UNIVERZITA KARLOVA V PRAZE

2. LÉKAŘSKÁ FAKULTA

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Genetické aspekty Crohnovy choroby

Dizertační práce

Praha 2011

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Souhlasím – Nesouhlasím

V Praze, 8.4.2011.

Jméno – Příjmení (hůlkovým písmem)

Podpis

*Nehodící se škrtněte

Poděkování

Rád bych poděkoval svému školiteli MUDr. Ondřeji Cinkovi, Ph.D. za předání velmi cenných vědomostí a vedení postgraduálního studia. Dále bych chtěl poděkovat všem spoluautorům.

Dizertační práce vznikala v rámci grantů Ministerstva zdravotnictví České republiky (MZOFNM2005), interního grantu Fakultní nemocnice Motol a Grantové agentury Univerzity Karlovy v Praze (GA UK 7660/2007).

Obsah

1 Úvod

1.1 Definice

Zánětlivá střevní onemocnění (IBD, inflammatory bowel diseases, dříve ozačované jako idiopatické střevní záněty) jsou chronická zánětlivá onemocnění gastrointestinálního traktu, která byla empiricky definována na základě klinických, histologických, endoskopických a radiologických nálezů [1]. Crohnova choroba (CD, Crohn´s disease) a ulcerózní kolitida (UC, ulcerative colitis) jsou dva póly zánětlivých střevních onemocnění. Formy onemocnění, které nelze zařadit ani do jedné z výše jmenovaných jednotek, jsou nazývány nezaraditelná zánětlivá střevní onemocnění (IBDU) nebo po histologickém ověření resekátu střeva kolitida indeterminovaná (IC, indetermined colitis). Jedná se tedy o skupinu onemocnění, která mají společné určité klinické i etiopatogenetické rysy, ale liší se charakterem a lokalizací zánětu, a dále také léčebnými postupy.

1.2 Historie

Nozologické jednotky Crohnova choroba a ulcerózní kolitida vznikly až ve dvacátém století, ale popisy odpovídající těmto chorobám se objevují již mnohem dříve. Protože příznaky typické pro IBD jsou společné také pro v minulosti častější infekční onemocnění střeva, je obtížné bez patologického popisu tato onemocnění odlišit. První známky typické pro IBD byly popsány při pitvách v patnáctém století Antoniem Benivienem. Ludvík XIII. během života trpěl chronickým

průjmem, teplotami a pravděpodobně měl perianální píštěle a abscesy. Záznam z posmrtné pitvy by mohl odpovídat ileocékální tuberkulóze či CD [2]. Poměrně přesvědčivý popis toho, co dnes nazýváme Crohnovou chorobou, byl publikován Combem v roce 1831. Termín ulcerózní kolitida byl zaveden v roce 1888 Hale –Whitem, ačkoli ne všechny případy popsané tímto autorem byly skutečně UC. Anglický lékař Samuel Wilks v roce 1889 popsal v zánětem postižené stěně střeva „pyoid corpuscles“ (granulomy). V roce 1901 ve své práci Lartigau naznačil, že granulomy nemusí mít nutně tuberkulózní původ [3]. Teprve v roce 1931 píše B.B. Crohn Americké gastroenterologické asociaci, že objevil novou chorobu, která by se měla jmenovat terminální ileitida. Práce byla v rozšířené verzi publikována v JAMA 1932 [4]. Jako autoři byli uvedeni Burel B. Crohn, Leon Ginzburg a Gordon D. Oppenheimer (obr. 1). Ačkoli jejich jednotlivý podíl na vytvoření nové klinicko-patologické jednotky je kontroverzní [5], od roku 1933 začalo být používáno pro toto onemocnění označení regionální ileitida Crohn.

Obrázek 1. Dr. Oppenheimer, Dr. Crohn a Dr. Ginzburg

1.3 Výskyt

Výskyt IBD se významně liší v jednotlivých zemích a u jednotlivých etnik. Publikovaná data o výskytu zánětlivých střevních onemocnění se různí podle období, kdy byla daná kohorta sledována. V devadesátých letech se incidence CD dle dostupných prací celosvětově pohybovala od 0 po 15 nových případů na 100 000 obyvatel a rok, prevalence od 3,6 po 214 na 100 000 obyvatel [6]. V případě UC je udávána incidence v rozmezí 1,2 až 20,3 na 100 000 obyvatel a rok

a prevalence od 7,6 po 246 na 100 000 obyvatel. Obecně je výskyt vyšší u kavkazské populace a v zemích severní Evropy a Severní Ameriky. V poslední době incidence CD i UC vzrostla [6, 7]. Nedávná práce ukazuje, že incidence roste rychleji u CD než u UC [8]. Incidence IBD u dětí kavkazského etnika se pohybuje kolem 4 / 100 000 [8]. V České republice byla stanovena v roce 2001 incidence CD u dětí do 15 let na 1,26 / 100 000 [9]. IBD patří mezi pět nejčastějších chronických onemocnění zažívacího traktu a náklady na léčbu se v USA ročně pohybují okolo 1,8 miliardy dolarů [10].

1.4 Etiopatogeneze

Zánětlivá střevní onemocnění vznikají na základě neadekvátní zánětlivé odpovědi na intestinální bakteriální flóru u geneticky predisponovaných jedinců.

Zevní faktory

Mezi faktory zevního prostředí, které by se mohly podílet na vzniku IBD, patří kouření, strava, užívaní antibiotik, nesteroidní protizánětlivé léky (NSAID), stres a infekce [6]. Infekce a NSAID mohou narušit slizniční bariéru [11]. O roli stravy a stresu při vzniku IBD je známo jen velmi málo. U železa a hliníku byla popsána zvýšená bakteriální virulence [12]. Kouření má na tato dvě onemocnění opačný vliv: u nekuřáků a bývalých kuřáků je vyšší riziko vzniku UC, naproti tomu kuřáci mají zvýšené riziko vzniku CD. Přesný mechanismus působení kouření na vznik IBD není znám. Za mediátory byly označeny nikotin, CO a hypoxie [13].

Některé teorie označují za původce IBD patogenní bakterie. Pouze pro dvě z nich existují seriózní experimentální důkazy. Již v roce 1984 bylo na základě nálezu v resekátech střeva u pacientů s CD mezi etiologické činitele zařazeno *Mycobacterium avium subspecies paratuberculosis* (MAP) [14]. Při nedávné studii byla inzerční sekvence DNA (IS900) nalezena ve tkáni střeva u 52% pacientů s CD, ale jen u 2% pacientů s UC a u 5% zdravých kontrol [15]. V publikovaných pracích kolísá pozitivita MAP u pacientů s CD od 0 do 100%. Tato teorie má však mnohé slabiny: chybí histochemický průkaz MAP ve tkáni a detekovaná DNA nálož MAP ve tkání je příliš nízká [16]. Proti této teorii svědčí také fakt, že pacienti většinou profitují z imunosupresivní léčby. Předpokládali bychom rovněž, že by se IS900 mělo preferenčně nalézat v místech nejčastějšího postižení u pacientů s CD, což se však nepotvrdilo. Je tedy pravděpodobnější, že tato relativně běžná bakterie selektivně kolonizuje poškozenou sliznici střeva u pacientů s CD, a není tedy za její vznik přímo zodpovědná [17]. Dalším podezřelým etiologickým činitelem je adherentně invazivní kmen E. coli, který byl prokázán častěji u pacientů s Crohnovou chorobou – zvláště s časným relapsem – než u kontrol [18]. Bylo také popsáno současné působení tohoto kmene a *NOD2* polymorfismů [19].

Střevní mikroflóra a její interakce s genotypem pacienta

Zásadní roli při vzniku a rozvoji IBD hraje interakce mezi střevní mikroflórou a sliznicí střeva. Při vzniku a rozvoji IBD se uplatňují tyto složky: mikrobiální flóra, bariéru tvořící epiteliální buňky a lymfatický gastrointestinální systém. U pacientů s CD a UC byly nalezeny odlišnosti ve složení mikrobiální flóry [20]. Také permeabilita paracelulárního prostoru slizniční bariéry a regulace tight junctions je u těchto pacientů defektní [21]. Tyto změny však mohou být jen důsledkem zánětu ve střevní sliznici. Dalším důležitým mechanismem podílejícím se na porušení

epiteliální bariéry je poškození pohárkových buněk secernujících hlen a poškození Panethových buněk, které produkují antimikrobní peptidy, alfa-defensiny [22]. Gen *ATG16L1*[1] zapojený do autofagie – procesu odstranění intracelulárních komponent – ovlivňuje morfologii a genovou expresi Panethových buňek [23].

Komunikace mezi mikrobiální flórou střeva a lymfatickým systémem je zprostředkována pomocí četných receptorů patřících do skupiny rozeznávající molekulární vzor (pattern-recognition receptors). Mezi ně patří skupina povrchových toll-like receptorů a intracelulárních receptorů skupiny NOD (nucleotide-binding oligomerization domain containing). Jejich porucha vede ke snížené imunitní odpovědi na intraluminální bakterie. NOD2[2] je intracelulární senzor muramyldipeptidu – složky bakteriální stěny [24]. Je exprimován v Panethových buňkách, epiteliálních buňkách, makrofázích, dendritických buňkách a endoteliálních buňkách. Aktivace intaktního NOD2 proteinu vede k aktivaci NF-kappaB a MAP kinázové kaskády signalizační dráhy, která vede k produkci TNF[3] a IL1B[4] [25]. Při chronické stimulaci dochází k negativní regulaci prozánětlivých cytokinů. Tato funkce NOD2 proteinu je porušena, jestliže je NOD2 poškozen [26]. Přesný mechanismus, jak snížená funkce NOD2 proteinu vede ke zvýšenému riziku vzniku CD, není znám.

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[1] *ATG16L1* (autophagy related 16-like 1) je asociován s CD také v české populaci.
[2] *NOD2* (nucleotide-binding oligomerization domain containing 2) obsahuje varianty, které jsou v našem souboru nejsilněji asociované s CD.
[3] *TNF* (tumor necrosis factor): nebyla nalezena asociace s CD v české populaci.
[4] IL1B (interleukin 1, beta): nebyla nalezena varianta, která by byla asociována s CD.

Vzorky bakterií jsou pravidelně přenášeny přes epiteliální buňky a M buňky Peyerových plaků pomocí imunoglobulinů [27] a dendritických buněk [28]. Aktivované antigen prezentující buňky (APC) předkládají antigenní peptidy T buňkám v sekundárních lymfatických orgánech střeva (Peyerových placích, mezenteriálních lymfatických uzlinách a izolovaných lymfatických uzlících). Při této interakci hraje zásadní roli vazba mezi antigenem vystaveným na MHC a TCR receptorem a dále vazba kostimulačních molekul (CD80 a CD28 nebo CD80 a CTLA4[5]). Signál poté může být přenášen do jádra mimo jiné přes protein PTPN22[6]. K rozvoji zánětu vede porušení rovnováhy mezi T-regulačními lymfocyty (Treg (FoxP3+)) a efektorovými subpopulacemi Th buněk (Th1, Th2, Th17). V případě CD je zvýšená hladina IL-17 (Th17 reakce), IFNgama a TNFalfa (Th1 reakce). Ve sliznici kolon při UC dochází ke zvýšení Th17 cytokínů a Th2 cytokínů (IL4, 5, 13). Proliferace a přežívání Th17 lymfocytů je zprostředkována vazbou IL23 – produkovaného APC – s IL23 receptorem (IL23R[7]) [29]. Po aktivaci exprimují T buňky (za pomocí kyseliny retinové) integrin alfa4beta7[8] a chemokinový receptor CCR9 a stávají se tak „intestino-tropní“. Také porucha funkce TGF-beta1, cytokinu Treg lymfocytů, způsobuje chronický zánět střeva [30]. Porucha IL-10[9], dalšího cytokinu, který podporuje vývoj Treg lymfocytů, může také vést k rozvoji CD [31] a UC [32]. Treg buňky jsou dále ovlivňovány geny zapojenými do autofagie [33]. Role B-lymfocytů je méně známá. O jejich aktivaci svědčí

[5] *CTLA4* (cytotoxic T-lymphocyte-associated protein 4). Přímá asociace s CD nebyla v kavkazské populaci prokázána. V našem souboru jsme nalezli možnou interakci s geny *NOD2* a *IL23R* při vzniku CD.
[6] *PTPN22* (protein tyrosine phosphatase, non-receptor type 22). V našem souboru jsme neprokázali rozdíl ve frekvencích alel či genotypů mezi pacienty s CD a kontrolním souborem.
[7] *IL23R* (interleukin 23 receptor) je asociován s CD také v české populaci.
[8] Integrin alfa 4 beta 7: humanizovaná protilátka proti alpha 4 beta 7 integrinu, natalizumab se zdá být efektivní v léčbě CD. Vzhledem k výskytu progresivní multifokální leukoencefalopatie v souvislosti s podáváním tohoto preparátu není pro rutinní léčbu dostupný.
[9] IL 10 (interleukin 10). Asociace IL10 s ulcerózní kolitidou byla potvrzena několika pracemi, asociace s CD je kontroverzní.

zvýšené hladiny antimikrobních protilátek, které se používají k diagnostice IBD. Postupně dochází k akumulaci leukocytů ve tkáni střeva a zánět se amplifikuje.

1.5 Klinický obraz

Onemocnění může začínat v kterémkoliv věku, typicky se však začne projevovat ve druhé nebo třetí dekádě. Crohnova choroba postihuje většinou ileum a kolon, může však zasáhnout kteroukoliv část trávicího traktu. Zánět je diskontinuální. Ulcerózní kolitida vždy postihuje rektum a může zasáhnout také část nebo i celé kolon. Zánět je kontinuální. Zatímco u UC je zánět omezen na sliznici, v případě CD prochází transmurálně. Pro CD jsou dále typické granulomy, striktury a fistule [34]. Tyto popsané odlišnosti platí spíše pro dospělé pacienty, u dětí je situace často složitější a rozlišení mezi oběma jednotkami bývá obtížné. Pro potřebu lepšího rozlišení forem IBD v dětském věku byla stanovena tzv. Portská kritéria [35]. I jednotlivé formy zánětlivých střevních onemocnění (CD, UC, IBDU) mají své podtypy. První genetické studie a širší rozšíření serologického panelu u pacientů s IBD přispěly k modifikaci klasifikace Vídeňské na tzv. Montrealskou klasifikaci, která je zachycena v tabulce 1[36].

Tabulka 1. Montrealská klasifikace Crohnovy choroby.

Věk v době diagnózy (%)		
A1 (< 17 let)		
A2 (17 - 40 let)		
A3 (> 40 let)		
Lokalizace[1]	Modifikace při postižení GIT (L4)	
L1 (terminal ileum)	L1 + L4	Term. Ileum + horní GIT
L2 (kolon)	L2 + L4	Kolon + horní GIT
L3 (ileokolon)	L3 + L4	Ileokolon + horní GIT
L4 (Horní GIT)	-	
Chování choroby	Modifikace při perianálním postižení	
B1 (nestrikturující/ nepenetrující)	B1p	nestrikturující/ nepenetrující + perianální postižení
B2 (strikturující)	B2p	strikturuící + perianální postižení
B3 (fistulující)	B3p	fistulující + perianální postižení

1.6 Léčba

Strategie léčby

Hlavní cíle terapie zánětlivých střevních onemocnění zahrnují indukci, udržení remise, prevenci komplikací, zlepšení a udržení kvality života a omezení indikací pro chirurgickou terapii [37]. Stávající doporučené postupy jsou postaveny na navození a udržení remise [38, 39]. Dle většiny stávajících doporučení je navození remise založeno na „step-up“ modelu dle tíže onemocnění [37]. Dospělí pacienti s lehkým onemocněním jsou většinou léčeni lokálními kortikosteroidy

(budesonid), případně aminosalicyláty (ačkoli je jejich efekt zvláště u CD značně kontroverzní). Pacienti se středně závažným onemocněním jsou při indukci léčeni kortikosteroidy a ti pacienti, jejichž choroba je refrakterní k celkově podávaným kortikosteroidům, pak biologickou léčbou (infliximab, adalimumab). Pro pacienty s UC je v indukční léčbě možno užít také cyklosporin. Po navození remise je indikována léčba imunosupresivní – nejčastěji v podobě azathioprinu. Chirurgická léčba je rezervována pro pacienty, u nichž farmakoterpie selhává, a dále pro určité typy postižení. V poslední době je hojně diskutován „top-down" nebo „early-agressive" model [40]. Přesné indikace tohoto postupu však dosud nejsou jednoznačně určeny.

Léčba zánětlivých střevních onemocnění v dětském věku se dosti liší od léčby pacientů dospělých. Většinu dětských pacientů je nutné indikovat k dlouhodobé imunosupresivní léčbě azathioprinem, který se zdá v dětském věku účinnější [41]. Doba podávání celkových kortikoidů, vzhledem k vedlejším účinkům a vlivu na vyvíjejícího se jedince, zvláště pak při časté poruše růstu, má být co nejkratší. Lokálně podávaný budesonid se při dlouhodobé terapii jeví jako nedostatečně účinný [42]. Ačkoli byla exkluzivní enterální výživa prokázána jako srovnatelně účinná alternativa farmakoterapie v indukci remise u dětských pacientů s CD, není její užití všeobecně rozšířeno.

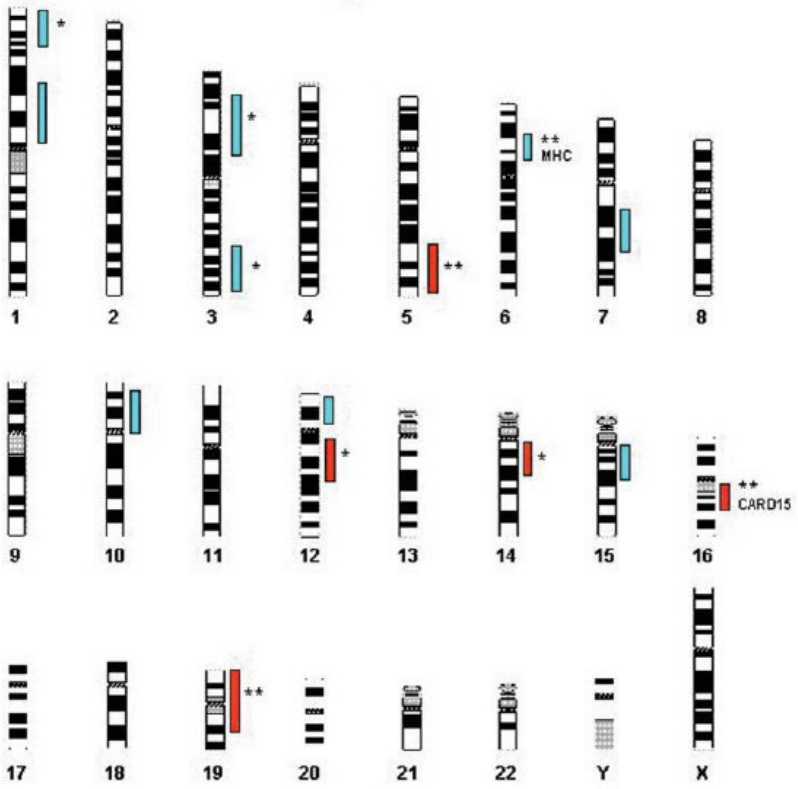
Biologická léčba

V současné době jsou v různých zemích a pro různé věkové kategorie dostupné tři preparáty z tzv. biologické terapie IBD. Prvním preparátem na trhu byl infliximab. Jedná se o IgG1 chimerní protilátku proti TNF (část Ig je lidského a část myšího původu). V případě adalimumabu

se jedná o protilátku plně humanizovanou. Certolizumab pegol je Fab' fragment, který je vázán na polyethylenglykol, a vykazuje tak lepší farmakokinetické vlastnosti. Ve Spojených státech amerických je dále k dispozici natalizumab (monoklonální protilátka proti α4 integrinu), jehož použití brání vzácná komplikace – progresivní multifokální leukoencefalopatie způsobená reaktivací JC viru [43]. V ČR je pro děti zatím registrován pouze infliximab, použití adalimumabu je možné jen v rámci probíhající klinické studie. Mechanismus účinku infliximabu a adalimumabu není zcela objasněn. Na základě *in vitro* a částečně také *in vivo* provedených experimentů bylo navrženo několik možných úrovní působení. Za nejdůležitější je považována neutralizace solubilní formy TNF (sTNF). Zmíněné dva preparáty se však váží i na transmembránový TNF (tmTNF) a blokují tak vazbu s TNFRI/II. Dalším mechanismem účinku je tzv. „reverzní signalizace", při které dojde k fosforylaci serinových zbytků na cytoplasmatické části tmTNF a signál je převeden do buňky, jež tento receptor vystavuje. Předpokládán je účinek přes aktivaci E-selektinů [44]. U infliximabu byla také prokázána suprese proliferace zastavením buněčného cyklu T buněk v G0/G1 fázi [45]. Dalším možným důsledkem reverzní signalizace je kompetice sdílených molekulárních signalizačních drah, například signalizace toll-like receptory vyvolaná endotoxinem [46, 47]. Vzhledem k tomu, že jak molekula infliximabu, tak humanizovaná molekula adalumimabu obsahují Fc fragment, předpokládá se, že jsou schopny vyvolat cytotoxickou reakci způsobenou komplementem (CDC) i cytotoxickou reakci závislou na protilátkách (ADCC). Význam těchto reakcí je však zatím nejasný [48]. Antagonisté TNF mohou indukovat apoptózu u lymfocytů a makrofágů dvěma možnými způsoby: 1) neutralizací sTNF, jež může vést k omezení antiapoptotických signálů přes protein p55 [49]; 2) zkříženou vazbou a signalizací přes tmTNF. Tento posledně zmíněný mechanismus však pravděpodobně nebude tím hlavním [50].

