UNIVERZITA KARLOVA

2. LÉKAŘSKÁ FAKULTA

Molekulární epidemiologie dětského diabetu 1. typu

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Obsah

1 Co je v této habilitační práci

Stejně jako dosavadní práce našich předchůdců, tak i práce mých kolegů a práce moje směřovala zejména k tomu, aby motolská pediatrická skupina byla v mezinárodním kontextu považována za **standardní dětské diabetologické centrum**. To neznamená jenom, aby byly děti řádně diagnostikovány a léčeny, ale i to, abychom znali parametry výskytu diabetu i jeho komplikací, abychom věděli, jak na tom naši pacienti jsou, a v neposlední řadě i to, abychom si vybrali jedno či dvě témata z dětské diabetologie, kterým se budeme dlouhodoběji věnovat a o nichž budeme publikovat.

V řadě publikací, které jsou součástí této habilitační práce, je naše snaha vidět. Před rokem 2000 nebylo z naší země publikováno o epidemiologických parametrech dětského diabetu zhola nic. Nevědělo se, kolik jej je, nevědělo se, jaké jsou genetické predisponující faktory, jak je to s autoimunitními komplikacemi; neprobíhal žádný systematický výzkum v této oblasti. V tomto smyslu byl úkol od nadřízených jasný a jeho plnění také - první řada publikací mapuje **výskyt a charakteristiku českého dětského diabetu**, aniž si - s výjimkou snad jedné práce - klade za cíl nějaké obecné vědecké blaho. Články se nečtou úplně snadno, protože neobsahují žádná převratná moudra a jsou mnohdy obecně docela nudné, jako ostatně epidemiologické články bývají.

Od roku 2006 se k řešeným tématům přidaly **viry u dětského prediabetu**. Zpočátku to byly zejména metodické aspekty práce, poslední články jsou však poměrně velké vnořené studie případů a kontrol zaměřené na vliv pikornavirů při vzniku a rozvoji prediabetické autoimunity.

Stranou této habilitační práce zůstávají další skupiny publikací - jednak publikace kolegy Dřevínka a jeho skupiny, na nichž jsem se podílel technickou pomocí (ty jsou zejména o *Burkholderia cepacia* u cystické fibrózy), dále publikace o virech u transplantovaných (kde byla moje role vymezena zavedením a udržením většiny z metod) a nakonec publikace o některých monogenních chorobách, kde jsem spoluautorem proto, že jsem techniky laboratorní i techniky psaní učil postgraduální studenty, kteří byli autory těchto prací.

Tato habilitační práce **začíná** celkovým pohledem na diabetes 1. typu optikou deskriptivní, analytické i molekulární epidemiologie - tato část se přitom jen málo zabývá mojí vlastní prací; **v druhé části** jsem pak vybral z článků, jejichž jsem spoluautorem, několik okruhů, které stručně diskutuji.

2 Epidemiologie dětského diabetu 1. typu

Dětský **diabetes 1. typu (DM1)** je prototypem autoimunitní choroby z mnoha různých hledisek. V následujícím textu se budu snažit nastínit, proč tomu tak je a co to může přinášet pro poznání autoimunitních chorob obecně.

I přes mnoho desetiletí výzkumu dodnes zejí v našich znalostech o etiologii a patogenezi DM1 velmi podstatné mezery. V současnosti jsme přitom svědky rychlého vzestupu incidence DM1, který nejenže nedokážeme zastavit nebo zpomalit, ale který nedokážeme ani vysvětlit. To, že obecně uznané vysvětlení příčin vzestupu incidence DM1 není k dispozici, ovšem neznamená, že neexistují alespoň jednotlivé dílky skládanky, která jednoho dne pravděpodobně poskytne ucelený obraz, jenž může překvapit svou jednoduchostí.

Epidemiologie přispívá k nalézání jednotlivých dílků skládanky zcela zásadně. Jako věda o výskytu nemocí a jeho příčinách není pouze popisnou, ale i analytickou a experimentální disciplínou. Diabetes 1. typu má přitom **unikátní rysy**, které umožnily, že se stal prototypem pro studium multifaktoriálních chorob – diagnóza je jednoznačná a terapie je zpravidla centralizovaná; choroba se v polovině případů manifestuje v dětství, kdy mohou být odebrány vzorky genetického materiálu od rodičů; pacienti jsou zpravidla vysoce motivováni ke spolupráci, existují pacientské organizace a diabetické registry.

Epidemiologie DM1 je v současnosti vědou mnoha tváří. Na jedné straně není stále jasné, kolik DM1 vůbec v některých populacích je, počítaje v to například i populaci USA. Na druhé straně diabetologové s epidemiology vedou velmi ambiciózní intervenční studie s cílem nalézt prevenci DM1.

V dalším textu stručně seznámím s výskytem choroby a z epidemiologického hlediska se zmíním o současném stavu výzkumu jeho příčin a prevence.

2.1 Diagnóza diabetu 1. typu

Diabetes mellitus 1. typu se v posledních letech diagnostikuje u 250 až 300 českých dětí za rok. Znamená to pro ně především zvýšení mortality [1] a nepříjemnou a obtěžující léčbu. Ačkoli z hlediska výše mortality jsme v té šťastnější části světa i Evropy [2], zůstává mortalita zvýšena zejména u těch, u koho se DM1 manifestoval časně, tedy v dětství – je tomu tak patrně pro delší trvání expozice hyperglykémii. V dětství se přitom podle hrubých odhadů manifestuje asi polovina případů DM1.

Diabetes se nyní **diagnostikuje a klasifikuje** podle kritérií Světové zdravotnické organizace [3] nebo Americké asociace diabetu (American Diabetes Association) [4]. Klasifikace diabetu vymezuje klinické třídy onemocnění a diabetes mellitus 1. typu je jednou z nich[1]. Je charakterizován jako diabetes, který vzniká následkem destrukce beta buněk Langerhansových ostrůvků pankreatu buďto autoimunitním zánětem, nebo idiopatickým procesem. Protože idiopatický diabetes 1. typu se v europoidních populacích skoro nevyskytuje, diabetes 1. typu v naší populaci lze považovat za výhradně autoimunitní.

Diabetes u dětí má při diagnóze zpravidla silně rozvinuté **příznaky**: ve velké multicentrické studii u evropských dětí při diagnóze diabetu měly v průměru čtyři z deseti dětí diabetickou ketoacidózu, osm až devět z deseti ketonurii, drtivá většina má anamnézu polyurie a okolo poloviny i anamnézu únavy a ztráty hmotnosti [5]. Podíl dětí s ketoacidózou se neliší mezi věkovými skupinami, ale liší se významně mezi populacemi: inverzně koreluje s incidencí diabetu. Tato inverzní korelace může souviset s lepší organizací zdravotní péče v zemích, kde je diabetu více – tyto země zpravidla patří mezi ty bohatší; stejným směrem ukazují i výsledky srovnání závažnosti stavu diabetických dětí při diagnóze ve Švédsku proti Litvě [6]. Další

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[1] Neméně zajímavým typem je také skupina Maturity onset diabetes of the young (MODY). Jsem spoluautorem několika prací o MODY, ale tato habilitační práce se jimi nezabývá mimo stručného shrnutí na konci.

možností je, že obecné povědomí o diabetu je vyšší u obyvatel zemí s vyšším výskytem dětského diabetu a děti přicházejí k lékaři časněji. Ať už jsou příznaky rozvinuté více nebo méně, DM1 je choroba, která vede buďto k léčbě inzulínem nebo – naštěstí výjimečně - k úmrtí dítěte na ketoacidózu. Z hlediska epidemiologického je proto DM1 jednou z nejlépe zachytitelných chorob, jedinec ji buďto má – pak se o ní ví – nebo nemá.

2.2 Incidence a prevalence diabetu 1.typu u dětí

Deskriptivní epidemiologie popisuje pozorování výskytu DM1: kolik je nových případů dětského diabetu, jak jsou rozloženy v čase, co je to za děti, jak to vypadá s trendy výskytu diabetu. Ukážu stručně, jak je tomu u nás, a použiji k tomu data, které sesbíral a analyzoval Český registr dětského diabetu dvacet let své činnosti, které se odrážejí v několika publikacích [7-9]. [2]

2.2.1 Incidence diabetu

Parametrem, který se používá pro popis výskytu DM1 v dětství, je hlavně incidence. V dalším textu budeme za děti považovat jedince ve věku 0-14 ukončených let věku, čili ty, kdo ještě neoslavili patnácté narozeniny. **Tabulka 1** obsahuje pro minulá léta počty nově diagnostikovaných pacientů, počet dětí 0-14 ukončených let v populaci a incidenci s pětadevadesátiprocentním intervalem spolehlivosti. **Obrázek 1** incidenci vykresluje – tentokrát se jedná o tři kategorie věku při manifestaci, kde je velmi dobře zachycen nedávný zlom ve vzestupném trendu incidence. V nejmladší věkové kategorii nejenže incidence nestoupá, ale dokonce klesá.

Co o výskytu DM1 u nás **víme**?

1. Incidence diabetu se za minulých patnáct let vyšplhala z 6,8 / 100 000 / rok 1989 až na 18,5 / 100 000 / rok 2009. Dětská populace se podstatně ztenčuje, absolutní počet případů ale přesto stoupá. U nás je průměrný meziroční nárůst incidence o 0,85 případu / 100 000 / rok[2] (tato dvojka je mocnitel, nikoli odkaz), jak už jsem se zmínil hned za prvním obrázkem: incidence tedy vzroste o 0,85 za každý rok[3].

2. Poměrně vysoké absolutní počty nově diagnostikovaných pacientů dovolují odhadnout incidenci se solidní přesností, intervaly spolehlivosti jsou úzké.

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[2] Výskyt DM1 kvantifikujeme, jako většinu onemocnění, pomocí dvou ukazatelů: incidence a prevalence. **Prevalence** ukazuje výskyt *všech* onemocnění DM1 v populaci, aniž by rozlišovala, zda se jedná o nově diagnostikovaná onemocnění nebo o onemocnění již trvající. U diabetu jako choroby celoživotní, ale v dětském věku pacienta neusmrcující, se stírá rozdíl mezi okamžikovou a intervalovou prevalencí – prevalenci DM1 získáme podělením počtu dětských pacientů středním stavem dětské populace. U nás bylo například k poslednímu prosinci 2003 1576 dětských pacientů s DM1, dětská populace měla 1 554 475 členů, čili prevalence DM1 byla 1,01 / 1000 dětí. **Incidence** popisuje, kolik *nových* onemocnění vzniklo v dané populaci v daném časovém období. Vypočítá se jako podíl nových případů v dané populaci a daném časovém intervalu proti součtu osobočasů v riziku v dané populaci a daném časovém intervalu. U vzácnějších onemocnění sledovaných ve velké populaci se jmenovatel zlomku prostě nahradí středním stavem populace násobeným délkou času sledování. Např. v roce 1994 bylo u nás nově diagnostikováno 210 dětí s DM1, přičemž velikost dětské populace byla ten rok 1 948 024 jedinců, incidence tedy činila 10,8 / 100 000 / rok.

[3] Je ve střední a východní Evropě něco specifického, co ji činí výjimečnou z hlediska výskytu diabetu 1. typu? Dlouho se tradovalo, že právě extrémně rychlý nárůst incidence diabetu je specifický pro Východ a mělo se za to, že to má souvislost se změnami po pádu komunismu. Možná to jsou opravdu chybějící červi v perníku a shnilé maso, které dnes na rozdíl od dob minulých nezatěžují imunitní systém (jak níže uvádí v jednom editorialu z doby, kdy ještě nebyl mým šéfem, můj současný šéf prof. Lebl) - a tento imunitní systém se pak obrací proti autoantigenům. Spíše ale na východních populacích nic zvláštního není.

> Před rokem 1990 maminky ředily kojenecká mléka vodou z komunálního vodovodu (měla být převařená) a jen výjimečně minerálkou z vratné skleněné lahve. Dnešní nadměrná nabídka balených sterilních vod mnoho mikrobiálních antigenů nepřináší … V dalších měsících a letech života pily naše děti vodovodní vodu ze sifonové lahve s naředěným sirupem, jedly mléčné výrobky se zárodky bakterií a plísní, balený perník se mnohdy hýbal červy. O další antigeny jsme je připravili dokonalým sortimentem sterilních džusů, limonád, mléka, mléčných výrobků, pečiva. V kuchyních mateřských školek i škol se dnes používají jednorázové rukavice a jsou nainstalovány bezdotykové baterie; před inspektory EU neobstojí praktiky, které na osudu nespotřebované vepřové kýty popsal Vladimír Páral v jednom ze svých románů ze 70. let minulého století: „ … a čtrnáctý den se umele na zavářku do polévky, která se sní vždycky.“ Naše dnešní děti mnoho mikrobiálních antigenů ani nesnědí, ani nevypijí, a většina z nich se s nimi díky drogistické nabídce čisticích prostředků nesetká ani jinde v domácím prostředí.

3. Byl vidět signifikatní nárůst incidence. Tento nárůst byl v absolutních číslech stálý, vzestup byl v průměru 0,85 případu / 100 000 / rok[2].

4. V posledních pěti letech ovšem incidence stagnuje, přičemž v nejmladších věkové kategori dokonce klesá.

Obrázek 1. Incidence diabetu 1. typu v letech 1989 až 2009 v české dětské populaci podle kategorie věku při manifestaci.
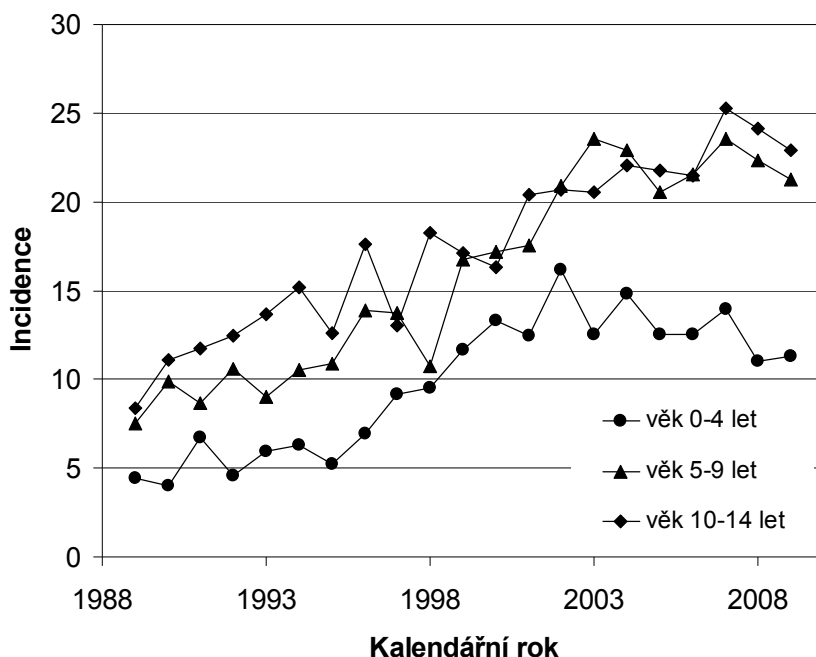
Jak je to s rozložením **incidence DM1 podle věku při manifestaci**? **Obrázek 2** zachycuje rozložení za prvních patnáct let sledování. Vidíme, že incidence stoupá s věkem do staršího předškolního věku. Poté jsou vidět dva vrcholy odpovídající pubertě dívek a chlapců, kde je zvýšená potřeba inzulínu a tedy i zvýšená incidence DM1. Pubertální vrchol incidence přichází u dívek asi o jeden až dva roky dříve než u chlapců. Rádi bychom věděli, co se děje po patnáctém roce věku, ale v České republice neexistuje žádný registr, který by nám to řekl – dlužno dodat, že ustavení a provoz takového registru by byl úkol nepoměrně obtížnější než vedení registru dětského DM1. Nejbližší data jsou k dispozici z Rakouska – zmínka o něm je na jiném místě.

Vlevo na obrázku 2 je vidět predominanci chlapců ve věkové skupině 0-4 let při manifestaci. Tato predominance nemá žádné vysvětlení; ačkoli je vzácná, může nás uklidinit, že nejsme jediná populace, ve které se vyskytuje. Obvykle bývá predominance mužů až u DM1 manifestujícího se v dospělosti (a už tím je DM1 výjimečný mezi autoimunitními onemocněními). Ve skupině 5-9 let je naopak signifikantní - avšak méně nápadná - převaha dívek; v nejvyšší věkové kategorii i v celém souboru je poměr pohlaví vyrovnaný.

Tabulka 1. Incidence diabetu 1. typu v české dětské populaci, 1989 - 2009

Rok	Nové případy DM1 za rok	Dětská populace k 31.12. (osoboroky pozorování)	Věkově standardizovaná incidence / 100 000 / rok	95% CI
1989	157	2 252 709	6,8	5,7 - 7,9
1990	189	2 193 682	8,3	7,2 - 9,6
1991	196	2 120 802	9,0	7,8 - 10,4
1992	195	2 064 545	9,2	7,9 - 10,6
1993	196	2 009 752	9,5	8,2 - 10,9
1994	212	1 948 024	10,7	9,3 - 12,2
1995	186	1 893 259	9,6	8,3 - 11,0
1996	244	1 842 679	12,8	11,2 - 14,5
1997	219	1 795 032	12,0	10,4 - 13,6
1998	232	1 751 471	12,9	11,2 - 14,6
1999	265	1 707 205	15,2	13,4 - 17,1
2000	263	1 664 434	15,6	13,8 - 17,6
2001	282	1 631 771	16,8	14,9 - 18,9
2002	310	1 589 766	19,3	17,2 - 21,5
2003	297	1 554 475	18,9	16,8 - 21,1
2004	307	1 526 946	19,9	17,8 - 22,2
2005	277	1 501 331	18,3	16,2 - 20,5
2006	274	1 479 514	18,5	16,4 - 20,8
2007	307	1 476 923	20,9	18,7 - 23,3
2008	278	1 480 007	19,2	17,0 - 21,5
2009	269	1 494 370	18,5	16,4 - 20,7
celkem	5155*	36 978 697*	15,6	15,1 - 16,0

*) celkový počet nových diabetických pacientů a celkový počet osoboroků sledování

Sezonalita výskytu DM1 je zřejmá a signifikantní u pacientů manifestovaných ve věku 10-14 let. Pacienti manifestovaní v nižším věku sezonalitu nevykazují. Význam sezonality manifestace je velmi omezený, protože o diabetu se rozhoduje fakticky měsíce až léta před manifestací. Mimo to je studium sezonality manifestace nudné, svým charakterem a metodami patří spíše do předminulého než minulého století a nebudu se jí proto dále zabývat.

2.2.2 Prevalence diabetu 1. typu

V populaci s rychle se měnící incidencí, jako je ta naše, neodráží prevalence dynamiku změn výskytu DM1. Do roku 2005 jsme navíc měli k dispozici jen velmi nepřesné odhady prevalence dětského diabetu – od dovršení patnácti let Registru ale již víme poměrně dobře, kolik dětí s DM1 u nás je. Zjednodušeně řečeno, prevalence se pohybuje okolo 1 / 1000 dětí (z věkové skupiny 0-14 dokončených let). Je dobře známá zanedbatelná prevalence u nejmenších dětí a postupně vzrůstající prevalence od staršího předškolního věku. Prevalence je ovlivněna incidencí, úmrtností a strukturou populace. Zatímco úmrtnost dětských diabetických pacientů je u nás naštěstí zanedbatelná, dva zbývající parametry se rychle mění. Co se děje s prevalencí, budu diskutovat v odstavci Projekce výskytu diabetu.

Obrázek 2. Věkově specifická incidence pro chlapce a pro dívky, Česká republika, 1989-2003. Věkově specifická incidence v naší populaci sleduje téměř "učebnicové" rozložení: stoupá do staršího předškolního věku, poté jsou vidět vrcholy odpovídající pubertě u dívek a u chlapců. Pak incidence klesá.
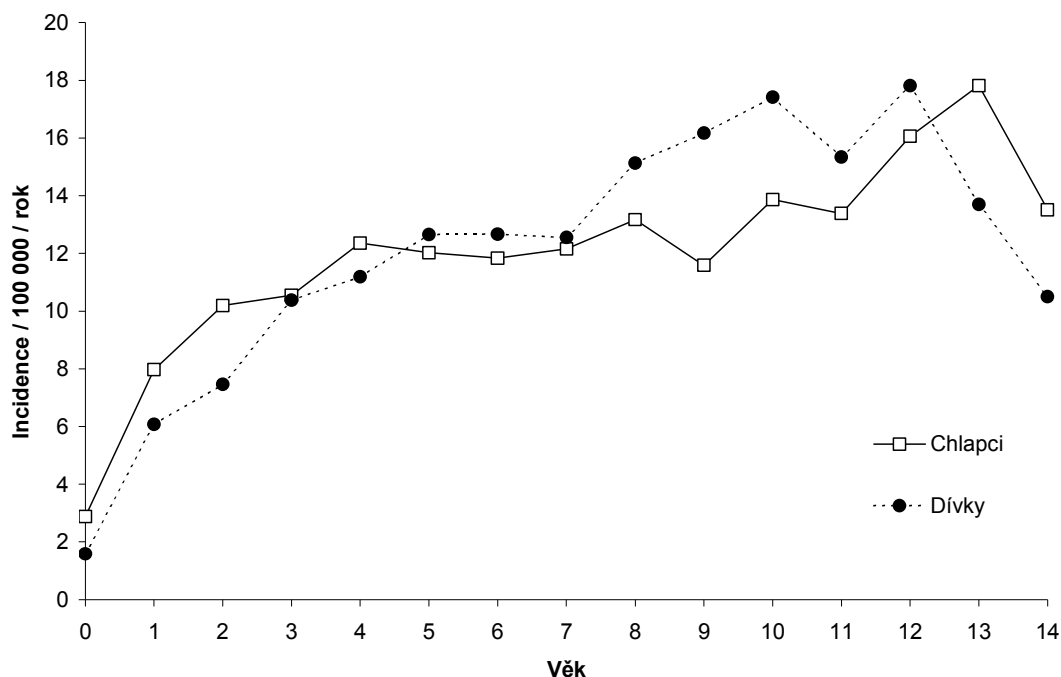
2.2.3 Diabetes u mladých dospělých

Zatímco diabetu u dětí do patnácti let je věnována velká pozornost, jsou k dispozici registry s kompletností záchytu zpravidla přes 95% a každý vzestup incidence je obsáhle komentován, o diabetu po patnáctých narozeninách se toho ví podstatně méně. Je pro to několik důvodů: u pacientů po patnáctých narozeninách může být nástup diabetu méně akutní. Mimo DM1 je u mladých dospělých nalézán i diabetes 2. typu nebo gestační diabetes. A hlavně – zatímco DM1 u dětí je léčen dětskými diabetology, u mladých dospělých jej může léčit internista, praktik, gynekolog i další lékaři. Ustavení a provoz diabetického registru pro dospělé je proto podstatně složitější úkol a jen málo zemí jej úspěšně zvládlo. Česká republika registr dospělých diabetických pacientů nemá a za současné legislativy ani patrně nikdy mít nebude. Nejbližší populací, ze které jsou k dispozici data o DM1 u mladých dospělých, je populace rakouská [10]. V Horních Rakousích je incidence u dospělých mezi 15 a 30 lety věku srovnatelná s incidencí u dětí do 15 let věku. Autoři potvrdili též známou vyšší incidenci u mladých mužů ve srovnání s mladými ženami. Nejvyšší incidence okolo 10 / 100 000 / rok byla vidět mezi pátým a dvacátým rokem.

Je pravděpodobné, že **ze všech případů DM1 se u dětí manifestuje asi polovina**, zatímco ta druhá polovina se manifestuje u dospělých. Diabetes u mladých dospělých je patrně trochu jiný než u dětí: dospělí mají nejen pomalejší průběh inzulitídy a méně akutní začátek, ale i menší frekvenci genetických rizikových faktorů a vyšší frekvenci faktorů, které jsou u dětí protektivní. Jasný fenotyp dětského DM1 se s postupujícím vyšším věkem manifestace více a více zamlžuje až k diabetu, kterému se z nedostatku lepšího názvu říká LADA (latent autoimmune

diabetes of adults, latentní autoimunitní diabetes dospělých). Tento diabetes - dle mého názoru žádná samostatná nosologická jednotka - pravděpodobně není nic jiného než **pomalu progredující autoimunitní diabetes 1. typu**, který se dá splést na chvíli s diabetem 2. typu – lze tak alespoň usuzovat z nemožnosti definovat nějaký jasný rys, který by diabetes LADA oddělil jako samostatnou jednotku nejen od diabetu 2. typu, ale též od diabetu 1. typu. Spolus s autorem excelentního review na toto téma [11] si představme stejně rychle progredující autoimunitní inzulitídu u dvou dospělých: jednoho obézního lenocha, druhého štíhlého sportovce. Obézní lenoch má vyšší inzulínovou rezistenci, deficit sekrece inzulínu se u něj projeví dříve a nikoli dramaticky: pacient patrně po řádném vyšetření spadne do škatulky LADA. Štíhlý sportovec má inzulínovou rezistenci nízkou, jeho sekrece inzulínu mu stačí až do fáze opravdu pokročilé destrukce beta buněk, kdy se ovšem jeho diabetes projeví jako klasický diabetes 1. typu. Oba přitom mají stejně rychle progredující ztrátu beta buněk.

Rozdíly v incidenci diabetu mezi různými národy jsou u mladých dospělých menší než u dětí, jak ukázala třeba mezinárodní studie v devíti zemích Evropy [12][4]. Dosud nejméně deset různých studií poukázalo na to, že na rozdíl od mnoha jiných autoimunitních chorob, DM1 v dospělosti vykazuje predominanci mužského pohlaví; velikost této predominance se pohybuje od 1,3 do 2,15 [13]. Není jasné, proč tomu tak je: nejsnadnější vysvětlení, které se nabízí, je chybná klasifikace DM1 do škatulky gestačního diabetu – tím by se predominance mužů vytvořila snadno. Není však pravděpodobné, že by špatná klasifikace DM1 dosáhla takových rozměrů. Spíše se dá očekávat, že vidíme důsledek hormonálních rozdílů u mladých dospělých, včetně těch rozdílů, které mají za následek změny v rozložení tělesného tuku a v inzulínové rezistenci.

2.3 Vzestup incidence diabetu

2.3.1 Současná data

V současnosti jsme svědky **vzestupu incidence diabetu nejen v naší populaci, ale i celosvětově**. Víme, že celosvětově incidence dětského diabetu meziročně stoupá o bezmála tři procenta [14], s výjimkou střední Ameriky a karibského regionu. O Evropě jsou díky studii EURODIAB[5] známa přesnější a spolehlivější data než o zbytku světa. Poslední zpráva iniciativy EURODIAB za roky 1989-2003 [15] uvádí, že průměrný meziroční vzestup incidence byl 3,9%, přičemž pokud by současné trendy trvaly, bude v roce 2020 dvakrát více nových dětí s diabetem do 5 let věku a prevalence do 15 let věku vzroste o 70%.

Tradičně zajímavý je také pohled na rychlost vzestupu incidence vynesenou proti průměrné incidenci v dané populaci (obr. 3). Zde je velmi jasně vidět, že "východní" země začínaly s nižší incidencí, ale snaží se zbytek Evropy "dohnat" vyšším relativním vzestupem[6].

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[4] To má přímý praktický význam: je třeba opatrnosti při extrapolaci rozdílů ve výskytu DM1 do dospělé populace. To, že jedna populace má pětkrát vyšší incidenci *dětského* DM1, neznamená, že současně bude mít i pětkrát vyšší incidenci DM1 *u dospělých.*

[5] Náš **Český registr dětského diabetu** se k EURODIABU připojil po krátkém čase činnosti (data z Česka nebyla do první publikace EURODIABU o incidenci diabetu v Evropě ještě zahrnuta). V současnosti se český registr dělí s registrem Bádenska – Württemberska o pozici registru s nejvíce pacienty, nicméně je zcela jasné, že o tuto pozici přijde v souvislosti s podceněnou důležitostí systémů kontroly kvality péče v naší zemi.

[6] Je logické, že právě tyto populace, které začínaly na nižších počátečních číslech incidence, jsou schopny vyprodukovat zcela hrozivá relativní čísla nárůstu. Meziroční nárůst se totiž obvykle nevyjadřuje v absolutních jednotkách (případy / 100 000 / rok^2), ale v procentech proti minulému roku. Čím nižší je počáteční incidence, tím vyšší je pak tento procentní nárůst – a naopak. Tím je skutečný vývoj značně zkreslen. Ve východní Evropě se tedy neděje nic strašlivého, jen tam dříve DM1 u dětí bylo málo a nyní jej stále přibývá. Proč bylo v této části světa diabetu zpočátku málo, může spekulativně objasnit třeba jedna z dostupných hypotéz spojených s výskytem některých HLA genotypů, o níž bude řeč dále.

Obrázek 3. Průměrný meziroční vzestup incidence versus průměrná incidence dětského diabetu v participujících evropských registrech. Povšimněte si logaritmického měřítka na vodorovné ose. Uvedeno se souhlasem prvního autora [15].
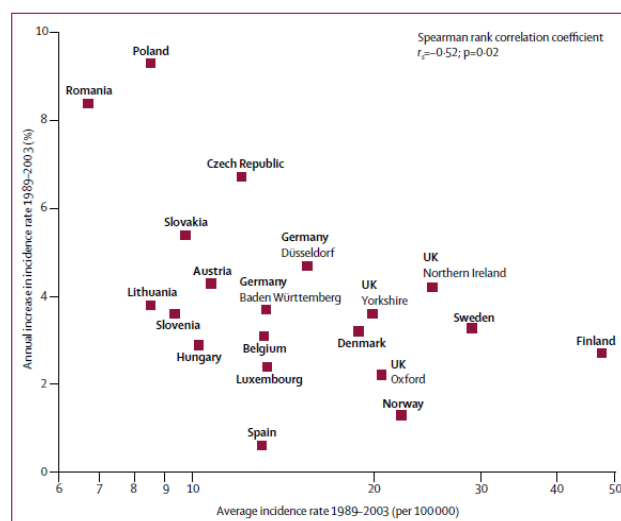
2.3.2 Historická data

Pokud promítneme současné tempo růstu incidence diabetu do minulosti, vidíme, že před čtyřiceti padesáti lety by DM1 neexistoval vůbec nebo by se vyskytoval jen velmi zřídka. To však není pravda. **Incidence diabetu nemohla tímto tempem růst dlouho**, křivka se musela obrátit směrem vzhůru relativně nedávno. K tomu, abychom mohli říci kdy, nám chybí údaje o incidenci v minulosti. Naštěstí skandinávské veřejné zdravotnictví zanechalo diabetologii dědictví v podobě několika publikací o incidenci dětského diabetu (tedy diabetu 1. typu) z doby před půl stoletím – a výsledky těchto studií se dají bez větší škody považovat za validní minimálně pro Evropany a populace evropského původu. Znalost trendů incidence má zásadní důležitost: pokud je zvyšování incidence rychlé a relativně nedávné, musí být způsobeno změnami prostředí, což dává jistou nevelkou naději na efektivní prevenci, až příslušné faktory budou identifikovány a odstraněny.

Edwin Gale v jedné ze svých publikací velmi působivě ukazuje, jak stařičká data z dávno zapomenutých článků a padesát let archivovaných dizertací mohou pomoci objasnit, jak to se vzestupem incidence diabetu je [16]. V dalších odstavcích shrnuji klíčové body tohoto nesmírně zajímavého a čtivého článku.

Zdá se, že dětský diabetes vykazoval **nízkou a stabilní incidenci v první polovině dvacátého století**, zatímco v jeho druhé polovině incidence rostla, aby se nyní růst v některých zemích zastavil. Dobře provedená retrospektivní studie incidence diabetu v Oslo mezi lety 1925 – 1954 ukazuje, že dětský diabetes tehdy nevykazoval žádný vzrůst incidence a průměrná incidence byla 4,1 / 100 000 / rok. Ze severního Švédska, Vasterbötten, je k dispozici studie, která pro roky 1938-1942 ukazuje incidenci 10,2 / 100 000 / rok, zdaleka nejvíce v té době – není divu, že i později se oblast řadila k těm s nejvyšší incidencí – ale pořád aspoň třikrát méně než dnes. Z Finska jsou spolehlivá data k dispozici až po válce, z roku 1953 z Helsink, 12,5 / 100 000 / rok - to je asi čtvrtina současné incidence.

Zejména norské zdroje naznačují, že vzestup incidence začal v padesátých letech či později. Studie z let 1925 – 1954 byla prodloužena do roku 1964 a náhle incidence z původních 4,1 / 100 000 / rok vzrostla pro roky 1955 – 1964 na 8,4 / 100 000 / rok. Od té doby až do devadesátých let incidence pořád rostla – a pak se růst zastavil. Křivka inicidence v čase tedy má tvar jakéhosi S. Oploštění křivky incidence a žádný její další růst hlásily v jedné fázi

sledování i Norsko, Švédsko a Dánsko, nikoli však Finsko. Je možné, že incidence odráží v takovém případě dosažení nové dočasné rovnováhy v těchto populacích.

Je zajímavé se zamyslet, **odkud pochází** vzestup incidence dětského diabetu – je to opravdový vzestup incidence v dětství, s kterým současně neklesá incidence v dospělosti? Není to spíše přesunutí manifestace diabetu do časnějšího období? Data z některých populací ukazují, že by blíže pravdě mohla být druhá alternativa: predisponovaní jedinci, kteří mají diabetes dostat, jej dostanou prostě dříve, věk manifestace se sníží a vidíme více nových dětských pacientů – zákonitě se pak incidence u mladých dospělých nezvyšuje [17] nebo se dokonce paralelně snižuje, jak bylo vidět ve dvou populacích: ve švédské [18] a v belgické [19].

2.4 Rozdíly ve výskytu diabetu

2.4.1 Mezipopulační rozdíly

Rozdíly v incidenci diabetu mezi populacemi jsou **zcela mimořádné**. Příklady incidencí z populací Evropy i ostatních kontinentů tyto propastné rozdíly dokumentují. Zpráva projektu Diabetes Mondiale (DiaMond) [14] uvádí jako země s nejnižším výskytem diabetu u dětí do patnácti let Venezuelu, Peru, Pákistán, Čínu, Dominikánskou Republiku a Paraguay. Naopak nejvyšší výskyt byl zaznamenán ve Finsku, na Sardinii, ve Švédsku, Kuvajtu, Norsku, Kanadě a Velké Británii. Jakkoli lze zpochybnit nízké incidence z Venezuely a Paraguaye (kde není záchyt verifikován nezávislým druhým zdrojem), z Peru (kde je studie omezena jen na hlavní město), nezpochybnitelné jsou výsledky z Číny. Data z mnoha provincií ukazují, že incidence dětského diabetu se zde pohybuje i pod 1 / 100 000 / rok. Čína je však také místem velkých – skoro padesátinásobných - rozdílů v incidenci: zatímco v provincii Zunyi činí 0,1 / 100 000 / rok, ve Wuhan je to 4,5 / 100 000 / rok. Ustavení incidenčních registrů v Číně na začátku devadesátých let nyní umožňuje nahlédnout, jak hluboké jsou rozdíly v incidenci diabetu mezi světovými populacemi – rozdíl mezi finským a čínským dítětem v pravděpodobnosti, že dostane diabetes, je až čtyřistanásobný.

2.4.2 Vysvětlení mezipopulačních rozdílů – ekologické studie[7]

Vysvětlení pro rozdíly v incidenci diabetu je několik. Můžeme snadno vyloučit metodické chyby: primární zdroje záchytu jsou v dobře vedených registrech kontrolovány nezávislými zdroji sekundárními a kompletnost kalkulována pomocí capture-recapture metody. Rozdíly v incidenci pak mohou být způsobeny buďto genetickými rozdíly mezi populacemi, nebo rozdíly v expozici faktorům vnějšího prostředí, nebo nejpravděpodobněji obojím. Diabetes 1. typu je polygenní multifaktoriální choroba, čili choroba velmi špatně uchopitelná z hlediska definice podílu jednotlivých faktorů. Je známo, že genetická složka přináší asi polovinu rizika a negenetická složka druhou polovinu. Z genetické složky rizika je asi polovina determinována polymorfismy v genech hlavního histokompatibilitního systému (MHC, též HLA) [20], kdežto zbytek je rozložen

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[7] Ekologická studie se zaměřuje na porovnávání skupin místo porovnávání jedinců. Důvodem pro provedení ekologické studie bývá zejména chybění individuálních dat pro jednu nebo více sledovaných proměnných.

Ekologické proměnné jsou vlastnosti skupin lidí (včetně populací), organizací, nebo míst. Mohou jimi být průměry nebo proporce pozorování nějakých jedinců (podíl kuřáků v populaci, podíl lidí s hypertenzí mezi zaměstnanci podniku, průměrný výdělek rodiny v zemi, výskyt diabetu v okrese), jejich prostředí (průměrná doba slunečního svitu za rok v dané zemi, průměrné znečištění ovzduší pevnými částicemi v daném městě, kyselost pitné vody ve městě), nebo globální proměnné, které ani nemají analogii na individuální úrovni (typ zdravotnického systému, hustota obyvatel).

Interpretace výsledků ekologických studií je problematická. Je pochopitelné, že vyvozovat z výsledků ekologické studie závěry o vlivu nějaké expozice na individuální riziko nemoci je velmi ošidné a nesprávné. Proč jsou tedy ekologické studie tak populární? Protože jsou levné a snadné, protože některé proměnné se špatně měří na úrovni jedince, ale lze je změřit na úrovni populace a protože se výsledky snadno analyzují. Jsou velmi užitečným nástrojem epidemiologie - s vědomím, že závěry, které učiníme o skupinách jedinců, je dobře také interpretovat jako údaje o skupinách a ne o jedincích samotných. Ekologické studie se dobře hodí ke generování hypotéz – naopak se ale zcela nehodí k jakýmkoli závěrům o kauzalitě a nelze pomocí nich ani kvantifikovat nalezenou asociaci expozice a nemoci.

mezi mnoho non-MHC genů, které z jisté části stále zůstávají nepoznány, i přes recentní velký pokrok přinesený celogenomovými asociačními studiemi.

Negenetické riziko je prozkoumáno podstatně hůře: je sice mnoho kandidátních faktorů, ale žádný z nich není silný a ani zdaleka se svým efektem neblíží faktorům genetickým.

V situaci, kdy dobře nevíme, čím je diabetes způsoben, je značně obtížné pokoušet se vysvětlovat, **čím jsou způsobeny rozdíly v jeho incidenci**. Těchto velikých rozdílů na poměrně malé ploše Evropy se ovšem dá využít ke studiu etiologie DM1: lze srovnávat geografické rozdíly v incidenci diabetu proti rozdílům různých charakteristik příslušných zemí. Studie, kde populace je jednotkou, se nazývají ekologické. Jakkoli jsou ekologické studie poměrně hrubým nástrojem pro detekci etiologických faktorů nemocí, v některých situacích mohou být velmi dobrým vodítkem nebo dokonce jediným schůdným řešením. Bylo publikováno několik zajímavých ekologických studií, které se snaží o přiblížení k etiologickým faktorům diabetu – o dvou z nich se sluší zmínit podrobněji.

Projekt EURODIAB se pokusil o korelaci incidence s negenetickými charakteristikami populací: autoři provedli **ekologické korelační a regresní analýzy mezi incidencí a ukazateli geografickými, zdravotními a socio-ekonomickými** [21]. Analýzou 34 populací byla nalezena pozitivní signifikantní korelace incidence DM1 s hrubým národním produktem, se spotřebou mléka a kávy, s očekávanou délkou života a slabě i se zeměpisnou šířkou. Negativně korelovala incidence s kojeneckou úmrtností. Pro některé z těchto indikátorů je možné konstruovat více či méně pravděpodobná vysvětlení – je ale nutné zdůraznit, že toto bezesporu nejsou přímé etiologické činitele v patogenezi diabetu. Z ekologické studie navíc nelze zjistit, zda se nejedná o falešnou asociaci (například na severu jsou pilnější občané a méně korupce než na jihu, proto je na severu vyšší HDP; a jen tak mimochodem na severu je také více nějakého jiného – s pílí či korupcí zcela nesouvisejícího – činitele způsobujícího diabetes). Nalezené korelace tak rozhodně nelze pokládat za průkaz kauzality a zůstávají jen zajímavými směrovkami ukazujícími do oblastí slibujících nález skutečné asociace. Stejně zajímavé jako pozitivní nálezy je i to, co asociované s incidencí diabetu není: ačkoli autoři odhalili slabou korelaci se zeměpisnou šířkou, incidence nekoreluje s klimatologickými proměnnými jako průměrné roční srážky, doba slunečního svitu, průměrné teploty vzduchu. To ukazuje, že za severojižní gradient incidence je zodpovědné něco jiného než klima – autoři poukazují na možný vliv genetických rozdílů.

Genetické faktory studovala Ronningen a spolupracovníci [22]. Autoři vzali incidenční data studie EURODIAB a data o rozložení genotypů HLA-DQB1 a –DQA1 v obecné populaci z 11. HLA workshopu. Pokusili se nalézt korelaci mezi frekvencí výskytu různých genotypů v nediabetické obecné populaci a mezi incidencí diabetu. Srovnáním 25 populací dospěli k tomu, že výskyt DM1 u dětí koreluje s výskytem rizikových genotypů HLA-DQ2/DQ8 (HLA-DQB1*02-DQA1*05 / DQB1*0302-DQA1*03) a DQ4/DQ8 (DQB1*0402-DQA1*04 / DQB1*0302-DQA1*03). Naopak nenalezli žádnou podobnou korelaci pro výskyt genotypů, které jsou známy jako ochranné (genotypů nesoucích alelu DQB1*0602). Z dat, která byla běžně dostupná z veřejných zdrojů, tak autoři byli schopni jednoduchým způsobem poukázat na jednu z možných příčin severojižního gradientu incidence diabetu v Evropě: je to vysoký výskyt dvou rizikových HLA genotypů v severských populacích a naopak jejich nízký výskyt v populacích jižních, zvláště balkánských (**obrázek 4**) Co naopak potvrdit nemohli, byly předchozí zprávy z menších studií o možném vztahu populační frekvence alel s bez aspartátu na pozici 57 řetězce DQB1 k incidenci diabetu [23, 24] – to je v souladu s převažujícími názory o překonanosti teorií připisujících jednotlivým aminokyselinám řetězců HLA-DQB1 a DQA1 samostatný význam v definici rizika DM1.

Obrázek 4 Incidence diabetu v evropských populacích koreluje s výskytem dvou genotypů: HLA-DQ2/DQ8 a DQ4/DQ8 v populaci. Standardizovaná incidence je uvedena na 100 000 / rok. Příklady zemí s nejvyšší, střední a nejnižší incidencí odlišeny barevně. Překresleno s laskavým svolením autorky [22].

DQ2/DQ8

DQ2/8 genotype frequency in the general population
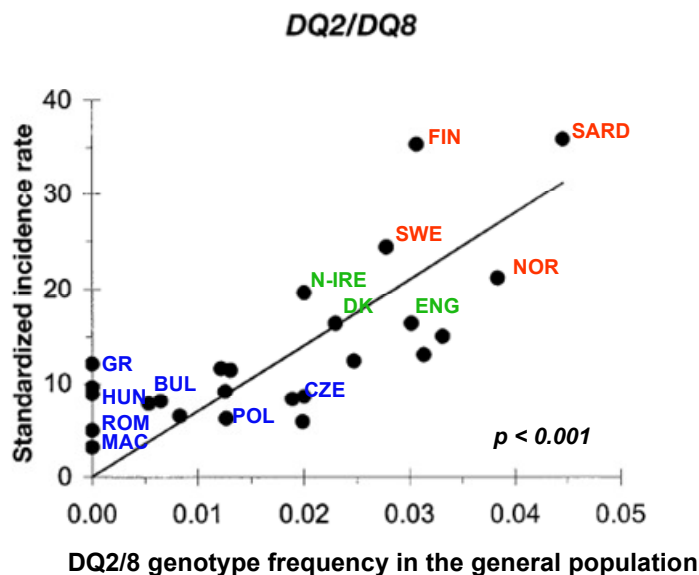
2.4.3 Vysvětlení mezipopulačních rozdílů – srovnávání populací

Mimo ekologických studií na mnoha různých populacích je další možností, jak poznávat příčiny rozdílů v incidenci diabetu, srovnávat geneticky příbuzné populace, které se liší v incidenci diabetu 1. typu. U nás ve střední Evropě, kde genetické rozdíly mezi populacemi jsou často těžko k postřehnutí a kde se incidence diabetu nikde silně neliší, nejsou takové studie efektivní. Naopak sever Evropy je ke srovnávání jak stvořený.

Populace **islandská a norská** jsou si geneticky blízké, ale na Islandu je incidence dětského diabetu méně než poloviční ve srovnání s Norskem. Islandská populace pochází z Norska: Island byl nejprve pustý, pak tam v osmém století pobývalo několik irských mnichů, v polovině devátého století proběhly průzkumné cesty připisované Noru Naddoddurovi, Švédu Gardaru Svavarssonovi a norskému Vikingovi jménem Flóki Vilgedarson – a konečně roku 870 nevlastní bratři Ingólfur Arnarson a Hjörleifur Hródmarsson emigrovali z Norska na Island a usadili se tam i s rodinami, otroky a družinou. Důvodem emigrace byl konflikt s norským králem, který je potrestal za jejich násilné činy propadnutím statků.

Islandská a norská populace jsou si dodnes geneticky podobné: i frekvence haplotypů HLA 2. třídy a charakteristiky genetické asociace DM1 se podobají a rozdíl v incidenci DM1 může být jen obtížně připisován malým nalezeným genetickým rozdílům [25]. V čem si populace až tak podobné nejsou, jsou expozice vlivům prostředí – od nepříliš obvyklého zvyku, kdy se u příležitosti obzvláště významných svátků pojídají celé uzené skopové hlavy (i s očima) či beraní varlata naložená v syrovátce, přes odlišné klima, až po fakt, že většina pitné vody vykazuje podstatně odlišné parametry než voda v Norsku - první a nejzjevnější odlišností budiž, že islandská voda obecně pekelně páchne sírou. Epidemiologové o odlišnostech mezi Islandem a Norskem v incidenci i expozici různým faktorům vědí a některé studie se zaměřily na vysvětlení možných příčin. Jedna z hypotéz říká, že výskyt DM1 u potomků by mohl mít spojitost s tradicí pojídání velkého množství uzeného skopového (obsahujícího N-nitroso sloučeniny), na což bylo poprvé poukazováno před třiceti lety [26]. Tato teorie je podpořena i experimentálními daty: potomstvo myší, které byly krmeny islandským uzeným skopovým, vykazovalo ve zvýšené míře známky poškození beta buněk pankreatu [27].

Další hypotéza byla poněkud běžnějšího rázu – islandské krávy jsou jiné než skandinávské krávy; islandské krávy jsou populačním izolátem. Vypadají značně jinak než krávy kontinentální, sami Islanďané žertují, že jejich krávy jsou menší proto, aby se nepřevracely na kamenitých pastvinách. Nejenže *vypadají* jinak, ale odlišné je i jejich mléko: liší se v zastoupení proteinových frakcí [28]. Povzbuzeni tímto nálezem, autoři provedli rozsáhlou studii, která se pokusila korelovat incidenci diabetu na Islandu a čtyřech severských zemích proti tomu, kolik se na obyvatele vypije v mléce bovinního sérového albuminu (který se považuje za diabetogenní), imunoglobulinů a laktoferinu (které jsou považovány za protektivní). Spotřeba těchto tří mléčných proteinů nekorelovala s incidencí diabetu [29], takže tím nelze vysvětlit nízkou incidenci DM1 na Islandu. Odlišnost islandské proti norské incidenci zůstává tak další z dosud nevysvětlených záhad epidemiologie diabetu.

Populace **finská a estonská** jsou si též blízko – nejenže je dělí jen Finský záliv, jsou si příbuzné geneticky a jazykově. Přesto v letech 1980 – 1988 byla incidence diabetu v Estonsku asi třetinová v porovnání s Finskem [30]. Navíc – zatímco ve Finsku incidence stoupala, v Estonsku nebyl v té době žádný vzestup patrný. V roce 1991 Estonci znovu získali svoji nezávislost na Rusku. Když je porovnána incidence mezi obdobím před a po získání nezávislosti, je patrný signifikantní vzestup – nyní incidence stoupá jak ve Finsku, a tak i v Estonsku [31]. Vysvětlení není tak jednoduché, jak by se mohlo nabízet: je sice možné, že vzestup mohl být způsoben nástupem prosperity, západního životního stylu, zlepšováním zdravotní péče a prohlubováním socioekonomických rozdílů v estonské populaci. Byla by to analogie vzestupu incidence v zemích střední a východní Evropy. Nicméně ekonomické změny v Estonsku byly provázeny také změnami ve složení populace: neestonské menšiny po roce 1991 zčásti emigrovaly a populace se náhle zmenšila o 15%. Tyto neestonské menšiny přitom mají nižší výskyt diabetu než rodilí Estonci, takže pozorovaný vzestup může být částečně artefakt.

Ještě hlubší a zajímavější jsou **rozdíly mezi finskou a ruskou Karelií**. Karelie je poněkud nešťastné území, které se historicky přelévá mezi Ruskem a Finskem za značného řinčení zbraní a ztrátách na životech. Nyní je hezčí část Karelie součástí Ruska. Diabetology zajímají tyto dvě oblasti proto, že se enormně liší ve výskytu diabetu: v době, kdy Finsko mělo incidenci 41,4, byla incidence v ruské Karelii 7,4 / 100000 / rok. Přitom zeměpisné charakteristiky jsou velmi podobné, populační složení také, neexistují velké rozdíly ve spektru HLA alel ani v hladinách vitamínu D, nejsou dokonce ani příliš velké rozdíly v prevalenci autoprotilátek svědčících o prediabetu [32-35]. Něco nicméně zapřičiňuje pozorovaný extrémní rozdíl v incidencích - a patrně to není též extrémní rozdíl v hrubém národním produktu na hlavu (ve Finsku 32790 a v Rusku 3410 dolarů za rok).

Obrázek 5. Ruská Karelie a Finsko. Nejvýraznější přeshraniční ekonomická propast v současné Evropě.
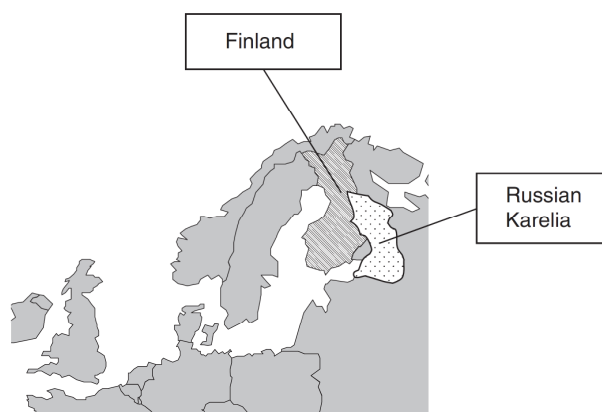
Zásadním pozorovaným rozdílem je prevalence významných infekčních agens: v ruské části Karelie je u školáků mnohem vyšší prevalence protilátek proti viru Coxsackie B4 (94% versus 77% ve Finsku), *Helicobacter pylori* (73% proti 5%), *Toxoplasma gondii* (24% verus 5%) a viru hepatitidy A (24% versus 2%) [36]. Výsledek této studie neznamená, že by zrovna vyjmenovaná agens měla mít nezbytně něco společného s diabetem, ale ukazuje na obecně nižší úroveň hygieny v Rusku (což pro ty z nás, kdo tam byli, asi není příliš překvapením).

Právě obecná úroveň hygieny může být tím faktorem, který rozhoduje: finští diabetologové již v roce 2000 přišli s konceptem tzv. *polio-hypotézy* [37] (o ní je řeč ještě na jednom místě), který vykresluje paralelu mezi zvýšeným výskytem paralytických forem poliomyelitidy po zlepšení hygienických podmínek v druhé třetině minulého století a mezi nyní se zvyšující incidencí diabetu. Pokud existuje diabetogenní virus, pak zlepšení hygieny vede k tomu, že první kontakt s tímto virem probíhá v pozdějším věku, když už dítě není chráněno přenesenými protilátkami od matky. Navíc se zlepšenou hygienou klesá i séroprevalence u matek. Pak - pokud se jedná o enterovirus (tedy virus, jehož prototypem je poliovirus) - čelíme obdobné situaci jako u poliomyelitidy. Virus nezůstane omezen na lymfatickou tkáň střeva a faryngu, ale pronikne dále, způsobí velkou virémii a najde si svůj cílové buňky, v kterých se ochotně pomnoží: v případě poliomyelitidy jsou to šedé buňky předních rohů míšních, v případě diabetu jsou to beta buňky.

Infekce enteroviry ostatně patří mezi faktory nejčastěji podezřívané ze spuštění nebo z akcelerace autoimunitní inzulitídy. Výskyt enterovirů byl srovnáván už i v souvislosti s rozdíly incidence DM1 mezi Finskem a ostatními zeměmi, zejména baltskými. Projekt EPIVIR porovnával na populační úrovni incidenci diabetu proti výskytu protilátek proti enterovirům v obecné populaci kojenců a školáků, a to ve Finsku, Švédsku, ruské Karélii, Estonsku, Lotyšsku, Německu a Maďarsku [32]. Ukázalo se, že prevalence protilátek proti enteroviru je nižší u dětí v zemích s vysokou incidencí diabetu (Finsko, Švédsko), než je v zemích s nižší incidencí diabetu (ostatní země ve studii). Výsledek je stejný jako to, co bylo nalezeno v podstatně menší o pět let starší studii srovnávající finské děti s lotyšskými [38].

Dalším, potenciálně velmi podstatným rozdílem, je vakcinace proti polioviru: ve Skandinávii a Finsku je vakcinace prováděna inaktivovanou vakcínou, kdežto v Litvě, Lotyšsku a Estonsku se očkovalo či očkuje zejména živou polio vakcínou. Proliferativní odpověď lymfocytů a jejich produkce IFN-gama jako reakce proti antigenům polio 1 a Coxsackie B4 byla významně vyšší u estonských než u finských devítiměsíčních dětí [39]. Po perorální polio vakcíně se tedy pravděpodobně vyvíjí vyšší odpovídavost buněčné imunity, která zkříženě reaguje i s ostatními enteroviry. Jak bylo zmíněno v předchozím odstavci, neznamená to ochranu proti infekci enterovirem – ve Finsku má anamnestických protilátek méně dětí. Může to však znamenat ochranu před napadením beta-buňky zatím neznámým diabetogenním kmenem enteroviru.

2.4.4   Rozdíly uvnitř populací

Když bereme při sledování incidence DM1 jako jednotku populaci celého státu, zanedbáváme záměrně její strukturu. V populaci tak zanedbáváme genetické rozdíly i rozdíly v expozici negenetickým faktorům. Zanedbáváme rozdíly mezi socioekonomickými třídami, městem a venkovem, hustě či řídce obydlenými oblastmi, mezi severnějšími či jižnějšími částmi země, mezi horami a nížinami. Celý stát není ale tvořen lidmi, kteří stejně bydlí, chodí do podobné práce, setkávají se s podobným počtem lidí, mají stejná domácí zvířata, pobývají stejnou dobu na slunci, pijí stejně kyselou pitnou vodu. Všechny tyto namátkou vybrané faktory jsou potenciální rizikové faktory DM1 nebo spíše jejich nepřímé indikátory, zástupná měřítka expozice (proxy measures).

Na to, zda opravdu existují regionální rozdíly v incidenci diabetu, se zaměřily četné studie: regionální rozdíly byly nalezeny například v Norsku, Švédsku, severní Anglii, Maďarsku, Německu a v obou populacích s nejvyšším rizikem diabetu: na Sardinii i ve Finsku [40-47]. Naopak nebyly nalezeny např. mezi čtyřmi relativně vzdálenými oblastmi Francie nebo v Japonsku [48, 49]. Uvedené práce konstatovaly, zda mezi geografickými regiony rozdíly existují nebo neexistují – lišily se přitom značně ve své statistické síle a designu studie. Schopnost

detekovat přítomné regionální rozdíly závisí na nejen na velikosti skutečného rozdílu, ale i na délce sledování a incidenci DM1 v populaci (tedy na počtu případů, které za sledované období zachytíme), na velikosti regionů a na dalších faktorech. Naopak falešného nálezu signifikatního rozdílu tam, kde ve skutečnosti není, se lze dočkat zejména při nestejné spolehlivosti zdrojů záchytu diabetu – tedy pokud v některém regionu je záchyt méně kompletní než v jiném.

Pokročilejší než pouhé konstatování přítomnosti nebo nepřítomnosti regionálních rozdílů, je **zaměřit se na testování možných příčin těchto rozdílů**. Lze provést ekologickou studii s krajem, okresem nebo volebním okrskem jako jednotkou: stačí identifikovat pro každého nově manifestovaného pacienta s DM1, kde přesně se manifestoval, a současně pro příslušné oblasti získat data od státní správy – jde třeba o data ze sčítání obyvatel: o velikosti sídla, o hustotě osídlení, o míře geografické izolace, o prosperitě regionu, či třeba o procentu domácností s automatickou pračku. Čím menší je geografická jednotka takovéto studie, tím více odpovídá její "průměrná" charakteristika tomu, jak lidé opravdu žijí. Na druhou stranu je pro malé jednotky třeba dlouhého pozorování, aby byl získán smysluplný počet případů.

Většina z takových studií sledovala vliv hustoty obyvatel (město versus vesnice) a průměrného počtu lidí v obydlí. Vysoká hustota osídlení je ve většině studií spojena s nižší incidencí DM1 [50-54], v některých je tomu ale naopak [55, 56]. Jak navzájem smířit tyto zcela protikladné výsledky? Možná výsledkem z další země: od našich sousedů, z Rakouska, odkud vyšly výborně provedené studie srovnávající data z malých oblastí odpovídajících velikostí asi našim okresům. Autoři rozdělili 1449 dětí s diabetem manifestovaným v letech 1989 – 1999 podle poštovních směrovacích čísel bydliště do 99 okresů [57]. Nalezli západo-východní gradient incidence uvnitř Rakouska. Zjistili též, že distribuce rizika DM1 podle hustoty obyvatel má v Rakousku zvláštní charakter: okresy se střední hustotou obyvatel mají vyšší riziko než okresy s vysokou nebo nízkou hustotou obyvatel. Autoři usuzují, že tento fenomén ukazuje na vliv socio-demografických faktorů a faktorů prostředí, které dosud nebyly zkoumány v jiných studiích. Není to jen fráze na konec článku: k tématice se důkladně vracejí o tři roky později, kdy analýzu téhož souboru obohatili o další proměnné: zajímali se i o podíl dětí do patnácti let v populaci, o koncentraci nitrátů v pitné vodě, o kojeneckou úmrtnost a podíl obyvatel zaměstnaných v průmyslu. Statistická signifikance hustoty obyvatel se v tomto novém modelu ztratila – riziko diabetu bylo zvýšeno hlavně s nižším podílem dětí do patnácti let v populaci a slabě též s vyšší koncentrací nitrátů v pitné vodě [58].

Nález inverzního vztahu mezi rizikem DM1 a podílem dětí do patnácti let je plně ve shodě s pozorováním, že časné začlenění do dětského kolektivu v jeslích nebo školce snižuje riziko; navíc, stejně jako v naší zemi, 30% dětí v Rakousku je jedináčky a 40% má jen jednoho sourozence. Publikace ze stejného roku navíc ukazuje, že v podobně vedené ekologické analýze je incidence diabetu asociovaná i s body mass indexem novorozenců v daném okrese [59]. To je v souladu s výsledky velkých populačních studií, o kterých bude řeč dále.

2.5 Projekce výskytu diabetu

V minulém oddíle jsme se podívali do minulosti. Nastínil jsem, že se nacházíme uprostřed silného vzestupu incidence diabetu. Bylo by dobré vědět, kolik u nás bude diabetických dětí, pokud se vzestup nezastaví. Též je důležité vědět, co by se stalo s výskytem diabetu, pokud bude vyvinuta prevence této choroby, která oddálí manifestaci nebo zabrání části případů.

2.5.1 Parametry ovlivňující modely výskytu

Poněkud mechanistický model prevalence diabetu u celé populace je na **obrázku 6**. Je velmi zjednodušený: na přítoku do prevalenčního kotlíku jsou nově manifestované případy - incidence vynásobená velikostí populace. V praxi to znamená, že sílu přítoku do kotlíku ovlivňuje velikost populace, její věková struktura, a velikost (věkově specifické) incidence. V dětském věku je to konkrétně tak, že v posledních letech klesala porodnost, čili se snižuje velikost dětské populace, a současně stoupá incidence DM1. V naší zemi patrně nedojde k ekonomicky významnému zvýšení *počtu* dětských diabetických pacientů, spíše poroste *prevalence* - s ohledem na věkovou strukturu současné dětské populace.

Obrázek 6. **Mechanistický model vztahu mezi prevalencí, incidencí a mortalitou u DM1.**
Schématicky se dá vztah mezi incidencí a prevalencí znázornit jako tzv. *prevalence pool*,
překládaný jako "prevalenční kotlík". K určení velikosti prevalence přispívají: incidence, věková
struktura populace, uzdravení (tedy trvání choroby), úmrtí. U DM1 odpadá možnost uzdravení.
Ve stabilní stavu se "přítok" rovná "odtoku" – existuje však dostatek údajů o tom, že je
současná populace stabilnímu stavu značně vzdálena.

Velikost populace x
incidence

- populace se zmenšuje

- incidence se zvyšuje

Mortalita; závislé na
struktuře populace
nemocných a na efektivitě
terapie.
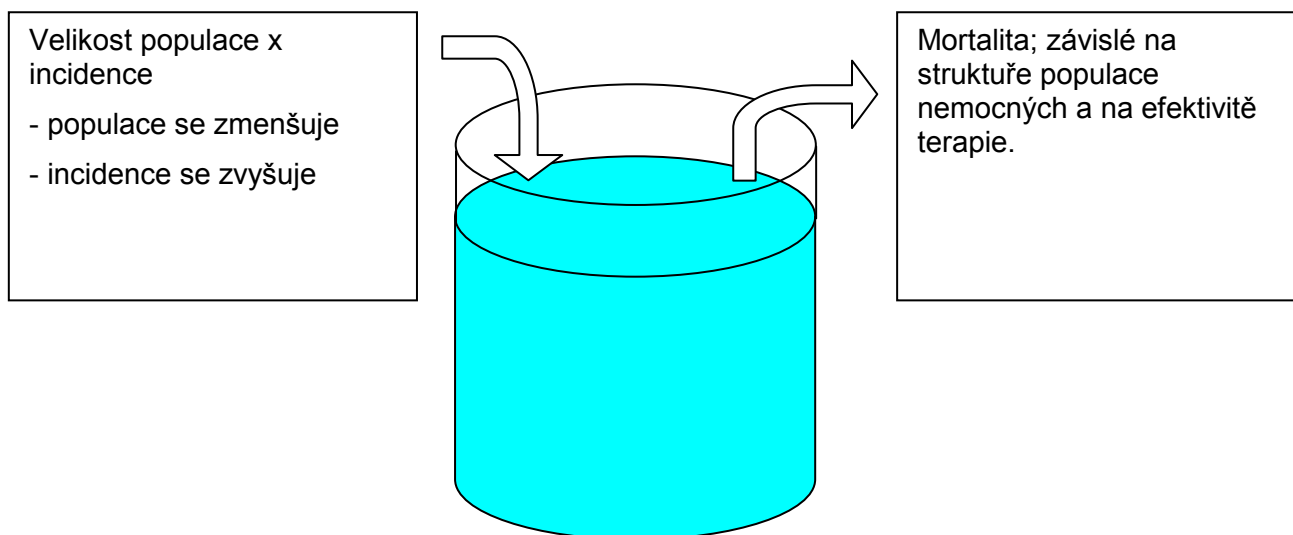
Z prevalenčního kotlíku *dětské populace* se dostane dítě tak, že dospěje a obrazně řečeno je
přelito do prevalenčního kotlíku dospělých. Úmrtnost u dětských diabetiků můžeme zanedbat
(víme, že standardizovaný poměr mortality - SMR - u dětí s diabetem je 2,0 proti dětem z
obecné populace [60]). U celé populace je to podstatně složitější. Diabetes 1. typu je
celoživotní záležitost, je nevyléčitelný. Délka života populace s DM1 je nižší než populace
nediabetické. Přítok do kotlíku se zvyšuje (zvyšuje se incidence), ale o méně, než by se
zvyšoval, pokud by populace jako celek nestárla.

Zatímco nárůst přítoku do kotlíku je relativně pomalý, faktorem, který bude nejvíce ovlivňovat
prevalenci, bude očekávaná délka života diabetické populace. Pokud se podaří udržet trend z
minulých desetiletí, trend prodlužující se očekávané délky života u pacientů s DM1, bude DM1
v populaci patrně silně přibývat. Dat o mortalitě diabetických pacientů zatím však není tolik, aby
predikce byly jednoznačné a univerzální [61, 62].

2.5.2 Prevence a její možný vliv na výskyt diabetu

Prevence DM1 není v současnosti možná. Neexistuje žádný bezpečný a efektivní způsob, o
kterém by bylo známo, že oddaluje nástup diabetu při běžící inzulitídě nebo snižuje proporci
pacientů s inzulitídou mezi jedinci s vysokým genetickým rizikem. Údaje ze zvířecích modelů
naznačovaly, že by bylo možné předejít nástupu diabetu podáváním léků, které pozmění
průběh destrukce beta buněk pankreatu. Před několika lety byly ukončeny dvě velké
intervenční studie, ENDIT a DPT-1, které bohužel tyto možnosti u lidí nepotvrdily.

ENDIT byla multicentrická randomizovaná studie sledující 552 nediabetických prvostupňových
příbuzných pacientů s DM1. Tito příbuzní byli vybráni pomocí testování na autoprotilátky proti
beta buňkám ostrůvků (ICA), které ukazovaly na probíhající inzulitídu – ta obvykle vede k DM1.
Účastníci studie byli randomizováni do dvou skupin: jedni dostávali nikotinamid, druzí placebo.
Incidence diabetu byla po pěti letech sledování u obou skupin shodná – nikotinamid v použitém
dávkování jako prevence DM1 nefunguje [63].

