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Studijní obor: **Biochemie a patobiochemie**

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Význam biosyntetické a katabolické dráhy cholesterolu u nádorových a zánětlivých onemocnění

The importance of biosynthetic and catabolic pathway of cholesterol in inflammatory and tumor diseases

Disertační práce

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ABSTRAKT

Předkládaná práce se zabývá významem meziproduktů biosyntetické a katabolické dráhy cholesterolu. Cílem první části práce bylo zjistit, zda statiny (inhibitory HMG-CoA reduktasy) mají protinádorový účinek a zda se účinky jednotlivých statinů liší.

Druhá část práce je zaměřena především na využití 7α-hydroxycholest-4-en-3-onu (C4), nadějného markeru aktivity cholesterol 7α-monooxygenasy (CYP7A1) a malabsorpce žlučových kyselin.

Na experimentálním modelu adenokarcinomu pankreatu jsme potvrdili protinádorové účinky statinů. Ty se však mezi jednotlivými statiny v závislosti na jejich fyzikálně-chemických vlastnostech značně lišily. Naše data ukazují, že nejpravděpodobnějším (i když ne jediným) mechanismem protinádorového účinku statinů je snížení prenylace signalizačních proteinů, převážně Ras protoonkogenu.

Podařilo se nám zavést spolehlivou metodu stanovení C4 a s její pomocí prokázat, že -203A>C polymorfismus v genu kódujícím CYP7A1 může ovlivňovat její aktivitu, že za diurnální variabilitou aktivity CYP7A1 pravděpodobně stojí insulin a že insulinová rezistence u pacientů s nealkoholovou steatosou jater dochází k poškození zpětnovazebé inhibice CYP7A1, která může následně přispět k progresi onemocnění. Konečně jsme demonstrovali důležitost laboratorního stanovení malabsorpce žlučových kyselin u pacientů s Crohnovou chorobou.

Klíčová slova: Crohnova choroba, CYP7A1, cholesterol, karcinom pankreatu, malabsorpce žlučových kyselin, statiny

ABSTRACT

This thesis focuses on the importance of intermediate products of biosynthetic and catabolic pathway of cholesterol. The aim of the first part of the thesis is mainly to investigate, whether statins (HMG-CoA reductase inhibitors) possess antitumor properties and to compare the differences in antitumor potential of individual statins.

The other part of the thesis aims at the utilization of 7α-hydroxycholest-4-en-3-one (C4), a promising marker of cholesterol 7α-monooxygenase (CYP7A1) activity and bile acid malabsorption.

We demonstrated antitumor effect of statins on an experimental model of pancreatic cancer. Individual statins, however, differed significantly in their efficacy, depending on their physico-chemical properties. Our data suggests, that the most likely (but not the only) mechanism of antitumor effect of statins is decreased prenylation of signaling proteins, especially Ras protooncogene.

We set up a reliable method for measurement of C4, which facilitated our research in CYP7A1 regulation. We demonstrated, that promoter polymorphism -203A>C might affect CYP7A1 activity, that diurnal variability of CYP7A1 activity might be triggered by insulin, and that insulin resistance in patients with non-alcoholic fatty liver disease impedes the feedback regulation of CYP7A1, which may lead to disease progression. Finally, we demonstrated the importance of laboratory investigation of bile acid malabsorption in Crohn's disease patients.

Keywords: bile acid malabsorption, cholesterol, Crohn's disease, CYP7A1, pancreatic cancer, statins

PODĚKOVÁNÍ

Rád bych poděkoval především svému školiteli, profesoru Liboru Vítkovi, který mě přivedl ke studiu metabolismu cholesterolu a žlučových kyselin a v průběhu studia mě trvale motivoval a podporoval. Doktoru Martinu Hřebíčkovi a docentu Milanu Jirsovi patří můj dík za zasvěcení do tajů molekulární biologie, profesoru Milanu Lukášovi a doktorce Daně Ďuričové za příkladnou spolupráci na klinických studiích, inženýrce Heleně Gbelcové a celému kolektivu Hepatologické laboratoře (především pak inženýrce Miroslavě Zelenkové, inženýru Jaroslavu Zelenkovi a magistře Lence Dudkové) za vydatnou pomoc v laboratoři. Za finanční podporu účasti na zahraničních kongresech bych chtěl poděkovat nadaci „Nadání Josefa, Marie a Zdeňky Hlávkových“ a České společnosti klinické biochemie.

Konečně bych chtěl poděkovat své rodině za trpělivost a podporu v průběhu celého studia.

OBSAH

1. ÚVOD

1.1. Biosyntetická dráha cholesterolu

1.1.1. Biosyntéza cholesterolu

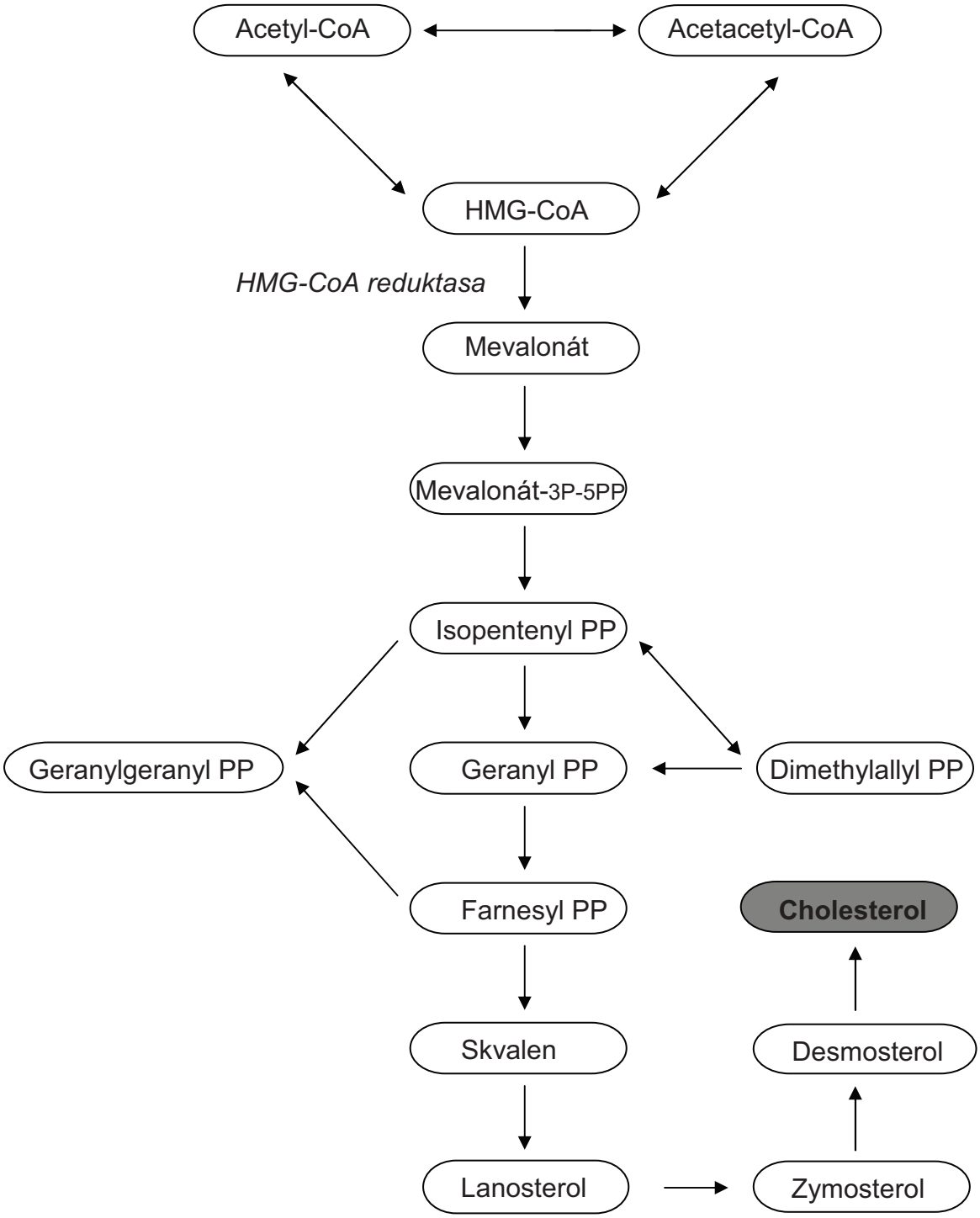
Cholesterol je steroidní látka, vyskytující se ve všech savčích buňkách. Slouží jako stavební součást buněčných membrán a zároveň jako výchozí látka pro syntézu steroidních hormonů, vitaminu D a žlučových kyselin. Část cholesterolu pochází z potravy (typicky 200-500 mg/24 hod), část je syntetizována *de novo* (většinou v rozmezí 400-700 mg/24 hod)[1]. Poměr přijatého a *de novo* syntetizovaného cholesterolu závisí na složení potravy. Je-li chudá na cholesterol, vychýlí se ve prospěch syntézy a naopak[1,2]. Ačkoli je schopnost syntetizovat cholesterol vlastní buňkám všech tkání, ve většině z nich je tato aktivita potlačena účinkem cholesterolu pocházejícího z jater či potravy. Hlavními producenty cholesterolu tak jsou játra, střevo a kůže[2].

Výchozím substrátem pro syntézu cholesterolu je acetyl-CoA, ze kterého v cytoplazmě vzniká 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Ten je v endoplazmatickém retikulu dále přeměňován HMG-CoA reduktasou na mevalonát, ze kterého vzniká aktivní isopren. Postupným spojením šesti isoprenových jednotek vzniká skvalen a následně v několika krocích cholesterol (Obr. 1).

1.1.2. Transport cholesterolu v organismu

Ve vodě nerozpustný cholesterol (ať už jako volný či esterifikovaný) musí být v organismu transportován v lipoproteinových částicích. Cholesterol vstřebaný z tenkého střeva se do cirkulace dostává v chylomikrech, rozměrných částicích bohatých na triacylglyceroly (TAG) s charakteristickým apoproteinem B48 na povrchu.

Obrázek 1: *Biosyntéza cholesterolu*



CoA, koenzym A; HMG, 3-hydroxy-3-methylglutaryl; 3P, 3-fosfo; PP, pyrofosfát

Po odštěpení většiny TAG lipoproteinovou lipasou periferních tkání jsou zbytky chylomiker (tzv. remnanty) s cholesterolem vychytány játry, kde spolu s nově syntetizovaným cholesterolem tvoří jaterní „pool“ cholesterolu. Z jater cholesterol odchází jako součást VLDL (very low density lipoproteins), které postupnou ztrátou TAG (opět vlivem periferní lipoproteinové lipasy) zmenšují svůj objem, zvyšují svou hustotu a koncentraci cholesterolu. Částice VLDL se tak postupně přes IDL (intermediate density lipoproteins) mění až na LDL (low density lipoproteins). Tyto na cholesterol velmi bohaté částice (tvoří až 45% obsahu) jsou hlavním zdrojem cholesterolu pro veškeré tkáně. Ty si přísun cholesterolu regulují počtem LDL-receptorů, které vystavují na své membráně. Zpětný (reverzní) transport cholesterolu z periferních tkání do jater obstarávají HDL (high density lipoproteins). Tyto částice nesou lecithincholesterolacyltransferasu, která esterifikuje volný cholesterol v poloze 3. Esterifikovaný cholesterol se přesouvá z hydrofilnějšího obalu lipoproteinové částice do hydrofobního jádra, a tak utváří koncentrační gradient, který umožňuje efektivní přesun cholesterolu z tkání a ostatních lipoproteinů do HDL. Část esterifikovaného cholesterolu je za pomoci přenašeče předána LDL a VLDL částicím, zbytek je vychytán játry. Cholesterol pocházející z HDL je určen především k vyloučení žlučí, narozdíl od cholesterolu z LDL, který slouží pro potřeby organismu (ať už jako výchozí látka pro syntézy či jako stavební kámen membrán)[3,4]. Ve vodě nerozpustný cholesterol je ve žluči solubilizován pomocí micel, tvořených fosfolipidy a žlučovými kyselinami. Dojde-li ke změně poměru fosfolipidů, žlučových kyselin a cholesterolu, může cholesterol precipitovat a stát se podkladem cholesterolového žlučového kamene[5]. Část žlučového cholesterolu se spolu s cholesterolem z potravy vstřebává v tenkém střevě a v chylomikrech opět vstupuje do cirkulace (zhruba 30-60 % z celkové střevní hotovosti cholesterolu)[1], zatímco část je působením střevní mikroflóry přeměněna na koprostanol a jiné steroidy, které jsou vyloučeny stolicí.

1.1.3. Inhibice mevalonátové dráhy syntézy cholesterolu

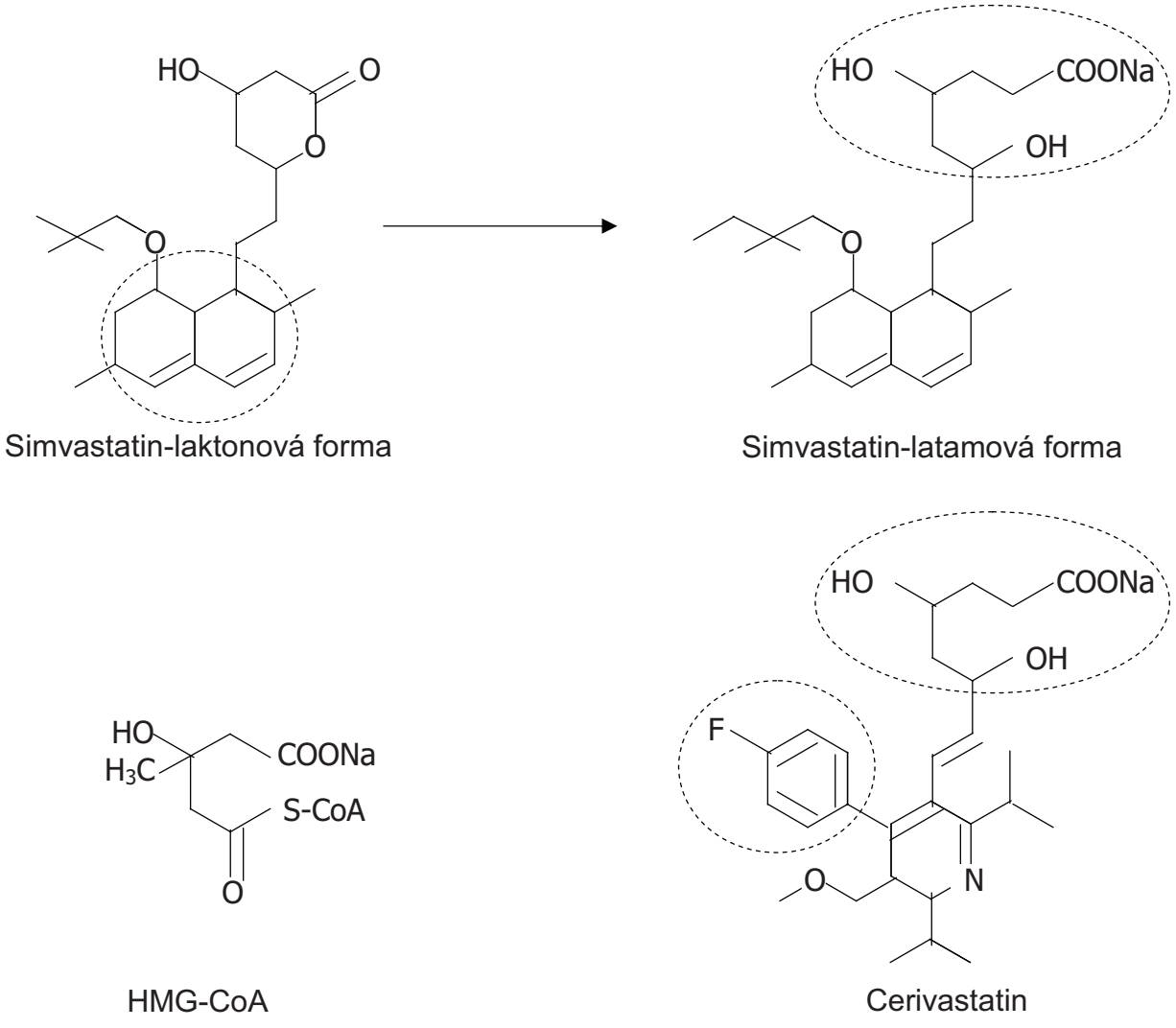
Metabolismus cholesterolu a možnosti jeho ovlivnění jsou již dlouhou dobu v centru pozornosti, kvůli úzkému vztahu hypercholesterolemie a aterosklerosy, jedné z nejčastějších příčin morbidity a mortality ve vyspělých zemích. Rizikový faktor představuje především LDL cholesterol, jehož nadbytek je vychytáván makrofágy. Ty se po akumulaci značného množství cholesterolu mění v pěnové buňky, které tvoří podstatnou část ateromového plátu v cévní stěně. K farmakologickému ovlivnění hladin cholesterolu máme v současné době k dispozici několik skupin léčiv. K nejdůležitějším patří statiny (inhibitory HMG-CoA reduktasy – ty budou podrobněji popsány v následující kapitole), fibráty, ovlivňující především hladiny triacylglycerolů s výrazně slabším účinkem na hladiny cholesterolu, pryskyřice, které přerušením enterohepatální cirkulace žlučových kyselin urychlují odsun cholesterolu z organismu (viz dále) a ezetimib, snižující střevní resorpci sterolů.

1.1.4. Inhibitory HMG-CoA reduktasy

HMG-CoA reduktasa, která přeměňuje HMG-CoA na mevalonát (Obr. 1), je klíčovým regulačním enzymem syntézy cholesterolu. Její aktivitu tlumí (na úrovni transkripce, posttranskripční či posttranslační modifikace) nejen cholesterol, jeho deriváty a meziprodukty jeho syntézy[6-8], ale například i glukagon či glukokortikoidy, zatímco insulin, estrogeny či hormony štítné žlázy ji zvyšují[9,10]. V sedmdesátých letech minulého století byly z plísně *P. citrinum* izolovány vysoce účinné inhibitory HMG-CoA reduktasy[11], statiny. Statiny, ať už přírodní či jejich syntetické deriváty, se záhy (lovastatin byl jako první schválen americkou FDA v roce 1987) staly běžně používanými účinnými a poměrně bezpečnými léky pro terapii hypercholesterolemie. O jejich masovém rozšíření svědčí i fakt, že v roce 2004 byl ve Velké Británii schválen jejich prodej bez lékařského receptu.

Na základě chemické struktury můžeme statiny rozdělit do dvou skupin. Struktura zástupců první skupiny (simvastatinu, lovastatinu a pravastatinu) je odvozena z dekahydronaftalenu, zatímco pro zástupce druhé skupiny (rosuvastatin, atorvastatin, cerivastatin, fluvastatin a pitavastatin) je typická fluorofenylová skupina (Obr. 2).

Obrázek2: *Struktura statinů*



Podle chemické struktury lze statiny rozdělit do dvou skupin. Do první řadíme statiny, jejichž kostru tvoří dekahydronaftalen (jejím zástupcem je zde simvastatin), zatímco statiny druhé skupiny nesou fluorofenylovou skupinu (zde cerivastatin). Simvastatin a lovastatin se podávají ve formě prekurzoru (jako lakton), který je v organismu hydrolyzován na účinnou laktamovou formu. Na obrázku je ukázána struktura HMG-CoA, kterou statiny napodobují.

Postranní skupiny na centrálním cyklu definují polaritu jednotlivých substancí, a tím i jejich farmakokinetiku. Ač odbourávání všech statinů probíhá v játrech pomocí cytochromu P450, liší se enzymy zúčastněné v přeměně jednotlivých statinů. Hydrofilní pravastatin je navíc vylučován z velké části močí v nezměněné podobě.

1.1.5. Antiproliferační účinky statinů

Ačkoli jsou statiny předepisovány především pacientům s hypercholesterolemií, snižování sérové koncentrace cholesterolu není jejich jediným účinkem. Celá řada experimentálních, epidemiologických či klinických studií si všímá možného terapeutického využití statinů napříč obory. Za lze jmenovat například stabilizaci ateromového plátu[12] a zlepšení funkce endotelu koronárních tepen u pacientů s aterosklerozou[13], zpomalení progrese některých onemocnění ledvin[14], protizánětlivé účinky[15,16] či zlepšení výsledků orgánových transplantací[17,18] atd. Tato kapitola bude věnována především antiproliferačním (či přesněji protinádorovým) účinkům statinů

Rychle rostoucí tkáně vykazují výrazně vyšší aktivitu HMC-CoA reduktasy ve srovnání s tkáněmi, kde buněčné dělení probíhá pomaleji (jedinou výjimku tvoří metabolicky velmi aktivní hepatocyty). Toto je přičítáno nejen zvýšené potřebě cholesterolu pro stavbu novotvořených membrán, ale též potřebou mevalonátu pro iniciaci replikace DNA[19,20]. Tato fakta, spolu s pozorováním deregulace syntézy cholesterolu u maligních i premaligních buněk, poukazují na významnou roli mevalonátové cesty syntézy cholesterolu u nádorových onemocnění[20]. HMG-CoA reduktasa tak představuje nový atraktivní cíl protinádorové terapie.

K velmi slibným výsledkům došli už v roce 1985 Maltese a spolupracovníci[21], kteří prokázali protinádorové účinky mevinolinu (lovastatinu) na myším neuroblastomu. Logickým pokračováním pak bylo zkoumání účinků ostatních statinů na různých modelech nádorových onemocnění a v klinických studiích. V celé řadě z nich byly popsány, někdy velmi výrazné,

protinádorové účinky statinů, ať už v případě kolorektálního[22-25] či hepatocelulárního karcinomu[26], karcinomu plic[27,28], ledvin[29] či pankreatu[30-36]. Na druhou stranu však existuje řada klinických studií, které neprokázaly účinnost statinů v prevenci například karcinomu prsu a prostaty[37] či kolorektálního karcinomu[38-43]. Situaci ještě více komplikují publikované údaje o možných kancerogenních účincích statinů[44]. Závěry této práce provedené na krysách nejspíše nejsou relevantní, neboť hlodavci reagují na podání statinů zcela jinak než vyšší savci[45,46]. Prokancerogenní efekt statinů je tak dnes považován spíše za nepravděpodobný[47-49].

Několik velmi rozsáhlých metaanalýz se pokusilo sjednotit značně nekonzistentní výsledky jednotlivých klinických studií, ale protinádorový účinek statinů v nich potvrzen nebyl[50-60]. Většina klinických studií a metaanalýz má značné slabiny, které mohou výrazně zpochybnit jejich výpovědní hodnotu. Jedná se zejména o nerozlišování jednotlivých statinů, jejichž struktura a fyzikálně-chemické vlastnosti zdaleka nejsou shodné (viz výše), dále krátká doba sledování a celkový návrh studií, které byly buď retrospektivní či v případě prospektivních byl pro/proti nádorový efekt sledován jen jako druhotný cíl či vedlejší účinek[61,62]. Problematický je též výběr vhodných kontrol. Sledujeme-li v epidemiologické studii pacienty užívající statiny, jedná se primárně o jedince s hypercholesterolemií. Ideální kontrolní soubor by tedy představovali hypercholesterolemičtí jedinci, kteří statin neužívají. Takové kontroly z pochopitelných důvodů k dispozici nejsou[63]. Dalšími vlivy, které nejsou ve většině studií zohledněny a zcela jistě znesnadňují či přímo znemožňují korektní interpretaci dat, jsou fenotypová a genotypová variabilita jedinců či buněk. Na buněčných liniích mnohočetného myelomu bylo ukázáno, že variabilita míry dysregulace mevalonátové dráhy syntézy cholesterolu v nádorových buňkách určuje míru odpovědi na statinovou terapii[64]. Podobně sekvenční varianty genu kódujícího HMG-CoA reduktasu jsou odpovědné za rozdílnou protinádorovou účinnost statinů[65].

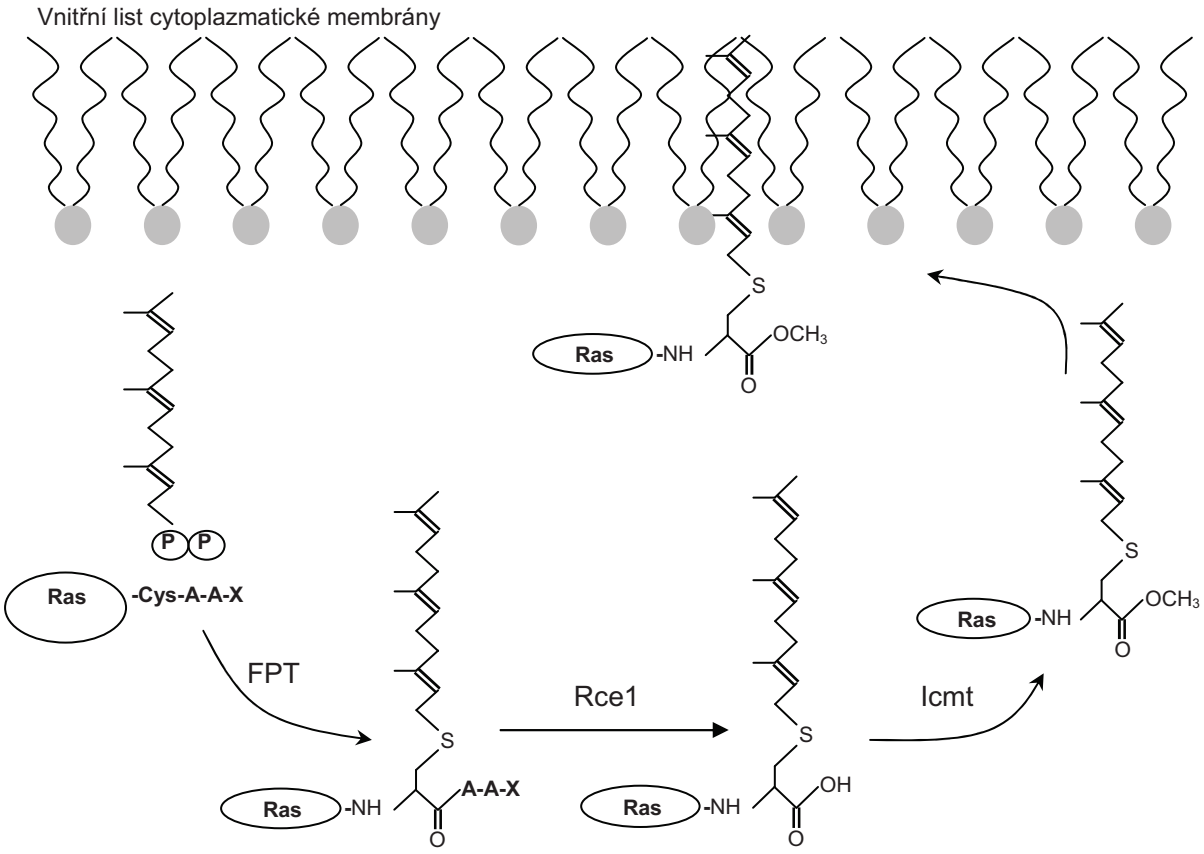
Oproti původním představám, že za protinádorovými účinky statinů stojí deplece cholesterolu, dnes víme, že účinky statinů na lidský organismus jsou mnohem rozmanitější, často nezávislé na snižování sérové koncentrace cholesterolu[66]. Pravděpodobně k nejdůležitějším mechanismům patří deplece meziproduktů mevalonátové dráhy syntézy cholesterolu (Obr. 1), především farnesylpyrofosfátu (FPP) či geranylgeranylpyrofosfátu (GGPP). Oba se uplatňují v modifikaci některých intracelulárních proteinů a zajišťují jejich ukotvení v membránách (viz dále) (Obr. 3). Na farnesylaci/geranylaci jsou tak závislé například receptory růstových hormonů, lamin A/B a Ras či Rho proteiny, které jsou důležitým článkem mitogenních signalizačních kaskád, uplatňují se v regulaci transkripce či zajišťují buněčnou adhezi[67-69].

1.1.6. Ras proteiny

Ras proteiny, někdy též označované jako $p21^{ras}$, fungují jako hlavní převodní stanice růstových a diferenciačních signálů. Jsou to malé regulační GTPasy, které v aktivním stavu (tj. v komplexu s GTP) interagují s intracelulárními efektory. Hydrolýza GTP vede ke zrušení interakce, a tím k ukončení signalizace. U nádorových onemocnění nacházíme velmi často mutaci ve dvanáctém kodonu, kde záměna původního glycinu za jinou aminokyselinu (s výjimkou prolinu) vede k výraznému (zhruba o 90%) zpomalení GTPasové aktivity Ras proteinu. Mutovaný Ras protein navíc nereaguje na endogenní aktivátory GTPas, které za normálních okolností urychlují hydrolýzu až 100 000x. Protrahovaná aktivace Ras proteinu tak vede k amplifikaci růstových signálů, což může být příčinou nekontrolovaného růstu[70].