1.7 Genetika IBD

Výskyt zánětlivých střevních onemocnění je oproti běžné populaci u příbuzných pacientů s IBD zvýšen. Poměr rizika rekurence CD pro sourozence proti riziku v obecné populaci (λ_S) byl odhadnut v rozmezí od 15 do 35, dle designu jednotlivých studií [51–55]. Vliv genetických faktorů byl u Crohnovy choroby dokázán při studiích na dvojčatech. Dánská práce uvádí konkordanci CD u monozygotních 5/10 a u dizygotních dvojčat 0/27 [56]. Prokázána byla také vyšší prevalence UC u příbuzných pacientů s CD [51, 52]. Pomocí vazbových studií s použitím mikrosatelitních markerů bylo vytipováno několik chromozomálních regionů, kde by se geny podílející se na vzniku IBD mohly nacházet [57-66]. Tyto lokusy byly označeny IBD1 až IBD9. Postupně pak v těchto regionech byly identifikovány jednotlivé geny, nebo se asociace v těchto lokusech nepotvrdila. Z těchto původních názvů se zachoval pouze IBD5. Na obrázku 2 jsou zobrazeny hlavní regiony, červeně signifikantní a modře „podezřelé“ z podílu na vzniku IBD [67].



Obrázek 2. Grafické znázornění hlavních chromozomálních regionů podezřelých z asociace s IBD nalezených pomocí vazebných studií. Červeně označeny regiony se signifikantní vazbou, modře s pravděpodobnou. Hvězdičkou jsou označeny regiony, jejichž vazba byla potvrzena následnými studiemi (MHC, hlavní histokompatibilní komplex). Převzato z [67].

NOD2

Nejvíce prozkoumaným genem v souvislosti s CD je nucleotide-binding oligomerization domain containing 2 (*NOD2)*. To, jak byla asociace objevena a potvrzena, je popsáno v úvodu naší publikace 2.1 [68]. Z počátku se pro tento gen používalo označení *NOD2*, tedy stejné jako pro protein. Krátce byl za oficiální název přijat *CARD15* (caspase recruitment domain family, member 15), který byl opět změněn na původní označení *NOD2*.

IBD5

Ačkoli je asociace IBD5 lokusu známa již poměrně dlouho, kauzální varianty a konkrétní gen zodpovědné za podíl na vzniku IBD stále nejsou nalezeny. Celkový přehled o výzkumu IBD5 lokusu uvádíme v publikaci 2.4 [69].

Další zkoumané geny před érou celogenomových asociačních studií

Ještě nedávno byly IBD řazeny do skupiny autoimunitních onemocnění; nyní vzhledem k novým poznatkům z oblasti imunologie a genetiky IBD, jsou spíše používány termíny jako imunitně podmíněná onemocnění. Přesto je zřejmé, že tato onemocnění mají alespoň v určité fázi (blíže viz Etiopatogenze) s autoimunitními chorobami mnoho společných rysů. Bylo tedy logické, že za další kandidátní geny byly voleny ty již dříve prokázaně asociované s autoimunitami. Typickým příkladem je gen *CTLA4* (cytotoxic T-lymphocyte-associated protein 4), jehož biologická role je relativně dobře známa a jehož asociace byla u mnohých autoimunitních nemocí prokázána [70]. Přehled o tomto genu ve vztahu k CD je podrobně popsán v naší publikaci 2.3 [71]. Dalším podobným kandidátním genem je *PTPN22*. I tímto genem jsme se v našich pracích zabývali – viz. publikace 2.1 [68]. V lokusu původně označeném jako IBD3, zahrnujícím geny komplexu MHC, leží také gen pro TNF-alfa. Analýzu jeho asociace jsme provedli v počátku naší práce. Stručný přehled věnovaný tomuto genu je popsán v úvodu přiložené publikace 2.1 [68]. Později publikovaná meta-analýza naznačuje, že jednotlivé varianty v genu *TNFA* jsou důležitější pro asijskou populaci, kde by varianta c.-1031T>C mohla mít mírný ochranný efekt při vzniku CD [72].

Éra celogenomových asociačních studií

V další fázi celosvětového výzkumu genetiky zánětlivých střevních onemocnění se hlavním nástrojem staly celogenomové asociační studie (genome–wide association study, GWAS). Hned pomocí první z nich se podařilo identifikovat varianty ve dvou genech zodpovědných za CD: *IL23R* a *ATG16L1*. Také těmito dvěma geny jsme se v naší práci zabývali a jejich přehled je uveden v publikaci 2.2 [73].

Od roku 2006 bylo v souvislosti s CD publikováno osm GWAS [74–82] a jedna meta-analýza [83]. Výsledky těchto prací jsou stručně shrnuty v tabulce 2. Upraveno dle [84].

Tabulka 2. Souhrn pomocí GWAS nalezených genů/lokusů a jejich další potvrzení.

Gen/lokus	Poprvé objevena asociace	Replikace asociace
TNSF15	[81]	[83, 85–88]
ATG16L1	[74]	[73, 76, 79, 80, 83, 89–108]
IL23R	[75]	[73, 76, 80, 82, 89–93, 95, 99, 105, 108–113]
IRGM	[76, 77]	[91, 93, 107, 114–116]
PTPN2	[76, 77]	[91]
NKX2-3	[76, 91]	[91, 114, 117]
MST1/BSN	[76, 77]	[82, 91, 93, 118]
IL12B	[76, 77]	[91, 93]
1q24	[76, 77]	[91, 93, 114]
10q21	[77]	[91, 93, 114, 117]
NCF4	[77]	[116]
5p13.1	[80]	[77, 78, 91, 114]
1q32	[83]	[93]
7p12	[83]	[93]
8q24	[83]	[93]
LRRK2/MUC19	[83]	[93]
TNFRSF6B	[90]	[92]
STAT3	[93]	[119, 120]
CDKAL1	[93]	[121]
JAK2	[93]	[120]
1q31	[76, 77]	Ne
FLJ45139	[76, 77]	Ne
NELL1	[78]	Ne
1q31	[76, 77]	Ne
FLJ45139	[76, 77]	Ne
NELL1	[78]	Ne
10p12	[122]	Ne
PHOX2B, FAM92B	[77]	Ne
PTPN22, *ITLN1,* 6q21, *CCR6,* 10p11, C11orf30, 13q14, *ORMDL3*, 21q21, *ICOSLG*, *ECM1*	[83]	Ne
21q22	[90]	Ne

Do stejné skupiny genů jako již dříve zmíněný *ATG16L1* patří i *IGRM* (immunity related GTPase related family, M). I tento gen se účastní autofagie. Na rozdíl od ATG16L1, který se účastní patogeneze CD tím, že reguluje Panethovy buňky [23], IGRM se podílí na autofagii intracelulárních baktérií [123]. Gen *BSN* (bassoon - presynaptic cytomatrix protein) kóduje protein, který je zapojen do organizace presynaptického cytoskeletu. Je exprimován především v neuronech mozku. Z těchto důvodů se jako pravděpodobnější kandidátní gen jeví *MST1* (macrophage stimulating 1) ležící v těsné blízkosti genu pro BSN. *MST1* kóduje makrofágy stimulující protein (MSP), jenž ovlivňuje schopnost pohybu a fagocytózy makrofágů [124]. Protein kódovaný *PTPN2* (Protein tyrosine phosphatase N2) je aktivovaný INF-gamma a současně omezuje INF- gamma indukovanou signalizaci, a tak může přispívat k porušení funkce střeva jako bariéry [125]. Gen *IL12B* (interleukin 12B) kóduje podjednotku p40, která je společná pro IL23 i IL12. Porucha *IL12B* tak může zasahovat do Th1 i Th17 reakce.

2 Závěr

2.1 Shrnutí publikovaných prací

Pohled na genetiku Crohnovy choroby se v posledních deseti letech zcela změnil. Postupně byly objevovány jednotlivé geny a později v nich také konkrétní varianty, které přispívají ke vzniku CD. Před rokem 2001 nebyl znám žádný gen podílející se na vzniku CD, dnes jich je (alespoň jednou potvrzeno) přes 20. Tempo, kterým přibývají, je dáno především změnou technik, které se používají při identifikaci SNP u polygenních onemocnění – místo vazebných studií se nyní používají GWAS. Tento projekt jsme zahájili v době, kdy byl znám jen jeden gen (*NOD2)* a jeden lokus (IBD5) podílející se na vzniku CD. V první práci **(příloha 1 [68])** jsme se proto soustředili na frekvence variant v genu *NOD2* a dále na geny, které byly podezírány na základě asociace s jinými imunitně podmíněnými chorobami. V rámci této práce jsme potvrdili asociaci tří variant v genu *NOD2*, přičemž nejsilněji asociována byla varianta 1007fs. Potvrdili jsme také asociaci s postižením terminálního ilea. Nalezli jsme rozdíl mezi dětmi a dospělými ve frekvenci této varianty a prokázali její vliv na věk manifestace. Zajímavým zjištěným byla vysoká prevalence (jak u českých pacientů, tak u českých kontrol) této varianty mezi evropskými populacemi, podobně jako v ostatních částech středoevropského regionu. Podařilo se nám upozornit na fakt, že hledání jednoduchého jiho-severního gradientu v rámci prevalence 1007fs varianty by bylo zjednodušující a zavádějící. V této práci jsme se v souvislosti s nástupem biologické léčby inhibitory TNF zabývali také asociací s genem *TNFA*. Frekvence této varianty se však mezi případy a kontrolami nelišily. Stejně tak jsme nenalezli asociaci s genem *PTPN22*.

Krátce po prvních GWAS jsme se začali věnovat genům *IL23R* a *ATG16L1* **(příloha 2 [73])**. Potvrzení asociace těchto dvou genů v české populaci jsme publikovali v roce 2009. Podařilo se nám nalézt dosud nepopsaný rozdíl v zastoupení variant *IL23R* u pacientů, kteří měli postižený horní gastrointestinální trakt.

Velmi podrobně jsme se zabývali také asociací s genem *CTLA4* **(příloha 3 [71]).** Prací o asociaci mezi CD a *CTLA4* bylo publikováno, vzhledem k předpokládanému patofyziologickému mechanismu, až překvapivě málo a většina jednoduchou asociaci nenalezla. Ani v rámci chorob, u kterých byl vliv tohoto genu jasně prokázán, není zřejmé, která varianta je za asociaci zodpovědná. Proto, kromě již testovaných variant, jsme volili takové, které se zdají být nejtěsněji vázané ke kauzální variantě u ostatních imunitně podmíněných onemocnění. Avšak ani v našem souboru nebyla nalezena asociace mezi *CTLA4* a CD. Jako prvním se nám ale podařilo najít interakci mezi efektem tohoto genu a genů *NOD2* a *IL23R*. Také jsme jako první popsali rozdíl ve frekvencích variant *NOD2* genu mezi dětmi a dospělými v kavkazské populaci a nalezli asociaci s ileokolickou formou onemocnění.

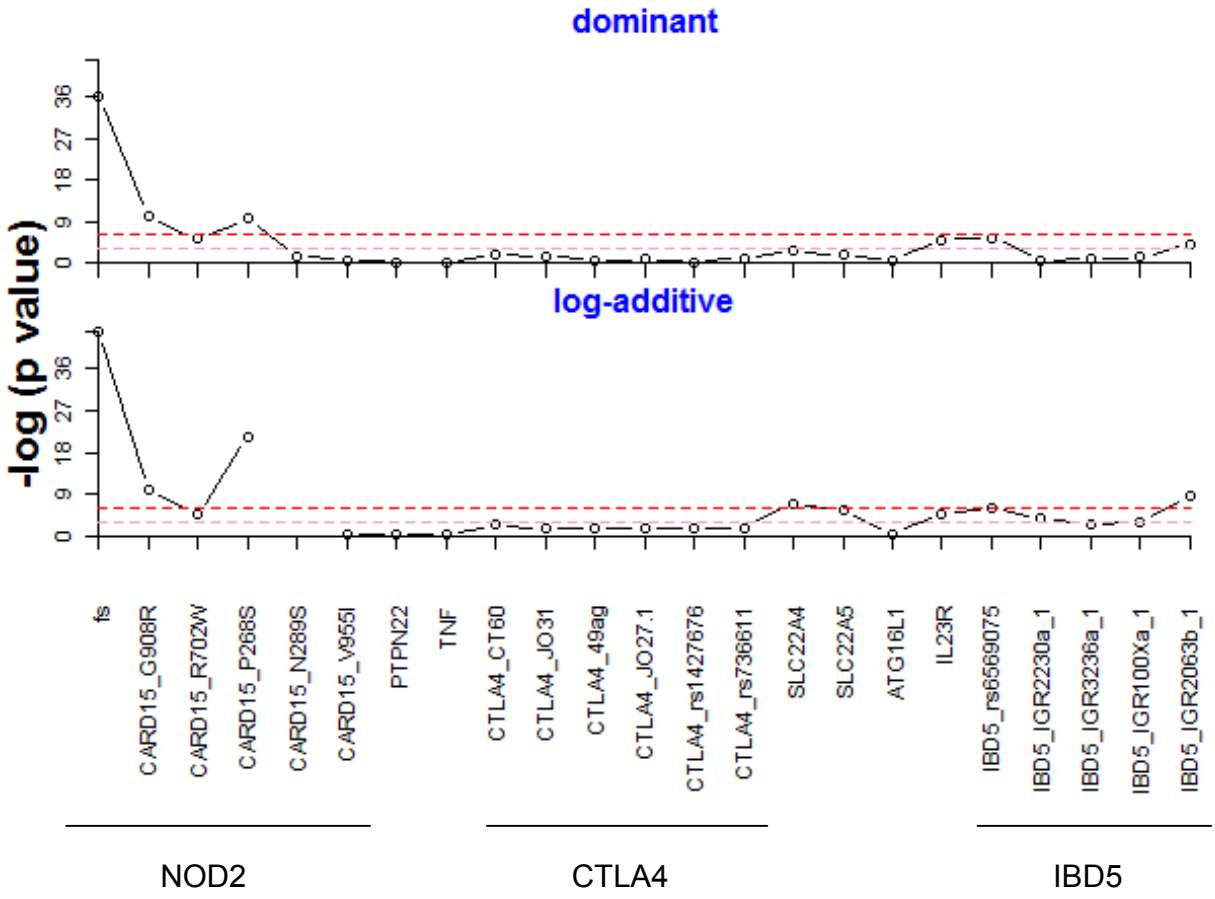
V práci publikované v *Inflammatory bowel disease* jsme se vrátili k již dávno testovanému IBD5 lokusu **(příloha 4 [69])**. Ač vazba na 5q31 patřila mezi vůbec první nalezené, stále nebylo jasné, která varianta v tomto složitém, velmi polymorfním úseku chromozomu je tou kauzální. Již sice byla publikována práce, která kauzální varianty identifikovala, pozdější studie toto ale nepotvrdily. Chtěli jsme tedy zjistit, jaké jsou asociace v české populaci a ověřit, zda asociace nalezené varianty v rámci GWAS skutečně reprezentuje asociaci dříve popsaného IBD5 haplotypu. V české populaci se nám podařilo prokázat, že na IBD5 existují dva nezávislé lokusy,

které jsou nezávisle asociované s CD a že SNP popsané v celogenomových asociačních studiích nejsou plně zodpovědné za asociaci dříve popsaného haplotypu IBD5 s CD. Potvrdili jsme také, že varianty označené za kauzální v české populaci nejsou asociovány nezávisle na rizikovém IBD5 haplotypu.

Připojili jsme se také k dánské skupině pod vedením profesorky Munkholmové v práci, zabývající se genetickými aspekty účinnosti a nutností kontinuálního podávání biologické léčby u dětí **(příloha 5 [126])**. V rámci zkoumaných variant v genech *TNF*, *CASP9*, *FASLG*, *LTA*, *NOD2* nebyla nalezena asociace s neúčinností či dependencí na infliximabu. Tato práce ukazuje, že 66% dětí se stává na infliximabu závislá, a není tedy u nich možné biologickou léčbu vysazovat. Za zevní nepříznivý faktor ve smyslu dependence bylo označeno perianální postižení. Genetické prediktory se identifikovat nepodařilo.

2.2 Použití získaných výsledků v klinické praxi

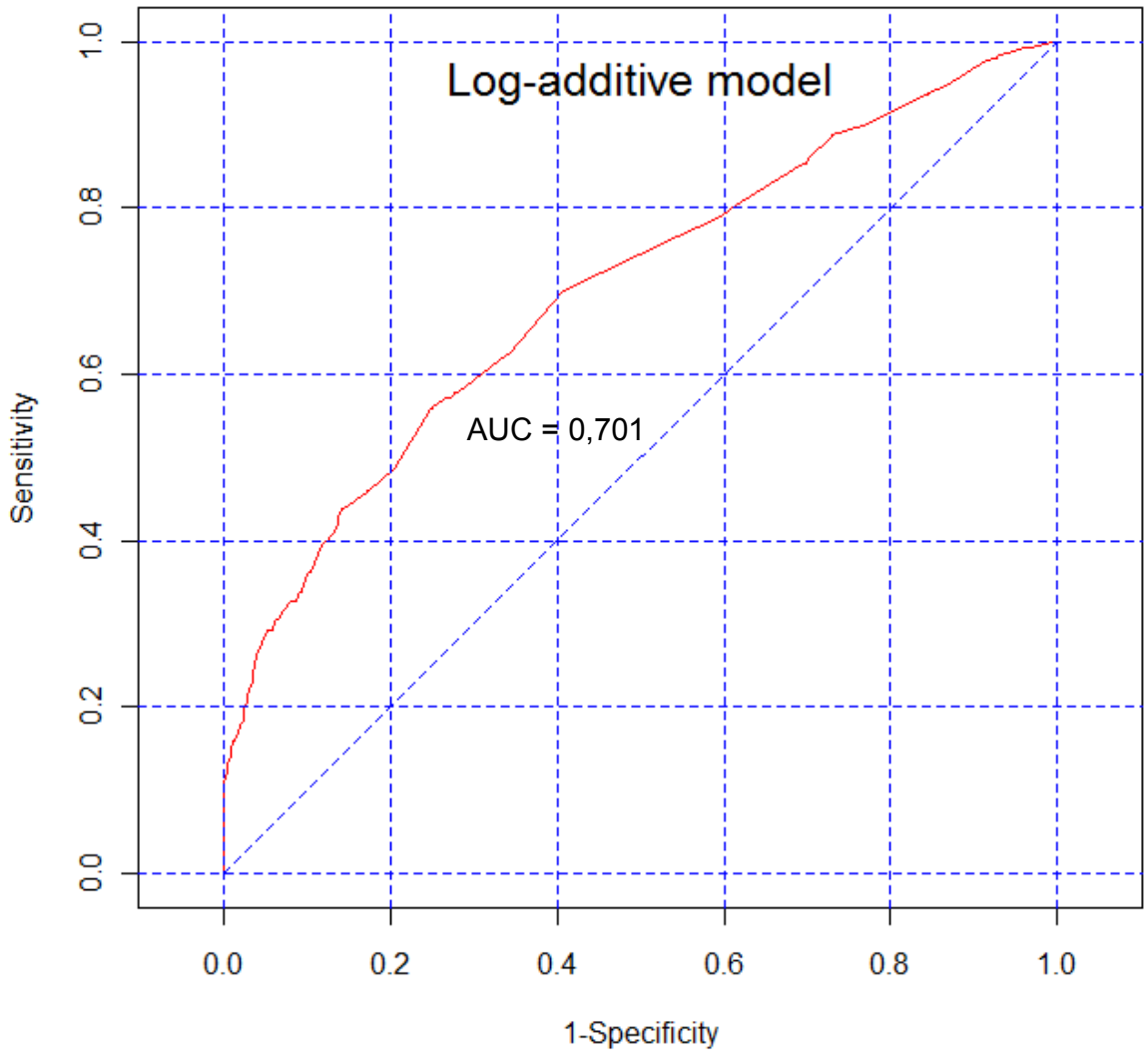
V rámci zde předkládaných publikací jsme zmapovali většinu nejdůležitějších kandidátních genů asociovaných s CD. Asociace jednotlivých variant je shrnuta na obrázku 3. Na obrázku 4 je zobrazena ROC křivka, která ukazuje sensitivitu a specificitu při použití jednotlivých variant k identifikaci CD na základě genetických faktorů. Ačkoli některé publikované práce naznačují, že genetické faktory je možné již rutině používat jako pomocnou metodu k diagnostice CD, z hodnot sensitivity a specificity je zřejmé, že síla těchto testů je stále nízká.



Obrázek 3. Asociace jednotlivých testovaných variant s CD v dominantním a log aditivním modelu (data získaná na našem souboru). Na ose x zobrazeny jednotlivé testované varinty, na ose y jejich signifikace v logaritmické stupnici. Z obrázku je patrné, že v české populaci jsou nejvýznamnějšími geny NOD2 a lokus IBD5.

Dalším polem, kde by se v brzké době mohlo uplatnit testování jednotlivých variant v klinické praxi, je identifikace vysoce rizikových pacientů a individuální úprava terapie. Například nyní intenzivně diskutovaný model „top-down“ terapie by byl indikován na základě modelu pracujícího se souborem rizikových a ochranných variant a dalších klinických a laboratorních

parametrů. Toto souvisí také s představou rozdělení IBD a tedy i CD na genetické podtypy s odlišnými terapeutickými postupy.



Obrázek 4. ROC křivka. Na ose x zobrazena senzitivita, na ose y specificita při použití kombinace jednotlivých testovaných variant.

Zatím teoretickou možností užití našich výsledků je identifikace rizikových pacientů před manifestací onemocnění. Pak by byl možný terapeutický zásah, který by nedovolil nebo by zbrzdil rozvoj tohoto onemocnění. Avšak i včasná identifikace pacienta by jistě vedla k včasné léčbě, u dětí tolik důležité a zabránila by rozvoji některých s růstem a vývojem souvisejících komplikací.

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4 Přílohy - vlastní publikace k tématu

Příloha 1

Hradsky, O., et al., *Variants of CARD15, TNFA and PTPN22 and susceptibility to Crohn's disease in the Czech population: high frequency of the CARD15 1007fs.* Tissue Antigens, 2008. **71**(6): p. 538-47. IF (2008) = 2,076.

Příloha 2

Dusatkova, P., et al., *Association of IL23R p.381Gln and ATG16L1 p.197Ala With Crohn Disease in the Czech Population.* J Pediatr Gastroenterol Nutr, 2009. IF (2009) = 2,183.

Příloha 3

Hradsky, O., et al., *The CTLA4 variants may interact with the IL23R- and NOD2-conferred risk in development of Crohn's disease.* BMC Med Genet. **11**: p. 91. IF (2009) = 2,840.

Příloha 4

Hradsky, O., et al., *Two independent genetic factors responsible for the associations of the IBD5 locus with Crohn's disease in the Czech population.* Inflamm Bowel Dis. 2010 Nov 8. [Epub ahead of print]. IF (2009) = 4,643.