DPT-1 byla studie sledující efekt inzulínu na prevenci DM1, buďto v injekční formě nebo formě perorální. Podobně jako ve studii ENDIT se jednalo o prvostupňové příbuzné pacientů s DM1. Studie měla dvě větve: účastníci s předpovězeným pětiletým rizikem DM1 vyšším než 50% byli zařazeni do větve testující parenterální inzulín, účastníci s předpovězeným pětiletým rizikem 26-50% byli zařazeni do větve testující perorální inzulín. V parenterální větvi studie skončilo 339 účastníků. Tato větev byla otevřená, pacienti byli randomizováni buďto do skupiny, která byla pouze důkladně sledovaná, nebo do skupiny, která jako intervenci dostávala 0,25 jednotky ultralente inzulínu na kilogram ve dvou denních dávkách, plus každoroční čtyřdenní kontinuální infúzi inzulínu. Střední doba sledování byla 3,7 roku. Podíl pacientů, kteří dostali diabetes, se ve skupině s intervencí nelišil od skupiny s pouhým sledováním. Větev studie sledující efekt perorálního inzulínu 7,5 mg/den proti placebu měla 372 členů, střední doba sledování byla 4,3 roku. Podíl pacientů, kteří dostali diabetes, se nelišil mezi skupinami s inzulínem a s placebem [64].

Ve velmi stručném komentáři k negativním výsledkům těchto intervenčních studií podotýká Ping Wang [65], že napodobení strategie, která vedla k úspěšné prevenci u zvířecího modelu, může být správné jen tehdy, pokud jsou splněny tři skupiny podmínek. V první řadě musí zvířecí model dostatečně odpovídat DM1 u člověka – u NOD myši tomu tak mnohdy není. Pak musí být prevence uplatněna předtím, než inzulitída vstoupí do fáze, odkud již není návratu – a možná, že inzulitída s rozvinutými protilátkami je již příliš daleko. Za třetí – dávky použitého léku musejí být dostatečně vysoké k dosažení terapeutického efektu. Pokud srovnáme dávky inzulínu přepočtené na kilogram používané k prevenci DM1 u NOD myší a dávky ze studie DPT-1, je zde zřejmé omezení. Podobný, ale mnohem podrobnější přehled příčin, proč je těžké dosáhnout prevence DM1, podává v komentáři z prosince 2003 Desmond Schatz [66]. Několik z jeho závěrů zasluhuje zvláštní pozornost: možná, že se dosavadní intervenční studie zaměřovaly na pacienty, kteří měli příliš vysoké riziko diabetu, inzulitída progredovala příliš rychle a imunitní systém nešlo odvrátit od jeho rozhodnutí zničit beta buňky ostrůvků. K tomu, abychom tento proces zachytili dostatečně brzy, nejsou poznání ani technické prostředky zatím dostatečné. Autor proto podtrhuje užitečnost intervenčních studií u čerstvě manifestovaných pacientů s DM1: tyto intervenční studie se zaměří na ochranu zbývajících beta-buněk. Čerstvě manifestovaní pacienti diabetes už mají, čili odpadá nutnost screeningu rizikové populace a není zde žádná falešné pozitivita screenigového testu. Reziduální masa beta buněk je malá a bude tak jako tak časem zničena – pacient může z její přechodné ochrany jen profitovat. Sice nelze očekávat žádný dlouhotrvající klinický prospěch, natožpak vyléčení diabetu, ale tato strategie může poskytnout cenná vodítka o tom, jaké preventivní strategie by mohly fungovat u těch, kdo k diabetu teprve směřují. Několik takových studií v současnosti probíhá či bylo recentně dokončeno (včetně jedné, kde jsem byl v kolektivu investigátorů - ale intervence nefungovala [67]).

Z publikovaných výsledků dosud dokončených intervenčních studií je zřejmé, že **prevence je složitá a nelze v žádném kratším horizontu očekávat její rutinní zavedení**. Její zavedení je nadějí pro děti ohrožené diabetem, pro jejich osobní osud. Jednoduchá úvaha však ukazuje, že prevence není nadějí pro populaci jako celek, není nadějí na snížení prevalence DM1 v populaci. Představme si zcela nerealisticky – jako přílišní optimisté - že se lze naprosto zbavit diabetu např. u dětských prvostupňových příbuzných pacientů s DM1. O této rizikové skupině se ví a je relativně snadné všechny její členy sledovat. Prvostupňoví příbuzných diabetických pacientů tvoří asi 5-10% nově diagnostikovaných dětí.

Co by se stalo s prevalencí DM1, kdyby náhle nastal desetiprocentní výpadek incidence? Prakticky nic, jak lze ověřit simulací v jakémkoli tabulkovém kalkulátoru. I kdybychom zanedbali, že by se tento deficit doplnil za dva až tři roky současného růstu incidence DM1, je zde ještě obrovská populace dříve dětských diabetiků, kteří již dospěli. Jak jsme se již zmínili, díky současné terapii je jejich očekávaná délka života patrně vyšší než by byla třeba před deseti lety. Prodloužení života dospělých pacientů s DM1 bude čím dál zásadnější. Všichni diabetologové i pacienti si jej dlouho přáli - takže bude dobře se na zvýšení prevalence DM1 svým způsobem těšit.

3 Genetika dětského diabetu 1. typu

Diabetes 1. typu je **typický příklad polygenního multifaktoriálního onemocnění**. Je to choroba multifaktoriální proto, že v patogenezi se setkávají jak genetické, tak negenetické vlivy, které spolu složitým a dosud ne zcela jasným způsobem interagují. Polygenní je proto, že na vnímavosti (míře rizika) se podílí až čtyřicet genů či genových komplexů [68] a bezpochyby i další geny dosud neodhalené.

Diabetes 1. typu patří mezi polygenní choroby **s prahovým efektem, mezi dichotomické, diskontinuální znaky**: pacient diabetes buďto má, nebo nemá, diagnóza je určena glykemickými kritérii. Škála genetického rizika DM1 je však kontinuální a rozložení míry tohoto rizika v populaci pravděpodobně sleduje Gaussovu křivku. K tomu přistupuje navíc variabilita prostředí, u nějž lze předpokládat podobné gaussovské rozložení míry rizika nebo protektivity. Vzájemnou kombinací vlivů genetických a vlivů prostředí (navíc v čase) je určeno, zda bude překročen práh, za nímž započne autoimunní inzulitída vedoucí dosud neodvratně k DM 1. typu.

Vznik diabetes mellitus 1. typu je výsledkem interakce genetických a negenetických faktorů: tradičně se má za to, že genetické faktory jsou zodpovědné za přibližně jednu polovinu rizika a negenetické za tu zbylou (obrázek 7). Toto přiblížení je však velmi zjednodušující, ačkoli pochází ze studií familiálních případů DM1. Poměr mezi genetickou a negenetickou složkou etiologie je případ od případu odlišný, je závislý na populaci, na věku a na mnoha dalších faktorech.

Obrázek 7. Diabetes jako skládanka.
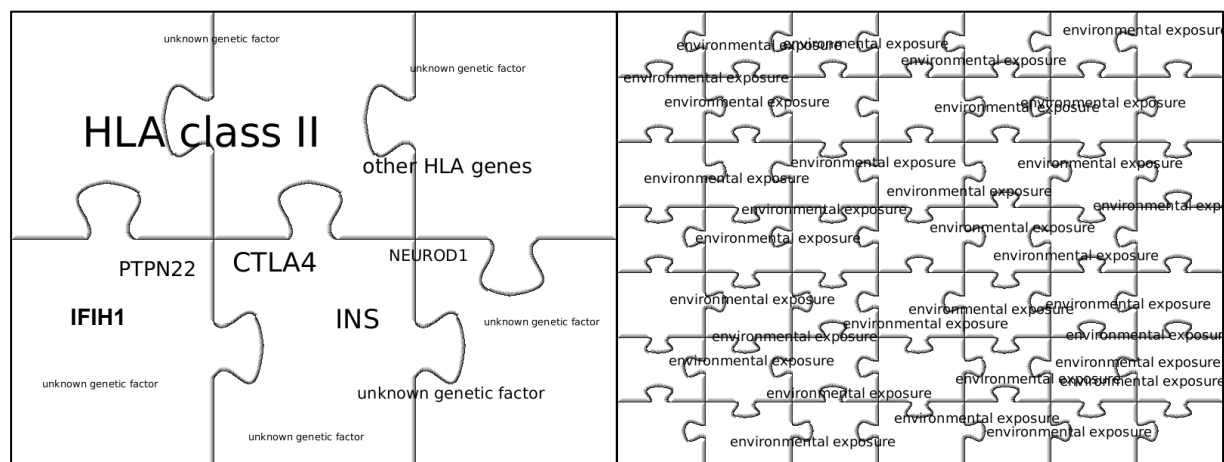
Kapitola stručně shrnuje poznatky o genetice diabetu 1. typu, provádí čtenáře důkazy o důležitosti negenetických faktorů a nakonec se u hlavních z těchto faktorů krátce zastavuje.

3.1 *Míra dědičnosti predispozic k DM1*

Sama dědičnost predispozic k DM1 je nezpochybnitelná: konkordance mezi jednovaječnými dvojčaty se pohybuje v různých studiích od 23% do 53% [69, 70], zatímco u dizygotních dvojčat je to od 2,5% do 11%. Míra hromadění v rodinách (familální clustering) je poměr mezi rizikem rekurence choroby u sourozence dítěte s chorobou a kumulativní incidencí choroby v obecné populaci. Poměr závisí na incidenci choroby a na její heritabilitě. Míra familiálního clusteringu je tedy dobrým indikátorem toho, do jaké míry jde variabilita mezi fenotypem jedinců na vrub jejich genotypu. Pro sourozence diabetických pacientů (λ_s) v americké populaci je to 15: poměr mezi rizikem rekurence sourozenců (6%) a kumulativní incidence DM1 v populaci (0,4%). Hodnota λ_s je populačně-specifická, protože populačně-specifická je i heritabilita a kumulativní incidence: u

nás je riziko rekurence u sourozenců něco přes 2,5% [71] a kumulativní incidence do 15 let věku je 0,1%.

Genetické riziko u příbuzných diabetického probanda souvisí s počtem alel sdílených s tímto probandem: nejvyšší riziko mají monozygotní dvojčata (sdílející vždy 100% alel), nižší je pozorováno u dětí probanda (50 % alel) nebo u sourozenců probanda (průměrně 50% alel). S rostoucí vzdáleností diabetického probanda v rodokmenu strmě klesá i riziko diabetu. Na základě pozorování rizik byl už před dvaceti lety navržen model, kde diabetes je vázán na *jeden hlavní lokus s několika lokusy menšího významu, které působí epistaticky* [72].

3.2   Jak si genetické riziko představit

Síla asociace se zpravidla vyjadřuje pomocí **poměru šancí**, který je u vzácných chorob velmi dobrým odhadem relativního rizika. Je to číslo, které přibližně ukazuje, kolikrát je vyšší riziko onemocnění u osoby, která danou genovou variantu nese, proti osobě, která ji nenese. Protože uvedené výpočty zpravidla vycházejí ze srovnání pacientů s DM1 proti populačním kontrolám, je nutno získané hodnoty rizika vztahovat na toto srovnání.

Vycházejme z známého rizika DM1 v české dětské populaci (asi 1 / 1000 do 15 let věku), a vypočítejme, jaká bude pozitivní prediktivní hodnota nosičství nejrizikovějšího definovaného genotypu pro DM1. V naší populaci je nejrizikovější genotyp HLA-DQB1*0302-DQA1*03 / DQB1*02-DQA1*05, spolu s absencí protektivní alely inzulínového genu. Tento genotyp nese 0,34% zdravé obecné populace a 23% pacientů, kteří dostali DM1 v dětském věku. Jednoduchým výpočtem dostaneme, že i když vybereme z obecné populace právě ty děti, které nesou nejrizikovější definovatelnou kombinaci genů, dostane z nich diabetes do patnácti let jen asi 6%. Zbylých 94% dětí s tímto genotypem zůstane bez diabetu. Zjevně není možné použít takové genetické vyšetřování pro solidně míněnou predikci rizika DM1 mezi dětmi v obecné populaci.

Pokud chceme **lepší pozitivní prediktivní hodnotu**, musíme se zabývat skupinou, která má vyšší prevalenci DM1. Jsou to prvostupňoví příbuzní diabetických pacientů: zejména jejich sourozenci, ale i děti a rodiče. Zde pozitivní prediktivní hodnota tohoto nejvíce rizikového genotypu není 6%, ale pohybuje se (u sourozenců) až někde okolo 30-40%. Sourozenci diabetických dětí mají nezřídka sami diabetes a můžeme se domnívat, že tomu tak je proto, že sdílejí mezi sebou průměrně polovinu genů a také že jsou vychováváni v podobném prostředí. Jenže nejméně polovinu genetického rizika jsme kvantifikovali vyšetřením dvou nejdůležitějších genetických faktorů a pořád je tu tak hluboký rozdíl dvěma dětmi se stejným výsledkem zmiňovaného genetického vyšetřování - mezi dítětem bez rodinné anamnézy DM1 a s ní. Neudržitelné je přičítat tento rozdíl na vrub negenetickým faktorům – tedy za rozdíl mezi oněmi 6% a 40% patrně mohou geny, na které naším vyšetřením nevidíme: mohou to být další faktory ńesené na haplotypech HLA (mimo DQ a DR oblast, ostatně podobné genetické faktory už prokázány byly [73-75]) i faktory mimo HLA – mnoho genů přispívajících každý malou částí ke genetickému riziku diabetu.

3.3   K čemu je genetika DM1

Logickou otázkou je, k čemu potřebujeme vyšetřování genetického rizika diabetu. Jeho význam by se dal vidět v několika rovinách. Jakékoli **studie etiopatogeneze** DM1, observace přirozeného průběhu prediabetu nebo dokonce studie možné intervence oddalující hrozící diabetes, by se měly odehrávat v geneticky definovaném souboru jedinců. Pokud víme, že lidé se navzájem liší v riziku DM1 více než o tři řády, je logickým požadavkem mít skupinu se stejně velkým (pokud možno co nejvyšším) genetickým rizikem DM1.

Dalším důvodem je naděje, že nám geny **řeknou něco o patogenezi choroby**. Definováním genetického rizika v mnoha různých populacích se pak dobíráme k informaci o tom, co je primárně asociováno s rizikem DM1 a co je asociováno jen díky vazbové nerovnováze nebo nepoznané populační heterogenitě. Primárně (kauzálně) asociované geny nám pak mohou říci více, jak diabetes vzniká a – jak doufáme – i o tom, jak jej zastavit. Polymorfismy asociované s

DM1 způsobují jak odlišnosti ve struktuře jimi kódovaných molekul, tak i odlišnosti v jejich expresi. Obojí může být zdrojem cenných poznatků o etiopatogenezi.

3.4 Proporce genetického a negenetického rizika se v čase mění

Vzestup incidence diabetu je příliš rychlý na to, aby se dal objasnit změnou genetického podkladu v populaci, nějakým zvýšením proporce rizikových genotypů. Rychlý vzestup incidence je pravděpodobně **způsoben změnou expozice nějakému negenetickému faktoru**. Pokud takové zvýšení expozice vede ke zvýšení počtu diabetiků v populaci, je zřejmé, že by se měla změnit i zastoupení rizikových genotypů mezi těmito pacienty: čím vyšší je tlak prostředí směrem k rozvoji diabetu, tím nižší bude zastoupení rizikových genotypů mezi diabetiky, protože diabetes budou dostávat i geneticky méně predisponovaní jedinci. Potud hypotéza. Tuto hypotézu se pokusily ověřit dvě studie, které srovnaly rozdělení rizikových a protektivních genetických faktorů mezi pacienty, kteří dostali diabetes před delším časem, proti rozdělení těchto genetických faktorů mezi nedávno manifestovanými diabetiky.

Ukázalo se, že **vliv genetických faktorů za léta oslabil**: k tomu, aby člověk dostal diabetes, je nyní třeba méně genetických predisponujících faktorů než v minulosti; obdobně, diabetes dostávají ve zvýšené míře i lidé nesoucí ochranné genetické faktory. V roce 2003 byla publikována studie z relativně uzavřené populace Finska [76], srovnávající genetické faktory mezi skupinou 367 pacientů diagnostikovaných mezi roky 1939 a 1965 ("starší" skupina) a skupinou 736 pacientů manifestovaných mezi 1999 a 2001 ("současná" skupina). "Starší" skupina pacientů pocházela ze studie o nefropatii u diabetu, současná skupina byla konsekutivně identifikována při diagnóze diabetu. Věk při diagnóze diabetu byl mezi skupinami srovnatelný – takto byl eliminován možný zavádějící faktor spojený s vyšším výskytem rizikových faktorů u časně manifestovaných dětí. Autoři nalezli, že ve "starší" skupině versus oproti "současné" skupině bylo signifikantně více rizikového haplotypu DR4-DQB1*0302 (77.1% proti 71.1%) a rizikového haplotypu DR3-DQB1*02-DQA1*05 (48.8% proti 40.9%). Naopak, "starší" skupina měla méně protektivního haplotypu DR15-DQB1*0602 (0.3% proti 2.6%) a protektivního haplotypu DR*1301-DQB1*0603 (1.9% proti 4.9%). Skupiny se nelišily v distribuci non-HLA rizikových faktorů, polymorfismů inzulínového genu a CTLA4.

Obdobná britská studie z roku 2004 [77] srovnávala genetické faktory mezi "starší" skupinou 194 nositelů zlaté medaile za padesát let s diabetem 1. typu a "současnou" skupinou pocházející z dětí manifestovaných v letech 1985-2002. Pacienti "starší" skupiny byli všichni diagnostikováni před patnáctým rokem věku v letech 1922-1946. Ke každému pacientovi "starší" skupiny byli vybráni tři "současní" pacienti tak, aby se shodovali ve věku při manifestaci a v pohlaví. Autoři nalezli výrazný rozdíl v zastoupení nejrizikovějšího genotypu DR4-DQA1*03-DQB1*0302 / DR3-DQA1*05-DQB1*02 mezi "starší" (47%) a "mladší" (35%) skupinou, nicméně nenalezli rozdíl v zastoupení protektivního haplotypu DR15-DQB1*0602.

Rizikové haplotypy tedy patrně ztratily kus své rizikovosti, a možná i protektivní ztratily kus své protektivity – oboje s časovým odstupem sotva nějakých padesáti let. Je to pravda, nebo vidíme nějaký artefakt? Ve studii srovnávající současné pacienty s těmi, kdo se manifestovali před padesáti nebo třiceti lety je místo pro mnoho potenciálních chyb spojených s výběrem a testováním té "starší" skupiny. Část pacientů "starší" skupiny je nedostupná, protože pacienti již dříve na svůj diabetes zemřeli, zejména na renální selhání a makrovaskulární komplikace. Asociace komplikací tohoto druhu s HLA-faktory nikdy nebyly prokázány, což ovšem neznamená, že neexistují. Další problém, méně závažný, je recentní nárůst imigrace, ale vzhledem k tomu, že imigranti neevropského původu jsou zpravidla v genetických studiích identifikováni při odběru anamnézy, neměli by podstatně ovlivňovat genetické frekvence v "současné" skupině.

3.5 HLA je komplex genů s nejsilnějším vlivem na riziko DM1

Míra rizika DM 1. typu je determinována polygenně, ale je zřejmé, že jeden z genových komplexů, HLA (hlavní histokompatibilitní systém člověka), hraje hlavní roli s podílem přibližně 50% [78]. Podíl HLA na genetické etiologii diabetu byl odhadnut z podílu na familiárním

clusteringu v rodinách s více než jedním diabetickým dítětem. Jedním z důkazů pro vedoucí roli HLA je, že riziko diabetu u HLA-identického sourozence diabetického dítěte je 12,9%, zatímco u HLA haploidentického sourozence je to 4,7% a u neidentického pouze 1,8% [79]. V lidském genomu neexistuje žádná jiná oblast s tak velkým vlivem na riziko DM 1. typu, jako má HLA. Svým podílem se mu vzdáleně blíží pouze gen inzulínový a gen *PTPN22*.

Obrázek 8. Organizace vybraných genů v oblasti MHC II. třídy. Fyzická vzdálenost genů je přibližně proporcionální, délka kódujících oblastí genů však neodpovídá skutečnosti. Na obrázku jsou zachyceny geny kódující jednotlivé klasické molekuly HLA 2. třídy (plné), pseudogeny jim podobné (slabě šrafované) a geny TAP a LMP (šedé obdélníčky).
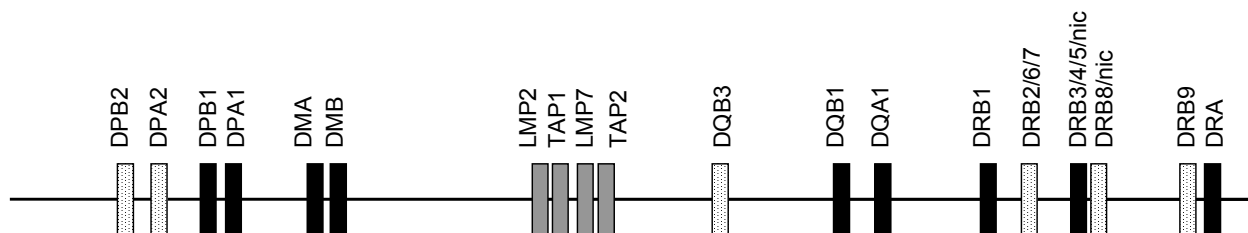
3.5.1 Struktura a funkce klasických HLA molekul

Hlavní histokompatibilitní komplex (MHC, major histocompatibility complex; u lidí HLA, human leukocyte antigens) byl nejprve popsán jako soubor genů, jejichž produkty jsou klíčové pro histokompatibilitu, tedy schopnost transplantátu přihojit se v organizmu jiného jedince. Klasické HLA molekuly (tedy ty, co prezentují antigen) jsou specializované receptory na buněčných membránách, které váží fragmenty peptidových antigenů a prezentují je T lymfocytům.

Jakkoli je funkce HLA molekul v histokompatibilitě nejdůležitější z klinického hlediska, je to pouze vedlejší důsledek jejich primární funkce - prezentace antigenu. Molekuly HLA I. třídy prezentují peptidy zejména intracelulárního původu, a to CD8 (cytotoxickým) T lymfocytům. Asociace HLA I. třídy s DM1 je prokázaná, avšak omezená, a proto se jimi zde dále zabývat nebudu.

Molekuly HLA II. třídy prezentují peptidy CD4-pozitivním T lymfocytům. Prezentují peptidy, které se dostaly do endocytických váčků buňky pomocí fagocytózy, receptory zprostředkované endocytózy či nespecificky pinocytózou; jedná se tedy o proteiny původu extracelulárního a membránového. HLA II. třídy jsou konstitutivně exprimovány pouze na profesionálních antigen prezentujících buňkách, tedy na makrofázích, dendritických buňkách a B-lymfocytech; jejich exprese může však být indukována i na jiných buňkách, jako jsou např. aktivované T lymfocyty. Exprese HLA-DR molekuly je obecně asi o řád vyšší než HLA-DQ, která má zase o řád vyšší expresi než HLA-DP.

3.5.2 Pojmy molekulární genetiky HLA

Oblast kódující HLA molekuly se nachází na krátkém raménku 6. chromozómu (6p21.3). Definice jejího rozsahu není dodnes ujednocena: "klasicky" udávaný rozsah je přibližně 3,6 Mbp, což je něco přes 1% lidského genomu. Asi 40% genů nalezených v této velmi hustě zaplněné oblasti má vztah k imunitnímu systému, zejména k prezentaci antigenů. Evolučně se toto nahromadění jeví účelně a není pravděpodobné, že by bylo náhodné. Komplex HLA je rozdělen do 3 částí. HLA I. třídy (zejména geny HLA-A, -B a -C) leží telomericky, HLA II. třídy (HLA-DR, -DQ a -DP) na centromerickém konci komplexu a mezi nimi leží soubor genů různé funkce nepřesně shrnovaných pod pojem HLA III. třídy. Velmi zjednodušené schéma polohy vybraných genů v HLA II. třídy uvádí **obrázek 8**.

<u>HLA I. třídy</u> obsahuje geny kódující α-řetězce klasických HLA-A, -B a -C i neklasických HLA-E, -F a -G (β2-mikroglobulin je kódován mimo HLA, nepolymorfním genem na 15. chromozómu). Mimo to obsahuje několik desítek dalších genů.

<u>HLA II. třídy</u> obsahuje geny kódující α- i β-řetězce molekul HLA-DR, -DQ a -DP, z nichž geny pro HLA molekuly DQ a DR jsou zásadní pro výši rizika DM1. Geny pro příslušné α-řetězce jsou umístěny v bezprostřední blízkosti svých odpovídajících genů pro β-řetězce. Všechny tři molekuly mají jen jeden gen pro α-řetězec, v případě DP a DQ polymorfní, v případě DR bez signifikantního polymorfismu; β-řetězec je vždy polymorfní. Gen pro β-řetězec je v případě DQ a DP jeden, v případě DRB je počet genů pro β-řetězec závislý na haplotypu: DRB1 je přítomen vždy, u některých haplotypů je přítomen i gen druhý (DRB3, DRB4 nebo DRB5), kódující další druh β-řetězce, jenž je na povrchu buněk také přítomen, ale zpravidla v nižším množství než genový produkt DRB1.

V oblasti mezi DMB a DQB1 se nalézají čtyři geny mající úzkou souvislost s přípravou antigenu pro prezentaci: z nich TAP2, LMP2 a LMP7 jsou polymorfní (takže by teoreticky jejich polymorfismy mohly ovlivňovat repertoár peptidů prezentovaných na HLA), avšak asociace těchto polymorfismů s DM1 je pouze sekundární, díky vazebné nerovnováze s HLA II. třídy [80, 81].

<u>HLA III. třídy</u> je rozsáhlá genová oblast ležící mezi HLA I. a II. třídy. Obsahuje geny, které mají imunologickou funkci, ale nepodílejí se na prezentaci antigenu, i další geny bez funkce v imunitním systému.

<u>Polymorfismus genů HLA</u>

Polymorfismus je takový druh alelické sekvenční varianty, kdy se alespoň jedna vzácnější alela vyskytuje s populační prevalencí vyšší než 1%. HLA systém je *nejpolymorfnější známou oblastí lidského genomu*: v říjnu 2010 bylo známo v II. třídě HLA 924 různých alel HLA-DRB1, 35 HLA-DQA1, 127 HLA-DQB1, 28 HLA-DPA1 a 142 HLA-DPB1 alel (http://hla.alleles.org). V jednotlivé populaci se nevyskytují všechny tyto alely, kupř. pro lokus DRB1 bývá přítomno 20-30 různých alel. Přesto je míra polymorfismu mimořádná a má dalekosáhlé praktické důsledky pro transplantační medicínu, epidemiologii, antropologii a soudní lékařství[8].

Mimo popisované *mnohotné alelie* (vysokého počtu alel genu) je polymorfismus HLA charakteristický ještě dalšími jedinečnými rysy. Jedním z nich je neobvykle vysoká genetická

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[8] Na tomto místě je vhodné stručně se zmínit o **nomenklatuře HLA:** je poměrně komplikovaná, protože typizační techniky se během let s poznáním nových metod měnily, každá z nich přinášela novou informaci a současně byly objevovány nové alely či celé lokusy.

<u>Geny a jejich alely</u> se dlouhá léta označovaly jménem svého lokusu následovaného hvězdičkou a čtyřmi (či více) číslicemi: **prvé dvě** odpovídají (většinou) serologické specificitě příslušného genového produktu, zatímco **další dvě** určují podtyp. Kupříkladu DRB1*0402 označuje jednu z alel lokusu DRB1 serologicky reagujících jako DR4. Číselný kód může být i pětimístný, pak se jedná o tentýž peptidový řetězec a poslední číslo označuje variantu silentní (tiché) mutace nemající vliv na sekvenci aminokyselin. Pokud není znám příslušný subtyp, používají se pouze první dvě číslice (např. DQB1*02). **Nedávno** se přešlo na mírně odlišný systém, který ovšem lépe vyjadřuje biologický charakter polymorfismů: v názvu alel nyní vidíme několik skupin číslic oddělených dvojtečkami. Diabetologové nevzali tuto změnu zatím ve své většině na vědomí, proto i já užívám staré nomenklatury.

<u>Názvosloví molekul, tedy genových produktů</u> je založeno na názvosloví alel kódujících příslušné řetězce. Molekuly mají být označovány názvem a v závorce uvedenou specifikací řetězců označené řeckými písmeny. Příkladem budiž DQ(α1*05, β1*0201) nebo DR(α, β1*0402) - v případě DR odráží nomenklatura přítomnost monomorfního α řetězce. V poslední době je zejména v medicínské literatuře často užívána genetická nomenklatura, tedy molekuly se označují stejně jako alely, jimiž jsou kódovány.

<u>Serologická nomenklatura</u> vychází z názvu příslušného lokusu následovaného číslem: např. DR4. Serologicky může být však rozlišena jen menší část variant molekul, takže kupř. serologická specificita DR4 zahrnuje okolo 20 sekvenčních variant molekuly DRB1, které mohou být dále rozlišeny geneticky. Nomenklatura založená na <u>T-buněčných specificitách</u> (Dw pro DQ a DR, DPw pro DP) není dnes aktuální, protože typizace pomocí homozygotních lymfocytárních linií byla plně nahrazena genetickými metodami.

vzdálenost mezi jednotlivými alelami HLA genů. Alely se zřídka liší jen v jednom nukleotidu, naopak nejčastější počet substitucí mezi dvěma náhodně vybranými alelami je 10-20. Rozdělení polymorfismů uvnitř HLA genu navíc není rovnoměrné: polymorfismy se z největší části vyskytují v oblasti vážící antigen (t.j. kódované 2. a 3. exonem α řetězce u HLA I. třídy a 2. exonem α či β řetězce HLA II. třídy), kde jsou tzv. kapsy - v nich interagují postranní aminokyselinové řetězce antigenu s řetězcem HLA. Vzájemná kompatibilita rozhoduje o síle vazby. Polymorfismy tedy rozhodují o repertoáru peptidů prezentovaných na jednotlivých alelických formách HLA.

Po alelických polymorfismech na DNA úrovni je dalším potenciálním zdrojem různosti molekul HLA II. třídy kombinace α a β řetězců z odlišných haplotypů na peptidové úrovni - tedy při sestavování HLA molekuly. Alely HLA nesené spolu na tomtéž chromozómu (v haplotypu) kódují vždy řetězce schopné spolu vytvořit stabilní α-β dimer. Mimo této *cis* kompletace řetězců je v některých případech stabilní i *trans* kompletace α řetězce z jednoho s β řetězcem z druhého chromozómu. Některé z takto vytvořených heterodimerů α-β jsou však nestabilní (tzv. nepermisivní kombinace) a proto jsou exprimovány výrazně méně než molekuly kódované *cis* kombinací. Teoreticky je dalším možným zdrojem diverzity HLA molekul II. třídy kombinace α a β řetězců různých genů (t.j. např. DQα1-DPβ1), avšak ta se nezdá být funkčně významnou.

3.5.3 Asociace DM1 s HLA

První údaje o asociaci některých antigenů HLA s "juvenilním" diabetem byly publikovány před cca pětatřiceti lety. Tehdy několik studií ukázalo, že HLA-B15 a B8 jsou pozitivně asociovány s DM1, kdežto B7 vykazuje negativní asociaci [82-85]. Tato asociace se později ukázala jako sekundární, přítomná díky vazebné nerovnováze s haplotypy HLA II. třídy DRB1*04-DQB1*0302 a DRB1*03-DQB1*02. Odhalení primárního etiologického faktoru v oblasti DRB1-DQA1-DQB1 bylo však velmi problematické vzhledem k silné vazebné nerovnováze, která v oblasti panuje.

Dnes je široce přijímán názor, že riziko diabetu je **primárně** určeno genotypem HLA-DQB1, -DQA1 s přispěním subtypů DRB1*04, pokud je alela DRB1*04 přítomna. V tomto smyslu shrnují dostupné poznatky i dva mezinárodní HLA workshopy [86, 87].

V oblasti DQB1 jsou významnými rizikovými faktory především alely DQB1*0302 (zejména v severských populacích) a DQB1*02 (zejména v jižních populacích). První hypotéza o možných příčinách rizikovosti nebo protektivity se zaměřila na oblast 57. aminokyseliny DQB1 řetězce. Zatímco protektivní molekuly nesou aspartát, rizikové nesou jinou aminokyselinu [88, 89]. Ačkoli aminokyselina na 57. pozici je zásadní pro vazbu peptidu a jeho prezentaci [90], byla teorie dalšími poznatky vyvrácena: jednak proti ní mluví výsledky mezirasových studií (japonští pacienti mají zvýšenou frekvenci alel, které nesou 57Asp [91]), stejně jako existence četných 57Asp pozitivních pacientů v kavkazských populacích [92]. Do teorie nezapadají ani některé genotypy, které přes homozygozitu 57nonAsp/57nonAsp přinášejí velmi nízké riziko diabetu (DQB1*0302/DQB1*02-DQA1*02, DQB1*02-DQA1*02/DQB1*02-DQA1*02 a další). Další studie ukazují jiný možný mechanismus rizika přinášeného HLA-DQ: riziko je přinášeno některými cis nebo trans kódovanými heterodimery HLA-DQ α/β, přičemž jejich efekt nelze jednoduše vysvětlit přítomností či absencí jednotlivé aminokyseliny v daném řetězci [93, 94]. Již od 11. HLA workshopu konaného počátkem 90. let je akceptováno, že rizikovost či protektivita je přinášena celým heterodimerem DQ [86]. Zatímco v severní Evropě jsou nejdůležitější genotypy obsahující haplotyp HLA-DR4-DQA1*03-DQB1*0302 v různých kombinacích, na jihu Evropy tuto roli zastává haplotyp DR3-DQA1*05-DQB1*02. Obdobně se chovají homozygotní kombinace z těchto haplotypů: první ze jmenovaných je důležitý v severní, druhý v jižní Evropě [86, 87].

Silným protektivním faktorem v oblasti DQB1 je alela DQB1*0602. Tato alela je nesena v kavkazské populaci na haplotypu DQB1*0602-DQA1*0103-DRB1*1501 (DR2). Alela DQB1*0602 je jediná z alel běžných DR2 haplotypů, která je přítomna na silně protektivních haplotypech konzistentně v populacích kavkazských, asijských, afrických i mexických [87], což

dokazuje její primární roli. Protektivita DQB1*0602 se jeví dominantní nad vlivem ostatních molekul a chrání před destruktivním průběhem inzulitídy: osoby s DQB1*0602 mohou mít autoprotilátky proti GAD65, ale tyto protilátky u nich mohou přetrvávat mnoho let, aniž by značily destrukci β-buněk.

DQA1 je stejně jako DQB1 primární faktor, ačkoli to není z analýz kavkazských populací ihned zřejmé. Jeden z důkazů může být mezirasové srovnání afro-karibské populace, kde DRB1*07-DQB1*02 haplotyp přináší riziko DM1, zatímco tento haplotyp nepřináší riziko u kavkazské rasy. Rozdíl mezi těmito dvěma haplotypy je právě v DQA1 alele: v afro-karibské populaci je to DQA1*0301, kdežto v kavkazské je to DQA1*02.

Riziko asociované s polymorfismy HLA-DQ **v naší populaci** je zachyceno v **tabulce 2**, srovnané s rizikem přinášeným variantami inzulínového genu. U nás jsou s DM1 asociovány signifikantně pozitivně alely DQB1*0302, DQB1*02, DQA1*03, kdežto negativně jsou asociovány alely DQB1*0602, DQB1*0301, DQB1*0603, DQB1*0503, DQA1*01 a DQA1*02 [95].

Efekt molekuly DQ je **modifikován subtypem alely DRB1*04** nesené na DQB1*0302-DQA1*03-DRB1*04 haplotypu. Různé subtypy DRB1*04 jsou asociovány s rizikem, které se navzájem zásadně liší. DRB1*0403 a DRB1*0406 jsou známy jako silně protektivní faktory (např. proto DQB1*0302 není v Japonsku rizikové: většina Japonců nesoucích DQB1*0302 nese též DRB1*0403 nebo *0406 [96]), DRB1*0404 je neutrální a riziko stoupá v řadě DRB1*0402 - DRB1*0401 - DRB1* 0405 [75, 97-99]. Neexistuje žádná aminokyselina v řetězci DRB1, která by výše uvedený fenomén vysvětlila, efekt je patrně výsledkem vzájemné kombinace aminokyselin v pozicích 37, 57, 71, 74 a 86. Rizikovost či protektivita některých z těchto alel není univerzální ani v rámci kavkazské rasy: v Evropě existují velké rozdíly jak v rozložení DRB1*04 subtypů jak v obecné, tak v diabetické populaci, a to i mezi sousedními národy. **U nás** je s DM1 asociovaný jedině subtyp DRB1*0403: tento subtyp přináší poměrně silnou ochranu proti DM1.

Míra genetického rizika se odráží nejen v tom, zda jedinec diabetem onemocní, ale **i ve věku manifestace diabetu**. DM1 je v tomto ohledu klinicky heterogenní onemocnění. Je známo, že mezi pacienty manifestujícími se v raném dětství je více některých rizikových alel, haplotypů či genotypů než u pacientů manifestujících se později nebo v dospělosti [87]. Ani protektivní efekt alely DQB1*0602 není u pacientů manifestujících se v dospělosti tolik patrný [100].

Na genetickém riziku DM1 se podílejí i geny uvnitř HLA, ale mimo oblast DQ a DR. Riziko modifikují zejména některé alely HLA-B [75], a dále geny vázané s mikrosatelity v oblasti HLA III. třídy [101, 102] i telomericky od HLA I. třídy [73]. Význam těchto nálezů zatím není zcela objasněn.

Velice zajímavé jsou analýzy haplotypů definovaných jednonukleotidovými polymorfismy napříč oblastí HLA: takový druh analýz byl umožněn teprve s nástupem vysokokapacitních metod. Ukazuje se, že **extrémně konzervované haplotypy** táhnoucí se přes celou oblast HLA jsou podstatně častější, než se myslelo, a že takovéto halotypy mohou být relevantní pro definici rizika diabetu [103-106]. To se vědělo už dlouho o ancestrálním haplotypu DR3-B8-A1 (tzv. haplotyp 8.1), který byl v minulosti zmiňován nejen v souvislosti s diabetem, ale i s celiakií.

Informace o haplotypech je také důležitá pro stanovení identity mezi diabetickým pacientem a jeho sourozencem při odhadu rizika rekurence diabetu u dosud zdravého sourozence - to, že mají sourozenci shodný genotyp na dvou lokusech, neznamená bez znalosti dalších genotypů v rodině nebo bez analýzy dalších markerů, že jsou HLA-identičtí. Přitom právě HLA-identita v nejrizikovějším genotypu pro DM1 mezi diabetickým dítětem a jeho sourozencem propůjčuje tomuto sourozenci extrémně vysoké riziko diabetu [107], ve studii DAISY přesahující 50% do 15 let věku. Autoři tím přispěli k objasnění paradoxu, že sourozenci diabetických dětí se stejným rizikovým HLA-DQ, -DR genotypem mají vyšší riziko než děti diabetického rodiče. Vysvělení leží ve faktorech mimo klasické antigeny HLA, které jsou na takových rizikových haplotypech neseny.

Tabulka 2. Asociace HLA-DQ a inzulínového genu s DM1 v české dětské populaci. Tučně jsou uvedeny poměry šancí pro alely, jejichž asociace je signifikantní po korekci na počet testů. Data jsou z předchozí publikace naší skupiny [108].

	Pacienti % n=332	Kontroly % n=292	OR [95% CI]
DQB1*			
02	**61**	**32**	3,4 [2,5 - 4,8]
0301	**6,3**	**41**	0,10 [0,06 - 0,16]
0302	**67**	**17**	10 [6,9 – 15]
0303	4,2	7,2	**0,57 [0,28 - 1,1]**
0304	2,4	0,3	**7,2 [0,89 - 58]**
0401	0,0	0,0	**0,88 [0,05 – 14]**
0402	5,1	7,2	**0,70 [0,36 - 1,4]**
0501	17	17	**1,0 [0,68 - 1,6]**
0502	6,6	7,5	**0,87 [0,47 - 1,6]**
0503	**2,4**	**8,2**	0,28 [0,12 - 0,62]
0601	0,0	1,4	**0,17 [0,02 - 1,5]**
0602	**1,8**	**25**	0,06 [0,02 - 0,13]
0603	**4,5**	**17**	0,23 [0,13 - 0,43]
0604	4,5	6,5	**0,68 [0,34 - 1,4]**
DQA1*			
01	**36**	**68**	0,27 [0,19 - 0,37]
02	**9,0**	**23**	0,33 [0,21 - 0,53]
03	**73**	**25**	8,0 [5,6 – 12]
04	5,1	6,8	**0,73 [0,38 - 1,4]**
05	**58**	**49**	1,4 [1,0 - 1,9]
06	0,0	0,3	**0,44 [0,04 - 4,9]**
INS			
HphI -/x	24	48	0,34 [0,24 - 0,48]

3.6 DM1 a non-HLA geny

HLA (neboli gen IDDM1) dokáže vysvětlit cca 50% nahromadění případů diabetu v rodinách [109]. Těch zbylých padesát procent je naštěstí rozděleno lépe, než například u běžné polygenní obezity, kde se nezdá existence nějakého "většího" genu pravděpodobná.

U diabetu 1. typu stále platí onen model jednoho velkého genu (HLA) a několika "malých". Naštěstí mezi těmi malými je dostatek těch, jejichž efekt je ještě detekovatelný. Účelem mapování genetického rizika diabetu není primárně jeho prevence - protože už vyšetření HLA dokáže docela spolehlivě poukázat na jedince čelící vysokému riziku vzniku inzulitídy - ale hlavně získání náhledu na patogenezi choroby - tak jako například nalezená asociace polymorfismu genu *IFIH1* nám ukazuje směrem k virové etiologii prediabetu.

Konečným cílem hledání genetických faktorů DM1 je identifikovat variantu v kódující nebo regulační oblasti určitého genu, která je zodpovědná za modifikaci rizika choroby. U monogenních chorob má toto hledání konečný výsledek - nalezení konkrétního genu a konkrétní množiny mutací segregujících s chorobou. U multifaktoriálních onemocnění tomu tak není, protože zde jednoduše neexistuje žádný gen výhradně za chorobu zodpovědný, pro vznik choroby nezbytný a postačující. U polygenních onemocnění tedy nedává takováto analýza nikdy konečné odpovědi na otázku, kde je příslušný etiologický gen, protože ani v přítomnosti nejrizikovější alely daného genu diabetes vzniknout nemusí a naopak ani ta nejochrannější varianta proti DM1 nechrání všechny - není zde žádná kompletní penetrance.

Mapování non-HLA genů lze rozdělit poměrně ostrou linií na dobu před celogenomovými asociačními studiemi (GWAS - genome-wide association study) a po nich. Před GWAS je to testování kandidátních genů nebo mapování pomocí vazby a následné upřesnění lokalizace pomocí asociace, po GWAS je to množství nových asociačních dat získaných z hustého setu jednonukleotidových markerů - nicméně o použití těchto poznatků zatím není úplně jasno.

Zajímavé je, že z dosud identifikovaných asociovaných non-HLA polymorfismů je funkce a příčina asociace zřejmá snad jen u genu inzulínového, zatímco pro žádný z ostatních non-HLA genů se nepodařilo dostatečně přesvědčivě prokázat jasnou roli **jednoho konkrétního polymorfismu** v etiologii DM1 - u těch důležitějších genů existuje konsensus, který z polymorfismů je nejlépe asociován, i teorie, proč je asociován, ale z frekvence publikací navrhujících alternativní vysvětlení je zřejmé, že ke konečnému závěru je daleko: to je případ dlouhé diskuse o *CTLA4* (kde není jasný ani ten "nejlepší" polymorfismus), o *PTPN22* (kde sice známý je, ale pro jeho asociaci jsou dvě zcela protichůdná vysvětlení), o *IFIH1* i dalších.

3.6.1 Mapování pomocí vazby

V minulosti, před příchodem celogenomových *asociačních* studií, bylo jedinou alternativou k testování jednotlivých kandidátních genů provedení **neparametrické vazbové analýzy** celého genomu **pomocí souborů párů diabetických sourozenců**. Mapování pomocí vazby si klade otázku, kde v lidském genomu leží geny přinášející riziko DM 1. typu, aniž by se zpočátku zajímalo o to, jaké geny to jsou a jaké jejich varianty riziko přinášejí. Pro DM 1. typu (stejně jako jiné multifaktoriální polygenní choroby) se používá neparametrických metod vazbové analýzy, tedy metod, které nepředpokládají žádný klasický způsob dědičnosti, které nevyžadují znalost penetrance, alelických frekvencí ani frekvence choroby v populaci. Proti jiným multifaktoriálním chorobám je toto mapování snadnější o to, že DM 1. typu má jasnou klinickou definici (na rozdíl od např. schizofrenie) a že obrazem rychlosti průběhu autoimunitní inzulitidy je věk manifestace (na rozdíl od nesnadných kritérií průběhu např. u roztroušené sklerózy)[9].

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[9] **Neparametrické metody vazbové analýzy** hledají takové chromosomální segmenty, které jsou sdíleny diabetickými sourozeneckými páry častěji, než by odpovídalo náhodě. Pokud si vybereme jakýkoli segment jakéhokoli chromosomu (mimo gonosomů), obecně budou sourozenci budou v tomto úseku identičtí v obou parentálních kopiích s pravděpodobností 25 %, identičtí v jedné z nich s pravděpodobností 50 % a nebudou mít ani jednu parentální kopii mezi sebou identickou s pravděpodobností 25 % – neboli poměr mezi páry sdílejícími 2,1 a 0 parentálních haplotypů bude 1:2:1. Pokud se jedná o sourozence s diabetem a v tomto chromosomálním úseku se nalézá nějaký gen přinášející riziko diabetu, budou tento úsek mezi sebou sourozenci sdílet s vyšší pravděpodobností, než by odpovídalo náhodě, a poměr 1:2:1 se posune vlevo ve prospěch více sdílených haplotypů. Statistická analýza se provádí softwarem, který je schopen adjustovat výsledek na to, zda jsou k dispozici vzorky rodičů (tedy lze rozhodnout, zda sourozenci sdílejí haplotypy identické původem) nebo vzorky rodičů nejsou k dispozici a identita haplotypů mezi sourozenci může být i důsledek výskytu haplotypu v populaci (identita stavem).

Prvním praktickým problémem mapování pomocí vazby vždy byla dostupnost sourozeneckých párů s DM 1. typu. Jen v opravdu velkých státech lze identifikovat dostatečný počet takových párů, většinou se prováděly analýzy na vzorcích z mnoha populací. Druhým problémem byl výběr markerů – v celogenomových vazbových studiích se zpočátku testovalo sdílení přibližně tří stovek mikrosatelitových markerů rozesetých po celém genomu v přibližně shodném odstupu. Tyto markery nemají žádný funkční význam, slouží jen k identifikaci původu chromosomálního úseku. Pro každý z těchto markerů lze pak získat poměr mezi pravděpodobností získaných dat za předpokladu vazby proti předpokladu žádné vazby. Dekadický logaritmus tohoto poměru je tzv. LOD-skóre (logarithm of the odds). Interpretace LOD-skóre je mnohem obtížnější pro set markerů než pro jeden marker a kritéria byla stále do jisté míry diskutována. Pro celogenomovou studii byla používaná kritéria, která navrhli roce 1995 Lander a Kruglyak:

V lidském genomu bylo touto cestou nalezeno okolo 16 oblastí s možnou vazbou s DM1 (lokusy jsou označeny *IDDM1 - IDDM18,* s vynecháním čísel 14 a 16), ale u většiny z nich nedosahuje statistická signifikance hodnot odpovídajících signifikanci v celém genomu [109-116]. Lokus IDDM1 je HLA, IDDM2 odpovídá inzulínovému genu.

Veliké množství odlišných nalezených lokusů odráží hlavní problém studií vazby: nedostatečná statistická síla menších studií má za následek množství navzájem zcela diskordantních výsledků. Například dvě rozsáhlé studie založené na analýze sourozeneckých párů s diabetem [115, 116] se neshodly na jediném lokusu mimo IDDM1.

Úspěch hledání nových genů se vztahem k riziku DM1 je nejvíce závislý na velikosti souboru. Kombinace dat z různých studií je velmi komplikovaná, až nemožná. Efektivním přístupem je získat co největší vzorek z co nejvíce různých populací a otestovat jej jednou metodikou, jedním setem markerů.

K naplnění tohoto cíle bylo ustaveno **genetické konsorcium** (Type 1 diabetes genetic consortium, www.t1dgc.org). Konsorcium se snažilo shromáždit co nejvíce vzorků od sourozeneckých párů s DM1 (dospělých i dětských), nicméně je zřejmé, že vedlo současnou válku prostředky minulých dob - když už jsme měli všechny vzorky za velkého a velmi drahého úsilí shromážděny, ukázalo se, že efektivnější by byla příprava na celogenomové asociační studie, které žádné vzácné rodiny s dvěma diabetickými sourozenci vůbec nepotřebují. Nicméně i tyto staré prostředky vedly k docela solidním publikacím. Všechny pacienty, co za námi přijeli, může uklidnit vědomí, že krev nedali nadarmo. Namátkou ze speciálního čísla *Genes Immunity* např. tyto publikace [117-119].

3.6.2 Celogenomové asociační studie

Jak je uvedeno výše, testování asociace jednotlivých polymorfismů bylo donedávna omezeno na geny identifikované mapováním pomocí vazbové analýzy nebo na geny kandidátní. Dosah vazbové nerovnováhy, na níž mapování pomocí asociace závisí, je omezený, a technické důvody přitom donedávna zabraňovaly testovat tak vysoký počet polymorfismů, aby byl pokryt celý genom.

Nástup velkokapacitních čipových metod v posledních pěti letech však možnosti testování genetických asociací multifaktoriálních onemocnění radikálně změnil: dnes je běžně dostupná

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kritéria vazby rozdělili podle výše dosažené signifikance od naznačené vazby (LOD-skóre očekávané jako náhodný jev jednou v celogenomové studii) až po vysoce pravděpodobnou vazbu (hodnota LOD-skóre, která se v celogenomové studii dosáhne náhodou s pravděpodobností 0,001) a potvrzenou vazbu (což je signifikantní vazba potvrzená další studií příslušné kandidátní oblasti).

Fyzický dosah vazby je - ve srovnání s dosahem vazbové nerovnováhy - značný, a proto i chromozomální segment, kterou příslušný mikrosatelit "označuje", je rozsáhlý. Dalším krokem je proto zpřesňování lokalizace pozorované vazbové nerovnováhy. U monogenních chorob lze set markerů zhušťovat na »podezřelých« místech genomu tak dlouho, až je nalezen gen za chorobu zodpovědný. U multifaktoriálních chorob, jako je DM 1. typu, tomu tak není, zejména proto, že neexistuje žádný gen postačující i nezbytný pro rozvoj DM 1. typu.

Zřídkakdy se tak podaří identifikovat chromosomální region úžeji než asi 20 cM (což je velmi zhruba asi 20 Mbp). Dalším krokem je mapování pomocí asociace (vazbové nerovnováhy) – chromosomy obsahují segmenty, jakési ostrovy, kde mohou být alely spolu v poměrně těsné vazbové nerovnováze. Tyto ostrovy (bloky) jsou zpravidla od sebe odděleny ostrým předělem místo toho, aby síla vazbové nerovnováhy plynule klesala se vzdáleností. Následkem je, že vazbová nerovnováha se dá využít často až do vzdálenosti mezi 50 kbp až 1 Mbp, a to i k mapování. Markery v oblasti se testují na asociaci s chorobou a oblast se postupně zužuje. Testy asociace jsou tedy citlivější než testy vazby, ale nepokrývají tak dlouhý chromosomální segment.

Když už je množina asociovaných polymorfismů úzká a asociace každého z nich je podobně silná, nastupuje poměrně obtížná identifikace toho primárního z nich. Znamená to jednak úsilí o replikaci asociace s DM 1. typu v různých populacích s různou skladbou haplotypů těchto polymorfismů, jednak hledání přijatelného vysvětlení funkce, kterou daný polymorfismus má. Toto vysvětlení by mělo být podpořené testy *in vitro* či prací se zvířecími modely, zejména pak s myšmi, u nichž byla modifikována funkce příslušného genu. Bližší specifikace postupů není předmětem této práce.

technologie pro simultánní testování několika miliónů jednonukleotidových polymorfismů u každého vzorku, polymorfismů rozmístěných víceméně pravidelně v celém genomu. To znamená, že není nutné nyní předpokládat, kde by asociovaný gen mohl v genomu nacházet, protože jsou testovány najednou všechny geny. A jsou testovány pomocí asociace, nikoli pomocí vazby - což je z hlediska přesnosti mapování propastný rozdíl, stejně jako z hlediska dostupnosti materiálu: zatímco pro mapování pomocí vazby jsou nutné relativně vzácné sourozenecké páry, kde oba sourozenci mají diabetes, pro celogenomové asociační studie postačí dostatečný počet pacientů a populačních kontrol.

Na druhou stranu s sebou velké množství testovaných polymorfismů přineslo problémy s designem a interpretací studií [120]. Není zejména zřejmé, jakým způsobem by měly být korigovány hodnoty statistické významnosti a kde je její hranice pro mnohonásobné testování – přitom tyto úvahy jsou zcela zásadní pro každou práci, která nechce upadnout v zapomnění spolu s ostatními nereprodukovatelnými studiemi.

Dalším problémem je výběr testovaných polymorfismů z jejich celkové množiny v lidském genomu - některá oblast nebo gen může trpět nedostatečným pokrytím. Jak bude uvedeno níže, již se ukázalo, že některé poměrně silně asociované geny sítem celogenomového asociačního testování propadly a jejich asociace - předtím opakovaně prokázaná - nebyla replikována. Další – čistě praktické otázky – souvisejí s velikostí souborů nutných k takovým studiím: stále více se ukazuje nutnost řádné spolupráce nejen na národní, ale i mezinárodní úrovni, aby mohlo být shromážděno dostatečné množství vzorků od fenotypicky dobře charakterizovaných pacientů.

První velké celogenomové asociační studie byly publikovány v roce 2007 ([121-123]), s tím, že předběžná analýza z jedné z nich byla publikována již v roce 2006 [124]. Tyto studie v první řadě potvrdily asociaci několika již známých genů: oblasti *HLA, INS, CTLA4, PTPN22*. Nově byla objevena asociace genu *IFIH1*, který kóduje receptor pro virovou RNA, oblasti genu *KIAA0350*, o jehož funkci se mnoho neví, a několika dalších oblastí. Současný pohled na množinu genů asociovaných s diabetem ukazuje **obrázek 9**.

Obrázek 9. Polymorfismy mimo HLA asociované s diabetem 1. typu: současný pohled.
Graf uvádí relativní riziko mezi homozygoty. Z [125].

34
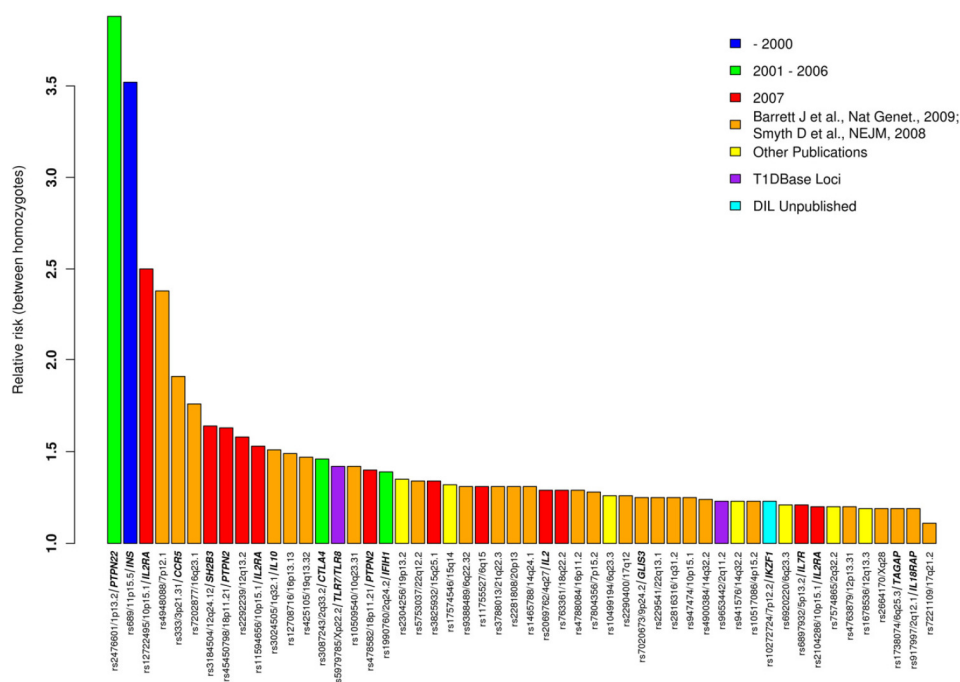
Dosavadní výsledky ukazují několik zásadních faktů: celogenomové asociační studie jsou velmi efektivním nástrojem, avšak jsou proveditelné pouze s dostatečnými zdroji nejen finančními, ale zejména zdroji pacientských vzorků. Kontrolní skupiny pro tyto studie mohou být populační a mohou být společné pro několik chorob. Zásadní je následná replikace nálezu v nezávislé skupině pacientů a kontrol, protože ani velmi striktní kritéria pro statistickou významnost nechrání tyto studie před falešnou pozitivitou. Celogenomové studie mohou nalézt nové oblasti asociace, ale nemohou vyloučit asociaci tam, kde ji nevidí (jedné z nich se totiž nepodařilo nalézt asociaci v oblasti inzulínového genu, druhé v oblasti *CTLA4*). Popsané problémy jsou překonatelné - dosud publikované celogenomové studie budou bezpochyby následovány dalšími, které použijí hustší set jednonukleotidových polymorfismů, rozsáhlejší soubory pacientů a pokročilejší statistické metody. Zdá se, že právě celogenomové asociační studie znamenají průlom v genetice multifaktoriálních onemocnění.

3.6.3   Příklady konkrétních non-HLA genů asociovaných s DM1

3.6.3.1   Inzulínový gen

Inzulínový gen je druhý gen po HLA, pro nějž byla asociace s DM1 identifikovaná. Má několik polymorfismů vně své kódující sekvence – po sérii studií, které vynaložily velké úsilí na mapování toho kauzálního z nich [126-129], se ukázalo, že kauzálním je variabilní počet tandemových opakování v promotoru. Variabilní počet tandemových opakování v promotoru ovlivňuje výši transkripce genu, a to nejen v β-buňce, ale i v thymu. Tím mění pravděpodobnost úspěšného ustavení centrální tolerance vůči inzulínu a tak zvyšuje nebo snižuje riziko diabetu. Protektivní varianta inzulínového genu se chová dominantně nad alternativní variantou rizikovou. Přítomnost protektivní varianty přináší v naší populaci asi třikrát nižší riziko DM1 proti její nepřítomnosti (OR=0,34; 95%CI 0,24-0,48) [108] a obdobně se chová i v četných jiných populacích. Síla jeho asociace se dá srovnat se slaběji asociovanými HLA alelami (**tabulka 2**). Je tedy vhodné vyšetřování polymorfismu inzulínového genu zahrnovat do odhadů genetického rizika při predikci diabetu.

3.6.3.2   CTLA4

Molekula CTLA4 je povrchová molekula T lymfocytů vysoce homologní s CD28. Funguje jako důležitý negativní regulátor imunitní odpovědi. Je exprimována zejména na aktivovaných T lymfocytech. Stejně jako CD28 se váže na molekuly B7.1 (CD80) a B7.2 (CD86) buňky prezentující antigen, ale na rozdíl od CD28 suprimuje odpověď T-lymfocytů indukcí apoptózy. Geneticky upravené myši, kterým CTLA4 chybí, hynou na polyklonální aktivaci T-lymfoblastů.

Molekula CTLA4 je kódována genem na dlouhém raménku druhého chromozómu (2q33), pro jehož oblast byla prokázána asociace s DM1 (oblast IDDM12). V oblasti se nalézají dva kandidátní geny, kódující molekuly CTLA4 a CD28. V oblasti jsou čtyři běžně vyšetřované polymorfismy, které vykazují asociaci s DM1 v některých populacích: +49*A/G polymorfismus v prvém exonu CTLA4, SNP CT60, mikrosatelitový $(AT)_n$ repeat v 3'-netranslatované oblasti genu a epidemiologicky málo prozkoumaný dimorfismus -318 C/T [130]. Polymorfismus +49*A/G býval v různých populacích nejčastěji testován na asociaci s DM1, následován testy $(AT)_n$ mikrosatelitu. Oba jsou však velmi pravděpodobně pouhými markery: závažným příspěvkem k diskusi o etiologickém polymorfismu uvnitř CTLA4 oblasti byla velmi často citovaná práce na obrovském vzorku 3600 rodin, kterou Ueda et al mapovali etiologickou variantu do 6,1 kbp nekódující oblasti genu [131]. Recentně se objevily další práce na toto téma, využívající moderní vysokokapacitní metodiky - nicméně závěr se nezměnil: není znám ani kauzální polymorfismus, ani možný mechanismus asociace (např. [132]).

Rozsáhlá metaanalýza publikovaných studií asociace CTLA4 ukázala (jakkoli tato varianta není kauzální), že alela +49*G je signifikantně s DM1 asociovaná (OR=1.45, 95%CI 1.28 – 1.65) [133], ale mezi studiemi je podstatná heterogenita též nepřímo dokládající, že alela je pouhým

markerem. V naší populaci není s DM1 asociován ani tento marker [134], ani další, nyní velmi populární polymorfismus CT60 (nepublikovaná data).

3.6.3.3 PTPN22

Protein tyrozin fosfatáza non-receptor typu 22 (PTPN22, také nazývaný Lyp) je další negativní regulátor signalizace T lymfocyty. Lyp je jeden z nejsilnějších inhibitorů aktivace T buněk. Jeden z polymorfismů v genu je kódující a vede k záměně aminokyseliny na pozici 620, což patrně vede k změně ve funkci proteinu.

Zdá se, že *PTPN22* vykazuje druhou nejsilnější asociaci s autoimunitními onemocněními obecně, hned po HLA komplexu [125]. Zprávy o asociaci se začaly objevovat v roce 2004, a to o asociaci s DM1 [135], revmatoidní artritídou [136], systémovým lupus erytematodes [137] a Gravesovou chorobou [138]. Za nedlouhý čas, který od první zprávy uplynul, se zdá být prokázané, že *PTPN22* silně modifikuje riziko některých autoimunitních onemocnění, zatímco jiná imunopatologická onemocnění s ním asociovaná nejsou (sclerosis multiplex, Crohnova choroba, celiakie). Není stále jasné, jak vysvětlit efekt substituce R620W, ačkoli o její funkční podstatě jsou k dispozici přesvědčivá data [139]. Navíc je zřejmé, že v některých populacích je substituce na pozici 620 velmi vzácná, ale s diabetem signifikantně asociují jiné polymorfismy, pro něž jsou funkční vysvětlení také k dispozici [140]. V naší populaci je substituce v pozici 620 s diabetem asociovaná relativně silně [141].

3.7 Další zdroje variability

V polovině minulého desetiletí se ukázalo, že v lidském genomu je polymorfního mnohem více, než se očekávalo: s rozvojem technik byly objeveny variace v počtu kopií genů a jejich částí, drobné (hluboce submikroskopické) delece, inverze, translokace a duplikace (review např. v [142]). Náhle stáli genetici zabývající se polygenními chorobami před zcela novou oblastí, jež bezesporu dávala variacím v lidském genomu nový rozměr, nicméně dávala též nový rozměr požadavkům na techniku a finance.

Než bylo dáno menším laboratořím začít s vyšetřováním takovéhoto zdroje diverzity mezi jedinci, ukázala naštěstí velká studie Welcome Trust Case Control Consortium [143] pro několik multifaktoriálních onemocnění, že **copy number variants** (CNV) nejsou významně asociovány s těmito onemocněními (včetně diabetu 1. typu) a že existující techniky jejich případnou asociaci pokrývají (u diabetu to platí o HLA). V HLA jsou CNV známé a jejich charakter je součástí daného haplotypu, proto není důvod předpokládat, že se jedná o něco nového, na známých haplotypech nezávislého.

Dalším zdrojem variability je methylace lidského genomu; data jsou obecně u diabetu 1. typu poměrně vzácná a tato práce se methylací nezabývá.

4 Negenetické příčiny diabetu 1. typu

Pro zásadní úlohu negenetických faktorů v patogenezi diabetu mluví několik faktů, o kterých již byla řeč: konkordance monozygotních dvojčat je neúplná, existují lokální epidemie DM1, incidence DM1 u mnoha geneticky stabilních populací setrvale stoupá a zdá se, že podíl genetických faktorů na etiologii diabetu s časem klesá. Mezi další nepřímé důkazy patří též hromadění případů DM1, u nějž se krátce zastavím. V další části se pak budu věnovat jednotlivým negenetickým faktorům, které mohou ovlivňovat riziko DM1.

4.1 Hromadění (clustering) případů DM1 v čase a v místě

Je-diabetes chorobou, na níž se podstatným způsobem podílejí negenetické vlivy, a liší-li se negenetické vlivy místně a časově, měly by se případy diabetu hromadit. Skutečně se tak děje: případy se hromadí v čase diagnózy (sezonalita diagnózy) i místě diagnózy (lokální epidemie diabetu), clustering je ovšem vidět i mnoho let před nástupem diabetu – v době školní docházky a dokonce v místě a čase narození.

4.1.1 Sezonalita manifestace diabetu

To, že se diabetes manifestuje více na podzim a v zimě než v pozdním jaru a v létě, je známo a první zprávy o tom byly publikovány již ve dvacátých letech minulého století. Je nesčetné množství prací dokládající sezónní výkyvy v incidenci diabetu. Tyto výkyvy jdou patrně na vrub sezónním infekcím: ty urychlí dlouho trvající proces destrukce beta buněk směrem k manifestaci diabetu. Nejsou patrně totožné s vlivy, které inzulitídu spouštějí - inzulitída probíhá měsíce až roky před manifestací diabetu. U malých dětí je patrné, že inzulitída probíhá rychleji než u větších dětí, menší děti mají vyšší zastoupení rizikových genotypů, častěji mají při manifestaci diabetu ketoacidózu – a sezónnost manifestace diabetu u nich zpravidla chybí. Je tomu tak proto, že destrukce beta buněk probíhá příliš rychle na to, aby mohla být signifikantně akcelerována infekcí. Naopak u dětí manifestovaných mezi desátým a patnáctým rokem je sezonalita zřejmá, jak ukazují i data z naší populace. Jak jsem již uvedl, výzkum sezonality manifestace u choroby, která fakticky začíná měsíce až léta před svou manifestací, považuji za poněkud samoúčelný a jeho metody navíc za nepříliš přínosné.