Pro správnou funkci Ras proteinů je nezbytné umístění v buněčné membráně, kterého je dosaženo vytvořením hydrofobní farnesylové „kotvy“ na C konci. Farnesyltransferasa rozpozná tzv. CAAX sekvenci (C=cystein, A=libovolná alifatická aminokyselina, X=serin či methionin) Ras proteinu a thioéterovou vazbou připojí farnesyl na cystein.

Obrázek 3: *Farnesylace Ras proteinů*



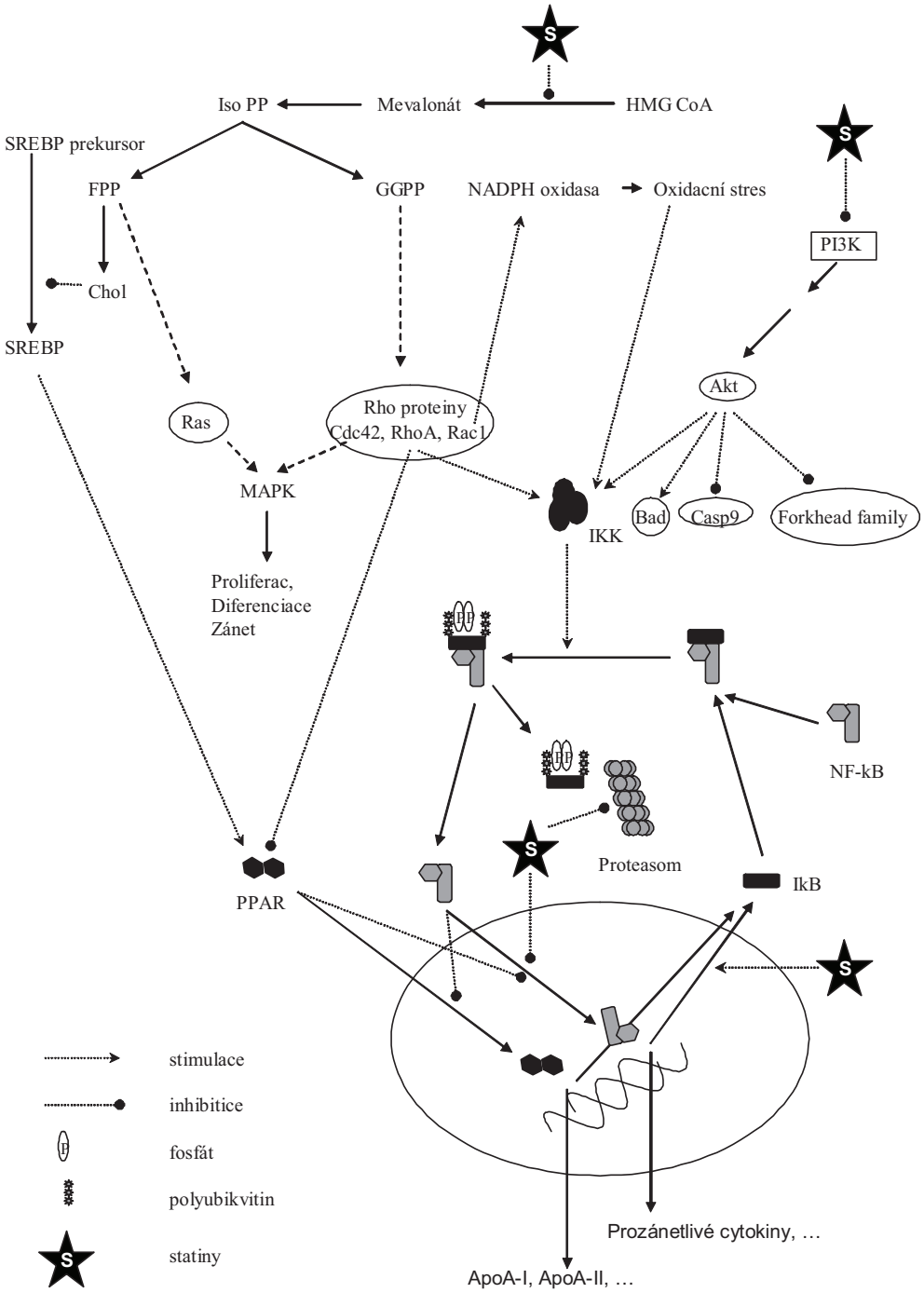
Farnesylproteintransferasa (FPT) připojí farnesyl na cystein v blízkosti C konce Ras proteinu. Ras a a-faktor konvertující enzym (Rce1) poté odštěpí koncové tři aminokyseliny a umožní tak methylaci karboxylové skupiny cysteinu, kterou zajišťuje isoprenylcysteinkarboxylmethyltransferasa (Icmt). Takto modifikovaný Ras protein přechází do plazmatické membrány, ve které je ukotven pomocí hydrofobního farnesylu. Některé Ras proteiny jsou kromě farnesylace modifikovány i palmitoylací.

A-alifatická aminokyselina, X-serin či methionin

Poté dojde k odštěpení koncových tří aminokyselin a methylesterifikaci koncového cysteinu (Obr. 3). U některých Ras proteinů navíc dochází k palmitoylaci dalších cysteinů v blízkosti C konce[70,71].

Vzhledem k tomu, že mutované *Ras* geny představují nejčastěji nalézané onkogeny v lidských nádorových tkáních[72], byl protinádorový účinek statinů spojován převážně se sníženou tvorbou farnesylpyrofosfátu, následovanou sníženou farnesylací a utlumením funkce Ras proteinů. Nicméně dnes je již zcela zřejmé, že protinádorové účinky statinů jsou velmi pestré a zahrnují modifikaci výše zmíněných proteinů (receptorů růstových hormonů, laminu A/B a Rho proteinů)[67,69,73]s ovlivněním příslušných signalizačních kaskád, výrazně modulují aktivitu nukleárního faktoru kappa B a zasahují i do klíčové signalizační kaskády Akt [46] (Obr. 4).

Obrázek 4: *Účinky statinů na buněčnou signalizaci*



Statiny ovlivňují buněčnou signalizaci na mnoha místech. K nejdůležitějším patří ovlivnění přenosu signálu Ras a Rho proteiny, modulace signalizační kaskády Akt a transkripčního faktoru NFκB. FPP,farnesylpyrofosfát; GGPP, geranylgeranylpyrofosfát; IκB, inhibitor NFκB; IKK, IκB kinasový komplex; IsoPP, isopentenyl pyrofosfát; NFκB, nukleární receptor kappa B; PPAR, peroxisome proliferator-activated receptor; PI3K, fosfatidylinositol 3-fosfát kinasa; SREBP, sterol regulatory element-binding protein. Upraveno dle[46].

1.2. Katabolická dráha cholesterolu

1.2.1. Biosyntéza žlučových kyselin

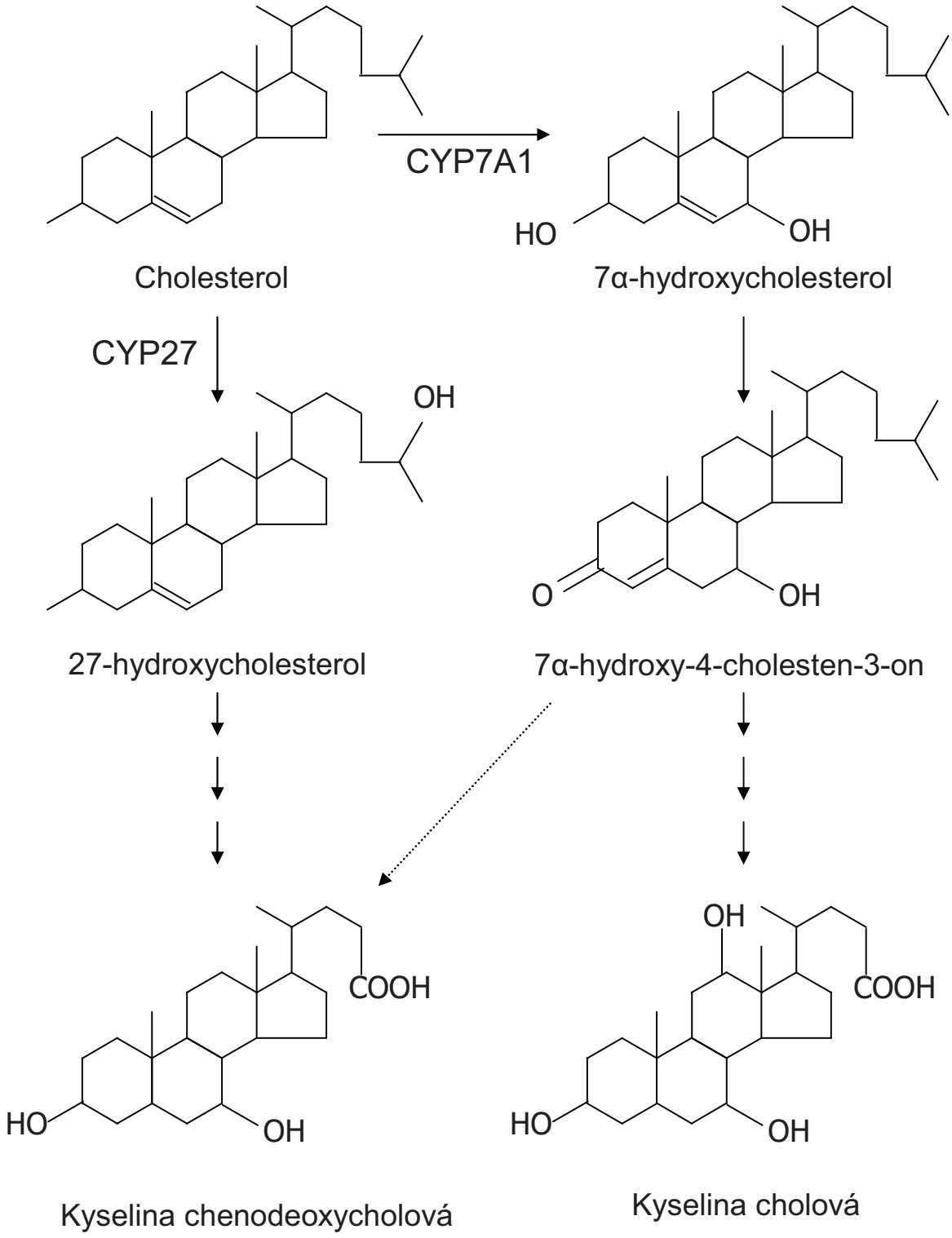
Savčí buňky nedisponují enzymatickou výbavou pro otevření cyklopentanoperhydrofenantrenového kruhu, a tak nedokážou molekulu cholesterolu odbourat. Odstraňování cholesterolu z organismu se děje v játrech buď přímým vyloučením cholesterolu do žluči (viz výše) nebo přeměnou na žlučové kyseliny. Ač v živočišné říši hraje konverze cholesterolu na žlučové kyseliny hlavní úlohu, u člověka a většiny savců je více cholesterolu (zhruba dvě třetiny) vyloučeno přímo[74].

Žlučové kyseliny vznikají buď neutrální či kyselou syntetickou cestou (Obr Syntéza ŽK). Neutrální (též klasickou) biosyntetickou cestu zahajuje cholesterol 7α-monooxygenasa (cholesterol 7α-hydroxylasa, CYP7A1, 1.14.13.17) která hydroxyluje cholesterol za vzniku 7α-hydroxycholesterolu. Ten se dehydrogenací mění na 7α-hydroxy-4-cholesten-3-on (C4), ze kterého v několika dalších krocích, zahrnujících i zkrácení postranního řetězce, vzniká kyselina cholová. Kyselá (též alternativní) biosyntetická cesta začíná hydroxylací cholesterolu v pozici 27 (tj. v postranním řetězci, nikoli na steroidním jádře), pokračuje oxidací a zkrácením postranního řetězce a v několika dalších krocích vzniká kyselina chenodeoxycholová (Obr. 5).

1.2.2. Regulace CYP7A1

Syntéza žlučových kyselin musí být, vzhledem k jejich povaze, velmi pečlivě regulována. Děje se tak na úrovni CYP7A1. Hlavním zpětnovazebným inhibitorem jsou samotné žlučové kyseliny, které snižují expresi CYP7A1 hned několika mechanismy[75]. V hepatocytech se váží na jaderný receptor FXR (farnesoid X receptor) a s jeho pomocí aktivují SHP (short heterodimer partner), který zabraňuje transkripci *CYP7A1*[76].

Obrázek 5: *Biosyntéza žlučových kyselin*



Neutrální (též zvaná klasická) cesta začíná hydroxylací cholesterolu v pozici 7 účinkem CYP7A1 a končí vytvořením kyseliny cholové. Kyselá (alternativní) cesta začíná hydroxylací cholesterolu v pozici 27 a vede k tvorbě chenodeoxycholové kyseliny. Tečkovaně vyznačená cesta se uplatňuje v případě defektu 12α hydroxylace v neutrální cestě[77].

Dalšími předpokládanými posly inhibiční signalizace žlučových kyselin jsou PXR (pregnane X receptor) a kináza JNK (c-Jun N terminal kinase)[78], nicméně detailní mechanismus není dopodrobna prozkoumán. Zatím posledním popsaným faktorem, zprostředkujícím zpětnovazebnou inhibici žlučovými kyselinami je FGF19 (fibroblast growth factor 19), který je po stimulaci žlučovými kyselinami tvořen v ileocytech a uvolňován do cirkulace. Na povrchu hepatocytu reaguje s receptorem FGFR4 (fibroblast growth factor receptor 4), který aktivuje MAPK (mitogen-activated protein kinase) kaskádu, jejíž součástí jsou ERK1/2 (extracellular signal-related kinase) a JNK, vedoucí v konečném důsledku k inhibici *CYP7A1*[79,80]. Zatím ne zcela prozkoumaným regulačním mechanismem zpětnovazebného působení žlučových kyselin je ovlivnění stability transkriptu *CYP7A1*. V 3'UTR (3' untranslated region) oblasti byla identifikována cílová místa dvou mikro-RNA (miR-122a a miR-422a), které urychlují degradaci mRNA a jejichž exprese je zvyšována žlučovými kyselinami[80]. Opačný účinek (tj. stabilizaci mRNA) vykazuje Apobec-1 (Apolipoprotein B editing enzyme), jehož vazebné místo v 3' UTR oblasti *CYP7A1* je v těsné blízkosti vazebných míst pro zmíněné mikro-RNA[80,81].

Exprese lidského *CYP7A1* nejspíš není, narozdíl od myšího homologu, ovlivňována cholesterolem. Pravděpodobnou příčinou je neschopnost promotoru lidského CYP7A1 vázat LXRα/RXR (liver X receptor α a retinoid X receptor)[82], nicméně otázka vlivu cholesterolu na expresi *CYP7A1* stále ještě není definitivně dořešena[83,84].

Dalšími endogenními regulátory CYP7A1 jsou některé hormony. Insulin při krátkodobé aplikaci silně aktivuje transkripci CYP7A1, zatímco při dlouhodobější aplikaci má účinek opačný[85], stejně jako glukagon[86]. Hormony štítné žlázy u lidí nejspíše nemají, narozdíl od hlodavců, vliv na aktivitu CYP7A1[87,88].

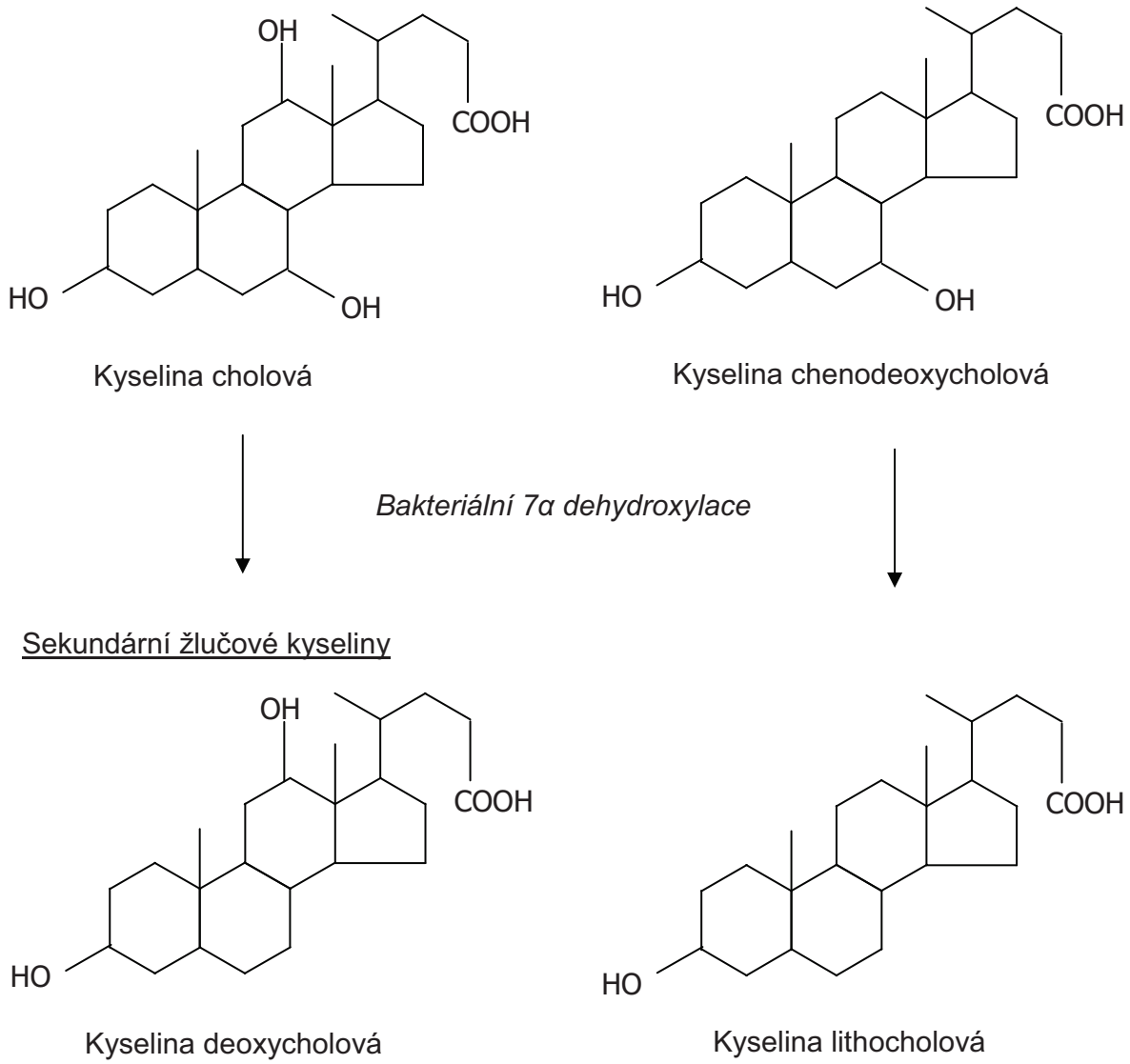
1.2.3. Enterohepatální cirkulace žlučových kyselin

Vzniklé žlučové kyseliny jsou pak konjugovány s glycinem či taurinem a secernovány do žluči. Konjugace žlučových kyselin zvyšuje jejich polaritu a s tím spojenou rozpustnost ve vodě (či ve žluči). Se ztrátou hydrofobicity přicházejí žlučové kyseliny o schopnost pasivního prostupu membránami, ke kterému dále potřebují transportéry. Glyko a taurokonjugáty žlučových kyselin jsou odolné vůči dekonjugaci žaludečními, pankreatickými či střevními enzymy[89], a tak nedochází k výraznému vstřebávání žlučových kyselin v proximální části trávicího traktu (duodenu, jejunu či proximálním ileu), kde chybí transportér konjugovaných žlučových kyselin. Díky tomu dosahují žlučové kyseliny v lumen trávicí trubice koncentrací, dostatečných pro jejich funkci.

Jakožto potentní detergenty emulgují tuky a tvorbou micel výrazně zefektivňují trávení a vstřebávání tuků a lipofilních látek (včetně cholesterolu) a dokonce i proteinů[90]. Navíc též inhibují růst bakterií v tenkém střevě, ač mechanismus tohoto účinku není zcela znám[91]. Nelze opomenout ani jejich, zatím ne kompletně zmapovanou, roli ligandů jaderných (FXR) či membránových (GPBAR1, G-protein coupled bile acid receptor 1) receptorů, uplatňujících se v regulaci buněčných pochodů a exprese genů[92-94]. Po splnění své funkce je převážná většina konjugovaných žlučových kyselin (až 98 %) vstřebána zpět do krve pomocí aktivního transportéru (ileal sodium/bile acid cotransporter), který se nachází v distální části ilea[95,96]. Vstřebané žlučové kyseliny jsou unášeny portálním řečištěm do jater, kde jsou aktivně vychytány jaterními buňkami a následně resecernovány do žluči. Nevstřebané žlučové kyseliny se dostávají do tlustého střeva, kde činností anaerobních bakterií dochází k jejich dekonjugaci a dehydroxylaci v pozici 7α, za vzniku hydrofobnějších toxických sekundárních žlučových kyselin (kyselina deoxycholová a lithocholová) (Obr. 6). Část dekonjugovaných žlučových kyselin se ještě pasivním transportem vstřebá z tlustého střeva, ostatní odcházejí stolicí. Za normálních okolností střevem denně projde cca 30 g žlučových kyselin, tj.

desetinásobek celkové hotovosti v organismu. Denní ztráty žlučových kyselin přitom tvoří necelých 500 mg a jsou plně hrazeny syntézou *de novo*.

Obrázek 6: *Struktura žlučových kyselin*



Primární žlučové kyseliny jsou v játrech syntetizovány z cholesterolu. V tlustém střevě jsou činností bakterií dehydroxylovány v poloze 7 za vzniku sekundárních žlučových kyselin. Počet hydroxylových skupin určuje polaritu a míru toxicity jednotlivých žlučových kyselin. Kyselina cholová (se třemi hydroxylovými skupinami) je tak nejpolárnější a nejméně toxická, zatímco kyselina lithocholová (s jedinou hydroxylovou skupinou) je nejvíce hydrofobní a zároveň nejtoxičtější.

1.2.4. Malabsorpce žlučových kyselin

Dochází-li ke ztrátám větším, hovoříme o malabsorpci žlučových kyselin (MŽK). Nejčastěji k ní dochází při postižení funkce terminálního ilea – ať už po resekci, zánětlivém či radiačním poškození, při biliární píštěli, případně při podávání sekvestrantů žlučových kyselin. V závislosti na tíži a rozsahu postižení klesá účinnost resorpce žlučových kyselin z 98 až k pouhým 10 %, dochází ke zmenšení hotovosti žlučových kyselin v organismu a výrazně se zrychlí jejich syntéza ve snaze o dorovnání deficitu[97]. Schopnost či neschopnost jater hradit ztráty žlučových kyselin pak určuje charakter symptomů.

Pokud játra dokážou vzniklé ztráty hradit, projeví se především nadbytek žlučových kyselin v tlustém střevě. Žlučové kyseliny vyvazují vápenaté ionty ve střevním obsahu, a tak omezují tvorbu nerozpustného (a tudíž nevstřebatelného) šťavelanu či bilirubinátu vápenatého. Dochází tak ke zvýšenému vstřebávání bilirubinu a rozpustných šťavelanů, které musí být následně vyloučeny buď žlučí (bilirubin) nebo močí (šťavelany), kde se mohou stát základemžlučových či ledvinových kamenů[98-100]. K tvorbě pigmentových žlučových kamenů přispívají žlučové kyseliny též zvýšenou tvorbou micel, podporujících vstřebávání bilirubinu[99]. Vysoká koncentrace žlučových kyselin v tlustém střevě též vede k průjmům – na zvýšení sekrece elektrolytů tlustým střevem mají vliv především žlučové kyseliny se dvěma hydroxylovými skupinami – chenodeoxycholová a deoxycholová[97,101,102]. Konečně nelze opomenout fakt, že vyšší koncentrace žlučových kyselin (převážně sekundárních) mohou vést k poškození DNA a následně k apoptóze[103]. Dochází tak k selekci buněk rezistentních k programované buněčné smrti, což může ve výsledku vést ke vzniku nádorového onemocnění[104].

V případě, že játra nedokážou hradit extrémní ztráty žlučových kyselin, projeví se především jejich nedostatek v tenkém střevě. Snižuje se vstřebávání tuků a ostatních lipofilních látek, vedoucí nejen k jejich deficitu, ale též k průjmům, vyvolaným přítomností

nevstřebaných tuků v tlustém střevě. Nevstřebané mastné kyseliny dále vyvazují vápenaté ionty, a přispívají tak ke vzniku ledvinových či žlučových kamenů (viz výše)[98].

2. CÍLE

2.1. Syntetická dráha cholesterolu

Vlivem statinů na nádorová onemocnění se zabývala celá řada studií a u všech dostupných statinů byly prokázány protinádorové účinky. K jednoznačným závěrům se však nepodařilo dojít, neboť v mnoha jiných studiích tyto účinky potvrzeny nebyly. Za účelem nalezení konečné odpovědi bylo provedeno několik rozsáhlých metaanalýz, ani ty však protinádorové účinky statinů nepotvrdily[50-60]. Závěry zmiňovaných metaanalýz značně oslabuje fakt, že souhrnně analyzovaly incidenci všech nádorových onemocnění, neodlišovaly jednotlivé statiny, sbíraly data ze studií zaměřených primárně na vliv statinů na kardiovaskulární onemocnění či probíhaly velmi krátce.

Cílem této části disertační práce tak bylo porovnat protinádorové účinky jednotlivých statinů na několika liniích lidského adenokarcinomu pankreatu jak *in vitro,* tak *in vivo* (na myších xenotransplantovaných lidskými nádorovými liniemi) a pokusit se vysvětlit případné rozdíly v účinnosti jednotlivých statinů. Adenokarcinom pankreatu byl vybrán proto, že většina karcinomů pankreatu nese aktivační mutaci v *K-ras* protoonkogenu[105] a zabránění farnesylace *K-ras* proteinu (například pomocí statinů), vedoucí k jeho deaktivaci, se tak jeví jako vhodný terapeutický přístup (viz práce „**Differences in antitumor effects of various statins on human pancreatic cancer**“).

2.2. Katabolická dráha cholesterolu

Komplikace spojené s malabsorpcí žlučových kyselin lze do značné míry eliminovat (například podáním sekvestrantů žlučových kyselin). Pro úspěšnou terapii je však nezbytné tento symptom u pacienta nejprve identifikovat. Jelikož konverze cholesterolu na 7α-hydroxycholesterol (katalyzovaná CYP7A1) je klíčovým regulačním krokem neutrální dráhy biosyntézy žlučových kyselin a podléhá jejich negativní zpětné vazbě, může měření aktivity

CYP7A1 sloužit nejen jako marker homeostázy cholesterolu, ale i jako ukazatel syntézy (a tím i malabsorpce) žlučových kyselin. Bylo opakovaně prokázáno, že sérové koncentrace C4 korelují s mírou aktivity CYP7A1[106-109] a závažností malabsorpce žlučových kyselin[110,111]. Vzhledem k tomu, že ostatní metody stanovení MŽK, jako například stanovení exkrece žlučových kyselin stolicí[112], měření sérových koncentrací 7α-hydroxycholesterolu či lathosterolu[113,114], dechové testy s použitím ^{14}C cholylglycinu nebo ^{14}C cholyltaurinu[115,116] či retenční test s použitím ^{75}Se-homotaurocholové kyseliny (^{75}SeHCAT)[110,117] jsou značně pracné, drahé, nespolehlivé či v některých zemích dokonce zakázané pro použití v humánní medicíně[111], sérové koncentrace C4 se zdají být ideálním ukazatelem malabsorpce žlučových kyselin. Současné metody stanovení C4 se však nehodí pro použití v klinické medicíně vzhledem k problematické extrakci analytu při 64°C[106,109] či použití poměrně nedostupných přístrojů[118]. Naším cílem tudíž bylo zavést a zvalidovat metodu vhodnou i pro rutinní stanovení C4 (viz práce „**Improved HPLC analysis of serum 7α-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption**“).

