endokrinních a parakrinních faktorů [32, 33].

Dosud bylo identifikováno již celkem 77 endokrinně-parakrinně působících látek uvolňovaných z tukové tkáně, označovaných jako adipokiny. Je velmi zajímavé, že tyto molekuly jsou produkovány ve velké míře také placentární tkání a že se přímo účastní regulace řady reprodukčních funkcí. Je možné se domnívat, že tyto molekuly ovlivňují jak prenatální růst plodu, tak jeho postnatální adaptaci ve smyslu ovlivnění vývojové plasticity. Soubor všech adipokinů, tzv. adipokinom, společně s lipidovými působky uvolňovanými adipocyty vytváří společně tzv. sekretom adipocytů [34].

Základní adipokiny účastnící se daných regulačních okruhů a jejich subcelulární efektory jsou uvedeny na obrázku 2.

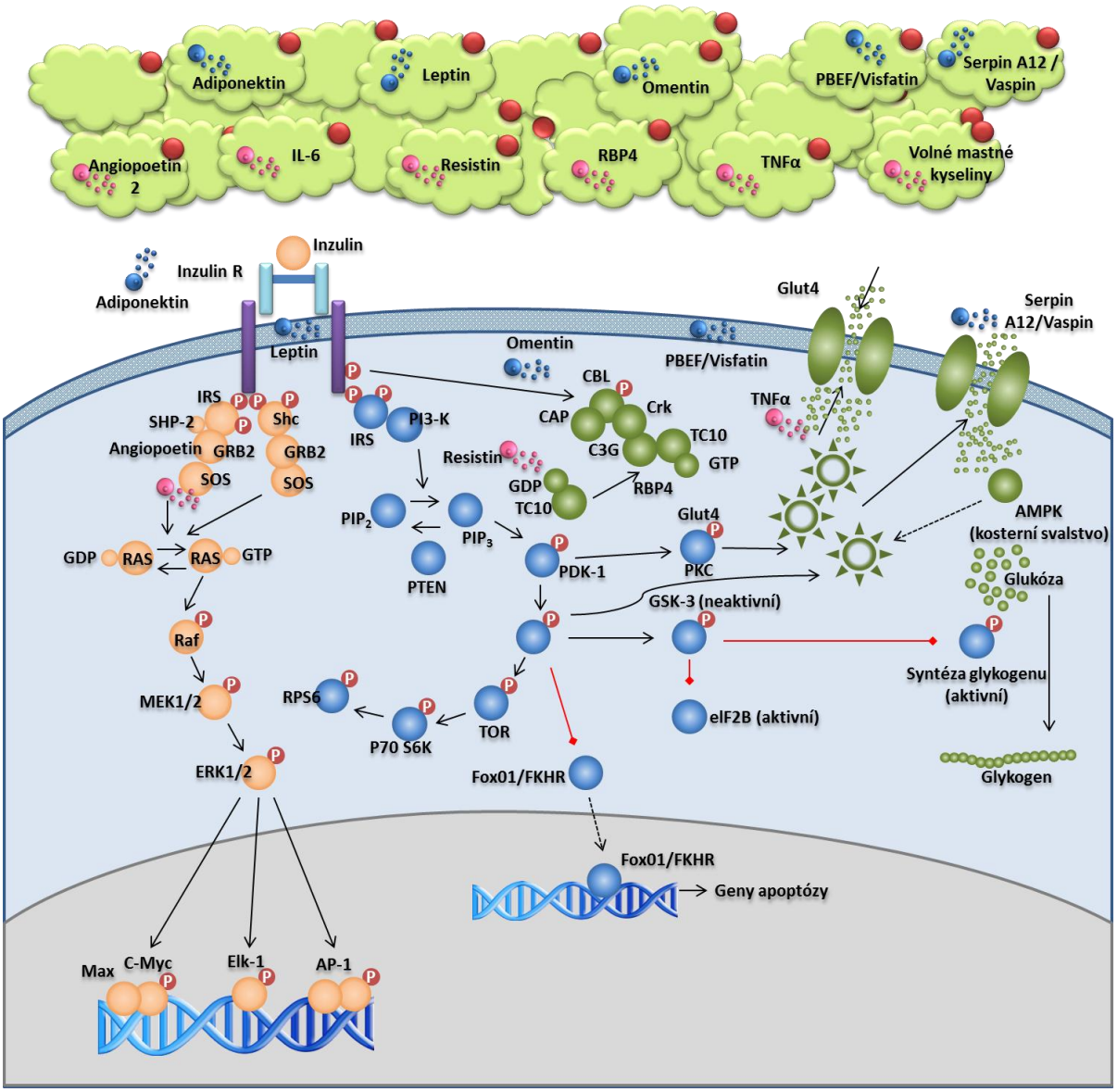
1.6. VZTAH ADIPOKINŮ, ADIPOZITY PLODU A FUNKCÍ IMUNITNÍHO SYSTÉMU

Některé důkazy naznačují, že na nutriční stav plodu i postnatální zdraví dítěte mají vliv transgenerační efekty. Studie na myších v 70. letech 20. století ukazují, že malnutrice matky může vést k permanentnímu imunodeficitu u potomstva, který nelze zvrátit optimální výživou v postnatálním období. Potomstvo těchto myší přitom také vykazuje abnormality imunitních funkcí, zvláště tehdy, když u matky došlo k výrazné karenci zinku v těhotenství [35, 36]. Tento transgenerační efekt byl také popsán u člověka – bylo pozorováno, že kouření babičky během těhotenství má za následek vyšší výskyt astmatu u vnoučat, bez ohledu na to, zda matka dětí kouří či nikoli [37].

I když konkrétní mechanismy i celková závažnost alterace imunitních funkcí u plodu/kojence z důvodu suboptimální adipozity matky v těhotenství, která do značné míry odráží její nutriční stav ovlivněný podmínkami okolního prostředí, nám nejsou v současnosti známy, existují jasné důkazy o tom, že podvýživa nebo nevhodné složení výživy v časném postnatálním

Obrázek 2.

Základní adipokiny účastnící se regulace adipozity v těhotenství a jejich intracelulární efektory, adaptováno dle: 2012, R&D Systems, Inc.



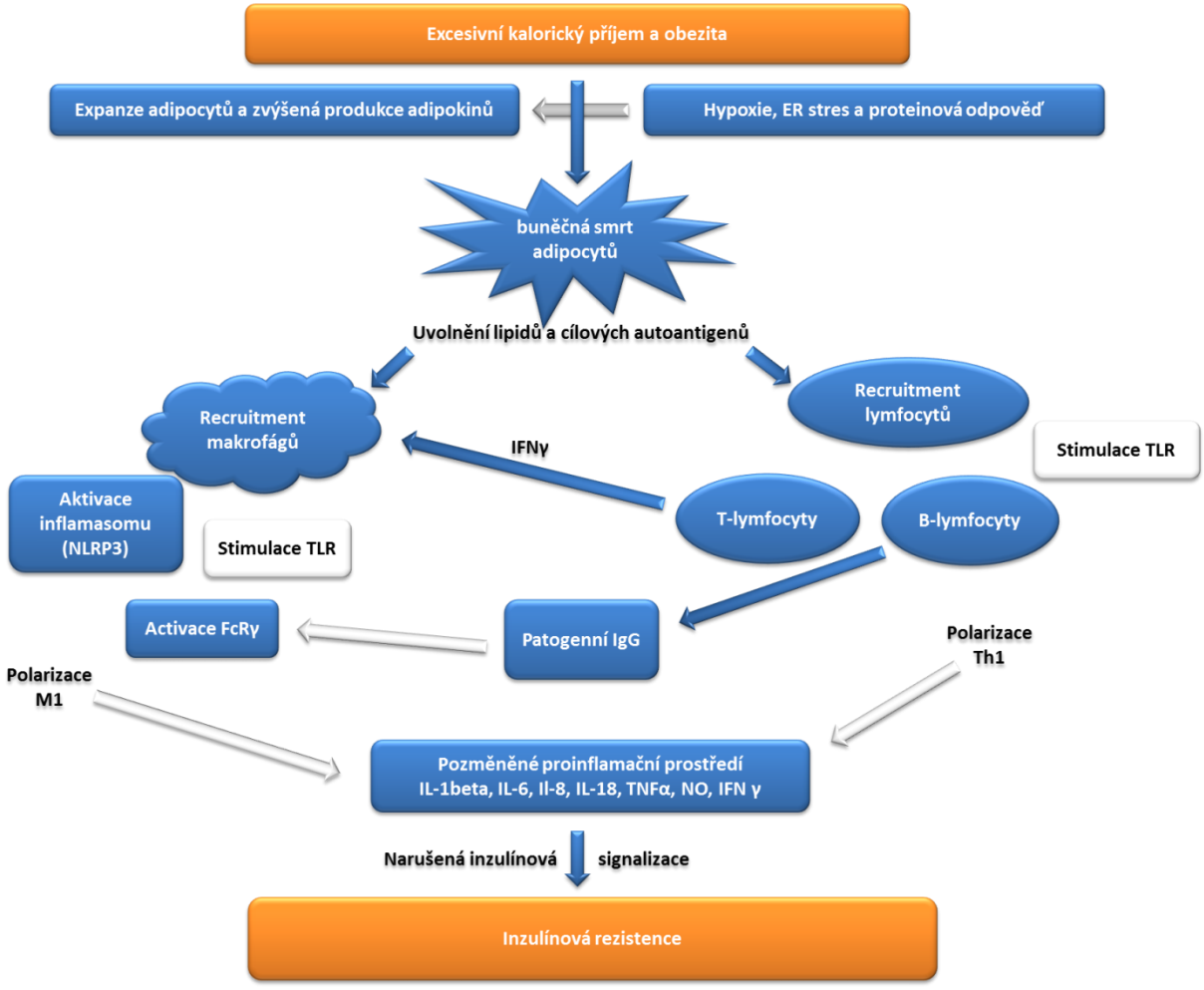
období může vést k trvalým strukturálním i funkčním změnám na úrovni imunitního systému [38, 39]. Kohortové studie provedené v Gambii v „hladovém“ období prokázaly sníženou velikost i funkci thymu u podvyživených dětí – u těchto dětí byly pozorovány zvýšené hladiny CD8+ T buněk a NK buněk a toto procento silně korelovalo s rizikem úmrtí v dětském a adolescentním věku na infekci [40]. Teorie vývojové plasticity přitom dobře vysvětluje pozorování z Gambie, kdy doba narození dítěte umožňuje poměrně přesně

odhadnout související kojeneckou/dětskou mortalitu. Děti narozené v „hladovém období“ jsou daleko častěji obětmi infekčních chorob než děti narozené během období hojnosti. Důležité je také pozorování, že mléko matek v „hladovém období“ obsahovalo významně nižší množství IL-7 než mléko matek v období hojnosti, což poukazuje na možnou významnou úlohu mateřského mléka při zprostředkovávání poporodní adaptace dítěte.

Základní schéma, jakým způsobem spolu souvisí adaptační imunitní odpověď a aktivita bílé tukové tkáně v podmínkách energetického dostatku, je uvedeno na obrázku 3.

Obrázek 3.

Souvislost mezi adaptivní imunitní odpovědí a aktivitou bílé tukové tkáně v podmínkách nadměrného příjme energie, adaptováno dle Winer et al [41].



1.7. ADIPOKINY, HYPOXIE A REGULACE GLYKÉMIE

Faktor indukovaný hypoxií (HIF) představuje zásadně důležitý transkripční faktor, který je indukován jednak environmentálními změnami (např. vysoká nadmořská výška), jednak patologickými stavy spojenými s hypoxií (např. nádorovými chorobami) [42, 43]. Vyskytuje se ve formě heterodimeru sestávajícího z podjednotek α a β; hladiny proteinu HIFα korelují se základní tkáňovou biologickou dostupností kyslíku, určitá hladina proteinu HIFβ (označovaného také jako protein ARNT) je exprimována konstitutivně. Při normoxii dochází k hydroxylaci proteinu HIFα, ubikvitinizaci a degradaci a tento proces je zprostředkován prolylhydroxylázami (PHD) a ubikvitin-E3-ligázou pVHL, což je produkt von Hippel-Lindau (VHL) genu [42, 43]. Při hypoxii se PHD suprimují, což vede ke stabilizaci HIFα proteinu a k transkripčnímu působení HIFα/β na úrovni indukce exprese genů, které klasicky regulují metabolickou adaptaci, cévní růst a přežití buněk.

Z toho důvodu je logické zjištění, že hypoxie představuje i jeden ze zásadních stimulátorů fetálního růstu, kde de facto dochází k prorůstání fetálních tkání „za kyslíkem“, tedy z míst o nižší koncentraci kyslíku do míst o vyšší koncentraci, kde se následně exprese hypoxií indukovatelných prorůstových genů tlumí [44].

Mezi geny citlivé na hypoxii patří řada genů kódujících adipokiny, např. leptin [45], angiopoietinu podobný protein 4 (Angptl4), interleukin-6 (IL-6), faktor inhibující migraci makrofágů (MIF) a cévní endoteliální růstový faktor (VEGF) [46-48]. V několika studiích u myší i u člověka bylo prokázáno, že nízký pO2 stimuluje v diferencovaných adipocytech i preadipocytech tvorbu leptinu [48-50], přičemž množství leptinu secernované preadipocyty je podstatně nižší.

Je všeobecně známo, že základním místem integrace signálů a regulace energetického příjmu (potravy), energetického výdeje a tělesné hmotnosti je hypothalamus [51-55]. Na úrovni hypothalamu přitom dochází ke kombinaci autokrinních, parakrinních i endokrinních

mechanismů signalizace. Odpovídající neuronální regulační děje zahrnují kromě hormonální signalizace typu leptinu a inzulínu i signalizační kaskády zahrnující samotné nutrienty, např. glukózu, aminokyseliny a mastné kyseliny [56]. Oproti dlouhodobé regulaci tělesné hmotnosti hypotalamickou hormonální signalizací je hypothalamické vnímání glukózy rychlé a podle nejnovějších prací za ně zodpovídají hypothalamické neurony exprimující proopiomelanokortin (POMC) [57]. Nedávno bylo prokázáno, že hypothalamická signalizace hladiny glukózy je zprostředkovaná aktivací HIF a vede k upregulaci POMC genu [58] a že HIF komplex hraje zásadní úlohu v regulaci glukóza-dependentní hypothalamické kontroly příjmu potravy a energetické rovnováhy. V této přelomové práci bylo zjištěno, že jako nutriční senzor na úrovni hypothalamu působí právě HIF a že hladina oxémie přímo ovlivňuje homeostázu příjmu potravy a energetické rovnováhy v reálném čase. Tato regulace navazuje dále na STAT3-dependentní expresi POMC genu u leptinové signalizace s následnými dlouhodobými účinky na příjem potravy a tělesnou hmotnost [59, 60].

Toto pozorování dále podporuje nedávná studie prokazující [58], že chronická hypoxie má u ryb vliv na příjem potravy, expresi leptinu a jeho receptoru a jejich mRNA na úrovni klíčových hypothalamických genů regulujících příjem potravy a aktivitu hypothalamo-pituitární osy. Ryby, které byly vystaveny po dobu 8 dní 10 % saturaci O_2, byly chronicky anorektické a konzumovaly přibližně o 79 % méně potravy než normoxické ryby. Autoři svá pozorování uzavírají tím, že snížení biologické dostupnosti O_2 (nikoli samotný příjem potravy) stimuluje u ryb expresi leptinu [61].

Tyto výsledky jsou v souladu s empirickými pozorováními, že u onemocnění provázených chronickou hypoxií bývá velká tendence pacientů k anorexii a hmotnostnímu úbytku. Hypotéza, že hypoxie a integrace tělesné energetické homeostázy na úrovni hypothalamu spolu úzce souvisejí, umožňuje vysvětlit řadu fyziologických i patofyziologických pozorování.

1.8. ADIPOKINY, EPIGENETICKÉ REGULACE, MIKROVEZIKULY

U potkanů bylo popsáno, že adipocyty secernují mikrovezikuly známé jako adipocytární vezikuly (ADM), které mají angiogenní aktivitu [62]. Ve studii, kterou publikoval Ogawa et al. v roce 2010, autoři udávají, že tyto vezikuly obsahují RNA bez přítomnosti typických 28S a 18S ribozomálních RNA a popisují přítomnost přibližně 7000 mRNA a 140 mikroRNA v těchto vezikulách [63]. Autoři práce si také povšimli, že mikrovezikuly secernované z tukové tkáně do séra obsahují velké množství genových transkriptů kódujících adipokiny, např. adiponektin a resistin. Na základě toho je možné domnívat se, že adipocytární mikrovezikuly mohou představovat nástroj mezibuněčné komunikace spojující centrální regulační okruhy zahrnující hypothalamické zpětnovazebné okruhy a konkrétní regulace exprese na tkáňové úrovni prostřednictvím diferenční exprese miRNA. Lze očekávat, že adipokiny zde hrají úlohu jakéhosi metabolického prostředníka.

V nedávné studii bylo zjištěno, že v mikrovezikulách detekovaných v plazmě a v monocytech periferní krve se současně exprimuje až 71 miRNA. U většiny miRNA, které jsou exprimovány v mikrovezikulách do séra/plazmy, se již dříve předpokládalo, že regulují buněčnou diferenciaci krevních buněk, metabolické dráhy a imunitní funkce [64].

1.9. VARIABILITA GENŮ KÓDUJÍCÍCH DANÉ PEPTIDY/PROTEINY

Přesná povaha vztahu mezi genetickou variabilitou v genech kódujících adipokiny, konkrétními hladinami daných adipokinů v kritických fázích vývoje a epigenetickými efekty není v současnosti známa. Dá se očekávat, že genetická variabilita v konkrétních lokusech ovlivňuje konstitutivně udržovanou hladinu adipokinů v této tkáni. Ta je ovšem pod kontrolou řady dalších faktorů, včetně environmentálních, jako je např. výživa. Konkrétní hladiny v konkrétní fázi ontogenetického cyklu tedy nelze spolehlivě označit za výslednici čistě genetických vlivů.

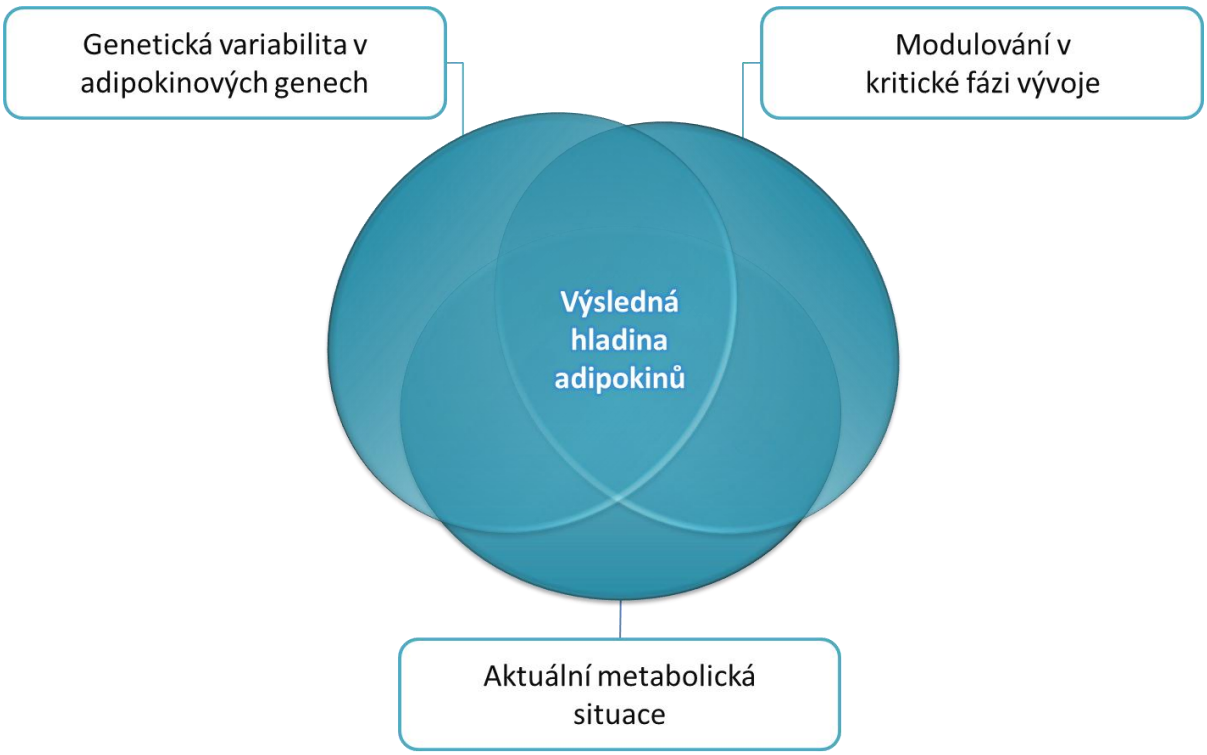
Navrhuji zde spíše pohlížet na výsledné hladiny adipokinů jako na výsledek určité triangulace:

- genetických faktorů včetné konkrétní interindividuální variability reprezentované zejména SNP v těchto lokusech;
- modulačních faktorů působících v konkrétních kritických fázích vývoje, např. v prenatálním období;
- výsledné aktuální metabolické situace, která je charakterizována celkovou adipozitou a nutričním stavem organismu v hodnoceném časovém bodě.

Uvedený princip je graficky zachycen na obrázku 4.

Obrázek 4.

Hladina adipokinů jako výsledek genetických a epigenetických faktorů



Poznání genetické variability konkrétních genů kódujících adipokiny pak představuje první etapu charakterizace těchto obtížně hodnotitelných a často transgeneračních efektů.

1.10. VÝBĚR KANDIDÁTNÍCH PEPTIDŮ/PROTEINŮ

Z výše zmíněných skutečností vyplývá, že adipokiny jsou elegantními kandidáty na pozici efektorů modulování vývojové plasticity na úrovni intermediárního metabolismu. Podléhájí přitom systémovému zpracování ze strany CNS i lokálním regulačním pochodům až na úroveň epigenetických dějů typu místní metylace nebo časoprostorové synchronizace exprese miRNA. Adipokiny představují logickou a konzistentní spojku mezi auto- a parakrinními účinky na lokální tkáňové úrovni a celkovým řízením složitými zpětnovazebnými okruhy na úrovni CNS.

2. VÝZKUMNÉ PROJEKTY

2.1. VÝCHODISKO

Základním východiskem mé práce zaměřené na úlohu adipokinů (eventuálně kooperujících orexigenních/anorexigenních molekul) v metabolickém modelování jsou následující skutečnosti:

- o efektech adipokinů v prenatálním/časném postanatálním období toho s výjimkou leptinu není mnoho známo;
- k dispozici nejsou téměř žádné informace o úloze adipokinů v časném postnatálním období, a to ve smyslu jejich sekrece do mateřského mléka i následného ovlivňování adipozity plodu;
- malé množství dostupných informací pochází z fyziologických studií, není přesně definována úloha adipokinů při zprostředkování nepříznivých účinků narušeného fetálního prostředí (např. u preeklampsie) na rizika u potomstva později v životě.

2.2. CÍLE PROJEKTŮ

Realizované projekty měly stanoveny následující základní cíle:

I. Sledování vybraných adipokinů u matek i dětí v peripartálním období.

II. Sledování vybraných adipokinů v mateřském mléce po porodu a v období 180denního období plného kojení.

III. Sledování těchto vybraných adipokinů u komplexních onemocnění později v životě (obezita, ischemická choroba srdeční, srdeční selhání).

IV. Sledování genetické variability vybraných adipokinů jako faktoru ovlivňujícího konstitutivně exprimované hladiny daného peptidu/proteinu a přispívajícího tedy k interindividuálním rozdílům ve vnímavosti ke komplexním nemocem.

2.3. MATERIÁL A METODY

Do této práce jsem zařadila sedm adipokinů a souvisejících molekul (leptin, adiponektin, agouti-related peptid, faktor aktivující B buňky, visfatin, mozkový natriuretický faktor, endokannabinoidní receptor typu I) u kterých základě jejich fyziologické funkce existuje předpoklad, že mohou hrát významnou úlohu v rámci vývojové plasticity prenatálního a časného postnatálního období.

S výjimkou leptinu, jehož úloha v reprodukci je již relativně dobře charakterizována, není o zkoumaných molekulách a jejich úloze v organizaci vývojové plasticity ve fetálním a časném postnatálním období mnoho známo. U řady adipokinů, včetně těch v naší studii, není či nebylo známo, zda jsou u člověka secernovány do mateřského mléka, případně jaká je dynamika jejich hladin během časného i pozdnějšího poporodního období. Proti sobě pak stavíme naše výsledky pozorování z prenatálního/časného postnatálního období a výsledky pozorování u komplexních chorob na populaci z téhož geotrafického regionu o několik generací později.

Pro sledování dlouhodobých regulací, které mohou být často realizovány až transgeneračně, jsme zvolili dva soubory pacientů:

I. Soubor pro prenatální/častné postnatální modelování pro sledování fyziologických/ patofyziologických závislostí při přechodu z fetálního do vnějšího prostředí; zde jsme svolili tyto subkohorty:

i. Zdravé matky s fyziologickým těhotenstvím, koncepcí i porodem, od kterých byla získána i pupečníková krev.
ii. Zdravé matky s fyziologickým těhotenstvím, koncepcí a porodem sledované po dobu 180 dní plné laktace, od kterých byly získávány vzorky mateřského mléka.
iii. Matky s preeklampsií (model prenatální hypoxie – hyponutrice).

iv. Matky s gestačním diabetem (model prenatální hyperglykémie).

II. Soubor pacientů s komplexními nemocemi, které jsou v populaci časté, u kterých se předpokládá významná úloha prenatálního/časného postnatálního modelování, konkrétně se jednalo o následjící subkohorty:

i. Pacienti s ischemickou chorobou srdeční včetně end-stage fenotypu chronického srdeční selhání a komplikací typu restenóza ve stentu po PCI.
ii. Pacienti s obezitou.
iii. Srovnávací populace odpovídající věku a pohlaví s normálním BMI bez dalších komorbidit.

U kohort I i II jsme sledovali tytéž adipokiny a cílem bylo vyhodnotit, jestli existuje souvislost mezi hladinami v pupečníkové krvi/mléce/periferní krvi matky a adipokinovým fenotypem komplexních chorob pozdě v dospělosti.

Detaily jednotlivých kohort i metodiky jednotlivých prací jsou popsány v následující sekci.

2.4. VÝSLEDKY

Viz část dokumentu s komentovaným souborem prací.

2.5. ZÁVĚR

Výchozím předpokladem této práce je významná úloha adipokinů při zprostředkovávání prenatálního modelování na úrovni intermediárního metabolismu. Tyto regulace lze považovat za důležitý článek spojující celulární-subcelulární regulace a meziorgánové interakce. Druhým výchozím bodem byla pozorování jiných výzkumných skupin, které došly k závěru, že v podmínkách nejsilnějších prenatálních stimulů typu hypoxie a hypoglykémie dochází ke změně exprese adipokinů. Třetím předpokladem bylo, že interakce mezi matkou a dítětem zprostředkovaná adipokiny nekončí porodem, ale pokračuje dále během celého

období kojení, kdy matka dítě dále „modeluje“ kontinuální a adaptivně se měnící dodávkou adipokinů do mateřského mléka. Čtvrtým, finálním východiskem je předpoklad, že adipokiny, resp. významná interindividuální variabilita v jejich konstitutivní expresi nastavená zčásti epigeneticky v kritických fázích prenatálního/časného postnatálního období, hrají významnou úlohu i později v dospělosti, při vzniku komplexních onemocnění. To by mělo dát specifický vznik pozorovatelnému fenotypu s ohledem na adipokinovou expresi.

Lze spekulovat o tom, že anomální vzorce sekrece/exprese adipokinů získané během prenatálního/časného postnatálního života se udržují až do hluboké dospělosti a v podmínkách příhodného okolního prostředí mohou facilitovat vznik komplexních chorob. V takovém případě by ovšem měla existovat korelace mezi dysregulacemi pozorovanými už peripartálně a metabolickými projevy v pozdní dospělosti, tedy pokud předpokládáme účast podobných mechanismů na úrovni intermediárního metabolismu. Zajímalo nás tedy mimo jiné, jakým způsobem se odrážejí pozorování získaná na prenatálních kohortách směrem k pozorováním u komplexních chorob v dospělosti.

Naše studie ukazují následující:

I. Při srovnání genetické variability v genech pro leptin a adiponektin ve skupinách žen s fyziologickým těhotenstvím a preeklampsií jsme nepozorovali rozdíly v distribuci genotypů ani alel. Pozorovali jsme konzistentní trend směrem k nízké porodní hmotnosti plodu při přítomnosti T alely polymorfismu v genu pro adiponektin ADIPOQ T94G. Tato alela byla sledována i u rozsáhlé kohorty pacientů s obezitou, kde nebyly pozorovány významné asociace ani s antropometrickými parametry, ani s parametry nutričními.
II. Při zkoumání hladiny faktoru aktivujícího B-lymfocyty (BAFF) v peripartálním období jsme zjistili, že hladiny BAFF v periferní krvi matek v peripartálním období

jsou významně nižší u žen s preeklampsií oproti zdravým rodičkám. Prokázali jsme také vůbec poprvé v literatuře, že u matek s fyziologickým těhotenstvím je BAFF ve významném množství přítomen v mateřském mléce během celých 6 měsíců po porodu, přičemž hladina BAFF je extrémně vysoká v kolostru a pak záhy klesá k nižším hodnotám. Tato hladina není závislá na cirkulující plazmatické hladině BAFF u matky. Při zkoumání hladiny BAFF u o několik desetiletí starší obézní kohorty jsme pozorovali, že hladiny BAFF jsou významně vyšší u obézních oproti neobézním kontrolám, přičemž BAFF byl významně korelován s procentuálním zastoupením tělesného tuku i obvodem pasu, a to jak u obézních, tak u neobézních jedinců.

III. Při zkoumání visfatinu jsme jako první skupina v literatuře prokázali přítomnost visfatinu v mateřském mléce, přičemž průměrná koncentrace v mateřském mléce přesahuje koncentraci v mateřském séru přibližně 100krát a tento poměr přetrvává během celého sledovaného období 180 dní po porodu. Nízkou hladinu visfatinu v kolostru lze navíc použít pro predikci hmotnostního vývoje novorozence s tím, že čím vyšší hladina je visfatinu v kolostru, tím výraznější je hmotnostní úbytek novorozence v poporodním období. Při sledování obézní kohorty byl visfatin inverzně korelován s leptinem a nejvyšších hladin dosahoval visfatin u obézních mužů, zatímco hladiny u obézních žen byly překvapivě velmi nízké.

IV. Pozorovali jsme významné rozdíly v distribuci genotypu rs806368 v genu pro endokannabionidní receptor typu I (CNR1) při srovnání pacientek s preeklampsií a kontrol, kdy u pacientek s preeklampsií byl významně nižší podíl homozygotek CC, přičemž u kohorty obézních dospělých vyššího věku tento polymorfismus vykazoval nezávislou predikční úlohu pro diastolický krevní tlak a procentuální zastoupení svalové hmoty na tělesné hmotnosti.

Lze tedy uzavřít, že genotypická/fenotypická variabilita exprese sledovaných adipokinů během peripartálního období (jak u matky, tak u plodu/novorozence/kojence) má svůj určitý korelát v přítomnosti významných asociací těchto adipokinů s komplexními chorobami u populace starší o 2-3 generace z téhož geografického regionu (viz podkapitoly 4.1-4.9). Jaký je konkrétní podklad těchto souvislostí, je nyní předmětem dalšího intenzivního výzkumu.

2.6. REFERENCE

1. Entringer S, Buss C, Swanson JM, Cooper DM, Wing DA, Waffarn F et al. Fetal programming of body composition, obesity, and metabolic function: the role of intrauterine stress and stress biology. J Nutr Metab. 2012;2012:632548.
2. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science. 2004;305(5691):1733-1736.
3. Kermack WO, McKendrick AG, McKinlay PL. Death-rates in Great Britain and Sweden: Expression of Specific Mortality Rates as Products of Two Factors, and some Consequences thereof. J Hyg (Lond). 2010;34(4):433-457.
4. Forsdahl A. Are poor living conditions in childhood and adolescence an important risk factor for arteriosclerotic heart disease. Br J Prev Soc Med. 1977;31(2):91-95.
5. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986;1(8489):1077-1081.
6. Neel JV. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? Am J Hum Genet. 1963;14:353-362.
7. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia. 1992;35(7):595-601.
8. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia. 1993;36(1):62-67.
9. Barraclough CA, Gorski RA. Evidence that the hypothalamus is responsible for androgen-induced sterility in the female rat. Endocrinology. 1961;68:68-79.
10. Ferguson MW, Joanen T. Temperature of egg incubation determines sex in Alligator mississippiensis. Nature. 1982;296(5860):850-853.

11. Thoman EB, Levine S. Hormonal and behavioral changes in the rat mother as a function of early experience treatments of the offspring. Physiol Behav. 1972;5(12):1417-1421.
12. Vaag AA, Grunnet LG, Arora GP, Brons C. The thrifty phenotype hypothesis revisited. Diabetologia. 2012;55(8):2085-2088.
13. Poulsen P, Vaag AA, Kyvik KO, Moller Jensen D, Beck-Nielsen H. Low birth weight is associated with NIDDM in discordant monozygotic and dizygotic twin pairs. Diabetologia. 1997;40(4):439-446.
14. Lucas A. Programming by early nutrition in man. Ciba Found Symp. 1991;156:38-50.
15. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev. 2005;85(2):571-633.
16. Lindstrom J. Early development and fitness in birds and mammals. Trends Ecol Evol. 1999;14(9):343-348.
17. Wadhwa PD, Glynn L, Hobel CJ, Garite TJ, Porto M, Chicz-DeMet A et al. Behavioral perinatology: biobehavioral processes in human fetal development. Regul Pept. 2002;108(2-3):149-157.
18. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA et al. Developmental plasticity and human health. Nature. 2004;430(6998):419-421.
19. Gluckman PD, Hanson MA, Spencer HG, Bateson P. Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. Proc Biol Sci. 2005;272(1564):671-677.
20. Gluckman PD, Hanson MA. Developmental plasticity and human disease: research directions. J Intern Med. 2007;261(5):461-471.
21. Barker DJ. Developmental origins of adult health and disease. J Epidemiol Community Health. 2004;58(2):114-115.
22. Gicquel C, El-Osta A, Le Bouc Y. Epigenetic regulation and fetal programming. Best Pract Res Clin Endocrinol Metab. 2008;22(1):1-16.
23. Mathew V, Ayyar SV. Developmental origins of adult diseases. Indian J Endocrinol Metab. 2012;16(4):532-541.
24. Christensen BC, Marsit CJ. Epigenomics in environmental health. Front Genet. 2012;2:84.
25. American College of Obstetricians and Gynecologists. Intrauterine growth restriction. Washington, DC: American College of Obstetricians and Gynecologists. 2000.
26. American College of Obstetricians and Gynecologists. Fetal macrosomia. Washington, DC: American College of Obstetricians and Gynecologists. 2000.

27. Chen HY, Chauhan SP, Ward TC, Mori N, Gass ET, Cisler RA. Aberrant fetal growth and early, late, and postneonatal mortality: an analysis of Milwaukee births, 1996-2007. Am J Obstet Gynecol. 2011;204(3):261.
28. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. BMJ. 2000;320(7240):967-971.
29. Jaquet D, Gaboriau A, Czernichow P, Levy-Marchal C. Insulin resistance early in adulthood in subjects born with intrauterine growth retardation. J Clin Endocrinol Metab. 2000;85(4):1401-1406.
30. Huxley RR, Shiell AW, Law CM. The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of the literature. J Hypertens. 2000;18(7):815-831.
31. Valsamakis G, Kumar S, Creatsas G, Mastorakos G. The effects of adipose tissue and adipocytokines in human pregnancy. Ann N Y Acad Sci. 2010;1205:76-81.
32. Barbour LA, McCurdy CE, Hernandez TL, Kirwan JP, Catalano PM, Friedman JE. Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes. Diabetes Care. 2007;30:S112-S119.
33. Brelje TC, Scharp DW, Lacy PE, Ogren L, Talamantes F, Robertson M et al. Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. Endocrinology. 1993;132(2):879-887.
34. Zhou H, Xiao Y, Li R, Hong S, Li S, Wang L et al. Quantitative analysis of secretome from adipocytes regulated by insulin. Acta Biochim Biophys Sin (Shanghai). 2009;41(11):910-921.
35. Chandra RK. Antibody formation in first and second generation offspring of nutritionally deprived rats. Science. 1975;190(4211):289-290.
36. Beach RS, Gershwin ME, Hurley LS. Gestational zinc deprivation in mice: persistence of immunodeficiency for three generations. Science. 1982;218(4571):469-471.
37. Li YF, Langholz B, Salam MT, Gilliland FD. Maternal and grandmaternal smoking patterns are associated with early childhood asthma. Chest. 2005;127(4):1232-1241.
38. Moore SE, Cole TJ, Collinson AC, Poskitt EM, McGregor IA, Prentice AM. Prenatal or early postnatal events predict infectious deaths in young adulthood in rural Africa. Int J Epidemiol. 2000;28(6):1088-1095.

39. McDade TW, Beck MA, Kuzawa CW, Adair LS. Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. J Nutr. 2001;131(4):1225-1231.
40. Ngom PT, Collinson AC, Pido-Lopez J, Henson SM, Prentice AM, Aspinall R. Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers' breast milk. Am J Clin Nutr. 2004;80(3):722-728.
41. Winer S, Winer DA. The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance. Immunol Cell Biol. 2012;90(8):755-762.
42. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721-732.
43. Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev. 2007;17(1):71-77.
44. Patterson AJ, Zhang L. Hypoxia and fetal heart development. Curr Mol Med. 2010;10(7):653-666.
45. Trayhurn P, Wang B, Wood IS. Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity. Br J Nutr. 2008;100(2):227-235.
46. Lolmede K, de Saint Front Durand V, Galitzky J, Lafontan M, Bouloumie A. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. Int J Obes Relat Metab Disord. 2003;27(10):1187-1195.
47. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes. 2007;56(4):901-911.
48. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Pflugers Arch. 2007;455(3):479-492.
49. Grosfeld A, Zilberfarb V, Turban S, Andre J, Guerre-Millo M, Issad T. Hypoxia increases leptin expression in human PAZ6 adipose cells. Diabetologia. 2002;45(4):527-530.
50. Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR et al. Intermittent hypoxia increases insulin resistance in genetically obese mice. J Physiol. 2003;552(PT 1):253-264.
51. Schwartz MW, Porte D Jr. Diabetes, obesity, and the brain. Science. 2005;307(5708):375-379.

52. Cone RD. Anatomy and regulation of the central melanocortin system. Nat Neurosci. 2005;8(5):571-578.
53. Ahima RS, Flier JS. Leptin. Annu Rev Physiol. 2000;62():413-437.
54. Coll AP, Farooqi IS, O'Rahilly S. The hormonal control of food intake. Cell. 2007;129(2):251-262.
55. Barsh GS, Farooqi IS, O'Rahilly S. Genetics of body-weight regulation. Nature. 2000;404(6778):644-651.
56. Jordan SD, Konner AC, Bruning JC. Sensing the fuels: glucose and lipid signaling in the CNS controlling energy homeostasis. Cell Mol Life Sci. 2010;67(19):3255-3273.
57. Karnani M, Burdakov D. Multiple hypothalamic circuits sense and regulate glucose levels. Am J Physiol Regul Integr Comp Physiol. 2010;300(1):R47-R55.
58. Zhang H, Zhang G, Gonzalez FJ, Park SM, Cai D. Hypoxia-inducible factor directs POMC gene to mediate hypothalamic glucose sensing and energy balance regulation. PLoS Biol. 2011;9(7):E1001112.
59. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006;443(7109):289-295.
60. Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature. 2000;404(6778):661-671.
61. Bernier NJ, Gorissen M, Flik G. Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp. J Exp Biol. 2012;215(PT 13):2273-2282.
62. Aoki N, Yokoyama R, Asai N, Ohki M, Ohki Y, Kusubata K et al. Adipocyte-derived microvesicles are associated with multiple angiogenic factors and induce angiogenesis in vivo and in vitro. Endocrinology. 2010;151(6):2567-2576.
63. Ogawa R, Tanaka C, Sato M, Nagasaki H, Sugimura K, Okumura K et al. Adipocyte-derived microvesicles contain RNA that is transported into macrophages and might be secreted into blood circulation. Biochem Biophys Res Commun. 2010;398(4):723-729.
64. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One. 2008;3(11):E3694.

3. OREXIGENNÍ/ANOREXIGENNÍ PEPTIDY/ADIPOKINY A JEJICH ÚLOHA V PRENATÁLNÍM MODELOVÁNÍ

Orexigenní/anorexigenní peptidy/adipokiny v prenatálním/časném postnatálním období:

I. Adipokines as Important Factor Related to the Prenatal Programming of Nutritional Behaviour (přehledová práce)

II. Práce zkoumající genové polymorfismy

   i. Leptin u preeklampsií (Is There Any Link Between Severe Pre-Eclampsia And Defined Polymorphisms In Leptin And Adiponectin Genes?)

   ii. Leptin u gestačního diabetu (Associationof Leptin Genetic Polymorphism -2548 G/A With Gestational Diabetes Mellitus)

   iii. CNR u preeklampsie (A Common Variation In The Cannabinoid 1 Receptor (CNR1) Gene Is Associated With Pre-Eclampsia In The Central European Population)

III. Práce zkoumající hladiny plazma matky/pupečníková krev

   i. BDNF (Brain-Derived Neurotrophic Factor (BDNF) And Ciliary Neurotrophic Factor (CNTF) In Maternal Plasma And Umbilical Cord Blood From Pre-Eclamptic And Physiological Pregnancies)

   ii. AGRP (Comparison Of Agouti-Related Peptide Levels In Peripheral Blood Of Postpartum Pre-Eclamptic And Non Pre-Eclampticwomen And In Umbilical Cord Blood From Their Pregnancies)

IV. Práce zkoumající mléko

   i. BAFF (B-cell activating factor (BAFF) is present in umbilical cord blood in healthy and pre-eclamptic pregnancies as well as in human breast milk with specific dynamics during 180d lactation)

   ii. Visfatin (Visfatin Is Secreted Into The Breast Milk And Is Correlated With Weight Changes Of The Infant After The Birth)

3.1. ADIPOKINES AS IMPORTANT FACTOR RELATED TO THE PRENATAL PROGRAMMING OF NUTRITIONAL BEHAVIOR

Resumé

Tato práce se zabývá celkovým přehledem současného chápání úlohy adipokinů při rozvoji nejrůznějších těhotenských komplikací a jejich vlivem na fenotyp potomstva z těchto těhotenství, zejména s ohledem na prenatální programování nutričního chování.

Článek (monografie In Advances in Medicine and Biology. Nova Science Publishers, 1. vyd., 2012, ISBN: 978-1-62100-843-9).

Adipokines as Important Factor Related to the Prenatal Programming of

Nutritional Behaviour

Julie Bienertova-Vasku* and Petr Bienert
Department of Pathological Physiology, Faculty of Medicine,
Masaryk University, Kamenice 5, A19, Brno, Czech Republic

Abstract

It has been suggested previously that some of the obesity-associated behavioral patterns as well as metabolic pathways are "programmed" during fetal development. The concept of "developmental metabolic programming" is based on the hypothesis that nutritional as well as metabolic status of the mother during the pregnancy affects the metabolism control in the child, thus resulting in a unique phenotype allowing for better adaptation of the infant to the environmental conditions. However, very little is known about factors that are responsible for this programming. The products of the white adipose tissue, so called adipokines, could be elegant candidates for controlling the process of fetal programming as they mostly correlated with the total fat mass and have pleiotropic effects both at parakrine and endocrine levels. It has been reported recently that e.g. leptin, adiponectin, resistin and visfatin as well as numerous other adipokines are associated with specific nutritional or metabolic conditions resulting in a wide range of adaptive phenotypes across the population. Moreover, placenta has been identified as a major source of adipokines, which also lead to the recognition of

* E-mail: jbienert@med.muni.cz.

prenatal period as a highly specific, unique metabolic state resulting in specific feeding and metabolic patterns of the infant. This review covers the major aspects of adipokines in relation to prenatal fetal modelling of the nutritional behaviour.

Keywords: Adipokines; early life programming; DNA methylation; miRNA, leptin; visfatin

Introduction

A long period of time between the action of environmental factor and the onset of subsequent disease has been widely accepted in the etiology of specific types of cancer. However, this phenomenon has been for a long time not recognized in the etiology of other non-cancer conditions, such as obesity, cardiovascular diseases or behavioural patterns [1], probably as a result of a less obvious cause-result relationship here. Recently, numerous clinical as well as experimental studies based both on large sets of epidemiologic data and laboratory experiments have shown that prenatal as well as early postnatal life plays a crucial role in influencing later susceptibility to certain metabolic diseases, e.g. diabetes [2-4].
The induction of persistent epigenetic changes by prenatal environmental factors is supposed to be a mechanism contributing to observed relationship between prenatal development and later life health in humans [2]. It has been demonstrated that placental disorders or maternal starvation can permanently change DNA methylation patterns and thus might by involved in development of diabetes and hypertension [5-7]. However, the mechanisms by which a phenomenon that onsets in early life can have long lasting effects on the function of a cell and therefore on the metabolism of an organism decades later are largely unclear [8]. The mechanisms, as defined by Tarry-Adkins JL [8] include: 1) permanent structural alterations of an organ resulting from abnormal concentration of a crucial factor during a critical period of development; 2) persistent epigenetic modifications (e.g. DNA methylation and histone modifications) leading altered gene expression and 3) permanent alterations in regulations of cellular aging (e.g. enhanced oxidative stress can lead to DNA damage, especially to telomeres, thus contributing to such effects). We would add also 4) transient as well as permanent changes induced by altered miRNA expression that might result also in persistent epigenetic imprint making an individual more prone to certain diseases in adulthood.

Placenta in Prenatal Modelling

The placenta, specific organ playing a critical structural and functional role during pregnancy, provides a key link in sequence of events leading to successful intrauterine programming of late life health [9]. Placenta development is characterized by precisely regulated spatio-temporally programmed epigenetic processes that, if impaired, lead to alteration in gene expression of different cell types and thereby affecting birth outcome. At every stage of fetal development there is a sequence of de novo methylation and chromatin remodelling that indicates the tissue structure and function through a precisely tuned pattern involving the switching on and off of specific gene expression [10]. The failure to complete critical spatiotemporal checkpoints during which the specific epigenetic programs must be completed may be irreparable and can have long-term (life-long or fatal) consequences for the fetus. The results of the study by Chavan Gautam [9] demonstrate that methylation patterns can also have implications for the consequences of prematurity since premature birth may impede the normal spatio-temporal pattern of gene expression affecting later development of the infant after birth, and for fetal programming of adult diseases since preterm babies are known to be

at increased risk of neurodevelopmental and metabolic disorders in later life. It can be assumed that every individual is under constant pressure of "programming factors", both intrauterine and environmental that consequently provide a specific, absolutely unique imprint in order to render the individual as adaptable to surrounding environment as possible.
In humans, periconceptional exposure (e.g., exposure around conception and during the first trimester) to the Dutch famine, a famine at the end of WWII, was associated with persistent differences in DNA methylation of various important loci involved in growth and metabolism, including IGF2, GNASAS, INSIGF and LEP [11]; the individuals who were prenatally exposed to famine in 1944-1945 had 6 decades later less DNA methylation of the imprinted IGF2 gene compared with their unexposed, same-sex siblings. Epigenetic differences were found among individuals who were exposed to famine early in gestation and had a normal birth weight; exposure to famine late in gestation was associated with low birth weight, as expected, but not with epigenetic changes. Tobi et al [12] investigated methylation of 15 loci implicated in growth and metabolic disease in the same group of individuals who were prenatally exposed to a war-time famine in 1944-45. Methylation of INSIGF gene was lower among individuals who were periconceptionally exposed to the famine (n = 60) compared with their unexposed same-sex siblings; methylation was also different for GNASAS and LEP gene, in men.
Another mode of alteration may be through the aberrant expression of microRNA (miRNA), 18–25 nucleotide long non-coding RNAs involved in post-transcriptional gene regulation [13-4]. miRNA base-pair to the 3'-untranslated region of target mRNA and are capable of effectively silencing gene expression by a mechanism of either translational repression or direct mRNA degradation. The finding by Maccani et al [15] that low expression of *miR-16* and *miR-21* in the placenta is associated with poor fetal growth may have many important implications as dysregulation of *miR- 16* in the placenta may lead to aberrant expression of its targets and may lead to functional and developmental abnormalities in the placenta that might result in reduced infant birth weight. This provides another line of evidence for possible robust epigenetic changes that the fetus undergoes during its prenatal development and makes more studies to provide a detailed research into miRNA roles in pregnancy a necessity.

Adipokines

Fetal adipose tissue development is regulated by the complex interaction of transcription factors, nutrients, and adipocytokines released both by placenta and maternal white adipose tissue [16]. Adipocytokines are mainly endocrine substances secreted mainly (but not exclusively) by the white tissue that has recently proved to be a highly active endocrine organ, secreting plenty of substance involved in energy homeostasis as well as tissue growth/death [17]. These adipocytokines are expressed and secreted by adipocytes and the human placenta during fetal life and are highly likely to play a major role in the etiopathogenesis of insulin resistance and cardiovascular disease [16]. The also provide a concise logical link between alterations of DNA methylation patterns in the early prenatal life and metabolic consequences decades later.

Leptin

Leptin is primarily synthesised in white adipose tissue and acts as an endocrine signal of energy reserves to the hypothalamus and other tissues in the coordination of appetite and metabolism with nutrient availability [18]. Leptin expression in the fetus is altered by

different intrauterine conditions, and these responses vary according to the nature of the stressor [19]. An upward trend in leptin levels has been reported through the three trimesters, which seems to parallel the increase of percent fat throughout gestation. In addition, placental leptin production makes a substantial contribution to maternal circulating levels during pregnancy [20]. Circulating leptin levels are high in the fetus at term, probably representing an important feedback modulator of substrate supply, and subsequently for adipose tissue status during late gestation [21]. However, leptin levels rapidly and dramatically decline after birth in healthy neonates [16, 21, 22].

As mentioned previously, altered methylation patterns are characteristic for individuals exposed to adverse environmental conditions, such as famine, during critical developmental windows [12], and these effect seem to be highly time- and sex-specific. For example, the famine exposed women presented with an increase in body mass index [22] and various lipids in blood [23], irrespectively of the precise gestational timing of the exposure. However, the results from the Dutch Famine study indicate that an increased risk of coronary artery disease was specific for exposure to famine in early gestation [25]. As for the LEP gene, there was no indication for a significant interaction between the famine association with DNA methylation and the gestational timing of the exposure, even though the methylation difference was significant only following periconceptional famine exposure [12]. In men in this study, LEP methylation was associated with prenatal famine irrespective of the timing of exposure and the observation that the methylation changes in relation to the prenatal environment may be sex-specific is in agreement with the sex-specific methylation changes found in offspring of sheep that were folate- and vitamin B12-restricted during periconception [25]. How these sex-specific associations can appear is currently unknown, but interactions between sex hormones and the expression of DNA methyltransferases may be the crucial factor [26]. The presumption that leptin gene methylation could be associated with metabolic patterns later in life was confirmed by the study by Jousse et al [18] who investigated the effects of perinatal undernutrition/starvation on methylation of LEP gene and observed that the nutritional stress resulted in the removal of methyls at CpGs located in the promoter of leptin gene, causing a permanent specific modification in the dynamics of the expression of leptin [27].

Visfatin

Visfatin is identical to pre-B-cell colony enhancing factor (PBEF, NAMPT), a cytokine involved in B-cell precursor maturation [28]. Visfatin is expressed in the human fetal membranes during normal gestation and parturition in the absence of infection and shows its effects on the expression of interleukin (IL)-6 and IL-8 [29].

Recent studies on the NAD(+) biosynthetic enzymes in the salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide mononucleotide adenylyltransferase 1 (NMNAT-1), have revealed important functions for these enzymes in SIRT1-dependent transcription regulation which is of interest with respect to the fact that SIRT1 can modulate chromatin function through direct deacetylation of histones as well as by promoting alterations in the methylation of histones and DNA, leading to the repression of transcription [30]. SIRT1 also participates in the recruitment of other nuclear enzymes to chromatin for histone methylation and DNA CpG methylation, thus making SIRT1 an elegant candidate for linking visfatin circulating levels with epigenetic regulation.

Adiponectin

It has been reported that decreased serum levels of adiponectin, an insulin sensitivity-related adipocytokine, in early pregnancy are able to predict an increased risk of GDM [31]. Also, it has been reported that the human placenta produces and secretes adiponectin, and that adiponectin and its receptors are differentially regulated by cytokines and their expression altered in women with gestational diabetes mellitus [32]. In addition, contrary to adults, some studies demonstrated a positive correlation between cord blood adiponectin levels and birth weight [33-34]. Recently, it has been reported that serum adiponectin levels at 11-13 weeks are increased in women that develop early pre-eclampsia by a mechanism unrelated to impaired placentation [35]. The relationship between circulating levels of adiponectin and fetal growth is, however, unclear. The results from studies investigating adiponectin levels in cord blood are highly contradictory [34, 36] and it is highly likely that any relationships between fetal growth and circulating adiponectin levels are covered by the negative correlation between maternal total fat mass and adiponectin plasma levels. In other words, it is highly difficult to separate possible causal effect of adiponectin on fetal growth from robust negative correlation of adiponectin levels with total maternal fat mass.

Fatty Acid Binding Protein

Adipocyte fatty acid binding protein (AFABP, also known as aP2 and FABP4) has recently been described as a novel adipokine associated with insulin resistance, T2DM, and cardiovascular disease. It was reported that the serum levels of AFABP were significantly increased in overweight and obese subjects as compared with lean controls and were correlated positively with waist circumference, blood pressure, and insulin resistance [37]. The results of study by Kralisch et al [38] indicate that maternal AFABP levels are significantly increased in GDM which might contribute to increased metabolic and cardiovascular risk in these patients and that body weight, renal function, and triacylglycerides are independently associated with serum AFABP concentrations [38]. This is highly interesting in the light of experiments carried out by Li et al [39] who demonstrated that transfection of the fibroblast-like preadipocytes with miRNA-143 antisense inhibitor induced a significant suppression of differentiation, and indicated decreased storage of lipid droplets and down-regulated expression of key adipocytes regulatory genes, such as CCAAT/enhancer-binding protein-α (C/EBPα) and AFABP. On the contrary, cells proliferation was increased with *miR143* inhibitor transfection, which provides the first evidence for stimulation of endogenous *miR143* in the differentiation of mammalian intramuscular fat, which in part contribute to the regulated expression of adipocyte genes. It is highly likely that expression of miR143 is being precisely regulated during pregnancy, although no experimental data are available yet, where it participates in control of differentiation of the human fat cells. Therefore, it can be hypothesized that AFABP could generally represent a good candidate for an intermediate factor in mediating persistent epigenetic changes during the prenatal development.

Resistin

Resistin is a metabolic hormone secreted by human adipocytes and mononuclear cells that has been postulated to play important roles in regulating energy homeostasis [40-42]. Resistin is expressed also in human placenta [43]. In a small cohort of cord blood samples obtained from pregnancies with intrauterine growth restriction (IUGR), macrosomia or normal pregnancies [44], umbilical serum levels and placental expression of resistin had positive correlation with

maternal serum resistin and negative correlation with birth weight. In addition, maternal serum resistin levels were inversely correlated with birth weight. In another study by Seol et al [45], maternal serum resistin levels were significantly elevated in women with preeclampsia compared to normal pregnant women and non-pregnant women. There was no significant difference in placental resistin expression and therefore it could be suggested that the source of the resistin in circulation is rather the maternal fat than placenta itself.

Conclusion

The presence of substantially increased concentrations (compared to the adults) of numerous adipocytokines in cord blood and placental and/or fetal tissues supports the hypothesis of adipocytokines involvement in the prenatal programming of postnatal development. The concentrations of adipokines in the fetus respond to a range of nutritional and endocrine stimuli, and therefore may represent a signal to changing environmental factors, thus providing a basis for establishment of neural pathways important for energy balance in later life. The precise mechanisms through which the epigenetic changes are induced are largely unclear. DNA methylation and miRNAs represent good candidates for possible mediators of these long-term, unique and highly individual effects. A deeper understanding of exact underlying mechanisms mediating prenatal modelling of postnatal metabolic pathways as well as more insight into early-life-based pathophysiology of civilization diseases may lead to individually-tailored, precisely-targeted therapy in the future.

Acknowledgement

The work was supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO,CZ.1.05/2.1.00/03.0101).

References

[1] Gluckma, P. D., Hanso, M. A., Coope, C. and Thornbur, K. L. (2008). Effect of in utero and early-life conditions on adult health and disease. *The New England Journal of Medicine*, 359, 61-73.

[2] Tob, E. W., Heijman, B. T., Kreme, D., Putte, H., Delemarre-va, de Waa H. A., Finke, M. J. and Wi, J. M. (2011). DNA methylation of IGF2, GNASAS, INSIGF and LEP and being born small for gestational age. Epigenetics *: Official Journal of the DNA Methylation Society*, 6, 171-176.

[3] Barre, R. and Zierat, J. R. (2011). DNA methylation in metabolic disorders. *The American Journal of Clinical Nutrition*, 93, 897S-8900.

[4] Lin, C. and Groo, L. (2009). Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes*, 58, 2718-2725.

[5] MacLenna, N. K., Jame, S. J., Melny, S., Pirooz, A., Jerniga, S., Hs, J. L. and Jank, S. M. (2004). Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. *Physiological Genomics*, 18, 43-50.

[6] K, X., Le, Q., Jame, S. J., Kellehe, S. L., Melny, S., Jerniga, S. and Y, X. (2006). Uteroplacental insufficiency affects epigenetic determinants of chromatin structure in brains of neonatal and juvenile IUGR rats. *Physiological Genomics*, 25, 16-28.

[7] Simmon, R. A. (2007). Developmental origins of beta-cell failure in type 2 diabetes: the role of epigenetic mechanisms. *Pediatric Research*, 61, 64R-67R.

[8] Tarry-Adkin, J. L. and Ozann, S. E. (2011). Mechanisms of early life programming: current knowledge and future directions. *The American Journal of Clinical Nutrition.*

[9] Chavan-Gauta, P., Sundran, D., Pisa, H., Nimbarg, V., Mehendal, S. and Josh, S. (2011). Gestation-dependent changes in human placental global DNA methylation levels. *Molecular Reproduction and Development*, 78, 150.

[10] Hale, C. N. and Barke, D. J. (2002). The thrifty phenotype hypothesis. *British Medical Bulletin*, 60, 5-20.

[11] Heijman, B. T., Tob, E. W., Stei, A. D., Putte, H., Blau, G. J., Susse, E. S. and Slagboo, P. E. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 17046-17049.

[12] Tob, E. W., Lume, L. H., Talen, R. P., Kreme, D., Putte, H., Stei, A. D. and Slagboo, P. E. (2009). DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Human Molecular Genetics*, 18, 4046-4053.

[13] Le, R. C., Feinbau, R. L. and Ambro, V. (1994). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75, 843-854.

[14] D, T. and Zamor, P. D. (2007). Beginning to understand microRNA function. *Cell Research*, 17, 661-663.

[15] Maccan, M. A., Padbur, J. F. and Marsi, C. J. (2011). miR-16 and miR-21 Expression in the Placenta Is Associated with Fetal Growth. *PloS one*, 6, E21210.

[16] Kies, W., Petzol, S., Topfe, M., Garte, A., Bluhe, S., Kapelle, T. and Korne, A. (2008). Adipocytes and adipose tissue. Best practice and research. *Clinical Endocrinology and Metabolism*, 22, 135-153.

[17] Meie, U. and Gressne, A. M. (2004). Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clinical Chemistry*, 50, 1511-1525.

[18] Ahim, R. S. and Flie, J. S. (2000). Leptin. *Annual Review of Physiology*, 62, 413-437.

[19] Forhea, A. J. and Fowde, A. L. (2009). The hungry fetus? Role of leptin as a nutritional signal before birth. *The Journal of Physiology*, 587, 1145-1152.

[20] Hendle, I., Blackwel, S. C., Meht, S. H., Whitt, J. E., Russel, E., Soroki, Y. and Cotto, D. B. (2005). The levels of leptin, adiponectin, and resistin in normal weight, overweight, and obese pregnant women with and without preeclampsia. *American Journal of Obstetrics and Gynecology*, 193, 979-983.

[21] Schubrin, C., Sieble, T., Kratzsc, J., Englar, P., Blu, W. F., Trie, K. and Kies, W. (2000). Leptin serum concentrations in healthy neonates within the first week of life: relation to insulin and growth hormone levels, skinfold thickness, body mass index and weight. *Clinical Endocrinology*, 51, 199-204.

[22] Stei, A. D., Kah, H. S., Rundl, A., Zyber, P. A., va, der Pal-de Brui K. and Lume, L. H. (2007). Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine. *The American Journal of Clinical Nutrition*, 85, 869-876.

[23] Lume, L. H., Stei, A. D., Kah, H. S. and Romij, J. A. (2009). Lipid profiles in middle-aged men and women after famine exposure during gestation: the Dutch Hunger Winter Families Study. *The American Journal of Clinical Nutrition*, 89, 1737-1743.

[24] Painte, R. C., d, Rooi S. R., Bossuy, P. M., Simmer, T. A., Osmon, C., Barke, D. J. and Bleke, O. P. (2006). Early onset of coronary artery disease after prenatal exposure to the Dutch famine. *The American Journal of Clinical Nutrition*, 84, 322-327.

[25] Sinclai, K. D., Allegrucc, C., Sing, R., Gardne, D. S., Sebastia, S., Bispha, J. and Thursto, A. (2007). DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 19351-19356.

[26] Yamagat, Y., Asad, H., Tamur, I., Le, L., Maekaw, R., Taniguch, K. and Taketan, T. (2009). DNA methyltransferase expression in the human endometrium: down-regulation by progesterone and estrogen. *Human Reproduction* (Oxford, England), 24, 1126-1132.

[27] Jouss, C., Parr, L., Lambert-Langlai, S., Mauri, A. C., Averou, J., Bruha, A. and Carrar, V. (2011). Perinatal undernutrition affects the methylation and expression of the leptin gene in adults: implication for the understanding of metabolic syndrome. *The FASEB Journal : official Publication of the Federation of American Societies for Experimental Biology*.

[28] Fukuhar, A., Matsud, M., Nishizaw, M., Segaw, K., Tanak, M., Kishimot, K. and Matsuk, Y. (2005). Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* (New York, N.Y.), 307, 426-430.

[29] Ognjanovi, S. and Bryant-Greenwoo, G. D. (2002). Pre-B-cell colony-enhancing factor, a novel cytokine of human fetal membranes. *American Journal of Obstetrics and Gynecology*, 187, 1051-1058.

[30] Zhan, T. and Krau, W. L. (2010). SIRT1-dependent regulation of chromatin and transcription: linking NAD(+) metabolism and signaling to the control of cellular functions. *Biochimica Et Biophysica Acta*, 1804, 1666-1675.

[31] William, M. A., Qi, C., Muy-River, M., Vadachkori, S., Son, T. and Luth, D. A. (2004). Plasma adiponectin concentrations in early pregnancy and subsequent risk of gestational diabetes mellitus. *The Journal of Clinical Endocrinology and Metabolism*, 89, 2306-2311.

[32] Che, J., Ta, B., Karteri, E., Zervo, S., Digb, J., Hillhous, E. W. and Vatis, M. (2006). Secretion of adiponectin by human placenta: differential modulation of adiponectin and its receptors by cytokines. *Diabetologia*, 49, 1292-1302.

[33] Kotan, Y., Yokot, I., Kitamur, S., Matsud, J., Nait, E. and Kurod, Y. (2004). Plasma adiponectin levels in newborns are higher than those in adults and positively correlated with birth weight. *Clinical Endocrinology*, 61, 418-423.

[34] Lindsay, R. S., Walker, J. D., Havel, P. J., Hamilton, B. A., Calder, A. A. and Johnstone, F. D. (2003). Adiponectin is present in cord blood but is unrelated to birth weight. *Diabetes Care*, 26, 2244-2249.

[35] Nand, S., Y, C. K., Giurcanean, L., Akoleka, R. and Nicolaide, K. H. (2011). Maternal serum adiponectin at 11-13 weeks of gestation in preeclampsia. *Fetal Diagnosis and Therapy*, 29, 208-215.

[36] Mazaki-Tov, S., Kanet, H., Parient, C., Hem, R., Schif, E. and Siva, E. (2005). Cord blood adiponectin in large-for-gestational age newborns. *American Journal of Obstetrics and Gynecology*, 193, 1238-1242.

[37] Fasshaue, M., Seege, J., Waldeye, T., Schre, S., Eber, T., Kratzsc, J. and Lossne, U. (2008). Serum levels of the adipokine adipocyte fatty acid-binding protein are increased in preeclampsia. *American Journal of Hypertension*, 21, 582-586.

[38] Kralisc, S., Stepa, H., Kratzsc, J., Verlohre, M., Verlohre, H. J., Drynd, K. and Lossne, U. (2008). Serum levels of adipocyte fatty acid binding protein are increased in gestational diabetes mellitus. *European Journal of Endocrinology/European Federation of Endocrine Societies*, 160, 33-38.

[39] L, H., Zhan, Z., Zho, X., Wan, Z., Wan, G. and Ha, Z. (2010). Effects of MicroRNA-143 in the differentiation and proliferation of bovine intramuscular preadipocytes. *Molecular Biology Reports.*

[40] Jamaluddi, M. S., Weakle, S. M., Ya, Q. and Che, C. (2011). Resistin: Functional roles and therapeutic considerations for cardiovascular disease. *British Journal of Pharmacology.*

[41] Higgin, M. and M, Auliff F. (2010). A review of maternal and fetal growth factors in diabetic pregnancy. *Current Diabetes Reviews*, 6, 116-125.

[42] Brian, D. D. and Malamitsi-Puchne, A. (2009). Reviews: adipocytokines in normal and complicated pregnancies. *Reproductive Sciences* (Thousand Oaks, Calif.), 16, 921-937.

[43] Lappa, M., Ye, K., Permeze, M. and Ric, G. E. (2005). Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies. *The Journal of Endocrinology*, 186, 457-465.

[44] Wan, J., Shan, L. X., Don, X., Wan, X., W, N., Wan, S. H. and Zhan, F. (2010). Relationship of adiponectin and resistin levels in umbilical serum, maternal serum and placenta with neonatal birth weight. *The Australian and New Zealand Journal of Obstetrics and Gynaecology*, 50, 432-438.

[45] Seo, H. J., O, M. J., Ye, M. K., Ki, A., Le, E. S. and Ki, H. J. (2010). Comparison of serum levels and the placental expression of resistin between patients with preeclampsia and normal pregnant women. Hypertension in pregnancy: *Official Journal of the International Society for the Study of Hypertension in Pregnancy*, 29, 310-317.

3.2. IS THERE ANY LINK BETWEEN SEVERE PRE-ECLAMPSIA ANDDEFINED POLYMORPHISMS IN LEPTIN AND ADIPONECTIN GENES?

Resumé

Patofyziologické pozadí preeklampsie, jedné z nejzávažnějších těhotenských komplikací, zůstává i přes zjevné pokroky současné vědy stále nejasné. Nedávno se objevila hypotéza, že důležitou roli při vzniku preeklampsie hraje narušená regulace rozsáhlé sítě adipokinů, produkovaných kromě tukové tkáně i placentou. Genetické pozadí exprese jednotlivých adipokinů přitom může být důležitým určujícím faktorem jejich sekrece.