4.1.2 Místní epidemie diabetu

Místní rozdíly v incidenci diabetu byly dobře popsány a zmiňuji se o nich v odstavci "Rozdíly uvnitř populací" v tomto textu. Tyto rozdíly v incidenci mezi regiony pravděpodobně odrážejí riziko vzniku inzulitídy a proto jsou cenným vodítkem při studiu etiopatogeneze diabetu. Mimo těchto regionálních rozdílů dlouhodobějšího charakteru však byly popsány i lokální epidemie diabetu, náhlá nahromadění případů v čase a místě [144-146]. Tyto lokální epidemie mohou odrážet krátkodobější působení nějakého faktoru, a to jak při spuštění inzulitídy, tak při manifestaci diabetu. Pro výzkum etiopatogeneze diabetu mají však tyto epidemie pouze omezený význam.

4.1.3 Clustering případů DM1 v období dlouho před diagnózou

Zatímco lokální epidemie diabetu mohou odrážet vliv prostředí na manifestaci diabetu i vliv prostředí na vznik inzulitídy, clustering případů diabetu dlouho před manifestací ukazuje na vliv negenetických faktorů při spuštění nebo akceleraci inzulitídy. Dalhquist se spolupracovníky ukázali, že případy diabetu mohou tvořit signifikantní místně-časové skupiny již při narození, což ukazuje na možný vliv faktorů působících buďto *in utero* nebo časně postnatálně [147]. Podobně bylo prokázáno hromadění případů v čase a v místě základní školní docházky – to ukazuje, že i v tomto období působí nějaké environmentální vlivy vedoucí k nastartování nebo akceleraci inzulitídy [148]. Které to jsou faktory, mohou ale odhalit jen studie jednotlivých kandidátních expozic.

4.1.4   Sezonalita narození

Představme si nějaký faktor spolupůsobící v patogenezi DM1, jehož výskyt se během roku mění a který působí perinatálně nebo časně postnatálně. Pak by se mělo spolupůsobení takového faktoru nějak odrazit v rozdílech v riziku DM1 mezi dětmi narozenými v období, kdy je expozice tomuto faktoru vysoká, proti období, kdy je nízká. Jinými slovy, děti s diabetem by měly mít jiný vzorec sezónnosti narození než děti bez diabetu.

Roční období, kdy se dítě narodí, je asociované s mnoha různými chorobami – nejsou to jen učebnicové příklady jako astma, atopie a schizofrenie. I u diabetu jsou odchylky v sezonalitě narození proti obecné populaci, jak ukázaly menší studie z Velké Británie [149], Nizozemí [150], Bádenska-Württemberska [151], Berlína [152], ze Slovinska [153] a z Irska [154]. Inspirována těmito zprávami z jednotlivých populací, mezinárodní pracovní skupina zkoumala odchylky sezonality narození ve velkém souboru 15 246 dětí ze 14 evropských diabetických registrů [149, 155]. Studie potvrdila předtím nalezené odchylky sezonality v britských populacích [156], ale ukázala také, že v dalších zatím nestudovaných zemích mimo Velkou Británii nejsou žádné podobné odchylky detekovatelné. To platí i o naší populaci, z níž pochází druhý největší studovaný soubor této studie.

Interpretace rozdílů v sezonalitě může být velmi rozdílná. Zatímco někteří budou preferovat teorii o vitamínu D ze slunečního svitu, jiní mohou hovořit o sezonalitě enterovirových nebo jakýchkoli jiných infekcí. Zvláště teorie o působení infekcí může být atraktivní – ať už budeme předpokládat, že enterovirová infekce modifikuje riziko diabetu už *in utero* nebo že se jedná o expozici enteroviru v jakémsi časovém okně někdy v prvých měsících postnatálně. To, že se celkové pravděpodobnost expozici enteroviru v prvním roce života signifikantně liší podle ročního období narození, bylo již opakovaně diskutováno, počítaje v to i náš nedávný příspěvek k tomuto tématu [157].

4.2   Jednotlivé negenetické faktory asociované s DM1

Existují četné negenetické faktory s prokázaným nebo alespoň diskutovaným vlivem na riziko DM1 – přehled uvádíme v **tabulce 3**. Většina z těchto faktorů je asociována sekundárně, tedy nejsou to přímé etiologické činitele, ale indikátory (proxy measures), měřítka nějaké další expozice, která je v kauzálním vztahu k DM1.

4.2.1   Infekční vlivy

4.2.1.1   První zprávy

Prvé zprávy o vlivu infekčních agens na riziko DM1 přicházejí z výzkumu dětí s **kongenitální rubeolou**: studie publikovaná v roce 1967 detailně vyšetřila 50 pacientů, kteří se narodili s kongenitální rubeolou po epidemii v Novém Jižním Walesu v letech 1939-1943. Ke všem různým problémům měl jeden z těchto pacientů měl i diabetes mellitus [158]. Dnes je známo, že u pacientů s kongenitální rubeolou se DM1 vyskytuje ve vysokém procentu a že se jedná o diabetes s typickými autoimunitními rysy, včetně přítomnosti autoprotilátek a genetické asociace [159, 160]. Vzhledem k očkování není spojení rubeoly a diabetu naštěstí epidemiologicky relevantní, ale slouží jako dobrý důkaz toho, že viry obecně mohou diabetes způsobovat.

Tabulka 3. Vybrané negenetické faktory asociované s rizikem DM1.

Infekce
enteroviry (např. *Coxsackievirus typ B*)
virus zarděnek
Nutriční faktory
délka kojení
kravské mléko ve stravě
nitráty a nitrity
deficit vitamínu D
Faktory perinatálního období a časného dětství
vyšší věk matky
nižší pořadí narození
porod císařským řezem
inkompatibilita krevních skupin
omezený kontakt s jinými dětmi

4.2.1.2 Enterovirus

Podstatně důležitější jsou zprávy z let 1969 a 1973 o souvislosti **enteroviru** s DM1 [161, 162]. Autoři nalezli v séru recentně diagnostikovaných pacientů s DM1 neutralizační protilátky proti viru Coxsackie B4 častěji, než odpovídalo ostatní dětské populaci. Coxsackie B viry byly také nalezeny v pankreatu osob, které zemřely těsně po manifestaci DM1 [163, 164]. Tak začala dlouhá a dosud neskončená honba za enterovirem, který by mohl způsobovat diabetes.

Enteroviry jsou malé neobalené RNA viry. Jejich jednořetězcová pozitivní RNA je poměrně krátká, okolo 7,5 kb, kapsida je dvacetistěn o velikosti okolo 20 nm. Enteroviry patří mezi pikornaviry, kam jsou zařazeny spolu s rhinoviry, virem hepatitídy A a parechoviry. Enteroviry se dělí na polioviry, Coxsackie A, Coxsackie B viry, ECHO viry a číslované enteroviry od 68 nahoru. Enteroviry s námi velmi úspěšně žijí; přenášejí se fekálně-orální cestou, přežívají v odpadních vodách, přirozených vodních rezervoárech, mohou dokonce i téct s vodou z kohoutku. Promořenost populace je vysoká, například ve školkách je – podle sezóny - vždy okolo pětiny až dvacetiny dětí, které nějaký enterovirus vylučují do své stolice. Na to, kolik enterovirů mezi lidmi cirkuluje a jak mnoho lidí je v každou chvíli infikováno, způsobují pozoruhodně málo onemocnění. Meningitidy, myokarditidy, chabé paralýzy a další choroby asociované s enteroviry jsou ve skutečnosti velmi neobvyklými projevy enterovirové infekce.

Mnoho z pikornavirů má poměrně vyhraněný tropismus k nějaké tkáni: rhinoviry k nosní sliznici, hepatovirus k játrům, poliovirus k šedým buňkám předních rohů míšních. Dalo by se

předpokládat, že existuje i nějaký enterovirus, který vykazuje tropismus k beta buňce pankreatu. Ten by mohl být za některých okolností diabetogenní [165-167].

Potenciálních vysvětlení je několik:

1. Teorie molekulárních mimikrů předpokládá zkříženou reaktivitu mezi antigenem enteroviru a proteinem beta-buňky ostrůvků. U geneticky predisponovaného jedince by se pak imunitní odpověď proti enteroviru obrátila směrem k beta-buňce a rozvinul by se autoimunitní proces. Příkladem je podobnost proteinu GAD65 beta-buňky a proteinu 2C enteroviru [168]. Dnes se nicméně nepovažuje tato teorie za příliš pravděpodobnou.

2. Onen hypotetický pankreatotropní kmen zaútočí na beta-buňku a obnaží její struktury, do té doby skryté imunitnímu systému (kryptické antigeny). Stane se tak buď přímou lýzou beta-buňky virem nebo tak, že infikovanou beta-buňku pohltí makrofág. Kryptické antigeny imunitní systém nezná, nemá proti nim centrální toleranci a proto rozvine autoimunitní reakci.

3. Pokud virus způsobí chronický zánět periinzulární tkáně, mohou beta-buňky hynout vlivem volných radikálů tvořených při tomto zánětu – tzv. innocent bystander theory.

4. Konečně jakákoli infekce může urychlovat již vzniklou inzulitídu tím, že navozuje stav inzulínové rezistence a zatěžuje tak zbytkovou masu beta-buněk.

Pokud jsou enteroviry opravdu spouštěči či akcelerátory inzulitídy, měly by být prokazatelné při jejím začátku častěji než by odpovídalo náhodě. Studie, které se tímto průkazem zabývaly, jsou vynikajícím způsobem shrnuty ve starším přehledném článku patrně nejlepšího současného odborníka na enterovirus a diabetes [169]; od té doby přibylo několik relevantních studií, jejichž výsledky shrnuje naše skupina v příslušných vlastním pracech, které jsou přiloženy. Z dosud provedených studií vyplývá, že spojení mezi enterovirem a DM1 je velice pravděpodobné, avšak jeho charakter je více než nejasný. Sérologické studie jsou četné a četné jsou i jejich pozitivní výsledky – nicméně jejich design a zvláště výběr kontrolních skupin je vesměs epidemiologicky nesprávný a získané závěry zpochybňuje. Někteří respektovaní autoři jsou toho názoru, že většina starších sérologických studií mlhu okolo vztahu mezi diabetem a enterovirem nijak nerozptýlila [170, 171].

Novější práce užívají přímou detekci enteroviru pomocí RT-PCR. Ukázaly, že enterovirus je přítomen **u čerstvě diagnostikovaných diabetických dětí** častěji než u kontrol. Jako materiál byly použity plná krev nebo sérum [172, 173], plazma a stolice [174] nebo periferní mononukleáry [175]. Zajímavá je velmi recentní práce ze studie DAISY, kde výskyt enterovirové RNA v séru (na rozdíl od výskytu ve stolici) signifikantně zvyšuje riziko progrese od pozitivity autoprotilátek ke vzniku klinického diabetu [176]. Podobě tomu bylo ve finské studii [177].

O krok dříve je **detekce enteroviru při vzniku autoimunitní inzulitídy nebo v těsné časové souvislosti s ním**. Autoimunitní inzulitída je detekovatelná pomocí kombinace protilátek proti komponentám beta-buňky. Finská studie DIPP (Type 1 Diabetes Prediction and Prevention Project) poukázala na vyšší počet enterovirových infekcí u prediabetických dětí (čili u dětí s autoprotilátkami proti beta-buňkám) ve srovnání s kontrolami bez autoprotilátek: enterovirus byl detekován buďto pomocí RT-PCR ze séra [178, 179] nebo pomocí kombinace výsledků serologie a RT-PCR ze stolice [180]. Podobně i další finská studie nalezla vyšší frekvenci enterovirové RNA v séru prediabetických dětí ve srovnání s kontrolami [181]. Jiné studie mimo Finsko však takové jednoznačné výsledky neposkytly. Ve studii DAISY (Diabetes AutoImmunity Study in the Young) z USA byla enterovirová infekce stejně častá u prediabetických dětí jako u kontrol [182].

Navíc – malá studie, které se zaměřila na děti po infekci virem Coxsackie B, ukázala, že známky prediabetické autoimunity, které se rozvinuly u některých dětí po této infekci, byly pouze přechodné, k diabetu nevedly a zmizely do roka [183]. Zjevně je k objasnění role enterovirů při vzniku DM1 třeba dalších dobře navržených studií z rozdílných světových

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populací. Takovéto studie by měly pozorovat kohorty zdravých dětí s co nejvyšším genetickým rizikem DM1, a to nejlépe od narození nebo časného dětství.

4.2.1.3 *Hygienická hypotéza a nepřímé indikátory expozice infekcím*

Koncept **hygienické hypotézy** předpokládá, že časné setkání imunitního systému dítěte s běžnými infekčními agens chrání dítě proti rozvoji atopií a autoimunit. Časnost a velikost expozice běžným infekcím může být měřena pomocí několika nepřímých indikátorů (*proxy measures*). Jeden z těchto indikátorů může být **návštěva předškolních zařízení**, která je obecně negativně asociovaná s DM1 (metaanalýza dostupných studií v [184]). V české populaci jsme negativní asociaci DM1 s návštěvou předškolních zařízení našli, ale velmi slabou [188]. Dalším indikátorem je **velikost sídla**, ve kterém dítě žije: tato velikost jednak souvisí s hustotou obyvatel, jednak do jisté míry i se socio-ekonomickými rozdíly mezi městským a venkovským obyvatelstvem. O velikosti sídla i hustotě obyvatel je řeč časněji v této kapitole, v oddíle o rozdílech incidence diabetu uvnitř populací. Velikost sídla může souviset ještě s jedním důležitým zdrojem infekčních agens: se **zvířaty**. Expozice běžným infekčním agens nemusí pocházet jen od dětí, může být i od domácích zvířat – a to zcela nezávisle na míře sociálních kontaktů dítěte. Negativní asociace DM1 s kontaktem se zvířaty byla nalezena v nedávné studii z Velké Británie [185], ale ne v jiných studiích [186, 187] – mezi negativní počítaje i naši studii v české populaci [188]. Oddělení případného efektu sociálních kontaktů od efektu kontaktů se zvířaty je komplikováno tím, že proporce domácností chovajících nějaké domácí zvíře je v nepřímé úměře k počtu obyvatel sídla, kde rodina žije.

4.2.2 Nutriční faktory

Publikace ze studií o nutričních expozicích přinášejících riziko DM1 se počítají již na stovky. Je překvapivé, že přes toto těžko zvládnutelné množství dat je dispozici tak málo odpovědí na základní otázky: přináší něco z toho, co jako kojenci nebo batolata požíváme, riziko diabetu? Jak vysoké toto riziko je? Je modifikováno dalšími expozicemi? Nedostatek odpovědí na tyto otázky je patrně způsoben tím, že teprve nedávno se rozběhlo několik adekvátně navržených silných studií sledující děti s dostatečným (a přitom relativně homogenním) genetickým rizikem DM1, od velmi útlého věku a dostatečně dlouho. Lze očekávat, že studie jako DAISY, TEDDY nebo TRIGR (Trial to Reduce IDDM in the Genetically at Risk) poskytnou na naše otázky lepší odpovědi, ale než budou tyto ambiciózní projekty opravdu dokončeny, musíme spoléhat na dostupná data.

Kojení versus přípravky z kravského mléka ve výživě kojenců jsou velmi populárním tématem diskusí – a jejich vliv na riziko diabetu je často vytržen z kontextu a značně přeceněn. Kravské mléko se od mateřského mléka liší celkovou vyšší koncentrací proteinů, přítomností beta-laktoglobulinu, bovinního sérového albuminu a bovinního inzulínu, jehož primární struktura se liší od lidského ve třech aminokyselinových zbytcích. Již od osmdesátých let jsou známy výsledky studií na krysím modelu diabetu 1. typu, které ukázaly, že krysy krmené proteiny kravského mléka měly asi trojnásobnou incidenci diabetu proti krysám krmeným semi-syntetickou dietou bez mléčných proteinů [189]. Lze tedy předpokládat, že riziko DM1 u kojených se může lišit od rizika DM1 u dětí, které dostávají přípravky umělé mléčné výživy - v drtivé většině založené na kravském mléku.

Epidemiologické studie zabývající se asociací diabetu a kojení u lidí nejprve poukázaly na pozitivní korelaci mezi množstvím přijatých produktů kravského mléka v dětství a incidencí diabetu [190, 191]. Závěry těchto a podobných studií ukazují, že zdraví sourozenci diabetických dětí, kteří během dětství požívají více než půl litru kravského mléka denně, mají v porovnání s dětmi, jež denně takové množství kravského mléka denně nepřijímají, riziko diabetu přibližně trojnásobné. Když byla tato asociace sledována u případů a kontrol nesoucích nejrizikovější HLA genotyp (DQB1*02/0302), relativní riziko vystoupilo až na 5,4 [192].

S příjmem kravského mléka úzce souvisí otázka, kdy je vhodná první expozice dítěte proteinům kravského mléka a jaká je tedy doporučená doba výlučného krmení mateřským mlékem. Opakovaně byla popsána inverzní korelace mezi délkou kojení a rizikem diabetu [193, 194]. Diabetické děti jsou kravskému mléku exponovány dříve než děti nediabetické (sledování

expozice během prvních tří měsíců života) [195]. Gerstein [194], který kvantifikuje tato rizika, však uzavírá, že kojení hraje jen malou protektivní úlohu - jeho nedostatek a brzká expozice kravskému mléku nenese riziko pro diabetes vyšší než 1,5. Domněnka, že by při časném setkání s proteiny kravského mléka (do ukončeného druhého měsíce života) rozhodoval o možné pozdější manifestaci diabetu rizikový HLA genotyp, nebyla potvrzena. Při porovnání skupin, do nichž byly tyto děti s časnou expozicí bílkovinám kravského mléka rozděleny podle rizikovosti jejich HLA genotypů, nebyl nalezen žádný signifikantní rozdíl [192].

Další známkou možného vlivu expozice kravského mléka jsou nálezy signifikantně zvýšených titrů protilátek proti bovinnímu laktoglobulinu ve třídě IgA [196] a protilátek proti bovinnímu sérovému albuminu ve třídě IgG u diabetických dětí v porovnání s dětmi nediabetickými [197]. Údaje o přítomnosti protilátek u diabetických pacientů mohou být však stejně zavádějící jako v případě sérologických metod u enterovirů, proto Saukkonen [197] své výsledky vztahoval k rizikovým HLA genotypům. Dle této práce jsou hladiny IgG protilátek proti bovinnímu sérovému albuminu signifikantně zvýšeny u diabetických dětí v porovnání s nediabetickými sourozenci shodného genotypu. Významná humorální odpověď na proteiny kravského mléka je tedy asociována s rozvojem diabetu nezávisle na genetickém pozadí určeném HLA genotypem. Z výsledků citovaných studií vyplývá, že riziko vzniku diabetu se výrazně zvyšuje s vysokým příjmem produktů kravského mléka v dětství; časná expozice proteinům kravského mléka v kojeneckém věku ani doba kojení riziko pravděpodobně zásadně nemění.

Přesný mechanismus působení bílkovin kravského mléka v diabetogenním procesu nebyl dosud přesně objasněn - bylo však formulováno několik hypotéz [166]:

1. BSA hypotéza vychází z mechanismu molekulárních mimikrů. Sedmnáct aminokyselin dlouhý peptidový fragment ABBOS bovinního sérového albuminu (BSA) má podobnou antigenní strukturu s ostrůvkovým antigenem ICA69 [198], proti němuž by se mohla imunitní odpověď obrátit. Tato údajná zkřížená reaktivita je však některými autory zpochybňována [199].

2. Enzymatické štěpení β kaseinu v trávicím traktu vede k produkci β kasomorfinu-7, u něhož byl v laboratorních podmínkách prokázán imunosupresivní účinek [200]. Ten může inhibovat navození aktivní imunologické tolerance vůči perorálně přijímaným zevním antigenům.

3. Příjem bovinního inzulínu, který je přítomen v kravském mléku, může za určitých okolností vnímavého jedince imunizovat a posléze indukovat autoimunitní odpověď proti inzulínu vlastnímu (tedy znovu princip molekulárních mimikrů) [201] .

4. Sliznice trávicího traktu pro své zrání v časném postnatálním období může vyžadovat dostatek určitých látek obsažených právě v mateřském mléce (např. růstové faktory či cytokiny). Nepřijímá-li organismus tyto působky v žádaném množství, funkční vyzrání sliznice je narušeno a namísto fyziologické vlastnosti slizniční imunity - tolerance exogenních antigenů - nastupuje spíše imunizace. Tato nežádoucí imunologická odpověď organismu se může obrátit jak proti vlastním antigenům, například humánnímu inzulínu vyskytujícímu se v mateřském mléce, tak proti antigenům cizím, jejichž epitopy mohou vyvolat zkříženou reaktivitu a rozvinout autoimunitní proces. Podle této teorie [202] je zmíněný průběh imunizace vlastní geneticky vnímavým jedincům, kteří navíc byli krátce kojeni.

Kojení je považováno za ochranný faktor proti DM1. Možných mechanismů je mnoho: kojení chrání proti infekcím, protože matka dítěti předává IgA protilátky, mateřské mléko obsahuje cytokiny a růstové faktory, které modulují zrání lymfoidní tkáně střeva, mateřské mléko obsahuje lidský inzulín, takže se může snáze navodit periferní tolerance.

Dřívější zavedení přípravků z kravského mléka je zpravidla paralelně doprovázeno kratší dobou kojení. Dodnes však nejsou k dispozici jasná data o tom, která z těchto dvou proměnných je asociovaná s DM1 primárně a která sekundárně [203]. Definitivní odpovědi alespoň z některých populací se snad dočkáme s výsledky studie TRIGR [204], která testuje efekt kravského mléka u geneticky selektované kohorty novorozenců a kojenců s rizikem DM1.

Při studiu etiopatogeneze diabetu 1. typu je ověřován vliv nejen kravského mléka proti kojení, ale i jiných dietetických faktorů, mezi něž patří například **nitráty**. Tyto látky obsažené

v zelenině, v masných výrobcích či ve vodě mohou ve formě N-nitroso-sloučenin přímo poškozovat β buňky, jak bylo dokázáno na nitroso-sloučenině streptozotocinu [191]. O islandském uzeném jehněčím se ostatně zmiňujeme na jiném místě.

Deficit vitamínu D je dalším z možných nutričních faktorů. U myšího modelu diabetu bylo prokázáno, že 1,25-dihydroxy-vitamín D snižuje výskyt DM1 [205]. U těchto NOD myší je ale známo, že výskyt diabetu snižuje lecjaká expozice [206], takže až epidemiologické studie u lidí poukázaly na skutečnou negativní asociaci diabetu s podáváním vitamínu D kojencům: nejprve se tak stalo v menší studii případů a kontrol [207], později to bylo potvrzeno ve velké kohortě ročníku 1966 v jednom z krajů Finska, pro níž byla k dispozici data o podávání vitamínu D [208]. Poměrně silný je tento efekt v metaanalýze dostupných studií z roku 2008 [209], kde je podávání vitamínu D asociováno s poklesem rizika o 29% - do této metaanalýzy se ovšem naše poměrně velká studie případů a kontrol nedostala, protože vitamín D nezmiňovala ani v abstraktu ani názvu, ačkoli nesignifikantní tendenci k snížení rizika diabetu jsme pozorovali také [188].

Vitamín D neovlivňuje jen metabolismus vápníku; má také prokázaný imunomodulační účinek [205, 210] a právě tento účinek by mohl být logickým vysvětlením protektivního efektu. Zdánlivě přehledná situace se nedávno zkomplikovala se zjištěním rozsáhlé norské populační retrospektivní studie, že podávání oleje z tresčích jater v prvním roce života má protektivní efekt proti DM1, kdežto podobný efekt pro jiné zdroje D vitamínu v norské populaci chybí [211]; obdobně je pro dítě protektivní podávání oleje z tresčích jater (ale nikoli multivitamínových přípravků) v průběhu těhotenství [212]. Není proto vyloučeno, že skutečný efekt je zde přinášen omega-3 mastnými kyselinami s dlouhým řetězcem, které jsou v tomto rybím tuku obsaženy.

### 4.2.3 Faktory perinatálního období a časného dětství

Faktory perinatálního období v etiopatogenezi DM1 se dobře studují. Některé z nich jsou zachyceny v záznamech o porodu, které jsou pak přeneseny do různých více či méně dostupných databází populační evidence (věk matky a otce, vzdělání matky a otce, porodní hmotnost, porodní délka, pořadí porodu, způsob porodu, velikost sídla bydliště a další) nebo nejsou předmětem žádného zkreslení (věk rodičů, pořadí narození); jiné faktory jsou pro rodiče důležité natolik, že si je pamatují – alespoň tedy matky - bez většího zkreslení desítky let (způsob porodu, porodní hmotnost a délka, celková doba kojení). Ačkoli se dají údaje o expozici dají docela dobře sbírat, problematické je, co vlastně ukazují. Většina jich totiž jsou sekundární indikátory jiných expozic (*proxy measures*). Je těžko si představit, že samo vzdělání matky může ovlivnit riziko diabetu, že by se dnem obdržení vysokoškolského diplomu zvýšila pravděpodobnost diabetu u dítěte. Naopak dobře představitelné je, že zde působí nějaký vzorec chování, který mají vysokoškolačky nebo obecně lidé z vyšší socioekonomické třídy: ten se projevuje v změně nějaké expozice primárně asociované s DM1.

Věk rodičů a zejména **věk matky při porodu** je proměnnou, která se dá snadno sbírat a snadno studovat. Většina studií se shoduje na tom, že zvyšující se věk matky je asociovaný s vyšším rizikem DM1 [213-219], v jiných studiích bylo zvýšené riziko omezeno jen na matky pokročilého věku nad 35 let [220-223]. Riziko asociované s mateřským věkem může navíc záviset na pořadí narození dítěte: v norské kohortě nebyla žádná asociace s věkem matky pro prvorozené – ta se objevila až pro vyšší pořadí narození [219]. Vyšší věk matky nefunguje jako rizikový faktor univerzálně ve všech populacích: studie z Pittsburghu v USA ukázala vyšší prevalenci diabetu nejen u dětí starších matek, ale i u dětí velmi mladých matek [222]. Existuje i jedna studie, kde riziko DM1 je spojeno jen s nízkým věkem matky: tato studie pochází z Indie, kde byl asociován s rizikem DM1 u potomka mateřský věk pod 25 let [224]. Efekt věku matky přehledně shrnuje metaanalýza dostupných kvalitních studií, jejíž jsem spoluautorem [225], **obrázek 10**.

Vyšší věk matky se tedy zdá být faktorem skutečně asociovaným s DM1, alespoň tedy v europoidních populacích. Menší shoda panuje o **pořadí narození** dítěte. Více studií ukázalo nižší pořadí narození jako faktor asociovaný s DM1 [215, 220, 222, 224, 226], méně studií pak ukázalo opak [213, 214, 227]. V obecné populaci je obvykle vyšší pořadí narození dítěte

spojeno s vyšším věkem matky, takže si lze představit, že by riziko spojené s pořadím narození bylo sekundárním jevem při asociaci DM1 a věku matky. Skutečnost je – zdá se – jiná. Výsledky studií, kde prvorozenost i vyšší věk matky byly asociovány s DM1, ukazují zcela jiným směrem [220, 222, 228]: ačkoli se děti, které dostanou DM1, rodí o něco starším matkám, mají nižší pořadí narození než obecná populace. V české populaci je popisovaný efekt přítomen [228]. Možná vysvětlení se dají konstruovat víceméně libovolně z teorií, které jsou pro etiopatogenezi diabetu k dispozici. Můžeme si představit například starší matky jedináčků, chránící své děti před expozicí běžným infekčním agens.

Obrázek 10. Shrnutí efektu věku matky na riziko diabetu potomka: každých pět let věku matky zvyšuje riziko DM1 u potomka o 5%. Z naší publikace [225].
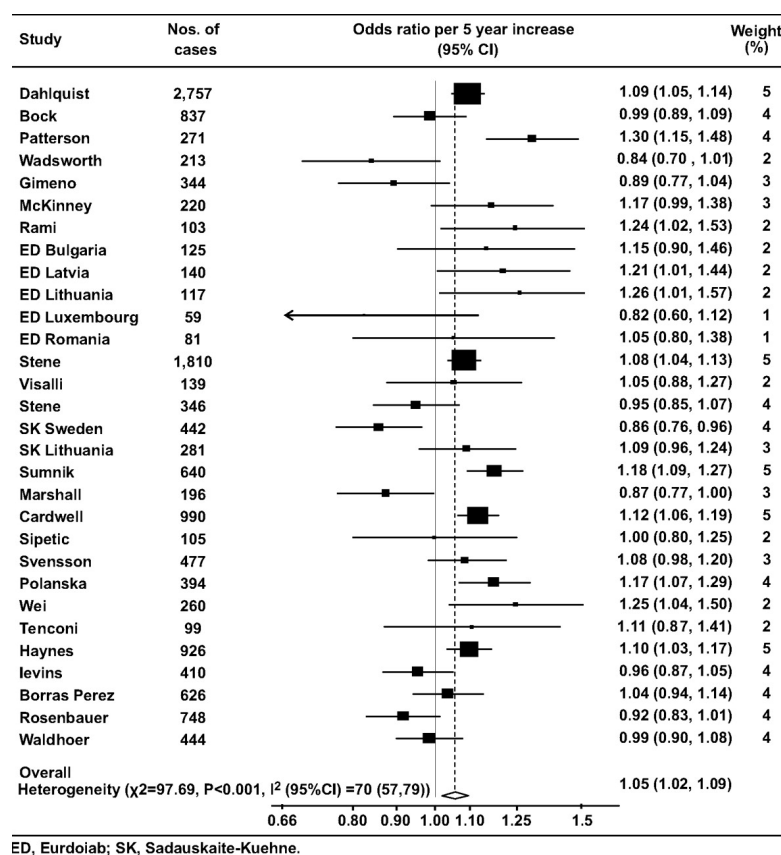
Dalším snadno zjistitelným parametrem jsou **porodní délka a hmotnost**. Velká populační studie z Norska nedávno ukázala vyšší riziko DM1 u dětí s vyšší porodní hmotností [229] a jiná velká studie případů a kontrol z několika evropských populací ukázala nižší riziko DM1 u dětí s nižší porodní hmotností a délkou [230]. Jakkoli nejsou tyto nálezy platné univerzálně (např. [217, 227]), mohou poukazovat na vliv intrauterinního prostředí na riziko multifaktoriální nemoci jako DM1. Ve velmi elegantně provedené studii přitom bylo prokázáno, že efekt porodní hmotnosti není vysvětlitelný rozdíly mezi porodní hmotností dětí s různě rizikovými HLA-DQ genotypy – tyto rozdíly byly opačné [231]. Pro tyto parametry je k dispozici metaanalýza dostupných studií, jejímž jsem spoluautorem [232]: děti s vyšší porodní hmotností mají mírně, avšak signifikantně vyšší riziko DM1.

Způsob porodu je další ze snadno měřitelných proměnných, jejichž efekt se velmi špatně vysvětluje. Porod císařským řezem je asociován s DM1 v některých [220, 233], ale ne všech studiích [216, 230, 234]. I v naší populaci je vidět (byť statisticky nesignifikantní [188]) tendence k asociaci s diabetem: ta byla dobře pozorovatelná v naší společné metaanalýze [235]: s císařským řezem se zvyšuje riziko diabetu v průměru o 23%. Příčinou může být kupříkladu setkání s nemocničními bakteriemi jako prvními místo s bakteriemi mateřských porodních cest. Interpretovat nálezy takovýchto studií opravdu není snadné, zvlášť proto, že indikace pro císařský řez se významně liší mezi jednotlivými porodnickými školami, tedy jak mezinárodně, tak uvnitř populace. Navíc v některých populacích se podíl císařských řezů zvyšuje – v naší se za patnáct let přibližně zdvojnásobil (obrázek 11). Zda v naší populaci indikace pro císařský řez zahrnují faktory primárně asociované s rizikem DM1 u dítěte, lze přitom pouze spekulovat.

Obrázek 11. Proporce císařských řezů u porodů v naší zemi. Zdroj dat: ÚZIS
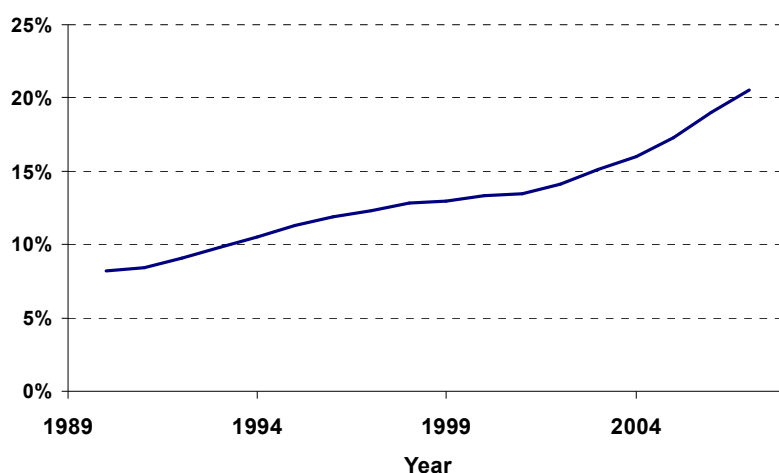
4.3   Srovnání síly genetických a negenetických faktorů

V předchozím textu je několikrát zmíněno, že genetické a negenetické faktory se na riziku diabetu podílejí přibližně shodně, asi tak polovinou. Polovinu genetického rizika pak přinášejí polymorfismy uvnitř komplexu HLA. Individuální *genetické* riziko spojené s HLA lze docela snadno vyšetřit – tak se obecná populace dá rozčlenit od lidí, kteří dětský DM1 téměř dostat nemohou (ti, co nesou HLA-DQB1*0602, asi čtvrtina obecné populace), až po ty, kdo mají riziko v řádu blížícím se deseti procentům (ti, co nesou genotyp HLA-DQB1*0302-DQA1*03/DQB1*02-DQA1*05, asi necelé půlprocento populace). Riziko DM1 se tak v naší populaci liší mezi lidmi nesoucími nejrizikovější a nejochrannější genetické faktory asi desettisíckrát [95, 108].

Nic podobně silného mezi *negenetickými* faktory neexistuje. Negenetické rizikové faktory jsou jednotlivě slabé – je jich nicméně velké množství. Riziko je rozdrobeno mezi špatně definované faktory, jejichž jednotlivý příspěvek je nízký – riziko asociované s přítomností nebo nepřítomností faktoru se zřídka liší více než dvakrát až pětkrát, u většiny expozic je to však ještě mnohem méně. Koncept poměru genetických a negenetických faktorů poměrně dobře ilustruje skládanka na obrázku 7.

Ze srovnání efektu genetických faktorů, kde jeden jediný genový komplex rozhoduje o desettisíckrát vyšším nebo nižším riziku, a faktorů negenetických, kde jednotlivý faktor rozhodne maximálně jen o několikanásobném vzestupu rizika, dojdeme k několika závažným závěrům: **1.** Negenetické faktory je třeba studovat s přihlédnutím k faktorům genetickým.

Současné moderní projekty sledují zpravidla děti od samého narození – a jsou to děti s vysokým rizikem DM1 vyplývajícím buďto z rodinné anamnézy nebo z genetického screeningu. **2.** Při konzultacích s rodiči pacientů informovanými o negenetických rizikových faktorech (kojení, návštěva školky) je třeba zdůraznit, že za diabetes svého dítěte nemohou a že případná expozice dítěte známým rizikovým faktorům zvýšila pravděpodobnost diabetu jen nepatrně. **3.** Je třeba se zdržet jakýchkoli rad k prevenci DM1, protože v současnosti neexistuje žádná bezpečná efektivní prevence – žádné preventivní strategie nemohou být navrženy na základě nálezu slabých asociací negenetických faktorů, ale jen na výsledcích dostatečně silných prospektivních intervenčních studií. Dvě nejautoritativnější takové studie přitom efekt kandidátních preventivních opatření nepotvrdily, jak se zmiňujeme dříve v této kapitole. Zda se na tom něco změní s dokončením dalších intervenčních studií, se dozvíme během dalších let.

4.4   Vzestup incidence DM1 a negenetické faktory

Protože incidence DM1 stoupá a tento vzestup nelze přičíst na vrub změnám v genetických vlastnostech populace, příčiny vzestupu incidence je nutno vidět v negenetických faktorech. Existuje několik hypotéz, proč incidence DM1 stoupá. Tři z nich je dobré zmínit, už proto, že se jejich znalost předpokládá při četbě současné literatury o etiopatogenezi DM1.

Hygienická hypotéza se objevila již v souvislosti s vysvětlením příčin vzestupu alergií a atopie. U diabetu – jakkoli je jeho imunopatologická podstata jiná než je podstata astmatu nebo atopie – se ujala také. Vychází z pozorování, že DM1 se vyskytuje více ve společnostech se západním životním stylem a že diabetes může být častější u dětí, které byly v časném dětství vystaveny nižší expozici infekcím. Je pravděpodobné, že k vývoji zralého a vyváženě reagujícího imunitního systému je třeba, aby byl imunitní systém v raném dětství adekvátně stimulován – zejména běžnými infekčními agens. Pokud tomu tak není, může vzniknout dysbalance vedoucí například k autoimunitní inzulitidě a DM1.

Hypotéza předpokládá, že se z našeho prostředí s postupujícími roky a zvyšujícím se životním standardem vytratila nějaká dříve běžná infekční expozice. Může to bezesporu být i obyčejný roup, jak se domnívá Edwin Gale ve svém dobře vypointovaném článku [236]. Jiní se však domnívají, že se nejedná o parazita, ale o virus.

Na takový virus, jehož oběh v populaci se mohl podstatně snížit, poukazuje **polio hypotéza** (nebo hypotéza polioviru – ale tento překlad není úplně správný, protože o poliovirus jako takový nejde) - hypotézy si mohli pozorní čtenáři všimnout už dříve v tomto textu (*v odstavci 2.4.3*). Autory hypotézy jsou členové finské skupiny zkoumající enteroviry a DM1 [37]. Hypotéza byla touto skupinou opakovaně publikována. Je založená na předchozích dobře známých zkušenostech s epidemiologií poliomyelitidy: před érou očkování se frekvence poškození motorických neuronů virem zvyšovala paralelně s tím, jak se snižovala frekvence infekce poliovirem v populaci. Do konce 19. století byla infekce poliovirem endemická a paralytické formy vzácné. Pak frekvence infekcí značně poklesla se zlepšením hygienických podmínek, ale frekvence paralytických forem neočekávaně vzrostla. Stejně tak bylo riziko paralytických forem vyšší v zemích s nízkým výskytem infekce, než v zemích s vysokým výskytem infekce.

Vysvětlením je opožďování prvního kontaktu s poliovirem: pokud je frekvence infekcí v populaci nízká, poprvé se jedinec s poliovirem potká pozdě ve svém životě – u starších dětí či dospělých obecně enterovirové infekce probíhají hůře, nemluvě o tom, že nejsou chráněni přenesenými mateřskými protilátkami. Poliovirus je enterovirus s afinitou k motoneuronům předních rohů míšních – pokud by existoval nějaký enterovirus s afinitou k beta buňkám Langerhansových ostrůvků, schopný spustit prediabetickou autoimunitu, lze si dobře představit analogii: pokud má dítě štěstí a potká se s ním časně, když je ještě chráněno mateřskými protilátkami, nejenže se jej snadněji zbaví, ale vytvoří si i vlastní imunitu proti tomuto viru. Pokud se nakazí později, je vyšší pravděpodobnost velké virémie a dosažení a poškození cílové tkáně – beta-buněk. Představme si, že je lepší hygienou omezena cirkulace viru v populaci. Pak se jednak začnou děti s virem potkávat později a jednak se některé dívky a ženy nepotkají s tímto virem vůbec. To znamená, že si protilátky nevytvoří a nemohou je v další generaci předat svým dětem.

Třetí z dnes velmi populárních hypotéz o důvodu zvyšující se incidence diabetu je **hypotéza akcelerátoru**. V roce 2001 Terrence Wilkin publikoval práci [237], kde uvádí tři faktory spolupůsobící na patogenezi diabetu obou typů – rychlost apoptózy beta-buněk, inzulínová rezistence a autoimunita proti beta-buňkám. Ve své sjednocující teorii postuluje, že konečný efekt – závislost na inzulínu – je tentýž u obou typů diabetu a že zvyšující se inzulínová rezistence má za následek zvyšující se výskyt obou typů diabetu v populaci. Diabetes 1. typu a 2. typu jsou shodné mimo autoimunity, kterou má DM1 jako zásadní rys navíc. Inzulínová rezistence je asociovaná s viscerální adipozitou. Ačkoli genotypy vedoucí k této adipozitě mohly v hladové minulosti poskytovat selekční výhodu, nyní nám škodí, protože jsme zavaleni přebytkem stravy a nedostává se nám pohybu. U DM1 inzulínová rezistence urychluje průběh inzulitídy směrem k diabetu – a pacienti se manifestují dříve než by se manifestovali s nižší inzulínovou rezistencí. Hypotéza akcelerátoru je relativně snadno testovatelná a dostalo se jí podpory z několika, ale ne všech studií; nyní se zdá, že souhlas nebo nesouhlas s touto hypotézou nabírá spíše religiózních než vědeckých rozměrů. Protože naštěstí nedisponujeme žádnými daty, která by umožňovala se k nějakému táboru přidat, v této práci se hypotézou akcelerátoru nezabývám.

5 Závěr

Pro molekulárního epidemiologa, který byl zvyklý provádět asociační studie kandidátních genů, představoval samozřejmě příchod vysokokapacitních metod problém: jeho práce se tím zcela zásadně mění, protože celogenomové metody vyžadují velmi rozsáhlé soubory a jedinec stojící mimo silné konsorcium nezvládne nic. Asociační studie, jak jsme je znali z minulého desetiletí, definitivně skončily. To je třeba si velmi rychle uvědomit, než dobré časopisy přestanou naše asociační studie zcela akceptovat.

Kde je pak prostor pro epidemiologa, který ovládá molekulární metody, ale nechce provádět práce jen na infekčních agens? Podle mého názoru je třeba tématicky přejít na rozhraní mezi mikrobiologií a neinfekční epidemiologií, kde lze sledovat, jak právě tato infekční agens spouštějí nebo potencují rozvoj autoimunitních chorob. O to jsem se pokusil v práci s pikornaviry. Jedná se o velmi dynamickou oblast, kam může ne-mikrobiolog přinést nový pohled: agens je prostě jako jakákoli jiná expozice, kterou hodnotím kvalitativně nebo kvantitativně. Tam, kde se snaží mikrobiolog zpravidla agens izolovat a komplexně popsat, lze mnohdy úspěšně použít pouhou jednoduchou detekci přímo z dodaného materiálu - což ovšem platí za předpokladu řádného navržení studie, v jejímž rámci tato detekce probíhá. Právě design studie kompenzuje nedokonalost detekčních metod.

Další oblast, kterou nelze ignorovat, je informatika. Současně s tím, jak stroje přebírají dříve kvalifikovanou práci v laboratoři, se musí těžiště znalostí biologa přesouvat k tomu, co stroje nedokáží a ani sečtělí studenti neumí: jednak k algoritmizaci běžných procesů v laboratoři, jednak k systematickému využití databázových systémů. Spektrum volně dostupných nástrojů je nepřeberné, pokud nebudeme ignorovat jednoduché programování. Z již připravených softwarových modulů můžeme při základní znalosti algoritmizace poslepovat překvapivě silné nástroje, které nám dávají značnou převahu nad těmi kolegy, kteří pro analýzu svých výsledků ještě stále pouze klikají v tabulkových procesorech, ignorujíce hrozivě narůstající objemy dat generovaných jejich laboratořemi.

Diabetes 1. typu je fascinující choroba, kterou lze velmi efektivně studovat - už proto, že poskytuje dostatek negativních emocí, které motivují k jejímu poznávání. Smířil-li jsem se s tím, že existuje jen mzivá pravděpodobnost, že udělám jakýkoli "velký objev" či vytvořím nějakou skvělou prevenci této choroby, mohu snad drobnou a systematickou prací pomoci pokroku vědy a konečně možná i přispět ke zlepšení individuálních osudů pacientů.

6 Diskuse vybraných vlastních výsledků

Z přibližně 70 článků indexovaných v listopadu 2010 na MEDLINE, jejichž jsem autorem nebo spoluautorem, vybírám ilustraci k několika okruhům problémů. Důležitější články přikládám v plném znění k této práci[10].

6.1 Pikornaviry a prediabetes

Jedno z nosných témat, jímž jsem se v minulých cca osmi letech zabýval, byla detekce enterovirů a některých dalších virů v norské kohortě dětí s nejvyšším genetickým rizikem diabetu 1. typu. Práce má za konečný cíl přispět k objasnění podílu této skupiny virů na prediabetu.

6.1.1 Pilotní studie detekce enteroviru

První z článků popisoval pilotní studii, která prokazovala schůdnost detekce enteroviru ze vzorků stolice sériově odebíraných od dětí s nejvyšším genetickým rizikem, identifikovaných při narození z norské obecné populace.

> ▪ Cinek O, Witsø E, Jeansson S, Rasmussen T, Drevinek P, Wetlesen T, Vavrinec J, Grinde B, Rønningen KS. Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. **J Clin Virol**. 2006 Jan;35(1):33-40. **(přiloženo in extenso, příloha I)**

Věnovali jsme se popisu logistiky, techniky extrakce a detekce a kvantitativním aspektům detekce. Extrakce RNA, detekce i typizace enteroviru probíhala v naší laboratoři v Praze. Naše detekční metoda má exogenní vnitřní kontrolu - přidaný fragment RNA viru západonilské horečky ve fágové partikuli. Tyto pseudovirové partikule procházejí celým procesem zamíchány do prvního pufru přidávaného ke vzorku stolice, čímž poskytují ideální informaci o efektivitě extrakce, reverzní transkripce a PCR, včetně informace o míře inhibice. Tento aspekt našeho technického řešení byl nový a efektivně kontroluje předpokládaný vysoký výskyt inhibitorů ve stolici.
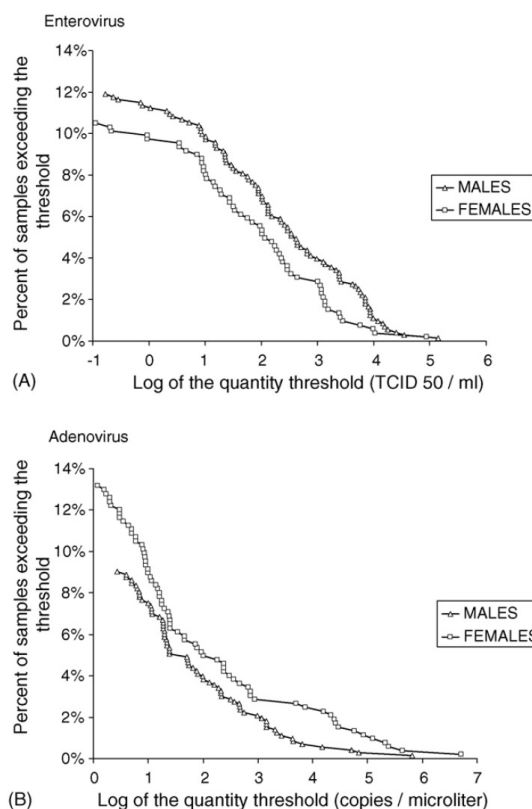

Fig. 1. Distribution of quantity of enterovirus (A) and adenovirus (B) in males and females. The amount of viruses in stool samples was measured by quantitative PCR. The horizontal axis shows the decadic logarithm of the viral concentration in sample ($\log_{10}$ of $TCID_{50}$/ml for enterovirus or $\log_{10}$ of copies/μl for adenovirus); the vertical axis shows the proportion of samples exceeding this quantity. Individual samples are plotted as triangles (samples from males) or squares (samples from females).

Na 1257 vzorcích od 113 dětí ukazujeme rozložení kvantity enteroviru a adenoviru (ten byl vybrán jako příklad viru, který nikdy nebyl podezírán z podílu na vzniku DM1). Na přetištěném obrázku vpravo nahoře je dobře vidět kvantitativní rozložení.

Studovali jsme věkovou závislost výskytu enterovirové infekce (vpravo dole): zde se prolínají dva fenomény - jednak obecně známá silná sezonalita enteroviru v mírném pásmu, jednak roční období, kdy se děti

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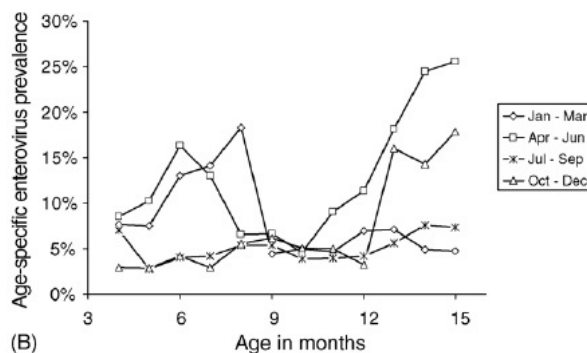
[10] Obrázky a tabulky kopírované do této kapitoly nečísluji ani nepopisuji, protože jsou většinou přímo kopírovány z publikovaných prací a číslo už mají. Odkazuji se na ně v textu pomocí jejich umístění.

narodí (čtyři různé křivky).

Dívali jsme se i na symptomy spojené s infekcemi: zatímco adenovirus nějaké symptomy měl, rozložení symptomů bylo zcela nezávislé na výskytu enteroviru. Protože se jeho okamžiková prevalence ve stolici pohybovala někde od 5% do 20%, lze s jistou dávkou nepřesnosti říci, že je to většinou neškodný virus, který někdy způsobí komplikace u jedince, který má smůlu. Podniknout kroky k definici této "smůly" z hlediska viru i z hlediska hostitele se dále snaží některé naše práce.

Neomezili jsme se jen na detekci viru, chtěli jsme znát i jeho sérotyp. Protože efektivita kultivace je různá sérotyp od sérotypu, je drahá a navíc ze stolice jde poměrně špatně, rozhodli jsme se jít rychlejší a modernější cestou: testování genu pro virový VP1 protein přímo z RNA ze stolice. Znalost sérotypu (nebo skupiny možných sérotypů) nám byla užitečná zejména v rozlišování nových proti pokračujícím infekcím - tak bylo možné určit skutečnou incidenci infekčních epizod.

6.1.2   Sérotypové rozložení enteroviru z pilotní studie

Další dva články byly napsány během PhD studia mladší kolegyně Elisabet Witso z Norska.

- Witsø E, Palacios G, <u>Cinek O</u>, Stene LC, Grinde B, Janowitz D, Lipkin WI, Rønningen KS. High prevalence of human enterovirus A infections in natural circulation of human enteroviruses. **J Clin Microbiol**. 2006 Nov;44(11):4095-100. Epub 2006 Aug 30. **(přiloženo in extenso, př. II)**

- Witsø E, Palacios G, Rønningen KS, <u>Cinek O</u>, Janowitz D, Rewers M, Grinde B, Lipkin WI. Asymptomatic circulation of HEV71 in Norway. **Virus Res**. 2007 Jan;123(1):19-29. **(přiloženo in extenso, př. III)**

Oba dva vznikly v úzké spolupráci s kolegy z New Yorku. Důvody mého spoluautorství (ačkoli jsem v inkriminované době nebyl ani v Oslo ani v New Yorku) jsou jednoduché: jednak podíl na psaní článku, ale jednak a hlavně - určování sérotypu každého ze vzorků jsem na počátku provedl s kolegy v Praze, autorka s ostatními spolupracovníky provedla hlubší typizaci pomocí mnohočlenného setu primerů. Jejím cílem byly co nejpřesnější fylogenetické analýzy, které ostatně první článek docela pěkně prezentuje.

Druá z prací ukazuje enterovirus 71 (HEV71) v jiném světle než učebnice. Norskem prošla epidemie HEV71 (obrázek vpravo), která celkem mohla zasáhnout skoro 50 tisíc dětí pod tři roky věku. Přesto se nezvýšila incidence encefalitid a dalších sledovaných chorob, které se obecně HEV71 připisují. Možná, že to jde na vrub rozdílům v sekvenci našeho kmene proti ostatním - zvláště změnám v oblasti virové polymerázy.
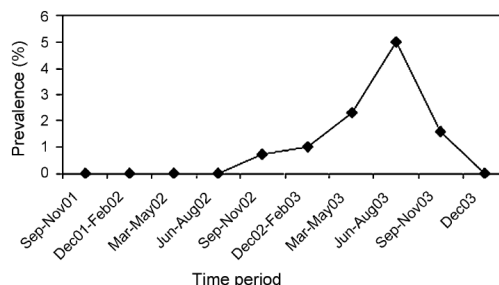

Fig. 1. Prevalence of HEV71 in stool samples from 113 asymptomatic children over the period September 2001 through December 2003.

6.1.3   Prediktory výskytu enteroviru

Výsledky získané během dalšího testování enterovirů u dětí s nejvyšším genetickým rizikem, i dalších dětí, které nenesly rizikový genotyp, ale byly sledovány shodně, jsme použili k testování faktorů asociovaných s rizikem enterovirové infekce.

- Witsø E, <u>Cinek O</u>, Aldrin M, Grinde B, Rasmussen T, Wetlesen T, Rønningen KS. Predictors of sub-clinical enterovirus infections in infants: a prospective cohort study. **Int J Epidemiol**. 2010 Apr;39(2):459-68. **(přiloženo in extenso, př. IV)**

Na straně závislé proměnné byl výskyt enteroviru - celkově se jednalo o 639 dětí a jejich skoro 4300 vzorků stolice, testovaných opět v naší laboratoři v Praze-Motole.

Testovanými prediktory byly proměnné jako počet sourozenců, návštěva školky, kojení, ale v neposlední řadě i HLA genotyp. Vcelku očekávaně se vyšší riziko enterovirové infekce pojilo s počtem sourozenů, s kontaktem s ostatními dětmi; stejně cenné je zjištění, že asociace chyběla se zdrojem vody pro rodinu, nebo s držením domácího zvířete. Kojení bylo marginálně protektivním faktorem, ale jen v prvním trimestru života - v jednom roce věku se efekt již ztratil zcela.

Table 2 ORs from univariable and multivariable logistic regression analysis for predictors of enterovirus infection in monthly longitudinal stool samples from 639 children aged 3–12 months, Norway, 2001–2006 ($n = 4279$)

Covariates	Univariable Unadjusted OR[a]	Multivariable Adjusted OR[b] (95% CI)	P-value
Number of breastfeeds per day (continuous)			
Effect at age 3 months	0.91 (0.87–0.94)	0.85 (0.79–0.92)	<0.001
Effect at age 12 months	(as above)	1.01 (0.92–1.11)	0.79
Number of siblings[c,d]			
0	1.0	1.0	
⩾1	1.55 (1.24–1.94)	1.89 (1.18–3.01)	0.01
Had sibling(s) in daycare at age 3 months[c]			
No[e]	1.0	1.0	
Yes, effect at age 3 months	2.45 (1.89–3.18)	2.46 (1.42–4.25)	0.001
Yes, effect at age 12 months	(as above)	1.74 (1.02–2.96)	0.04
Daytime company of other children (number of children, continuous)	1.08 (1.05–1.12)	1.04 (1.00–1.07)	0.05
Household water supply[c,d]			0.16[f]
Public waterworks	1.0	1.0	
Well or borehole	1.42 (0.83–2.43)	1.58 (0.89–2.80)	
Private waterworks	0.77 (0.38–1.57)	0.53 (0.24–1.19)	
Small lake or brook	0.81 (0.20–3.38)	0.72 (0.15–3.45)	
Ownership of pets (dog/cat)[c,d]			
No	1.0	1.0	
Yes	0.85 (0.60–1.20)	0.72 (0.50–1.03)	0.07

[a]OR adjusted for random effects. Constant over age of sampling.
[b]OR mutually adjusted for all predictors listed in the table and the confounders HLA risk for type 1 diabetes, gender, age, season, year, county of residence, maternal age and education, and random effects. The interaction effects are equivalently reported as the OR of the main effect at two time points (age 3 and 12 months). P values are two sided.
[c]Reported at delivery or at 3-month questionnaire of index child.
[d]The missing data category ('NA') for respective covariate was retained in the multivariable analysis but is for simplicity excluded from the table, because results for these categories are not easily interpreted anyway.
[e]Sibling(s) is not attending daycare or child has no siblings.
[f]Overall P-value (for categorical variables with more than two categories).

Studie vlivu HLA genotypu na výskyt enterovirových infekcí nakonec ukázala cosi poměrně neočekávaného: sice mezi vysokorizikovým a všemi ostatními genotypy je rozdíl jen na hranici statistické signifikance, ale pokud genotypy stratifikujeme, dobereme se dobře vyjádřeného trendu. Ilustraci podává náš recentní poster (Immunology of Diabetes Society Meeting, 2010, Korea - článek je v přípravě): zde z něj uvádím jednu samovysvětlující tabulku.

Table 1. ORs from logistic regression analysis for the effect of HLA on enterovirus infection in monthly longitudinal stool samples from 819 children (403 children without the highest genetic risk) aged 3–12 months ($n = 6373$).

	Frequency of enterovirus infection (%)	Unadjusted OR	Adjusted OR[a] (95% CI)	P-value
HLA genetisk risiko for T1D				0.014[b]
Protective (at least one *DQB1*06:02* allele)	140/1048 (13.4%)	1.0 (Ref.)	1.0 (Ref.)	
Neutral[&]	125/984 (12.7%)	0.95 (0.73-1.23)	1.04 (0.71-1.54)	
High/moderate risk[#]	127/1115 (11.4%)	0.83 (0.64-1.08)	0.87 (0.59-1.27)	
Very high risk[*]	342/3258 (10.5%)	0.76 (0.62-0.94)	0.73 (0.53-1.00)	

[*]*HLA-DRB1*04:01-DQA1*03-DQB1*03:02 (DQ8)/DRB1*03:01-DQA1*05-DQB1*02 (DQ2).*
[#]*DQ2/DQ2, DQ2/X, DQ8/DQ8, DQ8/X.*
[&]All other genotypes, including weakly protective groups (*DQA1*-DQB1**): 01:02-06:03, 01:03-06:03, 02:01-03:01, 02:01-03:03.
[a]OR mutually adjusted for age (months), season-month and no of other children in the family. P values are 2 sided.
[b]Overall P-value (test for trend).

6.1.4   Logistika studie

Další práce popisuje design studie, sběr vzorků a výskyt autoprotilátek v této studii.

- Stene LC, Witsø E, Torjesen PA, Rasmussen T, Magnus P, Cinek O, Wetlesen T, Rønningen KS. Islet autoantibody development during follow-up of high-risk children from the general Norwegian population from three months of age: design and early results from the MIDIA study. **J Autoimmun**. 2007 Aug;29(1):44-51.

Důvod mého spoluautorství se datuje poměrně dlouho do minulosti, kdy jsem se podílel na zavedení genotypizačních metod pro identifikaci dětí s nejvyšším genetickým rizikem z norské obecné novorozenecké populace - to je popsáno v tomto článku z roku 2000.

- Cinek O, Wilkinson E, Paltiel L, Saugstad OD, Magnus P, Rønningen KS. Screening for the IDDM high-risk genotype. A rapid microtitre plate method using serum as source of DNA. **Tissue Antigens**. 2000;56(4):344-9.

Obdobný důvod mělo autorství na další publikaci, tentokrát o mateřských parametrech jako prediktorech objevení se prediabetických autoprotilátek.

- Rasmussen T, Stene LC, Samuelsen SO, Cinek O, Wetlesen T, Torjesen PA, Rønningen KS. Maternal BMI before pregnancy, maternal weight gain during pregnancy, and risk of persistent positivity for multiple diabetes-associated autoantibodies in children with the high-risk HLA genotype: the MIDIA study. **Diabetes Care**. 2009 Oct;32(10):1904-6.

6.1.5 Výskyt parechoviru

Parechovirus ("par-ECHO-virus") je rod z rodiny *Picornaviridae*, tedy na úrovni rodu *Enterovirus*. Obsahuje několik lidských parechovirů a virus Ljungan. Lidských parechovirů rok od roku přibývá, nicméně jejich patogenetické schopnosti nejsou zcela jasné a ani výskyt nebyl až do naší studie systematicky studován pomocí molekulární detekce.

- Tapia G, Cinek O, Witsø E, Kulich M, Rasmussen T, Grinde B, Rønningen KS. Longitudinal observation of parechovirus in stool samples from Norwegian infants. **J Med Virol**. 2008 Oct;80(10):1835-42. **(přiloženo in extenso, př. V)**

V této studii jsme použili vzorky 51 dětí nesoucích vysokorizikový genotyp pro diabetes, které byly párovány s 51 dětmi nesoucími jiný genotyp (t.j. s nižším rizikem diabetu). Celkem bylo analyzováno 1941 vzorků stolice; výsledky byly vztaženy k systematicky sbíraným informacím z pravidelně vyplňovaných dotazníků.

Parechovirus jsme pozorovali v něco přes desetině vzorků. Kvantity vykazovaly plynulé rozložení od něco přes jednu kopii na mikrolitr, až po 100 000 kopií na mikrolitr extrahované nukleové kyseliny.
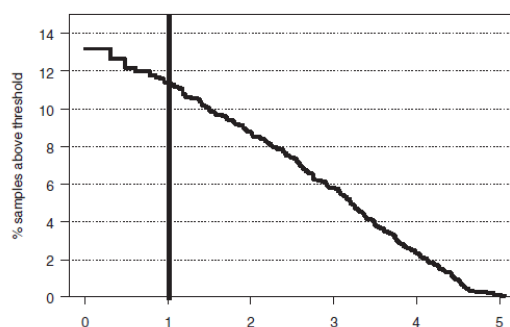

Fig. 1. Distribution of the concentration of human parechovirus found in the stool samples. The quantity is expressed as the number of viral genomes per µl of extracted RNA. Only samples exceeding 10 copies/µl extracted RNA (log = 1, marked with a vertical line) were included as positive in further analyses.

Pozorovali jsme výraznou sezonalitu infekcí a také věkovou závislost. Nenalezli jsme asociaci se symptomy: stejně jako u enterovirů se nezdá, že by jednotlivé infekce bylo možno spojit s teplotou, kašlem, rýmou, průjmem nebo zvracením.

Výskyt parechoviru nezávisel na genetickém riziku diabetu, nicméně stejně jako u enteroviru existovala *tendence* k tomu, že děti s vysokorizikovým genotypem měly o poznání nižší výskyt viru. U parechoviru byl efekt nesignifikantní, p=0.07 - navíc po řádné adjustaci na všechny zavádějící faktory efekt zcela vymizel (p=0.16). Než bude možný efekt genotypu odmítnut, je třeba si uvědomit, že v těchto dvou studiích jsme srovnávali jedince s nejvyšším definovatelným HLA rizikem pro diabetes proti skupině všech ostatních genotypů. I zde, podobně jako jsme učinili u enteroviru, by stálo za to podívat se blíže jednotlivé úrovně genetického rizika.

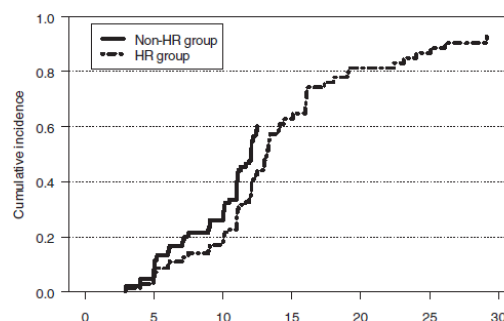Fig. 4. Cumulative incidence of parechovirus by the genetic risk for type 1 diabetes. The children carrying the high-risk diabetes genotype (HR) were followed until 36 months of age, while the children carrying other HLA genotypes (non-HR) were only followed between 3 and 12 months. The end of the periods are not included due to a less complete dataset. The difference between the two groups was not significant (P = 0.07).

6.1.6 Absence *Ljungan* viru ve stolici norských dětí

- Tapia G, Cinek O, Rasmussen T, Grinde B, Rønningen KS. No Ljungan virus RNA in stool samples from the Norwegian environmental triggers of type 1 diabetes (MIDIA) cohort study. **Diabetes Care**. 2010 May;33(5):1069-71. **(přiloženo in extenso, př. VI)**

Ljungan virus je parechovirus nalézaný u hlodavců, kde způsobuje diabetes, myokarditidy a intrauterinní úmrtí. U lidí byla infekce asociována s intrauterinním úmrtím, SIDS, anencefalií a poněkud spekulativně i s diabetem 1. typu. Naše studie se snažila Ljungan virus najít ve stolici u dětí, které měly nejvyšší genetické riziko diabetu a projevy prediabetické autoimunity, i u dalších dětí, ať již s nejvyšším genetickým rizikem nebo jinými genotypy. Protože Ljungan virus je parechovirus, ve stolici by se pravděpodobně měl vyskytovat.

Vyšetřili jsme jednak 2054 vzorků ze studie případů a kontrol (děti s prediabetickými autoprotilátkami versus děti bez těchto autoprotilátek, 2:1, vyrovnané podle doby místa narození, všichni stejného vysokorizikového genotypu), plus předtím vyšetřených 1941 vzorků od 102 dětí ze studie parechoviru. Rozložení vzorků je zachyceno na obrázku vpravo.

Figure 1: Age distribution of samples

Ljungan virus jsme ve stolici nenašli, ani v jednom vzorku; přitom efektivita assaye byla doložena konzistentně detekovanou exogenní vnitřní kontrolou i pozitivními kontrolami v každém běhu PCR.
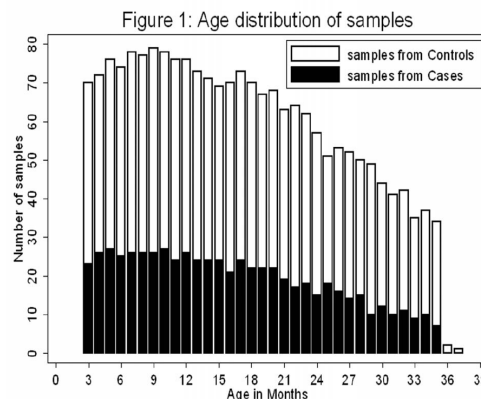
Nelze sice vyloučit, že právě virus Ljungan se množí jinde než ostatní parechoviry, takže by nebylo možné jej nalézt ve stolici, nicméně není to příliš pravděpodobné. Aby mohl virus být odpovědný alespoň za část případů diabetu, musel by se u těchto dětí vyskytovat. Infekce mohou být také geograficky specifické; spíše se však zdá, že infekce virem Ljungan je výjimečný jev pozorovaný u člověka pouze v materiálech z potratů.

