MASARYKOVA UNIVERZITA

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GENETICKÉ FAKTORY MODIFIKUJÍCÍ PRŮBĚH PRIMÁRNÍCH PORUCH TVORBY PROTILÁTEK

habilitační práce

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Obsah

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1 Úvod

1.1 Monogenní a komplexní dědičnost u primárních imunodeficiencí

Příčinou **monogenních** poruch je defekt jednoho genu, který zásadním způsobem postihuje některou komponentu nebo funkční dráhu v rámci imunitního systému a který také dominuje klinickému a/nebo laboratornímu obrazu onemocnění. Ostatní genetické a vnější faktory se uplatňují v menší míře, přesto mohou významně modifikovat klinickou manifestaci onemocnění, a tak i v případě monogenních onemocnění se můžeme setkat s celou škálou rozličných fenotypových projevů. Naprostá většina primárních imunodeficiencí (PID; primary immunodeficiencies) patří mezi monogenní choroby (Al-Herz et al., 2014).

Na fenotypu **komplexních** imunopatologických stavů se podílí více genů kódujících jednotlivé složky imunitního systému nebo příslušné regulační proteiny. Významnou roli hraje jejich vzájemná interakce a také interakce s faktory vnějšího prostředí. V rámci těchto genů se vyskytují polymorfizmy, což jsou sekvenční varianty přítomné u více než 1 % jedinců v dané populaci, které mohou nebo nemusí být funkčně významné. Bylo prokázáno, že některé imunopatologické stavy jsou asociovány s funkčními polymorfizmy ve zmíněných genech. Takové polymorfizmy představují pro jejich nositele zvýšené riziko vzniku příslušné imunopatologie. Z primárních imunodeficiencí má pravděpodobně komplexní povahu s velmi výraznou genetickou komponentou většina případů běžné variabilní imunodeficience (CVID, common variable immunodeficiency), jen menší část CVID (asi 3%) byla doposud charakterizována jako následek defektu jednoho genu, tedy jako monogenní onemocnění (Salzer et al., 2012). Opačná je situace u autoimunitních či alergických onemocnění, kde jen u několika stavů byly popsány kauzální defekty jednoho genu, ve většině případů se ukazuje komplexní povaha těchto chorob.

Funkčně významné varianty genů malého účinku samy o sobě nevedou ke vzniku onemocnění, ale ve vzájemné kombinaci a v interakci mezi sebou navzájem či s epigenetickými a zevními vlivy mohou onemocnění způsobit. Zároveň mohou být v roli genů modifikujících průběh chorob, kdy ovlivňují tíži a rozsah projevů, prognózu nebo odpověď na zavedenou léčbu.

1.2 Vztah genotypu a fenotypu u primárních imunodeficiencí

Klinická manifestace PID je velmi pestrá. K fenotypovým projevům patří kromě infekčních komplikací také lymfoproliferativní onemocnění, autoimunitní choroby, alergie či autoinflamatorní symptomy (Boisson et al., 2015). Infekce, stejně jako např. maligní onemocnění, přitom mohou být nejen důsledkem, ale také příčinou stavů imunodeficience. Vztah genotypu a fenotypu je v případě PID komplikovaný a často poměrně málo předvídatelný. **Stejný klinický obraz může být způsoben defekty různých genů a naopak defekt jednoho genu může vést k různým klinickým manifestacím**.

Částečně lze variabilitu v tíži fenotypových projevů vysvětlit typem mutace v genu zodpovědném za vnik onemocnění. V případě velkých delecí či duplikací nebo u mutací, které vedou k předčasnému ukončení syntézy proteinového řetězce, ať už v důsledku nukleotidové substituce (*nonsense* mutace), posunu čtecího rámce vlivem krátkých delecí či inzercí (*frameshift* mutace) či poruchy sestřihu mRNA (*splicing* mutace), lze obecně předpokládat větší funkční dopad než u mutací vedoucích k záměně aminokyseliny (*missense* mutace) nebo k výpadku či vložení jedné až několika aminokyselin (*inframe* delece či inzerce). U většiny PID ovšem nebyla mezi typem mutace a fenotypovými projevy nalezena významná korelace (Gaspar et al., 2000; Holinski-Feder et al., 1998; Tao et al., 2000).

Rozdílnou klinickou manifestaci logicky podmiňují mutace vyskytující se v rámci jednoho genu, které vedou ke ztrátě nebo zeslabení funkce proteinu (mutace typu „loss-of-function“) nebo naopak k posílení funkce nebo vzniku nové funkce kódovaného proteinu (mutace typu „gain-of-function“). Například mutace v genu *WASP* vedoucí k deficitu proteinu jsou zodpovědné za vznik Wiskottova-Aldrichova syndromu nebo trombocytopenie vázané na chromozom X, zatímco mutace typu gain-of-function ve stejném genu způsobují těžkou X-vázanou kongenitální neutropenii (Thrasher and Burns, 2010).

S rostoucím počtem známých mutací v jednotlivých genech roste i počet případů, kdy detekované mutace jsou spojeny s atypickým fenotypem, často v podobě mírného nebo dokonce asymptomatického průběhu onemocnění. V některých případech se zase významně liší klinická manifestace u jednotlivých členů téže rodiny, tedy u nositelů stejné mutace, z čehož plyne, že do hry vstupují **další genetické faktory**, epigenetické vlivy a snad i faktory zevního prostředí, **které modifikují fenotyp PID**.

Příkladem, kdy různé nebo i totožné mutace v jednom genu vedou k odlišným fenotypovým projevům, jsou defekty v molekulách RAG1, resp. RAG2. Jedná se o regulační proteiny důležité v procesu V(D)J rekombinace. Mutace v genech *RAG1* a *RAG2* byly původně popsané u T-B- formy těžké kombinované imunodeficience (SCID, severe combined immunodeficiency) (Schwarz et al., 1996). Hypomorfní mutace, které snižují, ale nikoliv ruší expresi RAG1 a RAG2, byly následně

detekovány u pacientů s odlišnou formou choroby, tzv. Omennovým syndromem, charakterizovaným absencí cirkulujících B lymfocytů a zároveň přítomností oligoklonálních, aktivovaných, leč funkčně neplnohodnotných T lymfocytů (Villa et al., 1998). Zajímavé je, že oba fenotypy se mohou vyskytovat současně v rámci jedné rodiny nebo u nositelů stejné mutace z různých rodin, což ukazuje, že charakter mutace není v těchto případech jediným určujícím faktorem fenotypového projevu (Corneo et al., 2001). Později byly nalezeny ještě další dva klinické fenotypy související s mutacemi v genech *RAG1/*2, a to kombinovaná imunodeficience spojená s expanzí γδ populace T lymfocytů a cytomegalovirovou infekcí a kombinovaná imunodeficience provázená kožními granulomatózními lézemi (Niehues et al., 2010). Aby byla situace ještě méně přehledná, fenotyp Ommenova syndromu byl zjištěn i v souvislosti s defekty v dalších genech, jako *DLCRE1C*, *IL2RG*, *IL7R* a *ADA* (Villa et al., 2008).

Zmíněné příklady jen dokumentují velkou heterogenitu popisovanou se vzrůstající frekvencí při klinické, imunologické a genetické charakterizaci pacientů s PID. Vezmeme-li v úvahu překvapivou šíři fenotypových projevů PID včetně zcela atypických prezentací, dojdeme ke spekulaci, že kauzální mutace v předmětných genech mohou být mnohem častější, než se v současné době domníváme, a že se tedy kumulativně nejedná o tak vzácná onemocnění, za která byla většinou považována.

1.3 Přínos a význam molekulárně genetické diagnostiky PID

Molekulárně genetické metody zaznamenaly v posledních 25 letech nebývalý rozvoj a zásadním způsobem změnily pochopení primárních imunodeficiencí. Přispěly k poznání fyziologických i imunopatologických mechanizmů imunitní odpovědi, vedly k indukci významných objevů, změnily pohled na klasifikaci PID a umožnily vývoj nových léčebných postupů.

1.3.1 Historické souvislosti

Za počátek éry moderní imunologie je považován rok 1952, kdy byl C. O. Brutonem uveřejněn popis případu 8-letého chlapce, kterého postihla ve 4 letech septická artritida kolene a který následně prodělal četné epizody pneumokokové sepse, dvakrát pneumokokovou pneumonii a opakované ataky otitidy. Pomocí tehdy nové techniky elektroforézy byla odhalena absence gamaglobulinové frakce v séru a pacient neodpovídal na vakcinaci tvorbou specifických protilátek. Po intramuskulárních injekcích gamaglobulinů se jeho stav výrazně zlepšil (Bruton, 1952). Později byla u podobně postižených chlapců prokázána dědičnost onemocnění vázaná na chromosom X. Sledováním pacientů s X-vázanou formou agamaglobulinemie (XLA) se ukázalo, že protilátky jsou

důležité zejména v obraně proti infekcím způsobeným opouzdřenými bakteriemi jako *Str. pneumoniae*, *Str. pyogenes* či *Haemophilus influenzae*, zatímco tuberkulóza, mykotické infekce či infekce virem spalniček, zarděnek nebo planých neštovic se u nich ve zvýšené míře nevyskytovaly. Již předtím, v roce 1950, popsali Glanzmann a Riniker dva pacienty s anamnézou těžkých infekcí, exantému a obtížně zvladatelného průjmu, kteří podlehli závažné infekci způsobené kvasinkou *Candida albicans* ve druhém roce života (Glanzmann and Riniker, 1950). U obou byla zaznamenána hluboká lymfopenie. Definice rozdílných populací lymfocytů T a B přišla v 60. letech minulého století a právě infekce pozorované u pacientů s těžkou kombinovanou imunodeficiencí (SCID) ukázaly spektrum patogenů, k jejichž eliminaci jsou důležité T lymfocyty. V 70. letech byly popsány absence B lymfocytů u pacientů s XLA, deficit adenosin deaminázy (ADA) u části pacientů se SCID či absence cytochromu b u jedinců s chronickou granulomatózní nemocí (CGD). K obrovskému nárůstu poznatků pak došlo s nástupem metod molekulární genetiky. V 80. letech byly detekovány mutace v genu pro ADA a phox91 u pacientů s CGD a od počátku 90. let jsme byli svědky exploze v identifikaci genů zodpovědných za vznik primárních imunodeficiencí (PID), mezi prvními genů pro Brutonovu tyrosinkinázu u XLA, pro CD40 ligand u hyperIgM syndromu, pro gama řetězec receptoru pro IL-2 u X-vázané formy SCID a pro WAS protein u Wiskottova-Aldrichova syndromu (WAS). Rychle následovaly další a dnes známe více než 260 geneticky definovaných PID (Al-Herz et al., 2014). Přesto, že již byl osekvenován celý lidský genom, a přes rychlý rozvoj metod od polymerázové řetězové reakce (PCR) až k sekvenování příští generace (NGS, Next Generation Sequencing), stále existují imunodefekty se silnou genetickou komponentou, u nichž nebyla genetická podstata dosud jasně vysvětlena. To je i případ relativně časté běžné variabilní imunodeficience (CVID), kde byla monogenní podstata odhalena jen u malé části pacientů.

1.3.2 Indukce nových objevů

Jedním z efektů, který s sebou nese poznání genů zodpovědných za danou poruchu, je indukce nových důležitých objevů. To lze demonstrovat na několika příkladech.

Odhalení mutací v genu pro společný γ_c řetězec interleukinových receptorů ukázalo příčinu těžké kombinované imunodeficience vázané na chromozom X (X-SCID), která je charakterizována defektním vývojem T lymfocytů i NK buněk (Noguchi et al., 1993b; Puck et al., 1993). V době objevu byl γ řetězec považován za součást receptoru pouze pro IL-2. Bylo ovšem známo, že absence IL-2 sama o sobě nepůsobí tak závažné poškození vývoje T lymfocytů, které by vysvětlovalo fenotyp X-SCID (Schorle et al., 1991). To vedlo ke spekulacím, že IL-2 není jediným cytokinem, který se uplatňuje v patogenezi tohoto onemocnění. Další výzkum skutečně potvrdil, že stejný γ řetězec je sdílen také

receptory pro IL-4, 7, 9, 15, a později i pro IL-21 (Asao et al., 2001; Giri et al., 1994; Kimura et al., 1995; Kondo et al., 1993; Noguchi et al., 1993a; Russell et al., 1993). Bylo tedy zřejmé, že jeden nebo více z uvedených cytokinů hrají klíčovou úlohu ve vývoji T lymfocytů a NK buněk. Nezastupitelným pro správný vývoj T lymfocytů, ale nikoliv NK buněk, se ukázal být IL-7 (He and Malek, 1996). To později korespondovalo se skutečností, že v periferní krvi jedinců s izolovaným defektem řetězce α receptoru pro IL-7 byly NK buňky přítomny v normálním počtu (T-B+NK+ SCID) (Puel et al., 1998). Jako rozhodující pro vývoj NK buněk byl identifikován IL-15 (Mrozek et al., 1996; Waldmann and Tagaya, 1999) , což zase odpovídalo tomu, že u defektu γ řetězce sdíleného receptorem pro IL-7 i IL-15 chybějí funkční T lymfocyty i NK buňky (T-B+NK- SCID) (Rosen FS, 1999). Mutace v genu pro α podjednotku receptoru specifického pro IL-2 způsobuje zcela jiný typ postižení, a to lymfoproliferativní chorobu. To poukazuje na důležitou úlohu IL-2 v procesu apoptózy aktivovaných T lymfocytů (Sharfe et al., 1997).

Dalším příkladem indukce nových objevů může být odhalení příčiny autosomálně recesivní (AR) formy T-B+ SCID. Vzhledem ke znalosti vazby proteinkinázy JAK3 na intracelulární doménu γ řetězce (Asao et al., 1994; Russell et al., 1994), jehož defekt hraje roli při manifestaci X-vázané formy stejného syndromu, stal se gen *JAK3* kandidátním genem pro AR formu onemocnění. Mutační analýza genu *JAK3* provedená na základě této dedukce potvrdila správnost takového přístupu (Russell et al., 1995).

Jiným příkladem jsou vrozené formy agamaglobulinemie. Jako příčina X-vázané agamaglobulinemie (XLA) byl popsán defekt Brutonovy tyrosinkinázy, která má významnou úlohu při vývoji B lymfocytů a je součástí signalizace přes pre-B a B buněčný receptor (pre-BCR, BCR) (Tsukada et al., 1993; Vetrie et al., 1993). Při pátrání po příčinách AR formy agamaglobulinemie byly u myší postupně detekovány defekty v genech kódujících jednotlivé složky pre-BCR a BCR komplexu (řetězec μ, Igα, zástupný lehký imunoglobulinový řetězec λ5), resp. další molekuly pre-BCR a BCR signalizační dráhy (BLNK). Uvedené poruchy byly následně demonstrovány i u člověka, stejně jako defekt v řetězci Igß nebo v podjednotce p85α molekuly PI3 kinázy (Boisson et al., 2013; Ferrari et al., 2007; Minegishi et al., 1999a; Minegishi et al., 1998; Minegishi et al., 1999b; Yel et al., 1996).

Na uvedených příkladech je vidět to, co je opakovaně zmiňováno v odborné literatuře, že vrozené, primární poruchy imunitního systému jsou experimenty přírody, představující jakési modelové situace, jejichž objevení, sledování a zkoumání významnou měrou přispělo a stále přispívá k hlubšímu poznání mechanizmů a souvislostí v rámci imunitního systému a k nacházení nových způsobů léčby imunodeficiencí. Přesný popis defektu a chybějící funkce na úrovni klinické a laboratorní stimuloval objevy nových buněčných subpopulací, receptorů či signálních drah a pomohl definovat jejich přirozenou úlohu v nespecifické i adaptivní imunitě. Vývoj šel postupně od klinických pozorování přes

charakterizaci defektu na buněčné úrovni až po poznání molekulární podstaty na úrovni proteinů a nukleových kyselin. Ne náhodou byly první úspěšná transplantace kostní dřeně (1968) a později první úspěšná genová terapie (2000) provedeny u pacientů s primární imunodeficiencí (X-vázanou formou SCID) (Cavazzana-Calvo et al., 2000; Gatti et al., 1968).

Pochopení vzácných dědičných imunodeficiencí na molekulární úrovni má navíc význam i pro diagnostiku a léčbu imunodeficiencí získaných, které jsou výrazně častější.

1.3.3 Klasifikace PID

Díky rozvoji a využití molekulárně biologických technik každoročně exponenciálně roste počet geneticky definovaných PID. Jednou za 2 roky dochází k revizi a aktualizaci klasifikace PID, kterou provádí expertní panel při Mezinárodní unii imunologických společností (IUIS, the International Union od Immunological Societies) (Al-Herz et al., 2014). Od založení systému klasifikace je uplatňován stejný princip členění PID, který se ve světle stávajícího poznání zdá být již nevyhovující a bude pravděpodobně vyžadovat koncepční revizi (Maggina and Gennery, 2013; Parvaneh et al., 2013). PID jsou členěny do kategorií podle dominantně postižené složky imunitní odpovědi. Řada nemocí ovšem genotypově i fenotypově spadá do více kategorií. Na jedné straně vidíme odlišné či překryvné fenotypy podmíněné stejnými genetickými defekty, na straně druhé se potkáváme se shodnými klinickými a laboratorními projevy různých genetických defektů. Díky rozšiřujícímu se spektru a heterogenitě popsaných nemocí postihujících imunitní systém, ale i další systémy, se tak současný klasifikační systém stává méně přehledným a matoucím. Jistou pomoc v orientaci v tak heterogenní skupině klinických projevů a genetických poruch představuje, zejména pro klinické imunology, fenotypový přístup k existujícímu klasifikačnímu schématu (Bousfiha et al., 2013).

Hlavním účelem klasifikace by měla být definice jednotlivých klinických jednotek tak, aby pro ně mohla být definována i optimální léčebná strategie. Výběr optimální léčby by měl vycházet z velkých mezinárodních studií, které shromáždí dostatečný počet případů pro každou kategorii onemocnění, aby mohly být validně porovnány jednotlivé léčebné přístupy. Určení zodpovědného genetického defektu u každého pacienta je a bude základem správné klasifikace onemocnění a jejich potřebné kategorizace, z níž bude vycházet volba léčby nejvhodnější pro daného pacienta.

1.3.4 Praktické aspekty molekulárně genetické diagnostiky

Jsou dva zásadní důvody, proč u pacienta s podezřením na imunodeficit zvažovat genetické vyšetření. Molekulárně genetická analýza s nálezem kauzální mutace znamená definitivní potvrzení diagnózy

PID, což má význam zejména v situacích, kdy klinická manifestace a/nebo výsledky imunologického vyšetření jsou atypické, nekompletní nebo nejasné. Znalost konkrétního genotypu může být důležitá pro stanovení prognózy a zejména pro určení optimální léčebné strategie. Druhým důvodem je možnost genetického poradenství v rodině včetně stanovení rizika narození dalšího postiženého dítěte. Pokud je znám přesný genetický defekt, je možné určit přenašečství u rodinných příslušníků a dostupná je i prenatální, případně preimplantační diagnostika.

U většiny genů v případě PID se setkáváme s jedinečnými mutacemi, popsanými jen u malého počtu rodin, nebo se zcela novými mutacemi. Proto není možné zaměřit se při analýze na konkrétní mutace, ale je nutné vyšetřovat celé geny. Metody detekce mutací v příslušných genech na úrovni nukleových kyselin se kontinuálně vyvíjejí, jejich základem je většinou polymerázová řetězová reakce (PCR). Vývoj postupuje od screeningových metod určení oblasti genu s pravděpodobným výskytem mutace, na něž navazovalo stanovení sekvence této oblasti, přes v současnosti nejvíce rozšířené sekvenování všech kódujících oblastí daného genu Sangerovou metodou, až po přicházející velkokapacitní sekvenování příští generace (NGS), jehož pomocí získáme v krátkém čase sekvence rozsáhlých oblastí genomu. Pro detekci velkých delecí genu se nejvíce používá metoda MLPA (multiplex ligation-dependent probe amplification).

Klíčová je ovšem správná interpretace výsledku, kdy je nutno rozhodnout, zda nově detekovaná, dosud nepopsaná sekvenční změna má kauzální vztah k dané chorobě. U popsaných mutací se můžeme orientovat podle publikovaných údajů. U nově detekovaných mutace se řídíme typem mutace (mutace způsobující předčasné ukončení syntézy proteinu mají zpravidla vyšší pravděpodobnost, že jsou funkčně významné, než mutace spočívající v záměně aminokyseliny), polohou mutace, segregací mutace s fenotypem v rámci postižené rodiny, případným výskytem detekované změny u zdravých jedinců či výsledky *in silico* predikčních testů. Největší hodnotu mají funkční analýzy, kdy prokážeme dopad mutace na kvalitu či množství mRNA a/nebo kvalitu, množství či funkci proteinu.

Zároveň je potřeba mít na paměti, že negativní výsledek mutační analýzy neznamená automaticky, že daný gen není defektní. Falešně negativní výsledek může být způsoben umístěním mutace v intronu, mimo analyzované oblasti genu, nebo nedokonalostí použitých technik.

Molekulárně genetické testy již dávno nejsou jen otázkou výzkumu, ale zaujímají pevné místo i v rutinní diagnostice PID. Výsledky však musí být interpretovány obezřetně.

1.4 Primární poruchy tvorby protilátek

Primární protilátkové imunodeficience jsou nejčastější vrozené poruchy imunity. Dříve se soudilo, že se jedná převážně o poruchy ležící v B lymfocytární linii, ale postupně byly odhaleny funkční defekty T lymfocytů nebo buněčných komponent nespecifické imunity, které rovněž vedou k poruše tvorby a/nebo funkce protilátek. Jak bylo obecně zmíněno výše, i zde platí, že molekulárně genetický výzkum přinesl objev nových aspektů vývoje B lymfocytů a produkce protilátek, včetně kontroly tvorby autoprotilátek a účasti v obraně proti patogenům. K poruchám tvorby protilátek se řadí defekty zasahující do vývoje, migrace, přežití a aktivace B lymfocytů, do mechanizmů izotypového přesmyku i cytokinové signalizace B lymfocytů, i defekty na úrovni T lymfocytů či antigen prezentujících buněk (Durandy et al., 2013).

Protilátkové imunodeficience, jak je uvádí mezinárodní klasifikace (Al-Herz et al., 2014), jsou shrnuty v tabulce č. 1. První skupinu onemocnění představují agamaglobulinemie s absencí všech imunoglobulinových izotypů v séru a dramaticky sníženými nebo chybějícími B lymfocyty v periferní krvi. Nejvýznamnějším zástupcem v této skupině je X-vázaná Brutonova agamaglobulinemie způsobená defektem Brutonovy tyrozin kinázy, klíčového enzymu vývoje B lymfocytů, mnohem vzácnější jsou autozomálně recesivní formy choroby s defekty složek pre-B či B buněčné signalizace (těžkého řetězce μ, Igα, Igβ, BLNK, λ5 či recentně přiřazené podjednotky p85α PI3 kinázy či transkripčního faktoru E47) (Boisson et al., 2013; Ferrari et al., 2007; Minegishi et al., 1999a; Minegishi et al., 1998; Minegishi et al., 1999b; Tsukada et al., 1993; Vetrie et al., 1993; Yel et al., 1996). S výjimkou defektu řetězce μ však nebyl žádný AR defekt popsán u více než 10 nepříbuzných pacientů.

Do druhé skupiny, charakterizované významným snížením alespoň 2 imunoglobulinových tříd s normálním či sníženým počtem B lymfocytů v periferní krvi, je zařazena běžná variabilní imunodeficience (CVID, common variable immunodeficiency), která je heterogenní skupinou onemocnění zahrnující pravděpodobně celý komplex genetických variant. Z kategorie CVID již byly vyčleněny stavy, kdy byly detekovány defekty jednoho genu zodpovědné za vznik daného fenotypu nebo s ním významně asociované, jako jsou defekty genů *ICOS*, *CD19*, *CD81*, *CD20*, *CD21*, *LRBA*, *TWEAK*, *NFKB2*, *TNFRSF13B* a *TNFRSF13C* (Alangari et al., 2012; Castigli et al., 2005; Grimbacher et al., 2003; Chen et al., 2013; Kuijpers et al., 2010; Lopez-Herrera et al., 2012; Salzer et al., 2005; Thiel et al., 2012; van Zelm et al., 2006; van Zelm et al., 2010; Wang et al., 2013; Warnatz et al., 2009). U genů *TNFRSF13B* a *TNFRSF13C* kódujících molekuly TACI a BAFF receptoru, které mají úlohu v přežití a udržení homeostázy B lymfocytů, se nyní spíše než o kauzálním významu mutací v těchto genech hovoří o jejich roli jako faktorů modifikujících průběh onemocnění (Durandy et al., 2013). Všechny

zmíněné defekty vysvětlují méně než 10% případů CVID, po odečtení defektu TACI se dostaneme jen asi ke 3% objasněných případů. Některé defekty (CD20, CD21) byly dosud popsány jen u 1 pacienta.

Třetí skupinu nemocí představují choroby s významným snížením hladin IgG a IgA, při normálních či dokonce zvýšených hladinách IgM a normálním počtu B lymfocytů. Jedná se o nemoci označené jako hyperIgM syndrom, kdy nejvýznamnější zástupce, X-vázaná forma onemocnění způsobená defektem CD40 ligandu, je zároveň řazena do skupiny kombinovaných imunodeficiencí (spolu se vzácným AR defektem molekuly CD40). Dalšími zástupci jsou AR defekty genů *AID* a *UNG*, všechny molekuly defektní v rámci této skupiny hrají zásadní roli v izotypovém přesmyku při dozrávání B lymfocytů v lymfatických uzlinách (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Ferrari et al., 2001; Fuleihan et al., 1993; Imai et al., 2003; Korthauer et al., 1993; Revy et al., 2000).

Tabulka 1. Přehled protilátkových primárních imunodeficiencí podle klasifikace IUIS (převzato z (Al-Herz et al., 2014))

Onemocnění	Genetický defekt, předpokládaná funkce proteinu, patogeneze	Dědič-nost	Sérové koncentrace imunoglobulinů	Hlavní symptomy	Klasifikace OMIM
1. Zásadní redukce všech tříd imunoglobulinů s významným poklesem nebo absencí B lymfocytů					
(a) Deficience Btk	**Mutace *BTK*, cytoplazmatické tyrozin kinázy, aktivované přemostěním BCR**	**X**	**U většiny pacientů všechny třídy imunoglobulinů sníženy, u části pacientů detekovatelné hladiny imunoglobulinů**	**Závažné bakteriální infekce, normální počet pro-B lymfocytů**	**300300**
(b) Deficience těžkého řetězce μ	Mutace *IGHM*, základní složky pre-BCR	AR	Všechny třídy imunoglobulinů sníženy	Závažné bakteriální infekce, normální počet pro-B lymfocytů	147020
(c) Deficience λ5 [a]	Mutace *IGLL1*, části zástupného lehkého řetězce pre-BCR	AR	Všechny třídy imunoglobulinů sníženy	Závažné bakteriální infekce, normální počet pro-B lymfocytů	146770
(d) Deficience Igα [a]	Mutace *CD79A*, složky pre-BCR a BCR	AR	Všechny třídy imunoglobulinů sníženy	Závažné bakteriální infekce, normální počet pro-B lymfocytů	112205

(e) Deficience Igβ [a]	Mutace *CD79B*, složky pre-BCR a BCR	AR	Všechny třídy imunoglobulinů sníženy	Závažné bakteriální infekce, normální počet pro-B lymfocytů	147245
(f) Deficience BLNK [a]	Mutace *BLNK*, strukturního proteinu vážícího BTK	AR	Všechny třídy imunoglobulinů sníženy	Závažné bakteriální infekce, normální počet pro-B lymfocytů	604515
(g) Deficience regulační podjednotky p85α PI3 kinázy [a]	Mutace *PIK3R1*, kinázy zahrnuté v signalizaci u více buněčných typů	AR	Všechny třídy imunoglobulinů sníženy	Závažné bakteriální infekce, pro-B lymfocyty snížené nebo chybí	171833
(h) Deficience transkripčního faktoru E47 [a]	Mutace *TCF3*, transkripčního faktoru důležitého provývoj B lymfocytů	AD	Všechny třídy imunoglobulinů sníženy	Opakované bakteriální infekce	147141
(i) Myelodysplázie s hypogama-globulinemií	Může být monosomie 7, trisomie 8, nebo kongenitální dyskeratóza	Variabil-ní	Jedna nebo více tříd mohou být sníženy	Infekce; snížený počet pro-B kymfocytů	Nepřiděleno
(j) Thymom s imunodeficiencí	Neznámý	-	Jedna nebo více tříd mohou být sníženy	Bakteriální a oportunní infekce; autoimunitní komplikace; snížený počet pro-B lymfocytů	Nepřiděleno
2. Zásadní redukce alespoň dvou imunoglobulinových tříd s normálním nebo nižším počtem B lymfodytů					
(a) Běžná variabilní imunodeficience (CVID)	**Neznámý**	**Variabil-ní**	**Nízký IgG a IgA a/nebo IgM**	**Heterogenní klinický fenotyp: většina pacientů s opakovanými infekcemi, u části polyklonální lymfoproliferace, autoimunitní cytopenie a/nebo granulomatózní onemocnění**	**Nepřiděleno**

(b) Deficience ICOS [a]	Mutace *ICOS*; kostimulační molekuly exprimované na T lymfocytech	AR	Nízký IgG a IgA a/nebo IgM	Opakované infekce, autoimunitní komplikace, gastroenteritidy, u části granulomy	604558
(c) Deficience CD19 [a]	Mutace *CD19*; transmembránového proteinu amplifikujícího signál z BCR	AR	Nízký IgG a IgA a/nebo IgM	Opakované infekce, u části glomerulonefritida	107265
(d) Deficience CD81 [a]	Mutace *CD81*; transmembránového proteinu amplifikujícího signál z BCR	AR	Nízký IgG, nízký nebo normální IgA a IgM	Opakované infekce, u části glomerulonefritida	186845
(e) Deficience CD20 [a]	Mutace *CD20*; povrchového B lymfocytárního receptoru zavzatého do vývoje B lymfocytů a diferenciace plazmatických buněk	AR	Nízký IgG, normální nebo zvýšený IgM a IgA	Opakované infekce	112210
(f) Deficience CD21 [a]	Mutace *CD21*; molekuly známé také jako komplementový receptor 2, spoluvytvářející komplex CD19	AR	Nízký IgG; snížená odpověď na pneumokoky	Opakované infekce	614699
(g) Deficience TACI [a]	Mutace *TNFRSF13B* (TACI); člena TNF receptorové rodiny přítomného na B lymfocytech jako receptor pro BAFF a APRIL	AD nebo AR nebo komp-lexní	Nízký IgG a IgA a/nebo IgM	Variabilní klinický fenotyp	604907

(h) Deficience LRBA	Mutace *LRBA;* lipopolysaccharide responsive beige-like anchor proteinu	AR	Snížný IgG a IgA u většiny pacientů	Opakované infekce, nespecifické střevní záněty, autoimunitní komplikace; EBV infekce	606453
(i) Deficience BAFF receptoru [a]	Mutace *TNFRSF13C* (BAFF-R); člena TNF receptorové rodiny přítomného na B lymfocytech jako receptor pro BAFF	AR	Nízký IgG a IgM	Variabilní klinický fenotyp	606269
(j) Deficience TWEAK [a]	Mutace *TNFSF12*, k TNF vztaženého slabého induktoru apoptózy (Tweak)	AD	Nízký IgM a IgA; nedostatečná odpověď na pneumokoky	Pneumonie, bakteriální infekce, veruky; trombocytopenie, neutropenie	602695
(k) Deficience NFKB2 [a]	Mutace *NFKB2*, základní komponenty nekanonické cesty NF-κB	AD	Nízký IgG a IgA a IgM	Opakované infekce	164012
(l) Syndrom WHIM: veruky (warts), hypogama-globulinemie, infekce, myelokathexe	Mutace *CXCR4;* receptoru pro CXCL12; typu gain-of-function	AD	Panhypogamaglobulinémie, B lymfocyty sníženy	Veruky/infekce HPV, neutropenie, snížený počet B lymfocytů, hypogamaglobulinemie	193670
3. Významné snížení sérových koncentrací IgG a IgA s normálním/zvýšeným IgM a normálním počtem B lymfocytů (hyperIgM syndrom)					
(a) Deficience CD40L	Mutace *CD40LG* (*TNFSF5*, *CD154*)	X	IgG a IgA snížený; IgM normální nebo zvýšený; počet B lymfocytů normální nebo zvýšený	Bakteriální a oportunní infekce, neutropenie, autoimunitní komplikace	300386
(b) Deficience CD40 [a]	Mutace *CD40* (*TNFRSF5*)	AR	Nízký IgG a IgA; normální nebo zvýšený IgM	Bakteriální a oportunní infekce, neutropenie, autoimunitní komplikace	109535

(c) Deficience AID	Mutace genu *AICDA*	AR	IgG a IgA snížený; IgM zvýšený	Bakteriální infekce, zvětšené uzliny a zárodečná centra	605257
(d) Deficience UNG	Mutace *UNG*	AR	IgG a IgA snížený; IgM zvýšený	Zvětšené uzliny a zárodečná centra	191525
4. Izotypové deficience či deficience lehkých řetězců, obvykle s normálním počtem B lymfocytů					
(a) Mutace nebo delece těžkého řetězce imunoglobulinů	Mutace nebo chromozomální delece 14q32	AR	Chybí jedna nebo více podtříd IgG a/nebo IgA, může chybět IgE	Může mít asymptomatický průběh	Nepřiděleno
(b) Deficience řetězce κ [a]	Mutace *IGKC;* konstantní oblasti pro lehký řetězec κ	AR	Všechny imunoglobuliny mají lehký řetězec λ	Asymptomatický průběh	147200
(c) Izolovaná deficience podtříd(y) IgG	Neznámý	Variabilní	Snížení jedné nebo více podtříd IgG	Obvykle asymptomatický průběh; malá část má sníženou protilátkovou odpověď na specifické antigeny a trpí opakovanými virovými/bakteriálními infekcemi	Nepřiděleno
(d) Deficience IgA spolu s podtřídou/ podtřídami IgG	Neznámý	Variabilní	Snížení IgA a jedné nebo více podtříd IgG	Opakované bakteriální infekce	Nepřiděleno
(e) Deficience PRKC δ [a]	Mutace *PRKCD;* člena rodiny protein kinázy C, kritické molekuly pro regulaci buněčného přežití, proliferace a apoptózy	AR	Nízký IgG; IgA a IgM zvýšeny	Opakované infekce; chronická infekce EBV; lymphoproliferace, autoimunita "SLE-like" (nefrotický a antifosfolipidový syndrom)	615559

(f) Aktivovaná PI3K-δ	Mutace *PIK3CD*; katalytické domény p110 PI3K (typu gain-of-function)	AD	Snížený IgG2 a slabší protilátková odpověď na pneumokoky a hemofily	Respirační infekce, bronchiektázie; autoimunitní komplikace; chronické infekce EBV, CMV	602839
(g) Selektivní deficience IgA	**Neznámý**	**Variabilní**	**IgA snížený/chybí**	**Obvykle asymptomatický průběh; mohou být přítomny opakované infekce se sníženou odpovědí na polysacharidové antigeny; zvýšené riziko autoimunitních nebo alergických projevů; vzácně progreduje do CVID; koexistence s CVID v rodinách**	**137100**
5. Deficience specifických protilátek s normálními koncentracemi imunoglobulinů a normálním počtem B lymfocytů					
	Neznámý	Variabilní	Normální	Snížená schopnost produkovat protilátky na specifické antigeny	Nepřiděleno
6. Tranzientní hypogamaglobulinemie dětského věku s normálním počtem B lymfocytů					
	Neznámý	Variabilní	IgG a IgA sníženy	Normální schopnost tvorby protilátek na vakcinační antigeny, zpravidla nebývají závažnější infekční komplikace	Nepřiděleno

X = dědičnost vázaná na chromozom X; AR = autozomálně recesivní dědičnosti; AD = autozomálně dominantní dědičnost; BTK = Bruton tyrosine kinase; BLNK = B cell linker; AID = activation-induced cytidine deaminase; UNG = uracil-DNA glycosylase; ICOS = inducible costimulator; Ig(κ) = lehký řetězec κ imunoglobulinu.

[a] Dosud v literatuře popsáno nejvíce 10 nepříbuzných případů.

Defekty *CD40L* a *CD40* zařazeny zároveň i do kategorie kombinovaných imunodeficiencí.

V další skupině jsou zahrnuty izotypové deficience a deficience lehkých řetězců, obvykle s normálním počtem B lymfocytů, včetně nejčastější primární imunodeficience vůbec, selektivního deficitu IgA (IgAD, IgA deficiency). Část nemocných se selektivním deficitem IgA progreduje do CVID a nejméně u části nemocných se tak předpokládá společný genetický základ obou chorob (Hammarstrom et al., 2000; Latiff and Kerr, 2007).

Poslední 2 skupiny představují porucha tvorby specifických protilátek s normálními koncentracemi imunoglobulinů a normálním počtem B lymfocytů, resp. transientní hypogamaglobulinemie dětského věku s normálním počtem B lymfocytů.

Ve své práci jsem se věnoval molekulárně genetickému podkladu nejčastějších protilátkových imunodeficiencí, X-vázané formy agamaglobulinemie, CVID a selektivního deficitu IgA, a to nejen z hlediska kauzálních mutací, ale především z hlediska genetických faktorů modifikujících průběh těchto onemocnění.

1.4.1 X-vázaná agamaglobulinemie

X-vázaná agamaglobulinemie (XLA, X-linked agammaglobulinemia; MIM#300755) je vzácná závažná imunodeficience postihující 1 ze 190.000 živě narozených chlapců, způsobená defektem enzymu BTK, Brutonovy tyrozin kinázy, který je důležitý pro správný vývoj B lymfocytů a myeloidních buněk. Defekt BTK stojí za 85% primárních agamaglobulinemií dětského věku, zatímco zbylých 15%, vyznačujících se autozomálně recesivním typem dědičnosti, je způsobeno převážně defekty v dalších molekulách téže signalizační dráhy pre-B a B buněčného receptoru. Důsledkem defektu BTK je absence B lymfocytů v periferní krvi a imunoglobulinů v séru. Onemocnění se typicky manifestuje ve druhém půlroce života, po vymizení mateřských protilátek z cirkulace dítěte. Chlapci trpí opakovanými respiračními infekty způsobenými zejména opouzdřenými bakteriemi, pneumokoky, hemofily a stafylokoky. Nejčastěji se objevují záněty středouší, bronchitidy a sinusitidy, ale můžeme se setkat i s pneumoniemi, průjmy, kožními infekcemi, artritidami, osteomyelitidami, sepsemi či meningitidami. Virové infekce nejsou typické, s výjimkou enterovirových meningoencefalitid. Infekce gastrointestinálního traktu mohou být způsobeny parazitem *Giardia lamblia*. Obávanou komplikací je rozvoj trvalého plicního postižení s bronchiektáziemi. Jedinou efektivní léčbou je substituce protilátek podávaných ve formě intravenózních či subkutánních infúzí preparátů IgG v pravidelných 3-4 týdenních, resp. týdenních intervalech. Důležité je včasné stanovení diagnózy, aby léčba mohla být zahájena včas, tj. před tím, než dojde k ireverzibilnímu poškození plicní tkáně (Conley and Howard, 2002; Ochs and Smith, 1996).

1.4.2 Běžná variabilní imunodeficience a selektivní deficit IgA

Selektivní deficit IgA je nejčastější primární imunodeficiencí s frekvencí výskytu asi 1:400 až 1:600. Vyznačuje se nízkými koncentracemi IgA v séru, opakovaně pod 0,07 g/l, bez dalších laboratorních abnormalit, ve věku alespoň 4 let. Většina jedinců s IgAD nemá žádné zdravotní problémy, ale u části z nich se mohou objevit častější respirační nebo gastrointestinální infekce nebo autoimunitní komplikace. Vzácně se vyskytují protilátky proti IgA, které mohou být zdrojem závažných komplikací při podání krevních derivátů s obsahem IgA. IgAD má silnou genetickou komponentu, zvýšeně se vyskytuje v rodinách a u malé části pacientů se rozvine CVID. Právě společný výskyt s CVID v rodinách a dokumentovaný přerod IgAD v CVID dává základ úvah o společném genetickém pozadí obou onemocnění (Hammarstrom et al., 2000).

Běžná variabilní imunodeficience se vyskytuje u 1 z 25.000 osob a je tak nejčastější závažnou primární poruchou imunity (Jolles, 2013). Mnohaletá sledování ukázala, že CVID významně snižuje dlouhodobé přežívání pacientů. Geneticky se jedná o velmi heterogenní skupinu onemocnění. Jen asi u 3% pacientů byl popsán genetický defekt zodpovědný za manifestaci onemocnění, jak je uvedeno výše. CVID je charakterizována nízkými koncentracemi IgG a IgA, s normálními nebo sníženými koncentracemi IgM. Počet B lymfocytů je snížený nebo může být normální. Základním kamenem diagnostiky je porucha tvorby specifických protilátek na vakcinační podněty. CVID může být diagnostikována v jakémkoliv věku s výjimkou dětí do 4 let. Na rozdíl od většiny PID je toto onemocnění typicky zjištěno v dospělém věku a určení diagnózy po 50. roku věku není výjimečné.

Onemocnění se manifestuje opakovanými závažnými infekčními komplikacemi, ale neméně významné jsou také neinfekční projevy choroby. Z infekčních komplikací, jako u ostatních protilátkových deficiencí, dominují respirační a gastrointestinální infekty opouzdřenými bakteriálními patogeny, typicky sinusitidy, bronchitidy, pneumonie a gastroenteritidy. Opakovanými respiračními infekcemi trpí 90% pacientů. Nicméně ukazuje se, že větší negativní vliv na přežívání pacientů mají neinfekční komplikace nemoci, jako jsou autoimunitní a lymfoproliferativní manifestace, především cytopenie (trombocytopenie, autoimunitní hemolytické anemie a/nebo neutropenie), granulomy, lymfocytární intersticiální pneumonie nebo nevysvětlitelné lymfadenopatie či enteropatie. U pacientů s CVID je také zvýšené riziko maligních komplikací. Pacienti s alespoň jednou neinfekční komplikací měli během sledovaného období 11x vyšší riziko úmrtí oproti pacientům trpícím pouze infekčními chorobami (Chapel et al., 2008; Resnick et al., 2012).

Osou léčby je v současné době stejně jako u XLA podávání preparátů obsahujících IgG, které dokáží uspokojivě držet pod kontrolou zejména infekční komplikace choroby. Bližší poznání faktorů

určujících rozvoj neinfekčních komplikací, včetně genetických vlivů, by mělo zásadní význam pro určení prognózy pacientů s CVID a pro nové možnosti prevence a léčby těchto komplikací.

Kapitola 1 byla sepsána s využitím následujících textů:

T. Freiberger: Molekulární genetika primárních poruch imunity. Alergie, 6, 2004, č. 4, pp. 23-33. (Cena ČSAKI za nejlepší přehledný vzdělávací článek za rok 2004 publikovaný v časopise Alergie).

T. Freiberger: Genetická analýza v diagnostice vrozených poruch imunity. In Jeseňák M., Bánovčin P. a kol., Vrodené poruchy imunity, A-medi management, 2014.

2 Určení kauzální mutace u pacientů s primárními poruchami tvorby protilátek

V genetické laboratoři Centra kardiovaskulární a transplantační chirurgie v Brně se dlouhodobě a systematicky věnujeme molekulárně genetickým aspektům PID, a to z pohledu diagnostiky i výzkumu. Diagnostická vyšetření prováděná pro celou ČR, v některých případech i pro kolegy ze zahraničí, jsou uvedena v tabulce č. 2. Z onemocnění zahrnujících primárně poruchu tvorby protilátek sem patří X-vázaná (Brutonova) agamaglobulinemie, AR agamaglobulinemie z deficitu těžkého řetězce μ a z deficitu PI3 kinázy, běžná variabilní imunodeficience, selektivní deficit IgA, syndrom aktivované PI3kinázy delta a X-vázaný hyper IgM syndrom, který je zároveň řazen do kategorie kombinovaných imunodeficiencí s postižením také T lymfocytů.