Cílem studie bylo prozkoumat, zda jsou časté polymorfismy v genu pro leptin (LEP) a adiponektin (ADIPOQ) spojeny s rozvojem preeklampsie a souvisejících znaků (gestační hypertenze, proteinurie, intrauterinní růstové retardace plodu).

Studie zahrnovala celkem 123 žen s preeklampsií a 150 zdravých kontrolních žen podobného věku a parity, u kterých byl genotypizován polymorfismus LEP -2548 G/A a ADIPOQ T94G.

Mezi oběma skupinami nebyly rozdíly v distribuci genotypů ani alel sledovaných polymorfismů. Ve skupině preeklamptických těhotenství byl ovšem polymorfismus ADIPOQ T94G spojen s výskytem nízké porodní hmotnosti a matky, které byly nosičkami T alely, měly přibližně třikrát vyšší pravděpodobnost narození dítěte s nízkou porodní hmotností ($p = 0.004$).

Na základě výsledků naší studie se nezdá, že by sledované polymorfismy byly důležitými genetickými determinantami preeklamsie, je ovšem možné, že polymorfismus ADIPOQ T94G se účastní regulace tělesné hmotnosti novorozenců, zvláště u preeklamptických těhotenství.

doi:10.1111/j.1447-0756.2008.00794.x J. Obstet. Gynaecol. Res. Vol. 34, No. 5: 858–864, October 2008

Is there any link between severe pre-eclampsia and defined polymorphisms in leptin and adiponectin genes?

Julie Bienertová-Vašků[1], Zuzana Dostálová[2], Kateřina Kaňková[1], Petr Bienert[1], Anna Vašků[1] and Vít Unzeitig[2]

[1]*Department of Pathophysiology, Faculty of Medicine, Faculty of Medicine, Masaryk University, and* [2]*Department of Obstetrics and Gynaecology, Faculty Hospital Brno, Brno, Czech Republic*

Abstract

Aim: The pathophysiology of pre-eclampsia, one of the leading causes of maternal mortality worldwide, still remains unclear. Recently, it has been suggested that impaired regulation of complex interactions among various adipokines plays an important role in the development of pre-eclampsia. The aim of this study was to investigate whether the two common polymorphisms of the leptin (*LEP*) and adiponectin (*APM1*) genes are associated with the development of pre-eclampsia and its related traits (gestational hypertension, proteinuria and various measures of reduced fetal growth) in the Czech pre-eclamptic population.

Methods: The case–control study comprised a total of 123 pre-eclamptic women and 150 healthy controls of similar age and parity distribution. They were genotyped for the LEP −2548G/A (5′-untranslated region) and APM1 T94G (exon 2) polymorphisms using polymerase chain reaction.

Results: The allele frequency of the LEP −2548G polymorphism was 0.541 in the pre-eclamptic group *versus* 0.583 in the control group ($P = 0.578$); the frequency of the APM1 94G polymorphism was 0.073 and 0.079 ($P = 0.628$), respectively. No significant associations were detected between either of the two single nucleotide polymorphisms or any of the parameter biomarkers related to pre-eclampsia, such as gestational hypertension or proteinuria. However, the APM1 T94G polymorphism was significantly associated with a low birth weight in pre-eclamptic pregnancies, with mothers carrying the T-allele having an almost three-fold increase in the likelihood of giving birth to a child with a low birth weight for its gestational age (odds ratio, 2.7; 95% confidence interval, 0.18–5.9; $P = 0.004$).

Conclusions: The APM1 T94G and LEP −2548G/A polymorphisms do not seem to be major genetic determinants of susceptibility to pre-eclampsia in the Czech Caucasian population. However, evidence has been provided for possible APM1 T94G involvement in controlling the birth weight of children from pre-eclamptic pregnancies, thus supporting the hypothesis of T94G involvement in controlling the birth weight of newborns.

Key words: adiponectin, leptin, polymorphism, pre-eclampsia.

Introduction

During the last few years, many reports on the physiological importance of adipokines, especially leptin, adiponectin and resistin, during pregnancy have been published.[1–3]

Pre-eclampsia is a pregnancy-associated condition that is characterized by increased vascular resistance, proteinuria, oedema and coagulopathy; it has been generally linked to endothelial cell malfunction of the maternal vasculature.[4] Potentially related to the important role of adipokines in implantation and

Received: June 13 2007.
Accepted: January 10 2008.
Reprint request to: Dr Julie Bienertová Vašků, Institute of Pathophysiology, Faculty of Medicine, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic. Email: jbienert@med.muni.cz

angiogenesis, pre-eclampsia is characterized by shallow trophoblastic invasion, the sudden onset of maternal hypertension and dramatic increases in both maternal and fetal leptin concentrations.[5–12] So far, it remains unclear whether the dramatic hyperleptinemia in pre-eclampsia is a compensatory response to the decreased nutrient and oxygen supply to the underperfused placenta or the possible cause of it.[13]

Leptin is a 167-amino acid hormone that is synthesized mainly by adipocytes. It is released into the blood in proportion to the size of adipose tissue, which is consistent with the role of leptin as a signal of the energy stores.[14] It has already been reported that the expression of leptin increases markedly during pregnancy. This increase occurs during the first trimester before any perceptible increase in body weight, and this relationship suggests that its expression levels are modulated by other factors, possibly unrelated to the amount of body fat. Leptin concentrations rise along with those of estrogen and are correlated in early pregnancy with those of human chorionic gonadotrophin.[1] Muy-Rivera *et al*.[15] observed that pregnant women with elevated plasma leptin levels have a 3.8-fold increased risk of developing pre-eclampsia. These authors also investigated the association between a tetranucleotide repeat (TTTC)(n) polymorphism in the 3-flanking region of the leptin gene and plasma leptin levels in pre-eclampsia. They reported that the risk of developing pre-eclampsia was higher in women who carried this polymorphism when compared with those who did not. However, as this study was undertaken in a very small population sample, further investigation into this association will be necessary to assess the potential of the (TTTC)(n) polymorphism in the 3-flanking region of the *leptin* gene in pre-eclampsia development.

In 2000, Mammès *et al*.[16] reported that a single nucleotide polymorphism (SNP), LEP −2548G/A, located within the 5′-untranslated region of the *leptin* gene and immediately adjacent to sequences implicated in the regulation of *leptin* gene transcription, is associated with obesity phenotypes in the French population. Because it is located in an important regulatory sequence of the *leptin* gene, we postulated that this SNP has the potential to influence *leptin* gene expression in early pregnancy and could account for the shallow trophoblastic invasion in the placentae from pre-eclamptic pregnancies.[6–8]

Adiponectin is a circulating protein hormone that is secreted mainly by adipocytes[17] and has previously been associated with obesity,[18–20] metabolic syndrome[21] and type 1 and 2 diabetes.[22] Chow *et al*.[23] report that hypoadiponectinemia is a potential risk factor for hypertension in the non-diabetic Chinese population. Bråkenhielm *et al*.[24] have recently reported that adiponectin plays an important role in angiogenesis, employing caspase-mediated endothelial cell apoptosis, which represents a possible mechanism of adiponectin involvment in the pathogenesis of pre-eclampsia. Common polymorphisms in the *APM1* gene are considered to participate in controlling insulin resistance and/or components of the insulin resistance syndrome. Yang *et al*.[25] reported on the association of the T94G polymorphism in exon 2 of the *APM1* gene and insulin resistance. The common haplotypes of SNP +45 and SNP +276 polymorphisms of the *APM1* gene have been shown to be associated with increased susceptibility to pre-eclampsia.[26]

In this investigation, we studied the possible influence of the functional polymorphism LEP −2548 G/A within the promoter of the *leptin* gene (dbSNP ID rs7799039) and the APM1 T94G polymorphism on exon 2 of the *adiponectin* gene (dbSNP rs2241766) on the risk of developing pre-eclampsia in a highly homogenous cohort of central European Caucasian women. To date, no such study focusing on these two polymorphisms and their potential in pre-eclampsia has been undertaken and published. The specific aims of the study were: (i) to compare allele and/or genotype frequencies of these two SNPs between cases of pre-eclampsia and controls; and (ii) to identify the potential genetic risk variants for developing pre-eclampsia. Finally, we attempted to ascertain a genetic risk factor (allele or genotype) for an adverse pregnancy outcome, namely pre-term birth (<37th gestational week), associated with pre-eclampsia or a low birth weight.

Methods

Subjects

A total of 123 pre-eclamptic women were enrolled in the association study, together with 150 randomly selected healthy pregnant women of similar parity whose pregnancies were uncomplicated and spontaneous. Pre-eclampsia was defined according to the guidelines of the Czech Gynecological and Obstetrical Society: development of hypertension after the 20th week of pregnancy (systolic blood pressure, ≥140 mmHg; and/or diastolic blood pressure, ≥90 mmHg; measured at rest on two consecutive occasions at least 24 h apart) in previously normotensive

angiogenesis, pre-eclampsia is characterized by shallow trophoblastic invasion, the sudden onset of maternal hypertension and dramatic increases in both maternal and fetal leptin concentrations.[5–12] So far, it remains unclear whether the dramatic hyperleptinemia in pre-eclampsia is a compensatory response to the decreased nutrient and oxygen supply to the underperfused placenta or the possible cause of it.[13]

Leptin is a 167-amino acid hormone that is synthesized mainly by adipocytes. It is released into the blood in proportion to the size of adipose tissue, which is consistent with the role of leptin as a signal of the energy stores.[14] It has already been reported that the expression of leptin increases markedly during pregnancy. This increase occurs during the first trimester before any perceptible increase in body weight, and this relationship suggests that its expression levels are modulated by other factors, possibly unrelated to the amount of body fat. Leptin concentrations rise along with those of estrogen and are correlated in early pregnancy with those of human chorionic gonadotrophin.[1] Muy-Rivera *et al*.[15] observed that pregnant women with elevated plasma leptin levels have a 3.8-fold increased risk of developing pre-eclampsia. These authors also investigated the association between a tetranucleotide repeat (TTTC)(n) polymorphism in the 3-flanking region of the leptin gene and plasma leptin levels in pre-eclampsia. They reported that the risk of developing pre-eclampsia was higher in women who carried this polymorphism when compared with those who did not. However, as this study was undertaken in a very small population sample, further investigation into this association will be necessary to assess the potential of the (TTTC)(n) polymorphism in the 3-flanking region of the *leptin* gene in pre-eclampsia development.

In 2000, Mammès *et al*.[16] reported that a single nucleotide polymorphism (SNP), LEP −2548G/A, located within the 5′-untranslated region of the *leptin* gene and immediately adjacent to sequences implicated in the regulation of *leptin* gene transcription, is associated with obesity phenotypes in the French population. Because it is located in an important regulatory sequence of the *leptin* gene, we postulated that this SNP has the potential to influence *leptin* gene expression in early pregnancy and could account for the shallow trophoblastic invasion in the placentae from pre-eclamptic pregnancies.[6–8]

Adiponectin is a circulating protein hormone that is secreted mainly by adipocytes[17] and has previously been associated with obesity,[18–20] metabolic syndrome[21] and type 1 and 2 diabetes.[22] Chow *et al*.[23] report that hypoadiponectinemia is a potential risk factor for hypertension in the non-diabetic Chinese population. Bråkenhielm *et al*.[24] have recently reported that adiponectin plays an important role in angiogenesis, employing caspase-mediated endothelial cell apoptosis, which represents a possible mechanism of adiponectin involvment in the pathogenesis of pre-eclampsia. Common polymorphisms in the *APM1* gene are considered to participate in controlling insulin resistance and/or components of the insulin resistance syndrome. Yang *et al*.[25] reported on the association of the T94G polymorphism in exon 2 of the *APM1* gene and insulin resistance. The common haplotypes of SNP +45 and SNP +276 polymorphisms of the *APM1* gene have been shown to be associated with increased susceptibility to pre-eclampsia.[26]

In this investigation, we studied the possible influence of the functional polymorphism LEP −2548 G/A within the promoter of the *leptin* gene (dbSNP ID rs7799039) and the APM1 T94G polymorphism on exon 2 of the *adiponectin* gene (dbSNP rs2241766) on the risk of developing pre-eclampsia in a highly homogenous cohort of central European Caucasian women. To date, no such study focusing on these two polymorphisms and their potential in pre-eclampsia has been undertaken and published. The specific aims of the study were: (i) to compare allele and/or genotype frequencies of these two SNPs between cases of pre-eclampsia and controls; and (ii) to identify the potential genetic risk variants for developing pre-eclampsia. Finally, we attempted to ascertain a genetic risk factor (allele or genotype) for an adverse pregnancy outcome, namely pre-term birth (<37th gestational week), associated with pre-eclampsia or a low birth weight.

Methods

Subjects

A total of 123 pre-eclamptic women were enrolled in the association study, together with 150 randomly selected healthy pregnant women of similar parity whose pregnancies were uncomplicated and spontaneous. Pre-eclampsia was defined according to the guidelines of the Czech Gynecological and Obstetrical Society: development of hypertension after the 20th week of pregnancy (systolic blood pressure, ≥140 mmHg; and/or diastolic blood pressure, ≥90 mmHg; measured at rest on two consecutive occasions at least 24 h apart) in previously normotensive

women, and the onset of proteinuria (>300 mg of urinary protein/L over 24 h). Women with chronic hypertension preceding pregnancy were excluded from the study. Furthermore, women with polycystic ovary syndrome, hirsutism or menstrual cycle disturbances and those previously treated for infertility were excluded from both the study and control groups. The gestational age was repeatedly confirmed by standard ultrasound examination. All participants in the study originated from the very homogenous Czech Caucasian population.

A structured questionnaire and individual medical records were used to obtain covariate information on maternal age, height, pre-pregnancy and end-pregnancy weight, personal and family reproductive history and the medical history of first- and second-degree family members along with smoking history and socioeconomic status. Infant weight, length, prenatal history and medical history of neonates were also obtained from the medical documentation. Pregnancy weight gain, pre-conception body mass index (BMI) and BMI at the time of delivery were calculated.

This study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno, and was performed in adherence to the Declaration of Helsinki Guidelines. Each participant gave her written informed consent, which has been archived.

Genotyping

The white cell fraction from a peripheral venous blood sample (5 mL) was used to extract DNA according to the standard procedure using proteinase K. The SNPs, LEP –2548G/A (dbSNP ID rs7799039) 2548 bp upstream from the beginning of the exon 1 and APM1 T94G (dbSNP rs2241766) on exon 2, were detected according to previously published methods.[16,25] Briefly, each 12 μL reaction volume contained 10 ng genomic DNA, 1.5 μL 10X polymerase chain reaction (PCR) buffer, 1.5 μL $MgCl_2$, 200 μM dNTP, 2 pmol of each primer and 0.4 U of Taq DNA polymerase (Fermentas). The reactions were performed using an XP Cycler. The PCR amplification conditions were as follows: LEP –2548G/A polymorphism, 95°C for 5 min, 94°C for 30 s, 50°C for 45 s, 72°C for 50 s for 35 cycles and 72°C for 10 min; APM1 T94G polymorphism, 94°C for 6 min, 94°C for 30 s, 65°C for 15 s, 72°C for 40 s for 30 cycles and 72°C for 10 min. The reliability of genotyping was assessed by double-genotyping of approximately 20% of the samples, and no differences were found in independent assays. Negative controls were included in each reaction batch to exclude possible false-positives.

Statistical analysis

Differences in genotype distributions and their consistency with the Hardy–Weinberg equilibrium were compared using the χ^2-test, and differences in allele frequencies were compared using Fisher's exact test. The Hardy–Weinberg was measured for both SNPs using an online program on the website of Human Genetics, Munich (http://ihg.gsf.de). Differences in parameters studied between the two groups were tested using the Mann–Whitney test. The Bonferroni correction was used to correct for multiple comparisons where appropriate. Logistic regression analysis was used to investigate the independence of the association between the quantitative variables (age, pregravid maternal weight, maternal height, week of delivery and parity were included as independent variables) and pregnancy complications (preterm birth, low birth weight) using the alleles of examined polymorphisms; the analysis was adjusted for potential confounders (smoking, socioeconomic status). We used ANOVA and ANCOVA to examine the individual effects of the SNPs on pre-eclampsia-related parameters. The Statistica version 7.0 computer statistical package was used for analysing the data.

Results

The baseline clinical and anthropometric characteristics of the study subjects are summarized in Table 1. Pre-eclampsia cases had significantly higher pre-conception BMI ($P < 0.001$) and gave birth to children with a significantly lower birth weight than the control subjects ($P < 0.001$).

Relationship between LEP –2548G/A and APM1 T94G and pre-eclampsia risk

The Hardy–Weinberg equilibrium evaluated for both SNPs expressed a significant deviation from that of the cases cohort ($P = 0.02$). No significant differences in genotype distributions of both the LEP –2548G/A and the APM1 T94G were observed when comparing the pre-eclampsia cases and the controls (Figs 1,2). When assuming a codominant model of inheritance, no differences in distribution of genotypes between pre-eclampsia cases and controls were observed ($P =$ not

Table 1 Baseline patient characteristics

	Cases ($n = 123$)	Controls ($n = 150$)	*P*-values
Maternal age (years)	30.24 ± 5.4	28.6 ± 5.15	NS
pre-conception body weight (kg)	71.30 ± 16.16	68.16 ± 14.23	<0.001
BMI at the time of delivery (kg/m^2)	31.15 ± 7.5	29.337 ± 6.5	NS
Pregnancy weight gain (kg)	14.34 ± 6.70	13.17 ± 5.24	NS
Birth weight (g)	2537 ± 785 g	3060 ± 747 g	<0.001
Birth length (cm)	47.1 ± 4.7	48.9 ± 3.7	NS
Week of gestation at delivery	37	39	NS
Primiparity/multiparity	45/78	61/89	NS

BMI, body mass index; NS, not significant.

Table 2 Genotype distribution of examined polymorphisms in cases and controls

Polymorphism	Total (n)	Pre-eclampsia cases (n)	Controls (n)	*P*-values
LEP –2548G/A Codominant model				0.376
AA	48	26	22	
AG	142	61	81	
GG	83	36	47	
Recessive model				0.108
GG + GA N (%)	225	97	128	
AA N (%)	48	26	22	
AMP1 T94G Co-dominant model				0.650
GG	4	2	2	
TG	32	12	20	
TT	237	109	128	
Recessive model				0.610
TT + TG N (%)	269	121	148	
GG N (%)	4	2	2	

P-values were obtained by comparison between pre-eclampsia cases and controls.

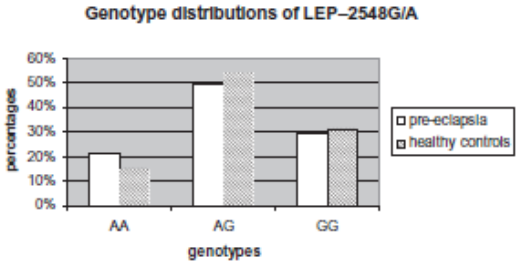


Figure 1 Genotype distributions of LEP –2548 G/A between cases and controls.

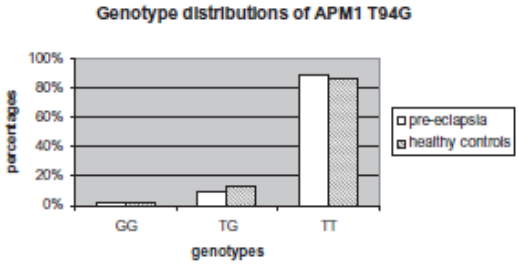


Figure 2 Genotype distributions of APM1 T94G between cases and controls.

significant). Furthermore, when assuming a recessive model of inheritance, no significant differences between pre-eclampsia cases and controls were observed either (Table 2). The LEP –2548G/A did not express a significant deviation from the Hardy–Weinberg equilibrium both in the cases and the controls; neither the genotypes nor the alleles of this polymorphism were significantly associated with increased pre-eclampsia risk.

Relationship between LEP –2548G/A and APM1 T94G and hypertension and proteinuria

We used ANOVA to examine the individual effects of both LEP –2548G/A and APM1 T94G on blood

pressure and proteinuria, and then used stepwise linear regression to consider the joint effects of both polymorphisms on these traits. None of the examined genetic variants expressed any effect on neither blood pressure or proteinuria (P = not significant).

Relationship between LEP −2548G/A and APM1 T94G and anthropometric data

Since both leptin and adiponectin are recognized to play an important role in adipose tissue signalling, we also investigated the potential of the examined polymorphisms to influence the adipose tissue status of pregnant women in the study. In the multivariate regression modeling, none of both examined polymorphisms served as the independent predictor for increased BMI or the absolute body weight (P = not significant).

Relationship between LEP −2548G/A and APM1 T94G and adverse pregnancy outcome

We evaluated the effect of the two SNPs studied on the risk of adverse pregnancy outcome defined as (a) preterm birth associated with pre-eclampsia (<37th gestational week); and (b) low birth weight, both in cases and controls. Genotypes of the LEP −2548G/A and APM1 T94G, together with maternal preconception weight, height and parity, represented input variables. Of the independent variables, maternal height was in the cases significantly correlated with both low birth weight and preterm birth risks ($r = -0.266$, $P = 0.03$, $P = 0.03$, respectively). As parity is recognized as an important risk factor for pre-eclampsia, we also analyzed the pre-eclamptic multiparous women separately from nulliparous ones, with no statistically significant differences. Furthermore, when using ANCOVA to estimate the potential influence of examined polymorphisms on birth weight of children from the pre-eclamptic pregnancies, the APM1 T94G showed an independent influence on birth weight in relation to gender of the child ($P = 0.04$, beta = 0.227), suggesting it controls birth weight in female newborns ($P = 0.002$, beta = 0.542) rather than in males. The observed effect was independent on the pregnancy weight-gain of the mother. Further analysis revealed that there was a significant trend toward low birth weight for gestational age associated with the T-allele (OR, 2.7; 95% CI, 0.18–5.9; $P = 0.004$) in the cases. However, no effect of the T-allele of APM1 T94G toward the lower birth weight was observed in the controls.

Discussion

Although it has been only a few years since the discovery of leptin and adiponectin, and initial recognition of their association with satiety and energy homeostasis, it is now evident that these adipokines also play crucial roles in reproductive biology. Pre-eclampsia is currently recognized as a complex pathophysiological condition that is characterized by aberrant endometrial invasion and hyperleptinemia in the maternal peripheral circulation.[6–12] Moreover, hyperleptinemia has been proposed as a potential biomarker for placental insufficiency.[8] Expression of LEP mRNA transcripts in the placentas of pre-eclamptic women is enhanced; however, it is not paralleled by adipose tissue specific upregulation, which further suggests causal leptin involvement in placental pathology.[27]

This study examined the possible relationship between LEP −2548G/A and APM1 T94G polymorphisms and pre-eclampsia risk in a sample of pre-eclamptic women originating from the very homogenous central European Czech population. It has been reported previously by our research group that the A-allele of the LEP −2548G/A polymorphism can contribute to gestational diabetes, despite the absence of genotypic associations with the condition.[28] This finding suggests that the *leptin* gene may play a role in the pathogenesis of insulin resistance in pregnancy. However, the results of the current study showed no significant associations between the LEP −2548G/A polymorphism and examined parameters related to pre-eclampsia.

Recently, it has been suggested that adiponectin gene variability can play an important role in modulating insulin sensitivity. Yang *et al.*[25] reported that the G-allele of the APM1 T94G polymorphism is significantly associated with increased insulin sensitivity: subjects carrying the G-allele seem to be more insulin sensitive than T-allele carriers. Jeng[29] recently reported that individuals carrying the TT genotype of the APM1 T94G polymorphism had reduced plasma adiponectin and higher plasma plasminogen activator inhibitor 1 (PAI-1) levels in a Chinese population. In that study, the hypertensive patients had significantly lower plasma adiponectin (9.7 ± 11.1 *vs* 11.5 ± 10.0 μg/ml, $P = 0.04$) and higher plasma PAI-1 ($P < 0.001$) levels than those measured in the normotensive subjects. In addition, Jeng *et al.* described that the frequency of the adiponectin TT genotype, (38.7 *vs* 33.5%) and the T-allele (0.620 *vs* 0.585) was significantly greater in the hypertensive group than in the normotensive subjects.

Collectively, these findings provide supporting evidence that the APM1 T94G polymorphism has functional properties.

In the current study, a significant linkage disequilibrium of the APM1 T94G polymorphism was observed in those individuals who developed pre-eclampsia. The APM1 T94G polymorphism was also an independent predictor for birth weight of the newborn according to gender, independent of maternal pregnancy weight gain. This finding is in full concordance with the results of previous studies on APM1 T94G polymorphism and its role in controlling insulin sensitivity, which is crucial for fetal growth and weight gain during gestation. Our results are also in agreement with those reported by Yang *et al*.[25] for the T-allele. They reported that the T-allele was more frequent in individuals who had low insulin sensitivity, and it was also significantly associated with a risk of low birth weight in our pre-eclamptic cohort. The T94G polymorphism of the *adiponectin* gene is a silent mutation for Gly15 (GGT to GGG). Yang and colleagues speculated that it might be in linkage disequilibrium with other genetic alterations, and that it is probably a regulatory mutation. Based on our findings, this hypothesis is to be supported.

Although substantial differences in allele and genotype frequencies across populations of differing geographical origins could be expected, the allele frequencies of the two polymorphisms closely resemble those of other European Caucasians as well as non-Caucasian populations, namely, German, French and Japanese populations.[16,30,31] Consistency in the genotype–phenotype patterns across different populations also contributes to the hypothesis of a possible functional impact of the examined genes.

Collectively, our results do not clearly support the idea of isolated involvement of LEP –2548G/A and AMP1 T94G polymorphisms in increased susceptibility to pre-eclampsia. However, we report here that the AMP1 T94G polymorphism expresses a significant influence on the birth weight of neonates, dependent on the gender of the child. Further work is necessary to determine the exact role of the APM1 T94G polymorphism in controlling the insulin sensitivity of pre-eclampsia, as well as its linkage disequilibrium with other functional variants.

Acknowledgement

The study was supported by a grant (FRVS 881-2006) from the Ministry of Education, Youth and Physical Education of the Czech Republic.

References

1. Henson MC, Castracane VD. Leptin in pregnancy. *Biol Reprod* 2006; **74**: 218–229.
2. Henson MD, Castracane VD. Leptin: roles and regulation in primate pregnancy. *Semin Reprod Med* 2002; **20**: 113–122.
3. Henson MC, Castracane VD. Leptin in primate pregnancy. In: Henson MC, Castracane VD, (eds). *Leptin and Reproduction*. New York: Kluwer Academic Press/Plenum Press Publishers, 2003: 239–263.
4. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Pre-eclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* 1989; **161**: 1200–1204.
5. Hoggard N, Haggartz P, Thomas L, Lea RG. Leptin expression in placental and fetal tissues: does leptin have a functional role? *Biochem Soc Trans* 2001; **29**: 57–63.
6. Lepercq J, Guerre-Millo M, Andre J, Cauzac M, Haguel-de Mouzon S. Leptin: a potential marker of placental insufficiency. *Gynecol Obstet Invest* 2003; **55**: 151–155.
7. Christou H, Connors JM, Ziotopoulou M *et al*. Cord blood leptin and insulin-like growth factor levels are independent predictors of fetal growth. *J Clin Endocrinol Metab* 2001; **86**: 935–938.
8. Anim-Nyame N, Soorana SR, Steer PJ, Johnson MR. Longitudinal analysis of maternal plasma leptin concentrations during normal pregnancy and further increased in pre-eclampsia. *Hum Reprod* 2000; **15**: 2033–2036.
9. Teppa RJ, Ness RB, Crombleholme WR, Roberts JM. Free leptin in increased in normal pregnancy and further increased in pre-eclampsia. *Metabolism* 2000; **49**: 1043–1048.
10. Linnemann K, Malek A, Schneider H, Fusch C. Physiological and pathological regulation of feto/placento/maternal leptin expression. *Biochem Soc Trans* 2001; **29**: 86–90.
11. Chappell LC, Seed PT, Briley A *et al*. A longitudinal study of biochemical variables in women at risk of pre-eclampsia. *Am J Obstet Gynecol* 2002; **187**: 127–136.
12. Odegard RA, Vatten LJ, Nilsen ST, Salvesen KA, Austgulen R. Umbilical cord plasma leptin is increased in pre-eclampsia. *Am J Obstet Gynecol* 2002; **186**: 427–432.
13. Tommaselli GA, Pighetti M, Nasti A *et al*. Serum leptin levels and uterine Doppler flow velicometry at 20 weeks gestation as markers for the development of pre-eclampsia. *Gynecol Endocrinol* 2004; **19**: 160–165.
14. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425–432.
15. Muy-Rivera M, Ning Y, Frederic IO, Vadachkoria S, Luthy DA, Williams MA. Leptin, soluble leptin receptor and leptin gene polymorphism in relation to pre-eclampsia risk. *Physiol Res* 2005; **54**: 167–174.
16. Mammes O, Betoulle D, Aubert R, Herbeth B, Siest G, Fumeron F. Association of the G-2548A polymorphism in the 5′ region of the LEP gene with overweight. *Ann Hum Genet* 2000; **64**: 391–394.
17. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995; **270**: 26746–9.
18. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab* 2002; **13**: 84–89.

19. Weyer C, Funahashi T, Tanaka S *et al*. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 2001; **86**: 1930–1935.
20. Bouatia-Naji N, Meyre D, Lobbens S *et al*. ACDC/adiponectin polymorphisms are associated with severe childhood and adult obesity. *Diabetes* 2006; **55**: 545–550.
21. Kim SM, Cho KH, Park HS. Relationship between plasma adiponectin levels and the metabolic syndrome among Korean people. *Endocr J* 2006; **53**: 247–254.
22. Lu HL, Wang HW, Wen Y, Zhang MX, Lin HH. Roles of adipocyte derived hormone adiponectin and resistin in insulin resistance of type 2 diabetes. *World J Gastroenterol* 2006; **12**: 1747–1751.
23. Chow WS, Cheung BM, Tso AW *et al*. Hypoadiponectinemia as a predictor for the development of hypertension: a 5-year prospective study. *Hypertension* 2007; **49**: 1455–1461.
24. Brăkenhielm E, Veitonmäki N, Cao R *et al*. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA* 2004; **101**: 2476–81.
25. Yang WS, Hsiung CA, Ho LT *et al*. Genetic epistasis of adiponectin and PPARgamma2 genotypes in modulation of insulin sensitivity: A family-based association study. *Diabetologia* 2003; **46**: 977–983.
26. Saarela T, Hiltunen M, Helisalmi S, Heinonen S, Laakso M. Adiponectin gene haplotype is associated with pre-eclampsia. *Genet Test* 2006; **10**: 35–39.
27. Haugen F, Ranheim T, Harsem NK, Lips E, Staff AC, Drevon CA. Increased plasma levels of adipokines in pre-eclampsia: Relationship to placenta and adipose tissue gene expression. *Am J Physiol Endocrinol Metab* 2006; **290**: E326–E333.
28. Bienertová-Vašků JA, Vašků A, Dostálová Z, Bienert P. Association of leptin genetic polymorphism –2548 G/A with gestational diabetes mellitus. *Genes Nutr* (in press).
29. Jeng JT. Plasma adiponectin, T94G gene polymorphism and PAI-1 in patients with and without hypertension. *Cardiology* 2007; **107**: 30–37.
30. Nieters A, Becker N, Linseisen J. Polymorphisms in candidate obesity genes and their interaction with dietary intake of n-6 polyunsaturated fatty acids affect obesity risk in a sub-sample of the EPIC-Heidelberg cohort. *Eur J Nutr* 2002; **41**: 210–221.
31. Hamajima N, Saito T, Matsuo K, *et al*. Genotype frequencies of 50 polymorphisms for 241 Japanese non-cancer patients. *J Epidemiol* 2002; **12**: 229–236.

3.3. ASSOCIATIONOF LEPTIN GENETIC POLYMORPHISM -2548 G/A WITH GESTATIONAL DIABETES MELLITUS

Resumé

Leptin je klíčovým adipokinem produkovaným ve velkém množství tukovou tkání, ale bylo zjištěno, že je ve velkém množství produkován i placentou. Je známo, že má vliv i na růst plodu. Cílem této studie bylo prozkoumat možnou souvislost mezi polymorfismem -2548 G/A v promotoru genu pro leptin a těhotenskými onemocněními charakterizovanými mimo jiné možnou poruchou růstu plodu, jako je gestační diabetes (makrosomie) nebo preeklampsie (intrauterinní růstová retardace). Tato studie se zaměřuje na srovnání genetického pozadí matky i dítěte a vyhodnocení jejich vlivu na výsledný fenotyp daného těhotenství.

Do studie bylo zařazeno 49 preeklamptických žen, 53 žen s fyziologickým těhotenstvím a 48 pacientek s gestačním diabetem a novorozenci z daných těhotenství. Byla zkoumána distribuce genotypů a alel polymorfismu LEP -2548 G/A.

Byla pozorována statisticky významně vyšší vnímavost ke gestačnímu diabetu v přítomnosti alely A a dále byla pozorována nejvyšší úroveň výskytu spontánních potratů v anamnéze u nosiček GG genotypu, zatímco u heterozygotek AG byl výskyt spontánních potratů nejnižší.

Výsledky této studie podporují hypotézu, že genetické pozadí na úrovni genu pro leptin může determinovat genetickou vnímavost k některým těhotenským patologiím typu gestačního diabetu či preeklampsie.

Genes & Nutrition Vol. 1, No. 2, pp. 117-124, 2006
ISSN 1555-8932 print,

ASSOCIATION OF LEPTIN GENETIC POLYMORPHISM -2548 G/A WITH GESTATIONAL DIABETES MELLITUS

Julie Anna Bienertová Vašků[1], Anna Vašků[1], Zuzana Dostálová[2], Petr Bienert[1]

[1]Institute of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic, 662 43; and [2]Clinic of Obstetrics and Gynecology, Masaryk University affiliated Hospital, Brno, Czech Republic 602 00

[Received December 23, 2005; Accepted May 9, 2006]

ABSTRACT: *The aim of this study was to investigate possible associations of -2548 G/A polymorphism in leptin gene promoter and pregnancy-associated diseases with abnormal fetal growth such as preeclampsia and gestational diabetes. The study was also focused on whether it is rather maternal or fetal variants that determines the pathological growth status. Peripheral or cord blood samples obtained from 49 preeclamptic women and their 39 newborns, 53 healthy controls and their 53 healthy newborns and 48 patients with gestational diabetes mellitus were evaluated for leptin gene (LEP) locus -2548 genotypes. The significantly higher risk for gestational diabetes mellitus was observed in the presence of an allele (AA and AG genotypes) against carriers of GG genotype (OR=2.84, 95%CI 1.14-7.07, p=0.02). There is a significant risk of diabetes mellitus associated to A allele (OR=1.79, 95%CI 1.02-3.14, p=0.03). Furthermore, evaluations of preeclamptic patients' data revealed a significant association of genotype distribution and delivery and spontaneous abortion rate, where the GG carriers performed the highest pregnancy rate while the AG carriers performed the lowest spontaneous abortion rate. Our results support the hypothesis for -2548 G/A leptin gene polymorphism involvement in ethiopathogenesis of pregnancy-associated diseases with abnormal fetal growth, especially gestational diabetes mellitus.*

KEY WORDS: Gestational Diabetes Mellitus, Leptin, Polymorphism, and Preeclampsia

Corresponding Author: Dr. Julie Anna Bienertová Vašků, Institute of Pathological Physiology, Faculty of Medicine, Masaryk University, Komenského nám. 2., Brno, Czech Republic, 662 46; E-mail: Vasku.Julie@seznam.cz

INTRODUCTION

Leptin, a small peptide produced by adipocytes, is implicated in a great number of endocrine regulations, including obesity, satiety regulation and fertility. This 167 amino acid protein transcribed from the *ob* gene, was originally cloned in the mouse during research directed at identifying the molecular defect in an obesity-prone strain, the *ob/ob* mouse (Zhang Y et al, 1994). Human leptin gene is located to 7q31 and contains three exons. At first, leptin was considered to be a signaling molecule limiting food intake and increasing energy expenditure (Zhang et al, 1994). This was essentially supported by the fact that rodents with genetic (Campfield et al, 1995; Halaas et al, 1995; Pelleymounter et al, 1995; Stephens et al, 1995; Weigle et al, 1995) or diet induced (Campfield et al, 1995) obesity that were injected with leptin had manifested with decrease in bodyweight and improvement of metabolic parameters. Furthermore, hypothalamus has been identified as the most probable critical target for the satiety effect of leptin that can be transported through the blood/brain barrier via a saturable transport system (Baumann et al, 1996; Golden et al, 1997).

Recently, human placentas have been identified as a major source of leptin and the existence of placenta specific upstream enhancer indicates that placenta leptin may be regulated differently to that of adipose origin (Green et al, 1995; Bi et al, 1997; Masuzaki et al, 1997). Furthermore, the placenta leptin localization suggests it could be released into both maternal and fetal blood. The localization of the leptin receptor on the maternal side of the placenta supports the hypothesis that placenta leptin may have an autocrine role on the placenta itself as well as an endocrine role in the mother (Lea et al, 2000). The recent reports suggest that leptin may exert physiological effects on the placenta and conception, including fetal and placenta angiogenesis, fetal growth and development, embryonic hematopoiesis (Holness et al, 1999; Henson and Castracane, 2000).

Taking into account that hyperinsulinemia and hypoxia induce partially overlapping pathophysiological disturbances during pregnancy and both of them are known to induce leptin secretion, we may ask what elements of the leptin promoter are responsible for these effects. Recently, evidence was provided that insulin

and hypoxia act as agonists on the human leptin transcription but on two different regulatory elements. It has been published that hypoxia induces leptin transcription by a hypoxia-inducible-factor-1 (HIF-1) dependent mechanism (Meissner et al, 2003) by identifying at least one hypoxia-responsive element, located -120 bp to -116 bp in the leptin promoter being involved in this HIF-1-mediated effect on the transcriptional regulation. Moreover, it was reported (Grosfeld et al, 2001) that the human leptin promoter carries a potential insulin response element, located in the region from -720 bp to -150 bp. Therefore, it could be suggested that placenta leptin synthesis can be stimulated by the combination of local (e. g., hypoxia) and generalized factors (e. g., hyperinsulinism). This is in agreement with recent finding that leptin gene expression and production are markedly elevated in placenta of diabetic women treated with insulin. The previous findings provide strong evidence that leptin production can be regulated in uteri and emphasize the role of placenta leptin in human pregnancy (Lepercq et al, 1998). In keeping with these findings, disturbances in leptin plasma levels occur not only in diabetic pregnancies with tendency to fetal macrosomia, but also in pregnancies representing the opposite trend – intrauterine growth retardation (IUGR, Jaquet et al, 1998).

As previously described, DNA polymorphisms in leptin gene (LEP) are linked to extreme obesity (Jaquet et al, 1998). Unlike the other polymorphic sites, the G-2548A polymorphism in the 5′ region of the LEP gene was reported not only to be associated with overweight (Clement et al, 1996; Mammes et al, 2000) but also to have a strong influence on leptin gene expression and adipose tissue secretion (Hoffstedt et al, 2002). Thus, we suppose it might also influence leptin levels during pregnancy, especially when taking into account that the polymorphic site is located approximately 1800 bp from the insulin response element within the leptin promoter.

From the personal history of patients whose fetuses suffer from IUGR we knot that some women are prone to have IUGR pregnancy while in others pregnancies with IUGR can alternate with normal birth weight pregnancies. As IUGR can in these cases be considered a maladaptive maternal-fetal genotype, attention was focused on investigating possible genetic background of intrauterine growth restriction in preeclampsia not only on mothers but also on the newborns from these pregnancies.

Based on these observations and the fact the G-2548A polymorphism has proved to influence leptin gene expression, possible associations between the -2548 G/A leptin gene polymorphism variants and pregnancy associated diseases implicating abnormal leptin status such as preeclampsia and gestational diabetes mellitus were set out to be identified.

MATERIALS AND METHODS

Subjects

Forty-nine women (group A; median age 29, age range 19-46 years) with preeclampsia, thirty-nine newborns of these preeclamptic women (group B, median of birth weight 1950 g, birth weight range 700-2300 g, 23 of these newborns were diagnosed intrauterine growth restriction (IUGR)), fifty-three healthy women with non-complicated single pregnancy without positive history of pregnancy or delivery complications and without serious internal disease (group C; median age 28, age range 21-45 years), fifty-three newborns of these healthy women (group D, median of birth weight 3650g, birth weight range 2350-4600 g) and forty-eight pregnant women with gestational diabetes mellitus (group E; median age 30, age range 24-39 years) were enrolled in study. To ensure homogeneity of the genetic background, the healthy controls, originating from a regional Czech population, were enrolled by random selection. Women with polycystic ovary syndrome, hirsutism or menstrual cycle disturbances and women previously treated for infertility were excluded from both the study and control groups.

In nine cases of children coming from preeclamptic pregnancies we did not succeed in obtaining the cord blood sample. All individuals in the study were Caucasians; the pregnant women were followed-up and their children delivered at Gynecology and Obstetrics Clinic, University Hospital Brno, Czech Republic.

All individuals in the study had given informed consent prior to their inclusion in the study expressing their agreement to the fact their blood samples and blood samples of their children would be included in the study. The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University Brno.

Glucose and diagnostic criteria for GDM

A standard OGTT protocol was used. After a 12-h overnight fasting, venous plasma samples were collected fasting, 1-h and 2-h post-oral 75-g glucose. The diagnosis of GDM was based on the criteria of the World Health Organization (plasma glucose thresholds mmol/l: 0h, 7.0; 2h, 7.8). This OGTT was performed routinely between 24 and 28 weeks gestation, but occasionally performed at other stages of gestations if clinically warranted. Women with type 1 or type 2 diabetes diagnosed before the pregnancy were excluded from the study.

Diagnostic criteria for preeclampsia

Preeclampsia was defined as the development of hypertension and new-onset proteinuria (>300 mg of urinary protein in 24 h) in women with no proteinuria at baseline. Hypertension was defined according to current guidelines that accept 140 and/or 90mmHg of systolic and diastolic pressure, respectively, or higher, as hypertension, when measured on two consecutive occasions at least 24 h apart. Women with chronic hypertension were excluded from the study.

Intrauterine growth restriction diagnostic criteria

IUGR was defined as infants whose birth weight is below the 10th percentile of birth weight adjusted for sex and

gestational age; children chromosomal abnormalities, fetal infections and multigestations were excluded from the study.

Genotyping of LEP -2548 G/A by PCR-Restriction Fragment Length Polymorphism (RFLP)

After venous blood sample (5-10 ml) or umbilical cord blood sample (1-5 ml) collection from each subject, white cell fraction was used to extract DNA according to standard procedure using proteinase K. The -2548 G/A polymorphism of the LEP gene was analyzed by RFLP as previously described (Mammes et al, 2000). The method was performed by PCR amplification using following primers: forward 5′-TTTCCTGTAATTTTCCCGTGAG and reverse 5′-AAAAGCAAAGACAGGCATAAA.

The PCR was followed by incubation of the resulting 242 bp at 37°C/overnight with *Hin6I* restriction enzyme, an isosquizomer of *Cfo I.* The restricted fragments were separated by electrophoresis on 2% agarose gels with ethidium bromide staining. The polymorphism was defined by presence (G allele) or absence (A allele) of a restriction site. To assess genotyping reliability double sampling in more than 20% of the samples was performed and found no differences. A quality control and negative controls were always used to identify possible false positive.

Statistical Analysis

The differences in genotype and allelic distributions as well as consistency of genotype distribution with Hardy-Weinberg equilibrium were tested using the $\div^2$ test. Statistical differences between mean values of groups were evaluated using the unpaired ANOVA test (Kruskal-Wallis). The observed number of each genotype was compared with that expected for a population in Hardy-Weinberg equilibrium using $\div^2$ test. The cases were analyzed according to age, weight, BMI, parity, delivery rate and spontaneous abortion history. The data analysis was performed using Statistica v. 6.0 (Statsoft Inc., Tulsa, USA) program package. The expected genotype distributions for LEP -2548 G/A under Hardy-Weinberg equilibrium were calculated for both patients and controls and they were compared with the observed distributions. This strategy confirmed whether the observed distributions were consistent with the Haedy-Weinberg equillibrium or not.

RESULTS

Borderline significant differences in genotype distributions between patients with preeclampsia and healthy controls (p=0.06) and patients with gestational diabetes mellitus and healthy controls (p=0.07) were found. This is presented in Table 1 as well as the genotype distributions and allelic frequencies in the two groups of children. The significantly higher risk for gestational diabetes mellitus was observed in presence of A allele (AA and AG genotypes) against carriers of GG genotype (OR=2.84, 95%CI 1.14-7.07, p=0.02).

The main preeclamptic patient characteristics according to LEP -2548 G/A genotypes are shown in Table 2. A statistically significant association between number of pregnancies and genotype distributions was found (p=0.04).

A statistically significant association between the number of spontaneous abortions in anamnesis of the patient and genotype distribution of examined polymorphism (p=0.03) was observed. In Table 3, we present the main personal characteristics of healthy women according to LEP -2548 G/A genotypes. No statistically significant differences were found between LEP -2548 G/A genotypes and age, weight or BMI. No statistically significant association of personal characteristic with a genotype of LEP -2548 G/A polymorphism in the group of gestational diabetes mothers was observed (Table 4).

TABLE 1. The frequency distribution of the genotypes of LEP -2548 polymorphism among mothers and children

MOTHERS	GG	GA	AA	PG	G	A	PA
Preeclampsia	12/24.5%	24/49.0%	13/26.5%		0.490	0.510	
HCMs	21/39.6%	24/45.3%	8/15.1%	0.06*	0.623	0.377	0.02**
GDs	9/18.8%	28/58.3%	11/22.9%		0.479	0.521	
HCMs	21/39.6%	24/45.3%	8/15.1%	0.07*	0.623	0.377	0.04**
GDs	9/18.8%	28/58.3%	11/22.9%		0.479	0.521	
Preeclampsia	12/24.5%	2449.0%	13/26.5%	0.775*	0.490	0.510	0.882**

CHILDREN	GG	GA	AA	PG	G	A	PA
CPG	12/30.8%	20/51.3%	7/17.9%		0.564	0.436	
HCC	15/28.3%	25/47.2%	13/24.5%	0,771*	0.519	0.481	0.543**

HCMs – healthy controls mother, GDs – gestational diabetes mother, CPG – newborn from preeclamptic pregnancy, HCC – healthy control child

** - Analysis for linear trend according to the presence of null, one or two A-alleles*

*** - Chi-square test*

P_g- probability of difference in genotype distributions between preeclampsia and HCMs, GDs and HCMs and GDs and preeclampsia

P_a- probability of difference in allelic frequencies between preeclampsia and HCMs, GDs and HCMs and GDs and preeclampsia

TABLE 2. Preeclamptic women Characteristics and LEP -2548 G/A Genotypes

	AA	AG	GG	P-VALUE
Age /means	29.40±4.24	30.08±4.44	33.80±6.01	0.136
Weight /means	71.00±19.19	73.56±19.18	67.00±11.38	0.579
BMI /means	25.78±6.84	25.80±5.86	24.23±3.88	0.783
Spontaneous abortion rate /median, (min+max)	1.0 (0.0-2.0)	0.0 (0.0-1.0)	0.0 (0.0-1.0)	0.03
Number of pregnancies/median, (min+max)	1.5 (1.0-5.0)	1.0 (0.0-4.0)	2.5 (1.0-6.0)	0.04

LEP – leptin, BMI – body mass index, Mean ± SEM, P- probability of difference in genotype distributions

TABLE 3. Healthy women Characteristics and LEP -2548 G/A Genotypes

	AA	AG	GG	P-VALUE
Age	30.25±7.28	30.04±4.48	26.67±4.09	0.070
Weight	65.75±14.35	70.43±11.19	71.76±11.47	0.581
BMI	23.18±3.75	25.14±3.88	25.66±3.43	0.321

LEP – leptin, BMI – body mass index, Mean ± SEM

TABLE 4. Diabetic women Characteristics and LEP -2548 G/A Genotypes

	AA/MEANS	AG/MEANS	GG/MEANS	P-VALUE
Age	31.63±4.80	30.50±3.98	29.88±1.96	0.686
Weight	77.18±27.54	71.00±17.32	73.77±20.53	0.976
BMI	27.40±9.43	25.38±5.65	26.67±6.66	0.974

LEP – leptin, BMI – body mass index, Mean ± SEM

TABLE 5. Comparative study of Genotype Frequencies in Normal Controls from Different Ethnic Populations

STUDY GROUP REF	NO.	GENOTYPES AA	AG	GG	P-VALUE	
Caucasian-European France	314	63 (20.1)	165 (52.5)	86 (27.4)	0.185*/0.635**	Mammes et al, 2000
Japanese	237	144 (60.8)	86 (36.3)	7 (3.0)	<0.001/<0.001	Hamajima et al, 2002
Caucasian-European Germany	152	24 (15.8)	84 (55.3)	44 (28.9)	0.338*/0.341**	Nieters et al, 2002
Caucasian-European Czech - Mothers	53	8 (15.1)	24 (45.3)	21(39.6)	0.331***	
Caucasian-European Czech - Children	53	13 (24.5)	25 (47.2)	15 (28.3)	referent	

Chi-square analysis comparing reported genotype frequencies with the present study

** - genotype frequencies of mothers*

*** - genotype frequencies of children*

**** - genotype frequencies of mothers against children*

DISCUSSION

GMD represents a heterogeneous disorder with both genetic and environmental component, as women with GMD tend to have more often family history of diabetes and as GMD is more frequent in some ethnic groups, independent of BMI (Dornhorst et al, 1992).

The physiological role of leptin in GMD pregnancy has yet to be entirely clarified. Leptin is known to be produced in large amounts by the placenta, which may explain why some researches described correlation between leptin levels and preeclampsia. However, in most of the studies, the results were not adjusted for maternal age or BMI. Previously, there were several conflicting reports on leptin levels in GMD pregnancy, with authors reporting similar (Simmons and Brier, 2002), reduced (Festa et al, 1999) or elevated (Hoffman et al, 1998) leptin levels in GMD women compared to healthy pregnancy controls.

The -2548 G/A polymorphism was first described as a sequence variant in the 5′ flanking region of the leptin gene (Li et al, 1999) and was previously associated to obesity. In 2002, it was reported that this polymorphism increase gene expression and adipose secretion of leptin (Hoffstedt, 2002).

As large for gestational age (LGA) infants coming mainly from diabetic pregnancies as well as those with IUGR associated rather with preeclampsia were reported to have different leptin expression profiles (Meissner et al, 2003; Jaquet et al, 1998; Hoffman et al, 1998), the possible associations of promoter polymorphism LEP gene -2548 G/A variants and maternal states with abnormal fetal growth such as preeclampsia and gestational diabetes mellitus were investigated.

Based on our data, it can be suggested that the AA and AG genotype carriers (that are supposed to have higher transcriptional activity of the LEP gene) have a significantly higher risk for gestational diabetes mellitus against those carrying the GG genotype, which supports the hypothesis for leptin involvement in ethiopathogenesis of GMD. Furthermore, evaluation of preeclamptic patients′ anamnestic data revealed a statistically significant association of genotype distribution and delivery and spontaneous abortion rate, where the GG carriers had the highest pregnancy rate while the AG carriers had the lowest spontaneous abortion rate. This suggests the leptin could also be involved in fertility regulation.

As it is known that women with a history of GMD have a high risk of progression to type 2 diabetes mellitus, it would be interesting to know whether A or G allele could be protective or risky in respect to type 2 diabetes risk. However, it definitely requires further investigation and patients′ follow-up to estimate whether the GG carriers are in higher risk of in the elderly or not, even if it is known that hyperleptinaemia in type 2 diabetes often goes hand in hand with insulin resistance, which supports the hypothesis leptin is involved in GMD as well as type 2 diabetes ethiopathogenesis. Still, we have to take into account that GMD as well as type 2 diabetes definitely are complex multifactorial disorders with both genetic and environmental component. A number of epidemiological studies examined serum leptin concentration in diabetic and non-diabetic subject. For example, among US Pima Indians, subjects suffering from type 2 diabetes had lower leptin concentrations than non-diabetic subjects, independently of percentage body fat (Fox et al, 1999). Even if it has been discussed previously that insulin resistance associated with GMD results in increased leptin secretion (Malmstrom et al, 1996, Utriainen et al, 1996), the prognosis of GMD patients in respect to type 2 diabetes still remains unclear.

The allelic distribution of -2548 G/A polymorphism of leptin gene promoter varies essentially among the European and Japanese population (Table 5). These findings proved to be consistent with allele frequencies reported recently for the German and French population (Mammes et al, 2000, Nieters et al, 2002). The A allele in the Japanese population showed essentially higher frequency (60.8%, Hamajima et al, 2002) than in the Czech Republic, which confirms previous findings.

A larger clinical study should be undertaken with a larger population sample to investigate the real meaning of correlations between leptin polymorphisms and preeclampsia, IUGR and gestational diabetes, supporting evidence for leptin gene polymorphism as a genetic factor on gestational diabetes risk. This is believed to be the first study focused on association between -2548 G/A leptin gene polymorphism and risk for gestational diabetes or preeclampsia.

REFERENCES

Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.F. and Tartaglia, L.A. (1996) The full-length leptin receptor has signaling capabilities of interleukin 6-type receptors. *Proceedings of the National Academy of Sciences of the United States of Americ*a 16:8374-8

Bi, S., Gavrilova, O. and Gong, D.W. (1997) Identification of a placental enhancer for the human leptin gene. *The Journal of biological chemistry* 48:30583-8.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R. and Burn, P. (1995) Recombinant mouse OB protein; evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546-9

Clement, K., Garner, C., Hager, J., Philippi, A., LeDuc, C., Carey, A., Harris, T.J., Jury, C., Cardon L.R., Basdevant, A., Demenais, F., Guy-Grand, B., North, M. and Froguel, P. (1996) Indication for linkage of the human OB gene region with extreme obesity. *Diabetes* 5:687-90

Dornhorst, A., Paterson, C.M., Nicholls, J.S., Wadsworth, J., Chiu, D.C., Elkeles, R.S., Johnston, D.G. and Beard, R.W. (1992) High prevalence of gestational diabetes in women from ethnic minority groups. *Diabetic Medicine* 9:820-5

Festa, A., Shnawa, N., Krugluger, W., Hopmeier, P., Schernthaner, G. and Haffner, S.M. (1999) Relative hypoleptinaemia in women with mild gestational diabetes mellitus. *Diabetic Medicine* 8:656-62.

Fox, C., Esparza, J., Nicolson, M., Bennett, P.H., Schulz, L.O. and Valencia, M.E. (1999). Plasma leptin concentrations in Pima Indians living in drastically different environments. *Diabetes Care* 3:413-7.

Golden, P.L., Maccagnan, T.J. and Pardridge, W.M. (1997) Human blood-barrier leptin receptor; binding and endocytosis in isolated human brain microvessels. *Journal of Clinical Investigation* 1:14-8

Green, E.D., Maffei, M. and Braden, V.V. (1995) The human obese (Ob) gene – RNA expression pattern and mapping on the physical, cytogenetic, and genetic maps of chromosome . *Genome Research* 1:5-12

Grosfeld, A., Turban, S., Andre, J., Cauzac, M., Challier, J.C., Hauguel-de Mouzon, S., Guerre-Millo, M. (2001) Transcriptional effect of hypoxia on placental leptin. *Federation of European Biochemical Societies Letters* 3:122-6.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K. and Friedman, J.M. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269:543-6

Hamajima, N., Saito, T., Matsuo, K., Suzuki, T., Nakamura, T., Matsuura, A., Okuma, K. and Tajima, K. (2002) Genotype frequencies of 50 polymorphisms for 241 Japanese non-cancer patients. *Journal of epidemiology* 3:229-36.

Henson, M.C. and Castracane, V.D. (2000) Leptin in pregnancy. *Biology of reproduction* 5:1219-28

Hoffman, L., Nolan, C., Wilson, J.D., Oats, J.J. and Simmons, D. (1998) Gestational diabetes mellitus—management guidelines - The Australasian Diabetes in Pregnancy Society. *The Medical journal of Australia* 2:93-7.

Hoffstedt, J., Eriksson, P., Mottagui-Tabar, S. and Arner, P. (2002) A polymorphism in the leptin promoter region (-2548 G/A) influences gene expression and adipose tissue secretion of leptin. *Hormone and metabolic research* 7:355-9

Holness, M.J., Munns, M.J. and Sugden, M.C. (1999) Current concepts concerning the role of leptin in reproductive function. *Molecular and cellular endocrinology* 1-2:11-20.

Jaquet, D., Leger, J., Levy-Marchal, C., Oury, J.F. and Czernichow, P. (1998) Ontogeny of leptin in human fetuses and newborns: effect of intrauterine growth retardation on serum leptin concentrations. *The Journal of clinical endocrinology and metabolism* 4:1243-6.

Lea, R.G., Howe, D., Hannah, L.T., Bonneau, O., Hunter, L. and Hoggard, N. (2000) Placental leptin in normal, diabetic and fetal growth-retarded pregnancies. *Molecular human reproduction* 8:763-9.

Lepercq, J., Cauzac, M., Lahlou, N., Timsit, J., Girard, J., Auwerx, J. and Hauguel-de Mouzon, S. (1998) Over expression of placental leptin in diabetic pregnancy: a critical role for insulin. *Diabetes* 5:847-50.

Li, W.D., Reed, D.R., Lee, J.H., Xu, W., Kilker, R.L., Sodam, B.R. and Price, R.A. (1999) Sequence variants in the 5' flanking region of the leptin gene are associated with obesity in women. *Annals of human genetics* Pt 3:227-34

Malmstrom, R., Taskinen, M.R., Karonen, S.L. and Yki-Jarvinen, H. (1996) Insulin increases plasma leptin concentrations in normal subjects and patients with NIDDM. *Diabetologia* 8:993-6.

Mammes, O., Betoulle, D., Aubert, R., Herbeth, B., Siest, G. and Fumeron, F. (2000) Association of the G-2548A polymorphism in the 5' region of the LEP gene with overweight. *Annals of human genetics* 5:391-4.

Masuzaki, H., Ogawa, Y. and Sagawa, N. (1997) Non-adipose tissue production of leptin: Leptin as a novel placenta-derived hormone in humans. *Nature Medicine* 9:1029-33

Meissner, U., Ostreicher, I., Allabauer, I., Rascher, W. and Dotsch, J. (2003) Synergistic effects of hypoxia and insulin are regulated by different transcriptional elements of the human leptin promoter. *Biochemical and biophysical research communications* 2:707-12

Nieters, A., Becker, N. and Linseisen, J. (2002) Polymorphisms in candidate obesity genes and their interaction with dietary intake of n-6 polyunsaturated fatty acids affect obesity risk in a sub-sample of the EPIC-Heidelberg cohort. *European journal of nutrition* 5:210-21

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T. and Collins, F (1995). Effect of the obese gene product on body weight regulation in ob/ob mice. *Science* 269:540-3

Simmons, D., Breier, B.H. (2002) Fetal overnutrition in polynesian pregnancies and in gestational diabetes may lead to dysregulation of the adipoinsular axis in offspring. *Diabetes Care* 9:1539-44

Stephens, T.W., Basinski, M., Bristow, P.K., Bue-Valleskey, J.M., Burgett, S.G. and Craft, L. (1995) The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530-2

Utriainen, T., Malmstrom, R., Makimattila, S. and Yki-Jarvinen, H. (1996) Supraphysiological hyperinsulinemia increases plasma leptin concentrations after 4 h in normal subjects. *Diabetes* 10:1364-6.

Weigle, D.S., Bukowski, T.R., Foster, D.C., Holderman, S., Kramer, J.M. and Lasser, G. (1995) Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. *Journal of Clinical Investigation* 4:2065-70.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425-32

3.4. A COMMON VARIATION IN THE CANNABINOID 1 RECEPTOR (CNR1) GENE IS ASSOCIATED WITH PRE-ECLAMPSIA IN THE CENTRAL EUROPEAN POPULATION

Resumé

Nedávno se objevila hypotéza, která tvrdí, že přísně regulovaná exprese endogenních kanabinoidů může hrát velmi důležitou roli v časném vývoji placenty. Cílem této studie bylo proto prozkoumat souvislost mezi třemi jednonukleotidovými polymorfismy v endokanabinoidním receptoru typu I (CNR1 – rs1049353,rs12720071 a rs806368) a souvisejícími haplotypy a preeklampsií, onemocněním charakterizovaným mělkou invazí deciduálních arterií a obecně poruchami placentace.

Tato studie zahrnuje celkem 115 žen s preeklampsií a 145 zdravých žen s fyziologickým těhotenstvím, přičemž všechny ženy pocházely ze stejného středoevropského regionu.

Byly pozorovány významné rozdíly v distribuci genotypu rs806368 při srovnání pacientek s preeklampsií a kontrol, kdy u pacientek s preeklampsií byl významně nižší podíl homozygotek CC. Ve vícerozměrné analýze sloužil polymorfismus rs806368 jako významný prediktor rozvoje preeklampsie. Haplotypová analýza odhalila existenci čtyř častých haplotypů, přičemž haplotyp CAA byl méně častý u preeklampsie než u kontrol. Analýza regresních modelů potvrdila nezávislou predikční roli haplotypu AAC pro nástup preeklampsie.

Jedná se o první studii popisující vztah mezi jednonukleotidovými polymorfismy v genu pro CNR1 a rizikem preeklampsie. Ačkoli je sledovaný soubor relativně málo rozsáhlý, výsledky naší studie ukazují, že rs806368 se může chovat jako jeden z markerů vnímavosti k preeklampsii u středoevropské populace.

European Journal of Obstetrics & Gynecology and Reproductive Biology 155 (2011) 19–22

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

A common variation in the cannabinoid 1 receptor (CNR1) gene is associated with pre-eclampsia in the Central European population

Julie Bienertova-Vasku [a,*], Petr Bienert [a], Zuzana Dostalova [b], Josef Chovanec [b], Anna Vasku [a], Vladimir Vasku [a]

[a] Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic
[b] Clinic of Gynaecology and Obstetrics, University affiliated Hospital, Brno, Czech Republic

ARTICLE INFO

Article history:
Received 14 April 2010
Received in revised form 8 July 2010
Accepted 2 November 2010

Keywords:
Endocannabinoids
CNR1
Pre-eclampsia
Polymorphism
Haplotype

ABSTRACT

Objective: Recently it has been proposed that tightly regulated levels of endogenous cannabinoids play a fundamental role in early placental development. The aim of this study was to investigate associations of three single-nucleotide polymorphisms (SNPs) in the cannabinoid 1 receptor (CNR1) gene (rs1049353, rs12720071 and rs806368) and their inferred haplotypes with pre-eclampsia, a severe pregnancy-associated condition characterized by abnormal development and remodeling of spiral decidual arteries.
Study design: The case-control study comprised a total of 115 pre-eclamptic women and 145 healthy pregnant controls, all originating from the Central-European Czech population. Using PCR-based methods, we tested rs1049353, rs12720071 and rs806368 in the CNR1 gene and haplotypes were constructed.
Results: Statistically significant difference in genotype distributions of rs806368 ($p_g < 10^{-3}$) was observed when comparing the cases and the controls; the cases presenting with significantly lower proportion of CC homozygotes. In multivariate modeling, the rs806368 served as a predictor for pre-eclampsia development ($\beta = 0.15$; $p = 0.04$). Haplotype analysis revealed presence of four common haplotypes; the CAA haplotype being less frequent in pre-eclamptic cases compared to the controls ($p < 0.008$). Analysis of regression models confirmed the independent prediction role of AAC haplotype for pre-eclampsia onset ($\beta = -0.18$; $p = 0.03$).
Conclusion: This is the first study focusing on the relationship between SNPs in the CNR1 gene and pre-eclampsia risk. Although limited by a relatively small sample size, the study indicates that rs806368 in the CNR1 gene may act as a susceptibility marker for pre-eclampsia in humans.

1. Introduction

The endocannabinoid system (ECS), including cannabinoid receptors 1 and 2 (CB1 and CB2), endogenous ligands ("endocannabinoids"), regulating enzymes and transporter molecules, has been detected from the earliest embryonal stages, implantation and throughout pre- and postnatal development [1,2]. The ECS seems to play an essential role not only during the crucial developmental stages, i.e. early embryonal development, the early phase of fetal brain development, and regulation of suckling in newborns and infants, but also in adulthood, where it has pleiotropic modulatory and regulatory effects on various neuro-endocrinological pathways.

Successful implantation and pregnancy progression are characterized by low plasma levels of anandamide (AEA), the crucial endocannabinoid [3,4], while in labour in humans the levels of this molecule dramatically rise [3]. It has been suggested that for successful pregnancy progression, a higher plasma AEA level at ovulation and a significantly lower level during implantation are required [5]. On the contrary, high levels of anandamide in early pregnancy [6] were reported to be associated with spontaneous miscarriage.

Following implantation, the uterus undergoes continuous extensive remodeling in order to provide an adaptive environment for the successful development of the embryo [7]. The ongoing process of tissue remodeling of the uterus represents a crucial condition for successful pregnancy, and its failure might result in development of pre-eclampsia, but very little is known about the molecular mechanisms underlying this process.

The recent study by Fonseca et al. [7] indicates that a tightly regulated level of endocannabinoids might play a crucial role not only in the early implantation period but also in the subsequent

* Corresponding author. Fax: +420 549 494 340.
E-mail addresses: jbienert@med.muni.cz, Vasku.julie@seznam.cz (J. Bienertova-Vasku).

0301-2115/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ejogrb.2010.11.004

spatio-temporal placental development. The results of immunohistochemical analysis indicated that CB1 is expressed mainly in decidual cells throughout gestation, suggesting that functioning of CB1 is related to the further phases of pregnancy following implantation. The differential spatio-temporal expression patterns of CB1 and CB2 in the pregnant uterus suggest that there is an uncoupling of these two types of anandamide binding receptors in mid/late gestation that are related to changes in cell phenotype.

Pre-eclampsia is a severe pregnancy-associated condition with relatively high morbidity and mortality that is characterized by abnormal development and remodeling of spiral decidual arteries, resulting later in pregnancy in impaired blood supply to the fetus and maladaptive maternal systemic response. Since the ECS plays an important role during the early stages of pregnancy which are crucial for optimum decidual spiral artery development, dysregulation of this system could be involved in the pathogenesis of pre-eclampsia.

The aim of the study therefore was to investigate possible associations of three common polymorphisms in the cannabinoid 1 receptor (CNR1) gene, 1359 A/G (rs1049353), 3813 A/G (rs12720071) and 4895 C/T (rs806368), and their inferred haplotypes with pre-eclampsia risk and its related risk factors and fetal parameters.

2. Materials and methods

2.1. Subjects

At the Clinic of Gynaecology and Obstetrics of the University Affiliated Hospital Brno, a total of 115 pre-eclamptic women were enrolled into this study, together with 145 healthy pregnant women of similar parity whose pregnancies were physiological, uncomplicated and spontaneous. The controls were recruited after the index cases in order to reduce possible bias. Gestational age was confirmed by first-trimester ultrasound in all subjects; gestational age at recruitment to the study was 27–40 weeks for the cases and 38–41 weeks for the controls.