6.1.7 Studie případů a kontrol o vlivu enteroviru a parechoviru na riziko prediabetické autoimunity

Dva naše recentní články publikované v Diabetes Care a Pediatric Diabetes ukazují první výsledky ze studie MIDIA: analýzu případů s prediabetickými autoprotilátkami proti kontrolám shodného genotypu, data a místa narození, ale bez prediabetu.

Byly sledovány pozitivita a kvantita enterovirů a parechovirů ve stolici: vzorky stolice, jak již bylo zmíněno výše, byly odebírány v pravidelných měsíčních intervalech. Testování probíhalo v naší laboratoři v Praze.

- Tapia G, Cinek O, Rasmussen T, Witsø E, Grinde B, Stene LC, Rønningen KS. Human Enterovirus RNA in monthly fecal samples and islet autoimmunity in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study. **Diabetes Care**. elektronicky 2010 Oct 7, v tisku. **(přiloženo in extenso, př. VII)**

- Tapia G, Cinek O, Rasmussen T, Grinde B, Stene LC, Rønningen KS. Longitudinal study of parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study, **Pediatric Diabetes**, v tisku **(přiloženo in extenso, př. VIII)**

Subjekty ve studii MIDIA jsou děti identifikované novorozeneckým screeningem podle přítomnosti genotypu HLA-DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02. Poté byla tato kohorta (911 dětí) sledována pomocí odběru krve na autoprotilátky (čtvrtletně v prvním roce, pak ročně; více často při pozitivitě) a měsíčně odebíranými vzorky stolice od 3. do 35. měsíce.

Z 911 dětí mělo **27 pozitivní autoprotilátky** proti komponentám ostrůvků: kritériem byly dvě pozitivní autoprotilátky ze tří testovaných (anti-GAD65, anti-IA2, antiinzulínové) v alespoň dvou nezávislých následných náběrech. Ke každému takto definovanému případu byly přiřazeny dvě kontroly, které byly vybrány podle doby sledování (tím i doby narození, protože sledování probíhá od narození) a okresu pobytu. Tím byla snížena pravděpodobnost zavádějících faktorů

času a místa, které souvisí s místními epidemiemi pikornavirů. Kontrol bylo 53 (rodiče jednoho z dětí vystoupili během analýzy ze studie).

Autoprotilátky jako marker ostrůvkové autoimunity jsme stanovovali, jak bylo popsáno v naší výše uvedené publikaci [238] a enterovirus byl testován stejně jako v našich všech předchozích pracech.

Frekvence **enteroviru se nelišila** u případů (43/339 vzorků, 12,7%) proti kontrolám (94/692, 13,6%); **ani frekvence parechoviru nebyla odlišná** mezi případy (13,0%) a kontrolami (11,1%).

Analýzy podskupin neukázala žádné potenciální asociace mimo nejkratšího časového okna 3 měsíců před objevením se autoprotilátek a výskytu parechoviru ve stolici (20,8% u případů versus 8,8% u kontrol, odds ratio = 3.2, P=0,022).

Níže přetiskuji tabulku z článku o enteroviru, kde ukazujeme (nezvýšené) riziko prediabetu spojené s pozitivním vzorkem stolice a s novou infekční epizodou (definovanou jako série pozitivních vzorků stolice).

Table 2. Frequency of human enterovirus RNA in fecal samples collected prior to islet autoimmunity.

	Cases	Controls	Odds ratio (95% CI)*	
	(n=27 subjects)	(n=53 subjects)	Unadjusted	Adjusted†
Enterovirus RNA **negative** samples	296	598	1.00 (reference)	1.00 (reference)
Enterovirus RNA **positive** samples	43 (12.7%)	94 (13.6%)	1.01 (0.59 - 1.72)	1.09 (0.61 - 1.96)
Total	339	692		
New enterovirus infection episode: No	296	598	1.00 (reference)	1.00 (reference)
New enterovirus infection episode: Yes	30 (9.2%)	65 (9.8%)	0.94 (0.59 - 1.52)	0.92 (0.54 - 1.57)
Total ‡	326	663		

* Odds ratio with 95% confidence interval (CI) estimated from logistic mixed effects logistic regression models with random intercept for each subject to control for intra-individual correlation (no significant random intercept in model for enterovirus episodes, but highly significant in model for enterovirus positivity). The unadjusted odds ratio in ordinary logistic regression ignoring intra-individual correlation in infections was 0.92.
† Adjusted for sex, calendar month of sample collection, year of sample collection (2001-3, 2004-6 or 2007-8), age (continuous), number of siblings (0 vs. >=1), breast-feeding, first degree family history of type 1 diabetes (yes/no).
‡ Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

Absence asociace s enterovirem ve stolici je obzvláště zajímavá s ohledem na diskrepanci s výsledky serologických studií. Naše data ze stolice - ve shodě s jinými studiemi [180, 182] - poukazují na to, že objasnění možné asociace mezi enteroviry a ostrůvkovou autoimunitou musí spočívat jinde než v místě primární replikace viru, jinde než ve střevě. Patrně existují některé vlastnosti určitých kmenů viru nebo vlastnosti hostitele, které jsou nutné k tomu, aby byly prolomeny linie imunitní obrany, aby vznikla signifikantní virémie a virus následně pronikl do cílových orgánů - v tomto případě pankreatu a jeho ostrůvků. Pokud to není sérotyp viru a doba jeho přítomnosti ve stolici, co pravděpodobně způsobuje vyšší výskyt protilátek proti enteroviru u dětí s autoimunitní inzulitídou proti kontrolám? Nejpravděpodobněji je to **virová nálož v krvi a trvání virémie**, čímž se zabývá náš recentně zahájený projekt.

Děti ve studii a jejich vzorky stolice (přetištěno z výše uvedeného článku o studii enteroviru)

Na obrázku je zachyceno 27 trojic případů a kontrol - trojice jsou srovnány sestupně podle věku při diagnóze prediabetické autoimunity a časová osa je vyrovnána k datu diagnózy prediabetické autoimunity. Sledování každého dítěte je znázorněno pomocí obdélníku okolo série kroužků odpovídajících jednotlivým vzorkům stolice. Levý okraj obdélníku odpovídá narození, pravý okraj odpovídá poslednímu získanému vzorku stolice (buďto 35. měsíci života, kdy se přestaly vzorky stolic odebírat, ebo 17.8.2008, kdy byl odebrán poslední vzorek analyzovaný v této studii).

Prázdné kroužky odpovídají negativním vzorkům stolice, šedé nízké až střední kvantitě enterovirové RNA, černé pak vysoké kvantitě enterovirové RNA ve stolici. Trojúhleníky ukazují datum diagnózy autoimunity, t.j. první ze vzorků s GAD, IA2 či IAA, který byl následován opakovanou pozitivitou na dvě nebo více z těchto autoprotilátek. Černé čtverce odpovídají datu diagnózy diabetu; diabetes jsme pozorovali do 1.9.2009 u 10 z těchto dětí. Kontrola 2 ze skupiny 27 zrušila svou účast ve studii, proto v analýze její data chybí.

54
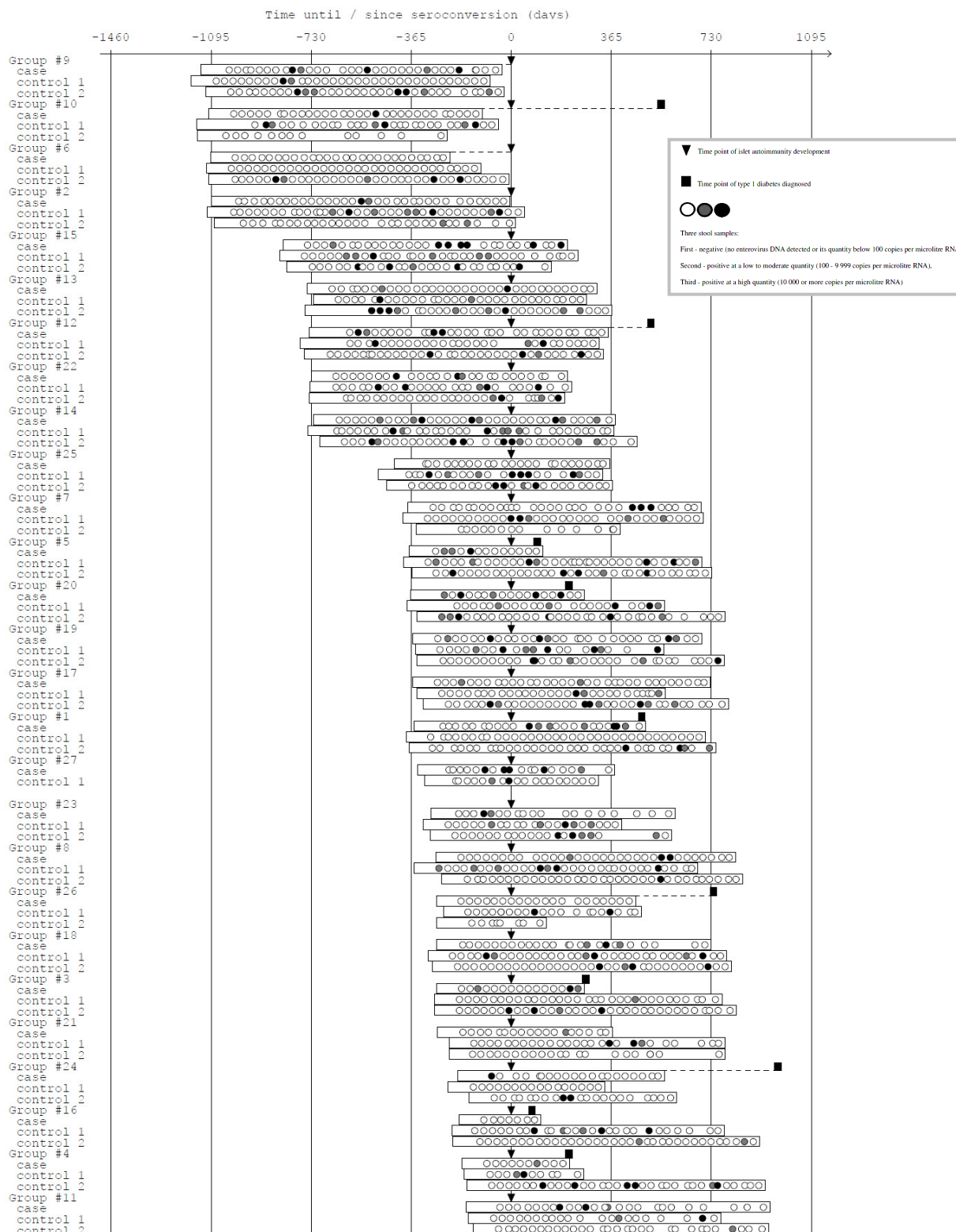
6.2 Genetické asociační studie

Od roku 2000 až do loňského roku jsme prováděli asociační studie různých multifaktoriálních pediatrických onemocnění, hlavně diabetu 1. typu a s ním asociovaných autoimunitních komplikací.

6.2.1 Diabetes 1. typu

Česká dětská populace byla stran asociace s diabetem charakterizována poměrně důkladně. Provedli jsme asociační studii s HLA 2. třídy ...

- Cinek O, Kolouskova S, Snajderová M, Sumník Z, Sedláková P, Drevínek P, Vavrinec J, Ronningen KS. HLA class II genetic association of type 1 diabetes mellitus in Czech children. **Pediatr Diabetes**. 2001 Sep;2(3):98-102. **(přiloženo in extenso, př. IX)**

... i s *CTLA 4*, *NEUROD1*, inzulínovým genem a *PTPN22*; posledně jmenovaný přikládám in extenso - jedná se o odpověď na tezi, že jiné polymorfismy než R620W určují riziko diabetu

- Cinek O, Drevínek P, Sumník Z, Bendlová B, Kolouskova S, Snajderová M, Vavrinec J. The CTLA4 +49 A/G dimorphism is not associated with type 1 diabetes in Czech children. **Eur J Immunogenet**. 2002 Jun;29(3):219-22.

- Cinek O, Drevínek P, Sumník Z, Bendlová B, Sedláková P, Kolouskova S, Snajderová M, Vavrinec J. NEUROD polymorphism Ala45Thr is associated with Type 1 diabetes mellitus in Czech children. **Diabetes Res Clin Pract**. 2003 Apr;60(1):49-56.

- Cinek O, Hradsky O, Ahmedov G, Slavcev A, Kolouskova S, Kulich M, Sumnik Z. No independent role of the -1123 G>C and+2740 A>G variants in the association of PTPN22 with type 1 diabetes and juvenile idiopathic arthritis in two Caucasian populations. **Diabetes Res Clin Pract**. 2007 May;76(2):297-303. **(přiloženo in extenso, př. X)**

Článek o inzulínovém genu vyhrál výroční cenu Časopisu lékařů českých pro nejlepší práci:

- Cinek O, Drevínek P, Sumník Z, Bendlová B, Vavrinec J. Asociace variant inzulinového genu s diabetes mellitus 1. typu v české dětské populaci. **Cas Lek Cesk**. 2004;143(5):318-22.

Inzulínového genu se týkala ještě jedna práce, jejímiž hlavními autory byli kolegové z Endokrinologického ústavu, já jsem práci poskytl technickou podporu.

- Vanková M, Vrbíková J, Hill M, Cinek O, Bendlová B. Association of insulin gene VNTR polymorphism with polycystic ovary syndrome. **Ann N Y Acad Sci**. 2002 Jun;967:558-65.

V naší laboratoři jsme měli hosta z Azerbajdžánu, který zde pod naším vedením provedl asociační studii u jejich populace.

- Ahmedov G, Ahmedova L, Sedlakova P, Cinek O. Genetic association of type 1 diabetes in an Azerbaijanian population: the HLA-DQ, -DRB1*04, the insulin gene, and CTLA4. **Pediatr Diabetes**. 2006 Apr;7(2):88-93.

6.2.2 Juvenilní idiopatická artritida

Provedli jsme technicky poměrně obtížnou asociační studii JIA s polymorfismy cytokinových genů - typizace byla prováděna pomocí PCR-SSP. Získané technické dovednosti jsme pak použili ve studii o genetickém riziku celiakie u diabetických dětí. Studie o JIA patří k našim lépe citovaným, čemuž se dodnes poněkud divím.

- Cinek O, Vavrincová P, Striz I, Drevínek P, Sedláková P, Vavrinec J, Slavcev A. Association of single nucleotide polymorphisms within cytokine genes with juvenile idiopathic arthritis in the Czech population. **J Rheumatol**. 2004 Jun;31(6):1206-10.

6.2.3 Crohnova nemoc

V rámci supervize PhD studia Dr. Ondřeje Hradského a magisterské diplomové práce Mgr. Petry Dušátkové vznikly tři práce o Crohnově nemoci, které se vyznačují velmi dobře zvládnutou statistikou. Práce byly publikovány jako naše poslední asociační studie - poté již bylo zřejmé, že s nástupem vysokokapacitních metod se stává publikace klasických prostých studií kandidátních genů obtížnější.

- Hradsky O, Lenicek M, Dusatkova P, Bronsky J, Nevoral J, Valtrova V, Kotalova R, Szitanyi P, Petro R, Starzykova V, Bortlik M, Vitek L, Lukas M, Cinek O. Variants of CARD15, TNFA and PTPN22 and susceptibility to Crohn's disease in the Czech population: high frequency of the CARD15 1007fs. **Tissue Antigens**. 2008 Jun;71(6):538-47.

- Dusatkova P, Hradsky O, Lenicek M, Bronsky J, Nevoral J, Kotalova R, Bajerova K, Vitek L, Lukas M, <u>Cinek O</u>. Association of IL23R p.381Gln and ATG16L1 p.197Ala with Crohn disease in the Czech population. **J Pediatr Gastroenterol Nutr**. 2009 Oct;49(4):405-10.
- Hradsky O, Dusatkova P, Lenicek M, Bronsky J, Nevoral J, Vitek L, Lukas M, Zeniskova I, <u>Cinek O</u>. The CTLA4 variants may interact with the IL23R- and NOD2-conferred risk in development of Crohn's disease. **BMC Med Genet**. 2010 Jun 10;11:91.

6.3 Autoimunitní komplikace dětského DM1: výskyt a genetika

Na konci poslední dekády minulého století jsme provedli průřezovou studii s cílem zjistit prevalenci celiakie u našich diabetických dětí a testovat, zda se liší rozložení HLA u diabetických dětí s a bez celiakie. Zjistili jsme, že děti s diabetem a celiakií mají častěji HLA-DQB1*02-DQA1*05 než děti s diabetem, ale bez celiakie.

- Sumník Z, Kolousková S, <u>Cinek O</u>, Kotalová R, Vavrinec J, Snajderová M. HLA-DQA1*05-DQB1*0201 positivity predisposes to coeliac disease in Czech diabetic children. **Acta Paediatr**. 2000 Dec;89(12):1426-30.

K tématu jsme se vrátili za několik let, kdy jsme podstatně vylepšili design studie, dosáhli podstatného zvětšení souboru pomocí mezinárodní spolupráce a testovali více genetických faktorů, zejména cytokinových polymorfismů.

- Sumnik Z, <u>Cinek O</u>, Bratanic N, Kordonouri O, Kulich M, Roszai B, Arato A, Lebl J, Soltesz G, Danne T, Battelino T, Schober E. Risk of celiac disease in children with type 1 diabetes is modified by positivity for HLA-DQB1*02-DQA1*05 and TNF -308A. **Diabetes Care**. 2006 Apr;29(4):858-63. **(přiloženo in extenso, př. XI)**

Další práce se zabývala výskytem celiakie u sourozenců dětí s diabetem - tentokrát jsme si stačili s domácími pacienty, genetické vyšetřování bylo omezeno - ale také jsme skončili v o něco horším časopise.

- Sumnik Z, Kolouskova S, Malcova H, Vavrinec J, Venhacova J, Lebl J, <u>Cinek O</u>. High prevalence of coeliac disease in siblings of children with type 1 diabetes. **Eur J Pediatr**. 2005 Jan;164(1):9-12.

Zatím poslední ze série článků o celiakii pojednává o komorbiditě autoimunitní tyroiditis, DM1 a celiakie a jejich vlivu na kompenzaci diabetu.

- Sumnik Z, <u>Cinek O</u>, Bratanic N, Lebl J, Rozsai B, Limbert C, Paskova M, Schober E. Thyroid autoimmunity in children with coexisting type 1 diabetes mellitus and celiac disease: a multicenter study. **J Pediatr Endocrinol Metab**. 2006 Apr;19(4):517-22.

Okrajově jsme se zabývali také tyroiditidou a jejím genetickým rizikem u diabetických dětí.

- Sumník Z, Drevínek P, Snajderová M, Kolousková S, Sedláková P, Pechová M, Vavrinec J, <u>Cinek O</u>. HLA-DQ polymorphisms modify the risk of thyroid autoimmunity in children with type 1 diabetes mellitus. **J Pediatr Endocrinol Metab**. 2003 Jul-Aug;16(6):851-8.

6.4 Monogenní diabetes a jiné monogenní choroby

V naší laboratoři jsme zavedli kompletní vyšetřování všech genů, jejichž defekty se reálně u monogenních diabetů vyskytují. O tuto tématiku jsem se staral spíše jako technická podpora, jak při laboratorních pracech, tak při psaní některých publikací.

Ve dvou rodinách jsme nalezli mutaci v genu *NEUROD1*, která patrně významně modifikuje pravděpodobnost diabetu.

- Gonsorcíková L, Průhová S, <u>Cinek O</u>, Ek J, Pelikánová T, Jørgensen T, Eiberg H, Pedersen O, Hansen T, Lebl J. Autosomal inheritance of diabetes in two families characterized by obesity and a novel H241Q mutation in NEUROD1. **Pediatr Diabetes**. 2008 Aug;9(4 Pt 2):367-72.

Nalezli jsme také mutace v inzulínovém genu u pacientů s MODY:

- Boesgaard TW, Pruhova S, Andersson EA, <u>Cinek O</u>, Obermannova B, Lauenborg J, Damm P, Bergholdt R, Pociot F, Pisinger C, Barbetti F, Lebl J, Pedersen O, Hansen T. Further evidence that mutations in INS can be a rare cause of Maturity-Onset Diabetes of the Young (MODY). **BMC Med Genet**. 2010 Mar 12;11:42.

A charakterizovali jsme výskyt mutací v rozsáhlé sbírce MODY2 (glukokinázového) diabetu, včetně nálezu pravděpodobných ancestrálních mutací.

- Pruhova S, Dusatkova P, Sumnik Z, Kolouskova S, Pedersen O, Hansen T, <u>Cinek O</u>, Lebl J. Glucokinase diabetes in 103 families from a country-based study in the Czech Republic: geographically restricted distribution of two prevalent GCK mutations. **Pediatr Diabetes**. 2010 Mar 10. (v tisku, vyšlo zatím elektronicky)

Podali jsme zprávu o absenci mutací v genu PAX4 v poměrně rozsáhlém souboru MODY-X rodin.

- Dusatkova P, Vesela K, Pruhova S, Lebl J, <u>Cinek O</u>.Lack of PAX4 mutations in 53 Czech MODYX families. **Diabet Med**. 2010 Dec;27(12):1459-60

Publikovali jsme charakteristiku atypického klinického obrazu mutace v genu *ABCC8*

- Gonsorcikova L, Vaxillaire M, Pruhova S, Dechaume A, Dusatkova P, <u>Cinek O</u>, Pedersen O, Froguel P, Hansen T, Lebl J. Familial mild hyperglycemia associated with a novel ABCC8-V84I mutation within three generations. **Pediatric Diabetes**, (v tisku)

Podali jsme také zprávu o chybějícím efektu obvyklé terapie u jedné z geneticky nejtěžších forem DEND syndromu.

- Sumnik Z, Kolouskova S, Wales JK, Komarek V, <u>Cinek O</u>. Sulphonylurea treatment does not improve psychomotor development in children with KCNJ11 mutations causing permanent neonatal diabetes mellitus accompanied by developmental delay and epilepsy (DEND syndrome). **Diabet Med**. 2007 Oct;24(10):1176-8.

Ostatní endokrinní choroby byly spíše na okraji mého zájmu - dvě publikace se týkaly Pendredova syndromu:

- Banghova K, Al Taji E, <u>Cinek O</u>, Novotna D, Pourova R, Zapletalova J, Hnikova O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel PDS/SLC26A4 mutations. **Eur J Pediatr**. 2008 Jul;167(7):777-83.

- Banghova K, <u>Cinek O</u>, Al Taji E, Zapletalova J, Vidura R, Lebl J. Thyroidectomy in a patient with multinodular dyshormonogenetic goitre--a case of Pendred syndrome confirmed by mutations in the PDS/SLC26A4 gene. **J Pediatr Endocrinol Metab**. 2008 Dec;21(12):1179-84.

Jedna se týkala defektu genu pro kalcium sensing receptor

- Obermannova B, Banghova K, Sumník Z, Dvorakova HM, Betka J, Fencl F, Kolouskova S, <u>Cinek O</u>, Lebl J. Unusually severe phenotype of neonatal primary hyperparathyroidism due to a heterozygous inactivating mutation in the CASR gene. **Eur J Pediatr**. 2009 May;168(5):569-73.

A další publikace o monogenní chorobě se týkala steroid-rezistentního nefrotického syndromu, respektive efektu cyklosporinu u pacienta s touto diagnózou:

- Malina M, <u>Cinek O</u>, Janda J, Seeman T. Partial remission with cyclosporine A in a patient with nephrotic syndrome due to NPHS2 mutation. **Pediatr Nephrol**. 2009 Oct;24(10):2051-3.

6.5   *Výskyt dětského diabetu u nás*

Jako první jsme publikovali incidenční data z naší země. Tématiky incidence a jejích trendů se týkaly zatím tři články:

- <u>Cinek O</u>, Lánská V, Kolousková S, Sumník Z, Snajderová M, Rønningen KS, Vavrinec J. Type 1 diabetes mellitus in Czech children diagnosed in 1990-1997: a significant increase in incidence and male predominance in the age group 0-4 years. Collaborators of the Czech Childhood Diabetes Registry. **Diabet Med**. 2000 Jan;17(1):64-9.

- <u>Cinek O</u>, Sumnik Z, Vavrinec J. Continuing increase in incidence of childhood-onset type 1 diabetes in the Czech Republic 1990-2001. **Eur J Pediatr**. 2003 Jun;162(6):428-9. **(přiloženo in extenso, př. XII)**

- <u>Cinek O</u>, Sumník Z, Vavrinec J. Dětský diabetes mellitus v České republice: stále více a čím dál dříve. **Cas Lek Cesk**. 2005;144(4):266-71; discussion 271-2. Czech.

6.6   *Rizikové faktory dětského diabetu nahlížené studiemi případů a kontrol*

Provedli jsme poměrně velkou studii případů a kontrol, která měla za cíl testovat asociaci kandidátních negenetických faktorů s diabetem:

- Malcova H, Sumnik Z, Drevinek P, Venhacova J, Lebl J, <u>Cinek O</u>. Absence of breast-feeding is associated with the risk of type 1 diabetes: a case-control study in a population with rapidly increasing incidence. **Eur J Pediatr**. 2006 Feb;165(2):114-9. **(přiloženo in extenso, př. XIII)**

S využitím populačních dat jsme provedli také studii o věku matek a pořadí narození jako rizikových faktorech diabetu:

- Sumnik Z, Drevinek P, Lanska V, Malcova H, Vavrinec J, <u>Cinek O</u>. Higher maternal age at delivery, and lower birth orders are associated with increased risk of childhood type 1 diabetes mellitus. **Exp Clin Endocrinol Diabetes**. 2004 Jun;112(6):294-7.

Dále se naše data stala součástí několika metaanalýz těchto rizikových faktorů; tři z nich zatím byly publikovány:

- Cardwell CR, Stene LC, Joner G, Cinek O, Svensson J, Goldacre MJ, Parslow RC, Pozzilli P, Brigis G, Stoyanov D, Urbonaite B, Sipetić S, Schober E, Ionescu-Tirgoviste C, Devoti G, de Beaufort CE, Buschard K, Patterson CC. Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies. **Diabetologia**. 2008 May;51(5):726-35. **(přiloženo in extenso, př. XIV)**

- Cardwell CR, Stene LC, Joner G, Bulsara MK, Cinek O, Rosenbauer J, Ludvigsson J, Jané M, Svensson J, Goldacre MJ, Waldhoer T, Jarosz-Chobot P, Gimeno SG, Chuang LM, Parslow RC, Wadsworth EJ, Chetwynd A, Pozzilli P, Brigis G, Urbonaite B, Sipetic S, Schober E, Devoti G, Ionescu-Tirgoviste C, de Beaufort CE, Stoyanov D, Buschard K, Patterson CC. Maternal age at birth and childhood type 1 diabetes: a pooled analysis of 30 observational studies. **Diabetes**. 2010 Feb;59(2):486-94.

- Cardwell CR, Stene LC, Joner G, Davis EA, Cinek O, Rosenbauer J, Ludvigsson J, Castell C, Svensson J, Goldacre MJ, Waldhoer T, Polanska J, Gimeno SG, Chuang LM, Parslow RC, Wadsworth EJ, Chetwynd A, Pozzilli P, Brigis G, Urbonaite B, Sipetić S, Schober E, Ionescu-Tirgoviste C, de Beaufort CE, Stoyanov D, Buschard K, Patterson CC. Birthweight and the risk of childhood-onset type 1 diabetes: a meta-analysis of observational studies using individual patient data. **Diabetologia**. 2010 Apr;53(4):641-51.

6.7 Molekulární metody v detekci a typizaci patogenů mimo diabetologický výzkum

Díky supervizi postgraduálních studentů a pozdější spolupráci s nimi po dokončení studia jsem měl příležitost technicky a metodicky podporovat velice zajímavou práci Pavla Dřevínka na bakteriích u cystické fibrózy a Petra Hubáčka na virech u transplantovaých.

Metodiky zde použité jsou často blízko tomu, co bylo v jiných našich pracech použito pro diabetes, ať už je to kvantifikace RNA virů nebo sekvenace genů či alelicky specifické PCR. Publikace uvádím výčtem bez komentáře.

6.7.1 Viry u transplantovaných

- Hubácek P, Cinek O, Kulich M, Zajac M, Keslová P, Formánková R, Starý J, Sedlácek P. Kvantifikace EBV u dětí po alogenní transplantaci hematopoetických kmenových buněk. **Cas Lek Cesk**. 2006;145(4):301-6. Czech.

- Hubacek P, Sedlacek P, Keslova P, Formankova R, Stary J, Kulich M, Cinek O. Incidence of HHV7 in donors and recipients of allogeneic hematopoietic stem cell transplantation. **Pediatr Blood Cancer**. 2008 Apr;50(4):935; author reply 936.

- Hubacek P, Maalouf J, Zajickova M, Kouba M, Cinek O, Hyncicova K, Fales I, Cetkovsky P. Failure of multiple antivirals to affect high HHV-6 DNAaemia resulting from viral chromosomal integration in case of severe aplastic anaemia. **Haematologica**. 2007 Oct;92(10):e98-e100.

- Hubacek P, Hyncicova K, Muzikova K, Cinek O, Zajac M, Sedlacek P. Disappearance of pre-existing high HHV-6 DNA load in blood after allogeneic SCT. **Bone Marrow Transplant**. 2007 Oct;40(8):805-6.

- Hubacek P, Virgili A, Ward KN, Pohlreich D, Keslova P, Goldova B, Markova M, Zajac M, Cinek O, Nacheva EP, Sedlacek P, Cetkovsky P. HHV-6 DNA throughout the tissues of two stem cell transplant patients with chromosomally integrated HHV-6 and fatal CMV pneumonitis. **Br J Haematol**. 2009 May;145(3):394-8.

- Hubacek P, Muzikova K, Hrdlickova A, Cinek O, Hyncicova K, Hrstkova H, Sedlacek P, Stary J. Prevalence of HHV-6 integrated chromosomally among children treated for acute lymphoblastic or myeloid leukemia in the Czech Republic. **J Med Virol**. 2009 Feb;81(2):258-63.

- Hubacek P, Keslova P, Formankova R, Pochop P, Cinek O, Zajac M, Lochmanova J, Stary J, Sedlacek P. Cytomegalovirus encephalitis/retinitis in allogeneic haematopoietic stem cell transplant recipient treated successfully with combination of cidofovir and foscarnet. **Pediatr Transplant**. 2009 Nov;13(7):919-22.

6.7.2 Bakterie u cystické fibrózy

- Drevínek P, Hrbácková H, Cinek O, Bartosová J, Nyc O, Nemec A, Pohunek P. Direct PCR detection of Burkholderia cepacia complex and identification of its genomovars by using sputum as source of DNA. **J Clin Microbiol**. 2002 Sep;40(9):3485-8.

- Drevinek P, Cinek O, Melter J, Langsadl L, Navesnakova Y, Vavrova V. Genomovar distribution of the Burkholderia cepacia complex differs significantly between Czech and Slovak patients with cystic fibrosis. **J Med Microbiol**. 2003 Jul;52(Pt 7):603-4.

- Drevinek P, Vosahlikova S, Cinek O, Vavrova V, Bartosova J, Pohunek P, Mahenthiralingam E. Widespread clone of Burkholderia cenocepacia in cystic fibrosis patients in the Czech Republic. **J Med Microbiol**. 2005 Jul;54(Pt 7):655-9.

- Vosahlikova S, Drevinek P, Cinek O, Pohunek P, Maixnerova M, Urbaskova P, van den Reijden TJ, Dijkshoorn L, Nemec A. High genotypic diversity of Pseudomonas aeruginosa strains isolated from patients with cystic fibrosis in the Czech Republic. **Res Microbiol**. 2007 May;158(4):324-9.

- Drevinek P, Vosahlikova S, Dedeckova K, Cinek O, Mahenthiralingam E. Direct culture-independent Strain typing of Burkholderia cepacia complex in sputum samples from patients with cystic fibrosis. **J Clin Microbiol**. 2010 May;48(5):1888-91.

7 Seznam prací přiložených v plném rozsahu

Z výše uvedených prací přikládám v plném znění následující:

I. Cinek O, Witsø E, Jeansson S, Rasmussen T, Drevinek P, Wetlesen T, Vavrinec J, Grinde B, Rønningen KS. Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. **J Clin Virol**. 2006 Jan;35(1):33-40.

II. Witsø E, Palacios G, Cinek O, Stene LC, Grinde B, Janowitz D, Lipkin WI, Rønningen KS. High prevalence of human enterovirus A infections in natural circulation of human enteroviruses. **J Clin Microbiol**. 2006 Nov;44(11):4095-100. Epub 2006 Aug 30.

III. Witsø E, Palacios G, Rønningen KS, Cinek O, Janowitz D, Rewers M, Grinde B, Lipkin WI. Asymptomatic circulation of HEV71 in Norway. **Virus Res**. 2007 Jan;123(1):19-29.

IV. Witsø E, Cinek O, Aldrin M, Grinde B, Rasmussen T, Wetlesen T, Rønningen KS. Predictors of sub-clinical enterovirus infections in infants: a prospective cohort study. **Int J Epidemiol**. 2010 Apr;39(2):459-68.

V. Tapia G, Cinek O, Witsø E, Kulich M, Rasmussen T, Grinde B, Rønningen KS. Longitudinal observation of parechovirus in stool samples from Norwegian infants. **J Med Virol**. 2008 Oct;80(10):1835-42.

VI. Tapia G, Cinek O, Rasmussen T, Grinde B, Rønningen KS. No Ljungan virus RNA in stool samples from the Norwegian environmental triggers of type 1 diabetes (MIDIA) cohort study. **Diabetes Care**. 2010 May;33(5):1069-71.

VII. Tapia G, Cinek O, Rasmussen T, Witsø E, Grinde B, Stene LC, Rønningen KS. Human enterovirus RNA in monthly fecal samples and islet autoimmunity in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study. **Diabetes Care**. elektronicky 2010 Oct 7, v tisku.

VIII. Tapia G, Cinek O, Rasmussen T, Grinde B, Stene LC, Rønningen KS. Longitudinal study of parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study, **Pediatric Diabetes**, v tisku

IX. Cinek O, Kolousková S, Snajderová M, Sumník Z, Sedláková P, Drevínek P, Vavrinec J, Ronningen KS. HLA class II genetic association of type 1 diabetes mellitus in Czech children. **Pediatr Diabetes**. 2001 Sep;2(3):98-102.

X. Cinek O, Hradsky O, Ahmedov G, Slavcev A, Kolouskova S, Kulich M, Sumnik Z. No independent role of the -1123 G>C and+2740 A>G variants in the association of PTPN22 with type 1 diabetes and juvenile idiopathic arthritis in two Caucasian populations. **Diabetes Res Clin Pract**. 2007 May;76(2):297-303.

XI. Sumnik Z, Cinek O, Bratanic N, Kordonouri O, Kulich M, Roszai B, Arato A, Lebl J, Soltesz G, Danne T, Battelino T, Schober E. Risk of celiac disease in children with type 1 diabetes is modified by positivity for HLA-DQB1*02-DQA1*05 and TNF -308A. **Diabetes Care**. 2006 Apr;29(4):858-63.

XII. Cinek O, Sumnik Z, Vavrinec J. Continuing increase in incidence of childhood-onset type 1 diabetes in the Czech Republic 1990-2001. **Eur J Pediatr**. 2003 Jun;162(6):428-9.

XIII. Malcova H, Sumnik Z, Drevinek P, Venhacova J, Lebl J, Cinek O. Absence of breast-feeding is associated with the risk of type 1 diabetes: a case-control study in a population with rapidly increasing incidence. **Eur J Pediatr**. 2006 Feb;165(2):114-9.

XIV. Cardwell CR, Stene LC, Joner G, Cinek O, Svensson J, Goldacre MJ, Parslow RC, Pozzilli P, Brigis G, Stoyanov D, Urbonaite B, Sipetić S, Schober E, Ionescu-Tirgoviste C, Devoti G, de Beaufort CE, Buschard K, Patterson CC. Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies. **Diabetologia**. 2008 May;51(5):726-35.

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9. Cinek, O., Z. Sumnik, and J. Vavrinec, *Dětský diabetes mellitus v České republice: stále více a čím dál dříve.* Cas Lek Cesk, 2005. **144**(4): p. 266-71.
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11. Gale, E.A., *Latent autoimmune diabetes in adults: a guide for the perplexed.* Diabetologia, 2005. **48**(11): p. 2195-9.
12. Kyvik, K.O., et al., *The epidemiology of Type 1 diabetes mellitus is not the same in young adults as in children.* Diabetologia, 2004. **47**(3): p. 377-84.
13. Gale, E.A. and K.M. Gillespie, *Diabetes and gender.* Diabetologia, 2001. **44**(1): p. 3-15.
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Příloha I.

Cinek O, Witsø E, Jeansson S, Rasmussen T, Drevinek P, Wetlesen T, Vavrinec J, Grinde B, Rønningen KS.

Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes.

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Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes

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Abstract

Background: Enterovirus and adenovirus are common in infancy, causing mostly asymptomatic infections. However, even an asymptomatic infection may be associated with increased risk of development of certain chronic non-infectious diseases, as has been suggested for enterovirus and type 1 diabetes. Data on occurrence and course of the infections in infancy are therefore important for designing effective approaches towards study of the association.

Objectives: To estimate the frequency of enterovirus and adenovirus infections in Norwegian infants, to evaluate the duration of the infections, to investigate their association with symptoms, and to establish a robust procedure that will be used to study the association between these viruses and the development of auto-immunity leading to type 1 diabetes.

Study design: Parents of infants, recruited for a study on environmental triggers of type 1 diabetes, submitted monthly samples of infant faeces, as well as information on symptoms of infection. The samples were analysed for enterovirus and adenovirus using quantitative real-time PCR, and enterovirus-positive samples were sequenced.

Results: Enteroviruses were found in 142/1255 (11.3%), and adenoviruses in 138/1255 (11.0%) of stool samples. Approximately half of the infants were exposed to these viruses at least once during the first year of observation (period 3–14 months of age). The presence of adenovirus was associated with fever and with symptoms of cold but not with diarrhoea and vomiting. The enterovirus positivity was not associated with any symptoms.

Conclusions: The prevalence of enterovirus and adenovirus in longitudinally obtained faecal samples from infants is sufficiently high to enable studies of their association with chronic diseases. The present protocol for evaluating exposure to these viruses is well suited for large-scale efforts aimed at assessing possible long-term consequences, particularly in relation to type 1 diabetes.
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Keywords: Enterovirus; Adenovirus; PCR; Quantitative PCR; Infants; Faeces

1. Introduction

Enteroviruses are associated with a variety of paediatric diseases, yet the majority of infections are completely asymptomatic or cause only mild uncharacteristic symptoms. However, even an asymptomatic infection may be of importance in the aetiology of certain chronic non-infectious diseases. For type 1 diabetes, a link to enteroviruses seems likely (reviewed

in Hyoty and Taylor, 2002), but the nature and strength of such an association is still largely unknown.

Type 1 diabetes has a long preclinical stage lasting months to years, when the pancreatic beta cells are destroyed by auto-immune insulitis, a T cell mediated autoreactive process. Previous studies using serological evidence of infection at diabetes onset have not yielded consistent evidence for or against an aetiological role of enteroviruses, but many of these studies suffered from serious methodological problems (Green et al., 2004). More recent works using RT-PCR detection of enterovirus in whole blood or serum (Clements

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et al., 1995; Andreoletti et al., 1998), plasma and stool (Craig et al., 2003), or peripheral blood mononuclear cells (Yin et al., 2002) have demonstrated an increased frequency of enteroviruses in newly diagnosed diabetic patients compared to controls. The Finnish DIPP study has shown indications of increased number of enterovirus infections in children subsequently developing beta-cell auto-antibodies (i.e. pre-diabetic children) compared to matched control subjects by either detecting enterovirus RNA in sera (Lonnrot et al., 2000; Salminen et al., 2003), or using a combination of data from serology and RT-PCR detection in serum and stool (Salminen et al., 2004). Similarly, an excess of frequency of enterovirus RNA in serum of pre-diabetic individuals compared to controls was detected in another Finnish prospective study (Sadeharju et al., 2003). However, in the DAISY study from USA, enterovirus infection was equally frequent in individuals who became pre-diabetic as in controls (Graves et al., 2003). Moreover, a small study that focused on children after coxsackie B infection showed that the signs of diabetic auto-immunity found in some subjects were transient and disappeared within 1 year (Cainelli et al., 2000). Clearly, to elucidate the role of enterovirus in diabetes pathogenesis, further adequately designed and powered studies must be performed.

Such studies should observe cohorts of individuals at high genetic risk of type 1 diabetes, starting from birth or early infancy. While there is agreement on testing for pre-diabetic auto-immunity using auto-antibody markers, we currently lack a standard for evaluating the exposure to enterovirus. More knowledge on natural course of enterovirus infection is important when deciding on the source of material, detection methods and sample schedules.

To exclude the possibility that a putative association of enterovirus with pre-diabetes just reflects an increased general propensity towards viral infection in pre-diabetic individuals, other virus of similar routes of spread should be studied from the same samples. If an association is seen for enterovirus but not for the other virus, a specific involvement of enterovirus is probable. For this purpose, adenovirus seems to be a suitable target.

The aim of the present study was to prepare a robust methodology for PCR-based detection and to collect data on frequency of enterovirus and adenovirus infection in the Norwegian infants at the highest genetic risk of type 1 diabetes. This effort is required in order to set up a large-scale cohort study on enterovirus in diabetes pathogenesis.

2. Subjects and methods

2.1. Subjects

The subjects were participants in "Environmental Triggers of Type 1 Diabetes Study—the MIDIA Study", which is a sub-study of the population-wide Norwegian mother and child cohort. Both projects have been approved by the

Medical Ethics Committee and the Norwegian Data Inspectorate. The MIDIA study recruits newborns from the general population who screen positive for the genotype conferring the highest genetic risk for type 1 diabetes, the HLA-DQB1*02-DQA1*05-DRB1*03/DQB1*0302-DQA1*03-DRB1*0401. This genotype is carried by 2.7% of the population (Cinek et al., 2000). The infants included in the present study were recruited from either the South-Eastern part of Norway, or the county of Hordaland in Western Norway. The genetic screening identified 116 infants of whom 113 participated (60 males, 53 females) after the parents consented to follow-up. The parents submitted monthly stool samples (from month 3 to 36, the majority of the present samples being from 3 to 15 months old infants). From the 113 infants, we received 1257 out of the 1306 scheduled stool samples (96%). Two samples were accidentally destroyed during handling. The samples were obtained between September 2001 and October 2003.

The parents also kept a detailed weekly diary that – among other – included information on types and dates of symptoms suggesting an infection: cough and sneezing, diarrhoea or vomiting and fever above 38 °C. These diaries were regularly collected and converted into electronic records.

2.2. Collection and processing of stool samples

Parents were instructed to obtain a stool sample from the diaper using containers with a spoon attached under the lid. The containers were mailed using the national post service in pre-paid envelopes to the central laboratory, which meant 1–3 days in transit. If a stool sample did not arrive in time, the parents were reminded by a phone call.

The stool samples were suspended (one part stool to four parts buffer, w/v) in phosphate buffered saline with 0.5% bovine serum albumin, 50 IU/ml penicilin, 50 µg/ml streptomycin and 2.5 µg/ml fungizone, and subjected to vortexing and centrifugation at $4000 \times g$ for 30 min. The faecal supernatant was divided into aliquots and frozen at $-80\,^{\circ}\mathrm{C}$ until further processing. The DNA and RNA were co-purified from 140 µl of the supernatant using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. A low amount of West Nile Virus (WNV) Armored RNA (Ambion Diagnostics, Austin, TX, USA) was added into each lysis buffer vial of the kit to serve as an exogenous internal control for the extraction, reverse transcription and amplification. The nucleic acid was eluted into 100 µl sterile nuclease-free water. Positive and negative controls were extracted with each batch of samples: as a positive control, we used 2 µl of Enterovirus Armored RNA (Ambion Diagnostics, Austin, TX, USA) diluted in 138 µl normal saline; the negative control was normal saline.

2.3. Enterovirus testing

Enterovirus was detected using a one-step real-time RT-PCR reaction with an exogenous internal control. Two mi-

Table 1
Sequences of primers and probes used to detect enterovirus and adenovirus

Designation	$5'$–$3'$ Sequence
Enterovirus detection	
Ent-forward[a]	ccctgaatgcggctaatcc
Ent-reverse[a]	attgtcaccataagcagcca
Ent-probe[a]	FAM-aaccgactacttTgggtgtccgtgtttc-TAMRA
WNV-forward[b]	gctccgctgtccctgtga
WNV-reverse	cctctatccaaacacggttcca
WNV-probe	VIC-tggtccatccatgcagga-dark quencher (MGB probe)
Enterovirus confirmation	
Pol-forward[c]	cgtaacgcgcaagtctgtgg
NonPol-forward	gtcgtaatgggyaactcygcagcg
Adenovirus detection	
ADVs[d]	catgactttTgaggtggatc
ADV31s[d]	tatgacatttgaagttgacc
ADVas[e]	ccggccgagaagggtgtgcgcaggta
AdV-probe[d]	FAM-agcccaccctKctttat-dark quencher (MGB probe)

[a] Sequences from Verstrepen et al. (2001).
[b] Sequence from Briese et al. (2000).
[c] Sequence from Watkins-Riedel et al. (2002).
[d] Sequences from a joint project on adenovirus infection in children after bone marrow transplantation, published in Claas et al. (2005).
[e] Sequence from Echavarria et al. (1998).

croliters of the template were pipetted in duplicate to a $10\,\mu l$ reaction containing $1\times$ QuantiTect Probe RT-PCR master mix, $1\times$ QuantiTect RT mix (both components of the QuantiTect Probe RT-PCR Kit, Qiagen), 900 nM enterovirus reverse (Ent-reverse) primer, 300 nM enterovirus forward (Ent-forward) primer, 200 nM FAM-labelled enterovirus probe (Ent-probe), 50 nM WNV-forward, 50 nM WNV-reverse primer and 300 nM VIC-labelled WNV probe. The sequences of primers and probes are shown in Table 1. The reverse transcription and amplification were performed on an ABI 7700 machine with the Sequence Detector Software version 1.9. The amplification profile consisted of 30 min reverse transcription at $50\,^{\circ}C$, 15 min denaturation at $95\,^{\circ}C$, and 45 cycles of 15 s denaturation at $94\,^{\circ}C$ and 1 min combined annealing–extension at $60\,^{\circ}C$. The tubes were discarded unopened.

Fluorescence data were collected during the annealing–extension phase, and evaluated using the Spectral Compensation option of the software, which improves the discrimination between the two reporters. First, the FAM-dye layer with the enterovirus probe was evaluated; then the VIC-dye layer was inspected for WNV amplification in enterovirus-negative samples. The control WNV RNA gave a threshold cycle value of 29–31. If the WNV threshold cycle was more than 3.5 cycles above the average of the other enterovirus-negative samples in the run, the extraction and RT-PCR were repeated.

The enterovirus quantification was calibrated using five different enterovirus strains (echovirus 25, 30, Coxsackie B1, Coxsackie B6, enterovirus 71) grown and quantified as to $TCID_{50}/ml$ in green monkey kidney cells (GMK-AH1) or

human embryonic fibroblasts (HE) cell lines. The RNA was extracted from the cell culture supernatants using the same method as for the stool samples. A quantity calibrator was calculated as an average of the five strains, and related to each batch of the positive control enterovirus armored RNA. The sensitivity of detection was at the order of an equivalent of $0.1\ TCID_{50}/ml$ of the calibrator.

The primer-probe system for enterovirus detection has been extensively verified by its authors (Verstrepen et al., 2001). In addition to this verification, we also tested the specificity with rhinovirus serotypes 43 and 45 (courtesy of the National Institute of Public Health, Prague). These rhinovirus serotypes completely match the forward enterovirus primer and therefore would theoretically have the highest potential risk of cross-reactivity with this enterovirus assay. We detected no signals from rhinoviruses 43 and 45 even at high template concentrations. To further ensure specificity, all enterovirus-positive samples in this study were confirmed by an independent amplification using identical chemistry, probe and amplification profile, but another primer set: the Pol-forward, NonPol-forward and Ent-reverse primers, sequences given in Table 1.

2.4. Enterovirus sequencing

To distinguish between a prolonged infection by the same serotype and multiple consecutive infections, the sequences of the VP1 region were determined from products of a nested RT-PCR amplification according to Casas et al. (2001) or Welch et al. (2003). The RNA extracted from the stool samples served as the template, no viral cultures were performed. Obtained sequences were analysed using the on-line BLAST 2.2.9 search tool of GenBank, NCBI to assign serotypes. If VP1 amplification was not successful, decision on identity of the strains seen in consecutive samples was taken based on RT-PCR amplification and sequencing of the RNA polymerase region according to Casas et al. (2001) and pair-wise comparison using the algorithm implemented in the BioEdit alignment editor.

2.5. Adenovirus testing

Adenovirus (subtypes A to C) was detected using quantitative real-time PCR in a $15\,\mu l$ volume with $1\times$ Qiagen HotStar PCR buffer, $2.5\ mM\ MgCl_2$, 500 nM each ADVs, ADV31s and ADVas primers, 200 nM AdV-probe (sequences given in Table 1), $100\,\mu M$ each dNTP, $1.5\,\mu M$ 6-carboxy rhodamine (Molecular Probes) as a passive colour reference, 0.3 U HotStarTaq DNA polymerase (Qiagen) and $2\,\mu l$ of the template. The amplification was performed on an ABI 7700 machine with the Sequence Detector Software version 1.9; the amplification profile consisted of 15 min denaturation at $95\,^{\circ}C$, followed by 45 cycles of 15 s denaturation at $94\,^{\circ}C$ and 1 min combined annealing–extension at $60\,^{\circ}C$. The tubes were discarded unopened. The adenovirus quantity was determined using a spectrophotometrically quantified plasmid

with a 475 bp fragment of the 3′-end of the hexon gene (courtesy of Eric Claas, Leiden University Medical Centre, the Netherlands). The detection sensitivity was at the order of 1 plasmid copy/μl nucleic acid.

2.6. Statistical analysis and analysis of symptoms

The enterovirus and adenovirus frequencies were calculated in the whole dataset, and the distribution of viral quantity was plotted in a modified cumulative frequency plot showing the proportion of positive samples at a given quantity threshold. The quantity of viruses was compared between boys and girls using the Mann–Whitney test.

To reflect the prolonged nature of enterovirus and adenovirus shedding into stool, we counted the infection episodes rather than months with the virus when analyzing the seasonal distribution, the age-specific prevalence and the association with symptoms. A new enterovirus infection episode was defined as the first finding of enterovirus after a negative monthly sample, or the finding of a serotype different from the previous monthly sample. Where the VP1 sequence was not available, we used data from the pair-wise comparison of RNA polymerase region sequences. A new adenovirus infection episode was defined as the first finding of adenovirus after a negative monthly sample. A continuing infection was defined as a positive sample following another positive one (in enterovirus, both having the same type).

The seasonal distributions of the infections were evaluated by calculating calendar-month-specific frequency of enterovirus or adenovirus infection episodes. The age-specific frequencies of infection episodes were first calculated for the whole dataset and then separately for four strata of month of birth (January–March, April–June, July–September, October–December) to adjust for the known seasonality.

The association of infection with symptoms was investigated using a nested case–control approach. Data from weekly diaries kept by parents were inspected for the occurrence of cough and sneezing, vomiting or diarrhoea and fever above 38 °C in the period spanning 2 weeks before to 2 weeks after each stool sample was taken. Each stool sample then represented one person-month of observation with data on virus quantity in the middle of the period, and occurrence of the three symptoms during the 4 weeks surrounding the date of sample. The association was tested separately for each particular symptom. Each person-month with the symptom was matched to two randomly chosen other children's person-months without the respective symptom. The matching was done for calendar date of sample (±30 days) and for age of the child giving the sample and providing data (±1 month of age). The odds ratios were then calculated in a conditional logistic regression model, which included dichotomous independent variables of new enterovirus infection, continuing enterovirus infection, new adenovirus infection and continuing adenovirus infection, with symptom as the outcome variable. The interaction terms between the infections did not improve the model (likelihood ratio statistics) and interactions were therefore not included in the final model.

3. Results

Enterovirus RNA was detected in 142/1255 stool samples (11.3%; 95%CI 9.6–13.1%), while adenovirus DNA was detected in 138/1255 stool samples (11.0%; 95%CI 9.3–12.7%). There was no statistically significant difference between males and females in prevalence of enterovirus, while adenovirus was significantly less frequent in males (9.4%, 95%CI 7.3–11.6%) than in females (13.2%, 95%CI 10.3–16.1%), $P_{difference} = 0.037$. The quantity of enterovirus ranged from an equivalent of less than 1 $TCID_{50}$/ml to over 10^5 $TCID_{50}$/ml stool supernatant. The quantity of adenovirus ranged from several copies to over 10^6 copies/μl nucleic acid extract. The distributions of quantities of enterovirus and adenovirus are shown in Fig. 1, where the positivity rate is plotted against the quantity of virus in a modified cumulative frequency chart. The quantity distributions did not differ significantly between males and females.

The serotype was identified using VP1 sequencing in 104/142 (73%) enterovirus-positive stool samples. The polymerase region sequence was obtained in further 23/142 (16%) samples where VP1 amplification was not successful, while no sequence data were obtainable in the remaining 15/142 (11%) enterovirus-positive samples. The viral load of the latter samples was significantly lower (average logarithm of quantity in $TCID_{50}$/ml was 0.38, S.E. 0.24) than in the samples typable by either polymerase region sequencing (average log quantity 1.68, S.E. 0.24), or VP1 sequencing (average log quantity 2.50, S.E. 0.11).

Based on the results of sequencing, the 142 enterovirus-positive samples were assigned to 113 infection episodes. The 138 adenovirus-positive samples were assigned to 90 infection episodes. There were no significant correlations between the peak virus quantity during the episode of infection, and the duration of the episode in either of the viruses. There was also no correlation between the presence of enterovirus and adenovirus in the samples.

The seasonality of enterovirus and adenovirus infection episodes is shown in Fig. 2. Enteroviruses were most prevalent between July and November, and less common during winter and spring. Adenovirus displayed no clear seasonality pattern.

The age-specific frequency rates of enterovirus and adenovirus infection episodes for children aged 3–16 months are shown in Fig. 3. On panel A, the 3-months' moving averages of age-specific prevalence of infection episodes are plotted against the age of the infants in months. The enterovirus prevalence had an apparent trough between the age of 9 and 12 months, and an increase after the 12th month of age. The distribution was similar for boys and girls (data not shown).
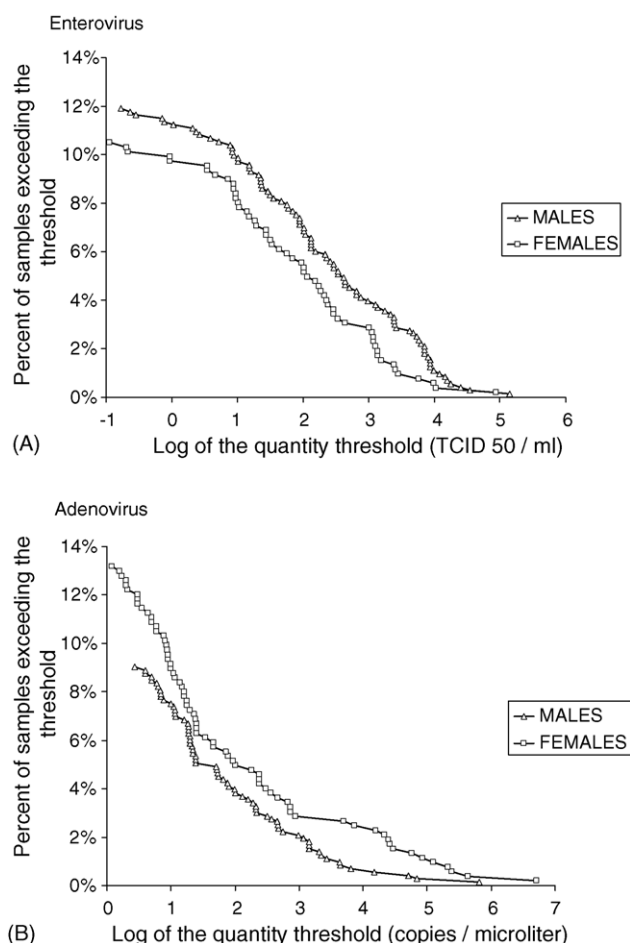
Fig. 1. Distribution of quantity of enterovirus (A) and adenovirus (B) in males and females. The amount of viruses in stool samples was measured by quantitative PCR. The horizontal axis shows the decadic logarithm of the viral concentration in sample ($\log_{10}$ of $TCID_{50}$/ml for enterovirus or $\log_{10}$ of copies/μl for adenovirus); the vertical axis shows the proportion of samples exceeding this quantity. Individual samples are plotted as triangles (samples from males) or squares (samples from females).

The first peak in enterovirus prevalence was most obvious in infants born during the first quarter of the year, while the increase after the age of 12 months was most pronounced in infants born in the second or fourth quarter of the year (Fig. 3, panel B). There was a significant heterogeneity in the number
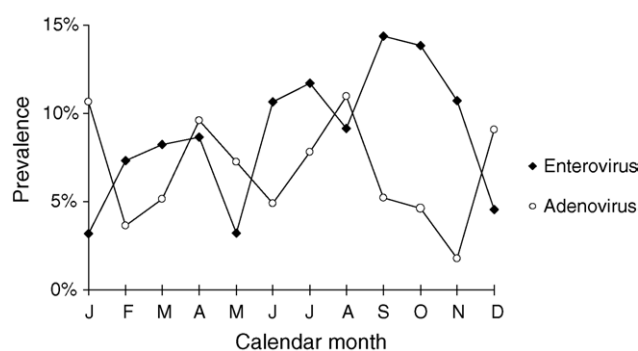
Fig. 2. Seasonality of enterovirus and adenovirus infection episodes.
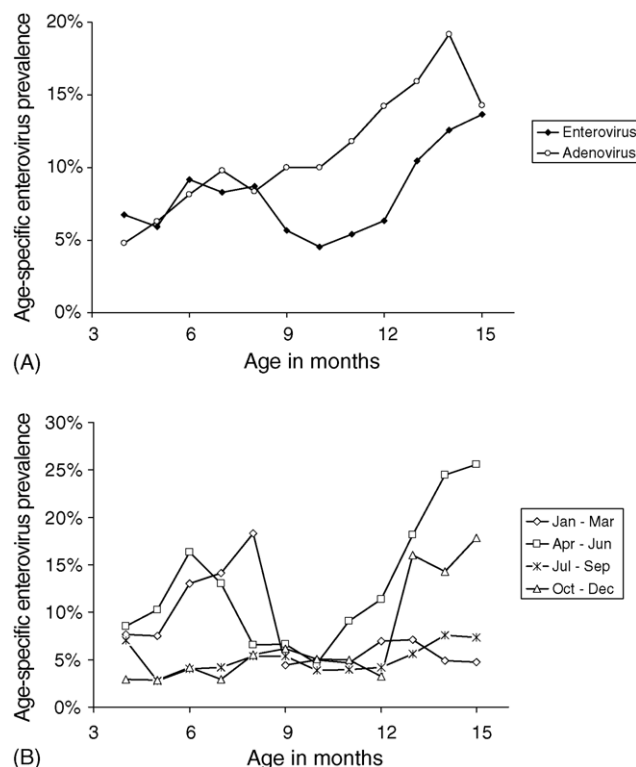
Fig. 3. Age-specific prevalence of enterovirus and adenovirus infection episodes. (A) The 3-months moving averages of enterovirus and adenovirus age-specific prevalence for the age of 3–16 months ($n = 1073$ person-months). (B) The 3-months moving average of enterovirus prevalence is shown separately for infants born in the four trimesters of the calendar year (based on the same data as in (A)).

of enterovirus infections when analysed by trimester of birth ($P = 0.007$).

The adenovirus prevalence increased significantly with age ($P = 0.006$ in chi-square linear trend test). As in the case of enterovirus, the age-specific prevalence curves did not differ significantly between males and females. There were no appreciable differences in adenovirus prevalence among infants born in different calendar trimesters ($P = 0.28$).

The numbers of enterovirus or adenovirus infection episodes were counted for the 57 infants (22 males and 35 females) who completed the first year of observation (i.e. provided samples from month 3 to month 14). As to both enterovirus and adenovirus, approximately half of the infants had at least one infection episode, while about one-fourth of the infants had two or more infection episodes (Fig. 4). There was no apparent relation between the number or duration of enterovirus and adenovirus infection episodes.

The results of the analysis of symptoms potentially attributable to infection are shown in Table 2. The first month of an adenovirus infection was significantly associated with fever ($OR = 3.1$, 95%CI 1.8–5.5) and with symptoms of cold ($OR = 2.3$, 95%CI 1.4–3.8). Neither enterovirus infection, nor subsequent months of adenovirus infection were significantly associated with any studied symptoms.
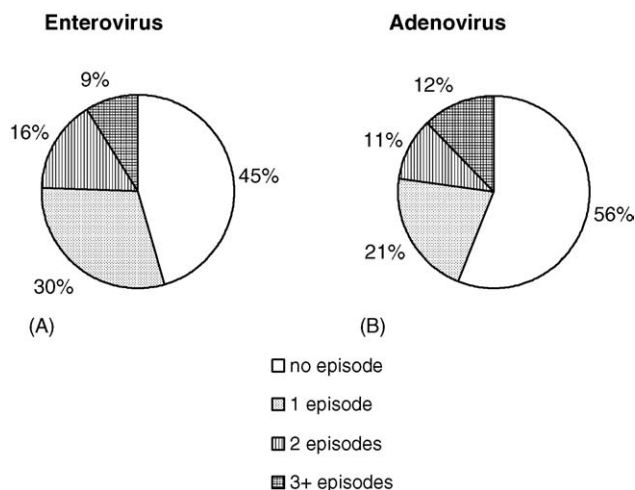
Fig. 4. Distribution of 57 infants by the number of enterovirus (panel A) or adenovirus (panel B) infection episodes during the first year of observation (a total of 684 person-months from infants aged 3–14 months).

Table 2
Association of fever, vomiting or diarrhoea and symptoms of cold with the presence of enterovirus and adenovirus in the stool sample

	OR (95%CI)	P
Fever (18.6% person-months)		
Enterovirus		
New infection	1.5 (0.8–2.7)	0.18
Continuing infection	0.4 (0.1–1.7)	0.20
Adenovirus		
New infection	3.1 (1.8–5.5)	1×10^{-4}
Continuing infection	1.9 (0.9–4.3)	0.10
Vomiting or diarrhoea (7.8% person-months)		
Enterovirus		
New infection	1.0 (0.4–2.4)	1.00
Continuing infection	0.7 (0.1–3.3)	0.62
Adenovirus		
New infection	1.0 (0.5–2.3)	0.95
Continuing infection	2.2 (0.6–7.2)	0.21
Cough or sneezing (22.4% person-months)		
Enterovirus		
New infection	1.0 (0.6–1.7)	0.99
Continuing infection	1.8 (0.6–5.6)	0.28
Adenovirus		
New infection	2.3 (1.4–3.8)	2×10^{-3}
Continuing infection	0.6 (0.3–1.4)	0.22

Symptoms were recorded for the period starting 2 weeks prior to and ending 2 weeks after the monthly stool sample. Odds ratios against no infection are given for a new infection (the first finding of virus in stool following a negative sample or following another virus serotype) and for a continuing infection (a positive sample following another positive one; in enterovirus, both having the same serotype).

4. Discussion

Enterovirus was detected in 11.3% of stool samples from young infants. The prevalence is slightly higher than what was found in a comparable Finnish study (72/878, 8.2%) (Salminen et al., 2004). Comparison to older studies is dif-

ficult, since for those with similar longitudinal sampling, molecular assays were not available. Indeed, the present estimate is considerably higher than frequencies obtained using cell culture techniques (Gelfand et al., 1963; Froeschle et al., 1966; Cooney et al., 1972).

As shown in Table 2, a clear association was found between adenovirus infection and fever or symptoms of cold (cough and sneezing). However, no association with symptoms (fever, cough/sneezing, vomiting/diarrhoea) was found for enterovirus infection. This is somewhat surprising considering that enteroviruses have been commonly implicated in these types of conditions. The observation suggests that the attribution of such symptoms to enterovirus may often be misleading. In fact, these viruses are so common in the faeces of healthy infants that one should be careful about their role in the aetiology of more serious diseases, particularly if relying solely on faecal material rather than on serum or cerebrospinal fluid.

Using quantitative real-time PCR, we detected entero- and adenovirus loads spanning over six orders of magnitude (Fig. 1). Quantification may add another aspect to the evaluation of enterovirus exposure, because the viral load may have a significant impact on the risk of eliciting beta-cell auto-immunity. Thus, in order to evaluate the true exposure of viral infections, one should preferably obtain both consecutive samples for a sufficient period of time, and quantify the virus. In this context, it is interesting to note that we did not detect any correlation between the peak quantity of enterovirus during an infection episode, and the duration of the episode (data not shown).

While the analysis of seasonality confirmed the known pattern for enterovirus and the limited seasonality in enteric adenoviruses (Fox et al., 1977), the analysis of age-specific frequency of enterovirus showed a somehow surprising pattern with a significant trough around the age of 10 months. This period of low enterovirus frequency was observed for both sexes, and in all strata of calendar trimester of birth. The latter point indicates that the seasonality of enterovirus infections cannot explain the trough. The cause of the increase following this trough may be related to the introduction of the child to the day care centre, which typically takes place at this age in Norway. An explanation for the higher prevalence between months 6 and 9 is not obvious, but may be related to the loss of passively transferred maternal antibodies. A similar increase has also been seen in a Finnish study using detection of viral RNA in serum (Sadeharju et al., 2003).