MŽK provází značnou část pacientů s Crohnovou chorobou, její diagnostice se však dosud věnuje poměrně malá pozornost. Adekvátní terapie (sekvestranty žlučových kyselin) je tak často nabízena jen pacientům po resekci distálního ilea, u nichž lze MŽK předpokládat. Zajímalo nás tudíž, zda se MŽK vyskytuje i u pacientů s Crohnovou chorobou, kteří resekci terminálního ilea nepodstoupili. Jelikož Walters a spolupracovníci[119] v nedávné studii prokázali, že nedostatečná sekrece FGF19 ileocyty může být příčinou MŽK a průjmů, bylo dalším cílem naší studie objasnit úlohu, jakou může FGF19 hrát v diagnostice MŽK (viz práce „**Bile acid malabsorption in inflammatory bowel disease: Assessment by serum markers**“).

Ačkoli je regulace CYP7A1 intenzivně zkoumána po řadu let, stále jsme daleko od pochopení tohoto komplexního děje. Při hledání genetických determinant aktivity *CYP7A1* se

pozornost velmi často obrací ke dvěma běžným polymorfismům v jejím promotoru (-203A>C a -469C>T). Navzdory mnoha velmi rozsáhlým studiím nepanuje shoda v tom, zda tyto polymorfismy mohou ovlivnit aktivitu CYP7A1 a případně i sérové koncentrace cholesterolu. Vzhledem k nejednoznačným výsledkům předchozích studií jsme předpokládali, že existuje-li rozdíl v aktivitě CYP7A1 mezi jednotlivými alelami, bude za fyziologických okolností velmi malý. V další studii jsme se proto zaměřili na kohortu pacientů s malabsorpcí žlučových kyselin, kde lze očekávat výrazně zvýšenou aktivitu CYP7A1 (viz práce **„CYP7A1 promoter polymorphism -203A>C affects bile salt synthesis rate in patients after ileal resection“)**.

Je známo, že aktivita CYP7A1 vykazuje značné výkyvy v průběhu dne – maxima dosahuje nedlouho po poledni[120]. V tomto období bychom však očekávali spíše pokles, způsobený návratem žlučových kyselin portální žilou do jater. Cílem další studie tak bylo sledovat diurnální variabilitu aktivity CYP7A1 u zdravých dobrovolníků a pokusit se identifikovat faktory, které na ni mají vliv. Pro zvýraznění efektu byla studie provedena též při zesílené (po podání chenodeoxycholové kyseliny) či oslabené (po podání cholestyraminu) enterohepatální cirkulaci žlučových kyselin (viz práce „**Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects**“).

V roce 2003 byl popsán FGF19 jako nový regulátor syntézy žlučových kyselin[79]. Dnes víme, že se podílí i na regulaci lipidového metabolismu v játrech[121-123] a zřejmě zvyšuje citlivost tkání k insulinu[124]. Zvýšená akumulace tuku v hepatocytech je podstatou nealkoholové steatosy jater (NAFLD), která se může rozvinout v nealkoholovou steatohepatitidu (NASH). K progresi NAFLD do NASH pravděpodobně stačí, navzdory původním představám, vliv samotných tukových depozit v hepatocytu[125]. Proto jsme se zaměřili na roli FGF19 v regulaci metabolických procesů u pacientů s NAFLD (viz práce „**The hepatic response to FGF19 is impaired in patients with non-alcoholic fatty liver disease and insulin resistance“).**

3. PUBLIKOVANÉ PRÁCE TVOŘÍCÍ PODSTATU DISERTACE

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3.2. Leníček M, Juklová M, Zelenka J, Kovář J, Lukáš M, Bortlík M, Vítek L. Improved HPLC analysis of serum 7α-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption. Clin Chem, 54 (6): 1087-1088, 2008.

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Differences in antitumor effects of various statins on human pancreatic cancer

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Statins are widely used for the treatment of hypercholesterolemia. However, their inhibitory action on HMG-CoA reductase also results in the depletion of intermediate biosynthetic products, which importantly contribute to cell proliferation. The aim of the present study was to compare the effects of the individual commercially available statins on experimental pancreatic cancer. The *in vitro* effects of individual statins (pravastatin, atorvastatin, simvastatin, lovastatin, cerivastatin, rosuvastatin and fluvastatin) on the viability of human pancreatic cancer were evaluated in CAPAN-2, BxPc-3 and MiaPaCa-2 cell lines. The *in vivo* experiments were performed on nude mice xenotransplanted with CAPAN-2 cells. The mice received oral treatments either with a placebo, or with the statins mentioned earlier in a daily dose corresponding to a hypocholesterolemic dose in humans. The effect of these statins on the intracellular Ras protein, trafficking in MiaPaCa-2 transfected cells, was also investigated. Substantial differences in the tumor-suppressive effects of all statins were detected in both *in vitro* and *in vivo* experiments. While simvastatin exerted the highest tumor-suppressive effects *in vitro*, rosuvastatin ($p = 0.002$), cerivastatin ($p = 0.002$) and fluvastatin ($p = 0.009$) were the most potent compounds in an animal model. All statins (except pravastatin) inhibited intracellular Ras protein translocation. In summary, substantial tumor-suppressive effects of various statins on the progression of experimental pancreatic adenocarcinoma were demonstrated, with marked differences among individual statins. These results support greatly the potential of statins for the chemoadjuvant treatment of pancreatic cancer.

Key words: pancreatic cancer; cholesterol; HMG-CoA reductase; statins; farnesylation; K-*ras* oncogene; mevalonate

Inhibitors of hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase (statins) are widely used for treatment of hypercholesterolemia. However, the effects of statins on human tissues are pleiotropic, involving inhibition of atherogenous plaque formation, platelet aggregation, the improvement of both endothelial function and fibrinolytic activity, or even direct protective effects of the statins upon mortality in acute myocardial infarction.[1,2] In addition, the inhibition of HMG-CoA reductase, a key enzyme in the cholesterol biosynthesis, also results in the depletion of several important intermediates, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which modify and target small GTPases to their site of action.[3] Farnesylated Ras proteins are associated with multiple mitogenic signal transduction pathways in response to growth factor stimulation,[4] including external signal regulating kinase 1/2 and phosphatidylinositol 3′-kinase/Akt signaling pathways to name the most important effectors.[5] On the other hand, geranylgeranylated proteins of the Ras homologous (Rho) family regulate signal transduction from membrane receptors in a variety of cellular events related to cell adhesion and invasion.[6] Accordingly, inhibition of farnesylation/geranylgeranylation became a plausible approach to modify cell proliferation in tumor tissues. During the last decade, the antiproliferative effects of statins were demonstrated in numerous *in vitro* as well as *in vivo* studies on various tumor cell lines including hepatocellular carcinoma,[7] lung,[8] colorectal[9] or pancreatic cancer.[10–15] In this regard, pancreatic cancer is of particular interest, since more than 90% of human pancreatic cancers bear activating mutations in the K-*ras* proto-oncogene.[16] These mutations result in the loss of GTPase activity (physiologically associated with the Ras protein as a negative feedback mechanism), which leads to protracted K-Ras activation. Suppression of this event *via* statin-mediated inhibition of K-Ras farnesylation thus seems to be a promising therapeutic approach. In fact, antitumor activities of statins were also demonstrated in some human studies,[17,18] and also interestingly in several human epidemiological studies, primarily focused on cardiovascular outcomes.[19–21]

The antiproliferative effects for all marketed statins have been described. However, some data suggest certain differences in the antitumor effects of individual statins. Wong *et al.*[22] demonstrated substantially higher inhibitory effects on the growth of acute myeloid leukemia cells of cerivastatin, compared to lovastatin, atorvastatin and fluvastatin. In another *in vitro* study on leukemia cell lines, simvastatin was the most effective statin; while, pravastatin had the weakest effect.[23] A similar tendency was also observed in osteosarcoma cell lines treated with simvastatin or pravastatin,[24] as well as in an *in vitro* breast cancer study by Mueck *et al.*[25] Based on incomplete and scattered data, it seems that individual statins act differently on various diseases and cell populations,[2] and this may be the reason for the inconclusive or controversial epidemiological data published so far. This might all result from the differences in the statins' structure, pharmacokinetics and biotransformation rates.[26] For instance, large variations in the modulation of hepatic cytochrome P450 activity have been documented among individual statins.[27]

Although, as pointed out earlier, statins have been extensively studied as possible chemotherapeutic agents, and no complex data have as yet been provided on the differences in antitumor activities of the individual statins. Therefore, the aim of the present study was to compare the tumor-suppressive effects of the various statins routinely used in clinical medicine upon the growth of human pancreatic cancer in both *in vitro* and *in vivo* experimental models.

The last two authors contributed equally to this paper.

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Material and methods

Material

For the *in vitro* study, the following pure forms of statins were used: pravastatin, atorvastatin, lovastatin, simvastatin, fluvastatin, cerivastatin and rosuvastatin (all obtained from Alexis; San Diego, CA except for simvastatin, kindly provided by Merck, Sharp and Dohme, NJ). Simvastatin was either used in its native (lactone) form (believed not to inhibit HMG-CoA reductase) or in the active (lactam) form, prepared as described previously.[28] Mevalonate, FPP and GGPP were purchased from Sigma (St. Louis, MO).

Cell cultures

The following pancreatic cancer cell lines were used for the *in vitro* studies: CAPAN-2, MiaPaCa-2 and BxPc-3 (ATCC, Manassas, VA). All cell lines were both maintained and grown in a humidified atmosphere containing 5% CO_2 at 37°C, in the following media, supplemented with 10% fetal bovine serum: MiaPaCa-2 in DMEM, BxPc-3 in RPMI 1640 and CAPAN-2 in McCoy's 5A medium with 1.5 mM L-glutamine containing sodium bicarbonate (1.5 g/l).

All statins in the *in vitro* study were used in the concentration range of 0–40 μM (0; 10; 20; 30 and 40 μM). Three hundred microliters of the cell suspension ($\sim 2.7 \times 10^5$ cells/ml) were used for inoculation of individual wells in the 6-well plate. Two milliliters of medium were added to each well, and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Statins from stock solutions in methanol (20 μl per well) were added 24 hr later to the final concentrations, indicated earlier. Twenty microliters of methanol were added to control wells. After 24, 48 and 72 hr of statin treatment, cells in triplicate were washed with PBS, harvested by 0.25% trypsin and resuspended. Both cell growth and viability were assessed by direct counting of 0.4% trypan blue dye-excluding cells.

Ras protein translocation assay

Reverse-transcription polymerase-chain reaction. Total RNA was isolated from HeLa (with wild type K-*ras* gene) and MiaPaCa-2 (with activation mutation in K-*ras* gene) cell lines by RNeasy Kit (Qiagen, MD), and wild type; and the mutated 570-bp K-*ras* cDNA genes, respectively, were amplified by reverse-transcription polymerase-chain reaction (RT-PCR) using Enhanced Avian HS RT-PCR kit (Sigma) and following primers:

5′-primer of K-*ras*: 5′- TTCAGATCTATGACTGAATATAA ACTTGTGGTAGTTGGAG -3′

3′-primer of K-*ras*: 5′- AAGGATCCTTACATAATTACACAC TTTGTCTTTGACTTC -3′

The PCR products were purified using QIAquick PCR Purification Kit (250) (Qiagen).

DNA constructs Owing to the C-terminal processing of Ras, both wild type and mutant *ras* DNA sequences were ligated into pEGFP-C1 vector (Clontech, CA), downstream of the coding sequence for the green fluorescent protein (GFP), to generate the pEGFP-K-Ras and pEGFP-K-RasG12C vectors, respectively. Vectors were amplified in the *Escherichia coli* DH5α cells (Invitrogen, CA) and verified by sequencing. Vectors were then used for expression of N-terminally tagged K-Ras with GFP (GFP-K-Ras) in MiaPaCa-2 cells.

Transfection and localization imaging MiaPaCa-2 cells were seeded in a single 6-well cell culture plate, with sterile glass coverslips 5 hr before transfection. Transfection with aforementioned plasmids was carried out by FuGene 6 (Roche, Basel, Switzerland) according to manufacturer instructions. After 24 hr the medium was changed, and statins to final concentration of 20 μM were added. After next 24 hr, cells were washed by PBS and fixed for 20 min with 4% formaldehyde in PBS. Actin filaments were stained with TRITC phalloidine (Sigma). The pEGFP-C1 vector was used as a control of transfection efficiency, and for observing the localization of the GFP protein (alone, with, and without the drug treatments). Intracellular localization of the individual proteins (GFP, GFP-K-Ras or GFP-K-RasG12C) and actin filaments was visualized by fluorescent microscopy, using QuickPHOTO CAMERA 2.1 processing software (Olympus, Tokyo, Japan).

Animal studies

The *in vivo* study was performed on nude mice (strain CD-1, Charles River WIGA, Sulzfeld, Germany) xenotransplanted subcutaneously with human pancreatic adenocarcinoma cell line CAPAN-2 (10^7 cells; $n = 6$ for each treatment group). After initiation of tumor growth (7–10 days after xenotransplantation; tumor size at the beginning of treatment was 0.27 ± 0.04 cm^3), the mice received oral treatment with a placebo (saline) or one of the following commercially available statins: pravastatin (Lipostat, Bristol-Myers Squibb, NY), atorvastatin (Sortis, Pfizer, NY), lovastatin (Mevacor, Merck, Sharp and Dohme), simvastatin (Zocor, Merck, Sharp and Dohme), cerivastatin (Cholstat, Laboratories Fournier, Paris, France) or fluvastatin (Lescol, Novartis, Basel, Switzerland); given in a daily dose approximately corresponding to the hypocholesterolemic dose used in humans (Fig. 3). Drugs were administered intragastrically once daily *via* gastric tube. The primary endpoint was the survival time. Simultaneously, an assessment of tumor size was performed by measurements of the 2 greatest perpendicular diameters of the subcutaneous tumors, measured every 3 days with a caliper.[29]

All aspects of the animal studies met the accepted criteria for the care and experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine Charles University in Prague.

Statistical analysis

Data are presented as the median and 25–75% range, or the mean ± SD. The statistical significance of differences between variables was evaluated by the Mann–Whitney Rank Sum test. The effect of statin treatment on the survival of animals was analyzed by a standard nonparametrical analysis (Mann–Whitney Rank Sum test), as well as Kaplan Meier Log Rank survival analysis with Holm–Sidak *posthoc* testing, when survival p-value was significant. Group mean differences in tumor size were measured by repeated measures analysis of variance (RM ANOVA) with Holm–Sidak *posthoc* testing, when p value was significant. When needed, log transform values of tumor size were used for comparisons to comply with normality and equal variance requirements. Differences were considered statistically significant when p values were less than 0.05.

Results

The in vitro *inhibitory effects of statins on viability of pancreatic adenocarcinoma cell lines*

Tumor-suppressive effects of individual statins were analyzed *in vitro* by experiments on pancreatic cancer-derived cell lines. In contrast to well-differentiated adenocarcinoma cells, CAPAN-2 and MiaPaCa-2 cells are poorly differentiated. Both of these cell lines harbor activating K-*ras* mutations in codon 12, prevalently associated with pancreatic cancer.[12] Generally, the presence of statins in the growth medium dramatically reduced the numbers of cancer cells (Figs. 1*a–f*, Table I). However, various statins exhibited significantly different inhibitory efficacy and we have also observed notable differences between the sensitivity among the individual cell lines. In the CAPAN-2 and MiaPaCa-2 cell lines, even the lowest tested concentrations (10 and 15 μM, respectively) of cerivastatin, simvastatin and lovastatin induced a cytostatic effect, evaluated as zero increase of the cell number, compared to the control at the time of statin application (Figs. 1*a–d*, Table I). The tumor-suppressive effect of fluvastatin and atorvastatin was slightly lower, corresponding to a 20 μM concentration. The tumor-suppressive effect of rosuvastatin was significantly lower than the aforementioned statins, and was observed at a concentration

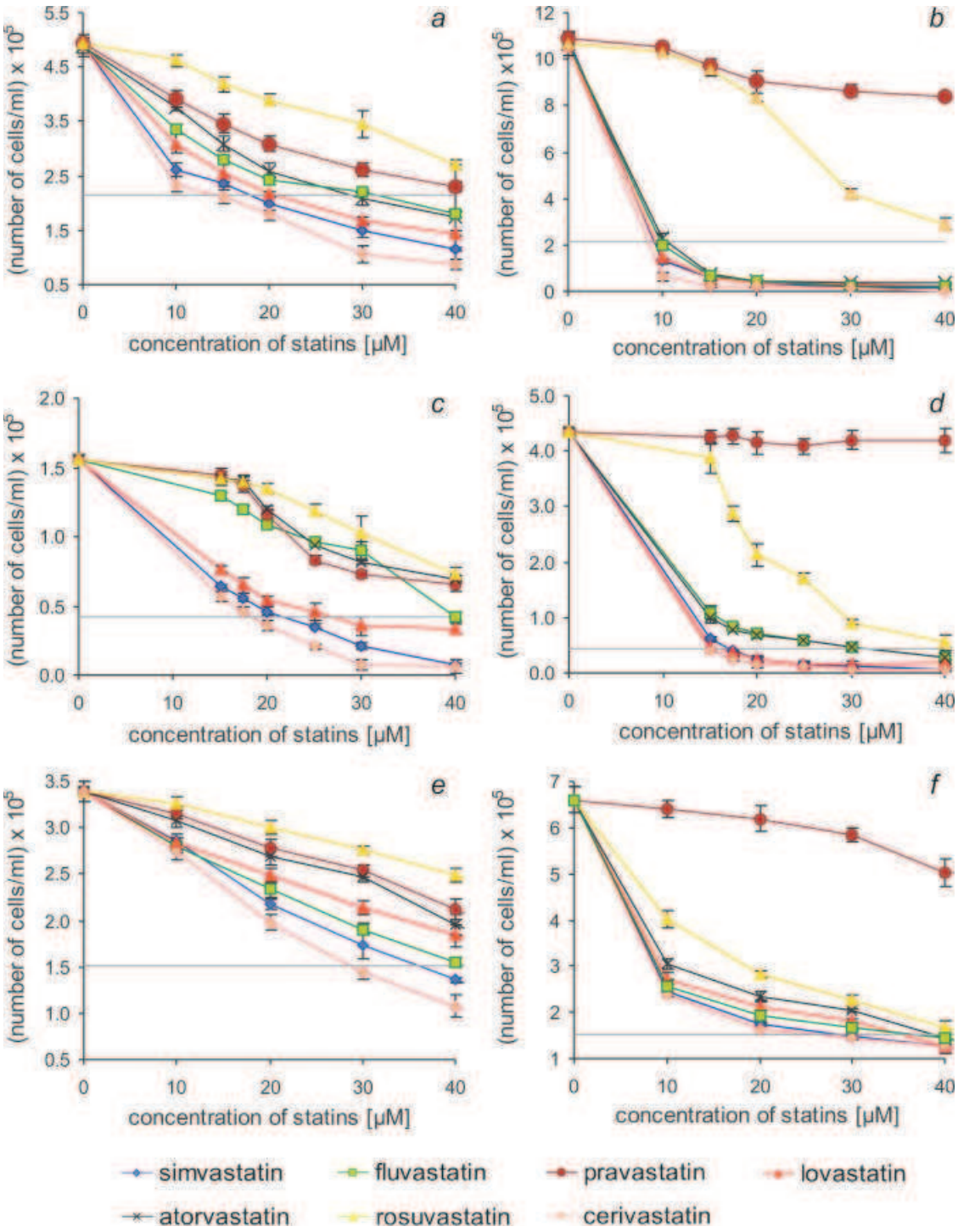

FIGURE 1 – Growth curves of pancreatic cancer cell lines cultured with different concentrations of individual statins. (*a*) CAPAN-2 cells, 24-hr incubation; (*b*) CAPAN-2 cells, 72-hr incubation; (*c*) MiaPaCa-2 cells, 24-hr incubation; (*d*) MiaPaCa-2 cells, 72-hr incubation; (*e*) BxPc-3 cells, 24-hr incubation; (*f*) BxPc-3 cells, 72-hr incubation. Gray horizontal line in each graph represents the initial cell number.

of 40 μM. Only slight cytotoxic effects (the prevalence of cell dying) were observed at the lower tested concentrations (Fig. 1). All statins (except pravastatin) exhibited an acute cytotoxic effect in concentrations higher than 40 μM, manifested by release of the cells into the growth medium and permeabilization of the plasma membrane, which was detected by trypan blue staining (data not shown).

BxPc-3 cells are moderately differentiated cancer cells producing the wild type K-*ras* proto-oncogene[30] and overexpressing cyclooxygenase-2.[31] This cell line was less sensitive to the tumor-suppressive effect of statins especially in the short-term experiments (24 and 72 hr, Figs. 1*e* and 1*f*, Table I; data for 48 hr not shown). This is well documented by an increase of IC_{50} values for individual statin, compared to these values for the other cell lines

TABLE I – THE EFFECT OF INDIVIDUAL STATINS ON GROWTH OF PANCREATIC CANCER

	IC_{50} (μM)								
	CAPAN-2			MiaPaCa-2			BxPc-3		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Rosuvastatin	39	30	26	36	27	20	56	50	13
Pravastatin	27	>40	>40	29	>40	>40	39	48	>40
Atorvastatin	22	7	5	27	21	10	37	33	10
Fluvastatin	21	5	5	26	12	9	29	27	7
Lovastatin	16	5	4	13	11	3	33	31	7
Simvastatin	14	6	3	12	10	5	26	23	6
Cerivastatin	12	3	2	10	9	5	22	21	8

TABLE II – THE EFFECT OF MEVALONATE, FPP AND GGPP ON THE TUMOR SUPPRESSIVE ACTION OF STATINS

	CAPAN-2 (% of control cells)	MiaPaCa-2 (% of control cells)	BxPc-3 (% of control cells)
Mevalonate 17 μM	76	–	–
Mevalonate 600 μM	98	92	81
FPP	103	–	–
GGPP	133	–	–
Pravastatin	88	91	88
Pravastatin + mevalonate 17 μM	91	–	–
Pravastatin + mevalonate 600 μM	98	112	87
Prava + FPP	90	–	–
Prava + GGPP	115	–	–
Lovastatin	6	2	9
Lovastatin + mevalonate 17 μM	32	–	–
Lovastatin + mevalonate 600 μM	86	93	90
Lovastatin + FPP	56	–	–
Lovastatin + GGPP	79	–	–
Atorvastatin	1	0	8
Atorvastatin + mevalonate 17 μM	23	–	–
Atorvastatin + mevalonate 600 μM	91	71	79
Atorvastatin + FPP	59	–	–
Atorvastatin + GGPP	76	–	–
Simvastatin	0	0	5
Simvastatin + mevalonate 17 μM	10	–	–
Simvastatin + mevalonate 600 μM	78	84	85
Simvastatin + FPP	29	–	–
Simvastatin + GGPP	75	–	–
Fluvastatin	3	0	6
Fluvastatin + mevalonate 17 μM	55	–	–
Fluvastatin + mevalonate 600 μM	105	99	67
Fluvastatin + FPP	41	–	–
Fluvastatin + GGPP	62	–	–

The cells were cultured for 72 hr in the presence of the substances stated above (concentration of statins = 30 μM, FPP, GGPP = 17 μM).

tested (Table I). Interestingly, all the tested compounds (except pravastatin) exhibited a comparable cytostatic effect for both the BxPc-3 cells and the other cell lines, after 72-hr exposure. A high proportion of the cell population exhibited signs of degeneration, such as granulation of cytoplasmic material, as well as decreased cell viability, according to trypan blue staining. A total cytostatic effect was observed at the 60 μM concentration of cerivastatin, simvastatin, lovastatin and fluvastatin (data not shown).

The viability of cancer cells was substantially prevented by all statins, except pravastatin, which only reduced the growth progression when administered at a high concentration (40 μM). Although pravastatin was generally the least effective statin after 48-hr and 72-hr exposure, compared to rosuvastatin, it exhibited a better inhibitory effect in the 24-hr experiment (Table I).

Addition of mevalonate, as well as FPP or GGPP, substantially abrogated the inhibitory growth effect of all statins (Table II), suggesting that the effect was caused by effective inhibition of farnesylation and not by the possible toxicity of statins. When mevalonate was added in a concentration far exceeding 20× that of statins, their tumor-suppressive effect was completely eliminated, while equimolar concentrations of mevalonate only had a partial effect (Table II). Surprisingly, both forms of simvastatin (*i.e.*, lactam and lactone) were found to have similar efficacy (data not shown).

The effect of individual statins on K-Ras protein translocation

Since K-Ras proteins translocation from cytoplasm to the cell membrane is dependent on its farnesylation, we investigated the potential effect of statins on this cellular event in MiaPaCa-2 pancreatic cancer cells transfected with pEGFP-K-Ras (HeLa, wild type) plasmids.

As demonstrated in Figure 2, all the statins tested at the 20 μM concentration efficiently inhibited K-Ras protein trafficking from cytoplasm to the cell membrane. The only exception was pravastatin, which did not influence this process at all. Similar effect of statins on the localization of K-ras carrying the activation mutation (MiaPaCa-2 G12C) was observed. Again, pravastatin did not have any effect (data not shown).

Antitumor effects of individual statins on human pancreatic adenocarcinoma cell line CAPAN-2 xenotransplanted to nude mice

All of the statins used significantly prolonged survival in mice suffering from human pancreatic adenocarcinoma. While the median survival rate of placebo treated animals was 31.5 [29–32] days (median [25–75%]), the mice treated with statins survived significantly longer, depending on statin used (39–106.5 days, Fig. 3). Compared to placebo-treated animals, mice treated with pravastatin

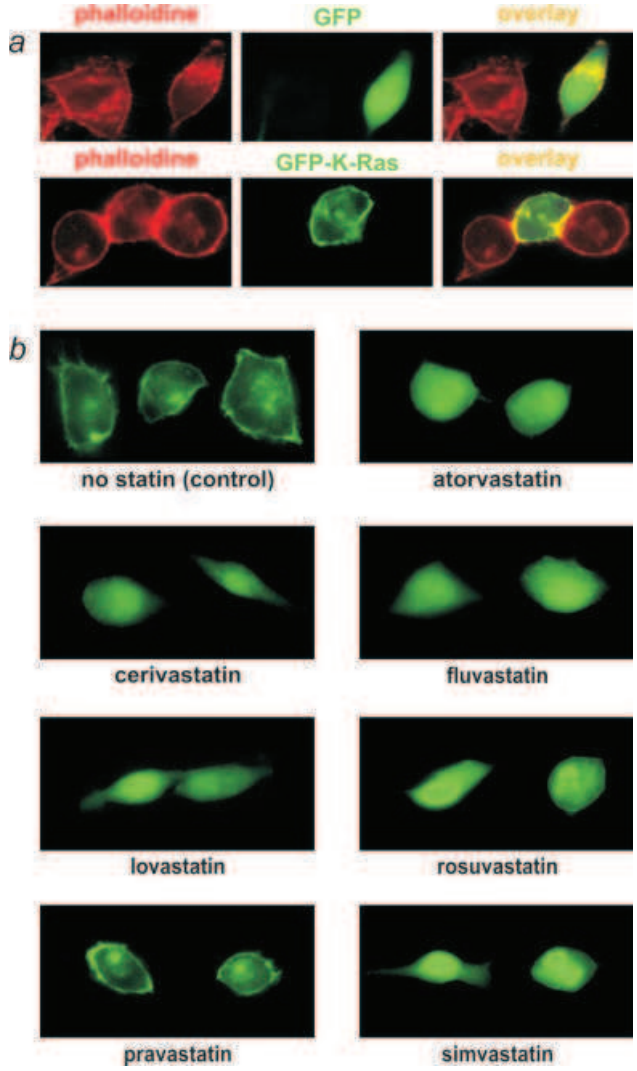

FIGURE 2 – Localization of GFP-K-Ras in MiaPaCa-2 pancreatic cancer cells. (*a*) Cells expressing GFP or GFP-K-Ras cultured without statin were stained with TRITC phalloidine for visualization of actin, localized predominantly at the cytoplasmic membrane. (*b*) The effect of individual statins (20 μM) on GFP-K-Ras localization in MiaPaCa-2 pancreatic cancer cells transfected by pEGFP-K-Ras plasmids.

survived 39.5 [36–42] days ($p = 0.01$), while survival rates in the atorvastatin and simvastatin group were 39 [36–45] ($p = 0.04$) and 42 [39–44] days ($p = 0.002$), respectively. The highest survival rate was found in the mice treated with cerivastatin (106.5 [47–122] days, $p = 0.002$) and rosuvastatin groups (104.5 [65–122] days, $p = 0.002$). Substantial anticancer effects were also observed in a fluvastatin group (56 [42–246] days, $p = 0.009$), where 2 complete remissions were even recorded. Partial tumor regressions accompanied with central necrosis were also observed in several mice treated with cerivastatin and rosuvastatin. The significance of these results was also confirmed in a Kaplan–Meier survival analysis, demonstrating that the longest survivals in animals were those treated with rosuvastatin, cerivastatin, fluvastatin and simvastatin (Fig. 3). However, marked differences in the survival rate were also observed among these most effective statins. After detailed analysis of the survival data, significant differences among the most and the least effective statins were detected (Table III).