Pre-eclampsia was defined according to the recent criteria of the International Society for the Study of Hypertension in Pregnancy [8]. These criteria include no previous history of hypertension, cardiovascular, or renal disease before pregnancy and blood pressure values exceeding 140/90 mmHg after the 20th week of gestation, confirmed by two consecutive readings at least six hours apart, with blood pressure reverting to normal within two months after delivery. The subjects were considered to have a physiological pregnancy if they did not have obstetrical, medical, or surgical complications of pregnancy, and delivered a term (>38 weeks) neonate with a birth weight above the 10th percentile for gestational age [9].

Intrauterine growth restriction (IUGR) was defined as an estimated birth weight below the 10th percentile of a reference group. Doppler velocimetry of material uterine arteries, umbilical cord, and middle cerebral arteries of the fetus was performed on a routine basis in the pre-eclamptic cohort. Pregnancy weight gain, pre-conception body mass index (BMI) and BMI at the time of delivery were calculated based on information obtained from the study subjects.

Women with pre-eclampsia superimposed on chronic hypertension preceding pregnancy were included in the study; on the contrary, subjects who had chronic hypertension of any etiology before pregnancy without superimposed consequent pre-eclampsia were not included in the study, as they might carry such genetic variants that are protective against pre-clampsia development.

This study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno, and was performed in adherence to the Declaration of Helsinki Guidelines. Each participant gave her written informed consent, which has been archived.

2.2. Genotyping

DNA for analyses was extracted from 5 mL of the patient's peripheral blood using the standard protocol based on proteinase K. Genotyping of the polymorphisms was performed as described previously [10,11] using a standard PCR-based methodology with following restriction fragment length polymorphism (RFLP). Restricted fragments were separated by electrophoresis on 2% agarosis gels with ethidium bromide staining. To assess genotyping reliability, we performed double sampling in more than 20% of the samples and found no differences. We always used quality control, and negative controls were used to identify possible false-positives. The genotyping success was 100% for all included single-nucleotide polymorphisms (SNPs).

2.3. Statistics

The genotype distributions were tested for Hardy–Weinberg equilibrium by a set of chi-square tests. Allelic frequencies were estimated by "counting method" and differences in allele frequencies between case and control subjects were tested by likelihood ratio χ^2 tests for 2×2 tables (two alleles, case vs. control subjects). Where applicable, it was first determined whether the variable presented a normal distribution using the Kolmogorov–Smirnov test, and in cases of skewed variables, logarithmic transformation was performed. To identify genetic as well as non-genetic variables that may contribute to predicting the specific phenotype, we carried out a forward stepwise logistic regression, a sequential procedure of adding one input variable at a time to build up a regression model in which the dependent variable (i.e. probability of presence or absence of pre-eclampsia) is represented as the logistic function of linear variables (anthropometric and clinical data and genotypes of three investigated SNPs). Odds ratios were calculated for the multiple logistic regression analysis models; we adjusted for covariates including age (continuous), BMI (<23, 23–24.9, 25–29.9, 30–34.9, or ≥35 kg/m^2), smoking (never, past, and current) and parity.

The data analysis was performed using Statistica v. 8.0 (Statsoft Inc., Tulsa, OK, USA) program package at the significance level defined as $p < 5\%$. Analysis of haplotypes was performed using the Haploview program package [http://www.broad.mit.edu/haploview/].

3. Results

The clinical characteristics of the study population are shown in Table 1. Maternal age, parity, maternal pre-conceptional as well as end-pregnancy BMI, gestational age at delivery, birth weight and smoking status were found to be significantly different between the two cohorts ($p < 0.05$). In addition, 22 (19%) of the 115 preeclamptic patients delivered a fetus with IUGR, defined as birth weight below the 10th percentile for gestational age.

Table 2 shows the allele and genotype distribution of CNR1. For all of the investigated polymorphisms except for 4895 C/T (rs806368) in the case cohort, genotype distributions conformed to Hardy–Weinberg equilibrium. The determination of the pairwise linkage disequilibrium (LD) indicated that there was a strong LD between the polymorphisms rs806368 and rs12720071 ($D' > 0.8$) in the cases as well as in the controls.

3.1. 1359 A/G (rs1049353)

No significant differences in allele or genotype frequencies were observed between the cases and the controls. As only a few

Table 1
Baseline characteristics of the pre-eclamptic subjects and control subjects with physiological pregnancies.

	Cases (n = 115)	Controls (n = 145)	p
Maternal age (years)	30.24 ± 5.47	27.43 ± 5.26	0.0001
Pre-conception BMI (kg/m^2)	26.07 ± 6.12	22.34 ± 3.99	0.01
End-pregnancy BMI (kg/m^2)	32.14 ± 6.99	26.92 ± 3.96	0.0003
Pregnancy weight gain (kg)	14.34 ± 6.70	12.56 ± 4.24	NS
Birth weight (g)	2537 ± 785	3395 ± 486	≤0.000001
Week of gestation at delivery (median/range)	38 (28–41)	40 (38–41)	0.0001
Week of gestation at recruitment (median/range)	34 (27–40)	39 (38–40)	0.001
IUGR (n)	22	0	≤0.000001
Primiparity/multiparity	50/65	79/66	NS
Smokers/non-smokers	34/81	20/125	0.01

BMI, body mass index; IUGR, intrauterine growth restriction; NS, not significant, results are given as mean ± SD, if not otherwise stated.

Table 2
Differences in genotype distributions and allele frequencies in the investigated cohorts.

SNP	Number and frequency (%) of genotypes				p[a]	OR	(95% CI)
	Pre-eclampsia		Controls				
1359 A/G (rs1049353)							
AA	61	(53)	94	(65)	0.19	0.81	0.55–1.23
AG	49	(43)	47	(41)	0.15	1.32	0.82–2.10
GG	5	(4)	4	(4)	0.75	1.26	0.36–4.46
3813 A/G (rs12720071)							
AA	91	(79)	114	(79)	0.55	1.01	0.70–1.65
AG	23	(20)	29	(20)	0.56	1.00	0.55–1.82
GG	1	(1)	2	(1)	0.59	1.26	0.08–20.38
4895 C/T (rs806368)							
CC	39	(34)	82	(57)	**0.02**	**0.60**	0.38–0.94
CT	71	(62)	52	(36)	**0.009**	**1.72**	1.12–2.66
TT	5	(4)	11	(7)	0.22	0.57	0.19–1.70

[a] Significances of differences in the number of genotypes were established by comparing the numbers on each line with the additional numbers on both other lines using conventional Fisher's exact test.

cases of GG homozygotes were identified, the analysis was performed according to a G dominant model (AG + GG vs. AA). When assuming the G dominant model of inheritance, significant differences were observed between the studied cohorts, the carriers of the G allele (AG + GG) being more frequent in the cases (p = 0.004). Univariate analysis, adjusted for covariates, revealed no significant associations of the 1359 A/G polymorphism with any of the investigated quantitative traits.

3.2. 3813 A/G (rs12720071)

No significant differences in allele or genotype frequencies were observed for the 3813 A/G polymorphism. We also pooled the GG homozygotes and AG heterozygotes to perform the analysis under a G dominant model (AG + GG vs. AA) and no significant associations were observed. Univariate analysis also did not reveal any significant relationship with investigated traits, such as pre-conceptional BMI, end-pregnancy BMI, pregnancy weight gain, birth weight of the infant or the gestational week of delivery.

3.3. 4895 C/T (rs806368)

A strong deviation from the Hardy–Weinberg equilibrium was observed in the cases for the CNR1 4895 C/T. Moreover, significant difference in genotype distributions was observed when comparing the cases and controls (p = 0.0002). The CC homozygote genotype was significantly less frequent in the cases compared to the controls (34% in the cases vs. 57% in the controls, p = 0.002), which is indicative of a possible protective role of the C allele against pre-eclampsia development. Therefore, an analysis was performed assuming the dominant model (CC + CT carriers vs. TT carriers). The mean end-pregnancy BMI was significantly higher in pooled C allele carriers CC and CT (32.06 ± 6.09 kg/m^2) compared to T allele carriers (24.60 ± 4.8). In the multivariate regression modelling, the 4895 C/T polymorphism expressed a significant prediction role on the end-pregnancy BMI, whereas this association retained its significance after appropriate adjustments and this association was independent of pregnancy weight gain. The mean values of end-pregnancy BMI related to 4895 C/T are given in Table 3.

3.4. Haplotype analysis

Haplotype analysis revealed presence of four common haplotypes in both the cohorts: ($f \geq 1\%$) $C_{4895}A_{3813}A_{1359}$ (CAA), $T_{4895}A_{3813}A_{1359}$ (TAA), $C_{4895}A_{3813}G_{1359}$ (CAG), $T_{4895}G_{3813}A_{1359}$ (TGA) (Table 3). The CAA haplotype seems to be less frequent in pre-eclamptic cases compared to controls ($p < 0.008$); on the contrary, the CAG and TAA haplotypes were significantly more frequent in the pre-eclampsia cases ($p < 0.04$ and $p < 0.03$, respectively). Analysis of regression models confirmed the independent prediction role of CAA haplotype for pre-eclampsia onset ($\beta = -0.18$; p = 0.03). None of the polymorphisms or haplotypes was associated with IUGR development or any other investigated traits.

Table 3
Mean values of end-pregnancy BMI in relation to 4895 C/T polymorphism in CNR1 locus.

Genotypes of 4895 C/T	Cases (n = 115)	Controls (n = 145)	p
CC	32.65 ± 11.75	26.88 ± 4.19	0.13
CT	31.89 ± 5.23	26.54 ± 3.47	0.002
TT	24.60 ± 4.8	29.14 ± 3.79	0.12

Differences between the cohorts calculated using Mann–Whitney U-test.

4. Comment

Several lines of evidence support the hypothesis that disturbances in the endogenous cannabinoid system are associated with aberrant early placental development [12–14]. Undoubtedly, there is a wide range of individual genetic variants, both maternal and fetal, that are more or less likely to contribute to pre-eclampsia development, whereas endocannabinoids theoretically seem to represent suitable candidate genes for pre-eclampsia susceptibility modulators. Yet it is rather uncertain which genes of the endocannabinoid system are directly involved in the molecular pathophysiology of pre-eclampsia, and their interactions are far from being clear.

The endocannabinoid system has been investigated mainly in relation to the implantation period in the endometrium of the mouse model [15–17], but only a limited number of reports is available that refer to the later placentation stages. Fonseca et al. [7] reported that cannabinoid receptor expression, mainly CB1, in the smooth muscle cells of blood vessels occurs until day 12 of pregnancy, and they suggested that anandamide, acting through the vascular CB1 that is expressed till the end of the pregnancy, might have an important role in regulation of fetal–placental vasoreactivity. In addition, anandamide seems to produce vasorelaxation in different vascular beds in an endothelium-dependent and endothelium-independent manner [1]. It has been also suggested that although anandamide has been shown to exert some of its effects directly on vascular smooth muscle via the CB1 receptor [18], a direct action of anandamide on the endothelium is possible [7]. Since anandamide is thought to have adverse effects on pregnancy and embryonic development, the activity of the degradative enzyme anandamide hydrolase may be crucial for prevention of excessive concentrations of anandamide in the uterus, and thus prevention of pregnancy failure or female infertility [19]. It has been reported that decreased anandamide hydrolase activity and expression in peripheral lymphocytes is an early (<8 weeks of gestation) marker of spontaneous abortion which might prove useful as a diagnostic tool for large-scale, routine monitoring of gestation [19].

To the best of our knowledge, this is the first study so far focusing on genetic variability in the CNR1 gene and pre-eclampsia risk. In the framework of the presented study, we observed significantly different genotype distributions of CNR1 4895 C/T in pre-eclamptic cases compared to controls. There was a significant decrease in the frequency of CC homozygotes in the cases, the heterozygote CT genotype being on the contrary significantly more frequent in cases compared to the controls (OR = 1.72, 95% CI 1.12–2.66, $p = 0.009$). There was a strong Hardy–Weinberg disequilibrium (HWD) in the cases, which is highly indicative of a possible valid marker-disease association. The functional background to the observed associations is unclear as the CNR1 4895 C/T is located in the untranslated 3′ region and therefore the most probable explanation is a strong linkage with another, functional variation.

After controlling for possible confounding effects from 1359 A/G to 3813 A/G; the polymorphism 4895 C/T was still associated with increased risk of pre-eclampsia development. Therefore, in CNR1, there might be a risk locus for pre-eclampsia that is in strong linkage with 4895 C/T. Interestingly, we did not observe the strong LD of 4895 C/T with rs1049353, which is the well-known 1359G/A (Thr453Thr) variant at CNR1, as described elsewhere [20]. As there is no evidence of functional impact of 4895 C/T related to pre-eclampsia, we propose a hypothesis that another pre-eclampsia related locus might be in strong linkage with rs4895 C/T.

To conclude, we observed a strong association of CNR1 4895 C/T with pre-eclampsia in the Central-European caucasian population. Based on our results, we propose that the C allele in the homozygous state might be associated with decreased susceptibility to pre-eclampsia. To properly understand this observation, replication of the study on a larger population sample in a multicentric design is highly advisable as well as further studies using denser marker sets that will be focused on localization of the disease-related loci near rs806368 and on investigation of the exact mechanisms of physiological–pathophysiological effects of this SNP.

Acknowledgements

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript, in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors.

The present study was supported by the Ministry of Education of the Czech Republic (Grant no. 881/2006). The authors are grateful to Svatava Tschöplova for an outstanding technical assistance.

References

[1] Taylor AH, Ang C, Bell SC, Konje JC. The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy. Hum Reprod Update 2007;13(5):501–13.
[2] Habayeb OM, Taylor AH, Bell SC, Taylor DJ, Konje JC. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology 2008;149(10):5052–60.
[3] Habayeb OM, Taylor AH, Evans MD, et al. Plasma levels of the endocannabinoid anandamide in women – a potential role in pregnancy maintenance and labor? J Clin Endocrinol Metab 2004;89(11):5482–7.
[4] Liu WM, Duan EK, Cao YJ. Effects of anandamide on embryo implantation in the mouse. Life Sci 2002;71(14):1623–32.
[5] El-Talatini MR, Taylor AH, Konje JC. Fluctuation in anandamide levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer women, and its hormonal regulation. Hum Reprod 2009;24(8):1989–98.
[6] Habayeb OM, Taylor AH, Finney M, Evans MD, Konje JC. Plasma anandamide concentration and pregnancy outcome in women with threatened miscarriage. JAMA 2008;299(10):1135–6.
[7] Fonseca BM, Correia-da-Silva G, Taylor AH, Konje JC, Bell SC, Teixeira NA. Spatio-temporal expression patterns of anandamide-binding receptors in rat implantation sites: evidence for a role of the endocannabinoid system during the period of placental development. Reprod Biol Endocrinol 2009;27(7):121.
[8] Davey DA, MacGillivray I. The classification and definition of the hypertensive disorders of pregnancy. Am J Obstet Gynecol 1988;158(4):892–8.
[9] Alexander GR, Himes JH, Kaufman RB, Mor J, Kogan M. A United States national reference for fetal growth. Obstet Gynecol 1996;87(2):163–8.
[10] Russo P, Strazzullo P, Cappuccio FP, et al. Genetic variations at the endocannabinoid type 1 receptor gene (CNR1) are associated with obesity phenotypes in men. Clin Endocrinol Metab 2007;92(6):2382–6.
[11] Gadzicki D, Müller-Vahl K, Stuhrmann M. A frequent polymorphism in the coding exon of the human cannabinoid receptor (CNR1) gene. Mol Cell Probes 1999;13(4):321–3.
[12] Trabucco E, Acone G, Marenna A, et al. Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage. Placenta 2009;30(6):516–22.
[13] Paria BC, Song H, Wang X, et al. Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 2001;276(23):20523–8.
[14] Chamley LW, Bhalla A, Stone PR, et al. Nuclear localisation of the endocannabinoid metabolizing enzyme fatty acid amide hydrolase (FAAH) in invasive trophoblasts and an association with recurrent miscarriage. Placenta 2008;29(11):970–5.
[15] Schmid PC, Paria BC, Krebsbach RJ, Schmid HH, Dey SK. Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. Proc Natl Acad Sci U S A 1997;94(8):4188–92.
[16] Horne AW, Phillips 3rd JA, Kane N, et al. CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy. PLoS One 2008;3(12):e3969.
[17] Wang H, Xie H, Guo Y, et al. Fatty acid amide hydrolase deficiency limits early pregnancy events. J Clin Invest 2006;116(8):2122–31.
[18] Gebremedhin D, Lange AR, Campbell WB, Hillard CJ, Harder DR. Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca^{2+} channel current. Am J Physiol 1999;276(6 (Pt 2)):H2085–93.
[19] Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C, Finazzi-Agrò A. Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet 2000;355(9212):1326–9.
[20] Zuo L, Kranzler HR, Luo X, Covault J, Gelernter J. CNR1 variation modulates risk for drug and alcohol dependence. Biol Psychiatry 2007;62(6):616–26.

3.5. BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) AND CILIARY NEUROTROPHIC FACTOR (CNTF) IN MATERNAL PLASMA AND UMBILICAL CORD BLOOD FROM PRE-ECLAMPTIC AND PHYSIOLOGICAL PREGNANCIES

Resumé

Neurotrofiny představují důležité stimulátory vývoje nervové tkáně a hrají pravděpodobně stěžejní úlohu při prenatálním a časném postnatálním modelování vývoje nervové tkáně u savců. Není překvapivé, že jsou produkovány i placentou. Cílem této studie bylo prozkoumat cirkulující hladiny ciliárního neurotrofního faktoru (CNTF) a mozkového neurotrofního faktoru (BDNF) v séru matek a umbilikální krvi u těhotenství postižených preeklampsií a zdravých kontrol.

Do studie bylo zařazeno 12 matek s preeklampsií a 34 žen s fyziologickým těhotenstvím a byla zkoumána hladina BDNF a CNTF v umbilikální krvi, respektive periferní krvi matky.

Hladina BDNF byla významně vyšší v umbilikální krvi z preeklamptických těhotenství, kdy u kontrolní skupiny byl významný rozdíl i v hladině BDNF mezi periferní krví matky a umbilikální krví ($p<0,001$). Hladina CNTF v umbilikální krvi byla významně vyšší u preeklamptických těhotenství než u kontrol ($p = 0.03$).

Tyto významné rozdíly v expresi proteinů CNTF a BDNF mezi preeklamptickými těhotenstvími a kontrolami mohou ukazovat na možné zapojení těchto proteinů při vytváření anomálních vzorců postanatálního modelování vývoje nervové tkáně. To je konzistentní i s dalšími empirickými pozorováními u dětí prenatálně zatíženými podobnými patologiemi.

Brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) in maternal plasma and umbilical cord blood from pre-eclamptic and physiological pregnancies

Julie Bienertova-Vasku[*,†], Petr Bienert*, Filip Zlamal*, Josef Tomandl**, Marie Tomandlova**, Zuzana Dostalova[††], Pavel Ventruba[††], Anna Vasku*

**Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic*
†Clinic of Paediatric Oncology, Masaryk University affiliated Hospital, Brno, Czech Republic
***Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno, Czech Republic*
[††]Clinic of Gynaecology and Obstetrics, Masaryk University affiliated Hospital, Brno, Czech Republic

Corresponding author. Tel.: +420 739300485; fax: +420 549494340.
E-mail address: jbienert@med.muni.cz (J. Bienertova-Vasku)

Running title

BDNF and CNTF are present in maternal plasma as well as in umbilical cord blood

Keywords
Brain-Derived Neurotrophic Factor; Ciliary Neurotrophic Factor; Pre-eclampsia

Abstract

The aim of the study was to investigate circulating levels of ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) in maternal serum and umbilical cord blood from respective pregnancies in pre-eclampsia and the controls. A total of 12 pre-eclampsia cases and 34 healthy controlswere enrolled in the study and the maternal peripheral blood - umbilical cord blood duos were examined for BDNF and CNTF levels.BNDF levels were significantly higher in umbilical cord blood from pre-eclamptic pregnancies, there was also significant difference between maternal plasma and umbilical cord blood levels of BDNF($p<0.001$) in the controls. The CNTF levels in umbilical cord blood (CNTF-UCB) were significantly higher in PE cases than in the controls ($p = 0.03$).Significant differences were observed in expression of BDNF and CNTF proteins in maternal peripheral blood and umbilical cord blood between pre-eclampsia cases and the healthy controls.

Introduction

Pre-eclampsia (PE) is a severe pregnancy-associated condition characterized by elevated blood pressure and proteinuria with onset after 20 weeks of gestation. It affects up to 5% of pregnancies(Robillard et al., 2011) and represents one of the leading causes of maternal as well as fetal mortality worldwide. The pathophysiology of PE still remains to be elucidated; most of PE cases are associated with the first pregnancy – primiparity; it has been suggested, however,that it is rather the first pregnancy with a particular father – primipaternity than primiparity that induces pre-eclampsia in susceptible women (Chaouat et al., 2005). In this view, pre-eclampsia seems to be partially underlined by specific genetic background (Hiby et al., 2004; Saftlas et al, 2005; Shelling et al., 2011), making the mother more prone to pathological response to paternal antigens of the fetus, which results in impaired trophoblastic invasion, typical for PE.

Neurotrophins (NT) are known to be important factors in the survival, maintenance and differentiation of neuronal tissue (Barde, 1990; Nguyen et al., 2009). The NT family consists of numerous nerve growth factors including nerve growth factor (NGF),brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) or neurotrophin-4 (NT-4). Brain-derived neurotrophic factor (BDNF) is as a typical member of the neurotrophin family known to activate the high-affinitytyrosine kinase B (TrkB) receptor together with the panneurotrophin low-affinity co-receptor p75 (Barbacid, 1995).As signalingmediated by BDNF through its receptor TrkB was reported toplay an important role in embryo implantation, subsequentplacental development and fetal growth by increasing trophoblastcell growth and survival in mice (Kawamura et al., 2009), it is highly likely to play an important role in pathophysiology of impaired placentation characteristic for pre-eclampsia. In their study, Kawamura demonstrates an augmentingrole of the BDNF/TrkB signaling pathway in

trophoblast celldevelopment which further underscores the importance of BDNF-related autocrine/paracrinesystem during peri- and postimplantation.

So far, only one study was performed that focused on possible correlations between BDNF in maternal blood and umbilical cord blood under physiological conditions (Malamitsi-Puchner et al., 2004), whereas the authors concluded that higher BDNF levels in mothers may reflect the mature nervous and immune systems of mother, they also observed higher BDNF levels in fullterm neonates than in the prematures ones, possibly due to overall higher maturity of the fullterm neonates. In another study, Fujita et al. (Fujita et al., 2011) focused on differences in BDNF levels between PE and non-PE cases; however, they do not report any association between BDNF maternal plasma levels and umbilical cord blood.

The results of study by Akahori et al. (Akahori et al., 2010) suggest that also the ciliary neurotrophic factor (CNTF) could be associated with pre-eclampsia, as its levels in PE are significantly lower than in healthy pregnancies, which the authors explain by the relationship between CNTF and insulin resistance, which is typical for most of the PE cases, however, they did not investigate CNTF levels in umbilical cord blood and/or their relationship to maternal plasma levels.

Taken together, the relationship between BDNF/CNTF maternal plasma and umbilical cord blood levels in PE and non-PE pregnancies is unclear as well as the source of BDNF in fetal circulation. As the animal experiments suggest that maternal BDNF reaches the fetal brain through uteroplacentalbarrier and might contribute to its development (Kodomari et al., 2009),wehypothesized that there might be a correlation betweenthe levels of BDNF in umbilical cord blood and maternalperipheral blood. Based on previously reported associations of BDNF with the total fat mass in mice (Godar et al., 2011; Wang et al., 2010), we also hypothesized that the birth weightof infants, roughly reflecting the fat mass, might be correlatedwith BDNF and/or CNTF levels in the umbilical cord blood as BDNF/CNTF have

distinct effects on regulation of total body adiposity. In order toinvestigate this, we compared the mothers-neonates duoswith uncomplicated, physiological pregnancies with those affected by pre-eclampsia, either with intrauterine growthrestriction (IUGR) or without it.

Material and methods

Study subjects

A total of 12 pre-eclampsia cases and34 randomly selectedhealthymothers originating from the Caucasian Central-European population that gave birth at the Clinic of Gynecology and Obstetrics of the Masaryk University affiliated Hospital in Brno, Czech Republic, whose conception, pregnancy anddelivery were physiological, were enrolled in the study. The duos of maternal peripheral blood sample obtained during 2 h pre-partum from peripheral vein and umbilical cord blood sample obtained from umbilical artery immediately afterthe childbirth were collected. Pre-eclampsia was defined according to theconsensual criteria of the International Society for the Study ofHypertension in Pregnancy (blood-pressure values exceeding140/90mmHg after the 20th week of gestation, confirmed bytwo consecutive readings at least six hours apart and theconcomitant onset of proteinuria (>300 mg of urinary protein/Lover 24 h). The study was approved by the Committee for Ethicsof Medical Experiments on Human Subjects of the MasarykUniversity, Brno, Czech Republic, and was performed in adherence to theDeclaration of Helsinki Guidelines.

The inclusion criteria for normal pregnancy cohort were: 1) spontaneous conception, 2) singleton pregnancy, 3) delivery of a term neonate with a birth weight above the 10^{th} percentile and 4) normal oral 75-g glucose tolerance test between week 24 and 28 of gestation based on the criteria set up by the World Health Organization (WHO). The exclusion criteria for the study were: 1) multiple pregnancy, 2) in vitro-assisted reproduction, 3) pre-existing hypertension disease, 4) gestational diabetes.

Biochemical analysis

BDFN and CNTF plasma levels were measured using a commercially available Milliplex MAP multiplex Human Brain-derived Protein Panel (Millipore Corp., Billerica, MA), which allows the simultaneous measurement of the analytes. Plasma samples of umbilical cord blood were 3 times diluted before assay using the Assay buffer. All analyses were performed in duplicates. Intra- and inter-assay precisions were less than 10 %.

Statistics

We used logarithmic transformation of non-normally distributed BDNF and CNTF levels for all analyses in all study groups. Generally, we performed Pearson's correlation testing between BDNF or CNTF and clinical and anthropometric parameters and we report thePearson's r coefficients, giving the strength of an association in the range of -1 and +1 and the Spearman's coefficient where appropriate. We also evaluated differences betweenthe groups using two-tailedMann Whitney test, dependent t-test andWilcoxon matched pairs test for the maternal plasma/umbilical cord blood duos,followed by linear regression analyses with BDNF or CNTF as dependent variable and birth weight of the infant as independent variables.The data analysis wasperformed using the Statistica v. 9.0 (Statsoft Inc., Tulsa,OK, USA) program package. The conventional values of $p < 0.05$ were considered statistically significant.

Results

BDNF

The baseline characteristics of the study subjects are summarizedin Table 1. There were no significant differences in circulating levels of BDNF in maternal plasma between the pre-

eclampsia cases and the controls (p = 0.623). When comparing BDNF levels in umbilical cord blood, significant differences were observed between the pre-eclampsia cases and the controls (p = 0.04). In the healthy pregnancies, the BDNF levels in umbilical cord blood (BDNF-UCB; mean ± SD: 1357 ± 1718 pg/mL) were significantly lower than in maternal peripheral blood (BDNF-BP; mean ± SD: 5864 ± 2836 pg/ mL, p = 0.000002; Fig. 1) ; the BDNF-UCB levels in the pre-eclamptic cohort (mean ± SD: 3236 ± 3153 pg/mL) were significantly different from BDNF-BP levels (mean ± SD: 6473 ± 3591 pg/mL, p = 0.02), whereas the levels also tended to be lower in the umbilical cord blood in the pre-eclamptic subjects.In the univariate regression modelling, the BDNF-BP levels were not proportionally correlated with BDNF-UCB levelseither in the pre-eclampsia cohort (R = -0.3279, p = 0.2981), or in the controls (R = 0.0740, p = 0.6775).

The BDNF-BP or BDNF-UCB levels were not proportionally correlated with maternal pre-pregnancy weight; neither in the pre-eclamptic women (R = -0.3217, p = 0.3087) nor in the controls (R = 0.0374, p = 0.8333) the analysis of BMI at time of birth also did not reveal and significant associations except for the correlation of BDNF-BP levels in the controls with BMI at the time of birth: r = 0.3727, p = 0.03. In the multiple regression modeling, BDNF-UCB levels were significantly correlated with maternal weight at the time of delivery (R = 0.617; p = 0.03). No correlations of BDNF-BP or BDNF-UCB levels with pre-pregnancy weight or BMI at the time of delivery or birth weight of the infant were observed.

CNTF

There were no significant differences in CNTF levels in maternal plasma (CNTF-BP) between the PE cases and the controls (p = 0.13), however, the CNTF levels in umbilical cord blood (CNTF-UCB) were significantly higher in PE cases (mean ± SD: 8446 ± 8950 pg/ml) compared to the controls (2526 ± 3105 pg/mL; p = 0.03; Fig. 2). Generally, the CNTF-UCB

levels substantially exceeded the levels in maternal plasma in both study cohorts. The CNTF-BP levels in the pre-eclamptic pregnancies weren't (mean ± SD: 2321 ± 2280 pg/mL) significantly different from CNTF-BP levels in the controls (mean ± SD: 4464 ± 5633 pg/mL, p = 0.14). When comparing CNTF-BP levels and CNTF-UCB levels, no significant differences were observed either in pre-eclamptic (p = 0.09) or control cohort (p = 0.14).

In the control subjects, CNTF levels in maternal plasma and umbilical cord blood were significantly correlated (r = 0.4549, p = 0.007). The CNTF-BP or CNTF-UCB levels were not proportionally correlated with maternal pre-pregnancy weight in pre-eclamptic women (R = -0.0455, p = 0.888), nor in the controls (R = 0.1467, p = 0.407).

The multivariate regression modelling didn't reveal any significant correlations of CNTF with pre-conceptional BMI, pregnancy weight gain or birth weight of the infant as dependent variables. In the umbilical cord blood from pre-eclamptic pregnancies, there was a significant correlation between BDNF and CNTF levels (r = 0.7167, p = 0.009).

BDNF and CNTF and week of gestation

In the regression modeling, no association was observed between the week of gestation at the time of delivery and BDNF, resp. CNTF levels; also the comparison of groups of PE women giving birth in the week 33-36 and 37-39 of their pregnancy did not reveal any significant associations.

Discussion

In this study on a small, homogenous cohort of Caucasian, Central-European women with or without PE, we studied the BDNF and CNTF levels in maternal plasma and umbilical cord blood.

BDNF, as a typical representant of neurotrophins, plays an important role in proliferation, differentiation and survival of neurons during development and is also capable of modulating synaptic activity and plasticity in many groups of mature neuron (Lebrun et al., 2006). Recently, it has been proposed that neurotrophins play an important role in development of feto-placental interface(Mayeur et al., 2011) and it is becoming apparent that this ligand signalingsystem, originally found to be essential for the development anddifferentiation of the neuronal system, is also essential for development of preimplantation embryos as well as trophoblast invasion and trophoblast survival (Seifer et al., 2002; Kawamura et al., 2005; Martins da Silva et al., 2005) After implantation,BDNF suppresses apoptosis in the embryo and promotes earlyembryonic development through its receptorTrkB (Kawamura et al., 2007). Little is known about the dynamics of BDNF level throughout pregnancy – BDNF plasma levels were reported to rise during the pregnancy, peaking in the third trimester (Chouthai et al., 2003), in this study the average BDNF values are reported to be 2190 ± 356 pg/mL after the 36th week of gestation, which could be relatively consistent with our observation of 1357 ± 1718 pg/mL in the umbilical cord blood from physiological pregnancies at the time of birth. In another study that was performed on 45 parturients with their fullterm or preterm neonates, the umbilical cord blood levels of BNDF were reported to be approx. 2472 (1179 – 13296 pg/mL) in the fullterm neonates and 1854 (1351-2094 pg/mL) in the pre-term newborns (Malamitsi-Puchner et al., 2004), which is also roughly in accordance with our findings. In our study, the BDNF-UCB levels in non-PE subjects were significantly lower than those in the pre-eclamptic subjects (3236 ± 3153 pg/mL). Even though the PE neonates were delivered in an earlier stage of gestation than the newborns from the physiological pregnancies, which makes them more prone to low BDNF levels, the BDNF levels were higher in the umbilical cord blood from PE pregnancies, which is consistent with the findings reported by Fujita et al. from the Japanese population (Fujita et al., 2011). However, the BDNF levels reported by

Fujita et al.were substantially lower (623.8 pg/mL [330.9-1024.0)than those observed in our study or in the studies conducted by Malamitsi-Puchner (Malamitsi-Puchner et al., 2004) or Chouthai (Chouthai et al., 2003). It can be hypothesized that the samples in Fujita's study were sampled at earlier stage of pregnancy (the time of sampling was located somewhere in the third trimester), the number of cases in our study was also higher. The observed variability of BDNF levels between above mentioned studies is also to be at least partially attributed to different ethnicity of the investigated cohorts.

Fujita et al.(Fujita et al., 2011) reported that BDNF/TrkBsignaling had anti-apoptotic effects against oxidative stress in JEG-3 cell lines, suggesting a protective role ofBDNF/TrkB in human villous tissue under unfavorable conditions in utero. Therefore, it could be hypothesized that the activation of BDNF/TrkB system atthe feto-maternal interface under unfavorable conditions could lead to development of pre-eclampsia. However, the role of BDNF at the late pregnancy remains to be elucidated.

In their study, Kodomari et al reported that maternal BDNF was capable to reach the mouse fetal brain via placenta, in a dose dependent manner (Kodomari et al., 2009). However, we did not confirm the baseline presumption that BDNF levels in maternal plasma and umbilical cord blood are correlated and we suggest the mechanisms of regulation of BDNF levels in the umbilical cord blood are more complicated than just simple utero-placental transmission.

Our study is the first to report a significant difference between CNTF levels in umbilical cord blood from pre-eclamptic and physiological pregnancies, which could be explained by an important role played by CNTF in regulation of insulin resistance. Pregnancy is generally accepted as a state of physiological insulin resistance and the treatment of insulin resistance using ciliary neurotrophic factor (CNTF) has been suggested for severe insulin-resistance-associated disorders where CNTF exerts a leptin-like effect, even in leptin-resistant states (Lambert et al., 2001). This effect is mediated through PTP-1B that constitutes a key

divergent element between leptin/insulin and CNTF signaling pathway (Benomar et al., 2009), thus providing a possible mechanism explaining the efficacy of CNTF in leptin-resistant states. So far, nothing is known about the possible transmission of maternal CNTF through utero-placental barrier to the fetus. In our study, the CNTF levels in umbilical cord blood exceeded the levels in maternal blood, both in PE and non-PE subjects, and therefore it we suggest that the fetus itself produces significant quantities of CNTF, however, the source of the CNTF production in the fetus remains to be elucidated. In their study on the Japanese population, Akahori et al.(Akahori et al., 2010) observed decreased CNTF levels in PE pregnancies, and suggest this effect could be associated with relative hemoconcentration in PE. In our study, we did not observe any differences in maternal CNTF levels between the both cohorts, however, the sampling was performed in different periods of gestation in both studies (approx. week 32 in Akahori's study vs. week 37 for PE and week 40 for non-PE cases in our study). More research into the dynamics of CNTF levels in physiological as well as pathological pregnancy seems to be necessary as well in order to elucidate the source of circulating CNTF both in maternal and umbilical cord blood.

It is well known that maternal disease or pathological condition can affect fetaldevelopment and neurogenesis. In this study, we investigated possible relationship between BDNF/CNTF levels in maternal plasma and umbilical cord blood and observed certain associations between BDNF/CNTF levels in maternal peripheral blood and umbilical cord blood from the physiological and pre-eclamptic pregnancies. To conclude, more research is necessary into the role of BNDF/CNTF in dramatic metabolic changes in maternal as well as fetal organism in the peripartum period.

Acknowledgement

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript, in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors. The present study was supported by the projects MZMOU2005 and RECAMO CZ.1.05/2.1.00/03.0101.

Declaration of Interest

The authors report no declarations of interest

References

[1] Akahori Y, Takamoto N, Masumoto A, Inoue S, Nakatsukasa H, Masuyama H, et al. 2010. Circulating levels of ciliary neurotrophic factor in normal pregnancy and preeclampsia. Acta Med Okayama. 64: 129-36.
[2] Barbacid M. The Trk family of neurotrophin receptors. 1995. J Neurobiol 25: 1386-403.
[3] Barde YA. 1990. Trophic factors and neuronal survival. Neuron 2: 1525-34.
[4] Benomar Y, Berthou F, Vacher CM, Bailleux V, Gertler A, Djiane J, et al. 2009. Leptin but not ciliary neurotrophic factor (CNTF) induces phosphotyrosine phosphatase-1B expression in human neuronal cells (SH-SY5Y): putative explanation of CNTF efficacy in leptin-resistant state. Endocrinology 150: 1182-91.
[5] Chaouat G, Ledee-Bataille N, Dubanchet S. 2005. Immunological similarities between implantation and pre-eclampsia. Am J Reprod Immunol 53:222-9.
[6] Chouthai NS, Sampers J, Desai N, Smith GM. 2003. Changes in neurotrophin levels in umbilical cord blood from infants with different gestational ages and clinical conditions. Pediatr Res. 53: 965-69
[7] Fujita K, Tatsumi K, Kondoh E, Chigusa Y, Mogami H, Fujii T, et al. 2011. Differential expression and the anti-apoptotic effect of human placental neurotrophins and their receptors. Placenta 32:737-44.
[8] Godar R, Dai Y, Bainter H, Billington C, Kotz CM, Wang CF. 2011. Reduction of high-fat diet-induced obesity after chronic administration of brain-derived neurotrophic factor in the hypothalamic ventromedial nucleus. Neuroscience 194: 36-52.
[9] Hiby SE, Walker JJ, O'shaughnessy KM, Redman CW, Carrington M, Trowsdale J, et al. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. J Exp Med 200: 957-65.
[10] Kawamura K, Kawamura N, Mulders SM, Sollewijn Gelpke MD, Hsueh AJ. 2005. Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. Proc Natl Acad Sci U S A 102: 9206-11.
[11] Kawamura K, Kawamura N, Sato W, Fukuda J, Kumagai J, Tanaka T. 2009. Brain-derived neurotrophic factor promotes implantation and subsequent placental development by stimulating trophoblast cell growth and survival. Endocrinology 150: 3774-82.

[12] Kawamura K, Kawamura N, Fukuda J, Kumagai J, Hsueh AJ, Tanaka T. 2007. Regulation of preimplantation embryo development by brain-derived neurotrophic factor. Dev Biol. 311: 147-58.
[13] Kodomari I, Wada E, Nakamura S, Wada K. 2009. Maternal supply of BDNF to mouse fetal brain through the placenta. Neurochem Int 54: 95-8.
[14] Lambert PD, Anderson KD, Sleeman MW, Wong V, Tan J, Hijarunguru A, et al. 2001. Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. Proc Natl Acad Sci U S A. 98: 4652-7.
[15] Lebrun B, Bariohay B, Moyse E, Jean A. 2006. Brain-derived neurotrophic factor (BDNF) and food intake regulation: a minireview. Auton Neurosci 126-127: 30-8.
[16] Malamitsi-Puchner A, Economou E, Rigopoulou O, Boutsikou T. 2004. Perinatal changes of brain-derived neurotrophic factor in pre- and fullterm neonates. Early Hum Dev 76: 17-22.
[17] Martins da Silva SJ, Gardner JO, Taylor JE, Springbett A, De Sousa PA, Anderson RA. 2005. Brain-derived neurotrophic factor promotes bovine oocyte cytoplasmic competence for embryo development. Reproduction 129: 423-34.
[18] Mayeur S, Lukaszewski MA, Breton C, Storme L, Vieau D, Lesage J. 2011. Do neurotrophins regulate the feto-placental development. Med Hypotheses 76: 726-8.
[19] Nguyen N, Lee SB, Lee YS, Lee KH, Ahn JY. 2009. Neuroprotection by NGF and BDNF against neurotoxin-exerted apoptotic death in neural stem cells are mediated through Trk receptors, activating PI3-kinase and MAPK pathways. Neurochem Res. 34: 942-51.
[20] Robillard PY, Dekker G, Chaouat G, Hulsey TC, Saftlas A. 2011. Epidemiological studies on primipaternity and immunology in preeclampsia--a statement after twelve years of workshops. J Reprod Immunol 89: 104-17.
[21] Saftlas AF, Beydoun H, Triche E. 2005. Immunogenetic determinants of preeclampsia and related pregnancy disorders: a systematic review. Obstet Gynecol 106: 162-72.
[22] Seifer DB, Feng B, Shelden RM, Chen S, Dreyfus CF. 2002. Brain-derived neurotrophic factor: a novel human ovarian follicular protein. J Clin Endocrinol Metab 87: 655-9.
[23] Shelling AN. 2012. Mutations in inhibin and activin genes associated with human disease. Mol Cell Endocrinol359(1-2): 113-20.
[24] Wang C, Godar RJ, Billington CJ, Kotz CM. 2010. Chronic administration of brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus reverses obesity induced by high-fat diet. Am J Physiol Regul Integr Comp Physiol. 298:R1320-R1332.

Figure 1 - Relationship between the BDNF circulating levels in maternal peripheral blood and umbilical cord blood in physiological pregnancies and pre-eclampsia

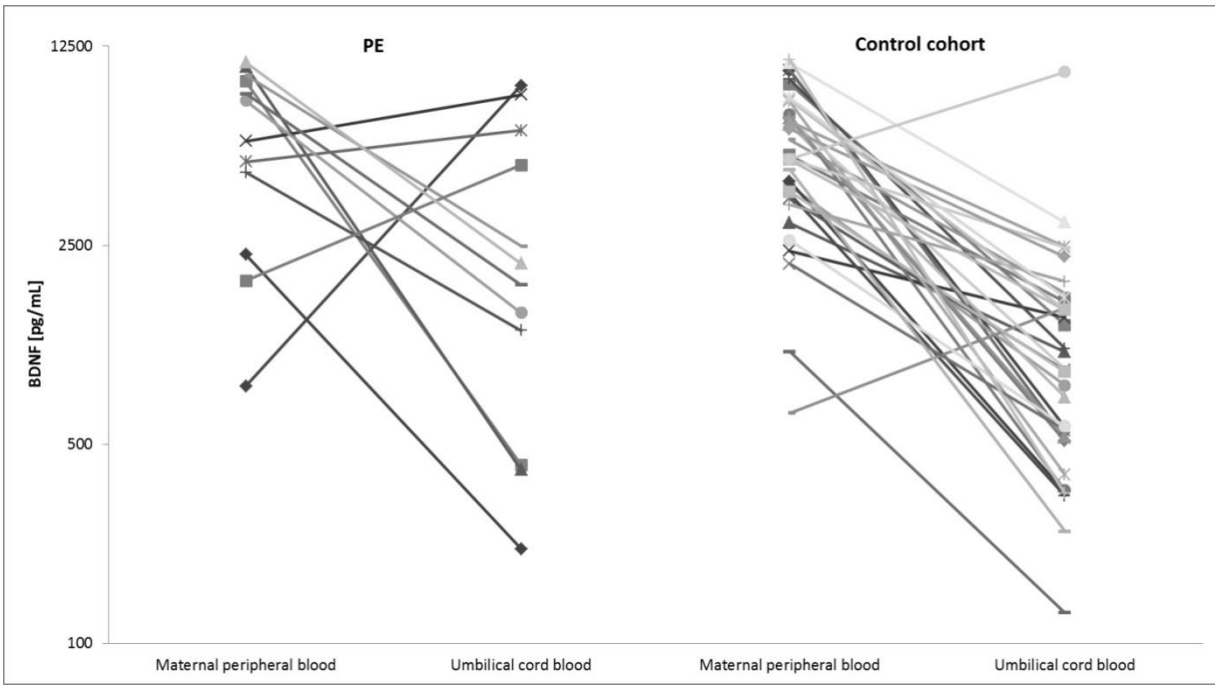


Figure 2 - Relationship between the CNTF circulating levels in maternal peripheral blood and umbilical cord blood in physiological pregnancies and pre-eclampsia

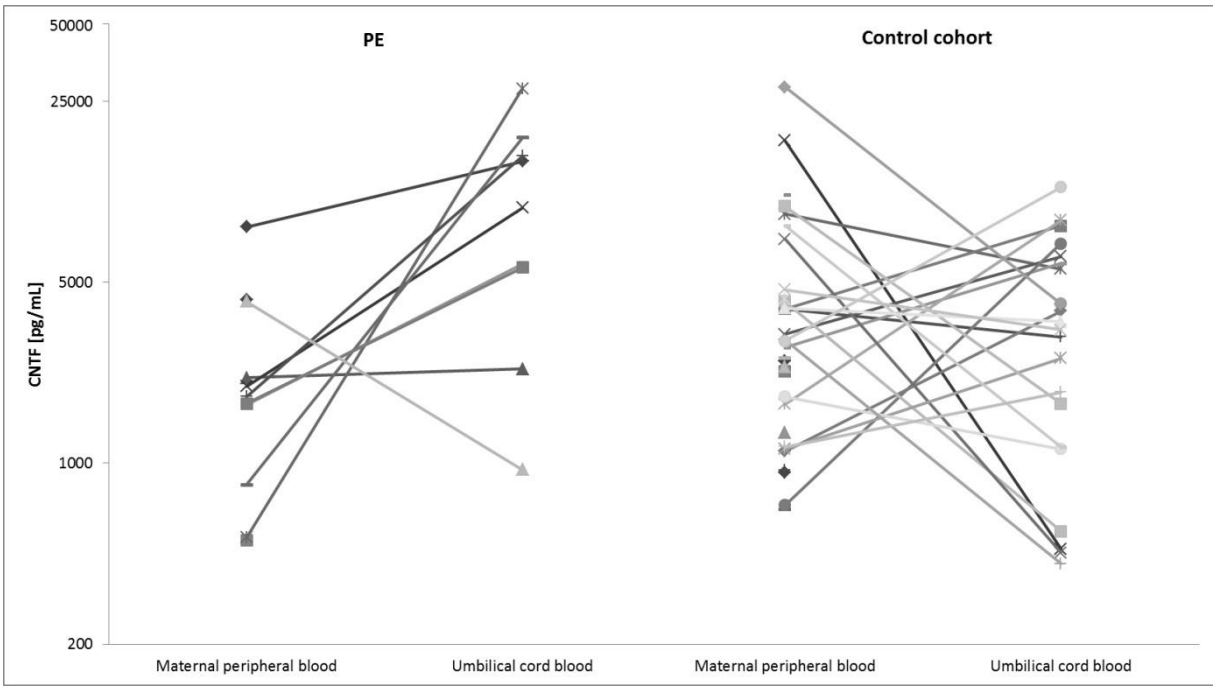


Table I. Baseline characteristics of the pre-eclamptic subjects and the control subjects with physiological conception, pregnancy and delivery

	Cases (n = 12)	**Controls (n = 34)**	**p**
Maternal age (yrs)	32.6 ± 4.0	29.3 ± 4.9	0.0128[+]
HELLP/early/late pre-eclampsia*	0/1/11	0	NA
Pre-conception BMI (kg/m^2)	26.7 ± 6.5	24.9 ± 4.0	0.5820[+]
BMI at the time of delivery (kg/m^2)	28.4 ± 5.7	28.2 ± 9.0	0.3731+
Pregnancy weight gain (kg)	12.3 ± 5.0	15.2 ± 7.0	0.063+
Birth weight (g)	2075 ± 435	3687 ± 337	0.000008[+]
Week of gestation at delivery	36.4 ± 1.9	40.4 ± 1.0	0.000000002[+]
IUGR (n)	3	0	0.0145[+++]

*BMI, body mass index; IUGR, intrauterine growth restriction; NS, not significant, results are given as mean ± SD, * early/late pre-eclampsia defined as onset of disease ≤ 34th week of gestation / > 34th week of gestation*

[+] *Mann-Whitney test*

[++] *chi-squared test*

[+++] *Fisher's exact test*

3.6. COMPARISON OF AGOUTI-RELATED PEPTIDE LEVELS IN PERIPHERALBLOOD OF POSTPARTUM PRE-ECLAMPTIC AND NON PRE-ECLAMPTICWOMEN AND IN UMBILICAL CORD BLOOD FROM THEIR PREGNANCIES

Resumé

Fyziologická úloha agouti-related peptidu (AgRP) v regulaci apetitu a energetické rovnováhy organismu zprostředkovávaná zejména melanokortinovou signalizační drahou je známa už řadu let. Kromě svých dobře prozkoumaných účinků na úrovni CNS má AgRP ovšem svoje účinky i v periferii – patofyziologický podklad těchto regulací je však zatím nejasný. Cílem této studie bylo prozkoumat hladinu AgRP v umbilikální krvi a periferní krvi matek s fyziologickým těhotenstvím a s preklampsií.

Do studie bylo zařazeno 12 matek s preeklampsií a 32 náhodně vybraných zdravých matek s fyziologickým těhotenstvím a porodem. Vzorek krve matky byl získán během dvou hodin před porodem a po porodu dítěte byl získán vzorek pupečníkové krve dítěte.

Průměrná hladina AgRP v pupečníkové krvi přesahovala nejvyšší hodnoty udávané u obézních dospělých více než 200krát, což může naznačovat extrémně robustní peripartální potravní „drive“ u novorozenců. Tento trend byl podobný u novorozenců z preeklamptických i fyziologických těhotenství.

Tato studie ovšem dále ukazuje, že plazmatická hladina AgRP v periferní nebo pupečníkové krvi je významně odlišná při srovnání matek s placentální dysfunkcí, konkrétně preeklampsií, a zdravých kontrolních žen.

AgRP může hrát významnou úlohu v anomálním metabolickém a nutričním programování potomstva z preeklamptických těhotenství, což by alespoň z části vysvětlovalo „thrifty“ fenotyp pozorovaný u těchto dětí.

DIABETES RESEARCH AND CLINICAL PRACTICE 89 (2010) e53–e55

Contents lists available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation

Brief report

Comparison of agouti-related peptide levels in peripheral blood of postpartum pre-eclamptic and non pre-eclamptic women and in umbilical cord blood from their pregnancies

Julie Bienertova-Vasku [a,*], *Petr Bienert* [a], *Josef Tomandl* [b], *Jolana Lipkova* [a], *Filip Zlamal* [a], *Zuzana Dostalova* [c], *Anna Vasku* [a]

[a] Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic
[b] Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic
[c] Clinic of Gynaecology and Obstetrics, Masaryk University Affiliated Hospital, Brno, Czech Republic

ARTICLE INFO

Article history:
Received 12 May 2010
Accepted 1 June 2010
Published on line 29 June 2010

Keywords:
Agouti-related peptide
Pre-eclampsia
Pregnancy
Umbilical cord blood
ELISA

ABSTRACT

Plasma levels of agouti-related peptide (AgRP) were reported to continuously rise during ongoing pregnancy in rats. The aim of the study was to investigate the maternal pre-partum peripheral plasma levels of AgRP and levels in umbilical cord blood in pre-eclamptic and physiological pregnancies in humans.

1. Introduction

Recently, a lot of attention has been paid to the physiological role of agouti-related peptide (AgRP) in regulation of appetite and energy balance, mediated mainly via melanocortin-4 receptor (MC4R) and melanocortin-3 receptor (MC3R). Apart from the essential effects of AgRP in the central nervous system, AgRP also circulates in plasma, however, its role in the periphery is far from being understood. Recently, it has been reported that plasma AgRP [1] is elevated in obese men compared to non-obese men, which might be indicative of some peripheral involvement in transduction of adiposity signals, however, this was not confirmed by other studies [2,3]. Nevertheless, most of the studies reported a consistent correlation of AgRP levels with the total body fat mass [1,2].

Considering the proposed relationship between total fat mass and circulating levels of AgRP in the adults, we hypothesized that there might be a relationship between the levels of AgRP in umbilical cord blood and maternal peripheral blood. We also hypothesized that the birth weight of infants, roughly reflecting the fat mass, might be correlated with AgRP levels in the umbilical cord blood. In order to investigate this, we compared the mothers-neonates duos with uncomplicated, physiological pregnancies with those

* Corresponding author. Tel.: +420 739 300 485; fax: +420 549 494 340.
E-mail address: jbienert@med.muni.cz (J. Bienertova-Vasku).
0168-8227/$ – see front matter
doi:10.1016/j.diabres.2010.06.003

Table 1 – Baseline characteristics of the pre-eclamptic subjects and the control subjects with physiological conception, pregnancy and delivery.

	Cases ($n = 12$)	Controls ($n = 32$)	p
Maternal age (yrs)	29.8 ± 4.19	29.98 ± 5.31	NS
HELLP/late/early pre-eclampsia*	0/9/3	–	–
Pre-conception BMI (kg/m^2)	24.92 ± 3.33	25.09 ± 3.98	NS
BMI at the time of delivery (kg/m^2)	31.15 ± 7.5	29.34 ± 6.5	NS
Pregnancy weight gain (kg)	14.34 ± 6.70	13.17 ± 5.24	NS
Birth weight (g)	1944 ± 735	3581 ± 460	$p \leq 0.00001$
Week of gestation at delivery	35	40	$p \leq 0.0001$
IUGR (n)	7	0	$p \leq 0.0004$
Primiparity/multiparity	5/7	20/12	NS
Smokers/non-smokers	2/10	3/29	NS

BMI, body mass index; IUGR, intrauterine growth restriction; NS, not significant, results are given as mean ± SD.
* Early/late pre-eclampsia defined as onset of disease ≤34th week of gestation/>34th week of gestation.

affected by pre-eclampsia, either with intrauterine growth restriction (IUGR) or without it, using pre-eclampsia associated IUGR as a model of aberrant body composition in neonates not carrying any karyotype abnormalities.

2. Material and methods

2.1. Subjects

A total of 12 pre-eclamsia cases and 32 randomly selected healthy mothers with physiological conception, pregnancy and delivery were enrolled in the study. The maternal peripheral blood sample was obtained during 2 h pre-partum and the sample of umbilical cord blood was obtained immediately after the childbirth. Pre-eclampsia was defined according to the consensual criteria of the International Society for the Study of Hypertension in Pregnancy (blood-pressure values exceeding 140/90 mmHg after the 20th week of gestation, confirmed by two consecutive readings at least six hours apart and the concomitant onset of proteinuria (>300 mg of urinary protein/L over 24 h). The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects of the Masaryk University, Brno, CR and was performed in adherence to the Declaration of Helsinki Guidelines.

2.2. Biochemical analysis

AgRP plasma levels were measured using a commercially available ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA). Plasma samples of umbilical cord blood were 11 times diluted before assay using the Calibrator Diluent RD6-10 included in the kit. All analyses were performed in duplicates. The analyses were repeated twice in 30% of peripheral blood samples and in the whole set of umbilical cord blood samples to confirm the obtained results.

2.3. Statistics

We used logarithmic transformation of non-normally distributed AGRP levels for all analyses in all study groups. Generally, we performed Pearson's correlation testing between AgRP and clinical, and anthropometric parameters and we report the Pearson's r coefficients, giving the strength of an association in the range of −1 and +1. We also evaluated difference between the groups using two-tailed, independent-sample t tests, followed by linear regression analyses with AgRP as dependent variable and birth weight, birth length and gender of the infant as independent variables. The data analysis was performed using the Statistica v. 8.0 (Statsoft Inc., Tulsa, OK, USA) program package. The conventional values of $p \leq 0.05$ were considered statistically significant.

3. Results

The baseline characteristics of the study subjects are summarized in Table 1. The plasma levels of AgRP in the umbilical cord blood ($AgRP^{UCB}$) in the pre-eclamptic cohort (mean 983.35 ± 1048.93 pg/mL) and non-pre-eclamptic cohort (mean 2340.29 ± 1138.07 pg/mL) were almost 11-fold, respectively 19-fold higher, compared with AgRP levels in peripheral blood ($AgRP^{BP}$) of pre-eclamptic subjects (mean 88.04 ± 32.47 pg/mL) and the control subjects (mean 124.09 ± 38.92 pg/mL, Fig. 1).

The $AgRP^{BP}$ levels were not proportionally correlated with maternal body weight either in pre-eclamptic women ($r = -0.23$, $p = 0.34$) nor in the controls ($r = -0.10$, $p = 0.52$); the analysis of BMI also did not reveal and significant associations.

The $AgRP^{BP}$ levels were proportionally correlated with AgRP concentrations in umbilical cord blood in the pre-eclampsia cohort ($r = 0.64$, $p = 0.02$), however, not in the controls (−0.07, $p = 0.70$).

The $AgRP^{UCB}$ from pre-eclamptic pregnancies were significantly lower than in the control pregnancies ($p < 0.001$). Furthermore, the $AgRP^{UCB}$ were proportionally correlated with birth weight of the infant in the pre-eclamptic cohort ($r = -0.23$, $p = 0.03$), however, not in the control cohort ($r = 0.22$, $p = 0.20$). No significant prediction role of AgRP levels, both $AgRP^{PB}$ and $AgRP^{UCB}$, for IUGR development was observed in univariate regression modelling adjusted for age, BMI and smoking status of the mother.

4. Discussion

Olofsson et al. [4] have recently reported that the hypothalamic expression of AgRP is associated with cyclic changes in

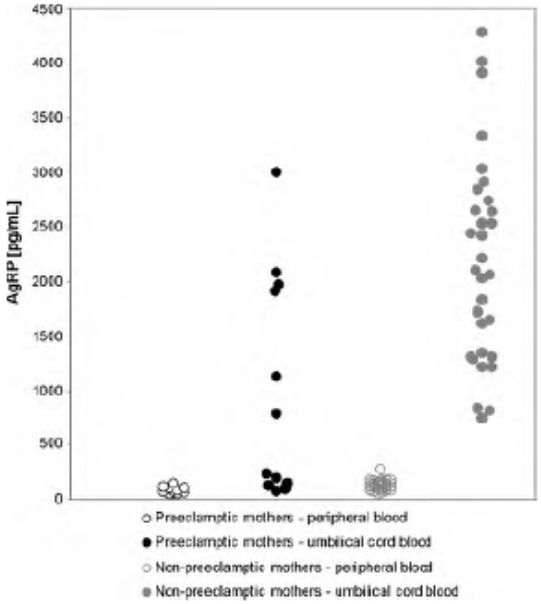


Fig. 1 – Plasma levels of AgRP in peripheral blood and umbilical cord blood in pre-edamptic and non-pre-eclamptic cohort ($p < 0.0009$).

feeding across the estrous cycle in mice suggesting that neurons co-expressing AgRP are functionally required for the cyclic changes in feeding across estrous cycle. In a recent study by Szczepankiewicz et al. it has been reported that AgRP is expressed in the placenta in rats and that AgRP levels consecutively rise during ongoing pregnancy [5]. In another snímal study by Caminos et al. [6], the placental AgRP expression remained unchanged between 12 and 16 days of gestation reaching the highest levels at the end of the gestation period ($p < 0.001$) in rats.

The average AgRP levels in umbilical cord blood samples in our study exceeded the highest AgRP levels reported in obese adults [1] almost 200 times, which might be an evidence of extreme robustness of peripartum feeding drive in neonates, both in pre-eclamptic and non pre-eclamptic offspring and this finding is well in accordance with previous findings on rats.

The present study demonstrates that plasma levels of AgRP in peripheral or umbilical cord blood are significantly different when comparing mothers with placental dysfunction – pre-eclampsia – and healthy control women. We hypothesize that AgRP could play an important role in aberrant peripartum modeling of the pre-eclamptic newborns, resulting e.g. in "thrifty" feeding patterns of such neonates. However, this hypothesis should be investigated on larger population samples of different ethnicity in a variety of study designs, mainly prospective ones, following also the weight gain and feeding patterns of the infants in relation to postnatal AgRP circulating levels.

Acknowledgements

The study was supported by the Ministry of Education of the Czech Republic (grant no. 881/2006) and by a project from the Danone Institute of the Czech Republic (DANONE/2007) focused on physiological and pathophysiological variability of adipokines.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

[1] Katsuki A, Sumida Y, Gabazza EC. Plasma levels of agouti-related protein are increased in obese men. J Clin Endocrinol Metab 2001;86(5):1921–4.
[2] Hoggard N, Johnstone AM, Faber P, Gibney ER, Elia M, Lobley G, et al. Plasma concentrations of alpha-MSH, AgRP and leptin in lean and obese men and their relationship to differing states of energy balance perturbation. Clin Endocrinol (Oxf) 2004;61(1):31–9.
[3] Shen CP, Wu KK, Shearman LP, Camacho R, Tota MR, Fong TM, et al. Plasma agouti-related protein level: a possible correlation with fasted and fed states in humans and rats. J Neuroendocrinol 2002;14(8):607–10.
[4] Olofsson LE, Pierce AA, Xu AW. Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. Proc Natl Acad Sci USA 2009;106(37):15932–7.
[5] Szczepankiewicz D, Pruszyńska-Oszmałek E, Kaczmarek P, Skrzypski M, Andrałojć K, Wojciechowicz T, et al. Changes of agouti-related protein in hypothalamus, placenta, and serum during pregnancy in the rat. J Endocrinol 2009;202(1):35–41.
[6] Caminos JE, Bravo SB, González CR, Garcés MF, Cepeda LA, González AC, et al. Food-intake-regulating-neuropeptides are expressed and regulated through pregnancy and following food restriction in rat placenta. Reprod Biol Endocrinol 2008;2(6):14.

3.7. B–CELL ACTIVATING FACTOR (BAFF) IS PRESENT IN UMBILICAL CORD BLOOD IN HEALTHY AND PRE-ECLAMPTIC PREGNANCIES AS WELL AS IN HUMAN BREAST MILK WITH SPECIFIC DYNAMICS DURING 180D LACTATION

Resumé

Aktivační faktor B-buněk (B-cell activating factor, BAFF) je členem TNF super rodiny membránových proteinů – jedná se o nedávno definovanou molekulu ovlivňující přežití, maturaci a diferenciaci periferních B buněk. Nedávno byla ovšem publikována i práce popisující uvolňování BAFF z adipocytů, což naznačuje, že se může jednat o molekulu, u které se kříží endokrinní regulace adiposity a imunitní regulace v rámci specifické imunity. Cílem této studie bylo prozkoumat hladinu BAFF v mateřském séru a pupečníkové krvi u preeklamptických a fyziologických těhotenství a stanovit, zda se BAFF secernuje do mateřského mléka a jaká je dynamika jeho hladin během šestiměsíčního období po porodu.

Do části studie zkoumající hladiny BAFF v umbilikální krvi a mateřské periferní krvi bylo zařazeno 12 žen s preeklampsií a 34 žen s fyziologickým těhotenstvím a byly od nich peripartálně získány dvojice vzorků mateřská periferní krev / pupečníková krev. Dále byla do studie zařazena kohorta 10 laktujících žen, které byly sledovány po dobu 180 dnů po porodu a ve stanovených časových intervalech (den porodu, den 1-3, den 12-14, den 28-30, den 88-90 a den 178-180) od nich byly odebírány dvojice vzorků periferní krev / mateřské mléko.

Pozorovali jsme významné rozdíly v hladinách BAFF v séru z periferní krve matky mezi preeklamptickou a kontrolní kohortou; hladiny BAFF v pupečníkové krvi přitom byly výrazně vyšší než v mateřském séru. Jako první ve světové literatuře jsme prokázali, že BAFF se konzistentně secernuje do mateřského mléka během celého sledovaného období, přičemž hladiny v kolostru byly extrémní, s tendencí k rychlému poklesu v období následujícím po porodu.

Jedná se o vůbec první studii zkoumající cirkulující hladiny BAFF u preeklampsie. Navíc v této studii vůbec poprvé podáváme zprávu o přítomnosti BAFF v mateřském mléce. Vzhledem k výrazným pleiotropním efektům BAFF lze očekávat, že BAFF může hrát významnou úlohu při stimulaci imunitní maturace novorozence v časném poporodním období. Zároveň je pravděpodobné, že umožňuje efektivní komunikaci mezi mateřským organismem a imunitním systémem novorozence.

B–cell activating factor (BAFF) is present in umbilical cord blood in healthy and pre-eclamptic pregnancies as well as in human breast milk with specific dynamics during 180d lactation

Julie Bienertova-Vasku[1,5], Petr Bienert[1], Filip Zlamal[1], Josef Tomandl[2], Zuzana Dostalova[3], Zbynek Splichal[1], Pavel Ventruba[3], Vojtech Thon[4], Anna Vasku[1]

[1]Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2]Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3]Clinic of Gynaecology and Obstetrics, University Hospital Brno, Brno, Czech Republic [4]
[4]Department of Immunology, St. Anne's University Hospital, Brno, Czech Republic
[5]Clinic of Paediatric Oncology, University Hospital Brno, Brno, Czech Republic

Corresponding author: *Corresponding author. Tel.: +420 739300485; fax: +420 549494340. E-mail address: jbienert@med.muni.cz (J. Bienertova-Vasku)*

Abstract

Background, aims: The TNF family member protein B-cell-activating factor (BAFF) is a recently defined molecule influencing peripheral B-cell survival, B-cell maturation and immunoglobulin class-switch recombination. The aim of this study was to investigate role of BAFF in maternal serum and umbilical cord blood in pre-eclampsia and physiological pregnancy, to establish whether BAFF is secreted into breast milk and to determine the dynamics of BAFF levels in maternal serum and in breast milk during the six-month-long period of full breastfeeding.

Study design, subjects, outcome measures: Pairs of maternal peripheral blood and umbilical cord blood samples were obtained from 12 pre-eclamptic and 34 physiological pregnancies in order to analyze circulating BAFF levels. A second cohort of 10 healthy lactating women was recruited for analyses of BAFF levels in breast milk and maternal serum who were followed-up for 180 d following delivery.

Results: BAFF levels in maternal serum differedsignificantly between pre-eclamptic and healthy mothers. BAFF levels in umbilical cord blood significantly exceeded those of maternal serum. The analysis of BAFF levels in breast milk throughout the entire study period revealed a consistent presence of BAFF in breast milk, whereas levels in the colostrum were extreme and subsequently dropped.

Conclusion: This is the first study to investigate the circulating levels of BAFF in pre-eclampsia thus far. This is also the first study to report the presence of BAFF in human breast milk;BAFF may be helpful in the maturation of an infant's immune system in GALT throughout the 180d period following delivery.

Keywords:
BAFF; cord blood; breast milk; lactation; pre-eclampsia

Introduction

The B-cell-activating factor (BAFF), also known as BLyS, TNFSF13B, TALL-1, zTNF4 and THANK, is a member of the tumor necrosis factor (TNF) superfamily which influences peripheral B-cell survival, selection of autoreactive B-cells, maturation and immunoglobulin class-switch recombination and which therefore has a wide range of potential clinical implications[1]. Overexpression of BAFF in animal models leads to B-cell hyperplasia, lymphoproliferation, hypergammaglobulinemia and symptoms of autoimmunity [2] while BAFF-deficient animals show defects in peripheral B-cell maturation as well as decreased levels of circulating immunoglobulins[3]. As the signaling through the BCR and B cell activating factor receptor (BAFF-R or BR3) differentially regulates apoptosis within early transitional (T1) and late transitional (T2; CD21int-T2) B-cells during selection processes to create mature B lymphocytes[4], the BAFF-cascade could be an elegant therapeutic target in numerous pathophysiological conditions including B-lineage neoplasms, autoimmunity, allergy or infections.

Apoptosis, i.e. “programmed cell death”, is a cellular suicide mechanism which plays a crucial role in the maintenance of homeostasis and self-tolerance[5]. Apoptosis also plays a central part in the trophoblast life cycle of the placenta[6]. Normal trophoblast development begins with the proliferation and differentiation of a mononucleated cytotrophoblast[7], followed by its fusion into an overlying multinucleated syncytiotrophoblast, and finally resulting in the formation of syncytial knots, whereas the initiation of apoptosis in the cytotrophoblast is tightly associated with the described process of syncytiogenesis and often referred to as trophoblast turnover[8].