Určení kauzální mutace v genu zodpovědném za vznik onemocnění vede k potvrzení diagnózy a k možnosti vyšetření nosičství mutace u příbuzných a genetického poradenství při plánování rodičovství. Znalost přesného molekulárního defektu umožní, při dostupnosti informací o průběhu onemocnění u skupiny pacientů se stejným typem postižení, přesnější stanovení prognózy a užití optimální léčby, samozřejmě s nutným zvážením individuální situace pacienta. U nových dosud nepopsaných mutací, které jsou prokazatelně funkčně významné, připívá jejich stanovení k objasnění mechanizmů, jimiž mutace vedou k porušení funkce proteinu, případně k pozorovanému fenotypu onemocnění. V souvislosti s vyšetřením genu *BTK* u pacientů s agamaglobulinemií jsme publikovali několik prací.

2.1 X-vázaná agamaglubulinemie u dětí s komunitní pneumonií

Primární imunodeficience jsou vzácná onemocnění a infekční komplikace, včetně pneumonií, patří ke klíčovým fenotypovým projevům. X-vázaná agamaglobulinemie (XLA) se manifestuje přibližně u 1 ze 190.000 narozených chlapců, asi v 50% případů se onemocnění projeví v prvním roce života, 90% případů se klinicky projeví do 5 let věku. Asi polovina pacientů prodělá před stanovením diagnózy alespoň jednu epizodu pneumonie. Medián stanovení diagnózy je 26 měsíců. Včasné určení diagnózy a zahájení léčby je velmi důležité, protože dramaticky snižuje riziko infekčních komplikací dolních cest dýchacích, které vedou k ireverzibilnímu poškození plic, zejména vznikem bronchiektázií, a jsou hlavní příčinou snížení délky a kvality života u pacientů s XLA (Conley and Howard, 2002; Ochs and Smith, 1996).

Tabulka 2. Přehled diagnostických molekulárně genetických vyšetření u pacientů s PID prováděných v genetické laboratoři CKTCH v Brně

Onemocnění	MIM	Gen	Dědičnost	Poznámka
X-vázaná agamaglobulinemie	300 755	*BTK*	X	
AR agamaglobulinemie - deficience těžkého řetězce μ	147 020	*IGHM*	AR	
Syndrom aktivované PI3 kinázy delta (defekt katalytické domény p110)	615 513	*PIK3CD*	AD	mutace gain-of-function
Syndrom aktivované PI3 kinázy delta 2 (defekt regulační podjednotky p85α)	616 005	*PIK3R1*	AD	mutace gain-of-function
X-vázaný hyper IgM syndrom	300 386	*CD40L* (*TNSF5*)	X	
Běžná variabilní imunodeficience a selektivní deficit IgA [a]	240 500 609 529	*TNFRSF13B*	(AD)	gen modifikující průběh onemocnění
X-vázaná těžká kombinovaná imunodeficience	102 700	*IL2RG*	X	
AR těžká kombinovaná imunodeficience, Omennův syndrom	179 615 179 616	*RAG1, RAG2*	AR	
X-vázaná chronická granulomatózní nemoc	300 481	*CYBB*	X	
Wiskott-Aldrichův syndrom	301 000	*WASP*	X	
X-vázaná trombocytopenie	313 900	*WASP*	X	
X-vázaná těžká kongenitální neutropenie	300 299	*WASP*	X	mutace gain-of-function
X-vázaný lymfoproliferativní syndrom 1	308 240	*SH2D1A*	X	
X-vázaný lymfoproliferativní syndrom 2	300 079	*BIRC4*	X	
Hyper IgE syndrom	147 060	*STAT3*	AD	často mutace *de novo*
MonoMAC syndrom/deficience DCML (dendritických buněk, monocytů a některých lymfocytů); Embergerův syndrom, familiární MDS/AML (myelodysplastický syndrom/ akutní myeloidní leukémie)	614 172 614 038 614 286 601 626	*GATA2*	AD	
Hereditární angioedém	106 100	*SERPING1*	AD	

Deficit C2 složky komplementu	217 000	*C2*	AR	
Deficit lektinu vázajícího manózu [a]	154 545	*MBL2*	AR/AD	gen modifikující průběh onemocnění

Tučně jsou zvýrazněny primární imunodeficience patřící do kategorie protilátkových imunodeficiencí. Pacienti s deficitem CD40L jsou současně řazeni do kategorie kombinovaných imunodeficiencí.

X = dědičnost vázaná na chromozom X; AR = autozomálně recesivní dědičnosti; AD = autozomálně dominantní dědičnost

[a] Nejedná se o vyšetření kauzálních genů.

Nedávné studie ukázaly, že diagnostika PID je často zpožděna. Poradní panel Nadace „Jeffrey Modell" navrhl sadu 10 varovných příznaků, jejichž zaznamenání, i jednotlivě, by mělo lékaře upozornit na možnou PID (www.info4pi.org). Bylo ovšem dokumentováno, že zejména v případě protilátkových deficiencí nemá uplatnění zmíněné sady 10 varovných příznaků dostatečnou senzitivitu a XLA tak často unikne pozornosti. Přitom diagnostika XLA je založena na jednoduchém vyšetření, stanovení koncentrací imunoglobulinů v séru. Při zachycené hypogamaglobulinemii by měla být vyšetřena koncentrace B lymfocytů v periferní krvi a jejich absence u chlapců pak znamená velmi pravděpodobnou diagnózu XLA.

Naproti tomu komunitní pneumonie se vyskytuje výrazně častěji, v evropské populaci ve věku 0-5 let s incidencí 33/10.000 (Harris et al., 2011). Významná část pacientů, 7-13% vyžaduje hospitalizaci, což činí komunitní pneumonii jednou z nejčastějších příčin hospitalizace v dětském věku (Rudan et al., 2008). Je tedy zřejmé, že jen malá část pneumonií vzniká na podkladě PID obecně či na podkladě XLA. Prevalence PID (XLA) u dětských pacientů s komunitní pneumonií však nebyla dosud systematicky studována.

V naší 3 leté prospektivní observační studii jsme se zaměřili na určení etiologie komunitní pneumonie u dětí hospitalizovaných s touto diagnózou ve FN Motol. Diagnóza pneumonie byla ve sledovaném období potvrzena radiologickým vyšetřením u 254 dětí, z toho u 131 chlapců. Prováděné vyšetření imunoglobulinů odhalilo kompletní agamaglobulinemii u 2 chlapců, u nichž byla následně prokázána i absence B lymfocytů v periferní krvi. U obou byla diagnóza XLA potvrzena molekulárně genetickým vyšetřením genu *BTK*, když v jednom případě byla popsána nová mutace, c.721-249_1026+366del, znamenající rozsáhlou deleci exonů 8-10, a v druhém případě byla detekována známá kauzální mutace spočívající v záměně aminokyseliny na pozici 28, p.Arg28Cys. Teprve po potvrzení diagnózy si

matka druhého chlapce vzpomněla na případ úmrtí bratra své matky v 18 letech věku v důsledku těžké infekce, když do té doby prodělal několik pneumonií, encefalitidu a byl u něj zjištěn i mozkový absces. U obou chlapců byla bez odkladu zahájena substituční terapie imunoglobuliny.

V obou případech byla diagnóza závažné primární protilátkové deficience stanovena při první závažnější manifestaci respirační infekce na základě vyšetření imunoglobulinů, které však v těchto situacích není rutinně prováděno, ani není součástí doporučení věnovaných diagnostice a léčbě dětí s komunitní pneumonií. Citlivý diagnostický pomocník, pozitivní rodinná anamnéza, byla sice v jednom případě přítomna, ale odhalena byla až po stanovení diagnózy. Frekvence XLA ve vyšetřovaném souboru byla výrazně vyšší než by vyplývalo s frekvencí výskytu komunitní pneumonie a XLA v populaci. Může se jednat o chybu malých čísel, ale může to také znamenat, že XLA je významně poddiagnostikované onemocnění. Na základě provedené studie jsme navrhli, aby jednoduché vyšetření imunoglobulinů bylo prováděno u všech případů komunitní pneumonie v dětském věku vyžadujících hospitalizaci. Molekulárně genetické vyšetření může být využito jako účinný nástroj k potvrzení diagnózy, jehož výsledky je možno využít při genetickém poradenství v postižených rodinách.

Následuje plný text článku:

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X-linked Agammaglobulinemia in Community-acquired Pneumonia Cases Revealed by Immunoglobulin Level Screening at Hospital Admission

X-gekoppelte Agammaglobulinämie in Patienten mit ambulant erworbener Pneumonie, entdeckt durch Immunglobulinbestimmung bei der Krankenhausaufnahme

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Key words:
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- immunodeficiency
- humoral immunity
- community-acquired pneumonia
- childhood

Schlüsselwörter
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- humorale Immunität
- ambulant erworbene Pneumonie
- Kindesalter

Abstract

In children with primary immunodeficiencies, the onset of symptoms precedes the diagnosis and the initiation of appropriate treatment by months or years. This delay in diagnosis is due to the fact that while these disorders are rare, some of the infections seen in immunodeficient patients are common. Defective antibody production represents the largest group among these disorders, with otitis, sinusitis and pneumonia as the most frequent initial manifestation. We performed a prospective study of humoral immunity in children hospitalized due to community-acquired pneumonia in tertiary care hospital. Out of 254 patients (131 boys, 123 girls, median age 4.5 years) recruited over 3 years, we found 2 boys (age 11 and 21 months) lacking serum immunoglobulins and circulating B cells. Subsequent genetic analysis confirmed diagnosis of X-linked agammaglobulinemia. Despite their immunodeficiency, the pneumonia was uncomplicated in both patients and did not call for immunological evaluation. However, the immunoglobulin screening at admission allowed for an early diagnosis of the immunodeficiency and timely initiation of immunoglobulin substitution, the key prerequisite for a favorable course of the disease.

Conclusions: Simple and inexpensive immunoglobulin measurement during the management of hospitalized children with community-acquired pneumonia may help in early identification of patients with compromised humoral immunity and prevent serious complications.

Zusammenfassung

Bei Kindern mit primärer Immundefizienz kann die Latenz zwischen klinischer Erstmanifestation und der definitiven Diagnosestellung Monate oder sogar Jahre betragen. Diese Verzögerung in der Diagnosestellung ist u.a. der Tatsache geschuldet, dass angeborene Immundefekte seltene Erkrankungen sind, während einige der Infektionen, zu denen primäre Immundefekte prädisponieren, häufig auftreten. Eine der größten Gruppen innerhalb dieser Erkrankungen stellen Defekte in der Antikörperproduktion dar, die in vielen Fällen als Otitis, Sinusitis und Pneumonie manifest werden. Wir stellen die Ergebnisse einer prospektiven Studie zur humoralen Immunität bei Kindern vor, die mit ambulant erworbener Pneumonie in einem Krankenhaus der maximalen Versorgungsstufe hospitalisiert wurden. Unter 254 Patienten (131 Jungen, 123 Mädchen, Durchschnittsalter 4,5 Jahre), die über einen Zeitraum von 3 Jahren rekrutiert wurden, waren 2 Jungen (11 bzw. 21 Monate alt) mit einem Mangel an Serumimmunglobulinen und erniedrigten B-Lymphozyten. In der genetischen Analyse konnte bei beiden Patienten eine X-chromosomal-vermittelte Agammaglobulinämie diagnostiziert werden. Die Bestimmung der Immunoglobuline bei stationärer Aufnahme erlaubte frühe Diagnosestellung des Immundefekts und einen zeitnahen Beginn der Immunglobulinsubstitution. Beides sind wesentliche Voraussetzung für einen günstigen Krankheitsverlauf.

Schlussfolgerung: Die einfache und kostengünstige Bestimmung von Serumimmunglobulinen während der stationären Behandlung von Kindern mit ambulant erworbenen Pneumonien kann dabei helfen, Patienten mit Defekten der humoralen Immunität frühzeitig zu identifizieren, und schwere Komplikationen verhindern.

Bibliography
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Introduction

The European incidence of community-acquired pneumonia (CAP) is estimated to be 33/10000 in those aged 0–5 years, and 14.5/10000 in those aged 0–16 years as reviewed by Harris et al. [5]. Those numbers certainly vary across the continent. For example the incidence of CAP among children younger 5 years in Schleswig-Holstein has been shown to be much higher (137–169/10000) [12]. Hospital admission is required in 7–13% of cases [8], making CAP one of the most frequent causes for hospitalization in childhood.

Primary immunodeficiency disorders (PID) are rare diseases, often presenting early in life. Affected children frequently suffer from symptoms related to their disease months or years before the diagnosis is made and the appropriate treatment initiated. Among PID, the largest group is represented by defective antibody production, with pneumonia being one of the most prevalent initial symptoms. The prevalence of PID in children with CAP is unknown as reflected in the recently published CAP guidelines [1,5].

A 3-year prospective observational study (2006–2009) was designed to address the etiology and humoral immune status in children admitted to a tertiary-care hospital for CAP. The study was approved according to the Declaration of Helsinki by local Ethical Committee and written parental informed consent was obtained. The inclusion criteria (CAP was defined as a previously well child, not hospitalized 14 days before admission, admitted with fever, symptoms of lower respiratory tract disease and chest X-ray finding compatible with the diagnosis of pneumonia, assessed by a skilled senior pediatric radiologist [9]) were fulfilled in 254 children (131 boys, 123 girls, median age 4.5 years). In all patients complete blood count, standard biochemistry panel, microbiological tests, as well as measurement of immunoglobulin levels and antibodies specific for vaccination antigens were performed on admission. Within this cohort several patients with humoral immunity abnormalities were identified. A detailed description of all results from the study is the subject of another manuscript in preparation. In this paper we concentrate on 2 patients with complete agammaglobulinemia.

Case 1

A previously healthy 21-month-old boy presented to emergency department with fever, laryngeal cough, dyspnea and diarrhea. 3 days before admission he had finished oral antibiotic treatment for uncomplicated CAP. The boy was born at term by Caesarean section from an uneventful dizygotic twin pregnancy after *in vitro* fertilization. Weight at birth was 2890 g, postnatal adaptation uncomplicated. The boy was not breast-fed, he received full vaccination including live attenuated bacillus Calmette-Guérin vaccine (BCG) and live attenuated Polio vaccine as well as measles-mumps-rubella vaccine, all without adverse reactions.

Initial investigations revealed high C-reactive protein (CRP), normal white blood count (WBC) with marked monocytosis, normal lymphocyte count and profound neutropenia (**Table 1**). Chest X-ray (CXR) showed peribronchial infiltrations. *Pseudomonas aeruginosa* was cultivated from stools, urine, as well as upper respiratory tract secretion and blood. Neither immunoglobulins nor circulating B cells were detectable in the serum. Since the agammaglobulinemia was diagnosed, the boy received intravenous immunoglobulins (IVIg, 500 mg/kg/dose) and intravenous antibiotics (3rd generation cephalosporins, aminoglycosides) for the following 2 weeks. His clinical condition improved, and the laboratory parameters subsequently normalized. Regular IVIg substitution every 3 weeks at 500 mg/kg/dose was initiated and he has been doing well since then.

In the meantime, a mutation in *BTK* confirming the diagnosis of XLA was detected (c.721-249_1026+366del, a newly described large deletion of exons 8–10).

Case 2

An 11-month-old boy presented with a history of 3-day fever and elevated inflammatory markers. Because of his young age he was admitted to the pediatric ward for further evaluation.

The boy was born at term following an uneventful pregnancy with normal weight (3100 g). Anorectal atresia was diagnosed soon after birth; colostomy was performed. At 3 months of age he required treatment with intravenous antibiotics for pyelonephritis ascribed to the previous surgical intervention and vesicoureteral reflux. At the age of 6 months plastic surgery of the

	Patient 1	Patient 2
Parameters at admission		
age	21 months	11 months
symptoms	fever, cough, dyspnoe, diarrhoea	fever
chest X-ray	peribronchial infiltration	bilateral peribronchial and paracardial infiltration
immunoglobulin levels (IgG, A, M)	Not detectable	Not detectable (IgE 28 IU/ml)
B cells	Not detectable	Not detectable
C-reactive protein (mg/L)	>160	129
white Blood Cells (10^9/L)	5,5	10
lymphocytes	37%	43%
granulocytes	5%	2%
monocytes	58%	55%
Treatment	antibiotics, IVIg	antibiotics, IVIg
Complications	bacteriaemia (*Pseudomonas aeruginosa*)	none
History known prior diagnosis of XLA		
personal	unremarkable	congenital anorectal atresia
family	unremarkable	unremarkable
Molecular defect in *BTK*	c.721-249_1026+366del	c.214C>T, p.Arg28Cys

Table 1 Characteristics of our patients diagnosed with X-linked agammaglobulinemia.

Table 2 Empirical 10 warning signs of primary immunodeficiency introduced by Jeffrey Model Foundation Medical Advisory Board (www.info4pi.org)

≥ 4 new ear infections within 1 year
≥ 2 serious sinus infections within 1 year
≥ 2 months of oral antibiotic treatment with little effect
≥ 2 episodes of pneumonia within 1 year
failure of an infant to gain weight or grow normally
recurrent, deep skin or organ abscesses
persistent thrush in mouth or fungal infection on skin
need for intravenous antibiotics to clear infections
≥ 2 deep-seated infections, including septicaemia
a family history of PID

anal region was performed; the colostomy was closed at 10 months. He received full vaccination, including live attenuated BCG without adverse reactions. He was still breastfed on admission. Family history taken at the time of admission was unremarkable.

On admission the patient's CXR demonstrated bilateral peribronchial and paracardial infiltration corresponding with pneumonia. No microorganism was isolated from the blood culture. The inflammatory markers (CRP, WBC) were elevated with marked monocytosis, normal lymphocyte count, and profound neutropenia (**Table 1**). Serum immunoglobulins were undetectable (only IgE was 28 IU/ml). Circulating B cells were absent. The patient was treated with intravenous penicillin for 6 days followed by oral treatment for another 3 days. Due to agammaglobulinemia he received a 400 mg/kg/dose of IVIg shortly after admission. Since then, his fever waned and the laboratory parameters normalized. Regular IVIg substitution every 3 weeks of 500 mg/kg/dose was started and he is doing well with a low rate of mild respiratory infections since then.

Diagnosis of XLA was verified by identification of a molecular defect in *BTK* in the patient as well as in his mother, her sister and their mother (p.Arg28Cys, a missense mutation repeatedly described in XLA patients).

After confirming the diagnosis of PID, the patient's mother recalled a remarkable family history. A brother of patient's maternal grandmother had suffered from recurrent pneumonias, encephalitis and a brain abscess and had died at the age of 18 years of severe infection of unknown origin.

Discussion

XLA (MIM 300755) is a rare (2 XLA cases in 1 million boys [11]) humoral immunodeficiency caused by a defect in the gene coding for Bruton's tyrosine kinase (BTK), a cytoplasmic enzyme important in the development of B lymphocytes and myeloid cells. Patients typically suffer from severe and recurrent bacterial infections and they present with low or absent serum immunoglobulins and low or absent circulating B cells. Half of the patients develop symptoms during the first year of life, 90% patients before 5 years of age. Median age at diagnosis of XLA is 26 months. However, some patients despite pronounced clinical symptoms are diagnosed significantly later. About 50% of XLA patients have at least 1 episode of pneumonia prior to diagnosis. Recurrent lower respiratory tract infections lead to a rapid development of irreversible injury of pulmonary tissue, particularly bronchiectasis, reducing quality of life as well as life expectancy [2, 7, 13].

PID are considered to be underdiagnosed or diagnosed with a significant delay [4]. In 1992 the Jeffrey Model Foundation Medical (JFM) Advisory Board introduced empirical warning signs for PID (**Table 2**, www.info4pi.org) that should improve the situation while guiding early diagnosis of PID. Surprisingly, a low reliability of these signs when diagnosing patients with defects in antibody production has been documented. In a retrospective study of 430 patients with defined diagnosis of PID, Subbarayan et al. [10] showed that only a family history positive for PID and antibiotic treatment for more than 2 months help to detect a patient with impairment of antibody production. The recent consensual German interdisciplinary guidelines for diagnosis of PID [3, 6] also highlight increased infection susceptibility as a leading sign of PID. However, in contrast to JFM warning signs, no precise figures, e.g. on frequency of infection episodes or length of therapy, are given. Individual evaluation is emphasized as the differentiation between physiological and pathological situations may differ due to age, social and environmental issues. Generally, further evaluation is only warranted if increased infection susceptibility is suspected. Among the tested laboratory parameters, main emphasis is placed on lymphocyte and neutrophil counts as well as serum immunoglobulin levels. Sensitivity of those guidelines in revealing PID and antibody deficiency, respectively, remains to be evaluated.

The underlying severe humoral immunodeficiency in our patients was detected at their first serious respiratory tract infection thanks to a screening serum immunoglobulin level measurement. The other remarkable abnormalities in the routine laboratory tests were the transient neutropenia and monocytosis **Table 1**, known phenomenon in patients with XLA [2, 7, 13]. However, we suppose that in the pediatric community a transient neutropenia would be more likely regarded as a symptom associated with a sepsis or a viral infection rather than antibody deficiency and would not warrant further immunological work up. Neutropenia listed among the warning signs of PID [3, 6] is presumably associated with symptoms of innate immunity impairment and is of persistent or cyclic character. Also the most sensitive marker of PID – the remarkable family history of the patient 2 – was revealed only after the diagnosis of XLA had already been confirmed by molecular genetic analysis. Thus, we presume that only using JMF warning signs of PID or recent guidelines for PID diagnosis [3, 6], diagnosis of XLA would have be delayed in our patients.

The frequency of 2 boys with a diagnosis of XLA among 131 boys hospitalized with CAP in our cohort is extremely high and presumably does not reflect the real epidemiological situation in the Central and Eastern Europe [11]. However, we believe that our findings may draw attention to the immune status evaluation of children hospitalized with CAP as it has not yet been reflected in the published guidelines for pediatric pneumonia [1, 5].

Admission for CAP could be perceived as a chance for screening investigation of antibody production disorders in a selected pediatric population. As a minimal screening method we propose to measure serum IgG level in all children admitted to the hospital for CAP, especially in boys younger than 5 years in whom neutropenia and/or monocytosis in differential blood count is detected. If the IgG level is below 2 SD for age, lymphocyte immunophenotyping should be performed together with careful reevaluation of the medical history with emphasis on early deaths in the broad family, infectious diseases and failure to thrive. If the B-cell count is normal, secondary causes of hypogammaglobulinemia should be excluded and/or humoral

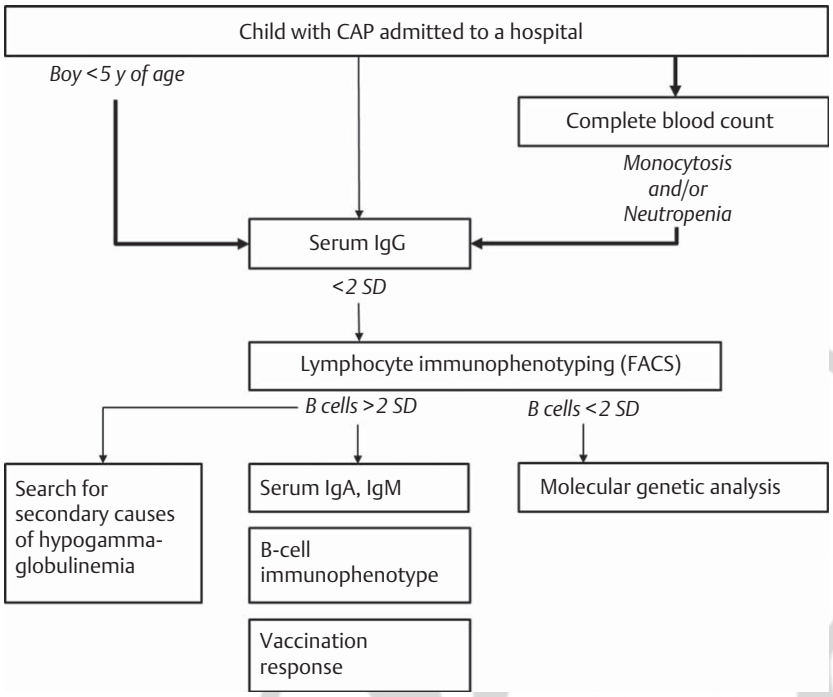

Fig. 1 Minimal algorithm for screening of antibody production disorders (with focus on X-linked agammaglobulinemia) in children admitted to hospital for community-acquired pneumonia.

immunity further investigated based on the clinical phenotype. In case of absent or very low B-cell counts molecular genetic analysis should follow (**Fig. 1**). This approach would reveal patients with agammaglobulinemia, hypogammaglobulinemia and some patients with dysgammaglobulinemia. Certainly, humoral defects that do not affect the total serum IgG level cannot be detected by this simple screening approach. If other signs of immunodeficiency are present or progressively develop, detailed evaluation of immune system should be prompted irrespective of the initial serum IgG level.

The suggested approach may increase the chance of early diagnosis and treatment of antibody production disorders and thus prevent significant later morbidity and mortality and contribute to a better quality of life of the patients.

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References

1 *Bradley JS, Byington CL, Shah SS et al.* The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America. Clin Infect Dis 2011; 53: e25–e76
2 *Conley ME, Howard V.* Clinical findings leading to the diagnosis of X-linked agammaglobulinemia. J Pediatr 2002; 141: 566–571
3 *Farmand S, Baumann U, von Bernuth H et al.* [Interdisciplinary AWMF guideline for the diagnostics of primary immunodeficiency]. Klin Padiatr 2011; 223: 378–385
4 *Gathmann B, Binder N, Ehl S et al.* The European internet-based patient and research database for primary immunodeficiencies: update 2011. Clin Exp Immunol 2012; 167: 479–491
5 *Harris M, Clark J, Coote N et al.* British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. Thorax 2011; 66 (suppl 2): ii1–23
6 *Krudewig J, Baumann U, Bernuth von H et al.* Interdisciplinary AWMF guideline for the treatment of primary antibody deficiencies. Klin Padiatr 2012; 224: 404–415
7 *Ochs HD, Smith CI.* X-linked agammaglobulinemia. A clinical and molecular analysis. Medicine (Baltimore) 1996; 75: 287–299
8 *Rudan I, Boschi-Pinto C, Biloglav Z et al.* Epidemiology and etiology of childhood pneumonia. Bulletin of the World Health Organization 2008; 86: 408–416
9 *Stein RT, Marostica PJ.* Community-acquired pneumonia: a review and recent advances. Pediatr Pulmonol 2007; 42: 1095–1103
10 *Subbarayan A, Colarusso G, Hughes SM et al.* Clinical features that identify children with primary immunodeficiency diseases. Pediatrics 2011; 127: 810–816
11 *Toth B, Volokha A, Mihas A et al.* Genetic and demographic features of X-linked agammaglobulinemia in Eastern and Central Europe: a cohort study. Molecular immunology 2009; 46: 2140–2146
12 *Weigl JA, Bader HM, Everding A et al.* Population-based burden of pneumonia before school entry in Schleswig-Holstein, Germany. Eur J Pediatr 2003; 162: 309–316
13 *Winkelstein JA, Marino MC, Lederman HM et al.* X-linked agammaglobulinemia: report on a United States registry of 201 patients. Medicine (Baltimore) 2006; 85: 193–202

2.2 X-vázaná agamaglubulinemie u dítěte s Klinefelterovým syndromem

XLA je vzácná závažná porucha tvorby protilátek, která je vázaná na chromozom X. Klinefelterův syndrom (KS) je nejčastější vrozená aberace pohlavních chromozomů, která spočívá v přídatném chromozomu X u jedinců mužského pohlaví (karyotyp 47,XXY) (Nielsen and Wohlert, 1990). Spojení obou diagnóz u jednoho nositele bylo popsáno poprvé v našem případě.

Klinefelterův syndrom se vyskytuje s incidencí 1:500, zatímco XLA postihuje jen 1 chlapce ze 190.000. Klinická manifestace přídatného X chromozomu může být velmi subtilní, nositelé této aberace mají vyšší postavu, nevyskytují se u nich žádné dysmorfické rysy, mohou mít hypospadii, kryptorchismus, typická je absence spermatogeneze a androgenová deficience. Diagnóza je často odhalena až při vyšetřování infertility. Jen asi 10% jedinců s KS je diagnostikováno před dosažením puberty a asi 75% zůstává nediagnostikováno po celý život. Onemocnění vázané na chromozom X se u nich projeví tehdy, pokud postihuje oba X chromozomy (Lahlou et al., 2011; Tuttelmann and Gromoll, 2010).

U chlapce rumunského původu s negativní rodinnou anamnézou a normálním vývojem bylo v rámci vyšetřování pro pneumonii v 6 letech provedeno vyšetření imunoglobulinů, které odhalilo závažnou hypogamaglobulinemii, následně byla dokumentována absence B lymfocytů v periferní krvi a bylo vysloveno podezření na XLA. Stojí za zmínku, že při postupu podle platných doporučení by vyšetření imunoglobulinů nebylo provedeno a došlo by přinejmenším k oddálení určení diagnózy a opoždění v zahájení adekvátní léčby. Z důvodu unilaterálního kryptorchismu doporučil klinický genetik psychologické vyšetření, které ukázalo mírné intelektuální a emoční opoždění. Následné vyšetření karyotypu vedlo k diagnóze KS. Paralelně byla molekulárně genetickým vyšetřením detekována missense mutace v SH2 doméně genu *BTK* na obou X chromozomech. Matka byla potvrzena jako nositelka mutace.

Přítomnost mutace na obou X chromozomech svědčí nejspíše pro chybu ve druhém meiotickém dělení v průběhu oogeneze. Tento typ non-disjunkce nesouvisí s vyšším věkem matek (Harvey et al., 1990), což odpovídalo našemu případu, kdy matka měla v době porodu 25 let.

Tak jako u žen dochází u jedinců s KS k inaktivaci jednoho X-chromozomu. Nicméně asi 5% genů uniká inaktivaci a dalších 10% genů může vykazovat variabilní obraz inaktivace (Carrel et al., 1999). Navíc asi 1% genů leží v pseudoautozomálních oblastech. Jakkoliv se v našem případě jedná nejspíše o náhodnou koincidenci obou onemocnění, vyvstávají úvahy, zda produkty zmíněných genů mohou ovlivnit klinickou manifestaci X-vázaného onemocnění u jedinců s KS. První závažná manifestace PID u našeho chlapce nastala v 6 letech, ale je obtížné spekulovat o případném protektivním účinku extra X chromozomu před rozvojem infekčních komplikací, protože data z lidských studií ani ze zvířecích

modelů nejsou k této problematice podle našich vědomostí ve světovém písemnictví k dispozici. Bude také zajímavé sledovat chlapce z hlediska rozvoje autoimunitních komplikací, jejichž vyšší výskyt není v souvislosti s XLA popisován, ale je známo, že riziko autoimunitních chorob je významně asociováno s ženským pohlavím a tedy hypoteticky s přítomností dvou X chromozomů (Gleicher and Barad, 2007).

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ASTUTE CLINICIAN REPORT

X-Linked agammaglobulinemia in a child with Klinefelter's syndrome

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Abstract Bruton's agammaglobulinemia is a rare X-linked humoral immunodeficiency manifesting with recurrent bacterial infections early in life. Klinefelter's syndrome caused by an additional X chromosome is the most common sex chromosome disorder. A previously unreported association of these two conditions is described here.

Keywords X-linked agammaglobulinemia · Klinefelter's syndrome · immunodeficiency

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Introduction

X-linked agammaglobulinemia (XLA) (MIM#300755) is a rare humoral immunodeficiency, occurring in 1 of 190.000 male births. It is caused by a defect in the gene coding for Bruton's tyrosine kinase (BTK), a cytoplasmic enzyme important in the development of B lymphocytes and myeloid cells. Patients typically suffer from severe and recurrent bacterial infections and they present with low or absent serum immunoglobulins and circulating B cells. Half of the patients develop symptoms during the first year of life, 90 % patients before 5 years of age. Median age at XLA diagnosis is 26 months. Typical symptoms are recurrent sinopulmonary, enteric and skin infections, as well as arthritis. Apart from bacterial agents, patients are endangered by enteroviral infections that may be fatal. Patients are treated with lifelong immunoglobulin substitution [1, 2].

Klinefelter's syndrome (KS) is the most common chromosome aneuploidy in males, with an incidence of 1:500 [3]. Patients carry an additional X chromosome (47,XXY), they are typically tall, with sparse body hair, narrow shoulders, broad hips, and normal to slightly decreased verbal intelligence. There is no facial dysmorphism and the phenotype may vary from absent spontaneous puberty to normally virilized men. KS men may manifest subtle clinical signs, such as hypospadias, small phallus, small firm testis or cryptorchidism, absent spermatogenesis and androgen deficiency. Osteoporosis, varicose veins, thromboembolic disease and diabetes mellitus are more frequent in these patients [4–6]. Only about 10 % of KS men are diagnosed before puberty and just around 25 % during their lifetime [7]. The diagnosis is often determined when the KS men consult physicians about their infertility. Although there are no specific therapeutic recommendations for KS, guidelines on the treatment of testosterone deficiency in general can also be used for these

patients [8]. In rare cases, viable sperm can be obtained from individual testicular tubules by biopsy and used for in vitro fertilization.

To the best of our knowledge an association between XLA and KS has never been reported, a case is briefly recorded here.

Case report

The patient is a Caucasian boy, term born and with an apparently normal growth and development until the age of 6 years. He is the first-born child of the family. Since he started kindergarten at the age of 3 until the age of 6, just two episodes of bronchitis and several common upper airways infections were reported. Then at 6 years of age he presented with fever, cough and malaise and right upper lobe pneumonia was diagnosed. Profound hypogammaglobulinemia was detected (IgG 2.58 g/l, IgA 0.03 g/l, IgM 0.27 g/l) by chance and flow cytometric evaluation of peripheral blood lymphocytes showed markedly decreased numbers of $CD19^{pos}$ cells (2 cells/μl, 0.05 % of lymphocyte count), while other lymphocyte subsets were normal. Family history was negative.

The physical examination of the boy was unremarkable except for hypoplastic tonsils and unilateral cryptorchidism. A clinical geneticist recommended a psychological evaluation, which indicated a mild intellectual and emotional developmental delay. KS was suspected and confirmed by karyotype analysis and fluorescence in situ hybridization (see Fig. 1).

Hypogammaglobulinemia and B-cell lymphopenia prompted *BTK* gene sequencing, a missense mutation p.His362Arg in the exon 12 was detected on both of the patient's X chromosomes (see Fig. 2). The same mutation was found in his mother, but not maternal grandmother.

Intravenous immunoglobulin (IVIg) substitution was initiated (0.4 g/kg/4 weeks) with prompt positive clinical response. Due to increased frequency of upper respiratory tract infections a year later, the substitution had to be increased up to 0.6 g/kg/4 weeks. Since then the patient has been free of significant infections and thrived. At the age of 10 years endocrinology consult is planned to evaluate possible hormonal replacement therapy.

Discussion

Despite not supported by guidelines [11–13], a screening of immunoglobulin levels was performed in our patient and it led to detection of profound hypogammaglobulinemia and subsequently to a diagnosis of XLA. Of note, as the course of pneumonia was uncomplicated and the family history was negative, immunodeficiency had not been suspected. Without immunoglobulin screening, the diagnosis of XLA and proper treatment would have been delayed. Similar observation was reported by Vancikova et al. [14] who suggested examining immunoglobulin levels in young boys suffering from pneumonia, even if uncomplicated and occurring for the first time.

Unilateral cryptorchidism and mild intellectual and emotional delay raised suspicion of KS. Diagnosis was confirmed by two independent methods.

In KS the redundant X chromosome results sporadically from either meiotic (meiosis I, II) nondisjunction in germinal cells or from mitotic non-disjunction in the developing zygote. The presence of the same *BTK* mutation on both X chromosomes indicates that the non-disjunction accident leading to KS probably occurred in the second meiotic division of oogenesis (maternal isodisomy). The maternal age is not supposed to play a role as the association of disjunction errors with increasing age of mothers has been attributed only to meiosis I errors [15]. It corresponds to mother's age of 25 at the time of delivery. Less likely the error could have occurred during an early postzygotic mitotic division [16].

In female somatic cells one X chromosome is transcriptionally inactive, in order to equalize the dosage of X-encoded genes to that of male cells. The similar process occurs in KS individuals. However, around 5 % of X-linked genes escape inactivation and an additional 10 % show variable pattern of inactivation [17]. Moreover, about 1 % of genes on X chromosome are located in the pseudoautosomal regions that behave like an autosome and recombine during meiosis. The KS phenotype reflects two or three active copies of X-Y homologous genes from the pseudoautosomal regions as well as the genes which escape X inactivation [4]. Products of these genes may have influenced the clinical manifestation of XLA in our patient.

The preferred (skewed) inactivation of X chromosome carrying the defective *BTK* allele occurs in B cells of female carriers [18]. Similarly, in some cases the heterozygous state (presence of unaffected extra X chromosome in KS patient with X-linked disease) protects the patient from either an increased susceptibility to infections [19] or a lethal effect of the defective allele [20]. However, both X chromosomes are mutated in our case and a random inactivation can be expected.

KS is generally not associated with immunodeficiency, though older surveys report increased incidence of respiratory tract problems in these patients [21, 22]. Our patient was asymptomatic until the age of six and one can speculate whether an additional X chromosome provided some protection against infection. Unfortunately, no data from human or murine studies on this issue is currently available.

Rare cases of KS associated with X-linked immunodeficiencies, other than XLA, have been reported. Individual patients suffered from chronic granulomatous disease (CGD) [21] and X-linked lymphoproliferative syndrome [23]. In addition, two cases of KS brothers of patients with CGD and properdin deficiency, carrying on one of their two X-chromosomes a causal mutation in the *CYBB* and *PFC* gene, respectively, have been published [19, 24]. Interestingly, one

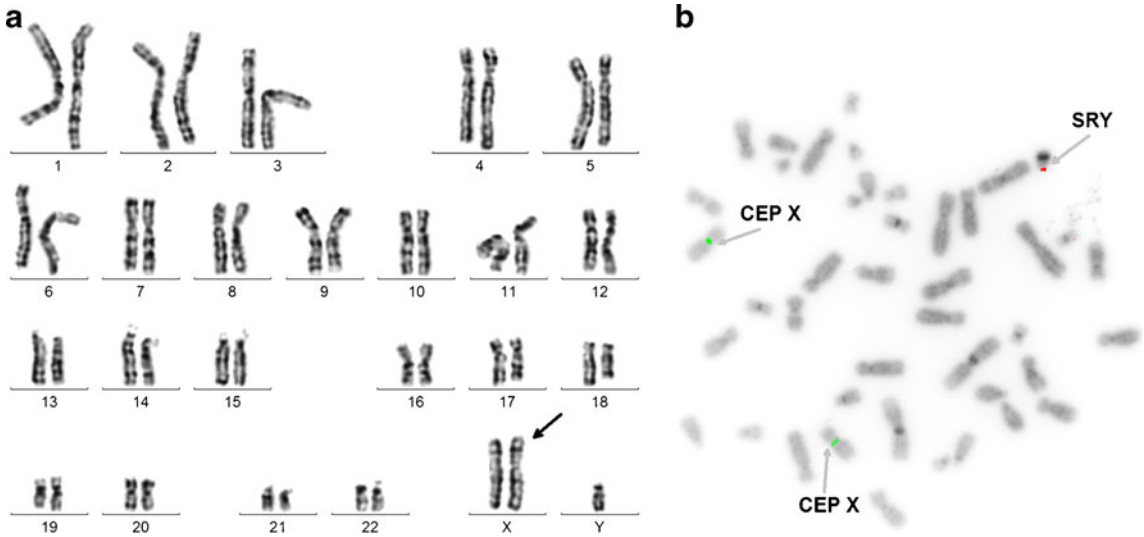

Fig. 1 **a**. Karyotype showing the presence of two X and one Y chromosomes. Peripheral blood specimens were collected and cytogenetic analysis was performed on GTG-banded metaphase spreads obtained from phytohaemagglutinin (PHA)-stimulated lymphocyte cultures. The harvesting of cultures was done after a 72-hour incubation period. **b**. FISH (fluorescence in situ hybridization), 47,XXY.ish X(DXZ1x2), Y(SRY+). Fluorescence in situ hybridization was carried out on metaphase spreads according to supplier's protocol, using probes for the centromeric region of the X chromosome (DXZ1) and the SRY region (Yp11.2) and the Y heterochromatic region (DYZ1) on the Y chromosome (Cytocell Aquarius® SRY kit). (Color Online)

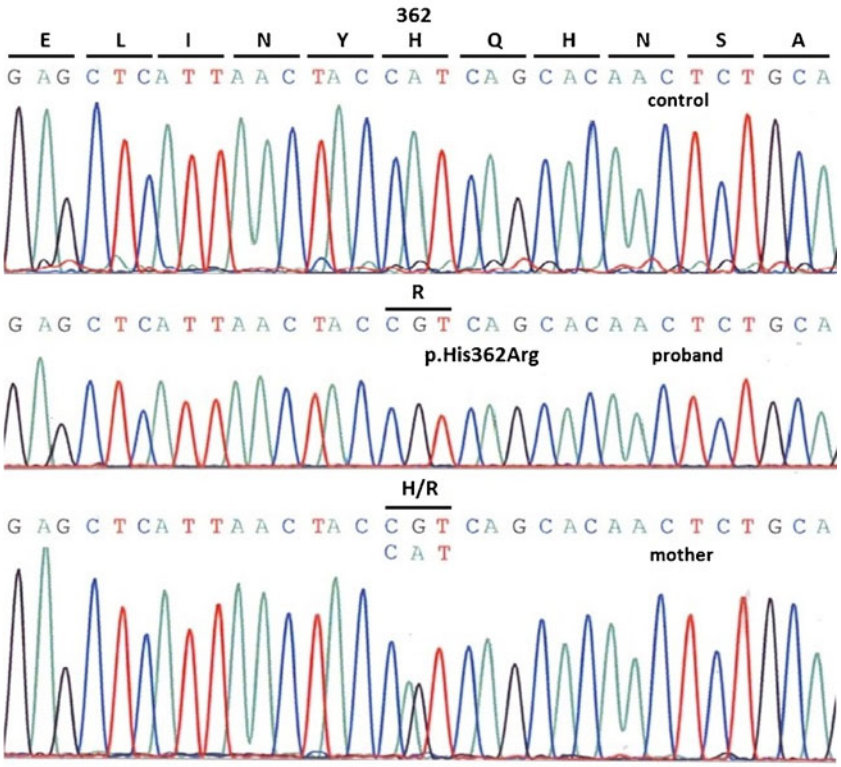

Fig. 2 Electropherograms showing missense mutation p.His362Arg in SH2 domain of the *BTK* gene in the proband (*middle*) compared to control sequence (*top*). Mutation is present on both proband's X chromosomes. Mother (*bottom*) is heterozygous for this mutation. This mutation can be considered as disease causing. It was reported in association with classical XLA phenotype, its pathogenicity was predicted using *in silico* tools (Polyphen, http://genetics.bwh.harvard.edu/pph/; PMut, http://mmb2.pcb.ub.es:8080/PMut/; SIFT, http://sift.jcvi.org/; data not shown), and previously published functional analyses of six missense mutations, located in the same region as p.His362Arg, showed impaired phosphotyrosine binding in all of them and conformation change of the SH2 domain in some of these mutations, including the one that affects the same codon as the one defective in our patient [9, 10]

patient with Wiskott-Aldrich syndrome was treated by hematopoietic stem cell transplantation from his brother with KS [25].