Enterovirus and enteric adenoviruses are known to persist in stool for a considerable period of time. Consequently, the positive samples clustered in time not only due to seasonality, but also due to the numerous infections lasting for more than one month. Interestingly, the multiple consecutive positive samples were not only prolonged episodes of infection with the same serotype (Fig. 5A), but frequently also successive infections with various enterovirus serotypes (as exemplified in Fig. 5B). Sequencing of a sufficiently informative region of viral RNA is crucial in deciding on whether the infection
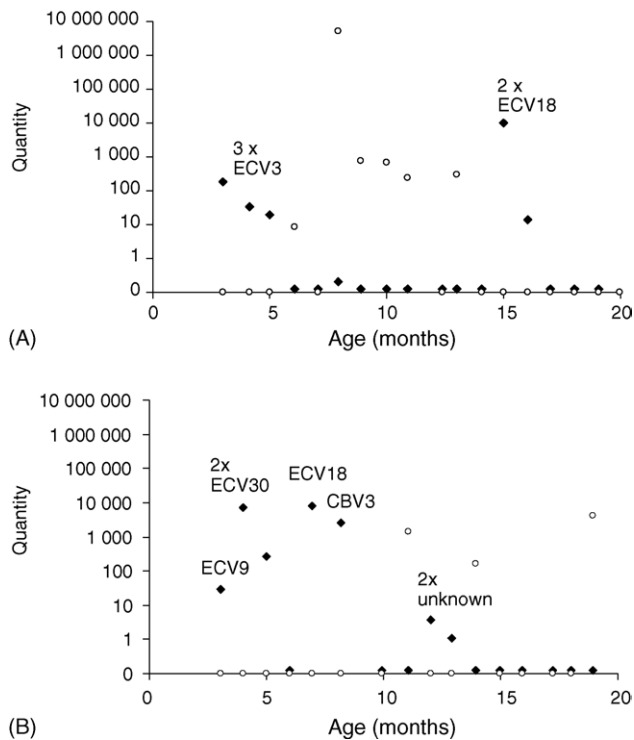
Fig. 5. Genotype and quantity of virus in two infants with multiple positive samples. The quantity of enterovirus (TCID$_{50}$/ml) and adenovirus (copies/μl) are plotted using the same scale. (A) An infant having two episodes of enterovirus infection separated by a period of adenovirus positive samples. The two enterovirus infections were found to be echovirus (ECV) 3 and 18. (B) An infant infected with four different enterovirus serotypes – ECV9, ECV30, ECV18, coxsackie B (CBV) 3 – as well as two low-quantity samples of unknown serotype.

is new or prolonged. This effort was successful in samples with high viral load, but not in all low-quantity samples, as indicated by our 11% failure rate.

Because stool contains many potential inhibitors of reverse transcription and PCR, the quality of data benefits from the use of an internal control. This is in accordance with recent calls for mandatory inclusion of internal controls in diagnostic PCR assays (Hoorfar et al., 2003). We therefore modified the method by Verstrepen et al. (2002) by adding an exogenous internal control in the first step of RNA extraction. The West Nile Virus Armored RNA was chosen because of its commercial availability, and because this target does not naturally occur in Norway. The quantity of the internal control was set to achieve threshold cycle of approximately 30. To maintain preferential enterovirus amplification, the concentration of WNV-specific primers was one order of magnitude lower than the enterovirus-specific primers. Inhibition was defined as an increase in threshold cycle value of more than 3.5 cycles compared to the average of the run. The frequency of inhibition was reasonably low (3.6%), and in all instances it could be resolved by repeated extraction and RT-PCR.

As the bowel is the primary site of enterovirus replication, one may expect a reasonable amount of virus in the case of an ongoing infection. Rather than sensitivity, the present detection method was therefore designed for simplicity, for safety towards contamination and for specificity. The latter point is particularly relevant as the closely related rhinoviruses are frequently found in stool samples from infants (Salminen et al., 2004). Consequently, we used a one-tube, closed RT-PCR system, and added a relatively low volume of RNA extract. Moreover, the inclusion of an exogenous internal control may decrease the sensitivity, even though the target and primer concentrations were chosen for minimal interference with the enteroviral PCR. The present method was approximately two orders of magnitude less sensitive than an optimal, nested real-time RT-PCR (Watkins-Riedel et al., 2002), data not shown. The reported enterovirus prevalence may therefore be an underestimate. However, the biological relevance of minute viral quantities is likely to be low. Moreover, most of the samples had enteroviral quantities more than two logs above the threshold of the present assay (Fig. 1A), suggesting that even if the threshold had been lower, the estimated prevalence would not have been dramatically different.

In conclusion, the study demonstrates that the present strategy for obtaining monthly samples of stool is well accepted by the parents, as reflected by the high response rate, and that the diagnostic methodology can be used to effectively and reliably screen large number of samples for virus. Although the quantitative, one-step, closed RT-PCR with an exogenous internal control has lower sensitivity for enterovirus detection compared to an optimally designed nested PCR, the lower sensitivity is outweighed by the safety from contamination and from PCR inhibition. Quantification offers another aspect of evaluating the exposure, as the viral load may be relevant to eliciting pre-diabetic auto-immunity, or other secondary consequences of being exposed to these viruses.

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Příloha II.

Witsø E, Palacios G, Cinek O, Stene LC, Grinde B, Janowitz D, Lipkin WI, Rønningen KS.

High prevalence of human enterovirus A infections in natural circulation of human enteroviruses.

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High Prevalence of Human Enterovirus A Infections in Natural Circulation of Human Enteroviruses[∇][†]

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Human enterovirus (HEV) infections can be asymptomatic or cause only mild illness; recent evidence may implicate HEV infection in type 1 diabetes mellitus and myocarditis. Here, we report the molecular characterization of HEV obtained in serial monthly collections from healthy Norwegian infants. A total of 1,255 fecal samples were collected from 113 healthy infants beginning at age 3 months and continuing to 28 months. The samples were analyzed for HEV nucleic acid by real-time PCR. Fifty-eight children (51.3%) had HEV infections. One hundred forty-five positive samples were typed directly by nucleotide sequencing of the VP1 region. HEV-A was detected most frequently, with an overall prevalence of 6.8%. HEV-B was present in 4.8% of the samples and HEV-C in only 0.2% of the samples. No poliovirus or HEV-D group viruses were detected. Twenty-two different serotypes were detected in the study period: the most common were EV71 (14.5%), CAV6 (10.5%), CAV4 (8.9%), E18 (8.9%), and CBV3 (7.3%). These findings suggest that the prevalence of HEV infections in general, and HEV-A infections in particular, has been underestimated in epidemiological studies based on virus culture.

The genus *Enterovirus* belongs to the family *Picornaviridae*. Although the majority of *Human enterovirus* (HEV) infections are asymptomatic, enterovirus infections can cause upper respiratory illness, febrile rash, aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis, and neonatal sepsis-like disease (35). HEV may also be implicated in the pathogeneses of severe chronic diseases, including type 1 diabetes mellitus (33), myocarditis and congestive cardiomyopathy (20), and neuromuscular diseases (8).

HEVs contain a linear (7.4-kb) single-stranded RNA genome comprising a 5′ and a 3′ noncoding region and a single, long open reading frame coding for a polyprotein of about 2,200 amino acids. HEVs are classified, based on molecular and biological properties, into five species (19, 36): (i) *Poliovirus* (types 1 to 3), (ii) *Human enterovirus A* (HEV-A) (CAV2 to CAV8, CAV10, CAV12, CAV14, CAV16, and EV71), (iii) *Human enterovirus B* (HEV-B) (CAV9, CBV1 to CBV6, E17, E9, E11 to E21, E24 to E27, E29 to E33, and EV69), (iv) *Human enterovirus C* (HEV-C) (CAV1, CAV11, CAV13, CAV15, CAV17 to CAV22, and CAV24), and (v) *Human enterovirus D* (HEV-D) (EV68 and EV70). Several new serotypes (EV73 to EV78 and EV89 to EV91) were recently described (28, 29, 31, 32).

Neutralization tests of cultured virus may not be sufficient to identify some serotypes (26). Many coxsackievirus A strains can be isolated and propagated only in suckling mice (24). To address the need for a robust and universal typing system, many investigators have turned to the use of molecular methods (4, 30, 41). The introduction of molecular sequencing has extended the potential of virus surveillance and epidemiological studies by facilitating the identification of genotypes (14).

Most previous data on HEV circulation have been obtained from analysis of specimens from individuals with disease. Studies of enterovirus circulation in healthy populations antedate the advent of molecular technologies (12, 13, 16–18, 21, 23, 38, 39).

We have reported that 11.3% of fecal samples obtained prospectively from 113 healthy Norwegian infants were positive for enterovirus RNA (11). Here, we present the results of a comprehensive molecular characterization of these samples.

MATERIALS AND METHODS

Study design and subjects. Participating children were enrolled in a prospective study (MIDIA) to investigate potential environmental triggers of type 1 diabetes mellitus. The MIDIA study recruits newborn infants at their first visit to health care centers. Candidates are screened for the HLA genotype conferring the highest risk of type 1 diabetes (HLA-DQB1*02-DQA1*05-DRB1*03/DQB1*0302-DQA1*03-DRB1*0401), which is carried by 2.7% of newborns (10). Over the period from September 2001 to October 2003, 113 of 116 infants identified as genetically susceptible were enrolled (60 males; 53 females). No more than one child in any nuclear family was enrolled. Beginning at 3 months of age and followed up to 28 months (for the first children recruited), monthly stool samples were obtained. Specimens were collected from September 2001 through November 2003; 96% (1,257/1,306) of the scheduled stool samples were received. Two samples were accidentally destroyed during handling. Eighty-nine percent of the stool samples were collected from children residing in the counties of Akershus (southeast Norway) and Hordaland (west coast), two counties separated by more than 400 km.

Processing of stool samples. Total nucleic acids were extracted from 140 µl of 1,255 stool suspensions using the QIAamp Viral RNA Mini Kit (QIAGEN). HEV nucleic acid was detected and quantitated using a one-step real-time reverse transcription (RT)-PCR targeting the 5′ untranscribed region (11).

TABLE 1. Oligonucleotide primers used for VP1 characterization of enteroviruses

Primer	5′–3′ sequence[a]	Enterovirus specificity	Positions[b]	Use[c]
ENV71SP-F1	GCACAGGTYTCNGTNCCRTTYATGTC	HEV-A	2987–3012	PCR/seq.
ENV71SP-R1	CATGCCCTGACRTGYTTCATYCTCAT	HEV-A	3207–3182	PCR/seq.
VP1-2S-CAV16	GCAGTACATGTATGTCCCSCCMGGSG	HEV-A	2878–2903	PCR
VP1-2A-CAV16	TCGCACCCCTGGGCRGTGGTGGA	HEV-A	3507–3485	PCR
VP1-2S-CAV2	CAGTACATGTAYGTCCCNCCYGGRG	HEV-A	2879–2903	PCR/seq.
VP1-2A-CAV2	CCGGTCTGACAATTACATCGAGC	HEV-A	3537–3515	PCR/seq.
VP1-2S-CAV3	CAGTACATGTAIGTTCCACCTGGTG	HEV-A	2879–2903	PCR/seq.
VP1-2A-CAV3	CCCGTCTGGCAGTTGCATCGAGC	HEV-A	3537–3515	PCR/seq.
VP1-2S-CAV4	CARTACATGTATGTGCCACCYGGRG	HEV-A	2879–2903	PCR
VP1-2A-CAV4	CCTGTTTGGCAATTACAGCGGGC	HEV-A	3537–3515	PCR
VP1-2S-CAV6	CAGTACATGTAYGTRCCRCCRGGTG	HEV-A	2879–2903	PCR
VP1-2A-CAV6	CCAGTCTGGCAGTTACATCGAGC	HEV-A	3537–3515	PCR
VP1-2S-CAV10	CAGTATATGTATGTNCCTCCNGGGYG	HEV-A	2879–2903	PCR
VP1-2A-CAV10	CCTGTCTGACAGTTGCACCGAGC	HEV-A	3537–3515	PCR
VP1-2S-CAV12	CAGTACATGTTIGTGCCICCTGGTG	HEV-A	2879–2903	PCR
VP1-2A-CAV12	CCAGTCTGACAATTGCATCGAGC	HEV-A	3537–3515	PCR/seq.
VP1-2S-ENV71	CARTAYATGTTTGTNCCSCCYGG	EV71	2879–2903	PCR
VP1-2A-ENV71	TCACAACCYTGRGCRGTGGTAGA	EV71	3507–3485	PCR
VP1-CAV6-F	CGGTGTTCGCAAAATTGAGT	CAV6	2958–2977	PCR
VP1-CAV6-R	TCACATCCTTGAGCAGTAGTGG	CAV6	3507–3486	PCR/seq.
VP1-CAV10-F	GCCCCTAAACCGACTGGTAG	CAV10	2903–2922	PCR
VP1-CAV10-R	ACCCCTGTGCAGTGGTAGAG	CAV10	3503–3484	PCR
VP1-HEV71-F	CCAAGCCAGACTCCAGAGAA	EV71	2907–2926	PCR/seq.
VP1-HEV71-R	ATTACAGCGGGCAATTGTGT	EV71	3526–3507	PCR/seq.
VP1-CBV3-F	CGGTGCCAGATAAGGTTGAC	CBV3	2907–2926	PCR/seq.
VP1-CBV3-R	TCTGGCTATTGTATCGCATCC	CBV3	3520–3500	PCR
VP1-EV18-F	GGCCAAGGTGGATAGTTACG	EV18	2914–2933	PCR
VP1-EV18-R	ACACCTGGCGATGGTATCAC	EV18	3523–3504	PCR

[a] I, inosine; Y, C or T; W, A or T; R, A or G; K, G or T.
[b] Relative to the genome of coxsackievirus A6 strain Gdula (GenBank accession number AY421764).
[c] seq., sequencing.

Molecular typing. HEV serotypes of samples positive in real-time PCR were determined by VP1 sequencing of RNA (4, 44). In instances where samples could not be serotyped by this approach (39 of 145 samples), the RNA polymerase region was amplified and sequenced (4). Based on the RNA polymerase sequence, serotype- or species-specific VP1 primers were designed (Table 1) (4, 44). In total, 14 additional nesting primer sets (localized inside the sequence product of the first amplification) were designed, facilitating serotype assignment of 91.7% of the enterovirus-positive samples.

First-round VP1 amplification products obtained by the method of Casas et al. (4) or separate random-hexamer-primed RT uncoupled from the first round of PCR were used as templates with the new sense and antisense primers. PCR was carried out in a total volume of 20 μl with 10 pmol of each primer (Table 1), 1 to 2 μl of the RT-PCR products or cDNA, 2 μl 10× PCR buffer II (Applied Biosystems), 2.5 mmol $MgCl_2$, 0.5 mmol deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase (Perkin-Elmer). The thermal-cycling program consisted of 5 min of denaturation at 94°C, followed by 40 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 50 to 55°C, and 1 min of extension at 72°C.

Nucleotide sequence determination and analysis. Direct product sequencing was performed using the amplification primers. Both strands were sequenced by automatic methods (BigDye version 3.1 and ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA). In instances where sequences could not be obtained directly, the products were cloned into a pGEM-T-Easy vector (Promega) and five clones from each sample were sequenced on both strands.

Raw sequence data were analyzed with Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI) to obtain the final consensus sequence. Ambiguous nucleotides were resolved by resequencing them.

Phylogenetic analysis. The VP1 capsid coding sequences determined in this study were included in a phylogenetic analysis with reference strains of all enterovirus serotypes. Multiple sequence alignments were performed with ClustalX version 1.82 (42). Phylogenetic analysis was performed using the Kimura two-parameter model as a model of nucleotide substitution and the neighbor-joining method to reconstruct the phylogenetic tree (MEGA program version 3.0 [22]). The statistical significance of the phylogenies constructed was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets.

Infection episodes and coinfections. A new infection episode was defined as detection of a new genotype after a monthly sample was negative for that genotype. Coinfection was defined as the simultaneous presence of two or more different genotypes in the same stool sample.

Statistical analysis. The software packages used were Stata version 9.0 (Stata Corporation, College Station, TX) and SPSS version 12.0.1 (SPSS, Chicago, IL). The overall prevalence for each species over the whole study period was computed with 95% confidence intervals (CI). The confidence intervals for prevalence were computed based on standard errors estimated by bootstrapping, using each child as a unit to provide a simple adjustment for dependence between repeated infections (15). Relationships of sex, year, season, and geographic location with prevalence for the main HEV species were assessed.

Associations of viral load and duration of infection, sex, age, and enterovirus species were assessed in linear-regression models, using the viral load of the first positive sample in an infection period. The potential dependence in data from repeated measurements for each child was handled in the regression models by generalized estimating equations using the xtgee procedure in STATA (Stata cross-sectional time-series reference manual, release 8, StataCorp, Stata Press, College Station, TX).

The cumulative incidence of first infection during follow-up for individual children at different ages was estimated using Kaplan-Meier survival analysis. Differences between groups were tested using the log rank test. A P value of 0.05 or less was considered to be statistically significant.

Nucleotide sequence accession numbers. The 3′-end VP1 sequences reported here were deposited in the GenBank sequence database under accession no. DQ317159 to DQ317293. The RNA polymerase sequences were reported under accession no. DQ315510 to DQ315562.

RESULTS

Molecular typing and phylogenetic analysis. The HEV type was identified in 92% of the samples (133/145) by VP1 sequencing and in 6.2% (9 cases) by RNA polymerase sequenc-

TABLE 2. Enterovirus serotypes and species detected in feces specimens taken from healthy children aged 3 to 28 months in Norway[a]

Species	Serotype	No. of episodes	% (n = 124)
HEV-A	CAV2	3	2.4
HEV-A	CAV4	11	8.9
HEV-A	CAV5	7	5.6
HEV-A	CAV6	13	10.5
HEV-A	CAV10	7	5.6
HEV-A	CAV14	6	4.8
HEV-A	CAV16	1	0.8
HEV-A	HEV71	18	14.5
HEV-A	HEV-A[b]	4	3.2
HEV-B	CAV9	4	3.2
HEV-B	CBV1	5	4.0
HEV-B	CBV3	9	7.3
HEV-B	CBV4	3	2.4
HEV-B	CBV5	4	3.2
HEV-B	EV3	2	1.6
HEV-B	EV5	1	0.8
HEV-B	EV9	3	2.4
HEV-B	EV11	1	0.8
HEV-B	EV13	2	1.6
HEV-B	EV18	11	8.9
HEV-B	EV25	1	0.8
HEV-B	EV30	1	0.8
HEV-B	HEV-B[b]	2	1.6
HEV-C	CAV19	2	1.6
Untyped	HEV	3	2.4

[a] In the period from September 2001 to November 2003. The frequencies of different serotypes are reported as numbers of infection episodes.
[b] Species were identified by pairwise comparison of RNA polymerase sequences.

ing. In total, 97.9% (142/145) of the real-time PCR-positive samples could be assigned to the species level. The VP1 sequencing and phylogenetic analysis allowed further subtyping. The three samples that were not identified had low viral loads. The positive samples represented 124 discrete infection episodes. A total of 22 different serotypes were detected (Table 2). The most prevalent serotypes were EV71 (14.5%), CAV6 (10.5%), CAV4 (8.9%), E18 (8.9%), and CBV3 (7.3%). Whereas CBV3 and E18 were detected throughout the study period, CAV4, CAV5, CAV6, CAV10, and CBV1 were detected only during short intervals. CAV16, E5, E11, E25, E30, E3, E13, and a CAV19-like strain were represented in only one or two infection episodes. The circulation of specific serotypes was not restricted to a single county or municipality.

Individual serotypes of the VP1 sequences were compared phylogenetically with all corresponding sequences of the respective serotypes in GenBank. Three serotypes (of the 22 analyzed) segregated in different clusters (CAV4, CAV9, and E18 [Fig. 1A to C, respectively]), indicating that there have been at least two introductions of genotypes into the population for each of these serotypes. Two distinct clusters were observed for the CAV4-positive samples (Fig. 1A). Seven CAV4 sequences clustered together with sequences from Spain (1999) or Japan (2000 to 2004); six sequences segregated with a strain that circulated in Spain in 1998 (Fig. 1A). Some CAV9 sequences (Fig. 1B) were clustered with isolates from Cyprus (2002) and Spain (1999); one clustered with an unrelated cluster closer to an isolate from the United Kingdom

(1962 to 1989). E18 sequences clustered with sequences isolated in Japan (2001 to 2004) or Spain (1995 to 1997), the United States (1996), and Sweden (1999) (Fig. 1C). Although more than one strain was found to be cocirculating in certain periods, as seen for CAV4 (Fig. 1A), the two clusters of CAV9 and E18 seem to have been introduced to the cohort at different times (with one exception) (Fig. 1B and C). The circulation of subgroups for each of the serotypes did not appear to be geographically restricted (Fig. 1A to C).

Prevalence and epidemiology of HEV-A and HEV-B infections. HEV was detected in 145 of 1,255 samples (11.6% of samples; 95% CI, 9.8% to 13.5%). HEV-A was detected most frequently, with an overall prevalence of 6.8% (95% CI, 5.0% to 8.6%); HEV-B was present in 4.8% of the samples (95% CI, 3.2% to 6.4%). HEV-C was present in only three samples (0.2%). No poliovirus or HEV-D group viruses were detected. Of 124 infection episodes, 70 (57%) were classified as HEV-A; 49 (40%) were classified as HEV-B (Table 2). HEV-B infections were more prevalent than HEV-A infections during the interval from March through August 2002 (2.9% to 4.4% versus 1.4% to 2.6%); however, during the interval from December 2002 through November 2003, HEV-A infections were more prevalent than HEV-B infections (3.9% to 10.4% versus 1.6% to 6.4%). From September through November 2002, HEV-A and HEV-B prevalence rates were similar (7.8%). The highest prevalence of HEV-A was observed from June through August 2003 (10.4%); the highest prevalence of HEV-B was observed from September through November 2002 (7.8%). The characteristic seasonal variation of HEV infections, with peak incidence in late summer and autumn, was seen for both HEV-A and HEV-B (data not shown).

Figure 2 shows the age-specific prevalences of HEV-A and HEV-B infections for children aged 3 to 23 months. The 3-month moving averages of the age-specific prevalences of positive samples are plotted against the ages of the children in months. In both species, the highest prevalence was in the second year of life.

The probability of having at least one HEV infection by the age of 12 months was approximately 40%; 90% of the children had been infected by the age of 2 years (see Fig. S1 in the supplemental material). There were no significant differences in prevalence of HEV-A or HEV-B between boys and girls or between the two main counties included in the study (data not shown).

Prolonged excretion, viral load, and coinfections. In 28 of the 124 infection episodes, the same viral serotype was observed in two or more consecutive samples: 23 cases of two consecutive months, 4 cases of three consecutive months, and 1 case of four consecutive months. Prolonged duration of infection was associated with a higher viral load in the first sample, but not with sex, viral species, or season. The initial viral load was on average eightfold higher in infections that persisted for at least 2 months than in single-month episodes (P = 0.001). A trend toward shorter duration of infection with increasing age was not statistically significant (P = 0.078).

Stool samples from 10 children showed evidence of coinfection with more than one HEV. Eight samples contained two serotypes; two samples had three serotypes. In six samples, the coinfections were limited to HEV-A; in three cases, there was coinfection with HEV-A and HEV-B; in one case, there was
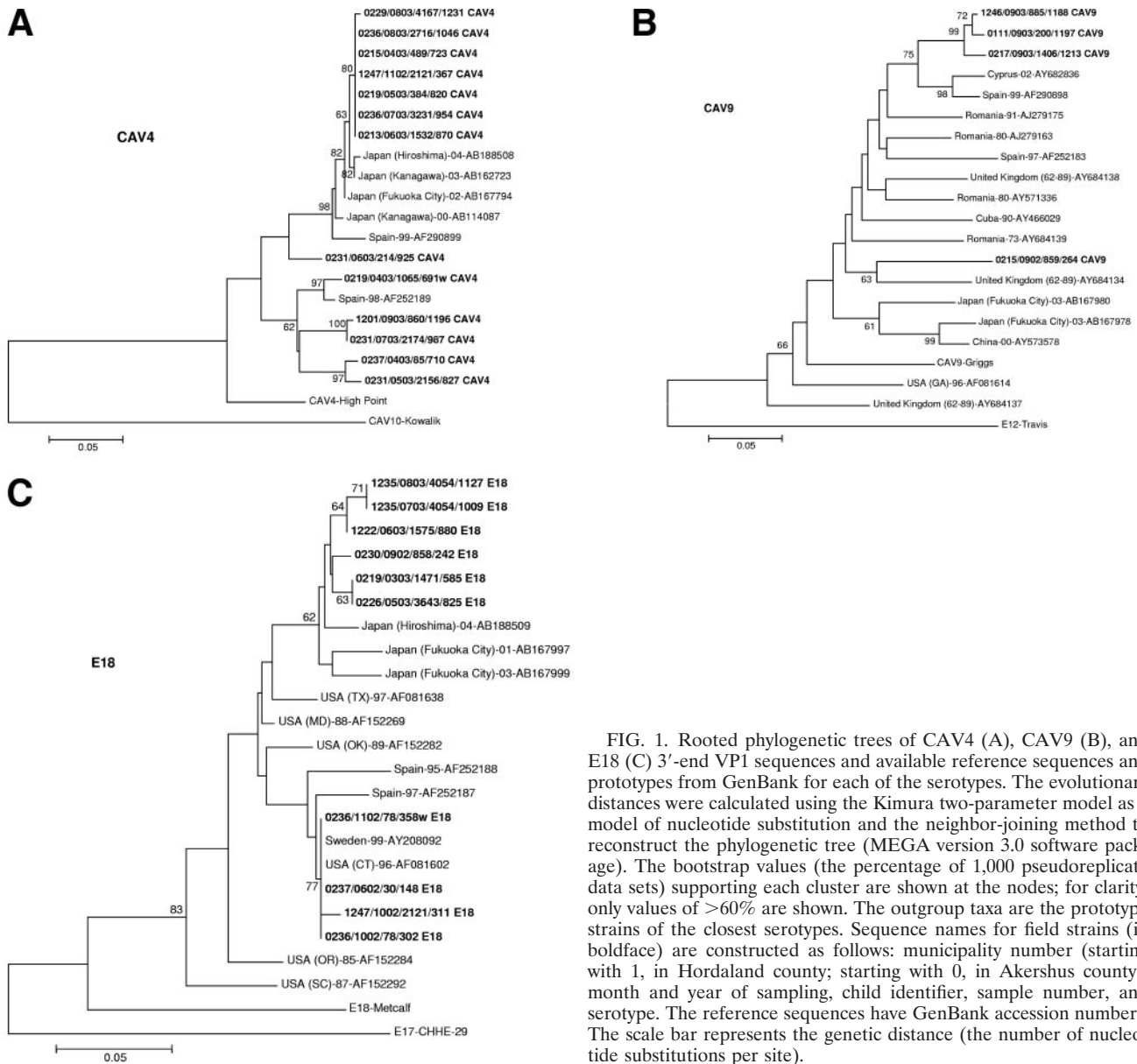
A

CAV4

B

CAV9

C

E18

FIG. 1. Rooted phylogenetic trees of CAV4 (A), CAV9 (B), and E18 (C) 3′-end VP1 sequences and available reference sequences and prototypes from GenBank for each of the serotypes. The evolutionary distances were calculated using the Kimura two-parameter model as a model of nucleotide substitution and the neighbor-joining method to reconstruct the phylogenetic tree (MEGA version 3.0 software package). The bootstrap values (the percentage of 1,000 pseudoreplicate data sets) supporting each cluster are shown at the nodes; for clarity, only values of >60% are shown. The outgroup taxa are the prototype strains of the closest serotypes. Sequence names for field strains (in boldface) are constructed as follows: municipality number (starting with 1, in Hordaland county; starting with 0, in Akershus county), month and year of sampling, child identifier, sample number, and serotype. The reference sequences have GenBank accession numbers. The scale bar represents the genetic distance (the number of nucleotide substitutions per site).

coinfection with HEV-A and HEV-C. The viral loads in the coinfected samples were not significantly different than in samples containing only a single serotype.

DISCUSSION

HEV infections were prospectively assessed in stools of healthy children by using a molecular typing strategy. HEVs were detected in 11.6% of the stool samples; 98% of the HEV-positive samples were identified to the species level. HEV-A species were detected in 6.8% of the samples; HEV-B was detected in 4.8% of the samples. Although these findings contrast with recent studies of HEV epidemiology based on culture and serology that reported only infrequent HEV-A infections in symptomatic (2, 5–7, 9, 25, 27, 37, 43) or healthy (13, 16, 21, 23, 38) children, they are consistent with earlier

studies in which suckling mice were employed for in vivo assays and HEV-A infections were observed to be relatively frequent in healthy children (12, 17, 39). In a 1958 and 1959 study of stool samples obtained from 2,084 healthy children less than 5 years of age in London, the prevalence rates for HEV-A (CAV2, -4, -5, -6, -8, -10, and -12) and HEV-B (CBV3 and -4) isolates were 5.4% and 1.2%, respectively (17). In a study of clinical samples (primarily stools) obtained in 1966 and 1967 from 625 healthy children in Australia, the prevalence rates for HEV-A (CAV2, -3, -4, -8, and -10) and HEV-B (CBV2, -4, and -5 and CAV9) isolates were 12.1% and 4.3%, respectively (12). The Australian samples were collected in a single nursery; thus, the extent to which they represent the prevalence in the general population is uncertain. Finally, a Malaysian study of stool samples collected in 1971 and 1972 from healthy children less than 7 years of age found prevalences for HEV-A of 5.3%
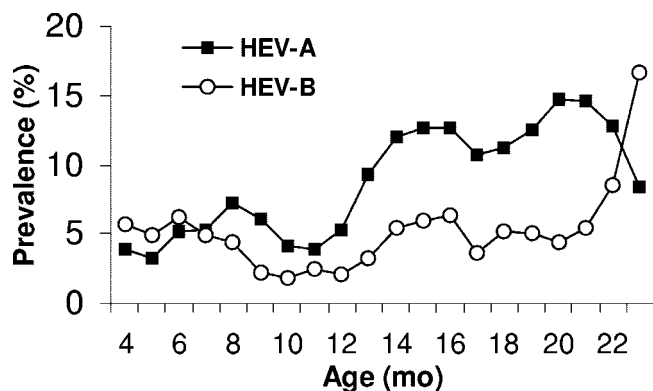
FIG. 2. Three-month moving average of the age-specific prevalences of HEV-A- and HEV-B-positive samples for ages 3 to 23 months ($n = 1,237$ person-months). The curve was truncated at 24 months, due to few observations (18 stool samples) from 24 to 28 months of age. A logistic regression model was applied to adjust for the potential mutual confounding of age and season or year. Adjusting for season or year indicated that confounding by these factors was minimal or did not change the effect of age in the logistic-regression model.

and for HEV-B of 3.4% (39). No species identification was reported.

We speculate that differences in the prevalences of individual HEV serotypes between our study and others reflect methodological differences. Whereas in vitro culture methods appear to be more sensitive for HEV-B, in vivo culture methods are more sensitive for HEV-A. To our knowledge, there are no previous studies reporting the prevalence of HEV-A in clinical samples using molecular methods. Our data indicate similar or equal sensitivities for both HEV-A and HEV-B. Thus, the findings reported here may represent accurate levels of circulating HEV serotypes.

Our analysis of seasonality confirmed the established pattern for HEV infections, where the highest prevalence in the Northern Hemisphere occurs between July and November (11). This observation held for both HEV-A and HEV-B (data not shown).

Analysis of age-specific frequencies of HEV-A and HEV-B showed an apparent trough between the ages of 9 and 12 months, with a distinct increase after 12 months of age (Fig. 2). These findings are consistent with those of Gamble et al. (17), in which the frequencies of both HEV-A and HEV-B infections were lower in the first year of life.

Phylogenetic analysis of the C-terminal domain of the VP1 gene has facilitated the differentiation of circulating serotypes and the association of specific HEV genotypes with disease (1, 3, 34, 40). Some serotypes are associated with global epidemics (1). During the period from 2001 to 2003, E13 and E18 were reported as the more common serotypes causing outbreaks of aseptic meningitis in the United States and elsewhere (6, 37). Most of the infections were E18 (8.9% versus 1.6%). Our VP1 sequence analysis revealed the circulation of several strains of the same serotype (Fig. 1).

HEV coinfections were commonly observed in the Norwegian infants followed in the present study. Although coinfections have been described in the developing world (23), they are only infrequently reported in children in industrialized or temperate areas (13).

We found no association between sex and either the frequency or duration of infection. These findings are in contrast with those reported for HEV-associated diseases, where infections were more frequent in males than in females, particularly for severe diseases, such as meningitis and carditis (35).

Our results suggest that the prevalence of HEV-A infections has been underestimated in recent studies that have relied on cell culture for detection. The diagnosis of HEV-A infections could be considerably improved by the use of molecular methods that facilitate detection and genotypic analysis.

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Příloha III.

Witsø E, Palacios G, Rønningen KS, Cinek O, Janowitz D, Rewers M, Grinde B, Lipkin WI.

Asymptomatic circulation of HEV71 in Norway.

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Asymptomatic circulation of HEV71 in Norway

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Abstract

Widespread circulation of human enterovirus 71 was discovered in a prospective study of fecal samples obtained from healthy Norwegian children. Molecular characterization of the virus determined that it belonged to genotype C1. Complete sequencing of this strain, HEV71 804/NO/03, revealed differences in the 5′UTR and polymerase with respect to more pathogenic genotypes that may explain its reduced neurovirulence.
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Human enterovirus 71 (HEV71) is associated with outbreaks of hand-foot-and-mouth disease (HFMD), aseptic meningitis and encephalitis. Like poliovirus, HEV71 has affinity for anterior horn cells (Chumakov et al., 1979) and is the most common non-polio enterovirus associated with poliomyelitis-like paralysis (Melnick, 1984). Since its initial isolation in California in 1969, HEV71 has caused epidemics in Australia, Europe, Asia, and the United States (Palacios and Oberste, 2005). More recently, HEV71 caused brain-stem encephalitis during HFMD outbreaks in Malaysia, in 1997 (Cardosa et al., 2003; Chan et al., 2000) and in Taiwan in 1998 (Ho et al., 1999; Lin et al., 2003). The molecular epidemiology of HEV71 has been widely studied (Brown et al., 1999; Cardosa et al., 2003; Chu et al., 2001; Herrero et al., 2003; McMinn et al., 2001; Shimizu et al., 2004). There are two major HEV71 genogroups (B and C) co-circulating worldwide (the HEV71 prototype strain BrCr, isolated in 1969, is the only known example of genogroup A). Genogroups B and C have been subdivided into genotypes: B1–B5 and C1–C4, respectively (Brown et al., 1999; Cardosa et al., 2003; McMinn et al., 2001; Mizuta et al., 2005). Here we

report asymptomatic circulation of HEV71 in Norway. Phylogenetic analysis of VP1 sequences revealed a single circulating strain of genotype C1.

Stool samples were obtained on a monthly basis from 113 healthy infants (60 males, 53 females) in a prospective cohort study focused on environmental triggers of type 1 diabetes. Newborns (6 weeks old) were recruited at their first visit to health care centres. Stool samples and clinical data were obtained monthly from September 2001 to November 2003 (Cinek et al., 2006), beginning at age 3 months and continuing up to 28 months. Total nucleic acids were extracted and analyzed for human enterovirus (HEV) RNA using real-time PCR; 11.3% were positive (Cinek et al., 2006).

The serotype of the HEV positive samples (145/1255) were determined by VP1 nucleotide sequencing. HEV71 was detected in 16.8% (19/113) of the children in the cohort (10 boys and 9 girls, median age 14.0 months, 75% <18 months old). Positive samples were detected from children residing in the following counties: Akershus (south-east), Nord-Trøndelag (central) and Hordaland (west coast). This finding suggests a wide geographical distribution. VP1-2A sequences of HEV71 Norwegian strains (200–630 nt in length) were deposited in the GenBank under accession numbers DQ317216, DQ317217, DQ317218, DQ317219, DQ317220, DQ317221, DQ317222, DQ317223, DQ317224, DQ317225, DQ317226, DQ317227,

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DQ317228, DQ317229, DQ317230, DQ317231, DQ317232, DQ317233, DQ317234, DQ317235, DQ317236, DQ317237, and DQ317238.

HEV71 was found to be circulating widely in a restricted period of time, from October 2002 until October 2003, with peak prevalence in July 2003 (seven cases) (Fig. 1). HEV71 infection was not associated with fever, symptoms of upper respiratory or gastrointestinal complaints as reported by parents (Cinek et al., 2006).

A representative isolate of the Norwegian strain (HEV71 804/NO/03) was recovered from a suspension stool sample, identified with HEV71-specific antibody and titrated in green monkey kidney cells (GMK-AH1) and in a human cervical adenocarcinoma cell line (HeLa). Viral RNA was extracted; reverse transcribed and amplified using primers
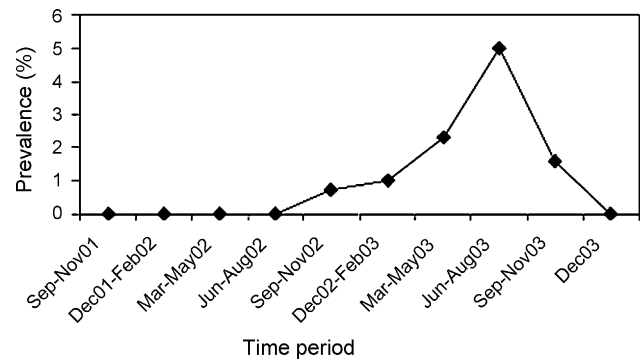
Fig. 1. Prevalence of HEV71 in stool samples from 113 asymptomatic children over the period September 2001 through December 2003.
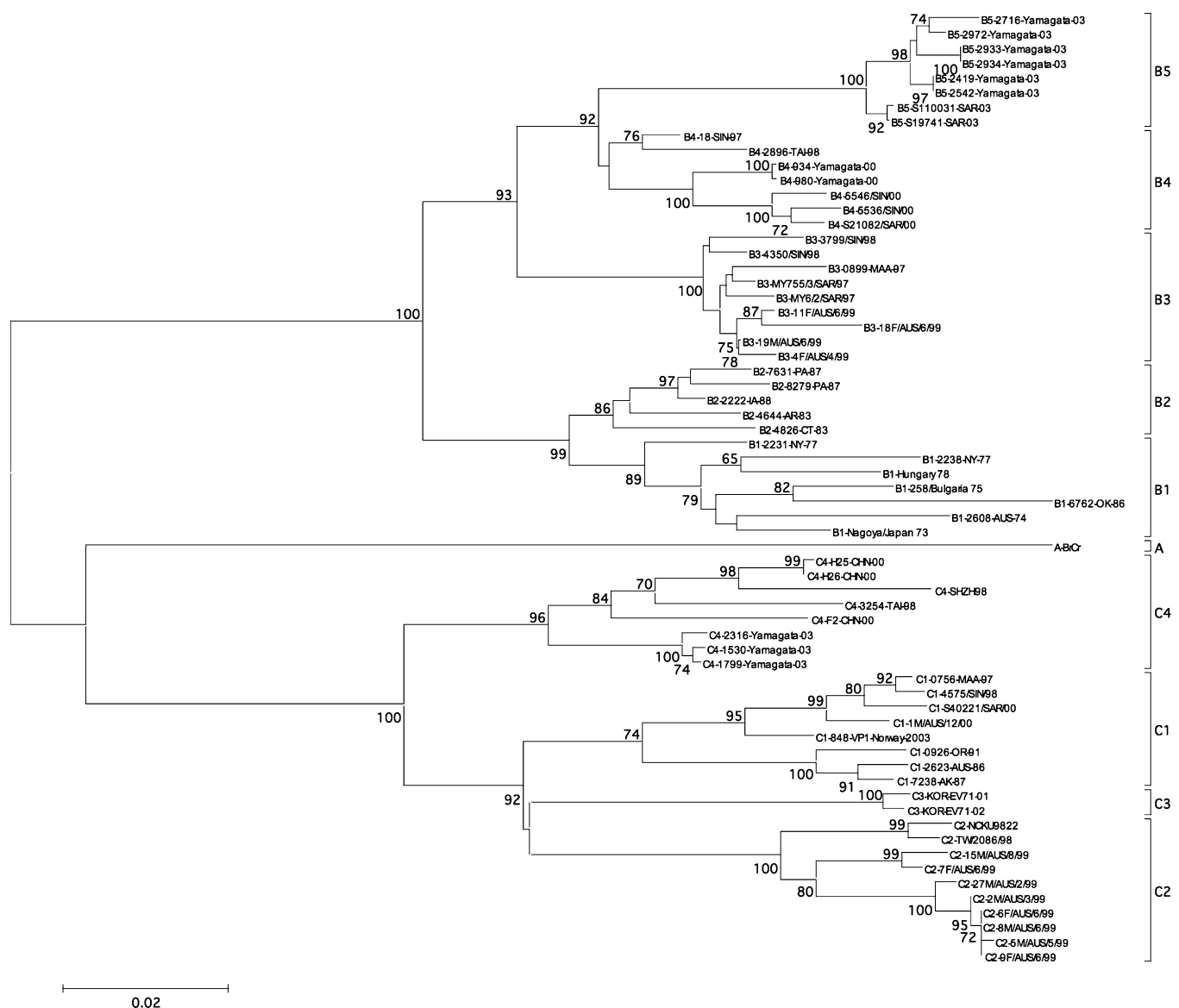

Fig. 2. Phylogenetic relationships of the sequence of the complete VP1 gene of HEV71 804/NO/03. The evolutionary distances were calculated using the Kimura two-parameter model as a model of nucleotide substitution and the Neighbour-Joining method to reconstruct the phylogenetic tree (MEGA version 3.1 software package). Numbers above the branches are bootstrap values (percentage of 1000 pseudo-replicate datasets) supporting each cluster. Genogroups A, B and C and respective sublineages are denoted in the figure. The scale bar represents the genetic distance (nucleotide substitutions per site).

Table 1

Number of hospitalizations or outpatient visits in the age group 0–3 years old associated with a diagnosis of encephalitis, HFMD and herpangina in the period 1999–2004

Diagnosis	ICD-10 code[a]	1999	2000	2001	2002	2003	2004	Total
Enteroviral encephalitis	A85.0+	0	0	2	0	0	0	2
Other viral encephalitis, not elsewhere classified	A85	3	6	1	6	5	10	31
Unspecified viral encephalitis	A86	8	7	7	8	10	5	45
Hand, foot and mouth disease (HFMD)	B08.4	5	4	25	14	9	8	65
Herpangina	B08.5	0	1	2	4	8	5	19
Total		18	24	46	35	34	28[b]	239

[a] ICD, international classification of diseases.

[b] One case is counted twice because it was associated with two diagnoses (A85.0 + B08.5).

designed on the basis of previously published HEV71 strains (Appendix A). High Fidelity PCR Master Mix (Roche) reagents were utilized to minimize introduction of mutations during amplification. Products were cloned into pGEM-T-Easy vector (Promega). Sequencing was performed on both strands, using Big Dye terminator cycle sequencing reagents and the ABI 3730 XL Sequencer (Applied Biosystems, Foster City, CA). Raw sequence data was analyzed with Sequencher (version 4.2, Gene Codes Corporation, Ann Arbor, MI). Ambiguous nucleotides were resolved by re-sequencing. To avoid introduction of mutations by cell culture adaptation, new primers were designed based on the sequence obtained, and direct amplification and sequencing was performed to obtain the HEV71 sequence directly from the stool sample. The complete genome sequence of the Norwegian HEV71 strain was deposited in the GenBank under the accession number DQ452074.

The complete VP1 sequence of the isolate and 64 reference sequences collected from GenBank (Appendix B) were used to reconstruct the phylogenetic tree (Fig. 2) employing neighbor joining and the Kimura model of nucleotide substitution in the program MEGA (version 3.1) (Kumar et al., 2004). The statistical significance was evaluated by bootstrap re-sampling of the sequences 1000 times. The Norwegian strain was classified into genotype C1. Phylogenetic analysis of partial VP1 nucleotide sequences from HEV positive samples revealed that a single strain of HEV71 was circulating in Norway (data not shown).

We sought to investigate if the circulation of this strain was associated with an increase in the frequency of hospitalizations related to HEV71 infection. Clinical records of HFMD, herpangina and encephalitis were obtained from the Norwegian Surveillance System for Communicable Diseases and the Norwegian Patient Register. First, we focused in the analysis of cases

Table 2

Comparative nucleotide sequence analysis of the HEV71 804/NO/03 and representative HEV71 strains of different lineages

Gene/region	Percentage of nucleotide identity between HEV71 804/NO/03 and					
	C2 Tainan/5746/98	C4 SHZH03	B2 MS/7423/87	B4 5865/SIN/00	A BrCr	CAV16-G10
5′UTR	91.2	85.8	83.5	83.5	81.8	79.9
P1 region						
VP4	85.0	86.5	82.6	83.6	83.1	65.7
VP2	89.2	87.0	84.4	82.5	81.5	68.2
VP3	88.7	88.8	81.3	81.8	82.4	71.6
VP1	90.2	89.9	83.8	83.2	82.8	64.2
P2 region						
2A	85.3	82.4	81.6	79.3	77.3	79.6
2B	86.9	75.1	74.7	74.7	76.1	79.8
2C	91.0	79.4	78.5	78.7	81.2	80.0
P3 region						
3A	89.9	74.0	75.2	75.6	79.1	75.6
3B	89.4	75.8	77.3	74.2	74.2	75.8
3C	90.2	76.5	75.8	75.0	74.7	77.4
3D	89.6	77.0	79.1	79.4	78.1	79.0
3′UTR	92.9	77.4	91.7	90.5	95.2	78.6
Overall	89.0	82.0	81.0	80.0	80.0	75.0

Nucleotide sequences of HEV71 reference strains were retrieved from GenBank (accession numbers AF304457, AY465356, U22522, AF316321, U22521 and U05876).

with EV71 diagnosis. Only one case of encephalitis was reported in children below the age of 3 years in 2003; no cases of herpangina or HFMD were reported. In other age groups, the virus was also barely detected: 1 case of HFMD in 2002, 3 in 2003. In previous years, there was one case with HFMD in 2001, none in 2000, and 3 or 4 cases with encephalitis along with 6 cases of HFMD in 1999. Since only a small proportion of HEV detected in clinical samples were serotyped, it is possible that some cases of symptomatic HEV71 infection were not detected. However, there was no increase in the numbers of hospitalizations of patients with encephalitis, HFMD or herpangina recorded during the period of this study in the same age range (Table 1). Thus, we conclude that the majority of HEV71 infections were either asymptomatic or associated with only mild disease.

The comparison of genomic sequences of HEV71 strains derived from fatal and non-fatal cases have been used to investigate the presence of neurovirulent determinants (AbuBakar et al., 1999; McMinn et al., 2001; Shih et al., 2000; Siafakas et al., 2005; Yan et al., 2001). Whereas genotype C1 epidemics in Malaysia, Singapore and Western Australia have been associated primarily with HFMD (McMinn et al., 2001), genotype C2 outbreaks in Malaysia and Taiwan have been associated with severe and fatal neurologic disease (Cardosa et al., 2003; Herrero et al., 2003; McMinn et al., 2001). The differences in neurovirulence between the C1 and C2 genotypes observed in these outbreaks may provide clues to EV71 pathogenicity determinants (McMinn, 2002). However, there is reason for caution in rendering this interpretation given a recent report wherein genotype C2 outbreaks in Japan were associated only with HFMD (Mizuta et al., 2005).

We compared the entire genome of HEV71 804/NO/03 with the HEV71 prototype BrCr (genotype A), HEV71 MS/73 (genotype B2), HEV71 5865/SIN/00 (genotype B4), HEV71 SHZ03 (genotype C4), Tainan/5746/98 (genotype C2) and CAV16 prototype G10. Overall, HEV71 804/NO/03 shared 89% nucleotide identity with the highly virulent C2 strain Tainan/5746/98 and ~81% identity with other HEV71 genotypes, while the identity to CAV16 was 75% (Table 2). Non-structural genes are typically better conserved than structural genes. Indeed, enteroviruses are typed using the structural gene VP1. Interestingly, analysis of HEV71 804/NO/03 and other EV71 strains revealed higher homology in P1 region than in the non-structural genes P2 and P3 regions. The exception was C2-Tainan/5746/98 where close to 90% homology to HEV71 804/NO/03 was observed throughout the genome. Table 3 indicates amino acid differences between HEV71 804/NO/03 and the C2-consensus sequence. Interestingly, most of the changes are located at non-structural sites (30 out of 37, 81.1%; rate of amino acid substitution of structural genes: 0.011 ± 0.002; non-structural genes: 0.028 ± 0.015) and the higher number in the polymerase gene (11 out of 37, 29.8%). With the exception of the mutations in the polymerase described below, we could not find any relation between these changes and changes affecting pathogenesis or viral replication. However, only a reverse genetics based model of these changes will allow prediction of their function, provided that the hypothesis of differences in neurovirulence between C2 and C1 genotypes is correct.

Table 3
Amino acid differences between the genomes of HEV71 804/NO/03 and the C2-consensus sequence

Position	Coding region	C1-Norway-804-2003	C2-consensus[a]
195	VP2	I	V
293	VP2	F	Y
354	VP3	H	T
416	VP3	N	S
587	VP1	Q	R
596	VP1	N	D
854	VP1	T	A
928	2A	S	N
930	2A	R	M
944	2A	V	I
945	2A	Y	F
946	2A	I	V
1042	2B	H	S
1053	2B	R	K
1095	2B	I	V
1187	2C	L	M
1282	2C	V	I
1368	2C	D	E
1406	2C	S	R
1427	2C	S	N
1430	2C	A	V
1479	3A	E	D/V[b]
1487	3A	T	S
1496	3A	S	N
1581	3C	H	R
1603	3C	R	K
1806	3D	R	K
1828	3D	I	M
1859	3D	R	K
1928	3D	T	A
1936	3D	V	I
1992	3D	E	D
2077	3D	K	R
2127	3D	K	R
2141	3D	N	S
2167	3D	T	A
2182	3D	F	Y

[a] Variations observed between HEV71 804/NO/03 and majority (>66%) of C2 full genome sequences (AF304457, AF304458, AF304459, AF136379, AF176044, AF119796 and AF119795).

[b] Five strains had a E → D change. Two (AF119796 and AF119795) had a E → V mutation.

The potential secondary structure of the $5'$ untranslated region ($5'$UTR) was modeled in an attempt to elucidate the existence of structural elements and motifs associated with neurovirulence within the EV71 genome. Neurovirulent phenotype determinants have been localized to the enterovirus $5'$UTR (De Jesus et al., 2005; Evans et al., 1985; Gromeier et al., 1996, 1999; Rinehart et al., 1997; Romero and Rotbart, 1995; Szendroi et al., 2000). The highly structured internal ribosome entry site (IRES) of the $5'$UTR contains seven stem-loop structural domains (I–VII) which are essential for cap-independent initiation of translation and replication. Mutational studies of the loop sequence and the putative stem structure immediately $5'$ to the loop in domain V have indicated the importance of this region to virus transcription and initiation of translation (Borman et al., 1994; Nicholson et al., 1991). Mutations within domain V
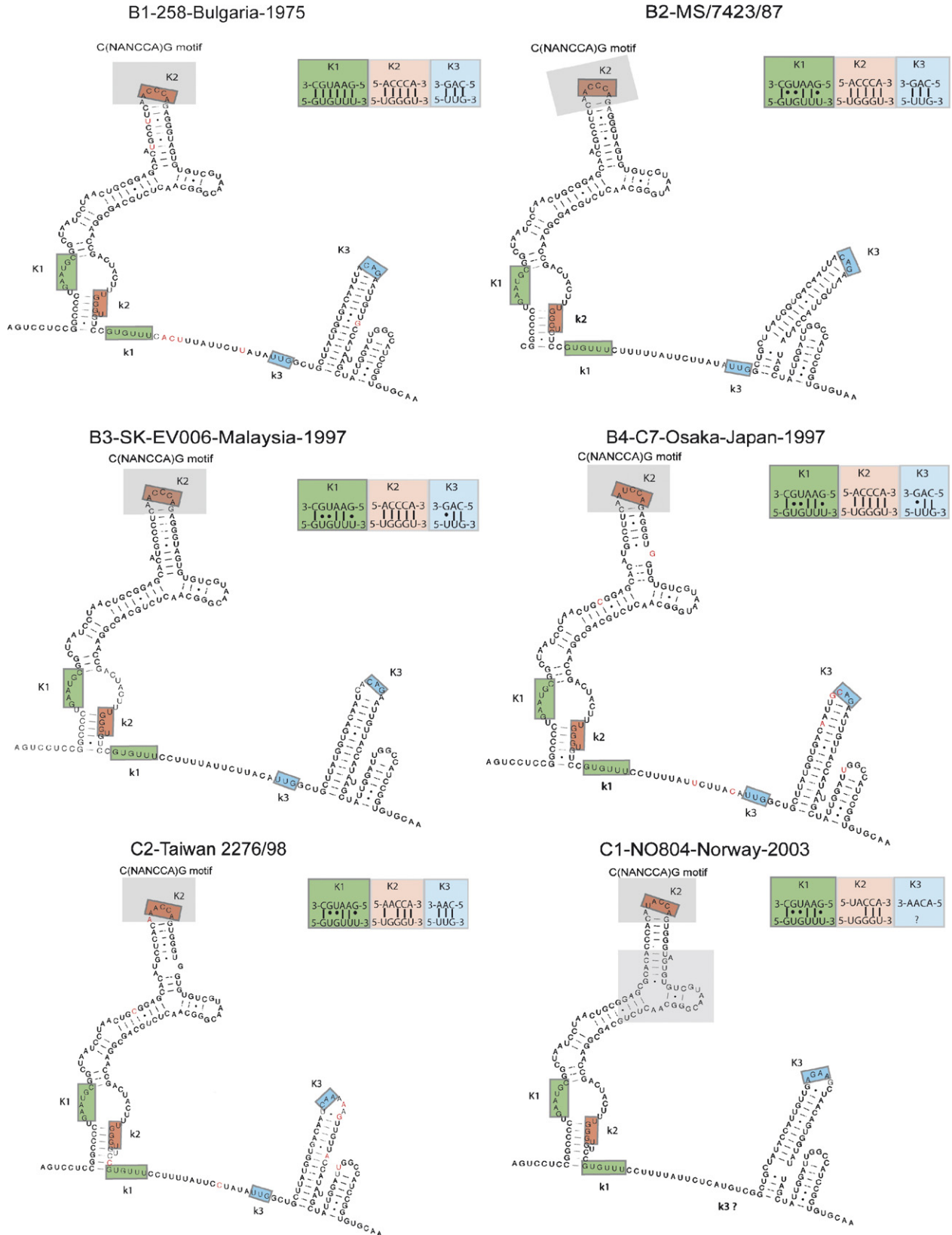
Fig. 3. Predicted RNA secondary structures of IRES domains V, VI and VII of B1-258-Bulgaria-1975 (AB059821), B2-MS/7423/87 (U22522), B3-SK-EV006-Malaysia-1997 (AB059826), B4-C7-Osaka-Japan-1997 (AB059825), C2-Taiwan 2276/98 (AF117634) and HEV71 804/NO/03. RNA structures were predicted based on the lowest free energy, using the Zuker algorithm as implemented in RNA structure (version 3.71). The three protrusions depicted for each viral RNA structure corresponds from the left to right to domains V, VI and VII. Conserved motifs are highlighted in color. Nucleotide positions found to be non-conserved in an alignment of all available sequences of the region are highlighted in red. Conserved tertiary "K" motifs are also denoted in the figure. K and k motifs paired by their numbers align together in the tertiary folding structure of the IRES.

of the genome of poliovirus (PV) Sabin attenuated strains (at nt 480, 481 and 472 for Sabin 1, 2 and 3, respectively), act as a temperature sensitive (ts) determinants and as major determinants of attenuation. The corresponding area containing those

mutations is the central loop (shaded in grey for genotype C1 in Fig. 3). In addition, a temperature sensitive mutant of HEV71-BrCr, including a U to C mutation at position 491, resulted in a marked reduction in virus growth (Arita et al., 2005). Interest-
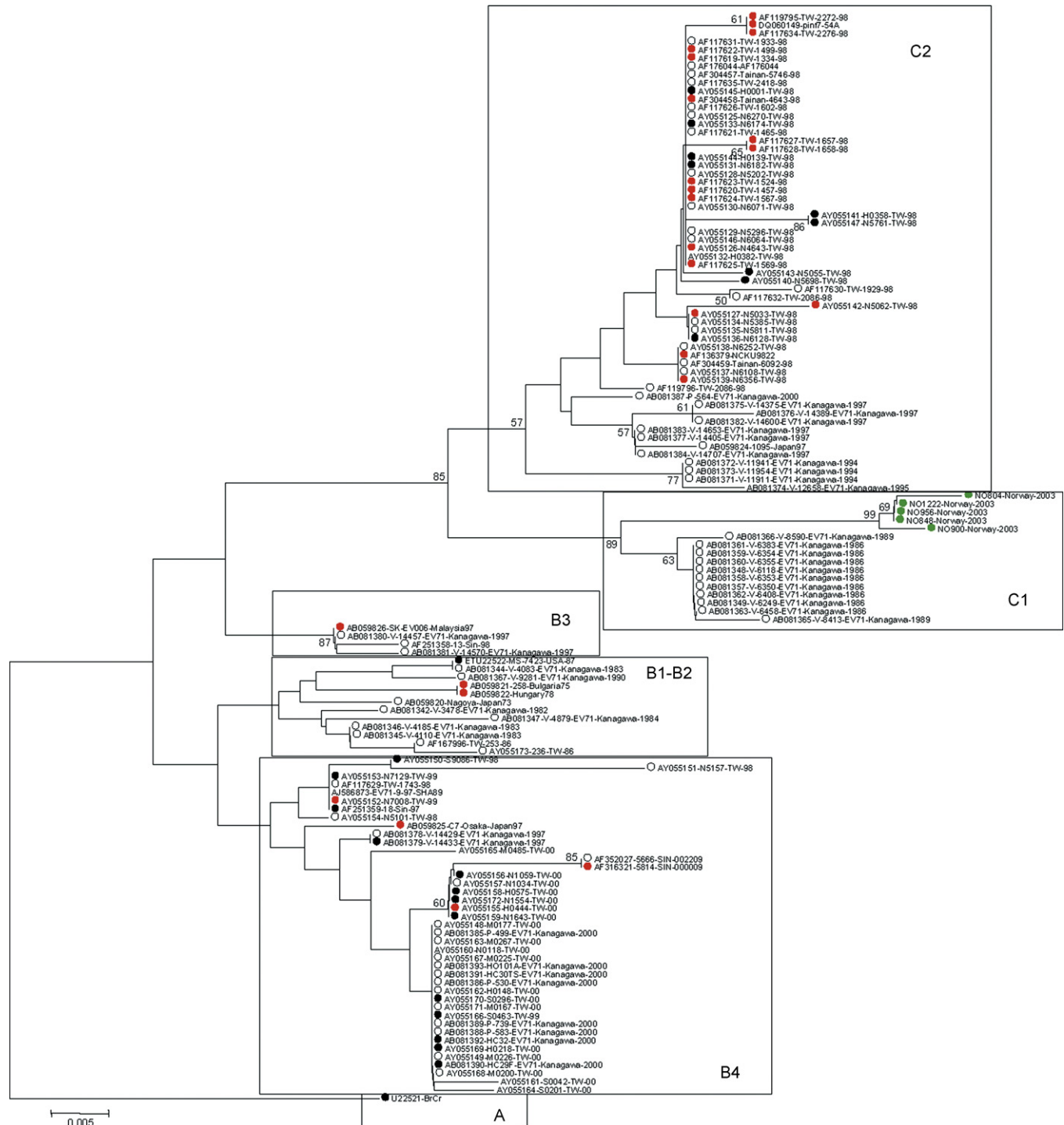
Fig. 4. Phylogenetic relationships of the sequence of the domains V, VI and VII of HEV71 804/NO/03 and all available 5′UTR sequences. The evolutionary distances were calculated using the Kimura two-parameter model as a model of nucleotide substitution and the Neighbour-Joining method to reconstruct the phylogenetic tree (MEGA version 3.1 software package). Numbers above the branches are bootstrap values (percentage of 1000 pseudo-replicate datasets) supporting each cluster. Genogroups A, B and C and respective sublineages are denoted in the figure. The scale bar represents the genetic distance (nucleotide substitutions per site). All published 5′UTR sequences were considered along their associated clinical outcome: (●) represent sequences associated with fatal outcome; (●) central nervous system involvement; (○) herpangina or HFMD; (●) asymptomatic.

ingly, a series of conserved covariant mutations in this domain in the Norwegian C1 strain resulted in a slight shortening of the central loop that clearly differentiated this genotype from all other known genotypes (Fig. 3) (multiple 5′UTR sequences were obtained to confirm this finding). Other genotype C1 sequences also had this modification in the structure of the central loop (data not shown).

A 5′UTR secondary structure feature known to be highly conserved amongst the HEV is the C(NANCCA)G motif (loop in parenthesis) in the secondary structure domain V (Siafakas et al., 2005). The first A in this motif is mutated to U in HEV71 804/NO/03. The changes observed in domain V might have direct impact on the tertiary folding structure of the IRES and its interaction with the 18S ribosomal RNA. Tertiary structure elements occur in the 3′ end of a segment termed a "ribosome landing pad". Those structures, identified as K1, K2 and K3 (Fig. 3), involve highly conserved sites in enteroviruses and rhinoviruses (Nicholson et al., 1991). K2 is suggested as a potential
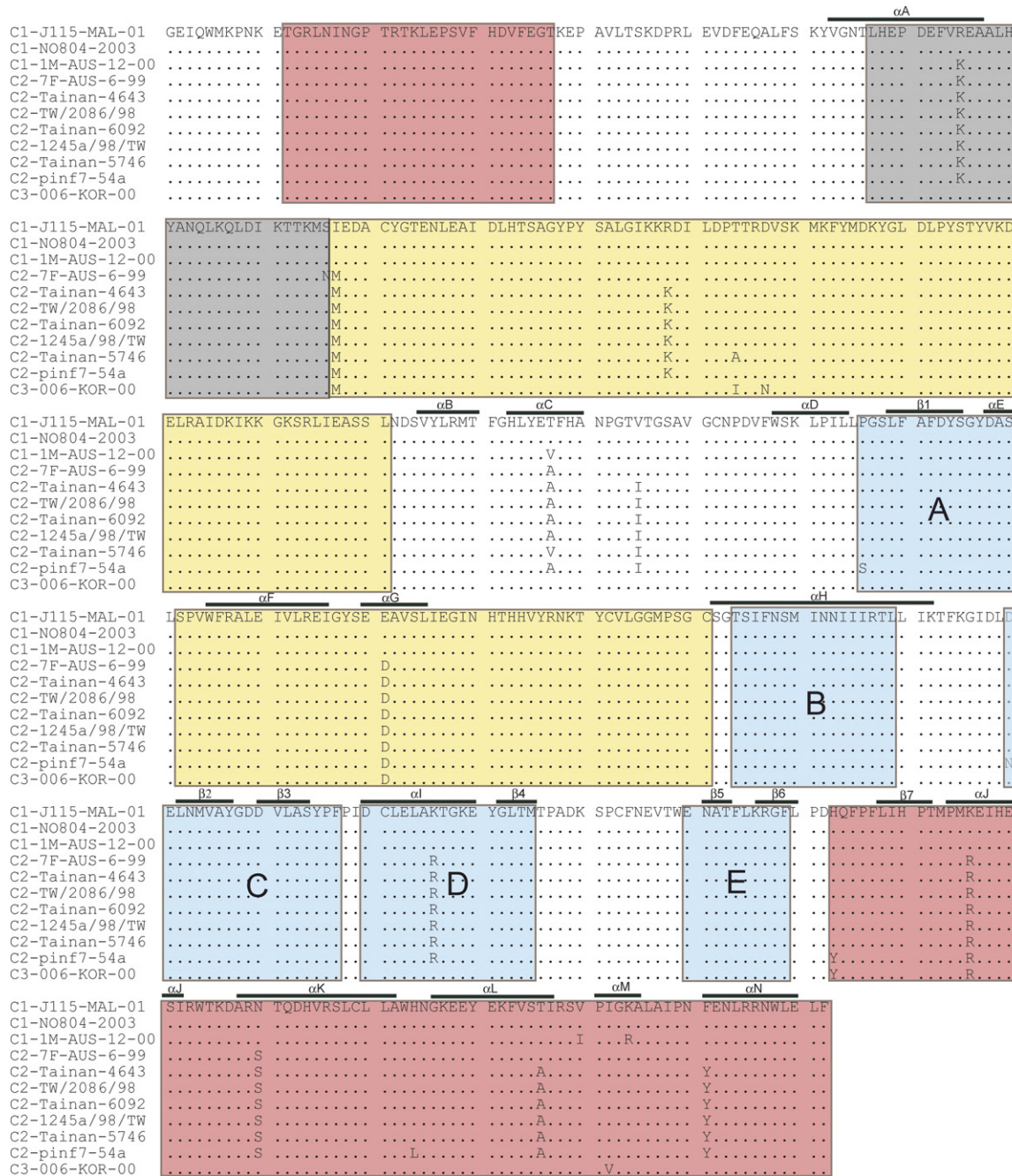
Fig. 5. Structural alignment of available HEV71 RNA-dependent RNA polymerase (3Dpol) sequences of genogroup C. Conserved residues are indicated as (·). The thumb subdomain is highlighted in grey and pink. The grey area appears beneath the fingers subdomain in the crystal structure of the polymerase. The finger subdomains are highlighted in yellow. The palm subdomains are highlighted in light blue. Black bars above the alignment represent alpha or beta helix structures.

binding site for the interaction between 40S ribosomes and/or transacting factor(s) and viral mRNAs. The tertiary folding structure K2 is theoretically destroyed by the A → U mutation in the C(NANCCA)G motif (Fig. 3) of HEV71 804/NO/03. The proposed K3 tertiary folding structure would also be theoretically destroyed by mutations of these conserved sites in HEV71 804/NO/03.