Příloha 5

Duricova, D., et al., *Infliximab dependency in children with Crohn's disease.* Aliment Pharmacol Ther, 2009. **29**(7): p. 792-9. IF (2009) = 4,357.

Tissue Antigens ISSN 0001-2815

Variants of *CARD15*, *TNFA* and *PTPN22* and susceptibility to Crohn's disease in the Czech population: high frequency of the *CARD15* 1007fs

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Key words
age at onset; clinical characteristics; genetic association; *TNFA*

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Received 16 October 2007; revised 13 January 2008, 21 February 2008; accepted 16 March 2008

doi: 10.1111/j.1399-0039.2008.01047.x

Abstract

Crohn's disease (CD) has been shown to be associated with the variants in the *CARD15* gene as well as in other genes involved in the immune response. The frequencies of the variants profoundly differ among populations and so does the associated risk. We examined the associations of variants in the *CARD15*, *TNFA* and *PTPN22* genes with pediatric-onset and adult-onset CD in the Czech population. Genotype, phenotype and allelic frequencies were compared between 345 patients with CD (136 pediatric-onset and 209 adult-onset patients) and 501 unrelated healthy controls. At least one minor allele of the *CARD15* gene was carried by 46% patients and only 21% control subjects (OR = 3.2, 95% CI 2.4–4.4). In a multiple logistic regression model, the strongest association with CD was found for the 1007fs variant (OR = 4.6, 95% CI 3.0–7.0), followed by p.G908R (OR = 2.9, 95% CI 1.5–5.7) and p.R702W (OR = 1.7, 95% CI 1.0–2.9), while no independent association was found for the remaining variants in the *CARD15* gene (p.268S, p.955I and p.289S), for the p.R620W variant in the *PTPN22* gene or for the g.−308G>A variant in the *TNFA* gene. The age at CD onset was strongly modified by positivity for the 1007fs allele: it was present in 42% pediatric-onset and only 25% adult-onset patients. In conclusion, we report a high frequency of the minor allele of the *CARD15* 1007fs polymorphism in the Czech population and a strong effect of this allele on the age at disease onset.

Introduction

Crohn's disease (CD) is one of the two common forms of inflammatory bowel disease (IBD). The disease results from the action of environmental factors in genetically susceptible individuals: the genetic susceptibility is determined by polymorphisms in the *CARD15* gene, as well as other candidate genes, including the ones newly shown by the genome-wide association studies (1, 2).

The *CARD15* gene encodes a nucleotide-binding oligomerization domain containing 2 (NOD2) protein, which recognizes muramyl dipeptide, a component of bacterial wall, and thus plays an important role in the innate immunity (3). It was first described by Hugot et al. (4) who identified a locus linked with CD on chromosome 16, which was then designated *IBD1*. Since then, the *CARD15* gene has been characterized in this locus and its three variants (1007fs, p.G809R and p.R702W) have been shown to account for the association with both pediatric-onset and adult-onset CD (5–7), as well as with its subtypes (8–12), or with its complications (12–14).

Three main theories have been proposed to explain why variants in *CARD15* gene contribute to development of CD [reviewed in (15)]. The first postulates that peptidoglycan (PGN) derived from intestinal microflora is recognized by

toll-like receptor 2 (TLR2) and also NOD2 protein. When *CARD15* is mutant, PGN-mediated nuclear factor-κB (NF-κB) activation is not negatively regulated trough TLR2. This leads to a clonal expansion of pathogenic Th1 cells. According to the second theory, muramyl dipeptide derived from bacteria in bowel activates NOD2 in the Paneth cells. When NOD2 is intact, secretion of α-defensins is induced; these antimicrobial peptides downregulate the population of commensal bacteria in the lumen. Consequently, when NOD2 is deficient, the lack of α-defensin production leads to a bacterial overgrowth and an immunological response to microflora. According to the third theory, muramyl dipeptide is recognized in antigen-presenting cells by the NOD2 protein. In this model, mutations in CARD15 lead to gain of function and subsequently to hyperactivation of NF-κB.

Other susceptibility genes than *CARD15* were searched for in later studies. Genome-wide linkage analyses identified several additional loci (16), including the *IBD3* on chromosome 6p21. This locus corresponds to the human leukocyte antigen (HLA) region that includes the *TNFA* gene frequently studied in CD. The *TNFA* gene encodes for tumor necrosis factor-α, a potent proinflammatory cytokine. Its g.−308A variant has been shown to be associated with higher promoter activity compared with the g.−308G variant (17) and therefore seems to be a plausible candidate for susceptibility to autoimmune diseases. Another general autoimmunity gene is the protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) that encodes a lymphoid-specific phosphatase (Lyp), one of the powerful downregulator of the T-cell response. There is very strong evidence that a missense substitution g.1858C>T encoding for amino acid substitution p.R620W is associated with a risk of multiple autoimmune diseases (18), but so far, it seems that this does not hold for CD (19–22).

The aim of the study was to examine the associations of candidate variants in the *CARD15*, *TNFA* and *PTPN22* genes in pediatric-onset and adult-onset CD in the Czech population and to find differences in the association between these two groups.

Subjects and methods

Subjects

In a case–control design, we studied 345 Czech-born CD patients and compared them with 501 unrelated healthy Czech control subjects. All subjects were of European Caucasian ancestry.

The patients were recruited at two institutions: the pediatric patients with CD were recruited at the Department of Pediatrics, University Hospital Motol, Prague, having been diagnosed before or at the age of 18 years according to the Porto criteria (23); the patients with adult-onset CD were recruited at the 4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague, having been diagnosed at or over the age of 19 years, using the standard clinical, radiological, endoscopic and histological criteria (24). The recruitment covered all patients who were cared of by the two institutions in October 2005; newly manifested patients were consecutively added until October 2007. There were 136 pediatric-onset and 209 adult-onset patients, and their clinical characteristics are detailed in Table 1.

The 501 controls included 296 unrelated healthy children (208 boys and 88 girls) consecutively recruited from patients who underwent minor surgical interventions at the Department of Pediatric Surgery of the University Hospital Motol, Prague, and 205 adult healthy volunteers (115 males and 90 females). The median age at sampling was 8 years in children and 38 years in adult volunteers. Subjects with any chronic medication, abnormal basic biochemical workup, increased erythrocyte sedimentation rate or abnormal electrocardiogram were carefully excluded.

Table 1 Demographic and clinical characteristics of patients[a]

	Total ($n = 345$)	Pediatric-onset CD ($n = 136$)	Adult-onset CD ($n = 209$)
Sex, male/female	154/191	71/65	83/126
Age at diagnosis, median (interquartile range)	21 (14–30)	13.5 (12–16)	28 (23–36)
A1 (<17 years)	108 (31%)	108 (79%)	0 (0%)
A2 (17–40 years)	201 (58%)	28 (21%)	173 (83%)
A3 (>40 years)	36 (10%)	0 (0%)	36 (17%)
Disease duration, median (interquartile range)	5 (2–10)	4 (2–8)	6 (3–11)
Localization			
L1 (terminal ileum)	73 (22%)	24 (18%)	49 (25%)
L2 (colon)	54 (16%)	15 (11%)	39 (20%)
L3 (ileocolon)	205 (62%)	96 (71%)	109 (55%)
L1–3 (not determined)	13	1	12
Upper GI	57 (17%)	21 (15%)	36 (17%)
Disease behavior			
B1 (nonstricturing / nonpenetrating)	130 (40%)	71 (55%)	59 (30%)
B2 (stricturing)	132 (40%)	34 (26%)	98 (49%)
B3 (penetrating)	66 (20%)	25 (19%)	41 (21%)
B1–3 (not determined)	17	6	11
B4 (perianal disease)	114 (33%)	35 (26%)	79 (38%)
Extraintestinal manifestation[b]	49 (14%)	16 (12%)	33 (16%)
Need for surgery[c]	177 (51%)	43 (32%)	134 (64%)

CD, Crohn's disease; GI, gastrointestinal.

[a] The clinical characteristics are given according to the Montreal classification (26).

[b] Extraintestinal manifestation: peripheral arthritis, ankylosing spondylitis, sacroiliitis, episcleritis and iritis, erythema nodosum, pyoderma gangrenosum and sclerosing cholangitis.

[c] Abdominal surgery for complication of CD (resection).

Ethical considerations

The study was approved by the institutional ethics committees, and a written informed consent was obtained from all participants or their guardians.

Determination of genotypes

Genomic DNA was extracted from peripheral blood with a routine salting-out method (95% samples) or from saliva (5% samples). Saliva was collected using Oragene DNA Self-Collection Kit according the manufacturer's protocol (DNA Genotek Inc., Ottawa, Ontario, Canada). The success rate for genotyping of DNA extracted from saliva was identical to that extracted from blood: 97% samples were successfully called in the first amplification run.

We analyzed six single nucleotide polymorphisms (SNPs) in the *CARD15* gene – 1007fs (rs5743293), p.G908R (rs2066845), p.R702W (rs2066844), p.P268S (rs2066842), p.N289S (rs5743271) and p.V955I (rs5743291) – one SNP in the *PTPN22* gene – p.R620W (rs2476601) – and one SNP within *TNF* region – g.−308G>A (rs1800629). The genotypes of the SNPs were determined using the TaqMan SNP genotyping assays. The 10 μl polymerase chain reaction (PCR) mixture contained a 0.5× to 1× mix of primers and the probe (TaqMan SNP Genotyping Assay; Applied Biosystems, Foster City, CA) and contained either 1× TaqMan Universal PCR Master Mix (Applied Biosystems) or a mixture of 1× PCR buffer, 4 mM $MgCl_2$, 10% glycerol, 150 μM each deoxyribonucleotide triphosphate (Sigma–Aldrich, Munich, Germany), 1 μM 6-carboxy-X-rhodamin (Molecular Probes/Invitrogen, Carlsbad, CA) and 0.2 unit HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany). PCRs were performed on an ABI 7300 Machine (Applied Biosystems). The end point readings were evaluated according to the manufacturer's instructions. For better discrimination of alleles, some run files were also inspected. To ensure consistency between runs, samples of known genotypes were repeated in every analysis.

Statistical analysis

Allelic frequencies were determined by gene counting. The Hardy–Weinberg equilibrium was checked by comparing observed with expected genotype frequencies and tested using exact tests. Association of particular SNP with the CD was expressed as odds ratio with 95% confidence interval and tested by chi-squared test with continuity correction and by Fisher's exact test. Haplotype analysis was performed by estimating the haplotype frequencies using an expectation–maximization algorithm. The association of the variants with the condition, including potential interactions, was further examined in a multivariate logistic model. Logistic regression was employed also to analyze the association between clinical phenotypes (outcomes) and three genetic predictors (positivity for *CARD15* 1007fs, p.G908R and p.R702W). The regression was performed first with the three predictors adjusted for the disease duration, then adjusted also for age at diagnosis and for sex. The population attributable risk (PAR) was calculated under the assumption of rare occurrence of the disease (25). Analyses were performed using the Stata package version 9.2 (Stata Corp., College Station, TX).

Results

Genotype, phenotype and allelic frequencies in patients and control subjects

The frequencies of the variants are listed in Table 2. The minor alleles of 1007fs, p.G908R and p.P268S variants in the *CARD15* gene were significantly associated with CD. The strongest association was found for the minor allele at 1007fs with a phenotypic frequency (carriage rate) of 32% in patients and 10% in controls (OR = 4.2, 95% CI 2.9–6.2 in univariate analysis). Homozygosity for the 1007fs greatly increased the CD risk, as we observed 8.4% homozygotes for the 1007fs allele among patients but no one among control subjects. Association was significant also for the p.G908R and p.P268S, while the difference between cases and controls in the p.R702W did not reach statistical significance in the univariate analysis. No association with CD was found for the minor alleles at the p.V955I and p.N289S in the *CARD15* gene, at the p.R620W variant in the *PTPN22* gene or at the g.−308G>A variant in the *TNFA* gene.

Presence of minor alleles of any of the 1007fs, p.G908R or p.R702W variants conferred a significant risk of CD: at least one such allele was carried by 46% patients and only 21% controls (OR = 3.2, 95% CI 2.4–4.4). The risk was strongly dose dependent, and we observed an increase in risk from OR = 2.3 (95% CI 1.7–3.2) for one minor allele to OR = 17 (95% CI 7.1–40) for two or more minor alleles. The PAR for carriage of one or more of the three CARD15 mutations was 31%, while for the carriage of 1007fs alone it was 24%.

Linkage disequilibrium and haplotype frequencies of 1007fs, p.G908R, p.R702W and p.P268S

There was a significant linkage disequilibrium among some of the studied variants (for its parameters, see Figure 1). We carried out a haplotype analysis: reconstructing the haplotypes of four variants that associated with CD in the univariate analyses at a *P* value less than 0.10. The estimated frequencies of the reconstructed haplotypes are shown in Table 3. The wild-type haplotype consisting of wild-type alleles at 1007fs, p.G908R, p.R702W and p.P268S was present on 56% of chromosomes in patients with CD and on 72% of chromosomes in control subject. The risk associated with the haplotypes was expressed relative to the baseline of

Table 2 Frequencies of the genotypes, and risk associated with phenotypic and allelic positivity of the variants[a]

Polymorphism	Genotype frequencies (n_{cases} = 345, $n_{controls}$ = 501)			Phenotypic frequencies and associated risk[b]	Allelic frequencies and associated risk[c]
CARD15					
1007fs	−/−	−/C	C/C	Phenotype C+	Allele C
Cases	235 (68%)	81 (23%)	29 (8.4%)	110 (32%)	139 (20%)
Controls	451 (90%)	50 (10%)	0	50 (10%)	50 (5.0%)
OR (95% CI)				4.2 (2.9–6.2)	4.8 (3.4–6.9)
p.G908R	G/G	G/R	R/R	Phenotype R+	Allele R
Cases	317 (92%)	24 (7.0%)	4 (1.1%)	28 (8.1%)	32 (4.6%)
Controls	485 (97%)	15 (3.0%)	1 (0.2%)	16 (3.2%)	17 (1.7%)
OR (95% CI)				2.7 (1.4–5.4)	2.8 (1.5–5.5)
p.R702W	R/R	R/W	W/W	Phenotype W+	Allele W
Cases	304 (88%)	40 (12%)	1 (0.3%)	41 (12%)	42 (6.1%)
Controls	459 (92%)	42 (8.4%)	0	42 (8.4%)	42 (4.2%)
OR (95% CI)				1.5 (0.91–2.4)	1.5 (0.93–2.4)
p.P268S	P/P	P/S	S/S	Phenotype S+	Allele S
Cases	128 (37%)	134 (39%)	83 (24%)	217 (63%)	300 (43%)
Controls	256 (51%)	209 (42%)	36 (7.2%)	245 (49%)	281 (28%)
OR (95% CI)				1.8 (1.3–2.4)	2.0 (1.6–2.4)
p.V955I	V/V	V/I	I/I	Phenotype I+	Allele I
Cases	308 (89%)	36 (10%)	1 (0.3%)	37 (11%)	38 (5.5%)
Controls	437 (87%)	63 (13%)	1 (0.2%)	64 (13%)	65 (6.5%)
OR (95% CI)				0.82 (0.52–1.3)	0.84 (0.54–1.3)
p.N289S	N/N	N/S	S/S	Phenotype S+	Allele S
Cases	340 (99%)	5 (1.5%)	0	5 (1.5%)	5 (0.72%)
Controls	491 (98%)	10 (2.0%)	0	10 (2.0%)	10 (1.0%)
OR (95% CI)				0.72 (0.19–2.3)	0.72 (0.19–2.3)
PTPN22					
R620W	R/R	R/W	W/W	Phenotype W+	Allele W
Cases	275 (80%)	66 (19%)	4 (1.2%)	70 (20%)	74 (11%)
Controls	398 (79%)	100 (20%)	3 (0.6%)	103 (21%)	106 (11%)
OR (95% CI)				1.0 (0.69–1.4)	1.0 (0.73–1.4)
TNFA					
g.−308G>A	G/G	G/A	A/A	Phenotype A+	Allele A
Cases	262 (76%)	76 (22%)	7 (2.0%)	83 (24%)	90 (13%)
Controls	381 (76%)	110 (22%)	10 (2.0%)	120 (24%)	130 (13%)
OR (95% CI)				1.0 (0.72–1.4)	1.0 (0.74–1.4)

[a] Genotypic and phenotypic frequencies are calculated from the number of subjects (345 cases and 501 controls), and allelic frequencies from the number of chromosomes (690 in cases and 1002 in controls). The genotype distributions in controls were examined for the Hardy–Weinberg equilibrium and neither of the polymorphisms significantly deviated ($P > 0.10$ in exact tests).

[b] The phenotypic frequency (carriage rate) of the minor allele, with the respective risk.

[c] The allelic frequency of the minor allele, with the respective risk.

this all-wild-type haplotype. Five additional haplotypes were present in cases or control subjects, with a frequency above 0.5%. The highest risk (OR = 4.9, 95% CI 3.4–7.1) was conferred by the '1007fs – 908G – 702R – 268S' haplotype, carried on 19% patient chromosomes and 4.9% control chromosomes. The risk of CD was conferred only by haplotypes containing the 1007fs, 908R or 702W alleles, while the risk of the haplotype was not further modified by presence of the 268S allele.

Multiple logistic regression models

A stepup building of a multiple regression model where the outcome was the presence of CD and the predictors were variants associated with CD in univariate analyses with a $P < 0.10$ showed that three of the *CARD15* variants were independently associated with CD: the strongest association was found for the 1007fs variant (OR = 4.6, 95% CI 3.0–7.0), followed by p.G908R (OR = 2.9, 95% CI 1.5–5.7) and p.R702W (OR = 1.7, 95% CI 1.0–2.9). There was no independent association of the p.P268S variant.

Genotype–phenotype analysis

Using a case-only design, we tested whether the phenotypic characteristics of the patients are dependent on carriage status of the minor alleles at CARD15 1007fs, p.G908R or p.R702W.

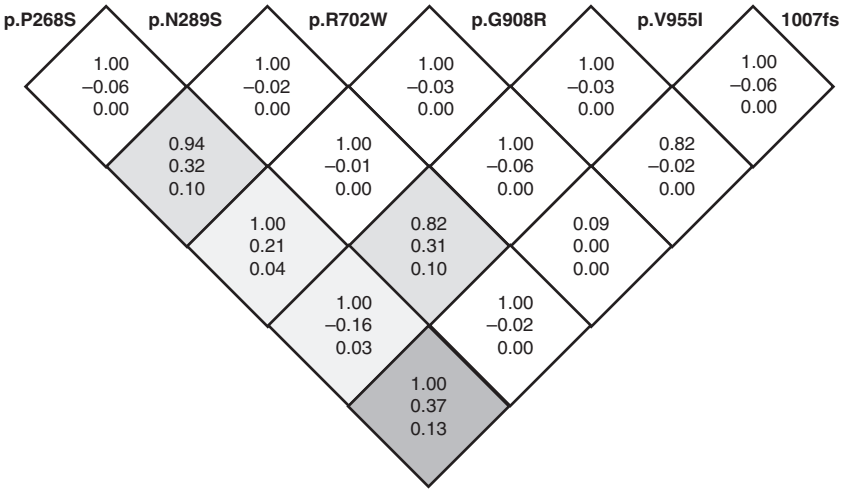

Figure 1 Linkage disequilibrium between the *CARD15* variants in the Czech population. Values are D', r and r^2. Grayscale according to the values of r^2.

Figure 2 shows the distribution of the patients by the age at diagnosis of CD, drawn separately by the phenotypic positivity (carrier status) of the 1007fs variant. Patients carrying 1007fs were diagnosed significantly earlier than those without this variant ($P = 0.021$, Mann–Whitney rank sum test). Consequently, we analyzed our data similarly to other studies that divided patients into pediatric-onset and adult-onset groups. The frequency of 1007fs carriers profoundly differed between pediatric-onset patients (42%) and adult-onset patients (25%), $P = 0.001$. Frequencies of phenotypic positivity (carriage rates) of other tested variants in pediatric-onset and adult-onset patients are listed in Table 4. In addition, stratification of patients into groups according to the Montreal Classification (A1, A2 and A3) (26) and application of linear regression showed that the carriers of 1007fs variant developed CD earlier than the others ($P = 0.008$). No difference between pediatric-onset and adult-onset group was found for additional variants in the *CARD15* gene when adjusted for the effect of the 1007fs, and the same applied also to the p.R620W variant in the *PTPN22* gene and the g.−308G>A variant in the *TNFA* gene.

Table 5 shows logistic regression with the outcomes of clinical characteristics other than the age at diagnosis and the three primarily associated variants in *CARD15* as the predictors. The ileal involvement was associated with positivity for *CARD15* 1007fs both in the pediatric-onset and in the adult-onset patients. We observed also a tendency toward a protective effect of *CARD15* 1007fs on the likelihood of perianal disease, which is, however, not significant after being corrected for the number of independently performed tests. The *CARD15* variants were not associated with any of the other tested clinical characteristics: localization in the upper gastrointestinal tract, the stricturing or penetrating behavior of the disease, extraintestinal manifestation or the need for abdominal surgery.

Discussion

Numerous studies have investigated the genetic association of CD with the *CARD15* gene and have shown that the magnitude of the risk profoundly differs among various populations (27). Here, we report a study on the genetic risk

Table 3 Haplotype analysis: the frequencies in patients and control subjects and the risk associated with the haplotype[a]

Composition of the haplotype	Patients with CD (690 chromosomes)	Healthy controls (1002 chromosomes)	OR (95% CI)
fs '-' - 908G - 702R - 268P	383 (56%)	720 (72%)	1.00 (reference)
fs '-' - 908G - 702R - 268**S**	101 (15%)	175 (18%)	1.1 (0.82–1.4)
fs '-' - 908G - 702**W** - 268P	1 (0.1%)	1 (0.1%)	
fs '-' - 908G - 702**W** - 268**S**	38 (5.5%)	39 (3.9%)	1.8 (1.1–3.0)
fs '-' - 908**R** - 702R - 268**S**	29 (4.2%)	17 (1.7%)	3.2 (1.7–6.3)
fs 'C' - 908G - 702R - 268P	5 (0.7%)	0 (0.0%)	*
fs 'C' - 908G - 702R - 268**S**	128 (19%)	49 (4.9%)	4.9 (3.4–7.1)
fs 'C' - 908G - 702**W** - 268**S**	3 (0.4%)	1 (0.1%)	

* indicates that the excess in cases was significant ($P = 0.0052$ in Fisher's exact test).