Autoimmune complications frequently occur in patients with primary immunodeficiencies. Women are in general more prone to autoimmune disease development [26]. Due to the possible gene dose effect of immunity-related genes and immune-related regulatory micro RNA [27, 28] on the X chromosome, the KS men share a similar risk of developing autoimmunity as women, as has been clearly shown for SLE [29]. It would be of interest to learn whether the possibly increased susceptibility to autoimmunity is counterbalanced in our patient by the absence of B cells.

The association of Klinefelter's syndrome and X-linked agammaglobulinemia in the same patient has not been reported so far and represents coincidental occurrence. Both diseases arose independently, however, *BTK* gene defect and additional X-chromosome might interfere in phenotype development, particularly in terms of autoimmune complications.

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Conflict of interest The authors declare no conflict of interests.

References

1. Winkelstein JA et al. X-linked agammaglobulinemia: report on a United States registry of 201 patients. Medicine (Baltimore). 2006;85(4):193–202.
2. Ochs HD, Smith CI. X-linked agammaglobulinemia. A clinical and molecular analysis. Medicine (Baltimore). 1996;75(6):287–99.
3. Nielsen J, Wohlert M. Sex chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in Arhus, Denmark. Birth Defects Orig Artic Ser. 1990;26(4):209–23.
4. Tuttelmann F, Gromoll J. Novel genetic aspects of Klinefelter's syndrome. Mol Hum Reprod. 2010;16(6):386–95.
5. Lahlou N et al. Clinical and hormonal status of infants with nonmosaic XXY karyotype. Acta Paediatr. 2011;100(6):824–9.
6. Fruhmesser A, Kotzot D. Chromosomal variants in Klinefelter syndrome. Sex Dev. 2011;5(3):109–23.
7. Bojesen A, Juul S, Gravholt CH. Prenatal and postnatal prevalence of Klinefelter syndrome: a national registry study. J Clin Endocrinol Metab. 2003;88(2):622–6.
8. Swerdloff RS, Wang CCL. Review of guidelines on diagnosis and treatment of testosterone deficiency. In: Nieschlag E, Behre HM, editors. Testosterone: action, deficiency, substitution. 4th ed. Cambridge: Cambridge University Press; 2012. p. 408–20.
9. Brooimans RA, van den Berg AJ, Rijkers GT, Sanders LA, van Amstel JK, Tilanus MG, et al. Identification of novel Bruton's tyrosine kinase mutations in 10 unrelated subjects with X linked agammaglobulinaemia. J Med Genet. 1997;34(6):484–8.
10. Mattsson PT, Lappalainen I, Bäckesjö CM, Brockmann E, Laurén S, Vihinen M, et al. Six X-linked agammaglobulinemia-causing missense mutations in the Src homology 2 domain of Bruton's tyrosine kinase: phosphotyrosine-binding and circular dichroism analysis. J Immunol. 2000;164(8):4170–7.
11. Harris M, Clark J, Coote N, Fletcher P, Harnden A, McKean M, et al. British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. Thorax. 2011;66 Suppl 2:ii1–ii23.
12. Bradley JS, Byington CL, Shah SS, Alverson B, Carter ER, Harrison C, et al. The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the pediatric infectious diseases society and the infectious diseases society of america. Clin Infect Dis. 2011;53(7):e25–76.
13. de Vries E. European society for immunodeficiencies (ESID) members. Patient-centred screening for primary immunodeficiency, a multistage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol. 2012;167:108–19.
14. Vancikova Z et al. X-linked agammaglobulinemia in community-acquired pneumonia cases revealed by immunoglobulin level screening at hospital admission. Klin Padiatr. 2013;225:1–5.
15. Harvey J et al. The parental origin of 47, XXY males. Birth Defects Orig Artic Ser. 1990;26(4):289–96.
16. Jacobs PA, Hassold TJ, Whittington E, Butler G, Collyer S, Keston M, et al. Klinefelter's syndrome: an analysis of the origin of the additional sex chromosome using molecular probes. Ann Hum Genet. 1988;52(Pt 2):93–109.
17. Carrel L et al. A first-generation X-inactivation profile of the human X chromosome. Proc Natl Acad Sci U S A. 1999;96(25):14440–4.
18. Allen RC et al. Application of carrier testing to genetic counseling for X-linked agammaglobulinemia. Am J Hum Genet. 1994;54(1):25–35.
19. Schejbel L et al. Properdin deficiency associated with recurrent otitis media and pneumonia, and identification of male carrier with Klinefelter syndrome. Clin Immunol. 2009;131(3):456–62.
20. Buinauskaite E et al. Incontinentia pigmenti in a male infant with Klinefelter syndrome: a case report and review of the literature. Pediatr Dermatol. 2010;27(5):492–5.
21. Sanders DY, Goodman HO, Cooper MR. Chronic granulomatous disease in a child with Klinefelter's syndrome. Pediatrics. 1974;54(3):373–5.
22. Daly JJ, Hunter H, Rickards DF. Klinefelter's syndrome and pulmonary disease. Am Rev Respir Dis. 1968;98(4):717–9.
23. Harris A, Docherty Z. X-linked lymphoproliferative disease: a karyotype analysis. Cytogenet Cell Genet. 1988;47:92–4.
24. Gill HK, Kumar HC, Cheng CK, Ming CC, Nallusamy R, Yusoff NM, et al. X-linked chronic granulomatous disease in a male child with an X-CGD carrier, Klinefelter brother. Asian Pac J Allergy Immunol. 2013;31(2):167–72.
25. Balci YI, Turul T, Daar G, Anak S, Devecioglu O, Tezcan I, et al. Hematopoietic stem cell transplantation from a donor with Klinefelter syndrome for Wiskott-Aldrich syndrome. Pediatr Transplant. 2008;12:597–9.
26. Gleicher N, Barad DH. Gender as risk factor for autoimmune diseases. J Autoimmun. 2007;28:1–6.
27. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. Nat Rev Immunol. 2010;10(8):594–604.
28. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. Bioessays. 2011;33(11):791–802.
29. Sawalha AH, Harley JB, Scofield RH. Autoimmunity and Klinefelter's syndrome: when men have two X chromosomes. J Autoimmun. 2009;33(1):31–4.

2.3 Ostatní

Dále byly publikovány následující kazuistiky, u nichž byl dokumentován přínos molekulárně genetického vyšetření genu *BTK*:

E. Pařízková, P. Rozsíval, T. Freiberger, D. Komárek: X-vázaná agamaglobulinémie (Brutonova nemoc) – tři kazuistiky a molekulárně genetické studie jejich rodin. Čes.-slov. Pediat., 59, 2004, č. 3, pp. 119-122.

Z. Havlicekova, M. Jesenak, T. Freiberger, P.Banovcin: X-linked agammaglobulinemia caused by new mutation in BTK gene: A case report. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2014; 158(3): 470-473.

3. Význam genů modifikujících průběh primárních protilátkových imunodeficiencí

Na rozdíl od určení mutací v kauzálních genech je analýza genů modifikujících průběh onemocnění zatím záležitostí spíše výzkumnou. Je ovšem zřejmé, že i u monogenních chorob existuje celá řada dalších genetických faktorů, které mají zásadní význam z hlediska věku manifestace onemocnění a tíže symptomů, spektra klinických projevů, prognózy, odpovědi na různé typy léčby a dalších fenotypových aspektů nemocí. U komplexních polygenních chorob je poznání jednotlivých genetických variant v kandidátních genech, jejich vzájemných interakcí a interakcí se zevními vlivy klíčové pro pochopení patogeneze choroby a pro vývoj cílené léčby. Předpokládá se, že podrobnější genetické vyšetření bude v budoucnu základem perzonalizované medicíny, kdy u každého pacienta bude možné stanovit individuální rizika rozvoje jednotlivých komplikací, prognózu a vybrat léčbu na míru, která bude mít optimální účinnost při minimálním riziku nežádoucích reakcí.

3.1 HLA systém

HLA systém je neobyčejně komplexní a polymorfní. Komplexnost spočívá ve velkém množství genů, které systém zahrnuje. Polymorfnost je dána vysokým počtem alel, které se mohou na jednotlivých genových lokusech vyskytovat. Jednotlivé alely kódují molekuly, které jsou schopny vázat různé peptidové fragmenty s různou efektivitou. Některá polymorfní místa jednotlivých HLA antigenů ovlivňují sílu vazby a spektrum proteinů prezentovaných T-lymfocytům, jiná přímo modulují interakci komplexu HLA-antigenní peptid s T buňkou. Každá HLA molekula váže v jedné chvíli pouze jeden peptidový fragment, celkově však může prezentovat celou řadu antigenních peptidů. Omezená HLA výbava každého jedince spolu s širší specificitou HLA molekul pro vazbu antigenů podporují hypotézu, že finální úzká specificita imunitní odpovědi je dána převážně antigenními receptory T-lymfocytů. Na druhou stranu peptidy vážící se ke konkrétní HLA molekule sdílejí určité rysy, které nemohou být ve výbavě peptidů vážících se k jiné HLA molekule. Člověk je ovšem obdán dostatečným počtem různých HLA molekul, které pokrývají prakticky celé spektrum antigenů, se kterými se potkává.

HLA typizace velkých skupin pacientů s různými onemocněními ukázala, že některé alely se vyskytují významně častěji u pacientů s daným postižením než v obecné populaci. Nejčastěji se jedná o choroby, v jejichž etiologii hraje důležitou roli imunitní systém, zejména o autoimunitní onemocnění, ale i o maligní nebo infekční onemocnění, může se ale jednat i o choroby, u nichž zatím žádná významnější etiopatogenetická vazba k imunitnímu systému prokázána nebyla (narkolepsie s vůbec nejsilněji dokumentovanou asociací s HLA, maniodepresívní choroba a další). Vzhledem k tomu, že přítomnost HLA antigenů je vrozená, zatímco takto asociované choroby jsou „získané“, je přesnější

říci, že lidé s určitými HLA antigeny mají zvýšené riziko vzniku některých onemocnění. Z uvedených studií byla odhadována relativní rizika vzniku daného onemocnění u nositelů konkrétního antigenu, resp. alely HLA systému. Jako relativní riziko je označován poměr mezi rizikem vzniku určité choroby u nositele definovaného rizikového faktoru (v tomto případě určitého antigenu HLA systému) a rizikem vzniku této choroby v běžné populaci.

U některých chorob byly zaznamenány velmi silné asociace. Na druhou stranu dosud nebyla popsána situace, kdy se nemoc rozvinula u všech jedinců s daným HLA antigenem. Byly ovšem identifikovány choroby, kdy výrazná většina jedinců, u nichž se nemoc rozvinula, skutečně sdílela konkrétní HLA antigen. To ukazuje na skutečnost, že přítomnost specifického HLA antigenu může být důležitou, ale nikoliv jedinou nebo nezbytnou podmínkou vzniku daného onemocnění. K rozvoji choroby je nutná účast dalších faktorů, jak genetických, tak i faktorů vnějšího prostředí.

Ačkoliv existuje několik hypotéz popisujících předpokládanou úlohu HLA systému v rozvoji autoimunity, přesný model objasňující patogenetické mechanismy autoimunitních procesů ve spojení s HLA molekulami dosud vytvořen nebyl. Asi nejvíce atraktivní hypotéza počítá se specifickou prezentací peptidu, který je spouštěčem daného onemocnění, určitou konkrétní HLA molekulou přímo v místě manifestace onemocnění, přičemž jiná HLA molekula vazby a prezentace tohoto peptidu schopna není. Může jít o autentický nebo modifikovaný vlastní peptid nebo i o cizorodý antigen. Odpověď T lymfocytů pak vede k poškození tkáně a vzniku onemocnění. Jiná hypotéza hovoří o selhání mechanizmu negativní selekce autoreaktivních T lymfocytů v thymu. Například, pokud určitá HLA molekula nenaváže nějaký tělu vlastní antigen s dostatečně vysokou afinitou, nezralý T lymfocyt reagující s tímto antigenem unikne negativní selekci a vyzraje ve funkčně kompetentní buňku schopnou autoimunitní reakce. Nebo se může jednat o ovlivnění repertoáru T-buněčných receptorů, včetně populace regulačních T lymfocytů (Treg), což může také vést ke vzniku a proliferaci autoreaktivních T lymfocytů. V úvahu je nutno vzít i fakt, že asociace genů *HLA* s daným onemocněním může být v některých případech dána pouze tím, že oblast HLA leží v blízkosti genu, který má skutečně kauzální vztah k tomuto onemocnění. Taková asociace je tedy zdánlivá a dochází k ní v důsledku vazebné nerovnováhy mezi určitými *HLA* alelami a mutacemi v kauzálním genu (Gregersen and Behrens, 2006; Shiina et al., 2004).

Kapitola 3.1 byla sepsána s využitím následujících textů:

T. Freiberger: Hlavní histokompatibilitní systém člověka. In Litzman a kol., Základy vyšetření v klinické imunologii, MU Brno, 2009.

T. Freiberger: Genetika imunopatologických stavů. In Souček a kol., Vnitřní lékařství, Grada, 2011.

3.1.1 Asociace HLA s CVID a IgAD

Většina případů CVID a IgAD je sporadických, ale familiární výskyt obou onemocnění byl také přesvědčivě a opakovaně dokumentován. Asi ve 20% publikovaných případů se CVID a IgAD vyskytly v rámci jedné rodiny, přičemž CVID bylo zpravidla zaznamenáno v rodičovské generaci a IgAD u jejich dětí. U některých pacientů byl popsán přechod IgAD do CVID, což by svědčilo pro společný genetický základ obou onemocnění. Až na výjimečné případy u CVID (asi 3%) nebyl dosud identifikován kauzální gen pro vznik CVID nebo IgAD a v současné době se soudí, že v obou případech se majoritně jedná spíše o komplexní polygenně podmíněná onemocnění. Byly uskutečněny asociační studie popisující souvislost obou nemocí s určitými sekvenčními variantami v některých genech, přičemž nejsilnější asociace byly detekovány u genů HLA systému (Cunningham-Rundles and Bodian, 1999; Ferreira et al., 2012; Ferreira et al., 2010; Hammarstrom et al., 2000; Jolles, 2013; Kralovicova et al., 2003; Vorechovsky et al., 1995; Yel, 2010).

Také naše skupina prokázala na začátku tisíciletí souvislost mezi neutrálními aminokyselinami na 57. pozici HLA řetězce DQβ a sporadickou i familiární formou IgAD, zatímco negativně nabitá kyselina asparagová na této pozici představovala protektivní účinek před vznikem onemocnění.

Podrobněji je studie popsána v následující publikaci:

T. Freiberger J. Litzman, E. Vondrušková: Úloha kyseliny asparagové na 57. pozici HLA-DQ beta řetězce u sporadické a familiární formy selektivní deficience IgA. Čas. lék. čes., 140, 2001, č. 24, s. 770-773.

3.2 Manózu vázající lektin

Lektin vázající manózu (MBL), dříve nazývaný také protein vázající manózu (MBP), je důležitou součástí vrozené imunity. Z hlediska struktury se řadí do rodiny tzv. kolektinů a je jedním ze 30 proteinů komplementu. Jeho syntéza probíhá v játrech. Mezi hlavní funkce MBL patří aktivace komplementu tzv. lektinovou cestou. Dále se vazbou na cukerné složky povrchu mikroorganismů podílí na opsonizaci infekčních agens, hraje určitou úlohu při modulaci zánětu, ovlivňuje uvolňování cytokinů monocyty, přispívá k odstraňování alergenů a účastní se apoptózy.

Sérová hladina MBL je geneticky determinována. Gen *MBL2* je lokalizován na chromosomu 10q11.2-q21. Deficit, resp. snížená hladina MBL je způsobena strukturálními mutacemi v kodonech 52, 54 a 57 prvního exonu genu MBL. Tyto mutace se označují písmeny D (vzácnější), B (tato je v Evropě nejčastější, je zastoupena asi u čtvrtiny Evropanů) a C (v Evropě vzácná, častá na africkém kontinentu); zatímco normální varianta je označována písmenem A. Nezanedbatelný vliv na sérovou hladinu MBL má také polymorfismus v promotorové oblasti genu *MBL2*. Popsané varianty jsou označovány -550 H/L, -221 X/Y a +4 P/Q, přičemž běžně se vyskytují promotorové haplotypy LXP, LYP, LYQ a HYP. Haplotyp HYP je spojen s normální až vysokou hladinou MBL, naopak haplotyp LXP určuje významně sníženou hladinu MBL (Kilpatrick, 2002; Turner, 2003).

MBL je proteinem akutní fáze, jeho hladina se tedy mírně zvyšuje v průběhu akutního infektu. Pozitivní vliv na sérovou hladinu MBL má růstový hormon, naopak glukokortikoidy hladinu MBL snižují (Hansen et al., 2001; Naito et al., 1999).

Deficit MBL je spojen se zvýšenou náchylností k infekčním onemocněním, způsobeným především extracelulárními patogeny a patogeny vyvolávajícími respirační infekce v raném dětství. Hladina MBL však nekoreluje se závažností klinických příznaků a k manifestaci imunodeficitu dochází většinou ve chvíli, kdy se objeví ještě další porucha, např. deficit podtříd IgG. Projevy imunodeficitu jsou častější u dětí, u dospělých je většinou kompenzován jinými mechanismy imunitního systému (Turner, 2003).

MBL deficit má modulační vliv na průběh některých infekčních a autoimunitních onemocnění. U pacientů se systémovým lupus erythematosus byla zjištěna vyšší frekvence mutantních alel genu MBL a nižší sérové hladiny proteinu (Garred et al., 2001). U pacientů s revmatoidní artritidou deficit MBL zhoršuje jejich prognózu (Graudal et al., 2000; Saevarsdottir et al., 2001). Spekuluje se o etiologickém vlivu deficitu MBL na rozvoj atopické dermatitidy (Brandrup et al., 1999). Zajímavé, i když poněkud kontroverzní, jsou další zjištění týkající se MBL. Například u pacientů infikovaných virem HIV, kteří mají zároveň strukturální mutaci *MBL2* genu, je popisován rychlejší průběh nemoci a vyšší mortalita (Garred et al., 1997). Při nižší hladině MBL byla u pacientů s hepatitidou C detekována horší odpověď na léčbu interferonem (Matsushita et al., 1998). U pacientů s cystickou fibrózou (CF) koreluje deficit

MBL s horšími plicními funkcemi, nepříznivou prognózou při chronické infekci *Pseudomonas aeruginosa* a s častějším výskytem infekce *Burkholderia cepacia* (Garred et al., 1999). Dále je u pacientů s CF popisována spojitost deficitu MBL s vyšší incidencí jaterní cirhózy (Gabolde et al., 2001). Další autoři zaznamenali u pacientů s deficitem MBL vyšší pravděpodobnost těžšího průběhu při infekci *Plasmodium falciparum* (Luty et al., 1998). Existují dílčí důkazy pro etiologickou spojitost nízké hladiny MBL a vyšší frekvence výskytu opakovaných potratů (Kilpatrick et al., 1995). Ojediněle se hovoří o vyšším výskytu mutantních alel *MBL*2 genu u pacientů s těžkou aterosklerózou (Madsen et al., 1998).

Relativně vysoká frekvence mutantní alel v populaci naznačuje, že nízká hladina MBL by mohla mít i určitý protektivní účinek. Bylo zjištěno, že nižší hladiny MBL chrání proti mykobakteriálním infekcím a viscerální leishmanióze (Hoal-Van Helden et al., 1999; Santos et al., 2001). Je tedy velice pravděpodobné, že MBL napomáhá intracelulárním parazitům, kteří používají receptory komplementu při průniku do buňky.

Prognóza pacientů s deficitem MBL je obecně velice dobrá. Jak je výše uvedeno, může však přispívat ke komplikovanějšímu průběhu různých onemocnění, případně zhoršovat jejich prognózu.

V kapitole 2.2 byl použit text následující publikace:

A. Janda, J. Bartůňková, R. Špíšek, T. Freiberger: Deficit lektinu vázajícího manózu. Čes.-slov. Pediat., 60, 2005, č. 2, pp 79-80.

3.2.1 Stanovení frekvence alel a haplotypů *MBL2* v české populaci

V genetické laboratoři CKTCH v Brně jsme zavedli novou metodu genotypizace *MBL2* a stanovili frekvenci výskytu jednotlivých alel a haplotypů podmiňujících parciální a úplný deficit MBL v české populaci. Polymorfizmy v oblasti promotoru genu *MBL2* byly detekovány pomocí mutačně specifické PCR, varianty v kódující oblasti byly analyzovány metodou multiplexní PCR se vznikem specifických amplikonů o délkách 128, 135 a 143 bp. Pro určení všech genotypových variant bylo zapotřebí jen 4-5 amplifikačních reakcí s vyhodnocením na 2%, resp. 4% agarózovém gelu. Naše metoda se ve srovnání s tehdy používanými genotypizačními technikami založenými na konvenční PCR ukázala být ekonomicky výhodným, méně pracným a přitom rychlým a spolehlivým nástrojem pro vyšetření *MBL2*.

Stanovili jsme frekvenci výskytu jednotlivých genotypových a haplotypových variant v souboru 359 jedinců obecné české populace a porovnali ji s ostatními populacemi. Velmi vzácný hyplotyp LYD,

který byl do té doby popsán jen u 1 osoby evropsko-brazilského původu a 3 polských dětí s opakovanými respiračními infekty, byl detekován u 7 jedinců. Rozložení jednotlivých variant v české populaci se stalo základem pro analýzu výskytu deficitních haplotypů ve specifických skupinách pacientů v dalších studiích.

Následuje plný text článku:

H. Skalníková, T. Freiberger, J. Chumchalová, H. Grombiříková, A. Šedivá: Cost-effective genotyping of human MBL2 gene mutations using multiplex PCR. J Immunol Methods, 295 (1-2), 2004, pp. 139-147. (TF korespondující autor).

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Research paper

Cost-effective genotyping of human *MBL2* gene mutations using multiplex PCR

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Abstract

Mannose-binding lectin (MBL) deficiency is associated with increased susceptibility to various infections and autoimmune disorders. It is caused by certain polymorphisms in the *MBL2* gene promoter and mutations in the coding region of the gene. In this report, we present a novel, rapid, efficient and cost-effective method of two multiplex polymerase chain reactions (PCRs) for the assessment of three structural point mutations within exon 1 at codons 52, 54 and 57. Three additional PCR reactions for the detection of promoter polymorphisms at positions −550 and −221 were performed. *MBL2* haplotypes in 359 individuals of the general Czech population were detected using this approach. The rare LYD haplotype was found in 1.1% of all alleles.

Keywords: MBL; Genotyping; Multiplex PCR; Slavic population

1. Introduction

Mannose-binding lectin (MBL) is a serum protein that can trigger complement activation and thus plays an important role in innate immunity. Low levels of MBL have been shown to be associated with impaired opsonization of pathogenic organisms. This defect then results in recurrent infections in childhood, more severe courses of infections in immunocompromised patients and is also implicated in several autoimmune diseases. The serum levels of MBL are influenced by the presence of promoter polymorphisms and mutations in the 1st exon of the *MBL2* gene (reviewed by Kilpatrick, 2002; Turner, 2003).

The gene encoding MBL (GenBank accession No. AL583855) is located in humans on 10q11.2-q21 and consists of four exons (Sastry et al., 1989). Two

Abbreviations: MBL, mannose-binding lectin; PCR, polymerase chain reaction; ARMS, amplification refractory mutation system; SSOP, sequence-specific oligonucleotide probes.

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principal sequence variants of the promoter region have been described: the nucleotide substitutions G to C in positions −550 (variant H to L) and −221 (variant X to Y). The haplotypes HY, LY and LX are associated with high, intermediate and low MBL serum levels, respectively (Madsen et al., 1995). The variant HX is extremely rare and was described only in three systemic lupus erythematosus patients of Black African ethnicity (Sullivan et al., 1996). It has not been reported so far in Caucasians. An additional substitution T to C at +4 position in the 5′ -untranslated region (variant Q to P) has been described and does not appear to dramatically reduce the MBL serum level (Madsen et al., 1998).

Three principal point mutations have been reported in the coding region of the *MBL2* gene and give rise to the allelic variants B, C and D (also called 0 variants), while the wild-type allele is designated A. The substitutions in codons 54 (allele B) and 57 (allele C) disrupt the amino acid repeat sequence (Glycine–X–Y) of the collagen-like region of MBL protein by the replacement of a glycine residue with an aspartic acid and a glutamic acid, respectively. The mutation in codon 52 (allele D) results in an arginine-to-cysteine substitution in the MBL protein and may lead to the formation of an additional disulphide bond. All three mutations hamper the oligomerization of MBL peptide and have a profound effect on MBL serum concentrations (reviewed by Petersen et al., 2001). Six common (HYA, LYA, HYD, LYB, LYC and LXA) and two rare (HXA and LYD) haplotypes have been reported so far (Sullivan et al., 1996; Boldt and Petzl-Erler, 2002; Garred et al., 2003).

Many approaches to the *MBL2* gene genotyping have been described, e.g., polymerase chain reaction (PCR) followed by restriction enzyme analysis (Madsen et al., 1994), hybridisation with sequence-specific oligonucleotide probes (SSOP; Madsen et al., 1994), amplification refractory mutation systems (ARMS; Davies et al., 1995; Mullighan et al., 2000; Steffensen et al., 2000), a combination of ARMS and SSOP (Crosdale et al., 2000; Boldt and Petzl-Erler, 2002), heteroduplex analysis (Jack et al., 1997), real-time PCR (Hladnik et al., 2002; Steffensen et al., 2003) and a 5′ nuclease assay using minor-groove-binder DNA probes (Van Hoeyveld et al., 2004).

Here, we describe a rapid and cost-effective method for genotyping the *MBL2* gene using multiplex PCR and its use to study *MBL2* allelic frequencies in the Czech population as a representative sample of Slavic populations.

2. Materials and methods

2.1. Samples

Samples of peripheral blood were obtained from (a) 255 healthy children with no history of immunodeficiency and (b) 104 umbilical cord blood samples of healthy neonates after obtaining informed consent from parent or legal guardian. The genomic DNA was extracted from leukocytes by established techniques.

The genotypes of all DNA samples used as positive controls for the presence of any of the 1st exon mutations were confirmed by PCR with CF and CR primers (see below), followed either by restriction analysis with BshNI and MboII (MBI Fermentas, Vilnius, Lithuania) in the case of the B and C alleles, respectively, or by direct sequencing in the case of allele D. Both restriction analyses were performed according to the manufacturer's instructions. Direct sequencing was performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA) on an ABI-310 sequencer (Applied Biosystems), also according to the manufacturer's instructions.

2.2. Primer design

The promoter polymorphisms were detected using the method of the double amplification refractory mutation system (ARMS; Newton et al., 1989). The primer sequences for the promoter genotyping (adapted from Steffensen et al., 2000) were modified in their length and by the addition of noncomplementary nucleotides at the fourth positions from the 3′ end of the primers, to increase the specificity of the reactions (see Fig. 1). Three separate amplifications with sequence specific sense and antisense primers were carried out to determine HY, LY and LX promoter haplotypes (reaction Nos. 1, 2 and 3, respectively, in Table 1). The amplification of a 796-bp segment of the third intron of the HLA-DRB1 gene with primers C3 (5′ GCA TCT TGC TCT GTG CAG AT 3′) and C5 (5′ TGC CAA GTG GAG CAC CCA A 3′) was

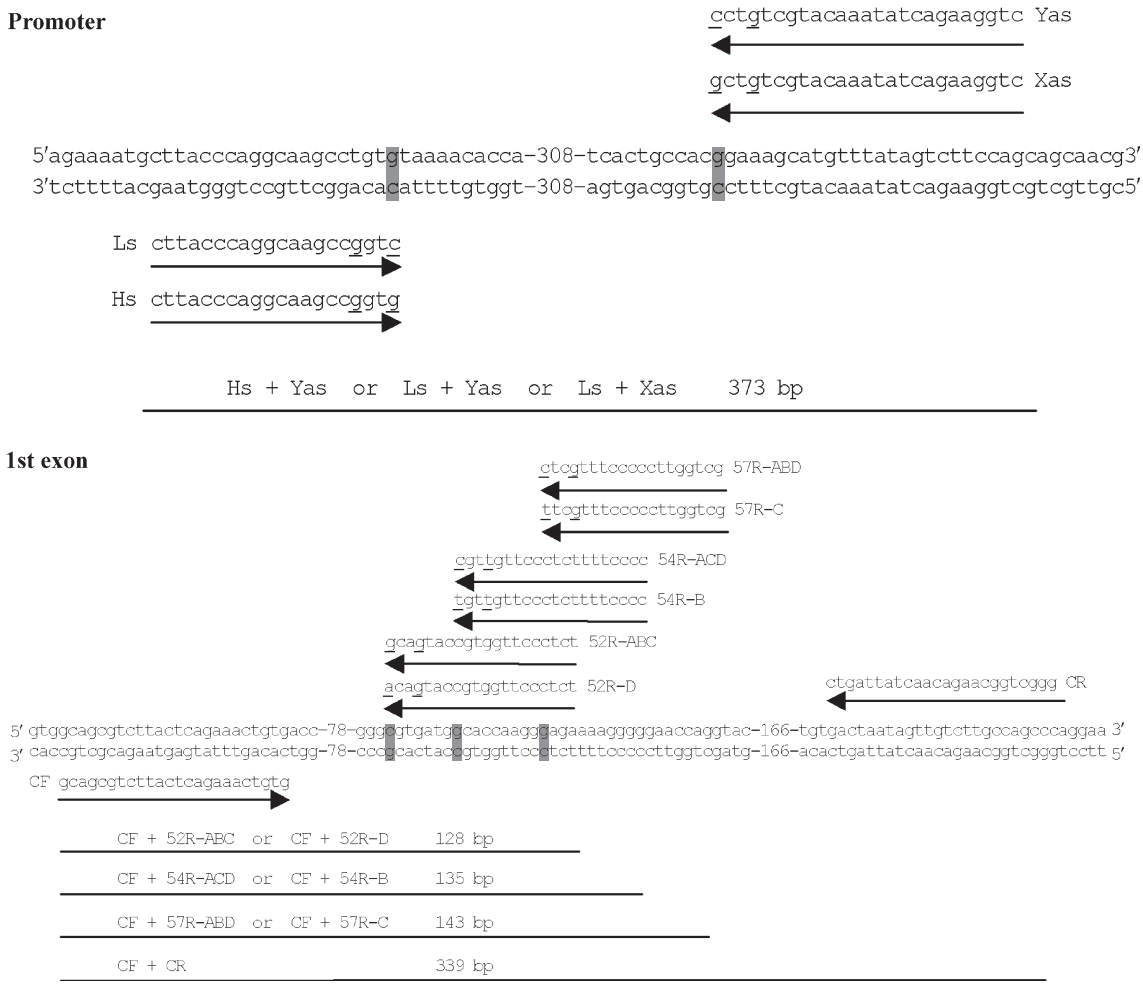

Fig. 1. The *MBL2* gene sequence, primer design and PCR products' lengths. The presented sequence of the HYA haplotype is based on GenBank DNA sequence, accession no. AL583855. The numbers within the sequence represent the number of nucleotides not displayed. The positions of nucleotide substitutions leading to the promoter polymorphisms −550 H/L, −221 X/Y and the 1st exon mutations at codons 52 (A/D), 54 (A/B) and 57 (A/C) are indicated in grey. The positions within the sequences of primer bases providing specificity are underlined. The primers C3 and C5 used as an internal control of amplification in the *MBL2* promoter genotyping are not shown.

performed as an internal control of amplification (Olerup and Zetterquist, 1992).

The multiplex PCR with sequence specific primers was used to genotype the 1st exon of the *MBL2* gene. The primers, including internal control of amplification, were designed using Oligo 4.0 software. Non-complementary nucleotides were placed at the fourth positions from the 3′ end of all the allele specific primers, to reach the higher specificity of the amplification (see Fig. 1). Three antisense primers, 54R-B, 57R-C and 52R-D specific for the B, C and D alleles, respectively, together with common primers CF (sense) and CR (antisense), were used in one reaction (reaction No. 4 in Table 1). The CR primer was added at a 20-fold lower concentration compared with the CF primer. Common CF and CR primers were designed partly to increase the amount of template DNA for specific reactions, partly to serve as an internal control of amplification. The PCR products of particular mutant alleles differed in their length (see Fig. 1 and Table 1). Reaction mix No. 5 (Table 1) contained three antisense primers, 52R-

Table 1
MBL2 genotyping in five PCR reactions

Gene region	Reaction No.	Primers used in PCR[a]	PCR product lengths in the presence of different haplotypes/genotypes		Mg^{2+} [b]	Taq[c]
Promoter	1	Ls (0.6)+Xas (0.6)+C3 (0.6)+C5 (0.6)	LX haplotype present	373+796 bp	1.0	0.75
			LX haplotype not present	796 bp		
	2	Ls (0.6)+Yas (0.6)+C3 (0.6)+C5 (0.6)	LY haplotype present	373+796 bp	1.0	0.75
			LY haplotype not present	796 bp		
	3	Hs (0.6)+Yas (0.6)+C3 (0.6)+C5 (0.6)	HY haplotype present	373+796 bp	1.0	0.75
			HY haplotype not present	796 bp		
1st exon	4	CF (0.4)+CR (0.02)+52R-D (0.2)+54R-B (0.4)+57R-C (0.4)	D/A or D/D genotype	128+339 bp	2.0	1.0
			B/A or B/B genotype	135+339 bp		
			C/A or C/C genotype	143+339 bp		
			D/B genotype	128+135+339 bp		
			D/C genotype	128+143+339 bp		
			B/C genotype	135+143+339 bp		
			A/A genotype	339 bp		
	5	CF (0.4)+CR (0.02)+52R-ABC (0.2)+54R-ACD (0.4)+57R-ABD (0.4)	D/D genotype	135+143+339 bp	2.0	1.0
			B/B genotype	128+143+339 bp[d]		
			C/C genotype	128+135+339 bp		
			A/A, A/B, A/C or A/D genotype	128+135+143+339 bp		

[a] Primer concentrations in μM are listed in parentheses.
[b] Concentration of Mg^{2+} in mM.
[c] Concentration of Taq DNA polymerase in U/reaction.
[d] In the case of B/B homozygotes, a 128-bp PCR fragment is not reliably detected (see Fig. 1 and Discussion).

ABC, 54R-ACD and 57R-ABD, specific for allele A in codons 52, 54 and 57, respectively. This setup was used to discriminate between A/0 heterozygotes and homozygotes for a given mutant allele, provided that such allele had been detected in the previous reaction (No. 4 in Table 1). Thus, theoretically, the PCR product of 128, 135 or 143 bp should be absent in the case of D/D, B/B or C/C homozygosity, respectively.

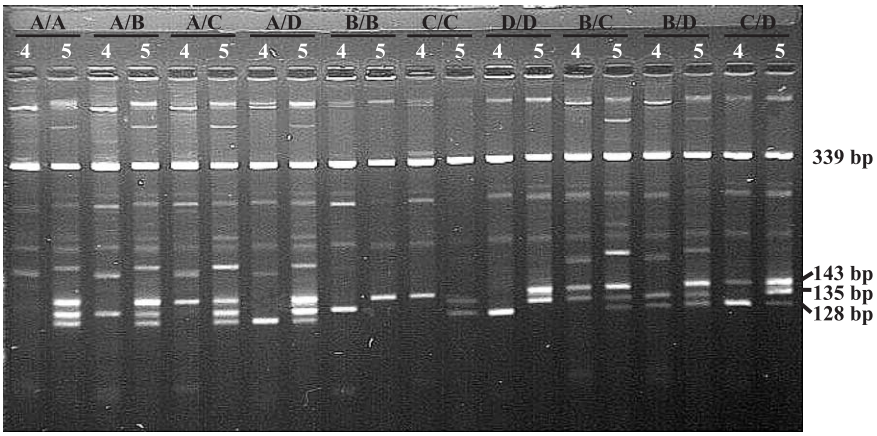

Fig. 2. Results of the 1st exon genotyping of different *MBL2* genotypes. The letters indicate *MBL2* genotypes. The numbers 4 and 5 correspond to the reactions in Table 1. The primers specific for B, C and D alleles were used in reaction No. 4, while primers specific for A allele in codons 52, 54 and 57 were used in reaction No. 5. The lengths of PCR products specific for the B, C and D alleles are 135, 143 and 128 bp, respectively. The common band amplified with CF and CR primers is 339 bp long (see also Table 1 and Fig. 1). A 4% MetaPhor agarose gel electrophoresis was used.

On the other hand, all three products should be detected in the presence of the A allele after PCR reaction No. 5 (see Table 1).

The sequences and sites of annealing of the primers, as well as the composition of primer mixes and lengths of the PCR products, are presented in Fig. 1 and Table 1.

2.3. PCR

The reactions were performed in 25-μl volumes that contained approximately 500 ng of genomic DNA and 0.02 to 0.6 μM of the specific primers in the presence of 1 to 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris, pH 8.4, 0.2 mg/ml albumin (BSA) and 0.75 to 1 U Taq DNA polymerase (Invitrogen, Paisley, UK; see Table 1).

All PCRs were initiated by a denaturation step at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 62 °C, and 30 s (in the case of the 1st exon analysis) to 60 s (in case of the promoter genotyping) at 72 °C. Reactions were completed by an extension step at 72 °C for 7 min.

2.4. Detection of PCR products

PCR products specific for the particular promoter polymorphisms and the 1st exon alleles were resolved by electrophoresis in 2% agarose (voltage 100 V for 60 min) or in 4% MetaPhor agarose (Cambrex, East Rutherford, NJ, USA; 110 V for 3 h), respectively. The gels were stained with ethidium bromide and visualised with UV light.

2.5. Estimation of haplotypes

The assignment of the haplotypes was based on the strong linkage disequilibrium between the promoter variants and the 1st exon alleles and the existence of frequent haplotypes HYA, LYA, HYD, LYB, LYC and LXA. All suspected or possible LYD haplotypes were evaluated by specific PCR (see below).

2.6. Detection of LYD haplotype

The primers 5′ CTCTGCCAGGGCCAACGTA 3′ (sense) and 5′ CCTCTGGAAGGTAAAGAATTG-CAG 3′ (antisense) were designed (using Oligo 4.0 software) to amplify a 914-bp DNA fragment in a sample that appeared likely to carry the LYD haplotype. The 50 μl PCR reaction mix contained approximately 1 μg of genomic DNA, 0.4 μM of each primer, 1.0 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris, pH 8.4, 0.2 mg/ml albumin (BSA) and 2.5 U Taq DNA polymerase (Invitrogen). The cycling conditions were 5-min denaturation at 95 °C, 40 cycles of 60 s at 95 °C,

Table 2
Complete genotype frequencies determined in the Czech population

Complete genotype	Absolute frequency (*n*)	Relative frequency (%)
Normal MBL plasma concentrations[a]		
HYA/HYA	30	8.36
HYA/LYA	70	19.50
HYA/LXA	66	18.38
LYA/LYA	24	6.69
LYA/LXA	39	10.86
Subtotal	229	63.79
Intermediate MBL plasma concentrations[a]		
HYA/LYB	27	7.52
HYA/LYC	0	0.00
HYA/HYD	9	2.51
HYA/LYD	1	0.28
LYA/LYB	22	6.13
LYA/LYC	3	0.84
LYA/HYD	11	3.06
LYA/LYD	1	0.28
LXA/LXA	15	4.18
Subtotal	89	24.79
Low MBL plasma concentrations[a]		
LXA/LYB	20	5.57
LXA/LYC	1	0.28
LXA/HYD	6	1.67
LXA/LYD	2	0.56
LYB/LYB	4	1.11
LYB/LYC	2	0.56
LYB/HYD	3	0.84
LYB/LYD	1	0.28
LYC/LYC	0	0.00
LYC/HYD	0	0.00
LYC/LYD	0	0.00
HYD/HYD	0	0.00
HYD/LYD	1	0.28
LYD/LYD	1	0.28
Subtotal	41	11.42
Total	359	100.00

[a] Plasma concentration groups established according to Schmiegelow et al., 2002.

60 s at 57 °C, and 60 s at 72 °C, with a final extension at 72 °C for 15 min. Then, the PCR product was purified using a Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pCR2.1 (TOPO TA cloning kit; Invitrogen) according to the manufacturer's instructions. Both strands of the insert were sequenced (as described above) to confirm the existence of the LYD haplotype sequence.

All DNA samples containing the D allele were further amplified using the LYD specific (Ls and 52R-D) or the HYD specific (Hs and 52R-D) primers to distinguish between the respective haplotypes. The composition of the reaction mix was the same as used for the promoter and the 1st exon genotyping but contained 2.0 mM $MgCl_2$ and 0.4 μM of each primer. The amplification conditions were 40 cycles of 95 (30 s), 66 (30 s) and 72 °C (40 s), followed by a final 7-min, 72 °C extension step. Both LYD and HYD haplotype-specific PCR reactions yielded 811-bp fragments after the respective reaction mixes were resolved by electrophoresis in 2% agarose gels.

3. Results

The DNA samples of 359 individuals were analyzed. For each sample, four PCR reactions were performed (three for promoter genotyping and one for the detection of mutant allele(s) of the 1st exon; reaction Nos. 1–4 in Table 1). One additional PCR amplification was carried out (reaction No. 5 in Table 1) if one mutant allele was detected within exon 1. All samples carrying the D allele were further analyzed for linkage with the LY or HY promoter variants. All potential variants of the 1st exon genotypes were tested and appeared to be clearly distinguishable (see Fig. 2).

The frequencies of individual genotypes in the Czech population studied and the estimated haplotype frequencies are summarized in Tables 2 and 3, respectively. The genotypes associated with normal (YA/YA or YA/XA), intermediate (YA/0 or XA/XA) and low (XA/0 or 0/0) plasma MBL concentrations were detected in 63.8%, 24.8% and 11.4% of individuals, respectively.

The D allele was previously described particularly as a part of the HYD haplotype. However, we have found seven individuals carrying the D allele who did not have the HY promoter haplotype, and three of them were LY homozygotes. Therefore, it appears that the D allele can be present in conjunction with both promoter variants (LY and HY) in the Czech population. The rare LYD haplotype was found in seven individuals, including one case in homozygous configuration, suggesting a relative frequency of 1.11±0.39% in the Czech population. The presence

Table 3
Haplotype frequencies in the Czech population compared with other Caucasian populations

	HYA	LYA	LXA	LYB	LYC	HYD	LYD	*n*
Australian (blood donors)[a]	0.265	0.267	0.218	0.144	0.030	0.076		236
British[b]	0.353	0.211	0.232	0.132	0.016	0.058	0	100
Czech[c]	0.325	0.270	0.228	0.116	0.008	0.042	0.011	359
Danish[d]	0.310	0.230	0.260	0.110	0.030	0.060	0	250
Danish[e]	0.285	0.280	0.195	0.135	0.020	0.085	0	100
Euro-Brazilian[f]	0.342	0.257	0.220	0.114	0.002	0.062	0.002	202
Polish[g]	0.513	0.256	0.128	0.090	0.006	0.006	0	78
Spanish[h]	0.329	0.322	0.185	0.091	0.011	0.062	0	137

n=Total number of genotypes analyzed.
[a] Minchinton et al., 2002.
[b] Mullighan et al., 2000.
[c] This study.
[d] Madsen et al., 1998.
[e] Steffensen et al., 2000.
[f] Boldt and Petzl-Erler, 2002.
[g] Cedzynski et al., 2004.
[h] Villarreal et al., 2001.

of the LYD haplotype was confirmed by cloning and sequencing of the particular PCR product. In all cases, the LYD haplotype was verified by ARMS.

4. Discussion

Multiplex PCR represents a fast and inexpensive method for the detection of specific DNA sequences. These advantages are particularly important if there is a need for analysis of a large number of samples and/or various regions of the gene, which is the case of *MBL2* genotyping.