The survival rate correlated well with the progression of tumor size. The increase in volume of CAPAN-2 tumors during treatment was significantly depressed in the statin-treated groups (Fig. 4*a*). Similar to the results of the survival analysis, significant differences were detected among the individual statins, with the best results found in fluvastatin and cerivastatin groups (Fig. 4*b*).

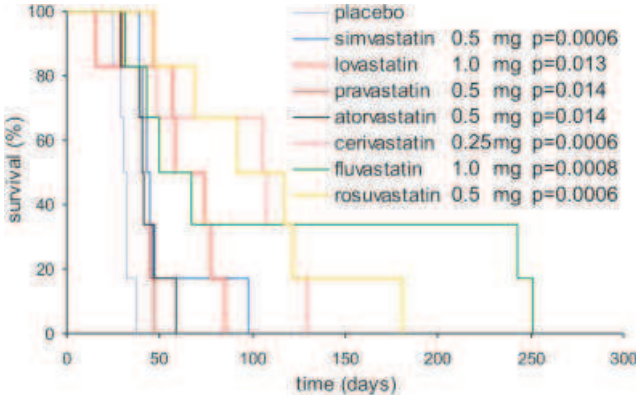

FIGURE 3 – The effect of statin therapy on survival of nude mice xenotransplanted with human pancreatic cancer cell line CAPAN-2. Dose = mg/kg body wt.

TABLE III – DIFFERENCES IN THE SURVIVAL RATE OF CAPAN-2 PANCREATIC CANCER-BEARING ANIMALS AMONG VARIOUS STATIN TREATMENTS

Statin treatment	*p* value
Rosuvastatin > pravastatin	0.0005
Rosuvastatin > atorvastatin	0.002
Rosuvastatin > simvastatin	0.013
Rosuvastatin > lovastatin	0.042
Cerivastatin > pravastatin	0.0005
Cerivastatin > atorvastatin	0.006
Cerivastatin > simvastatin	0.005
Cerivastatin > lovastatin	0.065
Fluvastatin > pravastatin	0.025
Fluvastatin > atorvastatin	0.060
Fluvastatin > simvastatin	0.165
Fluvastatin > lovastatin	0.582

Data from Kaplan–Meier Log-Rank Survival analysis.

Compared to initial values of tumor size, treatment with cerivastatin and rosuvastatin did not lead to significant tumor progression, as measured by tumor size before death ($p > 0.05$, data not shown); while treatment with fluvastatin resulted in a subtle, but again insignificant, diminishing of tumors (due to 2 remissions observed in this group). In all other treatment groups, tumor progression was delayed but not prevented.

Routine histological examination of pancreatic cancer treated with saline and fluvastatin did not show any major difference in any of the examined samples, despite the fact that placebo-treated animals exhibited rapid progression of the tumors (data not shown).

Discussion

Statins have been intensively studied drugs because of their deep impact on the human organism caused, in particular, by their cholesterol-lowering actions. However, their effects are pleiotropic and include potentially remarkable and clinically relevant antitumor effects. Indeed, the antiproliferative effects of statins were described for various experimental tumors, including pancreatic cancer.[10–15,32–35] The first study on the effect of statins upon pancreatic cancer was published as early as 1992 by Sumi *et al.*,[10] who described the substantial antiproliferative effect of lovastatin on both poorly and well-differentiated human pancreatic cancer cell lines *in vitro* as well as *in vivo*. However, only extremely high doses of lovastatin (60 mg/kg body wt./day) were used *in vivo*.[10] A similar effect on pancreatic cancer was also described for lova-

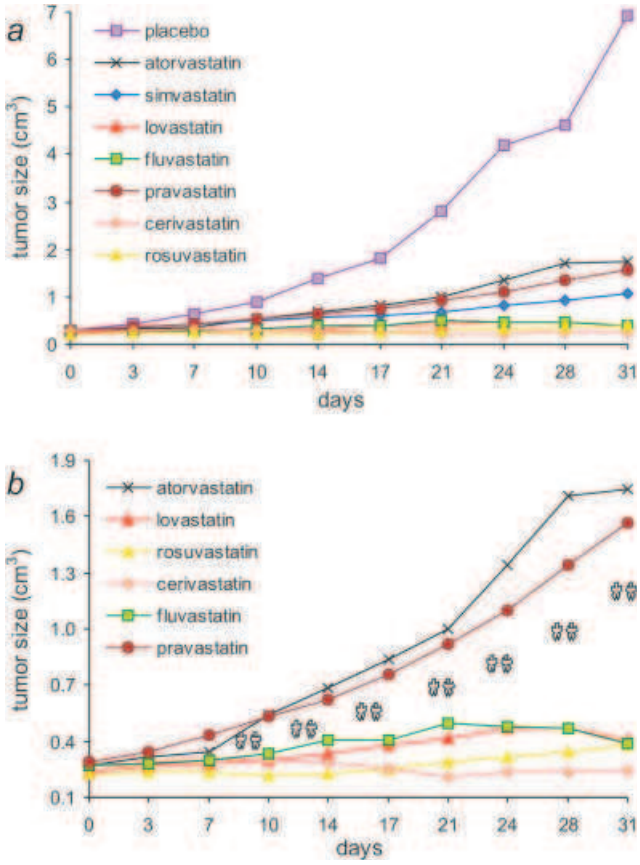

FIGURE 4 – (*a*) Tumor size after various statin treatments. (*b*) Differences in tumor size after various statin treatments. RM ANOVA with Holm–Sidak *posthoc* testing was used for comparison of measured variables. Although original data are depicted in the figures, statistical analyses were performed on log transform values to comply with normality and equal variance requirements. $^{**}p < 0.05$ for the difference between atorvastatin and pravastatin *vs.* the remaining (except simvastatin) statin groups.

statin by Mikulski *et al.*[11] In studies by Kusama *et al.*,[13,14] performed upon 3 human pancreatic cancer cell lines, micromolar concentrations of fluvastatin and lovastatin inhibited EGF-induced translocations of RhoA and cancer cell invasion in a dose-dependent manner. These effects were reversed by the addition of all-*trans*-geranylgeraniol, but not by the addition of all-*trans*-farnesol, suggesting involvement of Rho activation, and not Ras signaling, in the process of cancer cell invasion. In another *in vitro* study on 5 pancreatic cancer cell lines by Sumi *et al.*,[12] as well as in a study by Muller *et al.*,[32] lovastatin inhibited the proliferation of pancreatic cancer, even in the absence of activating point mutations in the K-*ras* gene. In addition, the chemoadjuvant effect of statins on experimental pancreatic cancer has been described in several studies, including the beneficiary action of fluvastatin and gemcitabine[34] or lovastatin and troglitazine, a PPAR-γ agonist.[35]

These data seem to be in contrast to recent observations on the inducing effects of statins on the heme oxygenase-1 (HO-1),[36] a potent bioactive enzymatic system supposed by some authors to have procancerogenic role.[37,38] However, opposing, *i.e.*, protective effects of HO-1 expression have been demonstrated by other authors for breast,[39] liver[40] and colon cancers.[41] In addition, both pravastatin and simvastatin in a concentration range of 50–250 μM were not able to anyhow modulate HO-1 activity in CAPAN-2 pancreatic cancer cells (unpublished data, R. Motterlini and L. Vítek).

The current study is, according to the authors' knowledge, the first study to have compared differences in the effects of the individual commercially available statins upon the viability and growth of human pancreatic cancer *in vitro* and *in vivo*. All of the statins used, except pravastatin, substantially inhibited the growth of all 3 different pancreatic cell lines *in vitro* (Fig. 1, Table I). However, the intensity of this effect was dependent on the type of statin used, with marked differences among the individual compounds. These inhibitory effects were partially prevented by concomitant addition of mevalonic acid, FPP or GGPP, indicating the contribution of downstream intermediates in cholesterol biosynthesis for growth and viability of pancreatic cancer cells (Table II). The effect of GGPP can be related to the activation of another protein family than Ras, *i.e.* the Rho family (Rho, Rac and Cdc42), where geranylgeranylation is the predominant mechanism, whereas Ras proteins are mainly farnesylated. However, both K- and N-ras can be geranylgeranylated in the cells treated with inhibitors of farnesyltransferase.[42]

The most potent statin under *in vitro* conditions was cerivastatin, followed by simvastatin and lovastatin. Slightly less effective were fluvastatin and atorvastatin. The effect of these statins far exceeded the effects of rosuvastatin and pravastatin. However, these findings did not entirely parallel *in vivo* results, in which fluvastatin, cerivastatin and rosuvastatin had much better tumor-suppressive responses (Fig. 3). This is very interesting, especially for fluvastatin, if we consider that fluvastatin has the lowest hypocholesterolemic effects among the statins on the market.[43] Marked differences in the impact of individual statins on cancer cell viability, with the worst effects found for pravastatin, may be due to many factors such as different chemical structures leading to changes in their pharmacokinetics and pharmacodynamics.[26] Except for pravastatin and rosuvastatin, all statins are lipophilic substances which certainly affect their behavior and bioavailability for cells and tissues in the organism (where the situation is far more complex). In fact, lipophilic statins lovastatin and simvastatin were found to inhibit HMG-CoA reductase (also in the cells of peripheral origin), while pravastatin exhibited this action only in cultured hepatocytes.[44] In contrast to pravastatin, rosuvastatin, the second hydrophilic statin, is able to inhibit HMG-CoA reductase in nonhepatic cells with 2 orders of magnitude higher efficacy.[45] However, it should also be noted that the half-life elimination of rosuvastatin is ~20 hr, compared to a half-life of all other statins between 2 and 3 hr. In addition, whereas lipophilic atorvastatin, fluvastatin, lovastatin and simvastatin were described to have direct proapoptotic effects,[46,47] pravastatin lacks this action.[47] These data are also consistent with the lack of inhibitory effects of pravastatin on K-Ras protein translocation in pancreatic cancer cells, as compared to other statins. Interestingly, the effect of rosuvastatin on K-Ras translocation, which exhibits the highest IC_{50} value, was comparable to the most effective statins (Table I, Figs. 1 and 2).

Differences in the biological behavior of statins are further evidenced by their antioxidant effects, since it has been demonstrated that fluvastatin exerts 2 orders of magnitude higher antioxidant capacity (which obviously might be of clinical relevance) as compared to pravastatin.[48] Although lactone prodrugs of lovastatin and simvastatin are ineffective for the inhibition for HMG-CoA reductase, these isomers are potent modulators of the 20S proteasome.[49] However, it is important to emphasize that both (open and lactone) forms were identically active against tumor viability in our *in vitro* experiments. Other factors, such as the aforementioned different bioavailability, protein binding, conversion to metabolites, as well as elimination half-life[26] may contribute to the observed diversity.

Our data are corroborated by an *in vitro* breast cancer study by Mueck *et al.*,[25] who also reported different tumor-suppressive effects of various statins. Interestingly, in this study, fluvastatin and simvastatin were also the most effective statins, whereas pravastatin had no effect up to 50 μM concentrations.[25] Negligible antitumor effects of pravastatin were also observed in other cancer studies,[22–24] as well as no antiviral effect having been demon-

strated for pravastatin compared to the strong antiviral activity on hepatitis C of the other tested statins.[50]

Genetic heterogeneity is another important factor which affects sensitivity of particular cancer cells to antiproliferative/proapoptotic effects of statins. This has been reported in a very recent paper by Wong *et al*.,[51] who demonstrated that only half of the 17 multiple myeloma cell lines tested was sensitive to lovastatin-induced apoptosis, while resistant cell lines had different genetic profile. Moreover, specific adaptation mechanisms may lead to selection of variant resistant cancer cell clones as was shown for pravastatin-treated human breast and gastric cell lines.[52] It is not clear whether other statins are more efficient in preventing development of statin resistance, but this may be further factor accounting for the weakest anticancer effects of pravastatin.

In vitro inhibitory concentrations of statins, similarly as in the majority of previous studies, may seem biologically irrelevant and not accounting for marked tumor-suppressive effects of much lower doses of statins *in vivo*. However, it must be stressed that in the human body statins may undergo biotransformation to become substantially more active inhibitors. All statins are metabolized in the liver where lovastatin, simvastatin, atorvastatin and cerivastatin share a common metabolic pathway through cytochrome P-450 3A4. Simvastatin is metabolized by hepatic CYP3A4 onto its dihydroxy acid form, whose antiproliferative effects were $\sim$1 order of magnitude higher, compared to simvastatin with IC_{50} (being 0.5–2.3 mg/l for 8 different pancreatic cancer cell lines tested).[33] Similar biotransformation into active hydroxyl metabolites was also described for lovastatin, atorvastatin and fluvastatin,[26] which is subjected to the action of cytochrome P-450 2C9. Pravastatin has multiple metabolic pathways. Since pancreatic cancer cell lines are not equipped with these biotransforming enzymes, it is not surprising that much higher doses must be used *in vitro* to mimic similar *in vivo* effects.

In support of the tumor-suppressive effects of statins, there are also the results of either randomized clinical trials or observational epidemiological studies, which were the subject of recent reviews and meta-analyses (for review, see Ref. 2). Although several epidemiological studies have proved the beneficial effects of statins on cancer risk,[2] these recent meta-analyses[53–56] did not confirm cancer protection in statin users. This fact might be due to a too short follow-up period, as well as a methodology bias of the epidemiologic studies on cardiovascular outcomes, which were not designed or statistically powered to appropriately evaluate any beneficial or detrimental effects on a relatively rare event (such as a subsequent malignancy). This also has been recognized as a possible explanation for the slightly increased cancer incidence in elderly individuals taking pravastatin in the PROSPER study.[57] Moreover, based on our *in vitro* and *in vivo* results, it seems that pravastatin has only negligible tumor-suppressive effects on pancreatic cancer. Interestingly, pravastatin also does not inhibit the proliferation of human breast cancer cells[25] or gastric carcinoma cells in culture,[58] but it does have beneficial effects on colon tumors in an animal model.[51] This may account for the negative results of the meta-analytic studies mentioned earlier. In fact, in a meta-analysis of 7 trials by Hebert *et al*.,[53] 43% (3 out of 7) of studies involved were those with pravastatin. The same was true for a study by Bjerre and LeLorier[54] (3 of 5 studies involved), CTT Collaborator's study[55] (5 of 14 studies involved), as well as in the most recent meta-analysis by Dale *et al*.[56] (10 of 20 studies). Interestingly, studies not demonstrating a drop of total cancer incidence or mortality also include data showing selective protective effects for certain types of cancer. This is true, for instance, for a large pravastatin clinical study CARE, in which a 43% decreased incidence of colorectal cancer was detected,[59] consistent with the experimental data for pravastatin mentioned earlier.[60] Similar results on the risk of colorectal cancer in patients mainly treated by pravastatin and simvastatin were also found in the study by Poynter *et al*.[61]

In conclusion, on the basis of our findings as well as results of other studies, we propose that the inhibitors of HMG-CoA reductase might be of potential for the chemoadjuvant treatment of pancreatic cancer. Indeed, promising results of a large, very recent study on the risk of pancreatic cancer in a population of almost half million US veterans support this.[62]

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Improved HPLC Analysis of Serum 7α-Hydroxycholest-4-en-3-one, a Marker of Bile Acid Malabsorption

To the Editor:

Serum concentrations of 7α-hydroxycholest-4-en-3-one (cholesten, bile acid synthesis intermediate) have been shown to correlate with the severity of bile acid malabsorption *(1, 2)*. Current techniques for holster quantification require sophisticated instrumentation *(3)* or solid-phase extraction (SPE) at 64 °C *(4)*. We developed and validated a method for measuring serum cholesten concentrations using routinely available HPLC instrumentation, focusing on the optimization of the SPE step.

Cholesten was purchased from Steraloids, and used to prepare serum calibrators containing 0–1000 μg/L. We added 30 ng of internal standard (7β-hydroxycholest-4-en-3-one, Steraloids) dissolved in 80 μL of methanol, to 1 mL of serum. Then 5 mL of chloroform:methanol (2:1, vol/vol, analytical grade, Penta) was added; the mixture was vortex-mixed vigorously, and centrifuged (2000*g*, 3 min, ambient temperature). The upper phase was discarded, and 2 mL of 125 mmol/L NaCl in 50% methanol (vol/vol) was added to the sample, vortex-mixed, and centrifuged as above. The lower phase was transferred to another tube and dried at 60 °C under nitrogen, dissolved in 1 mL of toluene (analytical grade, Penta), and loaded onto a Phenomenex Strata SI-1 100-mg silica precolumn that had been prewashed with 1 mL of isopropanol (Chromasolv, Merck), and equilibrated with 1 mL of hexane (Uvasol, Merck). After a washing step with 1 mL of hexane and 15 mL of isopropanol:hexane (0.4:99.6 vol/vol), cholesten was eluted with 1 mL of isopropanol, dried under nitrogen at 60 °C, and dissolved in 170 μL of acetonitrile:water (95:5 vol/vol). Then 150 μL of the sample was injected into the Agilent HP1100 HPLC system. The chromatographic parameters were: Tessek SGX C18 column (4 × 250 mm, 4 μm), acetonitrile:water (95:5, vol/vol) mobile phase, flow rate 1 mL/min, temperature 20 °C, detection/reference wavelength 241/360 nm *(4)*.

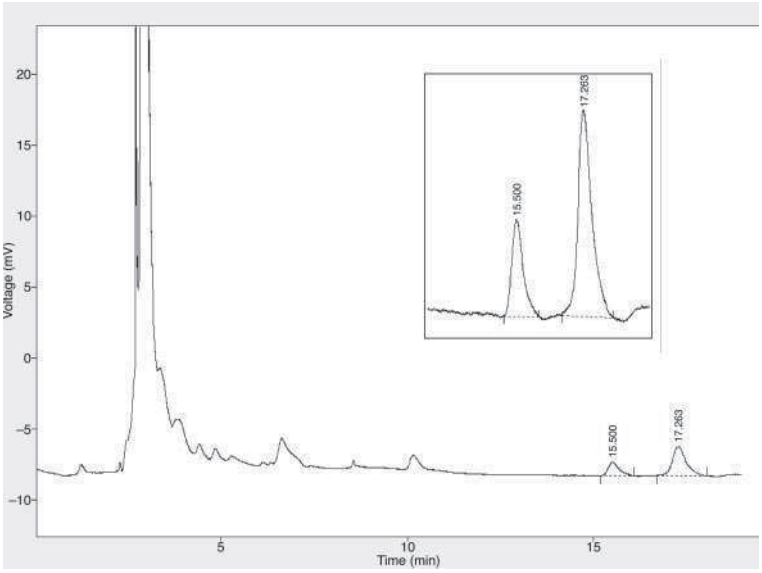

Fig. 1. Chromatogram of a serum sample containing 12 μg/L cholesten.
The inset provides a detailed view of the cholesten (first) and internal standard (second) peaks.

The method yielded a linear response (13 calibration points in triplicate) up to 1000 μg/L ($y = 0.9957x$, $R^2 = 0.9979$). A typical chromatogram is shown in Fig. 1. The detection limit, calculated as a concentration corresponding to a signal 3 SD above the mean for a calibrator free of analyte (n = 10), was 1.2 μg/L when 1 mL of serum was processed. Intraassay imprecision values (CV for 15 measurements of 3 specimens) were 2.8%, 3.2%, and 2.2% for samples with mean (SD) cholesten concentrations of 18.1 (0.5), 136.8 (4.3), and 237.7 (5.3) μg/L, respectively. We determined interassay imprecision by assaying 3 specimens 20 times (1 measurement per day) over a 3-month period. CVs were 5.1%, 4.3%, and 4.1% for samples with mean (SD) cholesten concentrations of 18.0 (0.9), 139.2 (6.0), and 244.4 (9.9) μg/L, respectively.

The average recovery, calculated as [(measured concentration − initial concentration)/added concentration], was 93%. The effects of hemoglobin, bilirubin, cholesterol, and triglycerides were estimated by the recovery of a known amount of analyte added to a serum sample with the interferent being tested. Recoveries (means of triplicates) were 97%, 90%, 108%, and 105% with added hemoglobin (5 g/L), bilirubin (96.2 μmol/L), cholesterol (9.1 mmol/L), or triglycerides (10.7 mmol/L), respectively.

To demonstrate the utility of this method, we measured serum cholesten concentrations in 2 groups: healthy volunteers [20 males, 30 females, mean (SD) age 39.6 (9.4) years] and patients who had undergone resection of terminal ileum, for whom malabsorption of bile acids would be expected owing to the loss of ileal bile acid transporter [22 males, 28 females, mean (SD) age

41.7 (13.5) years]. The study was approved by the local ethics committee. Compared to the healthy controls, median concentrations of cholesten (interquartile range) in patients with ileal resection were significantly higher [87.8 μg/L (range 42.1–150.5 μg/L) vs 11.9 μg/L (range 9.2–16.9 μg/L), $P < 0.001$, Mann–Whitney Rank-Sum test].

This method retains the advantages of other HPLC-based methods, while eliminating the need for temperature-controlled SPE. Silica cartridges offer higher binding capacity and the possibility of analyte extraction at ambient temperature, which, compared to previously used C8 cartridges, results in a wider linear range (up to 1000 μg/L vs 200 μg/L) *(4)*. Except for the chloroform:methanol extraction, the analysis can theoretically be automated. Additionally, the initial chloroform:methanol extraction ensures quantitative extraction of both cholesten and the internal standard and thus potential imprecision caused by either internal standard precipitation or incomplete extraction of protein-bound cholesten can be avoided.

The increased availability of a laboratory diagnosis of bile acid malabsorption is of considerable importance, especially for patients with chronic diarrhea and irritable bowel syndrome. These disorders belong to the most common gastrointestinal conditions, and it is estimated that bile acid malabsorption might be present in about half of these patients *(5)*. Because the majority of patients wit bile acid malabsorption respond to bile acid sequestrants *(5)*, targeted therapy, based on serum cholesten concentrations, should both improve outcomes and lower treatment costs.

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Unexpected Hemoglobin A Results after an Erythrocyte Exchange: Importance of Specimen Mixing

To the Editor:

Erythrocyte exchange, a procedure in which blood is removed and replaced with donor cells, is often used to prevent or treat severe vasocclusion in patients with sickling hemoglobinopathies. This procedure increases the percentage of hemoglobin A without dramatically increasing the hematocrit or viscosity. The efficacy of erythrocyte exchange is often measured by hemoglobin electrophoresis and densitometry to determine the posttransfusion percentages of hemoglobin A and S.

We performed a manual erythrocyte exchange on a 28-year-old pregnant woman with hemoglobin SD-Punjab. Five units of whole blood were withdrawn and replaced with packed erythrocytes and saline. The expected percentage of hemoglobin A in each unit withdrawn was calculated in an iterative fashion. We calculated the total volume of hemoglobin SD-Punjab erythrocytes using the patient's total blood volume estimated by nomogram, measured hematocrit, and assuming 100% hemoglobin SD-Punjab. The volume of erythrocytes removed was subtracted from the hemoglobin SD-Punjab erythrocyte volume tally, and the volume of erythrocytes administered (assumed to be an average of 160 mL) was added to the hemoglobin A erythrocyte volume tally. Volumes and percentages of hemoglobin A and SD-

Inflammatory Bowel Diseases

Bile Acid Malabsorption in Inflammatory Bowel Disease: Assessment by Serum Markers

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Title: Bile Acid Malabsorption in Inflammatory Bowel Disease: Assessment by Serum Markers

Short title: Bile acid malabsorption in Crohn's disease

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Abbreviations: BA, bile acid; BAM, bile acid malabsorption; C4, 7α-hydroxycholest-4-en-3-one; CD, Crohn's disease; FGF19, fibroblast growth factor 19; HPLC, high performance liquid chromatography; ^{75}SeHCAT, selenium homotaurocholic acid; SLC10A2, apical sodium-dependent bile acid transporter; UC, ulcerative colitis

Conflict of interest: Authors have nothing to disclose.

Authors´ contribution:

ML was involved in all analyses, data interpretation and drafting of the Ms.

DD, VK, and ML were responsible for examination of patients, material acquisition, and drafting of the Ms.

BG was involved in C4 analyses

ZS was responsible for all statistical analyses and critical reading of the Ms.

LV was responsible for study concept and design, study supervision, and critical reading of the Ms.

Abstract

Background: Bile acid malabsorption (BAM) is a common feature of Crohn's disease (CD). We aimed to determine whether BAM develops only in patients with a resected distal ileum or if it also occurs in patients that have not undergone surgery for CD.

Methods: The study included 347 patients with CD or ulcerative colitis (UC) and 119 healthy subjects (controls). BAM was assessed by measurement of serum levels of 7α-hydroxycholest-4-en-3-one (C4) and fibroblast growth factor 19 (FGF19). We surveyed members of the European Crohn's and Colitis Organization and International Organization for the Study of Inflammatory Bowel Disease to collect current information about BAM diagnosis.

Results: The severity of BAM was associated with resection of the distal ileum. Compared with controls, patients that received moderate or extensive ileal resection had significantly increased levels of serum C4 (12 vs. 62 vs. 243 μg/L, respectively; $P<.001$). However, BAM was also present in a substantial number of the patients with CD that were not treated by surgery who had ileitis or colitis (14% and 11%, respectively). There was an indirect, proportional relationship between levels of C4 and FGF19 ($P<.001$).

Conclusions: The most severe BAM occurs in CD patients after resection of the distal ileum, but BAM can occur in surgically untreated CD patients, regardless of disease localization. Laboratory tests for BAM should become a part of the algorithm for diagnosis of CD, to identify patients that might respond to therapies such as bile acid sequestrants. FGF19 appears to be a reliable marker of BAM.

Key Words: Bile acid malabsorption, Crohn´s disease, 7α-hydroxycholest-4-en-3-one; ileal disease, inflammatory bowel disease.

Introduction

Bile acid malabsorption (BAM) is a common feature of Crohn's disease (CD), which can lead to complications such as: diarrhea[1]; hyperoxaluria with increased kidney stone formation[2]; or biliary hypersecretion of bilirubin, leading to pigment gallstone formation[3]. Some of these complications can be prevented or mitigated. In cholerrheic diarrhea, sequestrants of bile acids (BA) are the therapeutics of choice[4]; while the oral supplementation of calcium salts[5], colestipol, aluminium hydroxide[6], or potassium citrate[7] might be useful in the prevention of kidney stone formation. The prerequisite of successful therapeutic intervention is either the laboratory or clinical diagnosis of BAM. Several diagnostic laboratory tests were introduced into clinical practice, including fecal BA determination, BA breath or retention tests or determination of plasma metabolites of the BA biosynthetic pathway[8-11]. The diagnostic and therapeutic value of a laboratory determination of BAM has been demonstrated in chronic diarrhea of unknown origin[11,12], collagenous colitis[13], and microscopic colitis[14]. With CD, the importance of BAM determination has been demonstrated in two small clinical studies[15,16]; however, its usefulness has not been confirmed in other studies[17,18].

The highly effective active resorption of conjugated BA occurs in the distal ileum, by the action of the ileal sodium/bile acid cotransporter (ISBT, alias IBAT, alias ASBT), encoded by *SLC10A2* gene[19]. The passive diffusion of unconjugated BA, which occurs throughout the whole large intestine, is much less effective. Therefore, the distal ileum plays a crucial role in the enterohepatic circulation of BA[20]. Recently, a haplotype block associated with the lower expression of ISBT has been described[21].

Upon stimulation with BA, ileal enterocytes secrete fibroblast growth factor 19 (FGF19), which subsequently down-regulates BA synthesis in the hepatocytes[22]. Thus, FGF19 represents an additional negative feedback regulatory mechanism for the production of

BA. Recently it has been suggested that defective FGF19-mediated down-regulation of BA synthesis, rather than insufficient BA absorption, is a possible cause of increased BA fecal excretion[23,24]. Simultaneously, FGF19 has been proposed as being a promising diagnostic marker for BAM[23].

The aim of our study was to find out whether the knowledge of the localization and extent of CD are sufficient for an estimation of BAM's severity; or whether laboratory testing should be employed in order to improve the diagnostic and therapeutic outcomes in these patients. The other aim was to analyze and compare serum levels of FGF19 and 7α-hydroxycholest-4-en-3-one (C4), a surrogate marker of BAM[10], in CD patients with well-defined disease localization.