[a] Risk was calculated for haplotypes whose frequencies exceeded 0.5% both in cases and in controls and expressed relative to the haplotype composed of wild-type alleles.

Minor alleles printed in bold.

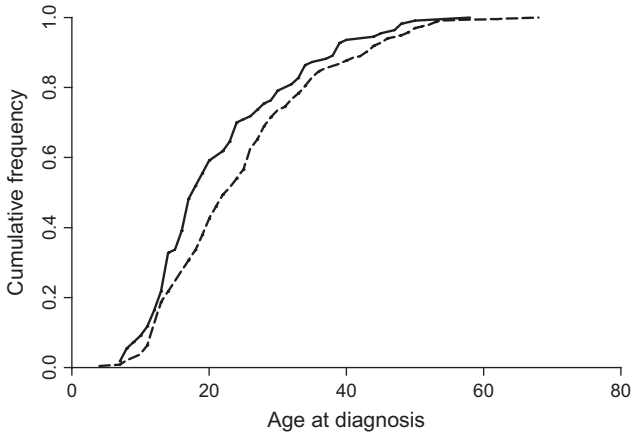

Figure 2 The *CARD15* 1007fs influences the age at disease onset. Cumulative frequency of patients by age at diagnosis, drawn separately for patients positive (full line) and negative (dashed line) for the *CARD15* 1007fs variant.

in a large group of Czech patients with both pediatric-onset and adult-onset CD.

Risk conferred by the CARD15 variants

An independent association with CD in the Czech population was confirmed for three variants in the *CARD15* gene: 1007fs, p.G908R and p.R702W; the effect of the latter being observable only after adjusting for the former two variants. Although patients and controls differed also in distribution of the P268S variant, both the haplotype analysis and the multiple logistic regression model confirmed that the variant is just a passive 'hitchhiker' on risk haplotypes, as has been well documented by other studies (12, 28).

Table 4 Differences between pediatric-onset and adult-onset patients: the phenotypic frequencies (carriage rates) of the minor alleles[a]

Frequency of the minor variant	Patients with pediatric-onset disease (%)	Patients with adult-onset disease (%)	*P* value
CARD15			
1007fs	42	25	0.001[a]
p.G908R	10	6.7	0.23
p.R702W	11	12	0.74
p.P268S	68	59	0.11
p.V955I	11	11	1.00
p.N289S	0.74	1.9	0.65
PTPN22			
p.R620W	19	21	0.68
TNFA			
g.−308G>A	28	22	0.19

[a] OR = 2.1, 95% CI 1.2–3.7 in a logistic regression case-only model (i.e. with CD patients only), where the outcome was 1 for pediatric-onset disease and 0 for adult-onset disease. The model was adjusted for other tested variants in the *CARD15* gene.

The allelic frequency of the 1007fs variant in the Czech population is 20% in CD patients and 5.0% in control subjects (OR = 4.8, 95% CI 3.4–6.9). To our knowledge, we report one of the largest proportions both in the case and in the control group (29). Indeed, a significant heterogeneity in the frequencies of the *CARD15* variants has been observed over Europe (Figure 3). In Northern European countries, the frequencies of the minor allele at *CARD15* 1007fs tend to be lower [CD *vs* controls – the Norwegians: 2.7–3.0% *vs* 1.2–1.5% (8, 30), the Finns 4.8% *vs* 1.7% (31), the Irish 3.0% *vs* 0.9% (32) and the Scots 4.6% *vs* 2.1% (32)], as compared with the Southern European populations who exhibit higher frequencies [CD *vs* controls – the Greeks: 27% *vs* 5.0% (33), the Italians: 6.3–9.3% *vs* 0.7–2.3% (34, 35), the Spanish: 4.5–14% *vs* 1–4.3% (9, 36, 37) and the Portuguese: 6.8% *vs* 1.6% (38)]. However, there is no simple gradient, as high frequencies have been observed also in Central Europe [CD *vs* controls – the Germans: 12.2–16.2% *vs* 2.1–4.3% (30, 39) and the Hungarians: 10.8–10.9% *vs* 2.2–2.5% (40, 41)] and in England [9.4% *vs* 1.6% (11)]. Consequently, there is no consistent trend in the risk conferred by *CARD15*, and although the frequencies of the minor allele of the 1007fs are very high both in the Czech patients and in the control subjects, the calculated OR of 4.2 (95% CI 2.9–6.2) is indeed almost identical to a value of 4.1 (95% CI 3.2–5.2), published in a meta-analysis of 37 studies from the Caucasian populations (27). The PAR of 24% for carriage of 1007fs in the Czechs ranks also high among the studies, being comparable with 17% and 18% observed in Germany (30, 39), but still lower than the PAR of 40% recently estimated from a medium-sized dataset in Greece (33).

It is apparent that large variations in the allele frequencies exist over Europe, although part of these variations may be attributable to selection of cases in university referral centers and to different distributions of the age at diagnosis. In type 1 diabetes, another immune-mediated disease, a model has been proposed where similar differences in the general population frequency of the highest risk genotype (HLA-DQ2/DQ8) partly explain the profound geographic variations in incidence of the disease (42). It, however, seems that no similar model works for CD, as recently shown by Hugot et al. (29).

Age at diagnosis

It has been postulated that the genetic factors play more important role in pediatric-onset patients than in adult-onset patients. However, results of multiple reports are inconsistent, partly because of differences in the methodology and genuine differences among population. Our data show that the 1007fs minor allele is associated with an earlier disease onset (Figure 2); this is well seen both when analyzing the data by rank-based methods and when

Table 5 Genotype–phenotype analysis[a]

	Predictors		
Outcome	*CARD15* 1007fs	*CARD15* p.G908R	*CARD15* p.R702W
Localization			
Any ileal involvement	5.2 (2.1–13)[b]	0.64 (0.24–1.7)	3.3 (0.96–11)
in pediatric-onset patients	3.8 (1.0–14)	0.72 (0.14–3.8)	NA[c]
in adult-onset patients	5.5 (1.6–19)	0.59 (0.16–2.1)	2.2 (0.62–8.1)
Upper GI involvement	1.1 (0.58–2.0)	0.84 (0.28–2.5)	0.55 (0.19–1.6)
Disease behavior			
B2 (stricturing), yes/no	1.14 (0.70–1.9)	0.61 (0.26–1.5)	1.3 (0.66–2.7)
B3 (penetrating), yes/no	1.1 (0.63–2.0)	2.1 (0.92–5.0)	0.75 (0.30–1.9)
Perianal disease, yes/no	0.56 (0.33–0.93)[d]	0.80 (0.34–1.9)	0.59 (0.27–1.3)
Extraintestinal manifestation	0.90 (0.46–1.7)	1.3 (0.45–3.5)	0.28 (0.07–1.2)
Need for surgery	1.0 (0.61–1.7)	1.1 (0.46–2.6)	1.1 (0.53–2.3)

GI, gastrointestinal.

[a] The data were analyzed using logistic regression, with the clinical phenotype as an outcome and positivity for *CARD15* 1007fs, p.G908R and p.R702W as predictors; the models are adjusted for the disease duration. Other outcomes than ileal involvement did not differ in their association among pediatric-onset and adult-onset groups; data are therefore not shown.

[b] OR adjusted also for age and sex is 5.0 (95% CI 2.0–12).

[c] Not analyzed, as neither pediatric-onset patient without ileal involvement carries the p.702W allele.

[d] $P > 0.05$ after correction for the number of tests performed.

choosing 18 years of age as a borderline between pediatric-onset and adult-onset disease.

In several studies, the risk effect of the three *CARD15* variants was found when mean age at diagnosis was compared, although this may often be incorrect because of the nature of the distribution of the age at onset. The effect was found in presence of two minor alleles (12), 1007fs minor allele (11) or at least one minor allele (43). Significant differences were found also when patients had been divided into age groups, and frequencies of these variants were compared, despite that individual investigators used different age limits: 40 years (according to Vienna classification) (37), 20 years (44) or quartiles (32).

However, the effect of the 1007fs is not universal, as documented by several well-powered studies that failed to detect its association with age. In Finland, the low frequency of the variants among cases precluded significant finding in otherwise a suitably sized case group (31). Tomer et al. failed to detect an effect on age within a group of pediatric-onset patients from the United States (45), and Abreu et al. did not detect an effect on age in a group of 201 U.S. Caucasian patients with a high proportion of Jewish ancestry (14), similarly as in two further studies in Jewish patients from Israel (46, 47).

Consequently, the minor allele frequency of 1007fs should vary more strongly among pediatric-onset groups from various populations than among adult-onset groups. Indeed, the published allelic frequencies of the 1007fs variant range from 26% in German pediatric-onset patients (7) to 1.7% in Swedish pediatric-onset patients (48). The frequency in our pediatric-onset group reached 42%, but meaningful comparison with pediatric populations from neighboring countries other than Germany is impossible because of low number of pediatric-onset patients in published studies.

Other clinical characteristics

We confirmed association between the ileal involvement and the positivity for *CARD15* 1007fs in both pediatric-onset

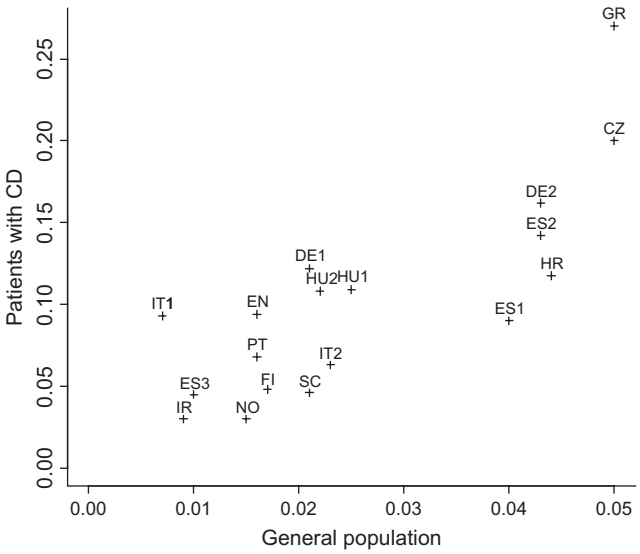

Figure 3 Profound differences among European populations in the frequency of the 1007fs minor variant. Frequencies are plotted for patients with CD and control subjects from various populations. Countries: NO, Norway (30); FI, Finland (31); SC, Scotland (32); IR, Ireland (32); EN, England (11); IT1, Italy (35); IT2, Italy (34); ES1, Spain (37); ES2, Spain (9); ES3, Spain (36); PT, Portugal (38); DE1, Germany (39); DE2, Germany (30); HU1, Hungary (40); HU2, Hungary (41); HR, Croatia (44); GR, Greece (33); CZ, Czech (this study).

and adult-onset patients, as has been repeatedly shown before (27, 49–51). This effect was marginally more pronounced in the adult-onset group. The risk of stricturing or penetrating forms was not increased by the presence of the minor variants within *CARD15*, which association has been reported in some (14, 52) but not other studies (7, 51).

There are several studies suggesting a significant contribution of *CARD15* variants to the need for abdominal surgery (9, 31, 41, 50). In contrast to these reports, surgery was not predicted by any of the *CARD15* polymorphisms in our dataset, similarly to other reports (12, 43, 51, 53). As Vermeire et al. (54) recently suggested, future studies should verify whether an earlier indication of infliximab in therapeutic practice might decrease the need for abdominal surgery. If so, such an association should attenuate over time.

Lack of association with TNFA g.−308G>A and PTPN22 p.R620W

In the present study, there was no difference in the *TNFA* *g.*−308G>A distribution between cases and control subjects, nor the frequencies differed between pediatric-onset and adult-onset patients. As the genotype distribution was almost identical in cases and control subjects, the g.−308G>A within *TNFA* promoter region is very unlikely to play an important role in the etiology of CD in our population. The study would nevertheless be capable of detecting a putative association at an OR $\geq$ 1.6 with a power of 80%. Within the *IBD3* locus, the first candidate was the classic HLA. Indeed, several weak associations of CD or its subphenotypes were found with HLA-DRB1*07 or other alleles (HLA-DRB1*0103, HLA-DRB3*0301, HLA-DRB1*04 and HLA-DRB1*1501) (55). Recently, *TNFA* gene emerged as the most extensively studied gene from the *IBD3* locus because of widespread use of anti-TNF antibodies in clinical practice. Ahmad et al. described a haplotype containing allele *TNFA* g.−308G as protective (RR = 0.6) (11). Ferreira et al. studied both children and adults and found significantly higher frequency of homozygotes for *TNFA* g.−308 A/A in patients in comparison with the control group and a significantly increased risk for developing CD (OR = 3.0, 95% CI 1.2–7.2) (38). Three recent studies have described association of *TNFA* polymorphisms in pediatric-onset patients. Levine et al. found that carriage of g.−863A variant was significantly more common in pediatric-onset patients than in control subjects (56). Sykora et al. found an association between *TNFA* g.−308G>A and complicated form of pediatric-onset CD but not with CD as a whole (57). Cucchiara et al. found an association between g.−308G>A and pediatric-onset patients with CD (58).

Similarly, the present study did not detect association of CD with the p.R620W polymorphism within *PTPN22*, although the study was adequately powered (detection of OR $\geq$ 1.6 with a power of 80%). The polymorphism is known to be associated with some but not all autoimmune diseases. Although an association with CD has not been detected as yet, studies in larger and multiple populations are warranted because of its uncertain biological role.

Conclusion

In conclusion, we present a study of genetic association of CD and polymorphisms within *CARD15, TNFA and PTPN22* genes, conferring the first data for the Czech population. We detected an unusually high frequency of the minor allele at *CARD15* 1007fs, and report a strong effect of this allele on the age at disease onset.

Acknowledgments

This study was supported by grants from the Czech Ministry of Health (MZ00064203), the University Hospital Motol, the Grant Agency of the Charles University in Prague (GAUK 7660/2007) and the Czech Ministry of Education no. 2B06155. The authors thank Dr Peter Lohse, the Ludwig-Maximilians-Universität Munich, Germany, for providing us with DNA samples for an external quality control.

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Journal of Pediatric Gastroenterology and Nutrition
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Association of *IL23R* p.381Gln and *ATG16L1* p.197Ala With Crohn Disease in the Czech Population

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ABSTRACT

Objectives: An association of variants in the genes encoding the interleukin 23 receptor (IL23R, p.Arg381Gln, rs11209026), and the autophagy-related gene 16-like 1 (ATG16L1, p.Ala197Thr, rs2241880) with Crohn disease (CD) was identified by whole genome association studies, and subsequently confirmed by other works. The aim of this study was to assess this association in the Czech population.

Subjects and Methods: In a case-control study 333 patients with CD (137 paediatric and 196 adult-onset) and 499 unrelated healthy controls were genotyped using TaqMan SNP assays.

Results: The IL23R p.381Gln allele was protective against CD in the Czech population (allelic frequency 3.2% in patients vs 5.5% in control subjects; OR 0.56, 95% CI 0.33–0.93, $P = 0.02$). *ATG16L1* p.197Ala allele conferred increased risk of CD (allelic frequency 60% in patients vs 51% in controls; OR 1.25, 95% CI 1.02–1.52, $P = 0.03$). There was no appreciable difference in the effect of the associated alleles across the strata of *CARD15*-conferred risk. The *IL23R* and *ATG16L1* variants did not influence the age at diagnosis, and in the genotype-phenotype analysis, the only detected association was a weak one between *IL23R* p.381Gln and involvement of the upper gastrointestinal tract (uncorrected $P = 0.031$).

Conclusions: We confirmed the role of *IL23R* and *ATG16L1* in the CD susceptibility in the Czech population, and found a weak protective effect of *IL23R* p.381Gln against upper gastrointestinal tract involvement. ***JPGN 49:405–410, 2009.***

Key Words: Age at onset—*ATG16L1*—Crohn disease—Genetic association—*IL23R*.

Crohn disease (CD), 1 of the 2 forms of inflammatory bowel disease (IBD), results from a combination of genetic predispositions and environmental exposures. The disease has long been known to associate with polymorphisms in several genes, in particular the *CARD15* (official gene symbol *NOD2*) gene and the risk haplotype in the *SLC22A4* and *SLC22A5* genes within the *IBD5* region (1). Association of several candidate genes were not replicated in follow-up studies (2,3). Recently, genome-wide association studies emerged as an effective instrument for detection of novel susceptibility loci. They found associations with CD in genes encoding interleukin-23 receptor (*IL23R*) on chromosome 1p31 (4) and the autophagy-related 16-like 1 gene (*ATG16L1*) on chromosome 2q37 (5). These findings were replicated in following genome-wide association studies published in 2007 (6–9). Testing the associations in further independent populations, and characterisation of the genotype-phenotype relation is the next step to strengthen the knowledge on aetiology of CD.

Since the reports from the genome-wide association studies, the p.Arg381Gln (c.1142G>A, rs11209026) substitution in the *IL23R* gene has been repeatedly confirmed to associate with CD (10–12). IL-23, a recently described proinflammatory cytokine (13), stimulates the secretion of IL-17, IL-21, and IL-22 from a subset of Th cells termed Th17 (14). IL-21 was described as an autocrine factor that induced higher production of itself and *IL23R* in naïve CD4(+) T cells (15). IL-17 and IL-22 seem to play an important role in intestinal inflammation in IBD, having been found in

Received April 24, 2008; accepted October 25, 2008.

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This study was supported by grants from the Czech Ministry of Health (MZ00064203), the University Hospital Motol, and the grant agency of the Charles University in Prague (GAUK 7660/2007).

The authors report no conflicts of interest.

TABLE 1. *Demographic and clinical characteristics of patients*

	Total (n = 333)	Pediatric-onset CD (n = 137)	Adult-onset CD (n = 196)
Sex, M/F	148/185	71/66	77/119
Age at diagnosis, median (interquartile range)	21 (14–30)	14 (12–16)	28 (23–35)
A1 (<17 y)	111	111	0
A2 (17–40 y)	190	26	164
A3 (>40 y)	32	0	32
Localization			
L1 (terminal ileum)	71 (21%)	23 (17%)	48 (24%)
L2 (colon)	50 (15%)	13 (9.5%)	37 (19%)
L3 (ileocolon)	208 (62%)	101 (74%)	107 (55%)
L1–3 not determined	1	0	1
Upper GI	57 (17%)	21 (15%)	36 (18%)
Disease behaviour			
B1 (nonstricturing/nonpenetrating)	137 (41%)	78 (57%)	59 (30%)
B2 (stricturing)	130 (39%)	31 (23%)	89 (45%)
B3 (penetrating)	62 (19%)	21 (15%)	41 (21%)
B 1–3 not determined	4	3	1
B4 (perianal disease)	109 (33%)	30 (22%)	75 (38%)
Extraintestinal manifestation*	49 (15%)	16 (12%)	33 (17%)
Need for surgery†	174 (52%)	42 (31%)	132 (67%)

The clinical characteristics are given according to the Montreal classification (24). GI = gastrointestinal.
* Extraintestinal manifestation: peripheral arthritis, ankylosing spondylitis, sacroilitis, episcleritis and iritis, erythema nodosum, pyoderma gangrenosum and sclerosing cholangitis.
† Abdominal surgery for complication of CD (resection).

increased quantities in the affected intestine (16,17). *IL23R* encodes one subunit of the IL-23 receptor complex, and its polymorphism affects IL-22 secretion from Th17 cells: significantly lower levels of IL-22 are secreted by individuals carrying the minor allele than those with the wild type (17).

Also, the G allele encoding the p.197Ala amino acid residue in the *ATG16L1* gene (c.1338G>A, p.Ala197Thr, rs2241880) has been demonstrated to increase the risk of CD in several follow-up populations (18–20). The protein is involved in autophagy, a process for degradation of cellular constituents (21) that forms an important part of innate immunity due to its ability to destroy intracellular pathogens that have escaped from the phagosome before fusion with lysosomes (22). The exact role of the autophagy in CD is not yet completely understood, and the same applies to the relevance of the amino acid change at position 197.

In this study, we examined the association of CD and its phenotypes with *IL23R* and *ATG16L1* polymorphisms in the Czech population, focusing on the differences among childhood-onset and adult-onset patients, and association with the clinical phenotypes.

SUBJECTS AND METHODS

In a case-control design, we compared 333 Czech-born patients with CD to 499 unrelated healthy Czech control subjects. All of the subjects were of European Caucasian ancestry. The patients and control subjects were recruited as previously described (22). Briefly, recruitment was performed from a paediatric centre (137 paediatric-onset patients diagnosed before or at the age of 18 years) and an adult centre (196 adult-onset patients diagnosed at or older than the age of 19 years) in Prague, Czech Republic. The demographic and clinical characteristics of the patients are listed in Table 1. The 499 controls included 295 unrelated healthy children (208 boys and 87 girls, median age 8 years, interquartile range 6–11 years) and 204 adult healthy volunteers (114 males, 90 females, median age 38 years, interquartile range 31–46 years). The study was approved by the institutional ethics committees, and a written informed consent was obtained from all of the participants or their guardians.

Determination of Genotypes

Genomic DNA was extracted from peripheral blood with a salting-out method (23) or from saliva collected into Oragene DNA Self-Collection Kit (DNA Genotek Inc, Ottawa, Ontario, Canada). One single nucleotide polymorphism (SNP) was analysed in the *IL23R* gene (p.Arg381Gln, rs11209026) and 1 in the *ATG16L1* gene (p.Ala197Thr, rs2241880), and previously obtained genotyping data were used for 3 polymorphisms in the *CARD15* gene (1007fs, G908R, R702W). The genotypes of the SNPs were determined using the TaqMan SNP genotyping assays run using the 1x TaqMan Universal PCR Master Mix on an ABI 7300 machine (TaqMan SNP Genotyping Assay by Applied Biosystems, Foster City, CA). The endpoint fluorescence readings were evaluated according to the manufacturer's instructions. For better

TABLE 2. *Genotype, allelic frequencies, carriage rates, and risk associated with allelic or carriage positivity of the variants*

	Genotype frequency $n_{CD} = 333$, $n_{Controls} = 499$			Allele frequency $2n_{CD} = 666$, $2n_{Controls} = 998$		Carriage rate $n_{CD} = 333$, $n_{Controls} = 499$	
IL23R p.Arg381Gln rs11209026	Arg/Arg	Arg/Gln	Gln/Gln	Arg	Gln	Arg+	Gln+
CD	312 (94%)	21 (6.3%)	0 (0%)	645 (97%)	21 (3.2%)	333 (100%)	21 (6.3%)
Control subjects	446 (90%)	51 (10%)	2 (0.4%)	943 (95%)	55 (5.5%)	497 (100%)	53 (11%)
OR (95% CI)					0.56 (0.33–0.93)		0.57 (0.33–0.96)
ATG16L1 p.Ala197Thr rs2241880	Thr/Thr	Thr/Ala	Ala/Ala	Thr	Ala	Thr+	Ala+
CD	68 (20%)	158 (48%)	107 (32%)	294 (44%)	372 (60%)	226 (68%)	265 (80%)
Control subjects	128 (26%)	239 (48%)	132 (27%)	495 (50%)	503 (51%)	367 (74%)	371 (74%)
OR (95% CI)					1.25 (1.02–1.5)		1.34 (0.96–1.9)

discrimination of alleles, some run files were also inspected. To ensure consistency between runs, samples of known genotypes were repeated in every analysis.