To date, three members of TNFSFs have been identified in human placenta[9-10]. The BAFF/BAFF-R signaling system is typically anti-apoptotic via induction of nuclear factor kappaB and Bcl-2, acting at the transition stage of B-lymphocyte development and inducing the Bcell differentiation markers CD21 and CD23[11]. It has been suggested that BAFF signaling through BAFF-R regulates the primary B-cell pool whereas BAFF and/or APRIL acting via the TACI receptor affects short-lived, proliferating B cells[11].

The sensitivity of immune cells in peripheral blood (i.e. lymphocytes, neutrophils, etc.) to undergo or evade apoptosis oscillates and the mechanism of apoptosis in these cells has been widely studied[12]. However, there is only one study of BAFF expression in B-cells from umbilical cord blood, reporting an increased *in vitro* rate of apoptosis of cultured B-cells from cord blood[13].

Pre-eclampsia is a severe pregnancy-associated condition, representing one of the major causes of mortality and morbidity worldwide[14]. Several studies reported an increase in placental apoptosis and formation of syncytial knots in pre-eclamptic pregnancies[15-18]. independently on Fas-Fas ligand expression[19].

So far, nothing is known about BAFF secretion and its possibledynamics of changes in human breast milk. Therefore, the objectives of this study included i) an investigation of BAFF levels in umbilical cord blood from physiological and pre-eclamptic pregnancies, ii) an investigation of maternal serum levels sampled the same time when the umbilical cord blood was withdrawn and iii) an investigation into whether BAFF is present in maternal milk and the examination of the dynamics of possible trends in BAFF secretion into milk.

Material and methods

Subjects

Preeclampsia-physiological pregnancy cohort

A total of 12 pre-eclampsia cases and 34 randomly recruited healthy mothers with physiological conceptions, pregnancies and deliveries, all with a Caucasian Central-European background, who delivered at the Clinic of Gynecology and Obstetrics of the University Hospital Brno, Brno, Czech Republic, were enrolled in the study. The pairs of maternal peripheral blood samples obtained during 2 h pre-partum and umbilical cord blood samples obtained immediately after childbirth were collected. Pre-eclampsia was defined according to the consensual criteria specified by the International Society for the Study of Hypertension in Pregnancy (blood-pressure values exceeding 140/90 mmHg after the 20^{th} week of gestation, confirmed by two consecutive readings at least six hours apart and the concomitant onset of proteinuria (> 300 mg of urinary protein/L over 24 h). The inclusion criteria for normal pregnancy cohort were: 1) spontaneous conception, 2) singleton pregnancy, 3) delivery of a term neonate with a birth weight above the 10^{th} percentile and 4) normal oral 75-g glucose tolerance test between weeks 24 and 28 of gestation based on the criteria specified by the World Health Organization (WHO). Exclusion criteria for the study were: 1) multiple pregnancy, 2) in vitro-assisted reproduction, 3) pre-existing hypertension disease, 4) gestational diabetes.

Lactation cohort

Breast milk and venous blood samples were obtained from another 10 healthy lactating women of Caucasian origin [mean age ± SD: 29.6 ± 3.4 y; mean preconceptional BMI ± SD: 23.2 ± 3.9 kg/m2] with uncomplicated, physiological pregnancies and appropriate-for-gestational age (AGA) neonates. The serum-milk sample pairs were collected at the time of birth and at days 1–3, 12–14, 28–30, 88–90 and 178–180 postpartum, with full lactation maintained during the entire 180-d period of the study (women who did not complete the 180-d duration of full lactation or introduced solid food before day 180 were excluded from the

study). These subjects were also enrolled at the Clinic of Gynecology and Obstetrics of the University Hospital Brno, Brno, Czech Republic. Inclusion criteria for the lactation cohort included (1) spontaneous conception; (2) uneventful, singleton pregnancy, (3) spontaneous, uncomplicated delivery and (4) a normal oral 75-g oral glucose tolerance test (OGTT) between 24 and 28 weeks of gestation based on the World Health Organization (WHO) criteria and (5) their children were appropriate-for-gestational age neonates. The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects of Masaryk University, Brno, Czech Republic, and was performed in adherence to the Declaration of Helsinki Guidelines.

Biochemistry

Umbilical cord blood and maternal venous blood sampling in pre-eclampsia cohort

Umbilical cord blood (5 ml) was withdrawn from the placenta immediately after childbirth;following clotting at room temperature and centrifugation, a serum specimen was collected into a plastic microtube and aliquots were stored at −80°C until analysis. Maternal venous blood (5 ml) was withdrawn duringthe two-hour pre-partum period and stored using the same procedure.

Breast milk

Milk samples were collected by manual expression from both breasts by the study subjects at given time points, always 2 h after previous breastfeeding or manual expression of milk;a total of 5 ml of milk was collected whenever possible (at timepoints 1 and 2, as much milk was sampled as possible). Samples were vortexed, divided into 200 µl aliquots and frozen at −80°C until analysis.

BAFF measurement

Concentrations of BAFF were measured in duplicate using a commercially available sandwich ELISA (R&D Systems, Inc. Minneapolis, USA) according to the manufacturer's instructions. Serum samples were diluted twice before assay using a calibrator diluent. Thawed milk samples were first delipidated by centrifugation at 12000×g for 15 min at 0 ºC and then diluted up to 40-fold in duplicate to assay range (62.5–4000 pg/ml) with calibrator diluent. Intra- and inter-assay coefficients of variation stood at < 6.0% and < 9.0%, respectively, with a detection limit of 3.38 pg/ml. The plates were read using a microplate reader (Spectra MAX 340PC, Molecular Devices, Sunnyvale, CA, USA), and the data were analyzed using Softmax Pro software.

Statistics

All statistical analyses were carried out using Statistica 8.0. Data were tested for normality using the Kolmogorov-Smirnov test and log-transformed where necessary. Pearson's correlation coefficient was used to determine whether linear associations were present and multivariate linear regression modeling was used to determine significant predictors of breast milk or serum BAFF concentration. Changes in BAFF serum and milk levels during the entire period of 180 days of lactation were examined by one-way ANOVA followed by Tukey's pairwise multiple comparison method. Differences in log values of whole milk or serum BAFF concentrations and their changes with the lactation periods were examined by one-way ANOVA followed by Tukey's pairwise multiple comparison method, with the assumption of homoscedasticity verified using Bartlett's test.

Results

Umbilical cord blood levels of BAFF in pre-eclamptic and physiological pregnancies

The basic characteristics of mothers with pre-eclampsia and physiological pregnancies arepresented in Table 1. While pre-eclampsia cases were significantly older than controls ($p = 0.01$), there were no differences in pre-conceptional BMI or BMI at the time of birth, the weight gain in pregnancy was significantly higher in PE cases ($p < 0.001$).

Comparison of BAFF circulating levels between pre-eclampsia cases and controls

There were significant differences in BAFF levels in maternal peripheral blood between physiological and pre-eclamptic pregnancies ($p = 0.03$) while BAFF levels were higher in physiological pregnancies than in pre-eclamptic pregnancies. No differences in BAFF cord blood levels were observed between the cohorts ($p = 0.91$). When comparing maternal serum levels and umbilical cord blood levels, highly significant results were observed both in the pre-eclamptic ($p < 0.002$) and physiological ($p < 0.001$) cohorts, in favor of higher serum levels in umbilical cord blood. The individual relationships between BAFF levels in maternal serum and umbilical cord blood serum duos in pre-eclampsia and the controls are summarized in Figure 1. When using linear regression modeling, no significant correlation of BAFF concentration in maternal blood and umbilical cord blood was observed either in pre-eclampsia cases ($r = 0.44$, $p = 0.15$) or in the controls ($r = 0.22$, $p = 0.20$).

BAFF levels in breast milk

The demographic and clinical characteristics of the lactation cohort are reported in Table 2. BAFF was consistently present in all evaluated milk samples in the study throughout the entire study period of 180 days; there was a continuous tendency towards a decrease of BAFF levels in milk and significant differences between investigated timepoints were also observed [$F(5;42) = 33.06$, $p < 0.001$], BAFF levels in breast milk throughout the study period are

given in Fig2. There were no significant differences in BAFF serum concentrations between the given time points [$F(5;38) = 3.09$, $p = 0.68$] Fig3. Furthermore, no correlation between BAFF levels in maternal serum and breast milk was observed either during the entire study period or when analyzing the individual timepoints. BAFF levels in breast milk exceeded those in maternal serum more than 25times (time point 1); during the 180-d duration of the study, the milk-serum concentrations equalized.

Discussion

The physiological role of BAFF in pregnancy and lactation is unknown. Apoptosis-inducing tumor necrosis factor (TNF) ligands and receptors have been reported in human placentas, including BAFF which was present primarily in villous cytotrophoblast cells[9]. The important role of BAFF in apoptosis has been previously defined in a study on B survival and selection through the BAFF/BAFF-R pathway, in addition to BCR-initiated signals[20]. BAFF/BAFF-R interaction activates the alternative NF-κB pathway through IKKα-mediated phosphorylation of p100 (NF-κB2) and results in its conversion to p52[21]which is capable of binding to the NF-κB family member c-Rel which has been previously shown to regulate Bcl-xL and A1 gene expression in B cells[22]. c-Rel, the prototype member of the NF-kB family, is expressed in numerous tissues in both adult and fetal hematopoietic organs, and is essential for the normal function of B and T cells, macrophages and dendritic cells[23].

To date, two studies focusing on BAFF levels in umbilical cord blood[13,24]have been performed – with contradictory results. In the study by Kessel et al.[13], BAFF serum levels in cord blood were reported to be lower than in peripheral blood of adult subjects, which the authors attribute to reported functional immaturity of cord blood macrophages (the expected source of the main proportion of BAFF). In the study by Kreuzaler et al.[24], sera from cord blood contained more BAFF (2.1 ng/ml; range, 0.6–4.5 ng/ml) than sera from the blood of the

corresponding mothers (0.6 ng/ml; range, 0.3–2.25 ng/ml), suggesting that BAFF concentrations are higher in individuals when the immune system and the B cell repertoire are not yet fully developed.

In this study, higher serum levels in umbilical cord blood were observed than in maternal peripheral blood from the respective pregnancy, independently of case-control status; average values of BAFF in maternal peripheral blood observed in the study (mean BAFF in maternal plasma 1.0 ± 0.3 ng/ml for the physiological cohort) were lower than those observed by Kessel et al. for non-pregnant subjects (mean BAFF 1.8 ± 0.5 ng/ml); moreover, there were largedifferences in BAFF levels in cord blood between our study (mean BAFF in cord blood 2.2 ± 0.7 ng/ml for the physiological cohort) and the study performed by Kessel et al. (mean BAFF in cord blood 0.68 ± 0.13 ng/ml). It must be mentioned that the study by Kessel et al. did not involve complete pairs of umbilical cord blood and maternal peripheral blood samples; the peripheral blood levels of BAFF were measured in 15 unrelated non-pregnant females and 15 males in their study, which could introduce a significant selection bias that was avoided in ourstudy.

The observed BAFF levels in maternal serum in this study were consistent with the circulating BAFF levels observed in non-pregnant non-obese women originating from the same population in another study of ours[25];this might indicate that pregnancy doesnot substantially increase maternal circulating levels of BAFF. The results of our study are highly concordant with the results of the study carried out by Kreuzaler et al.[24] as the observed concentrations of BAFF are almost identical between these two studies, possibly also due to the similar geographic origin of both study cohorts.

It has been recently suggested that the BAFF locus could act as a potential novel pre-eclampsia susceptibility gene[26]in Australian/New Zealand families, however, these results

were not replicated in Norwegian case/control cohort, however no physiological role for BAFF has been suggested so far.

This study has observed extremely high BAFF levels in maternal milk at the beginning of a 180-d study period starting with the day of birth; it may thus be hypothesized that extremely high BAFF levels in the colostrum could provide a strong B-cell maturation boost for the infant at the point of birth. As BAFF is involved in IgG, IgA and IgE isotype switching in B cells, which is a biological mechanism by which B cells change their antibody production from one isotype to another, the release of BAFF in maternal milk could influence the B cells of the mucosal lymfoid tissue (MALT) of the gut (GALT) of the newborns. It could contribute to the accumulation and maturation of B lymphocytes in the newborn as well as to the increase of intestinal production of secretory IgA antibodies in GALT.

To date, this study is the first to demonstrate that BAFF levels are significantly lower in maternal peripheral blood in pre-eclampsia compared to physiological pregnancy. Moreover, we are the first to demonstrate that BAFF is secreted into human breast milkand we show thatBAFF concentrations show a consistent decreasing tendency during the 6 months of full breastfeeding after birth. Although the population size of our study is limited, we report highly significant results which might provide a basis for further investigation of the role of BAFF in the stimulation of neonate/infant immunity/adiposity.

Conflict of interest statement

None declared.

Acknowledgements

This study was supported by the research grantsand projects: MZMOU2005 and RECAMO CZ.1.05/2.1.00/03.0101.

References

[1] Lied GA, Berstad A. Functional and clinical aspects of the B-cell-activating factor (BAFF): a narrative review. Scand J Immunol 2010;73:1–7.

[2] Ng LG, Mackay CR, Mackay F. The BAFF/APRIL system: life beyond B lymphocytes. Mol Immunol 2005;42:763–72.

[3] Mackay F, Leung H. The role of the BAFF/APRIL system on T cell function. Semin Immunol 2006;18:284–9.

[4] Castro I, Wright JA, Damdinsuren B, Hoek KL, Carlesso G, Shinners NP et al. B cell receptor-mediated sustained c-Rel activation facilitates late transitional B cell survival through control of B cell activating factor receptor and NF-kappaB2. J Immunol 2009;182:7729–37.

[5] Raff MC. Social controls on cell survival and cell death. Nature 1992; 356: 397–400.

[6] Tomas SZ, Prusac IK, Roje D, Tadin I. Trophoblast apoptosis in placentas from pregnancies complicated by preeclampsia. Gynecol Obstet Invest 2011;71:250–5.

[7] Baczyk D, Satkunaratnam A, Nait-Oumesmar B, Huppertz B, Cross JC, Kingdom JC. Complex patterns of GCM1 mRNA and protein in villous and extravillous trophoblast cells of the human placenta. Placenta 2004;25:553–9.

[8] Huppertz B, Frank HG, Reister F, Kingdom J, Korr H, Kaufmann P. Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments in vitro. Lab Invest 2000;79:1687–702.

[9] Phillips TA, Ni J, Hunt JS. Cell-specific expression of B lymphocyte (APRIL, BLyS)- and Th2 (CD30L/CD153)-promoting tumor necrosis factor superfamily ligands in human placentas. J Leukoc Biol 2003;74:81–7.

[10] Langat DL, Wheaton DA, Platt JS, Sifers T, Hunt JS. Signaling pathways for B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) in human placenta. Am J Pathol 2008;172:1303–11.

[11] Kalled SL. Impact of the BAFF/BR3 axis on B cell survival, germinal center maintenance and antibody production. Semin Immunol 2006;18:290–6.

[12] Treml LS, Crowley JE, Cancro MP. BLyS receptor signatures resolve homeostatically independent compartments among naive and antigen-experienced B cells. Semin Immunol 2006;18:297–304.

[13] Kessel A, Yehudai D, Peri R, Pavlotzky E, Bamberger E, Tov N et al. Increased susceptibility of cord blood B lymphocytes to undergo spontaneous apoptosis. Clin Exp Immunol 2006;145:563–70.

[14] Myers JE, Brockelsby J, The epidemiology of pre-eclampsia, in Baker PN, Kingdom JCP (eds), Pre-Eclampsia, Current Perspectives on Management. London, Parthenon, 2004

[15] Allaire AD, Ballenger KA, Wells SR, McMahon MJ, Lessey BA. Placental apoptosis in preeclampsia. Obstet Gynecol 2000;96:271–6.

[16] Austgulen R, Isaksen CV, Chedwick L, Romundstad P, Vatten L, Craven C. Pre-eclampsia: associated with increased syncytial apoptosis when the infant is small-for-gestational-age. J Reprod Immunol 2004;61:39–50.

[17] Heazell AE, Moll SJ, Jones CJ, Baker PN, Crocker IP. Formation of syncytial knots is increased by hyperoxia, hypoxia and reactive oxygen species. Placenta 2007;28:S33–S40.

[18] Huppertz B. Placental villous trophoblast: the altered balance between proliferation and apoptosis triggers preeclampsia. J Reproduktionsmed Endocrinol 2006;3:103–8.

[19] Mendilcioglu I, Karaveli S, Erdogan G, Simsek M, Taskin O, Ozekinci M. Apoptosis and expression of Bcl-2, Bax, p53, caspase-3, and Fas, Fas ligand in placentas complicated by preeclampsia. Clin Exp Obstet Gynecol 2011;38:38–42.
[20] Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. J Immunol 2004;173:2245–52.
[21] Claudio E, Brown K, Park S, Wang H, Siebenlist U. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. Nat Immunol 2002;3:958–65.
[22] Feng B, Cheng S, Hsia CY, King LB, Monroe JG, Liou HC. NF-kappaB inducible genes BCL-X and cyclin E promote immature B-cell proliferation and survival. Cell Immunol 2005;232:9–20.
[23] Clark JM, Aleksiyadis K, Martin A, McNamee K, Tharmalingam T, Williams RO et al. Inhibitor of kappa B epsilon (IkappaBepsilon) is a non-redundant regulator of c-Rel-dependent gene expression in murine T and B cells. PLoS One 2011;6:E24504.
[24] Kreuzaler M, Rauch M, Salzer U, Birmelin J, Rizzi M, Grimbacher B et al. Soluble BAFF levels inversely correlate with peripheral B cell numbers and the expression of BAFF receptors. J Immunol 2011;188:497–503.
[25] Bienertova-Vasku J, Bienert P, Zlamal F, Tomandl J, Forejt M, Tomandlova M et al. B-cell activating factor (BAFF) – a novel factor linking the immune status to diet. Cent Eur J Med 2012; in press.
[26] Fenstad MH, Johnson MP, Roten LT, Aas PA, Forsmo S, Klepper K et al. Genetic and molecular functional characterization of variants within TNFSF13B, a positional candidate preeclampsia susceptibility gene on 13q. PLoS One 2010;5.

Figure 1. Individual relationships between BAFF levels in maternal serum and umbilical cord blood in PE and the control cohort

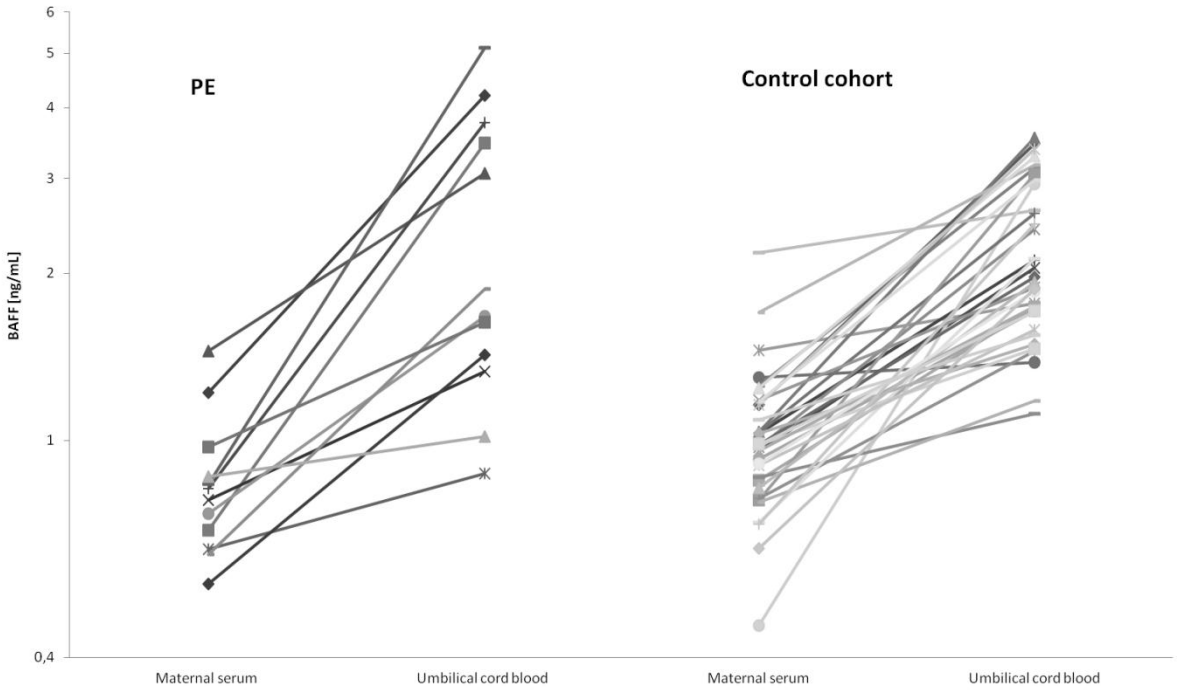


Figure 2: BAFF in breast milk at given timepoints of the study – individual trends

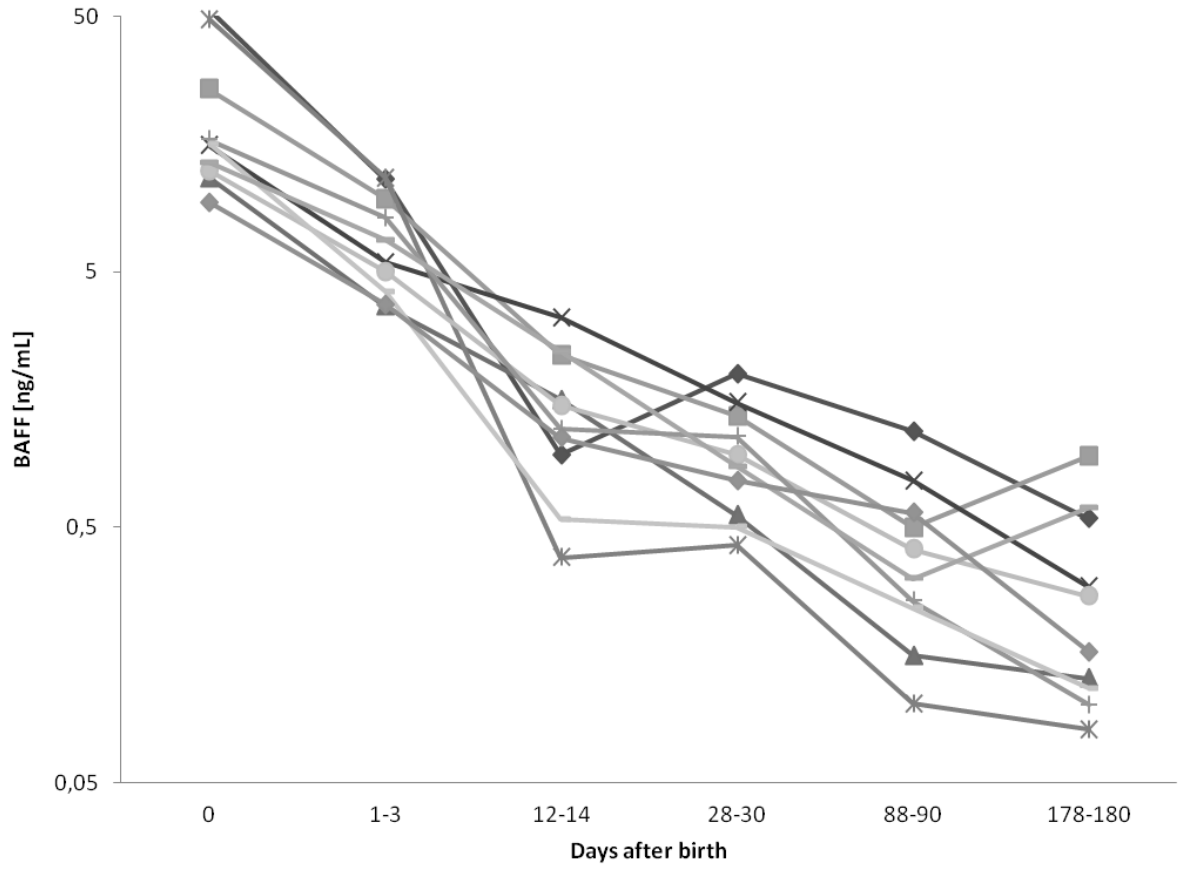


Figure 3: BAFF in maternal serum at given time points after birth

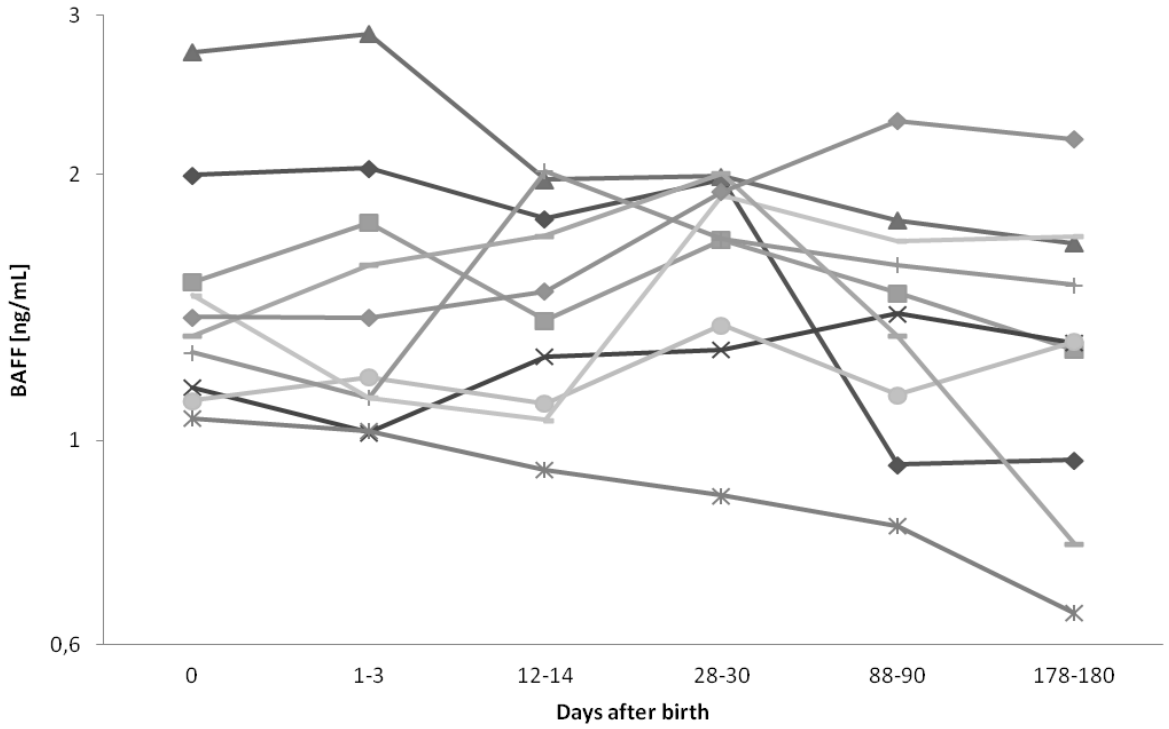


Table 1. Baseline characteristics of pre-eclamptic (PE) subjects and control subjects with physiological conception, pregnancy and delivery

	PE cases (n = 12)	Controls (n = 34)	p
Maternal age (yrs)	32.6 ± 4.0	29.3 ± 4.9	0.0128[+]
Pre-conception BMI (kg/m^2)	23.9 ± 6.5	24.9 ± 4.0	0.5820[+]
BMI at the time of delivery (kg/m^2)	28.4 ± 5.7	28.2 ± 9.0	0.3731[+]
Pregnancy weight gain (kg)	12.3 ± 5.0	5.2 ± 7.0	0.000063[+]
Birth weight (g)	2075 ± 435	3687 ± 337	0.000008[+]
Week of gestation at delivery	36.4 ± 1.9	40.4 ± 1.0	0.000000002[+]
IUGR (n)	3	0	0.0145[+++]
Primiparity/multiparity	2/10	22/12	0.0042[++]
BAFF level in maternal serum	843.4 ± 314.3	1021.4 ± 327.4	0.03[+]
BAFF level in umbilical cord blood serum	2453.3 ± 1352.1	2186.3 ± 698.4	0.91[+]

*BMI, body mass index; IUGR, intrauterine growth restriction; NS, not significant, results are given as mean ± SD, * early/late pre-eclampsia defined as onset of disease ≤ 34th week of gestation / > 34th week of gestation*

[+] *Mann-Whitney test*
[++] *chi-squared test*
[+++] *Fisher's exact test*

Table 2. Basic demographic and clinical findings in the lactation cohort (n = 10)

	Mean ± SD	Min	Max
Maternal age [y]	31.1 ± 3.7	26.4	36.6
Preconceptional BMI [kg/m^2]	23.9 ± 4.9	19.1	33.4
BMI before the birth [kg/m^2]	28.9 ± 5.2	22.0	38.9
Pregnancy weight gain [kg]	13.8 ± 4.2	7	19
Birth length of the infant [cm]	51.1 ± 2.0	48	55
Birth weight of the infant [g]	3645 ± 547	3060	4640
Menarche [y]	13.1 ± 1.1	11	14

3.8. VISFATIN IS SECRETED INTO THE BREAST MILK AND IS CORRELATED WITH WEIGHT CHANGES OF THE INFANT AFTER THE BIRTH

Resumé

Visfatin je velmi zajímavým adipokinem s mnoha metabolickými a imunoregulačními efekty. Předpokládá se, že díky svému inzulinomimetickému působení hraje významnou úlohu v regulaci pochodů v bílé tukové tkáni. Významné oscilace cirkulujících hladin visfatinu byly hlášeny i v období těhotenství. Dosud nebyly publikovány žádné zprávy o tom, zda se visfatin secernuje i do mateřského mléka.

Cílem této studie bylo prozkoumat, zda se visfatin u člověka uvolňuje do mateřského mléka, eventuálně prozkoumat dynamiku hladin visfatinu v mléce v 180denním poporodním období a dále prozkoumat korelaci hladin visfatinu v mléce s plazmatickými hladinami u matky. Do studie byly zařazeny dvojice vzorků mateřské mléko/sérum ve stanovených časových bodech: den porodu, den 1-3, den 12-14, den 28-30, den 88-90 a den 178-180 po porodu.

Naše výsledky ukazují, že: I. Visfatin je v hojném množství uvolňován do mateřského mléka během celého sledovaného poporodního období, II. Koncentrace visfatinu v mateřském mléce v různých časových bodech se značně liší, III. Koncentrace visfatinu v kolostru může být použita pro následnou predikci hmotnostního přírůstku novorozence (více či méně vyjádřený hmotnostní úbytek během prvních tří dnů po porodu). Naše data naznačují, že visfatin může hrát významnou roli v regulaci adipozity kojence po porodu prostřednictvím mateřského mléka.

DIAB-5191; No. of Pages 7
ARTICLE IN PRESS
DIABETES RESEARCH AND CLINICAL PRACTICE XXX (2011) XXX–XXX

Contents lists available at ScienceDirect
Diabetes Research and Clinical Practice
journal homepage: www.elsevier.com/locate/diabres

ELSEVIER
International Diabetes Federation

Visfatin is secreted into the breast milk and is correlated with weight changes of the infant after the birth

Julie Bienertová-Vašků [a,*], *P. Bienert* [a], *F. Zlámal* [a], *J. Tomandl* [b], *M. Tomandlová* [b], *Z. Dostálová* [c], *A. Vašků* [a]

[a] *Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, A18, 625 00 Brno, Czech Republic*
[b] *Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, A16, 625 00 Brno, Czech Republic*
[c] *Clinics of Gynecology and Obstetrics, Masaryk University Affiliated Hospital, Jihlavská 20, Brno, Czech Republic*

ARTICLE INFO

Article history:
Received 25 April 2011
Received in revised form
24 May 2011
Accepted 6 June 2011

Keywords:
Visfatin
Breast milk
Adiposity
Infant weight

ABSTRACT

Introduction: Visfatin is a recently identified adipokine with numerous metabolic and immunoregulatory properties that has been implicated in the regulation of the white adipose tissue (WAT) and significant changes in visfatin levels were reported during pregnancy. The aim of the study was to investigate dynamics of visfatin levels in maternal serum and human breast milk during a 180-d period after the delivery.
Materials and methods: : Breast milk and venous blood samples were obtained from 24 healthy lactating women with uncomplicated, physiological pregnancy and appropriate-for-gestational age neonates and serum–milk sample duos were collected at the time of birth, at the 1–3, 12–14, 28–30, 88–90 and 178–180 postpartum.
Results: Our study demonstrates that (1) visfatin is abundantly secreted into breast milk in humans, reaching approx. 100× higher concentrations compared to maternal serum; (2) visfatin concentrations in maternal serum show significant variations after the delivery and (3) visfatin concentration in colostrum could be used for prediction of the subsequent weight development (less/more severe weight loss during first 3 days after the birth) of the infant.
Discussion: Our data suggest that visfatin could play an important role in regulation of adiposity of the infatn after the birth.

1. Introduction

Lactation is a physiological state with major changes in energy homeostasis taking place in order to meet the energy as well as nutrient demands to maintain milk production. The huge metabolic demands associated with lactation are covered both by increased energy intake and excessive mobilization of energy from fat reserves, mainly from the white adipose tissue (WAT).

WAT is now strongly established as an active endocrine organ capable of producing a number of adipokines that serve as feedback signals to regulate WAT metabolism and have huge effects on food intake, energy expenditure, and carbohydrate and lipid metabolism including imporant implications for insulin sensitivity, thus having crucial effects in the intermediate metabolism [1,2].

Lactation is an immunologically unique state where immune factors are produced by the mother preferentially for the protection of the child. Several previous studies have focused on the immunological composition of human milk [3–6], however, much less information is available on maternal immune status during lactation. In a study by Zimmer [7], maternal peripheral blood lymphocytes showed dynamic,

* *Corresponding author.* Tel.: +420 739300485; fax: +420 549494340.
E-mail address: jbienert@med.muni.cz (J. Bienertová-Vašků).
0168-8227/$ – see front matter
doi:10.1016/j.diabres.2011.06.009

post-partum changes in the B-cell subpopulation and the relative percentage of CD19+ B-cells was significantly lower than control levels at 1–2 weeks and 1 month post-partum, but showed a significant, polynomial-linear rise over time, reaching control values by 2–4 months post-partum. In this study, the formula-feeding women presented with an earlier rise in the percentage of CD19+ cells, with relative percents always significantly higher than their lactating counterparts, which could be indicative of an involvement of another factor regulating population of B-lymphocytes during the perinatal period as well as during lactation.

Pre-B cell colony-enhancing factor/PBEF/Nampt, also known as visfatin, is a highly conserved, 52-kDa protein found in living species from bacteria to humans [Luc], having a unique structure and no obvious homology to other known proteins. Human visfatin was first identified by Samal et al. [8] as a protein that was secreted by activated lymphocytes in bone marrow stromal cells and acts synergistically with IL-7 and stem cell factor (SCF) to stimulate early stage B cell formation. Within the cell, visfatin functions as a nicotinamide phosphoribosyl transferase, the rate-limiting step in a salvage pathway of nicotinamide adenine dinucleotide (NAD) biosynthesis [9] and is also involved in two processes critical to cellular energetics: the synthesis of NAD and the uptake of energy substrates mediated by the actions of insulin [9]. PBEF gene was subsequently identified as one of the genes upregulated by distending the human fetal membranes *in vitro* [10], as visfatin is expressed in the normal fetal membranes and upregulated upon infection of the fetal membranes and it can be also speculated that visfatin plays a role in the cytokine network that facilitates normal, spontaneous labor and that initiates infection-induced preterm labor [11]. Engagement of the TNF superfamily member TALL-1 in B lymphocytes or B cell lymphoma cells induces expression of visfatin [12], and pre-B cells themselves also express visfatin following stimulation by IFN-gamma [13].

Recently, in a study by Folgueira et al. [14], PBEF was reported to be expressed in doxorubicin-responsive breast cancer, which could be, along with the report by Bae et al. [15] describing hypoxia-induced upregulation of visfatin mRNA in breast cancer cells, somewhat indicative of visfatin involvement in metabolism of mammary epithelials cells. Yonezawa et al. [16] performed a *in vitro* study on a bovine mammary epithelial cells, lactating bovine mammary gland and human breast cancer cell line, MCF-7, and observed visfatin mRNA as well as protein presence in the MCF-7 cells, bMEC and lactating mammary gland, which was the first demonstration of the presence of the visfatin protein in bovine milk, suggesting that the secretory epithelial cells could be the source of visfatin in the milk.

So far, no data on visfatin levels in human milk or the dynamics of visfatin levels in milk or maternal serum after delivery are available. In this study, we investigated visfatin levels in human milk in selected time points after delivery in order to examine the pattern of visfatin secretion to maternal milk within the 180d post-partum period. We also investigated visfatin concentration in maternal serum sampled at the same time points as breast milk, in order to compare visfatin dynamics in maternal serum and milk and we also investigated possible associations with maternal weight changes as well as infant weight gain after the birth.

2. Materials and methods

Between October 2009 and June 2010, breast milk and venous blood samples were obtained from 24 healthy lactating women who gave written informed consent with participation in the study. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine at Masaryk University (Brno, Czech Republic).

The study had a longitudinal design; couples of serum–milk samples were withdrawn at given time points from the same study subjects. Breast milk–serum samples were obtained on day of the childbirth (time point 0), further at day 1–3 (time point 1), 12–14 (time point 2), 28–30 (time point 3), 88–90 (time point 4) and 178–180 (time point 5), i.e. 6 times during the whole 180 days of the study. The basic anthropometric description of the study subjects is given in Table 1.

2.1. Study subjects

The presented study was designed to investigate changes in serum as well as milk concentrations of visfatin during a period of 180 days of full lactation after healthy, uncomplicated and physiological pregnancy and uncomplicated spontaneous delivery. Inclusion criteria in the study included (1) spontaneous conception; (2) uneventful, singleton pregnancy, (3) spontaneous, uncomplicated delivery and (4) a normal oral 75-g oral glucose tolerance test (OGTT) between 24 and 28 weeks of gestation based on the World Health Organization (WHO) criteria [17] and (5) their children were appropriate-for-gestational age (AGA) neonates. Exclusion criteria encompassed complicated pregnancy, pre-pregancy or pregnancy hormonal therapy, previous fertility treatment, surgical or induced delivery or multiple pregnancy or pre-conception BMI above 35 kg/m^2. All women in the study were primiparae.

Due to the demanding design of the study, we observed significant drop-out during duration of the study. The total of 24 women entered the study ($N = 24$; age [years] 29.8 ± 2.9; BMI [kg/m^2] 25.9 ± 3.7) and further continued to time point 1, out of whom the 16 subjects further continued to the time point 2 ($N = 16$; age [years] 24.4 ± 3.8, BMI [kg/m^2] 24.4 ± 3.8), 11 subjects continued to the time point 3 ($N = 11$; age [years] 25.6 ± 3.8, BMI [kg/m^2] 25.5 ± 3.8), 10 subjects continued to the time point 4 ($N = 10$; age [years] 25.5 ± 4.4, BMI [kg/m^2] 25.5 ± 4.4) and only 4 subjects finished the whole 180-d duration of the study, i.e. reached the time point 5 ($N = 4$; age [years] 26.4 ± 6.3, BMI [kg/m^2] 26.4 ± 6.3, ANOVA – age $\sim$ (0, 1, 2, 3, 4, 5): Bartlett's test: $p = 0.70$).

All the twenty-four women completed a questionnaire regarding their demographics and personal and family history with special attention paid to possible problems with fertility and previous fertility treatments.

2.2. Milk samples

Milk samples were collected by manual expression from both breasts by the study subjects at the given time points, always

Table 1 – Basic demografic findings and visfatin levels at the given timepoints.

	Mean ± SD	Min	Max
Demographics			
Maternal age [y]	29.8 ± 2.9	25.6	36.6
Preconceptional BMI [kg/m^2]	23.1 ± 4.3	17.7	33.7
BMI before the birth [kg/m^2]	27.9 ± 4.4	22.0	38.9
Pregnancy weight gain [kg]	13.7 ± 4.8	4.0	25.0
Birth length of the infant [cm]	50.3 ± 1.4	48.0	53.0
Birth weight of the infant [g]	3324 ± 283	2800	3710
Menarche [y]	13.0 ± 1.16	11.0	15.0
Visfatin levels			
Serum – at the birth [ng/mL]	10.7 ± 9.1	1.2	46.4
Serum – 1–3 days postpartum [ng/mL]	8.4 ± 9.8	1.7	46.8
Serum – 12–14 days postpartum [ng/mL]	3.5 ± 4.0	0.4	15.2
Serum – 28–30 days postpartum [ng/mL]	3.0 ± 2.3	0.7	7.1
Serum – 88–90 days postpartum [ng/mL]	3.4 ± 1.7	1.8	5.5
Serum – 178–180 days postpartum [ng/mL]	2.5 ± 1.1	1.1	4.5
Breast milk – at birth [ng/mL]	1208 ± 1508	129	5430
Breast milk – 1–3 days postpartum [ng/mL]	854 ± 863	124	3374
Breast milk – 12–14 days postpartum [ng/mL]	1777 ± 1204	243	5187
Breast milk – 28–30 days postpartum [ng/mL]	1851 ± 1824	96	5982
Breast milk – 88–90 days postpartum [ng/mL]	1269 ± 1124	199	2559
Breast milk – 178–180 days postpartum [ng/mL]	1244 ± 1211	335	4012

2 h after previous breastfeeding or manual expression of the milk, whereas the total of 5 ml of milk was collected where possible (at timepoints 1 and 2, as much milk was sampled as possible). Samples were vortexed, divided into 200 μl aliquots and frozen at −80 °C until analysis. Maternal venous blood (5 ml) was withdrawn at the same time point as the milk sample and after clotting at the room temperature and following centrifugation, a serum specimen was collected into a plastic microtube and aliquotes were stored at −80 °C until analysis. Concentrations of active visfatin in maternal serum and breast milk were determined using the human Nampt (visfatin/PBEF) ELISA (AdipoGen, Korea) according to manufacturer's instructions.

2.3. Biochemistry

For the measurements of the dimeric active form of visfatin in breast milk and serum, an enzyme immunoassay (ELISA) was used (AdipoGen, Korea). All milk and serum samples from the individual subjects were assayed in duplicates in the same test battery/batch/run. Thawed milk samples were first delipidated by centrifugation at 12000 × *g* for 15 min at 0 °C and then 300-fold diluted in singlet to assay range (0.125–8 ng/ml) with assay diluent. Intra- and inter-assay coefficients of variation (CV) for the visfatin in breast milk and serum were <4.5% and <5.9%, respectively, with a detection limit 30 pg/ml using 100 μl samples.

2.4. Statistics

All statistical analyses were carried out using Statistica 8.0. Data were tested for normality using the Kolmogorov–Smirnov test and log-transformed where necessary. Pearson's correlation coefficient was used to determine whether linear associations were present and multivariate linear regression modelling was used to determine significant predictors of breast milk or serum visfatin concentration. The changes in serum and milk visfatin levels during the whole period of 180 days of lactation were examined with one-way ANOVA followed by Tukey's pairwise multiple comparison method. Differences in the log values of whole milk or serum visfatin concentrations and their changes with the lactation periods were examined with one-way ANOVA followed by Tukey's pairwise multiple comparison method, with the assumption of homoscedasticity verified using Bartlett's test. If not otherwise stated, data are given as mean ± standard deviation (Std); p values less than 0.05 were considered significant in all tests.

3. Results

The basic demografic data are given in Table 1.

3.1. Distribution of visfatin milk concentrations across the study period

We observed consistent presence of visfatin in breast milk, whereas the average concentrations of visfatin in milk were approximately 100-higher than in maternal serum at the same time point (Fig. 1). Visfatin concentration in breast milk showed a lognormal distribution across the research period, no significant changes were in visfatin concentrations in maternal milk during the study period, although a tendency could be observed towards an increase of visfatin levels in early mature milk [$F(5, 69) = 1.743$, $p = 0.13$].

3.2. Visfatin concentrations in maternal serum

Analysis of visfatin levels in maternal serum across the whole study period revealed significant differences in visfatin serum concentrations along the given time points [$F(5, 80) = 8.3094$, $p = 0.000002$], Fig. 2. There were significant differences between the visfatin concentrations in maternal serum at

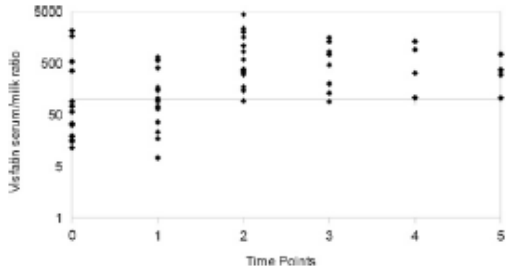


Fig. 1 – Serum/milk ratio of visfatin througout the study period. The graphic representation reveals that at the time of birth and shortly afterwards, there is more variation in the milk/serum ratio of visfatin, and the longer the elapsed time since delivery, the less variations is present. The trends in milk/serum ratio are opposing the trends in serum visfatin concentrations, the milk/serum ratio reaching the highest values at the time point 3 where serum visfatin levels are the lowest.

time point 0 and 2 ($p = 0.0001$), 0 and 3 ($p = 0.0006$), 0 and 5 ($p = 0.004$), 1 and 2 ($p = 0.012$), 1 and 3 ($p = 0.042$), whereas the serum visfatin levels showed a tendency towards a continuous decrease throughout the 180 days postpartum. When analyzing the individual trends of visfatin plasma levels in the study individuals, a tendency towards a decrease in visfatin plasma levels could be still observed (Fig. 3).

3.3. *Relationship between milk and serum visfatin and maternal BMI after the delivery*

No significant associations of milk and serum visfatin levels and maternal preconceptional BMI or BMI at the time of birth, however, there were significant correlations between visfatin concentration in milk at time point 0 and maternal BMI at the delivery ($r = -0.6771$, $p = 0.0156$).

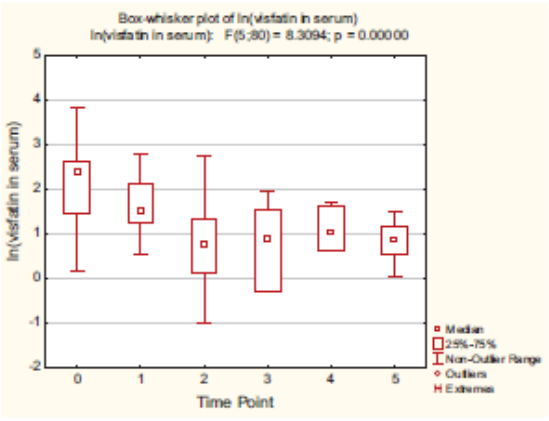


Fig. 2 – Dynamics of visfatin levels in maternal serum during the study period.

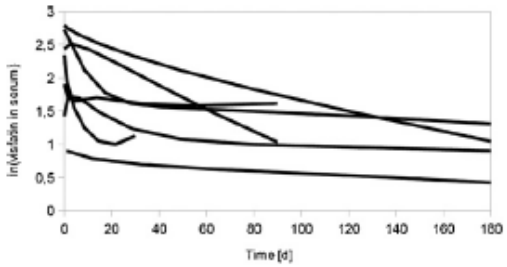


Fig. 3 – Visfatin in maternal serum at the given timepoints of the study – individual trends. *Women who completed at least 4 time points of the study were included into this graphic representation.

3.4. *Relationship between milk visfatin and weight gain of the infants*

The women with higher levels of milk visfatin in colostrum expressed a strong tendency towards a decrease of visfatin milk levels at the following time point, while there was a strong tendency towards an increase of visfatin in milk in the subgroup of women with lower levels of visfatin ($r = -0.696$, $p = 0.026$, Fig. 3), and this association was BMI-dependent.

The multiple linear regression modelling of the infant weight gain as the dependent variable and milk visfatin at time point 0 and birth weight of the infant as independent variables revealed a significant correlation of investigated parameters (multiple $R = 0.905$, $p = 0.032$), whereas there was no significant correlation of birth weight of the infant and the infant weight gain.

The subsequent increase of visfatin levels in women with lower visfatin concentration in colostrum was associated with less expressed weight loss of the infant after the birth, similarly, the decrease of visfatin concetration in women with higher visfatin levels in colostrum indicated more pronounced weight loss of the infant after the birth ($r = 0.796$, $p = 0.058$), independently on visfatin levels in maternal serum.

Furthermore, the lower level of visfatin in colostrum was indicative of lower weight loss of the infant between the birth day and time point 1, whereas the higher concentration of visfatin in colostrum was indicative of more pronounced weight loss of the infant in the next few days after the birth ($r = -0.679$, $p = 0.044$, Fig. 4).

4. Discussion

At present, the pathophysiological background to the regulations of adiposity in postpartum women as well as to regulation of infant adiposity through signaling molecules transmitted via breast milk is elusive. However, an abundant presence of various adipokines, such as leptin or adiponectin, in breast milk was described elsewhere [18–20], suggesting a complex network of adipokines is participating in regulating the adiposity status as well as nutritional behaviour of the infant.

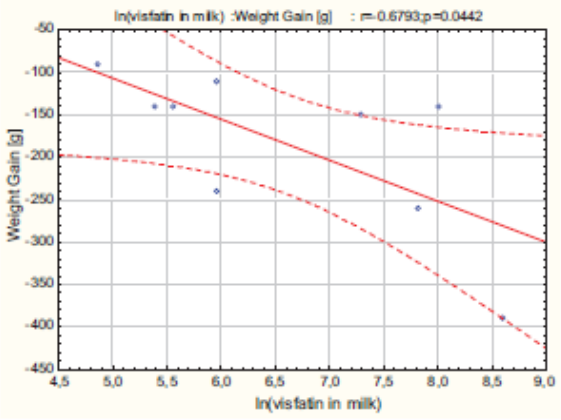


Fig. 4 – Relatioship between visfatin milk concentration and weight gain of the infant between the day of the birth and time point 1.

Visfatin is a remarkable adipokine with a wide range of effects, both at the subcellular and endocrine levels [21]. Visfatin mRNA as well as protein was reported to be present in cloned bovine mammary epithelial cells, lactating bovine mammary gland and human breast cancer cell line [16] and it seems that mammary epithelial cells express the visfatin protein and secrete it into the milk. Lemor et al. [22] observed changes related to transient period of lactation in the abundance of the mRNAs of visfatin in adipose tissue of high-yielding dairy cows. However, no study so far has investigated visfatin levels in human breast milk. Our study demonstrates that visfatin is consistently secreted into human milk throughout the first 6 month of lactation and that it achieves approximately 100× higher concentrations in milk than in maternal serum.

While very little is known about visfatin role in lactation in humans, the role of visfatin in pregnancy has been studied more extensively [23–25]. In their study on 100 women with gestational diabetes, Ferreira et al. [26] report reduced serum concentration of adiponectin and increased concentration of visfatin at 11–13 weeks of gestation in patients with gestational diabetes mellitus, which could be possibly explained by higher placental expression of visfatin in gestational diabetes, described elsewhere [27]. Mazaki-Tovi et al. [28] report that the acute pyelonephritis during pregnancy was associated with a high circulating maternal visfatin concentration which is consistent with the role of visfatin/PBEF in the regulation of the complex and dynamic crosstalk between inflammation and metabolism during pregnancy a also during delivery, as the same research group did observe in their another study that median visfatin concentration was higher in umbilical cord plasma of neonates appropriate for the gestation age born following a spontaneous labor at term than that of those who were born by an elective cesarean section [29], which could be suggestive of a role of visfatin in labor-associated inflammation. The absolute values of visfatin concentration in maternal serum at the time of birth in this study were consistent with our presented findings (mean value of 8.5 ng/mL for the study by Mazaki Tovi vs. 10.7 ng/mL in our study).

In another study, Mazaki-Tovi et al. [30] did not observe differences in maternal and neonatal circulating visfatin concentrations in patients with pre-eclampsia and a small-for-gestational age (SGA) neonates, suggesting that it is unlikely that the fetal circulation serves as the source of high maternal visfatin concentrations reported in patients with an SGA neonates. Based on these observations, a presence of a feedback mechanism secondary increasing visfatin levels in maternal serum in reaction to abnormal adiposity of the child could be suggested. In our study we observed an inverse relationship between maternal BMI and visfatin serum levels at the birth, indicating that the relationship between total adiposity of the mother and visfatin level would not be a simple correlation. In our cohort, women with lower visfatin concentration in serum at birth (typically presenting with preconceptional BMI of 28–30 kg/m^2) expressed a strong tendency towards a further increase of visfatin levels after delivery and children of these mothers were less likely to have a significant weight loss after the delivery. This suggests that dynamics of visfatin concentration in milk, namely the significant increase of visfatin conentration in breast milk, could act as a protective factor against a weight loss of the infant during the first days after the birth. On the contrary, children from the pregnancies where the preconceptional BMI of the mother was in the range of 22–25 kg/m^2 were more likely to have a serious weight loss after the delivery and there was a strong tendency towards a decrease of visfatin levels in milk in a few days after the delivery. Based on our data, visfatin concetration in maternal milk could be used a prediction marker for the subsequent weight loss of the infant during the first few days of life.

Why the children from the mother with higher preconceptional BMI were less likely to develop a significant weight drop after the birth, that was significantly associated with visfatin level in milk, is unclear. Taken our data together, it seems that increase of visfatin levels in the early postpartum period could be a protective factor against the weight loss of the infant and that this association is dependent on maternal BMI.

The role of visfatin in lipogenesis/lipolysis is unclear. It is clear that visfatin is produced abundantly in adipocytes and it is highly likely that there is a strong relationship between visfatin levels and lipid metabolism. It has been suggested that the relationship between visfatin and lipid profile represents a compensatory mechanism for diabetic dyslipidemia, as Sun et al. [31] reported that in rats, visfatin/PBEF/Nampt improved insulin sensitivity and exerted strong hypocholesterolemic effects at least partially through upregulation of the tyrosine phosphorylation of IRS-1 protein and the mRNA levels of PPARgamma and SREBP-2 [31]. Visfatin has insulinomimetic effects mediated through insulin receptor [32] and as insulin harbours strong antilipolytic properties, it could be hypothesized that visfatin in milk could have a insuling-mimicing antilipolytic effect on the adipose tissue of the child, thus preventing the child from a severe weight loss during the vulnerable period of the first days of its life where the production of sufficient amount of milk hasn't been established yet.

Our analyses are based on multiple measurements in women with physiological, uncomplicated pregnancies with AGA children and are reflecting the dynamics of visfatin levels in milk and serum over time under the physiological conditions. The major limitation of our study is the low number of study subjects and the significant drop-out of the study, possibly due to the demanding study design requiring 7 appointments, whereas only several mother finished the whole duration of 180 days of the study. It would be interesting to investigate larger population samples of different ethnicity and to measure serial changes of serum and milk visfatin levels in different subsets of study subjects, e.g. in mother–infant duos with pregnancy affected by pre-eclamsia, premature birth or intrauterine growth restriction of other etiology (e.g. karyotype abnormalities) to clarify the role of visfatin in regulation of maternal as well as infant adiposity after the birth.

5. Conclusion

Our study demonstrates that (1) visfatin is abundantly secreted into breast milk in humans, reaching approx. 100× higher concentrations compared to maternal serum; (2) visfatin concentrations in breast milk did not show significant variations while visfatin levels in maternal serum expressed a significant tendency towards a steady decrease during the whole period of 180 days after the delivery and (3) we observed a significant prediction role of visfatin concentration in colostrum for the subsequent weight development (less/more severe weight loss during first 3 days after the birth) of the infant, suggesting that visfatin could play an important role in regulation of infant adiposity through the maternal milk. The use of visfatin concentration in colostrum for prediction of the weight loss of the infant after the delivery thus can have potentially huge implication in the neonatal medicine as the neonates at increased risk of severe weight loss could benefit from more agressive treatment.

Larger population studies are imperative to reach a conclusion on role of visfatin in regulation of infant weight during the lactation period.

Acknowledgement

This study has been supported by the DANONE Institute of the Czech Republic (2010–2011).

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

[1] Trayhurn P, Wood IS. Signalling role of adipose tissue: adipokines and inflammation in obesity. Biochem Soc Trans 2007;33(Pt 5):1078–81.

[2] Ronti T, Lupattelli G, Mannarino E. The endocrine function of adipose tissue: an update. Clin Endocrinol (Oxf) 2006;64(4):355–65.

[3] Vigh E, Bódis J, Garai J. Longitudinal changes in macrophage migration inhibitory factor in breast milk during the first three months of lactation. J Reprod Immunol 2011 [epub ahead of print].

[4] Garofalo R. Cytokines in human milk. J Pediatr 2010;156:S36–40.

[5] Hanson LA. Session 1: feeding and infant development breast-feeding and immune function. Proc Nutr Soc 2007;66(3):384–96.

[6] Ballabio C, Bertino E, Coscia A, Fabris C, Fuggetta D, Molfino S, et al. Immunoglobulin-A profile in breast milk from mothers delivering full term and preterm infants. Int J Immunopathol Pharmacol 2007;20(1):119–28.

[7] Zimmer JP, Garza C, Heller ME, Butte N, Goldman AS. Relationship between serum prolactin, lactation and changes in maternal blood B-cell (CD19+) percents during the first 8 months post-partum. J Reprod Immunol 1996;30(2–3):81–95.

[8] Samal B, Sun Y, Stearns G, Xie C, Suggs S, McNiece I. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Mol Cell Biol 1994;14(2):1431–7.

[9] Luk T, Malam Z, Marshall JC. Pre-B cell colony-enhancing factor (PBEF)/visfatin: a novel mediator of innate immunity. J Leukoc Biol 2008;83(4):804–16.

[10] Nemeth E, Millar LK, Bryant-Greenwood G. Fetal membrane distention. II. Differentially expressed genes regulated by acute distention in vitro. Am J Obstet Gynecol 2000;182(1 Pt 1):60–7.

[11] Mazaki-Tovi S, Romero R, Kusanovic JP, Erez O, Gotsch F, et al. Visfatin/pre-B cell colony-enhancing factor in amniotic fluid in normal pregnancy, spontaneous labor at term, preterm labor and prelabor rupture of membranes: an association with subclinical intrauterine infection in preterm parturition. J Perinat Med 2008;36(6):485–96.

[12] Xu LG, Wu M, Hu J, Zhai Z, Shu HB. Identification of downstream genes up-regulated by the tumor necrosis factor family member TALL-1. J Leukoc Biol 2002;72(2):410–6.

[13] Patrone L, Damore MA, Lee MB, Malone CS, Wall R. Genes expressed during the IFN gamma-induced maturation of pre-B cells. Mol Immunol 2002 Jan;38(8):597–606.

[14] Folgueira MA, Carraro DM, Brentani H, Patrão DF, Barbosa EM, et al. Gene expression profile associated with response to doxorubicin-based therapy in breast cancer. Clin Cancer Res 2005;11(20):7434–43.

[15] Bae SK, Kim SR, Kim JG, Kim JY, Koo TH, Jang HO, et al. Hypoxic induction of human visfatin gene is directly mediated by hypoxia-inducible factor-1. FEBS Lett 2006;580(17):4105–13.

[16] Yonezawa T, Haga S, Kobayashi Y, Takahashi T, Obara Y. Visfatin is present in bovine mammary epithelial cells, lactating mammary gland and milk, and its expression is regulated by cAMP pathway. FEBS Lett 2006;580(28–29):6635–43.

[17] Prevention of diabetes mellitus. Report of a WHO study group. World Health Organ Tech Rep Ser 1994;844:1–100.

[18] Bronsky J, Mitrova K, Karpisek M, Mazoch J, Durilova M, Fisarkova B, et al. Adiponectin, AFABP, and leptin in human breast milk during 12 months of lactation. J Pediatr Gastroenterol Nutr 2011;52(4):474–7.

[19] Ilcol YO, Hizli ZB, Ozkan T. Leptin concentration in breast milk and its relationship to duration of lactation and hormonal status. Int Breastfeed J 2006;17(1):21.

4. OREXIGENNÍ/ANOREXIGENNÍ PEPTIDY/ADIPOKINY A JEJICH ÚLOHA PRO RIZIKO KOMPLEXNÍCH CHOROB POZDĚJI V DOSPĚLOSTI

V následujících podkapitolách jsou uvedeny práce z oblasti civilizačních onemocnění. Jsou rozděleny do dvou skupin na práce s kardiologickou a obezitologickou tématikou.

Práce s kardiologickou tématikou:

I. Are common leptin promoter polymorphisms associated with restenosis after coronary stenting?

II. Association between variants in the genes for leptin, leptin receptor and proopiomelanocortin with chronic heart failure in the Czech population.

III. Common polymorphism +45T/G in adiponectin gene as potential modulator of in-stent restenosis development.

Práce s obezitologickou tématikou:

IV. Relation between adiponectin 45 T/G polymorphism and dietary composition in the Czech population.

V. Genotype vs. nutrient association of common polymorphisms in obesity-related genes with food preferences and time structure of energy intake.

VI. Genotype vs. Nutrient association of common polymorphisms in obesity-related genes with food preferences and time structure of energy intake association of genetic variability in selected regions in visfatin (Nampt) gene with anthropometric parameters and dietary composition in obese and non-obese central-european population.

VII. Visfatin and its role in obesity development.

VIII. B-cell activating factor (BAFF) – a new factor linking immunity to diet?

IX. Variability in CNR1 locus influences protein intake and smoking status in the Central-European population.

4.1. ARE COMMON LEPTIN PROMOTER POLYMORPHISMS ASSOCIATED WITH RESTENOSIS AFTER CORONARY STENTING?

Resumé

Z hlediska cévního modelování hrají zásadní úlohu hypertrofie hladkých svalových buněk cév a neoptimální proliferace. Jeden z nejvýznamnějších adipokinů, leptin, má kromě četných endokrinních efektů i parakrinní účinky na úrovni periferie. Cílem této studie bylo prozkoumat, zda možné asociace dvou v populaci častých jednonukleotidových polymorfismů v genu pro leptin (LEP -2548 G/A a LEP -188C/A) souvisejí s restenózou po perkutánní koronární intervenci (PCI).

Do studie bylo zařazeno celkem 98 pacientů s implantací čistého kovového stentu, kteří podstoupili stentování do malých koronárních arterií (<3 mm). Při rekoronarografii po 6 měsících byla stanovena míra výskytu restenózy ve stentu, přičemž restenóza >50 % se objevila u 33,3 % nosičů A alely a 31,4 % nosičů CC polymorfismu LEP-188C/A a u 25 % pacientů s AA alelami, 32,7 % pacientů s AG genotypem a 30,4 % pacientů s GG alelami polymorfismu LEP-2548G/A.

Heterozygotní genotyp AG v LEP-2548G/A představoval vysoce významné riziko onemocnění více koronárních tepen oproti oběma homozygotním genotypům AA a GG (odds ratio = 4,038, 95% konfidenční interval 1,732 – 9,465; p = 0,0001).

Na základě naší studie lze sledované polymorfismy považovat za určité markery vnímavosti vůči výskytu restenózy ve stentu u ischemické koronarografované populace.

Heart Vessels (2007) 22:310–315
DOI 10.1007/s00380-007-0980-2

ORIGINAL ARTICLE

Julie Anna Bienertová-Vašků · Ota Hlinomaz
Anna Vašků

Are common leptin promoter polymorphisms associated with restenosis after coronary stenting?

Received: October 6, 2006 / Accepted: March 2, 2007

Abstract The hypertrophy of vascular smooth muscle cells as well as neointimal proliferation is critical in vascular remodeling, whereas leptin has proved to play an important role recently. The aim of the study was to investigate possible associations of two common leptin gene polymorphisms with restenosis after percutaneous coronary intervention (PCI). To study the association of two promoter polymorphisms, LEP –2548 G/A and LEP –188 C/A (dbSNP ID rs7799039 and rs791620) with neointimal proliferation in humans, 98 consecutive patients undergoing stenting into small coronary arteries (<3 mm) were genotyped. After a 6-month follow-up, the restenosis rate was estimated. Restenosis >50% occurred in 33.3% of patients carrying both A alleles, 33.3% of carriers of A and C alleles, and 31.4% of carriers of two CC alleles of LEP –188 C/A polymorphism; and in 25.0% of patients with AA, 32.7% with AG, and 30.4% with GG genotype of LEP –2548 G/A polymorphism. Interestingly, the heterozygote AG genotype of LEP –2548 polymorphism represented a highly significant risk for multiple-vessel disease when compared to both homozygote genotypes AA/GG (odds ratio = 4.038, 95% confidence interval: 1.732–9.465, $P_{corr} = 0.001$). Based on our findings, the AG genotype of LEP –2548 G/A polymorphism might be considered a genetic marker for multiple-vessel disease but not for restenosis after PCI. The role of the leptin gene polymorphisms as genetic markers of restenosis will require further investigation to elucidate the underlying pathophysiological consequences.

Key words Leptin · Restenosis · Gene · Polymorphism

J.A. Bienertová-Vašků (✉) · A. Vašků
Institute of Pathological Physiology, Faculty of Medicine, Masaryk University, Komenského nám. 2, 664 32, Brno, Czech Republic
Tel. +420-608147575; Fax +420-549494340
e-mail: vasku.julie@seznam.cz

O. Hlinomaz
First Department of Internal Medicine/Cardioangiology, St. Ann's Faculty Hospital, Brno, Czech Republic

Introduction

Coronary artery disease (CAD) represents the major cause of morbidity and mortality in developed countries and World Health Organization (WHO) statistics confirm that this trend will continue well into the future.[1] Coronary artery disease is considered a multifactorial disease as it aggregates in families; however, it does not segregate like a Mendelial single-gene disorder.

Apart from coronary artery bypass grafting, percutaneous transluminal coronary angioplasty (PTCA) and intracoronary stent placement stand for the established treatment strategy for CAD. Percutaneous transluminal coronary angioplasty was introduced as an alternative means of coronary revascularization in 1977[2] and it has readily become widely accepted as a safe and effective treatment for coronary artery disease. Unfortunately, various strategies have failed in preventing the major drawback to PTCA – restenosis of the treated vessel.

Intracoronary stenting has considerably reduced the incidence of restenosis.[3] Generally, restenosis occurs in approximately 30% of patients after PTCA and in 12% of patients after intracoronary stent placement.[4] At present when the treated lesions are much more complex the restenosis rate is even higher. Fortunately drug-eluting stents, discovered recently, further reduce the risk of restenosis. Morphological changes after coronary stenting mainly represent an early thrombus formation and acute inflammation, followed by neointimal hyperplasia, which seems to be almost the sole mechanism of restenosis after stenting.[5]

As genetic factors seem to considerably contribute to the risk of restenosis after PTCA and stent implantation, genetic epidemiology might provide insights into pathophysiological consequences of restenosis after stenting and should be also able to identify possible markers for predicting increased risk of restenosis. From this point of view, single nucleotide polymorphisms (SNPs), variants of a single nucleotide in DNA that can easily be identified, are most suitable for individual risk stratification that will enable tailoring of interventional treatment to the individual patient.