Materials and Methods

Subjects

A total of 347 patients with inflammatory bowel disease, as well as 119 healthy subjects participated in this study. Patients were recruited on the consecutive basis from the IBD Clinical and Research Center, ISCARE I.V.F. in Prague, and from the 4th Department of Internal Medicine, 1st Faculty of Medicine of Charles University in Prague. According to the localization of the disease, patients were divided into the following groups: ulcerative colitis (UC), CD only with colonic involvement, non-operated CD with an affected ileum, plus CD after moderate (≤70 cm), or after extensive (>70 cm) resections of the distal ileum (Table). Patients with other phenotypes, such as proximal gastrointestinal tract involvement, stomias, etc. (as well as individuals taking BA or BA sequestrants) were excluded. The healthy volunteers were blood donors, as well as employees of the 1st Faculty of Medicine of Charles University in Prague. The study's protocol conformed to all of the ethical guidelines of the 1975 Declaration of Helsinki, reflected in the *a priori* approval by the Ethics Committee of the Institution. Additionally, all subjects had signed informed-consent forms.

E-mail survey

To get a rough overview about the current strategy of diagnostic and therapeutic procedures, we sent a short e-mail questionnaire to 113 national representatives of ECCO (European Crohn's and Colitis Organization) as well as active members of the Directory of IO-IBD (International Organization for the Study of Inflammatory Bowel Disease). The questionnaire was designed to inquire about the availability of laboratory tests of BAM in their hospitals (if any), indications of BAM determination in CD, as well as the use of BA sequestrants in CD. All responses that were received within 2 months of sending were evaluated.

Biochemical analyses

Blood was drawn from subjects between 8 a.m. and 10 a.m. after overnight fasting to minimize the effect of postprandial and circadian variability of C4 levels[25,26]. Serum samples were stored in aliquots at -80°C until analyzed. The C4 concentration was measured by HPLC, as previously described[27]. Total and HDL cholesterol levels were measured on a Modular automatic analyzer (Roche Diagnostics, Switzerland), using commercially available kits (Cholesterol liquicolor, and HDL Cholesterol liquicolor, respectively; both Human GmbH, Germany). Serum FGF19 levels were measured by ELISA (FGF19 Quantikine ELISA kit, R&D Systems, USA) in 341 subjects, from whom an additional serum aliquot was available.

SLC10A2 genotyping

Genomic DNA was isolated from peripheral blood white cells by the standard salting-out procedure. The 129 C>T variant (NM_000452.1:c.129C>T, rs41281680) in the *SLC10A2* gene was typed by PCR-restriction fragment length polymorphism in all subjects, where DNA was available. Primers 5‘-CATCCCATGGAGAACATCACC-3‘, 5‘-CCAGCAATGAATGATCCGAAC-3‘were used for amplification of the target DNA region. The resulting 158 base pair long amplicon was digested with BsuRI. The primers were synthesized by Generi Biotech (Czech Republic). All other reagents were obtained from Fermentas (Lithuania).

Statistical analyses

The differences in distributions of variables of interest were analyzed by Kruskal-Wallis ANOVA, including post-hoc comparison. The results are presented as the means ± SD;

or medians (5 - 95%) in case of asymmetrically distributed data. The linear regression analysis was used to test the association between C4 and FGF19. Due to skewed distribution, the calculation is based on log transformed data. A ROC curve was constructed using a web based calculator (http://www.jrocfit.org). The Hardy-Weinberg equilibrium was tested by using the Chi square test. The level of significance for the entire study was set at $P<.05$. Analyses were performed using STATISTICA software (version 8).

Results

As expected, the severity of BAM, assessed by a serum C4 level, was mainly dependent on the inflammation/resection of the distal ileum (Table, Figure 1). Compared to healthy controls, patients after moderate or extensive ileal resection had on average a 5 and 20-fold increased serum C4 level, respectively (Table, Figure 1). Similarly, CD patients without intestinal surgery, suffering from ileal inflammation, had 2-fold higher serum C4 levels when compared to healthy controls (Table, Figure 1). On the other hand, serum C4 levels of UC patients or CD patients with colonic involvement alone did not differ significantly, when compared to controls (Table, Figure 1). Similar results were obtained when C4/total cholesterol or C4/non-HDL cholesterol ratios were used (as supposedly more accurate markers of BAM) (Table)[28,29].

Considerable inter-individual variability of serum C4 levels within groups was observed (Figure 1). For example, 12% of CD patients with ileal involvement had serum C4 levels above the median for CD patients after moderate ileal resection. Conversely, 21% of CD patients after moderate ileal resection had serum C4 levels below the median of those with ileal inflammation.

Considering that serum C4 levels under 50 µg/L are normal[11], abnormal values were observed in 4% of control subjects, in 11% of the patients with CD colitis, in 14% of CD patients with ileal involvement, and in 57% and 88% of CD patients after moderate and extensive ileal resection, respectively; and in 1% of the UC patients.

Effect of *SLC10A2* genetic variant

The 129 C>T variant, representing a novel haplotype block (possibly associated with the decreased ileal expression of SLC10A2[21]) was typed in 87% of the control subjects and 91% of the patients. Due to the low frequency of a minor allele, no significant effect on the

serum C4 levels could be observed (data not shown). The genotypes were in Hardy-Weinberg equilibrium.

FGF19 as a surrogate marker of BAM

In subjects with BAM serum levels of FGF19 were markedly lower, when compared to those without BAM. Analogous to the C4 levels, abnormal FGF19 levels were most frequently seen in subjects with affected terminal ileum (Table, Figure 1). Overall, the serum levels of FGF19 were indirectly proportional to levels of C4 (r=-0.0594, *P*<.001) (Figure 2).

The ROC curve was created by plotting FGF19 levels against the laboratory diagnosis of BAM, based on the serum level of C4 (Figure 3). Two different ROC curves have been constructed, with respect to different C4 cut-off values. The first one was 50 µg/L (severe BAM, corresponding to the 7-day ^{75}SeHCAT retention of approximately 5%; calculated according to Bajor *et al.*[30]). The other cut-off value of C4 used was 30 µg/L (mild BAM, corresponding to 7-day ^{75}SeHCAT retention of 10%).

Based on the ROC curve, the optimal value of serum FGF19 levels maximizing precision has been set to ≥60 ng/L. In this case, the sensitivity and specificity of FGF19 as a marker of BAM reached 80% and 68%, respectively (Fig. 3).

E-mail survey

Forty-one gastroenterologists from 27 countries responded in the survey. A response of having no access to any laboratory test of BAM was acknowledged by 24 (59%) experts in 18 (67%) countries. The remainder have the possibility to perform a ^{75}SeHCAT "selenium scan" (6 countries), measure the serum levels of 7α-hydroxy cholesterol (4 countries), perform fecal excretion tests of BA (3 countries), determine serum C4 levels (1 country), or to perform an unspecified breath test (1 country). Resection of the distal ileum is considered as

an indication for the laboratory determination of BAM by 3 (7%) of the experts; while 9 (22%) of those require BAM measurement in patients with therapy-resistant diarrhea. Patients with both distal ileal resection and therapy resistant diarrhea are referred to BAM measurement by 4 (10%) specialists. The remaining 25 (61%) respondents never indicated BAM measurement in their CD patients. BA sequestrants are most frequently used in CD patients after resection of the distal ileum with therapy resistant diarrhea (66% of responders), 22% of responders use them in CD patients with therapy resistant diarrhea (regardless of ileal surgery), 2% in all CD patients after ileal resection, and 12% use BA sequestrants in CD patients with confirmed BAM. In three countries, BA sequestrants are not indicated in CD patients.

Discussion

In the present study, we investigated BAM in patients with inflammatory bowel disease based on surrogate serum markers C4 and FGF19. We found BAM in 42% of the 276 CD patients; which confirms that BAM is a common feature of CD. The severity of BAM varied significantly with the localization of the disease. As expected, the most severe BAM was observed in CD patients after resection of the distal ileum and in those with ileal involvement; while the majority of CD patients with colonic inflammation or UC patients did not suffer from BAM. However, the inter-individual variability within groups was substantial. Up to 38% of the patients after ileal surgery did not manifest BAM. On the other hand, there were a high number (11%) of BA malabsorbing patients, even in the case of CD with only colonic involvement. This is in agreement with the results of Jung *et al.*[31] and Holzer *et al.*[32], who reported a diminished expression of SLC10A2, independent of the ileal inflammation in CD patients. Recently, a novel haplotype block, linked to a reduced ileal expression of SLC10A2, was described[21]. In our study, however, we did not observe any contribution of this genetic variation to the inter-individual variability of the serum levels of C4, presumably due to the low frequency of a minor allele. Collectively, these data suggest that although the disease localization plays a key role, other factors may significantly modify the severity of BAM. Therefore, a laboratory determination of BAM should be performed in all symptomatic patients, in order to identify possible candidates for therapy with BA sequestrants.

Recently, defective FGF19-mediated feedback inhibition of BA synthesis has been identified as a possible cause of chronic diarrhea[23,24]. This is consistent with a previous observation of Bajor *et al.*[30], who have not observed the anticipated diminished, but rather normal or increased, ileal BA resorption in BAM subjects. In our study, we have demonstrated the close indirectly proportional correlation between C4 and FGF19 levels; suggesting the great diagnostic potential of this analyte, consistent with results of previous

studies[23,33]. In addition, ROC curve analysis identified serum FGF19 levels as a specific and sensitive marker for BAM. However, more comprehensive studies utilizing the ^{75}SeHCAT retention test, stool frequency monitoring, and ideally the response to BA sequestrant therapy are needed to assess whether serum levels of FGF19 might be superior to those of C4 in identifying BA malabsorbing patients.

Sequestrants of BA are used as an efficient therapy of chronic diarrhea, when BAM is at least partially responsible for the symptoms. The efficacy of BA sequestrants has been shown to be dependent on the degree of BAM (although in some cases cholestyramine may exert antidiarrhoeal effect unrelated to its bile acid-sequestering action[9]). Williams *et al.*[12] studied patients with unexplained chronic diarrhea. All patients with severe BAM responded to cholestyramine; while those with moderate, or very mild BAM, either showed a less pronounced effect (38%), or no response at all, respectively. Similar results have also been observed in collagenous colitis[10], and in two small cohorts of CD patients[15,16].

Interestingly, more then two thirds of the gastroenterologists in our survey use BA sequestrants only in symptomatic patients after resection of the distal ileum. This means that as many as 13% of the non-operated CD patients, who malabsorb BA, will not receive BA sequestrants.

Although different methods for the assessment of BAM have been described, according to our survey, the overall availability, based on our survey, seems to be insufficient. Most of the gastroenterologists do not have any diagnostic tool for BAM assessment in their patients. In this regard, serum FGF19 shows a promising diagnostic potential. Nevertheless, the unavailability of BAM tests can be partially by-passed by a therapeutic attempt with BA sequestrants in symptomatic patients. However, asymptomatic (*i.e.* diarrhea-free) BA malabsorbers still might be at high risk of kidney stone or gallstone diseases. Without a determination of BAM, this possible risk would remain undetected.

The study was limited by the non-availability of detailed clinical data, such as the activity of the disease. Therefore, it was not possible to estimate the proportions of symptomatic/asymptomatic BA malabsorbers. Similarly, the suggested normal range of FGF19 levels should be considered as preliminary, and needs to be replicated and confirmed in further studies. It also should be noted that BAM in our study was based on assessment of surrogate serum markers, which might be in some cases influenced by other causes as well. Nevertheless, based on our as well as previous data, the overall clinical utility for BAM diagnosis of these markers seems to be very high.

In summary, we confirmed, that BAM cannot reliably be predicted, based solely on clinical data. Therefore, we suggest that a laboratory determination of BAM markers should be incorporated in the diagnostic algorithm of patients with CD. When such a test is not available, an attempt of therapeutic BA sequestrant administration should be offered to symptomatic patients, regardless of the disease's localization. It remains to be resolved, whether asymptomatic BAM should be treated in order to prevent kidney stone and gallstone diseases.

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Figure legend

Figure 1

Serum levels of C4 (Fig. 1A) or FGF-19 (Fig. 1B) in patients with inflammatory bowel disease, according to localization of the disease.

Data expressed as the median, 25 - 75% (boxes) and min-max (whiskers). Number of subjects in each group is indicated. Dashed line demarks the normal level of C4 (FGF19). Inset shows p-values. ns = not significant.

Figure 2

Relationship between serum levels of C4 and FGF19 in both patients and healthy subjects.

Data are log transformed.

Figure 3

ROC curves for FGF19 at different C4 cut-off values.

AUC = area under the curve.

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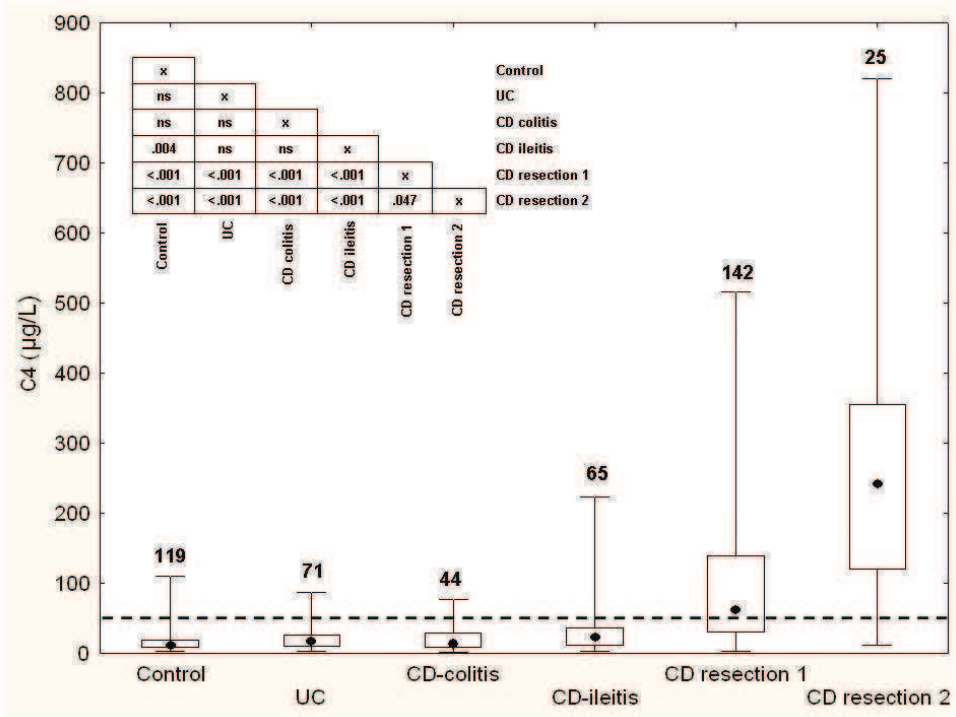
Table. Degree of BAM, according to disease localization

	Males	Age (years)	C4 (µg/L)	C4/total cholesterol (µg/mmol)	C4/non-HDL cholesterol (µg/mmol)	FGF-19 (ng/L)
Control subjects n=119 (102)*	53%	44±12	12 (3-43)	2.4 (0.7-8.5)	3.8 (1.0-12.8)	107 (39-402)
UC patients n=71 (43)*	51%	44±16	17 (4-42)	3.3 (0.7-8.9)	5.1 (1.0-12.0)	90 (20-444)
CD-colitis n=44 (40)*	36%	38±11	13 (3-68)	2.9 (0.7-13.9)	4.0 (1.3-19.0)	90 (19-259)
CD-ileitis n=65 (48)*	42%	34±11	24 (6-77)	5.4 (1.4-21.8)	7.5 (2.1-35.3)	58 (18-303)
CD-resection ≤70 cm n=142 (96)*	40%	42±13	62 (9-329)	15.4 (2.2-74.1)	23.6 (3.0-118.1)	37 (3-145)
CD-resection >70 cm n=25 (12)*	56%	56±10	243 (36-627)	66.6 (12.4-146.9)	92.9 (17.8-210.6)	21 (3-45)

*The number of subjects analyzed for FGF19 levels is shown in brackets.

Results are given as the mean±SD; or as the median (5 - 95%), when the data were not normally distributed.



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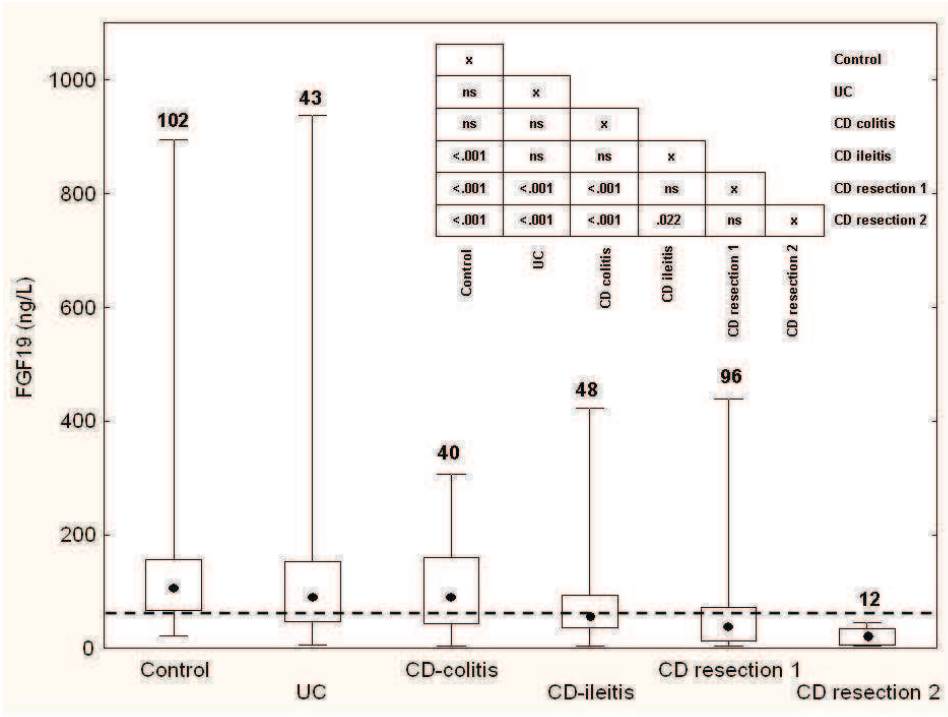

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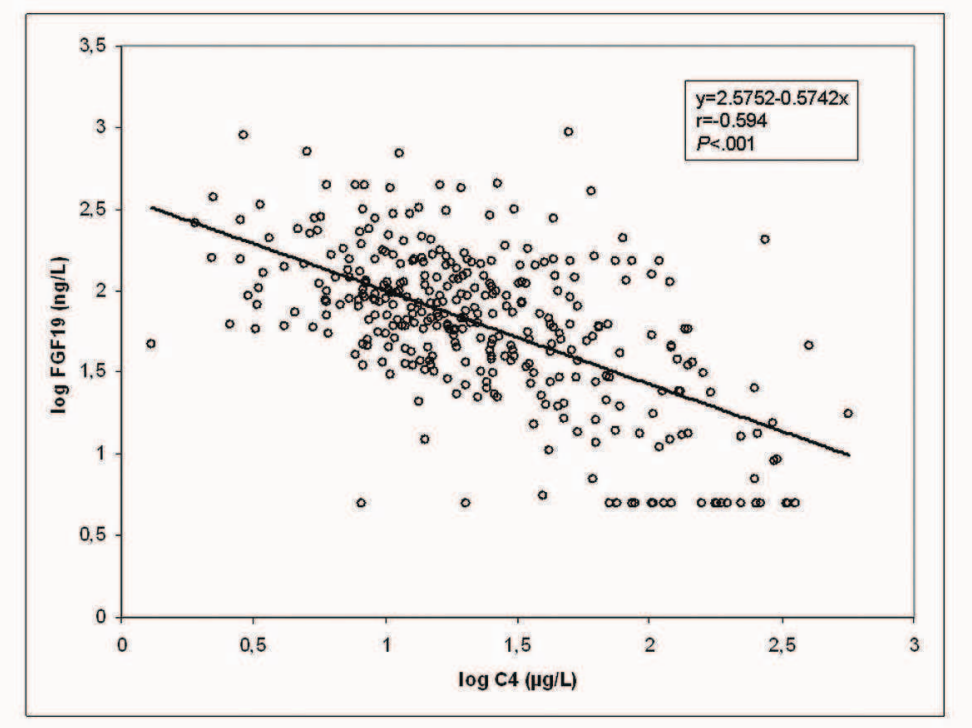

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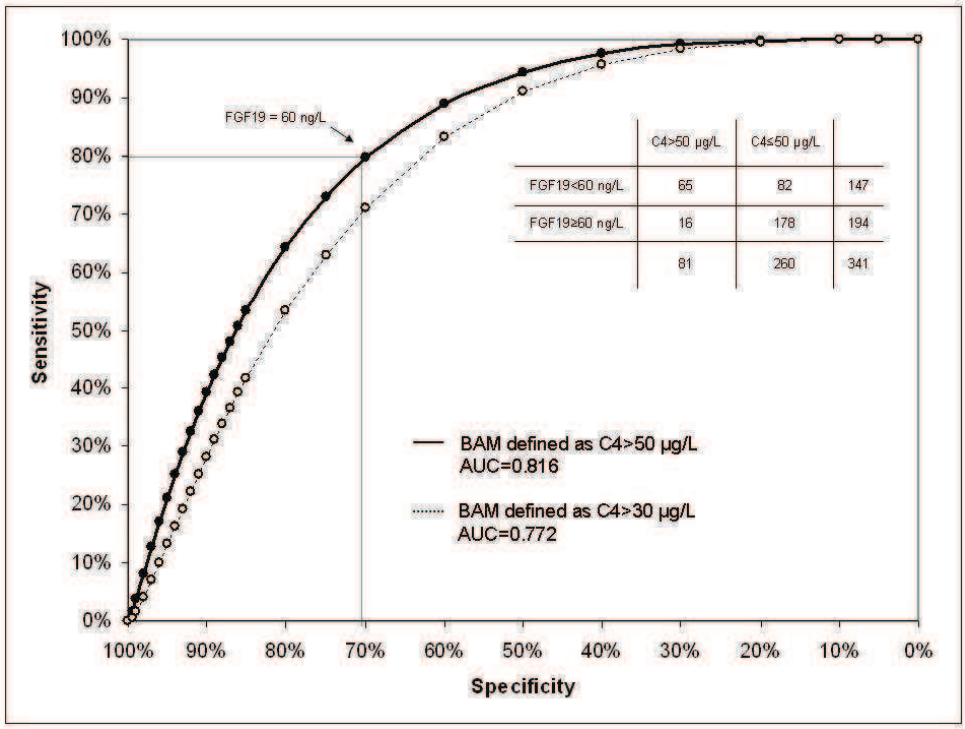

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CYP7A1 promoter polymorphism −203A>C affects bile salt synthesis rate in patients after ileal resection

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Abstract Cholesterol 7α-hydroxylase (CYP7A1) plays a crucial role in cholesterol metabolism and has been implicated in genetic susceptibility to atherosclerosis. Thus, an understanding of its transcriptional regulation is of considerable importance. We evaluated the effect of a common −203A>C polymorphism in the *CYP7A1* promoter region on the activity of CYP7A1, estimated as the ratios of serum 7α-hydroxycholest-4-en-3-one (C4) to either total or non-HDL-cholesterol. The study was performed on patients after resection of the distal ileum, leading to upregulation of CYP7A1 activity (n = 65). Healthy volunteers served as the control group (n = 66). Whereas higher CYP7A1 activity was associated with the −203A allele in the patient group (C4/cholesterol ratio, 29.0 vs. 14.8 μg/mmol, $P = 0.032$; C4/non-HDL-cholesterol ratio, 53.3 vs. 21.3 μg/mmol in −203AA and −203CC, $P = 0.017$, respectively), no differences were observed in the healthy controls. We conclude that under physiological conditions, the −203A>C polymorphism in the *CYP7A1* gene promoter region does not seem to have any clinically relevant effect. However, in patients with severe bile salt malabsorption, this polymorphism markedly affects CYP7A1 activity.—Leníček, M., V. Komárek, M. Zimolová, J. Kovář, M. Jirsa, M. Lukáš, and L. Vítek. ***CYP7A1* **promoter polymorphism −203A>C affects bile salt synthesis rate in patients after ileal resection. *J. Lipid Res.* 2008. 49: 2664–2667.**

Supplementary key words bile salt malabsorption • cholesterol • atherosclerosis • bile acid • metabolism

Conversion of cholesterol into bile salts (BSs) represents an important cholesterol-biotransforming pathway. BS biosynthesis is initiated by cholesterol 7α-hydroxylase (CYP7A1, EC 1.14.13.17), the rate-limiting enzyme in the classical BS biosynthetic pathway. The considerable contribution of CYP7A1 in the regulation of cholesterol metabolism has been confirmed in both animals and humans, where higher CYP7A1 activity correlated with lower cholesterol levels, and vice versa (1–6). Therefore, genetic determinants of its activity are of special interest. One of the most studied candidates is the −203A>C polymorphism (c.−267A>C, dbSNP rs3808607) in the promoter region of *CYP7A1*. This common variant represents the haplotype block, which covers a substantial part of the promoter and the first exon of *CYP7A1* (7).

Wang et al. (8) described an association between the −203A>C polymorphism and plasma LDL-cholesterol levels. Since then, numerous studies have been performed to examine the importance of this polymorphism or a linked variant, −469C>T (c.−533C>T, dbSNP rs3824260) in cholesterol metabolism regulation (9–14). The results were, however, inconsistent. Carriage of the −203C allele has been reported to be associated with increased LDL-cholesterol in men, but not in women (9), with increased serum levels of apolipoprotein A-I but not with apolipoprotein B-100 (10), and with significant LDL-cholesterol changes after dietary intervention (11, 12). In a study by Hegele et al. (13) the −203A allele was associated with higher LDL-cholesterol in Keewatin Inuits and lower HDL-cholesterol in Hutterites, whereas no association has been found in Sandy Lake Oji-Creek members. All of these studies correlated the presence of −203A>C variants with plasma cholesterol levels, rather than with CYP7A1 activity. Only Abrahamsson et al. (14) measured CYP7A1 activity or plasma levels of 7α-hydroxycholest-4-en-3-one (C4), a marker of CYP7A1 activity (15) in two small cohorts (of 21 and 30 subjects), respectively. Based on these observations, as well as on in vitro experiments, Abrahamsson et al. (14)

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Abbreviations: BS, bile salt; CYP7A1, cholesterol 7α-hydroxylase; C4, 7α-hydroxycholest-4-en-3-one.

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concluded that these polymorphisms contributed neither to CYP7A1 activity nor to the plasma LDL-cholesterol concentration in humans.

The aim of the present study was to ascertain, whether the −203A>C polymorphism in the *CYP7A1* promoter affects the activity of CYP7A1 in patients with a resection of the distal ileum, in whom the BS synthesis is grossly upregulated.

MATERIALS AND METHODS

Subjects

Two groups of subjects were included in the study. The first group consisted of healthy volunteers, employees of 1st Faculty of Medicine, Charles University in Prague, and blood donors. The other group consisted of inflammatory bowel disease patients with resection of the distal ileum. Owing to the lack of ileal bile acid transporter (ASBT, SLC10A2), BS malabsorption with a compensatory increase of CYP7A1 activity was expected in this group (6). Additionally, fibroblast growth factor 19, a supressor of CYP7A1, is produced in the small intestine under physiological conditions (16–18). After ileal resection, its production is expected to decrease, with subsequent derepression of CYP7A1. The length of the resected ileum was classified as extensive (≥70 cm) or small (<70 cm). Patients were recruited at the 4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague and at IBD Clinical and Research Center, ISCARE I.V.F. in Prague. Individuals taking BSs or BS sequestrants, which might influence the activity of CYP7A1, were excluded. Basic demographic characteristics of the subjects are shown in **Table 1**.

All subjects were genotyped for the −203A>C variant. The sera of homozygotes were further analyzed. The study was approved by the local Ethics Committee, and all participants gave their written informed consent prior to enrollment.

C4 and cholesterol measurements

Serum samples were obtained in the morning, aliquotted, and stored at −80°C until analysis. C4 concentration was measured by HPLC, as previously described (19). Briefly, 1 ml of serum and 30 ng of internal standard (7β-hydroxycholest-4-en-3-one; Steraloids, Newport, RI) were extracted by chloroform-methanol, purified on a Strata SI 100 mg precolumn (Phenomenex, Torrance, CA), and separated by HPLC (HP 1100 series, Agilent Technologies, Santa Clara, CA). The column (SGX C18, 4 × 250 mm, particles, 4 μM) was manufactured by Tessek, Prague, Czech Republic; mobile phase, acetonitrile-water (95:5, v/v), 1 ml/min, 20°C; detection/reference wavelength, 241/360 nm.