Statistical Analysis

Association of particular SNP with the CD was tested by analysis of cross-tabulation and expressed as odds ratios with 95% confidence intervals. An analysis was performed after stratification according to the *CARD15* genotype: 1 stratum (''*CARD15*+'') consisted of subjects who carried at least 1 minor allele at either of the 3 susceptibility SNPs (1007fs, G908R, R702W), the other stratum (''*CARD15*−'') consisted of subjects carrying none of the 3 minor variants. The stratified analysis was performed according to Mantel-Haenszel, and the Breslow-Day test was used to test homogeneity across strata. Genotype-phenotype interactions were studied using logistic regression having Montreal clinical classification categories (24) as an outcome, and presence of the genetic variants as predictors. The effect of polymorphisms on the age at diagnosis was tested using Mann-Whitney rank sum test. In all of the tests, $P < 0.05$ was considered statistically significant. Analyses were performed using the Stata package version 9.2 (StataCorp, College Station, TX).

RESULTS

Genotype, allelic, and phenotypic frequencies of *IL23R* p.Arg381Gln and *ATG16L1* p.Ala197Thr are presented with the respective OR and 95% CI in Table 2. Both studied SNPs were in Hardy-Weinberg equilibrium in control subjects ($P \geq 0.35$). The minor allele p.381Gln of *IL23R* gene was negatively associated with CD in the Czech population (minor allele frequency 3.2% in CD patients vs. 5.5% in control subjects, OR 0.56, 95% CI 0.33–0.93). Also, its carriage rate (phenotypic frequency) was significantly lower in the patients than in the control subjects (OR 0.57, 95% CI 0.33–0.96). The p.197Ala variant within the *ATG16L1* gene was significantly overrepresented in patients with CD (risk allele frequency 60% in patients with CD vs 51% in controls, OR 1.25, 95% CI 1.02–1.52). There was no appreciable difference in the effect of the associated alleles across the strata of *CARD15*-conferred risk, as shown in Table 3.

TABLE 3. *Stratification of* IL23R *p.Arg381Gln and* ATG16L1 *p.Ala197Thr by the* CARD15 *genotype*

		IL23R p.Arg381Gln carriage rate		*ATG16L1* p.Ala197Thr carriage rate	
CARD15 stratum		Arg+	Gln+	Ala+	Thr+
CARD15+[a]	CD (n = 154)	154 (100%)	13 (8.4%)	121 (79%)	106 (69%)
	Controls (n = 101)	101 (100%)	18 (18%)	78 (77%)	70 (69%)
	OR (95% CI)		0.43 (0.18–0.99)	1.11 (0.57–2.11)	0.96 (0.54–1.71)
CARD15−[b]	CD (n = 179)	179 (100%)	8 (4.5%)	144 (80%)	120 (67%)
	Controls (n = 398)	396 (99.5%)	35 (8.8%)	293 (74%)	297 (75%)
	OR (95% CI)		0.48 (0.19–1.10)	1.47 (0.94–2.34)	0.69 (0.46–1.04)
Adjusted OR[*]		0.46 (0.26–0.80)	1.34 (0.95–1.90)	0.77 (0.56–1.06)	
P, homogeneity test[†]		0.83	0.45	0.34	

CARD15+[a] = at least 1 minor allele within 3 common variants of *CARD15* (1007fs, G908R, R702W).
CARD15−[b] = no minor allele within 3 common variants of *CARD15* (1007fs, G908R, R702W).
[*] OR for carriers of the studied alleles adjusted for *CARD15* genotype using the Mantel-Haenszel test.
[†] Comparison of ORs for stratified cohorts using the Mantel-Haenszel test and the Breslow-Day test of homogeneity (*P* values were the same for both tests). Note that the power of the data set is severely limited as regards the ability to detect putative *CARD15* – *IL23R* or *CARD15* – *ATG16L1* interactions.

TABLE 4. *Genotype-phenotype analysis*

			Genotype frequencies *IL23R* p.Arg381Gln					Genotype frequencies *ATG16L1* p.Ala197Thr				
		Total	Arg/Arg	Arg/Gln	Gln/Gln	Carriers Gln	*P*	Ala/Ala	Ala/Thr	Thr/Thr	Carriers Ala	*P*
Localization	L1 (terminal ileum)	71 (21%)	68	3	0	4.2%	0.190	19	35	17	76%	0.396
	L2 (colon)	50 (15%)	44	6	0	12%		19	24	7	86%	
	L3 (ileocolon)	208 (63%)	196	12	0	5.8%		68	96	44	79%	
	Upper GI involvement											
	Yes	57 (17%)	57	0	0	0%	0.031*	18	27	12	79%	0.859
	No	275 (82%)	254	21	0	7.6%		88	131	56	80%	
Disease behavior	B1 (nonstricturing/nonpenetrating)	137 (42%)	129	8	0	6.2%	0.474	46	60	31	77%	0.560
	B2 (stricturing)	130 (39%)	120	10	0	7.8%		44	63	23	82%	
	B3 (penetrating)	62 (19%)	60	2	0	3.2%		16	32	14	77%	
	B4 (perianal disease)											
	Yes	109 (33%)	105	4	0	3.7%	0. 297	36	54	19	83%	0.381
	No	220 (69%)	204	16	0	7.3%		70	101	49	78%	
Extraintestinal manifestation	Yes	53 (16%)	48	5	0	9.4%	0.356	16	24	13	75.5%	0.458
	No	274 (84%)	258	16	0	5.8%		91	129	54	80%	
Surgery	Needed	174 (53%)	166	8	0	4.6%	0.183	57	86	31	82%	0.274
	Not needed	157 (47%)	144	13	0	8.3%		50	71	36	77%	

Genotype and carriage frequencies in patients with CD stratified by subphenotypes according to the Montreal classification (24). GI = gastrointestinal.
* The right limit of OR 95% CI 0.82.

Finally, the genotype-phenotype correlations were tested (Table 4) in a case-only model. First we inspected the effect on the age at diagnosis: the age was not related to presence of either the *IL23R* p.381Gln ($P = 0.48$) or the *ATG16L1* p.197Ala allele ($P = 0.74$, both by Mann-Whitney test). Then we tested the distribution of the *IL23R* and *ATG16L1* genotypes among categories of the Montreal classification using exact tests. The carriage of the minor allele within the *IL23R* gene is negatively associated with upper gastrointestinal involvement ($P = 0.031$), as no one of the patients with CD carrying the p.381Gln allele was affected by this phenotype. The association remained unchanged when the model was adjusted for sex, age, and presence of any of the risk variants in *CARD15* (OR 0.83, 95% CI 0.70–0.98, $P = 0.03$). Neither of the remaining clinical characteristics was associated with the genetic predictors.

DISCUSSION

Here we present the contribution of 2 currently discussed SNPs to CD in the Czech population. In agreement with most of the previous reports, the p.381Gln allele within *IL23R* was underrepresented in patients with CD as compared to healthy subjects. Also, the allele p.197Ala within the *ATG16L1* gene was associated with the disease, being more frequent in patients with CD compared to healthy controls; the association of the phenotypic positivity (carriage rate) for the *ATG16L1* p.197Ala allele, however, did not reach statistical significance. This may either reflect the relatively lower power of our study (80% to detect an allelic association of OR>1.3 as compared with OR>1.6 for a dominant model) or a decreased contribution of this ATG16L1 variant to the CD susceptibility in our population as compared with others—except in the Italian study, which did not find the association at all (25).

Often in multifactorial diseases, early-onset patients tend to have a stronger association with genetic factors than the late-onset patients do. We did not observe such a tendency when testing our data set either for *IL23R* or *ATG16L1* association. Testing the differences between childhood-onset and adult-onset patients does not seem to occur frequently in the published literature: for *IL23R*, such an analysis has been done only in a data set from Israel, with the result of an insignificant trend towards stronger association in earlier-onset disease (26). In *ATG16L1*, the situation becomes even more blurred with a study by Van Limbergen et al reporting a stronger association in the adult-onset than the childhood-onset patients from Scotland (19), whereas Prescott et al reported the expected pattern of a higher risk conferred by the risk allele in younger-onset patients recruited from London and Newcastle (20). To our best knowledge these are the only 2 studies so far reporting on the age at onset and *ATG16L1*-conferred susceptibility.

The genotype-phenotype analyses showed only a weak tendency towards a negative association of upper gastrointestinal involvement (L4) with the *IL23R* p.381Gln allele: it was present in none of the 57 patients with upper gastrointestinal involvement, in contrast to 7.6% patients without upper GI involvement ($P = 0.031$ without correction for the number of tests). Several published studies that investigated the relation between *IL23R* and upper GI involvement (12,26,27) did not observe such a trend. It should be noted that the size of our study group limits our ability to detect the associations with subphenotypes: the OR that can be detected with an 80% power ranges between 2.4 and 2.8 depending on the subphenotype and polymorphism. In general, the published literature on *IL23R* and *ATG16L1* has not shown any apparent genotype-phenotype correlation except 2 noncoding variants in *IL23R* (rs2201841 and rs10889677) that predisposed to stricturing behaviour of the disease in a Finnish cohort (28).

In conclusion, we confirmed the negative association of the *IL23R* p.381Gln allele and the positive association of the *ATG16L1* p.197Ala allele with Crohn disease. We also detected a weak negative association of the *IL23R* p.381Gln allele with the upper GI involvement.

Acknowledgments: Drs V. Valtrova, P. Szitanyi, R. Petro, M. Bortlik and V. Starzykova are gratefully acknowledged for providing us with samples and clinical data from their patients.

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Hradsky *et al. BMC Medical Genetics* 2010, **11**:91
http://www.biomedcentral.com/1471-2350/11/91

BMC Medical Genetics

RESEARCH ARTICLE Open Access

The *CTLA4* variants may interact with the *IL23R*- and *NOD2*-conferred risk in development of Crohn's disease

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Abstract

Background: The *CTLA4* (cytotoxic T-lymphocyte antigen 4) gene is associated with several immunopathologic diseases and because of its important immuno-regulatory role it may be considered also a plausible candidate for a genetic association with inflammatory bowel diseases. Previously published studies found no association of *CTLA4* with Crohn's disease itself, but some indicated an association with its subphenotypes. The aim of this study was to assess the association in the Czech population, using a set of markers shown to associate with other diseases.

Methods: Six polymorphisms within the *CTLA4* region were investigated in 333 patients with Crohn's disease and 482 unrelated healthy controls, all Caucasians of Czech origin. The genotypes of the SNPs were determined using the TaqMan SNP genotyping assays. Haplotypes were reconstructed using an expectation-maximization algorithm, and their association with the condition was assessed using log-linear modeling. Then, potential interactions were tested between the *CTLA4* variants and other genetic factors known to confer the disease susceptibility.

Results: No crude associations with Crohn's disease were found for the tested *CTLA4* variants under the log-additive or dominant models. However, when stratified for the genetic risk conferred by the variants in the *NOD2* (the p.Leu1007fsX1008, rs5743293) or the *IL23R* (p.R381Q, rs11209026), a significant negative association emerged for the minor alleles of *CTLA4* CT60 (rs3087243), JO31 (rs11571302), JO27-1 (rs11571297) polymorphisms. This negative association with *CTLA4* was apparent only in the strata defined by presence minor alleles at the *NOD2* rs5743293 (here the *CTLA4* CT60 A coffered an OR = 0.43, 95%CI 0.19 - 0.95 for the presence of CT60 A), or *IL23R* rs11209026 (here the OR for presence of CT60 A was 0.23, 95%CI 0.07 - 0.71). We observed this effect also for the haplotype consisting of minor alleles of the three tightly linked *CTLA4* markers. Furthermore, this haplotype was associated with the younger age at diagnosis (OR 1.52, 95%CI 1.09 - 2.11, p = 0.014).

Conclusions: A protective effect of a *CTLA4* haplotype was unmasked after stratification for the risk variants in the *NOD2* and *IL23R* genes, and may point towards the biological relevance of the molecule in the pathogenesis of the disease.

Background

Crohn's disease (CD) belongs to inflammatory bowel diseases (IBD) that are characterized by chronic, relapsing and recurrent inflammation of intestinal mucosa. The disease is thought to result from the action of environmental factors in genetically susceptible individuals. Three variants in the *NOD2* [1,2], IBD5 locus [3] and one variant in the *IL23R* [4] and in the *ATG16L1* [5] have been independently confirmed to be associated with CD, including associations found previously in the Czech population [6,7]. Recent studies, however, show that this list is far from being complete [5,8-12].

The *CTLA4* gene may also be considered as a plausible candidate for a genetic association with IBD. Its product, the cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is a T-cell suppressor which plays an essential

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role in the function of the CD25(+)CD4(+) regulatory cells that control the process of intestinal inflammation [13,14]. The *CTLA4* gene maps within the 2q33 region that has been found to carry suggestive linkage significance for IBD [15]. The *CTLA4* gene is associated with other immunopathologic diseases (type 1 diabetes, Graves' disease, Addison's disease, celiac disease, systemic lupus erythematosus, rheumatoid arthritis, vitiligo) [16]. Among the studied single nucleotide polymorphisms (SNPs), the CT60 (rs3087243) shows the most prominent associations, being followed by other three SNPs: JO31 (rs11571302), JO30 (rs7565213) and JO27-1 (rs11571297) [16]. A recent publication has shown evidence for association of another SNP within the *CTLA4* with type 1 diabetes, the rs1427676 [17]. Previously published papers about genetic association with CD tested three variants in the *CTLA4* gene: g.49A > G (rs231775), g.-318C > T (rs5742909) and the previously mentioned CT60, having found no association [18-21]. However, several works suggested that *CTLA4* variants may influence the phenotype of CD [18,19].

The aim of this study was to assess the association in the Czech population, using a set of markers previously shown to associate with other diseases.

Methods

Subjects

In a case-control design, 333 Czech patients were compared to 482 unrelated healthy Czech controls representing a general population sample from the same geographical region. We tested 137 pediatric-onset patients (71 boys, 66 girls) who developed CD under or at the age of 18 years and were diagnosed according to the Porto criteria [22], and 196 adult onset patients (77 males, 119 females) diagnosed according to endoscopic, radiological, histological and clinical criteria. Phenotypic classification was done according to the Montreal Classification [23]. The demographic and clinical characteristics of the patients are listed in Table 1 and Table 2. The control group included 482 individuals: 295 children, 187 adult; 311 males, 171 females; median age 12 years, interquartile range 7-34 years. The study was approved by the Ethics Committees of the authors' institutions, and a written informed consent was obtained from all participants or their guardians.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes with a routine salting out method, or from salivary samples using Oragene DNA Self-Collection Kit according the manufacturer's protocol (DNA Genotek Inc., Ottawa, Ontario, Canada). One SNP proximal to *CTLA4* (rs736611), one from within the gene (g.49G > A, rs231775), and four SNPs located distally from the coding part of *CTLA4* gene (rs3087243 also called CT60; rs11571302 called JO31; rs11571297 called JO27-1; and rs1427676) were selected based on available literature and genotyped using the TaqMan SNP genotyping assays (TaqMan SNP Genotyping Assay by Applied Biosystems, Foster City, CA, USA). The assays were run on an ABI 7300 machine (Applied Biosystems, Foster City, CA, USA) and evaluated according to manufacturer's instructions. To ensure consistency between runs, samples of known genotypes were repeated in every analysis. For testing interactions with other associated genes, we used genotypes generated in previously published studies on this sample set [6,7].

Statistical analysis

The Hardy-Weinberg equilibrium was checked by comparing observed to expected genotype frequencies in the control subjects, and tested using exact tests. Associations of particular SNPs with CD were evaluated using odds ratios (OR) with 95% confidence intervals (CI). Haplotype analysis was performed by estimating the haplotype frequencies by the expectation-maximization algorithm implemented in the R-project package 'haplo.stats' version 1.3.1. Association of haplotypes with the conditions was tested using log-linear modeling. Then, a potential interaction between the *CTLA4* variants and other genetic factors associated with the autoimmune conditions were tested. The statistical analysis was performed using the R-project package 'SNPassoc' version 1.5-2 [24].

Table 1: Demographic characteristics of patients and control subjects

	CD patients			Control subjects (n = 482)
	Total (n = 333)	Pediatric-onset CD (n = 137)	Adult-onset CD (n = 196)	
Sex, M/F	148/185	71/66	77/119	311/171
Age, median (interquartile range)	21 (14-30)1	14 (12-16)1	28 (23-35)1	12 (7-34)2

1: Age at diagnosis
2: Age at enrolment

Table 2: Clinical characteristics of patients

	Total (n = 333)	Pediatric-onset CD (n = 137)	Adult-onset CD (n = 196)
Age at diagnosis			
A1 (<17 years)	111	111	0
A2 (17 - 40 years)	190	26	164
A3 (>40 years)	32	0	32
Localization			
L1 (terminal ileum)	71 (21%)	23 (17%)	48 (25%)
L2 (colon)	50 (15%)	13 (9.5%)	37 (19%)
L3 (ileocolon)	208 (63%)	101 (74%)	107 (55%)
L 1-3 not determined	4	0	4
L4 (Upper GI)[1]	56 (17%)	20 (15%)	36 (18%)
Disease behavior			
B1 (nonstricturing/nonpenetraiting)	138 (42%)	79 (59%)	59 (30%)
B2 (stricturing)	129 (39%)	34 (25%)	95 (49%)
B3 (penetrating)	62 (18%)	21 (16%)	41 (21%)
B 1-3 not determined	4	3	1
B4 (perianal disease)	109 (33%)	32 (23%)	77 (39%)
Extraintestinal manifestation[2]	53 (16%)	21 (15%)	32 (17%)
Need for surgery[3]	173 (52%)	41 (30%)	132 (68%)

1: GI: gastrointestinal.
2: Either of: peripheral arthritis, ankylosing spondylitis, sacroilitis, episcleritis and iritis, erythema nodosum, pyoderma gangrenosum, or sclerosing cholangitis.
3: Abdominal surgery for complication of CD (resection)

Results

Crude associations

The frequencies of the variants and respective OR are listed in Table 3. No crude associations with CD were found for the tested SNPs under the log-additive or dominant models. The genotype distributions in control subjects conformed to Hardy-Weinberg equilibrium in all SNPs ($p > 0.20$) except the rs1427676 ($p = 0.014$ in exact tests) which was therefore excluded from all further analyses.

As the part of chromosome under the *CTLA4* gene is divided into the several blocks [16] we performed a haplotype analysis using the five SNPs; no crude association with CD was observed (data not shown).

Interaction of the CTLA4 SNPs with variants in IL23R and NOD2

We then tested possible interactions between variants in the *CTLA4* and polymorphisms in other genes previously associated with CD: *NOD2* gene p.Leu1007fsX1008 (c.3020insC), *IL23R* gene rs11209026 (c.1142G > A) [6,7], see Figure 1. This was done in dominant models using an R-project package 'SNPassoc' version 1.5-2 [24]. Significant interactions were observed between the three *CTLA4* variants (CT60, JO31, JO27-1) and *NOD2* p.Leu1007fsX1008, and the same variants in the *CTLA4* and *IL23R* rs11209026.

For a quantification of the *CTLA4* association stratified by the above *NOD2* and *IL23R* polymorphisms see Table 4 a Table 5: the minor alleles of the CT60, JO31 and JO27-1 within the *CTLA4* modified the risk of Crohn's disease in the stratum of subjects carrying the frameshift insertion p.Leu1007fsX1008 in *NOD2*, while no perceivable effect of *CTLA4* was found in the stratum of p.Leu1007fsX1008 wild-type homozygotes. Similarly, the three *CTLA4* variants clearly, albeit moderately, decreased the risk of CD in the stratum of subjects carrying minor alleles of rs11209026 within the *IL23R* (G/A and A/A), while no effect was observed in the *IL23R* wild-type homozygotes. The effect was observable also for the haplotype consisting of the three minor alleles of the variants in tight linkage disequilibrium, the CT60 "A", JO31 "T", JO27-1 "G" haplotype.

Table 3: Distribution of genotypes of the studied *CTLA4* polymorphisms[1]

Variants	Genotype frequency cases n = 333, controls n = 482			Dominant model[2]	Log-additive model[2]
rs736611	T/T	T/C	C/C	Genotype T/C + C/C	Allele C
CD	37%	48%	15%	63%	39%
Controls	34%	48%	18%	66%	42%
OR (95%CI)				0.89 (0.67 - 1.19)	0.86 (0.70 - 1.05)
g.49A > G (rs231775)	A/A	A/G	G/G	Genotype A/G + G/G	Allele G
CD	41%	46%	13%	59%	36%
Controls	40%	44%	16%	61%	38%
OR (95%CI)				0.85 (0.63 - 1.14)	0.89 (0.72 - 1.09)
CT60 (rs3087243)	G/G	G/A	A/A	Genotype G/A + A/A	Allele A
CD	33%	48%	19%	68%	43%
Controls	35%	48%	17%	65%	41%
OR (95%CI)				1.11 (0.82 - 1.50)	1.10 (0.90 - 1.35)
JO31 (rs11571302)	G/G	G/T	T/T	Genotype G/T + T/T	Allele T
CD	29%	50%	21%	71%	46%
Controls	31%	49%	20%	69%	44%
OR (95%CI)				1.12 (0.82 - 1.53)	1.09 (0.89 - 1.34)
JO27-1 (rs11571297)	A/A	A/G	G/G	Genotype A/G + G/G	Allele G
CD	29%	49%	22%	72%	47%
Controls	30%	50%	20%	70%	45%
OR (95%CI)				1.12 (0.82 - 1.53)	1.11 (0.91 - 1.36)

1: the rs1427676 polymorphism was excluded from the analyses as its distribution among healthy population did not conform to Hardy - Weinberg equilibrium.
2: Adjusted by gender

Genotype - phenotype analysis

Using a case-only design, we tested whether the phenotypic characteristics of the patients are dependent on carriage status of the minor alleles at *CTLA4* variants.