The typing of the *MBL2* promoter polymorphisms presented here was based on PCR with sequence specific primers described by Steffensen et al., 2000. Their procedure was very sensitive to Mg^{2+} concentrations, and therefore, we modified the allele specific primer sequences by a noncomplementary nucleotide at the fourth position from the 3′ end of the primer. Primers were also modified in their length. The combination of these refinements would appear to increase the robustness of the reactions. We did not include HX promoter variant detection because it has not yet been reported in Caucasians. We have also omitted analysis of the P/Q polymorphism at the +4 position because it does not significantly influence the MBL plasma level (Madsen et al., 1998).

Concerning the genotyping of the 1st exon variants, we focused on minimising the number of reactions that would be necessary for the detection of all common alleles. The method of multiplex PCR presented in this study permits the fast, efficient, reliable and cost-effective genotyping of the *MBL2* gene in a single tube and with only one additional reaction to test for homozygozity for the B, C and D alleles. Two more amplification reactions are necessary to discriminate between the LYD and HYD haplotypes in samples positive for the D allele. Thus, all frequently occurring haplotypes are determined using four to five PCR reactions. All of the designed reactions included an internal control of amplification.

By optimising the PCR conditions of the 1st exon genotyping, we were unable to completely prevent the generation of nonspecific PCR products without compromising the detection sensitivity for the studied alleles. However, the nonspecific PCR products differed significantly in length from the expected specific amplified fragments and, thus, did not interfere with the analysis (see Fig. 2). We have noted that the quality of PCR products was somewhat variable in various *MBL2* genotypes despite our efforts to optimize the PCR conditions. One explanation could be that this was due to the sequence overlap of certain primers that were used. In particular, we were unable to reliably detect a 128-bp specific product in B/B homozygotes in the reaction with A allele specific primers, while a 143-bp band was always present (reaction No. 5). It is conceivable that the presence of the mutant B allele in the homozygous state introduces an additional sequence mismatch that hinders the annealing of the 52R-ABC primer and, thus, the amplification of the 128 bp product, while the template for the 57R-ABD primer and, hence, the amplification of the 143-bp product, remain intact (see Figs. 1 and 2). Nevertheless, the patterns of all genotypes were clearly distinguishable (see Table 1, Fig. 2).

We are aware that our analysis provides only an estimate of the particular haplotypes based on previously described combinations of the promoter and the 1st exon *MBL2* gene variants. However, the estimates appear to be quite satisfactory and accurate for the vast majority of samples examined due to the strong linkage disequilibrium between the exon 1 and promoter polymorphisms. It is likely that only very rare previously undetected haplotypes may evade detection or be incorrectly analyzed.

One technical drawback of our method is the use of the MetaPhor agarose gels, which are expensive and complicated to handle compared with conventional agarose gels. However, the PCR products of 128, 135 and 143 bp could be unequivocally discriminated on the 4% MetaPhor agarose gels, which were still more convenient and provided better results in our hands than did polyacrylamide gels.

We have used the novel method of multiplex PCR described here to analyze the *MBL2* gene polymorphisms of DNA samples from 359 unrelated Czech residents as representatives of the Slavic population. The haplotype and genotype frequencies in comparison with those of other Caucasian populations are listed in Table 3. The rare haplotype LYD has been found only in one Euro-Brasilian person (Boldt et al., 2002) and three Polish children with recurrent respiratory infections (Cedzynski et al., 2004). We detected this haplotype in seven individuals (in one of them in

the homozygous state) and estimated its frequency as 1.1% in the Czech population. This is a significantly higher proportion than expected. It may suggest that the LYD haplotype is much more common in individuals of Slavic ancestry compared with other Caucasians.

In summary, our novel multiplex PCR method for *MBL2* genotyping is faster, needs less amplification reactions and is convenient when compared with previously used methods based on conventional PCR.

Acknowledgement

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References

Boldt, A.B.W., Petzl-Erler, M.L., 2002. A new strategy for Mannose-binding lectin gene haplotyping. Human Mutation 19, 296.

Cedzynski, M., Szemraj, J., Swierzko, A.S., Bak-Romaniszyn, L., Banasik, M., Zeman, K., Kilpatrick, D.C., 2004. Mannan-binding lectin insufficiency in children with recurrent infections of the respiratory system. Clinical and Experimental Immunology 136, 304.

Crosdale, D.J., Ollier, W.E., Thomson, W., Dyer, P.A., Jensenius, J., Johnson, R.W., Poulton, K.V., 2000. Mannose binding lectin (MBL) genotype distributions with relation to serum levels in UK Caucasoids. European Journal of Immunogenetics 27, 111.

Davies, E.J., Snowden, N., Hillarby, M.Ch., Carthy, D., Grennan, D.M., Thomson, W., Ollier, W.E.R., 1995. Mannose-binding protein gene polymorphism in systemic lupus erythematosus. Arthritis and Rheumatism 38, 110.

Garred, P., Larsen, F., Madsen, H.O., Koch, C., 2003. Mannose-binding lectin deficiency—revisited. Molecular Immunology 40, 73.

Hladnik, U., Braida, L., Boniotto, M., Pirulli, D., Gerin, F., Amoroso, A., Crovella, S., 2002. Single-tube genotyping of MBL-2 polymorphisms using melting temperature analysis. Clinical and Experimental Medicine 2, 105.

Jack, D., Bidwell, J., Turner, M., Wood, N., 1997. Simultaneous genotyping for all three known structural mutations in the human mannose-binding lectin gene. Human Mutation 9, 41.

Kilpatrick, D.C., 2002. Mannan-binding lectin: clinical significance and applications. Biochimica et Biophysica Acta 1572, 401.

Madsen, H.O., Garred, P., Kurtzhals, J.A., Lamm, L.U., Ryder, L.P., Thiel, S., Svejgaard, A., 1994. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics 40, 37.

Madsen, H.O., Garred, P., Thiel, S., Kurtzhals, J.A.L., Lamm, L.U., Ryder, L.P., Svejgaard, A., 1995. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. The Journal of Immunology 155, 3013.

Madsen, H.O., Satz, M.L., Hogh, B., Svejgaard, A., Garred, P., 1998. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and south America. The Journal of Immunology 161, 3169.

Minchinton, R.M., Dean, M.M., Clark, T.R., Heatley, S., Mullighan, C.G., 2002. Analysis of the relationship between Mannose-binding lectin (MBL) genotype, MBL levels and function in an Australian blood donor population. Scandinavian Journal of Immunology 56, 630.

Mullighan, C.G., Marshall, S.E., Welsh, K.I., 2000. Mannose binding lectin polymorphisms are associated with early age of disease onset and autoimmunity in common variable immunodeficiency. Scandinavian Journal of Immunology 51, 111.

Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C., Markham, A.F., 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Research 17, 2503.

Olerup, O., Zetterquist, H., 1992. HLA-DR typing by PCR amplification with sequence specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor–recipient matching in cadaveric transplantation. Tissue Antigens 39, 225.

Petersen, S.V., Thiel, S., Jensenius, J.Ch., 2001. The mannan-binding lectin pathway of complement activation: biology and disease association. Molecular Immunology 38, 133.

Sastry, K., Herman, G.A., Day, L., Deignan, E., Bruns, G., Morton, C.C., Ezekowitz, R.A.B., 1989. The human mannose-binding protein gene. Exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and localization to chromosome 10. Journal of Experimental Medicine 170, 1175.

Schmiegelow, K., Garred, P., Lausen, B., Andreassen, B., Petersen, B.L., Madsen, H.O., 2002. Increased frequency of mannose-binding lectin insufficiency among children with acute lymphoblastic leukemia. Blood 100, 3757.

Steffensen, R., Thiel, S., Varming, K., Jersild, C., Jensenius, J.Ch., 2000. Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. Journal of Immunological Methods 241, 33.

Steffensen, R., Hoffmann, K., Varming, K., 2003. Rapid genotyping of *MBL2* gene mutations using real-time PCR with fluorescent hybridization probes. Journal of Immunological Methods 278, 191.

Sullivan, K.E., Wooten, C., Goldman, D., Petri, M., 1996. Mannose-binding protein genetic polymorphisms in black patients with systemic lupus erythematosus. Arthritis and Rheumatism 39, 2046.

Turner, M.W., 2003. The role of mannose-binding lectin in health and disease. Molecular Immunology 40, 423.

Van Hoeyveld, E., Houtmeyers, F., Massonet, C., Moens, L., Van Ranst, M., Blanckaert, N., Bossuyt, X., 2004. Detection of single nucleotide polymorphisms in the mannose-binding lectin gene using minor groove binder-DNA probes. Journal of Immunological Methods 287, 227.

Villarreal, J., Crosdale, D., Ollier, W., Hajeer, A., Thomson, W., Ordi, J., Balada, E., Villarreal, M., Teh, L.-S., Poulton, K., 2001. Mannose binding lectin and FcγRIIa (CD32) polymorphism in Spanish systemic lupus erythematosus patients. Rheumatology 40, 1009.

3.2.2 MBL a CVID

Faktory ovlivňující klinický průběh a různé laboratorní abnormality běžné variabilní imunodeficience (CVID) nebyly dosud spolehlivě určeny. Publikované studie ukázaly spojitost určitých alelických variant genu pro vitamín D nebo interleukin-6 s imunofenotypovými abnormalitami pozorovanými u CVID a variant genů pro TNF a interleukin-10 se vznikem granulomatózních komplikací CVID (Mullighan et al., 1997; Mullighan et al., 1999; Rezaei et al., 2009), ale je zřejmé, že vliv na klinickou manifestaci mají i varianty v dalších genech. Gen *MBL2*, jehož varianty vedoucí k deficienci MBL byly popsány v souvislosti s vyšším rizikem invazivních infekčních komplikací nebo se závažnějším průběhem infekcí a plicního postižení u cystické fibrózy, je jedním z kandidátních genů pro vliv na fenotyp CVID. Tento gen byl u pacientů s CVID již dříve částečně zkoumán, Mullighan se spoluautory (Mullighan et al., 2000) ukázal časnější klinickou manifestaci choroby u pacientů s genotypy *MBL2* podmiňujícími deficit MBL, zejména u nositelů haplotypu LXA, Fevang a spoluautoři popsali negativní korelaci hladin MBL s frekvencí infekcí dolních cest dýchacích a přítomností bronchiektázií (Fevang et al., 2005), Andersen se spoluautory zaznamenali vyšší frekvenci závažných infekcí dolních cest dýchacích u heterozygotních nositelů deficit podmiňujících alel (Andersen et al., 2005) a Hamvas s kolegy na nevelkém vzorku pacientů demonstrovali, že u pacientů s hypogmaglobulinemií a kultivačně prokázanou mykoplazmovou infekcí je významně vyšší zastoupení *MBL2* deficitních haplotypů než v obecné populaci (Hamvas et al., 2005). Uvedené asociace však nebyly potvrzeny dalšími studiemi.

V naší studii bylo analyzováno 94 pacientů s diagnózou CVID ze dvou center, Ústavu klinické imunologie a alergologie LF MU a FN u sv. Anny v Brně a Divize revmatologie a klinické imunologie Univerzitní nemocnice ve Freiburgu, jako kontrolní byly použity vzorky 359 zdravých jedinců. U pacientů s CVID byly odhaleny statisticky významné asociace genotypů vedoucích k MBL deficienci s přítomností bronchiektázií, plicní fibrózou a respirační insuficiencí, zatímco s věkem začátku klinické manifestace onemocnění, věkem stanovení diagnózy, počtem pneumonií před zahájením léčby imunoglobuliny, hladinami imunoglobulinů před léčbou, frekvencí respiračních infekcí, splenomegalií, lymfadenopatií, výskytem granulomů či přítomností průjmů žádná souvislost zjištěna nebyla. Přinesli jsme tedy důkazy podporující možnou účast deficience MBL na vývoji chronických plicních změn u pacientů s CVID, zatímco role nízkých hladin MBL při vzniku extrapulmonálních komplikací či jejich souvislost s laboratorními abnormalitami potvrzeny nebyly. V případě konfirmace našich výsledků dalšími studiemi by bylo možno zvažovat možnost aplikace rekombinantního MBL pacientům s CVID k zabránění rozvoje plicních komplikací.

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Mannose-binding lectin gene polymorphic variants predispose to the development of bronchopulmonary complications but have no influence on other clinical and laboratory symptoms or signs of common variable immunodeficiency

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Summary

Mannose-binding lectin (MBL), activating protein of the lectin pathway of the complement system, is an important component of the non-specific immune response. *MBL2* gene polymorphisms, both in the coding and promoter regions, lead to low or deficient serum MBL levels. Low serum MBL levels were shown to be associated with serious infectious complications, mainly in patients in whom other non-specific immune system barriers were disturbed (granulocytopenia, cystic fibrosis). We have analysed two promoter (−550 and −221) and three exon (codons 52, 54 and 57) *MBL2* polymorphisms in a total of 94 patients with common variable immunodeficiency (CVID) from two immunodeficiency centres. Low-producing genotypes were associated with the presence of bronchiectasis ($P = 0{\cdot}009$), lung fibrosis ($P = 0{\cdot}037$) and also with respiratory insufficiency ($P = 0{\cdot}029$). We could not demonstrate any association of MBL deficiency with age at onset of clinical symptoms, age at diagnosis, the number of pneumonias before diagnosis or serum immunoglobulin (Ig)G, IgA and IgM levels before initiation of Ig treatment. No association with emphysema development was observed, such as with lung function test abnormalities. No effect of *MBL2* genotypes on the presence of diarrhoea, granuloma formation, lymphadenopathy, splenomegaly, frequency of respiratory tract infection or the number of antibiotic courses of the patients was observed. Our study suggests that low MBL-producing genotypes predispose to bronchiectasis formation, and also fibrosis and respiratory insufficiency development, but have no effect on other complications in CVID patients.

Keywords: common variable immunodeficiency, complement, lung disease

Introduction

Common variable immunodeficiency (CVID) is a primary hypogammaglobulinaemia affecting both sexes with clinical manifestation beginning at any age over 2 years [1]. Besides frequent and complicated respiratory tract infections (RTI), the patients suffer frequently from other symptoms – diarrhoea, autoimmune diseases, splenomegaly, lymphadenopathy and granuloma formation [2]. Although mutations in genes coding for inducible co-stimulator (ICOS), CD19, B cell-activating factor of the tumour necrosis factor (TNF) family receptor (BAFF-R), and possibly transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), were documented in some patients [3], in the majority of affected people the genetic background is unknown. Factors influencing the clinical course and various laboratory abnormalities of CVID are still unknown in general. Published studies of genetic polymorphisms have shown that the vitamin D receptor and interleukin (IL)-6 allelic polymorphisms were associated with immunophenotypic abnormalities in CVID patients, and particular variants of TNF and IL-10 alleles conferred susceptibility to granulomatous forms of CVID [4,5], but probably other disease-modifying genes are also involved.

Mannose-binding lectin (MBL) is an important component of the innate humoral immune response. It binds to polysaccharide groups on the surface of various microbes activating the lectin complement pathway, which is independent of previous antigen–antibody interaction. The gene coding for MBL, designated as *MBL2,* is located on chromosome 10. Various variants on exon 1 influencing serum MBL levels have been described. A single nucleotide mutation in codon 54 leads to Gly → Asp substitution (variant *B*), in codon 57 Gly → Glu (variant *C*) and in codon 52 Arg → Cys (variant *D*), while the normal, non-mutated allele is designated as *A* [6]. Homozygosity in the *A* allele leads to normal serum levels of MBL, while individuals heterozygous for one of the polymorphic alleles have decreased levels of MBL, reaching approximately one-tenth of the normal levels. Homozygotes or compound heterozygotes for mutated alleles have very low serum MBL levels, hardly detectable by conventional enzyme-linked immunosorbent assay [7], although marked interindividual variation can be documented [8]. Also, polymorphisms in the promoter were documented to lead to alteration of serum MBL levels. *H/L*, *Y/X* and *P/Q* polymorphisms at positions −550, −221 and +4 were described. When in *cis* position with the wild allele, *HYA*, *LYA* and *LXA* haplotypes are associated with high, low and deficient serum MBL levels respectively [9]. In summary, in healthy individuals various combinations of structural and promoter polymorphisms lead to a marked variation of up to 1000-fold in MBL concentrations [6].

The importance of MBL in anti-microbial defence has been documented by studies that showed increased occurrence of invasive infections caused by *Streptococcus pneumoniae* [10] and *Neisseria meningitidis* [11] in people with MBL deficiency. MBL deficiency increases the probability of attraction of human immunodeficiency virus infection [12,13], and possibly also shortens survival of patients in the acquired immune deficiency syndrome stage [12]. The frequency of complications of hepatitis B infection is also associated with *MBL2* genotypes [14,15].

Data concerning the influence of the *MBL2* genotype on the CVID phenotype are limited. Mullighan *et al.* [16] analysed *MBL2* polymorphisms in 163 CVID patients and 100 controls. They found that low MBL-producing alleles were associated with earlier clinical manifestations of CVID. This was most significant in patients with the *LXPA* haplotype. They also found that the *MBL2* +4 *Q* allele was associated with autoimmune manifestation. Fevang *et al.* [17] found that serum MBL concentrations correlated negatively with the frequency of lower RTI and the presence of bronchiectasis. Andersen *et al.* [18] observed an increased frequency of severe RTI before the initiation of immunoglobulin (Ig) treatment in patients heterozygous for *MBL2* exon 1 structural gene variants. *MBL2* exon 1 polymorphic variants were found in 16 of 23 of the patients with various forms of primary hypogammaglobulinaemia with a proven mycoplasma infection compared with two-thirds in the general population, showing that MBL deficiency predisposes to mycoplasma infections in hypogammaglobulinaemic patients [19]. None of these results were confirmed by additional studies.

In this study we have analysed *MBL2* exon 1 and promoter polymorphic markers in CVID patients from two immunodeficiency centres and correlated them with various clinical and laboratory parameters to assess to what extent the *MBL2* gene could be regarded as a disease-modifying gene in CVID.

Patients and methods

Ninety-four patients with CVID were included into the study: 51 females and 43 males aged 12–82 years [mean 45·4, standard deviation (s.d.) = 14·7]. Fifty-four patients were from the Department of Clinical Immunology and Allergology in Brno and 40 from the Department of Rheumatology and Clinical Immunology in Freiburg. None of them was known to have *ICOS* (tested in 51 patients) or *TNFRSF13C* (coding for BAFF-R; tested in six patients) mutations, while the *TNFRSF13B* (coding for TACI) mutation was documented in 10 of 87 patients tested.

Fifty-two patients fulfilled the European Society for Immunodeficiencies diagnostic criteria for CVID [1]. In 42 patients, mainly those whose treatment was initiated before the mid-1990s (the introduction of relevant tests in our laboratories), diagnosis was made by low Ig levels, clinically significant immunodeficiency and exclusion of other causes of hypogammaglobulinaemia.

Three hundred and fifty-nine healthy donors of Czech origin were used as control subjects for assessing the frequency of *MBL2* genotypes, as published previously [20].

The onset of the disease was defined as the age when the first episode of pneumonia or a marked increase in the frequency of RTI occurred. In patients without significant immunodeficiency symptoms, the date of statement of a CVID diagnosis was considered to be the beginning of the disease.

The presence of bronchiectasis and lung fibrosis was determined by high-resolution computerized tomography. Data were available in 66 patients. Respiratory functions were determined by spirometry; the data were available in 90 patients. Obstructive disease was graded as mild, moderate and severe if forced expiratory volume in 1 s was 60–79%, 45–59% and < 45% of the predicted value respectively. Restrictive lung disease was graded as mild, moderate and severe if vital capacity was 60–79%, 45–59% and < 45% of the predicted value, respectively. Splenomegaly was defined by the length of the spleen over 11 cm on ultrasonography.

Serum Ig levels were measured by radial immunodiffusion, turbidimetry or nephelometry, the method being dependent upon the year of diagnosis of the patients. In the case of 'immeasurable' Ig serum levels, a lower detection limit was used for calculation. B lymphocyte subpopulations were determined by flow cytometry, as published previously

Table 1. Comparison of age at onset, age at diagnosis, diagnostic delay and number of pneumonias in patients with genotypes associated with normal (N), low (L) and deficient (D) serum mannan-binding lectin levels.

	Normal ($n = 58$)	Low ($n = 19$)	Deficient ($n = 17$)	N *versus* L *versus* D *P*-value	N *versus* L+D *P*-value	N+L *versus* D *P*-value
Age at onset (years)	26·7 (13·7)	28·9 (17·2)	34·9 (15·8)	0·174*	0·122**	0·062**
Age at diagnosis (years)	32·9 (13·6)	35·9 (17·3)	38·4 (17·8)	0·460*	0·191**	0·240**
Diagnostic delay (years)	6·1 (7·6)	7·0 (8·0)	3·5 (4·4)	0·494*	0·866***	0·304***
Pneumonias before diagnosis	1·5 (2·7)	1·6 (3·4)	0·76 (1·0)	0·758*	0·889***	0·617***

The data are given as mean (standard deviation). *Kruskal–Wallis test was used for statistical evaluation; **t-test; ***Mann–Whitney test.

[21], and the patients were subdivided according to the 'Freiburg classification' [22].

The *MBL2* genotype determination was performed by multiplex polymerase chain reaction (PCR), as described previously [20]. Briefly, the promoter polymorphisms were detected using the double amplification refractory mutation system method. Three separate amplifications with sequence-specific sense and anti-sense primers were carried out to determine *HY*, *LY* and *LX* promoter haplotypes respectively. The first exon *MBL2* gene mutations were identified using the multiplex PCR method with sequence-specific primers. One reaction with primers specific to *B*, *C* and *D* alleles, and another reaction specific to the *A* allele in codons 52, 54 and 57, were performed. A 4% MetaPhor agarose gel electrophoresis was used to discriminate PCR products of 128, 135 and 143 base pairs. All reactions included internal control of amplification. Assignment of haplotypes was based on the strong linkage disequilibrium between the promoter variants and the first exon alleles, and the existence of the frequent haplotypes *HYA*, *LYA*, *HYD*, *LYB*, *LYC* and *LXA*. All suspected *LYD* haplotypes were confirmed by a separate long-chain PCR reaction with sequence-specific primers.

HYA/HYA, *HYA/LYA*, *HYA/LXA*, *LYA/LYA* and *LYA/LXA* genotypes were considered to be associated with normal levels of serum MBL; the patients with this genotype were labelled as 'normal' (N). Patients with *HYA/HYD*, *HYA/LYB*, *HYA/LYC*, *HYA/LYD*, *LYA/HYD*, *LYA/LYB*, *LYA/LYC*, *LYA/LYD* and *LXA/LXA* genotypes were considered to have low serum MBL levels forming the group 'low' (L), while *HYD/HYD*, *HYD/LYB*, *HYD/LYC*, *HYD/LYD*, *LYB/LYB*, *LYB/LYC*, *LYB/LYD*, *LYC/LYD*, *LYD/LYD*, *LXA/HYD*, *LXA/LYB*, *LXA/LYC* and *LXA/LYD* genotype holders were considered to have deficient MBL levels (the 'deficient' group: D).

Statistical analysis

Testing the difference in two continuous variables was performed using either the two-tailed *t*-test or the Mann–Whitney test according to normality of data, which was assessed by the Kolmogorov–Smirnov test. In the case of more than two variables tested, the Kruskal–Wallis test was used. Categorical variables were analysed using the appropriate test for the contingency tables, i.e. using Spearman's test for two variables with more than two levels and Fisher's exact test in the case of two variables with binary outcome. A standard level of statistical significance $\alpha = 0{\cdot}05$ was used, i.e. a P-value $< 0{\cdot}05$ was considered to be statistically significant. However, because of multiple hypotheses testing, standard Bonferroni correction was applied to the α-level resulting in the appropriate critical value. The statistical package STATISTICA (StatSoft, Inc., Tulsa, OK, USA), version 7, was used.

This study was approved by the Ethics Commission of the Centre for Cardiovascular Surgery and Transplantation in Brno. All patients and donors gave their written consent prior to genetic analysis.

Results

Fifty-eight CVID patients had genotypes associated with normal MBL levels, 19 patients had genotypes associated with low MBL levels and 17 patients exhibited genotypes associated with deficient MBL levels.

The frequency of *MBL2* genotype groups leading to normal, low and deficient MBL production in the general Czech population [20] and CVID patients did not show any significant differences (Spearman's test, data not shown), such as frequency of determined alleles or patients having at least one of the determined alleles (Fisher's exact test, data not shown).

State before CVID diagnosis

On comparing age at onset, age at diagnosis and the number of pneumonias before diagnosis, no significant differences were observed (see Table 1). There were no differences in serum IgG, IgA and IgM levels before the diagnosis comparing N *versus* L+D and N+L *versus* D groups (Mann–Whitney test, data not shown).

B cell analysis

There were no differences in numbers of patients with B cells < 1% of peripheral lymphocytes (six in normal, four in low and two in deficient groups; $P = 0{\cdot}278$, Spearman's test). In 76 patients in whom B cells were > 1% of peripheral lymphocytes the Freiburg classification was used, but no signifi-

Table 2. Presence of lung abnormalities on computed tomography scan. The results were available for only 66 patients. Generalized bronchiectases were defined as bronchiectases in more than three lung lobes. The results of the extent of bronchiectasis, the extent of fibrosis and the extent of emphysema are given as no/localized/generalized.

	Normal ($n = 43$)	Low ($n = 13$)	Deficient ($n = 10$)	N *versus* L *versus* D *P*-value	N *versus* L+D *P*-value	N+L *versus* D *P*-value
Presence of bronchiectasis	15	10	5	0·009*	0·022**	0·999**
Extent of bronchiectasis	28/10/5	3/5/5	5/3/2	0·006*	0·013*	0·768*
Presence of fibrosis	12	8	3	0·037*	0·102**	0·999**
Extent of fibrosis	31/10/2	5/6/2	7/2/1	0·048*	0·091*	0·800*
Presence of emphysema	7	2	2	0·959*	0·999**	0·668**
Extent of emphysema	36/4/3	11/0/2	8/2/0	0·913*	0·893*	0·870*

*Spearman's test; **Fisher's exact test. N, normal; L, low; D, deficient serum mannan-binding lectin levels.

cant differences in the frequency of groups Ia, Ib and II [22] were observed ($P = 0·894$ for all groups, Spearman's test).

Lung abnormalities

The association of *MBL2* genotype groups with the presence of bronchiectasis, lung fibrosis and emphysema is shown in Table 2; as can be seen, the presence of bronchiectasis and lung fibrosis was linked to defective *MBL2* genotype groups. On assessing lung function tests (see Table 3), no relation between restrictive and obstructive disease and *MBL2* genotype groups was observed, while respiratory insufficiency was associated mildly with the presence of defective *MBL2* genotype groups.

Respiratory tract infections

The number of RTI and antibiotic courses in our patients during 1 year prior to inclusion into this study is given in Table 4; no differences in the frequency of RTI or of antibiotic courses in the subgroups of CVID patients were observed. There was no difference in the number of patients with X-ray-proven pneumonia after the initiation of Ig treatment (nine in the N group, four in the L group, five in the D group) in all three groups of patients (Spearman's test, $P = 0·414$), not even when the groups were merged (N *versus* L+D: $P = 0·227$, N+L *versus* D: $P = 0·216$, Fisher's exact test). There was also no difference in the number of patients who were on permanent or seasonal antibiotic prophylaxis (eight patients in group N, three patients in group L and one patient in group D; Spearman's test for all groups: $P = 0·547$, Fisher's exact test for N *versus* L+D: $P = 0·760$, N+L *versus* D: $P = 0·688$). No significant difference was observed comparing patients with more than five infections in 1 year prior to inclusion into the study (four of 49 patients in group N, three of 18 patients in group L, one of 15 patients in group D; Spearman's test for all groups $P = 0·421$, Fisher's exact test: N *versus* L+D: $P = 0·708$, N+L *versus* D: $P = 0·999$).

Table 3. Lung function abnormalities in patients with common variable immunodeficiency. The results are given as normal/mild/moderate/severe in the degree of obstructive disease and degree of restrictive disease lines (see Patients and methods; 90 patients evaluated) and no/partial/global in the respiratory insufficiency degree line (88 patients evaluated).

	Normal	Low	Deficient	N *versus* L *versus* D *P*-value	N *versus* L+D *P*-value	N+L *versus* D *P*-value
Obstructive disease	29	5	8	0·132*	0·277**	0·789**
Degree of obstructive disease	27/18/8/3	13/2/2/1	8/3/5/0	0·274*	0·408*	0·620*
Restrictive disease	8	3	2	0·859*	0·999**	0·999**
Degree of restrictive disease	48/7/1/0	15/2/1/0	14/2/0/0	0·869*	0·904*	0·783*
Respiratory insufficiency	3	4	3	0·029*	0·041**	0·380**
Degree of respiratory insufficiency	51/2/1	14/4/0	13/2/1	0·034*	0·033*	0·291*

*Spearman's test; **Fisher's exact test. N, normal; L, low; D, deficient serum mannan-binding lectin levels.

Table 4. Number of respiratory tract infections (RTI) and antibiotic (ATB) courses during 1 year prior to inclusion into the study in subgroups of common variable immunodeficiency patients. Sufficient data were available in 83 patients. The data are given as mean (standard deviation).

	Normal ($n = 50$)	Low ($n = 18$)	Deficient ($n = 15$)	N *versus* L *versus* D *P*-value*	N *versus* L+D *P*-value**	N+L *versus* D *P*-value**
No of RTI infections	2·4 (2·1)	3·3 (2·6)	2·6 (1·5)	0·541	0·576	0·810
No. of ATB courses	1·6 (1·8)	1·8 (2·6)	1·5 (1·3)	0·761	0·655	0·901

*Kruskal–Wallis test; **Mann–Whitney test. N, normal; L, low; D, deficient serum mannan-binding lectin levels.

Table 5. Presence of splenomegaly, lymphadenopathy, autoimmune phenomena, granulomas and chronic diarrhoea in 94 patients with common variable immunodeficiency. N/L/D means positivity in the patients with genotypes associated with normal/low/deficient mannan-binding lectin levels respectively.

	N/L/D	N *versus* L *versus* D *P*-value	N *versus* L+D *P*-value	N+L *versus* D *P*-value
Splenomegaly	32/10/9	0·956*	0·999**	0·792**
Lymphadenopathy	45/11/16	0·561*	0·999**	0·106**
Autoimmune phenomena	15/5/6	0·658*	0·636**	0·551**
Granuloma	2/1/1	0·652*	0·635**	0·556**
Chronic diarrhoea	6/3/3	0·413*	0·629**	0·546**

*Spearman's test; **Fisher's exact test.

Other clinical indicators

The frequency of splenomegaly, lymphadenopathy, autoimmune phenomena, granuloma and chronic diarrhoea in CVID patients is given in Table 5. There were no significant differences between the groups studied.

Particular genetic variant analysis

We have analysed the relation of the polymorphic variants of *MBL2* determined with the presence of subsequent clinical or laboratory data: presence of pneumonia after initiation of Ig treatment, chronic diarrhoea, presence of bronchiectasis, fibrosis, emphysema, respiratory insufficiency, obstructive lung disease, restrictive lung disease, chronic diarrhoea, granuloma formation, autoimmune phenomena, splenomegaly and lymphadenopathy; more than five infections in 1 year prior to inclusion into the study. Fisher's test was used for statistical analysis. Only the presence of autoimmune phenomena in patients negative for allele *A* (present in five of eight *A*- patients, compared with 21 of 86 *A*+ patients, Fisher's test: $P = 0{\cdot}035$), and chronic diarrhoea for variant *D* (present in five of 17 *D*+ patients and in seven of 77 *D*– patients, $P = 0{\cdot}038$) exceeded $P < 0{\cdot}05$ but, because of multiple testing, only the resultant *P*-values < 0·002 should be considered statistically significant.

Discussion

The extensive variability of CVID stimulates searching not only for causative genes, but also for genes modifying the clinical course of the affected individual. One such disease-modifying gene in CVID might be *MBL2*. Previous studies have shown that MBL deficiency has only a minor, if any, influence on the morbidity or mortality of otherwise healthy people [23], but that it becomes symptomatic if other defence barrier(s) is/are disturbed, the best example being granulocytopenia during or after cytostatic treatment [24,25] or cystic fibrosis [26].

Much less clear is the association of MBL deficiency with various types of Ig production disturbances. A possible importance of MBL in patients with antibody deficiencies is supported by the observation that *MBL2* non-*A* variants were associated with increased otitis media episodes at the age of 12–24 months, but not later [27]. The age span mentioned is the life period when maternally derived antibodies have waned, but adequate adaptive immunity is not yet developed. On the other hand, Aittoniemi *et al.* [28] could not document any influence of serum MBL levels on the clinical state of IgA-deficient individuals.

Our study confirmed the previous observation [17] that in CVID patients low MBL levels were associated with the presence of bronchiectasis; also, the presence of fibrosis was associated with the presence of defective genotypes. Interestingly, observations in CVID patients are, to our knowledge, the first described associations of MBL deficiency with bronchiectasis development. Although the numbers of patients in the evaluated groups were too low to draw any unequivocal conclusions, it seems that it is predominantly the decrease in serum MBL level (in both patients from the L and D groups) that predisposed to bronchiectasis or fibrosis development. On the other hand, patients with MBL-deficient genotypes did not have higher proneness to the mentioned complications than the patients with low MBL-producing genotypes.

The association of *MBL2* genotype groups with respiratory insufficiency was also observed in our study, but this result could be questioned because of the low number of patients in whom respiratory insufficiency was present. On the other hand, we could not prove any influence of MBL status on the frequency of infections of patients under Ig treatment documented previously by others [17], or the frequency of antibiotic courses in CVID patients. Comparing our study and the above-mentioned studies, we have recorded all RTI in our patients, while in the above-mentioned study only lower RTI were documented [17]. As many of our patients were treated many years ago, we could not evaluate the number of lower RTI prior to Ig treatment, which was shown to be increased in patients heterozygous for structural polymorphisms associated with low MBL production [18]. However, when evaluating the number of pneumonias before making a CVID diagnosis, we could not document any difference among patients from different *MBL2* genotype groups.

Unlike the study by Mullighan *et al.* [16], we could not confirm the earlier clinical manifestation of CVID in patients with defective *MBL2* genotypes. Another study from Norway also showed no effect of MBL levels on the age of clinical manifestation of CVID[17]. Surprisingly, our data showed an even later (although not significant) manifestation of CVID in patients with low and mainly deficient MBL-producing genotypes. It is necessary to mention that the retrospective determinations of the onset of immunodeficiency symptoms, even when conducted by experienced physicians, are highly inaccurate in many cases. Also the fact that currently many patients are diagnosed much earlier than previously, even with mild clinical symptoms, should be taken into account as a possible difference from the above-mentioned study [16] published 8 years ago.

Several studies showed that MBL deficiency might be associated with autoimmune diseases such as systemic lupus erythematosus [29] or rheumatoid arthritis [30]. Mullighan *et al.* [16] found an association of autoimmune phenomena with the presence of the *MBL2* +4 *Q* polymorphism. Unfortunately, the polymorphism *MBL2* +4 was not determined in our study, as this polymorphism has only a minor impact on serum MBL levels [31]. Our study did not find any association of low or deficient *MBL2* genotype groups or the particular polymorphic variants evaluated with autoimmune phenomena in our CVID patients.

The MBL status in this study was determined only by *MBL2* genotyping, while the serum MBL level was not determined. This is a relatively common approach, as it allows simplification of the complex situation when the actual MBL level in a specific person is influenced not only by genetic background, but also by actual inflammatory status, as MBL reacts as an acute-phase protein [32]; thyroid hormones and the growth hormone were also shown to influence the production of MBL by hepatocytes [33]. Serum MBL in CVID patients may be influenced mainly by acute or chronic inflammation, which is common in these patients.

The observation about the influence of MBL deficiency on bronchiectasis and fibrosis development raises the question of whether, in patients with MBL deficiency (or holders of *MBL2* genotypes associated with abnormal serum MBL levels), a more intensive Ig regimen should be applied compared with patients with normal MBL levels. In our opinion the results of our study do not support this approach strongly. Although the results of MBL determination might be taken into account in such considerations, we still do not have clear evidence that the intensity of Ig treatment has a protective effect on bronchiectasis development in general, still less so in the case of MBL deficiency. Only a prospective large-scale study would be able to answer this question.

In general, our study showed that the presence of low or deficient MBL-producing genotypes in patients with CVID is associated with chronic changes of the bronchi and the lungs: bronchiectasis, fibrosis development and respiratory insufficiency. On the other hand, we could not document any influence of *MBL2* genotypes on the frequency of acute RTI, extrapulmonary manifestation or various laboratory parameters. It is supposed that various other disease-modifying genes and their mutual interactions as well as interactions with environmental factors must be involved in the variability of the disease.

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References

1 Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Clin Immunol 1999; **93**:190–7.
2 Hermaszewski RA, Webster AD. Primary hypogammaglobulinaemia: a survey of clinical manifestations and complications. Q J Med 1993; **86**:31–42.
3 Bacchelli C, Buckridge S, Thrasher AJ, Gaspar HB. Translational mini-review series on immunodeficiency: molecular defects in common variable immunodeficiency. Clin Exp Immunol. 2007; **149**:401–9.
4 Mullighan CG, Fanning GC, Chapel HM, Welsh KI. TNF and lymphotoxin-alpha polymorphisms associated with common variable immunodeficiency: role in the pathogenesis of granulomatous disease. J Immunol 1997; **159**:6236–41.
5 Mullighan CG, Marshall SE, Bunce M, Welsh KI. Variation in immunoregulatory genes determines the clinical phenotype of common variable immunodeficiency. Genes Immun 1999; **1**:137–48.
6 Dommett RM, Klein N, Turner MW. Mannose-binding lectin in innate immunity: past, present and future. Tissue Antigens 2006; **68**:193–209.
7 Kilpatrick DC. Mannan-binding lectin and its role in innate immunity. Transfus Med 2002; **12**:335–52.
8 Minchinton RM, Dean MM, Clark TR, Heatley S, Mullighan CG. Analysis of the relationship between mannose-binding lectin (MBL) genotype, MBL levels and function in an Australian blood donor population. Scand J Immunol 2002; **56**:630–41.
9 Worthley DL, Bardy PG, Mullighan CG. Mannose-binding lectin: biology and clinical implications. Intern Med J 2005; **35**:548–55.
10 Roy S, Knox K, Segal S, Griffiths D *et al.* MBL genotype and risk of invasive pneumococcal disease: a case–control study. Lancet 2002; **359**:1569–73.
11 Hibberd ML, Sumiya M, Summerfield JA, Booy R, Levin M. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group. Lancet 1999; **353**:1049–53.
12 Garred P, Madsen HO, Balslev U *et al.* Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. Lancet 1997; **349**:236–40.
13 Nielsen SL, Andersen PL, Koch C, Jensenius JC, Thiel S. The level of the serum opsonin, mannan-binding protein in HIV-1 antibody-positive patients. Clin Exp Immunol 1995; **100**:219–22.

14 Yuen MF, Lau CS, Lau YL, Wong WM, Cheng CC, Lai CL. Mannose binding lectin gene mutations are associated with progression of liver disease in chronic hepatitis B infection. Hepatology 1999; **29**:1248–51.
15 Thio CL, Mosbruger T, Astemborski J *et al*. Mannose binding lectin genotypes influence recovery from hepatitis B virus infection. J Virol 2005; **79**:9192–6.
16 Mullighan CG, Marshall SE, Welsh KI. Mannose binding lectin polymorphisms are associated with early age of disease onset and autoimmunity in common variable immunodeficiency. Scand J Immunol 2000; **51**:111–22.
17 Fevang B, Mollnes TE, Holm AM *et al*. Common variable immunodeficiency and the complement system; low mannose-binding lectin levels are associated with bronchiectasis. Clin Exp Immunol 2005; **142**:576–84.
18 Andersen P, Permin H, Andersen V *et al*. Deficiency of somatic hypermutation of the antibody light chain is associated with increased frequency of severe respiratory tract infection in common variable immunodeficiency. Blood 2005; **105**:511–17.
19 Hamvas RM, Johnson M, Vlieger AM *et al*. Role for mannose binding lectin in the prevention of Mycoplasma infection. Infect Immun 2005; **73**:5238–40.
20 Skalníková H, Freiberger T, Chumchalová J, Grombiríková H, Sedivá A. Cost-effective genotyping of human *MBL2* gene mutations using multiplex PCR. J Immunol Methods 2004; **295**:139–47.
21 Vlková M, Thon V, Sárfyová M *et al*. Age dependency and mutual relations in T and B lymphocyte abnormalities in common variable immunodeficiency patients. Clin Exp Immunol 2006; **143**:373–9.
22 Warnatz K, Denz A, Dräger R *et al*. Severe deficiency of switched memory B cells (CD27(+)IgM(−)IgD(−)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 2002; **99**:1544–51.
23 Dahl M, Tybjaerg-Hansen A, Schnohr P, Nordestgaard BG. A population-based study of morbidity and mortality in mannose-binding lectin deficiency. J Exp Med 2004; **199**:1391–9.
24 Neth O, Hann I, Turner MW, Klein NJ. Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. Lancet 2001; **358**:614–18.
25 Peterslund NA, Koch C, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. Lancet 2001; **358**:637–8.
26 Garred P, Pressler T, Madsen HO *et al*. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J Clin Invest 1999; **104**:431–7.
27 Wiertsema SP, Herpers BL, Veenhoven RH *et al*. Functional polymorphisms in the mannan-binding lectin 2 gene: effect on MBL levels and otitis media. J Allergy Clin Immunol 2006; **117**:1344–50.
28 Aittoniemi J, Koskinen S, Laippala P, Laine S, Miettinen A. The significance of IgG subclasses and mannan-binding lectin (MBL) for susceptibility to infection in apparently healthy adults with IgA deficiency. Clin Exp Immunol 1999; **116**:505–8.
29 Lee YH, Witte T, Momot T *et al*. The mannose-binding lectin gene polymorphisms and systemic lupus erythematosus: two case–control studies and a meta-analysis. Arthritis Rheum 2005; **52**:3966–74.
30 Graudal NA, Homann C, Madsen HO *et al*. Mannan binding lectin in rheumatoid arthritis. A longitudinal study. J Rheumatol 1998; **25**:629–35.
31 Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J Immunol 1998; **161**:3169–75.
32 Thiel S, Holmskov U, Hviid L, Laursen SB, Jensenius JC. The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. Clin Exp Immunol 1992; **90**:31–5.
33 Sørensen CM, Hansen TK, Steffensen R, Jensenius JC, Thiel S. Hormonal regulation of mannan-binding lectin synthesis in hepatocytes. Clin Exp Immunol 2006; **145**:173–82.

3.3 Neonatální Fc receptor

Neonatální Fc receptor (FcRn) patří do rodiny receptorů vázajících konstantní část těžkých řetězců imunoglobulinů třídy G. Je exprimován v širokém spektru buněk a tkání jako jsou epiteliální buňky tenkého střeva, endotel cév, hematopoetické buňky, epitel mléčné žlázy, epitel ledvin a syncytiotrofoblast. FcRn má v organismu tři základní funkce: 1) zajišťuje transfer protilátkové imunity z matky na potomstvo (nejprve byl popsán jako receptor přenášející imunoglobuliny G (IgG) z mateřského mléka přes epitel tenkého střeva u sajících krys a poté byl nalezen jeho lidský homolog v placentě, kde zprostředkovává přenos mateřských IgG na plod); 2) je zodpovědný za regulaci koncentrace IgG v séru, kde prodlužuje jejich biologický poločas; a 3) slouží jako senzor luminálních infekcí, když zodpovídá za přenos imunokomplexů z lumen respiračního či gastrointestinálního traktu na bazolaterální stranu epiteliálních buněk, kde jsou imunokomplexy převzaty dendritickými buňkami ke zpracování a prezentaci antigenů (Baker et al., 2009; Ghetie and Ward, 2000; Rodewald and Kraehenbuhl, 1984). Role FcRn v prezentaci antigenů se zdá být nezanedbatelná. Cervenak et al. na transgenních myších ukázali, že efekt nadprodukce FcRn v podobě zesílené imunitní odpovědi a s tím související zvýšené tvorby IgG byl dokonce výraznější než vliv zprostředkovaný zpomalením katabolismu IgG (Cervenak et al., 2011).