Total and HDL-cholesterol levels were measured on a modular automatic analyzer (Roche Diagnostics, Basel, Switzerland) using commercially available kits (CHOLESTEROL liquicolor and HDL CHOLESTEROL liquicolor, respectively, both by Human GmbH, Wiesbaden, Germany).

The activity of CYP7A1 was estimated using a serum C4/total cholesterol concentration ratio, which represents a more accurate marker of CYP7A1 activity than does serum C4 level alone (20). Additionally, the C4/non-HDL-cholesterol ratio was also used.

DNA analysis

Genomic DNA was isolated from peripheral blood white cells by a standard salting-out method (21). The −203A>C locus was genotyped by PCR-*Bsa*I restriction fragment length polymorphism; forward primer 5′-ATTAGCTATGCCCATCTTAAACAGG-3′ and reverse primer 5′-TAACTGGCCTTGAACTAAGTCCAC-3′ were used for PCR amplification of the corresponding DNA fragment.

Statistical analyses

Normally distributed data were compared using the Student *t*-test and presented as the mean (±SD), whereas skewed data were compared using the Mann-Whitney Rank Sum test and presented as the median (5–95%). The Hardy-Weinberg equilibrium was tested by the χ^2 test. Analyses were performed using STATISTICA software (version 8). The *P* value <0.05 was considered as significant.

RESULTS

As expected, C4/cholesterol as well as C4/non-HDL-cholesterol ratios in patients after ileal resection were higher than in controls (20.4 vs. 2.3 and 33.2 vs. 3.7 μg/mmol, respectively, $P < 0.001$ for both analyses). Both total and

TABLE 1. Effect of the −203A>C polymorphism on CYP7A1 activity

	−203AA	−203CC	*P*
Controls (n = 66)			
Gender (f/m)	7/16	28/15	**0.020**
Age (years)	41.0 ± 13.4	43.7 ± 11.8	0.470
C4/cholesterol	2.6 (1.0-5.5)	2.3 (0.6-7.5)	0.462
C4/non-HDL-cholesterol	4.2 (1.4-10.1)	3.6 (0.9-12.7)	0.548
All patients (n = 65)			
Gender (f/m)	21/16	15/13	1.000
Age (years)	41.3 ± 12.2	42.7 ± 12.6	0.810
C4/cholesterol	29.0 (3.5-106.4)	14.8 (2.4-68.9)	**0.032**
C4/non-HDL-cholesterol	53.3 (3.4-166.7)	21.3 (2.7-125.9)	**0.017**
Patients with resection <70 cm (n = 57)			
C4/cholesterol	24.3 (2.2-77.1)	13.5 (1.9-75.5)	0.112
C4/non-HDL-cholesterol	43.5 (3.3-128.2)	18.9 (2.7-125.9)	0.059
Patients with resection ⩾70 cm (n = 8)			
C4/cholesterol	108.9 (81.0-132.2)	26.0 (4.4-31.9)	**0.012**
C4/non-HDL-cholesterol	157.3 (118.1-186.1)	43.9 (9.1-48.6)	**0.029**

Results are given as mean ± SD, or as median (5–95%) when data were not normally distributed. Distribution of genotypes in both controls and patients followed Hardy-Weinberg equilibrium. The 7α-hydroxycholest-4-en-3-one (C4)/cholesterol and C4/non-HDL-cholesterol ratios are expressed in μg/mmol. Boldface indicates statistically significant results.

non-HDL serum cholesterol levels were significantly higher in controls than in patients (5.03 vs. 4.12 mmol/l and 3.25 vs. 2.69 mmol/l, respectively, $P < 0.001$ for both analyses).

In the group of all patients, the −203AA genotype was associated with a higher C4/cholesterol ratio, compared with the −203CC genotype (Table 1). Even more pronounced differences were observed in the subgroup of patients after extensive resection of the ileum; C4/cholesterol ratio in −203AA homozygotes was significantly higher than in −203CC patients (Table 1). The difference between the C4/cholesterol ratio in −203AA and CC homozygotes after short ileal resection showed the same tendency; however, it did not reach statistical significance (Table 1).

Similarly, C4/non-HDL-cholesterol ratios were significantly higher in −203AA than in −203CC homozygotes in all patients (**Fig. 1**), as well as in patients after extensive ileal resection (Table 1). In patients after short resection, the difference between −203AA and −203CC homozygotes did not reach statistical significance.

No significant effect of the −203A>C polymorphism in either C4/cholesterol or C4/non-HDL-cholesterol was found in the control group (Table 1).

DISCUSSION

Because of the key role of CYP7A1 in cholesterol metabolism, regulation of its activity is an important issue. Despite intensive research, the role of the −203A>C variant in the modulation of CYP7A1 activity has not yet been explained. To our knowledge, there is no direct evidence supporting the association of this variant with CYP7A1 activity in humans. Abrahamsson et al. (14) did not find any association between the −203A>C genotype and hepatic CYP7A1 activity in gallstone disease patients. The number of subjects in this study was, however, limited, and no −203AA homozygotes were included. Similarly, the authors did not observe any association between the −203A>C variant and serum levels of C4 as a marker of CYP7A activity in a cohort of 30 subjects with asymptomatic gallstone disease.

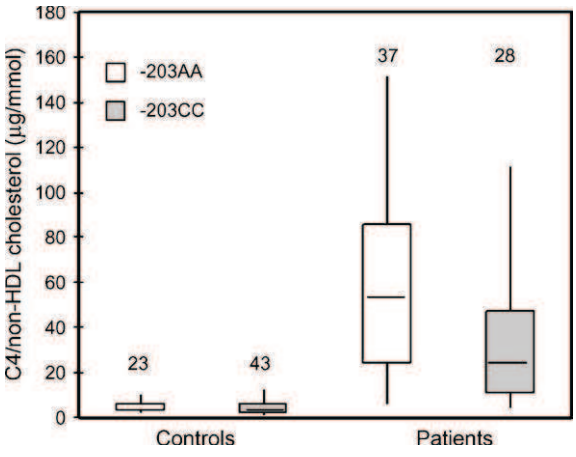

Fig. 1. 7α-Hydroxycholest-4-en-3-one (C4)/non-HDL-cholesterol ratio in −203AA and -203CC homozygotes. CYP7A1 activity estimated as C4/non-HDL-cholesterol ratio is higher in patients homozygous for the −203A allele. Data are expressed as median, 25–75% (boxes) and 5–95% (whiskers). Number of subjects is indicated.

Because direct measurement of CYP7A1 activity requires liver biopsy, less-invasive markers are preferred in humans. It has been shown that serum levels of C4 reflect CYP7A1 activity (15, 22). Honda et al. (20) suggested that the C4/cholesterol ratio would be a more accurate serum marker, because C4 is transported in lipoprotein particles carrying cholesterol. The authors experimentally confirmed that the C4/cholesterol ratio, as a marker of CYP7A1 activity, is superior to serum C4 levels. Based upon the fact that cholesterol for BS synthesis recruits predominantly from non-HDL-cholesterol (23, 24) and probably enters the circulation in the VLDLs (the first members of the metabolic lipoprotein cascade that includes all the lipoproteins other than HDL), the C4/non-HDL-cholesterol ratio (product/substrate) might theoretically be even more accurate.

In our study, we report an association of CYP7A1 activity with the −203A>C variant in patients after resection of the distal ileum. Owing to BS malabsorption and the possible lack of fibroblast growth factor 19, originating predominantly from the ileum, these patients have upregulated CYP7A1 activity (6, 16–18). Our patients, homozygous for the −203A allele, had an approximately 2-fold higher C4/cholesterol or C4/non-HDL-cholesterol ratio than did the −203CC homozygotes. The difference was more pronounced in patients after extensive resection. In patients after a small (<70 cm) resection of the ileum, both ratios were 2-fold higher in homozygotes for −203A,when compared with −203CC carriers. The differences, however, did not reach statistical significance. This is probably owing to the large heterogeneity of this group, in which patients after considerable resection as well as those with clinically negligible resection are included. In accord with Abrahamsson et al. (14), we did not observe significant differences in the C4/cholesterol or C4/non-HDL-cholesterol ratios between −203AA and −203CC homozygotes in the control group.

We hypothesize that under physiological conditions, the functional reserve of CYP7A1 is sufficient to override the effect of the promoter region, and that its variants do not contribute substantially to the CYP7A1 activity. However, when CYP7A1 is upregulated (e.g., in patients with ileal resection leading to malabsorption of BS), the −203C promoter is not able to increase the transcription level as much as the −203A is.

The higher activity of CYP7A1 leads to consumption of cholesterol within hepatocytes, which subsequently increases expression of LDL receptors and thus lowers the serum LDL-cholesterol levels (6, 25, 26). Similarly, lower CYP7A1 activity should result in higher serum cholesterol levels. In the present study, we did not focus on the serum cholesterol levels, because in the healthy population, the effect of the −203A>C polymorphism on cholesterol levels seems to be minor if any (8–14), and a large cohort needs to be collected in order to draw a relevant conclusion. Furthermore, in patients after ileal resection, the different nutritional status represents an important confounding

factor, which would mask the possible effect of the polymorphism on serum cholesterol levels.

In conclusion, our results suggest, that the −203AA genotype is associated with higher activity of CYP7A1 compared with −203CC in subjects with upregulated activity of CYP7A1. Under physiological conditions, however, the effect seems to be negligible.

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Regulation of Diurnal Variation of Cholesterol 7α-hydroxylase (CYP7A1) Activity in Healthy Subjects

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Summary

Cholesterol 7α-hydroxylase (CYP7A1), the key regulatory enzyme of bile acid synthesis, displays a pronounced diurnal variation. To better understand the regulation of CYP7A1 activity, three day-long examinations were carried out in 12 healthy men. The concentrations of 7α-hydroxycholest-4-en-3-one (C4), a surrogate marker of CYP7A1 activity, bile acids (BA), insulin, glucose, nonesterified fatty acids, triglycerides, and cholesterol were measured in serum in 90-min intervals from 7 AM till 10 PM. To lower and to increase BA concentration during the study, the subjects received cholestyramine and chenodeoxycholic acid (CDCA), respectively, in two examinations. No drug was used in the control examination. There was a pronounced diurnal variation of C4 concentration with a peak around 1 PM in most of the subjects. The area under the curve (AUC) of C4 concentration was five times higher and three times lower when subjects were treated with cholestyramine and CDCA, respectively. No relationship was found between AUC of C4 and AUC of BA concentration, but AUC of C4 correlated positively with that of insulin. Moreover, short-term treatment with cholestyramine resulted in about 10 % suppression of glycemia throughout the day. Our results suggest that insulin is involved in the regulation of diurnal variation of CYP7A1 activity in humans.

Key words

Cholesterol 7α-hydroxylase • Bile acid • Insulin • Glucose • Cholestyramine

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Introduction

Cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first key regulatory step in the synthesis of bile acids (BA) from cholesterol (Chiang *et al.* 1998, Gilardi *et al.* 2007). The synthesized bile acids are conjugated, exported into bile, stored in the gallbladder and, depending on metabolic needs, secreted into the intestine to facilitate fat absorption. Approximately 95 % is then reabsorbed and returned to the liver. BA returning from the enterohepatic cycle downregulate CYP7A1 activity and they exert such an effect at the level of gene transcription (Ramirez *et al.* 1994). BA bind to nuclear receptor – farnesoid X receptor (FXR) and turn on the expression of small heterodimer partner (SHP) which then blocks the CYP7A1 gene transcription. Alternatively, they can downregulate CYP7A1 by non-FXR-dependent pathway (Gilardi *et al.* 2007). Moreover, BA absorbed in the intestine activate synthesis of fibroblast growth factor 19 (FGF19), that signals to the liver through its receptor FGFR4 and synergistically downregulates CYP7A1 gene expression (Inagaki *et al.* 2005).

CYP7A1 activity varies during the day – the circadian variation of the enzyme activity is well documented in rodents (Noshiro *et al.* 1990) and displays a pronounced circadian variation in humans (Gälman *et al.* 2005). The peak of the enzyme activity in humans is observed shortly after midday, which is rather difficult to reconcile with the fact that at this time the inhibition by BA returning from the enterohepatic cycle should prevail.

Interestingly, it was recently demonstrated that

BA seem to directly affect the glucose metabolism through both FXR-independent and FXR-dependent pathways (De Fabiani *et al.* 2003, Ma *et al.* 2006).

Therefore, to better understand the mechanisms behind the regulation of CYP7A1 activity, we measured the diurnal variation of 7α-hydroxycholest-4-en-3-one (C4) concentration, a surrogate marker of CYP7A1 activity *in vivo* (Axelson *et al.* 1988), and changes of BA, insulin, glucose, nonesterified fatty acids (NEFA), triglycerides, and cholesterol concentration in healthy volunteers. The study was carried out under the conditions when BA synthesis was markedly increased or suppressed by short-term treatment with bile acid sequestrant cholestyramine or chenodeoxycholic acid (CDCA), respectively.

Methods

Subjects and study design

Twelve male volunteers (age: 32.1 ± 4.0 years, BMI: 25.7 ± 4.3 kg/m^2) were included into the study that consisted of three day-long examinations. One of these examinations served as a control examination (without any drug), while the other two examinations studied the effect of short-term administration of cholestyramine (Questran®, Bristol-Myers Squibb, Prague, Czech Republic, 16 g/day) and chenodeoxycholic acid (CDCA; Chenofalk®, Dr. Falk Pharma GmbH, Freiburg, Germany, 1-1.5 g/day dependent on the weight of the subject). One day before each of these examinations, the first blood sample was drawn at 7 AM (-24 h) and subjects received food for the whole day to standardize their intake before the study. On the day of examination, the first blood sample was drawn again at 7 AM (0 h) and the blood samples were then collected in 90-min intervals for 15 h till 10 PM. Again, subjects received food for the whole day and they had to eat at exactly defined intervals (breakfast at 7:15, snack at 9:45, lunch at 12:30, snack at 15:30 and dinner at 17:30). The amount of food was calculated to cover their energy requirements; the diet was relatively low in fat (25 % of energy intake). If the examination included the drug administration, the drugs were given to subjects on the day before the examination and also on the day of the examination. Questran® was given to subjects in two doses on both days – one with breakfast, the other one with dinner. Due to differences in pharmacokinetics, Chenofalk® treatment was started with dinner on the day preceding the examination and, on the day of examination, it was given to subjects in two doses at the same time as cholestyramine. The order of the examinations was randomized and they were carried out in three-week intervals at a minimum.

The study protocol was approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine and all the participants gave their informed consent.

Biochemistry

Cholesterol and triglycerides (TG) were measured using enzymatic kits from Roche Diagnostics GmbH, Mannheim, Germany, glucose using kits from PLIVA-Lachema Diagnostika, Brno, Czech Republic, nonesterified fatty acids using kits from Wako Chemicals GmbH, Neuss, Germany, and bile acids using enzymatic kits from Trinity Biotech plc, Bray, Ireland. Insulin was determined using IRMA kits from Immunotech, Prague, Czech Republic. Concentration of 7α-hydroxycholest-4en-3-one (C4) was determined by HPLC as described by Gälman *et al.* (2003) with a modification of C4 extraction procedure (Leníček *et al.* 2008).

Statistics

The differences between examinations were evaluated using ANOVA for repeated measures and if statistically significant differences were detected, a paired t test with Bonferroni correction was used to identify those differences. The differences between -24 h and 0 h were evaluated using a paired t test. The relationship between area under curve (AUC) of C4 and AUC of BA and insulin was tested using simple linear regression analysis.

Results

As expected, C4 concentration as a marker of CYP7A1 activity displayed marked diurnal variation (Fig. 1). The enzyme activity can vary in the order of magnitude within a few hours; the peak was reached around 1 PM in most of our subjects, but, as can be seen, the interindividual variation was rather very high. The one day treatment with cholestyramine resulted in a several fold increase in fasting C4 concentration on the day of examination and approximately a fivefold increase in the enzyme activity as assessed on the basis of AUC of C4 (Table 1, Fig. 2A). Conversely, the treatment with CDCA resulted in a decrease of C4 fasting concentration and AUC of C4 to approximately one third throughout

the day (Table 1, Fig. 2A). The BA concentration varied throughout the day with minor peaks reflecting food intake during lunch and dinner (Fig. 2B). The effects of both treatments on serum BA concentration were not so pronounced – there was a decrease in both fasting BA concentration and AUC BA after cholestyramine and, conversely, approximately a 30 % increase in AUC of BA after CDCA treatment (Table 1, Fig. 2B).

Table 1. The concentration of cholesterol, triglycerides, glucose, nonesterified fatty acids (NEFA), insulin, bile acids (BA), and 7α-hydroxycholest-4en-3-one (C4) at 7:00 AM on the day before the study (-24 h), at 7:00 AM on the day of the study (0 h) and 15-h area under the curve (AUC) of these variables (AUC [0-15 h]).

		-24 h	0 h	AUC [0-15 h]
Cholesterol	C	4.66±0.94	4.60±0.96	66.1±13.5
(mmol/l)	Q	4.73±0.76	4.59±0.86	64.4±11.5
	CDCA	4.65±0.91	4.63±0.91	66.8±12.5
Triglyceride	C	1.64±0.74	1.57±1.03	27.2±16.4
(mmol/l)	Q	1.56±0.95	1.76±0.86	27.5±14.9
	CDCA	1.62±0.85	1.61±0.84	28.3±12.2
Glucose	C	5.10±0.46	5.11±0.45	80.3±6.8[a]
(mmol/l)	Q	5.26±0.63	5.09±0.41	71.5±5.9[b]
	CDCA	5.45±0.96	4.96±0.59	74.9±9.2[a]
NEFA	C	0.37±0.27	0.43±0.15	3.32±0.98
(mmol/l)	Q	0.37±0.26	0.55±0.50	3.03±1.69
	CDCA	0.30±0.16	0.50±0.32*	2.31±1.49
Insulin	C	7.8±6.0	6.3±3.7	275±133
(IU/l)	Q	8.0±3.3	9.7±6.4	220±124
	CDCA	9.8±9.5	8.0±5.7	265±141
BA	C	13.4±6.1	11.0±4.5[a,b]	186±64[a]
(μmol/l)	Q	11.4±3.8	8.7±2.9*[a]	133±37[b]
	CDCA	12.6±4.7	13.1±4.0[b]	242±86[a]
C4	C	20.1±22.0	25.3±23.0[a]	303±168[a]
(μg/l)	Q	22.7±17.6	86.9±49.8**[b]	1512±784[b]
	CDCA	30.2±24.9	11.8±13.0**[c]	119±70[c]

Data are mean ± S.D. C – control experiment, Q – cholestyramine treatment, CDCA – chenodeoxycholic acid treatment. *, ** p<0.05, p<0.01 … -24 hours vs. 0 hours using paired t-test. a, b, c … the same letters are assigned to the examinations that do not differ when C, Q, and CDCA are compared using ANOVA for repeated measures (p<0.05).

The treatment with both drugs had no effect on both fasting value and AUC of cholesterol, TG, and insulin (Table 1, Fig. 1). The fasting concentration of NEFA rose after CDCA treatment but did not differ from the fasting concentration in cholestyramine or control examinations. However, although the treatment with both drugs had no effect on fasting glucose, the AUC of glucose after cholestyramine was approximately 10 % lower than in control experiment (p<0.05) (Table 1, Fig. 2D).

Table 2. Correlation coefficients r of the relationships between AUC of 7α-hydroxycholest-4-en-3-one (C4) and AUC of bile acids (BA) and AUC of insulin in subjects in control examination (C), after treatment with cholestyramine (Q) and chenodeoxycholic acid (CDCA).

	n	AUC of BA	AUC of insulin	
C	12	0.142	0.423	
Q	12	0.113	0.727	**
CDCA	12	0.011	0.729	**

** p<0.01.

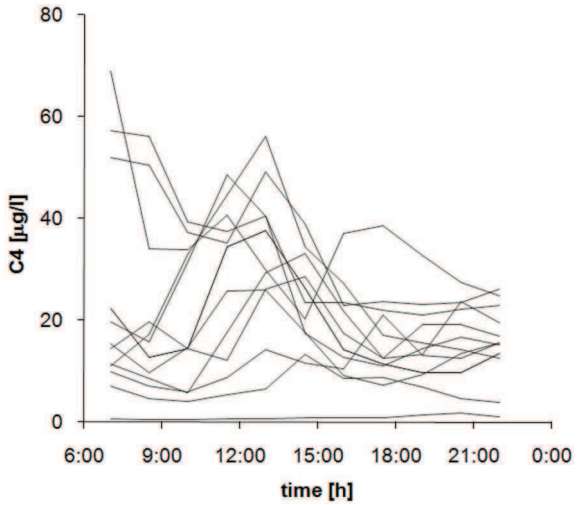

Fig. 1. Diurnal variation of 7α-hydroxycholest-4en-3-one (C4) concentration in twelve healthy subjects included into study during control examination (C).

To further analyze the relationship between possible regulatory role that BA and insulin can exert on BA synthesis, AUC of BA and AUC of insulin (as a measure of their bioavailability) were correlated to AUC of C4. As expected, there was a rather weak but statistically significant negative correlation between AUC BA and AUC C4 when all three examinations in all the subjects were included (r = –0.430, p<0.05). However, no such relationship could be found when the treatments were analyzed separately (Table 2). On the contrary, there was a strong positive correlation between AUC of insulin and AUC of C4 when subjects were treated with cholestyramine and/or CDCA (Table 2). Similar results were obtained when AUC's of BA and insulin obtained

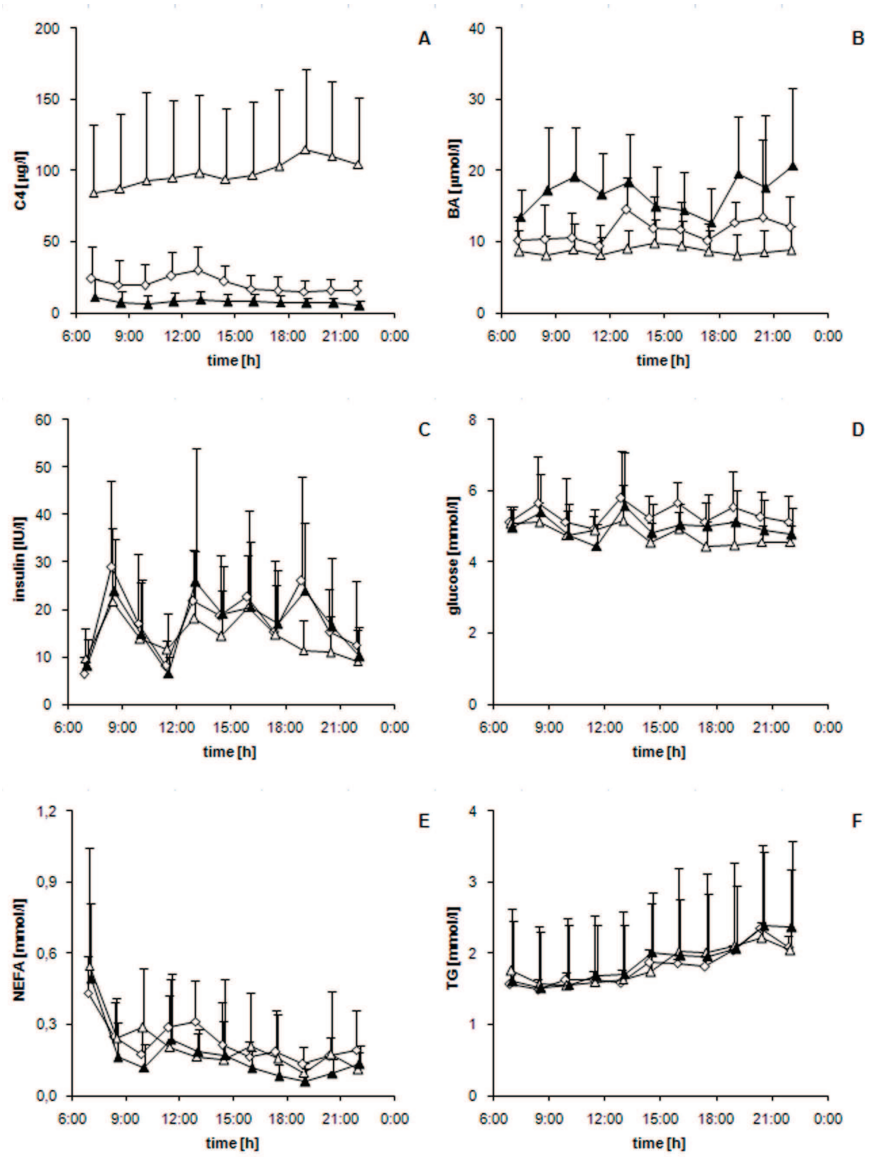

Fig. 2. The diurnal variation of concentration of 7α-hydroxycholest-4en-3-one (C4), bile acids (BA), insulin, glucose, nonesterified fatty acids (NEFA), and triglyceride in the control experiment (C), after short treatment with cholestyramine (Q) or chenodeoxycholic acid (CDCA). ◇ – control examination (C); Δ – short treatment with cholestyramine (Q); ▲ – short treatment with chenodeoxycholic acid (CDCA).

in shorter time intervals (7:00 to 11:30, 7:00 to 13:00, 7:00 to 14:30, 7:00 to 16:00) were correlated to AUC C4 (data not shown).

Discussion

In the present study we demonstrated that CYP7A1 activity displays a diurnal variation in healthy men with a peak around 1 PM. The treatment with cholestyramine reduces serum BA concentration and several fold upregulates the CYP7A1 activity and, conversely, the treatment with CDCA increases BA concentration and downregulates its activity. The AUC of C4 as a measure of bile acid synthetic capacity is positively correlated with AUC of insulin on cholestyramine and CDCA, but unexpectedly not with AUC of BA.

Our findings that C4 concentration as a marker of CYP7A1 activity displays a peak around a midday confirms the earlier findings from the study of five healthy volunteers (Gälman *et al.* 2005). However, it should be noted that three out of twelve subjects in our study show maximal C4 concentrations early in the morning (Fig. 1) and we should be cautious to generalize our conclusions about the CYP7A1 activity diurnal variation. Based on our data, some of the subjects may have displayed more than one peak of CYP7A1 activity a day and we cannot even exclude the possibility that in some subjects there may be a peak during the night like in rodents (Noshiro *et al.* 1990).

If we assume that the short-term administration of cholestyramine upregulates CYP7A1 gene expression to a maximum, then the comparison of areas under the C4 curves suggest that, on average, the enzyme activity

throughout the day varies around 20 % of the possible maximum and, thus, the enzyme activity is rather suppressed under physiological conditions (Table 1, Fig. 2A). On the other hand, it is quite surprising that there is no correlation between AUC of BA and that of C4 (Table 2). It may be that the effect of BA on CYP7A1 activity is at its maximum or minimum when the subjects are treated with drugs and this is not related to the concentration of BA anymore. However, the lack of such correlation in the control examination suggests that the effect of other factors on CYP7A1 may prevail. Moreover, given that CYP7A1 activity is rather low for most of the day, it may be difficult to see a further suppressive effect of BA. From the other factors that can override the effect of BA, insulin deserves a special attention. We could indeed demonstrate quite a strong positive correlation between AUC of C4 as a measure of CYP7A1 activity and AUC of insulin as a measure of its bioavailability at least when both drugs are used to manipulate the transhepatic flux of BA. Such an observation is in agreement with recent findings that insulin in physiological concentrations has a dual effect on CYP7A1 gene expression in human primary hepatocytes (Li *et al.* 2006). First, it induces a very rapid and pronounced activation of CYP7A1 gene expression likely due to the activation of p38 MAPK pathway (Xu *et al.* 2007). Then, after several hours, it inhibits gene expression through the effect of two insulin-regulated transcriptional factors – forkhead box O1 (FoxO1) and sterol regulatory element binding protein-1c (SREBP-1c) – on transactivation of the CYP7A1 gene (Li *et al.* 2006).

Therefore, we can hypothesize that insulin could be the first signal to induce CYP7A1 gene expression at the transition from fasting to postprandial phase. Afterwards, the effect of BA return from enterohepatic cycle, FGF19 signaling, and downregulating effect of insulin should prevail and lower the CYP7A1 activity. Although such a hypothesis fits with our data, we are fully aware of the fact that insulin is only one of many factors that are involved in the complex regulation of transition to the postprandial state. It was shown that the circadian variation of CYP7A1 activity is preserved even in fasting subjects that are allowed to drink only water (Gälman *et al.* 2005) which suggests that the other mechanisms such as signalling through albumin D-element-binding protein (DBP) (Lavery and Schibler 1993) or through FGF19 (Lundasen *et al.* 2006) may be involved in the regulation of bile acid synthesis during the day.