Table 6 shows logistic regression with the outcomes of clinical characteristics and the three *CTLA4* variants and the haplotype as the predictors. The pediatric-onset patients differed to the adult-onset patients in their frequencies of the minor allele at the CT60 (74% versus 63%, p = 0.03). The CT60, JO31 and JO27-1 SNPs, as well as their "ATG" haplotype retained their associations with the age at diagnosis after adjustment to the effect of the *NOD2* variant (p.Leu1007fsX1008). No difference between pediatric and adult-onset group was found for the other two variants in the *CTLA4* gene, data not shown.

The *CTLA4* variants were weakly associated with the ileal-only (L1) and ileocolonic involvement (L3) in a dominant manner, while no association was observed with any of the remaining clinical characteristics: localization in the upper gastrointestinal tract, the stricturing or penetrating behavior of the disease, perianal disease, extraintestinal manifestation, or the need for abdominal surgery (data not shown).

Discussion

The immunologic importance of the *CTLA4* gene is in striking contrast to the lack of knowledge on the functional relevance of its numerous polymorphisms. Consequently, many groups have investigated various polymorphisms located within various regions of the gene. The first published study on *CTLA4* variants in CD investigated the g.49A > G (rs231775) and g.-318C > T

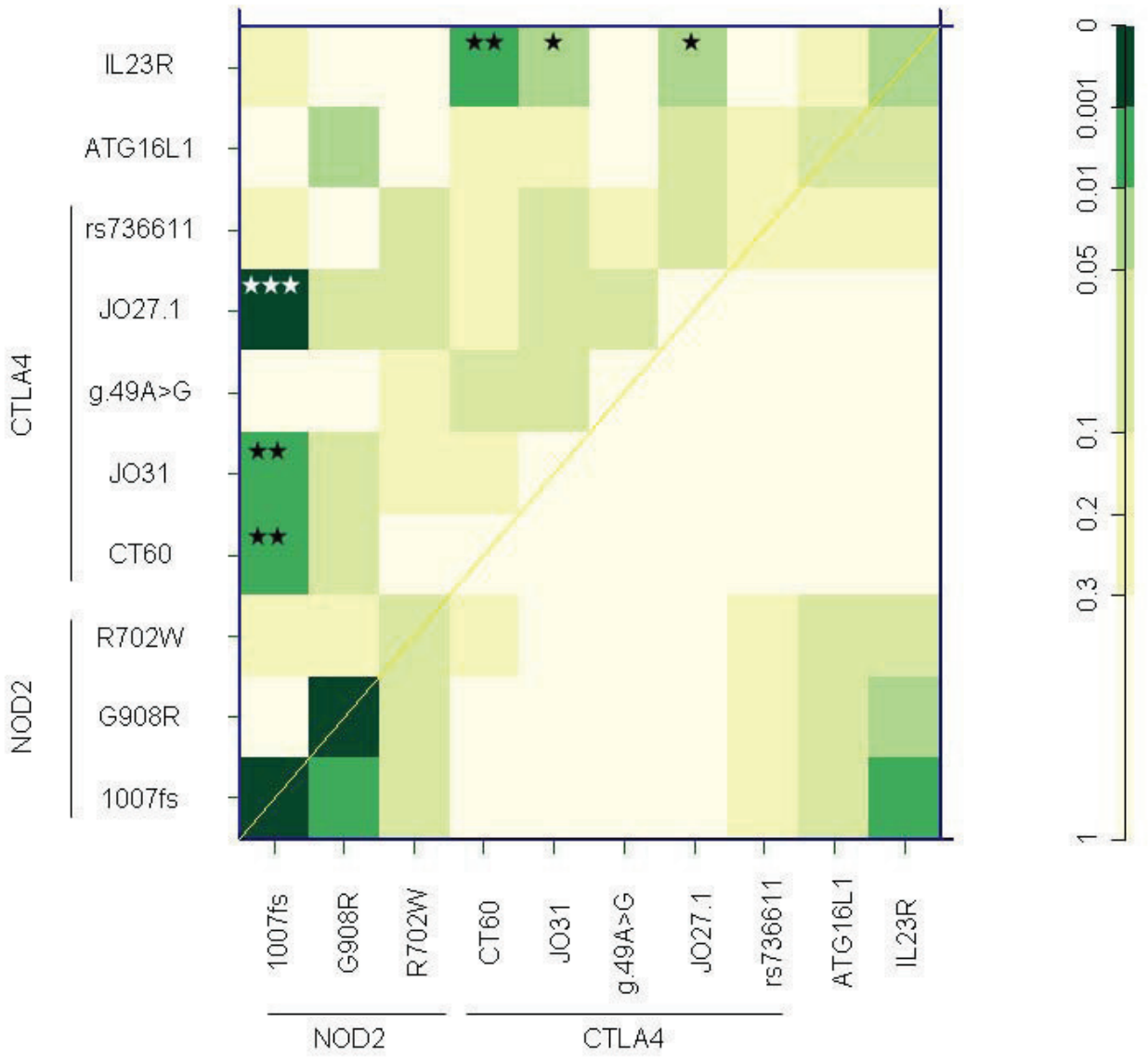

Figure 1 SNPs interaction in the dominant model. The upper triangle in matrix from this function contains the p values for the interaction (epistasis) log-likelihood ratio test (LRT). The diagonal contains the p values from LRT for the crude effect of each SNP. The lower triangle contains the p values from LRT comparing the two-SNP additive likelihood to the best of the single-SNP models [24]. P value of interactions: * P < 0.05; ** P < 0.01; *** P < 0.001.

(rs5742909) in the Dutch and the Chinese populations, finding only an association with the age of onset [18]. Similarly, in a Hungarian work, no association of g.49A > G with CD was detected [21]. Since the work by Ueda et al [16] had been published on dissecting the association of *CTLA4* with immunopathological diseases, further investigators focused on the CT60 polymorphism. This variant was studied in the Japanese [19] and the Spanish [20] populations, however no crude association with CD was detected. The G/G genotype of g.49A > G was associated with penetrating form of CD in the Japanese dataset [19]. No association within 2q33 chromosomal region has been found by genome-wide studies [4,5,8-12].

Thus, compelling evidence has been gathered against simple association of the disease itself with the polymorphisms of *CTLA4*. In line with these findings, we

Table 4: Stratified analysis of the effect conferred by the *CTLA4* CT60, JO31 and JO27-1 variants

***NOD2* stratum defined using the Leu1007fsX1008 polymorphism** [1)]	**CT60 (rs3087243), A carriage rate (genotypes A/G, A/A)**	**JO31 (rs11571302), T carriage rate (genotypes G/T, T/T)**	**JO27-1 (rs11571297), G carriage rate (genotypes A/G, G/G)**	**Haplotype "ATG"** [4]
***NOD2* "+"**				
cases, n = 108	67 (62%)	69 (64%)	70 (65%)	
controls, n = 48	38 (79%)	40 (83%)	42 (88%)	
OR (95%CI) [2)]	**0.43** (0.19-0.95)	**0.35** (0.15-0.83)	**0.26** (0.1-0.68)	0.62 (0.37 - 1.05)
***NOD2* "wt/wt"**				
cases, n = 224	157 (70%)	166 (74%)	168 (75%)	
controls, n = 434	277 (64%)	292 (67%)	294 (68%)	
OR (95%CI) [2)]	1.33 (0.94-1.88)	1.37 (0.96-1.96)	1.42 (0.99-2.03)	1.21 (0.96. - 1.53)
Heterogeneity between *NOD2*-defined strata [3)]	**p = 0.011**	**p = 0.0042**	**p = 0.0011**	**p = 0.043**

Strata of the risk conferred by the p.Leu1007fsX1008 polymorphism of the *NOD2* gene: the effect of *CTLA4* is apparent in the stratum with an increased *NOD2*-associated risk

1) *NOD2* "+": homozygous or heterozygous for the minor allele at the p.Leu1007fsX1008 polymorphism; *NOD2* "wt/wt": wild-type homozygote at the p.Leu1007fsX1008 polymorphism. The *NOD2* "+" category is associated with an increased risk of OR = 4.36, 95%CI 2.95 - 6.49 as compared to "wt/wt" category.

2) OR for the effect of the polymorphism in the specific stratum (*NOD2*"+" and *NOD2*"wt/wt"), adjusted for the effect of the *IL23R* p.381Gln variant and p.Gly908Arg, p.Arg702Trp in the *NOD2* gene. Results significant at $p < 0.05$ are in bold.

3) Heterogeneity in the effect conferred by the CTLA4 polymorphisms was assessed between NOD2-defined strata using the Mantel-Haenszel test of homogeneity.

4) The implemented expectation-maximization algorithm did not allow individual imputation and counting of haplotypes.

observed no crude association unless further genetic factors were taken into account. However, when *CTLA4* was considered as a modifier of the effects conferred by the *NOD2* and *IL23R* genes, possible interactions substantiated. Interactions in multifactorial immunopathological diseases are not infrequent: in CD, the interactions with the *NOD2* gene were detected in the IBD5 locus [25], IBD6 locus [15], *TNFA* [26], *DLG5* [27], *ATG16L1*[28], *IL23R* [29], *TLR4* [30] and in *CD14* [30]. The interaction was also found between IBD5 locus and *IL23R* [31] and between Toll-like receptor-9 polymorphisms and variants in *NOD2* and *IL23R* [32].

The interactions we found for the CT60, JO31, JO27-1 variants of *CTLA4* (or their haplotype) with the p.Leu1007fsX1008 variant of *NOD2* may imply that the effect of the strongest risk variant within the *NOD2* (p.Leu1007fsX1008) can be expressed better on the background of the common *CTLA4* haplotype. This is suggestive of a complex pattern of gene-gene interaction that may merit pursuing further functional studies. Similarly, the risk haplotype of *CTLA4* also interacts with the *IL23R* protective variants. This rather weak interaction can be also due to the relatively limited size of the dataset.

In addition to the modifying effect on the risk of the disease itself, we observed an association with the age at onset and the disease subphenotypes. Indeed, the impact of genetic factors in early-onset patients with CD seems to be stronger than in adult-onset patients (reviewed by de Ridder L et al. [33]). In our dataset, the age at diagnosis was associated with CT60, JO31 and JO27-1. Influence of *CTLA4* variants on the age at diagnosis has been previously described by Xia et al, although with a different SNP (g.-318C > T) [18]. Moreover, their patients were divided into groups where 40 years of age was the cut off, not 18 years as in our study.

The *CTLA4* was associated with the ileal and ileocolonic involvement in our case set: up to our knowledge, this is the first time when localization of CD is influenced by any variant within *CTLA4*. It should be however noted that these associations are weak, merit further investigation in other populations, and their clinical relevance can be only hardly envisaged. The ileal form of disease has been shown more common in adult-onset patients and more common in patients carrying minor variants of the *NOD2* gene. A possible explanation of the association of *CTLA4* with localization of disease might be found in the interaction between *CTLA4* and *NOD2* gene.

Similarly to Machida et al [19] we also tested whether the g.49A > G variant influences the occurrence of penetrating disease, but we were not able to confirm this association. However, the genetic background between Japanese and Czech populations differs markedly.

Table 5: Stratified analysis of the effect conferred by the CTLA4 CT60, JO31 and JO27-1 variants

IL23R **stratum defined using the p.Arg381Gln polymorphism** [1)]	**CT60 (rs3087243), A carriage rate (genotypes A/G, A/A)**	**JO31 (rs11571302), T carriage rate (genotypes G/T, T/T)**	**JO27-1 (rs11571297), G carriage rate (genotypes A/G, G/G)**	**Haplotype "ATG"** [4]
IL23R **"-"**				
cases, n = 21	10 (48%)	10 (48%)	10 (48%)	
controls, n = 50	42 (84%)	40 (80%)	40 (80%)	
OR (95%CI) [2)]	**0.23** (0.07-0.71)	**0.26** (0.08-0.85)	**0.24** (0.07-0.79)	**0.30** (0.11 - 0.81)
IL23R **"wt/wt"**				
cases, n = 312	214 (69%)	224 (72%)	227 (73%)	
controls, n = 432	276 (64%)	292 (68%)	296 (69%)	
OR (95%CI) [2)]	1.26 (0.91-1.74)	1.22 (0.87-1.71)	1.21 (0.86-1.69)	1.20 (0.96 - 1.51)
Heterogeneity between *IL23R*-defined strata [3)]	**p = 0.0061**	**p = 0.011**	**p = 0.011**	**p = 0.030**

Strata of the risk conferred by the p.Arg381Gln polymorphism of the *IL23R* gene: the effect of *CTLA4* is apparent in the stratum with an *IL23R*-associated protective effect

1) *IL23R* "-": homozygote or heterozygote for the *IL23R* p.381Gln allele; *IL23R* "wt/wt": wild-type homozygote at the p.Arg381Gln polymorphism. The *IL23R* "-" category is associated with a decreased risk of OR = 0.58, 95%CI 0.32 - 1.00 as compared to the "wt/wt" category.

2) OR for the effect of the polymorphism in the specific stratum (*IL23R* "-" or *IL23R* "wt/wt"), adjusted for the effect of p.Leu1007fsX1008, p.Gly908Arg, and p.Arg702Trp in the *NOD2* gene. Results significant at $p < 0.05$ are in bold.

3) heterogeneity in the effect conferred by the *CTLA4* polymorphisms was assessed between *IL23R*-defined strata using the Mantel-Haenszel test of homogeneity.

4) The implemented expectation-maximization algorithm did not allow individual imputation and counting of haplotypes.

Conclusions

We present a study of genetic association of polymorphisms within the *CTLA4* gene with CD and its subphenotypes, using a representative set of markers previously reported from other studies. We observed interactions of the *CTLA4* haplotype with variants in *NOD2* and *IL23R* genes, and detected an effect of three variants of the *CTLA4* on the age at diagnosis and localization of the disease.

Table 6: Genotype-phenotype analysis[1]

Outcome	**CT60 (rs3087243) allele A**	**JO31 (rs11571302) allele T**	**JO27-1 (rs11571297) allele G**	**"ATG" haplotype**
Pediatric age at diagnosis[2]	1.85 (1.12 - 3.03); **p = 0.014**	1.71 (1.03 - 2.85); **p = 0.035**	1.70 (1.02 - 2.84); **p = 0.039**	1.52 (1.09 - 2.11); **p = 0.014**
Ileal involvement (L1)	0.41 (0.24 - 0.70); **p = 0.0012**	0.45 (0.26 - 0.78); **p = 0.0052**	0.43 (0.24 - 0.74); **p = 0.0027**	0.70 (0.47 - 1.05); **p = 0.081**
Ileocolonic involvement (L3) [3]	1.97 (1.21 - 3.19); **p = 0.0059**	1.91 (1.16 - 3.13); **p = 0.010**	1.94 (1.18 - 3.20); **p = 0.0090**	1.54 (1.09 - 2.17); **p = 0.014**

1: OR with their 95%CI come from logistic regression analysis using dominant models, with the clinical phenotype as an outcome and *CTLA4* CT60, JO31, JO27-1 minor variants and ATG haplotype as predictors; the models are adjusted for the p.Leu1007fsX1008 variant in the *NOD2* gene.

2: Patients having been diagnosed before or at the age of 18 years.

3: Further, we tested interaction between *NOD2* p.Leu1007fsX1008 variant and the *CTLA4* ATG haplotype on development of L3. Comparing to wild haplotype on the background of the ATG haplotype the association of p.Leu1007fsX1008 *NOD2* high risk variant was significantly weaker. P-value of the interaction between ATG haplotype and *NOD2* p.Leu1007fsX1008 in the development of L3 was estimated 0.026.

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

Authors' contributions
OH, PD and MLe performed the experiments; JB, JN, LV, MLu coordinated and performed the collection of the samples and were also involved in editing the manuscript; OC and OH designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This study was supported by grants from the Czech Ministry of Health (MZOFNM2005), the University Hospital Motol and IGA - NR/9219-3/2007. Drs V. Valtrova, P.Szitanyi, R. Petro, M. Bortlik and V. Starzykova are gratefully acknowledged for providing us with samples and clinical data from their patients.

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Received: 9 November 2009 Accepted: 10 June 2010
Published: 10 June 2010

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Pre-publication history

The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2350/11/91/prepub

doi: 10.1186/1471-2350-11-91
Cite this article as: Hradsky *et al.*, The CTLA4 variants may interact with the IL23R- and NOD2-conferred risk in development of Crohn's disease *BMC Medical Genetics* 2010, **11**:91

Two Independent Genetic Factors Responsible for the Associations of the IBD5 Locus with Crohn's Disease in the Czech Population

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Background: The role of the IBD5 locus in development of Crohn's disease (CD) has not been clarified. In the Czech population we examined its genetic association using variants of the *SLC22A4* (rs1050152), *SLC22A5* (rs2631367), two single nucleotide polymorphisms (SNPs) shown to be associated with CD in genome-wide studies (rs6596075 and rs2188962), and four SNPs previously shown to tag the haplotype blocks 4, 7, 9, 10 of the IBD5 locus (IGR2063b_1, IGR2230a_1, IGR100Xa_1, IGR3236a_1).

Methods: The genotype, phenotype, and allelic frequencies were compared between 469 unrelated patients with CD (177 pediatric-onset, 292 adult-onset) and 470 unrelated healthy controls, all Caucasians of Czech ancestry.

Results: The most significant difference between patients and controls was found for the SNP rs6596075 (odds ratio [OR] = 0.70 for the G allele; 95% CI 0.52–0.94) in the dominant model and SNP IGR2063b_1 (OR = 1.38 for the G allele; 95% CI 1.14–1.67) in the log-additive model. We found a strong linkage disequilibrium across the IBD5 locus except rs6596075. The haplotype consisting of minor alleles of all tested SNPs except rs6596075 was carried by 31% patients and 23% control subjects (OR = 1.35, 95% CI 1.06–1.72). The association of variants in *SLC22A4* and *SLC22A5* was dependent on this risk haplotype, while the strong association of the rs6596075 was seemingly independent. In the analysis of subphenotypes we found only an association of the penetrating disease with rs6596075 (OR = 2.13; 95% CI 1.31–3.47).

Conclusions: Our study confirms the importance of IBD5 in determining CD susceptibility, and demonstrates that two independent genetic factors may be responsible for the association observed within this locus.

(Inflamm Bowel Dis 2010;000:000–000)

Key Words: Crohn's disease, IBD5, SLC22A4, SLC22A5, haplotype

Crohn's disease (CD) belongs to inflammatory bowel diseases (IBDs) characterized by chronic, relapsing, and recurrent inflammation of intestinal mucosa. CD is thought to result from the action of environmental factors in genetically susceptible individuals. The first identified gene associated with CD was *NOD2* (nucleotide-binding oligomerization domain containing 2)[1–3] which has been confirmed subsequently in many other populations,[4] including the Czech population.[5] Later, variants in the other genes such as *IL23R* (interleukin 23 receptor), *ATG16L1* (autophagy related 16-like1) have been independently confirmed to be associated with CD.[6–10] However, recent studies have shown that this list is far from complete.[11]

The association between CD and the IBD5 locus, located at position 5q31, was first identified by a genome-wide linkage study[12,13] and followed by further replication studies.[14–18] Fine mapping of the IBD5 locus led to the identification of a highly conserved 250-kb region that was in strong linkage disequilibrium.[12,13] A high-resolution analysis of the locus described its haplotype blocks.[19] Peltekova et al[20] suggested that 2 of the 11 variants on the risk haplotype, p.Leu503Phe (rs1050152) in the *SLC22A4* gene and c.-207G>C (rs2631367) within promoter region of *SLC22A5*, alter the functions of these organic cation transporters and are therefore responsible for the increased risk of CD.[20] Later, two articles postulated that these variants are not associated independently with the risk of IBD and that true causal variants still had not been identified.[21,22] The association of the haplotype composed by p.Leu503Phe (rs1050152) and c.-207G>C (rs2631367), however, has been widely replicated.[23–30]

Received for publication September 3, 2010; Accepted September 21, 2010.

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Supported by grants from the Czech Ministry of Health (MZOFNM2005) and GAUK 7660/2007.

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DOI 10.1002/ibd.21532

Published online in Wiley Online Library (wileyonlinelibrary.com).

TABLE 1. Demographic and Clinical Characteristics of Patients

	All Patients ($n = 469$)	Pediatric-onset CD ($n = 177$)	Adult-onset CD ($n = 292$)
Sex, M/F	211/258	89/88	122/170
Age at diagnosis, median (interquartile range)	22 (15–30)	14 (12–16)	28(23–36)
Disease duration, median (interquartile range)	9 (4–14)	7 (4–30)	9 (5–15)
Age at diagnosis (%)			
A1 (<17 years)	138 (29%)	138 (78%)	0
A2 (17–40 years)	280 (60%)	39 (22%)	41 (83%)
A3 (>40 years)	51 (11%)	0	51 (17%)
Localization[a]			
L1 (terminal ileum)	146 (31%)	45 (25%)	101 (35%)
L2 (colon)	69 (15%)	15 (8.5%)	54 (19%)
L3 (ileocolon)	252 (54%)	117 (66%)	135 (47%)
Upper GI[b]	70 (19%)	27 (24%)	43 (15%)
Disease behavior			
B1 (nonstricturing / nonpenetrating)	177 (38%)	88 (50%)	89 (30%)
B2 (stricturing)	193 (41%)	55 (31%)	138 (47%)
B3 (penetrating)	99 (21%)	34 (19%)	65 (22%)
B4 (perianal disease)	156 (33%)	49 (27%)	107 (37%)
Extraintestinal manifestation[c]	95 (20%)	34 (19%)	61 (21%)
Need for surgery[d]	253 (54%)	67 (38%)	186 (64%)

Pediatric patients defined by having the disease onset before or at the age of 18 years.
Clinical characteristics according to Montreal Classification.[35]
[a]Two patients have an isolated upper gastrointestinal involvement.
[b]GI: gastrointestinal involvement.
[c]Extraintestinal manifestation: peripheral arthritis, ankylosing spondylitis, sacroilitis, episcleritis, and iritis, erythema nodosum, pyoderma gangrenosum, and sclerosing cholangitis.
[d]Abdominal surgery for complication of CD (resection).