Interestingly, although the 5′UTRs of EV normally do not carry sufficient phylogenetic information to allow discrimination among serotypes (Casas et al., 2001), the analysis of all available HEV71 sequences for the domains V, VI and VII of the 5′UTR (Fig. 4) correlates with the genotype clustering of the VP1 gene (Fig. 2). Furthermore, the consensus predicted RNA secondary structure of the 5′UTR represented in Fig. 3 for each of the genotypes is conserved in all available sequences for each genotype (variable sites highlighted in red in the figure). The functional importance of this is that the RNA secondary structure of the 5′UTR, and especially the central loop modification in C1 (that clearly differentiates the C1 genotype from all other known genotypes) appears to correlate with clinical outcome. All 16 cases with C1 structure had a benign course; 26 of 57 cases with C2 structure presented with neurological disease; 17 of the 26 died. Three of 12 cases with B1–B2 structure had neurologic disease; 2 of 3 died. One of 4 cases with B3 structure had fatal neurologic disease. Seventeen of 41 cases with B4 structure had neurologic disease; 4 of the 17 had a fatal outcome (Fig. 4).

The crystal structure of the poliovirus RNA-dependent RNA polymerase (3Dpol) displays the characteristic "palm", "finger", and "thumb" subdomains, analogous to a right hand (Hansen et al., 1997). Within the palm subdomain of the polymerase is the RNA recognition motif (RRM) characteristic of RNA-dependent RNA polymerases. The palm subdomain contains motifs A–E present in many polymerases (for review see O'Reilly and Kao, 1998). Eleven amino acid changes were observed when comparing the 3Dpol gene between HEV71 804/NO/03 and other C2 strains (see Table 3). While the palm-motifs A, B, C and E were conserved, a K to R substitution in C2 strains was observed in the alpha I region of palm-motif D (Fig. 5). Two substitutions were also observed in the palm domain outside the palm-motifs. Four substitutions were observed in the thumb domain in positions involved in the alpha helix regions that are the core of the domain and three substitutions were observed in the finger domain. However, although structure prediction algorithms do not suggest that these changes modify the folding of the protein, we cannot exclude functional effects.

The finger and thumb subdomains of the polymerase play a role in modulating substrate recognition and proteolytic processing by the 3CD peptide. These subdomains have been implicated in oligomerization of the enzyme, a process that is critical to substrate binding and elongation of polypeptide chain. The crystal structure has revealed that 3Dpol molecules oligomerize along two interfaces: I and II. Interface-I-mediated dimerization (or oligomerization) has been pro-

posed to promote RNA binding. Interface II is formed largely by N-terminal peptide regions of the polymerase, of which only residues 13–37 (thumb) and 67–97 (beneath the finger domain) structural foldings are known. Given the unusual position of the 67–97 fragment in that interface of the crystal structure, it has been proposed that this N-terminal strand of the active polymerase is donated in trans after dimerization and is necessary for polymerase activity. A Y73H substitution of the Sabin strain of poliovirus 1 has been postulated as a neurovirulence determinant in poliovirus by impairing this dimerization (Paul et al., 2000). Indeed, substitutions in the amino acid position 73 (together with a change in position 363) of the HEV71-BrCr polymerase resulted in an attenuated neurological phenotype in a monkey model of infection (Arita et al., 2005). The HEV71 804/NO/03 polymerase presents an R to K substitution at position 75. Interestingly, the cynomolgous macaque model of HEV71 neurological infection demonstrates that the dual presence of 3Dpol substitutions and changes in the domain V of the 5′UTR increase the level of attenuation (Arita et al., 2005).

Our prospective study of HEV infection in Norwegian children over the period of September 2001 to November 2003 revealed the appearance of HEV71 in October 2002 and an incidence rate of HEV71 infection of 0.27 persons-years thereafter. Given a population at risk of approximately 180,000 (the number of infants below 3 years of age during the period of assessment), there may have been as many as 48,600 infants infected. The lack of any increase in neurological disease strongly suggests that the virus had low pathogenicity. It is striking therefore, that whereas all reports of C1 genotype infection in Asia are associated with HFMD with or without neurologic involvement, infection in Norway appears to be asymptomatic. The absence of disease in Norwegian children may reflect host factors such as genetic resistance, cross-reactive immunity, hygiene and nutritional status. However, viral factors are also plausible. There are no published sequences for the 5′UTR of C1 strains circulating in the US, Malaysia and Australia; nonetheless, direct comparison of sequences of C1 strains in Japan and Norway defines two clades (Fig. 4). Whether the absence of disease in Norwegian children reflects intrinsic viral properties or host factors remains to be determined.

Appendix A. Oligonucleotide primers used for amplification of HEV71

Primer (orientation)	5′–3′ sequence	Region	Position[a]
ENV71-sp-1 (F)	TTAAAACAGCTGTGGGTTGCACCCAC	5′UTR	1–26
EV167A (F)	CAAGCACTTCTGTATCCCCG	5′UTR	168–187
EV580B (R)	ATTGTCACCATAAGCAGCCA	5′UTR	584–603
ENV71-1510 (R)	TTGTTGGTCCYCARATTAATCCACTGRTGTGGGCA	VP2	1485–1519
ENV71-sp-1345 (F)	GAGTATGTCATTGGGACATGGCAGG	VP2	1338–1363
ENV71-sp-1809 (R)	GGTATRTGRATACACGGGTGGGGTG	VP3	1800–1825
ENV71-sp-1809 (F)	CACCCCACCCGTGTATYCAYATACC	VP3	1800–1825
ENV71-sp-2250 (R)	TGCGCTCTGTAGTGAGTGTTGCTGATCCATGG	VP3	2223–2254
ENV71-sp-2231 (F)	CCATGGATCAGYAACACTCAYTACAG	VP3	2223–2248
ENV71-sp-2250 (F)	CCATGGATCAGCAACACTCACTACAGAGCGCA	VP3	2223–2254
VP1-2S-ENV71 (F)	CARTAYATGTTTGTICCSCCYGG	VP1	2895–2917
HEV71-sp-2921 (F)	CCAAGCCAGACTCCAGAGAA	VP1	2923–2942
ENV71SP (F)	GCACAGGTYTCIGTICCCRTTYATGTC	VP1	3003–3028
VP1-2A-ENV71 (R)	TCACAACCYTGRGCRGTGGTAGA	2A	3462–3484
ENV71-3497 (F)	TGTAATTGTCAGACAGGGGTGTAT	2A	3498–3521
ENV71-4430 (R)	CATACAGGTTCAATACGGTGTTTGCTCTTGAACTGC	2C	4415–4450
ENV71-4405 (F)	TAATTACATGCAGTTCAAGAGCAAA	2C	4407–4430
ENV71-4958 (R)	TGTATCTCACCTTGGACTTSCTATC	2C	4959–4983
ENV71-4958 (F)	GATAGSAAGTCCAAGGTGAGATACA	2C	4959–4983
ENV71-4405 (R)	TTTGCTCTTGAACTGCATGTAATTA	2C	4407–4430
ENV71-4409 (R)	TGCTCTTGAACTGCATGTAATTATTCAT	2C	4401–4428
ENV71-5604 (R)	TTAGTATCAAGCGTTACCAGTGTGA	3C	5605–5629
ENV71-5604 (F)	TCACACTGGTAACTCTTGATACTAA	3C	5605–5629
ENV71-5790 (R)	GTTGGGAAATTGTACATCATAGTCC	3C	5791–5812
ENV71-5664 (F)	AAACAATTAGTCCTGCTAGTGATGC	3C	5665–5689
ENV71-6513 (R)	CCAAATGTCATTCTCAAGTACACTG	3D	6490–6514
ENV71-6655 (F)	TATGAYGCTAGYCTYAGYCCIGTGTGGTTCAG	3D	6648–6679
RPOL-1S (F)	YGARGCIWSIAGYYTIAAYGA	3D	6467–6487
RPOL-1A (R)	AWRTTRTTRATCATWGARTTRAAIAT	3D	6825–6850
EV7408B (R)	GCTATTCTGGTTATAACAAA	3′UTR	7392–7411

[a] Relative to the genome of HEV71/enterovirus 5865/sin/000009 (GenBank accession number AF316321).

Appendix B. Clinical isolates used in phylogenetic analysis of the complete VP1 gene of HEV71

Reference	Strain name	Year	Origin	Outcome[a]	Group	Nucleotide database accession no.
Brown and Pallansch (1995)	BrCr-CA-70	1970	USA	Encephalitis	A	U22521
Shimizu et al. (1999)	Nagoya/Japan 73	1973	Japan	NA	B1	AB059813
Brown et al. (1999)	2604-AUS-74	1974	Australia	Meningitis	B1	AF135883
Chumakov et al. (1979)	258/Bulgaria 75	1975	Bulgaria	Polio-like	B1	AB059814
Brown et al. (1999)	2238-NY-77	1977	USA	NA	B1	AF135876
Brown et al. (1999)	2231-NY-77	1977	USA	NA	B1	AF135870
Nagy et al. (1982)	Hungary 78	1978	Hungary	Polio-like	B1	AB059815
Brown et al. (1999)	4644-AR-83	1983	USA	NA	B2	AF135896
Brown et al. (1999)	4826-CT-83	1983	USA	NA	B2	AF135897
Brown et al. (1999)	6762-OK-86	1986	USA	NA	B1	AF135900
Brown et al. (1999)	2623-AUS-86	1986	Australia	HFMD	C1	AF135945
Brown et al. (1999)	7631-PA-87	1987	USA	Gastroenteritis	B2	AF009533
Brown et al. (1999)	8279-PA-87	1987	USA	NA	B2	AF009537
Brown et al. (1999)	7238-AK-87	1987	USA	Rash	C1	AF135952
Brown et al. (1999)	2222-IA-88	1988	USA	Fever	B2	AF009540
Brown et al. (1999)	0926-OR-91	1991	USA	NA	C1	AF009548
McMinn et al. (2001)	MY755/3/SAR/97	1997	Sarawak	HFMD	B3	AF376076
McMinn et al. (2001)	MY6/2/SAR/97	1997	Sarawak	ACS	B3	AF376075
Herrero et al. (2003)	0899-MAA-97	1997	Malaysia	Meningitis	B3	AY207642
Singh et al. (2000)	18-SIN-97	1997	Singapore	AFP	B4	AF251359
Brown et al. (1999)	0756-MAA-97	1997	Malaysia	NA	C1	AF135935
McMinn et al. (2001)	3799/SIN/98	1998	Singapore	HFMD	B3	AF376117
McMinn et al. (2001)	4350/SIN/98	1998	Singapore	HFMD	B3	AF376119
Li et al. (2005)	2896-TAI-98	1998	Taiwan	NA	B4	AF286516
McMinn et al. (2001)	4575/SIN/98	1998	Singapore	HFMD	C1	AF376120
Yan et al. (2000)	NCKU9822	1998	Taiwan	Fatal	C2	AF136379

Appendix B (*Continued*)

Reference	Strain name	Year	Origin	Outcome[a]	Group	Nucleotide database accession no.
Shih et al. (2000)	TW/2086/98	1998	Taiwan	Benign	C2	AF119796
Li et al. (2005)	3254-TAI-98	1998	Taiwan	NA	C4	AF286531
Cardosa et al. (2003)	SHZH98	1998	China	NA	C4	AF302996
McMinn et al. (2001)	11F/AUS/6/99	1999	Australia	Meningitis	B3	AF376089
McMinn et al. (2001)	18F/AUS/6/99	1999	Australia	HFMD	B3	AF376095
McMinn et al. (2001)	19M/AUS/6/99	1999	Australia	HFMD	B3	AF376096
McMinn et al. (2001)	4F/AUS/4/99	1999	Australia	GBS	B3	AF376105
McMinn et al. (2001)	7F/AUS/6/99	1999	Australia	Meningitis	C2	AF376108
McMinn et al. (2001)	15M/AUS/8/99	1999	Australia	HFMD	C2	AF376092
McMinn et al. (2001)	27M/AUS/2/99	1999	Australia	HFMD	C2	AF376102
McMinn et al. (2001)	2M/AUS/3/99	1999	Australia	Myelitis	C2	AF376103
McMinn et al. (2001)	6F/AUS/6/99	1999	Australia	Encephalitis	C2	AF376107
McMinn et al. (2001)	8M/AUS/6/99	1999	Australia	Myelitis	C2	AF376109
McMinn et al. (2001)	5M/AUS/5/99	1999	Australia	Meningitis	C2	AF376106
McMinn et al. (2001)	9F/AUS/6/99	1999	Australia	Ataxia	C2	AF376110
McMinn et al. (2001)	S21082/SAR/00	2000	Sarawak	HFMD	B4	AF376084
Singh et al. (2002)	5865/sin/000009	2000	Singapore	Fatal	B4	AF316321
Singh et al. (2002)	5666/sin/002209	2000	Singapore	Fatal	B4	AF352027
Mizuta et al. (2005)	934-Yamagata-00	2000	Japan	HFMD	B4	AB177809
Mizuta et al. (2005)	980-Yamagata-00	2000	Japan	HFMD	B4	AB213624
McMinn et al. (2001)	S40221/SAR/00	2000	Sarawak	HFMD	C1	AF376087
McMinn et al. (2001)	1M/AUS/12/00	2000	Australia	HFMD	C1	AF376098
Shimizu et al. (2004)	F2-CHN-00	2000	China	NA	C4	AB115491
Shimizu et al. (2004)	H25-CHN-00	2000	China	NA	C4	AB115492
Shimizu et al. (2004)	H26-CHN-00	2000	China	NA	C4	AB115493
Cardosa et al. (2003)	KOR-EV71-01	2001	Korea	NA	C3	AY125966
Cardosa et al. (2003)	KOR-EV71-02	2002	Korea	NA	C3	AY125967
Mizuta et al. (2005)	2542-Yamagata-03	2003	Japan	HFMD	B5	AB177815
Mizuta et al. (2005)	2716-Yamagata-03	2003	Japan	HFMD	B5	AB177816
Mizuta et al. (2005)	2419-Yamagata-03	2003	Japan	HFMD	B5	AB213647
Mizuta et al. (2005)	2933-Yamagata-03	2003	Japan	HFMD	B5	AB213648
Mizuta et al. (2005)	2934-Yamagata-03	2003	Japan	HFMD	B5	AB213649
Mizuta et al. (2005)	2972-Yamagata-03	2003	Japan	HFMD	B5	AB213650
Mizuta et al. (2005)	S110031-SAR-03	2003	Sarawak	NA	B5	AY258307
Mizuta et al. (2005)	S19741-SAR-03	2003	Sarawak	NA	B5	AY258313
Mizuta et al. (2005)	1530-Yamagata-03	2003	Japan	HFMD	C4	AB213638
Mizuta et al. (2005)	1799-Yamagata-03	2003	Japan	HFMD	C4	AB213641
Mizuta et al. (2005)	2316-Yamagata-03	2003	Japan	HFMD	C4	AB213644

[a] NA, not available; CNS, central nervous system; AFP, acute flaccid paralysis; ACS, acute cardiogenic shock; GBS, Guillain–Barré syndrome.

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Příloha IV.

Witsø E, Cinek O, Aldrin M, Grinde B,
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Predictors of sub-clinical enterovirus infections
in infants: a prospective cohort study.

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INFECTIOUS DISEASE

Predictors of sub-clinical enterovirus infections in infants: a prospective cohort study

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Background	Enterovirus infections are common, although most often sub-clinical. The present purpose was to assess the impact of breastfeeding and other factors on enterovirus infections in infancy.
Methods	A prospective observational study was carried out on a population-based cohort of 639 Norwegian infants aged 3–12 months. The outcome was enterovirus RNA measured in monthly stool samples. Data on underlying determinants, such as dietary feeding and household factors, were reported in parental questionnaires. Multivariable logistic regression was performed to allow for common confounders. Statistical analyses were performed by GLLAMM using Stata 9.2, which corrects for subject-specific random effects.
Results	The prevalence of enterovirus in stools was 11.1% (475/4279). Risk of enterovirus infection decreased with increasing number of daily breastfeeds; the effect was most pronounced at the age of 3 months [odds ratio (OR), 0.85; 95% confidence interval (CI) 0.8–0.9, $P < 0.001$], gradually declining thereafter, reaching no effect at 11 months. Increased risk was associated with having one or more sibling(s) (OR 1.89; 95% CI 1.2–3.0), particularly if they attended daycare (OR 2.46; 95% CI 1.4–4.2), and with increasing exposure to other children (OR 1.04; 95% CI 1.0–1.1). There was a tendency towards higher prevalence of infection when a household's drinking water came from a well, and a protective effect of owning a dog or cat.
Conclusions	Several factors may modify the risk for enterovirus infections in the first year of life. This study supports the protective effect of breastfeeding. The protection decreased with age and increased with dose of ingested milk.
Keywords	Enterovirus, breastfeeding, infant, multivariable logistic regression, prospective cohort study, predictors

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Introduction

Enterovirus infections are common in infancy, being usually asymptomatic and self-limiting.[1] They may, however, cause haemorrhagic conjunctivitis, aseptic meningitis, neonatal sepsis-like disease and acute flaccid paralysis.[2] Moreover, even asymptomatic infections may impact on health; enteroviruses have been postulated to trigger or accelerate type 1 diabetes.[3]

Breastfeeding may be an important protective factor for a variety of infectious diseases. It has been associated with reduced incidence of overall infections, gastrointestinal or respiratory tract infections in industrialized countries.[4] Increased duration, as well as exclusiveness of breastfeeding, have been associated with greater protective effects.[5–17] Moreover, the protection provided by breastfeeding on infant mortality to infectious diseases, declined steadily with age during infancy.[18] There is also evidence for protection from clinical infections extending years after termination of breastfeeding.[11,19]

Despite the clinical importance of enterovirus infections, only two studies have examined the effect of breastfeeding on these infections.[20,21] It remains unclear whether there are dose–response effects of breastfeeding, or whether the protection provided from breastfeeding depends on the age of the infant. In order to address these issues, infant feeding and health outcome data should be collected prospectively at frequent intervals. Breastfeeding is typically assessed as dichotomous (e.g. ever/never), trichotomous (exclusive vs partial vs none) or by duration. Prospective, population-based studies using a longer ordinal scale find significant dose–response effects as to the diagnosis of infections.[5,16,17] Moreover, a sensitive and specific method for assessing the infection is a prerequisite for investigating the predictors of infection. The cell culture techniques used for the detection of enteroviruses by most previous epidemiological studies suffer from a substantial bias, as the detection of various types of enteroviruses has varying sensitivities.[22] Finally, observational studies should control for confounding factors and effect modifiers associated with both putative predictors of infections (exposures) and infant infection (outcome). Although the available data on predictors of enterovirus infections are limited, enterovirus prevalence and transmissibility may depend on factors such as age, climate, geographic region, season, crowding, housing conditions, siblings and socio-economic status.[21,23–28]

In the present report, the above issues have been taken into consideration. The aim was to carry out an analysis of the independent impact of putative predictors, in particular breastfeeding, on sub-clinical enterovirus infections in the first year of life. In contrast to prior studies, the effect of breastfeeding was assessed prospectively via monthly recordings of a quantitative measure of daily breastfeeding on a continuous scale to assess dose–response relationships, age and long-term effects of breastfeeding. A reverse transcriptase polymerase chain reaction (PCR), targeted into a region well conserved across enteroviruses, was used to increase the sensitivity of detection. Other putative predictors were assessed simultaneously, with a proper adjustment for putative confounders.

Methods

Study design and sample size

Data from the present study were derived from the Environmental Triggers of Type 1 Diabetes Study (with the Norwegian acronym MIDIA), a prospective cohort study in Norway described in more detail elsewhere.[1,29] Briefly, newborns were recruited from the general population at their first visit at public healthcare centres. Genetic testing identified children carrying the type 1 diabetes high-risk genotype *DRB1*0401-DQA1*03-DQB1*0302/DRB1*0301-DQA1*05-DQB1*02* (often referred to as *DR4-DQ8/DR3-DQ2*) for further follow-up. The high-risk genotype is carried by 2.1% of the Norwegian newborns, with 6% risk of developing type 1 diabetes before the age of 15 years.[30–32] Beginning at 3 months of age and followed up to 12 months of age, monthly stool samples were taken by the parents, and data on putative predictors of infection were obtained from mailed questionnaires at ages 3, 6, 9 and 12 months. The mothers were requested to keep daily breastfeeding and supplementary feeding records, which could be used to help fill out the questionnaires. In addition, from 2004, children without the high-risk genotype were enrolled to the study and followed in the same manner. The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate approved the study, and written informed consents were obtained from the parents.

The analysis reported here focuses on data from 639 children (50.1% males) enrolled during the period from September 2001 to April 2006. The majority [62% (394/639)] of the children did not carry the high-risk genotype, and >70% of the children were residing in the counties of Akershus (southeast Norway) and Hordaland (west coast), two counties separated by >400 km.

Of the scheduled monthly stool samples, 95% were received. Prior to 2004, 113 children were recruited, and for these children enterovirus was analysed for (see 'Measures' section) in all stool samples. For the children recruited later, 69% of the samples were analysed at random due to reduced analysing capacity. Figure 1 shows the frequency of the number of longitudinal stool samples tested for enterovirus per child. Each child had at least one sample analysed. Fifty percent of children were followed until ≥8 months of age with corresponding data on enterovirus and
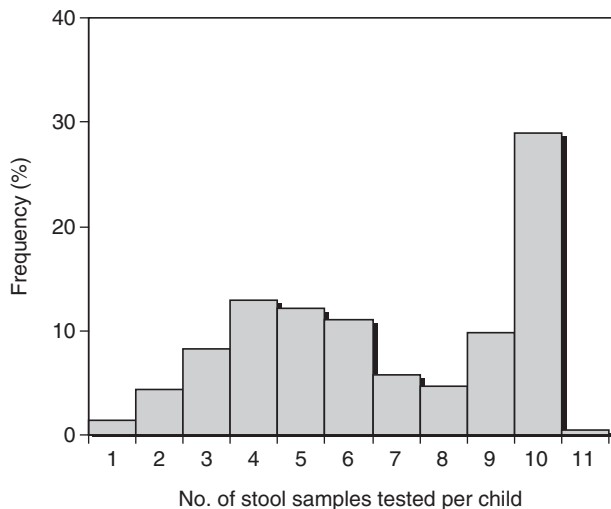
Figure 1 Frequency (%) of the number of longitudinal monthly stool samples tested per child among the 639 children in the study ($n = 4270$). Total 10 children had two samples tested instead of one, for one of the sample-months. One sample tested for a child, correspond to one month of follow-up; two samples correspond to 2 months of follow-up, and so on

questionnaire data, and 30% completed follow-up in the first year of life with 10 or 11 stool samples.

For ~5% of the analysed stool samples, there was no corresponding information on breastfeeding (see 'Measures' section) either because the whole questionnaire was missing, or because the mother did not answer the breastfeeding questions. The corresponding stool samples were not included in the present report, leaving a data set of 4279 observational person-months for statistical analysis.

Measures

Main outcome measure

Infection with enterovirus was the main outcome for this analysis. Total nucleic acids were purified from stool suspension and enterovirus nucleic acid was detected and quantified using a one-tube real-time reverse transcriptase PCR targeting to highly conserved sequences in the 5′ untranscribed region of the enterovirus genome designed for detection of all enteroviruses as previously described.[1] The analytical sensitivity and specificity of the assay's primer-probe system has been extensively verified by its authors.[33] An enterovirus infection was defined as the presence of enterovirus RNA in a stool sample above a given detection threshold (equivalent to a virus quantity $\geqslant 0.1$ median tissue culture infective dose (TCID50) of a selected strain).

Predictors of infection

The following variables were regarded to be of special interest as putative predictors of infection: infant breastfeeding, number of siblings, had sibling(s)

in daycare at age 3 months, daytime company of other children, household water supply and ownership of pets (cat or dog). The following variables were regarded as confounders: human leucocyte antigen (HLA) high-risk for type 1 diabetes, gender, age of child, season and year when stool sample was taken, county of residence and maternal age and education.

Demographic variables (sex, maternal age and education), data on siblings and siblings attending daycare, household water supply and pets were assessed from the parental questionnaires submitted at study entry, and treated as constant variables. Information on county of residence, breastfeeding, supplementary feeding (breast milk substitution, other non-human milk products, solid foods) and the daytime company of other children, was collected regularly (every or every third month). These were treated as time-dependent variables. The family's drinking-water source was categorized into public and private waterworks, or as other sources. Missing values in continuous predictors were imputed (Supplementary data available at *IJE* online). Categorical predictors with missing observations were extended by one extra category indicating missing.

Our main measure of breastfeeding was continuous or the average number of breastfeeds per day. Since this covariate was found to be inversely correlated to the frequency of supplementary feeding at all ages (Supplementary Table 1 available at *IJE* online), supplementary feeding was not included as a predictor in our model of infections. Other alternative models of breastfeeding were breastfeeding or predominantly breastfeeding as dichotomous variables. Categorization of breastfeeding into predominant breastfeeding was in accordance with the WHO classification system,[34] which defines predominant breastfeeding as when the infant's predominant source of nutrition is mother's milk. We also sought to investigate whether previous breastfeeding may protect against infection, when breastfeeding the same month was already accounted for. Thus, the predictor 'number of breastfeeds per day the previous month' was added to the first model. In another model, the accumulated value of the average number of breastfeeds per day from birth until the previous month was added. In this model, we assumed that the number of daily breastfeeds for the first 2 months in life was equal to the average number at age 3 months.

Statistical analysis

Univariable analysis was conducted to examine the association between enterovirus prevalence and the predictors defined previously. Multivariable logistic regression analyses were computed using a full model with all the covariates (predictors and confounders). All these covariates were kept in the main model, including those covariates not associated with infection. Further, as the effect of several covariates was expected to change as the child became older, the

model was extended by interaction terms (cross products) involving the children's age. Only the interaction terms associated with infection ($P < 0.05$) were retained in the final model as assessed by the likelihood ratio test.

To be more specific, let y_{ia} be the measurement of enterovirus infection at age-month a for child i, taking the values 1 (infection) or 0 (no infection). Furthermore, let p_{ia} be the probability of child i having an enterovirus infection at age a (i.e. $y_{ia} = 1$), conditioned on P corresponding covariates x_{aij}, $j = 1$, ..., p. To model the dependence among the repeated measurements of infection given the covariates, a subject-specific random intercept, β_{0i}, was included giving the model logit $(p_{ia}) = \log(p_{ia}/(1-p_{ia})) = \beta_{0i} + \beta_1 x_{ai1} + \ldots + \beta_p x_{aip}$, where β_{0i} is normal distributed with mean β_0 and variance δ^2, and the other βs are (fixed) regression coefficients. The inclusion of β_{0i} allows the probability of infection to vary between children, even after controlling for the covariates. Generalized Linear Latent and Mixed Models (GLLAMMs) were used to perform the analysis using Stata 9.2.[46] The covariates were entered simultaneously in the model. The correlation matrix of all the multivariable parameter estimates was assessed (by the 'correlate, _coef' postestimation command in Stata 9.2) to determine if there were any problems with multicollinearity of the independent variables in the model.

Results

The overall prevalence of enterovirus infection in stools was 11.1% (475/4279). Most children (54%) had at least one infection by the age of 12 months. The unadjusted prevalence of infection was lower in stool samples from breastfed infants compared with stool samples from non-breastfed infants, particularly
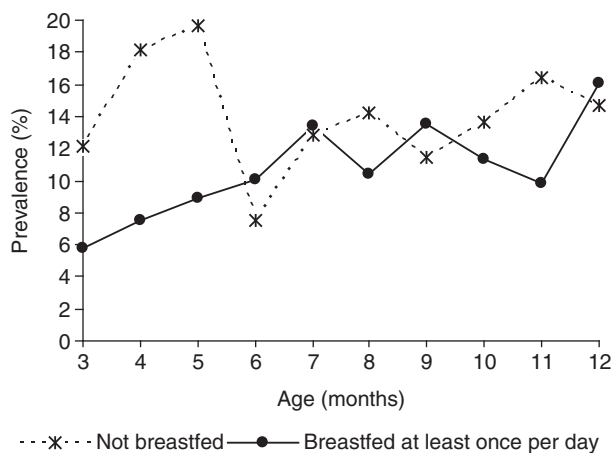
Figure 2 Prevalence of enterovirus in stool samples by age for 639 children categorized into either not breastfed or breastfed at least once per day ($n = 4279$)

before the age of 6 months (Figure 2). Of the mothers completing the questionnaires on breastfeeding, 3% (19/609) did not breastfeed at all, whereas 54% were still breastfeeding at 12 months postpartum. The frequency of predominant breastfeeding could be assessed at 3, 6, 9 and 12 months in 588 of the infants: 54% were predominantly breastfed for $\geqslant 3$ months and 11% were predominantly breastfed for $\geqslant 6$ months. The number of breastfeeds per day among breastfeeding infants ranged from 1 to 16 (mean = 5.5). The frequency of breastfeeding was lower in older infants (Table 1).

The frequency of daily breastfeeding was associated with the frequency of enterovirus infection, e.g. in stools from infants breastfed six or less times, the unadjusted prevalence of infection was 11.0%, compared with 8.5% in stools from those breastfed more than six times per day independent of age (Table 1). In stools from infants having one or more sibling(s) the prevalence of infection was 14.0%, compared with 5.8% for those not having any sibling(s) (Table 1).

Modelling the number of breastfeeds per day as a continuous variable in the multivariate logistic regression analysis showed a similar relation: at the age of 3 months the odds ratio (OR) per additional breastfeed was 0.85; 95% confidence interval (CI) 0.8–0.9, $P < 0.001$ (Table 2). The OR of having an infection decreased to 0.38 (0.85^6) if the number of breastfeeds per day at age 3 months was six, i.e. the risk was 2.6 times as high for an infant not breastfed compared with an infant breastfed six times per day. The protective effect of breastfeeding declined with age, being most pronounced at the age of 3 months and then gradually fading until about 11 months. Previous breastfeeding was not associated with protection from current enterovirus infection when current breastfeeding was accounted for (alternative models, not shown in Table 2). This was observable in two different models: one included the number of breastfeeds per day in the preceding month ($P = 0.44$); the other included the sum of daily breastfeeding from birth until the previous month ($P = 0.13$).

Other predictors of enterovirus infection included having at least one sibling (OR 1.89; 95% CI 1.2–3.0 compared with having no sibling), and in particular, if sibling(s) were attending daycare reported when the child was 3 months old. The effect of the latter covarieate decreased by increasing age of the infant. At the age of 3 months the OR was 2.46 (95% CI 1.4–4.2), and then the OR dropped to 1.74 at the age of 12 months. There was a tendency towards increased prevalence of infection with increasing daytime company of other children ($P = 0.053$) (Table 2). This effect became significant if the two related variables were deleted from the model, number of siblings and had sibling(s) attending daycare at age 3 months, although the OR was almost unchanged ($P = 0.009$). There were not any problems with multicollinearity of the independent variables in the model we applied.

Table 1 Proportion (%) and corresponding enterovirus prevalence (%) for categories of putative predictors and confounders ($n = 4279$ stool samples)

Covariates	Proportion of stool samples (% of 4279)	Enterovirus prevalence (%)
Putative predictors		
Number of breastfeeds per day		
0	27.3	13.9
0–6	45.0	11.0
>6	27.7	8.5
Number of breastfeeds per day at age ⩽6 months		
0	14.1	13.9
0–6	35.8	8.3
>6	50.1	7.7
Number of breastfeeds per day at age >6 months		
0	37.8	14.0
0–6	52.2	12.4
>6	10.0	11.7
Number of siblings[a]		
0	28.8	5.8
⩾1	57.2	14.0
NA	14.0	10.2
Had sibling(s) in daycare at age 3 months[a]		
No[b]	60.9	8.1
Yes	39.1	15.8
Daytime company of other children (number of children)		
0	37.3	7.7
0–3	56.3	12.6
>3.0	6.4	17.8
Household water supply[a]		
Public waterworks	87.4	11.2
Well or borehole	5.9	13.1
Private waterworks	3.9	7.9
Small lake or brook	1.0	9.8
NA	1.9	6.0
Pets (dog/cat)[a]		
No	59.0	12.0
Yes	20.0	9.9
NA	21.0	9.8
HLA high-risk for type 1 diabetes		
No	60.5	12.0
Yes	39.5	9.8
Gender		
Female	49.9	11.0
Male	50.1	11.2

(continued)

Table 1 Continued

Covariates	Proportion of stool samples (% of 4279)	Enterovirus prevalence (%)
Age (months)		
⩽6	44.1	8.8
>6	55.9	12.4
Season		
January	5.9	5.9
February	8.1	4.9
March	9.1	5.1
April	7.6	4.6
May	7.5	4.0
June	6.2	7.2
July	7.9	13.4
August	10.1	13.6
September	8.8	17.3
October	9.5	21.9
November	10.8	16.2
December	8.4	12.0
Year		
2005/2006	63.0	11.6
2004	17.0	11.8
2003	11.1	7.8
2002/2001	8.9	10.3
County of residence		
Akershus	53.4	11.5
Hordaland	18.1	11.0
Other counties	28.5	10.5
Maternal age (years)[a]		
⩽31	54.7	11.1
>31	45.3	11.1
Maternal education[a]		
High-school graduate or less (⩽12 years)	28.9	11.5
1–4 years in college	29.8	11.3
⩾4 years in college	16.1	11.3
NA	25.1	10.3

[a]Reported at delivery or at 3-month questionnaire of index child.
[b]Sibling(s) is not attending daycare or child has no siblings.
NA = Missing data for respective covariate.

The highest (in absolute value) correlation among all pairs of coefficients was –0.79 between the regression coefficients for stool sampling in year 2003 and the interaction term stool sampling in year 2003 with age. Also, changing the detection threshold for enterovirus positivity (to e.g. 10 and not 0.1) did not change the estimate of this predictor, e.g. towards a stronger

Table 2 ORs from univariable and multivariable logistic regression analysis for predictors of enterovirus infection in monthly longitudinal stool samples from 639 children aged 3–12 months, Norway, 2001–2006 ($n = 4279$)

Covariates	Univariable Unadjusted OR[a]	Multivariable Adjusted OR[b] (95% CI)	P-value
Number of breastfeeds per day (continuous)			
Effect at age 3 months	0.91 (0.87–0.94)	0.85 (0.79–0.92)	<0.001
Effect at age 12 months	(as above)	1.01 (0.92–1.11)	0.79
Number of siblings[c,d]			
0	1.0	1.0	
$\geqslant 1$	1.55 (1.24–1.94)	1.89 (1.18–3.01)	0.01
Had sibling(s) in daycare at age 3 months[c]			
No[e]	1.0	1.0	
Yes, effect at age 3 months	2.45 (1.89–3.18)	2.46 (1.42–4.25)	0.001
Yes, effect at age 12 months	(as above)	1.74 (1.02–2.96)	0.04
Daytime company of other children (number of children, continuous)	1.08 (1.05–1.12)	1.04 (1.00–1.07)	0.05
Household water supply[c,d]			0.16[f]
Public waterworks	1.0	1.0	
Well or borehole	1.42 (0.83–2.43)	1.58 (0.89–2.80)	
Private waterworks	0.77 (0.38–1.57)	0.53 (0.24–1.19)	
Small lake or brook	0.81 (0.20–3.38)	0.72 (0.15–3.45)	
Ownership of pets (dog/cat)[c,d]			
No	1.0	1.0	
Yes	0.85 (0.60–1.20)	0.72 (0.50-1.03)	0.07

[a]OR adjusted for random effects. Constant over age of sampling.
[b]OR mutually adjusted for all predictors listed in the table and the confounders HLA risk for type 1 diabetes, gender, age, season, year, county of residence, maternal age and education, and random effects. The interaction effects are equivalently reported as the OR of the main effect at two time points (age 3 and 12 months). P values are two sided.
[c]Reported at delivery or at 3-month questionnaire of index child.
[d]The missing data category ('NA') for respective covariate was retained in the multivariable analysis but is for simplicity excluded from the table, because results for these categories are not easily interpreted anyway.
[e]Sibling(s) is not attending daycare or child has no siblings.
[f]Overall P-value (for categorical variables with more than two categories).

effect (data not shown). In general, the results of the statistical analysis were insensitive to the exact choice of this threshold value.

Furthermore, there was a tendency towards a higher risk of infection for children whose household drinking-water source came from a private well or borehole compared with those whose source was public waterworks (overall $P = 0.16$). For children having a cat or a dog in the household compared with those not having these pets there was a tendency towards a lower risk of infection ($P = 0.07$) (Table 2).

There were some additional covariates, regarded as confounders, among them season and year of sampling, as shown in a complete table of all covariates included in the regression model (Supplementary Table 2 available at *IJE* online). Indeed, the prevalence of enterovirus in the population varied among calendar years, and risk of infection also varied by season October was the month with the highest risk

(OR 5.63; 95% CI 2.9–11.1). HLA-conferred susceptibility for type 1 diabetes, gender, socio-economic status and county of residence were not significantly associated with risk, although carrying the HLA high-risk genotype might warrant consideration in further studies (OR 0.67; 95% CI 0.5–1.0; $P = 0.050$).

There was a substantial heterogeneity of infection between children, visible also after adjusting for inequalities in covariates. The estimate of the mean β_0 of the subject-specific intercepts was –3.53 ($P < 0.0001$; 95% CI –4.8 to –2.2) and its corresponding standard deviation δ was 0.90, with standard error 0.19.

Discussion

The present data suggest that breastfeeding has a strong protective effect on enterovirus infection,

especially during early infancy. In children aged 3 months the risk decreased by 15% with each breast-feeding per day. The results support the protective effect of breastfeeding found in previous studies.[20,21] A study on neonatal (<1 months of age) enterovirus infections found that breastfeeding was associated with protection,[21] and a more recent study found that infants exclusively breastfed for >2 weeks had fewer enterovirus infections by the age of 1 year compared with those breastfed less.[20] An advantage of the present study compared with previous publications is the detection of virus directly from stool samples. This eliminates the bias of serological surveys in which maternal antibodies can disturb the measurement of enterovirus specific immunoglobulin (Ig) G in young children.

With a substantially larger number of subjects and samples than in previous studies[20,21] we were able to demonstrate that the protective effect of breastfeeding lasted until ~11 months of age, being most pronounced at 3 months and gradually vanishing thereafter. In this regard, it is interesting to note that in less developed countries, the protection provided by breastfeeding on infant mortality due to infectious disease decline steadily with age.[18] The weakening protection over age can be explained by a decline in the concentration of immune components in human milk,[35] or by the immaturity of the infant's own immune system at an early age.

A mechanism for the observed dose-dependent protective effect of breastfeeding is unknown, but some data indicate that the replication of the prototype enterovirus, poliovirus, in the intestine depends on the level of pre-existing secretory IgA antibodies from breast milk.[36] Dose–response effects have previously been reported for other infections,[4,5,14,16,17] but not for enterovirus. However, a recent study could not find any associations between breastfeeding intensity and infant visits for otitis media, respiratory and gastrointestinal illness or total illness visits, in a low-income, multi-ethnic population.[8] Low exclusive breastfeeding rates might be a reason for this finding.

The benefits of exclusive breastfeeding for health in infants have been widely described[4,6] and, moreover, the optimal duration of exclusive breastfeeding have been continuously debated.[5,10,12,13] The data presented here suggest that even a single dose of breast-milk per day, and continued breastfeeding past the recommended 6 months reduce enterovirus infections in infants. Long-term effects of breastfeeding could not be observed in the present study, indicating that the most important benefit of breastfeeding is the infant's immediate protection.

Having at least one sibling, especially one that attends daycare, was an important risk factor for enterovirus infection (Table 2). These findings are, in general, in accordance with previous reports.[21,23,24,28,37] The risk associated with having siblings in daycare decreased slightly with age, in line with the protective effect of breastfeeding decreasing over age. This may indicate a common explanation such as the immaturity of the child's immune system leading to increased susceptibility in early age; or it might be an artefact, because this predictor was reported at 3 months of age, and therefore may have changed when the infant became older.

Interestingly, infants whose drinking-water supply were private boreholes or wells had an increased frequency of enterovirus infections compared with those using public waterworks ($P > 0.05$). This finding is supported by other studies which have detected enterovirus contamination in drinking water wells around the world.[40–45] Enteroviruses have been found in surface and ground water throughout the world[26,27,30–32], even after chlorination and in the absence of fecal coliforms. However, little is known as to whether sources of drinking-water really constitute a common route of infections in the developed world.

When we excluded the covariates not related to infection in the multivariable analysis, except for HLA genetic risk group and 'the daytime company of other children' (had borderline significant P values) (Supplementary Table 2 available at *IJE* online), we confirmed that the results was approximately the same as the full model retaining all the predefined covariates.

Not all stools were analysed for enterovirus in children recruited to the study after 2004 due to reduced analysing capacity. Moreover, the proportion of unanalyzed samples is higher for the older children than for the younger ones, because children recruited late did not reach the age of 12 months by the end of the study period. Therefore, since breastfeeding decreases systematically with increasing age it is important to correct for age and sampling year in addition to other predictors of enterovirus infection, as we did in the statistical analysis, to generate unbiased results.

There were missing data for the number of siblings (14%) and pet ownership (21%) (Table 1). Simply deleting these observations could have introduced bias, if the (unknown) proportions of having siblings or having pets in the 'missing data' categories differ from the rest of the samples (Supplementary Table 2 available at *IJE* online). These observations were retained in the multivariable analysis with an extra 'missing data' category for each of the two variables. Using this strategy, no observations are lost and bias introduced by deleting data is avoided.

Additionally, substantial heterogeneity among children in the study was observed, which we controlled for when including a random intercept in our model. The inherent heterogeneity of the population may reflect yet undisclosed host genetic factors determining and modifying the susceptibility for infection.

This study has some limitations: the quantities of enterovirus in the stool samples varied over more than five orders of magnitude, whereas the outcome was dichotomized. Indeed, different quantities of

enterovirus may elicit different levels of response at the immunological and clinical level. We also did not assess if exposures were related to duration of an infection, only the frequency of positive stool samples, since we did not differentiate between new vs continuing infections. It is conceivable that the predictors may differ in their ability to start or maintain an enterovirus infection in an infant. In fact, breastfeeding may lower the duration of respiratory infection and diarrhoea.[6,13]

Monthly testing for enterovirus using nucleic acid extracted from stool is a valid strategy towards increasing sensitivity, as the gastrointestinal tract is the primary site of enterovirus replication. Monthly intervals are a compromise between sensitivity and the compliance of the parents. Only infection episodes with a very low viral load or short duration would escape the present detection scheme.

In conclusion, we have confirmed the previously observed protective effect of breastfeeding against sub-clinical enterovirus infection in otherwise normal infants during the first year of life. In addition, we observed a reduced protective effect in older children and the presence of a dose–response relationship, such as the higher the proportion of an infant's feeding that comes from human milk, the lower the prevalence of enterovirus infection. Long-term effects of breastfeeding could not be observed. A higher prevalence of infection was positively associated with having sibling(s), in particular if they attended daycare, and with the exposure to an increasing number of other children.

Supplementary data

Supplementary data are available at *IJE* online.

KEY MESSAGES

- The study revealed that daily breastfeeding has a pronounced protective effect on enterovirus infection in the first year of life.

- The protective effect of breastfeeding is strongest at an early age, and gradually declines with increasing age, reaching no effect at 11 months.

- The protection afforded by breastfeeding is dose dependent. The higher dose of ingested milk, the more protection.

- Having at least one sibling, in particular if they attend daycare, increases the risk of infection in early infancy.

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Longitudinal observation of parechovirus in
stool samples from Norwegian infants.

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Longitudinal Observation of Parechovirus in Stool Samples From Norwegian Infants

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Parechoviruses are assumed to be common infectious agents, but their epidemiologic and pathogenic properties are not well known. The aim of the present study was to assess the prevalence and molecular epidemiology of *Parechovirus* in Norwegian infants, as well as to investigate whether the presence of virus correlated with symptoms of infection. A group of 102 infants was longitudinally followed: 51 infants with a high genetic risk for type 1 diabetes (aged 3–35 months), and 51 children without this genotype (aged 3–12). Stool samples were obtained each month, and symptoms of infection were recorded regularly on questionnaires. Human parechovirus was detected in 11.3% of 1,941 samples examined by real-time RT-PCR. There was a distinct seasonality, peaking from September to December. By 12 months of age, 43% of the infants had had at least one infection, while 86% of the infants had encountered the virus by the end of the second year. Based on the VP1 sequence, human parechovirus 1 was the most prevalent type (76%), followed by human parechovirus 3 (13%), human parechovirus 6 (9%), an unclassified human parechovirus (1%), and human parechovirus 2 (1%). Ljungan virus, a murine parechovirus, was examined with a separate real-time RT-PCR, but no virus was detected. There was no significant association between infections and the following symptoms: coughing, sneezing, fever, diarrhea or vomiting. In conclusion, human parechovirus infects frequently infants at an early age without causing disease. *J. Med. Virol. 80:1835–1842, 2008.*
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KEY WORDS: human parechovirus; Ljungan virus; molecular epidemiology; quantitative PCR

INTRODUCTION

Parechovirus, a genus of the family *Picornaviridae*, consists of human parechovirus (HPeV) and Ljungan virus (LjV). Human parechovirus includes three well-known types that are considered common infectious agents: HPeV1 [Hyypia et al., 1992], HPeV2 [Ghazi et al., 1998; Oberste et al., 1998] and HPeV3 [Ito et al., 2004]; as well as three characterized more recently: HPeV4 [Benschop et al., 2006a], HPeV5 [Al Sunaidi et al., 2007], and HPeV6 [Watanabe et al., 2007]. Several studies have investigated the occurrence of parechovirus in ill persons [Stanway et al., 2000; Takao et al., 2001; Abed and Boivin, 2005; Boivin et al., 2005; Abed and Boivin, 2006; Benschop et al., 2006a; Blixt et al., 2007; Watanabe et al., 2007; Baumgarte et al., 2008; de Vries et al., 2008; Verboon-Maciolek et al., 2008], but beyond a few serological studies, the dissemination of virus in the healthy population has not been studied [Ito et al., 2004; Tauriainen et al., 2007].

LjV was first isolated from bank voles (*Clethrionomys glareolus*) [Niklasson et al., 1999], and is believed to be distributed globally in rodents [Johansson et al., 2003]. The virus is associated with several conditions in these animals, particularly type 1 diabetes [Niklasson et al., 2003a,b, 2006; Samsioe et al., 2006; Blixt et al., 2007]. LjV has not been isolated from humans, nor has its RNA been detected, although one study using immunochemistry showed the presence of LjV in formalin-fixed tissue from cases of intrauterine fetal deaths [Niklasson et al., 2007b].

The present investigation was part of a long-term project investigating environmental risk factors for

type 1 diabetes [Stene et al., 2007]. Parechoviruses are interesting candidates as they are related closely to enteroviruses, which accompany frequently diabetes onset and may participate in its early pathogenesis [Lonnrot et al., 2000; Kawashima et al., 2004; Roivainen, 2006; Elfaitouri et al., 2007]. The role of LjV in rodent diabetes suggests that parechoviruses should be studied in relation to human diabetes.

Type 1 diabetes is strongly associated with certain genotypes of the human leukocyte antigen (HLA) system [Ronningen et al., 1993; Caillat-Zucman et al., 1997]. An association between enterovirus infections and HLA genotype has also been suggested [Sadeharju et al., 2003], but no similar association has been indicated for parechovirus [Tauriainen et al., 2007]. If such an association exists, it will have consequences for the design and/or interpretation of studies investigating parechovirus as an environmental exposure leading to type 1 diabetes.

The objective of the present study was to investigate the molecular epidemiology of parechoviruses in a prospective cohort of Norwegian infants, aiming at the prevalence, viral load, symptoms of infection, distribution of genotypes, and the influence of the HLA genotype on the likelihood of infection.

MATERIALS AND METHODS

Subjects and Study Design

The children followed in this study participate in the "Environmental Triggers of Type 1 Diabetes study" [Stene et al., 2007]. The study was approved by The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate. The parents were asked to submit monthly stool samples from their infants from the 3rd to the 35th month, and to record information on the type and dates of symptoms of infection (coughing and sneezing, diarrhea, vomiting or fever).

The study involved two matched groups differing in their genetic risk of type 1 diabetes: the "high-risk group" included 51 children (24 males and 27 females) born in 2004 who were identified at birth to carry the HLA genotype conferring the highest risk of type 1 diabetes: DQB1*02-DQA1*05-DRB1*03/DQB1*0302-DQA1*03-DRB1*0401. Children carrying other HLA genotypes (27 males and 24 females) were recruited into a "non high-risk group." These non-high risk children originated from the same newborn screening scheme as the high risk group, and were matched 1:1 for date of birth (up to 30 days difference) and community of residence. They were followed up in the same manner with stool samples and questionnaires but only until 12 months of age. Of the expected 1980 samples and 482 questionnaires, 1941 (98%) samples and 476 (98%) questionnaires were received. The median endpoint of the follow-up was 31 months for the high risk children and 12 months for the children not at high risk.

Parechovirus Detection

Methods for the collection and processing of the monthly stool samples and RNA extraction are described elsewhere [Cinek et al., 2006]. To avoid false negative results, West Nile Virus (WNV) Armoured RNA (Ambion Diagnostics, Austin, TX) was used as an exogenous internal control of RNA extraction, RT and PCR. Parechoviruses were detected from extracted RNA using a two-step real-time RT-PCR reaction. The samples were reverse-transcribed with specific primers (Table I), 1μM LjV RT4, 1μM Par-1F and 0.5 μM WNV-R, using the Improm II RT kit (Promega, Madison, WI). One tube real-time PCR, using the HotStar Taq Polymerase chemistry (Qiagen, Hilden, Germany), was used to detect the 5′-UTR region of HPeV as well as the exogenous control. The following primers and probes were used: 500 nM Par-1F and Par-1R, 100 nM WNV-F and WNV-R, 200 nM HPeV probe, 200 nM WNW probe. The amplification was carried out on an ABI 7300 (Applied Biosystems, Foster City, CA) with the following thermal profile: 15 min at 95°C, followed by 45 cycles of 15 sec at 94°C and 1 min at 60°C (where fluorescence data were collected). If the WNV threshold cycle was too high, or the curve was not exponential, the sample was retested and re-extracted if necessary. For the detection of LjV, the method of Donoso et al. [2007] was used with 200 nM LV-F and LV-R primers, and 200 nM LjV probe. Dilutions of a sample with known quantity of HPeV1 from the QCMD (Quality Control in Molecular Diag-

TABLE I. Oligonucleotide Primers and Probes Used in the Study

	Sequence 5′−3′
Reverse transcription	
LV RT 4	TYARATTGGGMATRT
Parecho RT1A	ATACTTTACTGTGTCACA
Parecho RT1B	ATATTTCACTGTGTCACA
LV detection	
LV-F[a]	GCGGTCCCACTCTTCACAG
LV-R[a]	GCCCAGAGGCTAGTGTTACCA
LV-Probe[a]	FAM-TGTCSAGAGGTGAAAGC-dark quencher
HPeV detection	
Par-1F[b]	CACTAGTTGTAAGGCCCACGAA
Par-1R[b]	GGCCCCAGATCAGATCCA
Parecho Probe[b]	FAM-CAGTGTCTCTTGTTACCTGC-GGGTACCTTCT-TAMRA
HPeV genotyping	
VP1-parEchoF1[c]	CCAAAATTCRTGGGGTTC
VP1-parEchoR1[c]	AAACCYCTRTCTAAATAWGC
VP1-parEchoF1b	CCAAAYTCNTGGGGYTC
VP1-parEchoF0	TCMACTTGGATGAGGAARAC
VP1-parEchoR0A	AYAATWCCATAGTGCTTRTA
VP1-parEchoR0B	ATAATGCCATARTGTTTRTA
WNV detection	
WNV-F[d]	GCTCCGCTGTCVCCTGTGA
WNV-R[d]	CCTCTATCCAAACACGGTTCCA
WNV-Probe[d]	VIC-TGGTCCATCCATGCAGGA-dark quencher

[a]Donoso et al. [2007].
[b]Corless et al. [2002].
[c]Benschop et al. [2006b].
[d]Briese et al. [2000].

nostics, www.qcmd.org) were used as a standard for HPeV. For LjV, the standard was based on a transcript of a plasmid containing a cDNA clone of LjV prototype strain 87-012, kindly provided by Professor Lindberg (University of Kalmar, Sweden). The positive controls were detected consistently down to 10 copies/μl, and this level was therefore set as a positive threshold. Negative controls (water) were included in the extraction procedure, and additional negative controls were included in the individual RT-PCR reactions.

Parechovirus Genotyping and Phylogenetic Analysis

The VP1 region was sequenced in samples with more than 1,000 copies/μl. Nested PCR was used for amplification. After treatment with DNAse (Promega), the Access RT kit (Promega) was used for the first round (an RT-PCR with 0.8 μM external primers VP1-parEchoF0, VP1-parEchoR0A and -B (Table I), 1U AMV reverse transcriptase and 1 U *Tfl* DNA polymerase). The product was diluted 1:100 with water and subjected to the second round of the nested PCR (primers VP1-parEchoF1/F1B and VP1-parEchoR1), and 1 U aTaq polymerase (Promega). The thermal profile for the second round used the touch-down principle: denaturation 2 min at 96°C, then 10 cycles of 15 sec at 96°C, 30 sec at 60°C (decreasing by 0.5 degrees per cycle), and 60 sec at 72°C, followed by 30 cycles with the annealing temperature at 50°C, and a final extension for 2 min at 72°C. The products were bi-directionally sequenced on an ABI310 machine using BigDye Terminator 3.1 chemistry (Applied Biosystems, Foster City, CA) with the VP1-parEchoF1 and VP1-parEchoR1 primers. The sequences were analyzed using the on-line BLAST 2.2.17 search tool of GenBank, NCBI, to assign genotypes, and assembled and corrected manually using *Sequencher* v4.5 (Gene Codes Corporation, Ann Arbor, MI). Sequences in this study were deposited in the GeneBank, under the accession numbers: EU360509 to EU360577. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [Kumar et al., 2004]. The evolutionary distances were calculated using the Kimura two-parameter model as a model of nucleotide substitution and the Minimum evolution method, with a bootstrap value of 1,000.

Statistical Methods

To reflect the longitudinal character of the infections, infection episodes rather than single occasions of positive stool samples were counted when analyzing the seasonal distribution and the age-specific cumulative incidence. A new parechovirus infection episode was defined either as the first positive sample following a negative sample (both with a threshold of 10 copies/μl), or by the observation of a different parechovirus type compared to the previous month's sample. Cumulative distributions were estimated by the empirical distribution function (uncensored data) and Kaplan–Meier estimator (censored data). Distributions of infection

duration and intervals between episodes were estimated using the iterative convex minorant algorithm for interval-censored data. The effect of covariates on age-specific incidence was modeled by the Cox proportional model for recurrent event data incorporating left truncation and time-varying covariates. The covariates were gender, HLA genotype group (HR or non-HR), number of siblings (all fixed), calendar month, and occurrence of previous infection (time-varying). Age was chosen as the underlying time scale. The age-specific incidences were estimated from the estimated cumulative baseline hazard of the Cox model for recurrent data with previous infection status as a time-varying stratum. The cumulative hazard was differentiated numerically by fitting a smoothing spline with a large smoothing parameter. The effects of covariates on incidence were tested by Wald tests in the Cox model. The association of infection with symptoms was tested by exact Mantel–Haenszel test stratified on subject. All analyses were done in "R: A Language and Environment for Statistical Computing" [R Development Core Team, 2007].

RESULTS

Occurrence of Virus

The distribution of quantities of human parechovirus detected in the stool samples are shown in Figure 1. The quantity ranged from a single copy to more than 10^5 copies /μl RNA. Parechovirus, at a quantity exceeding the positivity threshold (10 copies/μl of extracted RNA), was detected in 220 of 1,941 samples (11.3%, CI 95%: 9.9–12.7). LjV was not detected in any of the samples.

Based on the quantity and sequencing data, the human parechovirus positive samples were assigned to 119 discrete infection episodes. Between 3 and 12 months of age, 44 of the 102 (43%) children had at least one human parechovirus infection episode, five (4.9%) had two infection episodes. About 10% of the episodes lasted more than 3 months, with one
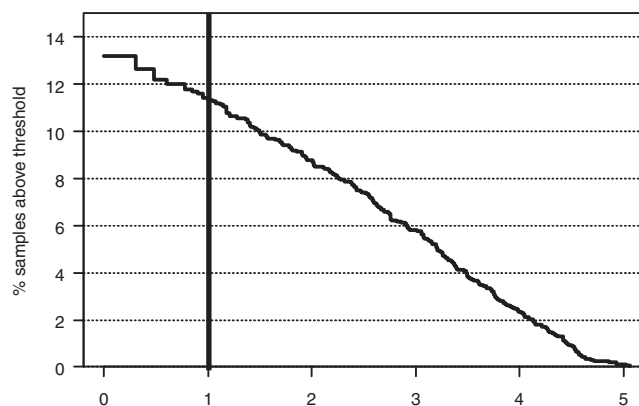

Fig. 1. Distribution of the concentration of human parechovirus found in the stool samples. The quantity is expressed as the number of viral genomes per μl of extracted RNA. Only samples exceeding 10 copies/μl extracted RNA (log = 1, marked with a vertical line) were included as positive in further analyses.

episode lasting 5 months. The estimated median duration of an infection episode was 51 days. The first sample contained typically more virus than subsequent samples from the same episode. The duration of the episodes correlated with the peak virus quantity; that is, of the episodes with peak quantity below 10^3 copies/μl, 75% were cleared within 50 days, while among those with peak quantity above 10^5, only 40% were cleared within 50 days.

Reinfections (repeated episodes, regardless of the type identified by sequencing) were rare during the first year, but occurred in 55% of the high risk group that was followed for 3 years. The median interval between the clearance of a previous episode and the start of a new one was 208 days. Approximately 10% of the subjects with repeated episodes were reinfected within 4 weeks, and 36% were reinfected within 2 months. Examples of subjects with repeated infections by different human parechoviruses are shown in Figure 2. There was no obvious case of reinfection with the same serotype. However, a reinfection would be expected to have a lower virus titer, so there was a bias against these samples being chosen for sequencing.

The cumulative incidence of human parechovirus infections increased steeply between 6 and 18 months of age (Fig. 4). By the third year, nearly all children (94%) had at least one infection. The incidence rates of the first infection increased with age: from about 11.6 infections per 100 person-months at the age of 4 months, to about 20–23 infections per 100 person-months at the age of >15 months. The reinfections had much lower rates (5–8 infections per 100 person-months) and were less age-dependent. A distinct seasonality (Fig. 3) was observed in both incidence (number of new infections per unit of time) and prevalence (total number of infected individuals divided by the total population at the given time). Most human parechovirus infections occurred from September to December, while a trough was noted between April and June.

Multivariant Analysis of Infection Risk

As shown in Figure 4, the children carrying the high risk genotype for type 1 diabetes had a tendency towards fewer initial infections during the first year compared to children carrying other genotypes (not high risk), but the difference was not statistically significant ($P = 0.07$). In a multivariate model for repeated infections, adjusted for age, calendar month, and previous infections, the relative risk of high risk children versus non-high risk children was 0.69 (95% CI: 0.42–1.15, $P = 0.16$). The multivariate model also showed that a previous infections decreased the future risk of reinfections by 75% (RR = 0.25, 95% CI 0.16–0.39, $P < 0.0001$). Neither the
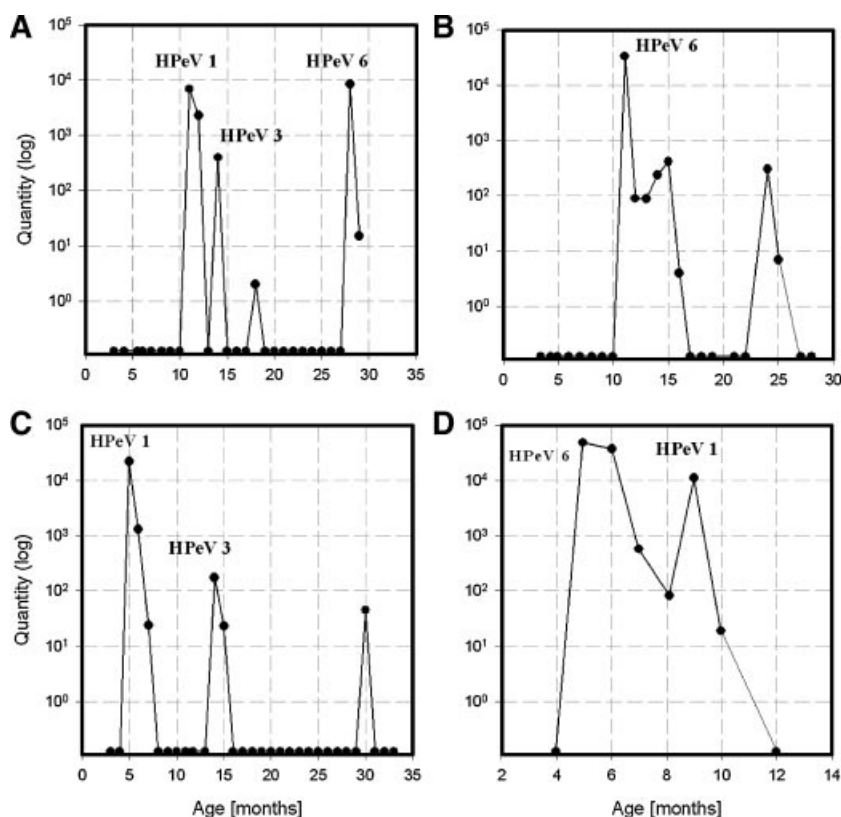

Fig. 2. Selected examples of parechovirus infections. Four infants with what could be considered typical HPeV infection histories; Viral peaks followed by clearance (**A–D**), several infections with different genotypes (A,C,D) and viral presence in stool over long periods (B,D). Note that mainly samples with more than 1,000 copies of virus (10^3) were sequenced.
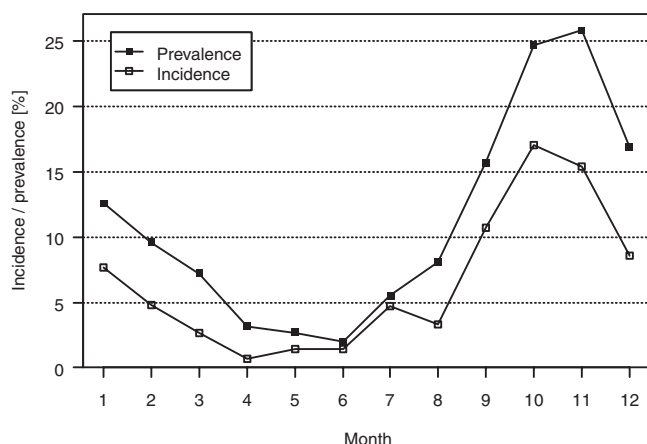
Fig. 3. Seasonality of parechovirus infections. Month 1 corresponds to January. The samples were collected over 2½ years (2004–2006). Similar seasonality was observed each year. Incidence is defined as the number of new infections per unit of time, prevalence is defined by total number of infected individuals divided by the total population at the given time.

gender of the subject, nor the number of their siblings affected the risk of infection. As shown in Figure 3, the effect of calendar month was prominent. Multivariant analysis demonstrated that the seasonal variation was highly significant ($P < 0.0001$).

Symptoms

The average prevalence of symptoms in months with respectively virus positive and negative fecal samples were: cold (coughing and sneezing) 13.2% versus 11.2%; fever above 38°C: 10.5% versus 9.4%; diarrhea: 5.9% versus 4.0%. Although the various symptoms were reported slightly more often during the months with virus present in the feces, in a Mantel–Haenszel analysis stratified on subject, none of the symptoms were associated significantly with parechovirus infection. There was no appreciable association between
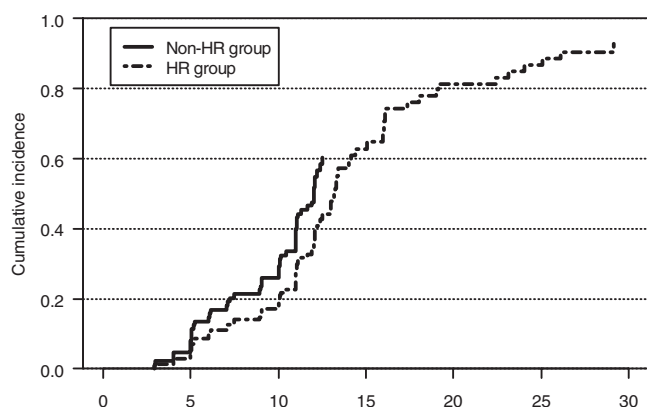
Fig. 4. Cumulative incidence of parechovirus by the genetic risk for type 1 diabetes. The children carrying the high-risk diabetes genotype (HR) were followed until 36 months of age, while the children carrying other HLA genotypes (non-HR) were only followed between 3 and 12 months. The end of the periods are not included due to a less complete dataset. The difference between the two groups was not significant ($P = 0.07$).

virus and symptoms even when the analysis was restricted to samples with higher levels of virus.

Viral Subtypes

Samples with quantities exceeding 1,000 copies/μl HPeV in the PCR reaction were subjected to VP1 sequencing. Sequence information of sufficient quality for use in phylogenetic analyses were obtained from 67 samples, which were aligned with reference strains from the databases and used to construct a phylogenetic tree (Fig. 5). No simultaneous infection by two strains were observed. HPeV1 was the most prevalent type (76%), followed by HPeV3 (13%), HPeV6 (9%), a yet uncharacterized type (1%), and HPeV2 (1%). Further analyses revealed no recognizable clustering of sequences from samples taken at the same location or time, indicating that the different strains were widely disseminated in Norway during the study. The only complete match of sequences from different samples came from consecutive samples from the same child.

DISCUSSION

Distribution and Characteristics of Human Parechovirus Infections

The present study demonstrates that HPeV is a common, almost ubiquitous virus, infecting infants from an early age. The high prevalence of HPeV is consistent with previous serological studies of children and adults [Joki-Korpela and Hyypia, 1998; Ito et al., 2004; Tauriainen et al., 2007]. The cumulative incidence of HPeV in the first year of life (43%) was higher in the present study compared to a recent Finnish study (20%) detecting virus specific antibodies [Tauriainen et al., 2007]. By the third year, however, the cumulative incidence reached similar levels in both studies (94% in the present study vs. 98%). The discrepancy for the first year may be related to a focus on antibodies directed at HPeV1 in the Finnish study or differences between Real Time RT-PCR and serology, but it may also reflect that infants produce less antibodies during their first year of life [Siegrist, 2007]. There could also be real differences between countries.