Results

To examine the hypothesis that variability of leptin gene promoter is involved in restenosis after stent implantation, a total of 98 patients after bare metal stent implantation were enrolled in the study. The genotype distribution of LEP −2548 G/A polymorphism in the patient cohort conformed to the Hardy–Weinberg equilibrium while genotype distributions of LEP −188 C/A polymorphism conformed to strong disequilibrium. When comparing the patients' genotype distributions of LEP −2548 G/A polymorphism to those of 200 healthy individuals, no statistically significant difference was found either in genotypes distributions (P_g = 0.38) or allelic frequencies (P_a = 0.46), the differences in genotype distributions as well as allelic frequencies of LEP −188 C/A polymorphism between the patients with restenosis and healthy individuals proved to be borderline statistically significant (P_g = 0.07, P_a = 0.06).

Follow-up angiography was performed at 6 months in 98 patients. The restenosis rate was 33.3% for AA, 33.3% for AC, and 31.4% for CC genotype of LEP −188 C/A polymorphism, and 25.0% for AA, 32.7% for AG, and 30.4% for GG genotype of LEP −2548 G/A polymorphism; when comparing genotype distributions and allelic frequencies of both examined polymorphisms between patients expressing restenosis and patients without restenosis after 6 months, no statistically significant differences were found (P_g = 0.68, P_a = 0.33 for LEP −188 C/A polymorphism; P_g = 0.54, P_a = 0.41 for LEP −2548 G/A polymorphism).

Relevant clinical and anthropological characteristics of study subjects are summarized in Tables 1 and 2. The angiographic and procedural data are listed in Tables 3 and 4. For the LEP −2548 G/A polymorphism, the mean diameter stenosis before percutaneous coronary intervention (PCI) was 82.6% ± 7.4% for the patients with two A alleles, 81.2% ± 13.2% for heterozygotes, and 80.3% ± 5.7% for carriers of two GG alleles. For the LEP −188 C/A polymorphism, the mean diameter stenosis before intervention was 85.3% ± 6.1% for AA carriers, 85.1% ± 7.8 for AC genotype, and 80.6% ± 10.8% for CC homozygotes. Due to the large variability of the parameter this difference did not prove to be statistically significant.

Consequently, multivariate logistic regression was used to analyze if a certain genotype of examined polymorphisms is a potential risk factor for adverse long-term PCI outcome. In the regression modeling, genotypes of either examined polymorphisms were not significantly associated with the baseline variables such as age, gender, hypercholesterolemia, insulin-dependent diabetes mellitus, non-insulin-dependent diabetes mellitus, the lesion length, or previous myocardial infarction history. In both examined polymorphisms no association with weight, height, waist-to-hip ratio, and body mass index (BMI) was observed.

Furthermore, we evaluated the effect of the two SNPs studied on the risk of adverse PCI outcome with special attention paid to the restenosis defined as ≥50% diameter reduction; reoccluded vessels were not excluded from the statistical analysis. The genotypes of the LEP −2548G/A and LEP −188 C/A together with patients' weight, BMI, cholesterol plasma levels, and number of affected vessels represented input variables. None of the independent variables was significantly correlated with restenosis.

The results suggested that the heterozygote genotype AG of LEP −2548 G/A polymorphism might be associated with the increased number of affected vessels. Further analysis was performed on the grouped genotype AA/GG as compared to heterozygote genotype AG. According to this analysis, the relative risk of AG genotype compared to homozygote genotypes AA/GG for multiple-vessel impairment was estimated to be 4.038 (95% confidence interval: 1.732–9.465; P_{corr} = 0.001; Fig. 1). No statistically significant association was observed between specific double genotypes and restenosis risk in a case–control approach as well as when comparing restenotic and non-restenotic males versus females.

Table 1. Baseline patients' characteristics in relation to LEP −2548 G/A genotypes

−2548 G/A	Overall		AA		AG		GG		*P* value
	n	%	*n*	%	*n*	%	*n*	%	
All patients	98	100.0	20	20.4	55	56.1	23	23.5	
Female	26	26.5	4	15.4	14	53.8	8	30.8	0.70
Hypertension	67	68.4	14	20.9	38	56.7	15	22.4	0.99
IDDM	8	8.2	1	12.5	5	62.5	2	25.0	0.86
NIDDM	26	26.5	5	19.2	15	57.7	6	23.1	0.99
Hypercholesterolemia	57	58.2	11	19.3	28	49.1	18	31.6	0.54
Stable angina	23	23.5	5	21.7	10	43.5	8	34.8	0.47
Previous MI	19	19.4	1	5.3	15	78.9	3	15.8	0.15
STEMI	41	41.8	9	22.0	24	58.5	8	19.5	0.88
Non-STEMI	4	4.1	0	0.0	4	100.0	0	0.0	0.22
CAD									
1-vessel	50	51.0	14	28.0	20	40.0	16	32.0	NA
2-vessel	26	26.5	4	15.4	18	69.2	4	15.4	NA
3-vessel	22	22.4	2	9.1	17	77.3	3	13.6	NA

IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; MI, myocardial infarction; STEMI, ST-segment elevation myocardial infarction; CAD, coronary artery disease; NA, not analyzed

Results

To examine the hypothesis that variability of leptin gene promoter is involved in restenosis after stent implantation, a total of 98 patients after bare metal stent implantation were enrolled in the study. The genotype distribution of LEP −2548 G/A polymorphism in the patient cohort conformed to the Hardy–Weinberg equilibrium while genotype distributions of LEP −188 C/A polymorphism conformed to strong disequilibrium. When comparing the patients' genotype distributions of LEP −2548 G/A polymorphism to those of 200 healthy individuals, no statistically significant difference was found either in genotypes distributions (P_g = 0.38) or allelic frequencies (P_a = 0.46), the differences in genotype distributions as well as allelic frequencies of LEP −188 C/A polymorphism between the patients with restenosis and healthy individuals proved to be borderline statistically significant (P_g = 0.07, P_a = 0.06).

Follow-up angiography was performed at 6 months in 98 patients. The restenosis rate was 33.3% for AA, 33.3% for AC, and 31.4% for CC genotype of LEP −188 C/A polymorphism, and 25.0% for AA, 32.7% for AG, and 30.4% for GG genotype of LEP −2548 G/A polymorphism; when comparing genotype distributions and allelic frequencies of both examined polymorphisms between patients expressing restenosis and patients without restenosis after 6 months, no statistically significant differences were found (P_g = 0.68, P_a = 0.33 for LEP −188 C/A polymorphism; P_g = 0.54, P_a = 0.41 for LEP −2548 G/A polymorphism).

Relevant clinical and anthropological characteristics of study subjects are summarized in Tables 1 and 2. The angiographic and procedural data are listed in Tables 3 and 4. For the LEP −2548 G/A polymorphism, the mean diameter stenosis before percutaneous coronary intervention (PCI) was 82.6% ± 7.4% for the patients with two A alleles, 81.2% ± 13.2% for heterozygotes, and 80.3% ± 5.7% for carriers of two GG alleles. For the LEP −188 C/A polymorphism, the mean diameter stenosis before intervention was 85.3% ± 6.1% for AA carriers, 85.1% ± 7.8 for AC genotype, and 80.6% ± 10.8% for CC homozygotes. Due to the large variability of the parameter this difference did not prove to be statistically significant.

Consequently, multivariate logistic regression was used to analyze if a certain genotype of examined polymorphisms is a potential risk factor for adverse long-term PCI outcome. In the regression modeling, genotypes of either examined polymorphisms were not significantly associated with the baseline variables such as age, gender, hypercholesterolemia, insulin-dependent diabetes mellitus, non-insulin-dependent diabetes mellitus, the lesion length, or previous myocardial infarction history. In both examined polymorphisms no association with weight, height, waist-to-hip ratio, and body mass index (BMI) was observed.

Furthermore, we evaluated the effect of the two SNPs studied on the risk of adverse PCI outcome with special attention paid to the restenosis defined as ≥50% diameter reduction; reoccluded vessels were not excluded from the statistical analysis. The genotypes of the LEP −2548G/A and LEP −188 C/A together with patients' weight, BMI, cholesterol plasma levels, and number of affected vessels represented input variables. None of the independent variables was significantly correlated with restenosis.

The results suggested that the heterozygote genotype AG of LEP −2548 G/A polymorphism might be associated with the increased number of affected vessels. Further analysis was performed on the grouped genotype AA/GG as compared to heterozygote genotype AG. According to this analysis, the relative risk of AG genotype compared to homozygote genotypes AA/GG for multiple-vessel impairment was estimated to be 4.038 (95% confidence interval: 1.732–9.465; P_{corr} = 0.001; Fig. 1). No statistically significant association was observed between specific double genotypes and restenosis risk in a case–control approach as well as when comparing restenotic and non-restenotic males versus females.

Table 1. Baseline patients' characteristics in relation to LEP −2548 G/A genotypes

−2548 G/A	Overall		AA		AG		GG		*P* value
	n	%	*n*	%	*n*	%	*n*	%	
All patients	98	100.0	20	20.4	55	56.1	23	23.5	
Female	26	26.5	4	15.4	14	53.8	8	30.8	0.70
Hypertension	67	68.4	14	20.9	38	56.7	15	22.4	0.99
IDDM	8	8.2	1	12.5	5	62.5	2	25.0	0.86
NIDDM	26	26.5	5	19.2	15	57.7	6	23.1	0.99
Hypercholesterolemia	57	58.2	11	19.3	28	49.1	18	31.6	0.54
Stable angina	23	23.5	5	21.7	10	43.5	8	34.8	0.47
Previous MI	19	19.4	1	5.3	15	78.9	3	15.8	0.15
STEMI	41	41.8	9	22.0	24	58.5	8	19.5	0.88
Non-STEMI	4	4.1	0	0.0	4	100.0	0	0.0	0.22
CAD									
1-vessel	50	51.0	14	28.0	20	40.0	16	32.0	NA
2-vessel	26	26.5	4	15.4	18	69.2	4	15.4	NA
3-vessel	22	22.4	2	9.1	17	77.3	3	13.6	NA

IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; MI, myocardial infarction; STEMI, ST-segment elevation myocardial infarction; CAD, coronary artery disease; NA, not analyzed

Table 2. Baseline patients' characteristics in relation to LEP –188 C/A genotypes

–188 C/A	Overall		AA		AC		CC		*P* value
	n	%	*n*	%	*n*	%	*n*	%	
All patients	98	100.0	4	4.1	11	11.2	83	84.7	
Female	26	26.5	0	0.0	3	11.5	23	88.5	NA
Hypertension	67	68.4	2	3.0	7	10.4	58	86.6	NA
IDDM	8	8.2	0	0.0	0	0.0	8	100.0	NA
NIDDM	26	26.5	1	3.8	3	11.5	22	84.6	NA
Hypercholesterolemia	57	58.2	1	1.8	7	12.3	49	86.0	NA
Stable angina	23	23.5	0	0.0	3	13.0	20	87.0	NA
Previous MI	19	19.4	0	0.0	3	15.8	16	84.2	NA
STEMI	41	41.8	2	4.9	2	4.9	37	90.2	NA
Non-STEMI	4	4.1	0	0.0	0	0.0	4	100.0	NA
CAD									
1-vessel	50	51.0	3	6.0	4	8.0	43	86.0	NA
2-vessel	26	26.5	1	3.8	3	11.5	22	84.6	NA
3-vessel	22	22.4	0	0.0	4	18.2	18	81.8	NA

Abbreviations: see Table 1

Table 3. Quantitative coronarography data in relation to LEP –2548 G/A genotypes

–2548 G/A	Genotype	*n*	Mean	SD	Q25	Q50	Q75	Min	Max
MLD before PCI (mm)	AG	55	0.4	0.2	0.3	0.5	0.6	0.0	0.9
	AA	20	0.4	0.2	0.3	0.4	0.6	0.0	0.7
	GG	23	0.5	0.2	0.4	0.5	0.5	0.2	0.8
	Sum	98	0.4	0.2	0.3	0.5	0.6	0.0	0.9
DS before PCI (%)	AG	55	81.1	13.0	74.3	81.2	90.0	18.6	100.0
	AA	20	82.6	7.4	76.3	81.8	88.8	72.9	100.0
	GG	23	80.3	5.7	75.7	80.1	84.8	71.1	92.7
	Sum	98	81.2	10.6	74.8	81.0	88.4	18.6	100.0
MLD after PCI (mm)	AG	55	2.5	0.4	2.3	2.5	2.8	1.7	3.8
	AA	20	2.4	0.4	2.2	2.5	2.7	1.8	3.0
	GG	23	2.5	0.4	2.3	2.6	2.7	1.6	3.0
	Sum	98	2.5	0.4	2.3	2.5	2.8	1.6	3.8
DS after PCI (%)	AG	55	8.0	7.3	1.1	6.5	13.3	0.0	26.8
	AA	20	7.2	5.5	3.2	6.4	10.6	0.0	17.4
	GG	23	6.9	6.9	0.4	5.1	9.6	0.0	22.2
	Sum	98	7.5	6.9	0.9	6.2	11.6	0.0	26.8
MLD after 6 months (mm)	AG	55	1.2	0.8	0.6	1.3	1.7	0.0	3.0
	AA	20	1.3	0.8	0.4	1.5	1.9	0.0	2.6
	GG	23	1.1	1.0	0.1	1.3	1.7	0.0	3.1
	Sum	98	1.2	0.8	0.4	1.4	1.7	0.0	3.1
DS after 6 months (%)	AG	55	40.9	28.8	23.5	35.2	57.0	0.0	100.0
	AA	20	35.1	30.4	12.9	31.1	39.9	0.0	100.0
	GG	23	39.1	35.5	13.4	26.4	70.0	0.0	100.0
	Sum	98	39.7	30.9	19.0	32.1	58.0	0.0	100.0

AA, AG and GG genotypes of –2548 G/A polymorphism in leptin gene promoter
MLD, minimal lumen diameter; PCI, percutaneous coronary intervention; DS, diameter stenosis; SD, standard deviation; before, before intervention; after, after intervention; after 6 months, after 6 months of follow-up

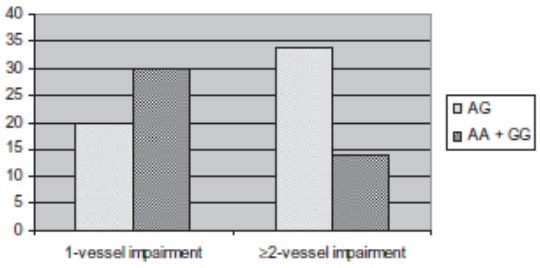


Fig. 1. Relative risk of AG genotype of LEP –2548 G/A polymorphism for multiple vessel impairment

Discussion

Physiological actions of leptin were previously demonstrated to regulate a variety of cardiac and vascular effects including angiogenesis, thrombosis, hemodynamics, and cardiac hypertrophy. Leptin contributes to the modulation of metabolism, breathing control, and inflammation, which all have been linked to cardiovascular health and disease. Thus, leptin might be involved both in local chronic inflammation and systemic immune response, which is well in accordance with new finding that atherosclerotic coronary artery disease seems to be accelerated in some patients with

Table 4. Quantitative coronarography data in relation to LEP –188 C/A genotypes

–188 C/A	Genotype	*n*	Mean	SD	Q25	Q50	Q75	Min	Max
MLD before PCI (mm)	CC	83	0.4	0.2	0.3	0.5	0.6	0	0.9
	AC	11	0.4	0.2	0.2	0.4	0.5	0	0.6
	AA	4	0.4	0.2	0.3	0.4	0.5	0.2	0.6
	Sum	98	0.4	0.2	0.3	0.5	0.6	0.0	0.9
DS before PCI (%)	CC	83	80.6	10.8	74.7	80.1	87.5	18.6	100.0
	AC	11	85.1	7.8	81.5	84.8	88.8	69.7	100.0
	AA	4	85.3	6.1	82.2	87.5	89.4	77.0	91.3
	Sum	98	81.2	10.6	74.8	81.0	88.4	18.6	100.0
MLD after PCI (mm)	CC	83	2.5	0.4	2.2	2.5	2.7	1.6	3.8
	AC	11	2.7	0.3	2.5	2.8	2.9	2.2	3.1
	AA	4	2.7	0.2	2.6	2.6	2.8	2.6	2.9
	Sum	98	2.5	0.4	2.3	2.5	2.8	1.6	3.8
DS after PCI (%)	CC	83	7.4	6.8	0.9	6.1	11.7	0.0	26.8
	AC	11	7.4	6.8	0.0	6.2	12.2	0.0	18.0
	AA	4	12.4	7.1	9.4	16.3	17.4	2.4	18.5
	Sum	98	7.5	6.9	0.9	6.2	11.6	0.0	26.8
MLD after 6 months (mm)	CC	83	1.2	0.8	0.3	1.4	1.7	0.0	3.1
	AC	11	1.5	0.8	1.2	1.3	1.8	0.0	3.0
	AA	4	1.4	1.0	0.9	1.8	2.1	0.0	2.3
	Sum	98	1.2	0.8	0.4	1.4	1.7	0.0	3.1
DS after 6 months (%)	CC	83	39.8	31.0	19.5	31.6	60.5	0.0	100.0
	AC	11	38.1	25.1	17.4	46.1	52.5	0.8	80.0
	AA	4	40.2	42.5	10.8	21.5	60.3	0.0	99.0
	Sum	98	39.7	30.9	19.0	32.1	58.0	0.0	100.0

AA, AC and CC genotypes of –188 C/A polymorphism in leptin gene promoter
Abbreviations: see Table 3

human immunodeficiency virus infection.[21] Recent results suggest that hyperleptinemia is independently related to poorer cardiovascular outcome.[22] Smooth muscle cell proliferation following the implantation of a coronary stent is the predominant factor contributing to restenosis, whereas recent findings confirm the role of leptin in vascular smooth muscle cell proliferation[23] and thus it can be hypothesized that variability of leptin gene promoter might be associated with altered vessel characteristics in restenotic patients.

In patients with angiographically confirmed coronary atherosclerosis, leptin predicts future cardiovascular events independent of other risk factors including lipid status or C-reactive protein.[24] In the West of Scotland Coronary Prevention Study that included only males, leptin was found to act as a moderate predictor of coronary events during a 5-year follow-up.[25] On the other hand, a smaller study using the Quebec Cardiovascular Study population, again restricted to male subjects, did not confirm those findings.[26] In patients with symptomatic coronary artery disease who developed in-stent restenosis after stent implantation, leptin levels were found to be increased and correlated to reduced serum nitric oxide levels.[17]

In vitro studies demonstrate direct effects of leptin on vascular and inflammatory cells that may promote atherothrombosis independent of the centrally mediated metabolic effect of leptin. Studies in mice have implicated altered leptin signaling in both arterial thrombosis and atherosclerosis.[27–29]

In this study, in both LEP –2548 G/A and LEP –188 C/A polymorphism genotypes, no association with restenosis (either binary or clinical), lumen diameters, or baseline patients' characteristics was observed when comparing the CAD patients to the healthy individuals. On the other hand, our data revealed a four-fold risk of AG genotype of LEP –2548 G/A polymorphism against both homozygote genotypes AA/GG for multiple-vessel impairment. Also, patients with AG genotype tended to suffer more frequently from myocardial infarction, although this association did not prove to be statistically significant. However, it cannot be easily concluded that AG genotype is a marker of severity of CAD as this would require a special study on numerous CAD cases. On the other hand, it has to be taken into account that the tissue leptin levels in AA homozygotes exceed those of GG/GA carriers more than 60%.[30] Provided that both constitutively increased plasma leptin levels in AA carriers and low plasma leptin in GG homozygotes are not as risky in relation to multiple-vessel disease as intermediate AG genotype, it is rather genetic variability of leptin gene than plasma levels that should be considered a marker of potential risk.

Limitations of the study include a significant lack of data on leptin phenotypic effects, which makes further functional studies a necessity to determine the exact genotype–phenotype correlation, putting special emphasis on leptin plasma levels. Moreover, the number of patients after PCI included in the study was rather low and thus it may be suggested that the results are affected by selection bias. However, it should be also mentioned that the present study was carried out on a highly static population from a wide region of Moravia, part of the Czech Republic, settled by a Slavonic population that can be assumed to be homogeneous. Therefore, the associations discussed cannot be

easily attributed to selection bias; the risk genotype AG of LEP−2548 G/A is likely to be truly associated with increased multiple vessel disease risk in this particular Czech population. To conclude, further comparative studies on different populations need to be carried out to elucidate the underlying genetic component of CAD as different phenotypic variations in CAD have been reported worldwide thus far.[31]

Despite the limitations mentioned above, this study clearly indicates that genetic variations in the leptin gene may act as an attractive susceptibility marker for multiple-vessel disease. In contrast, no association of variability in tandem region of leptin gene with restenosis after PCI has been observed. To investigate the possible utility of SNPs in the 5′-untranslated region of leptin gene as genetic markers for restenosis after PCI, further investigation appears necessary.

References

1. Murray CJ, Lopez AD (1997) Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. Lancet 349:1498–1504
2. Rapold HJ, David PR, Guiteras Val P, Mata AL, Crean PA, Bourassa MG (1987) Restenosis and its determinants in first and repeat coronary angioplasty. Eur Heart J 8:575–586
3. Gruntzig AR, Senning A, Siegenthaler WE (1979) Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. N Engl J Med 301:61–68
4. Sigwart U, Puel J, Mirkovitch V, Joffre F, Kappenberger L (1987) Intravascular stents to prevent occlusion and restenosis after transluminal angioplasty. N Engl J Med 316:701–706
5. Farb A, Sangiorgi G, Carter AJ, Walley VM, Edwards WD, Schwartz RS, Virmani R (1999) Pathology of acute and chronic coronary stenting in humans. Circulation 99:44–52
6. Thogersen AM, Soderberg S, Jansson JH, Dahlen G, Boman K, Nilsson TK, Lindahl B, Weinehall L, Stenlund H, Lundberg V, Johnson O, Ahren B, Hallmans G (2004) Interactions between fibrinolysis, lipoproteins and leptin related to a first myocardial infarction. Eur J Cardiovasc Prev Rehabil 11:33–40
7. Soderberg S, Ahren B, Eliasson M, Dinesen B, Olsson T (2002) The association between leptin and proinsulin is lost with central obesity. J Intern Med 252:140–148
8. Soderberg S, Olsson T, Eliasson M, Johnson O, Brismar K, Carlstrom K, Ahren B (2001) A strong association between biologically active testosterone and leptin in non-obese men and women is lost with increasing (central) adiposity. Int J Obes Relat Metab Disord 25:98–105
9. Soderberg S, Stegmayr B, Stenlund H, Sjostrom LG, Agren A, Johansson L, Weinehall L, Olsson T (2004) Leptin, but not adiponectin, predicts stroke in males. J Intern Med 256:128–136
10. Rajapurohitam V, Gan XT, Kirshenbaum LA, Karmazyn M (2003) The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. Circ Res 93:277–279
11. Paolisso G, Tagliamonte MR, Galderisi M, Zito GA, Petrocelli A, Carella C, De Divitiis O, Varricchio M (1999) Plasma leptin level is associated with myocardial wall thickness in hypertensive insulin-resistant men. Hypertension 34:1047–1052
12. Sweeney G (2002) Leptin signalling. Cell Signal 14:655–663
13. Bjorbaek C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers MG Jr, Flier JS (2001) Divergent roles of SHP-2 in ERK activation by leptin receptors. J Biol Chem 276:4747–4755
14. Bouloumie A, Marumo T, Lafontan M, Busse R (1999) Leptin induces oxidative stress in human endothelial cells. FASEB J 13: 1231–1238
15. Van Den Brink GR, O'toole T, Hardwick JC, Van Den Boogaardt DE, Versteeg HH, Van Deventer SJ, Peppelenbosch MP (2000) Leptin signaling in human peripheral blood mononuclear cells, activation of p38 and p42/44 mitogen-activated protein (MAP) kinase and p70 S6 kinase. Mol Cell Biol Res Commun 4:144–150
16. Schafer K, Halle M, Goeschen C, Dellas C, Pynn M, Loskutoff DJ (2004) Leptin promotes vascular remodeling and neointimal growth in mice. Arterioscler Thromb Vasc Biol 24:112–117
17. Piatti P, Di Mario C, Monti LD, Fragasso G, Sgura F, Caumo A, Setola E, Lucotti P, Galluccio E, Ronchi C, Orrigi A, Zavaroni I, Margonato A, Colombo A (2003) Association of insulin resistance, hyperleptinemia, and impaired nitric oxide release with in-stent restenosis in patients undergoing coronary stenting. Circulation 108:2074–2081
18. Oksanen L, Palvimo JJ, Janne OA, Kontula K (1998) Functional analysis of the C(-188)A polymorphism of the human leptin promoter. Hum Genet 103:527–528
19. Li WD, Reed DR, Lee JH, Xu W, Kilker RL, Sodam BR, Price RA (1999) Sequence variants in the 5′ flanking region of the leptin gene are associated with obesity in women. Ann Hum Genet 63 (Pt 3):227–234
20. Ribeiro R, Vasconcelos A, Costa S, Pinto D, Morais A, Oliveira J, Lobo F, Lopez C, Medeiros R (2004) Overexpressing leptin genetic polymorphism (–2548 G/A) is associated with susceptibility to prostate cancer and risk of advanced disease. Prostate 59: 268–274
21. Spieker LE, Karadag B, Binggeli C, Corti R (2005) Rapid progression of atherosclerotic coronary artery disease in patients with human immunodeficiency virus infection. Heart Vessels 20:171–174
22. Schulze PC, Kratzsch J (2005) Leptin as a new diagnostic tool in chronic heart failure. Clin Chim Acta 362:1–11
23. Li L, Mamputu JC, Wiernsperger N, Renier G (2005) Signaling pathways involved in human vascular smooth muscle cell proliferation and matrix metalloproteinase-2 expression induced by leptin: inhibitory effect of metformin. Diabetes 54:2227–2234
24. Wolk R, Berger P, Lennon RJ, Brilakis ES, Johnson BD, Somers VK (2004) Plasma leptin and prognosis in patients with established coronary atherosclerosis. J Am Coll Cardiol 44:1819–1824
25. Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N (2001) Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). Circulation 104:3052–3056
26. Couillard C, Lamarche B, Mauriege P, Cantin B, Dagenais GR, Moorjani S, Lupien PJ, Despres JP (1998) Leptinemia is not a risk factor for ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study. Diabetes Care 21:782–786
27. Yen TT, Allan JA, Pearson DV, Schinitsky MR (1977) Dissociation of obesity, hypercholesterolemia and diabetes from atherosclerosis in ob/ob mice. Experientia 33:995–996
28. Hasty AH, Shimano H, Osuga J, Namatame I, Takahashi A, Yahagi N, Perrey S, Iizuka Y, Tamura Y, Amemiya-Kudo M, Yoshikawa T, Okazaki H, Ohashi K, Harada K, Matsuzaka T, Sone H, Gotoda T, Naqai R, Ishibashi S, Yamada N (2001) Severe hypercholesterolemia, hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density lipoprotein receptor. J Biol Chem 276:37402–37408
29. Bodary PF, Westrick RJ, Wickenheiser KJ, Shen Y, Eitzman DT (2002) Effect of leptin on arterial thrombosis following vascular injury in mice. JAMA 287:1706–1709
30. Hoffstedt J, Eriksson P, Mottagui-Tabar S, Arner P (2002) A polymorphism in the leptin promoter region (–2548 G/A) influences gene expression and adipose tissue secretion of leptin. Horm Metab Res 34:355–359
31. Miwa K, Fujita M, Sasayama S (2005) Recent insights into the mechanisms, predisposing factors, and racial differences of coronary vasospasm. Heart Vessels 20:1–7

4.2. ASSOCIATION BETWEEN VARIANTS IN THE GENES FOR LEPTIN, LEPTIN RECEPTOR AND PROOPIOMELANOCORTIN WITH CHRONIC HEART FAILURE IN THE CZECH POPULATION

Resumé

Pacienti s chronickým srdečním selháním (CHF) vykazují znaky metabolického onemocnění typu progresivního úbytku svalového hmoty a akcelerované lipolýzy, tedy typické znaky terminální kachexie. Cílem této studie bylo zkoumat možné asociace definované variability v genech pro leptin (dbSNP ID rs7799039), proopiomelanokortin (dbSNP ID rs3754860 a dbSNP ID rs1009388) a leptinový receptor (dbSNP rs1137101) s CHF a vyhodnotit jejich potenciál jako genů vnímavosti pro CHF. Tato studie zahrnovala 372 pacientů s chronickým srdečním selháním (NYHA funkční třída II-IV, ejekční frakce < 40%), s ischemickou etiologií nebo dilatační kardiomyopatií.

Nebyly pozorovány case-control rozdíly ve frekvenci genotypů ani alel mezi pacienty s CHF a kontrolami. Jako další krok jsme konstruovali POMC haplotypy – nenalezli jsme významnou souvislost s body mass indexem (BMI), ejekční frakcí levé komory (LVEF), hypetrofií levé komory (LVH) ani současným výskytem diabetu mellitu. Vícerozměrné modelování ukázalo přibližně dvakrát vyšší riziko NYHA IV u Gln223Arg polymorfismu v genu pro leptinový receptor (odds ratio [OR] = 2,10, 95% konfidenční interval [CI] = 1,56–2,84), který také vykazoval nezávislou predikční úlohu pro LVEF u srdečního selhání všech etiologií dohromady (p = 0,002, OR = 4,05, 95% CI = 1,36–10,06). Role v populaci četných polymorfismů v patofyziologii CHF je nejasná. Na základě našich pozorování, lze Gln223Arg polymorfismus v genu pro leptinový receptor považovat za genetický marker CHF modulující průběh onemocnění jak u pacientů s ischemickou chorobou srdeční, tak u pacientů s dilatační kardiomyopatií.

Heart Vessels (2009) 24:131–137
DOI 10.1007/s00380-008-1090-5

ORIGINAL ARTICLE

Julie Anna Bienertová-Vašků · Lenka Špinarová
Petr Bienert · Anna Vašků

Association between variants in the genes for leptin, leptin receptor, and proopiomelanocortin with chronic heart failure in the Czech population

Received: March 27, 2008 / Accepted: July 3, 2008

Abstract Patients with chronic heart failure (CHF) express enhanced catabolic metabolism finally resulting in overall weight loss, whereas adipokines might play a crucial role in signaling among tissues. The aim of this study was to investigate the possible associations of defined variability in leptin (dbSNP ID rs7799039), proopiomelanocortin (dbSNP ID rs3754860 and dbSNP ID rs1009388), and leptin receptor gene (dbSNP rs1137101) with CHF and evaluate their potential as the CHF susceptibility genes. The case–control study comprised a total of 372 patients of Caucasian origin with chronic heart failure (New York Heart Association [NYHA] functional classes II–IV, ejection fraction (EF) <40%) and 407 healthy controls. They were genotyped for the leptin (LEP) –2548 G/A, leptin receptor (LEPR) Gln223Arg, and proopiomelanocortin (POMC) RsaI (5′-untranslated region) and C1032G variants (intron 1) using PCR-based methodology. No case–control differences in genotype as well as allele frequencies were observed between CHF patients and controls. We constructed POMC RsaI/C1032G haplotypes, having found no significant association with body mass index (BMI), left ventricle ejection fraction (LVEF), left ventricle hypertrophy (LVH) and diabetes mellitus (DM). Multivariate regression analyses revealed an approximately 2-fold risk for NYHA class IV associated with the LEPR Gln223Arg ($P = 0.0000001$, odds ratio [OR] = 2.10, 95% confidence interval [CI] = 1.56–2.84); it also displayed an independent prediction role for LVEF in heart failure cases of all etiologies ($P = 0.002$, OR = 4.05, 95% CI = 1.36–10.06). In subanalyses according to CHF etiology the LEPR Gln223Arg showed an independent prediction role for NYHA IV in IHD patients ($P = 0.0001$, OR = 2.50, 95% CI = 1.69–3.82) and both for NYHA IV($P = 0.007$, OR = 2.04, 95% CI = 1.20–3.84) and LVEF ($P = 0.004$, OR = 11.87, 95% CI = 2.08–55.6) in DCMP patients. The role of the polymorphic variants in the genes encoding for adipokines as potential CHF susceptibility genes is unclear. Based on our findings, the LEPR Gln223Arg polymorphism could be considered a disease susceptibility modulating factor both in ischemic heart disease or dilated cardiomyopathy patients.

Key words Adipokines · Polymorphisms · Leptin · Leptin receptor · Proopiomelanocortin · Chronic heart failure

J.A. Bienertová-Vašků (✉) · P. Bienert · A. Vašků
Institute of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic
Tel. +420-739300485; Fax +420-549494340
e-mail: jbienert@med.muni.cz

L. Špinarová
First Department of Internal Medicine/Cardioangiology, Faculty of Medicine and St Ann's Faculty Hospital, Brno, Czech Republic

Introduction

Chronic heart failure is characterized by progressive catabolic syndrome in the advanced stages of the disease. Recent research has emphasized the potential importance of peripheral changes in the development and progression of heart failure, where adipokines play a pivotal role.[1]

Leptin and leptin receptor (LEP, LEPR) and heart failure

Generally, physiological actions of leptin have been demonstrated to have important implications in the cardiovascular system including angiogenesis, thrombosis, neointimal proliferation, and cardiac hypertrophy. The adipocyte-derived hormone leptin has recently been reported to have predictive value for future cardiovascular events,[2–5] suggesting that hyperleptinemia is independently related to poorer cardiovascular outcome, which could be explained by the enhanced sympathetic activation in response to central stimulation of hypothalamic leptin receptors[6] being initially a supporting mechanism counteracting impaired cardiac function through positive inotropic effect in the failing myocardium mediated by sympathetic nervous outflow.[7] Hyperleptinemia was also reported to be associated with increased rate of in-stent restenosis following percutaneous coronary intervention (PCI).[8] Both ischemia and reperfusion induce leptin and leptin receptor gene expression.[9] Mammes et al.[10] were the first to report that the –2548 G/A variant in the

promoter of the leptin gene was associated with body mass index (BMI) reduction in overweight women and thus might express a functional effect in population. In the study by Guízar-Mendoza et al.,[11] the Gln allele carriers (Q/Q and Q/R) had also significantly higher heart sympathetic activity, body fat percentage, and leptin levels.

Proopiomelanocortin (POMC) and heart failure

Recently, evidence has been provided that in normal animals, leptin enhances cardiovascular dynamics but paradoxically decreases in obese animals.[12] There is strong evidence that much of leptin's action is mediated by its stimulation of POMC expression and the subsequent increase in POMC peptide products.[13] Proopiomelanocortin and POMC-derived peptides are important regulators in a number of central nervous pathways eliciting downstream events including food intake regulation as well as autonomic responses, acting both centrally to increase sympathetic nerve activity and cardiovascular tone[14] and decreasing cardiovascular tone by primary μ- or κ-receptor activation.[15]

The POMC gene is expressed in response to leptin signaling by neurons of the hypothalamic arcuate nucleus. The POMC propeptide is intracellularly processed by prohormone convertase 2, leading to the production of α-, β- and γ-melanocytes-stimulating hormones.

Obesity is generally recognized as one of the crucial risk factors in chronic heart failure development. Thus, it might be hypothesized that variability in quantitative trait loci determining leptin levels could play a role in chronic heart failure pathogenesis. Previously, the major quantitative trait locus (QTL) determining leptin levels was linked to the POMC region in several studies.[16,17]

Moreover, the circulating alpha-melanocyte stimulating hormone (α-MSH) was reported to be increased in CHF patients.[18] As this neuropeptide with potent anti-inflammatory properties inhibits tissue injury in a wide array of inflammation models, the potential treatment options of alpha-MSH in CHF patients are considered. Hence, it might be hypothesized that the genes encoding for proteins controlling alpha-MSH production such as POMC may play a role in an individual susceptibility to aberrant myocardial remodeling resulting in chronic heart failure development.

The RsaI polymorphism the in 5′-untranslated region (UTR) of POMC gene has been previously reported to be associated with the serum leptin and body fat in normal female population.[19] In addition, the 1032 C/G polymorphism within the first intron of the POMC gene has been reported to be associated with the waist-to-hip ratio.[20]

The possible contribution of leptin, leptin receptor, and proopiomelanocortin gene variability to the regulation of catabolic/anabolic processes in chronic heart failure still remains to be clarified. Cachexia has been in the chronic heart failure recognized as an negative prognostic factor; however, the underlying mechanisms of its onset and progression remain still elusive. In the framework of this study, we hypothesized whether the four previously mentioned SNPs with significant phenotypic effects associated to the body weight (LEP –2548 G/A, LEPR Gln223Arg, POMC RsaI, and POMC C1032G) could contribute to disease onset and modulated the disease progression of CHF. We thus conducted a preliminary survey of these four single nucleotide polymorphisms (SNPs) in the total of three genes in a population of 372 chronic heart failure patients and 407 healthy control subjects and investigated possible associations of this genetic variability in LEP, LEPR, and POMC genes with chronic heart failure pathogenesis.

Patients and methods

Subjects

The study cohort consisted of 372 consecutive patients (median of age 56 years, age range 21–91 years, 107 females/265 males) inclusive of chronic heart failure diagnosis (functional class New York Heart Association (NYHA) II–IV, ejection fraction median 25%, cardiothoracic index more than 50%) both of ischemic heart disease (IHD) or dilated cardiomyopathy (DCMP) origin. To estimate the population frequencies of the examined genotypes and alleles, the control cohort was recruited consisting of 407 healthy individuals of similar age and sex distribution, without clinical signs of cardiovascular diseases and without family history of early cardiovascular disease (median of age 51 years, age range 15.8–86 years, 218 females/189 males). The prevalence of diabetes mellitus and hyperlipidemia in the controls was approximately 6% and 8%, respectively, which corresponds well with the general prevalence in the Czech population. In all subjects, a complete medical history with respect to conventional cardiovascular risk factors was obtained.

All patients had chronic heart failure of at least of 3 months duration and were stable on unchanged medication for at least 1 month. All patients originated from the Czech Caucasian population and were recruited at the 1st Cardioangiological and 2nd Internal Departments, St. Anne's Hospital, Brno. This study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno (no. 64/93, 1993) and was performed in adherence to the Declaration of Helsinki Guidelines. Participants gave their written informed consent before they entered the study.

Clinical examination

Clinical examination, basic and special laboratory (including renal functions), were done early in the morning followed by echocardiographic examination (SONOS 5500, Hewlett-Packard; Andover, MA, USA). The echocardiographic measurements were performed using a 2.5-MHz phased array transducer; volumes were measured from apical four-chamber view and ejection fraction was calculated using the single plain Chapman method. Electro-

cardiography and standard X-ray (with evaluation of cardiothoracic index and pulmonary congestion by the method of Meszaros) were obtained. Hyperlipidemia was defined as plasma total cholesterol level of >200 mg/dl, plasma triglyceride level of >150 mg/dl, or current use of antilipidemics with the previously established diagnosis of hyperlipidemia.

The patients were treated with heart failure therapies including cardiac transplantation. After venous blood sample (5–10 ml) collection from each subject, white cell fraction was used to extract DNA according to standard procedure using proteinase K.

Single nucleotide polymorphism genotyping

The common noncoding polymorphism LEP –2548 G/A within the promoter of leptin gene (db ID rs7799039) was investigated as previously described.[10] Each 12-µl reaction contained 10 ng genomic DNA, 1.5 µl 10 × PCR Buffer–$MgCl_2$, 1.8 µl $MgCl_2$, 200 µM dNTP, 2 pmol each primer, and 0.4 U of Taq DNA polymerase (Fermentas; UAB, Lithuania). The reactions were performed using an XP Cycler (BIOER; Technology, Germany). Polymerase chain reaction (PCR) amplification conditions were as follows: 95°C for 5 min, 94°C for 1 min, 52°C for 1 min, 72°C for 1 min for 35 cycles, and 72°C for 5 min.

The LEPR Gln223Arg polymorphism within the exon 4 of leptin receptor gene (dbSNP ID rs1137101) was investigated as previously described.[21] Each 12-µl reaction contained 10 ng genomic DNA, 1.5 µl 10 × PCR Buffer–$MgCl_2$, 1.5 µl $MgCl_2$, 200 µM dNTP, 2 pmol each primer, and 0.4 U of Taq DNA polymerase (Fermentas). The reactions were performed using XP Cycler (BIOER). PCR amplification conditions were as following: 95°C for 3 min, 95°C for 30 s, 53.5°C for 30 s, 72°C for 30 s for 30 cycles, and 72°C for 5 min.

Furthermore, the subjects were genotyped for the following two SNPs of the POMC gene as previously described by Baker et al.[20]: the *Rsa*I polymorphism 1789 bp 5′ to the beginning of the first exon (dbSNP rs3754860) and the *Ava*I polymorphism within the first intron (C1032G, dbSNP 1009388). Each 12 µl reaction contained 10 ng genomic DNA, 1.5 µl 10 × PCR Buffer–$MgCl_2$,1.5 µl $MgCl_2$, 200 µM dNTP, 2 pmol each primer, and 0.4 U of Taq DNA polymerase (Fermentas). The reactions were performed using XP Cycler (BIOER). PCR amplification conditions were as following: RsaI polymorphism: 95°C for 5 min, 95°C for 30 s, 62°C for 45 s, 72°C for 30 s for 35 cycles, and 72°C for 10 min; AvaI: 95°C for 6 min, 95°C for 30 s, 59°C for 15 s, 72°C for 20 s for 30 cycles, and 72°C for 10 min.

Quality control measures included blinded analyses, replicating 20% of samples, and negative controls (water blanks) in every set of PCR reactions. The overall sample success rate was 96.3%, the sample success rate for LEP –2548 G/A, LEPRGln223Arg, POMC RsaI was approximately 98% (97.9%, 98.1% and 98.9%); the rate for POMC AvaI was approximately 92%.

Statistics

The observed number of each genotype was compared with that expected for a population in Hardy-Weinberg equilibrium using χ^2 test. Fisher's exact test with the Tukey–Kramer method of adjustment for multiple comparison were used for comparison of categorical variables. Differences between continuous variables were evaluated by analysis of variance (ANOVA) with corresponding post hoc test for more than two groups; multiple regression analysis was applied in all cases of associations between the genotypes and clinical parameters, significant in the univariate analysis. The data analysis was performed using the Statistica v. 7.0 (Statsoft; Tulsa, OK, USA) program package. To estimate the risk of chronic heart failure associated with the proopiomelanocortin SNPs, odds ratios were calculated using multiple regression analysis; for each odds ratio, *P* values and 95% confidence intervals were estimated. The data analysis was performed using the Statistica v. 7.0 (Statsoft) program package. Furthermore, we tested the association of statistically inferred haplotypes with continuous traits as described previously,[22,23] the probabilities of haplotype pairs being estimated by PHASE 2.0 software. The power calculations were performed using Quanto software.[24]

Results

A total of 779 unrelated Caucasian subjects (372 cases and 407 controls) were enrolled in the study. The basic demo-

Table 1. Baseline characteristics of the CHF cohorts

Variable	IHD patients	DCMP patients
LVEF (%)	26.4 ± 1	31.5 ± 1
NYHA (No. in class II/III/IV)	45/98/95	15/75/44
BMI (kg/m^2)	30.14 (16.78–38.34)	28.98 (16.14–33.14)
DM I (%)	8.8	7.4
DM II (%)	21.0	16.4
HLPP (%)	38.0	13.4
Renin (µU/ml)	4.12 (0.01–229)	8.50 (0.11–46.19)
Aldosterone (nmol/l)	0.21 (0.0–5.97)	0.40 (0.07–11.92)

Values are given as median (min–max range)
CHF, chronic heart failure; IHD, ischemic heart disease; DCMP, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association classification; DM I, type I diabetes; DM II, type II diabetes; HLPP, hyperlipoproteinemia

Table 2. Genotype distribution of examined polymorphisms in cases and controls

Polymorphism	Total *N*	Cases *n*	Controls *n*	*P* value*
LEP –2548 G/A				0.226
Co-dominant model				
AA	150	65	85	
AG	378	193	185	
GG	235	109	126	
Recessive model				0.128
GG/GA (*n*) (%)	613	302	311	
AA (*n*) (%)	150	65	85	
LEPR Gln223Arg				0.981
Co-dominant model				
QQ	222	114	108	
QR	334	169	165	
RR	158	81	77	
Recessive model				0.503
QQ/QR (*n*) (%)	556	283	273	
RR (*n*) (%)	158	81	77	
POMC Rsa I				0.680
Co-dominant model				
+ +	339	170	169	
+ –	344	161	183	
– –	84	41	43	
Recessive model				0.570
+ +/+ – (*n*) (%)	683	331	352	
– – (*n*) (%)	84	41	43	
POMC C1032G				0.537
Co-dominant model				
CC	467	228	239	
CG	265	130	135	
GG	31	12	19	
Recessive model				0.176
CC/CG (*n*) (%)	732	358	374	
GG (*n*) (%)	31	12	19	

LEP, leptin; LEPR, leptin receptor; POMC, proopiomelanocortin
* Hardy–Weinberg equilibrium tests (χ^2) in case and control groups, respectively

Table 3. Haplotype frequencies of POMC RsaI/C1032G haplotypes in CHF cases according to etiology and controls

	DCMP				IHD				Controls			
Haplotype RsaI/AvaI	+/C	+/G	–/C	–/G	+/C	+/G	–/C	–/G	+/C	+/G	–/C	–/G
Frequency	0.500	0.197	0.301	0.002	0.456	0.208	0.329	0.005	0.484	0.196	0.319	0.001

DCMP, dilated cardiomyopathy; IHD, ischemic heart disease; POMC, proopiomelanocortin

graphic, clinical and laboratory parameters of the cases are listed in Table 1.

Associations between examined single nucleotide polymorphisms in leptin, leptin receptor and proopiomelanocortin gene, and chronic heart failure

None of the investigated SNPs expressed deviation from Hardy–Weinberg equilibrium (tested by conventional χ^2 test). After assuming a codominant model, the genotype distributions of examined polymorphisms were not different between cases and controls (P = not significant [NS]). Moreover, when assuming a recessive model of inheritance, no significant differences between CHF cases and control were observed (Table 2). As some sex-linked differences in allele or genotypes of examined polymorphisms could have been expected, the analysis by sex was performed; however, no significant sex-dependent associations of genotype distributions or allele frequencies were observed (P = NS). Furthermore, we estimated the frequency of the POMC RsaI/C1032G haplotypes among chronic heart failure cases (subdivided into ischemic heart disease CHF patients and dilated cardiomyopathy patients) and the controls and did not observe any significant differences between cohorts studied (Table 3).

Effect of examined SNPs on ejection fraction and NYHA class

In the next step, we tested whether these SNPs had any effect on ejection fraction or CHF risk. No association of LEP –2548 G/A and POMC C1032G with ejection fraction of NYHA class was observed.

In regression modeling, the LEPR Gln223Arg showed an independent prediction role on chronic heart failure, with age being the strong predictor of it ($P < 0.001$), thus suggesting that a remarkable proportion of the gene effect on CHF risk is dependent upon aging. Therefore, we con-

Table 4. Multivariate analysis of the association of the clinical data and the LEPR Gln223Arg polymorphism

Variable	Heart failure, all etiologies		IHD heart failure		DCMP heart failure	
	Hazard ratio (95%CI)	*P*	Hazard ratio (95%CI)	*P*	Hazard ratio (95%CI)	*P*
Age	3.31 (0.61–17.98)	0.16	0.31 (0.01–8.77)	0.47	1.34 (0.05–13.02)	0.72
Age ≤56 years	1.29 (1.09–1.55)	<0.001	1.33 (1.02–3.72)	0.23	1.21 (0.87–1.77)	0.66
Age >56 years	1.10 (0.50–4.23)	0.21	1.42 (1.02–4.01)	0.47	1.54 (0.88–3.87)	0.88
Male sex	3.23 (1.87–5.58)	<0.0001	3.15 (1.68–5.91)	<0.001	3.5 (1.13–10.81)	0.02
NYHA IV	2.10 (1.56–2.84)	<0.0000001	2.50 (1.69–3.82)	<0.0001	2.04 (1.20–3.48)	0.007
LVEF	4.05 (1.63–10.06)	0.002	2.64 (0.81–8.22)	0.10	11.87 (2.08–55.6)	0.004

NYHA, New York Heart Association functional class. LVEF, left ventricular ejection fraction; CI, confidence interval; IHD, ischemic heart disease; DCMP, dilated cardiomyopathy

secutively tested the possible relation between age of CHF onset and LEPR Gln223Arg by subdividing the study group according to individual age (i.e., below or above 56 years, the median value of the entire CHF cohort). As supposed, these two subgroups differed significantly in LEP Gln223Arg allele frequency, the R allele being more frequent in CHF below 56 years of age ($P = 0.0002$, odds ratio [OR] = 1.29, 95% confidence interval [CI] = 1.089–1.549). Moreover, the strongest predictor of chronic heart progression in relation to LEPR Gln223Arg polymorphism was sex, with more than 3-fold increased risk of accelerated progression associated to male sex (Table 4).

In the next step, we analyzed the relationship between LEPRGln223Arg polymorphism and ejection fraction and NYHA class in all heart failure cases. Multivariate regression analyses revealed an approximately 2-fold risk for NYHA class IV associated to the examined polymorphism ($P = 0.0000001$, OR = 2.10, 95% CI = 1.56–2.84). LEPR Gln223Arg also displayed an independent prediction role for LVEF in all heart failure cases ($P = 0.002$, OR = 4.05, 95% CI = 1.36–10.06) (Table 4).

The possible diversity in CHF etiology and its relation to LEPR Gln223Arg was further tested by subdividing the study group according to CHF etiology (ischemic heart disease vs dilated cardiomyopathy), whereas the LEPR Gln223Arg showed an independent prediction role for NYHA IV in IHD patients ($P = 0.0001$, OR = 2.50, 95% CI = 1.69–3.82) and for NYHA IV ($P = 0.007$, OR = 2.04, 95% CI = 1.20–3.84) and LVEF ($P = 0.004$, OR = 11.87, 95% CI = 2.08–55.6).

In a multivariate analysis, the POMC RsaI polymorphism served as an independent predictor for hyperlipidemia in CHF patients ($P = 0.0004$), with the–allele being more frequent in CHF patients with hyperlipidemia (70.1% vs 65.6%, $P = 0.05$). Power calculations assuming 779 individuals with complete genotypic and phenotypic data suggest that a SNP with minor allele frequency (MAF) of 0.20 provides approximately 80% power at $\alpha = 0.05$ to observe a 0.22 change in standard deviation. This analysis indicates that the study has the power to detect clinically meaningful differences in quantitative measures of heart function.

The post hoc power calculations using Quanto and Gauderman[24] showed that our study (372 cases and 404 controls) had 98%; 98%; 99.6%, and 99.8% statistical power to detect an odds ratio of 2.0 for polymorphisms with an allele frequency of 0.44 (approximate for LEP –2548 G/A), 0.46 (approximate for LEPR Gln223Arg), 0.33 (approximate for POMC RsaI), and 0.21 (approximate for POMC AvaI) under the dominant model assuming the disease prevalence of 1%. When the subanalysis was performed by subdividing the cases cohort to ischemic heart disease patients ($n = 238$) and the patients with dilated cardiomyopathy ($n = 134$), the statistical power was 78% (LEP-2548 G/A), 76% (LEPR Gln 223 Arg), 86% (POMC RsaI), and 89% (POMC AvaI).

Discussion

Generally, there are numerous accepted adverse clinical prognostic markers for human heart failure, including male sex, African American race, age, disease severity, ischemic etiology, hypertension, and low body weight.[25–27] However, an increasing number of single nucleotide polymorphisms are being accepted as underlying causes for numerous cardiovascular disorders. Recently, it has been reported by our research group that the LEP –2548 G/A polymorphism is associated with multiple vessel disease,[28] the AG heterozygote carrying almost four-fold risk for multiple vessel disease against both homozygotes.

In this case–control study carried out in a Czech Caucasian population we provide evidence for association between the Gln223Arg variant in leptin receptor gene and chronic heart failure in the Czech Caucasian population. Based on our results, the LEPR Gln223Arg associates with age of onset in chronic heart failure in the studied population, independently of CHF etiology (ischemic heart disease vs dilated cardiomyopathy) or BMI of the patients. The significant trend toward earlier CHF onset was observed in R allele presence when compared to QQ+QR carriers, thus suggesting that LEPR Gln223Arg does modulate covariate factors associated with CHF disease. Moreover, the LEPR Gln223Arg polymorphism was also an independent predictor for LVEF in chronic heart failure patients, whereas this effect was significant in dilated cardiomyopathy patients, while the lower LVEF was associated with the Q allele ($P = 0.004$, OR = 11.87, 95% CI = 2.08–55.6).

The functional consequences of LEPR Gln223Arg polymorphism for various phenotypic traits are not entirely

clear. Several studies also reported conflicting information on obesity predisposition in individuals with specific leptin receptor gene polymorphisms.[29–31] Recently, van der Vleuten et al.[32] carried out a study on 644 individuals from 37 families and reported the Gln223Arg polymorphism within the leptin receptor gene to be associated with familial combined hyperlipidemia (FCH); the carriers of one or two R alleles had an increased risk of CHF, compared to subjects homozygous for the Q allele (OR = 1.6; 95% CI 1.0–2.4). Moreover, Guízar-Mendoza et al.[11] reported the higher prevalence of Q allele among subjects with higher insulin levels (0.72 vs 0.57; $P = 0.04$ for adolescents with insulin levels higher and lower than 100 pmol/l, respectively).

A major question concerns the possible mechanism by which LEPR Gln223Arg influences CHF onset and its progression. The LEPR Gln223Arg leads to an amino acid change, consecutively leading to a change from neutral to positive charge of the molecule, in the extracellular domain of the receptor that represents a typical leptin-binding site and it was suggested that a change of charge could significantly affect the functionality of the receptor.[33] Despite these obvious functional consequences, the reports on Gln223Arg in body weight regulation and its related disorders remain highly controversial.[34–37] It has been reported[7] that there is a relationship between increased leptin levels and the progressive functional impairment in advanced CHF, which is well in accordance with the finding that hyperleptinemia represents a negative prognostic factor in CHF patients. The LEPR Gln223Arg polymorphism leading to a charge change might considerably impair the ability of leptin to bind to its receptor and thus provide a phenotype of leptin resistance with inadequate leptin signaling, however this is in contrast with the study by Schulze and Kratzsch[7] that suggest that the leptin signaling is adequate in CHF patients.

The possible limitation of the present study includes a significant lack of data on phenotypic effects of examined polymorphisms, which makes further population-based studies on CHF patients, preferably using the nested design, necessary to determine the exact genotype–phenotype correlation, putting special emphasis on leptin, leptin receptor, and glucocorticoids plasma levels. Power to examine possible interactions of LEPR Gln223 genotype and clinical data of CHF patients was somewhat limited, however, by the lower proportion of subjects with dilated cardiomyopathy along with lower proportion of females in the study, leading to wide confidence intervals in some analyses. Even although for some analyses the CHF sample in the study was relatively large, we were still limited in our power to examine interactions between genotype and CHF phenotypes; the sample size needed to detect the sex-dependent interactions was typically at least five times larger than that needed to detect the main effect of a single variable. We had even less power to analyze these relations among females or males separately.

However, it should be also mentioned that the present study was carried out on a highly static population from a wide region of Moravia, part of the Czech Republic, settled by Slavonic population that can be assumed to be homogeneous. Therefore, we assume that the associations discussed cannot be easily attributed to selection bias; the RR homozygotes of LEPR Gln223Arg are likely to be truly at higher risk of earlier onset of CHF in the examined Czech population. To conclude, further comparative studies on different populations possibly employing the nested case–control approach have to be carried out to elucidate the underlying genetic component of CHF cachexia, as various phenotypic variations in relation to hyperleptinemia have been reported worldwide so far.[2–4]

Despite the limitations mentioned above, this study is the first to demonstrate a direct effect of a genetic variability in leptin receptor gene on disease progression in CHF both of ischemic and dilated cardiomyopathy origin. Nowadays, molecular evidence is being accumulated for different genetic traits predisposing for CHF. Hereafter, confirmed associations of CHF with some SNPs might help identify patients who are either at greater risk of developing CHF or whose CHF is likely to progress more rapidly, and such patients might benefit from a targeted aggressive therapeutic approach.

Acknowledgments The study was supported by the project DANONE 2007 from the DANONE Institute. We are grateful to Dana Polaskova for technical assistance.

References

1. Clark AL, Poole-Wilson PA, Coats AJ (1996) Exercise limitation in chronic heart failure: The central role for periphery. J Am Coll Cardiol 28:1092–1102
2. Soderberg S, Stegmayr B, Ahlbeck-Glader C, Slunga-Birgander L, Ahren B, Olsson T (2003) High leptin levels are associated with stroke. Cerebrovasc Dis 15:63–69
3. Soderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, Olsson T (1999) Leptin is associated with increased risk of myocardial infarction. J Intern Med 246:409–418
4. Soderberg S, Ahren B, Stegmayr B, Johnson O, Wiklund PG, Weinehall L, Hallmans G, Olsson T (1999) Leptin is a risk marker for first-ever hemorrhagic stroke in a population based cohort. Stroke 30:328–337
5. Soderberg S, Olsson T, Eliasson M, Johnson O, Ahren B (1999) Plasma leptin levels are associated with abnormal fibrinolysis in men and postmenopausal women. J Intern Med 245:533–543
6. Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI (1997) Receptor-mediated regional sympathetic nerve activation by leptin. J Clin Invest 100:270–278
7. Schulze PC, Kratzsch J (2005) Leptin as a new diagnostic tool in chronic heart failure. Clin Chim Acta 362(1–2):1–11
8. Piatti P, Di Mario C, Monti LD, Fragasso G, Sgura F, Caumo A, Setola E, Lucotti P, Galluccio E, Ronchi C, Origgi A, Zavaroni I, Margonato A, Colombo A (2003) Association of insulin resistance, hyperleptinemia, and impaired nitric oxide release with in-stent restenosis in patients undergoing coronary stenting. Circulation 108:2074–2081
9. Matsui H, Motooka M, Koike H, Inoue M, Iwasaki T, Suzuki T, Kurabayashi M, Yokoyama T (2007) Ischemia/reperfusion in rat heart induces leptin and leptin receptor gene expression. Life Sci 80 (7):672–680
10. Mammes O, Betoulle D, Aubert R, Herbeth B, Siest G, Fumeron F (2000) Association of the G-2548A polymorphism in the 5′ region of the LEP gene with overweight. Ann Hum Gen 64 (Pt 5): 391–394
11. Guízar-Mendoza JM, Amador-Licona N, Flores-Martínez SE, López-Cardona MG, Ahuatzin-Trémary R, Sánchez-Corona J (2005) Association analysis of the Gln223Arg polymorphism in the

human leptin receptor gene, and traits related to obesity in Mexican adolescents. J Hum Hypertens 19(5):341–346
12. Lu H, Duanmu Z, Houck C, Jen KL, Buison A, Dunbar JC (1998) Obesity due to high fat diet decreases the sympathetic nervous and cardiovascular responses to intracerebroventricular leptin in rats. Brain Res Bull 47(4):331–335
13. Dunbar JC, Lu H (2000) Proopiomelanocortin (POMC) products in the central regulation of sympathetic and cardiovascular dynamics: studies on melanocortin and opioid interactions. Peptides 21 (2):211–217
14. Saunders WS, Thornhill JA (1986) Pressor, tachycardic and behavioral excitatory responses in conscious rats following ICV administration of ACTH and CRF are blocked by naloxone pretreatment. Peptides 7(4):597–601
15. Bachelard H, Pitre M (1995) Regional haemodynamic effects of mu-, delta-, and kappa-opioid agonists microinjected into the hypothalamic paraventricular nuclei of conscious, unrestrained rats. Br J Pharmacol 115(4):613–621
16. Hager J, Dina C, Francke S, Dubois S, Houari M, Vatin V, Vaillant E, Lorentz N, Basdevant A, Clement K, Guy-Grand B, Froguel P (1998) A genome-wide scan for human obesity genes reveals a major susceptibility locus on chromosome 10. Nat Genet 20(3): 304–308
17. Rotimi CN, Comuzzie AG, Lowe WL, Luke A, Blangero J, Cooper RS (1999) The quantitative trait locus on chromosome 2 for serum leptin levels is confirmed in African-Americans. Diabetes 48(3): 643–644
18. Yamaoka-Tojo M, Tojo T, Shioi T, Masuda T, Inomata T, Izumi T (2006) Central neurotranspeptide, alpha-melanocyte-stimulating hormone (alpha-MSH) is upregulated in patients with congestive heart failure. Intern Med 45(7):429–434
19. Chen Y, Snieder H, Wang X, Kaviya B, McCaffrey C, Spector TD, Carter ND, O'Dell SD (2005)Proopiomelanocortin gene variants are associated with serum leptin and body fat in a normal female population. Eur J Hum Genet 13(6):772–780
20. Baker M, Gaukrodger N, Mayosi BM, Imrie H, Farrall M, Watkins H, Connell JM, Avery PJ, Keavney B (2005) Association between common polymorphisms of the proopiomelanocortin gene and body fat distribution: a family study. Diabetes 54(8):2492–2496
21. Echwald SM, Sorensen TD, Sorensen TI, Tybjaerg-Hansen A, Andersen T, Chung WK, Leibel RL, Pedersen O (1997) Amino acid variants in the human leptin receptor: lack of association to juvenile onset obesity. Biochem Biophys Res Commun 233(1): 248–252
22. Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978–989
23. Stephens M, Donnelly P (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 73:1162–1169
24. Quanto: Gauderman WJ (2002) Sample size requirements for association studies of gene-gene interaction. Am J Epidemiol 155: 478–484
25. Cowburn PJ, Cleland JG, Coats AJ, Komajda M. (1998) Risk stratification in chronic heart failure. Eur Heart J 19:696–710
26. Pulignano G, Del Sindaco D, Tavazzi L, Lucci D, Gorini M, Leggio F, Porcu M, Scherillo M, Opasich C, Di Lenarda A, Senni M, Maggioni AP (2002) Clinical features and outcomes of elderly outpatients with heart failure followed up in hospital cardiology units: data from a large nationwide cardiology database (IN-CHF Registry). Am Heart J 143:45–55
27. Kalantar-Zadeh K, Block G, Horwich T, Fonarow GC (2004) Reverse epidemiology of conventional cardiovascular risk factors in patients with chronic heart failure. J Am Coll Cardiol 43: 1439–1444
28. Bienertová-Vasků JA, Hlinomaz O, Vasků A (2007) Are common leptin promoter polymorphisms associated with restenosis after coronary stenting? Heart Vessels 22(5)310–315
29. Matsuoka N, Ogawa Y, Hosoda K, Matsuda J, Masuzaki H, Miyawaki T, Azuma N, Natsui K, Nishimura H, Yoshimasa Y, Nishi S, Thompson DB, Nakao K (1997) Human leptin receptor gene in obese Japanese subjects: evidence against either obesity-causing mutations or association of sequence variants with obesity. Diabetologia 40:1204–1210
30. van Rossum CT, Hoebee B, Seidell JC, Bouchard C, van Baak MA, de Groot CP, Chagnon M, de Graaf C, Saris WH (2002). Genetic factors as predictors of weight gain in young adult Dutch men and women. Int J Obes Relat Metab Disord 26(4):517–528
31. Chagnon YC, Wilmore JH, Borecki IB, Gagnon J, Perusse L, Chagnon M, Collier GR, Leon AS, Skinner JS, Rao DC, Bouchard C (2000) Associations between the leptin receptor gene and adiposity in middle-aged Caucasian males from the HERITAGE family study. J Clin Endocrinol Metab 85:29–34
32. van der Vleuten GM, Kluijtmans LA, Hijmans A, Blom HJ, Stalenhoef AF, de Graaf J (2006) The Gln223Arg polymorphism in the leptin receptor is associated with familial combined hyperlipidemia. Int J Obes 30(6):892–898
33. White DW, Wang DW, Chua SC Jr, Morgenstern JP, Leibel RL, Baumann H, Tartaglia LA (1997) Constitutive and impaired signalling of leptin receptors containing the Gln–Pro extracellular domain fatty. Proc Natl Acad Sci USA 94:10657–10662
34. Yiannakouris N, Yannakoulia M, Melistas L, Chan JL, Klimis-Zacas D, Mantzoros CS (2001) The Q223R polymorphism of the leptin receptor gene is significantly associated with obesity and predicts a small percentage of body weight and body composition variability. J Clin Endocrinol Metab 86:4434–4439
35. Mattevi VS, Zembrzuski VM, Hutz MH (2002) Association analysis of genes involved in the leptin-signaling pathway with obesity in Brazil. Int J Obes Relat Metab Disord 26(9):1179–1185
36. van Rossum CT, Hoebee B, van Baak MA, Mars M, Saris WH, Seidell JC (2003) Genetic variation in the leptin receptor gene, leptin, and weight gain in young Dutch adults. Obes Res 11(3): 377–386
37. Mergen H, Karaaslan C, Mergen M, Deniz Ozsoy E, Ozata M (2007) LEPR, ADBR3, IRS-1 and 5-HTT genes polymorphisms do not associate with obesity. Endocr J 54(1):89–94

4.3. COMMON POLYMORPHISM +45T/G IN ADIPONECTIN GENE AS POTENTIAL MODULATOR OF IN-STENT RESTENOSIS DEVELOPMENT

Resumé

Je zjevné, že existuje různá individuální vnímavost vůči rozvoji nejrůznějších komplexních onemocnění typu ischemické choroby srdeční i jejich komplikací či komplikací jejich léčby, např. restenózy ve stentu. Tato vnímavost je bezesporu částečně determinovaná geneticky. Nedávno byly publikovány zajímavé závěry naznačující, že nízké hladiny adiponektinu u pacientů při terapeutické koronarografii mají velkou predikční hodnotu pro pozdní rozvoj restenózy ve stentu. To je zjištění s potenciálně závažným terapeutickým dopadem. Daná studie se ovšem zaměřovala pouze na fenotyp onemocnění a neodlišovala, zda jsou nízké hladiny adiponektinu způsobeny konstitutivně nižší expresí adiponektinového proteinu, nebo zda jsou výsledkem samotného patofyziologického procesu. V naší studii jsme zkoumali možnou souvislost mezi polymorfismem +45T/G (rs2241766) v lokusu ADIPOQ s rizikem restenózy ve stentu u české populace pacientů s ischemickou chorobou srdeční podstupujících stentování malých koronárních tepen s použitím čistě kovového stentu. Alelické frekvence i frekvence genotypů byly srovnány s frekvencemi u zdravé české populace podobného věku a to ve stejném zastoupení obou pohlaví. Morfologie lézí byla hodnocena podle ACC/AHA. Ve vícerozměrné regresní analýze sloužil sledovaný polymorfismus jako nezávislý prediktor minimálního průměru lumen okamžitě po PCI – nezávisle na věku, pohlaví, BMI i minimálním průměru lumen byla alela T spojena s přítomností menšího luminálního průměru než alela G (beta = 0,34, p = 0,02).

Již dříve bylo prokázáno, že ač synonymní, polymorfismus +45T/G významně ovlivňuje hladiny adiponektinu v plazmě, což lze vysvětlit tím, že je v úzké vazbě s jiným, funkčním nesynonymním polymorfismem. Bylo přitom popsáno, že nosičství alely T predisponuje

k nízkým hladinám adiponektinu. To je v souladu s našimi pozorováními, že nosiči T alely, ať již v homozygotní nebo v heterozygotní formě, mají závažnější stenózy a častější výskyt restenózy ve stentu.

Naše studie tedy naznačuje, že adiponektin, respektive jeho genetická variabilita, může hrát důležitou úlohu při regulaci místních dějů v ischemické oblasti a může ovlivňovat i rychlost nástupu a případnou závažnost restenózy ve stentu.