Genome-wide association studies (GWAS) have also pointed out other SNPs in the IBD5 locus that are associated with an increased risk of CD.[9,10,31–33] A recent robust study has indicated that the polymorphism rs6596075 could tag the previously described risk IBD5 haplotype.[9] Furthermore, a meta-analysis that merged and inputted data from three large GWAS[7–9] pointed out another SNP, rs2188962, as the strongest disease-associated variant within IBD5.[11]

The aim of this study was to examine the association of CD with SNPs within IBD5, including the role of its presumed components within *SLC22A4* and *SLC22A5*. In addition, we also explored genotype–phenotype analysis and potential gene–gene interactions in a panel of Czech patients and healthy controls.

SUBJECTS AND METHODS

Subjects

In a case–control design, we compared 469 Czech unrelated CD patients to 470 unrelated healthy Czech controls. Of these, 177 were pediatric-onset patients (89 males, 88 females) who developed CD under or at the age of 18 years and were diagnosed according to the Porto criteria specified,[34] and 292 adult-onset patients (122 males, 170 females) diagnosed according to endoscopic, radiological, histological, and clinical criteria.[35] Phenotypic classification was done according to the Montreal Classification.[36] The demographic and clinical characteristics of the patients are listed in Table 1. The control group consisted of 299 children recruited from patients who underwent minor surgical interventions at the Department of Pediatric Surgery of the University Hospital Motol, Prague, and 171 adult healthy volunteers; 304 males, 166 females; median age 12 years, interquartile range 7–33 years, as reported previously.[5,37] All subjects were Caucasians of Czech ancestry.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes with a routine salting out method, or from salivary samples using the Oragene DNA Self-Collection Kit according to the manufacturer's protocol (DNA Genotek, Ottawa, Ontario, Canada). Variants in *SLC22A4* (p.Leu503Phe, rs1050152), *SLC22A5* (c.-207G>C, rs2631367), haplotype-tagging SNPs: IGR2063b_1, IGR2230a_1 (rs17622 208), IGRX100a_1, IGR3236a_1 (rs2301579), rs6596075 and rs2188962 were genotyped using the TaqMan SNP genotyping assays (TaqMan SNP Genotyping Assay; Applied Biosystems,

TABLE 2. Genotype and Allele Frequencies

SNP	Model	Variants	Patients	Controls	OR (CI95%)[a]	P-value
SLC22A4 (rs1050152)	Dominant	L/L	132 (28%)	161 (34%)		
		F/L-F/F	337 (72%)	309 (66%)	1.28 (0.97–1.70)	0.081
	log-Additive	F	445 (47%)	376 (40%)	1.33 (1.10–1.60)	0.0035
SLC22A5 (rs2631367)	Dominant	G/G	112 (24%)	133 (28%)		
		G/C-C/C	357 (76%)	337 (72%)	1.23 (0.91–1.66)	0.12
	log-Additive	C	483 (51%)	423 (45%)	1.28 (1.06–1.55)	0.0099
rs6596075	Dominant	C/C	362 (77%)	324 (69%)		
		C/G-G/G	107 (23%)	146 (31%)	0.70 (0.52–0.94)	0.018
	log-Additive	G	114 (12%)	160 (17%)	0.71 (0.55–0.93)	0.011
IBD5_IGR2230a_1 (rs17622208)	Dominant	G/G	133 (28%)	141 (30%)		
		G/A-A/A	336 (72%)	329 (70%)	1.03 (0.78–1.38)	0.82
	log-Additive	A	452 (48%)	407 (43%)	1.17 (0.97–1.42)	0.094
IBD5_IGR3236a_1 (rs2301579)	Dominant	C/C	121 (26%)	131 (28%)		
		C/A-A/A	348 (74%)	339 (72%)	1.12 (0.83–1.50)	0.46
	log-Additive	A	459 (49%)	426 (45%)	1.16 (0.96–1.40)	0.13
IBD5_IGR100Xa_1	Dominant	G/G	127 (27%)	141 (30%)		
		G/A-A/A	342 (73%)	329 (70%)	1.16 (0.87–1.54)	0.33
	log-Additive	A	451 (48%)	412 (44%)	1.19 (0.98–1.43)	0.073
IBD5_IGR2063b_1	Dominant	G/G	134 (29%)	169 (36%)		
		G/C-C/C	335 (71%)	301 (64%)	1.36 (1.03–1.80)	0.032
	log-Additive	C	442 (47%)	364 (39%)	1.38 (1.14–1.67)	0.00075
rs2188962	Dominant	C/C	135 (29%)	169 (36%)		
		C/T–T/T	334 (71%)	301 (64%)	1.36 (1.02–1.79)	0.033
	log-Additive	T	438 (47%)	368 (39%)	1.33 (1.10–1.61)	0.0029

[a]Adjusted by gender.

Foster City, CA) on an ABI 7300 machine (Applied Biosystems), and evaluated according to the manufacturer's instructions. To ensure consistency between runs, samples of known genotypes were repeated in every analysis. For testing interactions with other associated genes, we used genotypes generated in previously published studies on this sample set.[5,37]

Power Calculation

Power calculations indicated that a sample size of 469 patients and 470 controls gives the study an 80% power to detect an association with odds ratios (OR) >1.45 at the α level of 0.05. The power estimates for haplotype analysis widely differed due to the uncertainty of the assumptions, but generally indicated limited power to detect associations with haplotypes. To enable comparison with other published works with similarly sized datasets, we nevertheless ran the haplotype analysis.

Statistical Analysis

First, the Hardy–Weinberg equilibrium (HWE) was checked by comparing observed to expected genotype frequencies in the control subjects and tested using exact tests. Linkage disequilibrium testing was performed using the R-project package "genetics." Associations of particular SNPs with CD were evaluated using OR with 95% confidence intervals (95% CI), using the R-project package "SNPassoc" v. 1.5-2.[38] Haplotype analysis was performed by estimating the haplotype frequencies by the expectation-maximization algorithm implemented in the R-project package "haplo.stats" v. 1.3.1. Association of haplotypes with the conditions was tested using log-linear modeling. The potential interaction between variants and other genetic factors associated with the autoimmune conditions were tested using R-project package "SNPassoc" v. 1.5-2.[38] Regression analyses were adjusted for gender.

Ethical Considerations

The study was approved by the Ethics Committees of the authors' institutions and a written informed consent was obtained from all participants or their guardians.

RESULTS

Association of SNPs with CD

All tested SNPs were in HWE in the control population. Genotype frequencies with the respective ORs from a log-additive and a dominant model are shown in Table 2. The

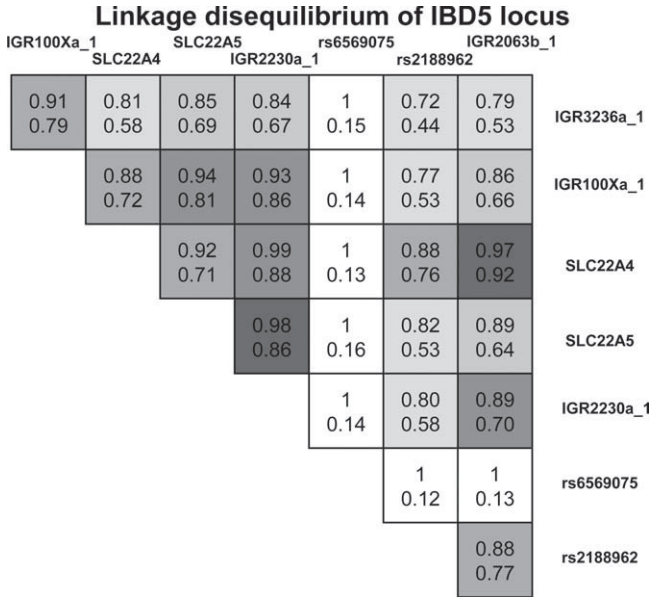

FIGURE 1. Color schema is displayed according to r^2. Data were obtained using the Czech population. D′ a scaled disequilibrium coefficient; r^2 correlation coefficient. The data were obtained using R-project package 'SNPassoc' v. 1.5-2.[37]

association with CD was found for variants p.Leu503Phe, rs6596075, rs2188962, and IG2063b_1 in a dominant model and p.Leu503Phe, c.-207G>C, rs6596075, IGR2230a_1, rs2188962, and IGR2063b_1 in log-additive model. The most profound difference between patients and controls was found for SNP rs6596075, allele G (OR = 0.70; 95% CI 0.52–0.94; $P = 0.018$) in the dominant, and for SNP IGR2063b_1, allele C (OR = 1.38; 95% CI 1.14–1.67; $P = 0.00075$) in the log-additive model.

Linkage Disequilibrium (LD)

The region over the *IBD5* locus has been previously divided into several blocks.[19] These established linkage disequilibria were present among the studied variants also in our dataset (Fig. 1). However, the correlation coefficient (r^2) showed that p.Leu503Phe, c.-207G>C, IGR3236a_1, IGR100Xa_1, IGR2230a_1, rs2188962, and IGR2063b_1 were in tighter SD ($r^2 \geq 0.53$), while the linkage disequilibrium of rs6596075 with other SNPs was significantly weaker (all $r^2 \leq 0.16$).

Haplotype Analysis

According to LD we then constructed haplotypes using SNPs except rs6596075 (consisting of IGR3236a_1, IGR100Xa_1, p.Leu503Phe *SLC22A4*; c.-207G>C *SLC22A5*, IGR2230a_1, rs2188962, and IGR2063b_1). Comparison of haplotype frequencies between cases and controls showed an association with CD for the haplotype composed of minor alleles (OR = 1.35; 95% CI 1.06–1.72; $P = 0.013$) (Table 3).

Because of the weak linkage disequilibrium to rs6596075, we investigated in a multiple regression analysis whether this polymorphism independently contributes to the risk of CD conferred by the above risk haplotype (Table 3). The effect of the risk haplotype remained virtually unchanged (OR = 1.28, 95% CI 1.00–1.64, $P = 0.046$), and the term for the rs6596075 (positivity for allele G, OR = 0.73, 95% CI 0.53–0.99, $P = 0.044$) indicated likely independence of the two genetic factors in determining the disease risk.

Similarly, we tested independence on the haplotype also for p.Leu503Phe in *SLC22A4* and c.-207G>C in *SLC22A5*. These two variants did not significantly add to the model with IGR2063b_1, the strongest associated SNP: when adjusted for IGR2063b_1, p.Leu503Phe yielded $P = 0.50$ and $P = 0.78$, while c.-207G>C yielded $P = 0.39$ and $P = 0.60$ in the dominant and log-additive models, respectively.

Genotype–Phenotype Analysis

In a case-only design, we tested possible association between genotype and available clinical data (age at

TABLE 3. Estimated Haplotype Frequencies and CD Odds Ratios in Patients and Controls

			Base Model[b]		Model Adjusted Also for the rs6596075 (C/G-G/G)[b,e]	
Haplotype[a]	Patients	Controls	OR (CI95%)	P	OR (CI95%)	*P*-value
GCGGCGC	42%	49%	Reference[c]		Reference[c]	
CTACTAA	37%	31%	1.33 (1.06–1.65)	0.012	1.26 (1.01–1.58)	0.044
Rare, pooled[d]	21%	20%	1.25 (0.98–1.60)	0.068	1.28 (1.00–1.63)	0.050

[a]The haplotypes were constructed from the IGR2063b_1, rs2188962, IGR2230a_1, c.-207G>C SLC22A5, p.Leu503Phe SLC22A4, IGR100Xa_1 and IGR3236a_1 polymorphisms.
[b]Adjusted by gender.
[c]The most frequent haplotype was used as the reference.
[d]Rare haplotypes (with frequency <5%) were pooled into this category.
[e]The term for the rs6596075 itself (C/G or G/G genotypes against the C/C) had an OR of 0.68 (CI95% 0.50–0.92), $P = 0.014$.

TABLE 4. Literature Describing Association Between IBD5 and Penetrating Form of CD

Author	Year	Classification	Variant	Quantification of Association
Noble (21)	2005	Vienna B2+B3	IGR2198 homozygosity	$P = 0.026$ (RR = 1.97)
			SLC22A5 (rs17622208)	$P = 0.029$ (RR = 1.78)
			SLC22A4 (rs1050152)	$P = 0.011$ (RR = 2.0)
			SLC22A4/5 TC haplotype	$P = 0.011$ (RR = 2.05)
Vermeire (40)	2005	Vienna B3	SLC22A4/5 TC haplotype	$P = 0.034$
Cucchiara (43)	2007	Montreal B3	SLC22A4/5 TC haplotype	$P = 0.038$, OR = 2.13 [95%CI 1.03–4.4]
Weersma (44)	2008	Montreal B2+B3	rs273900	$P = 0.020$
			rs272893	$P = 0.011$
Weersma (45)	2009	Montreal B3	IBD locus	No
Current study	2010	Montreal B3	rs6596075	$P = 0.0029$, OR=2.13 [95%CI 1.31–3.47]

diagnosis, localization, upper gastrointestinal localization, disease behavior, perianal disease, extraintestinal manifestation, need for surgery). The only association that was strong enough to persist after Bonferroni correction to the seven independently tested clinical characteristics was that of rs6596075 SNP with the penetrating form of disease: OR = 2.13; 95% CI 1.31–3.47; $P = 0.0029$ in the dominant, and OR = 1.80; 95% CI 1.16–2.80; $P = 0.010$ in the log-additive model. Penetrating form of disease was associated with rs6596075 also in a logistic regression analysis including disease duration, extraintestinal manifestation, need for surgery, and location of the disease. Other associations that were significant only before correction for multiple testing may also indicate a possible biological phenomenon: e.g., the rs6596075 with the extraintestinal manifestation (OR = 0.52; 95% CI 0.28–0.96 in the dominant, and OR = 0.56; 95% CI 0.32–0.99 in the log-additive model), with the L1 form of the disease (OR = 1.60; 95% CI 1.02–2.51 in the dominant model). Furthermore, a weak association of CD and upper gastrointestinal involvement was found for c.-207G>C in both models (dominant: OR = 1.93; 95% CI 1.00–3.73; log-additive: OR = 1.45, 95% CI 1.01–2.08) and with IGR100Xa_1 in log-additive model (OR = 0.68, 95% CI 0.47–0.98). We did not find any association between phenotypic characteristics and the above-defined risk haplotype.

Analysis of Interaction of the IBD5 Locus with *NOD2, IL23R*, and *ATG16L1*

No interactions were observed between variants within the IBD5 locus and polymorphisms in other genes previously associated with CD: *NOD2* gene, 1007fs, p.Gly908Arg, p.Arg702Trp; *IL23R* gene, rs11209026; *ATG16L1* gene, rs2241880.[5,37]

DISCUSSION

Although the IBD5 region is undoubtedly associated with risk of CD, the localization of the causal variants is still the subject of research. Peltekova et al[20] had shown that haplotypes consisting of at least one of the p.503Phe or c.-207C alleles on the nonrisk-associated IBD5 background (defined by IGR2078a_1) were more frequent in patients compared to controls, and based on this finding and functional studies, these variants in *SLC22A4* and *SLC22A5* genes were proposed to be causal. Similar to several previous studies,[21,22,29,39–41] we did not confirm this finding; the power to detect the associations was, however, borderline. In our dataset, neither variants in *SLC22A4* and *SLC22A5* were associated with CD independently of IGR2063b_1 as the strongest associated SNP within IBD5.

Interestingly, although the variant rs6596075 had been assigned by the Wellcome Trust Case Control Consortium as the SNP tagging the IBD5 risk haplotype,[9] we observed that it contributes to the risk of CD independently of the risk haplotype defined by other SNPs: this finding may imply that more yet-unidentified IBD5 variants could be causal. This finding has to be confirmed by larger studies sufficiently powered for precise haplotype analyses.

Another SNP that came out of a meta-analysis[11] of three GWAS,[7–9] the rs2188962, has recently been assigned as the most disease-associated variant from the IBD5 region. This variant lies in the gap between previously described block 4 and block 5.[19] We confirmed an association between CD and this SNP, yet there are indications that better markers exist of the risk conferred by IBD5: the association of the SNP IGR2063b_1 within block 4 was stronger and seems to add to the risk associated with rs2188962.

The IBD5 risk haplotype has been repeatedly shown to influence the clinical characteristics of CD.[14,15,17,24,25,42]

Regarding the age at diagnosis, we did not detect any difference between pediatric and adult-onset patients, although significant differences have been observed in equally sized datasets previously.[13,14,20,30] We attempted to replicate results by Mirza et al[14] analyzing the effect of IGR2063b_1 stratified by NOD2 risk variant, but found no interaction. Although same previous studies have found locus–locus interactions (with *NOD2, IL23R, ATG16L1*) in susceptibility to CD, no interactions have been detected in the present study.

The results of genotype–phenotype analyses in CD patients showed remarkable discrepancies for the IBD5 locus among various studies. Perianal, penetrating, and stricturing forms have been associated with IBD5 in several datasets.[17,21,41,43] After we applied a proper correction for the number of independent tests performed in our dataset, we could confirm only an association between the rs6596075 and the penetrating form of disease (for a list of previous relevant studies, see Table 4), while the risk haplotype was not associated with any clinical subtype of disease (including penetrating form). This may indicate an independent role of rs6596075 not only in development of CD but also in the disease course—such a role could underline the true biological character of the association.

In conclusion, the present study on IBD5 associations in the Czech population shows a prominent role of the rs6596075 and IGR2063b_1, and also confirms the association of the IBD5 risk haplotype. The association of the SNP rs6596075 was independent of the IBD5 risk haplotype, and increased the likelihood of penetrating disease. We could not confirm an association independent of the high-risk haplotype for the previously suggested variants p.Leu503Phe in the *SLC22A4* gene, c.-207G>C in the *SLC22A5,* and rs2188962.

ACKNOWLEDGMENTS

We thank Drs. V. Valtrova, R. Kotalova, P. Szitanyi, R. Petro, M. Bortlik, and V. Starzykova for providing the samples and clinical data from their patients.

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Alimentary Pharmacology & Therapeutics

Infliximab dependency in children with Crohn's disease

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Publication data
Submitted 14 May 2008
First decision 1 June 2008
Resubmitted 21 June 2008
Resubmitted 29 November 2008
Resubmitted 13 December 2008
Resubmitted 21 December 2008
Accepted 21 December 2008
Epub Accepted Article 13 January 2009

SUMMARY

Background
Recently, infliximab dependency has been described.

Aim
To assess frequency of ID in 82 consecutive Crohn's disease children treated with infliximab 2000–2006 and to describe clinical and genetic predictors of long-term infliximab response.

Methods
A phenotype model of infliximab dependency was used to assess treatment response: 'immediate outcome' (30 days after infliximab start) – complete/partial/no response. 'Long-term outcome': (i) prolonged response: maintenance of complete/partial response; (ii) infliximab dependency: relapse ≤90 days after intended infliximab cessation requiring repeated infusions to regain complete/partial response or need of infliximab >12 months to sustain response. Polymorphisms *TNF*-308 A>G, *TNF*-857 C>T, *Casp9* 93 C>T, *FasL*-844 C>T, *LTA* 252 C>T and *CARD15* (R702W, G908R, 1007fs) were analysed.

Results
Ninety-four per cent of children obtained complete/partial response. In long-term outcome, 22% maintained prolonged response, 12% had no response, while 66% became infliximab dependent. Perianal disease and no previous surgery were associated with infliximab dependency (OR 5.34, 95% CI: 1.24–22.55; OR 6.7, 95% CI: 1.67–26.61). No association was found with studied polymorphisms. The cumulative probability of surgery 50 months after starting infliximab was 10% in infliximab dependency, 30% in prolonged responders and 70% in nonresponders (P = 0.0002).

Conclusions
Sixty-six per cent of children became infliximab dependent. Perianal disease and no surgery prior to infliximab were associated with infliximab dependency phenotype.

Aliment Pharmacol Ther **29**, 792–799

doi:10.1111/j.1365-2036.2009.03926.x

INTRODUCTION

An increasing incidence of inflammatory bowel disease (IBD) has been described. Six to seven per cent of newly diagnosed patients are children below 15 years of age.[1–6] Medical therapy is symptomatic and with limited efficacy. Biologicals are currently the most potent therapeutic option in severe aggressive IBD in induction as well as in maintenance therapy.[7–11] Infliximab (IFX) has been shown to be efficacious in children with Crohn's disease (CD).[12] Drug dependency is well known in corticosteroid use and has been recently defined and described also in IFX treatment.[13, 14] A retrospective study of 24 children with CD treated with IFX showed that 42% of patients became IFX dependent (ID).[15] ID patients represented a subgroup of patients benefiting from the therapy, but requiring repetitive IFX infusions to sustain their initial response. Although the occurrence of adverse events is relatively low, the risk of severe and potentially fatal adverse events is still present and the long-term side effects due to relatively short clinical use of the drug are still being monitored.[16–21] Therefore, the need for predictors of IFX response defining the subgroup of patients benefiting from the therapy is essential.

The aim of the study was to assess the occurrence of ID phenotype in CD children treated with IFX. The secondary aim was to define clinical and genetic predictors of IFX outcome.

PATIENTS AND METHODS

Study population

A total of 82 consecutive paediatric patients with CD treated with IFX were included and followed up until the end of 2006. Forty-one originated from the Danish Crohn Colitis Database treated at two Departments of Pediatrics in Denmark (Hvidovre and Odense University Hospital) in the period from August 2000 until November 2006. Twenty-four of these children had already been included in a previously published study assessing the occurrence of ID.[15] Forty-one originated from the Czech Republic treated at two Departments of Pediatrics in Prague (Hospitals of the 1st and the 2nd Faculty of Medicine, Charles University) in the period from October 2002 to June 2006. The diagnosis of CD of all patients was assessed according to the international diagnostic criteria.[22] Data regarding age, disease duration, disease behaviour and localization, surgery, date of IFX infusions, infusion dose, treatment indication and concomitant medical therapy (azathioprine/mercaptopurine/methotrexate) were retrospectively retrieved from the files. The indication for IFX treatment was luminal disease (refractory or intolerant to treatment, corticosteroid dependency, with or without growth retardation) in 62 children and perianal disease in 20 children. A total number of 668 infusions (median: 7; range: 1–27) were given in a dose of 5 mg/kg. The treatment strategy and cessation of the therapy were individualized in accordance with the factors such as patient's condition and decision of parents–patient to stop the therapy due to a fear of long-term side effects. Twelve patients received only induction infusions (weeks 0, 2, 6), 38 children were treated with induction infusions followed by maintenance therapy (median: 10 infusions; range: 5–21) and 31 received IFX episodically-nonscheduled (median: 6; range: 1–27). In two patients on maintenance therapy, the dose of IFX was increased to 10 mg/kg later in the treatment course due to loss of efficacy. When IFX was stopped, a majority of children continued on immunomodulators, which were introduced prior to initiation or shortly after the start of IFX therapy. One patient was lost to follow-up. Demographic and clinical characteristics are shown in Table 1.