The cumulative incidence of HPeV increased steeply from 6 to 18 months of age (Fig. 4). This might be explained by the fact that children start to lose transplacental maternal antibodies around the age of 6 months, or that Norwegian children are introduced often to kindergartens at this age. HPeV had a distinct seasonality from September to December (Fig. 3), but the small number of non-HPeV1 infections makes it impossible to say whether this is true for all different types. A related study on enteroviruses using the same cohort, found that these viruses also peaked late in the year, but the seasonality was less distinct [Cinek et al., 2006]. In the present study, both the prevalence and seasonality were similar over the 3 years when the samples were obtained.

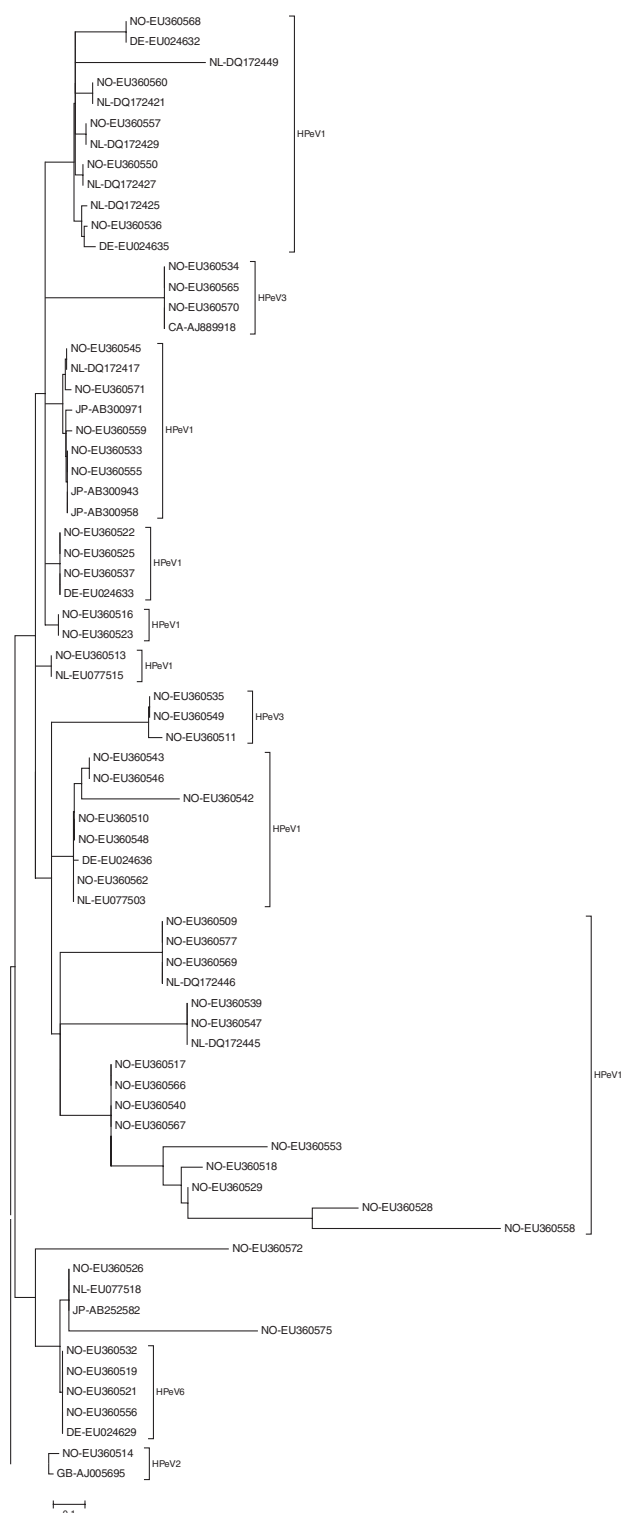Most infections started with a high viral load, which decreased subsequently to lower values. The decrease in

Fig. 5. Phylogenetic tree of representative Norwegian human parechovirus VP1 amino acid sequences compared with repretrative sequences from the rest of the world. The PCR product sequenced was 770 bp long. The sequences are denoted with their country of origin and GenBank accession number. HPeVs from Norway (NO) are reasonably dispersed with strains from other countries (The Netherlands, NL; Great Britain, GB; Canada, CA; Japan, JP). The Norwegian samples did not cluster according to the geographical location or the time the sample was obtained. The tree was constructed using the minimum evolution method, with a bootstrap value of 1,000.

viral activity suggests an active immunological response. In this respect it was interesting to note that previous HPeV infections seemed to protect against reinfection, that is, the incidence of first infections was much higher than that of reinfections. There was, however, insufficient data to specify to what extent this protection depended on the viral subtype. Neither gender nor the number of siblings affected the risk of infection. Previous reports suggest that HPeV is less common in older children [Benschop et al., 2006b; Baumgarte et al., 2008]. Thus, if the age difference between the siblings is more than 2 years, the more likely source of infection will be same age peers, such as those encountered in kindergarten.

A slight trend towards fewer HPeV infections was observed in children with the highest genetic risk for T1D (Fig. 4). More data are required to investigate such an association, and to find out which component of the genotype may be responsible for the putative association.

Symptoms of Infection

Unlike most studies on parechoviruses, the present data are from healthy children. Significant associations of HPeV infection with symptoms recorded by the parents (fever, diarrhea, vomiting or coughing/sneezing) were not observed. Almost all children had a history of infection by the third year of life, proving that HPeV is both common and normally harmless. Interestingly, a recent German study did not find any association between HPeV and enteritis in children [Baumgarte et al., 2008]. However, since the larger part of the infections found in this study are HPeV1 it is difficult to ascertain the pathogenic properties of any other HPeV. As such, these observations do not exclude the possibility that HPeVs may cause symptoms [Verboon-Maciolek et al., 2008], or serious complications [Ito et al., 2004; Abed and Boivin, 2005; Watanabe et al., 2007] in individual cases, but the very high prevalence in healthy children should be borne in mind when evaluating causality based on findings from stool samples.

Sequence Analysis

As seen in Figure 5, the HPeVs found in the present study, with samples taken from a few communities in Norway, displayed diversity close to what is observed worldwide. The different strains observed did not cluster in time or locality. These observations suggest that HPeV is disseminated efficiently worldwide, a conclusion that is also supported by the high prevalence.

The discovery of new HPeVs, and their subsequent detection in other parts of the world, demonstrates the advantages of molecular methods as compared with serological analyses [Ito et al., 2004; Boivin et al., 2005; Benschop et al., 2006a; Al Sunaidi et al., 2007; Baumgarte et al., 2008]. The present study confirmed the presence of four of the six known genotypes, as well as what may be classified as a novel genotype. The

absence of HPeV5 may be related to problems of VP1 amplification with the presently used primers [de Vries et al., 2008]. HPeV1, HPeV3, and HPeV6 seem to be the more common viruses worldwide, while subtype 2 and 4 may be rare. The HPeV1 and 3 seem to be the most similar types, and clustered closely to each other. As noted by Baumgarte et al. [2008], the contemporary variants of HPeV1 seem to be in evolutionary transition away from the prototype Harris strain. Further sequencing is required to determine whether the unclassified parechovirus reported in the present study should be grouped with type 1 or be classified as a novel genotype.

Ljungan Virus

So far no studies have been able to prove clearly that LjV can infect humans. The virus may be limited to rodents [Niklasson et al., 1999, 2007a; Johansson et al., 2003]. LjV might also not be found in human stool, and little is known about the eventual transmission route. The present data at least indicate that the virus is not common in the stool of Norwegian infants.

ACKNOWLEDGMENTS

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Příloha VI.

Tapia G, Cinek O, Rasmussen T, Grinde B, Rønningen KS.

No Ljungan virus RNA in stool samples from the Norwegian environmental triggers of type 1 diabetes (MIDIA) cohort study.

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No Ljungan Virus RNA in Stool Samples From the Norwegian Environmental Triggers of Type 1 Diabetes (MIDIA) Cohort Study

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OBJECTIVE — Ljungan virus (LjV) has been proposed as a potential environmental factor for type 1 diabetes. The objective was to test for any association of LjV with type 1 diabetes.

RESEARCH DESIGN AND METHODS — A nested case-control design was used to test for any association between the development of pre-diabetic autoimmunity and presence of LjV in stool samples ($n = 3,803$) in the Norwegian Environmental Triggers of Type 1 Diabetes (MIDIA) study. The children followed were 27 infants who developed pre-diabetic autoimmunity during or shortly after the sampling period, 54 matched control subjects, and 94 other children.

RESULTS — No LjV RNA was detected.

CONCLUSIONS — The results indicate that LjV is rare in young children. LjV does not seem to be involved in the development of human type 1 diabetes.

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L jungan virus (LjV), a rodent virus described by Niklasson et al. (1), has been associated with a variety of conditions in rodents, including type 1 diabetes (2), myocarditis (3), and intrauterine death (4). In humans, LjV has been associated with intrauterine fetal death (5), anencephaly (6), and sudden infant death syndrome (7) and has been suggested as a factor in type 1 diabetes (2). LjV belongs to the viral family *Picornaviridae*, genus *Parechovirus*. The genus also includes human parechovirus (HPeV), which is common in infancy and replicates mainly in the gut. A possible role of LjV in type 1 diabetes is of particular interest because of the strong association found in captive bank voles (2,3,8).

The aim of the study was to investigate the presence of LjV RNA in stool samples to find a possible association with type 1 diabetes.

RESEARCH DESIGN AND METHODS

— A nested case-control study was conducted, using 2,054 stool samples from 81 children (27 case subjects, 54 control subjects) all carrying the DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02 HLA genotype, which gives the highest risk for type 1 diabetes. Case subjects (10 boys, 17 girls) were defined on development of type 1 diabetes or of diabetes-associated autoimmunity (being positive for two or three autoantibodies: GAD, IA2, or IAA). Control children (31 boys, 23 girls) were matched for birth date and geographical residence. This dataset was merged with a previously published dataset investigating parechovirus infections (9) in 1,941 stool samples from 102 children (51 with the high-risk genotype and 51 without). In total, there were 3,803 unique samples from 175 children (86 boys, 89 girls), since some children participated in both studies. The children represented 16 of 19 municipalities in Norway.

Monthly stool samples, from 3 to 35 months of age, were collected by parents as previously described (10); the age distribution is shown in Fig. 1. The samples were collected between 2001 and 2008; because most children were followed for at least 1 year, there was an equal seasonal distribution of samples. The median follow-up was 30 months for the stool samples (range 9–37 months), with median end point (autoimmunity) of the cases at 20.5 months (range 6–43). The presence of LjV was examined by extraction of RNA/DNA, reverse transcription (RT), and real-time PCR as described by Tapia et al. (9), with minor modifications of the protocol due to introduction of a 96-well format and the use of the antisense primer described by Donoso Mantke et al. (11) as the RT primer. Two positive control subjects were included from extraction to PCR run (140 and 70 copies of a transcript from a plasmid containing a cDNA clone of LjV prototype strain 87-012, provided by Professor Lindberg, University of Kalmar, Sweden). A fragment of West Nile Virus RNA was spiked into the samples as an exogenous internal control (10). The study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

RESULTS — No LjV RNA was detected in any of the stool samples investigated, neither from the 27 children who had developed autoimmunity, nor from the 54 matched control subjects and the 94 healthy children previously tested. The positive LjV control was consistently detected, and the West Nile Virus Armored RNA used as an exogenous internal control in each sample was consistently positive.
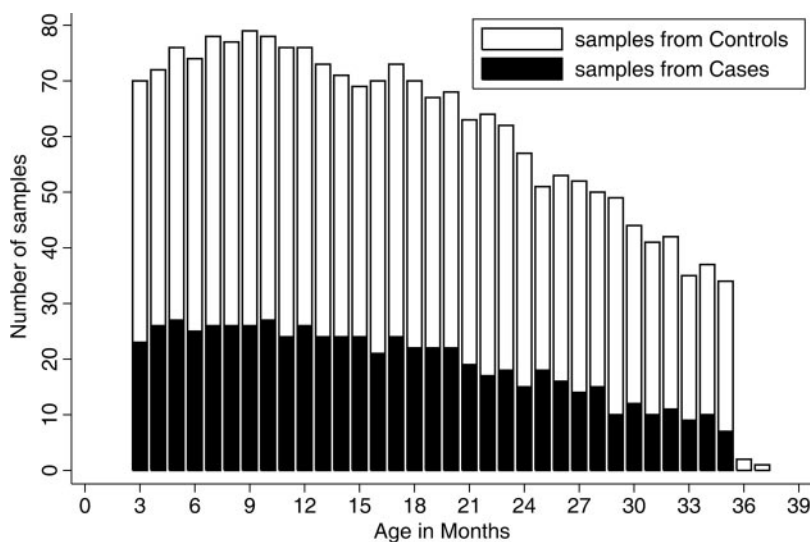
No Ljungan virus found in stool samples

Figure 1—*Age distribution of the samples. The number of samples from case children (■) and the total number of samples (□) distributed by month of age are shown.*

CONCLUSIONS — Considering the follow-up time, the number of tested samples, and that both pre-diabetic and healthy children were tested, these results indicate that LjV is very rare in the stool of Norwegian infants. The typical stool quantities of human enterovirus and HPeV in samples from the Norwegian Environmental Triggers of Type 1 Diabetes (MIDIA) cohort study were two to five orders of magnitude higher than the detection limit for LjV (9,12); presumably any appreciable replication of LjV in the gut would be detected. The detection of the exogenously added West Nile virus RNA safeguards against the presence of inhibitors and RNA degradation. The primers used consistently detected the positive LjV RNA controls included in each run and are expected to detect all strains of LjV. No change in sensitivity was detected with the introduction of a 96-well extraction method. The use of the antisense primer in the RT reaction could be presumed to increase the sensitivity at the cost of the formation of more spurious products, but was chosen to ensure that any possible positive sample would be detected. The lack of evidence for the presence of LjV suggests that this virus rarely infects the gut of Norwegian infants, and it seems unlikely that the virus is the cause of the autoimmunity observed in the present study.

Picornaviruses may also replicate outside the intestinal tract and may cause viremia or respiratory infections. Although one would expect a gastrointestinal route of infection in the case of LjV, the virus might reach the pancreas even after limited replication in the gut. Although data supporting LjV infections in humans have been published, there is so far no conclusive evidence. The arguably strongest evidence stems from prenatal studies (5,6). In these studies, the presence of virus has been suggested partly by serology and partly by PCR; but, as pointed out by Bergstrom et al. (13), different methods do not seem to give congruent results. Moreover, the PCR positivity observed in the earlier studies is not reported to have been confirmed by sequencing. Although the evidence suggests possible human LjV infections, the data also indicate that it is a rare event and primarily during the prenatal period.

The likelihood of infection may also be geographically specific and dependent on the cycles of its natural reservoir, which presumably are bank voles in Scandinavia. Although common in Norway, their prevalence in the communities from which infants in the present study was recruited is not known. Thus, the possibility that the infants investigated were never exposed cannot be ruled out.

In conclusion, although the present data do not rule out the possibility that LjV can cause type 1 diabetes, they do suggest that this virus is not a common risk factor in the etiology of the disease in Norway.

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Příloha VII.

Tapia G, Cinek O, Rasmussen T, Witsø E, Grinde B, Stene LC, Rønningen KS.

Human Enterovirus RNA in monthly fecal samples and islet autoimmunity in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study.

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Human Enterovirus RNA in monthly fecal samples and islet autoimmunity in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study

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Running title: Enterovirus and islet autoimmunity

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Objective: To test whether the frequency of human enterovirus RNA in fecal samples collected monthly from early infancy was associated with development of multiple islet autoantibodies in children with the highest risk HLA genotype.

Research Design and Methods: Individuals carrying the HLA-DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02 genotype were identified at birth and followed with monthly stool samples from age 3 to 35 months. Blood samples taken at age 3, 6, 9, 12 months, and then annually, were tested for autoantibodies to insulin, glutamic acid decarboxylase 65 and IA-2. Among 911 children, 27 developed positivity for ≥2 islet autoantibodies in ≥2 consecutive samples (cases). Two controls per case were matched by follow-up time, date of birth and county of residence. Stool samples were analyzed for enterovirus with a semi quantitative real-time reverse transcriptase PCR.

Results: The frequency of human enterovirus RNA in stool samples from cases prior to seroconversion (43/339, 12.7%) did not differ from the frequency in controls (94/692, 13.6%), P=0.97. Results remained essentially unchanged after adjustment for potential confounders, restriction to various time windows before seroconversion, infections in the first year of life, or after including samples collected after seroconversion. There was no difference in the average quantity of enterovirus RNA, or the frequency of repeatedly positive samples. The estimated relative risk for islet autoimmunity per enterovirus RNA positive sample during follow-up (nested case-control analysis) was 1.12, 95% CI: 0.66-1.91.

Conclusions: There was no support for the hypothesis that fecal shedding of enteroviral RNA is a major predictor of advanced islet autoimmunity.

Human enteroviruses have been considered as possible environmental triggers or accelerators of islet autoimmunity leading to type 1 diabetes (1; 2). They have been observed more frequently in patients recently diagnosed with type 1 diabetes as compared to controls, and there is also evidence of the virus in the pancreata of subjects who died shortly after the disease onset (3). However, an interpretation of virus occurrence at or shortly after diagnosis is difficult with respect to type 1 diabetes pathogenesis, because the autoimmune process starts months to years before its clinical manifestation.

Potential causal relations between infections and development of islet autoimmunity are best assessed in longitudinal birth cohorts testing viral infections at frequent intervals before and during the development of islet autoimmunity. Five such studies, including between 11 and 41 cases of islet autoimmunity, have so far published results on enterovirus using various methods and testing strategies (more details in discussion). The evidence for involvement of enterovirus in type 1 diabetes pathogenesis comes predominantly from the Finnish population (4-6), while a study from Colorado (7) and one from Germany (8) did not find any significant association.

In view of these conflicting results, we aimed to test whether the presence of human enterovirus in monthly fecal samples predicted development of repeated positivity for two or more diabetes associated islet autoantibodies in children with the HLA genotype conferring the highest risk for type 1

diabetes.

RESEARCH DESIGN AND METHODS

Subjects and study design. The children prospectively observed in this study participate in the Norwegian cohort entitled "Environmental Triggers of Type 1 Diabetes: The MIDIA study". The cohort was identified at birth from the general population based on genetic testing for the HLA genotype conferring the highest genetic risk of type 1 diabetes, *DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02*. Between 2001 and 2006, 911 children were included into the cohort. All subjects were followed up with stool samples, blood samples for autoantibody screening and structured questionnaires. The study was approved by The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Blood samples taken at ages 3, 6, 9, and 12 months and every 12 months thereafter were processed and the plasma tested for autoantibodies against glutamic acid decarboxylase 65 (GADA), protein tyrosine phosphatase IA2 (IA2A) and insulin (IAA), using radiobinding assays as described in detail earlier (9). Mailed questionnaires were administered at the same intervals. If a plasma sample was found positive for one autoantibody, the child was retested every six months; if two or three antibodies were positive, the child was retested every three months. The endpoint for this study, islet autoimmunity, was defined as positivity for two or more islet autoantibodies in two or more consecutive samples. Type 1 diabetes was diagnosed according to the WHO criteria. By December 2008, 27 of the 911 children in the cohort had reached the endpoint, and were assigned as cases. The median age at onset of islet autoimmunity was 12.0 months (range 5.4-37.4). Of the 27 case children, ten subjects were diagnosed with diabetes by September 1, 2009, at a median age of 23.1 months (range 8.7-54.2). The timing of autoantibody seroconversion and age at diagnosis for each of the cases is shown in Supplementary Table 1.

Two control subjects were randomly assigned per case, matched for the length of follow-up (at least as long as the time when the corresponding case developed multiple islet autoantibodies), date of birth within +/- 1 month (tolerating up to +/- 3 months if necessary) and county of residence (tolerating closest neighboring county if necessary). Children were ineligible as controls if they were repeatedly positive for one or more islet autoantibodies during follow-up. One control subject was transiently positive for a single autoantibody prior to the endpoint in the respective case, otherwise no controls developed positive autoantibodies (even after their case reached the endpoint). Data from one control child (matching group 27) are missing as the parents later withdrew from the study and refused any use of the collected data.

To test for enterovirus infections, we utilized stool samples obtained by the parents; they collected stool samples from their children every month from 3 to 35 months of age. These were sent by mail to our central laboratory, with a median transit time of 3 days. Parents also kept records of symptoms of infection in structured questionnaires. Of 704 planned blood samples, 637 were taken (91%); 2173 of 2482 scheduled stool samples (88%) and 492 of 547 questionnaires were received (90%). The median duration of follow-up with stool samples was 28 months (range 7-35 months). The characteristics of the study participants are described in Table 1.

Processing and molecular testing of stool samples The processing and testing of stool samples in this study has been described earlier (10). Briefly, the samples were received by postal service, diluted and centrifuged. The supernatants were frozen at -80°C until co-purification of RNA and DNA. The extraction protocol utilized the vacuum-processed 96-well QIAamp plates processed

under the QIAamp Viral RNA Mini protocol (Qiagen, Hilden, Germany). West Nile Virus (WNV) Armored RNA (Asuragen, USA) was added in a constant quantity to the lysis buffer which was used in the first step of the protocol. This exogenous internal control monitored the success of RNA extraction and detection. Testing for human enterovirus RNA was performed in duplicates in 20 µl volume one-step real-time reverse transcriptase PCR with primer-probe combination specific for the conserved 5'-untranslated region of human enteroviruses. This combination does not react with the *rhinovirus* species. Serial dilutions of Enterovirus Armored RNA (Asuragen, USA) were used to construct a seven-point standard curve from 24 to 10^5 copies/µl. The threshold of positivity used in this study was set to 100 copies/µl RNA, a quantity that could be consistently and reliably detected.

Statistical analysis. To optimize the use of information in repeated samples collected from each individual, we compared the percentage of enterovirus RNA positive samples collected from cases to those collected from controls, and tested this using a mixed effect logistic regression model with random intercept for each individual to account for potential intra-individual correlation (clustering) in risk of enterovirus positivity (xtmelogit in Stata11). The primary analysis involved only samples collected up to seroconversion for the cases and the corresponding age in the matched controls. In cases who first tested positive for a single autoantibody, this first occurrence of autoantibody positivity was regarded as the onset of autoimmunity. The estimated odds ratio (with 95% confidence interval) from this model is interpreted as the odds that a fecal sample is positive for enteroviral RNA given that it came from a child who later developed islet autoimmunity, relative to the odds that a sample is enterovirus positive given that it came from a control child. Planned

(secondary) sub-group analyses involved time windows of 6 and 12 months prior to seroconversion in cases (and corresponding ages in matched controls), samples collected prior to one year of age, and samples collected after seroconversion. We also adjusted for other variables by including them in the regression model, as reported in the results section. In separate analyses only the first enterovirus RNA positive samples among series of two or more consecutively positive samples was counted, assuming they were part of the same infectious episode.

We also analyzed the data according to a formal nested case-control study design using conditional logistic regression (accounting for the matched design with a fixed intercept for each matching group), modeling the cumulative number of enterovirus RNA positive fecal samples before seroconversion (grouped as 0, 1, 2, or ≥3) as the exposure variable. With the given study design, the measure of association from this analysis is interpreted as the relative risk of islet autoimmunity per increase in cumulative number of enterovirus RNA positive samples, with a corresponding 95% confidence interval.

RESULTS

The frequency of human enterovirus before development of autoimmunity. The frequency of human enterovirus RNA in stool samples prior to the development of islet autoimmunity did not differ between cases (12.7%) and controls (13.6%). Results were similar even after adjusting for age, sex, month of sampling, year of sample, number of siblings, breastfeeding, and first degree relatives with type 1 diabetes (Table 2). Similarly, no association was seen when analyzing only infections before 12 months of age (OR=1.02, 95% CI: 0.51-2.04), or various time window before seroconversion in cases: with a six months' window, the frequency was 20 / 142 (14.1%) in cases versus 42 / 308

(13.6%) in controls, (OR = 1.05, 95% CI 0.54 - 2.04), and with a twelve months' window the frequency was 31 / 214 (14.5%) in cases versus 62 / 454 (13.7%) in controls (OR = 1.09, 95% CI 0.62 - 1.92). The use of infectious episodes rather than number of positive stool samples (i.e. consecutive positive samples were deemed as a single episode) did not appreciably alter the above figures.

The results were similar when using a conditional logistic regression model estimating the odds ration per increase in infections prior to development of islet autoimmunity (OR = 1.12, 95%CI 0.66 - 1.91).

The quantity of human enterovirus RNA. The effect of viral load was assessed by dividing the positivity into two categories: low to moderate (quantity of 100 - 9 999 enterovirus copies / µl RNA) and high (10 000 or more enterovirus copies / µl RNA). No association with islet autoimmunity was found in this type of analysis (**Table 3**). In the 43 enterovirus-positive samples from the pre-autoimmunity period among cases, the median estimated human enterovirus quantity was 18000 copies / µl RNA compared to a median of 12000 copies / µl RNA among 94 enterovirus positive samples from matched controls from the corresponding periods (Mann-Whitney non-parametric test: P=0.37). Similar results were seen in the samples collected after the onset of autoimmunity. Among the 30 new enterovirus episodes during the pre-autoimmune period of cases, 13 (43.3%) were followed by at least one additional consecutive enterovirus positive sample, compared to 29 of 65 (44.6%) among the controls (chi-square test: P=0.73).

The occurrence of human enterovirus during the whole observation period. In total, we tested 2 044 stool samples from the cases (627) and controls (1417) in the study. Human enterovirus was detected in 80 / 627 (12.8%) samples from cases, and 210 / 1417 (14.8%)

samples from controls; the overall occurrence did not differ between cases and controls (OR = 0.84, 95% CI: 0.58 - 1.22). Looking only at samples taken after the start of islet autoimmunity gave similar results (OR = 0.74, 95% CI: 0.45 - 1.22). Only 11 subjects did not shed enterovirus in their stool during their entire observation period (4 cases and 7 controls). The remaining children had varying number of monthly samples positive, from only one (n=7) up to 8-9 (n=7). Infections and their distribution over the observational period in case and control subjects of the 27 matching groups are shown in Supplementary Figure 1 in the online appendix available at http://care.diabetesjournals.org.

Seasonal variation of infections. There was a pronounced seasonality of infections with a peak in autumn (October with 27% positive samples) and a smaller peak in July (with 24% positive samples), and a dip in March with 3% positive samples. Online Supplementary Figure 1 shows several episodes of increased density of infections that can be observed across the case-control matching group. The occurrence of infections was also age-dependent: a rise was noted from the fifth to ninth month of age, and during the first half of the second year of life.

Molecular typing of enterovirus strains using partial VP1 sequencing. Selected positive samples (97) had their VP1 genotypes determined in order to distinguish prolonged infections with one strain against multiple consecutive infections. The distribution of the 17 different serotypes found is shown in Online Supplementary Table 2. As the sequenced samples were not representative for the whole case-control dataset, direct comparison of serotype repertoire between cases and controls was not possible. A phylogenetic tree constructed from the dataset is shown in Online Supplementary Figure 2.

CONCLUSIONS

We tested enterovirus RNA in over 2,000 monthly fecal samples from children who developed repeated positivity for multiple islet autoantibodies and their matched controls, all with a single *HLA-DQ, -DR* genotype conferring the highest risk of type 1 diabetes. We found no evidence to support a higher frequency of enterovirus in cases than in controls either before or after seroconversion for islet autoantibodies. It must be kept in mind that the study population consists only of very young children; thus the conclusions might not apply to older individuals.

This study is the first to use a quantitative assay for testing the viral load, enabling us to distinguish between low- and high-quantity infections and follow the dynamics of the viral load. Our cohort includes only the highest risk *HLA-DQ, -DR* genotype, and is thus more genetically restricted than previously reported studies. The generalizability of our results might be questioned if the HLA genotype influenced the risk of enterovirus infection and/or immune response. However, preliminary results from our pilot study which also included a group without the high-risk HLA genotype indicated only a moderate difference in frequency of fecal enterovirus shedding (11). To our knowledge, none of the previous cohort studies of enterovirus and islet autoimmunity has found any significant difference in association depending on HLA genotype.

We have also used a strict definition of islet autoimmunity, requiring repeated positivity for two or three islet autoantibodies. This is known to be strongly predictive of type 1 diabetes in genetically susceptible children. The number of cases and sample size could indeed be increased with a less strict definition of autoimmunity. However, the power of the study might actually decrease by including subjects with milder autoimmunity who are less likely to eventually develop type 1 diabetes.

Regular monthly sampling from all participants, and high completeness are important strengths, as shedding duration is thought to be around 3-4 weeks (12); the necessity of frequent stool sampling is further supported by our earlier study showing that excretion usually lasted less than three months (13). Detection of viral RNA in serum would likely underestimate the true infection frequency, as enterovirus RNA is present in serum for much shorter period (12) than is the usual time span between blood samples. On the other hand, it is probable that viremia reflects more closely the spreading of the virus to the target organ, so frequent sampling of both stool and blood samples would be ideal.

Although the serotypes detected were not representative for all samples, we observed no preponderance of a strain, serotype or group in either cases or controls. Several serotypes previously reported as possibly diabetogenic (e.g. Coxsackie B), were observed both in cases and in controls. Although some types may seem more prevalent, this is caused mostly due to repeatedly positive stool samples from a small geographical area during a short period, reflecting local epidemics.

Two previous studies assessed fecal shedding of enterovirus RNA. The Finnish DIPP study utilized equally frequent sampling of stool as we did, reporting data from 12 cases of islet autoimmunity and 53 controls (14). The other study was DAISY in Colorado, where rectal swabs were collected in longer intervals (at ages 9, 12, 15 and 24 months, and then annually) from 26 cases and 39 controls (7). In both studies, there was no significant difference in the frequency of fecal enterovirus RNA shedding between cases with islet autoimmunity and controls, which is consistent with our findings. However, in contrast to us, the DIPP study reported that cases were more frequently positive in

consecutive samples than controls.

A publication from the DAISY study (7) and a separate publication from DIPP including 41 cases and 196 controls with 3-6 month sample intervals (4) also analyzed enterovirus RNA in serum. In both these studies there was no significant difference in the frequency of serum enterovirus RNA, but when combining serum RNA and a series of enterovirus antibodies as indicators of infection, there was a significant difference in the DIPP study, particularly in the six month interval before seroconversion in cases. Although we did not assess enterovirus RNA or antibodies in serum, no indication of a clustering of infections prior to seroconversion was found.

Two other Finnish studies reported a significant difference between cases of islet autoimmunity and controls in frequency of indicators of enterovirus infection in serum, namely the DiMe-study assessing 11 prediabetic siblings of patients with type 1 diabetes and 34 autoantibody negative controls (6), and the TRIGR-study assessing 19 cases and 84 controls from birth to 2 years of age (5). Note however, that enterovirus RNA in serum accounted for 23% of the identified infections (increases in enterovirus antibodies accounted for the remaining), and that the difference in enterovirus RNA was borderline (not) significant (14% vs. 8.4%, P=0.07). Finally, no significant association was found the German BABYDIAB study tested antibodies against Coxsackie viruses in blood samples collected at age of 9 months, and at 2, 5 and 8 years in 28 cases with persistent islet antibodies and 51 matched controls (8).

None of the previous studies contradicts our finding that fecal shedding of enterovirus RNA in general does not strongly predict islet autoimmunity. While moderate effects (OR of 1.5-2.0) cannot be ruled out from our data, the 95% confidence interval around the odds ratio estimated from our actual data suggest that strong associations (OR >2) are unlikely. However, we cannot exclude a possible role of a subgroup of enterovirus infections (particular strains), perhaps influencing viremia and ability to spread from the gut (the primary site of replication) to the target organ. This ability was seemingly unlinked to the viral load or duration of gut infections, as judged from our results. Other relevant factors may potentially influence the level and duration of viremia, and the ability to invade the islets and their beta cells.

In conclusion, there was no evidence to support a major role of frequency, timing or quantity of fecal enterovirus shedding in prediction of advanced islet autoimmunity, and no evidence that islet autoimmunity predicted increased susceptibility to fecal enterovirus shedding. Further research should be focused on the character of viremia, and the ability of enterovirus to invade the target pancreatic tissue, in much larger sample sets.

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Table 1. Characteristics of the cases and control subjects in this study

	Cases (n = 27)	Controls (n = 53)
Age at onset of islet autoimmunity*, Median months (range)	12.1 (5-37)	12.3 (5-37)
Female sex	17 (63%)	23 (43%)
Number of other children in the family (siblings, half-siblings, step siblings)		
None	5 (18.5%)	16 (30.2%)
One or more	22 (81.5%)	37 (69.8%)
First degree relative with diabetes		
None	17 (63%)	50 (94.3%)
Yes, of that:	10 (37%)	3 (5.7%)
- sibling only	3	0
- father only	3	2
- mother only	2	1
- multiple family members	2	0
Progression from islet autoimmunity to type 1 diabetes		
Yes	10	None
Stool Samples		
Total	627	1417
Prior to development of islet autoimmunity*	339	692

* For matched controls: before the age at which the corresponding case seroconverted for islet autoantibodies.

Table 2. Frequency of human enterovirus RNA in fecal samples collected prior to islet autoimmunity.

	Cases (n=27 subjects)	Controls (n=53 subjects)	Odds ratio (95% CI)* Unadjusted	Adjusted†
Enterovirus RNA **negative** samples	296	598	1.00 (reference)	1.00 (reference)
Enterovirus RNA **positive** samples	43 (12.7%)	94 (13.6%)	1.01 (0.59 - 1.72)	1.09 (0.61 - 1.96)
Total	339	692		
New enterovirus infection episode: **No**	296	598	1.00 (reference)	1.00 (reference)
New enterovirus infection episode: **Yes**	30 (9.2%)	65 (9.8%)	0.94 (0.59 - 1.52)	0.92 (0.54 - 1.57)
Total ‡	326	663		

* Odds ratio with 95% confidence interval (CI) estimated from logistic mixed effects logistic regression models with random intercept for each subject to control for intra-individual correlation (no significant random intercept in model for enterovirus episodes, but highly significant in model for enterovirus positivity). The unadjusted odds ratio in ordinary logistic regression ignoring intra-individual correlation in infections was 0.92.

† Adjusted for sex, calendar month of sample collection, year of sample collection (2001-3, 2004-6 or 2007-8), age (continuous), number of siblings (0 vs. >=1), breast-feeding, first degree family history of type 1 diabetes (yes/no).

‡ Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

Table 3. Semi quantitative testing of the stool samples.
Frequency of enterovirus infections with high and low viral load in the children who subsequently developed repeated positivity of multiple autoantibodies versus matched controls who did not develop autoimmunity.

	Cases (n=27 subjects)	Controls (n=53 subjects)
Enterovirus RNA negative	296	598
Enterovirus RNA positive, low-moderate quantity*	18 (5.3%)	46 (6.6%)
Enterovirus RNA positive, high quantity*	25 (7.4%)	48 (6.9%)
Total	339	692
Enterovirus RNA negative	296	598
New infection episode, low-moderate quantity*	11 (3.4%)	31 (4.7%)
New infection episode, high quantity*	19 (5.9%)	34 (5.1%)
Total†	326	663

* Negative: <100 copies per microliter EV RNA quantity, low-moderate: 100-9999, high: ≥10000 .
† Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

Příloha VIII.

Tapia G, Cinek O, Rasmussen T, Grinde B, Stene LC, Rønningen KS.

Longitudinal study of parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study,

Pediatric Diabetes, v tisku

Case Report

Longitudinal study of parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study

Tapia G, Cinek O, Rasmussen T, Grinde B, Stene LC, Rønningen KS. Longitudinal study of parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study.
Pediatric Diabetes 2010: 0: 000–000.

The objective of this study was to investigate a possible association between human parechovirus infections in early infancy, diagnosed in fecal samples, and the development of islet autoimmunity. In the 'Environmental Triggers of Type 1 Diabetes: The MIDIA study', newborns with the highest genetic risk for type 1 diabetes were identified and followed with regular fecal sampling and questionnaires. A nested case–control study, including 27 children who developed islet autoimmunity (repeatedly positive for two or three autoantibodies) and 53 children matched for age and community of residence was used. Monthly stool samples from these children were analyzed for human parechovirus using a semi-quantitative real-time polymerase chain reaction. There was no significant difference in the prevalence of human parechovirus in stool samples when cases and controls were compared: 13.0 and 11.1%, respectively. There was also not any difference as to the number of infection episodes. In analyses restricted to samples collected 3, 6 or 12 months prior to seroconversion for islet autoantibodies, there was a suggestive association in the shortest time window of 3 months (20.8 vs. 8.8%, odds ratio = 3.2, 95%, uncorrected p = 0.022). No symptoms were associated with human parechovirus infection. A subset of the positive samples (n = 31) were sequenced, suggesting that human parechovirus 1 was the dominant genotype. The present study does not support strong associations between human parechovirus infections and the signs of islet autoimmunity. The weak association of parechovirus present in the last 3 months before development of autoimmunity warrants further investigation.

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Environmental factors presumably contribute to the development of islet autoimmunity and ultimately type 1 diabetes. Viral infections are considered to be likely candidates for causing islet autoimmunity [reviewed in ref. (1)]. The Picornaviridae are among the most frequently studied viruses in relation to type 1 diabetes in humans and animal models. They are single-stranded RNA viruses that replicate mainly in the gut and are transmitted by the fecal–oral route. Other viruses in the gut, e.g., rotaviruses (2), have also been investigated and associated with islet autoimmunity. Human

parechoviruses belong to the relatively new genus Parechovirus within the family of Picornaviridae and are closely related to the enteroviruses, which have been implicated in type 1 diabetes (3). The Parechovirus genus includes eight genotypes of human parechovirus as well as the rodent parechovirus Ljungan virus, which has been proposed as having an association with type 1 diabetes in bank voles (4). Human parechovirus 1 and 2 were described in the 1950s, while genotypes 3–8 have been described quite recently (5–10). There are also VP1 sequences of five undescribed human

parechoviruses assigned as human parechovirus 9–14 (11). The most common human parechovirus is genotype 1, followed by human parechovirus 3 and 6, with the remaining genotypes being rare.

Human parechoviruses are common in infancy, and while the presence of virus in stool samples seems mostly asymptomatic, viral infections are also associated with various diseases (11). A previous report (12) did not find any association between human parechovirus 1 and type 1 diabetes by measuring specific antibodies against human parechovirus 1 in a nested case–control study in a cohort similar to the one used in the present study.

There are two reasons for investigating an association between parechovirus infections and the development of type 1 diabetes: First, the infections might be directly involved in triggering autoimmunity. Second, infection with common viruses can be used as a proxy for hygiene because the presence of virus is expected to correlate with exposure to a large variety of microorganisms, which, according to the hygiene hypothesis, is the preferred situation in order to develop a well-functioning immune system. Thus, the aim of the present study was to establish if there is any association between the development of islet autoimmunity and human parechovirus infections in infancy, as well as the viral load and associated symptoms of infection as indicators of infection severity.

Methods

Subjects and study design

The children followed in this study participate in 'Environmental Triggers of Type 1 Diabetes: The MIDIA study'. They were identified at birth to carry the human leukocyte antigen (HLA) genotype conferring the highest genetic risk of type 1 diabetes: DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02 (13). The parents were asked to submit monthly stool samples from their infants from the 3rd to the 35th month and to record information on the type and dates of symptoms of infection (coughing and sneezing, diarrhea, vomiting, or fever), and some indicators of diet, in questionnaires. In order to monitor the development of islet autoimmunity, the children were tested for autoantibodies against glutamic acid decarboxylase$_{65}$ (GAD), protein tyrosine phosphatase IA2 (IA2), and insulin (IAA) with blood samples taken every 3 months until the age of 1 yr and every 12 months thereafter. The testing is described in detail by Stene et al. (13). If a blood sample is found positive for one autoantibody, the child is retested every 6 months; if two or three antibodies are positive, the child is retested every 3 months. The end-point for this study was set as the development of autoimmunity

defined as at least two autoantibodies positive in at least two consecutive samples. The study was approved by The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

By 27 December 2008, children (10 boys and 17 girls) were confirmed positive for two or three autoantibodies and were assigned as cases. In cases who at first tested positive for a single autoantibody, the first occurrence of autoantibody positivity was regarded as the onset of autoimmunity and was used as the end-point. Two children per case were randomly selected from the cohort, matched for duration of follow-up, date of birth (±1 month, tolerating up to 3 months in a few cases where necessary), and county of residence. The family of one control child later withdrew and asked us to delete their data. In total, 80 children were included in the analysis (40 boys and 40 girls, born between 2001 and 2006). Of the scheduled samples and questionnaires, 637 of 704 blood samples (91%), 2173 of 2482 stool samples (88%), and 492 of 547 questionnaires (90%) were received. Of these, 2027 stool samples were tested for human parechovirus. The median age at onset of islet autoimmunity was 12.0 months (range 5.4–37.4 months) and the median duration of follow-up with stool samples was 28 months (range 7–36 months).

Statistical analysis and power calculations

The odds ratio (OR) for association between parechovirus infections and autoimmunity that could be detected with a two-sided test with alpha-level of 5 and 80–95% power under various scenarios was calculated. Assuming a total human parechovirus prevalence of 12% based on earlier data (14), 1800 samples, a true OR of 1.6, and comparing all samples, a power of 88% would be expected. Assuming a true OR of 2.0, and comparing samples prior to development of autoimmunity, gives an expected power of 93%. The association of parechovirus frequency with islet autoimmunity was tested and estimated using logistic regression with random intercept to take into account repeated measures within individuals. This was done using the xtmelogit procedure in STATA 11.

Virus detection

Methods for the collection, processing, and RNA extraction of the monthly stool samples are described elsewhere (15). The extraction protocol described therein was modified for the use of 96-well vacuum-processed QIAAmp plates (Qiagen) instead of single columns. To avoid false-negative results, West Nile Virus Armored RNA (Asuragen, USA) was added to the sample in the first step of extraction and used as an exogenous internal control for RNA extraction, reverse

transcription (RT), and real-time polymerase chain reaction (PCR). Briefly, the samples were reverse transcribed with specific primers for human parechovirus, using the Improm II RT kit (Promega), and a real-time RT–PCR was used to detect human parechovirus RNA from the extracted samples, described in detail previously (14). The amplification was carried out on an ABI 7300 (Applied Biosystems) with the following thermal profile: 15 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C (where fluorescence data were collected). If the threshold cycle of the internal control was too high, or the amplification curve was not exponential, the sample was re-extracted and retested. Positive and negative controls were included in the extraction procedure. Dilutions of a mix of positive samples with known quantities were used to quantify the human parechovirus present in the reactions. All samples were subjected to blind testing. The positive controls were consistently detected down to 10^1 copies/μL, and this level was therefore set as a positivity threshold. The real-time PCR method used is theoretically able to detect all the eight genotypes of human parechoviruses.

Genotyping of positive samples

A subset of the positive samples (n = 31) were genotyped by sequencing and comparison of the VP1 polymerase region, using primers designed by Benschop et al. (16). The products were obtained by a RT-step using the Improm II RT system, followed by a PCR with the VP1 region as the target, using GoTaq chemistry (Promega). Detailed protocols are available from the authors. Products were cleaned using AMPure chemistry run on an Biomek 3000 robot (both Beckman Coulter, Beverly, MA, USA), sequenced using BigDye Terminator v3.1 (Applied Biosystems) with the same primers as used in the PCR, purified using the CleanSEQ chemistry on the Biomek 3000 robot, and analyzed on an ABI3130XL (Applied Biosystems) capillary sequencer.

Results

A total of 2027 samples from 27 cases and 53 matched controls were analyzed, of which 11.6% were positive. The frequency of human parechovirus infections did not differ significantly between cases (13.0%) and controls (11.1%) when all samples were included [OR = 1.2, 95% confidence interval (CI) 0.90–1.6]. Restricting the analysis to samples taken prior to the development of islet autoimmunity (and the corresponding time point in the matched controls), the frequencies were 13.6% for cases and 11.4% for controls (OR = 1.2, 95% CI 0.7–1.9), summarized in Table 1. Interestingly, if a time window is used 3 months before

the development of islet autoimmunity, 16/77 samples (20.8%) from cases had an infection as opposed to 16/182 (8.8%) samples from controls (OR = 3.2, 95% CI 1.2–8.5, p = 0.022).

Counting only the first in a series of two or more consecutive positive samples from an infant (assuming they were part of the same infectious episode) gave no significant differences in the occurrence of human parechovirus episodes between cases and controls, and neither were there any significant difference in number of infections, number of infections in the first year of life or infections with symptoms (Table 1). The median age of first human parechovirus infection was 12.1 months in both the cases and interestingly, it seems like the children who developed islet autoimmunity had more infections lasting 2 or more months than those who did not after development of islet autoimmunity (OR = 2.4, 95% CI 1.1–5.3, p = 0.034). The median number of viral genomes was 608.5 RNA copies per microliter in the positive samples obtained from cases prior to the development of islet autoimmunity, compared to 469 RNA copies per microliter in controls; the difference was not statistically significant. There was no difference when including all the positive samples from both groups, either.

A subset of the viral samples (n = 31, 26 infectious episodes) were sequenced in order to identify the genotypes present. The following genotypes were found (number of infectious episodes in parenthesis): human parechovirus 1 (15/26), human parechovirus 2 (1/26), human parechovirus 3 (7/26) and human parechovirus 6 (3/26). The sequencing of samples taken in consecutive months supported the notion that these samples reflected a single infectious episode.

Discussion

Human parechovirus infection during infancy does not seem to predict islet autoimmunity, and there was no difference in frequency of infection in the first year of life or after seroconversion for islet autoantibodies. Neither was there any difference in the median ages of first infection. The present study supports and supplements the previously published data by Tauriainen et al. (12), which found no association of human parechovirus 1 and type 1 diabetes. However, we did find a higher frequency of infection in case children in the interval 3 months before seroconversion for islet autoantibodies (OR = 3.17, p = 0.022), which could warrant subsequent replication.

Although the data indicate that human parechoviruses are not alone a major cause of type 1 diabetes, it is still conceivable that these viruses may be involved in type 1 diabetes because of individual vulnerability or qualities of particular strains of virus. A possible correlation between one of the rarer

Table 1. Comparison of human parechovirus infections in stool samples from cases and controls

	Positive for human parechovirus RNA	Infection episodes (per child)	Infection episodes lasting >2 months (per child)	Positive samples with symptoms	Positive samples, no symptoms
Cases before end-point	44 (13.1%)	27 (1.0)	14 (0.52)	20/39 (51.3%)	19/39 (48.7%)
Controls before end-point	78 (11.4%)	43 (0.81)	25 (0.47)	27/73 (37.0%)	46/73 (63.0%)
OR (95% CI)	1.17 (0.79–1.74)	1.30 (0.79–2.15)	0.98 (0.55–1.79)	1.79 (0.81–3.74)	
Cases in total	82 (13.0%)	45 (1.66)	25 (0.93)	25/57 (43.9%)	32/57 (56.1%)
Controls in total	154 (11.1%)	96 (1.81)	43 (0.81)	46/133 (34.6%)	87/133 (65.4%)
OR(95% CI)	1.19 (0.90–1.60)	1.0 3(0.72–1.49)	1.42 (0.94–2.18)	1.47 (0.78–2.78)	
All	236 (11.6%)	1.76 (141 episodes)	0.85 (68 episodes)	71/190 (37.4%)	119/190 (62.6%)

OR, odds ratio; CI, confidence interval.
The end-point reflects the time when the cases developed autoimmunity. None of the comparisons made between cases and controls had significance, irrespective of whether the samples taken prior to the end-point or all the samples were considered.

parechovirus genotypes (other than the types seen in this study) and type 1 diabetes cannot be ruled out but seems unlikely. In addition, qualities that might make a human parechovirus diabetogenic do not necessarily correlate with genotype, viral load, symptoms, or other parameters presently investigated.

Although in some cases mild symptoms were reported for the same month that the infant shed human parechovirus RNA in the feces (fever, cold, or diarrhea), there was no significant correlations between symptoms and the presence of virus neither in cases nor in controls (data not shown). There was no correlation between symptomatic infections and the development of islet autoimmunity. Furthermore, the viral load observed was similar in both cases and controls, and there was no difference between cases and controls in the length of infections before development of islet autoimmunity. These observations further point against direct involvement of human parechoviruses. Interestingly, the cases seemed to have longer human parechovirus infections than control children after the development of islet autoimmunity. This could reflect an altered immune state where autoimmunity leads to susceptibility for human parechovirus. The stool samples were taken monthly, and the possibility of having an infection between two samples cannot be dismissed; however, in 50 of the 128 infectious episodes, viral RNA was detected in two or more consecutive months, suggesting that infections are likely to be detected by monthly sampling.

In conclusion, the role of human parechoviruses as environmental factors of type 1 diabetes seems to be very small, if any, but the interesting higher prevalence in cases in a 3-month period before development of islet autoimmunity warrants further research.

Acknowledgement

Conflict of interest

The authors report no conflicts of interest.

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Příloha IX.

Cinek O, Kolousková S, Snajderová M, Sumník Z, Sedláková P, Drevínek P, Vavrinec J, Ronningen KS.

HLA class II genetic association of type 1 diabetes mellitus in Czech children.

Pediatr Diabetes. 2001 Sep;2(3):98-102.

Original Article

HLA class II genetic association of type 1 diabetes mellitus in Czech children

Cinek O, Koloušková S, Šnajderová M, Šumník Z, Sedláková P, Dřevínek P, Vavřinec J, Ronningen KS. HLA class II genetic association of type 1 diabetes mellitus in Czech children.
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Abstract: To examine human leukocyte antigen (HLA) class II association of type 1 diabetes mellitus (DM) in Czech children, we performed a case–control study of 261 patients diagnosed before the age of 15 and 289 non-diabetic control children. Complete HLA-DQA1, DQB1 genotyping and DRB1*04 subtyping were carried out by polymerase chain reactions with sequence-specific primers. The effect of the DRB1*04 subtypes was studied in DRB1*04 alleles carried on DQB1*0302-DQA1*03 haplotypes. The risk was statistically evaluated by testing 2×2 tables, considering corrected p-values < 0.05 significant. The DQB1*0302 (odds ratio, OR = 9.0), DQB1*0201 (OR = 3.4) and DQA1*03 (OR = 7.5) alleles were significantly associated with diabetes risk, while the DQB1*0602 (OR = 0.02), DQB1*0301 (OR = 0.08), DQB1*0503 (OR = 0.13), DQB1*0603 (OR = 0.20), DQA1*01 (OR = 0.28) and DQA1*02 (OR = 0.26) alleles were significantly protective. Of the DQA1-DQB1 genotypes, we point out the extremely high risk of OR = 116 conferred by HLA-DQA1*05-DQB1*0201/DQA1*03-DQB1*0302. Among DRB1*04 subtypes, DRB1*0403 was significantly protective (OR = 0.05, CI 95% 0.01–0.45). Since none of the remaining DRB1*04 subtypes was associated with type 1 DM, our study may present another piece of evidence that the DRB1*0401 and DRB1*0404 alleles do not modify type 1 diabetes risk generally in European populations.

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Type 1 diabetes mellitus (DM) is an autoimmune disease with a well defined genetic association to the human leukocyte antigen (HLA) complex. A major part of the HLA-related risk or protection is conferred by the whole HLA-DQ molecule (1), with a contribution of certain DRB1*04 subtypes (2, 3), and at least two genes within HLA but outside class I and II (4, 5). The level of risk conferred by particular HLA alleles, haplotypes and genotypes varies among nations and ethnic groups (1). Knowledge about HLA-encoded diabetes susceptibility in a particular population is thus useful for disease pathogenesis studies at a population level, and is essential for the prediction of type 1 DM. Central and Eastern Europe is a region with considerable differences both in the incidence of type 1 diabetes (6) and in the HLA genetic characteristics of the populations (7). So far no international report has been published on HLA associations of type 1 DM in Czechs. The aim was therefore to characterize type 1 diabetes genetic susceptibility conferred by the HLA – DQB1, DQA1 genes and the DRB1*04 subtypes in the Czech population.

The Czech Republic is a Central European country. Its population of 10.3 million inhabitants is ethnically homogenous with more than 98% Caucasians. The incidence of type 1 DM incidence in children under the age of 15 is at an intermediate level among European countries with 10.1 new cases per 100 000 per yr (1990–97) (8).

Subjects, materials and methods

The study design was case–control. The case group consisted of 261 Czech diabetic patients (131 boys

and 130 girls) diagnosed with type 1 DM before the age of 15. The diagnosis was established based on glycemic criteria according to the World Health Organization (WHO), absolute insulin dependency and proneness to ketosis. The age at diagnosis was 7.6 ± 3.8 (mean $\pm$ SD); the numbers of patients diagnosed within the age intervals of 0–4, 5–9, and 10–14 yr were 82, 98, and 81, respectively. The patients were recruited over the period between November 1996 and May 2000. Their selection was either random at our outpatient unit or at our in-patient pediatric endocrinology department at the onset of disease.

The control group consisted of 289 non-diabetic Caucasian children aged 8.5 ± 3.9 yr. These children were consecutively recruited among patients undergoing minor surgical interventions in the period between January and October 1999 at the Department of Pediatric Surgery (the University Hospital Motol, Prague). Informed consent to genetic investigation and to storage of DNA was obtained. Children with any history of autoimmune disorders, connective tissue disorders, glucose intolerance, endocrine diseases, or with increased erythrocyte sedimentation rate were excluded. The study was approved by the local ethics committee.

DNA was extracted using the salting-out method (9). The HLA class II genotype was determined by polymerase chain reactions with a set of sequence-specific primer pairs (10, 11). Complete genotyping for 13 DQB1 alleles and six DQA1 alleles was performed in all subjects. The effect of DRB1*04 subtypes was studied by subtyping DRB1*04 alleles carried on the DQB1*0302-DQA1*03-DRB1*04 haplotypes.

The risk levels for particular alleles or genotypes were expressed as odds ratios (OR) and their confidence intervals calculated according to Woolf's formula. Haldane's correction was applied when no patient or control had the particular allele or genotype. Comparisons were made by testing the 2×2 table using the χ^2-test with Yate's correction or two-sided Fisher's exact test where appropriate. Age-at-diagnosis trends in frequency of genotypes were tested by the χ^2-test for trend. Correction for multiple testing was made for the number of alleles or genotypes found at each locus (for 13 DQB1 alleles, six DQA1 alleles, six DRB1*04 subtypes, and 68 different DQB1 genotypes), according to Bonferroni's method (12). Corrected p-values lower than 0.05 were considered significant.

Results

HLA-DQB1, -DQA1 alleles found to be significantly associated with type 1 DM are presented in Table 1: the DQB1*0302, DQB1*0201, and DQA1*03 alleles conferred an increased risk for diabetes, while the DQB1*0602, DQB1*0301, DQB1*0503, DQB1*0603 and DQA1*01, DQA1*02 alleles were protective. Of the 68 different DQB1 genotypes found in either patients or controls, four were significantly associated with type 1 DM (Table 2): the DQB1*0201/0302 and DQB1*0302/0302 genotypes conferred an increased risk, whereas the DQB1*0201/0301 and DQB1*0201/0602 were protective. Examining the DQA1-DQB1 genotypes, the highest risk of type 1 diabetes (OR = 116; 95% CI = 16–842, p $< 10^{-15}$) was found for the DQA1*05-DQB1*0201/DQA1*03-DQB1*0302 genotype, which was carried by 29% of the cases, but only 0.35% of the controls (one out of 289).

The allelic frequencies of the DRB1*04 subtypes, carried on the DQA1*03-DQB1*0302 haplotypes, are shown in Table 3. Six out of eight DRB1*04 subtypes which could be distinguished (0401–0408) were found either in diabetic patients or in controls. The DRB1*0403 allele was significantly protective (OR = 0.05; 95% CI = 0.01–0.45), while none of the remaining alleles was significantly associated after a correction for multiple testing.

We investigated a trend towards a decrease in DQB1*0201/0302 prevalence with an increasing age at type 1 DM diagnosis (13). Prevalences of DQB1*0201/0302 heterozygotes among children diagnosed at the age of 0–4, 5–9, and 10–14 yr were 37/82 (45%), 35/98 (36%), and 22/81 (27%), respectively. This trend was statistically significant (p = 0.017).

Discussion

The highest type 1 DM risk found in our study was conferred by the DQA1*05-DQB1*0201/DQA1*03-DQB1*0302 genotype: OR = 116 (95% CI = 16–842). Such an exceedingly high risk may have arisen partly due to a random error, since it is caused by the low prevalence of the genotype in our controls (1/289, 0.35%) rather than by the 29% prevalence in the patients. In a combined Caucasian dataset at the 11th HLA workshop, this genotype was shown to be carried by 26% of patients compared with 2.0% of healthy subjects (1). The upper limit of the 95% confidence interval for the prevalence in our controls is 1.6%, a level that would correspond better with the geographic origin of our population and would conform to a previous study of the Czech general population (14). Moreover, the HLA-DQA1 and DQB1 allelic frequencies of our controls did not significantly differ from the previous study (14).

Knowledge of type 1 diabetes HLA associations in a particular population is necessary for an effective prediction, since the type 1 diabetes genetic characteristics vary substantially among populations: in northern Europe, the risk is conferred primarily by the DQB1*0302 haplotypes while in southern Europe this role is played by the DQB1*0201-DQA1*05 haplotype

Table 1. Phenotypic frequencies and odds ratios (ORs) for the HLA-DQB1 and -DQA1 alleles significantly associated with type 1 diabetes mellitus (DM) in Czech children

Allele	Patients (n = 261)	Controls (n = 289)	OR (95% CI)	$p_{corrected}$
DQB1*				
0302	169 (65%)	49 (17%)	9.0 (6.0–13)	$< 10^{-15}$
0201	160 (61%)	92 (32%)	3.4 (2.4–4.8)	1×10^{-10}
0603	10 (4%)	49 (17%)	0.20 (0.10–0.39)	2×10^{-5}
0503	3 (1%)	23 (8%)	0.13 (0.04–0.45)	5×10^{-3}
0301	14 (5%)	117 (40%)	0.08 (0.05–0.15)	$< 10^{-15}$
0602	2 (1%)	72 (25%)	0.02 (0.01–0.10)	4×10^{-15}
DQA1*				
03	186 (71%)	72 (25%)	7.5 (5.1–11)	$< 10^{-15}$
02	26 (10%)	65 (22%)	0.38 (0.23–0.62)	8×10^{-4}
01	97 (37%)	196 (68%)	0.28 (0.20–0.40)	7×10^{-12}

n, number of individuals carrying the particular allele (% are phenotypic frequencies); 95% CI, 95% confidence interval.
p-values have been corrected for 13 DQB1 and 6 DQA1 alleles.

Table 2. DQB1 genotypes significantly associated with type 1 diabetes mellitus (DM)

DQB1* genotype	Patients DM (n = 261)	Controls (n = 289)	OR (95% CI)	$p_{corrected}$
0201/0302	94 (36%)	7 (2.4%)	22 (10–50)	$< 10^{-15}$
0302/0302	23 (9%)	2 (0.7%)	14 (3.2–59)	8×10^{-4}
0201/0301	2 (1%)	23 (8.0%)	0.09 (0.02–0.38)	8×10^{-3}
0301/0602	0 (0%)	17 (5.9%)	0.06 (0.01–0.44)	1×10^{-3}

n, number of individuals carrying the genotype (% of n); OR, odds ratio; 95% CI, 95% confidence interval.
The p-values have been corrected for 68 different DQB1 genotypes found either in the cases or in the controls.

Table 3. DRB1*0403 confers protection from type 1 diabetes mellitus (DM) in Czech children

DRB1*04	Patients (187 alleles)	Controls (51 alleles)	OR (95% CI)	$p_{corrected}$
0401	127 (68%)	29 (57%)	1.6 (0.85–3.0)	0.72
0402	24 (13%)	3 (6%)	2.4 (0.68–8.2)	0.83
0403	0 (0%)	4 (8%)	0.05 (0.01–0.45)	0.011
0404	24 (13%)	13 (25%)	0.48 (0.20–0.92)	0.25
0405	11 (5.9%)	2 (4%)	1.6 (0.33–7.1)	1.0
0406	0	0		
0407	0	0		
0408	1 (0.5%)	0	0.59 (0.05–6.3)	1.0

Allelic frequencies of particular DRB1*04 subtypes were compared between 187 DQB1*0302-DQA1*03-DRB1*04 haplotypes carried by the patients, and 51 DQB1*0302-DQA1*03-DRB1*04 haplotypes carried by the controls.
Data are numbers of alleles (% are allelic frequencies).
OR, odds ratio; 95% CI, 95% confidence interval.
The p-values have been corrected for six alleles.

(15). Our findings are currently being utilized in a diabetes prediction study in first-degree relatives (16). Although no screening of our general population is currently feasible, due to rather low incidence of type 1 diabetes as well as lack of effective preventive strategies, the present data suggest that the set of alleles screened for risk assessment could be similar to the strategies of DIPP (17) or DAISY (18) studies.

We sought to characterize the contribution of the DRB1*04 subtypes to HLA-encoded type 1 DM susceptibility (2, 3, 19–22). While it is generally agreed that DRB1*0403 acts as a dominantly protective factor (3, 19, 23, 24), the effect of other DRB1*04 subtypes varies substantially among populations, including Central and Eastern Europe (19, 25, 26). We decided to analyze the DRB1*04 alleles carried on the DQA1*03-DQB1*0302 haplotypes; this approach has been used in several major studies (2, 26). Our finding of a relatively strong protection conferred by the DRB1*0403 allele is confirmative of other studies (3, 19, 23, 24). The absence of a significant risk or protection for the remaining DRB1*04 subtypes may be another piece of evidence that the DRB1*0401 and DRB1*0404 subtypes, in particular, do not modify the risk of type 1 DM generally in European populations.

Of our four neighboring countries, data on DRB1*04-encoded type 1 DM susceptibility were available only for Germany and Poland. In Germans, whose HLA-DRB1*04 subtype distribution in the general population is not significantly different from that of Czechs (24), none of the DRB1*04 subtypes was significantly associated with either diabetes risk or protection (24). The Poles substantially differ from most of the European nations (including Czechs) in their DRB1*04 subtype distribution, having a rather high frequency of DRB1*0401 (conferring diabetes risk) and DRB1*0403 (conferring diabetes protection), while the DRB1*0404, 0405, 0406, and 0408 alleles are rare or absent (23, 27). No data from Slovakia [genetically our closest neighbor (28)] or Austria on type 1 DM genetic susceptibility have been published.

Both the cases and the controls can be considered representative of the Czech population. All of our cases diagnosed after 1989 have been ascertained by the population-based Czech Childhood Diabetes Registry (CCDR) established that year. A slight over-representation of children with a lower age at onset in the study (age at diagnosis 7.6 ± 3.8 yr) against the records of the CCDR (age at diagnosis 8.7 ± 3.7 yr, significant at $p < 0.01$) is probably due to specialization of our pediatric endocrinology department in critical care of young children and infants.

In conclusion, we present the first report on genetic susceptibility to type 1 DM in Czech children, showing an unusually high risk conferred by the DQA1*05-DQB1*0201/DQA1*03-DQB1*0302 genotype, and a considerable protective effect of the HLA-DRB1*0403 allele.

Acknowledgements

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Příloha X.

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No independent role of the -1123 G>C and+2740 A>G variants in the association of PTPN22 with type 1 diabetes and juvenile idiopathic arthritis in two Caucasian populations.

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No independent role of the −1123 G > C and + 2740 A > G variants in the association of PTPN22 with type 1 diabetes and juvenile idiopathic arthritis in two Caucasian populations

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Abstract

Introduction: The PTPN22 is a negative regulator of the T cell response. Its +1858C > T (R620W) polymorphism has been shown to associate with a risk for multiple autoimmune diseases, including type 1 diabetes (T1D) and juvenile idiopathic arthritis (JIA). The minor (susceptibility) allele is absent in Asian populations, but a recent study suggested an independent involvement of another polymorphism located within the promoter −1123 nucleotides relative to the translational start site.

Aims: We aimed to analyse the association of three PTPN22 polymorphisms in two distinct Caucasian populations, the Czechs (with T1D and with JIA) and Azeri (with T1D).

Methods: The single nucleotide polymorphisms (SNP) at positions −1123 (rs2488457), +1858 (rs2476601, the R620W substitution), and +2740 (rs1217412) were genotyped using TaqMan assays in 372 subjects with childhood-onset T1D, 130 subjects with JIA, and 400 control subjects of Czech origin, and in 160 subjects with T1D and 271 healthy controls of Azeri origin.

Results: In the Czechs, all three SNPs were in a tight linkage disequilibrium, while in the Azeri, the linkage disequilibrium was limited to between the promoter and 3′-UTR polymorphism, $D'(-1123, +2740) = 0.99$, $r^2 = 0.72$. Haplotype reconstruction via the expectation–maximization algorithm showed in both populations that only the haplotype containing the minor (W) allele at codon 620 was associated with T1D (OR = 2.26, 95% CI 1.68–3.02 in Czechs, OR = 14.8, 95% CI 2.0–651 in Azeri) or JIA (OR = 2.43, 95% CI 1.66–3.56 in Czechs). The haplotypes having the wild-type (R) allele at codon 620 and minor alleles at −1123 and/or +2740 were neutral as to the risk of autoimmune conditions in both populations.

Conclusions: In two different Caucasian populations, the Czechs and the Azeri, no independent contribution can be detected either of the −1123 promoter SNP or the +2740 3′-UTR SNP, and only the minor allele at PTPN22 codon 620 contributes to the risk of autoimmunity.

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1. Introduction

The protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) gene encodes a lymphoid-specific

phosphatase (Lyp), one of the regulatory protein tyrosine phosphatases expressed in the T cell. It is a powerful down-regulator of the T cell response. There is very strong evidence that a missense substitution C1858T encoding for amino acid substitution R620W is associated with a risk of multiple autoimmune diseases (meta-analysis in Ref. [1]). The polymorphism is the second only to HLA as to the width of associated conditions: they include type 1 diabetes (T1D), rheumatoid arthritis, systemic lupus erythematosus, juvenile idiopathic arthritis (JIA) and Graves disease [1].