Common polymorphism +45T/G in adiponectin gene as potential modulator of in-stent restenosis development

Julie Bienertova-Vasku [a,*], Petr Bienert [a], Ota Hlinomaz [b], Anna Vasku [a]

[a] Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic
[b] First Department of Internal Medicine/Cardioangiology, St. Ann's Faculty Hospital, Brno, Czech Republic

ARTICLE INFO

Article history:
Received 30 November 2009
Accepted 4 December 2009
Available online 31 December 2009

Keywords:
In-stent restenosis
Polymorphism
Adiponectin
Association study

Kitta et al. [1] have recently reported that low adiponectin levels have a predictive value for late in-stent restenosis (ISR) after percutaneous coronary intervention (PCI) with bare-metal stents in native coronary arteries. This is a very interesting finding with potential huge diagnostic and therapeutic implications. However, as the analyses were based exclusively on phenotypic data from the patients, it remains rather difficult to distinguish between constitutively increased adiponectin plasma levels due to specific individual genetic background (levels elevated/reduced already before the cardiac event) predisposing the patient for the primary disease as well as for the worse PCI outcome and post-hoc elevated/decreased plasma levels as a pathophysiological consequence of the primary disease. In principle, resolution between these two conditions might influence the prognostic value of the marker.

Therefore, we investigated in our study possible association of +45T/G (rs2241766) polymorphism at the adiponectin (APM1) locus with risk of ISR in the Czech ischemic heart disease (IHD) patients ($n=88$) undergoing bare-metal stenting into small coronary arteries (<3 mm). The selection of the SNP was based on the previously reported functional consequences that were validated also on the Czech population in our previous studies [2,3]. The study subjects were genotyped using the PCR-based methodology with following restriction analysis and the allele and genotype frequencies were established and compared to those of the healthy Czech individuals of similar age and gender distribution ($n=163$). Lesion morphology was interpreted according to the American College of Cardiology/American Heart Association (ACC/AHA) classification. In-stent restenosis was defined as ≥50% diameter reduction; reoccluded vessels were not excluded from the statistical analysis. In all patients, a follow-up coronarography was performed 6 months after the primary PCI. Extended quantitative coronarography data were collected at the moment of primary intervention and at the 6-month follow-up, no data on adiponectin plasma levels were available. Written informed consent was obtained from all patients before the study, and the study protocol, which was in accordance with the Declaration of Helsinki, was approved by the Ethics committee of the Masaryk University.

In the multivariate modelling, the APM1 +45T/G polymorphism served as independent predictor for minimal lumen diameter examined immediately after PCI ($\beta=0.34$, $p=0.02$, after correction for multiple comparisons), independently on age, gender, BMI and minimal lumen diameter before PCI; the T allele was associated with smaller minimal lumen diameter values than the G allele. The diameter of stenosis before PCI was identical between the TT and TG carriers, diameter of stenosis after PCI was higher in TG carriers (9.1%) compared to TT genotype (7.8%; $p=0.56$) as well as diameter of stenosis at the 6-months follow-up (in TG carriers 55.9% compared to 43.1% in TT carriers, $p=0.52$). The AMP 45T/G has been described to have functional impact on adiponectin plasma levels [4], whereas the carrier status of the T allele is associated with the lower adiponectin plasma levels. Based on our results, we propose the +45T/G polymorphism in the adiponectin gene to be considered a possible genetic marker for diameter of stenosis after PCI. However, we do not confirm the protective role of the G allele described elsewhere. Obviously, the study was underpowered and therefore it would be challenging to perform a genotype–phenotype study on a larger population sample to investigate the underlying consequences of the genetic determination of circulating adiponectin plasma levels in the patients before and after PCI.

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [5].

References

[1] Kitta Y, Takano H, Nakamura T, et al. Low adiponectin levels predict late in-stent restenosis after bare metal stenting in native coronary arteries. Int J Cardiol 2008;131:78–82.
[2] Bienertová-Vašků J, Bienert P, Forejt M, et al. Genotype× nutrient association of common polymorphisms in obesity-related genes with food preferences and time structure of energy intake. Br J Nutr 2009;4:1–8.
[3] Bienertova-Vasku J, Bienert P, Tomandl J, Forejt M, Vasku A. Relation between adiponectin 45T/G polymorphism and dietary composition in the Czech population. Diabetes Res Clin Pract 2009;3:329–31.
[4] Heid IM, Wagner SA, Gohlke H, et al. Genetic architecture of the APM1 gene and its influence on adiponectin plasma levels and parameters of the metabolic syndrome in 1,727 healthy Caucasians. Diabetes 2006;55:375–84.
[5] Coats AJ. Ethical authorship and publishing. Int J Cardiol 2009;131:149–50.

* Corresponding author. Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Kamenice 5 A18, 625 00, Czech Republic. Fax: +420 549 494 340.
E-mail address: jbienert@med.muni.cz (J. Bienertova-Vasku).

4.4. RELATION BETWEEN ADIPONECTIN 45 T/G POLYMORPHISM AND DIETARY COMPOSITION IN THE CZECH POPULATION

Resumé

Preference určitých typů potravy mohou přispět ke vzniku obezity a zdá se, že zastoupení jednotlivých makronutrientů ve volně preferované stravě má alespoň z části dědičnou komponentu. Excesivní příjem tuku ve stravě je úzce spojen s rizikem obezity a několik studií naznačuje, že preference tuků oproti jiným makronutrientům je zčásti dědičná. Tuková tkáň přitom sama produkuje řadu působků s parakrinně-endokrinními efekty, tzv. adipokinů, které mohou samy modulovat nativní potravní preference. Adiponektin představuje jeden z nedávno identifikovaných adipokinů – polymorfismus 45T/G (rs2241766) v exonu 2 genu ADIPOQ má podle literatury souvislost s plazmatickou hladinou adiponektinu i se zvýšeným rizikem obezity, inzulinovou rezistencí a diabetem 2. typu.

V této studii jsme zkoumali možný vliv tohoto polymorfismu na nemodifikované, nativní složení potravy u české štíhlé, obézní a extrémně obézní populace.

Ve vícerozměrném regresním modelování měl polymorfismus ADIPOQ 45T/G významnou predikční roli pro celkovou tukovou masu (ß = - 0,98, p = 0,02) u morbidně obézních jedinců. T alela sloužila také jako nezávislý prediktor vysokého příjmu proteinů ve stravě (beta = 1,87, p = 0,04) a tuků (ß = 2,75, p = 0,04), kdy homozygotní genotyp TT byl spojen s nejvyšším příjmem vlákniny v potravě (beta = 0,45, p = 0,04).

Vzhledem k tomu, že polymorfismus 45T/G v ADIPOQ genu byl již v minulosti asociován s funkčními efekty na úrovni plazmatické hladiny adiponektinu, lze očekávat, že exprese T alely může být spojena s různými odchylkami na úrovni intermediárního metabolismu, které ve výsledku vedou k určitým rizikovým potravním preferencím.

DIABETES RESEARCH AND CLINICAL PRACTICE 84 (2009) 329–331

Contents lists available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

Brief report

Relation between adiponectin 45 T/G polymorphism and dietary composition in the Czech population

Julie Bienertova-Vasku [a,*], *Petr Bienert* [a], *Josef Tomandl* [b], *Martin Forejt* [c], *Anna Vasku* [a]

[a] *Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Kamenice 5, Czech Republic*
[b] *Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno, Kamenice 5, Czech Republic*
[c] *Department of Preventive Medicine, Faculty of Medicine, Masaryk University, Brno, Tomesova 12, Czech Republic*

ARTICLE INFO

Article history:
Received 19 August 2008
Received in revised form
16 February 2009
Accepted 23 February 2009
Published on line 27 March 2009

Keywords:
Adiponectin
Polymorphism
Obesity
Food preferences

ABSTRACT

In this study on 138 Czech Caucasians, the ADIPOQ 45T/G polymorphism was associated with the dietary composition. As the GG homozygotes were associated with the increased intake of carbohydrates, we suggest that a proportion of the prodiabetogenic effect of the polymorphism might be due to its influence on eating behaviour.

The personal food preferences can either induce or retard the development of obesity. The selection of macronutrients in the diet appears to be, at least in part, heritable. Excessive dietary fat intake is strongly tied to obesity, and several studies suggest that a preference for fat and the resulting obesity are also partially genetically determined [1]. Recently, a novel adipocyte-derived hormone, adiponectin (ADIPOQ) was identified [2]; its silent 45 T/G polymorphism (rs2241766) in exon 2 (45 T/G, Gly15Gly) was reported to have functional effects on plasma adiponectin levels and was also associated with an increased risk of obesity, insulin resistance and type 2 diabetes [3]. In this study, we aimed to investigate the possible role of the ADIPOQ 45 T/G on food preferences and general eating patterns of lean, obese and extremely obese Czech Caucasians.

The baseline demographic, anthropometric, dietary and clinical characteristics of the study subjects in relation to gender are listed in Table 1. The anthropometric parameters were measured (BMI, lean body mass, fat mass, waist and hip circumferences, waist-to-hip ratio) and native 7-day food records were collected. After having obtained the informed consent, DNA for analyses was extracted from 5 mL of the patients' saliva collected after 3 h fasting. The genotypes of ADIPOQ 45 T/G were determined using PCR with following restriction analysis as described previously [4]. Plasma adiponectin levels were measured by a commercially available enzyme-linked immunosorbent assays (Raybiotech, Inc., Norcross, GA, USA) with a sensitivity less than 10 pg/mL. Samples were 50,000-fold diluted in singlet to assay range (4.1–1000 pg/mL) with standardized assay diluent. The observed number of each genotype was compared with that expected for a population in Hardy-Weinberg equilibrium using χ^2 test. Fisher's exact test with Tukey–Kramer's method of adjustment

* *Corresponding author*. Tel.: +420 739300485; fax: +420 549494340.
E-mail address: jbienert@med.muni.cz (J. Bienertova-Vasku).
0168-8227/$ – see front matter
doi:10.1016/j.diabres.2009.02.023

Table 1 – Baseline patients' characteristics.

Variable	Obese		Morbidly obese		Controls	
Body composition	Female	Males	Females	Males	Females	Males
N	38	8	38	8	38	8
Age (years)	49.04 ± 11.93	44.87 ± 9.85	50.17 ± 9.34	48.46 ± 15.36	48.28 ± 9.97	52.73 ± 13.38
BMI (kg/m^2)	34.44 ± 2.96	34.30 ± 2.92	45.73 ± 5.57	47.42 ± 4.40	25.85 ± 3.25	26.38 ± 2.83
Body fat (%)	42.00 ± 5.74	39.46 ± 3.61	51.84 ± 4.05	43.13 ± 3.29	34.76 ± 6.77	23.97 ± 5.51
Dietary intake						
Energy (MJ)	7.92 ± 2.46	10.45 ± 2.71	7.38 ± 1.91	10.69 ± 4.93	7.77 ± 1.81	10.26 ± 19.68
Protein (% energy)	14.80 ± 2.05	14.82 ± 2.95	15.47 ± 2.24	15.25 ± 3.67	14.28 ± 2.24	13.6 ± 1.80
Carbohydrates (%) energy)	49.23 ± 4.64	52.86 ± 8.38	49.13 ± 5.91	49.83 ± 2.62	50.28 ± 6.25	51.47 ± 9.22
Fat (% energy)	35.96 ± 4.72	32.3 ± 7.66	35.39 ± 4.75	34.58 ± 5.46	35.45 ± 5.93	34.41 ± 8.93
Adiponectin (μg/mL)	10.79 ± 6.78	12.85 ± 7.16	11.22 ± 6.72	12.99 ± 7.61	9.49 ± 5.71	3.85 ± 1.48

Values are expressed as mean ± SD, morbidly obese (BMI ≥ 40 kg/m^2), obese (30 ≤ BMI < 40 kg/m^2), lean controls (20 < BMI < 30 kg/m^2).

Table 2 – Standardized β coefficients (95% confidential intervals) for associations of total dietary carbohydrate intake with adiponectin plasma level by ADIPOQ 45 T/G polymorphism: multivariate regression analysis in the total study cohort.

Model	ADIPO 45 T/G polymorphism		P for interaction*
	TT	GG + TG	
1	−0.12 (−0.36–0.11)	0.34 (−0.16–0.84)**	0.04
2	−0.11 (−0.23–0.18)	0.25 (−0.06–0.44)	0.07
3	−0.12 (−0.35–0.11)	0.12 (−0.06–0.31)	0.12
4	−0.13** (−0.37–0.09)	0.05 (−0.15–0.26)	0.11

Model 1: total dietary carbohydrate intake adjusted for age, sex, fat and protein intake.
Model 2: Model 1 + fibre intake.
Model 3: Model 1 + waist-to-hip ratio.
Model 4: Model 1 + BMI.
* Interaction between total carbohydrate intake and ADIPOQ 45 T/G polymorphism.
** Significant association of total carbohydrate intake with plasma adiponectin in the model, $P < 0.05$.

for multiple comparisons was employed for comparison of categorical variables; the data analysis was performed using Statistica v. 8.0 (Statsoft Inc., Tulsa, OK, USA).

In the multivariate regression modelling, ADIPOQ 45T/G expressed an independent prediction role for the total fat mass ($\beta = -0.98$, $P = 0.02$) in the morbidly obese individuals along with the thickness of the triceptal skin fold ($\beta = -1.1$, $P = 0.001$) in the lean subjects.

Moreover, the T allele served also as an independent predictor for the highest protein intake ($\beta = -1.87$, $P = 0.03$) and fat intake ($\beta = -2.75$, $P = 0.04$), reported by the study subjects, whereas the TT genotype was associated with the highest absolute fat intake. In addition, we observed a strong association of T allele with high fibre intake in food consumed, significant also after adjustment for age, sex, carbohydrate intake, protein intake and BMI ($\beta = 0.45$, $P = 0.04$). The results of the multivariate analysis of the daily dietary intake of carbohydrates with respect to ADIPOQ 45T/G are presented in Table 2.

So far, no data on possible associations of ADIPOQ 45 T/G with the specific food preferences in either normal-weighed or the obese population are available. However, Cecil et al. [5] have reported recently that a genetic variant that confers a predisposition to obesity does not appear to be involved in the regulation of energy expenditure; the authors suggest that it may have a role in the control of food intake and food choice, which supports our hypothesis that the significant proportion of the "obesitogenic" effect of SNPs is due to food preferences rather than initial metabolic disturbances.

In the French study on DESIR cohort, ADIPOQ 45 T/G was significantly associated with the increased 3-year risk of developing hyperglycaemia and the risk was increased in GG carriers against the TT homozygotes [6]. In our study, we observed a significant association of the G allele with the carbohydrate intake as well as its negative association with the protein and fat intake, independently on the total amount of food consumed, thus implying that the GG carriers, reported to be more susceptible to hyperhlycaemia-type II diabetes, are more prone to increased carbohydrate intake than the T allele carriers. To conclude, the prodiabetogenic effect of the polymorphism seems to be at least partially associated with the increased carbohydrate preferences of the individuals.

Conflict of interest

The authors state that they have no conflict of interest.

Acknowledgements

Study was supported by grant of Ministry of Education of the Czech Republic No. 881/2006 and by project of DANONE/2007

by Danone Institute focused on genetic variability of adipokines in obese individuals.

REFERENCES

[1] D.R. Reed, A.A. Bachmanov, G.K. Beauchamp, M.G. Tordoff, R.A. Price, Heritable variation in food preferences and their contribution to obesity, Behav. Genet. 27 (4) (1997) 373–387.
[2] P.E. Scherer, S. Williams, M. Fogliano, G. Baldini, H.F. Lodish, A novel serum protein similar to C1q, produced exclusively in adipocytes, J. Biol. Chem. 270 (1995) 26746–26749.
[3] L. Bouchard, A. Tremblay, C. Bouchard, L. Pérusse, Contribution of several candidate gene polymorphisms in the determination of adiposity changes: results from the Québec Family Study, Int. J. Obes. (Lond.) 31 (2007) 891–899.
[4] O. Ukkola, E. Ravussin, P. Jacobson, L. Sjöström, C. Bouchard, Mutations in the adiponectin gene in lean and obese subjects from the Swedish obese subjects cohort, Metabolism 52 (2003) 881–884.
[5] J.E. Cecil, R. Tavendale, P. Watt, M.M. Hetherington, C.N. Palmer, An obesity-associated FTO gene variant and increased energy intake in children, N. Engl. J. Med. 359 (2008) 2558–2566.
[6] F. Fumeron, R. Aubert, A. Siddiq, D. Betoulle, F. Péan, S. Hadjadj, et al.,Epidemiologic Data on the Insulin Resistance Syndrome (DESIR) Study Group, Adiponectin gene polymorphisms and adiponectin levels are independently associated with the development of hyperglycemia during a 3-year period: the epidemiologic data on the insulin resistance syndrome prospective study, Diabetes 53 (2004) 1150–1157.

4.5. GENOTYPE VS. NUTRIENT ASSOCIATION OF COMMON POLYMORPHISMS IN OBESITY-RELATEDGENES WITH FOOD PREFERENCES AND TIME STRUCTURE OF ENERGY INTAKE

Resumé

Nativní stravovací preference a stravovací vzorce jsou v podmínkách okolního dostatku potravy klíčové při rozvoji excesivní akumulace tělesného tuku, tj. obezity. Ačkoli genetické pozadí obezity bylo již v mnoha studiích rozsáhle zkoumáno, o genetickém pozadí stravovacích vzorců a preferencí toho dosud víme velmi málo. V této studii se proto zaměřujeme na složení potravy jako na specifický znak související s obezitou; zejména sledujeme, zda definované jednonukleotidové polymorfismy v genech pro leptin (LEP), leptinový receptor (LEPR), adiponektin (ADIPOQ), interleukin-6 (IL6) a proopiomelanokortin (POMC) souvisejí s přirozenými, nemodifikovanými stravovacími preferencemi a antropometrickými znaky spojenými s obezitou u české populace 409 jedinců. SNP byly určeny na základě odpovídajících RFLP metod na bázi PCR. Potravní preference a stravovací vzorce u subjektů byly stanoveny pomocí sedmidenní záznamové metody. U části subjektů byly dále měřeny plazmatické hladiny adiponektinu, leptinu a solubilního leptinového receptoru.

Ve studii jsme pozorovali, že nezávisle na BMI jedinců souvisejí časté polymorfismy v genech LEP a LEPR se specifickými vzorci příjmu potravy, a to hlavně s ohledem na časovou strukturu stravování. Polymorfismus 19 A/G v genu pro leptin se dále projevil jako významný nezávislý prediktor celkového BMI, procenta tělesného tuku a součtu tloušťky kožních řas. Významně ovlivňoval i časovou strukturu denního příjmu energie. Polymorfismus RsaI v genu pro POMC byl asociován s procentem tělesného tuku. Polymorfismus +45T/G v genu ADIPOQ souvisel s tloušťkou kožních řas. S mnoha sledovanými parametry byl asociován i polymorfismus Gln223Arg v genu pro LEPR, který

byl významně asociován s diastolickým krevním tlakem, velikostí porcí jídla i plazmatickou hladinou adiponektinu.

V této studii jsme tedy pozorovali velký počet asociací mezi definovanou variabilitou v genech pro konkrétní adipokiny a antropometrickými i behaviorálními charakteristikami provázejími obezitu, a to zejména s ohledem na nemodifikované nutriční preference.

British Journal of Nutrition (2009), page 1 of 8
doi:10.1017/S0007114509991887

Genotype × nutrient association of common polymorphisms in obesity-related genes with food preferences and time structure of energy intake

Julie Bienertová-Vašků[1]*, Petr Bienert[1], Martin Forejt[2], Josef Tomandl[3], Zuzana Brázdová[2] and Anna Vašků[1]

[1]*Institute of Pathological Physiology, Medical Faculty, Masaryk University, Kamenice 5, Brno 625 00, Czech Republic*
[2]*Department of Preventive Medicine, Faculty of Medicine, Masaryk University, Tomesova 12, Brno 602 00, Czech Republic*
[3]*Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, Brno 625 00, Czech Republic*

(Received 9 March 2009 – Revised 25 June 2009 – Accepted 7 August 2009)

Personal food preferences can either enhance or suppress the development of obesity and the selection and proportion of macronutrients in the diet seem to have a heritable component. In the present study, we therefore focused on dietary composition as a specific trait related to obesity and we determined whether genetic variations in leptin (LEP), LEP receptor (LEPR), adiponectin (ADIPOQ), IL-6 and pro-opiomelanocortin (POMC) underlie specific native food preferences and obesity-related anthropometric parameters. The total of 409 individuals of Czech Caucasian origin were enrolled into the present study and 7 d food records were obtained from the study subjects along with selected anthropometric measurements. In a subset of study subjects, plasma levels of ADIPOQ, LEP and soluble LEPR were measured. Independently of the BMI of the individuals, common variations in LEP and LEPR genes were associated with specific eating patterns, mainly with respect to timing of eating. The LEP + 19A/G polymorphism served as an independent predictor for BMI, percentage of body fat and skinfold thickness and significantly affected the time structure of the daily energy intake. The POMC *Rsa*I polymorphism was associated with percentage of body fat. The ADIPOQ 45 T/G polymorphism was associated with the thickness of the subscapular skinfold. The LEPR Gln223Arg polymorphism was associated with multiple parameters, including diastolic blood pressure, meal sizes during the day and plasma ADIPOQ levels. In a separate analysis, soluble leptin receptor (sObR) plasma levels and LEP:sObR ratio were significantly correlated with systolic blood pressure ($\beta = -0{\cdot}66$, $P = 0{\cdot}002$; $\beta = -1{\cdot}23$, $P = 0{\cdot}02$) and sObR plasma levels also served as an independent predictor for diastolic blood pressure ($\beta = -0{\cdot}50$; $P = 0{\cdot}04$). To conclude, we report common allelic variants associated with specific feeding behaviour and obesity-related anthropometric traits. Moreover, we identified allelic variants that significantly influence the time structure of food intake during the day.

Adipokines: Polymorphisms: Obesity: Nutrition: Feeding behaviour

In Western societies, the prevalence of obesity has been steadily increasing for the last few decades. Obesity (Online Mendelian Inheritance in Man® (OMIM) no. 601665) is generally associated with an increased risk for cardiovascular disorders, diabetes, lipid disorders and some types of cancer. The disease is generally associated with specific lifestyle and dietary habits that interfere with the given genetic background of the individual and several studies have focused recently on the genetic background of these characteristics[1,2]. However, resolution of the genetic factors underlying the susceptibility to certain feeding or lifestyle behaviour is far from being completed.

Previously, various studies reported a heritable component for specific feeding behaviour[3–8]. However, the underlying mechanisms that could explain credibly the inheritance of food preferences are yet to be elucidated. Various adipokines and their genetic variability have been found to be associated with obesity and its related traits; however, the results are often contradictory[9–13].

Recently, it has been reported by de Krom *et al.*[14] that certain common allelic variants in leptin (LEP) and LEP receptor (LEPR) genes are specifically associated with distinctly different eating patterns, namely extreme snacking behaviour or excessive portion size[14]. On the other hand, Schulz *et al.*[15] propose strong evidence for lifestyle-based, i.e. environmental, influences based on their observations of Pima Indians and they suggest that the lifestyle associated with Westernisation plays a major role in the global epidemic of type 2 diabetes, independently of genetic background of an individual[15].

Although it has been reported that genetic variations underlie specific eating patterns[4] and that specific food preferences are considered to be a risk factor for obesity, only a few reports have focused on these genotype × nutrient associations[16], and none has examined thoroughly the relationship between the single nucleotide polymorphisms (SNP) in genes encoding for adipokines and the time structure of the daily energy intake.

Abbreviations: ADIPOQ, adiponectin; LEP, leptin; LEPR, leptin receptor; POMC, pro-opiomelanocortin; SNP, single nucleotide polymorphism; sObR, soluble leptin receptor.

***Corresponding author:** Dr Julie Bienertová-Vašků, fax +420 549494340, email jbienert@med.muni.cz

BJN British Journal of Nutrition

Our previous study on 185 healthy Caucasian volunteers of Middle-European origin did not reveal any significant associations of selected SNP in LEP, LEPR, adiponectin (ADIPOQ), pro-opiomelanocortin (POMC) and ghrelin genes with specific food behaviour. However, in further analyses, distinct trends were observed towards specific nutritional behaviour and therefore the cohort sample was substantially extended for the purposes of the present study.

The aim of the present case–control study on 409 unrelated individuals of Czech (middle-European) Caucasian origin was to analyse the possible associations of eight selected SNP in obesity-related genes with selected lifestyle and dietary characteristics of studied individuals.

Experimental methods

Study subjects

A total of 409 unrelated Czech Caucasian individuals were recruited for the present study in a mass media campaign addressing the population of the south Moravia region of the Czech Republic[17]. The participants were divided into two groups: obese and lean subjects. The inclusion and exclusion criteria were derived from Ma *et al.*[18]. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine at Masaryk University (Brno, Czech Republic). Written informed consent was obtained from all subjects and it was archived.

The first subgroup consisted of 252 obese individuals (BMI $\geq 30\,kg/m^2$; mean BMI 37·4 (SD 6·25) kg/m^2; median age 49·1 years; age range 18·6–73·9 years). The control group consisted of 157 healthy normal-weight control subjects with no history of childhood obesity or eating disorder (mean BMI 25·2 (SD 3·1) kg/m^2; median age 37·1 years; age range 18·1–67·5 years). A set of sixty-four morbidly obese patients was selected from the obese group (BMI $\geq 40\,kg/m^2$; mean BMI 45·5 (SD 5·6) kg/m^2; median age 52·4 years; age range 18·6–73·2 years). These individuals were available for plasma LEP, soluble LEPR (sObR) and ADIPOQ analyses and were precisely matched for age and sex with another sixty-four subjects (control or non-morbidly obese) that underwent the same set of biochemical analyses.

Anthropometric characteristics

All phenotypic measurements were performed by three specialists and included weight, height, BMI, lean body mass, fat mass, body fat, waist and hip circumferences, waist: hip ratio and skinfold thickness. Body composition was assessed by bioelectrical impedance analysis, using the single frequency bioimpedance analyser (BodyStat Ltd, Douglas, Isle of Man, UK), with the subject lying in a supine position. The measurement of height was performed with a calibrated stadiometer and weight (in light indoor clothes and without shoes) was measured with a precisely calibrated set of scales.

Dietary intake

Participants were furthermore advised to complete standardised 7 d food records. Food intake data were obtained from the study subjects and were further analysed, whereas the percentage of daily energy intake from carbohydrates, fat and protein as well as total energy and macronutrient intake were calculated using the Nutrimaster Diet Analysis software modified for the Czech population (Abbott Laboratories, Abbott Park, IL, USA). Special attention was paid to extreme snacking behaviour (defined as higher daily energy intake from snacks than 25 %), eventual dieting, extreme portion sizes and irregularity in eating. The structure of the daily energy intake was also investigated – a snacking index (established as a ratio of daily energy intake from snacks *v.* daily energy intake from the main meals) was calculated.

Candidate genes

The selection of particular SNP was based on: (1) population frequency in the European Caucasian population; (2) their known or potential functional or regulatory impact on feeding behaviour or association in the case of synonymous SNP; and/or (3) a previously described association with obesity or feeding behaviour.

Genotyping was carried out for eight SNP in five genes related to the production of adipokines, control of energy homeostasis, appetite and satiety regulation: LEP (rs2167270); LEPR (rs1137101); ADIPOQ (rs2241766, + 94T/G); IL-6 (rs1800797, rs1800795); POMC (rs3754860, rs1009388).

Genotyping

DNA for analyses was extracted from 5 ml of the patients' saliva using a standard technique employing proteinase K. Genotyping of each of eight investigated SNP in ADIPOQ, LEP, LEPR, IL6 and POMC genes was performed as described previously[19–26], using standard PCR-based methodology followed by restriction fragment length polymorphism with subsequent electrophoresis on the agarose gel with ethidium bromide staining. The DNA fragments were visualised by UV illumination using Image Analyser (AlphaImager™ 1220; Alpha Innotech Corp., San Leandro, CA, USA).

All reactions were performed using the XP BIOER Cycler (Bioer Technology Co. Ltd, Hangzhou, China), the overall genotyping success varied between 83·3 % (LEP +19A/G) and 99·7 % (LEPR Gln223Arg); missing genotypes were due to either consistent PCR dropout or depletion of template DNA. To assess genotyping reliability we performed double sampling in more than 20 % of the samples and found no differences. We always used quality control and negative controls were used to identify possible false-positives.

Determination of plasma leptin, soluble leptin receptor and adiponectin

Blood samples for total LEP, ADIPOQ and sObR plasma analyses were collected after overnight fasting and were immediately centrifuged at 1700 *g* for 20 min and then stored at −80°C until analysis. Plasma LEP and sObR levels were

measured by commercially available sandwich ELISA (R&D Systems, Minneapolis, MN, USA) with a sensitivity of 7·8 pg/ml and 0·057 ng/ml, respectively. Plasma samples for LEP and sObR were 100-fold and 5-fold diluted with calibrator diluent immediately before the assay, respectively. The intra- and inter-assay CV were less than 3·3 and 5·4 % for the LEP assay, and 6·1 and 8·6 % for the sObR assay, respectively. Plasma ADIPOQ levels were measured by a commercially available ELISA (RayBiotech, Norcross, GA, USA) with a sensitivity less than 10 pg/ml. Samples were 50 000-fold diluted in singlet to assay range (4·1–1000 pg/ml) with standardised assay diluent. The intra- and inter-assay CV were less than 10 and 12 %, respectively.

Statistics

The genotype distributions were tested for Hardy–Weinberg equilibrium by a set of χ^2 tests. Allelic frequencies were estimated by the 'counting method' and differences in allele frequencies between case and control subjects were tested by likelihood ratio χ^2 tests for 2×2 tables (two alleles, case *v.* control subjects). Where applicable, it was first determined whether the variable presented a normal distribution using the Kolmogorov–Smirnov test, and in cases of skewed variables, logarithmic transformation was performed. For descriptive purposes, mean values are presented using untransformed values. Results are expressed as mean values and standard deviations unless otherwise stated.

To identify genetic as well as non-genetic variables that may contribute to predicting the anthropometric phenotype or nutritional phenotype, we carried out a forward stepwise logistic regression, a sequential procedure of adding one input variable at a time to build up a regression model in which the dependent variable (i.e. presence or absence of obesity) is represented as the linear combination of independent variables (anthropometric and nutritional parameters and genotypes of eight investigated SNP). In this analysis, the codes of genotypes were used as quantitative variables (AA = 0, AB = 1, BB = 2).

OR were calculated using the multiple logistic regression analysis models; we adjusted for covariates including age (continuous), BMI (<23, 23–24·9, 25–29·9, 30–34·9, or ≥ 35 kg/m^2), sex, smoking (never, past, and current), alcohol intake (non-drinker and drinker (0·1–4·9, 5–10, or >10 g/d)), family history of obesity and menopausal status in females.

Using sample tertiles, the nutrient variables were categorised in three groups of equal size (the upper third, the middle third and the lower third) as described by Santos *et al.* [16]. Each nutrient variable was then included in logistic regressions as binary indicators leaving one category as the reference.

The data analysis was performed using the Statistica v. 8.0 (Statsoft Inc., Tulsa, OK, USA) program package. The values of $P<0·05$ were considered statistically significant.

Results

The baseline demographic, anthropometric, dietary and clinical characteristics of the study subjects in relation to sex are listed in Table 1. The allele frequencies of all examined SNP exceeded 0·05. The Hardy–Weinberg equilibrium test showed that the examined polymorphisms, except for the ADIPOQ 45T/G and ADIPOQ 94T/G polymorphisms in the obese group, were in Hardy–Weinberg equilibrium. Moreover, no significant differences both in genotype distributions and allele frequencies were observed when comparing the morbidly obese cohort (BMI > 40 kg/m^2) with the controls and the obese cohort (30 < BMI ≤ 40 kg/m^2) with the controls (BMI ≤ 30 kg/m^2) (Table 2).

Effect of single nucleotide polymorphisms on anthropometric characteristics (BMI, waist:hip ratio, total body fat, skinfold thickness)

In the next step, we tested whether these SNP had any effect on anthropometric characteristics related to obesity in the study subjects.

Univariate logistic regression analysis revealed that the subjects with the GG genotype of LEP + 19A/G had a 1·9 higher risk for the development of obesity compared with subjects with the LEP + 19 A allele (OR 1·9 (95 % CI 0·87, 3·02); $P=0·002$). Moreover, the AA homozygotes of the LEPR Gln223Arg polymorphism carried in the univariate analysis approximately half the risk for the development of increased diastolic blood pressure when compared with the G allele carriers (OR 0·49 (95 % CI 0·12, 1·32); $P=0·002$). In the multivariate regression modelling across all the study subjects that was based on the results of the univariate analysis, the LEP + 19A/G polymorphism expressed an independent prediction role for BMI ($\beta = -0·15$; $P=0·02$), while LEPR Gln223Arg was significantly correlated with diastolic blood pressure ($\beta = -0·15$; $P = 0·02$). When analysing the possible relationships between the SNP and the thickness of the skinfolds, LEP + 19A/G expressed a significant prediction role for the thickness of the triceptal skinfold ($\beta = -0·14$; $P=0·04$) and ADIPOQ + 94T/G was correlated with the thickness of the subscapular skinfold ($\beta = 0·13$; $P=0·04$). Moreover, LEP + 19A/G and POMC *Rsa*I expressed an independent prediction role for percentage of body fat in the multivariate analysis ($\beta = -0·17$, $P=0·008$; $\beta = 0·13$; $\beta = 0·13$, $P=0·03$, respectively).

Effect of single nucleotide polymorphisms on dietary characteristics and food preferences of the study subjects

In the univariate regression modelling, none of the examined polymorphisms served as an independent predictor for percentage of daily energy intake from macronutrients or abnormal eating patterns (extreme snacking behaviour, extreme portion sizes, night eating, irregular food intake; NS). When analysing the general eating behaviour patterns in the tertile analysis, the presence of extreme snacking behaviour was in the total studied cohort (obese plus non-obese individuals) associated with lower obesity risk (Table 3). This effect was also observed in females separately (OR 0·42 (95 % CI 0·23, 0·77); $P=0·003$), but not in males (OR 1·26 (95 % CI 0·42, 3·74); $P=0·44$).

When analysing the distribution of energy intake during the day, the ADIPOQ + 45T/G polymorphism was in the univariate regression modelling associated with the energy value of breakfast, defined as the first meal during the day ($\beta = 0·15$;

Table 1. Descriptive statistics of the patients' baseline characteristics
(Mean values and standard deviations)

Group...	Obese (*n* 252)				Morbidly obese (*n* 64)*				Controls (*n* 157)			
	Female		Males		Females		Males		Females		Males	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Subjects (*n*)	188		64		51		13		120		37	
Body composition												
Age (years)	50·1	11·4	46·4	12·2	51·6	10·6	48·7	13·0	38·8	13·2	36·8	14·0
BMI (kg/m^2)	37·5	6·3	37·0	6·0	45·3	5·2	46·9	5·3	25·0	3·3	25·7	2·5
Body fat (%)	46·3	5·9	32·8	6·7	52·4	4·1	41·9	3·8	31·5	7·0	19·5	6·0
Dietary intake												
Energy (kJ)	7848	2410	10791	3201	7344	1885	10357	3394	7799	1768	10747	2425
Protein (% energy)	15·6	3·5	14·8	2·7	15·6	3·0	15·6	3·5	14·2	2·5	13·4	1·9
Carbohydrates (% energy)	49·4	5·4	49·6	5·4	49·6	5·7	48·8	4·9	51·1	5·1	51·6	6·1
Fat (% energy)	35·0	4·9	35·6	5·2	34·8	4·9	35·6	5·3	34·7	4·7	34·8	5·9
Hormonal status†												
Leptin (ng/ml)	45·6	25·8	37·3	17·7	45·1	25·6	38·1	18·5	29·6	28·1	NA	
sObR (ng/ml)	20·4	4·3	16·8	4·0	20·2	4·3	16·3	4·0	27·7	5·2	NA	
Leptin:sObR ratio	2·4	1·4	2·2	0·8	2·4	1·3	2·3	0·8	1·1	1·0	NA	
Adiponectin (μg/ml)	9·3	5·1	8·4	6·6	9·8	5·1	8·8	6·7	9·7	5·6	NA	
Anthropometry												
Waist circumference (cm)	103·9	8·9	116·0	9·2	124·5	10·2	141·9	11·2	82·1	9·7	90·1	11·6
Hip circumference (cm)	119·2	7·6	114·1	6·1	139·1	12·6	137·0	10·8	102·3	7·9	100·1	9·2
Waist:hip ratio	0·9	0·1	1·0	0·1	0·9	0·1	1·0	0·1	0·8	0·1	0·9	0·1
Skinfold thickness (mm)												
Supraspinal skinfold	26·0	7·9	23·1	8·8	30·3	12·5	32·0	16·4	19·4	24·4	15·3	5·8
Subscapular skinfold	30·1	21·6	28·4	8·7	35·4	10·0	31·0	14·6	19·1	10·6	22·4	26·9
Bicepital skinfold	22·1	6·4	16·9	5·6	26·3	8·2	25·1	8·0	14·5	5·1	11·3	6·3
Tricepital skinfold	29·6	5·6	22·9	7·0	31·5	6·9	29·1	7·4	21·5	5·5	16·5	5·3
Sum of all skinfolds	107·7	27·5	91·3	20·0	122·8	30·3	117·2	33·3	74·2	31·5	65·4	32·8
Systolic blood pressure (mmHg)	135·2	19·5	141·0	17·5	140·0	24·7	140·3	17·0	121·9	17·9	125·8	12·5
Diastolic blood pressure (mmHg)	89·0	11·0	92·3	13·6	93·1	17·8	95·7	12·8	81·7	11·1	79·7	11·2

NA, not analysed.
*Subset of obese subjects.
†Analysed in a subset of individuals, consisting of the sixty-four morbidly obese subjects and sixty-four subjects from the other groups matched by age and sex.

Table 2. Distributions of genotypes and alleles of examined polymorphisms in the studied subpopulations*

Polymorphism	Genotypes				Alleles		
ADIPOQ rs2241766 (+45T/G) (synonymous coding, GGT → GGG, Gly → Gly)	TT	TG	GG	*P*	T	G	*P*
Obese cases	149 (81)	28 (15)	7 (4)	0·36	326 (89)	42 (11)	0·42
Morbidly obese cases	51 (80)	12 (19)	1 (1)	0·90	114 (84)	14 (16)	0·64
Controls	126 (82)	25 (16)	2 (2)	–	277 (91)	29 (9)	–
ADIPOQ +94T/G (synonymous coding, GGT → GGG, Gly → Gly)	TT	TG	GG	*P*	T	G	*P*
Obese cases	97 (61)	45 (28)	16 (11)	0·26	239 (76)	77 (24)	0·74
Morbidly obese cases	35 (61)	19 (33)	3 (6)	0·96	89 (78)	25 (22)	0·79
Controls	82 (59)	48 (35)	8 (6)	–	212 (77)	64 (23)	–
LEP rs2167270 (+19A/G) (5′ UTR)	AA	AG	GG	*P*	G	A	*P*
Obese cases	73 (40)	84 (46)	24 (14)	0·66	230 (63)	132 (37)	0·85
Morbidly obese cases	30 (45)	28 (42)	6 (13)	0·41	88 (69)	40 (31)	0·24
Controls	58 (37)	80 (52)	18 (11)	–	196 (63)	116 (37)	–
LEPR rs1137101 (+27265A/G) (synonymous coding, CAG → CGG, Gln → Arg)	AA	AG	GG	*P*	A	G	*P*
Obese cases	48 (26)	98 (53)	39 (21)	0·30	194 (52)	176 (48)	0·59
Morbidly obese cases	20 (31)	33 (51)	12 (18)	0·66	77 (57)	57 (43)	0·75
Controls	50 (32)	70 (45)	36 (23)	–	170 (54)	142 (46)	–
POMC rs3754860 (+1798C/T) (5′ UTR)	++	+ −	− −	*P*	+	−	*P*
Obese cases	76 (41)	90 (49)	19 (10)	0·68	242 (65)	128 (35)	0·40
Morbidly obese cases	23 (35)	34 (52)	8 (13)	0·34	80 (62)	50 (38)	0·16
Controls	71 (45)	73 (46)	13 (9)	–	215 (69)	99 (31)	–
POMC rs1009388 (+1032C/G) (intronic)	CC	CG	GG	*P*	C	G	*P*
Obese cases	111 (59)	64 (34)	12 (7)	0·75	286 (76)	88 (24)	0·89
Morbidly obese cases	40 (62)	21 (32)	4 (6)	0·91	101 (78)	29 (22)	0·86
Controls	96 (62)	48 (31)	12 (7)	–	240 (77)	72 (23)	–
IL-6 rs1800797 (−596A/G) (5′ UTR)	GG	GA	AA	*P*	G	A	*P*
Obese cases	56 (32)	90 (51)	31 (17)	0·94	202 (57)	152 (43)	0·75
Morbidly obese cases	22 (34)	28 (44)	14 (22)	0·54	72 (56)	56 (44)	0·94
Controls	46 (30)	80 (52)	28 (18)	–	172 (56)	136 (44)	–
IL-6 rs1800795 (−174G/C) (5′ UTR)	CC	CG	GG	*P*	C	G	*P*
Obese cases	54 (31)	92 (52)	30 (17)	0·88	200 (57)	152 (43)	0·67
Morbidly obese cases	20 (31)	30 (47)	14 (22)	0·82	70 (55)	58 (45)	0·93
Controls	43 (30)	75 (51)	28 (19)	–	161 (55)	131 (45)	–

ADIPOQ, adiponectin; LEP, leptin; UTR, untranslated region; LEPR, leptin receptor; POMC, pro-opiomelanocortin.
*Numbers in parentheses are the percentages of the genotypes present in the different groups. For some genotypes, only a 83–90 % success rate could be reached due to a less efficient PCR amplification.

Table 3. Association between the upper and lower tertiles of extreme snacking behaviour with obesity in the studied cohorts

	Obesity (+)		Obesity (−)				
	%	Total *n*	%	Total *n*	OR	95 % CI	*P*
Total					0·57	0·35, 0·95	0·019*
Upper tertile	75	248	59	155			
Lower tertile	93	248	42	155			
Male					1·26	0·42, 3·74	0·44
Upper tertile	13	61	7	36			
Lower tertile	28	61	19	36			
Female					0·42	0·23, 0·77	0·003*
Upper tertile	62	187	52	119			
Lower tertile	65	187	23	119			

*$P<0·05$.

$P=0·02$). In the multivariate modelling adjusted for age, sex and smoking, the LEPR Gln223Arg polymorphism was positively correlated with the energy value of the dinner ($\beta=0·13$; $P=0·04$), whereas the GG carriers expressed a trend toward higher energy intake later on, in their dinner. Moreover, the LEP + 19A/G polymorphism was correlated with the energy value of the supper ($\beta=0·13$; $P=0·05$), with the AG heterozygotes expressing a tendency toward the highest energy intake in their supper.

In the above-defined subset of patients, LEP, sObR and ADIPOQ plasma levels were analysed in relation to examined SNP. Neither ADIPOQ + 45T/G nor +94T/G was associated with ADIPOQ plasma levels either in the obese or lean individuals. The LEPR Gln223Arg polymorphism was correlated with the ADIPOQ plasma levels ($\beta=-0·28$; $P=0·03$), whereas the GG homozygotes were showing on average the lowest plasma ADIPOQ levels. The *Rsa* I polymorphism was associated with both plasma LEP levels ($P=0·007$) and the LEP:sObR ratio ($P=0·003$). Furthermore, the bivariate analysis was performed on examined polymorphisms to assess possible associations of LEP, sObR and the LEP:sObR ratio or ADIPOQ and dietary characteristics. To control for possible confounders, the results from the bivariate correlation analysis were consecutively explored using multivariate analysis using logarithmically transformed plasma LEP and sObR and LEP:sObR ratio regressed on total energy intake as well as on the energy intake provide by each macronutrient[19]. However, no significant associations were observed.

In a separate analysis, sObR plasma levels and the LEP:sObR ratio were significantly correlated with systolic blood pressure ($\beta=-0·66$, $P=0·002$; $\beta=-1·23$, $P=0·02$) and sObR plasma levels also served as an independent predictor for diastolic blood pressure ($\beta=-0·50$; $P=0·04$).

Discussion

The investigation of genotype × nutrient interactions is a general base for a better understanding of the multifactorial pathogenesis of complex diseases as well as for the identification of obesity-related traits. Unfortunately, the evaluation of genotype × nutrient associations seems to be extremely difficult, mainly because of the complicated epidemiology of obesity, too many candidate genes investigated and also because of the small power of the reported observed findings, altogether making the investigation of genotype × food preferences highly tangled and difficult to accomplish[16].

In the present study, we investigated the associations of eight SNP in adipose tissue-related genes in a cohort of 409 individuals with precisely quantified anthropometric and dietary characteristics and we employed different statistical models to precisely assess interactions defined as departures from the multiplicative risk ratios[16], thus strengthening our analysis.

The allele frequencies and genotype distributions within the present study closely resembled those previously reported in other Caucasian European populations[27–29]; no significant differences were observed both in allele and genotypes frequencies and these were highly similar to those reported for the HapMap CEU population (Utah residents with ancestry from northern and western Europe)[30].

Moreover, the results of the present study refer to the possible interaction between the carriers of the genetic variant of the LEP + 19A/G polymorphism and various anthropometric parameters including BMI, percentage body fat and skinfold thickness. Our findings are partially in accordance with the findings of Mizuta *et al.* [31], as we did not observe any association of the LEP + 19A/G polymorphism with plasma LEP levels either; however, we did not confirm any associations of LEP + 19A/G or LEPR Gln223Arg with sweet preference described by these Japanese investigators.

Analysis of the anthropometric parameters revealed a significant association of ADIPOQ + 94T/G with subscapular skinfold thickness. As reported by Yang *et al.* [32] the synonymous mutation ADIPOQ + 94T/G might affect steady-state mRNA levels by altering RNA splicing or stability[32]. This T/G or linked polymorphism nearby could therefore affect mRNA splicing or stability and lead to extensive metabolic consequences that might result in varying body fat distribution. The strong linkage disequilibrium with type 2 diabetes, measures of adiposity, and insulin levels found in the chromosomal region where the ADIPOQ gene is located[33–34] suggest that somewhere on this locus a common genetic variant should be located, such as the T-G polymorphism in the ADIPOQ gene, that expresses measurable effects on adiposity-related traits. As far as we know, the present study is the first to report an association of ADIPOQ + 94T/G with skinfold thickness.

Moreover, we observed a significant effect of the '−' allele of the POMC *Rsa*I polymorphism on the percentage of body fat, where the '− −' homozygotes presented with the highest percentage of body fat when compared with the '+' allele carriers. This is in contrast to the study by Baker *et al.*[22] who did not observe any effects of *Rsa*I on BMI or waist:hip ratio; moreover, we did not observe the association of the POMC C1032G polymorphism with the waist:hip ratio or BMI reported by these authors.

Surprisingly, the tertile analysis of the macronutrient intake revealed that the presence of extreme snacking behaviour was in the total studied cohort (obese cases plus non-obese individuals) associated with lower obesity risk, which contradicts the generally accepted model of maintaining steady body weight. The present results are in accordance with a study by Lioret *et al.*[35] on 748 French children, as the authors concluded that a combination of more frequent food intake occasions and lower contribution of the main meals to total daily energy intake is associated with a smaller risk of overweight in children. However, the observed patterns were not consistently expressed in males in the present study and thus conclusions on these eating patterns in relation to obesity risk require further investigation, possibly with a prospective design.

Furthermore, significant interactions with various dietary and circadian characteristics concerning the LEPR Gln223Arg polymorphism were observed. Recently, it has been reported by Guízar-Mendoza *et al.*[36] that adolescent individuals with the A allele (A/A and A/G) had higher heart sympathetic activity, body fat percentage and LEP levels. This is partially in contrast to our findings of higher plasma LEP levels in G allele carriers (GG + GA), also observed in our previous study[17]; however, the G allele carriers in the present study presented with lower body fat percentage, which is in accordance with the Mexican adolescent cohort. The finding of half the risk of AA carriers for diastolic blood pressure in our cohort is also in contrast to this Mexican study, where a sympathetic effect of the A allele was observed. The present study is so far the first to demonstrate any effect of LEPR Gln223Arg on the structuring of energy intake during the day, and thus it provides a basis for further investigation of the possible association of Gln223Arg with eating behaviour and involvement of this polymorphism in the regulation of circadian rhythmicity. In this context, the observed significant association of the LEPR Gln223Arg polymorphism with the blood pressure values is of particular importance.

We did not confirm the observations by Yannakoulia *et al.*[19] that the sObR is positively associated with energy intake from carbohydrates and negatively with energy intake from dietary fat, whereas the free LEP index was in this study on a Greek population negatively associated with energy intake from carbohydrates and positively with energy intake from dietary fat, thus contrasting with the present results. The present study also did not reveal any associations of free LEP and sObR with energy intake and the macronutrient composition of the diet reported by Yannakoulia *et al.*[19].

The main strength of the present study is the use of state-of-the-art methodology including 7 d food records for evaluating the subjects' dietary intake. The 7 d food records provide quantitatively accurate information on food consumed during the recording period by recording food while it is consumed, the problem of reporting bias or omission is lessened, whereas subjects are not restricted to selecting from a predetermined list of foods included in a FFQ[19]. Although confounding was appropriately controlled for through standard statistical procedures, there is always a possibility of residual confounding by other serum adipokines, genetic factors or unmeasured and unknown factors that have to be considered. Confirmation of the present results by future studies on different populations and above all the precise assessment of the adipokine genes in relation to the time structure of daily energy intake are warranted.

To conclude, the present population-based case–control study revealed significant associations of selected polymorphisms in genes encoding for adipokines both with percentage body fat, skinfold thickness and specific dietary composition and time patterns of feeding behaviour.

Acknowledgements

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript, in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors.

The present study was supported by the Ministry of Education of the Czech Republic (grant no. 881/2006) and by a project of the Danone Institute of the Czech Republic (DANONE/2007) focused on genetic variability of adipokines in obese individuals.

The authors are grateful to Dana Polášková, Petra Přikrylová, Svatava Tschoplová and Jarmila Laušová for technical assistance. We thank Kateřina Heczková, Zlata Piskáčková and Lenka Kučerová for their assistance in processing and analysing food records.

References

1. Marti A, Martinez-González MA & Martinez JA (2008) Interaction between genes and lifestyle factors on obesity. *Proc Nutr Soc* **67**, 1–8.
2. Uusitupa M (2005) Gene–diet interaction in relation to the prevention of obesity and type 2 diabetes: evidence from the Finnish Diabetes Prevention Study. *Nutr Metab Cardiovasc Dis* **15**, 225–233.
3. Kenchaiah S, Evans JC, Levy D, *et al.* (2002) Obesity and the risk of heart failure. *N Engl J Med* **347**, 305–313.
4. Sharma AM & Chetty VT (2005) Obesity, hypertension and insulin resistance. *Acta Diabetol* **42**, S3–S8.
5. Barsh GS, Farooqi IS & O'Rahilly S (2000) Genetics of bodyweight regulation. *Nature* **404**, 644–651.
6. Clément K, Vaisse C, Lahlou N, *et al.* (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392**, 398–401.
7. Farooqi IS, Wangensteen T, Collins S, *et al.* (2007) Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. *N Engl J Med* **356**, 237–247.
8. Krude H, Biebermann H, Luck W, *et al.* (1998) Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* **19**, 155–157.
9. Yiannakouris N, Melistas L, Yannakoulia M, *et al.* (2003) The −2548G/A polymorphism in the human leptin gene

promoter region is associated with plasma free leptin levels; interaction with adiposity and gender in healthy subjects. *Hormones* **2**, 229–236.
10. Vivenza D, Rapa A, Castellino N, *et al.* (2004) Ghrelin gene polymorphisms and ghrelin, insulin, IGF-I, leptin and anthropometric data in children and adolescents. *Eur J Endocrinol* **151**, 127–133.
11. Portolés O, Sorlí JV, Francés F, *et al.* (2006) Effect of genetic variation in the leptin gene promoter and the leptin receptor gene on obesity risk in a population-based case-control study in Spain. *Eur J Epidemiol* **21**, 605–612.
12. Chagnon YC, Wilmore JH, Borecki IB, *et al.* (2000) Associations between the leptin receptor gene and adiposity in middle-aged Caucasian males from the HERITAGE family study. *J Clin Endocrinol Metab* **85**, 29–34.
13. Slattery ML, Curtin K, Sweeney C, *et al.* (2008) Modifying effects of IL-6 polymorphisms on body size-associated breast cancer risk. *Obesity* **16**, 339–347.
14. de Krom M, van der Schouw YT, Hendriks J, *et al.* (2007) Common genetic variations in CCK, leptin, and leptin receptor genes are associated with specific human eating patterns. *Diabetes* **56**, 276–280.
15. Schulz LO, Bennett PH, Ravussin E, *et al.* (2006) Effects of traditional and Western environments on prevalence of type 2 diabetes in Pima Indians in Mexico and the U.S. *Diabetes Care* **29**, 1866–1871.
16. Santos JL, Boutin P, Verdich C, *et al.* (2006) Genotype-by-nutrient interactions assessed in European obese women. A case-only study. *Eur J Nutr* **45**, 454–462.
17. Bienertova-Vasku J, Bienert P, Tomandl J, *et al.* (2008) No association of defined variability in leptin, leptin receptor, adiponectin, proopiomelanocortin and ghrelin gene with food preferences in the Czech population. *Nutr Neurosci* **11**, 2–8.
18. Ma Y, Bertone ER, Stanek EJ III, *et al.* (2003) Association between eating patterns and obesity in a free-living US adult population. *Am J Epidemiol* **158**, 85–92.
19. Yannakoulia M, Yiannakouris N, Bluher S, *et al.* (2003) Body fat mass and macronutrient intake in relation to circulating soluble leptin receptor, free leptin index, adiponectin, and resistin concentrations in healthy humans. *J Clin Endocrinol Metab* **88**, 730–736.
20. Mammès O, Betoulle D, Aubert R, *et al.* (2000) Association of the G-2548A polymorphism in the 5′ region of the LEP gene with overweight. *Ann Hum Genet* **64**, 391–394.
21. Gaukrodger N, Mayosi BM, Imrie H, *et al.* (2005) A rare variant of the leptin gene has large effects on blood pressure and carotid intima-medial thickness: a study of 1428 individuals in 248 families. *J Med Genet* **42**, 474–478.
22. Baker M, Gaukrodger N, Mayosi BM, *et al.* (2005) Association between common polymorphisms of the proopiomelanocortin gene and body fat distribution: a family study. *Diabetes* **54**, 2492–2496.
23. Monteleone P, Tortorella A, Castaldo E, *et al.* (2006) No association of the Arg51Gln and Leu72Met polymorphisms of the ghrelin gene with anorexia nervosa or bulimia nervosa. *Neurosci Lett* **398**, 325–327.
24. Yang WS, Hsiung CA, Ho LT, *et al.* (2003) Genetic epistasis of adiponectin and PPARγ2 genotypes in modulation of insulin sensitivity: a family-based association study. *Diabetologia* **46**, 977–983.
25. Nakatani K, Noma K, Nishioka J, *et al.* (2005) Adiponectin gene variation associates with the increasing risk of type 2 diabetes in non-diabetic Japanese subjects. *Int J Mol Med* **15**, 173–177.
26. Rosmond R, Chagnon M, Bouchard C, *et al.* (2001) A missense mutation in the human melanocortin-4 receptor gene in relation to abdominal obesity and salivary cortisol. *Diabetologia* **44**, 1335–1338.
27. Pollin TI, Tanner K, O'Connell JR, *et al.* (2005) Linkage of plasma adiponectin levels to 3q27 explained by association with variation in the APM1 gene. *Diabetes* **54**, 268–274.
28. Souren NY, Paulussen AD, Steyls A, *et al.* (2008) Common SNPs in LEP and LEPR associated with birth weight and type 2 diabetes-related metabolic risk factors in twins. *Int J Obes (Lond)* **32**, 1233–1239.
29. Kämäräinen OP, Solovieva S, Vehmas T, *et al.* (2008) Common interleukin-6 promoter variants associate with the more severe forms of distal interphalangeal osteoarthritis. *Arthritis Res Ther* **10**, R21.
30. International HapMap Consortium (2005) A haplotype map of the human genome. *Nature* **437**, 1299–1320.
31. Mizuta E, Kokubo Y, Yamanaka I, *et al.* (2008) Leptin gene and leptin receptor gene polymorphisms are associated with sweet preference and obesity. *Hypertens Res* **31**, 1068–1077.
32. Yang WS, Tsou PL, Lee WJ, *et al.* (2003) Allele-specific differential expression of a common adiponectin gene polymorphism related to obesity. *J Mol Med* **81**, 428–434.
33. Kissebah AH, Sonnenberg GE, Myklebust J, *et al.* (2000) Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome. *Proc Natl Acad Sci U S A* **97**, 14478–14483.
34. Vionnet N, Hani E, Dupont S, *et al.* (2000) Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21–q24. *Am J Hum Genet* **67**, 1470–1480.
35. Lioret S, Touvier M, Lafay L, *et al.* (2008) Are eating occasions and their energy content related to child overweight and socio-economic status? *Obesity (Silver Spring)* **16**, 2518–2523.
36. Guízar-Mendoza JM, Amador-Licona N, Flores-Martínez SE, *et al.* (2005) Association analysis of the Gln223Arg polymorphism in the human leptin receptor gene, and traits related to obesity in Mexican adolescents. *J Hum Hypertens* **19**, 341–346.

4.6. ASSOCIATION OF GENETIC VARIABILITY IN SELECTED REGIONS IN VISFATIN (NAMPT) GENE WITH ANTHROPOMETRIC PARAMETERS AND DIETARY COMPOSITION IN OBESE AND NON-OBESE CENTRAL-EUROPEAN POPULATION

Resumé

Visfatin je významným adipokinem s inzulinomimetickým působením, který má řadu účinků na úrovni intracelulárního i intermediárního metabolismu. V této studii jsme zkoumali, zda a případně jak souvisí genetická variabilita v genu pro visfatin s antropometrickými determinantami obezity a složením potravy. Analyzovali jsme celkem 6 exonů v genu pro visfatin u 20 extrémně obézních jedinců (průměrný BMI 52,2 ± 5 kg/m^2) a frekvenci sledovaných SNP jsme dále ověřili na validační kohortě 605 jedinců. Identifikovali jsme přítomnost tří častých SNP – dvou v nekódujících oblastech přiléhajících ke sledovaným exonům (rs78411774 A/C, rs71564769 A/C) a jednoho synonymního SNP v exonu 7 (rs2302559 A/G). Poslední zmíněný polymorfismus byl významně korelován s hladinou visfatinu v celé populaci, včetně rozsáhlé validační kohorty s různým BMI ($p<0,001$). Navíc byla pozorována významná inverzní korelace hladin visfatinu a leptinu. Nebyla naopak pozorována asociace mezi zkoumanými SNP a sledovanými antropometrickými či nutričními parametry.

Jedná se o první studii, která popisuje, že polymorfismus rs2302559 má přímý vliv na cirkulující hladinu visfatinu.

Nutrients **2012**, *4*,1-x manuscripts; doi:10.3390/nu40x000x

OPEN ACCESS

nutrients

ISSN 2072-6643

www.mdpi.com/journal/nutrients

Article

Association of Genetic Variability In Selected Regions in Visfatin (NAMPT) Gene with Anthropometric Parameters and Dietary Composition in Obese and Non-Obese Central-European Population

Jiri Stastny[1], Julie Bienertova-Vasku[1,2,5,]*, Josef Tomandl[3], Marie Tomandlova[3], Filip Zlamal[1], Martin Forejt[4], Zbynek Splichal[1] and Anna Vasku[1]

[1] Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

[2] Department of Pediatric Oncology, University Hospital Brno, Cernopolni 22, Brno, Czech Republic

[3] Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

[4] Department of Preventive Medicine, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

[5] Central European Institute of Technology, Masaryk University, Kamenice 5 Brno, Czech Republic

* Author to whom correspondence should be addressed; E-Mail: jbienert@med.muni.cz; Tel.:; Fax: +420-549-494-340.

Received: / Accepted: /Published:

Abstract: Visfatin (NAMPT/PBEF) is a recently identified adipocytokine which harbors strong insulin-mimetic activity and was reported to be associated with obesity. However,nothing is known about whether visfatin is related to specific nutritional behavior which may result in obesity development. This is the first study focusing on genetic variability of the visfatin gene and its association with circulating visfatin, anthropometric parameters and dietary composition.We

analyzed a total of 6 exons and adjacent non-coding regions of the NAMPT gene in 20 extremely obese Czech individuals (mean BMI 52.2 ± 5.0 SD) using direct sequencing and a frequency of rs2302559 was established in the validation cohort of another 605 individuals with completed 7-day food records and complex anthropometric measurements. Serum levels of visfatin, leptin and leptin-receptor were measured in all sequenced individuals and in part of the validation cohort.Three common polymorphisms were identified, two in non-coding regions (rs78411774 A/C, rs71564769 A/C) and one synonymous SNP in exon 7 (rs2302559 A/G). The rs2302559 showed significant correlation with visfatin serum level throughout the entire study cohort ($p < 0.001$); there was a significant tendency towards higher visfatin levels in G allele carriers with GG homozygotes having the highest visfatin serum levels. Furthermore, a negative correlation was observed between visfatin and leptin serum level ($p = 0.01$). No association between investigated SNPs and anthropometric parameters or native dietary composition was observed.This is the first study to demonstrate that the rs2302559 polymorphism in the PBEF gene is related to circulating levels of visfatin. As the SNP is synonymous, we hypothesize it might be linked to another SNP in the PBEF gene which controls visfatin serum levels.

Keywords: Visfatin, NAMPT, SNP, dietary composition, anthropometric parameters, extreme obesity

---

1. Introduction

Visfatin is a recently identified adipokine [1] associated with obesity development, insulin-resistance, plasma lipids profile, atherosclerosis, inflammation and many other conditions. The visfatin/NAMPT gene is located on the 7q22.3 complement strand , contains 11 exons and spans a region of 34.7kb. The product of the gene was originally recognized by Samal *et al.* [2] in 1994 as a pre-B-cell colony-enhancing factor which was identified in peripheral blood lymphocytes and which induced lymphocyte maturation and inhibited apoptosis of neutrophils [3]. Subsequently, the same protein was identified as an intracellular enzyme further characterized as the nicotinamide phosphoribosyltransferase (Nampt), which is a key limiting step in the nicotinamide adenine dinucleotide (NAD+) biosynthesis pathway [4]. Furthermore, it was demonstrated that the extracellular visfatin also harbors a strong enzymatic activity and that it participates in the regulation of insulin secretion from pancreatic beta-cells [5].

In 2005, Fukuhara *et al.* [1] reported increased expression of the NAMPT gene product within visceral adipose tissue and named it accordingly: visfatin. In his observation of 101 males and females, a significant positive correlation between visfatin plasma levels and the amount of visceral adipose tissue was observed. On the other hand, only a mild association

between the circulating visfatin and subcutaneous adipose tissue was demonstrated in the study. The authors also observed an increase in visfatin plasma levels during obesity development. However, the most important finding of the study was the insulin-mimetic activity of visfatin via the insulin receptor. Similarly to insulin, visfatin induces phosphorylation of the insulin receptor (IR) and also IRS1 and IRS2 (insulin receptor substrate). Moreover, visfatin binds to PI3K (phosphatidylinositol 3-kinase) and induces phosphorylation of Akt (protein kinase B) and MAPK (mitogen-activated protein kinase). An interesting finding, making visfatin signaling an attractive therapeutic target in insulin resistance and T2DM, is the ability of visfatin to bind to IR on a different site than insulin. The importance of visfatin in metabolic pathways is supported by the fact that homozygous mice with visfatin deficiency die during early embryogenesis. Insulin-mimetic activity of visfatin was also observed in experiments on heterozygous mice with mutated NAMPT gene. Heterozygous mice had slightly increased plasma glucose levels in comparison with the wild type individuals. When the authors focused on visfatin effects in cultured cells, effects similar to insulin were observed. Not only had visfatin influenced the glucose uptake into 3T3-L1 adipocytes and L6 myocytes, it also suppressed glucose release from H4IIEC3 hepatocytes. Furthermore, visfatin stimulated the accumulation and synthesis of triglycerides in cultured mice preadipocytes, and thus is likely to influence the differentiation of adipose tissue in much the same ways as insulin does [1]. Visfatin is not exclusively expressed by adipocytes, there are others sources of visfatin in circulation [6]. In addition to adipocytes and leukocytes, visfatin is also expressed in hepatocytes [7] and skeletal muscles [8]. When a person is becoming obese, the amount of adipose tissue macrophages (ATM) in visceral fat increases and these macrophages become a major source of visfatin in circulation [9]. Obesity and its associated accumulation of white adipose tissue (WAT) is linked to a low-grade inflammation and it is associated with tissue hypoxia [10]. One of the possible explanations of increased visfatin expression under these conditions is a regulation of its expression through a hypoxia-inducible factor 1α (HIF1α), which is a transcription factor accumulated during hypoxia and plays a key role in adaptation to hypoxia. HIF1α is binding to two hypoxia responsive elements (HREs) of visfatin promoters in mice and promotes visfatin expression [11].

As mentioned above, there is a considerable amount of evidence that visfatin has pleiotropic endocrine, paracrine and autocrine effects. As visfatin harbors certain insulin-mimicking effects, it could influence specific nutritional behavior which may further result in obesity development. This is the first study to evaluate the association of visfatin concentration in relation to the genetic variability of the NAMPT gene, adipokines and anthropometric parameters related to obesity and native, unmodified, dietary composition in the Central-European population.

2. Material and Methods

2.1. Subjects

A total of 10 women and 10 men (mean age 47.8 y ± 11.6 [SD]) with extremely high BMI (mean BMI 52.2 [kg/m2] ± 5.0 [SD]) were selected from a large population cohort of 673 volunteers of Central-European Caucasian origin, more information on recruitment of the subjects is provided elsewhere [12]. Of the original cohort of 673 individuals, a total of 605 subjects were available for genotyping of the rs2302559 A/G polymorphism in the NAMPT gene to validate the population genotype and allele frequencies of this polymorphism. The complete native nutritional profiles (food frequency questionnaire (FFQ), 7-day food records) were obtained from all the study subjects along with anthropometric information (BMI, body fat percentage, skin fold thickness, waist and hip circumference, W-H ratio). Food intake data were obtained from the study subjects and further analyzed, whereas the percentage of daily energy intake from carbohydrates, fat and protein as well as total energy and macronutrient intake was calculated using the Nutrimaster Diet Analysis software modified for the Czech population (Abbott Laboratories, Abbott Park, IL, USA). Special attention was paid to extreme snacking behavior (defined as higher daily energy intake from snacks than 25 %), eventual dieting, extreme portion sizes and irregularity in eating. All phenotypic measurements were performed by the same specialists and included weight, height, BMI, lean body mass, fat mass, body fat, waist and hip circumferences, waist-to-hip ratio (WHR) and skin fold thickness. Body composition was assessed by means of bioelectrical impedance analysis, using a single frequency bioimpedance analyzer (BodyStat Ltd, Douglas, Isle of Man, UK) with the subject lying in a supine position. The measurement of height was performed with a calibrated stadiometer and weight (in light indoor clothes and without shoes) was measured on a precisely calibrated set of scales.