Healthy individuals, 182 from Denmark and 283 from the Czech Republic, served as controls in the genotype–phenotype association study.

Ethical consideration

This study was approved by The Danish Data Protection Agency and Ethics Committees of the 1st and the 2nd Faculties of Medicine, Charles University in Prague.

Assessment of ID

Clinical outcome of IFX therapy was assessed according to a modified phenotype model of ID developed and described previously.[15] The model aimed to fit more than 90% of all response patterns.

Immediate outcome: 30 days after the first infusion.
Complete response: Luminal disease: ≤2 stools/day (after surgery +2 stools). No blood, pus, mucus, abdominal pain and weight loss. Perianal disease:

Table 1. Demographic and clinical characteristics of 82 Crohn's disease children treated with infliximab (IFX)

	Danish	Czech	*P*
Number of patients	41	41	
Age			
Median (range)	14 (9–18)	15 (8–18)	0.58
Gender			
Male/female	18/23	22/19	0.38
Disease duration (years)			
Median (range)	2 (0–7)	2 (0–6)	0.50
Follow-up after the first IFX infusion (months)			
Median (range)	45 (3–75)	21 (6–50)	<0.001
Disease localization (%)			
Ileum	0	3 (7)	0.23
Colon	15 (37)	10 (24)	
Ileo-colon	17 (41)	20 (49)	
±Upper disease	9 (22)	8 (20)	
Disease behaviour (%)			
Inflammatory	24 (58)	11 (27)	0.01
Stricturing + penetrating	6 (15)	14 (34)	
+Perianal	11 (27)	16 (39)	
Intestinal surgery prior to IFX (%)	8 (20)	3 (7)	0.19
Indication of IFX			
Luminal disease	35 (85)	27 (66)	0.04
Perianal disease	6 (15)	14 (34)	
Concomitant immunosuppressive therapy (%) (azathioprine/ mercaptopurine/methotrexate)	36 (88)	39 (95)	0.49
Treatment regime			
Only induction (0, 2, 6 weeks)	6 (15)	6 (15)	0.04
Induction + maintenance (every 8 or 6 weeks)	14 (34)	24 (60)	
Episodic	21 (51)	10 (25)	
IFX infusions			
Total	314	254	0.18
Median (range)	7 (2–27)	8 (1–21)	

P-values were calculated by Mann–Whitney/chi-squared test.

closure of all fistulas evaluated by thumb pressure or patients announcement of 'no secretion'.

Partial response: Luminal disease: ≤4 stools/day (after surgery +2 stools). Blood, pus, mucus, abdominal pain less than daily, no fever and weight loss. Perianal disease: reduced secretion or discomfort from fistulas or closure of one or some of the fistulas.

No response: Luminal/perianal disease: no regression of symptoms with a need to shift to another immunomodulator and/or surgery within 3 months after initiation of IFX.

Long-term outcome: irrespective of the length of treatment.

Prolonged response: maintenance of complete or partial response.

ID: relapse within 90 days after intended treatment cessation requiring repeated IFX infusions to regain complete/partial response or need of IFX treatment >12 months to sustain complete/partial response.

No response: no regression of symptoms with a need to shift from IFX treatment to another immunomodulator and/or surgery.

Immunomodulator was defined as corticosteroids, azathioprine/mercaptopurine/methotrexate and other biological drugs. Surgery was classified as intestinal (resection, strictureplasty, colectomy) or perianal (incision of abscess, fistulotomy, advancement flap). Incision of perianal abscess as a possible consequence of healing process during IFX treatment was not considered as surgery. If re-infusion of IFX was given more than 1 year after the previous last infusion, the treatment was considered as the second, the third, etc. course. In the present study, the clinical outcome after the first treatment course was analysed.

Furthermore, clinical outcome of IFX therapy was evaluated one year after the last IFX infusion in patients who obtained prolonged response and ID.

Genetic polymorphisms

Association of polymorphisms *TNF*-308 A>G, *TNF*-857 C>T, *Casp9* 93 C>T, *FasL*-844 C>T, *LTA* 252 C>T and *CARD15* (R702W, G908R, 1007fs) was studied with response to IFX treatment.

Genomic DNA was isolated from peripheral blood by routine salting out procedure (Czech Republic) or from buccal swab (Denmark) using Qiagen DNA purification Kit (Qiagen, Hilden, Germany).

All polymorphisms were typed using PCR–restriction fragmented length polymorphism (RFLP). PCR was carried out in a total volume of 15 μL containing 25 ng of genomic DNA, 3 pmol of each primer (Generi Biotech, Hradec Kralove, Czech Republic), 1.5–6 mM $MgCl_2$, 200 μM dNTPs (each), 0.25 units of Taq polymerase and 1× Taq buffer (all from Fermentas, Lithuania). The mixture was incubated for 3 min at 95 °C followed by 30–40 cycles of 10–30 s at 95 °C, 15–20 s at 48–68 °C and 15–30 s at 72 °C. Final extension was 5 min at 72 °C.

About 7–10 μL of PCR product was digested with 1 unit of restriction endonuclease in appropriate buffer

(HpyCHIV was from New England Biolabs, Ipswich, MA, USA, other restriction endonucleases were from Fermentas, Lithuania) for at least 2 h. Digests were separated on 1.5–3.5% agarose gel (low EEO; Applichem, Darmstadt, Germany) in 0.5× TBE buffer and stained with ethidium bromide (Applichem, Germany). For details, see Table S1 (published online). Genotypes of controls were in Hardy–Weinberg equilibrium.

Statistical analysis

Empirical transition probabilities from immediate outcome to long-term outcome were calculated. Univariate logistic analyses were carried out analysing: (i) the probability of being complete or partial responder at immediate outcome and (ii) the probability of being prolonged responder and ID at long-term outcome. Fisher exact test was used, when appropriate. Chi-squared test and Fisher exact test were used to compare allelic and genotype frequencies and to analyse an association of present polymorphisms with response to IFX. Time to surgery was compared by the log-rank test. A significance level of 5% was chosen.

RESULTS

Response to IFX

One month after the first infusion (immediate outcome), 65 (79%) children obtained complete response, 12 (15%) partial response and five (6%) did not respond. In long-term outcome, 18 (22%) patients achieved prolonged response, 53 (66%) developed ID and 10 (12%) patients were nonresponders. The median number of infusions was 3 (range: 1–6) in prolonged responders, 10 (5–27) in ID and 2 (2–3) in nonresponders.

One year after the last IFX infusion, 23 (28%) children were still in remission, while 10 (12%) children lost their response, thus resulting in 20 (25%) nonresponders in total. Thirty-eight (47%) children were still in treatment or their observational time was <1 year after the last infusion (Figure 1).

Transition probabilities from immediate to long-term outcome showed that patients obtaining complete response had a probability of 20% of developing prolonged response, 75% probability of developing ID and 5% probability of becoming nonresponders. Those

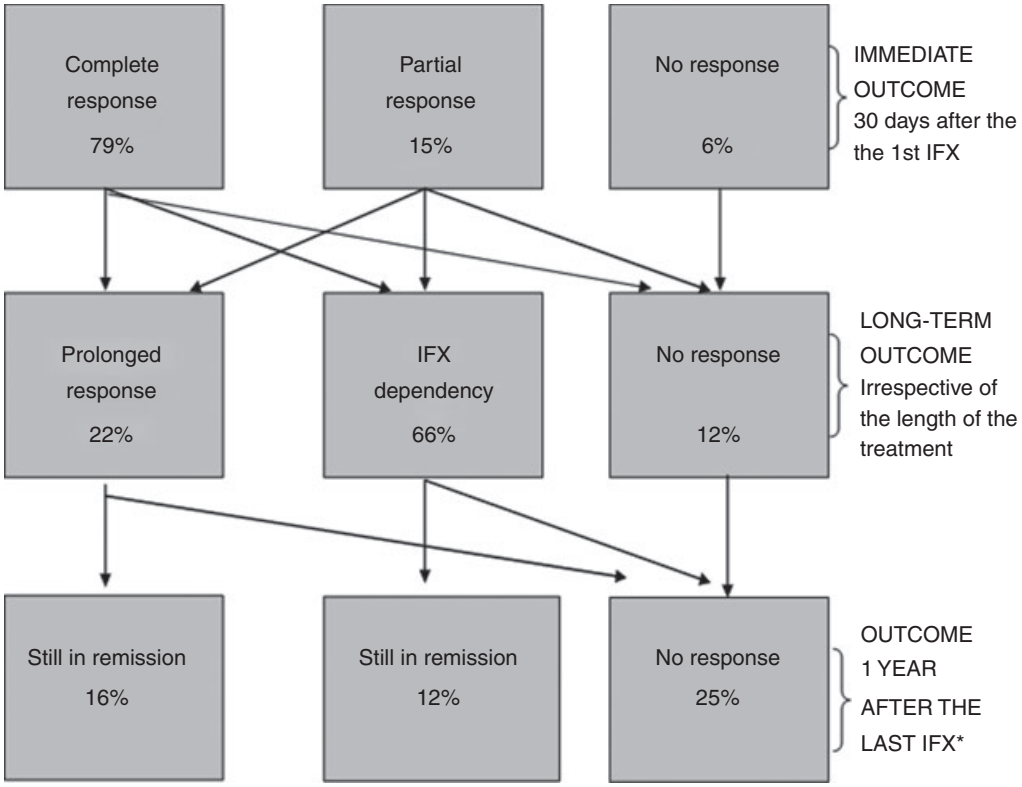


Figure 1. Immediate and long-term outcome of infliximab (IFX) treatment in 82 children with Crohn's disease.
* Thirty-eight (47%) children were still in treatment or their observational time was <1 year after the last IFX infusion.

with partial response had 42% probability of becoming prolonged responders, 42% of becoming ID and 16% of becoming nonresponders (Figure 2).

Clinical predictors

Patients with inflammatory disease behaviour were more likely to become prolonged responders or ID compared with those with stricturing/penetrating disease (OR ∞, 95% CI: 3.23–∞, $P = 0.003$). Intestinal surgery prior to IFX treatment was related to a lower probability to achieve prolonged response or ID (OR 0.05, 95% CI: 0.01–0.32; $P = 0.001$).

Complete responders developed more ID phenotype than partial responders (OR 3.9, 95% CI: 1.13–13.22; $P = 0.036$). Patients with perianal disease compared with luminal disease had higher probability to become ID (OR 5.34, 95% CI: 1.24–22.55; $P = 0.014$). Similarly, children with no intestinal surgery prior to IFX vs. those who underwent surgery were more likely to develop ID response (OR 6.7, 95% CI: 1.67–26.61; $P = 0.007$).

Country, gender, age, disease duration, localization and concomitant immunosuppressive therapy did not influence therapeutic outcome.

Genetic predictors

No association was found between studied polymorphisms and IFX outcome (Table S2, published online).

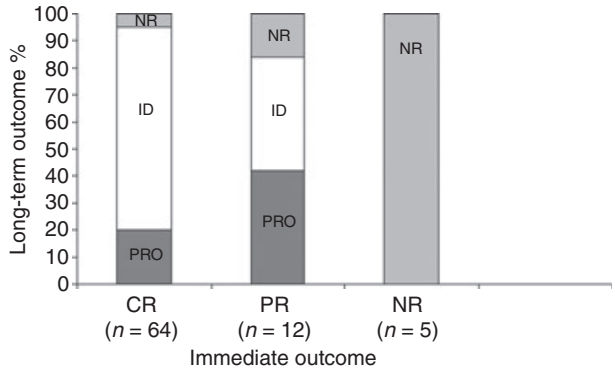


Figure 2. The probability of transition from immediate outcome (30 days after the first infusion) to long-term outcome (irrespective of the length of treatment) in 81 children with Crohn's disease treated with infliximab (one child was lost to follow-up). CR, complete response; PR, partial response; NR, no response; PRO, prolonged response; ID, infliximab dependency.

Significantly higher frequency of C allele of *LTA* 252 C>T polymorphism was observed in Czech patients compared with Czech controls (36% vs. 23%, $P = 0.016$). This association, however, was not confirmed in the Danish cohort. No significant differences in allele frequencies of *FasL*-844 C>T; *Casp9* 93 C>T; *TNF*-308 A>G and *TNF*-857 C>T polymorphisms were found.

Surgery

Complete and partial responders had significantly lower cumulative probability of undergoing intestinal surgery than nonresponders ($P = 0.0012$). The median time to the first surgery was 62 months in complete and partial responders compared to 25 months in nonresponders. The cumulative probability of surgery 50 months after the start of IFX was 10% in ID, 30% in prolonged responders and 70% in nonresponders ($P = 0.0002$). ID patients had significantly less surgeries compared with patients with prolonged response ($P = 0.036$) (Figure 3).

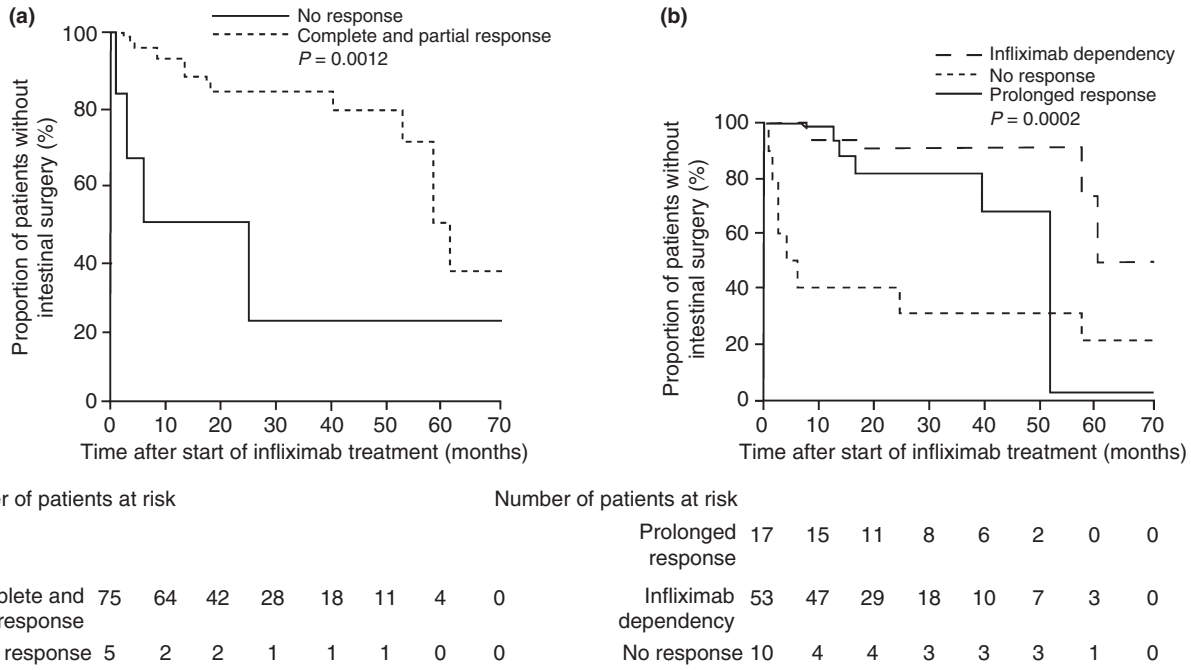
Safety

No cancer, death or severe adverse events, such as infections, occurred during the treatment with IFX or follow-up.

DISCUSSION

One type of ID patients was characterized by early relapse after the treatment cessation with a need for repetitive infusions to regain and sustain initial response. The other type was defined by inability to stop maintenance or episodic therapy within 1 year after the treatment start. In this study, we demonstrated the occurrence of ID in 66% of CD children treated with IFX. Children having perianal disease or no history of surgery prior to IFX were indicative of developing ID response. No association was revealed between selected polymorphisms and IFX response.

Thirty days after the first infusion, 94% of CD children obtained complete or partial response. These results are in agreement with previous studies showing a tendency of higher response rate in children compared with adult patients.[7, 12, 23] This high positive response was maintained also in the long-term as 22% of children were in remission without further need for IFX; however, 66% became ID. In the previously published studies, the frequency of ID with a magnitude



Number of patients at risk

Complete and partial response	75	64	42	28	18	11	4	0
No response	5	2	2	1	1	1	0	0

Number of patients at risk

Prolonged response	17	15	11	8	6	2	0	0
Infliximab dependency	53	47	29	18	10	7	3	0
No response	10	4	4	3	3	3	1	0

Figure 3. Proportion of Crohn's disease patients without intestinal surgery (resection, strictureplasty, colectomy) after the start of infliximab therapy (log-rank test). (a) Comparison with respect to immediate outcome (30 days after the first infusion); (b) Comparison with respect to long-term outcome (irrespective of the length of treatment); Significant difference found also between prolonged response and infliximab dependency ($P = 0.036$)

of 42–56% has been reported.[15, 24] A study evaluating long-term outcome of CD children after only three induction infusions or after induction + maintenance 1-year therapy revealed that 75% and 72% of children respectively relapsed within 1-year after the treatment cessation (median time to relapse 4 and 3 months). In the majority, IFX reintroduction was required to maintain clinical response.[25] These results are very indicative of the new response pattern of ID.

Corticosteroids are not effective in maintaining remission and their long-term use is often accompanied by serious adverse events.[26] Thus, corticosteroid dependency is an undesirable condition and the prompt withdrawal of the drug is required, especially in children where corticosteroids cause growth failure. Contrary to this, IFX is efficacious in maintenance therapy[7] and has rather a good safety profile as outlined in national and local cohorts.[19, 21, 27] From these perspectives, it seems that ID, in contrast to corticosteroid dependency, may be considered beneficial. However, severe adverse events are still matter of concern, especially in ID patients in whom the number of infusions is high and the drug is often combined with azathioprine.[16, 17, 20] Although neither cancer nor death occurred in our cohort, the median follow-up (45 and 21 months respectively) was too short and possible long-term adverse effects could not be excluded.

The transition probabilities from immediate to long-term outcome showed that 95% of children with immediate complete response and 84% with partial response had a benefit in long-term outcome. These results suggest that patients with partial as well as complete response may profit from the therapy not only in short term but also in long-term perspective.

Children with inflammatory behaviour had significantly better long-term outcome compared with those with stricturing/penetrating disease. This is in agreement with the finding that patients with no intestinal surgery prior to IFX (assumed non-stricturing/non-penetrating disease) had higher probability to become prolonged responders or ID. The explanation could be the anti-inflammatory activity of IFX. Occurrence of perianal fistula was predicative of ID phenotype. Perianal disease is characterized by varied complexity and severity, which influence the therapeutic success. Deep and permanent healing of all tracks is an important assumption of sustained response.[28] We speculate that superficial healing or premature closure with

remaining deep tracks could lead to early and recurrent relapses and thus contribute to ID. The limitation concerning validity of the results is relatively small number of patients involved in analysis. New studies are needed to confirm our findings.

As the frequencies of studied polymorphisms in background populations of Denmark and Czech Republic were different (results not shown), analyses were carried out separately.

No association between the polymorphisms and IFX outcome was found in our study. In a previous study, genes involved in apoptosis such as *FasL/Fas* system and *Casp9* have been shown to have an influence on therapeutic outcome.[29] A certain haplotype of lymphotoxin alpha has been reported to be responsible for a decreased response to IFX in CD.[30] Although no association was found with TNF-α promoter polymorphisms and *CARD15* variants,[31–33] studies showing their role in a disease course and disease behaviour[34–36] have suggested these polymorphisms as conceivable predictors of ID response. The small number of individuals involved could be a reason that possible association was not detected in our study. Further studies with large sample sizes are needed.

Biologicals were believed to change the natural course of the disease and thus decrease the need for surgery. A retrospective study evaluating risk factors for initial surgery in CD children showed that the treatment with IFX was associated with a decreased risk for the 1st surgery (HR 0.36, 95% CI: 0.20–0.64).[37] In contrast, another study proposed that IFX may only delay the need for surgery. Up to 60% of treated children required surgery within 12 months, 47% of those with initial complete response.[38] Our results showed lower cumulative probability of surgery in patients with prolonged response and ID compared with nonresponders. This observation confirms the validity of the definitions and indicates a long-term benefit of IFX. The follow-up is too short to conclude if IFX may change the disease course, but at least we can see that the need for surgery was postponed.

In conclusion, IFX had high efficacy in children with CD. Up to 66% developed ID and needed repetitive infusions to regain and sustain initial response. ID, contrary to corticosteroid dependency, was associated with a good overall clinical outcome, but one has to be aware of potential long-term adverse events. Perianal disease and status without previous surgery were found to be possible predictors of ID response. No genetic predictor was revealed. IFX seems to delay the necessity for surgery in those responding to therapy. Prospective trials with further assessment of the occurrence of ID are needed.

ACKNOWLEDGEMENTS

Declaration of personal interests: Dr Munkholm is a member of an advisory board for Schering-Plough, Denmark; Dr Lukos has served as a speaker, consultant and member of an advisory board for Abbott and Schering-Plough in Czech Republic; Dr Duricova and Dr Pedersen have received travel grants from Centocor for poster presentations. *Declaration of funding interests*: This paper was supported by following grants: (i) Grant No. NR/8963-3 and VZ 64203/6903 given by the Czech Ministry of Health; (ii) Grant No. 2B06155 given by the Czech Ministry of Education; (iii) V Binder and P Riis Foundation; (iv) Colitis Crohn Patient Foundation of Denmark; (v) Munkholm Foundation, Danish Crohn Colitis Database.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Details of polymorphisms' typing using PCR–RFLP

Table S2. Minor allele frequency (%) vs. infliximab outcome in children with Crohn's disease

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