Gen pro FcRn (*FCGRT*, alias *FCRN*) se nachází na 19. chromozomu, je dlouhý přibližně 15,5 kb a obsahuje sedm exonů. Kóduje protein strukturálně podobný proteinům hlavního histokompatibilitního komplexu I. třídy (MHC-I), který obsahuje tři extracelulární domény α1-α3, jednu doménu transmembránovou a jednu cytoplasmatickou (Simister and Mostov, 1989). K interakci pravděpodobně dochází mezi CH2-CH3 doménou IgG a α2 doménou FcRn a tato interakce je velice závislá na pH (Raghavan et al., 1995). IgG cirkulující v krvi je zřejmě endocytózou přemístěn do buněk cévního endotelu nebo do monocytů, v jejichž endosomech dochází k vazbě s FcRn při pH<6,5. Tím je IgG ochráněn před degradací v lysozomech a následně je transportován na buněčný povrch, kde je při neutrálním pH uvolněn zpět do cirkulace. Podobným mechanizmem je zajištěn transplacentární přenos IgG. Imunoglobulin G z maternálního oběhu je pasivně internalizován do syncytiotrofoblastu, v jehož v endosomech dochází k vytvoření vazby FcRn-IgG, která i v tomto případě chrání IgG před degradací. Následně endosomy fúzují s membránou na fetální straně syncytiotrofoblastu, kde dochází k disociaci IgG a FcRn, přičemž IgG vstupuje do fetální cirkulace a FcRn se vrací zpět na maternální stranu syncytiotrofoblastu (Baker et al., 2009).

3.3.1 FcRn a protilátkové imunodeficience

FCRN je díky své roli v katabolismu a tím pádem dostupnosti autologních nebo terapeutických protilátek jistě kandidátním genem s možným vlivem na průběh protilátkových PID, včetně odpovědi na substituční imunoglobulinovou léčbu. Případné změny v promotorové oblasti genu nebo záměny aminokyselin ve vazebné doméně, ale i na jiném místě proteinového řetězce, by mohly mít vliv na expresi, resp. funkci FcRn. Sachs et al. (Sachs et al., 2006) popsali polymorfismus typu VNTR (*variable number of tandem repeats*) v promotorové oblasti genu *FCRN* a ukázali jeho vliv na aktivitu promotoru, expresi mRNA i vazebnou kapacitu FcRn exprimujících buněk k IgG. Další přirozeně se vyskytující funkčně významné varianty genu *FCRN* zatím popsány nebyly (Gunraj et al., 2002; Ishii-Watabe et al., 2010). My jsme se problematikou sekvenční variability genu *FCRN* zabývali a nalezli jsme několik variant, které se vyskytovaly pouze u pacientů s primárními hypogamaglobulinemiemi a nikoliv ve vzorku obecné české populace. Neprokázali jsme ovšem žádnou asociaci frekventnějších polymorfismů, zejména VNTR, s fenotypovými projevy CVID, s poklesem IgG po léčebném intravenózním podání IgG (IVIG) u pacientů s CVID ani s expresí mRNA u pacientů s CVID. Zato jsme nalezli statisticky významnou souvislost mezi mírou exprese mRNA *FCRN* a poklesem IgG po IVIG, jakož i výskytem strukturních plicních abnormit u pacientů s CVID. Pacienti s generalizovanými bronchiektáziemi a plicní fibrózou měli významně nižší hladiny mRNA *FCRN* a rozsah bronchiektázií dokonce vykazoval negativní korelaci s hladinami mRNA *FCRN*. To by bylo v souladu s hypotézou o významné roli FcRn nejen v homeostáze IgG, ale i při prezentaci antigenů na slizničních površích. Na druhé straně jsme nenašli žádnou souvislost mezi expresí mRNA *FCRN* a věkem první klinické manifestace CVID, věkem stanovení diagnózy, hladinami IgG a frekvencí antibiotické léčby před zahájením substituční imunoglobulinové terapie nebo v jejím průběhu, přítomností plicních funkčních abnormalit, chronických průjmů, splenomegalie či lymfadenopatií.

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Association of FcRn expression with lung abnormalities and IVIG catabolism in patients with common variable immunodeficiency

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Abstract The neonatal Fc receptor (FcRn) acts as a key regulator of IgG homeostasis and is an important sensor of luminal infection. We analyzed the influence of FcRn expression on disease phenotype and the catabolism of therapeutically administered intravenous immunoglobulins (IVIG) in 28 patients with common variable immunodeficiency (CVID). Patients with generalized bronchiectasis and fibrosis had lower levels of *FCRN* mRNA compared to patients without these complications ($P=0.027$ and $P=0.041$, respectively). Moreover, *FCRN* mRNA levels correlated negatively with the extent of bronchiectasis and the rate of IgG decline after infusion of IVIG ($P=0.027$ and $P=0.045$, respectively). No relationship of *FCRN* expression with age at disease onset, age at diagnosis, diagnostic delay, IgG levels or frequency of infections before or during replacement immunoglobulin treatment, the presence of lung functional abnormalities, chronic diarrhea, granulomas, lymphadenopathy, splenomegaly or autoimmune phenomena was observed. Our results showed that FcRn might play a role in the development of lung structural abnormalities and in the catabolism of IVIG in patients with CVID.

Introduction

The neonatal Fc receptor (FcRn) is not only responsible for maternofetal immunoglobulin G (IgG) transfer but also

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serves as a key regulator of IgG and albumin homeostasis [1]. It protects these two proteins, which constitute 80% of the total plasma protein pool, from catabolism very efficiently. FcRn recycles an equivalent amount of albumin and even four times as much IgG as can be produced in a given time [2,3]. It binds endocytosed IgG at acidic pH (<6.5) within endosomes, diverts it from a degradative fate within lysosomes and instead transports it back to the cell surface where IgG is released at neutral pH (>7.0) [1]. Endothelial and hematopoietic cells expressing FcRn seem to play an equally important role in this process [4].

Another notable function of FcRn consists in antigen delivery. In an animal model, FcRn was shown to be involved in the transcytosis of monomeric serum IgG from the basolateral to the apical side of the epithelium, the binding of immune complexes formed in the lumen and their delivery to the lamina propria for antigen processing and presentation by mucosal dendritic cells [5]. Therefore, FcRn in the epithelium is probably able to sense luminal and epithelial infections and transmit evidence of these infections to the local and systemic immune apparatus. In contrast to the recycling pathway observed in epithelial and endothelial cells for monomeric IgG, multimeric immune complexes within dendritic cells are rapidly targeted to a degradative pathway leading to active antigen presentation [6].

Recently, a polymorphism in the promoter region of the human *FCRN* gene consisting of a variable number of 37-bp tandem repeats (VNTR) has been described. The allele with two tandem repeats (VNTR2) is associated with decreased promoter activity compared with the most common VNTR3 allele, and VNTR2 carriers have been shown to have lower *FCRN* mRNA levels and decreased binding capacity of monocytes to immobilized IgG than normal VNTR3/3 homozygotes [7].

Common variable immunodeficiency (CVID) is the most frequent clinically relevant primary immunodeficiency. It is a heterogeneous disorder with various genetic backgrounds and variable clinical manifestations, characterized by hypogammaglobulinemia (low IgG and IgA, normal or low IgM levels), recurrent sinopulmonary infections and impaired functional antibody responses, encompassing absent isohemagglutinins and poor responses to protein and/or polysaccharide vaccines. In addition to these, there can be other clinical findings, such as autoimmunity, granulomatous disease and neoplasia. A cornerstone of CVID therapy is an intravenous or subcutaneous IgG substitution [8]. Patients with CVID differ one from another in their IgG dose, and intervals of IgG infusions needed to maintain sufficient serum IgG concentrations and satisfactory clinical status.

Defects in the *ICOS*, *CD19*, *TNFRSF13C* and *TNFRSF13B* genes have been described in a minority of CVID patients [8]. Several candidate genes with potential disease-modifying effects have also been studied, and some have been shown to be associated with particular laboratory or clinical features of CVID [9–11]. It is likely that other genes influencing CVID phenotype are involved.

We hypothesized that *FCRN* expression, determined by VNTR polymorphism, influenced IVIG catabolism and clinical phenotype in patients with CVID due to the role of FcRn in both IgG protection from degradation and mucosal antigen presentation.

Materials and methods

Patients

Sixty-two patients with CVID from the Department of Clinical Immunology and Allergology in Brno were included into the study: 37 females and 25 males aged 15–79 years (mean 46.0, SD = 15.82). None of them was known to have *ICOS* (tested in 22 patients) or *TNFRSF13C* (coding for BAFF-R; tested in 4 patients) mutation, while *TNFRSF13B* (coding for TACI) mutations were documented in 4 out of 57 patients tested.

Thirty-eight patients fulfilled the ESID diagnostic criteria for CVID [12] while 24 patients were diagnosed by low immunoglobulin levels, clinically significant immunodeficiency and exclusion of other causes of hypogammaglobulinemia (as relevant tests had not been available in the laboratory before the mid-1990s, when replacement immunoglobulin treatment was initiated).

Limited numbers of patients (31 and 28) were available for post-infusion IgG concentrations and *FCRN* mRNA level measurements, respectively. However, there were no significant differences in phenotypic features between patients available and not available for analyses (data shown in Supplementary Table 1a for *FCRN* mRNA expression analysis).

Two hundred and two umbilical cord blood samples obtained from consecutively full-term newborns of Caucasian origin were examined to estabish allele frequencies in Czech population. All persons involved in the study provided a written statement of informed consent approved by the Ethics Committee of the Centre for Cardiovascular Surgery and Transplantation in Brno.

The onset of the disease was defined as the age when the first episode of pneumonia or a marked increase in the frequency of respiratory tract infection occurred. In patients without significant immunodeficiency symptoms, the date of statement of a CVID diagnosis was considered the beginning of the disease. The presence of bronchiectasis and lung fibrosis was determined by high-resolution computerized tomography (HRCT), and respiratory function was evaluated by spirometry. Twenty-seven patients suffered from bronchiectasis, 22 from fibrosis. Both bronchiectasis and fibrosis were detected in 16 patients. Obstructive disease was graded as mild, moderate, or severe if forced expiratory volume in 1 s (FEV_1) was 60–79%, 45–59%, or <45% of the predicted value, respectively. Restrictive lung disease was graded as mild, moderate, or severe if vital capacity (VC) was 60–79%, 45–59%, and <45% of the predicted value, respectively. Splenomegaly was defined a spleen length of over 11 cm on an ultrasonograph.

Fifty-two patients were on regular intravenous replacement treatment at a dose of 210–520 mg/kg/3–4 weeks. Three patients were on subcutaneous immunoglobulin treatment at a dose of 360–415 mg/kg/4 weeks. Four patients were not on immunoglobulin replacement treatment during the study, and three patients were on intramuscular immunoglobulin treatment at a dose of 0.3–1.5 g/week, because each patient had a satisfactory clinical condition.

IgG catabolism

Serum IgG levels were measured by nephelometry before IVIG infusion and on days +7 (D7) and +14 (D14) after IVIG

infusion. This interval was applied because previous research has shown that approximately half of the infused IgG disappears from blood by day 7 after infusion, due to not only catabolism but also to diffusion into extravascular space [13]. The consequent decrease before the next infusion is caused particularly by catabolism of IgG, in which FcRn plays an important role, so the D14/D7 ratio was used for analyses.

FCRN gene VNTR promoter polymorphism

For the VNTR-genotyping PCR reaction, we used HotStart Taq Master Mix (Qiagen, Hilden, Germany), 800 nM of each gene-specific primer (sequences available upon request), and 200 ng of genomic DNA. Heating the reaction at 95 °C for 15 min was followed by 35 cycles of 95 °C for 30 s, 57 °C for 40 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Reaction products were resolved in 1.5% agarose (Serva, Heidelberg, Germany).

FCRN mRNA quantitation by real-time RT-PCR

Peripheral blood mononuclear cells (PBMC) were isolated from heparin-treated whole blood by Ficoll-gradient centrifugation. A maximum of 10 million cells was used for RNA isolation with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The quality of isolated RNA was assured using spectrophotometry and agarose gel electrophoresis. Reverse transcription (RT) was carried out with a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, California). Quantitative PCR (qPCR) was performed with TaqMan Gene Expression Master Mix (Applied Biosystems, Forster City, California) on an ABI Prism 7000 PCR cycler (Applied Biosystems). Validated PCR primers and TaqMan probes were used as follows: FCGRT (assay ID: Hs01108967_m1, Fam-MGB) and GAPDH (TaqMan endogenous control, VIC-MGB), both purchased from Applied Biosystems. Reaction conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was analyzed in duplicate using average C_T values for *FCRN* expression determination. Relative expression changes of the *FCRN* gene were calculated using the delta-C_T method ($2^{-(\mathrm{Ct}\ FCRN - \mathrm{Ct}\ GAPDH)}$) as described in Nolan et al. [14].

Statistical analysis

Differences between two continuous variables were detected with the Mann–Whitney *U* test. Categorical variables were analyzed using the appropriate test for the contingency tables, i.e., Fisher's exact test in the case of two variables with a binary outcome and the maximal likelihood chi-square test in the case of two variables with multiple outcomes. Independence between two continuous variables was tested by Spearman's test. A standard level of statistical significance of $\alpha = 0.05$ was used. The results are presented without correction for multiple testing since no statistically significant results were found when this correction was applied (Bonferroni and false discovery rate (FDR) correction for multiple testing were adopted for this computation). The statistical package STATISTICA (StatSoft, Inc.), version 9, was used; FDR procedure for multiple testing was computed using R-project 2.8.1 and qvalue library.

Results

Frequency of VNTR polymorphism

The frequencies of individual VNTR alleles (VNTR1, 2, 3, 4 and 5) did not differ significantly between CVID patients (0.0, 8.9, 90.3, 0.8 and 0.0%) and the general Czech population (0.2, 6.4, 92.6, 0.7 and 0.0%), and they were similar to frequencies reported in German blood donors [7]. VNTR genotypes detected in CVID patients were as follows: 3/3 (82.3%), 2/3 (14.5%), 2/2 (1.6%), and 3/4 (1.6%). As functional significance was established only for VNTR alleles 2 and 3 [7] and just one carrier of the other allele (VNTR4) was found among our patients, all further analyses were done for VNTR3/3 homozygotes compared with VNTR2 allele carriers.

VNTR polymorphism and phenotypic features of CVID

No significant differences between VNTR3/3 homozygotes and VNTR2 allele carriers were found in clinical or laboratory characteristics before diagnosis, respiratory tract infections, lung structural and functional abnormalities or other phenotypic features of CVID, with the exception of diagnostic delay, which was longer in the latter group (Table 1). However, a difficulty to interpret these results in cases of absence of any event in the VNTR2 subgroup, as occurred in analysis of respiratory insufficiency and granuloma presence, should be noted.

VNTR polymorphism and IVIG kinetics

No significant differences in serum IgG concentration before IVIG infusion or in the IgG decrease after IVIG infusion (D14/D7 ratio, see Materials and methods) were noted between the analyzed subgroups of patients (Table 2).

FCRN gene expression and VNTR polymorphism

As we failed to find any influence of VNTR2 allele presence on CVID phenotype or IVIG catabolism, peripheral blood mononuclear cells were isolated from 28 CVID patients to analyze *FCRN* mRNA expression. However, we did not detect any difference in *FCRN* expression between VNTR3/3 homozygotes and VNTR2 allele carriers with CVID (Table 3).

FCRN gene expression and phenotypic features of CVID

We continued to analyze the relationship between *FCRN* expression and CVID phenotype. The results are documented in Tables 4 and 5. No correlation was found between *FCRN* levels and clinical or laboratory features before diagnosis of CVID, respiratory tract infections or lung functional abnormalities, although a tendency of lower *FCRN* mRNA levels in patients with respiratory insufficiency was noted ($P = 0.065$). However, in the analysis of lung structural abnormalities, *FCRN* mRNA levels were negatively correlated with the extent of bronchiectasis ($P = 0.027$), tended to be lower in patients with any bronchiectasis ($P = 0.085$) and were lower

Table 1 Comparison of phenotypic features of CVID in *FcRn* VNTR3/3 homozygotes and VNTR2 allele carriers.

Phenotypic feature	All CVID patients		VNTR3/3 homozygotes		VNTR2 carriers		*P*-value
	n		*n*		*n*		
Age; years	62	45.0 (22.0–70.0)	51	45.0 (22.0–70.0)	10	51.0 (18.0–70.0)	0.477**
Sex; males/females	62	25/37	51	20/31	10	5/5	0.727*
Before diagnosis							
Age at onset; years	61	30.0 (6.0–62.0)	50	28.0 (6.0–65.0)	10	34.5 (2.0–50.0)	0.367**
Age at diagnosis; years	61	33.0 (13.0–65.0)	50	33.0 (13.0–65.0)	10	41.5 (13.0–68.0)	0.226**
Diagnostic delay; years	61	3.0 (0.0–17.0)	50	2.5 (0.0–17.0)	10	5.5 (1.0–18.0)	**0.035****
Pneumonias during dg delay; *n*	60	1.0 (0.0–10.0)	49	1.0 (0.0–10.0)	10	1.0 (0.0–5.0)	0.574**
IgG at diagnosis; g/L	61	1.74 (0.12–4.34)	50	1.73 (0.12–4.39)	9	2.13 (0.06–3.19)	0.758**
Respiratory tract infections (RTI)							
Pneumonias on IVIG/SCIG; *n*	58	0.0 (0.0–3.0)	48	0.0 (0.0–3.0)	9	0.0 (0.0–1.0)	0.455**
RTI per year; *n*	59	2.0 (1.0–7.0)	48	2.0 (1.0–7.0)	10	3.0 (1.0–6.0)	0.315**
Lung structure abnormalities							
Presence of bronchiectasis; *n* (%)	58	27 (46.6)	48	23 (47.9)	9	3 (33.3)	0.488*
Extent of bronchiectasis; *n*/*n*/*n*	58	31/17/10	48	25/15/8	9	6/2/1	0.717***
Presence of generalized bronchiectasis; *n* (%)	58	10 (17.2)	48	8 (16.7)	9	1 (11.1)	1.000*
Presence of fibrosis; *n* (%)	58	22 (37.9)	48	18 (37.5)	9	3 (33.3)	1.000*
Extent of fibrosis; *n*/*n*/*n*	58	36/17/5	48	30/14/4	9	6/2/1	0.895***
Lung function abnormalities							
Presence of obstructive disease; *n* (%)	57	27 (47.4)	48	22 (45.8)	8	4 (50.0)	1.000*
Degree of obstructive disease; *n*/*n*/*n*/*n*	57	30/15/10/2	48	26/14/7/1	8	4/1/2/1	0.437***
Presence of restrictive disease; *n* (%)	57	10 (17.5)	48	8 (16.7)	8	2 (25.0)	0.623*
Degree of restrictive disease; *n*/*n*/*n*/*n*	57	47/8/2/0	48	40/6/2/0	8	6/2/0/0	0.519***
Presence of respiratory insufficiency; *n* (%)	55	9 (16.4)	46	8 (17.4)	8	0 (0.0)	0.336*
Others							
Presence of chronic diarrhea; *n* (%)	60	14 (23.3)	49	11 (22.4)	10	3 (30.0)	0.688*
Presence of splenomegaly; *n* (%)	59	37 (62.7)	48	30 (62.5)	10	7 (70.0)	0.733*
Presence of AI phenomena; *n* (%)	61	15 (24.6)	50	12 (24.0)	10	3 (30.0)	0.700*
Presence of granulomas; *n* (%)	60	5 (8.3)	50	5 (10.0)	9	0 (0.0)	1.000*
Presence of lymphadenopathy; *n* (%)	60	17 (28.3)	49	13 (26.5)	10	3 (30.0)	1.000*

The extent of bronchiectasis and the extent of fibrosis are given as numbers of patients with no/localized/generalized disease. The degrees of obstructive and restrictive disease are reported as numbers of patients with normal/mild/moderate/severe form of disease. The presence of any symptom/disease is given as the number of patients with this particular symptom/disease. Continuous variables are given as median (5th–95th percentile). AI = autoimmune. *P*-value: VNTR3/3 homozygotes vs. VNTR2 carriers; *two-tailed Fisher's exact test; **Mann–Whitney *U* test; ***maximal likelihood chi-square test. Considerable *P*-values are marked in bold.

in patients with generalized bronchiectasis ($P=0.027$). In addition, patients with fibrosis had lower *FCRN* mRNA levels compared to patients without fibrosis ($P=0.041$), and a tendency to negative correlation of *FCRN* mRNA levels with the extent of fibrosis was noted ($P=0.066$). No relationship between *FCRN* mRNA levels and other phenotypic features of CVID (presence of chronic diarrhea, splenomegaly, granulomas, lymphadenopathy or autoimmune phenomena) was documented. An additional analysis was performed to determine if *FCRN* mRNA levels were not related just to the absolute number of peripheral blood monocytes. No correlation was revealed between these two variables (Spearman's correlation coefficient $R=0.082$, $P=0.677$).

FCRN gene expression and IVIG kinetics

No correlation was found between *FCRN* mRNA level and pre-infusion IgG concentration in CVID patients. However, a

Table 2 Comparison of IgG concentration before IVIG infusion and IgG kinetics after IVIG infusion in *FcRn* VNTR3/3 homozygotes and VNTR2 allele carriers.

IgG and IVIG kinetics	All CVID patients		VNTR3/3 homozygotes		VNTR2 carriers		*P*-value
	n		*n*		*n*		
IgG before IVIG	31	5.82 (3.92–7.79)	26	5.70 (3.99–7.79)	4	6.51 (6.13–6.96)	0.329
D14/D7 ratio	30	0.797 (0.729–0.968)	25	0.816 (0.739–0.941)	4	0.776 (0.736–0.994)	0.448

D7 (D14) = IgG concentration +7 days (+14 days) after IVIG infusion; IgG concentration is given as median (5th–95th percentile) in g/L. *P*-value: VNTR3/3 homozygotes vs. VNTR2 carriers, Mann–Whitney *U* test.

Table 3 Comparison of *FcRn* mRNA expression in VNTR3/3 homozygotes and VNTR2 allele carriers.

FcRn mRNA expression	All CVID patients		VNTR3/3 homozygotes		VNTR2 carriers		*P*-value
	n		*n*		*n*		
FcRn/GAPDH	28	0.086 (0.058–0.131)	24	0.087 (0.058–0.131)	4	0.083 (0.062–0.101)	0.646

The results are given as median (5th–95th percentile) values of the *FcRn/GAPDH* ratio, which presents the mRNA level of the *FcRn* gene normalized to the housekeeping *GAPDH* gene in peripheral blood mononuclear cells. *P*-value: VNTR3/3 homozygotes vs. VNTR2 carriers, Mann–Whitney *U* test.

correlation was demonstrated between *FCRN* mRNA level and the decline in serum IgG concentration during the 2nd week after IVIG infusion ($P=0.045$; Table 6). The lower the *FCRN* mRNA expression, the more pronounced the decrease in IgG concentration, in CVID patients in the tracked period after IVIG infusion. When a relationship between the extent of bronchiectasis and the rate of IgG decline after infusion of IVIG was analyzed, a tendency to the lower 14D/7D IgG concentration ratio in patients with generalized bronchiectasis was shown (Spearman's correlation coefficient $R=-0.365$, $P=0.067$).

Discussion

CVID is a heterogeneous disease with complex immunological and clinical phenotypes. The heterogeneity in the clinical symptoms and immunological defects is supposedly underlain by the number of disease-causing and disease-modifying genes. An association with CVID has already been shown for several genes. Biallelic mutations in the *ICOS* [15], *CD19* [16] and *BAFF-R* [17] genes are responsible for CVID phenotype in a minority of patients. Defects in the *TNFRSF13B* gene, coding for TACI, are found in about 10% of CVID patients and increase the risk of disease development. However, these defects seem to be neither necessary nor sufficient to cause CVID [18]. Polymorphisms in several other genes (vitamin D receptor, interleukin (*IL*)*-6*, *IL-10*, *TNF*, and *MBL2*) have been shown to be associated with particular phenotypic features of CVID [9–11]. In this study, we analyzed the *FCRN* gene as a potential strong disease modifier because of its documented role in IgG catabolism and antigen delivery.

Table 4 Correlation of *FcRn* mRNA expression with quantifiable phenotypic features of CVID.

Phenotypic feature ($n=27$)	Spearman *R* coefficient	*P*-value
Before diagnosis		
Age at onset	0.024	0.904
Age at diagnosis	0.027	0.894
Diagnostic delay	−0.241	0.227
Pneumonias during dg delay	−0.212	0.289
IgG at diagnosis	0.142	0.481
Respiratory tract infections (RTI)		
Pneumonias on IVIG/SCIG	−0.066	0.742
RTI per year	−0.194	0.332
Lung structure abnormalities		
Extent of bronchiectasis	−0.424	**0.027**
Extent of fibrosis	−0.359	0.066
Lung function abnormalities		
Degree of obstructive disease	−0.127	0.529
Degree of restrictive disease	−0.214	0.283

The extent of bronchiectasis and the extent of fibrosis were characterized as no, localized and generalized (grades 0–2) and the degrees of obstructive and restrictive disease as normal, mild, moderate and severe (grades 0–3). *P*-value: determined by Spearman's test. Considerable *P*-values are marked in bold.

Recently, Sachs et al. described a promoter polymorphism of a variable number of tandem repeats (VNTR) in the *FCRN* gene. VNTR2 allele carriers have lower *FCRN* mRNA levels, and their monocytes show decreased binding capacity in vitro compared with VNTR3/3 homozygotes [7]. However, analyzing a number of phenotypic features, including IVIG catabolism, we did not find any difference in CVID patients carrying one or two VNTR2 alleles compared with VNTR3/3 homozygotes. The only exception was a longer time from the first symptoms to diagnosis in VNTR2 carriers, but this finding lost its statistical significance after Bonferroni or FDR correction. Moreover, there would be no known explanation for such a difference because VNTR2 allele carriers are expected to exhibit more rapid IgG catabolism and less efficient antigen delivery and thus shorter diagnostic delay than VNTR3/3 individuals.

Consequently, no difference in *FCRN* mRNA expression was detected between our CVID VNTR2 allele carriers and VNTR3/3 homozygotes. This result is contrary to the study by Sachs et al., who reported a significant difference, and even no overlapping values, between healthy VNTR2 carriers and VNTR3/3 homozygotes [7]. In that study, RNA was extracted from the $CD14^+$ fraction of peripheral blood mononuclear cells (PBMC), while all PBMC were used in our study. The *FCRN* gene is expressed in multiple blood cells, including monocytes [19], polymorphonuclear leukocytes [20] and, probably, B lymphocytes [21]. We consider both monocytes and all PBMC relevant for *FCRN* expression analyses, as hematopoietically derived cells contribute as much to FcRn mediated protection of IgG from catabolism as do vascular endothelial cells [4]. We showed that *FCRN* mRNA levels were not related to the absolute number of peripheral blood monocytes in our patients. The discrepancy in results could arise from low numbers of analyzed individuals: 5 subjects in both groups in the study of Sachs et al. and 24 patients with the VNTR3/3 genotype and 4 patients carrying a VNTR2 allele in our study. Another explanation could be the different regulation of *FCRN* expression in CVID patients which may be determined by other sequence changes within the *FCRN* gene or altered activity of other genes/proteins in CVID patients compared to healthy controls. However, if no difference was noted in *FCRN* expression in both analyzed subgroups (VNTR3/3 vs. VNTR2 allele carriers) it is not surprising that no difference in phenotypes between these subgroups was

Table 5 Comparison of *FcRn* mRNA levels in subgroups of patients with and without particular phenotypic features of CVID.

Phenotypic feature	Patients with		Patients without		*P*-value
	n		*n*		
Lung structure abnormalities					
Bronchiectasis	14	0.080 (0.055–0.105)	13	0.090 (0.060–0.138)	0.085
Generalized bronchiectasis	5	0.067 (0.055–0.083)	22	0.089 (0.060–0.131)	**0.027**
Fibrosis	8	0.073 (0.058–0.095)	19	0.088 (0.055–0.138)	**0.041**
Lung function abnormalities					
Obstructive disease	13	0.083 (0.058–0.138)	14	0.089 (0.055–0.112)	0.610
Restrictive disease	5	0.079 (0.062–0.095)	22	0.087 (0.058–0.131)	0.275
Respiratory insufficiency	4	0.064 (0.058–0.090)	23	0.086 (0.062–0.131)	0.065
Others					
Chronic diarrhea	5	0.086 (0.058–0.112)	22	0.085 (0.060–0.131)	0.779
Splenomegaly	20	0.085 (0.057–0.134)	7	0.086 (0.062–0.131)	0.507
AI phenomena	4	0.094 (0.078–0.112)	23	0.084 (0.058–0.131)	0.394
Granulomas	2	0.075 (0.055–0.095)	25	0.086 (0.060–0.131)	0.405
Lymphadenopathy	5	0.076 (0.062–0.101)	22	0.086 (0.058–0.131)	0.473

The results are given as median (5th–95th percentile) values of the *FcRn*/*GAPDH* ratio, which presents the mRNA level of *FcRn* normalized to the housekeeping *GAPDH* gene in peripheral blood mononuclear cells. *P*-value: determined by the Mann–Whitney *U* test. Considerable *P*-values are marked in bold.

found in the previous experiments (see Tables 1 and 2). Little is known about how *FCRN* is transcriptionally regulated. Undoubtedly, the regulation of *FCRN* expression is a complex process. Our results indicate that the influence of VNTR polymorphism on *FCRN* expression is not dominant in CVID patients.

When correlating *FCRN* mRNA levels with phenotypic features of CVID, we revealed several interesting and consistent associations, although their statistical significance disappeared after Bonferroni or FDR correction. Patients with generalized bronchiectasis or fibrosis had lower *FCRN* mRNA levels compared to patients without such abnormalities. Moreover, there was a negative correlation of *FCRN* mRNA levels with the extent of the bronchiectasis. In concordance with these findings, *FCRN* mRNA levels were negatively correlated with the rate of IgG decline in the 2nd week after infusion of IVIG. A decreased capacity of FcRn both to protect IgG from degradation and to transcytose immune complexes via the respiratory epithelium due to lower specific mRNA levels might contribute to bronchiectasis and lung fibrosis development. However, we did not find an association of *FCRN* expression with either the age of disease onset, age of diagnosis, diagnostic delay, the number of pneumonias before diagnosis or the frequency of infections, which could also be expected considering the presumed mechanism of FcRn function. It should be taken into account that the latter variables were mostly based on patient information, which did not have to be sufficiently exact for statistical evaluation, particularly when a limited number of patients were available for analysis. On the other hand, the presence and extent of bronchiectasis and fibrosis and the presence of respiratory insufficiency were clearly determined by HRCT and lung function tests.

Table 6 Correlation of *FcRn* mRNA expression with IgG concentration before IVIG infusion and IgG kinetics after IVIG infusion in CVID patients.

IgG and IVIG kinetics	Spearman *R* coefficient		*P*-value
	n		
IgG before IVIG	28	−0.123	0.532
D14/D7 ratio	27	0.390	**0.045**

D7 (D14) = IgG concentration +7 days (+14 days) after IVIG infusion. *P*-value: determined by Spearman's test. Considerable *P*-values are marked in bold.

FcRn deficiency or blockade confers protection from autoimmune humoral reactivity in a mouse model of autoimmune arthritis [22]. However, no association of *FCRN* expression with the presence of autoimmune phenomena was found in our CVID patients.

In our previous work we reported the association of low mannan-binding lectin (MBL) levels with the development of bronchiectasis, fibrosis and respiratory insufficiency but not other phenotypic features in patients with CVID [11]. However, we found no difference in *FCRN* mRNA levels between subgroups of patients with *MBL2* genotypes associated with normal, low and deficient MBL levels (data not shown). Therefore, low *FCRN* expression and low MBL levels seem to be independent risk factors for the development of lung complications in CVID patients.

Our findings show that FcRn may play a role in the development of lung structural abnormalities, which are the principal life-threatening complications in patients with CVID, as well as in the catabolism of therapeutically administered IVIG. Nevertheless, our results show borderline statistical significance and need to be interpreted carefully. In addition, some limitations of our study should be addressed in successive studies for better interpretation of the results. It would be definitely useful to analyze *FCRN* expression in people without an antibody deficiency suffering from bronchiectasis and/or lung fibrosis to show if altered *FCRN* levels may contribute mechanistically to pulmonary complications. It would also be valuable to examine *FCRN* expression directly at the site of

damage as local conditions in the lungs may not reflect truly the situation in peripheral blood. However, if our observations are confirmed in a larger cohort of patients in future studies, increasing *FCRN* expression might be a potential therapeutic target to prevent lung complications not only in CVID patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2010.05.006.

References

[1] K. Baker, S.W. Qiao, T. Kuo, K. Kobayashi, M. Yoshida, W.I. Lencer, R.S. Blumberg, Immune and non-immune functions of the (not so) neonatal Fc receptor, FcRn, Semin. Immunopathol. 31 (2009) 223–236.

[2] C.L. Anderson, C. Chaudhury, J. Kim, C.L. Bronson, M.A. Wani, S. Mohanty, Perspective — FcRn transports albumin: relevance to immunology and medicine, Trends Immunol. 27 (2006) 343–348.

[3] J. Kim, C.L. Bronson, W.L. Hayton, M.D. Radmacher, D.C. Roopenian, J.M. Robinson, C.L. Anderson, Albumin turnover: FcRn-mediated recycling saves as much albumin from degradation as the liver produces, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (2006) G352–G360.

[4] S. Akilesh, G.J. Christianson, D.C. Roopenian, A.S. Shaw, Neonatal FcR expression in bone marrow-derived cells functions to protect serum IgG from catabolism, J. Immunol. 179 (2007) 4580–4588.

[5] M. Yoshida, S.M. Claypool, J.S. Wagner, E. Mizoguchi, A. Mizoguchi, D.C. Roopenian, W.I. Lencer, R.S. Blumberg, Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells, Immunity 20 (2004) 769–783.

[6] S.W. Qiao, K. Kobayashi, F.E. Johansen, L.M. Sollid, J.T. Andersen, E. Milford, D.C. Roopenian, W.I. Lencer, R.S. Blumberg, Dependence of antibody-mediated presentation of antigen on FcRn, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 9337–9342.

[7] U.J. Sachs, I. Socher, C.G. Braeunlich, H. Kroll, G. Bein, S. Santoso, A variable number of tandem repeats polymorphism influences the transcriptional activity of the neonatal Fc receptor alpha-chain promoter, Immunology 119 (2006) 83–89.

[8] M.A. Park, J.T. Li, J.B. Hagan, D.E. Maddox, R.S. Abraham, Common variable immunodeficiency: a new look at an old disease, Lancet 372 (2008) 489–502.

[9] C.G. Mullighan, G.C. Fanning, H.M. Chapel, K.I. Welsh, TNF and lymphotoxin-alpha polymorphisms associated with common variable immunodeficiency: role in the pathogenesis of granulomatous disease, J. Immunol. 159 (1997) 6236–6241.

[10] C.G. Mullighan, S.E. Marshall, M. Bunce, K.I. Welsh, Variation in immunoregulatory genes determines the clinical phenotype of common variable immunodeficiency, Genes Immun. 1 (1999) 137–148.

[11] J. Litzman, T. Freiberger, B. Grimbacher, B. Gathmann, U. Salzer, T. Pavlik, J. Vlcek, V. Postranecka, Z. Travnickova, V. Thon, Mannose-binding lectin gene polymorphic variants predispose to the development of bronchopulmonary complications but have no influence on other clinical and laboratory symptoms or signs of common variable immunodeficiency, Clin. Exp. Immunol. 153 (2008) 324–330.

[12] M.E. Conley, L.D. Notarangelo, A. Etzioni, Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies), Clin. Immunol. 93 (1999) 190–197.

[13] S. Mankarious, M. Lee, S. Fischer, K.H. Pyun, H.D. Ochs, V.A. Oxelius, R.J. Wedgwood, The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin, J. Lab. Clin. Med. 112 (1988) 634–640.

[14] T. Nolan, R.E. Hands, S.A. Bustin, Quantification of mRNA using real-time RT-PCR, Nat. Protoc. 1 (2006) 1559–1582.

[15] B. Grimbacher, A. Hutloff, M. Schlesier, E. Glocker, K. Warnatz, R. Drager, H. Eibel, B. Fischer, A.A. Schaffer, H.W. Mages, R.A. Kroczek, H.H. Peter, Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency, Nat. Immunol. 4 (2003) 261–268.

[16] M.C. van Zelm, I. Reisli, M. van der Burg, D. Castano, C.J. van Noesel, M.J. van Tol, C. Woellner, B. Grimbacher, P.J. Patino, J.J. van Dongen, J.L. Franco, An antibody-deficiency syndrome due to mutations in the CD19 gene, N. Engl. J. Med. 354 (2006) 1901–1912.

[17] K. Warnatz, U. Salzer, M. Rizzi, B. Fischer, S. Gutenberger, J. Bohm, A.K. Kienzler, Q. Pan-Hammarstrom, L. Hammarstrom, M. Rakhmanov, M. Schlesier, B. Grimbacher, H.H. Peter, H. Eibel, B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 13945–13950.

[18] U. Salzer, C. Bacchelli, S. Buckridge, Q. Pan-Hammarstrom, S. Jennings, V. Lougaris, A. Bergbreiter, T. Hagena, J. Birmelin, A. Plebani, A.D. Webster, H.H. Peter, D. Suez, H. Chapel, A. McLean-Tooke, G.P. Spickett, S. Anover-Sombke, H.D. Ochs, S. Urschel, B.H. Belohradsky, S. Ugrinovic, D.S. Kumararatne, T.C. Lawrence, A.M. Holm, J.L. Franco, I. Schulze, P. Schneider, E.M. Gertz, A.A. Schaffer, L. Hammarstrom, A.J. Thrasher, H.B. Gaspar, B. Grimbacher, Relevance of biallelic versus monoallelic TNFRSF13B mutations in distinguishing disease-causing from risk-increasing TNFRSF13B variants in antibody deficiency syndromes, Blood 113 (2009) 1967–1976.

[19] X. Zhu, G. Meng, B.L. Dickinson, X. Li, E. Mizoguchi, L. Miao, Y. Wang, C. Robert, B. Wu, P.D. Smith, W.I. Lencer, R.S. Blumberg, MHC class I-related neonatal Fc receptor for IgG is functionally expressed in monocytes, intestinal macrophages, and dendritic cells, J. Immunol. 166 (2001) 3266–3276.

[20] G. Vidarsson, A.M. Stemerding, N.M. Stapleton, S.E. Spliethoff, H. Janssen, F.E. Rebers, M. de Haas, J.G. van de Winkel, FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis, Blood 108 (2006) 3573–3579.

[21] W. Mi, S. Wanjie, S.T. Lo, Z. Gan, B. Pickl-Herk, R.J. Ober, E.S. Ward, Targeting the neonatal fc receptor for antigen delivery using engineered fc fragments, J. Immunol. 181 (2008) 7550–7561.

[22] S. Akilesh, S. Petkova, T.J. Sproule, D.J. Shaffer, G.J. Christianson, D. Roopenian, The MHC class I-like Fc receptor promotes humorally mediated autoimmune disease, J. Clin. Invest. 113 (2004) 1328–1333.

Supplementary Table 1a: Comparison of phenotypic features of CVID in patients available and not available for *FcRn* mRNA expression analysis.

Phenotypic feature	Available for analysis		Not available for analysis		*P*-value
	n		n		
Age; years	28	46.5 (22.0 - 69.0)	34	41.5 (18.0 - 70.0)	0.538**
Sex; males/females	28	13/15	34	12/22	0.441*
Before diagnosis					
Age at onset; years	27	30.0 (5.0 - 48.0)	34	28.5 (6.0 - 65.0)	0.856**
Age at diagnosis; years	27	35.0 (14.0 - 55.0)	34	33.0 (13.0 - 68.0)	0.994**
Diagnostic delay; years	27	3.0 (0.0 - 17.0)	34	3.0 (0.0 - 18.0)	0.930**
Pneumonias during dg delay; n	27	1.0 (0.0 - 10.0)	33	1.0 (0.0 - 10.0)	0.194**
IgG at diagnosis; g/l	27	1.74 (0.12 - 4.48)	34	1.81 (0.07 - 4.34)	0.744**
Respiratory tract infections (RTI)					
Pneumonias on IVIG/SCIG; n	27	0.0 (0.0 - 1.0)	31	0.0 (0.0 - 3.0)	0.399**
RTI per year; n	27	2.0 (1.0 - 5.0)	32	3.0 (1.0 - 8.0)	0.212**
Lung structure abnormalities					
Presence of bronchiectasis; n (%)	27	14 (51.9)	31	13 (41.9)	0.598*
Extent of bronchiectasis; n/n/n	27	13/9/5	31	18/8/5	0.744***
Presence of generalized bronchiectasis; n (%)	27	5 (18.5)	31	5 (16.1)	1.000*
Presence of fibrosis; n (%)	27	8 (29.6)	31	14 (45.2)	0.283*
Extent of fibrosis; n/n/n	27	19/5/3	31	17/12/2	0.222***
Lung function abnormalities					
Presence of obstructive disease; n (%)	27	13 (48.1)	30	14 (46.7)	1.000*
Degree of obstructive disease; n/n/n/n	27	14/7/5/1	30	16/8/5/1	0.998***
Presence of restrictive disease; n (%)	27	5 (18.5)	30	5 (16.7)	1.000*
Degree of restrictive disease; n/n/n/n	27	22/5/0/0	30	25/3/2/0	0.191***
Presence of respiratory insuficiency; n (%)	27	4 (14.8)	28	5 (17.9)	1.000*
Others					
Presence of chronic diarrhoea; n (%)	27	5 (18.5)	33	9 (27.3)	0.544*
Presence of splenomegaly; n (%)	27	20 (74.1)	32	17 (53.1)	0.114*
Presence of AI phenomenona; n (%)	27	4 (14.8)	34	11 (32.4)	0.143*
Presence of granulomas; n (%)	27	2 (7.4)	33	3 (9.1)	1.000*
Presence of lymphadenopathy; n (%)	27	5 (18.5)	33	12 (36.4)	0.158*

The extent of bronchiectasis and the extent of fibrosis are given as numbers of patients with no/localized/generalized disease. The degrees of obstructive and restrictive disease are reported as numbers of patients with normal/mild/moderate/severe form of disease. The presence of any symptom/disease is given as the number of patients with this particular symptom/disease. Continuous variables are given as median (5^{th}–95^{th} percentile). AI = autoimmune. *P*-value: VNTR3/3 homozygotes vs. VNTR2 carriers; * two-tailed Fisher exact test; ** Mann-Whitney U test; *** maximal likelihood chi-square test.

3.3.2 FcRn a přenos IgG přes placentu

Analyzovali jsme také vliv popsaných funkčně významných polymorfizmů v promotorové oblasti genu *FCRN*, reprezentovaných variabilním počtem tandemových repetic (VNTR), na placentární přenos IgG. Vyšetření 103 párových vzorků mateřské a pupečníkové krve, odebraných při porodech v 38. až 41. týdnu gravidity, neprokázalo vliv jednotlivých polymorfních variant na gradient IgG mezi mateřskou a placentární krví.

Podrobný popis studie je uveden v následující publikaci:

T. Freiberger, B. Ravčuková, L. Grodecká, B. Kuřecová, J. Jarkovský, D. Bartoňková, V. Thon, J. Litzman: No association of FCRN promoter VNTR polymorphism with the rate of maternal-fetal IgG transfer. J Reprod Immunol 2010; 85(2): 193-7.