2.2. Biochemistry

The number of adipokines was measured in the serum samples of the study subjects using ELISA (leptin, leptin receptor, adiponectin, adipsin, agouti-related peptide) and visfatin circulation levels were determined in all sequenced individuals and in a subset of 128 individuals from the validation cohort, consisting of the sixty-four obese subjects and sixty-four subjects from the non-obese cohort matched by age and sex.

Blood samples for the serum analyses were collected following overnight fasting and were immediately centrifuged at 1700 g for 20 min and then stored at -80 °C until analysis. Serum levels of the respective adipokines were measured by commercially available sandwich ELISA (R&D Systems, Minneapolis, MN, USA), where intra-assay variability did not exceed 10 %.

2.3. DNA Analysis

Principally, the appropriate pairs of primers were designed using the ExonPrimer program for exons 3 to 8 based on the gene sequence obtained from the Ensembl database (www.ensembl.org); exon 1 and 3 were not analyzed. PCR reaction for the amplification of

the exons was carried out using the TouchDown PCR reaction (www.pcrlinks.com/variants/touchdown.htm).

The total of 6 exons were used for direct sequencing using the ABI PRISM 3130 sequencer with the BigDye Terminator v3.1 Cycle Sequencing Kit, used as per the manufacturer's instructions. The sequenced regions were further analyzed and compared with the database sequence (www.ensembl.org) using the SeqScape Software v. 2.5 from Applied Biosystems.

Genotyping of rs2302559 A/G was performed using the XP BIOER Cycler (Bioer Technology Co. Ltd, Hangzhou, China); overall genotyping success was 100 %. In order to assess genotyping reliability, double sampling in over 20 % of all samples was performed; no differences were found. Quality control and negative controls were always used in order to identify possible false-positives. PCR products were digested using the TaqI exonuclease and set on 2% agarose gel to visualize the respective fragments.

2.4. Statistics

All statistical analyses were carried out using Statistica 8.0. Data were tested for normality using the Kolmogorov-Smirnov test and log-transformed where necessary. Pearson's correlation coefficient was used to determine whether linear associations were present and multivariate linear regression modeling was used to determine significant predictors of visfatin concentration. Differences in the log values of serum visfatin concentrations and their association with dietary and anthropometric findings were examined with one-way ANOVA followed by Tukey's pairwise multiple comparison method, with the assumption of homoscedasticity verified using Bartlett's test.

In order to identify genetic as well as non-genetic variables which may contribute to predicting the anthropometric or nutritional phenotype, we carried out a forward stepwise logistic regression, a sequential procedure of adding one input variable at a time to build up a regression model in which the dependent variable (i.e. the presence or absence of obesity) is represented as the linear combination of independent variables (anthropometric and nutritional parameters and genotypes of three investigated SNPs). In this analysis, the codes of genotypes were used as quantitative variables (AA = 0, AB = 1, BB = 2).

Unless otherwise indicated, data are given as mean ± standard deviation (Std); p values less than 0.05 were considered significant in all tests.

3. Results

The basic descriptive statistic of the patients' baseline characteristics of the morbidly obese sequenced individuals are given in Table 1, descriptive characteristics of the validation cohort is provided in Table 2.

Table 1. Descriptive statistics of the patients' baseline characteristics (mean values and standard deviations)—the sequenced individuals.

	Morbidly Obese		
	Females	**Males**	***p***
Subjects (N)	10	10	
Age (years)	48.60 ± 10.38	46.92 ± 13.20	0,706
Body composition			
BMI	54.80 ± 4.06	49.66 ± 4.58	0.013
Body fat (%)	56.60 ± 2.29	43.33 ± 2.82	< 0.001
Dietary intake			
Energy (kJ/day)	7501 ± 2302	9005 ± 3435	0.364
Proteins (% energy)	16.35 ± 4.09	15.20 ± 3.16	0.345
Carbohydrates (% energy)	49.81 ± 6.87	50.69 ± 4.31	0.427
Fat (% energy)	34.00 ± 5.97	33.86 ± 5.21	0.762
Fluids intake (ml/day)	1982 ± 1080	1393 ± 792	0.186
Cholesterol (mg/day)	237.32 ± 93.19	210.79 ± 105.64	0.564
Biochemistry			
Visfatin (ng/mL)	1.80 ± 0.97	1.50 ± 0.89	0.289
Leptin (ng/mL)	62.66 ± 29.72	45.45 ± 18.63	0.253
Leptin receptor (ng/mL)	17.87 ± 2.44	17.70 ± 2.90	0.704
Adiponectin (μg/mL)	8.10 ± 5.04	9.36 ± 7.08	1
AgRP (ng/mL)	75.71 ± 14.24	80.28 ± 12.69	0.762
Adipsin (pg/mL)	3669 ± 1085	3363 ± 747	0.594
Anthropometry			
Waist circumference (cm)	136.35 ± 11.17	145.90 ± 11.99	0.096
Hip circumference (cm)	151.65 ± 13.67	142.00 ± 7.04	0.112
Waist-to-hip ratio	0.91 ± 0.10	1.03 ± 0.08	0.006
Skinfold thickness (mm)			
Supraspinal skinfold	39.71 ± 7.80	38.00 ± 9.12	0.816
Table 1. *Cont.*			
Subscapular skinfold	43.22 ± 9.92	36.00 ± 5.13	0.111
Biceptal skinfold	35.78 ± 9.55	24.70 ± 6.72	0.022
Triceptal skinfold	37.44 ± 6.78	28.30 ± 7.60	0.008
Sum of skinfold thickness	155.86 ± 21.74	131.14 ± 16.07	0.025
Blood pressure			
Systolic blood pressure (mmHg)	147.25 ± 18.81	138.90 ± 18.31	0.450
Diastolic blood pressure (mmHg)	100.75 ± 10.98	93.50 ± 12.98	0.307

Table 2. Descriptive statistics of the patients' baseline characteristics (mean values and standard deviations)—the validation cohort.

	Non-Obese		Obese		
	Females	**Males**	**Females**	**Males**	***p***
Subjects (N)	172	63	264	106	
Age (years)	39.96 ± 13.61	42.29 ± 14.84	51.25 ± 11.67	50.20 ± 12.57	< 0.001
Body composition					
BMI	24.88 ± 3.46	26.10 ± 2.42	37.08 ± 6.18	36.79 ± 5.70	< 0.001
Body fat (%)	31.28 ± 7.30	21.45 ± 6.07	45.85 ± 5.94	33.88 ± 6.72	< 0.001
Dietary intake					
Energy (kJ/day)	6203 ± 673	7862 ± 1265	6735 ± 764	9003 ± 1156	< 0.001
Proteins (% energy)	14.07 ± 2.55	13.53 ± 2.00	15.48 ± 3.37	14.91 ± 2.66	< 0.001
Carbohydrates (% energy)	51.46 ± 5.37	51.29 ± 5.87	49.24 ± 5.55	49.09 ± 4.84	< 0.001
Fat (% energy)	34.51 ± 4.80	34.98 ± 5.64	35.16 ± 5.04	35.96 ± 4.75	0.107
Fluids intake (ml/day)	1607 ± 688	1579 ± 764	1473 ± 628	1561 ± 690	0.911
Cholesterol (mg/day)	180.80 ± 83.69	261.86 ± 118.02	205.28 ± 91.16	278.53 ± 131.66	0.003
Biochemistry†					
Visfatin (ng/mL)	6.29 ± 21.90	3.34 ± 4.10	1.71 ± 1.73	15.02 ± 56.74	0.283
Leptin (ng/mL)	29.61 ± 28.10	23.25 ± 18.17	46.21 ± 26.33	37.26 ± 17.74	0.014
Leptin receptor (ng/mL)	27.68 ± 5.25	26.40 ± 3.11	20.15 ± 4.17	16.80 ± 4.05	< 0.001
Adiponectin (μg/mL)	9.24 ± 5.81	5.31 ± 2.50	9.01 ± 6.39	8.55 ± 7.41	0.996
AgRP (ng/mL)	89.05 ± 24.92	99.24 ± 24.03	77.48 ± 20	78.61 ± 11.99	0.003
Adipsin (pg/mL)	2196 ± 255	2325 ± 530	3191 ± 788	3148 ± 719	< 0.001
Anthropometry					
Waist circumference (cm)	81.08 ± 10.04	92.31 ± 9.56	109.17 ± 12.88	120.74 ± 14.08	< 0.001
Hip circumference (cm)	101.88 ± 7.64	101.24 ± 4.88	123.40 ± 12.65	118.20 ± 11.05	< 0.001
Waist-to-hip ratio	0.79 ± 0.07	0.91 ± 0.08	0.89 ± 0.07	1.02 ± 0.07	< 0.001
Skinfold thickness (mm)					
Supraspinal skinfold	16.61 ± 5.94	15.11 ± 6.36	27.24 ± 8.60	24.93 ± 9.43	< 0.001
Subscapular skinfold	18.03 ± 6.26	17.91 ± 7.02	30.61 ± 8.33	29.42 ± 9.60	< 0.001
Biceptal skinfold	13.92 ± 5.24	10.65 ± 6.06	23.13 ± 7.03	18.05 ± 7.09	< 0.001
Triceptal skinfold	20.83 ± 5.67	16.13 ± 6.53	30.33 ± 5.91	24.01 ± 7.59	< 0.001
Sum of skinfold thickness	69.39 ± 19.25	61.13 ± 20.31	110.55 ± 23.38	94.76 ± 25.95	< 0.001
Blood pressure					
Systolic blood pressure (mmHg)	120.06 ± 18.46	128.62 ± 13.16	136.95 ± 18.49	141.44 ± 21.62	< 0.001
Diastolic blood pressure (mmHg)	80.21 ± 11.71	80.57 ± 9.84	89.4 ± 11.54	90.95 ± 14.14	< 0.001

† Analysed in a subset of individuals, consisting of the sixty-four obese subjects and sixty-four subjects from the non-obese cohort matched by age and sex.

p value refers to the comparison between the lean and obese subject (males and females pooled together).

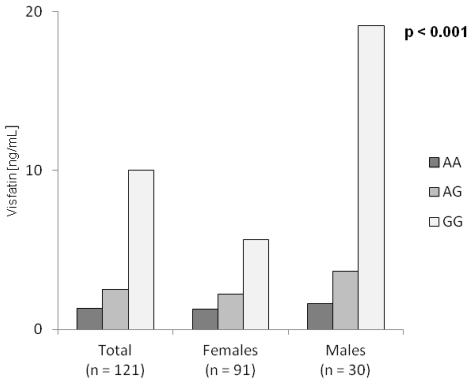
No novel unreported mutations were identified in our study. When analyzing the coding sequence of the NAMPT gene, three common single-nucleotide polymorphisms (SNPs) were identified: two in adjacent non-coding regions (rs78411774 T/G, rs71564769 A/C) and one synonymous SNP in exon 7 (rs2302559 A/G).

The rs78411774 located in the non-coding region adjacent to exon 2 presented as two T/T and five T/G individuals. Another SNP identified in the non-coding region of PBEF gene (rs71564769 A/C) showed a different sequence variation compared to the database sequence – C/G in two and G/G in 18 individuals (instead of the reported A/C).

3.1. rs2302559

In case of rs2302559, there were no differences in genotype distributions and allele frequencies of rs2302559 between obese and non-obese individuals, both cohorts were in HWE. In a total of 20 sequenced individuals, a significant association between rs2302559 and visfatin serum levels ($p < 0.001$) was observed. This initial observation that visfatin serum level is associated with rs2302559 was further confirmed on the larger population cohort of 128 investigated individuals with rs2302559 genotypes and visfatin serum levels available for analysis ($p = 0.01$), whereas there was a significant tendency towards higher visfatin serum levels in G allele carriers, the male GG homozygotes having the highest visfatin levels (Fig. 1).

Figure 1. Association of genotypes of rs2302559 with visfatin serum levels (mean values).



3.2. Association of Investigated SNPs with Anthropometric Characteristics

For the rs2302559 polymorphism, no significant association with any of the investigated anthropometric parameters (weight, height, BMI, lean body mass, fat mass, body fat, waist and hip circumferences, WHR and skin fold thickness) was observed, the other two polymorphisms (rs78411774 T/G, rs71564769 A/C) were not investigated in the large cohort, hence they were excluded from the analysis.

3.3. Association of Investigated SNPs with Nutritional Characteristics

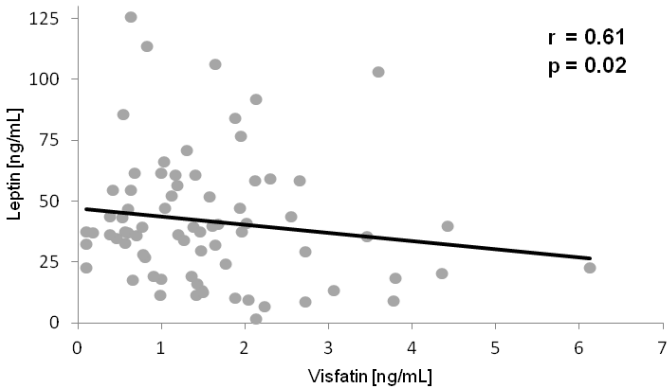
None of the investigated polymorphisms, rs78411774 A/C, rs71564769 A/C and rs2302559 A/G, showed – following BMI and gender adjustments – significant association with any of the investigated nutritional parameters based on the 7-day food records, including total energy intake, percentage of energy derived from the respective macronutrients, total intake of macronutrients in g, fluid intake, cholesterol intake and vitamin C intake. Lastly,

analysis of nutritional records of the studied individuals did not reveal any significant association of visfatin serum levels with dietary composition of these individuals.

3.4. Association of Visfatin Serum Levels with other Adipokines

Significant negative correlation was observed between visfatin and leptin serum levels (r = -0.61; p = 0.02) (Fig 2), but not between any of the remaining investigated adipokines.

Figure 2. Correlation between circulating visfatin and leptin levels.



4. Discussion

Visfatin is an adipokine which is being studied extensively across various fields of medicine – from obesitology and endocrinology to rheumatology, cardiology, etc. Visfatin is capable of influencing numerous metabolic pathways at the intra- as well as extracellular levels, which makes it an elegant candidate for an obesity-related marker.

In this study, we identified a synonymous SNP in exon 7 (rs2302559) which was significantly associated with visfatin serum levels in the examined extremely obese Central-European population as well as in the large population cohort (n = 605). The homozygotes with the GG genotype presented the highest serum levels of visfatin, while the carriers of the AA genotype had the lowest visfatin levels; it may be therefore suggested that higher visfatin serum levels are associated with the presence of the G allele. The frequencies in our study were significantly different from the frequencies observed by Tokunaga et al. [13] in their cohort of 200 diabetic and 200 non-diabetic individuals (the observed frequencies were: GG 83.5 %, AG 16.5 % and AA 0.0 % compared to ours: GG 30 %, AG 40 % and AA 30 %). The different frequencies between the Japanese and our Central-European population could be attributed to different ethnicity and BMI of the investigated subjects (52.2 ± 5.0 [kg/m2] in our population vs. 23.2 ± 3.5 [kg/m2] in the Japanese cohort).

Another SNP (rs71564769 A/C) identified in our study showed a different sequence variation compared to the database sequence – C/G in two and G/G in 18 individuals (instead of the reported A/C). The possible explanation is that this site is polymorphic which hasn't been reported so far.

It was reported previously that the visfatin serum level correlates with BMI (Berndt et al. 2005). In our study, we did not observe such association, i.e. visfatin serum levels were not associated with BMI or other investigated anthropometric traits. When investigating associations of the rs2302559 with anthropometric traits, the AG genotype showed the highest BMI (54.2 ± 6.4 [kg/m2]) compared to the AA (50.8 ± 4.8 [kg/m2]) and the GG (51.1 ± 1.8 [kg/m2]) genotypes. As rs2302559 is a synonymous SNP, its significant relationship with visfatin serum levels could be attributed to possible linkage with another SNP in the region that is functional. Our preliminary results obtained on the small cohort of sequenced individuals (n = 20) were confirmed on a larger population cohort, however, further studies on other populations with different ethnicity are necessary to elucidate the role of rs2302559 in influencing the visfatin plasma levels.

The principal role of visfatin in the intermediate metabolism is unclear. The mechanism of visfatin action through the insulin receptor [1] and its physiological meaning remains elusive. Under physiological conditions, adipose tissue does not produce high levels of visfatin and visfatin plasma levels increase during obesity development. The association between higher visfatin levels in plasma and T2DM [14] and obesity [15] were observed but its mechanism thus far remains unclear. As Fukuhara et al. [1] showed, visfatin exerts insulin-mimetic activity within adipose tissue and hepatocytes and also leads to intense adipogenesis with a subsequent increase in fat deposits – circulus vitiosus. Large population studies on cohorts of different ethnicities are necessary to further clarify the underlying mechanisms of visfatin association with insulin metabolism as well as obesity development.

So far, no information is available on the relationship between rs2302559 and anthropometric and/or dietary parameters. The pivotal study by Böttcher et al [16] reports that the ratio of visceral/sc visfatin mRNA expression is associated with three genetic polymorphisms in visfatin gene ($p < 0.05$). No correlation between rs2302559 and anthropometric parameters (waist, hip circumference or WHR) was observed in our study. The relationship between the native dietary composition derived from the 7-day food records and the rs2302559 polymorphism was also investigated in our study. Based on our results, examined SNP do not seem to be a determinant of the nutritional intake of the macronutrients or the total energy intake.

The logistic modeling of investigated adipokines (leptin, leptin receptor, adiponektin, adipsin, agouti-related peptide, visfatin) showed a negative correlation between serum levels of visfatin and leptin. Leptin plays a key role in energy intake regulation and energy expenditure and correlates with the amount of body fat. This correlation between visfatin and leptin seems to be logical as there is an increase of visfatin expression in obesity. However, there is no evidence of visfatin effect as an appetite inhibitor, thus mimicking leptin action in nucleus arcuatus. In our study, visfatin reached the highest levels in obese males, while the levels in obese females were very low. Moreover, visfatin levels in obese males showed considerable variation, which was not due to a few outlying values in this cohort but rather due to homogenously wide distribution in this population. Therefore, it could be hypothesized that there two opposite trends in visfatin levels in relation to increasing BMI, whereas in

females the visfatin levels are inversely correlated with increasing BMI, while in males the visfatin levels significantly rise along with an increase in BMI. However, at this moment we are unable to explain the considerable interindividual variability in visfatin levels in these population extremes of distribution. Therefore we suggest that in the extremely obese population, visfatin levels are gender-dependent. The increase of visfatin in obese individuals may be due to higher visfatin expression by adipose tissue macrophages as a result of low-inflammatory state [9], hypoxia [10] or simply as a correlate of a number of adipocytes [1].

The main strength of the present study is the use of state-of-the-art methodology including 7-day food records for evaluating the subjects' dietary intake in the context of the genetic variability of the PBEF gene and visfatin serum levels; a total of 605 individuals with available 7-day food records provide the necessary statistical power to our study. The 7-day food records provide quantitatively accurate information on food consumed during the recording period by recording food while it is consumed, the problem of reporting bias or omission is lessened, as subjects are not restricted to selecting from a predetermined list of foods included in a FFQ. Although confounding was appropriately controlled by means of standard statistical procedures, there is always a possibility of residual confounding by other serum adipokines, genetic factors or unmeasured and unknown factors which must be taken into consideration. On the other hand, the major limitation of our study are the significant differences in age between the cases and the controls that are due to the consecutive nature of the enrollment into the study (the subject were enrolled consecutively, not on the basis of their BMI or other anthropometric traits). The confirmation of the present results by future studies on different populations is warranted.

Conclusion

To the best of our knowledge, this is the first study to demonstrate that the rs2302559 polymorphism in the NAMPT gene is associated with visfatin serum levels suggesting that this synonymous polymorphism is associated with significant expressional effect, possibly due to the linkage with another, nonsynonymous SNP. On the other hand, our study failed to demonstrate significant effects of visfatin circulating levels or variability in investigated regions in PBEF gene including rs2302559 with native dietary composition of the individuals. To conclude, further studies on the functional impact of this polymorphism and its possible associations with other loci are imperative.

Ethical Standards

The present study was conducted according to guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee for Ethics of Medical Experiments on Human Subjects of the Faculty of Medicine of Masaryk University (Brno, Czech Republic). Written informed consent was obtained from all subjects and subsequently archived.

Conflict of Interest

The authors have nothing to disclose.

Acknowledgement

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors.

This study was supported by the Ministry of Education of the Czech Republic (grant No. 881/2006), and MZMOU2005 and RECAMO CZ.1.05/2.1.00/03.0101.

References

1. Fukuhara, A.; Matsuda, M.; Nishizawa, M.; Segawa, K.; Tanaka, M.; Kishimoto, K.; Matsuki, Y.; Murakami, M.; Ichisaka, T.; Murakami, H.; Watanabe, E.; Takagi, T.; Akiyoshi, M.; Ohtsubo, T.; Kihara, S.; Yamashita, S.; Makishima, M.; Funahashi, T.; Yamanaka, S.; Hiramatsu, R.; Matsuzawa, Y.; Shimomura, I. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* **2005**, *307*, 426-430.
2. Samal, B.; Sun, Y.; Stearns, G.; Xie, C.; Suggs, S.; McNiece, I. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol* **1994**, *14*, 1431-1437.
3. Jia, S.H.; Li, Y.; Parodo, J.; Kapus, A.; Fan, L.; Rotstein, O.D.; Marshall, J.C. Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. *J Clin Invest* **2004**, *113*, 1318-1327.
4. Rongvaux, A.; Shea, R.J.; Mulks, M.H.; Gigot, D.; Urbain, J.; Leo, O.; Andris, F. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol* **2003**, *32*, 3225-3234.
5. Revollo, J.R.; Korner, A.; Mills, K.F.; Satoh, A.; Wang, T.; Garten, A.; Dasgupta, B.; Sasaki, Y.; Wolberger, C.; Townsend, R.R.; Milbrandt, J.; Kiess, W.; Imai, S. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* **2007**, *6*, 363-375.
6. Friebe, D.; Neef, M.; Kratzsch, J.; Erbs, S.; Dittrich, K.; Garten, A.; Petzold-Quinque, S.; Bluher, S.; Reinehr, T.; Stumvoll, M.; Bluher, M.; Kiess, W.; Korner, A. Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans. *Diabetologia* **2011**, *54*, 1200-1211.
7. Garten, A.; Petzold, S.; Barnikol-Oettler, A.; Korner, A.; Thasler, W.E.; Kratzsch, J.; Kiess, W.; Gebhardt, R. Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes. *Biochem Biophys Res Commun* **2010**, *391*, 376-381.

8. Costford, S.R.; Bajpeyi, S.; Pasarica, M.; Albarado, D.C.; Thomas, S.C.; Xie, H.; Church, T.S.; Jubrias, S.A.; Conley, K.E.; Smith, S.R. Skeletal muscle NAMPT is induced by exercise in humans. *Am J Physiol Endocrinol Metab* **2010**, *298*, E117-E126.
9. Curat, C.A.; Wegner, V.; Sengenes, C.; Miranville, A.; Tonus, C.; Busse, R.; Bouloumie, A. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia* **2006**, *49*, 744-747.
10. Hosogai, N.; Fukuhara, A.; Oshima, K.; Miyata, Y.; Tanaka, S.; Segawa, K.; Furukawa, S.; Tochino, Y.; Komuro, R.; Matsuda, M.; Shimomura, I. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* **2007**, *56*, 901-911.
11. Segawa, K.; Fukuhara, A.; Hosogai, N.; Morita, K.; Okuno, Y.; Tanaka, M.; Nakagawa, Y.; Kihara, S.; Funahashi, T.; Komuro, R.; Matsuda, M.; Shimomura, I. Visfatin in adipocytes is upregulated by hypoxia through HIF1alpha-dependent mechanism. *Biochem Biophys Res Commun* **2006**, *349*, 875-882.
12. Bienertova-Vasku, J.; Bienert, P.; Forejt, M.; Tomandl, J.; Brazdova, Z.; Vasku, A. Genotype x nutrient association of common polymorphisms in obesity-related genes with food preferences and time structure of energy intake. *Br J Nutr* **2010**, *103*, 352-359.
13. Tokunaga, A.; Miura, A.; Okauchi, Y.; Segawa, K.; Fukuhara, A.; Okita, K.; Takahashi, M.; Funahashi, T.; Miyagawa, J.; Shimomura, I.; Yamagata, K. The -1535 promoter variant of the visfatin gene is associated with serum triglyceride and HDL-cholesterol levels in Japanese subjects. *Endocr J* **2008**, *55*, 205-212.
14. Chen, M.P.; Chung, F.M.; Chang, D.M.; Tsai, J.C.; Huang, H.F.; Shin, S.J.; Lee, Y.J. Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* **2006**, *91*, 295-299.
15. Berndt, J.; Kloting, N.; Kralisch, S.; Kovacs, P.; Fasshauer, M.; Schon, M.R.; Stumvoll, M.; Bluher, M. Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* **2005**, *54*, 2911-2916.
16. Böttcher,Y.; Teupser, D.;, Enigk, B.; Berndt, J.; Klöting, N.; Schön, MR.; Thiery, J.; Blüher, M.; Stumvoll, M.; Kovacs, P. Genetic variation in the visfatin gene (PBEF1) and its relation to glucose metabolism and fat-depot-specific messenger ribonucleic acid expression in humans. *J Clin Endocrinol Metab*. **2006**, *91(7)*, 2725-31.

4.7. VISFATIN AND ITS ROLE IN OBESITY DEVELOPMENT

Resumé

Visfatin, produkt NAMPT/PBEF genu, je adipokin se silnou inzulinomimetickou aktivitou realizovanou cestou inzulínového receptoru. Již v minulosti byl v některých studiích asociován s obezitou. Existují i důkazy o důležitých intracelulárních efektech visfatinu, který je homologní s nikotinamidfosforibosyltransferázou (NAMPT). Úkolem této práce je zhodnotit současný stav poznání na poli chápání patofyziologické úlohy visfatinu při vzniku excesivní akumulace bílé tukové tkáně.

Původně se mělo za to, že visfatin je čistě produktem tukové tkáně, který koreluje s celkovou masou bílé tukové tkáně v organismu. Další studie ale prokázaly, že tomu tak není – visfatin je produkován v celé řadě tkání i buněk, a to hlavně buňkami imunitního systému, např. makrofágy. Zásadní otázkou je, která z tkání organismu je hlavním zdrojem visfatinu v konkrétní fázi života a jaké důsledky to má z hlediska patofyziologie obezity. Současný konsensus říká, že visfatin je produkován zejména adipocyty a makrofázy tukové tkáně. Wang et al. naznačují významnou negativní korelaci mezi visfatinem a triglyceridy v plazmě. Navrhují hypotézu, že vztah visfatinu k lipidovému profilu je výsledkem spíše intracelulárních metabolických aktivit visfatinu na úrovni NAD metabolismu než souvislosti s metabolismem viscerálního tuku nebo inzulinovou rezistencí.

Zprávy týkající se hladin visfatinu u obézních populací tak i nadále zůstávají protichůdné a bude nutný další výzkum pro objasnění role visfatinu v regulaci excesivní akumulace bílé tukové tkáně u této populace.

G Model
DSX-276; No. of Pages 5
ARTICLE IN PRESS

Diabetes & Metabolic Syndrome: Clinical Research & Reviews xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Diabetes & Metabolic Syndrome: Clinical Research & Reviews

journal homepage: www.elsevier.com/locate/dsx

Review

Visfatin and its role in obesity development

Jiri Stastny *, Julie Bienertova-Vasku, Anna Vasku

Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5 A18, Brno 62500, Czech Republic

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Visfatin
NAMPT
Adipocytokines
Lipid metabolism
Obesity

ABSTRACT

Visfatin, a product of PBEF gene, is an adipocytokine that harbours strong insulin-mimetic activity and it has been reported previously to associate with obesity. Recent reports also provide evidence that Visfatin has also important intracellular effects as it is homologous with nicotinamide phosphoribosyltransferase (NAMPT). In this review, we summarize the main documented effects of Visfatin on metabolism in humans, with special emphasis put on the pathways associated with obesity.

Contents

1. Introduction

In this review, we would like to present the interesting and relatively long way of discovering a new protein – Visfatin – and primarily the effects that it has on obesity development, insulin resistance, lipid profile, inflammation and more other. So far, many of probable effects of this protein have been identified and many of them proved their importance in metabolic pathways and subsequently in development of many pathological conditions. Nevertheless, it has to be mentioned that most of these effects are still unclear or mostly controversial.

The product of Visfatin/PBEF gene was initially identified by Samal and his collegues in 1994 [1] as a cytokine subsequently named pre-B-cell colony-enhancing factor (PBEF) that is expressed in lymphocytes of peripheral blood and plays a role in lymphocytes maturation and inhibition of neutrophil apoptosis [2]. Subsequently, the same protein was confirmed as intracellular enzyme nicotinamide phosphoribosyltransferase (Nampt). It is involved in nicotinamide adenine dinucleotide (NAD) biosynthesis [3]. Finally, in January 2005 Visfatin was recognized as one of adipocytokines by Fukuhara et al. [4] in article in Science. Visfatin was reported to have numerous proinflammatory [5,6] and insulin-mimetic [4] effects, relationship to lipid profile [6–12], to insulin resistance [6], function of B cells in pancreas [13] and many other effects.

2. Fukuhara's discovery

Visfatin is a 52 kDa large protein, and its gene PBEF/Visfatin is located on chromosome 7q22.2. It consists of 11 exons and 10 introns and is 34.7 kb large. The history of this adipocytokine started in January 2005 with the article in Science [4]. In their

* Corresponding author. Tel.: +420 777932660; fax: +420 549494340.
E-mail address: opravdu.stastny@seznam.cz (J. Stastny).

article, Fukuhara et al. presented Visfatin as a newly identified adipocytokine with many possibly effects in physiological regulation in humans and possible role in development of some pathological conditions. The authors observed that Visfatin plasma levels were positively correlated with obesity development and had insulin-mimetic activity. However, this positive correlation between obesity development and increased Visfatin levels was not clearly confirmed as many following works showed [14,15]. The insulin-mimetic activity was observed during experiment with cultured cells, whereas Visfatin lowered plasma glucose level in mice. In experiment with heterozygous mice with mutated Visfatin gene, lightly higher plasma glucose levels were observed than in wild type individuals. Further it was described that Visfatin binds to the insulin receptor and activates it, however it binds to a different binding site than insulin. Therefore, Visfatin could act as an attractive target molecule with insulin-mimetic activity, which is non-competitive with insulin, in pharmacotherapy of insulin-resistant conditions [16]. It is not surprising that Fukuhara et al. were considering the role of Visfatin in some metabolic disorders related to glucose homeostasis. Surprisingly, the Fukuhara's paper has been withdrawn from Science in October 2007 as the investigation by the Committee for Research Integrity of Osaka University revealed that not all preparations of Visfatin were capable to bind to the insulin receptor [17].

3. Where is Visfatin expressed?

The Visfatin/PBEF gene is expressed in many tissues and it was originally identified as the pre-B-cell colony-enhancing factor (PBEF), expressed in leucocytes of peripheral blood, by Samal and his collegues in 1994 [1]. The adipokine-like character of the molecule was for the first time identified by Fukuhara et al. and the molecule was reported to be predominantly expressed in visceral fat tissue [4] However, the visceral fat is not the only tissue where Visfatin is expressed. Leucocytes of peripheral blood [1] or adipose tissue macrophages [18], hepatocytes [19] or skeletal muscles [20] also participate in Visfatin production and may influence Visfatin plasma levels. Friebe with collegues [21] recently published the paper, describing the leucocytes, especially granulocytes, as the major source of Visfatin plasma levels. However, the expression of Visfatin in visceral adipose tissue is not constant and it increases in obesity [14]. The one of possible explanations of this could be that the adipose tissue is more prone to be hypoxic in the obese individuals [22]. Segawa et al. suggest that Visfatin in adipocytes is upregulated by hypoxia through HIF1α (hypoxia-inducible factor 1α) dependent mechanism [23]. HIF1α is a transcription factor accumulated during hypoxia and it play a key role in adaptation to hypoxic state. They also showed that HIF1α is binding to two HREs (hypoxia responsive elements) of Visfatin promotore in mice and through them is Visfatin expression upregulated.

Interesting are also findings of Revollo et al. [13] where they showed that Visfatin/eNampt is secreted by cells actively but in non-classical secretory pathway, not through Golgi–ER secretory system.

4. The role of macrophages in Visfatin production

The obesity is considered a chronic low-inflammatory state. The research group by Curat et al. reported interesting findings in 2006 [18]. They recognized a population of CD14+ macrophages in visceral fat tissue that was increasing its size with increasing BMI. Within the adipose tissue, the adipose tissue macrophages, not adipocytes, was found to be predominant producers and releasers of Visfatin.

Similarly, Chang et al. [6] polished in 2010 their study describing a strong correlation between Visfatin and macrophage-specific CD68 and TNF-α gene expressions in human adipose tissues.

This supports the presumption that Visfatin is predominantly a pro-inflammatory substance and its growth in visceral obesity is caused mainly by visceral adipose tissue macrophages.

5. Regulation of Visfatin expression

Hormonal regulation of Visfatin expression is very complicated and has been studied extensively. Kralisch et al. [24] used various hormones, which are known to alter insulin sensitivity, to observe their effects on Visfatin expression at 3T3-L1 adipocytes in vitro. A hypothesis of influence of insulin on Visfatin expression was not confirmed, however, there was a decrease of Visfatin expression after TNFalpha, GH and isoproterenol stimulation. And increase after stimulation by dexamethasone.

In 2010, Mayi et al. studied the role of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARgamma) in regulation of Visfatin expression and secretion in adipose tissue macrophages. They used synthetic ligands of PPARgamma and they observed increased Visfatin expression and secretion in macrophages, but not in adipocytes [25], which they explain by proved attribute of PPARgamma – to have distinct functions in different cell types.The Visfatin expression in subcutaneous adipose tissue was not increased even by the TZD's, which also act as PPARgamma agonists [26]. Treatment by TZD's lead to an improvement of peripheral insulin resistance, but according this study, not through the increase in Visfatin expression and its insulin-mimetic activity. But there is a prediction, that positive influence of TZD's is because of a mobilization of lipids to subcutaneous adipose tissue as a defence against insulin resistance.

And a few months later Mayi et al. [27] observed in their experiment that liver X receptor (LXR) activation negatively regulates Visfatin expression in macrophages. Synthetic LXR ligands decreased Visfatin gene expression and the decrease of Visfatin mRNA was paralleled by a decrease of protein secretion.

Taken together these two studies showed opposite regulation of Visfatin expression in macrophages.

In the study on the OLEFT rats the expression of Visfatin mRNA in visceral fat tissue was elevated by rosiglitazone or fenofibrate treatments when compared to untreated rats. [28]

6. The role of Visfatin in regulation of insulin signalling

A very important is the finding of Visfatin insulin-mimetic activity. As we mentioned previously, at very first time has been this effect showed by Fukuhara et al. [4] in 2005. The insulin-mimetic activity was observed during various experiments with cultured cells, whereas Visfatin lowered plasma glucose level in mice. In experiment with heterozygous mice with mutated Visfatin gene, lightly higher plasma glucose levels were observed than in wild type individuals. When they focused on Visfatin effects in cultured cells the similar results to insulin were showed. Visfatin had not only influence on glucose uptake into 3T3-L1 adipocytes and L6 myocytes, but also suppressed glucose release from H4IIEC3 hepatocytes. And, what is important, stimulated accumulation and synthesis of triglycerides in cultured mice preadipocytes, so had an influence on differentiation of adipose tissue as insulin has. Fukuhara et al. went further and showed that molecule of Visfatin is binding on insulin receptor, but in distinct site from insulin. Visfatin induced the phosphorylation of insulin receptor, IRS1 and also IRS2 (insulin receptor substrate). Visfatin is binding to PI3K (phosphatidylinositol 3-kinase) to IRS1 and IRS2 and also induced phosphorylation of Akt (protein kinase B) and MAPK (mitogen-activated protein kinase).

Please cite this article in press as: Stastny J, et al. Visfatin and its role in obesity development. Diab Met Syndr: Clin Res Rev (2012), http://dx.doi.org/10.1016/j.dsx.2012.08.011

To over-feeding and normal-chow rats was injected pcDNA3.1-Visfatin plasmid and caused Visfatin over-expression. The increase of insulin-sensitivity and also a beneficial influence to cholesterolaemia through increased expression of SREBP2, PPARgamma and increased tyrosine phosphorylation of IRS1 were showed [29].

The insulin-mimetic activity of Visfatin was also observed in experiment with osteoblasts [30]. Human osteoblasts express IR but not Visfatin. After stimulation of osteoblasts, Visfatin induced the phosphorylation of IR, IRS1 and IRS2 and promotes glucose uptake, proliferation and type I collagen production. Taken together, the same effects as exerts insulin.

Despite of these findings, Revollo et al. came with another theory of physiological role of Visfatin. They proved that not the insulin-mimetic activity, but synthesis of NAD is more important in glucose homeostasis in vivo. They found that the heterozygous mice with mutated Visfatin gene and pancreatic beta cells had defects in MNM/NAD biosynthesis and also in glucose-stimulated insulin secretion. After chemical inhibition of Nampt by FK866 the defects in NAD synthesis and in glucose-stimulated insulin secretion were observed. After administration of exogenous MNM were these defects cancelled.

Diabete mellitus type 2 is a pathological condition with increasingly incidence worldwide, which is associated with obesity. The state of insulin resistance and its relation to Visfatin was also studied. Chen et al. [31] in 2006 showed a significantly positive correlation between level of circulating Visfatin and T2DM.

The similar results showed El-mesallamy et al. [32] in Egyptian T2DM obese and non-obese patients. By comparing these patients to healthy controls the increased plasma Visfatin levels were observed. The possible explanations for this relation are still unclear but there is a realistic presumption of direct pathophysiological linkage.

7. The differences within adipose tissue

Human body contains two types of adipose tissue – subcutaneous and visceral fat. Visceral fat mass is more tightly correlated with obesity-associated pathological conditions than overall adiposity. Visceral fat has significant relation to metabolic disorders, especially metabolic syndrome, and represents a strong risk factor [33], whereas the metabolic syndrome is associated with the central obesity, type 2 diabetes mellitus, insulin resistance, hypertension and higher cardiovascular risk. In numbers, it is associated with a 2-fold increase in cardiovascular events and a 1.5-fold increase in all-cause mortality rates [34]. The obesity has been also associated with increased accumulation of macrophages in visceral fat and the amount of macrophages is positively correlated with the total fat mass of the body [18,35].

Fukuhara et al. [4] showed in their experiment on 101 male and female human subjects that Visfatin plasma levels correlated strongly with the amount of visceral fat, but only mildly with the amount of subcutaneous fat. They also analyzed the mRNA Visfatin expression in the visceral and subcutaneous fat in KKAy mice which are models for obese diabetes type 2. In the time period, when these mice become obese (between 6 and 12 week of age), Fukuhara et al. found increased levels of plasma Visfatin which correlate with the increase of mRNA Visfatin expression in visceral fat. On the contrary, no significant changes in mRNA expression in subcutaneous and surprisingly in liver fat were observed.

Nevertheless, Berndt et al. [36] in October 2005 published the results of the study involving 189 subjects which was only partially with accordance with the findings by Fukuhara's group. Berndt et al. observed that Visfatin plasma concentrations were positively correlated with visceral and negatively with subcutaneous Visfatin mRNA expression and formulated the hypothesis that Visfatin mRNA expression in subcutaneous fat could be, at least partially, regulated by plasma Visfatin concentration. However, in a subgroup of 73 subjects in the study, there was no correlation between plasma Visfatin concentrations and visceral fat mass. The authors also reported that Visfatin plasma levels and mRNA expression in visceral fat corresponded with some criteria of obesity such as BMI and body fat content, but not with WHR and waist circumference. No difference between visceral and subcutaneous mRNA expression was also showed.

Pagano et al. [14] had also some very interesting findings that are partially in agreement and partially contradicting previous studies. These authors showed that plasma Visfatin levels are down-regulated in obesity as well as Visfatin mRNA expression in subcutaneous adipose tissue. Furthermore, they observed differences in Visfatin mRNA expression in various subcutaneous fat locations. In the fat sampled from the gluteal region, there was a significant negative correlation between Visfatin mRNA expression and BMI. If the sample of subcutaneous fat was taken from abdominal region, no correlation was observed. Maybe, there are some differences in Visfatin expression within subcutaneous adipose tissue. However, increased expression of Visfatin mRNA was found in visceral fat of obese subjects. In another recent study, the Visfatin gene expression in subcutaneous adipose tissue of normal-weight controls was significantly higher than in samples of obese adults [37], which is in agreement with most of the previous reports.

Jian et al. [15] in their study on Chinese subjects observed lower Visfatin plasma concentrations in obese subjects in compare with non-obese and over-weight ones. Furthermore, Visfatin plasma levels were negatively correlated negatively with BMI and positively with waist/hip ratio – but only in male subjects.

Varma et al. [26] observed that Visfatin mRNA expression in visceral and subcutaneous fat was dependent on BMI. The Visfatin expression in visceral fat was associated positively with BMI, whereas mRNA expression in subcutaneous fat decreased with BMI. However, the expression in the visceral and subcutaneous fat in non-obese showed no differences, which is well in agreement with Berndt et al. [36]. Moreover, Visfatin mRNA expression in subcutaneous fat was positively correlated with insulin sensitivity, as the insulin-resistant subjects had lower expression of Visfatin. However, the authors observed no correlation between Visfatin plasma levels and BMI which the authors concluded may be a consequence of different regulation of Visfatin mRNA expression in visceral and subcutaneous fat tissue.

Interesting findings were presented by Filippatos et al. [9] who observed higher Visfatin plasma concentrations in subjects with metabolic syndrome compared to the group of similar BMI, however without metabolic syndrome.

In another study [38] on relatively more study subjects (500 subject), plasma correlation of Visfatin was negatively correlated with BMI in male subjects, but not in females.

8. Visfatin in children

Childhood obesity is an increasing problem in developed countries and is associated with interrelated pathologies, such as hypertension and insulin resistance in this period. Visfatin plasma levels and their correlation with further parameters were studied, e.g. in obese and non-obese children [39]. The comparison of two cohorts of 30 obese and 30 non-obese children revealed that the levels are similar to those of the adult probands. Positive correlations were observed between Visfatin plasma levels and BMI and also between plasma Visfatin and insulin.

In another study [7] comparing Visfatin plasma levels in Chinese adolescents, a positive correlation was identified between plasma levels of Visfatin and HDL cholesterol in obese subjects, but

Please cite this article in press as: Stastny J, et al. Visfatin and its role in obesity development. Diab Met Syndr: Clin Res Rev (2012), http://dx.doi.org/10.1016/j.dsx.2012.08.011

not in the normal-weighted individuals. The Visfatin plasma concentrations were significantly higher in obese individuals, but were not directly correlated with BMI. The similar conclusion was also described by Haider et al. [40] who observed that Visfatin plasma concentrations were almost 2-fold elevated in obese in compare with non-obese. However, no relationship was detectable between Visfatin and other subject characteristics, hsCRP or the lipid profile in their study.

Kolsgaard et al. [38] compared in their study the total of 175 overweight and obese individuals aged 3–17 years. In their group of children with metabolic syndrome, Visfatin plasma levels were significantly higher than in other investigated groups. Visfatin plasma levels were also increased proportionally to the number of components of metabolic syndrome.

9. Visfatin and lipid profile

The physiological role of circulating Visfatin seems to be elusive. Some papers found positive associations of plasma Visfatin concentrations with HDL-cholesterol [7,8] and negative associations with triglycerides in non-diabetic Caucasian subjects [8]. The relationship with lipid metabolism seems to be not dependent on visceral obesity and insulin resistance and is probably linked to intracellular enzymatic function in NAD synthesis [8]. The authors concluded that circulating PBEF/NAMPT/Visfatin levels are an indicator of beneficial lipid profile in non-diabetic Caucasian subjects, whereas the relation to lipid metabolism does not depend on visceral obesity and insulin resistance, but may be linked to the enzymatic function of Visfatin in NAD metabolism.

However, when Filippatos et al. [9] compared a group of patients with metabolic syndrome with another group of patiens without metabolic syndrome, whereas all patiens in both groups had BMI over 28, the results were contradictory. Plasma Visfatin levels were positively correlated with triglycerides and negatively with HDL-cholesterol in this study.

In another work [38], plasma Visfatin levels were shown to be correlated positively with HDL-cholesterol levels and negatively with LDL-cholesterol levels only in female subjects, not in males.

Sun et al. [11] investigated the response of Visfatin plasma levels to short-term overfeeding in 61 healthy young men. Visfatin showed a total decrease of 19% and there was positive correlation between Visfatin plasma concentrations and triglycerides.

Visfatin also correlated positively to HDL-C in the Asian Indian men and women [12].

10. Conclusion

Visfatin is a recently identified adipocytokine that was originally considered to be a product of visceral adipose tissue correlated with its masss [4]. The following studies [18–21] as well as study by Samal et al. [1] proved, that the Visfatin is produced widely within various tissues in whole human body. An important observation is that Visfatin is produced and secreted by the cells of immune system, especially macrophages [18], which might play an important role in obesity and its associated comorbidities. The crucial question remains what is the main source of Visfatin responsible for its effects in obesity development? At the present, a prevailing hypothesis is that Visfatin is produced directly by adipocytes [4] of visceral fat tissue or the macrophages [18,26] present in the white adipose tissue during obesity development.

When different fractions of adipose tissue were examined, the stromal vascular fraction expressed significantly more Visfatin than the adipocyte fraction, which could be indicative of the fact that adipocytes are not the major source of Visfatin in the adipose tissue [26].

The hypothesis of the crucial role of macrophages in Visfatin production is further supported by the differences in Visfatin production among different types of adipose tissue. It seems that mRNA expression in visceral and subcutaneous fat seems to be virtually identical in non-obese individuals [26,36]. However, the differences are emerging in obese subjects. The visceral fat becomes a more potent producer of Visfatin [14], while the production of Visfatin in the subcutaneous tissue diminishes [36] or remains the same in obese individuals [4]. The question is whether the adipocytes are solely responsible for these changes or whether this is rather the role of macrophages, which are present in higher amount in visceral fat in obese individuals [18,35]?

Furthermore, the possible influence of gender has to be mentioned. In two studies [10,15], Visfatin plasma concentrations were significantly negatively correlated with BMI only in men. The different dispositions of adiposity in male and female are a widely recognized fact and this could allow for an interpretation of the findings from these studies. However, the negative correlation of Visfatin with BMI, without the gender influence, was reported in the study by Pagano et al. [14], positive correlations were observed in Berndt's work [36] and no correlation at all was observed in Varma's study [26], which makes the interpretation of the gender influences in Visfatin production a difficult issue.

BMI was also associated with Visfatin mRNA expression in visceral fat mass and these observations achieved statistical significance [14,26]. On the other hand, there was a negative correlation between BMI and mRNA expression in subcutaneous fat mass in these studies. The Visfatin mRNA expression in the adipose tissue is also related to the Visfatin plasma levels. Negative association of Visfatin plasma levels and Visfatin mRNA expression in subcutaneous adipose tissue was reported in some studies [14,36], conversely, there was a positive association with Visfatin expression in visceral fat mass [4,36].

Fukuhara et al. compared the Visfatin plasma levels with the total amount of visceral fat with positive results [4], however, Berndt et al. observed no correlations [36]. In both papers, the subjecfs visceral adiposity was estimated using the computed tomography.

A link between Visfatin plasma levels and lipid metabolism should be also discussed. Relatively many papers describe a positive association between plasma Visfatin and HDL-cholesterol [7,8,12], both in men and women, with an exception of the paper by Chen et al. [10], who observed positive association only in females and in addition to this reported negative realations of plasma Visfatin to LDL-cholesterol. Also, Flippatos et al. report negative correlation between HDLc and plasma Visfatin concentrations.

There is no unanimous consensus on the correlation between plasma Visfatin levels and triglycerides. The majority of researchers describe a positive correlation [6,9,11], but Wang et al. report significantly negative correlation and also suggest that relation of Visfatin to lipid profile is a result of its enzymatic properties in NAD metabolism rather than the dependence on visceral obesity or insulin resistance [8].

Visfatin was also studied in children where the plasma levels of Visfatin and BMI were showed positive correlation [39] or no correlation [7]. Almost all studies here found higher plasma Visfatin levels in obese children compared to the non/obese [7,38–40]. Kolsgaard et al. [38] also noticed that Visfatin plasma concentrations increased with number of metabolic syndrome components.

Conflict of interest

The authors have nothing to disclose.

Please cite this article in press as: Stastny J, et al. Visfatin and its role in obesity development. Diab Met Syndr: Clin Res Rev (2012), http://dx.doi.org/10.1016/j.dsx.2012.08.011

References

[1] Samal B, Sun Y, Stearns G, Xie C, Suggs S, McNiece I. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Molecular and Cellular Biology 1994;14(February (2)):1431–7.

[2] Jia SH, Li Y, Parodo J, Kapus A, Fan L, Rotstein OD, et al. Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. Journal of Clinical Investigation 2004;113(May (9)):1318–27.

[3] Rongvaux A, Shea RJ, Mulks MH, Gigot D, Urbain J, Leo O, et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. European Journal of Immunology 2002;32(November (11)):3225–34.

[4] Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science 2005;307(January (5708)):426–30.

[5] Moschen AR, Kaser A, Enrich B, Mosheimer B, Theurl M, Niederegger H, et al. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. Journal of Immunology 2007;178(February (3)):1748–58.

[6] Chang YC, Chang TJ, Lee WJ, Chuang LM. The relationship of visfatin/pre-B-cell colony-enhancing factor/nicotinamide phosphoribosyltransferase in adipose tissue with inflammation, insulin resistance, and plasma lipids. Metabolism Clinical and Experimental 2010;59(January (1)):93–9.

[7] Jin H, Jiang B, Tang J, Lu W, Wang W, Zhou L, et al. Serum visfatin concentrations in obese adolescents and its correlation with age and high-density lipoprotein cholesterol. Diabetes Research and Clinical Practice 2008;79(March (3)):412–8.

[8] Wang P, van Greevenbroek MM, Bouwman FG, Brouwers MC, van der Kallen CJ, Smit E, et al. The circulating PBEF/NAMPT/visfatin level is associated with a beneficial blood lipid profile. Pflugers Archiv 2007 Sep;454(6):971–6.

[9] Filippatos TD, Derdemezis CS, Kiortsis DN, Tselepis AD, Elisaf MS. Increased plasma levels of visfatin/pre-B cell colony-enhancing factor in obese and overweight patients with metabolic syndrome. Journal of Endocrinological Investigation 2007;30(April (4)):323–6.

[10] Chen CC, Li TC, Li CI, Liu CS, Lin WY, Wu MT, et al. The relationship between visfatin levels and anthropometric and metabolic parameters: association with cholesterol levels in women. Metabolism Clinical and Experimental 2007;56(September (9)):1216–20.

[11] Sun G, Bishop J, Khalili S, Vasdev S, Gill V, Pace D, et al. Serum visfatin concentrations are positively correlated with serum triacylglycerols and down-regulated by overfeeding in healthy young men. American Journal of Clinical Nutrition 2007;85(February (2)):399–404.

[12] Smith J, Al-Amri M, Sniderman A, Cianflone K. Visfatin concentration in Asian Indians is correlated with high density lipoprotein cholesterol and apolipoprotein A1. Clinical Endocrinology (Oxford) 2006;65(November (5)):667–72.

[13] Revollo JR, Körner A, Mills KF, Satoh A, Wang T, Garten A, et al. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. Cell Metabolism 2007;6(November (5)):363–75.

[14] Pagano C, Pilon C, Olivieri M, Mason P, Fabris R, Serra R, et al. Reduced plasma visfatin/pre-B cell colony-enhancing factor in obesity is not related to insulin resistance in humans. Journal of Clinical Endocrinology and Metabolism 2006;91(August (8)):3165–70.

[15] Jian WX, Luo TH, Gu YY, Zhang HL, Zheng S, Dai M, et al. The visfatin gene is associated with glucose and lipid metabolism in a Chinese population. Diabetic Medicine 2006;23(September (9)):967–73.

[16] Hug C, Lodish HF. Medicine. Visfatin: a new adipokine. Science 2005;307(January (5708)):366–7.

[17] Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, et al. Retraction. Science 2007;318(October (5850)):565.

[18] Curat CA, Wegner V, Sengenès C, Miranville A, Tonus C, Busse R, et al. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. Diabetologia 2006;49(April (4)):744–7.

[19] Garten A, Petzold S, Barnikol-Oettler A, Körner A, Thasler WE, Kratzsch J, et al. Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes. Biochemical and Biophysical Research Communications 2010;391(January (1)):376–81.

[20] Costford SR, Bajpeyi S, Pasarica M, Albarado DC, Thomas SC, Xie H, et al. Skeletal muscle NAMPT is induced by exercise in humans. American Journal of Physiology – Endocrinology and Metabolism 2010;298(January (1)):E117–26.

[21] Friebe D, Neef M, Kratzsch J, Erbs S, Dittrich K, Garten A, et al. Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans. Diabetologia 2011;54(May (5)):1200–11.

[22] Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes 2007;56(April (4)):901–11.

[23] Segawa K, Fukuhara A, Hosogai N, Morita K, Okuno Y, Tanaka M, et al. Visfatin in dipocytes is upregulated by hypoxia through HIF1alpha-dependent mechanism. Biochemical and Biophysical Research Communications 2006;349(October (3)):875–82.

[24] Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M, et al. Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes. Journal of Endocrinology 2005;185(June (3)):R1–8.

[25] Mayi TH, Duhem C, Copin C, Bouhlel MA, Rigamonti E, Pattou F, et al. Visfatin is induced by peroxisome proliferator-activated receptor gamma in human macrophages. FASEB Journal 2010;277(August (16)):3308–20.

[26] Varma V, Yao-Borengasser A, Rasouli N, Bodles AM, Phanavanh B, Lee MJ, et al. Human visutin expression: relationship to insulin sensitivity, intramyocellular lipids, and inflammation. Journal of Clinical Endocrinology and Metabolism 2007;92(February (2)):666–72.

[27] Mayi TH, Rigamonti E, Pattou F, Staels B, Chinetti-Gbaguidi G, Liver X. Receptor (LXR) activation negatively regulates visfatin expression in macrophages. Biochemical and Biophysical Research Communications 2011;404(January (1)):458–62.

[28] Choi KC, Ryu OH, Lee KW, Kim HY, Seo JA, Kim SG, et al. Effect of PPAR-alpha and -gamma agonist on the expression of visfatin, adiponectin, and TNF-alpha in visceral fat of OLETF rats. Biochemical and Biophysical Research Communications 2005;336(October (3)):747–53.

[29] Sun Q, Li L, Li R, Yang M, Liu H, Nowicki MJ, et al. Overexpression of visfatin/PBEF/Nampt alters whole-body insulin sensitivity and lipid profile in rats. Annals of Medicine 2009;41(4):311–20.

[30] Xie H, Tang SY, Luo XH, Huang J, Cui RR, Yuan LQ, et al. Insulin-like effects of visfatin on human osteoblasts. Calcified Tissue International 2007;80(March (3)):201–10.

[31] Chen MP, Chung FM, Chang DM, Tsai JC, Huang HF, Shin SJ, et al. Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. Journal of Clinical Endocrinology and Metabolism 2006;91(January (1)):295–9.

[32] El-Mesallamy HO, Kassem DH, El-Demerdash E, Amin AI. Vaspin and visfatin/Nampt are interesting interrelated adipokines playing a role in the pathogenesis of type 2 diabetes mellitus. Metabolism Clinical and Experimental 2011;60(January (1)):63–70.

[33] Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocrine Research 2000;21(December (6)):697–738.

[34] Tenenbaum A, Fisman EZ. "The metabolic syndrome. . .is dead": these reports are an exaggeration. Cerebrovascular Diseases 2011;10(January (1)):11.

[35] Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW. Obesity is associated with macrophage accumulation in adipose tissue. Journal of Clinical Investigation 2003;112(December (12)):1796–808.

[36] Berndt J, Klöting N, Kralisch S, Kovacs P, Fasshauer M, Schön MR, et al. Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. Diabetes 2005;54(October (10)):2911–6.

[37] Barth S, Klein P, Horbach T, Dötsch J, Rauh M, Rascher W, et al. Expression of neuropeptide Y, omentin and visfatin in visceral and subcutaneous adipose tissues in humans: relation to endocrine and clinical parameters. Obesity Facts 2010;3(August (4)):245–51 [Epub August 3, 2010].

[38] Kolsgaard ML, Wangensteen T, Brunborg C, Joner G, Holven KB, Halvorsen B, et al. Elevated visfatin levels in overweight and obese children and adolescents with metabolic syndrome. Scandinavian Journal of Clinical and Laboratory Investigation 2009;69(8):858–64.

[39] Davutoglu M, Ozkaya M, Guler E, Garipardic M, Gursoy H, Karabiber H, et al. Plasma visfatin concentrations in childhood obesity: relationships with insulin resistance and anthropometric indices. Swiss Medical Weekly 2009;139(January (1–2)):22–7.

[40] Haider DG, Holzer G, Schaller G, Weghuber D, Widhalm K, Wagner O, et al. The adipokine visfatin is markedly elevated in obese children. Journal of Pediatric Gastroenterology and Nutrition 2006;43(October (4)):548–9.

4.8. B-CELL ACTIVATING FACTOR (BAFF) – A NEW FACTOR LINKING IMMUNITY TO DIET?

Resumé

Faktor aktivující B lymfocyty (BAFF) je nedávno objeveným členem superrodiny ligandů TNF, u nějž bylo prokázáno, že kromě rozmanitých členů myeloidní řady je produkován i adipocyty. Již v minulosti byl intenzivně zkoumán u lymfoproliferativních chorob. Cílem této studie bylo prozkoumat vztah mezi plazmatickými hladinami BAFF a nemodifikovaným nativním složením stravy u obézní a neobézní české populace.

Vícerozměrné regresní modelování ukázálo, že hladiny BAFF slouží jako nezávislý prediktor celkové adipozity. Navíc byly korelovány i obvodem pasu a boků a také s celkovým zastoupením makronutrientů v nativní, nemodifikované stravě, kde hladiina BAFF korelovala s celkovým procentem energie odvozené ze sacharidů a tuků. Naše výsledky naznačují, že BAFF by mohl představovat elegantní molekulu spojující imunitní stav organismu s aktuální metabolickou odpovědí na stravu.

Cent. Eur. J. Med. • 7(3) • 2012 • 275-283
DOI: 10.2478/s11536-011-0153-7

VERSITA

Central European Journal of **Medicine**

B-cell activating factor (BAFF) – a new factor linking immunity to diet?

Research Article

Julie Bienertova-Vasku[1], Petr Bienert[1]*, Filip Zlamal[1], Josef Tomandl[2], Martin Forejt[3], Marie Tomandlova[2], Martin Vavrina[1], Jana Kudelková[1], Zbynek Splichal[1], Anna Vasku[1]

1 Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

2 Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

3 Department of Preventive Medicine, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

Received 24 May 2011; Accepted 9 January 2012

Abstract: B cell activation factor (BAFF) is a recently discovered member of the TNF ligand superfamily secreted by adipocytes, previously linked to autoimmune and lymphoproliferative disease. The aim of this study was to investigate the relationship between BAFF plasma levels and the non-modified, usual dietary composition as well as obesity-related anthropometric parameters in a cohort of 58 obese and non-obese Central-European Caucasian individuals. We found that BAFF had an independent predictive role for percentage of body fat; moreover, BAFF levels were correlated with waist and hip circumference. BAFF plasma levels were also significantly correlated with investigated dietary composition based on the 7-day food records, as the BAFF levels correlated with the percentage of energy derived from the carbohydrates and with energy derived from the dietary fat. Our results suggest that BAFF may play a role in linking the immune status and metabolic response to diet.

Keywords: *BAFF • Obesity • Dietary composition • Diet • Anthropometry*

1. Introduction

The B cell activation factor (BAFF), also known as THANK, TALL-1 or BLys, is a recently discovered member of the TNF ligand superfamily (TNFSF13B) [1,2]; it is best known for its role in the survival and differentiation of B cells. The biological effects of this type II transmembrane protein are mediated mainly via specific receptors: B cell maturation antigen (BCMA/TNFRSF17), transmembrane activation and calcium modulator and cyclophilin ligand interactor (TACI/TNFRSF13B), and BAFF receptor (BAFF-R/TNFRSF13C) that are present on B cells, plasma cells, but also on specific subpopulations of T cells [3,4]. BAFF is generally produced by myeloid pathway cells, malignant B cells, activated T cells, and the stromal cells of the bone marrow [2,5-7].

In humans, BAFF has been linked mainly to systemic autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis [8-10]. However, BAFF expression has also been reported to increase during adipocyte differentiation [11], whereas BAFF expression was augmented by TNF-α treatment and decreased by rosiglitazone treatment in that study. Kim *et al.* [11] have also reported BAFF secretion to be surprisingly lower in *ob/ob* mice sera compared with controls. Furthermore, those authors reported a considerable difference in mRNA and protein expression between epididymal tissue and visceral adipose tissue [11]. In a study by other authors, the presence of all known BAFF receptors (BAFF-R, BCMA, and TACI) was confirmed in adipocytes, and their expression was upregulated during adipocyte differentiation [12]. To

* *E-mail: jbienert@med.muni.cz*

summarize, it is highly likely that, apart from its role in the immune system, BAFF can be also considered an adipokine, which might have potentially huge consequences for comprehension of immune mechanisms within the white adipose tissue (WAT). As the BAFF expression is tightly related to the adipose tissue, it probably plays an important role in development of the low grade inflammation that is characteristic of obesity.

To date, little is known about the relationship between BAFF and the diet. Fabris *et al* [13] reported elevated BAFF plasma levels in a small cohort of patients with coeliac disease, and concluded that BAFF might play a pathogenic role in its development. Jee *et al.* [14] observed a relationship between food allergens and BAFF levels in a cohort of children with atopic dermatitis, suggesting that food allergens are more important for atopic dermatitis development than are aeroallergens; however, dietary composition was not investigated in their study.

Previously, it has been suggested that daily fasting serum leptin levels are different when comparing individuals on a diet with a high glycemic index and versus a low-glycemic-index diet [15]. Also, it has been reported that leptin, which is produced by adipocytes present mainly in the perilymphonodal adipose tissue, promotes differentiation of TH1 cells and secretion of pro-inflammatory cytokines (e.g. IFN-γ, TNFα) [16], and that it is highly likely that there is a direct link between the BAFF secretion and leptin plasma levels. In the study by Kim *et al.* [11] that measured BAFF serum levels in lean and ob/ob mice, BAFF secretion was surprisingly decreased in leptin-deficient ob/ob mice – which are generally resistant to the induction of autoimmune diseases. Therefore, we hypothesized that there could be a relationship between circulating BAFF plasma levels and diet composition, mainly in terms of proportion of fat and carbohydrates in the diet.

The aim of this study was to investigate the relationship between plasma levels of BAFF and the non-modified, usualdietary composition in an obese and non-obese Caucasian Central-European population and to evaluate possible associations of BAFF plasma levels with anthropometric parameters related to obesity.

2. Material and methods

2.1. Study subjects

We recruited 58 unrelated Czech Caucasian individuals for the present study in a mass media campaign, as described previously [17]; the inclusion and exclusion criteria have also been described elsewhere [18]. The study was conducted according to the guidelines of the Declaration of Helsinki; all procedures involving human subjects were approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine at Masaryk University (Brno, Czech Republic). Written informed consent was obtained from all subjects and was archived.

The study cohort was subdivided into two subgroups: the obese arm consisted of 36 obese individuals (BMI ≥ 30 kg/m^2; mean BMI 43.97 ± 6.39 kg/m^2; mean age 50.7 ± 12.8 years). The non-obese arm consisted of 22 healthy non-obese control subjects with no history of childhood obesity or eating disorder (mean BMI 23.14 ± 2.68 kg/m^2; mean age 40.3 ± 10.3 years). All the study subjects were available for leptin, soluble leptin receptor (sObR), and BAFF analyses.

2.2. Anthropometric characteristics

All phenotypic measurements were performed according to the standardized protocol and included weight, height, BMI, lean body mass, fat mass, body fat, waist and hip circumferences, waist-to-hip ratio, and skinfold thickness measured on four different loci. The quality of the measurement was repeatedly monitored during the study. Body composition was assessed by bioelectrical impedance analysis using the single frequency bioimpedance analyser (BodyStat Ltd, Douglas, Isle of Man, UK) with the subject in a supine position. The height measurement was performed with a calibrated stadiometer, and weight (in light indoor clothes and without shoes) was measured with a precisely calibrated set of scales.

2.3. Dietary intake

Participants were furthermore advised to complete 7-day food records augmented by the food frequency questionnaire of Willet *et al* [19] that has been validated for the Central-European population [20]. Food intake data were obtained from the study subjects and were further analyzed to establish the percentage of daily energy intake from carbohydrates, fat, and protein, as well as total energy and macronutrient intake. The nutritional analyses were performed using the Nutrimaster Diet Analysis software (Abbott Laboratories, Abbott Park, IL, USA). Special attention was paid to extreme snacking behaviour (defined as higher than 25% daily energy intake from snacks), eventual dieting, extreme portion sizes, and irregularity in eating. The structure of the daily energy intake was also investigated: a snacking

Table 1. Clinical, anthropometric and nutritional data in the obese and non-obese cohorts

	Non-obese (18 ≤ BMI < 30)		Obese (BMI > 30)		
	Female	Male	Female	Male	P-value
Subjects (n)	17	5	25	11	
Body composition					
Age (years)	40.2 ± 11.2	40.7 ± 7.9	52.9 ± 12.0	49.1 ± 14.7	< 0.001
BMI (kg/m2)	22.6 ± 2.5	25.0 ± 2.8	43.5 ± 5.7	45.2 ± 7.9	< 0.001
Body fat (%)	30.1 ± 6.7	16.2 ± 3.1	51.0 ± 6.3	39.3 ± 6.0	< 0.001
Dietary intake					
Energy (kJ)	5955 ± 632	8494 ± 1585	7140 ± 799	10050 ± 958	0.509
Protein (% energy)	14.1 ± 1.9	13.0 ± 1.0	15.8 ± 3.6	15.7 ± 3.3	0.007
Carbohydrates (% energy)	51.8 ± 4.8	53.5 ± 2.4	48.7 ± 6.7	48.7 ± 6.2	0.017
Fat (% energy)	34.2 ± 4.3	33.4 ± 2.7	35.5 ± 5.6	35.6 ± 5.1	0.220
Anthropometry					
Waist circumference (cm)	75.9 ± 7.8	87.4 ± 11.2	120.8 ± 12.4	135.8 ± 16.1	< 0.001
Hip circumference (cm)	96.6 ± 5.8	100.3 ± 8.3	134.7 ± 11.9	131.8 ± 15.1	< 0.001
Waist-hip ratio	0.79 ± 0.06	0.87 ± 0.07	0.90 ± 0.08	1.04 ± 0.08	< 0.001
Skinfold thickness (mm)					
Supraspinal skinfold	13.7 ± 4.2	10.4 ± 1.7	31.1 ± 10.9	31.4 ± 11.2	< 0.001
Subscapular skinfold	17.1 ± 5.1	15.0 ± 4.0	34.4 ± 9.2	35.9 ± 9.1	< 0.001
Bicepital skinfold	12.3 ± 3.7	8.0 ± 3.7	26.2 ± 8.1	24.9 ± 9.3	< 0.001
Tricepital skinfold	19.4 ± 5.2	13.4 ± 3.9	32.2 ± 5.7	29.5 ± 7.1	< 0.001
Sum of all skinfolds	62.5 ± 13.0	46.8 ± 9.4	122.6 ± 28.9	115.6 ± 34.2	< 0.001
Systolic blood pressure (mmHg)	112.3 ± 15.7	127.2 ± 8.8	144.0 ± 17.2	149.1 ± 19.9	< 0.001
Diastolic blood pressure (mmHg)	74.6 ± 9.1	80.2 ± 14.6	91.7 ± 12.8	97.5 ± 11.1	< 0.001
BAFF [pg/ml]	1007 ± 211	938 ± 118	1303 ± 333	1037 ± 210	

Results given as mean ± SD. P-value refers to the Mann-Whitney test for the comparison of the obese and non-obese cohort (males and females pooled together)

Table 2. Clinical and anthropometric data from the studied population in the study subgroups

	Non-obese 18 ≤ BMI < 30	Obese 30 ≤ BMI < 40	Morbidly obese BMI ≥ 40	Total	P-value
Gender M/F	5/17	2/6	9/19	16/42	
Age (years)	40.3 ±10.3^{A,B}	55.3 ± 9.7^{A}	50.8 ±13.5^{B}	47.4 ± 13.1	0.004
BMI (kg/m^2)	23.1 ± 2.7$^{\bullet}$	36.3 ± 4.2$^{\bullet}$	46.2 ± 5.1$^{\bullet A}$	36.1 ± 11.5	< 0.001
% body fat	27.0 ± 8.5$^{\bullet}$	40.6 ± 9.9	49.4 ± 6.6$^{\bullet}$	39.7 ± 12.9	< 0.001
Waist circumference (cm)	78.5 ± 9.7$^{\bullet b}$	111.9 ± 14.2$^{\bullet}$	129.2 ±13.3^{b}	107.6 ± 26.5	< 0.001
Hip circumference (cm)	97.5 ± 6.4$^{\bullet A}$	122.9 ±11.2^{A}	137.0 ±11.5$^{\bullet}$	120.0 ± 20.8	< 0.001
Waist-to-hip ratio	0.80 ± 0.07$^{\bullet A}$	0.91 ± 0.08^{A}	0.95± 0.11$^{\bullet}$	0.89 ± 0.11	< 0.001
Sum of skin fold thicknesses (mm)	58.9 ± 13.8$^{\bullet}$	99.3 ± 21.2	128.3 ± 24.1$^{\bullet}$	98.0 ± 37.4	< 0.001

Results given as mean ± SD. P-value refers to the Kruskal-Wallis test for the three groups (non-obese, obese, morbidly obese). The inter-group differences were tested using the Tukey-Kramer´s method with a correction for α.
$^{A, B}$ – $P < 0.05$ when comparing the pairs of the groups (non-obese x obese, non-obese x morbidly obese, obese x morbidly obese) on the same line;
^{a,b} – $P < 0.01$

are reported in Table 1, obese females had the highest BAFF levels; however, these observations lacked statistical significance.