Our findings that plasma glucose concentration is suppressed after a 24-h treatment with cholestyramine is in line with the observation that a 6-week treatment of diabetic patients with cholestyramine improved glycemia and glycated hemoglobin (Garg and Grundy 1994) and with the findings that bile acids may directly inhibit gluconeogenesis by affecting the transcription of its key regulatory enzyme, phosphoenolpyruvate carboxykinase (PEPCK) (De Fabiani *et al.* 2003). The mechanism of this effect is not FXR-dependent. Our data suggest for the first time that the effect of low transhepatic flux of bile acid on gluconeogenesis is immediate and likely does not include the extrahepatic effects because the variations of insulin and NEFA concentrations throughout the day were not affected by cholestyramine.

In conclusion, we observed a positive correlation between the area under the curve of insulinemia and that of 7α-hydroxycholest-4-en-3-one, the surrogate marker of CYP7A1 activity. Our findings support an idea that insulin may play a role in the regulation of bile acid synthesis in humans.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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The hepatic response to FGF19 is impaired in patients with nonalcoholic fatty liver disease and insulin resistance

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Academic Medical Center, Departments of [1]Gastroenterology and Hepatology, [3]Surgery, and [5]Radiology, and [6]AMC Liver Center, Amsterdam; [2]Department of Gastroenterology and Hepatology, Liver Unit, Vrije Universiteit Medical Center, Amsterdam, The Netherlands; and [4]Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Czech Republic

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Schreuder TC, Marsman HA, Lenicek M, van Werven JR, Nederveen AJ, Jansen PL, Schaap FG. The hepatic response to FGF19 is impaired in patients with nonalcoholic fatty liver disease and insulin resistance. *Am J Physiol Gastrointest Liver Physiol* 298: G440–G445, 2010. First published January 21, 2010; doi:10.1152/ajpgi.00322.2009.— Intestinal FGF19 has emerged as a novel endocrine regulator of hepatic bile salt and lipid metabolism. In patients with nonalcoholic fatty liver disease (NAFLD) hepatic lipid metabolism is deranged. A possible role of FGF19 in NAFLD has not been reported yet. In this study, we assessed intestinal FGF19 production and the hepatic response to FGF19 in NAFLD patients with and without insulin resistance [homeostasis model of assessment (HOMA) score $\geq$2.5 (n = 12) and HOMA score $<$2.5 (n = 8), respectively]. To this end, NAFLD patients received a standardized oral fat challenge. Postprandial excursions of triglycerides, bile salts, and FGF19 were monitored, and plasma levels of a marker for bile salt synthesis (7α-hydroxy-4-cholesten-3-one) were determined. Fasted FGF19 levels were comparable in a control group of healthy volunteers (n = 15) and in NAFLD patients (0.26 $\pm$ 0.28 vs. 0.18 $\pm$ 0.09 ng/ml, respectively, P = 0.94). Postprandial FGF19 levels in both controls and NAFLD patients peaked between 3–4 h and were three times higher than baseline levels. The areas under the postprandial FGF19 curve were similar in controls and in the HOMA score-based NAFLD subgroups. In NAFLD patients with HOMA score $<$2.5, the postprandial increase in plasma FGF19 was accompanied by a lowering of plasma levels of 7α-hydroxy-4-cholesten-3-one ($-$30%, P = 0.015). This anticipated decline was not observed in insulin-resistant NAFLD patients ($+$10%, P = 0.22). In conclusion, patients with NAFLD show an unimpaired intestinal FGF19 production. However, the hepatic response to FGF19 is impaired in NAFLD patients with insulin resistance (HOMA score $\geq$2.5). This impaired hepatic response to FGF19 may contribute to the dysregulation of lipid homeostasis in NAFLD.

enterohepatic signaling; postprandial response; 7α-hydroxy-4-cholesten-3-one; CYP7A1; bile salt uptake

INCREASED HEPATIC FAT ACCUMULATION underlies the development of nonalcoholic fatty liver disease (NAFLD). The spectrum of liver abnormalities in NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) when accompanied by inflammation and fibrosis. One-fifth of NASH patients ultimately develops cirrhosis (4). The etiopathology of NAFLD is far from understood. The progression of NAFLD toward NASH has traditionally been explained by a two-hit model in which initial accumulation of triglycerides (first hit) is followed by inflammatory and/or oxidative stress (second hit) resulting in steatohepatitis (8). More recently it has been suggested that hepatic deposition of saturated fat in particular may directly damage the liver and mediate progression of plain steatosis to NASH (12). The increasing prevalence of this obesity-related disorder and its health consequences necessitate further insight into the causes and consequences of hepatic lipid accumulation.

FGF19 belongs to a subfamily of FGFs that have an endocrine function and has emerged as a novel regulator of hepatic lipid homeostasis (5, 17). Initial studies in mice transgenic for FGF19 indicated a role for FGF19 in whole-body energy and lipid homeostasis (11, 32). Specifically, FGF19 transgenic mice had decreased adiposity and were resistant to high-fat diet-induced weight gain. Furthermore, transgenic FGF19 expression or infusion of recombinant FGF19 protein reduced hepatic lipid accumulation and improved insulin-sensitivity in *ob/ob* mice (11). The observed in vivo effects of FGF19 have been attributed in part to reduced hepatic expression of acetyl-CoA carboxylase 2 (*Acc2*), a negative regulator of mitochondrial fatty acid oxidation. Repression of *Acc2* in FGF19 transgenic mice may thus deplete hepatic lipid stores by promoting mitochondrial fatty acid degradation. In addition, a recent study revealed that FGF19 suppresses insulin-induced fatty acid synthesis in isolated hepatocytes (6). Thus FGF19 appears to influence both hepatic fatty acid oxidation and hepatic lipid synthesis.

It is currently unknown whether FGF19 plays a role in the etiopathogenesis of NAFLD. Reduced plasma FGF19 levels have been reported for patients with Type 2 diabetes mellitus, but the cause of this decrease is unknown (30). FGF19 is produced by the small intestine in a bile salt-stimulated manner (16). Binding of FGF19 to its cognate receptor on hepatocytes activates intracellular signaling pathways, resulting in repression of bile salt synthesis and, presumably, lipid synthetic pathways (6, 14, 16, 17). FGF19 can thus be viewed as a feedforward signal that informs the liver on dietary fat intake and return of bile salts. Reduced plasma FGF19 in Type 2 diabetic subjects may be due to impaired intestinal FGF19 production and may contribute to ongoing triglyceride synthesis and exacerbation of hepatic steatosis in subjects with NAFLD. In the present study, we examined the postprandial FGF19 response in subjects with NAFLD with or without insulin resistance.

EXPERIMENTAL PROCEDURES

Patients and study design. From a prospective database on NAFLD patients, 20 patients between the ages of 18 and 75 yr were recruited for participation in this study. Initial diagnosis

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of NAFLD was based on elevated alanine aminotransferase levels (≥ 45 IU/l), increased echogenicity of the liver on ultrasound examination, and exclusion of other chronic liver diseases defined as normal iron and copper studies and absence of hepatitis B surface antigen, hepatitis C antibodies, and autoimmune antibodies (antinuclear antibodies, antibodies to smooth muscle antigens and mitochondria). Patients with excessive alcohol intake, defined as more than 2 units/day in men and more than 1 unit/day in women, were excluded. Following study inclusion, all NAFLD patients underwent assessment of hepatic fat content by magnetic resonance spectroscopy (^{1}H-MRS).

Patients were divided into two groups according to severity of insulin resistance [homeostasis model of assessment (HOMA)] (2). Following overnight fasting, all study subjects received a standardized oral fat challenge consisting of 30 g cream (35 g fat per 100 ml) per square meter of body surface area (26). Blood samples were taken from an indwelling cannula placed in a cubital vein at baseline and at hourly intervals for up to 7 h after oral fat intake. During the sampling period, patients were withheld from additional food but had free access to water. Patients gave their informed consent to the protocol of the study, which was approved by the medical ethical committee of the Academic Medical Center in Amsterdam.

A previously described cohort consisting of 15 healthy volunteers with an irrelevant medical history (26) was used as a control group. These subjects underwent an identical oral lipid challenge with blood sampling at baseline and after 2, 3, 4, and 6 h.

Determination of hepatic fat content. The fat content in the liver of NAFLD patients was determined by ^{1}H-MRS. All measurements were performed on a 3.0-T Philips Intera scanner (Philips Healthcare, Best, The Netherlands). A voxel of 20 × 20 × 20 mm was positioned in the right hepatic lobe, avoiding vascular, biliary, and extrahepatic structures. Spectra were acquired by use of first order iterative shimming, a PRESS sequence with echo time/repetition time = 35/2,000 ms, and 64 signal acquisitions. The water (4.7 ppm) and fat (1.3 ppm) resonance peaks were integrated by use of jMRUI software (25), and relative fat content was expressed as a ratio of the fat peak area over the cumulative water and fat peak areas. Calculated peak areas of water and fat were corrected for T2 relaxations ($T2_{water}$ = 34 ms, $T2_{fat}$ = 68 ms, Ref. 9), and the percentage hepatic fat content was calculated according to Szczepaniak et al. (31).

Blood chemistry. Following collection of blood in EDTA tubes, plasma was prepared and analyzed for levels of glucose, insulin, C-reactive protein, and liver enzymes at baseline (t = 0 h). Triglycerides (TG), total cholesterol, FGF19 and total bile salts (Diazyme, Poway, CA) were assayed at baseline and at the respective sampling points after oral fat intake. Baseline interleukin-6 (IL-6) levels were determined by sandwich ELISA (Sanquin, Amsterdam, The Netherlands). 7α-Hydroxy-4-cholesten-3-one (C4) levels, a plasma marker for bile salt synthesis, were determined at baseline and at 4 and 5 h after oral fat intake as previously described (19). C4 levels were expressed relative to total cholesterol, because their ratio was shown to be a more accurate marker of bile salt synthesis (15).

Determination of plasma FGF19. Plasma FGF19 levels were determined by using an in-house developed sandwich ELISA described in detail elsewhere (F. G. Schaap, unpublished observations). Briefly, microtiter plates were coated with goat anti-human FGF19 antibody (AF969, R&D Systems, Minneapolis, MN). Samples and recombinant FGF19 standards (R&D Systems) were diluted in PBS containing 1.0% casein and 0.05% Tween-20. Captured antigen was detected with biotinylated goat anti-human FGF19 antibody (BAF969, R&D Systems) and streptavidin-horseradish peroxidase with tetramethylbenzidine as chromogenic substrate.

Data and statistical analysis. Area under the postprandial curve (AUC) was calculated by use of baseline-subtracted values with GraphPad Prism (GraphPad Software, La Jolla, CA). For comparison with the historical control population, data derived from time points that were missing (i.e., T = 1, 5, and 7 h) in the sampling scheme of the controls were omitted for AUC calculations (denoted as $AUC_{0-6\ h}$). Descriptive statistics are expressed as means ± standard deviation. Within each group, a paired t-test was used to evaluate changes from baseline during the oral fat challenge. Differences between groups (AUC or individual time points of the postprandial curves) were evaluated by Student's t-test, Mann-Whitney U-test, or one-way ANOVA with Bonferroni post hoc testing. Statistical analyses were performed with SPSS version 16.0 (SPSS, Chicago, IL). Statistical significance was accepted at $P < 0.05$.

RESULTS

Patient characteristics. Table 1 shows the characteristics of the controls and the NAFLD groups. As expected, NAFLD patients were obese (body mass index >30). Hyperglycemia and hyperinsulinemia in the NAFLD population were attributable to patients with HOMA score ≥2.5. Nine NAFLD patients fulfilled the American Diabetes Association criteria for Type 2 diabetes mellitus (2). The majority of these patients (6 of 9) was treated with metformin. Five diabetic patients had dyslipidemia [TG >1.7 mmol/l and/or HDL-cholesterol <0.9 mmol/l (1)]. Two patients were treated with statins.

Plasma FGF19 levels in NAFLD patients. Baseline FGF19 levels in NAFLD patients (0.18 ± 0.09 ng/ml) were comparable to values in the studied controls (0.26 ± 0.28 ng/ml, P = 0.94) and to values recently reported for an unrelated control population (0.28 ± 0.20 ng/ml) (28). Furthermore, baseline plasma FGF19 levels were identical (P = 1.00) in the HOMA-based NAFLD subgroups. Baseline FGF19 levels in NAFLD patients showed a weak inverse correlation (Spearman's r = −0.47, P = 0.038) with baseline plasma levels of 7α-hydroxy-4-cholesten-3-one (C4), a marker for bile salt synthesis. This appears in line with FGF19's role in negative regulation of bile salt synthesis. Baseline FGF19 levels showed no correlation with hepatic fat content or HOMA score (data not shown). Baseline bile salt levels were elevated in NAFLD patients with a HOMA score ≥2.5 compared with controls ($P < 0.001$) and NAFLD patients with a HOMA score <2.5 (P = 0.006) (Table 1).

Postprandial responses in NAFLD patients. Entry of dietary fat in the duodenum causes gallbladder contraction and inflow of bile salts into the intestinal lumen (13). Reabsorption of bile salts in the distal part of the small intestine activates the bile salt receptor farnesoid X receptor (FXR), resulting in enhanced transcription and portal release of FGF19 (16). The postprandial excursions of TG, bile salts, and FGF19 in control subjects and NAFLD patients are depicted in Fig. 1.

Packaging of digested dietary lipids into chylomicrons and their release into lymph causes an increase in plasma TG levels (Fig. 1*A*).

Table 1. *Characteristics and baseline values of controls, NAFLD patients, and the HOMA score-based subgroups*

	Controls	NAFLD			*P* Value
		All	HOMA <2.5	HOMA ≥2.5	
Men/women	15/0	14/6	6/2	8/4	1.00
Age, yr	50 ± 8	49 ± 11	47 ± 9	51 ± 12	0.51
BMI, kg/m^2	26.4 ± 3.5	30.6 ± 3.7	29.8 ± 4.5	31.3 ± 3.2	0.39
% hepatic fat	n.d.	16.0 ± 11.4	13.4 ± 11.8	17.9 ± 11.2	0.42
Glucose, mmol/l	n.d.	6.3 ± 2.3	5.5 ± 0.8	6.8 ± 2.9	0.18
Insulin, μmol/l	n.d.	118.6 ± 127.2	35.6 ± 13.3	173.8 ± 139.6	<0.001
HOMA-IR	n.d.	6.0 ± 10.9	1.2 ± 0.5	9.2 ± 13.3	<0.001
ALT, IU/l	n.d.	74 ± 37	69 ± 22	78 ± 44	0.62
γGT, IU/l	n.d.	102 ± 87	83 ± 44	114 ± 107	0.65
CRP	n.d.	3.6 ± 3.1	3.3 ± 3.1	3.9 ± 3.2	0.52
TC, mmol/l	5.10 ± 0.69	4.86 ± 1.01	4.78 ± 0.47	4.92 ± 1.27	0.77
TG, mmol/l	1.13 ± 0.35	1.88 ± 1.13	1.63 ± 1.01	2.05 ± 1.21	0.42
Bile salts, μmol/l	2.2 ± 1.4	4.2 ± 2.4	2.6 ± 0.3	5.3 ± 0.7	0.009
C4, ng/mg cholesterol	n.d.	12.2 ± 9.7	16.1 ± 3.6	9.7 ± 5.2	0.24
FGF19, ng/ml	0.26 ± 0.28	0.18 ± 0.09	0.18 ± 0.09	0.18 ± 0.09	1.00
IL-6, pg/ml	0.9 ± 0.6	1.8 ± 1.5	1.5 ± 1.4	2.1 ± 1.6	0.40

All values are expressed as means ± SD. **P* values are for comparison between the HOMA <2.5 and HOMA ≥2.5 subgroups. Part of the data in the control group has been published previously (26). NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; n.d., not determined; HOMA-IR, homeostasis model of assessment for insulin resistance; ALT, alanine aminotransferase; γGT, γglutamyltransferase; CRP, C-reactive protein; TC, total cholesterol; TG, triglycerides; C4, 7α-hydroxy-4-cholesten-3-one.

As reported earlier for the volunteer group (26), a postprandial increase in TG was first noted after 2 h. TG levels peaked after 3 h in controls and between 3 and 4 h in NAFLD patients. The total area under the postprandial TG curve ($AUC_{TG\ 0-6h}$) was elevated in NAFLD patients (4.1 ± 3.2 vs. 2.1 ± 1.4 mmol·l^{-1}·h^{-1} in controls, $P = 0.013$). During the ascending phase of the postprandial TG excursion, TG production (i.e., chylomicron synthesis) predominates. The area under the ascending part of the curve ($AUC_{TG\ 0-3h}$) was not different (1.4 ± 1.3 vs. 1.0 ± 0.6 mmol·l^{-1}·h^{-1} in NAFLD patients and controls, respectively, $P = 0.079$). In the descending phase of the postprandial TG curve, clearance of TG-rich lipoproteins prevails. The area under the descending part of the curve ($AUC_{TG\ 3-6h}$) was elevated in NAFLD patients (2.7 ± 2.1 vs. 1.1 ± 1.0 mmol·l^{-1}·h^{-1} in volunteers, $P = 0.002$). The latter appears in line with impaired TG clearance in insulin-resistant subjects (23). This notion is supported when ascending, descending and total AUC_{TG} values for the control and the HOMA-based NAFLD subgroups are compared. This analysis revealed that total and descending AUC_{TG} values are significantly elevated in the NAFLD HOMA ≥2.5 subgroup only (data not shown). Thus the observed difference in $AUC_{TG\ 0-6h}$ is likely due to impaired TG clearance in insulin-resistant NAFLD patients. This also indicates that gastrointestinal passage and handling of ingested lipids is similar in controls and both NAFLD subgroups.

Following an oral fat challenge, an incline in plasma bile salt levels is apparent after 1 h in NAFLD patients, with levels reaching a maximum after 3–4 h (Fig. 1*B*). In control subjects, which were first sampled after 2 h, bile salt levels peak after 2 h and return to baseline levels after 6 h. In contrast, bile salts remain elevated at this and at the final time point in NAFLD patients (Figs. 1*B* and 2*A*). After gallbladder contraction-induced entry into the duodenum, bile salts are efficiently reclaimed from the small intestinal lumen and released into the portal circulation. First-pass clearance of bile salts by the liver is highly efficient with little systemic spillover and is depending on the Na^+ taurocholate cotransporting protein (NTCP) (13). As can be appreciated from Fig. 1*B*, the postprandial bile salt excursion is different in controls and NAFLD patients. Bile salt levels were significantly higher in NAFLD subjects at time points $T = 0$ ($P = 0.004$), $T = 4$ ($P = 0.015$), and $T = 6$ h ($P = 0.001$), and this was entirely attributable to NAFLD patients with HOMA score ≥2.5 (data not shown). The area under the postprandial bile salt curve ($AUC_{BS\ 0-6\ h}$) tended to be elevated in NAFLD patients (67.3 ± 47.7 vs. 47.1 ± 29.4 μmol·l^{-1}·h^{-1} in controls, $P = 0.09$). NAFLD patients with HOMA score ≥2.5 (75.8 ± 59.9 vs. 54.5 ± 14.9 μmol·l^{-1}·h^{-1} in the HOMA <2.5 subgroup, $P = 0.43$) largely accounted for this tendency. Since these results suggest impaired hepatic bile salt uptake we measured interleukin-6 (IL-6), a known regulator of NTCP expression (3). NAFLD patients with HOMA score ≥2.5 showed elevated IL-6 levels compared with controls (2.1 ± 1.6 vs. 0.9 ± 0.6 pg/ml, respectively, $P = 0.005$, Table 1).

Following fat ingestion, a slight drop in plasma FGF19 level occurs after 1 h in NAFLD patients. Plasma FGF19 was significantly elevated after 2 h in control subjects and after 3 h in NAFLD patients (Fig. 1*C*). Postprandial FGF19 levels peaked between 3–4 h in controls and NAFLD patients and remained elevated in both groups until the last sampling point. Peak postprandial FGF19 levels were 3.0-fold higher compared with baseline levels in both controls and in NAFLD patients. The postprandial excursion of FGF19 closely followed that of bile salts, although some lagging was apparent. This is in line with bile salt-mediated induction of ileal FGF19 expression (14, 16). When analyzing individual time points, no significant differences in plasma FGF19 level were apparent between controls and NAFLD patients. The postprandial FGF19 AUC ($AUC_{FGF19\ 0-6\ h}$) was, however, significantly lower in NAFLD patients (1.4 ± 1.3 vs. 1.9 ± 1.4 ng·ml^{-1}·h^{-1} in controls, $P = 0.032$).

Influence of insulin-resistance on the postprandial FGF19 response in NAFLD patients. Both NAFLD subgroups displayed similar postprandial bile salt excursions (Fig. 2*A*) and had indistinguishable $AUC_{BS\ 0-7h}$ values (61.2 ± 17.8 vs. 85.1 ± 69.6 μmol·l^{-1}·h^{-1} in the HOMA <2.5 and HOMA ≥2.5 subgroups, respectively, $P = 0.47$). The postprandial FGF19

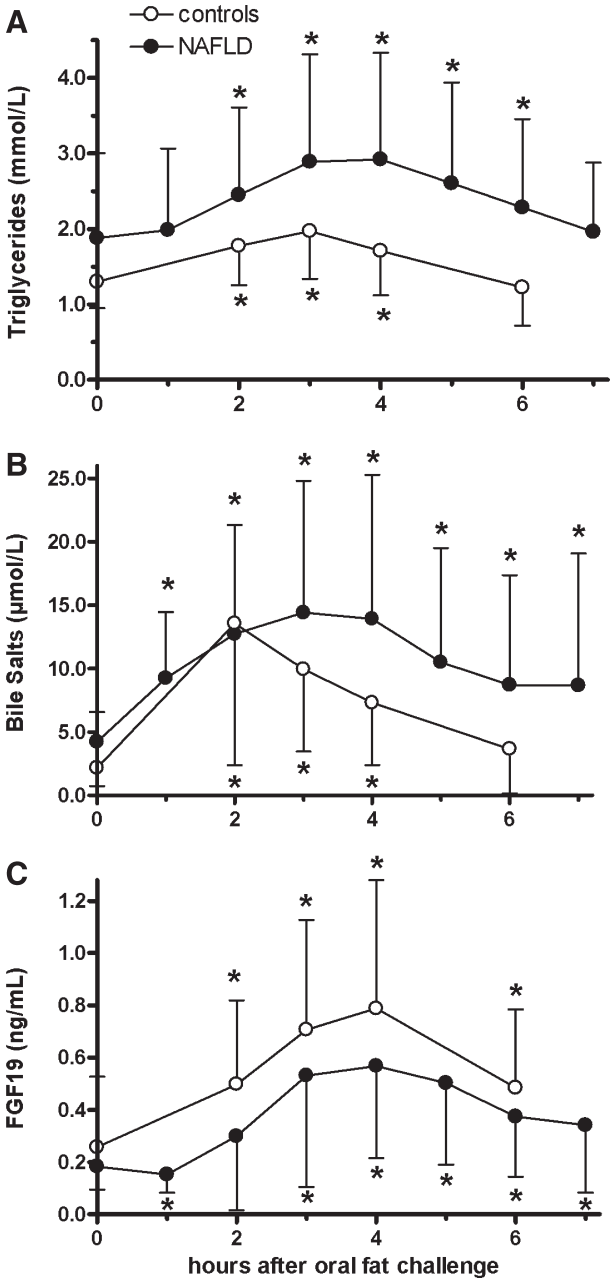

Fig. 1. Plasma levels of triglycerides, bile salts, and FGF19 increase after an oral fat challenge. Following overnight fasting, control subjects (○) and nonalcoholic fatty liver disease (NAFLD) patients (●) received a standardized oral fat challenge. Serial blood samples were drawn at the indicated time points, and plasma was assayed for triglycerides (*A*), total bile salts (*B*), and FGF19 (*C*). Data points are depicted as means ± SD. Asterisks denote significant changes from baseline values ($T = 0$) in the respective groups.

response in HOMA-based NAFLD subgroups is shown in Fig. 2*B*. Plasma FGF19 is significantly elevated above baseline levels after 3 h in the HOMA <2.5 subgroup ($P = 0.018$) and, albeit with borderline significance, in the HOMA ≥2.5 subgroup ($P = 0.051$). Postprandial FGF19 levels peaked at 3 and 4 h in the HOMA <2.5 and HOMA ≥2.5 subgroups, respectively. Mean FGF19 levels in the NAFLD subgroups were not different at any of the individual time points. The $AUC_{FGF19\ 0–7h}$ was similar in both NAFLD subgroups (1.6 ± 1.3 vs. 1.4 ± 1.5 $ng \cdot ml^{-1} \cdot h^{-1}$ in the HOMA <2.5 and HOMA ≥2.5 groups, respectively, $P = 0.49$). In addition, analysis of variance indicated that $AUC_{FGF19\ 0–6h}$ values were not significantly different in controls and the two NAFLD subgroups (ANOVA $P = 0.081$).

Elevation of plasma FGF19 is expected to result in diminished bile salt synthesis through downregulation of hepatic CYP7A1 mRNA (14, 16). Plasma C4, a marker for bile salt synthesis, was determined in NAFLD patients at baseline, and at 4 and 5 h following an oral fat challenge. Despite similar postprandial elevation of FGF19 in both NAFLD subgroups (Fig. 3*A*), C4 levels declined after 5 h only in the HOMA <2.5 group (−30%, $P = 0.015$) while remaining unchanged in the HOMA ≥2.5 group (+10%, $P = 0.22$) (Fig. 3*B*).

DISCUSSION

Recent findings indicate a role for the endocrine factor FGF19 in the regulation of hepatic lipid metabolism (6, 11, 32). Altered intestinal FGF19 production and/or altered hepatic responsiveness to FGF19 may accordingly contribute to the dysregulation of lipid homeostasis encountered in NAFLD. In this study, we evaluated the postprandial FGF19 response following an oral fat challenge in healthy volunteers and in NAFLD patients with and without insulin resistance. The major novel finding of this study is that the response of the liver to elevated plasma FGF19 levels is impaired in insulin-resistant NAFLD patients.

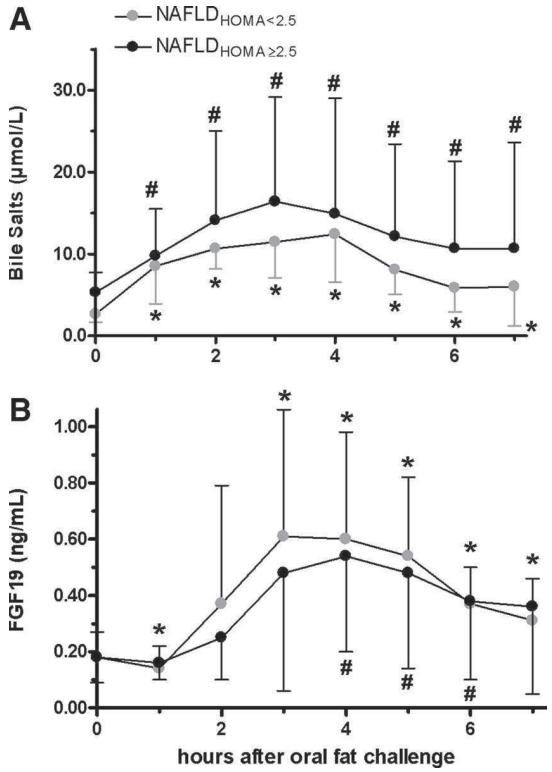

Fig. 2. Postprandial bile salt and FGF19 excursions in NAFLD patients are not affected by insulin resistance. The postprandial responses in NAFLD patients (Fig. 1) were analyzed separately in each homeostasis model of assessment (HOMA)-based subgroup. Postprandial excursions of both bile salts and FGF19 are similar in NAFLD patients with (HOMA ≥2.5, solid symbols) and without (HOMA <2.5, shaded symbols) insulin-resistance. Data points are depicted as means ± SD. * and #, Significant changes from baseline values ($T = 0$) in the HOMA <2.5 and HOMA ≥2.5 groups, respectively.