3.4 TACI

TACI je transmembránový aktivátor a interaktor s proteinem CAML (Transmembrane Activator and CAML Interactor), přičemž CAML je kalciový modulátor i ligand pro cyclophilin (CAlcium Modulator and cyclophilin Ligand). Molekula TACI sestává z 293 aminokyselin a je kódována genem *TNFRSF13B*, který leží na krátkém raménku 17. chromozomu, má 5 exonů a velikost 42,5 kb. TACI je jedním z členů rodiny receptorů TNF (tumour necrosis factor) exprimovaných na periferních B lymfocytech a podílejících se na jejich finální maturaci. Je receptorem pro molekuly BAFF (B-cell Activating Factor) a APRIL (A PRoliferation Inducing Ligand), které slouží jako regulátory aktivace, proliferace a diferenciace B lymfocytů s využitím signalizační cesty NF-AT (nukleární faktor aktivovaných T lymfocytů; Nuclear Factor of Activated T cells) a NF-κB. TACI pomáhá B lymfocytům v izotypovém přesmyku z IgM na IgG, IgA a IgE, zároveň posiluje vyzrávání a přežívání izotypově determinovaných paměťových B lymfocytů a plazmatických buněk a přispívá ke zvyšování afinity tvořených protilátek cestou somatických hypermutací (He et al., 2010; Ozcan et al., 2011).

V experimentech s knock-out *tnfrsf13b-/-* myšími modely bylo ukázáno, že TACI je negativním regulátorem aktivace a proliferace B lymfocytů a nezbytnou složkou imunitní odpovědi nezávislé na T lymfocytech, typu II (von Bulow et al., 2001).

3.4.1 Asociace TACI s CVID a IgAD

Genetická podstata CVID a IgAD nebyla dlouho objasněna a u většiny případů není objasněna dodnes. Velkou naději představovaly v době svého zveřejnění 2 nezávislé práce (Castigli et al., 2005; Salzer et

al., 2005), které na základě přístupu analýzy kandidátních genů detekovaly mutace v genu *TNFRSF13B* jak u pacientů s CVID, tak IgAD. Zprvu to vypadalo na opravdový průlom, protože se zdálo, že mutace v *TNFRSF13B* jsou zodpovědné za podstatnou část, až 10%, případů CVID. U nejčastějších mutací (p.Cys04Arg a p.Ala181Glu) byl prokázán negativní efekt na proliferaci B lymfocytů a izotypový přesmyk po stimulaci ligandem APRIL (Castigli et al., 2005; Salzer et al., 2005). Mutace nebyly v prvních studiích nalezeny u žádné z použitých zdravých kontrol, ale již v počátku byla zjištěna velká šíře fenotypového projevu u nositelů mutací v rodinách pacientů, od asymptomatického průběhu, přes IgAD, až po klasickou formu CVID.

Myší knock-out model TACI-/- s expanzí B lymfocytů, jejich hyperreaktivitou a těžkým lupus-like postižením (Seshasayee et al., 2003; Yan et al., 2001) neodrážel B lymfocytární ani klinický fenotyp pozorovaný u lidí.

Další studie navíc ukázaly přítomnost mutací u malé části zdravých jedinců kontrolních souborů a stále více se objevovaly zprávy o tom, že v řadě rodin nosičství mutace nekoreluje s fenotypem IgAD ani CVID (Poodt et al., 2009; Salzer et al., 2009; Sazzini et al., 2009; Speletas et al., 2011; Zhang et al., 2007). Opakovaně však byla dokumentována silná, statisticky významná asociace mutací v genu *TNFRSF13B* s CVID, zatímco u IgAD byla tato souvislost zpochybněna (Pan-Hammarstrom et al., 2007). Současný stav poznání podporuje roli genu *TNFRSF13B* jako faktoru, který představuje predispozici ke vzniku CVID, nikoliv IgAD, a může modifikovat průběh onemocnění, ale nezbytná je účast dalších genetických faktorů a zevních vlivů.

K poznání úlohy genu *TNFRSF13B* při vzniku CVID a/nebo IgAD přispěla i naše práce. Genovou mutaci jsme detekovali u 4 ze 70 pacientů s CVID (5,7%), 9 ze 161 pacientů s IgAD (5,6%), 1 ze 17 pacientů s jinou hypogamaglobulinemií nebo dysgamaglobulinemií (5,9%) a žádného ze 195 jedinců obecné české populace. U českých pacientů jsme tak zaznamenali statisticky významnou asociaci mutací *TNFRSF13B* jak s CVID (p=0,01), tak s IgAD (p=0,02), ovšem při kompilaci všech dat z dostupných publikací zůstala významná asociace ve srovnání s kontrolním souborem jen v případě CVID (9,9% vs. 3,2%, $p<10^{-6}$), zatímco u IgAD asociace ztratila statickou významnost (5,7% vs. 3,2%, p=0,145). Detekovali jsme v souhrnu 8 různých mutací, včetně nejčastějších mutací p.Cys104Arg a p.Ala181Glu a jedné dříve nepopsané missense varianty p.Arg189Lys. Popsali jsme také statisticky významnou asociaci tiché záměny p.Pro97Pro s CVID, a to nejen v souboru českých pacientů (alelická frekvence 4.3% vs. 0.2%, p=0.01), ale i v kompilovaném souboru všech populací (5.1% vs. 1.8%, p=0.003). Není vyloučeno, že tato tichá mutace, resp. polymorfizmus, může mít funkční význam cestou ovlivnění sestřihu mRNA.

Přesto, že jsme nenašli mutaci u žádného jedince v kontrolním souboru, naše pozorování přítomnosti mutace u asymptomatických členů rodin pacientů s CVID a/nebo IgAD a naopak nepřítomnosti mutace u klinicky zřejmých případů onemocnění jsou ve shodě s publikovanými daty a podporují hypotézu o roli *TNFRSF13B* jako faktoru modifikujícího vznik a průběh CVID, spíše než kauzálního faktoru vzniku choroby.

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Sequence variants of the *TNFRSF13B* gene in Czech CVID and IgAD patients in the context of other populations

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ABSTRACT

Mutations in the *TNFRSF13B* gene, encoding TACI, have been found in common variable immunodeficiency (CVID) and selective IgA deficient (IgAD) patients, but only the association with CVID seems to be significant. In this study, Czech CVID, IgAD and primary hypo/dysgammaglobulinemic (HG/DG) patients were screened for all *TNFRSF13B* sequence variants. The *TNFRSF13B* gene was mutated in 4/70 CVID patients (5.7%), 9/161 IgAD patients (5.6%), 1/17 HG/DG patient (5.9%) and none of 195 controls. Eight different mutations were detected, including the most frequent p.C104R and p.A181E mutations as well as 1 novel missense mutation, p.R189K. A significant association of *TNFRSF13B* gene mutations was observed in both CVID ($p = 0.01$) and IgAD ($p = 0.002$) Czech patients. However, when combined with all published data, only the association with CVID remained significant compared with the controls (9.9% vs. 3.2%, $p < 10^{-6}$), while statistical significance disappeared for IgAD (5.7% vs. 3.2%, $p = 0.145$). The silent mutation p.P97P was shown to be associated significantly with CVID compared with the controls in both Czech patients (allele frequency 4.3% vs. 0.2%, $p = 0.01$) and in connection with the published data (5.1% vs. 1.8%, $p = 0.003$). The relevance of some *TNFRSF13B* gene variants remains unclear and needs to be elucidated in future studies.

1. Introduction

Common variable immunodeficiency (CVID) and IgA deficiency (IgAD) are considered to be different phenotypic manifestations of similar or related genetic backgrounds [1]. Severe deficiency in production of specific antibodies accompanied by low levels of IgG, IgA and sometimes IgM, as seen in CVID, manifests clinically through recurrent and severe infections, predominantly of the respiratory tract, and frequent autoimmune complications [2,3]. In IgAD, the immunoglobulins other than IgA are not altered, and the disease is usually asymptomatic, although increased frequencies of autoimmune manifestations have been documented [4,5]. Despite the stated differences, both diseases seem to be closely related. CVID and IgAD may occur simultaneously within families, and IgAD may develop into CVID [6,7]. Although a common genetic background is assumed, the genes responsible for the majority of clinical cases of CVID and IgAD remain unknown. It was suggested that multiple genes may be responsible for the above mentioned clinical and laboratory findings.

Previous studies showed that both diseases are linked to gene(s) in the HLA region of chromosome 6q, and a potential gene locus named *IGAD1* was identified in the HLADQ/DR region [8,9]. Other studies located the gene(s) responsible for CVID on chromosomes 16q [10], 4q [11], and 5p [12]. Particular autoimmunity risk allelic variants were found to be significantly associated also with IgAD [13]. Since 2003, mutations in six genes encoding for receptor/signaling molecules have been recognized as associated with CVID.

Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; CRD, cysteine rich domain; CVID, common variable immunodeficiency; DGGE, denaturing grdient gel electrophoresis; ESID, European Society for Immunodeficiencies; HFDR, healthy first degree relatives; HG/DG, hypogamma/dysgammaglobulinemia; HRM, high resolution melting; IC, intracellular domain; IgAD, selective IgA deficiency; NCBI, National Center for Biotechnology Information; PAGID, Pan American Group for Immunodeficiency; SSPNN, splice site prediction by neural network; TACI, transmembrane activator and calcium modulating cyclophilin ligand interactor; TM, transmembrane domain; *TNFRSF13B*, tumor necrosis factor receptor superfamily 13B gene.

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These include *ICOS* [14], *TNFRSF13B* [15,16], *TNFRSF13C* [17], *CD19* [18], *CD81* [19] and *CD20* [20]. While biallelic mutations in the *ICOS*, *TNFRSF13C*, *CD19, CD81* and *CD20* genes were mostly detected in single autosomal recessive CVID families [21,22], heterozygous or homozygous mutations in the *TNFRSF13B* gene were found in a considerable proportion (approximately 10%) of the patients with CVID [21]. The *TNFRSF13B* gene encodes for the transmembrane activator and calcium modulating cyclophilin ligand interactor (TACI), which is a receptor present on B-cells that interacts with B-cell activating factor (BAFF) or a proliferation-inducing ligand (APRIL), serving as a regulator of B-cell activation, proliferation and differentiation and a trigger of immunoglobulin isotype switching [23,24]. However, *TNFRSF13B* mutations were found also in healthy or only mildly affected first degree relatives of CVID patients and even in unrelated healthy controls [21]. *TNFRSF13B* mutations were also detected in IgAD patients [15], but it was shown later that the frequency in IgAD was not significantly increased compared with the control populations [25,26]. Only two studies dealt with larger series of IgAD patients, but both studies were mainly focused on several of the most frequent mutations [25,26]. The exact role of *TNFRSF13B* mutations in the pathogenesis of CVID, IgAD or other hypogammaglobulinemias is still unclear.

The aim of this study was to screen for all *TNFRSF13B* mutations in Czech CVID and IgAD patients, to map the frequencies of these mutations in Slavonic patients and the general population and to contribute to defining a role for *TNFRSF13B* mutations particularly in IgAD development.

2. Materials and methods

2.1. Patients and controls

Seventy unrelated patients with CVID (58 non-familial and 12 familial cases), 173 unrelated patients with IgAD (149 sporadic cases, 12 familial cases and 12 cases related to CVID patients), an additional 14 IgAD patients related to familial IgAD individuals and 17 unrelated patients with primary hypo/dysgammaglobulinemia (HG/DG), followed in the Department of Clinical Immunology and Allergy, University St. Anne's Hospital in Brno, were included in the study. All of the CVID and IgAD patients fulfilled the ESID/PAGID diagnostic criteria [27]. The patients in the HD/DG group included 5 patients with Good syndrome and 12 patients with probable primary HG/DG not fulfilling diagnostic criteria for CVID, IgAD or other well-defined primary immunoglobulin deficiencies. Available relatives in the IgAD, CVID and HG/DG families with detected mutations were invited and tested for the particular mutations. All persons involved in the study were of the Czech origin and provided a written statement of informed consent approved by the Ethics Committee of the University St. Anne's Hospital in Brno. DNA from umbilical blood samples of 256 consecutively born healthy neonates were used as controls after obtaining a written consent from their mothers, which was approved by the Ethics Committee of the Centre for Cardiovascular Surgery and Transplantation in Brno.

2.2. Mutation and polymorphism analyses

DNA samples were amplified for further analyses using the primers and conditions depicted in Supplementary Table 1. Amplicons of exons 1 and 5 were subjected to single-strand conformation polymorphism (SSCP) electrophoresis, after restriction with AluI enzyme in the case of exon 5. Briefly, 10 μl of PCR products were denatured for 5 min at 100 °C and stored at 4 °C to avoid renaturation. Electrophoresis was performed on a special polyacrylamide gel designed for mutation detection (MDE; Sigma) at the concentration given by the manufacturer for 24 h at a constant voltage of 200 V and a temperature of 6.5 °C (apparatus INGENYphorU system). DNA fragments were then visualized by silver staining.

Amplicons of exons 2 and 3 (one of each of the primer pairs used contained a GC-rich 40 bp segment, a so-called "GC clamp") were subjected to denaturation gradient gel electrophoresis (DGGE) on a 6% polyacrylamide/TAE gel. The linear urea gradient was from 35% to 70% in the exon 2 experiments and from 30% to 80% in the exon 3 experiments (100% denaturant = 9.5 M urea). The resolution was carried out at a constant voltage of 110 V and a temperature of 65 °C overnight (INGENYphorU system). The heteroduplexes were visualized by UV light in the presence of ethidium bromide.

Exon 4 amplicons were analyzed by the high resolution melting (HRM) method. PCR was performed using a SensiMix™ HRM kit (Bioline) with EvaGreen as a fluorescent dye and 200 nM concentration of each primer. The PCR temperature conditions used were as follows: 95 °C for 10 min followed by 45 cycles of 93 °C for 20 s, 64 °C for 20 s and 72 °C for 15 s. The HRM analysis was run with temperature increments of 0.15 °C between 75 and 95 °C. The samples containing one of following mutations (R189K, A181E and R202H) were used as positive detection controls.

The amplicons that differed from the standard samples during analysis were purified with QIAquick PCR purification Kit (Qiagen) and sequenced using a BigDye terminator cycle sequencing kit (Life Technologies) on an ABI PRISM 3100 analyzer (Life Technologies) according to manufacturer's instructions.

All regions of the gene were tested in 42 unrelated CVID and 57 unrelated IgAD patients, and exons 3 and 4 were analyzed in the remaining patients and controls. All of the detected non-synonymous mutations were confirmed by independent amplification and sequencing. All of the detected synonymous and intronic variants and common polymorphisms were checked and examined by restriction analysis or the HRM method (p.V220A) in all of the patients and controls. The restrictions were performed overnight with 5 U of enzyme per reaction, and the resulting bands were visualized on agarose gels. Further reaction details are described in Supplementary Table 2. HRM analysis was performed using temperature increments of 0.15 °C between 75 and 95 °C after exon 5 PCR using the following conditions: 95 °C for 10 min, 45 cycles of 95 °C for 20 s, 66 °C for 20 s, 72 °C for 15 s.

To estimate the pathogenic effect of the described *TNFRSF13B* mutations on TACI expression and function, we employed several web based *in silico* software tools. For assessment of protein affection, we utilized PMut (http://www.mmb.pcb.ub.es/PMut), SIFT (http://www.blocks.fhcrc.org/sift/SIFT.html) and Polyphen (http://www.genetics.bwh.harvard.edu/pph), and for assessment of mutation influence on splicing, we used Splice site prediction by neural network (http://www.fruitfly.org/seq_tools/splice.html) and Sroogle (http://www.sroogle.tau.ac.il).

2.3. Statistical analysis

Statistical significance of differences in the frequencies of mutations and polymorphisms between groups was evaluated using two-tailed Fisher's exact test. The results of the tests are presented both as crude p-values and p'-values after Bonferroni correction (in cases when crude p-values were significant). p'-Values less than 0.05 were considered significant.

3. Results

The non-synonymous mutations in the *TNFRSF13B* gene detected in Czech patients are listed in Table 1. Mutations were found in 4 unrelated patients with CVID (5.7%), 3 of them with the

Table 1
Czech individuals with non-synonymous *TNFRSF13B* mutations.

Mutation			CVID unrelated patients (n = 70)		IgAD unrelated patients (n = 161)		IgAD patients related to IgAD(n = 13 in 12 families)		IgAD patients related to CVID(n = 15 in 12 families)		HG/DG unrelated patients(n = 17)		HFDR to CVID(n = 7)		HFDR to IgAD(n = 3)		HFDR to HG/DG(n = 2)		General Czech population (n = 207/exon 3/, n = 195/exon 4/)		$p1$	$p2$	$p3$
			Mutated		Mutated		Mutated		Mutated		Mutated		Mutated		Mutated		Mutated		Mutated				
cDNA nomencl.	Protein nomencl.	Exon/intron (domain)	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%			
c.204_205insA	p.L69TfsX12	E3 (CRD2)	0	0.0	1	0.6	0	0.0	0	0.0	0	0.0							0	0.0	1.000	0.438	1.000
c.215G > A	p.R72H	E3 (CRD2)	1[a]	1.4	0	0.0	0	0.0	0	0.0	0	0.0	2/4[d]	50.0					0	0.0	0.253	1.000	1.000
c.260T > A	p.I87N	E3 (CRD2)	1	1.4	0	0.0	0	0.0	0	0.0	0	0.0	0/1	0.0					0	0.0	0.253	1.000	1.000
c.310T > C	p.C104R	E3 (CRD2)	2[a]	2.9	3[b]	1.9	1[b]	7.7	1[c]	6.7	0	0.0	3/6[d]	50.0	0/1	0.0			0	0.0	0.063	–	1.000
					4[b]	2.5															–	**0.036**/ns	–
c.311G > A	p.C104Y	E3 (CRD2)	0	0.0	1	0.6	0	0.0	0	0.0	1	5.9					1/2	50.0	0	0.0	1.000	0.438	0.076
c.542C > A	p.A181E	E4 (TM)	0	0.0	2	1.2	0	0.0	0	0.0	0	0.0			1/2	50.0			0	0.0	1.000	0.204	1.000
c.566G > A	**p.R189K**	E4 (IC)	0	0.0	1	0.6	0	0.0	0	0.0	0	0.0							0	0.0	1.000	0.452	1.000
c.605G > A	p.R202H	E4 (IC)	1	1.4	0	0.0	0	0.0	0	0.0	0	0.0							0	0.0	0.264	1.000	1.000
Total number of individuals with mutations			4[a]	5.7	8[b]	5.0	1	7.7	1	6.7	1	5.9	5	71.4	1	33.3	1	50.0	0	0.0	**0.004**/**0.011**	–	0.076
					9[b]	5.6															–	**0.001**/**0.002**	–

$p1$, $p2$, $p3$ = p values for comparison of unrelated CVID ($p1$), IgAD ($p2$) and HG/DG patients ($p3$) with controls. A two-tailed Fisher's exact test was used to calculate p-values. If $p < 0.05$, Bonferroni correction for 8 mutations in particular mutations and for 3 tests in the total number of individuals with mutations was applied, p'-value after correction is reported behind a slash mark. HFDR = healthy first degree relatives in families with a mutation detected; HG/DG = hypogamma/dysgammaglobulinemia; ns = not significant; CRD = cysteine rich domain; TM = transmembrane domain; IC = intracellular domain. The novel mutation is marked in bold. The cDNA and protein nomenclature are according to NCBI nucleotide entry NM_012452 and NCBI protein entry NP_036584, respectively.

[a] One patient was a compound heterozygote p.C104R/p.R72H.

[b] Mutation was found in a relative to the IgAD proband, although the index case was not a carrier of the mutation; therefore, there are 4 unrelated families with the p.C104R mutation and 9 unrelated families with any mutation.

[c] This patient has the same mutation as the CVID index case.

[d] There were 4 healthy relatives of the p.C104R/p.R72H compound heterozygote, 2/4 were p.C104R carriers, and the remaining 2/4 were p.R72H carriers (see family A, Supplementary Fig. 1).

non-familial and 1 with the familial form of the disease. One CVID patient was a compound heterozygote for the p.C104R/p.R72H mutations. (Her healthy mother and son were p.R72H carriers, while 2 healthy daughters were p.C104R carriers, and the father was not available; see family A in Supplementary Fig. 1). Among the IgAD patients, mutations were detected in 8 out of 161 unrelated probands, all of them were non-familial cases, as well as 1 relative to the IgAD proband and 1 relative to the CVID proband. While the relative of the CVID patient was a carrier with the same mutation as the index case (see family C in Supplementary Fig. 1), in a different family, the mutation carrier was related to the IgAD index case who was found to be free of the mutation (see family D in Supplementary Fig. 1). Thus, in total, mutations were found in 9 unrelated IgAD patients (5.6%). A mutation was found also in 1 sporadic HG/DG case (5.9%), an asymptomatic 16 years old male with slightly decreased immunoglobulin (IgG 6.47 g/l, IgA 0.34 g/l and IgM 0.17 g/l). Eight different mutations were detected in our patients, including the most frequent p.C104R and p.A181E mutations as well as 1 novel missense mutation, p.R189K, located in the intracellular domain of the TACI protein. The latter was assessed as benign using the *in silico* prediction tools Pmut, SIFT and Polyphen (see Supplementary Table 6). No mutation was found in the control population. Thus, a significant association of the mutated *TNFRSF13B* gene was noted in the CVID and IgAD patients ($p' = 0.01$ and $p' = 0.002$, respectively) and a tendency to association was observed in the HG/DG group ($p = 0.08$). Mutations were also detected in 5/7 of the examined healthy first degree relatives of the CVID (71.4%), 1/3 of the IgAD (33.3%) and 1/2 of the HG/DG (50%) carriers (see families A, B, and C for CVID and families E and F for IgAD in Supplementary Fig. 1). Considering the particular mutations, a significant association and tendency to association was only found between p.C104R and IgAD and CVID ($p = 0.04$ and $p = 0.06$, respectively). However, this association disappeared after Bonferroni correction.

The synonymous, intronic and/or common *TNFRSF13B* variants detected in Czech individuals are given in Table 2. A novel synonymous variant, p.F178F, was identified in the control samples but not in the patients. A silent mutation, p.P97P, was detected more frequently in the CVID and IgAD patients than in the controls, although the significance disappeared after Bonferroni correction for the IgAD patients. A rare intronic variant in intron 3, c.445 + 31t > a, was found in a heterozygous state in only 1 CVID patient and 1 IgAD patient. A novel intronic variant localized in the neighborhood of the former one, c.445+34c > t, was found in 1 CVID patient and 2 IgAD patients. In 2 cases, both variants occurred together, and only c.445+34c > t was present in another case. None of these variants were found in the controls. Although *in silico* prediction tools (SSPNN and Sroogle) did not show any new splicing site resulting from these sequence changes, their influence on splicing via binding of splicing factor(s) cannot be completely excluded without analyzing mRNA, which was not available in these patients.

To connect our single center results with the published data, we conducted an overview of all published studies involving series of unrelated patients (summarized in Table 3, for details see Supplementary Tables 3 and 5). After correcting for the number of patients with all regions of the gene analyzed and for the number of compound heterozygotes, the *TNFRSF13B* gene was found to be mutated in 9.9% of the CVID patients, 5.7% of the IgAD patients and 4.1% of the HG/DG patients, while the controls carried the mutation in 3.2% of the cases. Statistically significant association of the mutated gene could only be demonstrated with CVID ($p' < 10^{-6}$). Several studies only screened for mutations in exons 3 and 4, particularly in the controls, because the vast majority of all detected mutations occurred in these exons. If only mutations in exons 3 and 4 were considered, mutations were found in 9.3%

Table 2
Czech individuals with synonymous, intronic and/or common *TNFRSF13B* variants.

cDNA nomencl.	Protein nomencl.	rs			CVID unrelated patients			IgADunrelated patients			HG/DGunrelated patients			General Czech population			*p*1	*p*2	*p*3
			Allele 1	Allele 2	Allele 1	Allele 2	Freq 1 (%)	Allele 1	Allele 2	Freq 1 (%)	Allele 1	Allele 2	Freq 1 (%)	Allele 1	Allele 2	Freq 1 (%)			
c.81G > A	p.T27T	rs8072293	a	g	110	30	78.6	211	105	66.8	29	5	85.3	369	143	72.1	0.131	0.118	0.112
c.291T > G	p.P97P	rs35062843	g	t	6	134	4.3	6	312	1.9	0	34	0.0	1	413	0.2	**0.001/ 0.012**	**0.047/ ns**	1.000
c.534C > T	**p.F178F**	–		t	c	0	140	0.0	0	322	0.0	0	34	0.0	1	389	0.3	1.000	1.000	1.000
c.659T > C	p.V220A	rs56063729	c	t	3	135	2.2	17	291	5.5	3	31	8.8	18	392	4.4	0.311	0.490	0.211
c.752C > T	p.P251L	rs34562254	t	c	18	122	12.9	38	276	12.1	7	27	20.6	41	365	10.1	0.349	0.402	0.080
c.831T > C	p.S277S	rs11078355	c	t	57	83	40.7	135	175	43.5	17	17	50.0	160	244	39.6	0.842	0.319	0.276
c.445+25a > c		rs2274892	c	a	51	89	36.4	154	164	48.4	18	16	52.9	174	238	42.2	0.234	0.099	0.280
c.445+31t > a		rs55955502	a	t	1	139	0.7	1	307	0.3	0	34	0.0	0	414	0.0	0.253	0.427	1.000
c.445+34c > t		–	t	c	1	139	0.7	2	316	0.6	0	34	0.0	0	414	0.0	0.253	0.188	1.000

*p*1, *p*2, *p*3 = *p* values for comparisons of unrelated CVID (*p*1), IgAD (*p*2) and HG/DG patients (*p*3) with controls. A two-tailed Fisher's exact test was used to calculate *p*-values. If $p < 0.05$, Bonferroni correction for 9 polymorphisms was applied, p'-value after correction is reported behind a slash mark. ns = not significant; HG/DG = hypogamma/dysgammaglobulinemia; freq1 = allele 1 frequency. Novel polymorphisms are marked in bold. The cDNA and protein nomenclature are according to NCBI nucleotide entry NM_012452 and NCBI protein entry NP_036584, respectively.

Table 3
Number of unrelated individuals with non-synonymous *TNFRSF13B* mutations.

Mutation	CVID unrelated patients					IgAD unrelated patients					HG/DG unrelated patients					Controls					*p*1	*p*2	*p*3	References[f]
	Mut[a]	all[b]	%[c]	Mut corr[d]	% corr[e]	Mut[a]	All[b]	%[c]	Mut corr[d]	% corr[e]	Mut[a]	All[b]	%[c]	Mut corr[d]	% corr[e]	Mut[a]	All[b]	%[c]	Mut corr[d]	% corr[e]				
c.61+1G > T	1	48	2.1	1.0	0.1				0.0	0.0				0.0	0.0	0	241	0.0	0.0	0.0	0.166			[38]
p.W40R	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	107	0.0	0.0	0.0	1.000		1.000	[37]
p.D41IfsX43	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	107	0.0	0.0	0.0	1.000		1.000	[37]
p.D41H + p.C100LfsX6	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	107	0.0	0.0	0.0	1.000		1.000	[37]
p.P42T	1	48	2.1	1.0	0.1				0.0	0.0				0.0	0.0	1	241	0.4	1.0	0.2	0.305			[38]
p.L69TfsX12	6	886	0.7	6.0	0.5	1	430	0.2	0.3	0.2	2	74	2.7	1.6	2.7	0	2108	0.0	0.0	0.0	**0.001**/ **0.019**	0.169	**0.001**/ **0.033**	[15,26,37,39,40], this study
p.R72H	7	701	1.0	7.0	0.6	1	238	0.4	0.6	0.4				0.0	0.0	11	1767	0.6	3.9	0.6	0.306	1.000		[26,39,41], this study
p.G76fsX3	1	106	0.9	1.0	0.1				0.0	0.0				0.0	0.0	0	62	0.0	0.0	0.0	1.000			[39]
p.Y79C	1	518	0.2	1.0	0.1				0.0	0.0	1	46	2.2	1.0	1.7	0	675	0.0	0.0	0.0	0.434		0.064	[37]
p.I87N	3	588	0.5	3.0	0.2	0	161	0.0	0.0	0.0				0.0	0.0	1	882	0.1	0.7	0.1	0.308	1.000		[37], this study
p.C89Y	1	118	0.8	1.0	0.1				0.0	0.0				0.0	0.0	0	198	0.0	0.0	0.0	0.373			[28]
p.C104R	60	1423	4.2	52.3	4.2	11	757	1.5	2.2	1.5				0.0	0.0	41	4225	1.0	6.1	1.0	**<10^{-7}**/ **<10^{-6}**	0.242		[15,16,25–26,28,37–42], this study
p.C104Y	1	588	0.2	1.0	0.1	1	161	0.6	0.9	0.6				0.0	0.0	0	882	0.0	0.0	0.0	0.400	0.154		[37], this study
p.E117G	1	118	0.8	1.0	0.1				0.0	0.0				0.0	0.0	0	198	0.0	0.0	0.0	0.373			[28]
p.R122W	1	268	0.4	1.0	0.1	1	240	0.4	0.6	0.4				0.0	0.0	5	1210	0.4	2.6	0.4	1.000	1.000		[26]
p.E140K	1	118	0.8	1.0	0.1				0.0	0.0				0.0	0.0	0	198	0.0	0.0	0.0	0.373			[28]
p.S144X	2	335	0.6	2.0	0.2				0.0	0.0				0.0	0.0	0	200	0.0	0.0	0.0	0.531			[16,41]
p.A149T	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	675	0.0	0.0	0.0	0.434		1.000	[37]
p.G152E	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	675	0.0	0.0	0.0	0.434		1.000	[37]
p.Y164X	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	675	0.0	0.0	0.0	0.434		1.000	[37]
p.L171R	4	397	1.0	4.0	0.3				0.0	0.0				0.0	0.0	0	360	0.0	0.0	0.0	0.126			[28,39,41]
p.C172Y	2	221	0.9	2.0	0.2				0.0	0.0				0.0	0.0	0	341	0.0	0.0	0.0	0.154			[38,41]
p.A181E	39	1439	2.7	33.6	2.7	11	723	1.5	2.3	1.5	1	76	1.3	0.8	1.3	25	3558	0.7	4.4	0.7	**<10^{-7}**/ **<10^{-5}**	**0.041**/ **ns**	0.424	[15,16,25–26,28,37,39–43], this study
p.R189K	0	70	0.0	0.0	0.0	1	161	0.6	0.9	0.6	0	17	0.0	0.0	0.0	0	195	0.0	0.0	0.0	1.000	0.452	1.000	this study
p.D191GfsX46	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	675	0.0	0.0	0.0	0.434		1.000	[37]
p.C193X	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	675	0.0	0.0	0.0	0.434		1.000	[37]
p.S194X	2	335	0.6	2.0	0.2				0.0	0.0				0.0	0.0	0	200	0.0	0.0	0.0	0.531			[16,41]
p.R202H	4	629	0.6	4.0	0.3	3	722	0.4	0.6	0.4	0	17	0.0	0.0	0.0	7	2899	0.2	1.5	0.2	0.116	0.428	1.000	[15,16,25,26,39,43], this study
p.V246F	1	518	0.2	1.0	0.1				0.0	0.0	0	46	0.0	0.0	0.0	0	107	0.0	0.0	0.0	1.000		1.000	[37]
Total[g]	147	1241		134.0	10.8	30	150		8.6	5.7	4	59		3.4	5.7	91	631		20.3	3.2				
Total corrected[h]	135	**1229**		**122**	**9.9**	30	**150**		**8.6**	**5.7**	3	**58**		**2.4**	**4.1**	91	**631**		**20.3**	**3.2**	**<10^{-7}**/ **<10^{-6}**	0.145	0.707	
Total exons 3.4[i]	141	1332		134.3	10.1	30	254		13.2	5.2	4	76		4.0	5.3	90	1394		41.4	3.0				
Total corrected exons 3.4[h]	129	1320		122.3	**9.3**	30	254		13.2	**5.2**	3	75		**3.0**	**4.0**	90	1394		**41.4**	**3.0**	**<10^{-11}**/ **<10^{-10}**	0.083	0.488	

*p*1, *p*2, *p*3 = *p* values for comparison of unrelated CVID (*p*1), IgAD (*p*2) and HG/DG patients (*p*3) with controls (in particular mutations uncorrected numbers used). A two-tailed Fisher's exact test was used to calculate *p*-values. If $p < 0.05$, Bonferroni correction for 29 different mutations in particular mutations and for 3 tests in total number of individuals with mutation was applied, *p*′-value after correction is reported behind a slash mark. ns = not significant; HG/DG = hypogamma/dysgammaglobulinemia.The cDNA and protein nomenclature are according to NCBI nucleotide entry NM_012452 and NCBI protein entry NP_036584, respectively.

[a] Number of individuals with mutations.

[b] Number of all individuals tested in studies in which the particular mutation was detected.

[c] Number of individuals with mutations/number of all individuals tested in studies in which the particular mutation was detected (in %).

[d] Number of individuals with mutations corrected for the number of individuals with all regions of the gene tested in available studies (including studies in which the particular mutation was not detected). All regions were tested in 1229 unrelated CVID, 150 unrelated IgAD, 58 unrelated HG/DG and 631 controls. For details, see Supplementary Table 5. ("mut corr" = "mut" if number of all individuals tested for the particular mutation ("all") is ⩽ than number of patients with all regions of the gene tested ("total all"); "mut corr" = ("mut"/"all") x "total all" if "all" > than "total all").

[e] Corrected number of individuals with mutations/all individuals with all regions of the gene tested in available studies (in %).

[f] Calculations of total mutation frequencies if more studies available are reported in Supplementary Table 3.

[g] Total numbers of individuals with mutations and all individuals with all regions of the gene tested in the available studies.

[h] Total numbers corrected for compound heterozygotes (12 in CVID group, 1 in HG/DG group).

[i] Total numbers of individuals with mutations in exon 3 and 4 and all individuals with exons 3 and 4 tested (counted from available studies). Exons 3 and 4 were tested in 1320 unrelated CVID, 254 unrelated IgAD, 76 unrelated HG/DG and 1394 controls. For details, see Supplementary Table 5.

Table 4
Allele frequencies of synonymous, intronic and/or common variants in the *TNFRSF13B* gene.

cDNA nomencl.	Protein nomencl.	rs			CVID unrelated patients			IgAD unrelated patients			HG/DG unrelated patients			Controls			*p1*	*p2*	*p3*	References[a]
			Allele 1	Allele 2	Allele 1	Allele 2	Freq 1 (%)	Allele 1	Allele 2	Freq 1 (%)	Allele 1	Allele 2	Freq 1 (%)	Allele 1	Allele 2	Freq 1 (%)				
c.81G > A	p.T27T	rs8072293	a	g	285	99	74.2	229	119	65.8	45	11	80.4	419	217	65.9	**0.005**/ **0.047**	1.000	**0.027**/ ns	[39,40], this study
c.291T > G	p.P97P	rs35062843	g	t	26	484	5.1	6	344	1.7	1	55	1.8	22	1176	1.8	**<0.001**/ **0.003**	1.000	1.000	[39,40,43,44], this study
c.534C > T	p.F178F		t	c	0	140	0.0	0	322	0.0	0	34	0.0	1	389	0.3	1.000	1.000	1.000	this study
c.659T > C	p.V220A	rs56063729	c	t	28	1200	2.3	34	786	4.1	3	53	5.4	105	3629	2.8	0.360	0.056	0.213	[26,39–40], this study
c.752C > T	p.P251L	rs34562254	t	c	119	1113	9.7	160	1342	10.7	9	47	16.1	611	4983	10.9	0.203	0.815	0.201	[15,25–26,39,40], this study
c.831T > C	p.S277S	rs11078355	c	t	135	249	35.2	149	193	43.6	24	32	42.9	192	336	36.4	0.727	**0.039**/ ns	0.383	[39,40], this study
c.445+25a > c		rs2274892	c	a	65	107	37.8	431	585	42.4	27	29	48.2	565	913	38.2	0.934	**0.038**/ ns	0.161	[25,40], this study
c.445+31t > a		rs55955502	a	t	1	139	0.7	1	307	0.3	0	34	0.0	0	414	0.0	0.253	0.427	1.000	This study
c.445+34c > t			t	c	1	139	0.7	2	316	0.6	0	34	0.0	0	414	0.0	0.253	0.188	1.000	This study

p1, *p2*, *p3* = *p* values for comparison of unrelated CVID (*p1*), IgAD (*p2*) and HG/DG patients (*p3*) with controls. A two-tailed Fisher's exact test was applied for *p*-value calculations. If $p < 0.05$, Bonferroni correction for 9 polymorphisms was applied, *p′*-value after correction is reported behind a slash mark. ns = not significant; HG/DG = hypogamma/dysgammaglobulinemia; freq1 = allele 1 frequency. The cDNA and protein nomenclature are according to NCBI nucleotide entry NM_012452 and NCBI protein entry NP_036584, respectively.

[a] Calculations of total polymorphism frequencies if multiple studies available are reported in Supplementary Table 4.

of the CVID patients, 5.2% of the IgAD patients and 4% of the HG/DG patients, while mutations were only detected in 3.0% of the controls. The association was again only highly significant for CVID ($p' < 10^{-10}$), although a tendency to association was noted for IgAD ($p = 0.08$). When considering individual mutations, a significant relationship was demonstrated between CVID and the p.L69TfsX12, p.C104R and p.A181E mutations ($p' < 10^{-6}$, $p' < 10^{-5}$ and $p' = 0.02$, respectively) as well as between HG/DG and the p.L69TfsX12 mutation ($p' = 0.03$). For IgAD, only a borderline association was shown for p.A181E, losing its statistical significance after Bonferroni correction.

The data published on synonymous, intronic and/or common variants of the *TNFRSF13B* gene in CVID, IgAD and HG/DG patients and in controls are summarized in Table 4 (for details see Supplementary Table 4). Surprisingly, in concordance with our results, the overall data confirmed a significant association between the p.P97P variant and CVID ($p' = 0.03$) and showed a significant association of the p.T27T variant with CVID ($p' = 0.05$). Other indicated associations lost their significance after Bonferroni correction.

4. Discussion

Our study demonstrated a lesser occurrence of mutations in CVID patients than available published data (5.7% vs. 9.9%) and similar occurrences of mutations with IgAD patients (5.6% vs. 5.7%) and HG/DG patients (5.9% vs. 4.1%). In contrast with the majority of other studies, we failed to detect any non-synonymous mutation in the general population (0.0% vs. 3.2%). Although mutations in the *TNFRSF13B* gene were detected at similar frequency in both CVID and IgAD Czech patients and were significantly associated with both diseases compared with the general Czech population, the combined analysis of all published data only confirmed an association between the *TNFRSF13B* mutations and CVID. In this combined analysis a slight tendency toward association was shown between the *TNFRSF13B* mutations and disease in IgAD patients (5.7% vs. 3.2%, $p = 0.145$).

Similar to many other studies, we detected mutations in a substantial number of the examined healthy first degree relatives of affected mutation carriers (71.4% with CVID and 33.3% with IgAD). Martinez-Pomar et al. found mutations even in 28 out of 31 examined healthy relatives, including 3 p.C104R homozygotes and 1 p.C104R/p.A181E compound heterozygote, although 2 of 3 asymptomatic p.C104R homozygotes had decreased plasma immunoglobulin levels, which might be the first step toward CVID development [28]. Interestingly, both studies on Spanish populations [25,28] showed considerably higher frequencies of the p.C104R mutation among healthy individuals (2.5% and 2.6%) than in any other population studied (all populations published <1.0%) (see Supplementary Table 3).

Several approaches can be used to establish the role of a particular gene in disease development and the functional significance of mutations in such a gene to abolish or severely affect protein function and be causative, including genetic knock-out animal models and *in vitro* experiments, functional and *in silico* studies of mutant variants and segregation studies of the mutated gene with disease phenotypes. The evidence suggests that TACI, encoded by the *TNFRSF13B* gene, acts as a negative regulator of murine B-cell activation, an indispensable component of T-cell independent type II responses and has a role in IgA class switching [29–31]. These defects are similar to the immunological abnormalities observed in CVID [21,32]. Moreover, in previous studies, mutations in the *TNFRSF13B* gene were clearly associated with CVID [15,16] (summarized in Table 3), and in many mutations, such as the most frequent alleles p.C104R and p.A181E, there was an impact on protein function, including affected NF-κB/NFAT signaling (summarized in Supplementary Table 6). In the mutations p.C104R and p.A181E,

stop codon introducing mutations and several other mutations, pathological significance was demonstrated. Different mutations, particularly missense ones, were suggested to be functionally silent using *in vitro* assays and/or *in silico* prediction tools [33–37]. However, as proposed pathological mutations were found in the general control population and healthy first degree relatives of CVID and IgAD patients, occasionally in a homozygous state [28], questions arise as to which *TNFRSF13B* mutations are truly pathogenic (disease causing) or if they act as susceptibility or disease-modifying mutations relevant only in co-existence with other gene defects.

The p.C104R and p.A181E mutations and premature stop codon introducing mutations appear to be relevant, either alone or more likely in combination with other genetic and/or environmental factor(s), although statistically significant association was only demonstrated with CVID for the p.C104R, p.A181E and p.L69TfsX12 mutations, mainly because other protein truncating mutations are very rare (see Table 3). The relevance of most missense mutations is unclear. Some of them, such as p.R72H and p.R202H, also detected in our CVID patients, were reported at similar frequencies in CVID patients and controls but were suggested to be possibly pathological based on several tests (see Supplementary Table 6). On the other hand, some mutations were predicted and/or tested to be benign but were detected in the CVID patients and not in the control samples, including p.E140K and p.E149T. Mutation p.R189K, found in our IgAD patient and not described previously, was also predicted to be benign but was not detected in the general population. Another mutation located in a neighboring codon, p.K188M, was suggested to be possibly deleterious using the β-aggregation test [37], although this mutation did not impair NF-κB signaling *in vitro* [34].

The relevance of *TNFRSF13B* silent mutations and intronic polymorphisms located outside conserved splicing sequences are not clear, but many of them are probably not associated with the disease. However, we detected the p.P97P variant to be significantly associated with CVID in Czech patients, and the same result also arose from data extracted from all of the available published papers (see Tables 2 and 4). To examine the possibility that this variant might affect splicing, we used the *in silico* tools SSPNN and Sroogle. We did not detect any obvious relevant changes in the predicted splice sites or in the splicing regulators. However, we cannot rule out that the variant affects a splicing regulator that is outside the scope of the predictors used or some other process that influences gene expression, such as RNA secondary structure and/or instability. Similarly, we cannot exclude the influence of two intronic changes detected in our CVID and IgAD patients and not in the controls, c.445 + 31t > a, c.445 + 34c > t, on splicing without mRNA based analyses. Unfortunately, mRNA and/or protein based analyses were not available within the scope of this study, but question of functional significance of the mentioned sequence variants, particularly p.P97P, should definitely be addressed in future studies.

In conclusion, we mapped *TNFRSF13B* gene variants in an extensive single center study of Czech CVID, IgAD and HG/DG patients and controls, and we connected our results with current published data. We showed the p.P97P silent variant, considered to be a non-relevant polymorphism, to be significantly associated with CVID. We detected one novel mutation in an IgAD patient and one novel rare intronic variant of unknown significance in CVID and IgAD patients. Further functional studies including transfection experiments will be necessary to establish if some of these *TNFRSF13B* gene variants are functionally relevant to disease and may play a role, in a combination with other factors, in CVID, IgAD or hypogammaglobulinemia development.