In unvariate linear modelling, BAFF was significantly correlated with waist circumference (R = 0.432, $P < 0.05$) and body fat (R = 0.447, $P < 0.05$); this data is presented in Figure 3. In multivariate regression modeling across the entire cohort, BAFF expressed an independent prediction role for BMI (β = 0.380, $P = 0.005$; all P values adjusted for age, gender, smoking status). Moreover, BAFF had an independent predictive role for percentage of body fat (β = 0.310, $P = 0.009$), waist

Table 1. Clinical, anthropometric and nutritional data in the obese and non-obese cohorts

	Non-obese (18 ≤ BMI < 30)		Obese (BMI > 30)		
	Female	Male	Female	Male	P-value
Subjects (n)	17	5	25	11	
Body composition					
Age (years)	40.2 ± 11.2	40.7 ± 7.9	52.9 ± 12.0	49.1 ± 14.7	< 0.001
BMI (kg/m2)	22.6 ± 2.5	25.0 ± 2.8	43.5 ± 5.7	45.2 ± 7.9	< 0.001
Body fat (%)	30.1 ± 6.7	16.2 ± 3.1	51.0 ± 6.3	39.3 ± 6.0	< 0.001
Dietary intake					
Energy (kJ)	5955 ± 632	8494 ± 1585	7140 ± 799	10050 ± 958	0.509
Protein (% energy)	14.1 ± 1.9	13.0 ± 1.0	15.8 ± 3.6	15.7 ± 3.3	0.007
Carbohydrates (% energy)	51.8 ± 4.8	53.5 ± 2.4	48.7 ± 6.7	48.7 ± 6.2	0.017
Fat (% energy)	34.2 ± 4.3	33.4 ± 2.7	35.5 ± 5.6	35.6 ± 5.1	0.220
Anthropometry					
Waist circumference (cm)	75.9 ± 7.8	87.4 ± 11.2	120.8 ± 12.4	135.8 ± 16.1	< 0.001
Hip circumference (cm)	96.6 ± 5.8	100.3 ± 8.3	134.7 ± 11.9	131.8 ± 15.1	< 0.001
Waist-hip ratio	0.79 ± 0.06	0.87 ± 0.07	0.90 ± 0.08	1.04 ± 0.08	< 0.001
Skinfold thickness (mm)					
Suprascpinal skinfold	13.7 ± 4.2	10.4 ± 1.7	31.1 ± 10.9	31.4 ± 11.2	< 0.001
Subscapular skinfold	17.1 ± 5.1	15.0 ± 4.0	34.4 ± 9.2	35.9 ± 9.1	< 0.001
Bicepital skinfold	12.3 ± 3.7	8.0 ± 3.7	26.2 ± 8.1	24.9 ± 9.3	< 0.001
Tricepital skinfold	19.4 ± 5.2	13.4 ± 3.9	32.2 ± 5.7	29.5 ± 7.1	< 0.001
Sum of all skinfolds	62.5 ± 13.0	46.8 ± 9.4	122.6 ± 28.9	115.6 ± 34.2	< 0.001
Systolic blood pressure (mmHg)	112.3 ± 15.7	127.2 ± 8.8	144.0 ± 17.2	149.1 ± 19.9	< 0.001
Diastolic blood pressure (mmHg)	74.6 ± 9.1	80.2 ± 14.6	91.7 ± 12.8	97.5 ± 11.1	< 0.001
BAFF [pg/ml]	1007 ± 211	938 ± 118	1303 ± 333	1037 ± 210	

Results given as mean ± SD. P-value refers to the Mann-Whitney test for the comparison of the obese and non-obese cohort (males and females pooled together)

Table 2. Clinical and anthropometric data from the studied population in the study subgroups

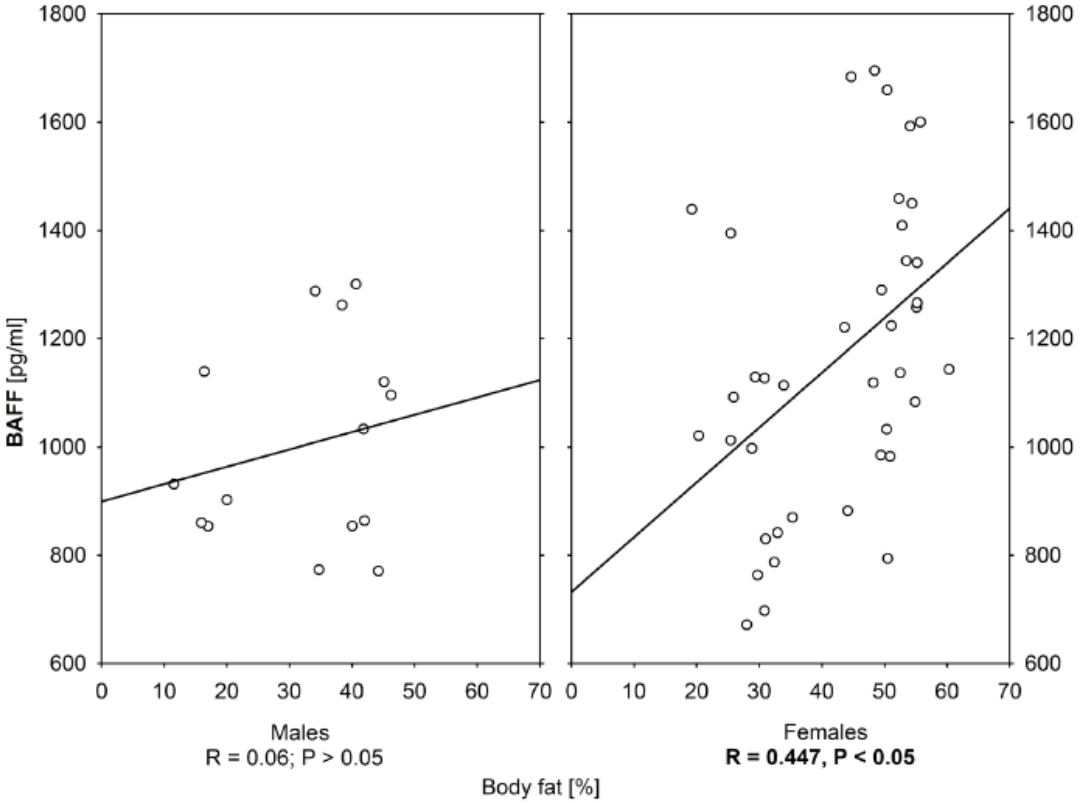
	Non-obese 18 ≤ BMI < 30	Obese 30 ≤ BMI < 40	Morbidly obese BMI ≥ 40	Total	P-value
Gender M/F	5/17	2/6	9/19	16/42	
Age (years)	40.3 ±10.3^{A,B}	55.3 ± 9.7^{A}	50.8 ±13.5^{B}	47.4 ± 13.1	0.004
BMI (kg/m^2)	23.1 ± 2.7^{a}	36.3 ± 4.2^{A}	46.2 ± 5.1^{a,A}	36.1 ± 11.5	< 0.001
% body fat	27.0 ± 8.5^{a}	40.6 ± 9.9	49.4 ± 6.6^{a}	39.7 ± 12.9	< 0.001
Waist circumference (cm)	78.5 ± 9.7^{a,b}	111.9 ± 14.2^{a}	129.2 ±13.3^{b}	107.6 ± 26.5	< 0.001
Hip circumference (cm)	97.5 ± 6.4^{a,A}	122.9 ±11.2^{A}	137.0 ±11.5^{a}	120.0 ± 20.8	< 0.001
Waist-to-hip ratio	0.80 ± 0.07^{a,A}	0.91 ± 0.08^{A}	0.95± 0.11^{a}	0.89 ± 0.11	< 0.001
Sum of skin fold thicknesses (mm)	58.9 ± 13.8^{a}	99.3 ± 21.2	128.3 ± 24.1^{a}	98.0 ± 37.4	< 0.001

Results given as mean ± SD. P-value refers to the Kruskal-Wallis test for the three groups (non-obese, obese, morbidly obese). The inter-group differences were tested using the Tukey-Kramer´s method with a correction for α.
^{A,B} – P < 0.05 when comparing the pairs of the groups (non-obese x obese, non-obese x morbidly obese, obese x morbidly obese) on the same line;
^{a,b} – P < 0.01

are reported in Table 1, obese females had the highest BAFF levels; however, these observations lacked statistical significance.

In unvariate linear modelling, BAFF was significantly correlated with waist circumference (R = 0.432, $P < 0.05$) and body fat (R = 0.447, $P < 0.05$); this data is presented in Figure 3. In multivariate regression modeling across the entire cohort, BAFF expressed an independent prediction role for BMI (β = 0.380, $P = 0.005$; all P values adjusted for age, gender, smoking status). Moreover, BAFF had an independent predictive role for percentage of body fat (β = 0.310, $P = 0.009$), waist

Figure 2. Correlation between BAFF and percentage of body fat by gender. The correlation coefficient was determined using the Pearson's test; the observed trend is indicated by the solid line.



circumference (β = 0.312, *P* = 0.01), and hip circumference (β = 0.392, *P* = 0.003), but not on waist-to-hip ratio (WHR) (β = 0.06, *P* = 0.547).

3.4. Correlation of BAFF levels with nutritional parameters

In the multivariate regression modeling, BAFF plasma levels were significantly correlated with percentage of energy derived from the carbohydrates (β = 0.38, *P* = 0.01), whereas BMI was also a significant predictor (β = −0.30, *P* = 0.04). BAFF plasma levels also served as an independent predictor for the proportion of energy derived from the dietary fat (β = −0.36, *P* = 0.02) independently of age, gender, smoking status, and BMI. No significant associations of BAFF levels with total daily energy intake, percentage of energy derived from proteins, dietary fibre intake, or dietary cholesterol intake were observed.

As BAFF plasma levels were significantly correlated with the percentage of energy derived from carbohydrates and fats, we investigated the differences in BAFF plasma levels between the highest, middle, and lowest tertile of carbohydrate/fat intake (Table 3). The results of the tertile analysis did not reveal any significant ORs of the categories we investigated (upper, lower tertile of carbohydrate/fat intake) for elevated BAFF in plasma. No association between BAFF plasma levels and abnormal eating behavior (irregular food intake, extreme portion sizes, increased snacking index) was observed.

3.5. Correlation of BAFF with leptin and soluble leptin receptor

In multivariate regression models for prediction of BMI using the available plasma levels of leptin, sObR, and BAFF, only BAFF plasma levels were significantly correlated with BMI (β = 0.438, *P* = 0.003). No significant correlation of BAFF plasma levels with plasma leptin or sObR was observed.

Furthermore, the bivariate analysis was performed to assess possible associations of LEP, sObR and the LEP:sObR ratio, and BAFF and dietary characteristics. To control for possible confounders, the results from the bivariate correlation analysis were consecutively explored using multivariate analysis with logarithmically

Figure 3. Correlation between BAFF and waist circumference by gender. The correlation coefficient was determined using the Pearson's test; the observed trend is indicated by the solid line.

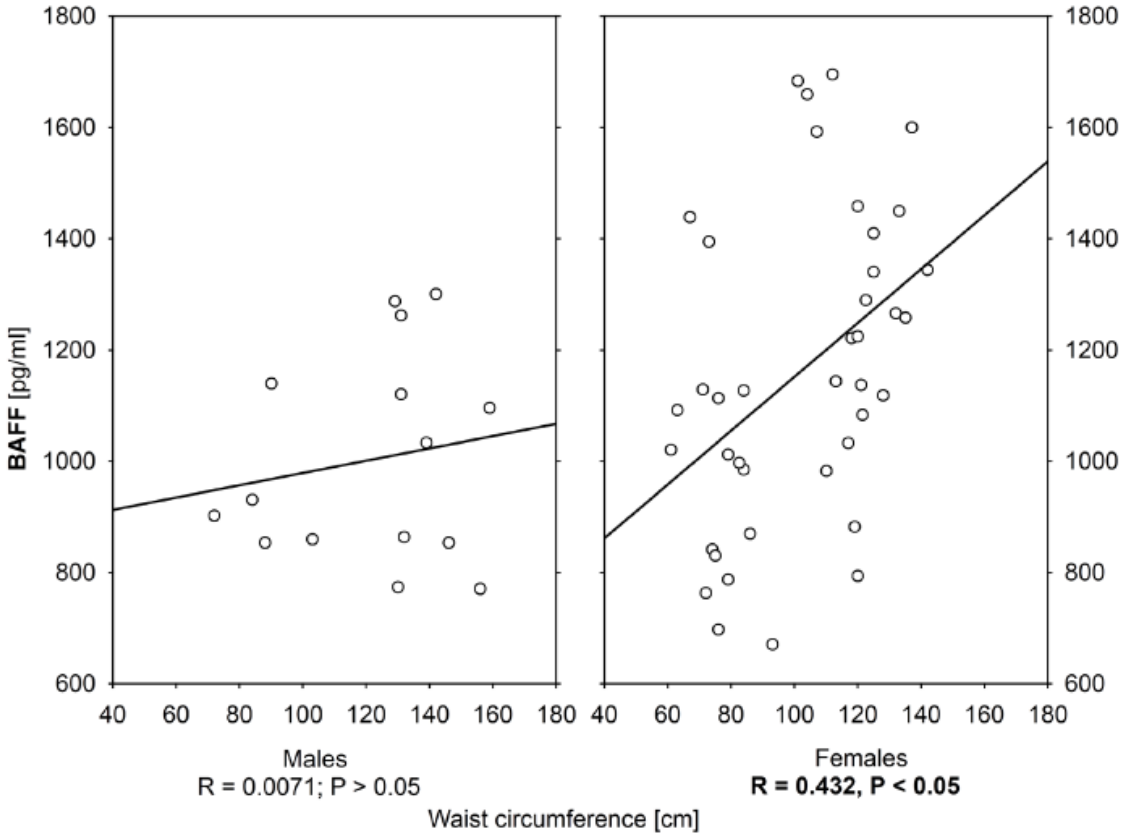


Table 3. Association between the upper and lower tertiles of fat and carbohydrate intake for elevated or normal BAFF plasma levels in the study cohorts

FATS	BAFF elevated	BAFF not elevated	OR	95% CI	P -value
Total	13	45	0.406	0.084–1.947	0.22
Upper tertile	3	16			
Lower tertile	6	13			
CARBOHYDRATES	**BAFF elevated**	**BAFF not elevated**	**OR**	**95% CI**	**P -value**
Total	13	45	0.254	0.044–1.475	0.11
Upper tertile	2	17			
Lower tertile	6	13			

OR – odds ratio,
CI – confidence interval, elevated values defined as BAFF plasma levels higher than the 97.5th percentile (1374 ng/ml) of the control plasma samples

transformed plasma LEP and sObR and LEP: the sObR ratio regressed on total energy intake as well as on the energy intake provided by each macronutrient. However, no significant associations were observed in these analyses.

4. Discussion

BAFF represents a fundamental cytokine of the T-independent IgA class-switching recombination system through the interaction with the common receptor TACI shared also by other proteins, such as APRIL [21]. In this

study, we report elevated BAFF levels among Central-European Caucasian patients with obesity, and we also demonstrate substantial dependence of differences in BAFF plasma levels on gender.

The role of BAFF in obesity is unclear, and it seems to be related to the role of BAFF in the low-grade inflammation typical of obesity. It has been recently demonstrated that BAFF is expressed in adipocytes and that BAFF expression is augmented via TNF-alfa treatment [11]. Moreover, all the three types of known BAFF receptors (BAFF-R, BCMA, and TACI) are expressed in adipocytes and are upregulated during adipocyte differentiation. In agreement with this, is the expression of BAFF mRNA and protein reported in *ob/ob* mice [11].

In a recent study by Hamada et al [22], BAFF levels in the sera and visceral adipose tissue (VAT) of obese mice were investigated. In obese mice, the BAFF levels were preferentially increased in VAT and sera compared with these levels in normal control mice. BAFF also induced alterations in the expression levels of genes related to insulin resistance in adipocytes *in vivo*. In addition, BAFF directly affected the glucose uptake and phosphorylation of insulin receptor substrate-1 in adipocytes. Therefore, the present authors conclude that paracrine BAFF and BAFF-receptor (BAFF-R) interaction in VAT can lead to impaired insulin sensitivity via inhibition of insulin signaling pathways and alterations in adipokine production.

It is difficult to define whether the observed high BAFF phenotype in obese individuals is a priori linked to obesity as a result of a specific shared genetic background, or whether it results from consecutive changes in cytokine profiles during obesity development. Previously, it has been suggested that limited BAFF signaling leads only marginally towards selection against higher affinity autoreactive B cells, whereas BAFF overexpression leads to broad tolerance escape and positive selection of autoreactive cells. In animal experiments, the B cells in BAFF/3H9 mice were elevated in number, used a broad L chain repertoire, including L chains generating high-affinity autoreactivity, and produced abundant autoantibodies [23], making BAFF an outstanding candidate for being a factor linking obesity and autoimmune diseases. It seems to be highly likely that increased BAFF levels favor the development of autoimmune and also lymphoproliferative diseases, which is also consistent with empiric observations that obese individuals are more prone to certain types of autoimmune diseases and cancer [24]. It has also been reported that the presence of an underlying IgA deficiency (IgAD) characterized by increased BAFF levels could represent a further risk factor for lymphoproliferative diseases in course of autoimmune diseases, such as systemic rheumatologic diseases, suggesting also a BAFF-targeted therapeutic interventions might be of advantage under specific circumstances, such as IgAD [25].

Our results show that plasma BAFF levels are gender-dependent, proportional to BMI, waist circumference, percentage of body fat, and significantly different between obese and non-obese individuals. This is consistent with observations that adipocytes are capable of producing BAFF and that they carry BAFF receptors capable of binding BAFF [3,4]. The pivotal role of BAFF in adipogenesis has recently been proposed [12] and the site-dependant differences in BAFF expression might possibly contribute to differences in body fat distribution, making patients with higher BAFF circulating levels more prone to develop abdominal obesity than the others.

In this study, we also observed multiple associations of BAFF plasma levels with dietary composition parameters, the possible explanation of which could be the presence of a leptin-BAFF axis, as proposed recently by Kim *et al.* [11]. Decreased responsiveness to leptin was documented in mice fed a high-fat diet (HFD), probably in relation to gender and/or duration of exposure to diet and/or the strain of mice [26,28]. The reduced activity of STAT-3 in lymphocytes of mice kept on an HFD is consistent with prior reports showing decreased STAT-3 activity in the hypothalamus of HFD mice [29]. This effect of an HFD has recently been found to be associated, at least in part, with an increased level of the suppressor of cytokine signaling 3 (SOCS3), acting also as an inhibitor of leptin signaling [29]. In the study by Papathanassoglou *et al.* [30], ObR/STAT-3-mediated signaling in T lymphocytes was decreased in the diet-induced obese mouse model of obesity and leptin resistance. This research group demonstrated that the leptin receptor (ObR) is expressed on normal mouse lymphocyte subgroups and that leptin plays a role in lymphocyte survival as it alters the ObR/STAT-3-mediated signaling in T cells in relation to the proportion of fat in the diet. On the whole, the data in the Papathanassoglou *et al.* study support the hypothesis that nutrition status acting via leptin-dependent mechanisms might significantly alter the strength and quality of the immune response, and based on our data, it can be suggested that BAFF is a missing piece in this pathway.

The major limitation of our study is its failure to find a significant association between circulating BAFF and leptin levels, which is detrimental to the study hypothesis. However, this study was performed on a small population sample, and we presume the effect could be observed on a larger cohort. Moreover, some of the variations in diet parameters could be at least partially attributed to seasonal variation, and therefore, another

study in a prospective design investigating food records and BAFF levels in various seasons of the year would be also advantageous. Finally, the lack of observation of an association between circulating BAFF and leptin levels does not exclude presence of other mechanisms linking the BAFF plasma levels to dietary composition observed in this study.

In conclusion, we report here multiple associations of BAFF plasma levels with anthropometric determinants of obesity as well as with dietary composition in the Caucasian Central-European population. The results provide some evidence that BAFF might play an important role in linking body composition and risk of autoimmune or malignant diseases.

References

[1] Moore PA, Belvedere O, Orr A, Pieri K, LaFleur DW, Feng P, Soppet D, Charters M, Gentz R, Parmelee D, Li Y, Galperina O, Giri J, Roschke V, Nardelli B, Carrell J, Sosnovtseva S, Greenfield W, Ruben SM, Olsen HS, Fikes J, Hilbert DM. BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 1999;285:260–263

[2] Nardelli B, Belvedere O, Roschke V, Moore PA, Olsen HS, Migone TS, Sosnovtseva S, Carrell JA, Feng P, Giri JG, Hilbert DM. Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood* 2001;97(1):198-204

[3] Mackay F, Browning JL. BAFF: a fundamental survival factor for B cells. *Nat Rev Immunol* 2002;2:465-475

[4] Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, Hession C, Schneider P, Sizing ID, Mullen C, Strauch K, Zafari M, Benjamin CD, Tschopp J, Browning JL, Ambrose C. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* 2001;293:2108-2111

[5] Novak AJ, Bram RJ, Kay NE, Jelinek DF. Aberrant expression of B-lymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. *Blood* 2002;100:2973-2979

[6] Scapini P, Nardelli B, Nadali G, Calzetti F, Pizzolo G, Montecucco C, Cassatella MA. G-CSF stimulated neutrophils are a prominent source of functional. *BLyS J Exp Med* 2003;197:297-302

[7] Lavie F, Miceli-Richard C, Quillard J, Roux S, Leclerc P, Mariette X. Expression of BAFF (BLyS) in T cells infiltrating labial salivary glands from patients with Sjogren's syndrome. *J Pathol* 2004;202:496-502

[8] Krumbholz M, Theil D, Derfuss T, Rosenwald A, Schrader F, Monoranu CM, Kalled SL, Hess DM, Serafini B, Aloisi F, Wekerle H, Hohlfeld R, Meinl E. BAFF is produced by astrocytes and up-regulated in multiple sclerosis lesions and primary central nervous system lymphoma. *J Exp Med* 2005;201:195–200

[9] Huard B, Arlettaz L, Ambrose C, Kindler V, Mauri D, Roosnek E, Tschopp J, Schneider P, French LE. BAFF production by antigen-presenting cells provides T cell co-stimulation. *Int Immunol* 2004;16:467–475

[10] Zhang J, Roschke V, Baker KP, Wang Z, Alarcón GS, Fessler BJ, Bastian H, Kimberly RP, Zhou T. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J Immunol* 2001;166:6-10

[11] Kim YH, Choi BH, Cheon HG, Do MS. B cell activation factor (BAFF) is a novel adipokine that links obesity and inflammation. *Exp Mol Med* 2009;41(3):208-216

[12] Alexaki VI, Notas G, Pelekanou V, Kampa M, Valkanou M, Theodoropoulos P, Stathopoulos EN, Tsapis A, Castanas E. Adipocytes as immune cells: differential expression of TWEAK, BAFF, and APRIL and their receptors (Fn14, BAFF-R, TACI, and BCMA) at different stages of normal and pathological adipose tissue development. *J Immunol* 2009;183(9):5948-5956

[13] Fabris M, Visentini D, De Re V, Picierno A, Maieron R, Cannizzaro R, Villalta D, Curcio F, De Vita S, Tonutti E. Elevated B cell-activating factor of the tumour necrosis factor family in coeliac disease. *Scand J Gastroenterol.* 2007;42(12):1434-1439

Acknowledgement

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript, in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors.

The present study was supported by a project of the Danone Institute of the Czech Republic (DANONE/2007) focused on genetic variability of adipokines in obese individuals.

The authors are grateful to Jolana Lipková and Marian Hlavna for excellent technical assistance. We thank to Katerina Heczkova, Zlata Piskackova and Lenka Kucerova for their assistance in processing and analysing food records.

[14] Jee HM, Kim KW, Hong JY, Sohn MH, Kim KE. Increased serum B cell-activating factor level in children with atopic dermatitis. *Clin Exp Dermatol.* 2010;35(6):593-598
[15] Agus MS, Swain JF, Larson CL, Eckert EA, Ludwig DS. Dietary composition and physiologic adaptations to energy restriction. *Am J Clin Nutr* 2000;71(4):901-907
[16] La Cava A, Matarese G. The weight of leptin in immunity. *Nat Rev Immunol* 2004;4: 371-379
[17] Bienertová-Vasků J, Bienert P, Forejt M, Tomandl J, Brázdová Z, Vasků A. A genotype x nutrient association of common polymorphisms in obesity-related genes with food preferences and time structure of energy intake. *Br J Nutr* 2010;103(3):352-359
[18] Ma Y, Bertone ER, Stanek EJ 3rd, Reed GW, Hebert JR, Cohen NL, Merriam PA, Ockene IS. Association between eating patterns and obesity in a free-living US adult population. *Am J Epidemiol* 2003;158:85–92
[19] Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, Hennekens CH, Speizer FE. Reproducibility and Validity of A Semiquantitative Food Frequency Questionnaire. *Am J Epidemiol* 1985;122: 51-65
[20] Boylan S, Welch A, Pikhart H, Malyutina S, Pajak A, Kubinova R, Bragina O, Simonova G, Stepaniak U, Gilis-Januszewska A, Milla L, Peasey A, Marmot M, Bobak M. Dietary habits in three Central and Eastern European countries: the HAPIEE study. *BMC Public Health* 2009;1;9: 439
[21] Fabris M, De Vita S, Visentini D, Fabro C, Picierno A, Lerussi A, Villalta D, Alessio MG, Tampoia M, Tonutti E. B-lymphocyte stimulator and a proliferation-inducing ligand serum levels in IgA-deficient patients with and without celiac disease. *Ann N Y Acad Sci.* 2009;1173:268-273
[22] Hamada M, Abe M, Miyake T, Kawasaki K, Tada F, Furukawa S, Matsuura B, Hiasa Y, Onji M. B cell-activating factor controls the production of adipokines and induces insulin resistance. *Obesity (Silver Spring).* 2011;19(10):1915-1922
[23] Ota M, Duong BH, Torkamani A, Doyle CM, Gavin AL, Ota T, Nemazee D. Regulation of the B Cell Receptor Repertoire and Self-Reactivity by BAFF. *J Immunol.* 2010;185(7):4128-4136
[24] Enzler T, Bonizzi G, Silverman GJ, Otero DC, Widhopf GF, Anzelon-Mills A, Rickert RC, Karin M. Alternative and classical NF-kappa B signaling retain autoreactive B cells in the splenic marginal zone and result in lupus-like disease. *Immunity* 2006;25(3):403-415
[25] Fabris M, Quartuccio L, Sacco S, De Marchi G, Pozzato G, Mazzaro C, Ferraccioli G, Migone TS, De Vita S. B-Lymphocyte stimulator (BLyS) up-regulation in mixed cryoglobulinaemia syndrome and hepatitis-C virus infection. *Rheumatology (Oxford)* 2007;46(1):37-43
[26] Harris RB, Bowen HM, Mitchell TD. Leptin resistance in mice is determined by gender and duration of exposure to high-fat diet. *Physiol Behav* 2003;78(4-5):543-555
[27] Della-Fera MA, Li C, Baile CA. Resistance to IP leptin-induced adipose apoptosis caused by high-fat diet in mice. *Biochem Biophys Res Commun* 2003;303(4):1053-1057
[28] Prpic V, Watson PM, Frampton IC, Sabol MA, Jezek GE, Gettys TW. Differential mechanisms and development of leptin resistance in A/J versus C57BL/6J mice during diet-induced obesity. *Endocrinology* 2003;144:1155–1163
[29] Münzberg H, Flier JS, Bjørbaek C. Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. *Endocrinology* 2004;145:4880–4889
[30] Papathanassoglou E, El-Haschimi K, Li XC, Matarese G, Strom T, Mantzoros C. Leptin receptor expression and signaling in lymphocytes: kinetics during lymphocyte activation, role in lymphocyte survival, and response to high fat diet in mice. *J Immunol* 2006;176(12):7745-7752

4.9. VARIABILITY IN CNR1 LOCUS INFLUENCES PROTEININTAKE AND SMOKING STATUS IN THE CENTRAL-EUROPEAN POPULATION

Resumé

Endokannabioidní receptor typu 1 (CB1) je kódován CNR1 genem. V nedávné době bylo zjištěno, že hraje důležitou úlohu při regulaci sytosti potravního chování – je schopen modulovat metabolickou odpověď i samotný příjem potravy.

Cílem naší studie bylo prozkoumat potenciál tří jednonukleotidových polymorfismů v CNR1 genu z hlediska nativních potravních preferencí ve vzorku typické středoevropské populace.

Do studie bylo zařazeno celkem 258 jedinců ze středoevropské populace a byly u nich genotypizovány polymorfismy rs1049353, rs12720071 a rs806368 v CNR1 genu. U jedinců ve studii bylo dále bioimpedanční metodou hodnoceno tělesné složení a změřeny různé antropometrické parametry subjektů (obvod pasu, obvod boků, tloušťka kožních řas). Jejich nativní stravovací zvyklosti byly vyhodnoceny na základě 7denní záznamové metody i pomocí FFQ dotazníku, sledováno bylo i kouření.

Alelické varianty a běžné haplotypy v CNR1 genu byly statisticky významně spojeny se zastoupením jednotlivých makronutrientů v nativní stravě jedinců, a to bez ohledu na jejich fyzickou aktivitu.

Běžný haplotyp v genu pro CNR1 byl dále spojen s výskytem kouření, resp. množstvím vykouřených cigaret za den i roky kouření.

Naše výsledky ve studované středoevropské populaci naznačují, že specifické genetické varianty v CNR1 genu můžeme považovat za markery vnímavosti u specifického složení stravy i vnímavosti ke kouření.

Research article

Variability in CNR1 locus influences protein intake and smoking status in the Central-European population

Julie Bienertova-Vasku[1], Petr Bienert[1], Lenka Slovackova[1], Lenka Sablikova[1], Zlata Piskackova[2], Martin Forejt[2], Zbynek Splichal[1], Filip Zlamal[1], Anna Vasku[1]

[1]Department of Pathological Physiology, Medical Faculty, Masaryk University, Kamenice 5, Brno, Czech Republic, [2]Department of Preventive Medicine, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, Czech Republic

Objectives: The endocannabinoid receptor 1 (CB1) is encoded by the CNR1 gene and has been recently recognized to play an important role in the regulation of satiety and feeding behaviour with a huge potential of modulating metabolic response and feeding control. The aim of the study was to investigate the potential of three selected single nucleotide polymorphisms (SNPs) in the CNR1 locus on native dietary composition in the Central-European Caucasian population.
Methods: A total of 258 unrelated individuals originating from the Central-European Caucasian population were enrolled into the study and rs1049353, rs12720071, and rs806368 polymorphisms in CNR1 locus were examined in these individuals using PCR-based methodology. Body composition was assessed using a bioimpedance method, various anthropometric parameters were investigated (waist and hip circumference, skin folds), and native dietary composition was analysed using 7-day food records as well as a food frequency questionnaire.
Results: Allelic variations and common haplotypes in the CNR1 gene were associated with the daily intake of proteins, fluids, and fibre, regardless of the physical activity of the individuals. The common haplotype in the CNR1 gene was associated with self-reported smoking (number of cigarettes per day, smoking years).
Discussion: Our results indicate that specific genetic variations in the CNR1 gene may act as susceptibility markers for specific dietary composition in the Central-European population.

Keywords: Endocannabinoids, SNP, Dietary composition, Macronutrients, Gene

Introduction

The endocannabinoid system (ECS) has been recognized to play a pivotal role in the regulation of feeding behaviour in mammals. Both its endogenous and synthetic agonists cause orexigenic behaviour at the central level and modulate cascade of peptides involved in the regulation of feeding behaviour; moreover, ECS significantly contributes to the hedonic appraisal of food intake and increasing attention is being paid to the role of ECS in peripheral metabolic regulation and the paracrine effects of ECS.

Animal experiments suggest that agonists of ECS can increase the rewarding value of food and diminish latency for intake as well as induce food intake in satiated animals whose motivation to eat is highly suppressed.[1,2] The ECS is strongly tied to the brain reward system and thus expresses pervasive influence on addictive behaviour. Indeed, it is generally well known that consumption of cannabis triggers voracious appetite, especially for sweet food.[1] In 1970, Tart[3] described that marijuana intoxication induces appreciation of new qualities of food. A subsequent study by Sofia and Knobloch[4] supported the hypothesis for tetrahydrocannabinol (THC) involvement in sweet preference regulation. More of these empirical observations could be explained following the discovery of specific ECS receptors found in food intake-controlling centres such as hypothalamic nuclei or the dorsal motor nucleus of the vagus nerve.[2,5,6] These receptors are generally expressed in peptidergic hypothalamic circuits regulating food intake, such as neurons in the arcuate nucleus (ARC) secreting pro-opiomelanocortin / cocaine- and amphetamine-regulated transcript (POMC/CART), neurons of the lateral hypothalamic nucleus (LHN) releasing

Correspondence to: Julie Bienertova-Vasku, Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno 625 00, Czech Republic. Email: jbienert@med.muni.cz

Melanin concentrating hormone (MCH) and orexin, and periventricular nucleus (PVN) neurons secreting corticotropin-releasing hormone (CRH).[7] The actual signalling in the endocannabinoid pathway is mediated via the binding of 2-arachidonylglycerol (2-AG) and ananadamide (AEA) to endocannabinoid receptor 1 (CNR1).[8] Subsequently, the CNR1 antagonists have been developed as potential therapeutic agents for the treatment of obesity and its comorbidities,[9–12] where these agonists were reported to induce weight loss, reduce food intake, improve glucose metabolism, and regulate plasma lipid levels in humans.[13]

Based on these findings, we postulated our primary hypothesis that the CNR1 locus, positioned 6q14–q15, controls dietary and behavioural characteristics in humans, representing possible risk factors for obesity development or modulating the severity of the disease. We presumed that rather than the risk of obesity itself as a metabolic disease, it could be the specific dietary habits which are associated with the CNR1 locus.

For the purpose of the study a total of three common polymorphisms in the CNR1 gene (rs1049353, rs12720071, and rs806368) were selected, all of which were previously reported to be associated with obesity-related anthropometric traits. The possible relationships between these polymorphisms and dietary composition, patterns of food intake, and specific patterns of physical activity in the study subjects were thus investigated.

Material and methods

Subjects

A total of 258 unrelated Czech Caucasian individuals were recruited for this study in a mass media campaign addressing the population of the South Moravian Region of the Czech Republic as described previously and – for the purposes of genotype and allele frequencies comparisons – assigned (according to their body mass index (BMI)) either to the non-obese or the obese cohort.[14] The inclusion and exclusion criteria were derived from Ma *et al.*[15] The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno and was performed in accordance with the Declaration of Helsinki Guidelines. Participants gave their written informed consent before they entered the study; the written consent forms were subsequently archived.

The obese subgroup consisted of 160 obese individuals (BMI > 30 kg/m^2; mean BMI 37.5 ± 6.4 kg/m^2, mean age 50.0 ± 11.5 years). The non-obese group comprised a total of 98 healthy normal-weight control subjects with no history of childhood obesity or eating disorders (20 < BMI < 30 kg/m^2; mean BMI 25.2 ± 3.2 kg/m^2, mean age 37.5 ± 12.4 years); for the purposes of some analyses, the non-obese group was further divided into a normal-weight (20 < BMI ≤ 25 kg/m^2), and overweight (25 < BMI ≤ 30 kg/m^2) cohort.

Data on personal or family history of obesity, birth weight, age at onset of obesity, eating disorders, age of menarche and menopause in women, family history of sterility, infertility, or stillbirth were obtained by a professional using a semi-structured interview. A positive family history for obesity was established as one obese relative with BMI ≥ 30 kg/m^2 in the close family (siblings, parents and their siblings, and grandparents). Both the obese cases and the non-obese controls underwent the same subset of examinations focusing on their anthropometric characteristics, dietary intake, and genetic background of both the individual and the family; the subjects of the study were also interviewed with respect to their smoking status and history.

Anthropometric characteristics

All phenotypic measurements were performed by a specialist and included weight, height, BMI, lean body mass, total body fat, waist and hip circumferences, waist-to-hip ratio (WHR), skin-fold thickness. Body composition was assessed by bioelectrical impedance analysis, using the single-frequency bio-impedance analyser BodyStat (Bodystat Ltd, Douglas, Isle of Man, UK) with the subject lying in a supine position.

Pedometer-determined physical activity

To roughly estimate the physical activity of the study individuals, all participants were provided with an Omron HJ113 E pedometer (OMRON Corp., Tokyo, Japan) and a daily step log in order to perform a 7-day record of their physical activity, simultaneously with the 7-day food records.

Participants were instructed to wear the pedometer throughout all waking hours for a period of 7 days, only removing it when bathing or showering. The correct position to wear the pedometer was on the waistband in line with the midline of the thigh and this position was demonstrated to participants at the beginning of the study. Each night before going to bed participants recorded the number of steps displayed in their log. The pedometer was then reset for each following day, according to the method described by Clemes *et al.*[16]

All participants were encouraged not to change their typical daily routine of work and leisure activities. On finishing the study, the participants were questioned as to whether they had suffered from any ill health or forgotten to wear the pedometer for an entire day or whether they made any changes to their daily routine, diet, or general activity during the study period.

Dietary intake

The dietary habits of the subjects were assessed simultaneously with physical activity records using a modified diet history method as described by Larsson

et al.,[17] which considers the entire diet, including cooking methods.

The method combined quantitative and semi-quantitative measurements of dietary intake, using a version of a food frequency questionnaire which surveys the regularly consumed foods during last 7 days. For each food item, usual intake frequency and portion size were given. Portion sizes were estimated using a booklet with pictures of the different food items with varying portion sizes. All cooked meals and beverages during 7 days were recorded in the menu book, including all ingredients of each meal. At the start of the study, the subjects were instructed individually by a dietician how to fill out the questionnaires. After 2–3 weeks later, the subjects returned to the dietician individually and their eating habits were consulted.

The dietician recorded the usual amount consumed by the subject of each food item in the food frequency questionnaire and the 7-day food record. Participants were furthermore advised to complete 7-day food records. Food intake data obtained from the study subjects were analysed and energy and nutrient intake were calculated using the Nutrimaster Diet Analysis software (Abbott Laboratories, Abbott Park, IL, USA) adjusted for the Czech population. Special attention was paid to extreme snacking behaviour, dieting, extreme portion sizes, and irregularity in eating.

Selected quantitative parameters were followed, including: total energy intake per day; per cent of energy intake in fat, carbohydrate, and protein; per cent of total energy intake per day and servings per day for each food group; cholesterol, saturated fat, and fibre intake; and grams of carbohydrates per day and the percentage of energy derived from carbohydrates.

Genotyping

DNA for analyses was extracted from 5 ml of the patients' saliva collected after a 3-hour-long fasting period. The selection of the polymorphisms was based on their previous association with obesity-related anthropometric traits. Genotyping of the polymorphisms was performed as described previously using a standard polymerase chain reaction (PCR)-based methodology with following restriction-fragment-length polymorphism.[18,19] Restricted fragments were separated by electrophoresis on 2% agarosis gels with ethidium bromide staining. To assess genotyping reliability, we performed double sampling in more than 20% of the samples and found no differences. We always used quality control, and negative controls were used to identify possible false-positives. The genotyping success was 100% for all of the included SNPs.

Statistics

The genotype distributions were tested for the Hardy–Weinberg equilibrium by a set of chi-square tests. Allelic frequencies were estimated by a 'counting method' and differences in allele frequencies between case and control subjects were tested by likelihood ratio chi-square tests for 2×2 tables (two alleles, case vs. control subjects). Where applicable, it was first determined whether the variable presented a normal distribution using the Kolmogorov–Smirnov test, and in cases of skewed variables, logarithmic transformation was performed. For descriptive purposes, mean values are presented using untransformed values. Results are expressed as mean ± SD unless otherwise stated.

In order to identify genetic as well as non-genetic variables which may contribute to predicting the anthropometric phenotype or nutritional phenotype, we carried out a forward stepwise logistic regression, a sequential procedure of adding one input variable at a time to build up a regression model in which the dependent variable (i.e. presence or absence of obesity) is represented as the linear combination of independent variables (anthropometric and nutritional parameters and genotypes of three investigated SNPs). In this analysis, the codes of genotypes were used as quantitative variables (AA = 0, AB = 1, BB = 2).

Odds ratios were calculated for the multiple logistic regression analysis models; we adjusted for covariates including age (continuous), BMI (<23, 23–24.9, 25–29.9, 30–34.9, or ≥35 kg/m^2), gender, smoking (never, past, and current), alcohol intake (non-drinker and drinker (0.1–4.9, 5–10, or >10 g/day), family history of obesity, and menopausal status in females.

Using sample tertiles, the nutrient variables were categorized in three groups of equal size (the upper third, the middle third, and the lower third) as described by Santos *et al.*[20] Each nutrient variable was then included in logistic regressions as a binary indicator leaving one category as reference.

Data analysis was performed using the Statistica v. 9.0 (Statsoft Inc., Tulsa, OK, USA) programme package at a significance level defined as $P < 5\%$. An analysis of haplotypes was performed using the Haploview program package (http://www.broad.mit.edu/haploview/).

Results

The general characteristics of the study participants with respect to their dietary composition are shown in Table 1. No significant differences were observed in total energy intake, percentage of energy derived from dietary proteins, carbohydrates or fats when comparing the cases, and the controls both together and separately according to their gender. Moreover, no significant differences were observed when dividing the study subjects into three tertiles in a tertile analysis.

The entire investigated population was found to be in Hardy–Weinberg equilibrium at the investigated CNR1 loci. The genotype frequencies in the total

Table 1 Baseline patients' characteristics

Variable	Obese (BMI ≥ 30)		Non-obese (BMI ≤ 30)	
	Females	Males	Females	Males
Body composition				
N	118	42	74	24
Age (years)	50.65 ± 10.92	48.44 ± 13.06	38.06 ± 12.74	35.81 ± 11.50
BMI (kg/m^2)	37.50 ± 6.40	37.50 ± 6.64	25.01 ± 3.44	25.76 ± 2.32
Body fat (%)	46.09 ± 5.83	32.84 ± 6.26	31.02 ± 7.32	19.12 ± 4.63
Dietary intake				
Energy (MJ)	7.77 ± 2.46	10.59 ± 3.56	7.76 ± 1.87	10.96 ± 2.49
Protein (daily intake in g)	69.77 ± 22.37	90.02 ± 31.14	65.31 ± 17.59	87.08 ± 23.50
Carbohydrates (daily intake in g)	228.90 ± 70.04	304.47 ± 110.22	232.60 ± 58.46	329.98 ± 86.12
Fat (daily intake in g)	71.57 ± 28.28	97.61 ± 37.36	71.79 ± 21.76	99.91 ± 30.00
Tertiles of carbohydrate intake *N* (%)				
1	50 (23)	8 (8)	26 (19)	2 (3)
2	80 (37)	22 (22)	62 (45)	8 (13)
3	84 (40)	69 (70)	51 (36)	54 (84)

study cohort were 55, 39, and 6% for AA, AG, and GG (1359 G/A); 83, 15, and 2% for AA, AG, and GG (3138 G/A); and 66, 30, and 4% for CC, CT, and TT (4895 C/T), respectively. Significant differences were observed for the CNR1 3813 A/G polymorphism genotype as well as allele frequencies when comparing the obese subjects (30 ≤ BMI) with the non-obese ones (BMI < 30) ($p_g = 0.02$, $p_a = 0.39$).

Haplotype analysis

Five common haplotypes with the general population frequency over 1% were estimated (Table 2). The linkage disequilibrium (LD) plot of the investigated SNPs is presented in Fig. 1. Five common haplotypes ($f \geq 1\%$) were estimated: $A_{1359}A_{3813}C_{4895}$ (AAC), $G_{1359}A_{3813}C_{4895}$ (CAG), $A_{1359}A_{3813}T_{4895}$ (AAT), $A_{1359}G_{3813}T_{4895}$ (AGT), and $A_{1359}G_{3813}C_{4895}$ (AGC). The established frequency of the $A_{1359}G_{3813}C_{4895}$ haplotype in the obese cohort was under 1%.

Table 2 Haplotype frequencies of CNR1 haplotypes in studied populations according to their obesity status

Haplotype 1359/3813/4895	Total	Obese	Non-obese
AAC	0.560	0.580	0.562
CAG	0.243	0.222	0.243
AAT	0.098	0.093	0.094
AGT	0.080	0.096	0.078
AGC	0.011	0.006	0.011

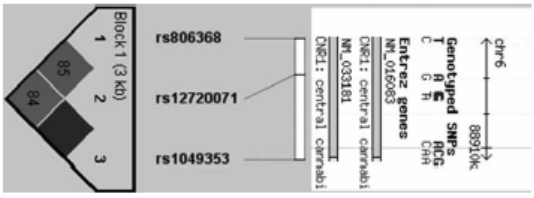


Figure 1 Haploview linkage disequilibrium plot of the *CNR1* single nucleotide polymorphisms rs1049353, rs12720071, and rs806368.

Effects of CNR1 polymorphisms on anthropometric characteristics (BMI, WHR, total body fat, skin-fold thickness)

In the next step, the entire cohort was tested with respect to whether any of the studied SNPs expressed significant effects on obesity-related anthropometric characteristics. For the CNR1 1359 G/A, a significant increase in the percentage of body water was observed among AA homozygotes (45.9%) and AG heterozygotes (46.7%) in comparison with GG carriers (50.5%) ($P = 0.03$). In the case of the CNR1 3813 G/A polymorphism, the AG heterozygotes presented with the highest thickness of the triceptal skin fold (an average thickness of 36 mm) when compared with the AA (25 mm) and GG (21.5 mm) homozygotes ($P = 0.05$). Moreover, significant differences in diastolic blood pressure were observed across the genotypes; the carrying of the 3813 *G* allele was associated with the highest diastolic BP both in GG homozygote (92 mmHg) and AG heterozygote genotypes (mean 93.5 mmHg), compared to AA (86.3 mmHg) ($P = 0.002$).

In the multivariate regression modelling across all study subjects, the CNR1 3813 G/A polymorphism expressed an independent prediction role on BMI, this association remained significant after adjustment for age and sex ($\beta = 0.14$, $P = 0.05$) but lost its significance following the introduction of the daily step count into the analysis ($\beta = -0.06$, $P = 0.21$). Furthermore, this SNP also expressed an independent prediction role on the total percentage of body fat ($\beta = 0.15$, $P = 0.05$), where the gender of subjects played a significant prediction role ($\beta = 0.49$, $P < 0.00001$), independent of the daily step count.

The AGC haplotype was associated with the highest thickness of the supraspinal skin fold (29.2 mm) and there was a significant difference when compared with the GAC haplotype with the lowest supraspinal fold thickness (19.2 mm, $P = 0.03$).

In the multivariate modelling of systolic blood pressure, both the CNR1 3813 G/A and CNR1

4895 C/T served as independent predictors for systolic blood pressure ($\beta = 0.23$, $P = 0.002$; $\beta = -0.19$, $P = 0.009$, respectively) along with the gender of subjects ($\beta = -0.13$, $P = 0.03$). The AGC haplotype was significantly associated with the highest average systolic blood pressure (mean 149 mmHg); on the other hand, the AAT haplotype was associated with the lowest systolic blood pressure (124 mmHg, $P = 0.03$).

Effects of CNR1 polymorphisms on smoking status and history of the study subjects

None of the polymorphisms investigated separately were significantly associated with current smoking status, previous smoking history, total years spent smoking per lifetime, or the calculated number of cigarettes per lifetime. The AGC haplotype was significantly associated with the average number of cigarettes per day in smokers (average count 30 per day in AGC carriers compared to the GAC haplotype with the lowest number of cigarettes per day (6 cigarettes per day, $P = 0.03$).

Effects of CNR1 polymorphisms on dietary characteristics of the study subjects

As the GG genotype of CNR1 3813 G/A was substantially less frequent, we assumed the G dominant mode of inheritance (GG + AG vs. AA). In multivariate regression modelling, both the CNR1 3813 G/A and CNR1 4895 C/T served as independent predictors for total daily intake of fluids ($\beta = -0.17$, $P = 0.02$; $\beta = 0.18$, $P = 0.02$, respectively). The CNR1 3813 G/A polymorphism served as an independent predictor for the total amount of proteins in food ($\beta = 0.15$, $P = 0.05$), whereas the GG homozygotes were associated with the lowest daily protein intake (59.5 g per day) when compared with AA and AG genotypes (72.7, respectively 78.6 g per day). However, none of the investigated polymorphisms served as a predictor for food intake of fat or carbohydrates, both in univariate and multivariate analyses.

The results of the tertile analysis of carbohydrate intake in relation to CNR 3813 G/A did not reveal any significant associations. No significant effects of any of investigated polymorphisms on average daily intake of carbohydrates were observed when dividing the study subjects into three subgroups according to the tertile of daily energy intake derived from carbohydrates.

Table 3 presents the stratified multivariate analyses of total carbohydrate intake across the whole study population of 258 individuals in relation to CNR1 genotypes. None of the presented models adjusted for age, sex, and percentage of body fat (model 1), plus total energy intake per day (model 2), or total carbohydrate intake (model 3) was significantly associated with the investigated genotypes except for the 3813 G/A polymorphism in model 1 ($P = 0.02$).

Table 3 Standardized beta coefficients (95% confidential intervals) for associations total protein intake by CNR1 1359 G/A, 3813 G/A, and 4895 C/T polymorphisms: multivariate regression analysis across the total study cohort

	CNR1 1359G/A			CNR1 3813 G/A			CNR1 4895 C/T		
Model	AG + GG	AA	*P* for interaction	GG + GA	AA	*P* for interaction	TC + CC	TT	*P* for interaction
1	−0.02 (−0.14–0.10)	0.02 (−0.10–0.14)	0.12	−0.11 (−0.35–0.11)	0.01 (−0.23–0.25)	0.02	0.05 (−0.08–0.18)	−0.03 (−0.16–0.10)	0.23
2	−0.04 (−0.11–0.03)	−0.05 (−0.12–0.02)	0.13	0.01 (−0.13–0.15)	−0.05 (−0.19–0.09)	0.44	−0.03 (−0.10–0.04)	−0.03 (−0.10–0.04)	0.45
3	−0.01 (−0.10–0.07)	−0.03 (−0.12–0.05)	0.43	−0.04 (−0.22–0.16)	0.01 (−0.19–0.16)	0.67	−0.02 (−0.11–0.07)	−0.03 (−0.11–0.07)	0.65

Model 1: total fat intake adjusted for age, sex, and % of body fat.
Model 2: model 1 + total energy intake per day.
Model 3: model 1 + total carbohydrate intake.

Table 4 Mean (SD) demographic data and step counts for normal-weight, overweight, and obese participants (along with the data for the complete cohort)*

	Normal weight ($n = 40$)	Overweight ($n = 58$)	Obese ($n = 160$)	Between-group differences (P value)	All ($n = 258$)
	32 females 8 males	42 females 16 males	118 females 42 males		192 females 66 males
Age (years)	33.3 (9.4)*,†	40.4 (13.4)*,°	50.1 (11.5)†,°	<0.001	45.3 (13.3)
Height (cm)	170.7 (9.2)	169.2 (9.2)	167.0 (9.3)	0.041	168.1 (9.4)
Weight (kg)	64.2 (9.0)*,†	78.7 (9.4)*,°	104.7 (20.5)†,°	<0.001	92.6 (23.5)
BMI (kg/m^2)	22.0 (2.1)*,†	27.4 (1.5)*,°	37.5 (6.5)†,°	<0.001	32.8 (8.1)
Mean steps/day	8779 (3011)*	8009 (2600)†	6679 (2892)*,†	<0.001	7303 (2957)

BMI, body mass index, calculated by weight in kilograms divided by height in metres squared.
Between-group differences were tested by the Kruskal–Wallis test.
*,†,°Significant differences between groups, following *post-hoc* analyses (all $P < 0.05$).

Table 5 Mean (SD) daily step counts calculated for each day of the week, for normal-weight, overweight, and obese participants (along with data for the complete sample)

	Normal weight ($n = 40$)	Overweight ($n = 58$)	Obese ($n = 160$)	Between-group differences (P value)	All ($n = 258$)
	32 females 8 males	42 females 16 males	118 females 42 males		192 females 66 males
Monday	9122 (3526)*	8631 (3798)	7093 (3876)*	<0.001	7753 (3888)
Tuesday	8329 (4658)	7838 (3398)	6725 (3643)	0.012	7224 (3807)
Wednesday	8683 (3957)*	8522 (3786)†	6864 (3719)*,†	<0.001	7518 (3849)
Thursday	9635 (4357)*	8407 (4165)	7037 (3897)*	<0.001	7748 (4134)
Friday	9210 (3978)*	8455 (3722)†	7139 (4170)*,†	<0.001	7756 (4112)
Saturday	9241 (6224)*	7043 (3864)	6253 (4672)*	<0.001	6896 (4880)
Sunday	7232 (3782)	7167 (4338)	5666 (3728)	0.007	6246 (3936)
Within-group differences	0.117	0.061	<0.001		<0.001
Within-group differences*	0.513	0.784	0.921		0.468

BMI, body mass index, calculated by weight in kilograms divided by height in metres squared.
Between-group differences were tested by the Kruskal–Wallis test.
*,† Significant differences between groups, following *post-hoc* analyses (all $P < 0.05$).

Furthermore, the AGC haplotype was also significantly associated with the lower protein intake per day (62.5 g per day) as opposed to AGT carriers with the highest protein intake (77.5 g per day, $P = 0.01$).

None of the investigated polymorphisms or haplotypes was associated with investigated eating patterns (time structure of daily energy intake, extreme snacking, and excessive portion sizes).

Effects of CNR1 polymorphisms on the patterns of physical activity of the study subjects

For the purposes of the analysis of physical activity as described by Clemes *et al.*,[16] the study participants were divided into three subgroups: normal weight ($20 < \text{BMI} \leq 25$), overweight ($25 < \text{BMI} \leq 30$), and obese ($\text{BMI} \geq 30$). The demographic characteristics of the three BMI groups, and of the complete sample, are presented in Table 4; the analysis of the day-of-the-week effect is summarized in Table 5. The mean step counts reported by the normal-weight, overweight, and obese groups differed significantly ($P < 0.001$). Generally, there was a significant trend for all three BMI groups to report a decrease in activity on Sunday ($P = 0.007$), the normal-weighted subjects were significantly younger than the obese participants and therefore a stratified analysis by age was

Table 6 Mean (SD) daily step counts calculated over 7-day study for normal-weight, overweight, and obese participants stratified by age group (along with the data for the whole cohort)

	Normal weight ($n = 40$)		Overweight ($n = 58$)		Obese ($n = 160$)		Between-group differences (P value)	All ($n = 258$)	
	N	Steps	*N*	Steps	*N*	Steps		*N*	Steps
16–29 (years)	18	7979 (3434)	14	8890 (2844)	7	7724 (3919)	0.310	39	8260 (3272)
30–45 (years)	17	9290 (2711)*	22	7223 (2046)	49	6473 (3098)*	<0.001	88	7205 (2968)
46–71 (years)	5	9925 (1797)	22	8234 (2816)	104	6705 (2728)	0.004	40	7085 (2815)
Within-group differences		0.131		0.182		0.511			0.158

BMI, body mass index, calculated by weight in kilograms divided by height in metres squared.
Between-group differences were tested by the Kruskal–Wallis test.
*Significant differences between groups, following *post hoc* analyses (all $P < 0.05$).

performed across the study cohorts (Table 6). In the multivariate modelling, the 4895 C/T polymorphism was associated significantly with the daily step count on a working day ($\beta = 0.16$, $P = 0.03$).

Discussion

Cannabis is one of the most common illegal drugs worldwide and the cannabinoids have been used in therapy for centuries, e.g. the first evidence of the use of cannabis for medical purposes was recorded in China in ca. 4000 BC. Cannabis has been used in therapy mainly for its potential in appetite stimulation, indeed, cannabis users often report persistent hunger and a craving for sweets, although this does not result in significant weight gain among cannabis users.[21]

Numerous authors have hypothesized that a number of genes influence the reward pathways and may contribute to a 'reward deficiency syndrome' associated with, e.g. alcohol and drug abuse.[22–24] As the food intake also represents a 'reward-controlled' system, we hypothesized that the common polymorphisms within the CNR1 gene could significantly influence dietary composition or patterns of food intake with special attention paid to the increased intake of carbohydrates and extreme snacking behaviour, independently of physical activity carried out by the individual. As all three investigated polymorphisms were previously reported to be associated with specific anthropometric traits, possible associations with various anthropometric determinants of obesity were also investigated.

This study presents multiple significant associations between the 1359 G/A polymorphism located in codon 453 of the coding exon in the CNR1 gene and 3813 G/A and 4895 C/T polymorphisms within the 3′ region of CNR1 gene and anthropometric parameters related to obesity as well as some of the investigated dietary parameters. The carriers of the 1359A allele presented with significantly higher body water percentage. The carriers of the 3813 G allele were significantly more frequent in the obese cohort and, moreover, the 3813 G allele was associated with BMI, percentage of body fat, and higher diastolic blood pressure; however, only until adjustments for the daily step count were made. In the dietary analysis, the 3813 G/A polymorphism served as an independent predictor for daily fibre intake and the total amount of proteins in food. The heterozygote AG genotype of 3813 G/A was significantly associated with the highest thickness of the triceptal skin fold, when compared with both the homozygote genotypes. It has been previously reported by Russo *et al.*[19] that the 3813 G allele was associated with waist circumference and BMI; this could be partially in accordance with our findings as we observed significant association of the allele with BMI. However, no association of 3813 A/G polymorphism with waist circumference was observed in our study. The 4895 C/T polymorphism expressed an independent prediction role on the percentage of body water and diastolic blood pressure; however, no association with BMI and waist circumference (WC) was observed. Thus, although our study supports the hypothesis of the involvement of CNR1 variability in influencing various anthropometric parameters, we do not confirm the relationship of investigated polymorphisms with waist circumference reported by Russo *et al.*[19]

Furthermore, haplotype analysis revealed multiple associations of the common AGC haplotype with behavioural and anthropometric characteristics (supraspinal skin fold thickness, systolic blood pressure, low protein intake, and number of cigarettes per day for smokers), these remained significant even after all relevant adjustments had been made. However, as the overall population frequency of the AGC haplotype was limited to approximately 1%, we considered this haplotype a rare population variant (although – based on our results – a highly risky one), its related behavioural impact definitely requiring further investigation. As the number of AGC cases was extremely low in our study, any conclusions regarding this variant must be considered rather speculative.

The limitations of the study include a significant lack of data on the phenotypic effects of the investigated polymorphisms, which makes further functional studies a necessity in order to determine the exact genotype–phenotype correlations. Moreover, the number of subjects included in the study was limited, which makes further studies on larger population samples necessary. On the other hand, it should also be mentioned that the study was carried out on a highly static, unrelated population from a wide region of Moravia (part of the Czech Republic) settled by a Slavonic population. In a large meta-analysis of the European populations, the Moravian population has been recently reported to be genetically slightly different from the rest of the Czech Republic, which could be explicable by the fact that Moravia has a long-shared history with the remainder of the Czech Republic, but is nevertheless separated from the rest of the country by the Czech-Moravian highlands, which hindered stronger intermixing in the past.[25] Therefore, we assume that the associations discussed cannot be easily attributed to selection bias. In a study by Muller *et al.*,[26] conducted on the German – geographically highly related – population of children and adolescents, no evidence in favour of the association of any of investigated CNR1 alleles, including the 1359 G/A polymorphism, with obesity was observed, which stands in contrast to the association of the 3813G/A polymorphism with obesity observed in our group.

Despite the above-mentioned limitations, this study indicates that common genetic variations in the endocannabinoid type-1 receptor gene may act as attractive susceptibility markers for specific dietary characteristics and obesity-related anthropometric traits.

Acknowledgements

The study was supported by a grant of Ministry of Education of the Czech Republic No. 881/2006 and by the DANONE/2007 project of the Danone Institute, focusing on the genetic variability of adipokines in obese individuals. The authors are grateful to Svatava Tschoplová for technical assistance and to all study participants for their participation in the project.

References

1 Arias Horcajadas F. Cannabinoids in eating disorders and obesity. Mol Neurobiol 2007;36(1):113–28.
2 Kirkham TC, Williams CM, Fezza F, Di Marzo V. Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol. Br J Pharmacol 2002;136(4):550–7.
3 Tart CT. Marijuana intoxication common experiences. Nature 1970;226(5247):701–4.
4 Sofia RD, Knobloch LC. Comparative effects of various naturally occurring cannabinoids on food, sucrose and water consumption by rats. Pharmacol Biochem Behav 1976;4(5):591–9.
5 Barna I, Zelena D, Arszovszki AC, Ledent C. The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: in vivo and in vitro studies in CB1 receptor knockout mice. Life Sci 2004;75(24):2959–70.
6 Derbenev AV, Stuart TC, Smith BN. Cannabinoids suppress synaptic input to neurones of the rat dorsal motor nucleus of the vagus nerve. J Physiol 2004;559(Pt 3):923–38.
7 Cota D, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M, *et al.* The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. J Clin Invest 2003;112(3):423–31.
8 Hillard CJ. Biochemistry and pharmacology of the endocannabinoids arachidonylethanolamide and 2-arachidonylglycerol. Prostaglandins Other Lipid Mediat 2000;61(1–2):3–18.
9 Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet 2005;365(9468):1389–97.
10 Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J. Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. J Am Med Assoc 2006;295(7):761–75.
11 Ravinet Trillou C, Arnone M, Delgorge C, Gonalons N, Keane P, Maffrand JP, *et al.* Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. Am J Physiol Regul Integr Comp Physiol 2003;284(2):R345–53.
12 Scheen AJ, Finer N, Hollander P, Jensen MD, Van Gaal LF. Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomised controlled study. Lancet 2006;368(9548):1660–72.
13 Despres JP, Golay A, Sjostrom L. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. N Engl J Med 2005;353(20):2121–34.
14 Bienertova-Vasku J, Bienert P, Tomandl J, Forejt M, Vavrina M, Kudelkova J, *et al.* No association of defined variability in leptin, leptin receptor, adiponectin, proopiomelanocortin and ghrelin gene with food preferences in the Czech population. Nutr Neurosci 2008;11(1):2–8.
15 Ma Y, Bertone ER, Stanek III EJ, Reed GW, Hebert JR, Cohen NL, *et al.* Association between eating patterns and obesity in a free-living US adult population. Am J Epidemiol 2003;158(1):85–92.
16 Clemes SA, Hamilton SL, Lindley MR. Four-week pedometer-determined activity patterns in normal-weight, overweight and obese adults. Prev Med 2008;46(4):325–30.
17 Larsson H, Elmstahl S, Berglund G, Ahren B. Evidence for leptin regulation of food intake in humans. J Clin Endocrinol Metab 1999;83(12):4382–5.
18 Gadzicki D, Muller-Vahl K, Stuhrmann M. A frequent polymorphism in the coding exon of the human cannabinoid receptor (CNR1) gene. Mol Cell Probes 1999;13(4):321–3.
19 Russo P, Strazzullo P, Cappuccio FP, Tregouet DA, Lauria F, Loguercio M, *et al.* Genetic variations at the endocannabinoid type 1 receptor gene (CNR1) are associated with obesity phenotypes in men. J Clin Endocrinol Metab 2007;92(6):2382–6.
20 Santos JL, Boutin P, Verdich C, Holst C, Larsen LH, Toubro S, *et al.* Genotype-by-nutrient interactions assessed in European obese women. A case-only study. Eur J Nutr 2006;45(8):454–62.
21 Hollister L. Marijuana (cannabis) as medicine. J Cannabis Ther 2001;1:5–28.
22 Blum K, Chen AL, Chen TJ, Rhoades P, Prihoda TJ, Downs BW, *et al.* LG839: anti-obesity effects and polymorphic gene correlates of reward deficiency syndrome. Adv Ther 2008;25(9):894–913.
23 Blum K, Braverman ER, Holder JM, Lubar JF, Monastra VJ, Miller D, *et al.* Reward deficiency syndrome: a biogenetic model for the diagnosis and treatment of impulsive, addictive, and compulsive behaviors. J Psychoactive Drugs 2001;32:I–IV.
24 Comings DE, Blum K. Reward deficiency syndrome: genetic aspects of behavioral disorders. Prog Brain Res 2000;126:325–41.
25 Nelis M, Esko T, Mägi R, Zimprich F, Zimprich A, Toncheva D, *et al.* Genetic structure of Europeans: a view from the North-East. PLoS One 2009;4(5):e5472.
26 Muller TD, Reichwald K, Wermter AK, Bronner G, Nguyen TT, Friedel S, *et al.* No evidence for an involvement of variants in the cannabinoid receptor gene (CNR1) in obesity in German children and adolescents. Mol Genet Metab 2007;90(4):429–34.