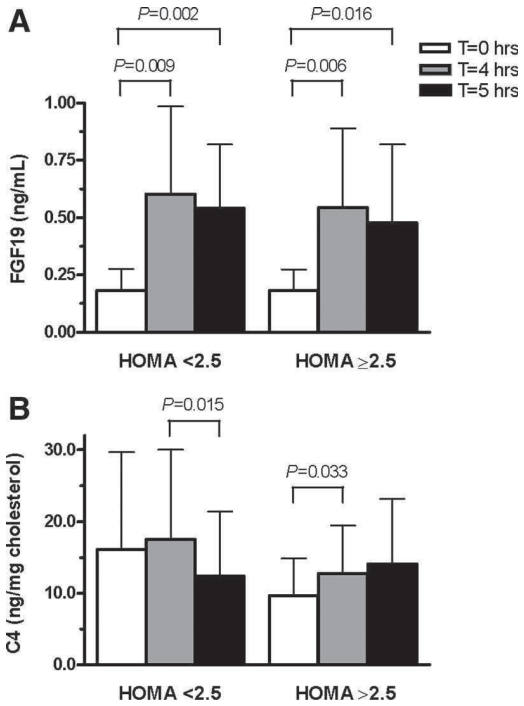

Fig. 3. Postprandial elevation of FGF19 in insulin-resistant NAFLD subjects is not accompanied by reduction of 7α-hydroxy-4-cholesten-3-one (C4) levels. Plasma FGF19 (*A*) and C4 (*B*) levels were determined at baseline (open bars) and at 4 h (shaded bars) and 5 h (solid bars) after an oral fat challenge. In NAFLD patients with normal HOMA score (HOMA <2.5), the postprandial increase in FGF19 levels was accompanied by an expected decline in C4 levels, a marker for bile salt synthesis. In contrast, C4 levels were unaffected in insulin-resistant NAFLD patients (HOMA ≥2.5) despite a similar postprandial elevation of FGF19 level. Data points are depicted as means ± SD.

FGF19 is an integral part of a regulatory mechanism by which bile salts negatively regulate their own synthesis (14, 16). Having fulfilled their function in digestion and absorption of dietary lipids in the proximal parts of the small intestine, bile salts are reclaimed in the terminal ileum (13). This results in activation of the bile salt-activated transcription factor FXR and transcriptional induction of FGF19, an ileal FXR target gene (16). Binding of FGF19 to its cell surface receptor on hepatocytes results in repression of bile salt synthesis via downregulation of CYP7A1 (14, 16). In this study we used a physiological stimulus, viz., a fatty meal, to induce gallbladder contraction and accordingly stimulate intestinal FGF19 production. The functional consequence of postprandial elevation of FGF19 levels was assessed by measurement of plasma C4, a marker for bile salt synthesis.

A standardized oral fat challenge resulted in elevation of bile salt levels after 1 h, followed by an increase in plasma FGF19 level after 2–3 h (Fig. 1). In line with an earlier study in volunteers receiving regular meals (21), FGF19 levels show a postprandial peak after 3–4 h. Healthy volunteers and NAFLD patients had comparable fasted FGF19 levels (0.26 ± 0.28 vs. 0.18 ± 0.09 ng/ml, respectively, $P = 0.94$) and displayed a similar postprandial FGF19 response. Peak postprandial FGF19 levels were 3.0-fold higher than baseline values in both groups. The $AUC_{FGF19\ 0-6h}$ appeared somewhat lower in NAFLD patients compared with the volunteers when considering the entire NAFLD group (1.4 ± 1.3 vs. 1.9 ± 1.4 $ng \cdot ml^{-1} \cdot h^{-1}$ in controls, $P = 0.032$) but was indistinguishable when comparing the volunteers and the two HOMA-based NAFLD subgroups by ANOVA ($P_{ALL} = 0.081$).

Postprandial elevation of plasma FGF19 is expected to result in repression of bile salt synthesis. Indeed, plasma C4 levels were decreased following postprandial peaking of FGF19 levels in NAFLD patients with a normal HOMA score (Fig. 3*B*). Interestingly, such decline in C4 levels was not observed in insulin-resistant (i.e., HOMA score ≥2.5) NAFLD patients. Because postprandial FGF19 levels in the HOMA-based NAFLD subgroups were similar at all individual time points, this leaves the possibility that the response of the liver to FGF19 is impaired in insulin-resistant NAFLD patients.

In addition to an apparently impaired hepatic response to FGF19 in NAFLD patients with a HOMA score ≥2.5, we noted an altered postprandial bile salt excursion in this patient group. Similar postprandial FGF19 excursions in controls and both NAFLD patient groups suggests that ileal bile salt reclamation proceeds similarly in these groups; it is thus tempting to speculate that the hepatic uptake of bile salts is reduced in NAFLD patients with HOMA score ≥2.5. Reduced first-pass clearance of bile salts by the liver would result in a higher systemic spillover of bile salts and consequently prolonged circulation times. Proinflammatory cytokines released from inflamed adipose tissue have been implicated in the development of hepatic insulin resistance (22), and among these IL-1β and IL-6 are known to reduce NTCP expression (3, 10). Elevated IL-6 levels in NAFLD patients with HOMA score ≥2.5 (Table 1) may thus have contributed to the altered postprandial bile salt excursion in this patient group.

Activation of the hepatic FXR/short heterodimer partner (SHP) axis by bile salts has been implicated in the regulation of bile salt synthesis (7, 27). Could reduced hepatic uptake of bile salts account for the absence of a postprandial decline in plasma C4 levels in NAFLD patients with HOMA score ≥2.5? Although this cannot be ruled out in the present study, several lines of evidence implicate the intestinal FXR/FGF19 axis as the principal mediator of bile salt-mediated repression of bile salt synthesis. Firstly, bypassing the small intestine through direct infusion of taurocholate in the portal or systemic circulation failed to downregulate Cyp7a1 expression in the rat (24). Secondly, in mice lacking intestinal *Fxr* the expression of *Cyp7a1* is unaffected by FXR agonists, whereas in mice lacking hepatic *Fxr, Cyp7a1* is effectively repressed following FXR agonism (18). Moreover, bile salt-mediated negative feedback control of *Cyp7a1* expression was lost in mice deficient for either *Fgf15* or its receptor *Fgfr4* (16). Thirdly, postprandial increases in plasma FGF19 levels rather than postprandial increases in bile salt levels are followed by a decline in plasma C4 levels (21). Taking into account the above findings, it is unlikely that reduced hepatic bile salt uptake underlies the absence of a postprandial decline in plasma C4 levels in NAFLD patients with HOMA score ≥2.5.

What mechanism could underlie the apparently abrogated hepatic response to FGF19 in insulin-resistant NAFLD patients? Altered expression of the FGF19-receptor FGFR4 and/or the obligate signaling cofactor βKlotho may be an underlying factor. A recent study revealed that expression of hepatic *Fgfr4* mRNA was reduced after prolonged fasting as well as in streptozotocin-induced diabetic mice, whereas insulin-treatment induced hepatic *Fgfr4* mRNA expression in mice (29).

Hepatic expression of βKlotho was affected by none of these treatments. No information is available on FGFR4 expression in the insulin-resistant human liver, although microarray analysis suggests that *Fgfr4* expression is somewhat reduced in the liver of insulin-resistant, leptin-deficient mice (20). Apart from altered signal transduction at the level of the plasma membrane, intracellular relay of the FGF19 signal may be affected in the insulin-resistant liver. FGF19 was recently shown to reduce insulin-stimulated fatty acid synthesis and lipogenic gene expression in hepatocytes (6). This suggests that FGF19 signaling interferes with insulin signaling. Whether such interference is mutual and whether it is maintained in the insulin-resistant state is unknown. Adding complexity to a possible cross-talk between FGF19 and insulin signaling pathways, recent studies in mice indicate that FGF19 signals, at least in part, through the insulin-activated PI3K pathway (29).

In conclusion, a reduced response of the liver to FGF19 in NAFLD patients with insulin resistance may result in further derangement of hepatic lipid homeostasis. Further studies will be required to address the mechanisms by which insulin resistance affects the hepatic response to FGF19 and whether this impaired response contributes to the pathology in NAFLD.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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4. DISKUSE

4.1. Syntetická dráha cholesterolu

V rámci studie „**Differences in antitumor effects of various statins on human pancreatic cancer**“ jsme demonstrovali velmi rozdílné protinádorové účinky inhibitorů HMG-CoA reduktasy. Tyto rozdíly závisely na použití různých statinů, daném typu nádorové linie, délce terapie, stejně jako na zvoleném experimentálním modelu (*in vitro* versus *in vivo*).

Nejsilnější protinádorový účinek *in vitro* vykazovaly cerivastatin, simvastatin a lovastatin, které daleko předčily téměř neúčinný pravastatin s rosuvastatinem. V *in vivo* experimentu dosáhly nejlepších účinků fluvastatin, cerivastatin a překvapivě i rosuvastatin, který v *in vitro* experimentu téměř nefungoval. Lze předpokládat, že za tento rozdíl může pomalá eliminace rosuvastatinu. Poločas rosuvastatinu v organismu je, ve srovnání s ostatními statiny (s výjimkou atorvastatinu), až o řád delší.

Ač byl u všech statinů prokázán protinádorový účinek, v případě pravastatinu šlo o účinek velmi slabý, který v třídenním *in vitro* experimentu chyběl úplně. Tento výsledek je v souladu s naším pozorováním, že pravastatin (jako jediný z testovaných statinů) nedokázal zabránit translokaci K-Ras proteinu do buněčné membrány. Díky hydroxylové skupině v poloze 3 patří pravastatin mezi relativně hydrofilní substance, které pro průnik do buňky potřebují přenašeč[126]. Do buněk, které tímto přenašečem nedisponují, se tak pravastatin nedostane. Oproti tomu hydrofobní statiny (jako například laktonová forma simvastatinu a lovastatinu) procházejí buněčnými membránami volně[127,128].

Ve srovnání s pankreatickými nádorovými liniemi CAPAN-2 a MiaPaCa-2, které nesou aktivační mutaci ve dvanáctém kodonu protoonkogenu K-Ras, odpovídaly buňky linie BxPc-3 (bez aktivační mutace) na podání statinů hůře. Tato fakta, společně s pozorováním, že podání mevalonátu, farnesyl pyrofosfátu či geranylgeranyl pyrofosfátu značně oslabuje účinek statinů, podporují předpoklad, že za protinádorovým účinkem statinů stojí deplece

meziproduktů mevalonátové dráhy syntézy cholesterolu a následně snížená prenylace proteinů, především K-Ras.

Simvastatin a lovastatin se při terapii hypercholesterolémie podávají ve své nativní podobě (tj v laktonové formě), ve které nemají inhibiční účinky na HMG-CoA reduktasu. Teprve v játrech dochází k přeměně těchto prekurzorů v účinné laktamy (Obr. Struktura statinů). Je velmi nepravděpodobné, že by v *in vitro* experimentech s pankreatickými nádorovými liniemi samovolně docházelo k přeměně laktonu v laktam, a tak byla terapeutika podávána v obou formách. Oproti očekávání byly i laktonové prekurzory účinné. To svědčí o dalším možném, na HMG-CoA reduktase nezávislém, mechanismu protinádorového působení statinů. Takovým mechanismem může být například inhibice proteasomu, která patří mezi známé, v klinické medicíně používané postupy[129]. Inhibiční či modulační působení statinů na proteasom již bylo prokázáno v dřívějších studiích[130,131].

Při výčtu vlastností, které mohou modifikovat protinádorové účinky statinů, nelze opomenout jejich antioxidační potenciál. Ve srovnání s pravastatinem je fluvastatin výrazně silnější antioxidant[132]. Není zcela zřejmé, jestli vysoký antioxidační potenciál fluvastatinu představuje výhodu či nikoli. Oxidační stres se zcela jistě podílí na vzniku nádorového onemocnění, proto můžeme očekávat příznivé preventivní působení fluvastatinu. Při cytostatické léčbě je však oxidační stres důležitým mechanismem účinku[133,134], a tak by podání antioxidačně působícího fluvastatinu v kombinaci s cytostatiky mohlo působit kontraproduktivně.

Statiny mají velmi pravděpodobně značný terapeutický potenciál, nicméně vzhledem ke značně komplexnímu mechanismu jejich protinádorového účinku je třeba dalších detailních studií, které je pomohou zahrnout do standardních terapeutických protokolů. Abychom tohoto dosáhli, potřebujeme velmi precizně navržené klinické studie. Jak se ukázalo, ani velmi rozsáhlé metaanalýzy epidemiologických studií nemusí přinést potřebné

údaje, pokud neberou na vědomí značnou rozdílnost jednotlivých statinů, odlišný charakter různých typů nádorových onemocnění, genetickou predispozici jedinců či rozdíl mezi preventivním a léčebným účinkem.

4.2. Katabolická dráha cholesterolu

V předkládaných pracích jsme se zaměřili na studium katabolické dráhy cholesterolu: její regulaci, funkci, interakci s ostatními metabolickými procesy a zároveň i na možnosti monitorování její aktivity. V práci nazvané „**Improved HPLC analysis of serum 7α-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption**“ se nám podařilo zavést a zvalidovat metodu, vhodnou pro kvantitativní stanovení C4 v séru či plasmě. Tato metoda si ponechává hlavní výhody ostatních HPLC metod (dobrá dostupnost, nízké náklady) a zároveň obchází jejich největší slabinu (komplikovanou extrakci na pevnou fázi při 64°C). Použité silikagelové předkolonky, ve srovnání s dříve používanými oktylovými předkolonkami, umožňují extrakci analytu za pokojové teploty, což představuje značné zjednodušení a umožňuje částečnou automatizaci procesu. Silikagelové předkolonky jsou též výrazně levnější a mají vyšší vazebnou kapacitu, díky které se až pětinásobně zvyšuje rozsah linearity. Použití chloroform-methanolu zajistí kvantitativní extrakci jak vnitřního standardu, tak C4. Tím se odstraní zdroje případných nepřesností, jakými jsou precipitace vnitřního standardu či vazba C4 na sérové proteiny[135]. Hlavní předností této metody je její jednoduchost a robustnost, díky které ji lze zavést do klinické praxe.

Široká dostupnost tohoto vyšetření by měla vést ke snadnější diagnostice MŽK, zejména u chronických průjmových onemocnění, a následně k její racionální terapii. Počet chronických průjmových onemocnění v USA byl v roce 1998 přes 15 milionů v případě chronických průjmů či přes 3 miliony v případě syndromu dráždivého tračníku, náklady na jejich diagnostiku a léčbu přesáhly 2 miliardy dolarů[136]. Odhaduje se, že MŽK může být

přítomna zhruba u třetiny těchto pacientů[137,138]. Cílená terapie je v tomto případě nesrovnatelně účinnější, než léčba konvenční[138], a tak lze očekávat poměrně výrazné zlepšení terapeutických výsledků.

V práci nazvané „**Bile acid malabsorption in inflammatory bowel disease: Assessment by serum markers**“ jsme publikovali výsledky studie, provedené na pacientech s Crohnovou chorobou. Podle očekávání jsme potvrdili častý výskyt MŽK – téměř každý druhý pacient vykazoval laboratorní známky (sérové koncentrace C4) tohoto symptomu. Závažnost malabsorpce byla, taktéž podle předpokladů, závislá především na postižení terminálního ilea. Překvapením však byl poměrně vysoký počet (11%) malabsorbujících i ve skupině s intaktním ileem. Na druhou stranu, více než třetina pacientů po resekci terminálního ilea měla normální sérové koncentrace C4. Tyto výsledky podporují tvrzení, že exprese ileálního transportéru žlučových kyselin je snížená u pacientů s Crohnovou chorobou a nezávisí na zánětlivém postižení ilea[139,140]. Z uvedeného plyne, že postižení terminálního ilea sice je nejvýznamnějším faktorem, určujícím míru MŽK, nikoli však jediným. Symptomatičtí pacienti (tj. s idiopatickým chronickým průjmem) by měli být indikováni k laboratornímu vyšetření MŽK. V případě, že toto vyšetření není dostupné, měl by následovat terapeutický pokus se sekvestranty žlučových kyselin.

Abychom zjistili, jak je MŽK u pacientů s Crohnovou chorobou diagnostikována a léčena v různých zemích, rozeslali jsme krátký dotazník vybraným specialistům. Zajímavým zjištěním bylo, že více než dvě třetiny gastroenterologů podávají sekvestranty žlučových kyselin pouze symptomatickým pacientům po resekci terminálního ilea. Neoperovaným pacientům, byť s MŽK (v našem souboru takových bylo více než 10%), není tato léčba vůbec nabídnuta.

Nedávno publikované práce poukazují na roli defektní signalizační kaskády FGF19-FGFR4 (vedoucí k nadměrné produkci žlučových kyselin) v patogenezi průjmových

onemocnění[119,141]. Zdá se tedy, že v některých případech není primárním důvodem malabsorpce žlučových kyselin jejich snížené vychytávání tenkým střevem, nýbrž jejich extrémně zvýšená produkce. Výrazný nadbytek žlučových kyselin překročí resorpční kapacitu tenkého střeva a nevstřebané žlučové kyseliny odcházejí z organismu spolu se stolicí. Tato teorie vysvětluje dřívější překvapivé pozorování Bajora a spolupracovníků[142], že vstřebávání žlučových kyselin ileem pacientů s MŽK je účinnější než u zdravých jedinců. Těsný vztah sérových koncentrací FGF19 a C4, pozorovaný v naší studii, poukazuje na možné využití stanovení sérových koncentrací FGF19 jako dalšího ukazatele míry MŽK.

C4 však neslouží jen jako marker MŽK, nýbrž především jako ukazatel aktivity CYP7A1. Toho jsme využili ve studii nazvané **„*CYP7A1* promoter polymorphism -203A>C affects bile salt synthesis rate in patients after ileal resection“**. V ní jsme prokázali, že běžný polymorfismus (-203A>C) v promotoru *CYP7A1* může ovlivňovat její aktivitu. Nepotvrdili jsme, že by se tak dělo za normálních okolností, avšak u pacientů s MŽK, kteří mají výrazně zýšenou aktivitu CYP7A1, byl efekt polymorfismu jasně patrný. Předpokládáme, že funkční rezerva CYP7A1 dokáže za fyziologických podmínek překonat vliv polymorfismu. Teprve v případě závažné MŽK, kdy se aktivita CYP7A1 pohybuje pravděpodobně na hranici svých možností, se může projevit „slabší“ varianta promotoru, asociovaná s alelou -203A. U nositelů „silnějšího“ promotoru (tj. alely -203C) by měla vyšší aktivita CYP7A1 vést k rychlejšímu odbourávání cholesterolu a zvýšené expresi LDL receptorů v hepatocytech a následně k poklesu sérového LDL cholesterolu[143-145]. Pozorování, že efekt polymorfismu je za fyziologických okolností zanedbatelný, vysvětluje, proč se jeho asociaci se sérovou koncentrací cholesterolu podařilo prokázat jen v některých rozsáhlých studiích[146,147]. Díky velikému počtu studovaných subjektů se v nich i velmi malý (klinicky irelevantní) rozdíl v koncentracích cholesterolu projevil jako statisticky významný.

Jelikož jsme v naší studii neprokazovali kauzalitu daného polymorfismu, můžeme hovořit pouze o asociaci polymorfismu s aktivitou CYP7A1. Úsek DNA přímo odpovědný za odlišnou aktivitu leží v oblasti promotoru či v prvním exonu CYP7A1, které tvoří poměrně rozsáhlý, silný haploblok.

Ve studii nazvané „**Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects**“ jsme podle předpokladů a ve shodě s výsledky malé studie švédských autorů[120] pozorovali značnou diurnální variabilitu koncentrace C4 (markeru aktivity CYP7A1), vrcholící asi hodinu po poledni. Tyto výkyvy jsou vázány na příjem potravy, kdy aktivita CYP7A1 postprandiálně stoupá. Toto nelze vysvětlit účinkem žlučových kyselin, neboť v tom případě bychom očekávali efekt zcela opačný. Pozitivní korelace mezi plochou pod křivkou (AUC) insulinu a C4 podporuje teorii, že za diurnální výkyvy aktivity CYP7A1 může insulin. Je známo, že insulin při krátkodobém působení rychle zvyšuje aktivitu CYP7A1, zatímco v dlouhodobém horizontu převládne jeho mírný inhibiční efekt. To je v souladu s pozorováním rychlého postprandiálního nástupu aktivity CYP7A1 s postupným odezníváním. Na tom se již podílí i inhibiční efekt žlučových kyselin, které se tou dobou portálním řečištěm vracejí do jater.

Zhruba u čtvrtiny studovaných subjektů však CYP7A1 dosahovala vrcholu již kolem sedmé hodiny ranní, tedy nalačno. Zde musíme připustit existenci jiného regulátoru aktivity CYP7A1 – to je ve shodě s pozorováním, že i u lačnících jedinců jsou přítomny diurnální výkyvy v aktivitě CYP7A1[120]. Reguluje-li insulin rychlost syntézy žlučových kyselin, zajímalo nás, zda existuje i vztah opačný - zda žlučové kyseliny ovlivňují insulinemii či glykemii. Ač je známo, že podávání cholestyraminu (sekvestrantu žlučových kyselin) snižuje glykemii a žlučové kyseliny inhibují glukoneogenezi, naše výsledky naznačují, že žlučové kyseliny nejspíš přímý vliv na glykemii nemají. Po podání cholestyraminu jsme sice pozorovali pokles glykemie, podání kyseliny chenodeoxycholové však opačný efekt nemělo.

Z toho lze usuzovat, že za hypoglykemizujícím účinkem cholestyraminu (a zřejmě i jiných sekvestrantů žlučových kyselin) stojí jiný, doposud neznámý mechanismus[148].

Nedávné práce poukazují na vztah FGF19 a regulace lipidového metabolismu v játrech[122-124]. Ve studii nazvané „**The hepatic response to FGF19 is impaired in patients with non-alcoholic fatty liver disease and insulin resistance“** jsme se proto zaměřili na úlohu signalizace FGF19 u pacientů s NAFLD. Jelikož FGF19 pravděpodobně ovlivňuje citlivost tkání k insulinu, porovnávali jsme NAFLD pacienty s či bez insulinové rezistence, posuzované podle HOMA (homeostasis model of assessment) skóre.

Pacienti s NAFLD mají výrazně vyšší bazální hladiny triacylglycerolů (TAG) a žlučových kyselin, nikoli však FGF19. Porovnáním vzestupných či sestupných částí AUC_{TAG} jsme dospěli k závěru, že vyšší koncentrace TAG jsou způsobeny zpomaleným vychytáváním TAG játry. Podobně i zvýšené hladiny žlučových kyselin padají pravděpodobně na vrub sníženému vychytání v hepatocytech, jelikož střevní produkce FGF19 se u sledovaných skupin neliší.

Po perorálním podání tuku dosahovaly, v souladu s očekáváním, koncentrace žlučových kyselin svého maxima asi 1 hodinu a FGF19 2-3 hodiny po zátěži. Zhruba po dalších 2 hodinách jsme očekávali pokles hladin C4, způsobený FGF19 zprostředkovanou inhibicí CYP7A1. Překvapivě se tento pokles dostavil pouze u NAFLD pacientů bez insulinové rezistence, nikoli však u insulin rezistentních (HOMA≥2,5). Tyto výsledky naznačují, že insulin rezistentní NAFLD pacienti mají porušenou (výrazně opožděnou či úplně chybějící) FGF19 signalizaci. Můžeme spekulovat, že se tak děje na úrovni receptoru FGFR4, neboť bylo prokázáno, že jeho myší homolog (Fgfr4) je indukován insulinem[149,150]. Data o vztahu lidského FGFR4 a insulinu však stále k dispozici nemáme. Neodpovídavost na FGF19 může u NAFLD dále prohlubovat dysregulaci lipidového metabolismu, a tak představuje potenciálně atraktivní cíl budoucí specifické terapie.

5. SOUHRN

Meziprodukty biochemických drah v organismu již dnes nejsou považovány jen za mezistupeň na cestě k finálnímu produktu. Ukazuje se, že jejich funkce může být v organismu značně pestrá a mnohdy i nepostradatelná. Kromě významu pro vlastní organismus také nelze opomenout jejich přínos například pro diagnostiku či výzkum celé řady onemocnění.

V předkládané práci jsme se zaměřili na biosyntetickou a degradační dráhu cholesterolu. Většina statinů, inhibitorů mevalonátové cesty biosyntézy cholesterolu, vykazovala výrazné protinádorové účinky na *in vitro* i *in vivo* modelu karcinomu pankreatu. Ač se na protinádorových účincích statinů podílí více mechanismů zároveň, klíčovou roli hraje deplece meziproduktů syntetické dráhy cholesterolu, převážně farnesylpyrofosfátu a geranylpyrofosfátu. Pozorované výrazné rozdíly v protinádorové účinnosti jednotlivých statinů jsou dané jejich odlišnými fyzikálně-chemickými vlastnostni, vlastnostmi jednotlivých nádorových linií i použitými experimentálními modely. To poukazuje na nutnost pečlivého návrhu klinických studií a vysvětluje často rozporuplné závěry rozsáhlých metaanalýz.

V katabolické dráze cholesterolu nachází zatím největší diagnostické uplatnění C4, jehož sérová koncentrace slouží převážně jako spolehlivý ukazatel aktivity CYP7A1, rychlosti syntézy a zároveň míry malabsorpce žlučových kyselin[111,151]. Rutinnímu diagnostickému využití tohoto analytu doposud bránila jeho problematická kvantifikace. Proto jsme zavedli a zvalidovali nový postup stanovení C4, který obchází klíčové slabiny předchozích metod.

Díky tomuto postupu jsme mohli stanovit sérové koncentrace C4 u rozsáhlé skupiny pacientů s nespecifickými střevními záněty. Zjistili jsme, že MŽK nelze spolehlivě určit jen z klinického obrazu. Její laboratorní vyšetření by se tudíž mělo stát běžnou součástí péče o pacienty s Crohnovou chorobou. Tam, kde není dostupné stanovení koncentrace C4, lze MŽK stanovit na základě sérových koncentrací FGF19.

Stanovení sérových koncentrací C4 jsme použili též při studiu regulace CYP7A1.

Ukázalo se, že alela -203A v promotoru CYP7A1 je asociována se slabší aktivitou CYP7A1. Díky značné funkční rezervě není tento vliv za fyziologických okolností patrný a objeví se teprve při výrazné aktivitě CYP7A1. Dále se nám podařilo potvrdit význam insulinu v aktivaci CYP7A1. Překvapivě jsme asi u čtvrtiny dobrovolníků pozorovali netypická ranní maxima aktivity CYP7A1, jejichž důvod zatím zůstává skryt.

V poslední studii jsme u pacientů s NAFLD, rezistentních na insulin, pozorovali porušenou odpovídavost hepatocytů na FGF19 signalizaci. Předpokládáme, že tato porucha může dále deregulovat lipidový metabolismus v jaterní tkáni a vést k rychlejší progresi onemocnění.

6. SEZNAM POUŽITÝCH ZKRATEK

Akt	kinasa, známá též jako protein kinasa B
Apobec-1	apolipoprotein B editing enzyme
AUC	plocha pod křivkou
C4	7α-hydroxy-4-cholesten-3-on
CYP7A1	cholesterol 7α-monooxygenasa
DNA	deoxyribonukleová kyselina
ERK1/2	kinasa (extracellular signal-related kinase)
FDA	Národní úřad pro potraviny a léky (USA)
FGF19	růstový faktor fibroblastů 19
FGFR4	receptor pro FGF19
FPP	farnesylpyrofosfát
FXR	farnesoid X receptor
GGPP	geranylgeranylpyrofosfát (GGPP)
GPBAR1	receptor pro žlučové kyseliny spřažený s G-proteinem (též označovaný jako TGR5)
GTP	guanosintrifosfát
HDL	lipoproteiny o vysoké hustotě
HMG-CoA	3-hydroxy-3-methyl-glutaryl-koenzym A
HOMA	homeostasis model of assessment
HPLC	vysokoúčinná kapalinová chromatografie
IDL	lipoproteiny o střední hustotě
JNK	kinasa (c-Jun N terminal kinase)
LDL	lipoproteiny o nízké hustotě
LXR	liver X receptor α
MAPK	kinasa (mitogen-activated protein kinase)
MŽK	malabsorpce žlučových kyselin
NAFLD	nealkoholová steatosa jater
NASH	nealkoholová steatohepatitida
PXR	pregnane X receptor
Ras	rodina protoonkogenů (z angl. „**ra**t **s**arcoma“)
Rho	rodina malých signalizačních proteinů (z angl. „**r**as **ho**mologous“)
RNA	ribonukleová kyselina
RXR	retinoid X receptor
^{75}SeHCAT	^{75}Se-homotaurocholová kyselina
SHP	short heterodimer partner
TAG	triacylglyceroly
3’UTR	3‘ nepřekládaná oblast
VLDL	lipoproteiny o velmi nízké hustotě

7. SEZNAM POUŽITÉ LITERATURY

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