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Appendix A. Supplementary data

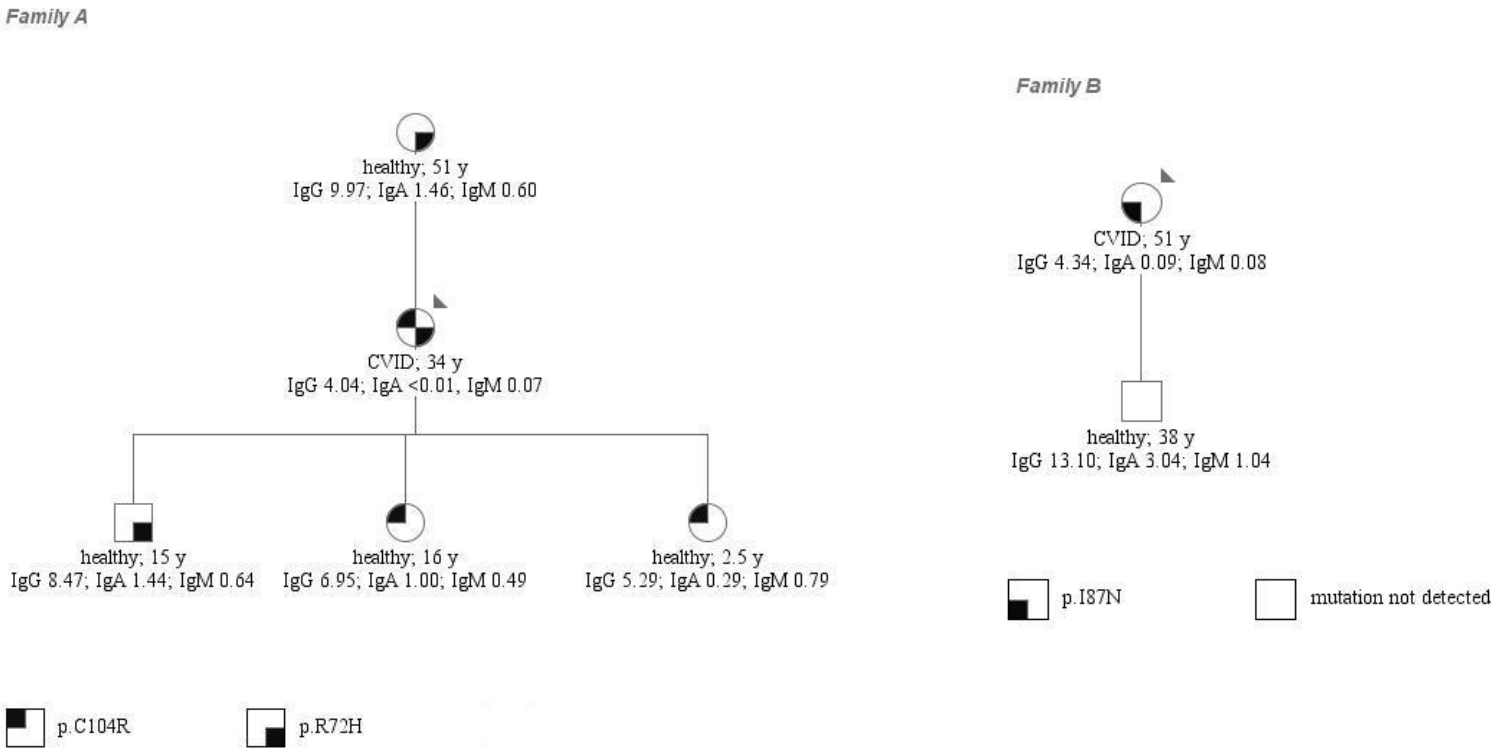
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2012.07.342.

References

[1] Hammarstrom L, Vorechovsky I, Webster D. Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). Clin Exp Immunol 2000;120:225.
[2] Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol 1999;92:34.
[3] Chapel H, Cunningham-Rundles C. Update in understanding common variable immunodeficiency disorders (CVIDs) and the management of patients with these conditions. Br J Haematol 2009;145:709.
[4] Latiff AH, Kerr MA. The clinical significance of immunoglobulin A deficiency. Ann Clin Biochem 2007;44:131.
[5] Yel L. Selective IgA deficiency. J Clin Immunol 2010;30:10.
[6] Litzman J, Sevcikova I, Stikarovska D, Pikulova Z, Pazdirkova A, Lokaj J. IgA deficiency in Czech healthy individuals and selected patient groups. Int Arch Allergy Immunol 2000;123:177.
[7] Vorechovsky I, Zetterquist H, Paganelli R, Koskinen S, Webster AD, Bjorkander J, et al. Family and linkage study of selective IgA deficiency and common variable immunodeficiency. Clin Immunol Immunopathol 1995;77:185.
[8] Kralovicova J, Hammarstrom L, Plebani A, Webster AD, Vorechovsky I. Fine-scale mapping at IGAD1 and genome-wide genetic linkage analysis implicate HLA-DQ/DR as a major susceptibility locus in selective IgA deficiency and common variable immunodeficiency. J Immunol 2003;170:2765.
[9] Ferreira RC, Pan-Hammarstrom Q, Graham RR, Fontan G, Lee AT, Ortmann W, et al. High-density SNP mapping of the HLA region identifies multiple independent susceptibility loci associated with selective IgA deficiency. PLoS Genet 2012;8:e1002476.
[10] Schaffer AA, Pfannstiel J, Webster AD, Plebani A, Hammarstrom L, Grimbacher B. Analysis of families with common variable immunodeficiency (CVID) and IgA deficiency suggests linkage of CVID to chromosome 16q. Hum Genet 2006;118:725.
[11] Finck A, Van der Meer JW, Schaffer AA, Pfannstiel J, Fieschi C, Plebani A, et al. Linkage of autosomal-dominant common variable immunodeficiency to chromosome 4q. Eur J Hum Genet 2006;14:867.
[12] Braig DU, Schaffer AA, Glocker E, Salzer U, Warnatz K, Peter HH, et al. Linkage of autosomal dominant common variable immunodeficiency to chromosome 5p and evidence for locus heterogeneity. Hum Genet 2003;112:369.
[13] Ferreira RC, Pan-Hammarstrom Q, Graham RR, Gateva V, Fontan G, Lee AT, et al. Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency. Nat Genet 2010;42:777.
[14] Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol 2003;4:261.
[15] Castigli E, Wilson SA, Garibyan L, Rachid R, Bonilla F, Schneider L. TACI is mutant in common variable immunodeficiency and IgA deficiency. Nat Genet 2005;37:829.
[16] Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, Schlesier M, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. Nat Genet 2005;37:820.
[17] Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci USA 2009;106:13945.
[18] van Zelm MC, Reisli I, van der Burg M, Castano D, van Noesel CJ, van Tol MJ, et al. An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med 2006;354:1901.
[19] van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest 2010;120:1265.
[20] Kuijpers TW, Bende RJ, Baars PA, Grummels A, Derks IA, Dolman KM, et al. CD20 deficiency in humans results in impaired T cell-independent antibody responses. J Clin Invest 2010;120:214.
[21] Bacchelli C, Buckridge S, Thrasher AJ, Gaspar HB. Translational mini-review series on immunodeficiency: molecular defects in common variable immunodeficiency. Clin Exp Immunol 2007;149:401.

[22] Eibel H, Salzer U, Warnatz K. Common variable immunodeficiency at the end of a prospering decade: towards novel gene defects and beyond. Curr Opin Allergy Clin Immunol 2010;10:526.
[23] He B, Santamaria R, Xu W, Cols M, Chen K, Puga I, et al. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. Nat Immunol 2010;11:836.
[24] Ozcan E, Rauter I, Garibyan L, Dillon SR, Geha RS. Toll-like receptor 9, transmembrane activator and calcium-modulating cyclophilin ligand interactor, and CD40 synergize in causing B-cell activation. J Allergy Clin Immunol 2011;128:601.
[25] Lopez-Mejias R, del Pozo N, Fernandez-Arquero M, Ferreira A, Garcia-Rodriguez MC, de la Concha EG, et al. Role of polymorphisms in the TNFRSF13B (TACI) gene in Spanish patients with immunoglobulin A deficiency. Tissue Antigens 2009;74:42.
[26] Pan-Hammarstrom Q, Salzer U, Du L, Bjorkander J, Cunningham-Rundles C, Nelson DL, et al. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. Nat Genet 2007;39:429.
[27] Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol 1999;93:190.
[28] Martinez-Pomar N, Detkova D, Arostegui JI, Alvarez A, Soler-Palacin P, Vidaller A, et al. Role of TNFRSF13B variants in patients with common variable immunodeficiency. Blood 2009;114:2846.
[29] Seshasayee D, Valdez P, Yan M, Dixit VM, Tumas D, Grewal IS. Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor. Immunity 2003;18:279.
[30] von Bulow GU, van Deursen JM, Bram RJ. Regulation of the T-independent humoral response by TACI. Immunity 2001;14:573.
[31] Yan M, Wang H, Chan B, Roose-Girma M, Erickson S, Baker T, et al. Activation and accumulation of B cells in TACI-deficient mice. Nat Immunol 2001;2:638.
[32] Salzer U, Grimbacher B. TACItly changing tunes: farewell to a yin and yang of BAFF receptor and TACI in humoral immunity? New genetic defects in common variable immunodeficiency. Curr Opin Allergy Clin Immunol 2005;5:496.
[33] Bacchelli C, Buckland KF, Buckridge S, Salzer U, Schneider P, Thrasher AJ, et al. The C76R transmembrane activator and calcium modulator cyclophilin ligand interactor mutation disrupts antibody production and B-cell homeostasis in heterozygous and homozygous mice. J Allergy Clin Immunol 2011;127:1253.
[34] Fried AJ, Rauter I, Dillon SR, Jabara HH, Geha RS. Functional analysis of transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) mutations associated with common variable immunodeficiency. J Allergy Clin Immunol 2011;128:226.
[35] Lee JJ, Jabara HH, Garibyan L, Rauter I, Sannikova T, Dillon SR, et al. The C104R mutant impairs the function of transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) through haploinsufficiency. J Allergy Clin Immunol 2010;126:1234.
[36] Lee JJ, Rauter I, Garibyan L, Ozcan E, Sannikova T, Dillon SR, et al. The murine equivalent of the A181E TACI mutation associated with common variable immunodeficiency severely impairs B-cell function. Blood 2009;114:2254.
[37] Salzer U, Bacchelli C, Buckridge S, Pan-Hammarstrom Q, Jennings S, Lougaris V, et al. Relevance of biallelic versus monoallelic TNFRSF13B mutations in distinguishing disease-causing from risk-increasing TNFRSF13B variants in antibody deficiency syndromes. Blood 2009;113:1967.
[38] Mohammadi J, Liu C, Aghamohammadi A, Bergbreiter A, Du L, Lu J, et al. Novel mutations in TACI (TNFRSF13B) causing common variable immunodeficiency. J Clin Immunol 2009;29:777.
[39] Castigli E, Wilson S, Garibyan L, Rachid R, Bonilla F, Schneider L, et al. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. Nat Genet 2007;39:430.
[40] Speletas M, Mamara A, Papadopoulou-Alataki E, Iordanakis G, Liadaki K, Bardaka F, et al. TNFRSF13B/TACI alterations in Greek patients with antibody deficiencies. J Clin Immunol 2011;31:550.
[41] Zhang L, Radigan L, Salzer U, Behrens TW, Grimbacher B, Diaz G, et al. Transmembrane activator and calcium-modulating cyclophilin ligand interactor mutations in common variable immunodeficiency: clinical and immunologic outcomes in heterozygotes. J Allergy Clin Immunol 2007;120:1178.
[42] Dong X, Hoeltzle MV, Hagan JB, Park MA, Li JT, Abraham RS. Phenotypic and clinical heterogeneity associated with monoallelic TNFRSF13B-A181E mutations in common variable immunodeficiency. Hum Immunol 2010;71:505.
[43] Waldrep ML, Zhuang Y, Schroeder Jr HW. Analysis of TACI mutations in CVID & RESPI patients who have inherited HLA B*44 or HLA*B8. BMC Med Genet 2009;10:100.
[44] Salzer U, Birmelin J, Bacchelli C, Witte T, Buchegger-Podbielski U, Buckridge S, et al. Sequence analysis of TNFRSF13b, encoding TACI, in patients with systemic lupus erythematosus. J Clin Immunol 2007;27:372.

Supplementary figure 1



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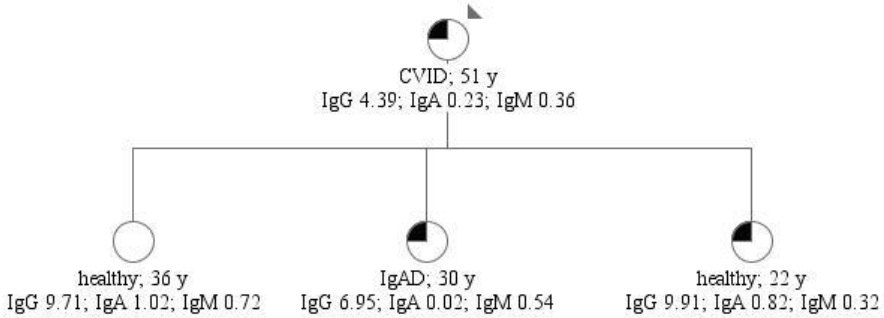

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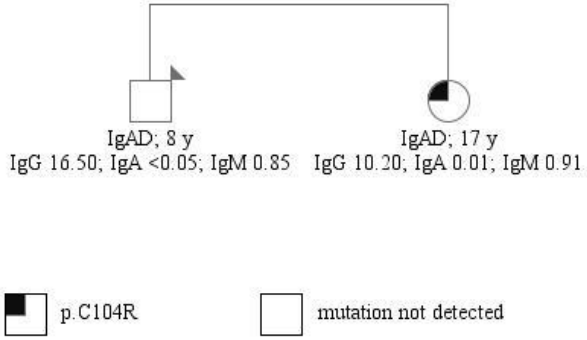

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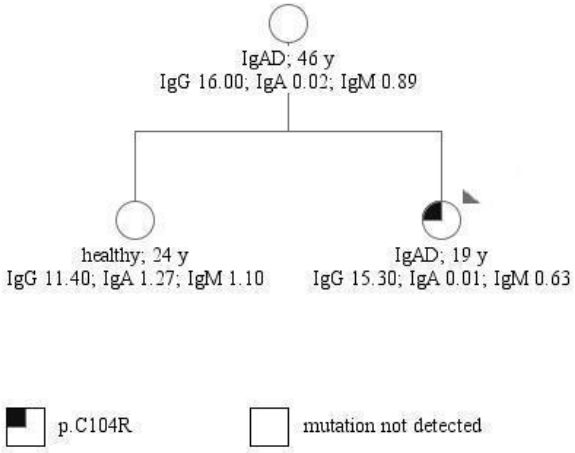

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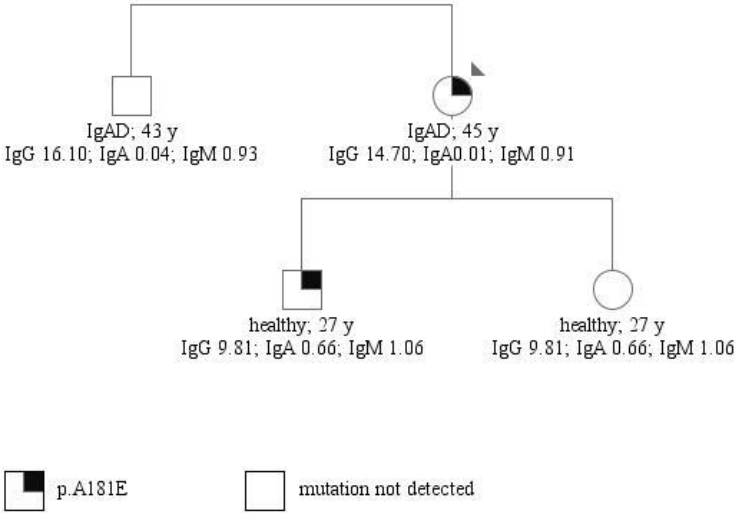

2

Legend:
The index cases are marked by arrow. Age and immunoglobulin levels in g/L at diagnosis in CVID/IgAD patients and at the first examination in healthy relatives are reported.

Supplementary table 1

Exon	Primers	Annealling temp.	Mg^{2+} conc.	Number of cycles	Amplicon Length
1	A: CACCCACCTGGGCTCCTGAGA B: CCCCCACGGCACTCAGGC	60°C	1.5 mM	35	282 bp
2	A: TCAGGGACAAGAGGCCGGGC B: ACTGTGGGGCCAGAGGGTGCTC	67°C	1.5 mM	35	280 bp
3	A: TGGTCAAACCCAGAGTTCCTGCTAG B: TCCCACGCTTTCTCACCCTGC	60°C	1.5 mM	40	406 bp
4	A: GGATGGGGGGATGTGGATTGCT B: TGACAGGACCGAGGGATGGCC	64°C (HRMA)	3.0 mM	45	346 bp
5	A: CCCACACCGTCACCCCTACC B: TCTTTCTCTCTCCCCTCCTCTCCAT	60°C	1.5 mM	35	380 bp

Primer sequences are given from 5' to 3'. A = forward primer; B = reverse primer.
For DGGE analysis a 40 bp "GC clamp" 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC TGT GGG GCC AGA GGG TGC TC 3' was joined to the 5' end of primers 2B and 3A.
HRMA = high resolution melting analysis.

Supplementary table 2

Exon	Polymorphism		Enzyme	Temp.	Minor allele bands	Wild type allele bands
2	c.81G>A	p.T27T	BslI	55 °C	149+110+22 bp	149+58+52+21 bp
3	c.291T>G	p.P97P	Bpu10I	37 °C	191+145+30 bp	156+145+35+30 bp
3	c.445+25a>c		NlaI	37 °C	317+49 bp	289+49+28 bp
3	c.445+31t>a		HphI	37 °C	327+23+12+4 bp	339+23+4 bp
3	c.445+34c>t		Tsp45I	37 °C	299+36+30 bp	274+36+31+25 bp
5	c.752C>T	p.P251L	TaqI	65 °C	190+190 bp	380 bp
5	c.831T>C	p.S277S	AciI	37 °C	270+110 bp	380 bp

Minor allele is considered allele 1 in Table 2 in the text.

Supplementary table 3: Number of unrelated individuals with non-synonymous *TNFRSF13B* mutation reported in multiple studies

		CVID unrelated patients			IgAD unrelated patients			HG/DG unrelated patients			Controls			Reference/ Total
c.DNA nomencl.	protein nomencl.	mut	all	%	mut	all	%	mut	all	%	mut	all	%	
c.204_205insA	p.L69TfsX12	1	19	5.3	0	16	0.0				0	50	0.0	[15]
		1	106	0.9							0	62	0.0	[39]
					0	237	0.0				0	358	0.0	[26] swedish
		1	157	0.6							0	756	0.0	[26] US
		3	518	0.6				0	46	0.0	0	675	0.0	[26, 37] (only german in [23])
		0	16	0.0	0	16	0.0	1	11	9.1				[40]
		0	70	0.0	1	161	0.6	1	17	5.9	0	207	0.0	this study
		6	886	0.7	1	430	0.2	2	74	2.7	0	2108	0.0	Total
c.215G>A	p.R72H	2	106	1.9							0	62	0.0	[39]
		1	115	0.9	1	238	0.4				0	535	0.0	[26] swedish
		1	154	0.6							4	318	1.3	[26] german
		2	153	1.3							7	752	0.9	[26] US
		1	173	0.6							0	100	0.0	[41]
		1	70	1.4	0	161	0.0	0	17	0.0	0	207	0.0	this study
		7	701	1.0	1	238	0.4				11	1767	0.6	Total
c.260T>A	p.I87N	2	518	0.4				1	46	2.2	1	675	0.1	[37]
		1	70	1.4	0	161	0.0	0	17	0.0	0	207	0.0	this study
		3	588	0.5	0	161	0.0				1	882	0.1	Total
c.310T>C	p.C104R	3	162	1.9										[16]
		2	19	10.5	1	16	6.3				0	50	0.0	[15]
		5	106	4.7							0	62	0.0	[39]
					1	239	0.4				8	1019	0.8	[26] swedish
		6	154	3.9							5	755	0.7	[26] US
		7	173	4.0							0	100	0.0	[41]
		25	518	4.8				1	46	2.2	6	675	0.9	[26, 37] (only german in [23])
					5	319	1.6				14	545	2.6	[25]
		1	48	2.1							2	241	0.8	[38]
		7	118	5.9							5	198	2.5	[28]
		1	39	2.6	0	6	0.0	0	2	0.0	0	114	0.0	[42]
		1	16	6.3	0	16	0.0	0	11	0.0	1	259	0.4	[40]
		2	70	2.9	4	161	2.5	0	17	0.0	0	207	0.0	this study
		60	1423	4.2	11	757	1.5				41	4225	1.0	Total
c.311G>A	p.C104Y	1	518	0.2				0	46	0.0	0	675	0.0	[37]
		0	70	0.0	1	161	0.6	1	17	5.9	0	207	0.0	this study
		1	588	0.2	1	161	0.6				0	882	0.0	Total
c.364C>T	p.R122W	1	114	0.9	1	240	0.4				4	892	0.4	[26] swedish

		0	154	0.0							1	318	0.3	[26] german
		1	268	0.4	1	240	0.4				5	1210	0.4	Total
c.431C>A	p.S144X	1	162	0.6							0	100	0.0	[16]
		1	173	0.6							0	100	0.0	[41]
		2	335	0.6							0	200	0.0	Total
c.512T>G	p.L171R	1	106	0.9							0	62	0.0	[39]
		2	118	1.7							0	198	0.0	[28]
		1	173	0.6							0	100	0.0	[41]
		4	397	1.0							0	360	0.0	Total
c.515G>A	p.C172Y	1	173	0.6							0	100	0.0	[41]
		1	48	2.1							0	241	0.0	[38]
		2	221	0.9							0	341	0.0	Total
c.542C>A	p.A181E	7	162	4.3										[16]
		1	19	5.3	0	16	0.0				0	50	0.0	[15]
		4	106	3.8							0	62	0.0	[39]
					7	232	3.0				12	865	1.4	[26] swedish
		6	155	3.9							4	755	0.5	[26] US
		4	173	2.3							0	100	0.0	[41]
		12	518	2.3				0	46	0.0	7	675	1.0	[37]
					1	292	0.3				1	544	0.2	[25]
		1	63	1.6										[43]
		1	118	0.8							0	198	0.0	[28]
		3	39	7.7	1	6	16.7	0	2	0.0	1	114	0.9	[42]
		0	16	0.0	0	16	0.0	1	11	9.1				[40]
		0	70	0.0	2	161	1.2	0	17	0.0	0	195	0.0	this study
		39	1439	2.7	11	723	1.5	1	76	1.3	25	3558	0.7	Total
c.581_582del CCinsAA	p.S194X	1	162	0.6							0	100	0.0	[16]
		1	173	0.6							0	100	0.0	[41]
		2	335	0.6							0	200	0.0	Total
c.602G>A	p.R202H	1	162	0.6										[16]
		1	19	5.3	0	16	0.0				0	50	0.0	[15]
		1	106	0.9							0	62	0.0	[39]
		0	115	0.0	2	240	0.8				0	1041	0.0	[26] swedish
											1	318	0.3	[26] german
		0	157	0.0							5	764	0.7	[26] US
					1	305	0.3				1	469	0.2	[25]
		1	70	1.4	0	161	0.0	0	17	0.0	0	195	0.0	this study
		4	629	0.6	3	722	0.4	0	17	0.0	7	2899	0.2	Total

HG/DG = hypogamma/dysgammaglobulinemia.
Counts from Total row are used in summary table 3.
cDNA and protein nomenclature according to NCBI nucleotide entry NM_012452 and NCBI protein entry NP_036584, respectively.

Supplementary table 4: Allele frequencies of synonymous. intronic and/or common variants in *TNFRSF13B* gene reported in multiple studies

c.DNA nomenclature	protein nomenclature	rs	allele 1	allele 2	CVID unrelated patients allele 1	CVID unrelated patients allele 2	CVID unrelated patients freq 1 (%)	IgAD unrelated patients allele 1	IgAD unrelated patients allele 2	IgAD unrelated patients freq 1 (%)	HG/DG unrelated patients allele 1	HG/DG unrelated patients allele 2	HG/DG unrelated patients freq 1 (%)	Controls allele 1	Controls allele 2	Controls freq 1 (%)	Reference/ Total
c.81G>A	p.T27T	rs8072293	a	g	154	58	72.6							50	74	40.3	[39]
					21	11	65.6	18	14	56.3	16	6	72.7				[40]
					110	30	78.6	211	105	66.8	29	5	85.3	369	143	72.1	this study
					285	99	74.2	229	119	65.8	45	11	80.4	419	217	65.9	Total
c.291T>G	p.P97P	rs35062843	g	t										19	641	2.9	[44]
					13	199	6.1							2	122	1.6	[39]
					7	119	5.6										[43]
					0	32	0.0	0	32	0.0	1	21	4.5				[40]
					6	134	4.3	6	312	1.9	0	34	0.0	1	413	0.2	this study
					26	484	5.1	6	344	1.7	1	55	1.8	22	1176	1.8	Total
c.659T>C	p.V220A	rs56063729	c	t	7	205	3.3							1	123	0.8	[39]
					17	829	2.0	17	463	3.5				62	2620	2.3	[26]
					1	31	3.1	0	32	0.0	0	22	0.0	24	494	4.6	[40]
					3	135	2.2	17	291	5.5	3	31	8.8	18	392	4.4	this study
					28	1200	2.3	34	786	4.1	3	53	5.4	105	3629	2.8	Total
c.752C>T	p.P251L	rs34562254	t	c										6	94	6.0	[15]
					15	197	7.1							13	111	10.5	[39]
					83	765	9.8	56	424	11.7				358	3028	10.6	[26]
								60	616	8.9				108	952	10.2	[25]
					3	29	9.4	6	26	18.8	2	20	9.1	85	433	16.4	[40]
					18	122	12.9	38	276	12.1	7	27	20.6	41	365	10.1	this study
					119	1113	9.7	160	1342	10.7	9	47	16.1	611	4983	10.9	Total
c.831T>C	p.S277S	rs11078355	c	t	68	144	32.1							32	92	25.8	[39]
					10	22	31.3	14	18	43.8	7	15	31.8				[40]
					57	83	40.7	135	175	43.5	17	17	50.0	160	244	39.6	this study

					135	249	35.2	149	193	43.6	24	32	42.9	192	336	36.4	Total

c.445+25a>c		rs2274892	c	a				263	403	39.5				391	675	36.7	[25]
					14	18	43.8	14	18	43.8	9	13	40.9				[40]
					51	89	36.4	154	164	48.4	18	16	52.9	174	238	42.2	this study
					65	107	37.8	431	585	42.4	27	29	48.2	565	913	38.2	Total

HG/DG = hypogamma/dysgammaglobulinemia; freq1 = allele 1 frequency.
Counts from Total row are used in summary table 4.
cDNA and protein nomenclature according to NCBI nucleotide entry NM_012452 and NCBI protein entry NP_036584, respectively.

Supplementary table 5: Number of patients screened for unknown *TNFRSF13B* mutations

CVID all regions	CVID EX3,4 only	IgAD all regions	IgAD EX3,4 only	HG/DG all regions	HG/DG EX3,4 only	general population all regions	general population EX3,4 only	methods	reference
162		0		0		100		sequencing	[16]
518		0		46		107	568	heteroduplex analysis. sequencing	[37]
19		16		0		50		sequencing	[15]
106		0		0		62		sequencing	[39]
0		55		0		0		sequencing	[26]
173		0		0		0		sequencing	[41]
118		0		0		198		?	[28]
0	63	0		0		0		sequencing	[43]
48		0		0		0		sequencing	[38]
39		6		2		114		sequencing	[42]
16		16		11		0		sequencing	[40]
42	28	57	104	0	17	0	195	SSCP. DGGE. HRM	this study
Total	Total EX3,4	Total	Total EX3,4	Total	Total EX3,4	Total	Total EX3,4		
1241	1332	150	254	59	76	631	1394		

HG/DG = hypogamma/dysgammaglobulinemia; EX3,4 – exon 3 and 4

Supplementary table 6: Summary on functional studies and *in silico* predictions of *TNFRSF13B* mutations significance

Mutation	Domain	Expression (cell type)	Ligand binding BAFF / APRIL (cell type)	APRIL induced proliferation / class switch (cell type)	NFκB activation (cell type)	*In silico* predictions for missense mutations Pmut / Polyphen / SIFT / summary [a]	Patient status	Comments	References
c.61+1G>T	EC	0 (B cells, EBV)	n.d. / 0 (EBV)	n.d.	n.d.	n.a.	hom.		[38]
p.W40R	CRD1	N (293T)	N (293T) / n.d.	n.d.	N	D / D / D / pathological	het.		[34, 37]
p.D41H	CRD1	N (293T)	N (293T) / n.d.	n.d.	N	PD / D / D / pathological	together with p.C100LfsX6 on one allele		[34, 37]
p.D41IfsX43	CRD1	N (EBV)	n.d. / N (EBV)	n.d.	n.d.	n.a.	het.		[37]
p.P42T	CRD1	n.d.	n.d.	n.d.	n.d.	PD / PD / B / possibly pathological	het.		[38]
p.L69TfsX12	CRD1	N (EBV/het., B cells/c.het.); 0 (293)	n.d. / N (EBV/het.)	n.d. / 0 (naive B cells/het., c.het.)	n.d.	n.a.	het. and c.het. with p.C104R		[15, 37]
p.R72H		n.d.	n.d.	n.d. / ↓↓ (PBL)		PD / PD / B / possibly pathological	het.		[16, 37, 41]
p.G76fsX3	CRD2	n.d.	n.d.	n.d.	n.d.	n.a.	het.		[39]
p.Y79C	CRD2	↓ (EBV, 293T)	↓↓ (EBV, 293T) / ↓↓ (EBV)	n.d.	0 (293T); (0 NFAT activation in 293T cells)	D / D / D / pathological	c.het. with p.I87N		[33-34, 37]
p.I87N	CRD2	N (EBV/het., 293T); ↓ (EBV/c.het.)	↓↓ (EBV/het.; 293T) / ↓ to ↓↓ (EBV/het.), 0 (EBV/c.het.)	n.d.	↓↓ (293T); (↓↓ NFAT activation in 293T cells)	D / D / D / pathological	het. and c.het. with p.Y79C		[33-34, 37]
p.C89Y	CRD2	n.d.	n.d.	n.d.	n.d.	D / D / D / pathological	het.		[28]
p.C100LfsX6	CRD2	n.d.	n.d.	n.d.	n.d.	n.a.	together with p.D41H on one allele		[37]

p.C104R	CRD2	N to ↓↓↓ (PBL/het.), ↓↓ (EBV/hom, het.), ↓ (293T)	0 (293T) / diverse [b]	diverse [c] / ↓↓ (naive B cells/het, PBL/het)	0 (293T)	D / D / D / pathological	hom., het. and c.het.	elevated TACI serum levels in mice; effect by haploinsufficiency	[15-16, 33-35, 37-38, 41]
p.C104Y	CRD2	↓↓ (EBV)	n.d. / 0 (EBV)	n.d.	n.d.	D / D / D / pathological	c.het. with p.C104R		[37]
p.E117G	stalk	n.d.	n.d.	n.d.	n.d.	D / PD / B / possibly pathological	het.		[28]
p.R122W	stalk	n.d.	n.d.	n.d.	n.d.	D / PD / B / possibly pathological	het.		[37]
p.E140K	stalk	n.d.	n.d.	n.d.	n.d.	B / PD / B / harmless	het.		[28]
p.S144X	stalk	0 (EBV)	n.d. / 0 (EBV)	n.d. / 0 (PBL)	n.d.	n.a.	hom.	0 class switching even after IL-10/BAFF stimulation	[16, 38, 41]
p.A149T	stalk	n.d.	n.d.	n.d.	n.d.	B / B / B / harmless	het.		[37]
p.G152E	stalk	n.d.	n.d.	n.d.	n.d.	D / PD / B / possibly pathological	c.het. with p.A181E		[37]
p.Y164X	stalk	↓↓ (EBV)	n.d. / 0	n.d.	n.d.	n.a.	c.het. with p.C104R		[37]
p.L171R	TM	↓↓↓ (293T)	0 (293T) / ↓↓ (EBV)	↓↓ (PBL) / ↓↓ (PBL)	0 (293T); (↓↓ NFAT activation in 293T cells)	PD / PD / D[d] / possibly pathological	c.het. with p.A181E		[34, 37, 41]
p.C172Y	TM	N (293T)	N (293T) / n.d.	0 (PBL)/ ↓↓ (PBL)	0 (293T); (↓↓ NFAT activation in 293T cells)	D / D / D[d] / pathological	het.	0 proliferative response of B cells after APRIL+IL-10 or CD40L + IL-10 stimulation	[34, 37-38, 41]
p.A181E	TM	N (EBV/het, PBL/het., 293T)	N (293T) / N (EBV/het.) to ↓↓ (PBL/het.)	some response (PBL/het.) / ↓↓ (PBL/het.)	N to 0 (293T)	D / B / D[d] / pathological	het. (+ c.het.)	elevated TACI serum levels - may have resulted from patients lymphoma	[15-16, 33-34, 37, 41]

p.K188M	IC	N (293T)	N (293T) / n.d.	n.d.	N (293T)	B / PD / D / possibly pathological			[34, 37]
p.R189K	IC	n.d.	n.d.	n.d.	n.d.	B / B / B / harmless	het.		this study
p.D191GfsX46	IC	↓↓ (EBV, 293T)	↓↓ (EBV)	n.d.	0 (293T)	n.a.	c.het. with p.C104R	0 BAFF-ligand NFκB activation (293T)	[33, 37]
p.C193X	IC	N to ↓↓ (EBV)	n.d. / N to ↓↓ (EBV)	n.d.	n.d.	n.a.	het.		[37-38]
p.S194X	IC	N to ↓↓ (EBV/het.)	n.d. / ↓↓ (EBV/het.), ↓ (EBV/c.het.)	0 / ↓↓ (PBL/c.het.)	n.d.; (↓↓ NFAT activation in 293T cells)	n.a.	het. + 1 c.het. with p.C104R	0 proliferative response of B cells after APRIL+IL-10 or CD40L + IL-10 stimulation	[16, 38, 41]
p.R202H	IC	N (293T, PBL)	N (293) / n.d.	n.d.	↓ (293T); (possibly NFAT activation block)	PD / PD / B / possibly pathological	het.		[15-16, 33, 37, 45]; (unpublished data in [45])
p.V246F	IC	N (293T)	N (293T) / n.d.	n.d.	N (293T)	PD / B / D[d] / possibly pathological	het.		[34, 37]

[a] Predictions done for the purpose of this study. Unified terminology for Pmut/Polyphen/SIFT results: D = damaging; PD = possibly damaging (in Pmut for pathological mutations with reliability score 0-4); B = benign. Suggested summary from *in silico* prediction tools used: pathological/ possibly pathological/ harmless.

[b] APRIL binding: Normal in EBV lines of some heterozygous patients, ↓↓ in others; ↓↓↓ in homozygous EBV lines.

[c] IgM/APRIL induced proliferation was detectable with one heterozygous EBV line, ↓↓ with another and 0 with the third one.

[d] Predictions that differ from what has been described in Salzer et al. [37] because they used their own multiple sequence alignment setting.

N = normal; 0 = absent/none/abolished; ↓= slightly reduced; ↓↓= reduced/decreased/impaired; ↓↓↓ = deeply reduced; n.d. = not determined; n.a. = not applicable; EBV = EBV-transformed B cell line; PBL = peripheral B-lymphocytes; het. = heterozygote; hom. = homozygote; c.het. = compound heterozygote.

Reference [45] is involved only in the Supplementary material:

45. Castigli E, Geha RS. Molecular basis of common variable immunodeficiency. J Allergy Clin Immunol. 2006;117(4):740-6; quiz 7. doi:S0091-6749(06)00296-X [pii] 10.1016/j.jaci.2006.01.038.

4. Diskuze a závěr

Rozvoj metod molekulární biologie v posledních 2 desetiletích znamenal obrovský pokrok v poznání genetické podstaty řady chorob. Začátkem tohoto století a tisíciletí bylo dokončeno sekvenování celého lidského genomu a celkový počet lidských genů je nyní odhadován na přibližně 22.000. Z odhadu počtu genů zahrnutých do vývoje a funkce leukocytů, který převyšuje 1.000, vyplývá, že i při předpokládané redundanci části genů můžeme počítat s existencí téměř 1.000 monogenních primárních poruch imunity, tedy těch, které jsou bazálně determinované defektem jednoho genu (Edvard Smith et al.). Pokud jsou tyto úvahy správné, jsou ještě více než 2/3 genů, jejichž defekt vede k poruše imunity, neznámé. Identifikace kauzální mutace ve známých genech, ale i identifikace nových genů podílejících se na správné funkci imunitního systému, se stává s technologickým pokrokem stále rychlejší a ekonomicky dostupnější. Zatímco získání sekvence prvního lidského genomu v rámci projektu započatého v roce 1990 trvalo 13 let a stálo 3 miliardy amerických dolarů, v roce 2012 bylo možno získat s využitím technologie NGS sekvenci jednoho lidského genomu za méně než 2 týdny za cenu menší než 1.000 amerických dolarů. Úzkým hrdlem je dnes spíše bioinformatická analýza obrovského množství získaných dat, a také funkční analýzy odhalených mutací, které prokáží jejich kauzální vztah ke zkoumanému onemocnění.

Objevení kauzálních mutací zodpovědných za vznik monogenních primárních imunodeficiencí v nových genech má význam pro hlubší poznání funkcí imunitního systému a pro vývoj nových léčebných přístupů dané poruchy, včetně genové terapie. Určení kauzální mutace u konkrétního pacienta má význam pro přesnější určení prognózy a volbu optimální terapie, jakož i pro genetické poradenství v postižených rodinách. Komplikovanější je situace u komplexních, polygenních chorob, kdy se na jejich vzniku podílí pravděpodobně kombinace určitých genetických variant v celé řadě genů, vzájemná interakce těchto variant, epigenetické vlivy a interakce genetických variant s faktory vnějšího prostředí. To je v oblasti primárních imunodeficiencí podle nejnovějších poznatků relevantní u nejčastější protilátkové imunodeficience, selektivního deficitu IgA, a nejčastější klinicky závažné protilátkové imunodeficience, CVID. Studium genetických faktorů podílejících se na vzniku těchto chorob, vzniku komplikací, jakož i faktorů ovlivňujících průběh onemocnění i odpověď na různé typy léčby, je zásadní pro vývoj účinných léčebných strategií. To platí i pro studium genetických faktorů modifikujících průběh monogenních chorob. Při obrovské heterogenitě klinických projevů při postižení stejného genu by znalost dalších genetických vlivů u konkrétního pacienta pomohla individualizovat léčbu ovlivněním faktorů, které se podílejí na vzniku komplikací a které ovlivňují negativně průběh choroby u konkrétního pacienta.

Z uvedených důvodů jsme se s kolegy ve svých pracích zaměřili nejen na detekování kauzálních mutací v genech zodpovědných za vznik monogenních PID, ale také na studium genů malého účinku modifikujících průběh nejen monogenních onemocnění, ale zejména se podílejících na vzniku a ovlivňujících manifestaci komplexních PID, jako je IgAD a CVID.

Závěrem bych rád uvedl, že využití metod molekulární genetiky v praxi musí ctít 3 základní pilíře lékařské etiky, jimiž jsou prospěšnost, autonomie a rovnost. Prospěšnost znamená, že testování musí mít pro pacienta přínos, autonomie znamená garanci práva každému jedinci svobodně a bez nátlaku se rozhodnout o navrhované lékařské péči a rovnost znamená zajištění rovné a spravedlivé léčby pro všechny pacienty.

Dramaticky se rozvíjející možnosti diagnostiky mohou vést k prodlevě mezi dobou, kdy jsme schopni stanovit diagnózu genetického onemocnění, a chvílí, kdy máme k dispozici efektivní nástroje, jak vzniku daného onemocnění zabránit nebo jak jej léčit. V případě imunodeficiencí se vědcům a lékařům daří tuto mezeru úspěšně zacelovat, připomeňme vývoj nových antibiotik, substituční léčbu imunoglobuliny, transplantaci hematopoetických kmenových buněk či genovou terapii. Otázka nabídky a zachování dostupnosti péče všem potřebným (rovnost) je ale jistě v souvislosti s nabídkou genetického testování na místě.

Další okruh etických problémů představuje testování genetické predispozice ke vzniku onemocnění u zdravých dosud asymptomatických jedinců. Na jednu stranu může znalost výsledku genetického testování vyvolat žádoucí změny životního stylu a případně vést k zahájení preventivních opatření k oddálení nástupu onemocnění, na druhé straně může představovat velkou psychickou zátěž, společenskou stigmatizaci a diskriminaci u zaměstnavatele nebo u pojišťovacích společností. Také v případě genetického testování u nemocí, u nichž není možná účinná prevence a neexistuje ani efektivní terapie, je diskutabilní, kdy je testování pro pacienta prospěšné a kdy přináší více škod než užitku.

Testování přenašečství u dětí zase otevírá otázku autonomie. Je nutné testovat už nyní nebo je možné počkat, až dítě dospěje a bude samo moci rozhodnout o provedení testů? Se stoupající silou genetické diagnostiky stoupá také potřeba dobré informovanosti na straně lékařů a poskytovatelů zdravotní péče, ale i na straně pacientů, jejich rodin a společnosti, aby informace získané genetickým testováním byly využívány rozumně a s maximální možnou mírou zisku pro pacienta.

5. Reference

Al-Herz, W., A. Bousfiha, J.L. Casanova, T. Chatila, M.E. Conley, C. Cunningham-Rundles, A. Etzioni, J.L. Franco, H.B. Gaspar, S.M. Holland, C. Klein, S. Nonoyama, H.D. Ochs, E. Oksenhendler, C. Picard, J.M. Puck, K. Sullivan, and M.L. Tang. 2014. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Front Immunol* 5:162.

Alangari, A., A. Alsultan, N. Adly, M.J. Massaad, I.S. Kiani, A. Aljebreen, E. Raddaoui, A.K. Almomen, S. Al-Muhsen, R.S. Geha, and F.S. Alkuraya. 2012. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. *J Allergy Clin Immunol* 130:481-488 e482.

Allen, R.C., R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, C.M. Disteche, D.K. Simoneaux, and et al. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 259:990-993.

Andersen, P., H. Permin, V. Andersen, L. Schejbel, P. Garred, A. Svejgaard, and T. Barington. 2005. Deficiency of somatic hypermutation of the antibody light chain is associated with increased frequency of severe respiratory tract infection in common variable immunodeficiency. *Blood* 105:511-517.

Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grosmaire, R. Stenkamp, M. Neubauer, and et al. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 72:291-300.

Asao, H., C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, and K. Sugamura. 2001. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol* 167:1-5.

Asao, H., N. Tanaka, N. Ishii, M. Higuchi, T. Takeshita, M. Nakamura, T. Shirasawa, and K. Sugamura. 1994. Interleukin 2-induced activation of JAK3: possible involvement in signal transduction for c-myc induction and cell proliferation. *FEBS Lett* 351:201-206.

Baker, K., S.W. Qiao, T. Kuo, K. Kobayashi, M. Yoshida, W.I. Lencer, and R.S. Blumberg. 2009. Immune and non-immune functions of the (not so) neonatal Fc receptor, FcRn. *Semin Immunopathol* 31:223-236.

Boisson, B., P. Quartier, and J.L. Casanova. 2015. Immunological loss-of-function due to genetic gain-of-function in humans: autosomal dominance of the third kind. *Curr Opin Immunol* 32:90-105.

Boisson, B., Y.D. Wang, A. Bosompem, C.S. Ma, A. Lim, T. Kochetkov, S.G. Tangye, J.L. Casanova, and M.E. Conley. 2013. A recurrent dominant negative E47 mutation causes agammaglobulinemia and BCR(-) B cells. *J Clin Invest* 123:4781-4785.

Bousfiha, A.A., L. Jeddane, F. Ailal, W. Al Herz, M.E. Conley, C. Cunningham-Rundles, A. Etzioni, A. Fischer, J.L. Franco, R.S. Geha, L. Hammarstrom, S. Nonoyama, H.D. Ochs, C.M. Roifman, R. Seger, M.L. Tang, J.M. Puck, H. Chapel, L.D. Notarangelo, and J.L. Casanova. 2013. A phenotypic approach for IUIS PID classification and diagnosis: guidelines for clinicians at the bedside. *J Clin Immunol* 33:1078-1087.

Brandrup, F., K.M. Homburg, P. Wang, P. Garred, and H.O. Madsen. 1999. Mannan-binding lectin deficiency associated with recurrent cutaneous abscesses, prurigo and possibly atopic dermatitis. A family study. *Br J Dermatol* 140:180-181.

Bruton, O. 1952. Agammaglobulinemia. *Pediatrics* 9:722-728.

Carrel, L., A.A. Cottle, K.C. Goglin, and H.F. Willard. 1999. A first-generation X-inactivation profile of the human X chromosome. *Proc Natl Acad Sci U S A* 96:14440-14444.

Castigli, E., S.A. Wilson, L. Garibyan, R. Rachid, F. Bonilla, L. Schneider, and R.S. Geha. 2005. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet* 37:829-834.

Cavazzana-Calvo, M., S. Hacein-Bey, G. de Saint Basile, F. Gross, E. Yvon, P. Nusbaum, F. Selz, C. Hue, S. Certain, J.L. Casanova, P. Bousso, F.L. Deist, and A. Fischer. 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 288:669-672.

Cervenak, J., B. Bender, Z. Schneider, M. Magna, B.V. Carstea, K. Liliom, A. Erdei, Z. Bosze, and I. Kacskovics. 2011. Neonatal FcR overexpression boosts humoral immune response in transgenic mice. *J Immunol* 186:959-968.

Conley, M.E., and V. Howard. 2002. Clinical findings leading to the diagnosis of X-linked agammaglobulinemia. *J Pediatr* 141:566-571.

Corneo, B., D. Moshous, T. Gungor, N. Wulffraat, P. Philippet, F.L. Le Deist, A. Fischer, and J.P. de Villartay. 2001. Identical mutations in RAG1 or RAG2 genes leading to defective V(D)J recombinase activity can cause either T-B-severe combined immune deficiency or Omenn syndrome. *Blood* 97:2772-2776.

Cunningham-Rundles, C., and C. Bodian. 1999. Common variable immunodeficiency: clinical and immunological features of 248 patients. *Clin Immunol* 92:34-48.

DiSanto, J.P., J.Y. Bonnefoy, J.F. Gauchat, A. Fischer, and G. de Saint Basile. 1993. CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. *Nature* 361:541-543.

Durandy, A., S. Kracker, and A. Fischer. 2013. Primary antibody deficiencies. *Nat Rev Immunol* 13:519-533.

Edvard Smith, C.I., H.D. Ochs, and J.M. Puck. Genetically Determined Immunodeficiency Diseases: A Perspective. In 'Oxford University Press', Oxford, UK.

Ferrari, S., S. Giliani, A. Insalaco, A. Al-Ghonaium, A.R. Soresina, M. Loubser, M.A. Avanzini, M. Marconi, R. Badolato, A.G. Ugazio, Y. Levy, N. Catalan, A. Durandy, A. Tbakhi, L.D. Notarangelo, and A. Plebani. 2001. Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* 98:12614-12619.

Ferrari, S., V. Lougaris, S. Caraffi, R. Zuntini, J. Yang, A. Soresina, A. Meini, G. Cazzola, C. Rossi, M. Reth, and A. Plebani. 2007. Mutations of the Igbeta gene cause agammaglobulinemia in man. *J Exp Med* 204:2047-2051.

Ferreira, R.C., Q. Pan-Hammarstrom, R.R. Graham, G. Fontan, A.T. Lee, W. Ortmann, N. Wang, E. Urcelay, M. Fernandez-Arquero, C. Nunez, G. Jorgensen, B.R. Ludviksson, S. Koskinen, K. Haimila, L. Padyukov, P.K. Gregersen, L. Hammarstrom, and T.W. Behrens. 2012. High-density SNP mapping of the HLA region identifies multiple independent susceptibility loci associated with selective IgA deficiency. *PLoS Genet* 8:e1002476.

Ferreira, R.C., Q. Pan-Hammarstrom, R.R. Graham, V. Gateva, G. Fontan, A.T. Lee, W. Ortmann, E. Urcelay, M. Fernandez-Arquero, C. Nunez, G. Jorgensen, B.R. Ludviksson, S. Koskinen, K. Haimila, H.F. Clark, L. Klareskog, P.K. Gregersen, T.W. Behrens, and L. Hammarstrom. 2010. Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency. *Nat Genet* 42:777-780.

Fevang, B., T.E. Mollnes, A.M. Holm, T. Ueland, L. Heggelund, J.K. Damas, P. Aukrust, and S.S. Froland. 2005. Common variable immunodeficiency and the complement system; low mannose-binding lectin levels are associated with bronchiectasis. *Clin Exp Immunol* 142:576-584.

Fuleihan, R., N. Ramesh, R. Loh, H. Jabara, R.S. Rosen, T. Chatila, S.M. Fu, I. Stamenkovic, and R.S. Geha. 1993. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci U S A* 90:2170-2173.

Gabolde, M., D. Hubert, M. Guilloud-Bataille, C. Lenaerts, J. Feingold, and C. Besmond. 2001. The mannose binding lectin gene influences the severity of chronic liver disease in cystic fibrosis. *J Med Genet* 38:310-311.

Garred, P., H.O. Madsen, U. Balslev, B. Hofmann, C. Pedersen, J. Gerstoft, and A. Svejgaard. 1997. Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. *Lancet* 349:236-240.

Garred, P., T. Pressler, H.O. Madsen, B. Frederiksen, A. Svejgaard, N. Hoiby, M. Schwartz, and C. Koch. 1999. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. *J Clin Invest* 104:431-437.

Garred, P., A. Voss, H.O. Madsen, and P. Junker. 2001. Association of mannose-binding lectin gene variation with disease severity and infections in a population-based cohort of systemic lupus erythematosus patients. *Genes Immun* 2:442-450.

Gaspar, H.B., M. Ferrando, I. Caragol, M. Hernandez, J.M. Bertran, X. De Gracia, T. Lester, C. Kinnon, E. Ashton, and T. Espanol. 2000. Kinase mutant Btk results in atypical X-linked agammaglobulinaemia phenotype. *Clin Exp Immunol* 120:346-350.

Gatti, R.A., H.J. Meuwissen, H.D. Allen, R. Hong, and R.A. Good. 1968. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet* 2:1366-1369.

Ghetie, V., and E.S. Ward. 2000. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. *Annu Rev Immunol* 18:739-766.

Giri, J.G., M. Ahdieh, J. Eisenman, K. Shanebeck, K. Grabstein, S. Kumaki, A. Namen, L.S. Park, D. Cosman, and D. Anderson. 1994. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *Embo J* 13:2822-2830.

Glanzmann, E., and P. Riniker. 1950. [Essential lymphocytophthisis; new clinical aspect of infant pathology]. *Ann Paediatr* 175:1-32.

Gleicher, N., and D.H. Barad. 2007. Gender as risk factor for autoimmune diseases. *J Autoimmun* 28:1-6.

Graudal, N.A., H.O. Madsen, U. Tarp, A. Svejgaard, G. Jurik, H.K. Graudal, and P. Garred. 2000. The association of variant mannose-binding lectin genotypes with radiographic outcome in rheumatoid arthritis. *Arthritis Rheum* 43:515-521.

Gregersen, P.K., and T.W. Behrens. 2006. Genetics of autoimmune diseases--disorders of immune homeostasis. *Nat Rev Genet* 7:917-928.

Grimbacher, B., A. Hutloff, M. Schlesier, E. Glocker, K. Warnatz, R. Drager, H. Eibel, B. Fischer, A.A. Schaffer, H.W. Mages, R.A. Kroczek, and H.H. Peter. 2003. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* 4:261-268.

Gunraj, C.A., B.J. Fernandes, and G.A. Denomme. 2002. Synonymous nucleotide substitutions in the neonatal Fc receptor. *Immunogenetics* 54:139-140.

Hammarstrom, L., I. Vorechovsky, and D. Webster. 2000. Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). *Clin Exp Immunol* 120:225-231.

Hamvas, R.M., M. Johnson, A.M. Vlieger, C. Ling, A. Sherriff, A. Wade, N.J. Klein, M.W. Turner, and A.D. Webster. 2005. Role for mannose binding lectin in the prevention of Mycoplasma infection. *Infect Immun* 73:5238-5240.

Hansen, T.K., S. Thiel, R. Dall, A.M. Rosenfalck, P. Trainer, A. Flyvbjerg, J.O. Jorgensen, and J.S. Christiansen. 2001. GH strongly affects serum concentrations of mannan-binding lectin: evidence for a new IGF-I independent immunomodulatory effect of GH. *J Clin Endocrinol Metab* 86:5383-5388.

Harris, M., J. Clark, N. Coote, P. Fletcher, A. Harnden, M. McKean, A. Thomson, and C. British Thoracic Society Standards of Care. 2011. British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. *Thorax* 66 Suppl 2:ii1-23.

Harvey, J., P.A. Jacobs, T. Hassold, and D. Pettay. 1990. The parental origin of 47,XXY males. *Birth Defects Orig Artic Ser* 26:289-296.

He, B., R. Santamaria, W. Xu, M. Cols, K. Chen, I. Puga, M. Shan, H. Xiong, J.B. Bussel, A. Chiu, A. Puel, J. Reichenbach, L. Marodi, R. Doffinger, J. Vasconcelos, A. Issekutz, J. Krause, G. Davies, X. Li, B. Grimbacher, A. Plebani, E. Meffre, C. Picard, C. Cunningham-Rundles, J.L. Casanova, and A. Cerutti. 2010. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat Immunol* 11:836-845.

He, Y.W., and T.R. Malek. 1996. Interleukin-7 receptor alpha is essential for the development of gamma delta + T cells, but not natural killer cells. *J Exp Med* 184:289-293.

Hoal-Van Helden, E.G., J. Epstein, T.C. Victor, D. Hon, L.A. Lewis, N. Beyers, D. Zurakowski, A.B. Ezekowitz, and P.D. Van Helden. 1999. Mannose-binding protein B allele confers protection against tuberculous meningitis. *Pediatr Res* 45:459-464.

Holinski-Feder, E., M. Weiss, O. Brandau, K.B. Jedele, B. Nore, C.M. Backesjo, M. Vihinen, S.R. Hubbard, B.H. Belohradsky, C.I. Smith, and A. Meindl. 1998. Mutation screening of the BTK gene in 56 families with X-linked agammaglobulinemia (XLA): 47 unique mutations without correlation to clinical course. *Pediatrics* 101:276-284.

Chapel, H., M. Lucas, M. Lee, J. Bjorkander, D. Webster, B. Grimbacher, C. Fieschi, V. Thon, M.R. Abedi, and L. Hammarstrom. 2008. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood* 112:277-286.

Chen, K., E.M. Coonrod, A. Kumanovics, Z.F. Franks, J.D. Durtschi, R.L. Margraf, W. Wu, N.M. Heikal, N.H. Augustine, P.G. Ridge, H.R. Hill, L.B. Jorde, A.S. Weyrich, G.A. Zimmerman, A.V. Gundlapalli, J.F. Bohnsack, and K.V. Voelkerding. 2013. Germline mutations in NFKB2 implicate the noncanonical NF-kappaB pathway in the pathogenesis of common variable immunodeficiency. *Am J Hum Genet* 93:812-824.

Imai, K., G. Slupphaug, W.I. Lee, P. Revy, S. Nonoyama, N. Catalan, L. Yel, M. Forveille, B. Kavli, H.E. Krokan, H.D. Ochs, A. Fischer, and A. Durandy. 2003. Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. *Nat Immunol* 4:1023-1028.

Ishii-Watabe, A., Y. Saito, T. Suzuki, M. Tada, M. Ukaji, K. Maekawa, K. Kurose, N. Kaniwa, J. Sawada, N. Kawasaki, T. Yamaguchi, T.E. Nakajima, K. Kato, Y. Yamada, Y. Shimada, T. Yoshida, T. Ura, M. Saito, K. Muro, T. Doi, N. Fuse, T. Yoshino, A. Ohtsu, N. Saijo, T. Hamaguchi, H. Okuda, and Y. Matsumura. 2010. Genetic polymorphisms of FCGRT encoding FcRn in a Japanese population and their functional analysis. *Drug Metab Pharmacokinet* 25:578-587.

Jolles, S. 2013. The variable in common variable immunodeficiency: a disease of complex phenotypes. *J Allergy Clin Immunol Pract* 1:545-556; quiz 557.

Kilpatrick, D.C. 2002. Mannan-binding lectin and its role in innate immunity. *Transfus Med* 12:335-352.

Kilpatrick, D.C., B.H. Bevan, and W.A. Liston. 1995. Association between mannan binding protein deficiency and recurrent miscarriage. *Hum Reprod* 10:2501-2505.

Kimura, Y., T. Takeshita, M. Kondo, N. Ishii, M. Nakamura, J. Van Snick, and K. Sugamura. 1995. Sharing of the IL-2 receptor gamma chain with the functional IL-9 receptor complex. *Int Immunol* 7:115-120.

Kondo, M., T. Takeshita, N. Ishii, M. Nakamura, S. Watanabe, K. Arai, and K. Sugamura. 1993. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science* 262:1874-1877.

Korthauer, U., D. Graf, H.W. Mages, F. Briere, M. Padayachee, S. Malcolm, A.G. Ugazio, L.D. Notarangelo, R.J. Levinsky, and R.A. Kroczek. 1993. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* 361:539-541.

Kralovicova, J., L. Hammarstrom, A. Plebani, A.D. Webster, and I. Vorechovsky. 2003. Fine-scale mapping at IGAD1 and genome-wide genetic linkage analysis implicate HLA-DQ/DR as a major susceptibility locus in selective IgA deficiency and common variable immunodeficiency. *J Immunol* 170:2765-2775.

Kuijpers, T.W., R.J. Bende, P.A. Baars, A. Grummels, I.A. Derks, K.M. Dolman, T. Beaumont, T.F. Tedder, C.J. van Noesel, E. Eldering, and R.A. van Lier. 2010. CD20 deficiency in humans results in impaired T cell-independent antibody responses. *J Clin Invest* 120:214-222.

Lahlou, N., I. Fennoy, J.L. Ross, C. Bouvattier, and M. Roger. 2011. Clinical and hormonal status of infants with nonmosaic XXY karyotype. *Acta Paediatr* 100:824-829.

Latiff, A.H., and M.A. Kerr. 2007. The clinical significance of immunoglobulin A deficiency. *Ann Clin Biochem* 44:131-139.

Lopez-Herrera, G., G. Tampella, Q. Pan-Hammarstrom, P. Herholz, C.M. Trujillo-Vargas, K. Phadwal, A.K. Simon, M. Moutschen, A. Etzioni, A. Mory, I. Srugo, D. Melamed, K. Hultenby, C. Liu, M.

Baronio, M. Vitali, P. Philippet, V. Dideberg, A. Aghamohammadi, N. Rezaei, V. Enright, L. Du, U. Salzer, H. Eibel, D. Pfeifer, H. Veelken, H. Stauss, V. Lougaris, A. Plebani, E.M. Gertz, A.A. Schaffer, L. Hammarstrom, and B. Grimbacher. 2012. Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. *Am J Hum Genet* 90:986-1001.

Luty, A.J., J.F. Kun, and P.G. Kremsner. 1998. Mannose-binding lectin plasma levels and gene polymorphisms in Plasmodium falciparum malaria. *J Infect Dis* 178:1221-1224.

Madsen, H.O., V. Videm, A. Svejgaard, J.L. Svennevig, and P. Garred. 1998. Association of mannose-binding-lectin deficiency with severe atherosclerosis. *Lancet* 352:959-960.

Maggina, P., and A.R. Gennery. 2013. Classification of primary immunodeficiencies: need for a revised approach? *J Allergy Clin Immunol* 131:292-294.

Matsushita, M., M. Hijikata, Y. Ohta, K. Iwata, M. Matsumoto, K. Nakao, K. Kanai, N. Yoshida, K. Baba, and S. Mishiro. 1998. Hepatitis C virus infection and mutations of mannose-binding lectin gene MBL. *Arch Virol* 143:645-651.

Minegishi, Y., E. Coustan-Smith, L. Rapalus, F. Ersoy, D. Campana, and M.E. Conley. 1999a. Mutations in Igalpha (CD79a) result in a complete block in B-cell development. *J Clin Invest* 104:1115-1121.

Minegishi, Y., E. Coustan-Smith, Y.H. Wang, M.D. Cooper, D. Campana, and M.E. Conley. 1998. Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. *J Exp Med* 187:71-77.

Minegishi, Y., J. Rohrer, E. Coustan-Smith, H.M. Lederman, R. Pappu, D. Campana, A.C. Chan, and M.E. Conley. 1999b. An essential role for BLNK in human B cell development. *Science* 286:1954-1957.

Mrozek, E., P. Anderson, and M.A. Caligiuri. 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood* 87:2632-2640.

Mullighan, C.G., G.C. Fanning, H.M. Chapel, and K.I. Welsh. 1997. TNF and lymphotoxin-alpha polymorphisms associated with common variable immunodeficiency: role in the pathogenesis of granulomatous disease. *J Immunol* 159:6236-6241.

Mullighan, C.G., S.E. Marshall, M. Bunce, and K.I. Welsh. 1999. Variation in immunoregulatory genes determines the clinical phenotype of common variable immunodeficiency. *Genes Immun* 1:137-148.

Mullighan, C.G., S.E. Marshall, and K.I. Welsh. 2000. Mannose binding lectin polymorphisms are associated with early age of disease onset and autoimmunity in common variable immunodeficiency. *Scand J Immunol* 51:111-122.

Naito, H., A. Ikeda, K. Hasegawa, S. Oka, K. Uemura, N. Kawasaki, and T. Kawasaki. 1999. Characterization of human serum mannan-binding protein promoter. *J Biochem* 126:1004-1012.

Niehues, T., R. Perez-Becker, and C. Schuetz. 2010. More than just SCID--the phenotypic range of combined immunodeficiencies associated with mutations in the recombinase activating genes (RAG) 1 and 2. *Clin Immunol* 135:183-192.

Nielsen, J., and M. Wohlert. 1990. Sex chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in Arhus, Denmark. *Birth Defects Orig Artic Ser* 26:209-223.

Noguchi, M., Y. Nakamura, S.M. Russell, S.F. Ziegler, M. Tsang, X. Cao, and W.J. Leonard. 1993a. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science* 262:1877-1880.

Noguchi, M., H. Yi, H.M. Rosenblatt, A.H. Filipovich, S. Adelstein, W.S. Modi, O.W. McBride, and W.J. Leonard. 1993b. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73:147-157.

Ochs, H.D., and C.I. Smith. 1996. X-linked agammaglobulinemia. A clinical and molecular analysis. *Medicine (Baltimore)* 75:287-299.

Ozcan, E., I. Rauter, L. Garibyan, S.R. Dillon, and R.S. Geha. 2011. Toll-like receptor 9, transmembrane activator and calcium-modulating cyclophilin ligand interactor, and CD40 synergize in causing B-cell activation. *J Allergy Clin Immunol* 128:601-609 e601-604.

Pan-Hammarstrom, Q., U. Salzer, L. Du, J. Bjorkander, C. Cunningham-Rundles, D.L. Nelson, C. Bacchelli, H.B. Gaspar, S. Offer, T.W. Behrens, B. Grimbacher, and L. Hammarstrom. 2007. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. *Nat Genet* 39:429-430.

Parvaneh, N., J.L. Casanova, L.D. Notarangelo, and M.E. Conley. 2013. Primary immunodeficiencies: a rapidly evolving story. *J Allergy Clin Immunol* 131:314-323.

Poodt, A.E., G.J. Driessen, A. de Klein, J.J. van Dongen, M. van der Burg, and E. de Vries. 2009. TACI mutations and disease susceptibility in patients with common variable immunodeficiency. *Clin Exp Immunol* 156:35-39.

Puck, J.M., S.M. Deschenes, J.C. Porter, A.S. Dutra, C.J. Brown, H.F. Willard, and P.S. Henthorn. 1993. The interleukin-2 receptor gamma chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum Mol Genet* 2:1099-1104.

Puel, A., S.F. Ziegler, R.H. Buckley, and W.J. Leonard. 1998. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genet* 20:394-397.

Raghavan, M., V.R. Bonagura, S.L. Morrison, and P.J. Bjorkman. 1995. Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants. *Biochemistry* 34:14649-14657.

Resnick, E.S., E.L. Moshier, J.H. Godbold, and C. Cunningham-Rundles. 2012. Morbidity and mortality in common variable immune deficiency over 4 decades. *Blood* 119:1650-1657.

Revy, P., T. Muto, Y. Levy, F. Geissmann, A. Plebani, O. Sanal, N. Catalan, M. Forveille, R. Dufourcq-Labelouse, A. Gennery, I. Tezcan, F. Ersoy, H. Kayserili, A.G. Ugazio, N. Brousse, M. Muramatsu, L.D. Notarangelo, K. Kinoshita, T. Honjo, A. Fischer, and A. Durandy. 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 102:565-575.

Rezaei, N., A.A. Amirzargar, Y. Shakiba, M. Mahmoudi, B. Moradi, and A. Aghamohammadi. 2009. Proinflammatory cytokine gene single nucleotide polymorphisms in common variable immunodeficiency. *Clin Exp Immunol* 155:21-27.

Rodewald, R., and J.P. Kraehenbuhl. 1984. Receptor-mediated transport of IgG. *J Cell Biol* 99:159s-164s.

Rosen FS, E.M., Roifman C, Fischer A, Volanakis J, Aiuti F, Notarangelo L, Kishimoto T, Resnick IB, Hammarstrom L, Seger R, Chapel H, Cooper MD, Geha RS, Good, RA, Waldmann TA, Wedgwood RJP. 1999. Primary immunodeficiency diseases. Report of an IUIS Scientific Committee. International Union of Immunological Societies. *Clin Exp Immunol* 118 Suppl 1:1-28.

Rudan, I., C. Boschi-Pinto, Z. Biloglav, K. Mulholland, and H. Campbell. 2008. Epidemiology and etiology of childhood pneumonia. *Bull World Health Organ* 86:408-416.

Russell, S.M., J.A. Johnston, M. Noguchi, M. Kawamura, C.M. Bacon, M. Friedmann, M. Berg, D.W. McVicar, B.A. Witthuhn, O. Silvennoinen, and et al. 1994. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science* 266:1042-1045.

Russell, S.M., A.D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M.C. Friedmann, A. Miyajima, R.K. Puri, W.E. Paul, and et al. 1993. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science* 262:1880-1883.

Russell, S.M., N. Tayebi, H. Nakajima, M.C. Riedy, J.L. Roberts, M.J. Aman, T.S. Migone, M. Noguchi, M.L. Markert, R.H. Buckley, and et al. 1995. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* 270:797-800.

Saevarsdottir, S., T. Vikingsdottir, A. Vikingsson, V. Manfredsdottir, A.J. Geirsson, and H. Valdimarsson. 2001. Low mannose binding lectin predicts poor prognosis in patients with early rheumatoid arthritis. A prospective study. *J Rheumatol* 28:728-734.

Sachs, U.J., I. Socher, C.G. Braeunlich, H. Kroll, G. Bein, and S. Santoso. 2006. A variable number of tandem repeats polymorphism influences the transcriptional activity of the neonatal Fc receptor alpha-chain promoter. *Immunology* 119:83-89.

Salzer, U., C. Bacchelli, S. Buckridge, Q. Pan-Hammarstrom, S. Jennings, V. Lougaris, A. Bergbreiter, T. Hagena, J. Birmelin, A. Plebani, A.D. Webster, H.H. Peter, D. Suez, H. Chapel, A. McLean-Tooke, G.P. Spickett, S. Anover-Sombke, H.D. Ochs, S. Urschel, B.H. Belohradsky, S. Ugrinovic, D.S. Kumararatne, T.C. Lawrence, A.M. Holm, J.L. Franco, I. Schulze, P. Schneider, E.M. Gertz, A.A. Schaffer, L. Hammarstrom, A.J. Thrasher, H.B. Gaspar, and B. Grimbacher. 2009. Relevance of biallelic versus monoallelic TNFRSF13B mutations in distinguishing disease-causing from risk-increasing TNFRSF13B variants in antibody deficiency syndromes. *Blood* 113:1967-1976.

Salzer, U., H.M. Chapel, A.D. Webster, Q. Pan-Hammarstrom, A. Schmitt-Graeff, M. Schlesier, H.H. Peter, J.K. Rockstroh, P. Schneider, A.A. Schaffer, L. Hammarstrom, and B. Grimbacher. 2005. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat Genet* 37:820-828.

Salzer, U., S. Unger, and K. Warnatz. 2012. Common variable immunodeficiency (CVID): exploring the multiple dimensions of a heterogeneous disease. *Ann N Y Acad Sci* 1250:41-49.

Santos, I.K., C.H. Costa, H. Krieger, M.F. Feitosa, D. Zurakowski, B. Fardin, R.B. Gomes, D.L. Weiner, D.A. Harn, R.A. Ezekowitz, and J.E. Epstein. 2001. Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. *Infect Immun* 69:5212-5215.

Sazzini, M., R. Zuntini, S. Farjadian, I. Quinti, G. Ricci, G. Romeo, S. Ferrari, F. Calafell, and D. Luiselli. 2009. An evolutionary approach to the medical implications of the tumor necrosis factor receptor superfamily member 13B (TNFRSF13B) gene. *Genes Immun* 10:566-578.

Seshasayee, D., P. Valdez, M. Yan, V.M. Dixit, D. Tumas, and I.S. Grewal. 2003. Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor. *Immunity* 18:279-288.

Sharfe, N., H.K. Dadi, M. Shahar, and C.M. Roifman. 1997. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. *Proc Natl Acad Sci U S A* 94:3168-3171.

Shiina, T., H. Inoko, and J.K. Kulski. 2004. An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue Antigens* 64:631-649.

Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 352:621-624.

Schwarz, K., G.H. Gauss, L. Ludwig, U. Pannicke, Z. Li, D. Lindner, W. Friedrich, R.A. Seger, T.E. Hansen-Hagge, S. Desiderio, M.R. Lieber, and C.R. Bartram. 1996. RAG mutations in human B cell-negative SCID. *Science* 274:97-99.

Simister, N.E., and K.E. Mostov. 1989. An Fc receptor structurally related to MHC class I antigens. *Nature* 337:184-187.

Speletas, M., A. Mamara, E. Papadopoulou-Alataki, G. Iordanakis, K. Liadaki, F. Bardaka, M. Kanariou, and A.E. Germenis. 2011. TNFRSF13B/TACI alterations in Greek patients with antibody deficiencies. *J Clin Immunol* 31:550-559.

Tao, L., M. Boyd, G. Gonye, B. Malone, and J. Schwaber. 2000. BTK mutations in patients with X-linked agammaglobulinemia: lack of correlation between presence of peripheral B lymphocytes and specific mutations. *Hum Mutat* 16:528-529.

Thiel, J., L. Kimmig, U. Salzer, M. Grudzien, D. Lebrecht, T. Hagena, R. Draeger, N. Voelxen, A. Bergbreiter, S. Jennings, S. Gutenberger, A. Aichem, H. Illges, J.P. Hannan, A.K. Kienzler, M. Rizzi, H. Eibel, H.H. Peter, K. Warnatz, B. Grimbacher, J.A. Rump, and M. Schlesier. 2012. Genetic CD21 deficiency is associated with hypogammaglobulinemia. *J Allergy Clin Immunol* 129:801-810 e806.

Thrasher, A.J., and S.O. Burns. 2010. WASP: a key immunological multitasker. *Nat Rev Immunol* 10:182-192.

Tsukada, S., D.C. Saffran, D.J. Rawlings, O. Parolini, R.C. Allen, I. Klisak, R.S. Sparkes, H. Kubagawa, T. Mohandas, S. Quan, and et al. 1993. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 72:279-290.

Turner, M.W. 2003. The role of mannose-binding lectin in health and disease. *Mol Immunol* 40:423-429.

Tuttelmann, F., and J. Gromoll. 2010. Novel genetic aspects of Klinefelter's syndrome. *Mol Hum Reprod* 16:386-395.

van Zelm, M.C., I. Reisli, M. van der Burg, D. Castano, C.J. van Noesel, M.J. van Tol, C. Woellner, B. Grimbacher, P.J. Patino, J.J. van Dongen, and J.L. Franco. 2006. An antibody-deficiency syndrome due to mutations in the CD19 gene. *N Engl J Med* 354:1901-1912.

van Zelm, M.C., J. Smet, B. Adams, F. Mascart, L. Schandene, F. Janssen, A. Ferster, C.C. Kuo, S. Levy, J.J. van Dongen, and M. van der Burg. 2010. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. *J Clin Invest* 120:1265-1274.

Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarstrom, C. Kinnon, R. Levinsky, M. Bobrow, and et al. 1993. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* 361:226-233.

Villa, A., L.D. Notarangelo, and C.M. Roifman. 2008. Omenn syndrome: inflammation in leaky severe combined immunodeficiency. *J Allergy Clin Immunol* 122:1082-1086.

Villa, A., S. Santagata, F. Bozzi, S. Giliani, A. Frattini, L. Imberti, L.B. Gatta, H.D. Ochs, K. Schwarz, L.D. Notarangelo, P. Vezzoni, and E. Spanopoulou. 1998. Partial V(D)J recombination activity leads to Omenn syndrome. *Cell* 93:885-896.

von Bulow, G.U., J.M. van Deursen, and R.J. Bram. 2001. Regulation of the T-independent humoral response by TACI. *Immunity* 14:573-582.

Vorechovsky, I., H. Zetterquist, R. Paganelli, S. Koskinen, A.D. Webster, J. Bjorkander, C.I. Smith, and L. Hammarstrom. 1995. Family and linkage study of selective IgA deficiency and common variable immunodeficiency. *Clin Immunol Immunopathol* 77:185-192.

Waldmann, T.A., and Y. Tagaya. 1999. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol* 17:19-49.

Wang, H.Y., C.A. Ma, Y. Zhao, X. Fan, Q. Zhou, P. Edmonds, G. Uzel, J.B. Oliveira, J. Orange, and A. Jain. 2013. Antibody deficiency associated with an inherited autosomal dominant mutation in TWEAK. *Proc Natl Acad Sci U S A* 110:5127-5132.

Warnatz, K., U. Salzer, M. Rizzi, B. Fischer, S. Gutenberger, J. Bohm, A.K. Kienzler, Q. Pan-Hammarstrom, L. Hammarstrom, M. Rakhmanov, M. Schlesier, B. Grimbacher, H.H. Peter, and H. Eibel. 2009. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. *Proc Natl Acad Sci U S A* 106:13945-13950.

Yan, M., H. Wang, B. Chan, M. Roose-Girma, S. Erickson, T. Baker, D. Tumas, I.S. Grewal, and V.M. Dixit. 2001. Activation and accumulation of B cells in TACI-deficient mice. *Nat Immunol* 2:638-643.

Yel, L. 2010. Selective IgA deficiency. *J Clin Immunol* 30:10-16.

Yel, L., Y. Minegishi, E. Coustan-Smith, R.H. Buckley, H. Trubel, L.M. Pachman, G.R. Kitchingman, D. Campana, J. Rohrer, and M.E. Conley. 1996. Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. *N Engl J Med* 335:1486-1493.

Zhang, L., L. Radigan, U. Salzer, T.W. Behrens, B. Grimbacher, G. Diaz, J. Bussel, and C. Cunningham-Rundles. 2007. Transmembrane activator and calcium-modulating cyclophilin ligand interactor mutations in common variable immunodeficiency: clinical and immunologic outcomes in heterozygotes. *J Allergy Clin Immunol* 120:1178-1185.

6. Příloha - publikace autora v oblasti primárních imunodeficiencí a imunologie

Analýza kauzálních genů u primárních imunodeficencí

- Poruchy tvorby protilátek

  E. Pařízková, P. Rozsíval, T. Freiberger, D. Komárek: X-vázaná agamaglobulinémie (Brutonova nemoc) – tři kazuistiky a molekulárně genetické studie jejich rodin. Čes.-slov. Pediat., 59, 2004, č. 3, pp. 119-122.

  Z. Vancikova, T. Freiberger, W. Vach, M. Trojanek, M. Rizzi, A. Janda. X-linked agammaglobulinemia in community-acquired pneumonia cases revealed by immunoglobulin level screening at hospital admission. Klin Padiatr 2013; 225(6):339-342.

  A.-V. Cochino, A. Janda, B. Ravcukova, V. Plaiasu, D. Ochiana, I. Gherghina, T. Freiberger: X-Linked agammaglobulinemia in a child with Klinefelter's syndrome. J Clin Immunol 2014; 34(2): 142-145.

  Z. Havlicekova, M. Jesenak, T. Freiberger, P.Banovcin: X-linked agammaglobulinemia caused by new mutation in BTK gene: A case report. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2014; 158(3): 470-473.

- Poruchy komplementu

  T. Freiberger, L. Kolářová, P. Mejstřík, M. Vyskočilová, P. Kuklínek, J. Litzman: Five novel mutations in C1 inhibitor gene leading to a premature stop codon in patients with type I hereditary angioedema. Human Mutation, 19 (4), 2002, p. 461. *TF koresp. autor.*

  J. Litzman, T. Freiberger, D. Bartoňková, M. Vlková, V. Thon, J. Lokaj: Early manifestation and recognition of C2 complement deficiency in the form of pyogenic infection in infancy. J Paediatr Child Health, 39 (4), 2003, pp. 274-277.

  P. Králíčková, E. Burešová, T. Freiberger, I. Tachecí: Hereditární angioedém - opomíjená diagnóza. Vnitř Lék 2010; 56(9): 927-931.

- Ostatní

  R. Formánková, P. Sedláček, T. Freiberger, J. Bartůňková, A. Šedivá, E. Mejstříková, P. Keslová, B. Ravčuková, V. Vávra, J. Litzman, E. Pařízková, Y. Jabali, H. Schneiderová, J. Starý: Wiskottův-Aldrichův syndrom – onemocnění vyžadující včasnou transplantaci kmenových buněk krvetvorby. Čes-slov Pediat 2009; 64, č. 3, pp. 106-114.

  D. Belada, L. Smolej, P. Stěpánková, P. Králíčková, T. Freiberger: Diffuse large B-cell lymphoma in a patient with hyper-IgE syndrome: Successful treatment with risk-adapted rituximab-based immunochemotherapy. Leuk Res 2010; 34: e232-e234.

  M. Suková, E. Mejstříková, E. Vodičková, R. Špíšek, R. Formánková, D. Sumerauer, T. Freiberger, P. Sedláček, J. Starý: Hemofagocytující lymfohistiocytóza. Vnitř Lék 2010; 56(suppl 2): 2S157-2S169.

V. Gulácsy, T. Freiberger, A. Shcherbina, M. Pac, L. Chernyshova, T. Avcin, I. Kondratenko, L. Kostyuchenko, T. Prokofjeva, S. Pasic, E. Bernatowska, N. Kutukculer, J. Rascon, N. Iagaru, C. Mazza, B. Tóth, M. Erdős, M. van der Burg, L. Maródi: Genetic characteristics of eighty-seven patients with the Wiskott-Aldrich syndrome. Mol Immunol 2011; 48(5): 788-792. *VG a TF contributed equally.*

E. Mejstříková, A. Janda, O. Hrušák, H. Bučková, M. Vlčková, M. Hančárová, T. Freiberger, B. Ravčuková, K. Veselý, L. Fajkusová, L. Kopečková, D. Sumerauer, E. Kabíčková, A. Šedivá, J. Starý, Z. Sedláček: Skin lesions in a boy with X-linked lymphoproliferative disorder. Comparison of 5 *SH2D1A* deletion cases. Pediatrics 2012; 129(2): e523-8.

A. Janda, L. Król, T. Kalina, V. Král, J. Pohořská, E. Mejstříková, O. Hrušák, P. Keslová, R. Formánková, H. Schneiderová, J. Litzman, T. Freiberger, A. Poloučková, A. Šedivá, V. Skalická, K. Beránková, D. Zemková, D. Jílek, P. Sedláček, J. Starý: X-vázaný hyper-IgM syndrom. Pacienti v České republice a přehled literatury. Alergie 2012; 1: 34-44.

E. Mejstříková, T. Freiberger, A. Šedivá, P. Čižnár, P. Švec, O. Hrušák, D. Sumerauer, E. Kabíčková, P. Keslová, R. Formánková, M. Suková, P. Sedláček, J. Starý, A. Janda: Přehled pacientů s diagnózou X-vázaného lymfoproliferativního onemocnění (XLP) diagnostikovaných v České republice a na Slovensku. Čs. pediat. 2013, 68(2), 67-77.

T. Svobodova, E. Mejstrikova, U. Salzer, M. Sukova, P. Hubacek, R. Matej, M. Vasakova, L. Hornofova, M. Dvorakova, E. Fronkova, F. Votava, T. Freiberger, P. Pohunek, J. Stary, A. Janda: Diffuse parenchymal lung disease as first clinical manifestation of GATA-2 deficiency in childhood. BMC Pulmonary Medicine 2015; 15:8.

Analýza genů modifikujících průběh primárních imunodeficiencí

- Poruchy tvorby protilátek

T. Freiberger J. Litzman, E. Vondrušková: Úloha kyseliny asparagové na 57. pozici HLA-DQ beta řetězce u sporadické a familiární formy selektivní deficience IgA. Čas. lék. čes., 140, 2001, č. 24, s. 770-773. *TF koresp. autor.*

Thon V, Vlková M, Freiberger T, Litzman J, Lokaj J: The expression of Fc gamma receptors on leukocytes and clinical course of common variable immunodeficiency (CVID). Scripta Medica, 78 (6), 2005, pp. 315-322.

J. Litzman, T. Freiberger, B. Grimbacher, B. Gathmann, U. Salzer, T. Pavlík, J. Vlček, V. Postránecká, Z. Trávníčková, V. Thon: *Mannose-binding lectin* gene polymorphic variants predispose to the development of bronchopulmonary complications but have no influence on other clinical and laboratory symptoms or signs of common variable immunodeficiency. Clin Exp Immunol 2008; 153: 324-325. *JL a TF contributed equally.*

T. Freiberger, L. Grodecká, B. Ravčuková, B. Kuřecová, V. Postránecká, J. Vlček, J. Jarkovský, V. Thon, J. Litzman: Association of FcRn expression with lung abnormalities and IVIG catabolism in patients with common variable immunodeficiency. Clin Immunol 2010; 136(3): 419-425. *TF koresp. autor.*

T. Freiberger, B. Ravčuková, L. Grodecká, Z. Pikulová, D. Stikarovská, S. Pešák, P. Kuklínek, J. Jarkovský, U. Salzer, J. Litzman: Sequence variants of the TNFRSF13B gene in Czech CVID and IgAD patients in the context of other populations. Hum Immunol 2012; 73(11):1147-54. *TF koresp. autor.*

- Poruchy komplementu

T. Freiberger, M. Vyskočilová, L. Kolářová, P. Kuklínek, O. Kryštůfková, M. Lahodná, J. Hanzlíková, J. Litzman: Exon 1 polymorphism of the B2BKR gene does not influence a clinical status of patients with hereditary angioedema. Human Immunology, 63 (6), 2002, pp. 492-494.

T. Freiberger, H. Grombiříková, B. Ravčuková, J. Jarkovský, P. Kuklínek, O. Kryštůfková, J. Hanzlíková, E. Daňková, O. Kopecký, R. Zachová, M. Lahodná, M. Vašáková, L. Grodecká, J. Litzman: No evidence for linkage between the hereditary angioedema clinical phenotype and the *BDKR1*, *BDKR2*, *ACE* or *MBL2* gene. Scand J Immunol 2011; 74(1): 100-106. *TF koresp. autor.*

- Ostatní

A. Janda, A. Šedivá, T. Freiberger, E. Vernerová, J. Bartůňková. Syndrom Churga-Straussové a deficit lektinu vázajícího manózu u dospělé pacientky. Alergie, 6, 2004, č. 4, pp. 49-52.

H. Skalníková, T. Freiberger, J. Chumchalová, H. Grombiříková, A. Šedivá: Cost-effective genotyping of human *MBL2* gene mutations using multiplex PCR. J Immunol Methods, 295 (1-2), 2004, pp. 139-147. *TF koresp. autor.*

A. Janda, J. Bartůňková, R. Špíšek, T. Freiberger: Deficit lektinu vázajícího manózu. Čes.-slov. Pediat., 60, 2005, č. 2, pp 79-80.

T. Freiberger, B. Ravčuková, L. Grodecká, B. Kuřecová, J. Jarkovský, D. Bartoňková, V. Thon, J. Litzman: No association of FCRN promoter VNTR polymorphism with the rate of maternal-fetal IgG transfer. J Reprod Immunol 2010; 85(2): 193-7. *TF koresp. autor.*

E. Potlukova, J. Jiskra, T. Freiberger, Z. Limanova, D. Zivorova, K. Malickova, D. Springer, L. Grodecka, M. Antosova, Z. Telicka, S.S. Pesickova, M. Trendelenburg: The production of mannan-binding lectin is dependent upon thyroid hormones regardless of the genotype: A cohort study of 95 patients with autoimmune thyroid disorders. Clin Immunol 2010; 136: 123-129.

E. Potlukova, T. Freiberger, Z. Limanova, J. Jiskra, Z. Telicka, J. Bartakova, D. Springer, M. Trendelenburg: Association between low levels of mannan-binding lectin and markers of autoimmune thyroid disease in pregnancy. PloS One 2013; 8(12): e81755.

Ostatní práce s imunologickou tematikou

J. Mayer, M. Krahulová, Z. Kořístek, H. Kubešová, T. Freiberger, L. Kozák, Z. Adam et al.: Allogenní transplantace periferních kmenových krvetvorných buněk po nemyeloablativním režimu. Čas lék čes, 138, 1999, č. 20, s. 624-627.

T. Freiberger: Molekulární genetika primárních poruch imunity. Alergie, 6, 2004, č. 4, pp. 23-33. *(Cena ČSAKI za nejlepší přehledný vzdělávací článek za rok 2004 publikovaný v časopise Alergie).*

I. Malkusová, P. Panzner, T. Freiberger, P. Zeman, V. Compel: Běžná variabilní imunodeficience (CVID) u pacienta s celiakií. Alergie 2007; 9(4): 347-350.

O. Ticha, M. Stouracova, M. Kuman, P. Studenik, T. Freiberger, J. Litzman: Monitoring of CD38*high* expression in peripheral blood CD8+ lymphocytes in patients after kidney transplantation as a marker of cytomegalovirus infection. Transplant Immunology 2010; 24(1): 50-56.

L. Grodecká, P. Lockerová, B. Ravčuková, E. Buratti, F.E. Baralle, L. Dušek, T. Freiberger: Exon first nucleotide mutations in splicing: evaluation of *in silico* prediction tools. PloS One 2014; 9(2): e89570.

E. Froňková, A. Klocperk, M. Svatoň, M. Nováková, M. Kotrová, J. Kayserová, T. Kalina, P. Keslová, F. Votava, H. Vinohradská, T. Freiberger, E. Mejstříková, J. Trka, A. Šedivá: The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. PloS One 2014; 9(12): e114514.

Učebnice, monografie, skripta s imunologickou tematikou:

T. Freiberger: Genetika imunopatologických stavů. In: Souček M: Vnitřní lékařství, 1. vyd. Praha, Brno: Grada 2011, 1788 s, ISBN 978-80-247-2110-1. Strany 7-13.

T. Freiberger: Infekce přenášené při transplantacích. In: Souček M: Vnitřní lékařství, 1. vyd. Praha, Brno: Grada 2011, 1788 s, ISBN 978-80-247-2110-1. Strany 48-51.

T. Freiberger: Genetická analýza v diagnostice vrozených poruch imunity. In Jeseňák M., Bánovčin P. a kol., Vrodené poruchy imunity, A-medi management, 2014.

T. Freiberger: Hlavní histokompatibilitní systém člověka. In Litzman a kol., Základy vyšetření v klinické imunologii, MU Brno, 2009, 2015.