Nevertheless, even in type 1 diabetes, the association of the minor W allele at codon 620 is not universal. In the Asian populations, *PTPN22* is probably non-polymorphic at this site – earlier studies found no W620 allele in Chinese and Japanese subjects [2,3]. The lack of the minor (620W) allele reported from multiple Asian populations is intriguing: it might still be possible that other polymorphisms within the PTPN22 are associated. Moreover, although there is ample evidence of a functional role of R620W, the mechanism is still incompletely understood. It is clear that the PTPN22 function is very important for down-regulation of lymphocyte activation, however two fundamentally different functional explanations have been proposed. The W620 variant of LYP was demonstrated ineffective in Csk binding and therefore disrupting the formation of LYP-Csk complex important in T cell inactivation [4]. On the other hand, it was also shown that this disease-predisposing variant inhibits stimulated interleukin-2 production by T cells and also that the W620 – encoded phosphatase has a higher catalytic activity, although it does not bind to Csk [5].

Recently, Kawasaki et al. [6] confirmed a complete lack of the minor allele at codon 620 in a large set of 1520 Japanese and 178 Koreans, but found two polymorphisms within the region that had the minor allele frequency over 1%: the −1123 promoter polymorphism, and the +2740 polymorphism within the 3′-UTR. They further studied the association of the −1123 SNP and reported an association with acute-onset diabetes in the Japanese and Korean patients. Furthermore, they deemed a significant association of this polymorphism in a dataset of families from the British Diabetes Warren Repository.

If the −1123 SNP was really a causal variant within the *PTPN22*, the association should be consistent among populations where *PTPN2*2 modifies the risk of autoimmune diseases. It should also modify the risk of autoimmunity regardless of the R620W genotype. To test this, we set out to determine three-SNP genotypes in sample sets of diabetic patients from two Caucasian populations, the Czechs and the Azeri, as well as in Czech patients with JIA, and compare them to unrelated controls from the same population. We hypothesized that if the −1123 SNP is primarily associated, haplotypes can be found in which the minor allele at position −1123 modifies the risk independently of R620W. The secondary aim of this study was to quantify the association and test it for potential interaction to other genetic susceptibility factors.

2. Subjects and methods

2.1. Subjects

In a case-control design, we studied 372 Czech patients with childhood-onset T1D, and 130 Czech patients with JIA, comparing them to 400 unrelated healthy Czech controls. Further, 160 Azerbaijanian patients with childhood-onset T1D were compared to 271 unrelated Azerbaijanian controls.

The Czech diabetic subjects were 372 children (188 males, 184 females) who developed T1D under the age of 15 years (median 7.4, interquartile range 4.1–10.9), and were treated at the Department of Paediatrics of the University Hospital Motol in Prague. Diagnosis was based on the WHO criteria, abrupt onset, and proneness to ketosis.

The 130 Czech patients with JIA (63 males, 67 females) were recruited at the outpatient Rheumatology Clinic of the University Hospital Motol in Prague. Patients were classified according to the criteria of the International League of Associations for Rheumatology (ILAR); 43 presented with oligoarticular, 72 with polyarticular, and 15 with systemic form of JIA. The median of age at disease onset was 7 years (interquartile range 7–11), and the follow up lasted 1–46 years, median 6.0 years.

The Czech control group consisted of 400 healthy unrelated subjects, either healthy children consecutively recruited from patients who underwent minor surgical interventions at the Department of Paediatric Surgery of the University Hospital Motol, Prague, or healthy blood donors. Control subjects with any history of autoimmune disorders, connective tissue disorders, glucose intolerance, endocrine diseases, or with increased erythrocyte sedimentation rate were excluded.

The Azerbaijanian patients were 160 subjects (81 males and 79 females) with childhood-onset T1D manifested under 19 years of age, fulfilling the WHO glycaemic criteria, having an abrupt onset of disease, proneness to ketosis, and an absolute insulin dependency. The median age at T1D onset was 9 years (interquartile range 6–12). Patients of other than Azeri descent were not included into the study. Non-diabetic Azeri control subjects (271, of them 79 males and 192 females, median age 21 years, interquartile range 19–21) were randomly selected among the students and staff of the Medical College in Baku. As in patients with T1D, other

self-reported descent than Azeri was an exclusion criterion. The criteria for recruiting patients and controls are described in detail elsewhere [7].

2.2. Determination of genotypes

Genotypes of three single nucleotide polymorphisms within the PTPN22 region were determined. These SNP were rs2476601, an G > A substitution (on the forward strand of the genome assembly, C > T on the reverse, coding strand) at position +1858 relative to translational start nucleotide, resulting in amino acid substitution R620W; rs2488457, a C > G substitution (on the forward strand of the genome assembly) in PTPN22 promoter, −1123 relative to translational start nucleotide; and rs1217412, an A > G substitution (on the forward strand of the genome assembly) in the PTPN22 5'-untranslated region, +2740 relative to translational start nucleotide.

The genotypes of the SNPs were determined using the TaqMan SNP genotyping assays. The PCR reaction contained in a volume of 10 μl 1 × PCR buffer, 4 mM $MgCl_2$, 10% glycerol, 150 μM each dNTP (Sigma–Aldrich), 1 μM 6-carboxy-X-rhodamin (Molecular Probes/Invitrogen), 0.5× to 1× mix of primers and the probe (TaqMan SNP Genotyping Assay by Applied Biosystems, Foster City, CA, USA), and 0.2 unit HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany). The thermal profile consisted of 15 min at 95 °C, followed by 45 cycles consisting of 15 s at 92 °C and 1 min at 60 °C where the fluorescence was collected. The PCR reactions were performed on an ABI 7300 machine (Applied Biosystems, Foster City, CA, USA), and evaluated according to manufacturer's instructions. To ensure consis-

tency between runs, samples of known genotypes were repeated in every analysis.

2.3. Statistical analysis

Allele frequencies were determined by gene counting. The Hardy-Weinberg equilibrium was checked by comparing observed to expected genotype frequencies, and tested using exact tests. Association of particular SNPs with the autoimmune conditions was expressed using odds ratios with 95% confidence intervals, and tested by either χ^2-tests, or exact tests. Haplotype analysis was performed by estimating the haplotype frequencies using an expectation–maximization (EM) algorithm implemented in the Stata program package version 9.2 (Stata Corp., College Station, Texas, USA). Association of haplotypes with the conditions was tested using log-linear modelling. The potential interaction between the PTPN22 genotypes and other genetic factors associated with the autoimmune conditions were tested using multiple logistic regression. To adjust for other genetic factors associated with the conditions, we used previously obtained data on the genotypes of HLA, insulin gene and CTLA4 in diabetic patients [7,8], or cytokine genotypes in JIA [9].

3. Results

3.1. T1D association in the Czechs

The genotype frequencies of the PTPN22 −1123 C/G, R620W and +2740 A/G polymorphisms are listed in

Table 1
Association of PTPN22 polymorphisms with T1D and with JIA in the Czechs

PTPN22 genotype	Czech subjects with T1D ($n = 372$)	Czech subjects with JIA ($n = 130$)	Czech control subjects ($n = 400$)
Position −1123			
G/G	179 (48%)	58 (45%)	244 (61%)
G/C	165 (44%)	59 (45%)	143 (36%)
C/C	28 (7.5%)	13 (10%)	13 (3.3%)
OR for the phenotypic positivity of the minor (C) allele	OR = 1.7, 95% CI 1.3–2.2	OR = 1.9, 95% CI 1.3–2.9	
Amino acid 620			
R/R	231 (62%)	79 (61%)	323 (81%)
R/W	127 (34%)	47 (36%)	72 (18%)
W/W	14 (3.8%)	4 (3.0%)	5 (1.3%)
OR for the phenotypic positivity of the minor (W) allele	OR = 2.6, 95% CI 1.8–3.5	OR = 2.7, 95% CI 1.8–4.2	
Position +2740			
A/A	166 (45%)	53 (41%)	231 (58%)
A/G	175 (47%)	63 (48%)	145 (37%)
G/G	31 (8.3%)	14 (11%)	21 (5.3%)
OR for the phenotypic positivity of the minor (G) allele	OR = 1.7, 95% CI 1.3–2.3	OR = 2.0, 95% CI 1.4–3.0	

Odds ratio (OR) is calculated as a comparison of the phenotypic frequency of the minor allele in subjects with the condition to the phenotypic frequency in the controls.

Table 1 for Czech patients with T1D, Czech patients with JIA, and healthy controls. The controls were first examined for the Hardy-Weinberg equilibrium and neither of the polymorphisms significantly deviated ($P_{corrected} > 0.30$ in exact tests). Then we assessed the linkage disequilibrium between the polymorphisms. There was a strong disequilibrium among the SNPs, the Lewontine's $D' = 1.0$ for all, and r^2 values being 0.89 for −1123 C/G to +2740 A/G, 0.42 for −1123 C/G to R620W, and 0.38 for +2740 A/G to R620W.

Minor alleles of all three polymorphisms were significantly associated with T1D in the Czechs, the association being strongest for the allele W at the codon 620 polymorphism, OR = 2.6, 95% CI 1.8–3.5. The analysis of estimated haplotype frequencies (Table 3) showed that T1D was associated only with the haplotype containing the W620 allele (OR = 2.26, 95% CI 1.68–3.02 relative to the haplotype consisting of three wild-type alleles). The haplotypes containing minor alleles at the promoter and/or 3′-UTR SNP together with the wild-type R620 allele were neutral as to the T1D risk. The association did not differ among genders.

The adjusted analysis in the Czech dataset was performed with all 372 cases, and a subset of 297 control subjects for whom the genotypes were previously determined for HLA-DQB1, –DQA1 and –DRB1, for insulin gene, NEUROD1 and CTLA4. The adjusted odds ratio was not significantly different from the unadjusted one, OR = 3.3, 95% CI 2.1–5.3. There was no statistically significant interaction between PTPN22 W620 and either of the other genetic factors.

3.2. JIA association in the Czechs

Minor alleles of all three polymorphisms were significantly associated also with JIA (Table 1), the OR for the W620 was 2.6, 95% CI 1.8–3.5. As in T1D, only the haplotype containing the W620 allele was associated (OR = 2.43, 95% CI 1.66–3.56), Table 3. The three subgroups of JIA (oligoarticular, polyarticular and systemic) did not significantly differ in the magnitude of association to the W620 allele, the odds ratio being 2.7, 2.7 and 2.8, respectively. An analysis of the PTPN22 association with JIA adjusted for the previously studied IL-4 −1098 T/G did not reveal a significant interaction.

3.3. T1D association in Azeri

The genotype frequencies of the three polymorphisms are listed in Table 2. All three polymorphisms in controls followed the Hardy-Weinberg equilibrium ($P_{corrected} > 0.10$ in exact tests). The −1123 C/G and +2740 A/G were in a linkage disequlibrium (Lewontine $D' = 0.99$, $r^2 = 0.72$, $p < 10^{-3}$), while the linkage disequilibria of the two polymorphisms to the R620W were absent, $D' = 0.14$ and 0.16, $r^2 = 0.0$, $p > 0.9$ for both.

Association with T1D was observed only in the R620W polymorphism, where the minor (W) allele was carried by 8 (5%) cases but only 2 (0.74%) controls, OR = 7.1, 95% CI 1.5–34 for phenotypic positivity of the W allele. Neither allele of the two remaining polymorphisms was associated with diabetes.

Table 2
Association of PTPN22 polymorphisms with T1D in the Azeri

PTPN22 genotype	Azeri subjects with T1D ($n = 160$)	Azeri control subjects ($n = 271$)
Position −1123		
G/G	106 (66%)	164 (61%)
G/C	47 (29%)	101 (37%)
C/C	7 (4.4%)	6 (2.2%)
OR for the phenotypic positivity of the minor (C) allele	OR = 0.78, 95% CI 0.52–1.2	
Amino acid 620		
R/R	152 (95%)	269 (99%)
R/W	7 (4.4%)	2 (0.74%)
W/W	1 (0.63%)	0
OR for the phenotypic positivity of the minor (W) allele	OR = 7.1, 95% CI 1.5–34	
Position +2740		
A/A	93 (54%)	140 (52%)
A/G	55 (35%)	119 (44%)
G/G	12 (7.5%)	12 (4.4%)
OR for the phenotypic positivity of the minor (G) allele	OR = 0.77, 95% CI 0.52–1.1	

Odds ratio (OR) is calculated as a comparison of the phenotypic frequency of the minor allele in subjects with the condition to the phenotypic frequency in the controls.

Table 3
PTPN22 haplotypes and their association with the autoimmune condition in the three datasets

PTPN22 haplotypes	T1D in Czechs			JIA in Czechs			T1D in Azeri		
	% cases (2n = 744)	% controls[a] (2n = 800)	OR [95% CI]	% cases (2n = 260)	% controls[a] (2n = 800)	OR [95% CI]	% cases (2n = 320)	% controls (2n = 542)	OR [95% CI]
−1123G R620 +2740A[b]	68.0	76.5	1.0, reference	65.0	76.5	1.0, reference	75.3	73.2	1.0, reference
−1123G R620 + 2740G	2.0	2.4	0.95 [0.48–1.90]	2.3	2.4	1.14 [0.45–2.91]	5.6	5.7	0.96 [0.52–1.75]
−1123G W620 + 2740A	0.1	0		0	0		0	0.2	
−1123G W620 + 2740G	0.1	0		0	0		0	0	
−1123C R620 + 2740A	0	0		0	0		0	0.2	
−1123C R620 + 2740G	9.1	10.9	0.95 [0.67–1.33]	11.5	10.9	1.25 [0.80–1.96]	16.3	20.5	0.77 [0.54–1.11]
−1123C W620 + 2740A	0	0		0	0		0	0	
−1123C W620 + 2740G	20.6	10.3	2.26 [1.68–3.02]	21.2	10.3	2.43 [1.66–3.56]	2.8	0.2	14.8 [2.0–651]

[a] The same control dataset is used for both analyses in the Czech population.
[b] The most frequent haplotype was used as a reference (baseline) for OR calculations.

The best fitted model of the haplotype frequency estimation is shown in Table 3, last three columns. As in the Czech population, the haplotype consisting of three wild-type (major) alleles is taken as reference. The risk of T1D is associated only with the haplotype carrying the W620 allele, while the haplotypes consisting of the wild-type R620 allele plus the minor alleles at positions −1123 and/or +2740 are neutral with respect to T1D risk. As there were only 10/431 individuals with the W620 allele in the Azeri dataset, neither the analysis adjusted for other genetic factors, nor gender-specific analyses were performed.

4. Discussion

The present study indicates a rather strong positive association of the W620 variant of *PTPN22* with T1D in a Czech and an Azeri populations, and with JIA in a Czech population, but no independent association of the −1123 G/C or +2740 A/G polymorphisms with the conditions. This is in contrast to findings on T1D reported by Kawasaki et al. [6] where the −1123 polymorphism was shown to associate with acute-onset T1D in a Japanese population, and with T1D in a Caucasian dataset from the BDA Warren repository. What the authors detected was an excess of heterozygotes among Japanese cases with acute-onset T1D and an insignificant increase of heterozygotes among Korean subjects with T1D, while there was actually no

difference in the overall phenotypic or allelic frequency between cases and controls. The authors interpret this as a case of molecular heterosis that is increasingly recognized in promoter polymorphisms. In the present report, no similar effect was observed, which however does not exclude the possibility of its presence in the Asian populations where it is not masked by the strong association of the W620 allele. Nevertheless, the analysis by Kawasaki et al. of a sample set from the British Diabetes Association Warren Repository (472 Caucasian individuals from 95 families) is not easy to follow. The authors concluded that the transmission distortion was stronger for the −1123 polymorphism, but the AFBAC point estimates for −1123G and +1858T are identical (both OR = 1.46), and for the classical TDT, only χ^2 and P values are presented. It is conceivable that a relatively frequent variant (−1123G) can give a lower P-value than a more rare variant (W620) solely because the TDT uses only heterozygous parents. The transmission distortion P-value may reflect rather the size of the sample set, than a true difference in an effect magnitude. In our view, the authors should have presented more details to support their notion of causality based on TDT performed in a population where a strong linkage disequilibrium between the tested variants may be assumed.

Here we present the *PTPN22* genetic association in two distinct populations of Caucasian origin, the Czechs living in Central Europe, and the Azeri, one of the

eastern-most Caucasian populations, living at the Caspian sea. The two populations differ significantly in the repertoire of the *PTPN22* haplotypes (Table 3). Although in both general populations about one-fifth of the haplotypes carry the minor allele at position −1123 together with the minor allele at position +2740, the proportion of R620W variants on these haplotypes varies. The R620 and W620 variants are equally frequent on the haplotype in healthy Czechs, but R620 prevails in healthy Azeri. Despite of this difference, in both populations only the haplotype carrying the W620 allele is associated with T1D. The haplotypes carrying R620 are neutral as to the T1D risk, regardless of whether they carry the minor allele at position +2740, or both at −1123 and at +2740. This clearly shows that neither the promoter, nor the 3′-UTR variant are causal in these sample sets. In Azeri – where W620 is so rare – this is mirrored also by neutrality of the −1123 and +2740 polymorphisms if analysed alone, independent of their haplotype. In the Czechs, however, the association of both −1123C and +2740G alleles is apparent, being secondary due to a linkage disequilibrium with W620.

The detected associations of the W620 variant with T1D and JIA are rather strong. The magnitude of the association with T1D in the Czechs is similar or insignificantly higher than what has been found elsewhere [1]. In the Czech dataset, no significant interactions with other genetic factors known to associate with T1D, nor any gender-specific effects were detected. This is in contrast to the report from the neighboring German population, where a strong association in females accounted for the whole effect of PTPN22 R620W on T1D risk owing to a difference in the genotype distribution among male and female diabetic subjects [10]. As we cannot detect any tendency towards a similar phenomenon in our genetically related population, the relatively small sample becomes one of the plausible explanations among those that the German authors propose.

Although the results of the JIA association studies may be influenced by the enormous heterogeneity of the condition, it seems that there is a genuine difference among populations in the magnitude of this association. In a large Finnish sample set, no association was found [11], while in a Norwegian and a UK populations *PTPN22* W620 was associated with an OR of 1.41 and 1.53, respectively. In our sample set, the overall association was somehow stronger (OR = 2.35, 95% CI 1.61–3.42 for the W620 allele), but our limited sample size does not allow us to draw any conclusion from this difference. Interestingly, the

systemic form of JIA showed no association in the UK sample set, while in the Czechs the condition was associated across the subtypes. The results of analysis stratified by the rheumatoid factor status are not available due to an insufficient number of positive individuals.

In conclusion, we report a haplotype analysis indicating that neither the −1123 G/C, nor the +2740 A/G polymorphisms are independently associated with the risk of T1D or JIA. The study also confers further evidence on the role of the *PTPN22* R620W polymorphism in determining the risk of T1D and of JIA.

Acknowledgements

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Příloha XI.

Sumnik Z, Cinek O, Bratanic N, Kordonouri O, Kulich M, Roszai B, Arato A, Lebl J, Soltesz G, Danne T, Battelino T, Schober E.

Risk of celiac disease in children with type 1 diabetes is modified by positivity for HLA-DQB1*02-DQA1*05 and TNF -308A.

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Risk of Celiac Disease in Children With Type 1 Diabetes Is Modified by Positivity for *HLA-DQB1*02-DQA1*05* and *TNF −308A*

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OBJECTIVE — The overlap between genetic susceptibility to celiac disease (CD) and to type 1 diabetes is incomplete; therefore, some genetic polymorphisms may significantly modify the risk of CD in subjects with type 1 diabetes. This study aimed to investigate whether the susceptibility to CD in diabetic children is modified by positivity for *HLA-DQB1*02-DQA1*05* and *DQB1*0302-DQA1*03* and by alleles of single nucleotide polymorphisms within the genes encoding *CTLA4*, transforming growth factor (*TGF*)-*β*, tumor necrosis factor (*TNF*)-*α*, interferon (*IFN*)-*γ*, interleukin (*IL*)-*1*, *IL-2*, *IL-6*, and *IL-10*.

RESEARCH DESIGN AND METHODS — Genotypic data were compared between 130 case subjects (children with type 1 diabetes and CD diagnosed using endomysium antibodies) and 245 control subjects (children with type 1 diabetes only, optimally two per case, matched for center, age at type 1 diabetes onset, and type 1 diabetes duration). The subjects were recruited from 10 major European pediatric diabetes centers performing regular screening for CD. The polymorphisms were determined using PCR with sequence-specific primers, and the risk was assessed by building a step-up conditional logistic regression model using variables that were significantly associated with CD in the univariate analysis.

RESULTS — The best-fitted model showed that risk of CD is increased by presence of *HLA-DQB1*02-DQA1*05* (odds ratio 4.5 [95% CI 1.8–11], for homozygosity, and 2.0 [1.1–3.7], for a single dose) and also independently by *TNF −308A* (1.9 [1.1–3.2], for phenotypic positivity), whereas IL1-*α* −889T showed a weak negative association (0.6 [0.4–0.9]).

CONCLUSIONS — The results indicate that the risk of CD in children with type 1 diabetes is significantly modified both by the presence of *HLA-DQB1*02-DQA1*05* and by a variant of another gene within the major histocompatibility complex, the *TNF −308A*.

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Celiac disease (CD) is the second most prevalent autoimmune condition accompanying type 1 diabetes, after autoimmune thyroid disease. In Europe, CD is ~10 times more frequent in patients with type 1 diabetes than in the respective general populations (1). The reason for the association has not yet been fully elucidated. The environment of the ongoing diabetic autoimmunity may be a stimulant to the development of CD, a disease that possesses autoimmune features. Also, the genetic susceptibility to both conditions substantially overlaps: a certain degree of susceptibility to both diseases is associated with the HLA molecules *DQB1*0302-DQA1*03* and *DQB1*02-DQA1*05*, encoded either in *cis* or *trans*. The *CTLA4* polymorphisms have also been implicated, as well as several polymorphisms within the genes encoding for cytokines.

However, the overlap of the genetic susceptibility is incomplete. This is well demonstrated in the *HLA-DQ* region: the main determinant of CD risk is *HLA-DQB1*02-DQA1*05*, whereas for type 1 diabetes in populations of central and northern Europe, it is *HLA-DQB1*0302-DQA1*03*. There are also strong indications that certain variants of the tumor necrosis factor (*TNF*)-*α* gene, another gene residing within the major histocompatibility complex, are associated with CD independently of *HLA-DQ* (2–5), whereas in type 1 diabetes, the *TNF* association is secondary to that of *HLA* (6,7), although this opinion is not universally accepted (8,9). *CTLA4 +49A/G* is a marker associated with both diseases: type 1 diabetes (as well as several other autoimmune diseases) is associated with the G allele (rev. in 10), whereas for CD, the studies on the association have yielded conflicting results.

Thus, CD and type 1 diabetes differ in the strength or direction of genetic association with particular alleles of the same genes. Such a difference might be useful for determining the level of CD risk among patients with type 1 diabetes. This idea is appealing because genetic testing

Table 1—*Characteristics of case subjects (diabetic children with CD diagnosed using endomysial antibodies) and matched control subjects (diabetic children without CD)*

	Case subjects	Control subjects
n	130	245
The Czech Republic (Prague)	34	69
Austria (Vienna)	23	46
Slovenia (Ljubljana)	21	29
Hungary (Pecs and Budapest)	25	48
Germany (Berlin and Hannover)	18	35
Portugal (Lisbon)	5	9
Slovakia (Kosice)	4	9
M:F ratio (n)	72:58	134:111
Ethnicity (n)		
European Caucasian	128	243
Other (Asian)	2	2
Age at type 1 diabetes onset [median (interquartile range)]	4.6 (2.8–8.2)	4.6 (2.2–7.7)
Type 1 diabetes duration at study [median (interquartile range)]	7.3 (4.3–11)	6.9 (3.9–11)
Results of small bowel biopsy in case subjects		
Results available	114 (88%)	
Total or subtotal atrophy	97	
Increased intraepithelial lymphocytes	13	
Normal	4	
Results not available	16 (12%)	
Biopsy not performed–typical symptoms present	3	
Biopsy refused by parents	5	
Unsuccessful biopsy	2	
Biopsy not indicated by physician	6	

might introduce ways to better target the CD antibody screening, ultimately leading to more efficient early detection of the condition.

The hypothesis of an *HLA-DQB1*02-DQA1*05*–associated susceptibility to CD in children with type 1 diabetes has been tested in several previous single-center studies. Most of these studies suffer from a low statistical power to detect a possible effect. To overcome this difficulty, we set out to undertake a sufficiently powered international multicenter case-control study aimed to answer the question whether *HLA-DQB1*02-DQA1*05, HLA-DQB1*0302-DQA1*03, CTLA4 +49A/G* polymorphisms, and polymorphisms within selected cytokine genes modify the risk of CD among children with type 1 diabetes. The aim was to test DNA from all available children with both type 1 diabetes and CD (case subjects) and to compare their genotypes with closely matched children with type 1 diabetes but without CD (control subjects). We decided to match for the following: center because genetic background differs among populations, age at diabetes onset because it is influenced by the *HLA-DQ* genotype, and diabetes duration because the cumulative risk of CD rises within years after the diagnosis of type 1 diabetes.

RESEARCH DESIGN AND METHODS

We performed a case-control study where the case subjects were children with both CD and type 1 diabetes and the control subjects were children with type 1 diabetes only, matched to the case subjects by center, age at type 1 diabetes onset, and duration of diabetes. Optimally, two control subjects should have been identified for each case. Ten centers from seven countries participated in the study. All participating children should have manifested with type 1 diabetes by their 15th birthday, and case subjects should have manifested with CD by their 18th birthday. All available diabetic children with CD cared for

by the center were approached and asked to participate. The nature of the study was described to the child, parents, or guardian before a written consent was obtained, in accordance with the procedures approved by the ethics committee at the particular center. Basic clinical and laboratory data were collected retrospectively from the patient's records. Two matched control subjects were identified for each case. First, the case was assigned to a 5-year band of age at type 1 diabetes onset. Then, two control children were selected at the same center from the same age-at-onset band, one being the child manifesting with type 1 diabetes just before and the other just next to the case. If the selected control subjects were not available or did not agree to participate, the selection went further to the next eligible control subject.

The characteristics of subjects are given in Table 1. The participating centers recruited 130 eligible case subjects with both type 1 diabetes and CD. The case subjects had been identified by endomysium antibody screening that was positive on two separate occasions and confirmed by small bowel biopsy in most instances. The case subjects were matched to 245 control subjects (for single case subjects, two matched counterparts could not be found). The control subjects were deemed free of CD based on the annual endomysium antibody testing. The case and control subjects were European Caucasians except for four subjects of Asian origin (one control subject from Vienna, one case subject from Berlin, and one case and one control subject from Hannover). Several exceptions were allowed from the matching criteria because the center had no better control subjects available: 25 (10%) control subjects were selected from the neighboring lower or higher band of age at type 1 diabetes onset, and seven control subjects were selected although they had manifested with type 1 diabetes after their 15th birthday, with the maximum age at onset being 17 years and 1 month.

Either genetic material was obtained from the center's DNA archives or EDTA-anticoagulated blood was drawn from the child, sent by mail to the central laboratory, and processed using the QiaAmp Blood DNA extraction kits (Qiagen, Germany). All DNA samples were quantified using a real-time assay for the human albumin gene, and 83 of 375 (22%) samples, which had an insufficient total DNA amount, were pre-amplified using the

Genomi-Phi whole-genome amplification kit (Amersham) according to the manufacturer's instructions. Because all downstream assays were based on PCR with sequence-specific primers (PCR-SSP), the quantity of the DNA was equalized among all samples by diluting, using data from real-time quantification of the human albumin gene.

The genotypes of HLA-DQB1, -DQA1 were determined using an in-house assay based on PCR-SSP. The performance of the assays is controlled by an exchange of samples with the Tissue Typing Laboratory IKEM in Prague, a laboratory accredited by the European Federation of Immunogenetics. The assay for *DQA1* and *DQB1* typing includes 24 reactions distinguishing the *DQA1*01–06* alleles and the *DQB1*02, 0301, 0302, 0303, 0304, 0401, 0402, 0501, 0502, 0503, 0601, 0602, 0603,* and *0604–9* alleles. Full-typing data were necessary to determine the dose of *DQB1*02-DQA1*05* and *DQB1*0302* in the individual. The *CTLA4 +49A/G* was determined using a previously reported PCR-SSP technique (11). The cytokine single nucleotide polymorphism (SNP) PCR-SSP typing was performed using a protocol with the sequence-specific primers developed for the cytokine component of the 13th International Histocompatibility Workshop and Conference (IHWC) (12). The laboratory was controlled within the 13th IHWC proficiency testing and achieved the accuracy of 98%. Seventeen SNPs were tested within 10 different genes for cytokines (interleukin [IL]-1α −889 T/C; IL-1β −511 C/T and +3962 C/T; IL-2 −330 T/G and +160 G/T; IL-6 −174 G/C and +565 G/A; IL-10 haplotypes of three SNPs at −1082, −819, and −590, ACC/ GCC/ATA; TNF −308 A/G and −238 G/A; TGFβ1 codon 10 C/T and 25 G/C; IFN-γ UTR 5644 A/T) and for IL-1–related molecules (IL-1R 1970 C/T, IL-1RA 11100 T/C). The design of the sequence-specific primers also allowed us to directly determine the phase of SNPs in haplotypes of TGF-β1, TNF, IL-2, IL-6, and IL-10.

The association of the variants with CD was first tested by conditional logistic regression for matched data. The outcome variable identified whether the individual was a case subject (patient with type 1 diabetes and CD, encoded as 1) or a control subject (patient with type 1 diabetes only, encoded as 0). The identifier of the matching group was used as the conditioning variable. The independent variables were the phenotype positivity

for the examined alleles or haplotypes in a univariate model. Then, the variables achieving a P value of the Wald statistics at <0.05 in the univariate analysis were used for step-up building of a conditional logistic regression model. The best-fitted model was determined using the likelihood ratio test statistics. All models were—apart from conditioning for the matching identifier—adjusted for the matching variables. Analyses were performed using the Stata 9.0 program (StataCorp, College Station, TX). The sample size provided an 80% power at $\alpha = 0.05$ to detect an effect size with an odds ratio (OR) of ≥ 1.83 for a genetic factor present in 50% of the control subjects (e.g., the HLA-DQB1*02-DQA1*05): this OR corresponds to a $\geq 65\%$ prevalence in case subjects. Similarly, the study had an 80% power to detect an OR >1.86 for a genetic factor with a 30% prevalence among control subjects or OR >2.07 for a genetic factor with a 70% prevalence.

RESULTS — The results of the univariate analysis of the case-control status versus phenotypic positivity of the studied factors are shown in Table 2. HLA-DQB1*02-DQA1*05 was found in 77% of the case subjects and 53% of the control subjects, being strongly associated with CD among type 1 diabetic subjects (P < 10^{-3}). The effect of DQB1*02-DQA1*05 is adequately described by a codominant model taking the gene dose into account; there is a proportional rise in risk with gene dose. HLA-DQB1*0302-DQA1*03 shows a weak negative association with CD. Among the cytokine genes, TNF −308A is strongly associated with CD (P < 10^{-3}), whereas the weak negative association of IL1α −889 disappears after correction for multiple testing.

The best-fitted regression model is shown in Table 3. Although HLA-DQB1*0302-DQA1*03 completely lost its effect after being adjusted for HLA-DQB1*02-DQA1*05, both cytokine SNP alleles retained the association observed in the univariate analysis. Homozygosity for HLA-DQB1*02-DQA1*05 increased the risk of CD roughly fourfold, whereas the presence of a single dose of DQB1*02-DQA1*05 doubled the risk relative to individuals carrying no HLA-DQB1*02-DQA1*05. In the final model, both HLA-DQB1*02-DQA1*05 and TNF −308A retained the statistical significance, indicating an independent effect: the risk was roughly doubled also by the presence of the −308A variant of the TNF. While the

association of HLA-DQB1*02-DQA1*05 was dose dependent, such an effect could neither be confirmed nor refuted for the TNF −308A dose. IL-1α −889T was associated with a marginal decrease in CD risk. No interaction terms between the variables could further improve the fit.

The results did not change after exclusion of four subjects having other than European Caucasian descent (e.g., the OR for a double dose of DQB1*02-DQA1*05 was 4.45 [95% CI 1.75–11.3], in the whole dataset vs. 4.27 [1.67–10.9], after exclusion of the subjects of non-European descent). Including sex into the analysis as one of the predictors neither improved the fit nor changed the results at the first two decimal positions of the risk estimate.

CONCLUSIONS — The present study clearly demonstrates that factors inside HLA significantly modify the risk of CD in children with type 1 diabetes. The HLA-DQB1*02-DQA1*05 increases the risk in a dose-dependent manner, with a significant contribution of TNF −308A.

The observed effect of HLA-DQB1*02-DQA1*05 may have been expected in populations where DQB1*02-DQA1*05 confers a lower risk of type 1 diabetes than CD but has seldom been documented. The first study to indicate that DQB1*02 is overrepresented among diabetic children with CD was published 10 years ago by Saukkonen et al. (13), based on a longitudinal observation of 775 diabetic children that identified 18 case subjects of CD diagnosed after type 1 diabetes onset. A later Czech study suggested that HLA-DQB1*0201-DQA1*05 is associated with a fourfold increased risk of CD in children with type 1 diabetes (14), based on data from 15 children with CD identified from a cohort of 345 diabetic children, of whom 186 were genotyped. However, the CI of the risk estimate was wide. The association was not replicated in an Italian study of 25 children with CD and type 1 diabetes compared with 79 children with type 1 diabetes only (15): the DQB1*0201-DQA1*05 frequency was 68% in the former group and 62% in the latter group. The authors concluded that the previously seen association may have been population specific, which is likely particularly for the Italian population where DQB1*0201-DQA1*05 is frequent among diabetic subjects. Another study comes from Australia (16): an increased frequency of HLA-DQB1*0201-DQA1*05

Table 2—Univariate analysis of genetic factors tested for association with CD in children with diabetes

	Case subjects (%)	Control subjects (%)	OR (95%CI)	P
n	130	245		
*HLA-DQB1*02-DQA1*05*				
Dominant model*	77	53	2.97 (1.78–4.97)	<0.001
Codominant model†				
Two doses of *DQB1*02-DQA1*05*	17	7	5.94 (2.54–14)	<0.001
Single dose of *DQB1*02-DQA1*05*	60	46	2.53 (1.48–4.34)	0.001
No *DQB1*02-DQA1*05*	23	47	1.0: reference	
*HLA-DQB1*0302-DQA1*03*	52	65	0.58 (0.36–0.93)	0.024
Cytokines and related molecules				
IL-1α −889 T/C				
T	38	49	0.64 (0.42–0.98)	0.041
C	94	90	1.69 (0.72–3.93)	0.23
IL-1β				
−511 C	88	91	0.84 (0.42–1.69)	0.64
−511 T	59	56	1.12 (0.72–1.74)	0.61
+3962 C	98	97	1.84 (0.47–7.2)	0.38
+3962 T	41	45	0.82 (0.54–1.26)	0.37
IL-1R 1970 C/T				
C	88	87	1.15 (0.58–2.30)	0.69
T	59	55	1.18 (0.76–1.87)	0.46
IL-1RA 11100 T/C				
T	89	93	0.59 (0.27–1.31)	0.20
C	53	59	0.81 (0.52–1.26)	0.36
IFN-γ UTR 5644 A/T				
A	84	82	1.19 (0.68–2.10)	0.54
T	72	72	1.00 (0.61–1.63)	1.0
TGF-β1				
Codon 10 C	68	60	1.35 (0.87–2.11)	0.12
Codon 10 T	81	84	0.77 (0.43–1.37)	0.37
Codon 25 G	100	99		0.99
Codon 25 C	12	15	0.75 (0.38–1.46)	0.40
TNF-α				
−308 G	90	93	0.59 (0.26–1.36)	0.22
−308 A	68	45	2.7 (1.7–4.31)	<0.001
−238 G	100	100		0.99
−238 A	5	10	0.44 (0.18–1.1)	0.080
IL-2				
−330 T	95	95	1.01 (0.34–2.7)	0.98
−330 G	52	47	1.22 (0.78–1.91)	0.38
+160 G	85	83	1.36 (0.74–2.48)	0.32
+160 T	54	63	0.67 (0.43–1.03)	0.068
IL-6				
−174 G	79	82	0.84 (0.5–1.44)	0.54
−174 C	71	70	1.2 (0.73–1.99)	0.46
nt565 G	82	86	0.76 (0.44–1.33)	0.34
nt565 A	67	68	1.05 (0.65–1.71)	0.83
IL-10 haplotype				
ACC	59	50	1.52 (0.98–2.34)	0.064
ATA	47	51	0.77 (0.49–1.19)	0.24
GCC	61	64	0.98 (0.68–1.52)	0.94
CTLA4				
+49A	86	80	1.44 (0.81–2.57)	0.21
+49G	59	64	0.9 (0.59–1.38)	0.64

The ORs come from conditional logistic regression models where the outcome variable was the presence of CD in the diabetic child (i.e., whether the individual is a case = 1 versus a control = 0). The conditioning variable was the identifier of the matched set (matching stratum). The model was adjusted for the matching variables of age at diabetes onset and duration of diabetes. The presented *P* values are not corrected for multiple testing. *In the dominant model, the predictor variable was 1 when any *DQB1*02-DQA1*05* was present (irrespective of dose) and 0 when no *DQB1*02-DQA1*05* was present. †In the codominant model, two indicator variables compare the levels of risk associated with the double dose and the single dose of *HLA-DQB1*02-DQA1*05*, relative to its absence.

Table 3—*The best-fit multiple regression model for risk of CD in children with type 1 diabetes*

Genetic factor	OR (95%CI)	Wald *P*
*HLA-DQB1*02-DQA1*05*		
Double dose*	4.45 (1.75–11.3)	0.002
Single dose*	2.04 (1.11–3.76)	0.022
TNF-α −308A	1.87 (1.10–3.20)	0.021
IL-1α −889T	0.58 (0.37–0.93)	0.023

The variables associated with CD in the univariate model were used to build a step-up multiple model. The predictor variables included two indicator variables showing a double and single dose of *HLA-DQB1*02-DQA1*05* and dichotomous variables showing the phenotypic positivity for a given allele of a cytokine SNP. The adequacy of inclusion of predictor variables into the model was tested using the log-likelihood ratio test. No interaction terms improved the model. The effect of *HLA-DQB1*0302-DQA1*03* observed in the univariate analysis was lost after adjusting for *HLA-DQB1*02-DQA1*05*. *Two indicator variables compare the levels of risk relative to an absence of *HLA-DQB1*02-DQA1*05*.

was seen in diabetic subjects with CD (10/13, 77%) compared with subjects with type 1 diabetes but not CD (70/118, 59%), but this difference was not statistically significant. A clear limitation to these studies is the low number of identified case subjects (diabetic children also having CD): a single-center study therefore has a very substantial risk of type II error, i.e., not refuting the false null hypothesis due to low statistical power. Negative results reported by some of the studies should therefore be interpreted with caution. Two articles were published by a group from Denver, Colorado, who used positivity for the transglutaminase antibody as an end point, thus increasing the number of case subjects: the studies indicate a significant contribution of genetic factors to CD risk in diabetic individuals, since an increased prevalence of transglutaminase antibodies was reported in diabetic children carrying *DQB1*02-DQA1*05* or being *DQB1*02-DQA1*05* homozygous (17,18). Possible weak points of these two reports are that the antibodies were measured only one time and that analysis on ethnicity is not reported in a population that is likely to contain ethnic minorities.

In the present report, we show a significant effect of *HLA-DQB1*02-DQA1*05* positivity that is probably a compound of the effects of *DQB1*02-DQA1*05* itself and other genetic factors carried on the *DQB1*02-DQA1*05* haplotypes, namely the *TNF −308A*. Whereas the pathogenic effect of *DQB1*02-DQA1*05* in CD is well established and the association is primary, the association of the *TNF −308A* may be due either to its functional significance as a primary risk modifier or to a linkage disequilibrium to another variant on the haplotype. The former alternative is plausible for TNF-α,

a potent proinflammatory cytokine involved in T-cell immunity. However, the data on the functional significance of its polymorphisms are conflicting (rev. in 19), and the *TNF −308A* may be just a passive marker traveling on a proinflammatory haplotype. The *TNF-α* gene lies between HLA class II and class I in what is termed the class III region. *TNF −308A* is known to be associated with the proinflammatory 8.1 ancestral haplotype, so other components of the haplotype may significantly contribute as well. There are many genes with known inflammatory roles in the close vicinity of TNF-α: lymphotoxin α, the heat-shock protein-70 complex, the complement components 4A and 4B, and others.

Although the *TNF −308A* remains associated when adjusted for *DQB1*02-DQA1*05* positivity, this fact does not allow us to deem the genetic association truly independent. Adjusting or matching only for *DQB1*02-DQA1*05* phenotypic positivity is not enough, because we do not control the other chromosome in heterozygotes carrying one dose of *DQB1*02-DQA1*05* (20). An alternative approach to dissecting the association may be to analyze only subjects positive for a fixed *HLA-DQ* genotype, e.g., the frequent *HLA-DQB1*02-DQA1*05/DQB1*0302-DQA1*03*. However, the power of this type of analysis of our dataset is low, because there are only 23 matching groups where a case and at least one control are positive for this genotype. In this small subset, we observed indications that the *TNF −308A* is associated with an increased risk of CD (OR 2.4 [95% CI 0.63–9.1], *P* = 0.20).

An optimal design for a future study verifying the independence of the *TNF −308A* effect in determining the CD risk among diabetic individuals would involve matching for the primarily associ-

ated *DQB1*02-DQA1*05* on both chromosomes. As seen in Table 2, there are not many *DQB1*02-DQA1*05* homozygotes, only 17% among the case subjects and 7% among the control subjects. Thus, for such a relatively rare combination of diseases, it would be especially difficult to recruit a sufficient number of *HLA-DQB1*02-DQA1*05* homozygous diabetic individuals with CD and to match them adequately with control subjects with type 1 diabetes but without CD.

The present study was the first to use matching for age at diabetes onset and duration of diabetes. This is because the age at onset is known to be influenced by the child's HLA genotype and because the cumulative incidence of CD rises with duration of exposure to type 1 diabetes. This careful matching, together with matching for study center, should eliminate possible bias due to the recent shift toward a younger age at type 1 diabetes onset observed in some populations. Another important reason for matching is the heterogeneity in the CD prevalence in diabetic children: the prevalences previously reported from populations in this study range from 3 to 12%, moreover with significant variations over time (14,21–25). Consequently, reliable estimations of absolute risks attributable to particular genotypes are impossible, although knowledge of them might be of interest for the clinician.

An important source of technical error was eliminated by equalizing the DNA content across the samples using real-time quantification. All the genotyping reactions we used were based on PCR with sequence-specific primers, a technique known to be sensitive to uneven DNA content. The adequacy of this approach is well documented by the high success rate in IHWC proficiency testing.

In conclusion, at least in some European populations, the risk of CD in children with type 1 diabetes is substantially modified by the child's genotype, with a contribution from both *HLA-DQB1*02-DQA1*05* and the *TNF −308A* variant. Although there had been previous indications that children with diabetes differ in their risk of CD based on their HLA genotype, our study has conferred the strongest evidence so far, having collected the largest adequately matched case-control dataset. Although an immediate transition of the knowledge into clinical use is unlikely, in the future, genetic testing may be useful for stratifying the frequency of

testing diabetic children for antibodies indicative of CD.

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Příloha XII.

Cinek O, Sumnik Z, Vavrinec J.

Continuing increase in incidence of childhood-onset type 1 diabetes in the Czech Republic 1990-2001.

RESEARCH LETTER

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Continuing increase in incidence of childhood-onset type 1 diabetes in the Czech Republic 1990–2001

Using a prospective (1990–2001) population-based registration of new cases of type 1 diabetes among children under the age of 15 years, we demonstrate a continuing increase in incidence, markedly shown in the lowest age-at-onset category.

Incidence rates of type 1 diabetes are known to rise significantly in most European populations. While this trend is modest or absent in populations of Scandinavia (Denmark, Norway, Sweden), it is markedly expressed in the countries of Eastern and Central Europe [3, 4, 5]. Here we report the type 1 diabetes incidence rates and their trends for the Czech Republic, 1990–2001.

Cases of childhood-onset type 1 diabetes (0–14 years of age) were registered over the period 1990–2001 by the population-based Czech Childhood Diabetes Register according to EURODIAB criteria [3]. Ascertainment rates were calculated by a capture-recapture method using the Association of Parents and Friends of Diabetic Children as the secondary data source. The ascertainment rate was calculated to be 97% for the primary data source, 76% for the secondary, and 99% for both data sources combined. Population data were taken from annual reports of the Czech Statistics Bureau. Apart from calculating crude incidence rates, the rates were also standardised on a virtual population with equal numbers of individuals in the age groups 0–4, 5–9 and 10–14 years. Trends in incidence were estimated using Poisson regression, sex differences were tested using heterogeneity tests, and cyclic trends of seasonality of onset were investigated according to Edwards [2].

A total of 2,644 cases were registered by the Czech Childhood Diabetes Register throughout the period 1990–2001. The average standardised incidence rate was 11.4/100,000/year. The rates did not significantly differ between sexes. The crude and adjusted incidence rates are shown in Table 1. The incidence rates in the age-at-onset groups were 7.6/100,000/year in 0–4 completed years at diagnosis, 12.0/100,000/year in 5–9, and 14.6/100,000/year in 10–14. Seasonality of onset was most apparent in children with diabetes onset at 10–14 years of age, with high numbers of new cases in September to November, and a trough in July and August ($P < 10^{-4}$).

A significant increase in incidence was seen in all categories of age at onset. The yearly increase in standardised incidence was 6.3% ($P < 10^{-3}$); the yearly age-specific increases were 10.3% ($P < 10^{-3}$), 6.0% ($P < 10^{-3}$), and 4.4% ($P = 0.009$) for the categories 0–4, 5–9, and 10–14, respectively. The absolute average yearly increase in incidence was, however, very similar in all age-at-onset categories (+0.73, +0.70, +0.79/100,000/year for the three categories). Consequently, the relative difference in incidence between the three age-at-onset bands has weakened over time: this is demonstrated in Fig. 1 where age-specific incidence rates are compared between the first and the second half of the observation period, i.e. between 1990–1995 and 1996–2001.

A significant increase in type 1 diabetes incidence in Czech children has been already demonstrated for the time period 1990–1997 [1]. The present data show that the incidence rate continued to rise for further 4 years, with a similar absolute increase in all three age-at-onset categories. The variations in diabetes incidence between populations are partly attributable to differences in genetic background of the population [8], and are linked to several indicators of national prosperity [7] as proxy measures of yet unknown factors. The explanations for the observed increase in incidence still remain rather speculative. The increase may be, for instance, partly attributed to the rise in the body mass index of Czech children [6], according to the 'accelerator hypothesis' which deems insulin resistance resulting from weight gain to be one of the leading causes of rising incidence of

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Table 1 Incidence of type 1 diabetes in Czech children 0–14 years of age, 1990–2001

Year	Number of cases	Population size	Crude incidence rate	CI 95%	Standardised incidence rate	CI 95%
1990	189	2,193,682	8.6	7.4–9.9	8.3	7.2–9.6
1991	195	2,120,802	9.2	7.9–10.5	9.0	7.7–10.3
1992	197	2,064,545	9.5	8.3–10.9	9.3	8.0–10.7
1993	195	2,009,752	9.7	8.4–11.1	9.5	8.2–10.9
1994	210	1,948,024	10.8	9.4–12.3	10.6	9.2–12.0
1995	187	1,893,259	9.9	8.5–11.3	9.7	8.3–11.1
1996	234	1,842,679	12.7	11.1–14.4	12.3	10.7–13.9
1997	211	1,795,032	11.8	10.2–13.4	11.6	10.1–13.3
1998	228	1,751,471	13.0	11.4–14.8	12.6	11.0–14.4
1999	265	1,707,205	15.5	13.7–17.4	15.2	13.4–17.1
2000	257	1,664,434	15.4	13.6–17.4	15.3	13.5–17.2
2001	276	1,631,771	16.9	15.0–19.0	16.5	14.5–18.5
1990–2001	2,644	22,622,656	11.7	11.2–12.1	11.4	11.0–11.9
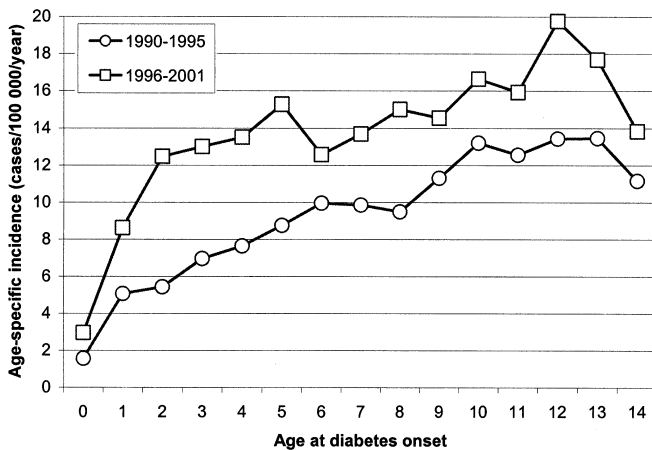
Fig. 1 Comparison of the age-specific incidence rates in 1990–1995 and in 1996–2001

both types of diabetes [9]. However, this hypothesis alone cannot explain why type 1 diabetes incidence increases so rapidly, especially in populations of Central and Eastern Europe. Continuous registering of new cases of childhood diabetes, and international comparison of the incidence rates and their trends may therefore yield some further clues towards understanding diabetes aetiology.

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Příloha XIII.

Malcova H, Sumnik Z, Drevinek P, Venhacova J, Lebl J, Cinek O.

Absence of breast-feeding is associated with the risk of type 1 diabetes: a case-control study in a population with rapidly increasing incidence.

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Absence of breast-feeding is associated with the risk of type 1 diabetes: a case–control study in a population with rapidly increasing incidence

Abstract There are indications that the effect of environmental factors on the risk of type 1 diabetes mellitus (T1DM) is increasing over time. This can be documented by the rapid increase of T1DM incidence in genetically stable populations. Our aim was to study an association of T1DM with the variable factors of the perinatal period and of early infancy, using data from children born over a period of changing exposure to some of the studied factors. A case–control dataset was analysed, consisting of 868 diabetic children and 1,466 anonymous controls, mostly schoolmates of the children with T1DM. The data were collected using structured questionnaires completed by parents. After performing univariate analyses, the associations were analysed using multiple logistic regression adjusted for potential confounders, including the year of birth. The risk of T1DM decreased with increasing duration of breast-feeding, while no breast-feeding was associated with an increased T1DM risk, OR=1.93 [95% CI: 1.33–2.80], breast-feeding for more than 12 months was protective, OR=0.42 [95% CI: 0.22–0.81], both being relative to the reference category of breast-feeding for 1–3 months. A short duration of day-care attendance (none or less than 1 year) was weakly associated with the risk of T1DM, OR=1.65 [95% CI: 1.05–2.62]. No association was detected between T1DM and signs of prenatal infections, perinatal stress factors, birth size and weight, indicators of crowding or the presence of a domestic pet in the household. Short breast-feeding, and short attendance to day-care is associated with risk of T1DM in Czech children.

Keywords Diabetes mellitus · Type 1 · Epidemiology · Risk factors · Infant nutrition

Abbreviations 95% CI: 95% confidence interval · OR: Odds ratio · T1DM: Type 1 diabetes mellitus

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Introduction

Type 1 diabetes mellitus (T1DM) is a consequence of immune-mediated destruction of pancreatic β-cells in a genetically predisposed individual. Controversy still exists on which environmental factors—and to what extent—are responsible for the disease's susceptibility. The effect of environmental factors is probably increasing; most European populations have experienced a continuous increase in T1DM incidence despite their genetic stability [1, 4]. This increase has been most pronounced in the populations of Central and Eastern Europe. In these populations, the putative environmental factors started to act later or with different strengths compared to the Western societies. The delay makes these populations interesting for investigating environmental factors in T1DM pathogenesis. In a previous paper, we addressed the T1DM risk associated with maternal age at delivery and birth order under the conditions of a rapid fall in natality and a swift increase of average maternal age [19].

In the present work, we aimed to investigate the associations of T1DM with several parameters of pregnancy, of the perinatal period and of early infancy, namely with signs of infection in pregnancy, with term and mode of delivery, birth weight and length, with infant feeding,

attendance to day-care, size of the community and the presence of domestic pet animals in the household.

Subjects and methods

The study design was case–control. Retrospectively obtained questionnaire data on the putative T1DM risk factors were compared between children with T1DM (cases) and their non-diabetic peers (control subjects). The study was approved by the Ethics Committee at the Motol University Hospital, Charles University, Prague, Czech Republic.

Logistics of the study

The study was conducted by the network of the population-based Czech Childhood Diabetes Register. Overall, 51 local pediatric diabetology centres took part in the study, with no geographical predominance compared with those who did not take part. The last 1,000 diabetic patients recorded by the Diabetes Register, aged less than 15 years at the onset of the disease, and under the age of 18 years at the time of the start of the study (March 2000), were invited to participate by their pediatric diabetologists. In addition, the diabetologists were encouraged to include children who manifested with T1DM since the last annual report to the Diabetes Register. The control subjects (schoolmates of diabetic children, unrelated to the case, non-diabetic and of approximately the same age) were chosen and their parents were approached directly by the parents of the patients. According to the criteria explained in letters to the parents, optimally, three control subjects should have been contacted.

The questionnaire

The data were obtained using structured questionnaires identical for the children with T1DM and for the non-diabetic control subjects. The questionnaire was designed to evaluate the following variables: signs of infections during pregnancy (fever above 38°C, diarrhoea and rash, reported separately for each trimester), perinatal parameters (term of delivery, mode of delivery, birth weight and length, neonatal jaundice), infant feeding (duration of breast-feeding and time of introduction of formula or other supplementary feeding) and vitamin D supplementation. In children who already completed their fifth year of life, we also evaluated other putative factors of the pre-school period related to diabetes risk: day-care attendance, size of the community and the presence of pet animals in the household. Most questions had the option of "I do not remember/I do not want to tell," which was recorded as "unknown" in the analysis.

Statistical analysis

The distribution of the investigated factors was first evaluated by univariate analyses and expressed as odds ratios (OR) with their 95% confidence intervals, and the continuous variables were analysed using the analysis of variance. Logistic regression models were built for mutually related exposures or for exposures that change over time, with adjustment to the calendar year. Discriminant functional analysis was used for the identification of the more important regressor among the correlated nutrition variables of breast-feeding and the introduction of supplementary feeding.

Results

A total of 1,143 questionnaires were distributed to the parents of the children with T1DM, who, in turn, approached the parents of 2,012 control subjects. We received 868 completed questionnaires from the children with T1DM and 1,466 from the control subjects; the details are shown in Table 1.

The results of the analysis of signs attributable to infections during pregnancy, of perinatal parameters and of

Table 1 Characteristics of the study group

	Children with T1DM	Control subjects
Response		
Number of distributed questionnaires	1,143	2,012
Number of returned questionnaires	868	1,466
Response rate	76%	73%
Case:control ratio	1 :	1.69
Sex		
Male	434	670
Female	434	796
Age at study		
Median (25th; 75th percentile)	13 (10; 16)	12 (9; 15)
Distribution of age at T1DM onset		
0–4 years	201 (23%)	
5–9 years	346 (40%)	
10–14 years	321 (37%)	

infant feeding are presented in Table 2. The signs attributable to infections during pregnancy were not associated with T1DM, and this held true also after separate analysis of trimesters (data not shown).

None of the term of delivery, mode of delivery, birth weight and length, or neonatal jaundice was associated with a subsequent risk of T1DM. The proportion of caesarean sections substantially increased over the period when the studied children were born, from 5.9% deliveries in the first quartile to 11.4% in the last quartile of the period. The association was, therefore, investigated also with an adjustment for the calendar year of birth, birth

Table 2 Distribution of studied parameters in children with T1DM and control patients

	T1DM	%	Controls	%	OR [95% CI]	
Signs attributable to infections during pregnancy						
Fever >38°C	78/693	11	149/1244	12	0.93 [0.70–1.25]	
Diarrhoea	20/689	3	34/1201	3	1.03 [0.59–1.80]	
Skin rash	12/749	2	38/1308	3	0.54 [0.28–1.05]	
Perinatal parameters						
Term of delivery						
Preterm (<week 38)	83	10	126	9	1.11 [0.83–1.48]	
Full term (week 38–42)	741	87	1,245	86	1.00, reference	
Postdate (>week 42)	29	3	73	5	0.67 [0.43–1.04]	
Unknown	15		22			
Mode of delivery						
Spontaneous	737	87	1,278	89	1.00, reference	
Forceps	18	2	29	2	1.08 [0.59–1.95]	
Caesarean section	78	9	107	7	1.26 [0.93–1.72]	
Unknown	35		52			
Neonatal jaundice						
Present	345	43	627	46	0.91 [0.77–1.09]	
Absent	451	57	748	54	1.00, reference	
Unknown	72		91			
Birth length and weight, median (25th; 75th percentile)						
Length	50 (49; 52)		50 (49; 52)		N.S.	
Weight	3,350 (3,028; 3,700)		3,350 (3,045; 3,650)		N.S.	
Infant feeding	**T1DM**	**%**	**Controls**	**%**	**Unadjusted OR [95% CI][a]**	**Adjusted OR [95% CI][b]**
Total duration of breast-feeding in completed months						
No breast feeding	79	11	113	8	1.23 [0.89–1.70]	1.93 [1.33–2.80]
1–3 months	268	36	471	35	1.00, reference	1.00, reference
4–6 months	197	27	343	25	1.01 [0.80–1.27]	1.11 [0.82–1.50]
7–9 months	110	15	211	16	0.92 [0.70–1.21]	0.96 [0.65–1.41]
10–12 months	54	7	120	9	0.79 [0.55–1.13]	0.94 [0.57–1.56]
>12 months	30	4	88	7	0.60 [0.39–0.93]	0.42 [0.22–0.81]
Unknown	130		120			
Time at introduction of formula or other supplementary feeding					Unadjusted OR [95% CI][c]	Adjusted OR [95% CI][b]
Month 1–3	317	42	496	35	1.26 [1.03–1.53]	1.11 [0.83–1.50]
Month 4–6	298	40	587	42	1.00, reference	1.00, reference
Month 7–9	114	15	251	18	0.89 [0.69–1.16]	0.96 [0.69–1.34]
Month 10+	25	3	69	5	0.71 [0.44–1.15]	0.90 [0.49–1.67]
Unknown	114		63			
Vitamin D supplementation during the first year of life[d]					OR [95% CI]	
Yes	672	93	1,181	95	1.00, reference	
No	47	6.5	66	5.3	0.80 [0.54–1.18]	
Unknown	149		219			

[a]Chi-square test for trend $P=3.9\times10^{-3}$
[b]Duration of breast-feeding and time at introduction of formula feeding adjusted for each other, for the maternal age and birth order, and for the year of birth
[c]Chi-square test for trend $P=8.5\times10^{-4}$
[d]Data for duration of supplementation not presented: 62% of cases and 60% of controls report that they do not remember

Table 3 Analysis of putative risk factors operating in the pre-school age. Only children five years of age or older were analysed

	T1DM	%	Controls	%	Unadjusted OR [95% CI]	Adjusted OR [95% CI][a]
Domestic pet in the household						
	395/789	50	676/1,291	52	0.91 [0.76–1.09]	0.88 [0.72–1.08]
Size of the community (number of inhabitants)						
Less than 1,000	164	21	263	20	0.98 [0.76–1.25]	0.98 [0.75–1.28]
1,000–9,999	183	23	313	24	0.92 [0.72–1.25]	0.93 [0.72–1.20]
10,000–99,999	261	33	409	32	1.00, reference	1.00, reference
100,000+	173	22	299	23	0.91 [0.71–1.16]	0.88 [0.68–1.14]
Unknown	37		48			
Total duration of day-care attendance in completed years[b]					Unadjusted OR [95% CI]	Adjusted OR [95% CI][c]
None or <1 year	39	6	53	4	1.58 [1.03–2.44]	1.65 [1.05–2.62]
1–2 years	154	25	357	28	0.93 [0.74–1.16]	1.04 [0.82–1.33]
3–4 years	345	57	741	58	1.00, reference	1.00, reference
5+ years	70	12	129	10	1.17 [0.85–1.60]	0.94 [0.67–1.32]
Unknown	59		52			

[a]The size of the community and the presence of domestic pets in the household are adjusted for each other, for the birth order, maternal age and year of birth of the child

[b]The total duration of day-care attendance was analysed only in diabetic children who manifested with diabetes after their fifth birthday, as the diabetes status affects the ability to attend day-care centres

[c]Adjusted for the size of the community in which the child lived, for maternal age, birth order and the calendar year of birth

weight and length; none of the variables was significantly associated with T1DM (data not shown).

Univariate analysis of infant feeding showed that the risk of T1DM proportionally decreases both with increasing duration of breast-feeding and with the age when formula or other supplementary feeding was introduced. In a logistic regression model with the two variables and adjustment for the calendar year of birth, the duration of breast-feeding turned out as the more important regressor, while the association of T1DM with the time of introduction of supplementary feeding was found secondary to that of breast-feeding. Further discriminant functional analysis confirmed these results.

In children older than five years, we analysed proxy measures of social contacts, as well as contacts with domestic animals (Table 3). Of the studied parameters, only a short or negative history of day-care attendance (less than 1 year) is associated with T1DM, being more frequent in diabetic children (6%) than in the controls (4%), and remaining borderline-significant also in the model adjusted for calendar year of birth, birth order, maternal age and size of the community in which the child lived. The adjustment for the calendar year of birth was necessary because the duration of day-care significantly shortened over time: the proportion of children attending day-care for four or more years was 41% in children born before 1989, compared to 22% in those born later.

We analysed whether the variables associated with T1DM influences also the age at which it is diagnosed. The association was investigated using a model with dichotomised age at diabetes onset (below and over median) as the outcome, and the factors previously associated in the univariate analyses as the predictors. Neither of the variables influenced significantly the age at diabetes onset (data not shown).

Discussion

We performed a large case–control study on type 1 diabetes mellitus (T1DM) environmental risk factors, finding significant association with parameters of infant nutrition and with attendance to day-care.

Infant nutrition

The present study shows an association between the lack of breast-feeding and T1DM risk, while breast-feeding over 12 months is associated with protection. The parallel effect of the early introduction of supplementary feeding is secondary to the effect of breast-feeding. Such associations have been known for a long time; nevertheless, the question on which of the exposures is primary has not yet been fully answered (reviewed in [20]). The situation is further complicated by the putative effect of exposure to cereals, as indicated by results from the DAISY birth cohort [10]. However, such a variable cannot be tested in a retrospective case–control study without the risk of a substantial recall bias. Although case–control data like ours may confer important pieces of the whole picture of nutritional risk factors, the final answer has to be provided by the ongoing prospective interventional studies as in the international TRIGR project [6].

Prenatal and perinatal variables

Delivery by caesarean section has been reported to significantly increase the risk of T1DM in some [8, 12], but not other, studies [3, 9, 18]. In the present study, the proportion of caesarean sections doubled over the study pe-

riod. This change was parallel in children with T1DM and in controls, indicating that even the newly introduced indications for caesarean section probably do not include factors primarily associated with T1DM.

The present study, as did other case–control studies (e.g. [2, 13]), failed to show a difference in the birth size of diabetic children compared to the non-diabetic population. However, the lack of association is not universal; in a large multicentre EURODIAB study [3], low birth weight and short birth length were protective, and a Norwegian-population-based record linkage study showed an increase in T1DM risk with higher birth weight [17].

Limitations of the retrospective approach are seen in the analysis of neonatal jaundice. Although we had collected data on the occurrence of neonatal jaundice, most parents cannot recollect whether phototherapy was instituted or not. The same is true about vitamin D supplementation: most parents remember having given this supplementation but only 40% can recollect for how long it was given.

Day-care attendance and other proxy measures of exposure to infectious agents

The concept of the hygiene hypothesis suggests that an early encounter of a young immune system with common infectious agents may protect against the development of autoimmunity and atopy. The early exposure to infections may be indicated by several proxy measures. One is day-care attendance, which has been reported to be negatively associated with T1DM (reviewed in [5]). In our study, the effect of none or very short day-care attendance held borderline significance—its epidemiological relevance seems low, given that only few children attend day-care for less than a year and that there is no decreasing trend in T1DM risk with longer day-care attendance.

The size of the community in which the family lived is another proxy measure of exposure to infectious agents. This parameter reflects the population density, and also, to a certain extent, the differences in socioeconomic status between urban and rural areas. In previous studies, a higher population density was associated with lower diabetes risk [11, 15]. Further, we investigated whether the families kept a domestic pet animal, since we hypothesized that the exposure to infections and allergens may also come from pets in the household, independently of the child's social mixing. Conceivably, the size of the community is strongly negatively correlated with the proportion of households owning a domestic pet—in control patients, a domestic pet was present in 84% of households in villages under 1,000 inhabitants, but only 42% households in cities with 100,000 or more inhabitants. Negative association between contact with pets and childhood diabetes has been recently found in a study from UK [7], but not in other studies [14, 16]. In the present work, neither the presence of a domestic pet, nor the size of the community was associated with the risk of T1DM. This may, amongst other factors, be attributed to the shallow socioeconomic

stratification in Czechoslovakia within the period when the children were born.

Study design

The present study is among one of the largest European case–control studies on the T1DM-related environmental factors, and it is the first report on this topic from our population. Although a prospective observation on genetically preselected individuals (as is currently being used by several studies) is obviously superior to our approach, retrospective case–control design is the only way to investigate the T1DM associations within certain time periods of interest. In our case, it was a rapid rise in T1DM incidence in a genetically stable population. Swift changes in exposure to some of the candidate factors are rather easy to see; over the period in which the studied children were born, the proportion of caesarean sections doubled, the length of day-care attendance shortened and the duration of breast-feeding lengthened. However, as also seen from our data, these changes cannot explain the rise of T1DM incidence in our population—there are undoubtedly other reasons, as yet unrecognised.

Conclusions

The present study shows a negative association of T1DM with breast-feeding, a weak positive association with none or short attendance to day-care and a lack of association with other factors, namely, the introduction of supplementary feeding and selected perinatal parameters.

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Příloha XIV.

Cardwell CR, Stene LC, Joner G, Cinek O, Svensson J, Goldacre MJ, Parslow RC, Pozzilli P, Brigis G, Stoyanov D, Urbonaite B, Sipetić S, Schober E, Ionescu-Tirgoviste C, Devoti G, de Beaufort CE, Buschard K, Patterson CC.

Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies.

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META ANALYSIS

Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies

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Abstract

Aims/hypothesis The aim of this study was to investigate the evidence of an increased risk of childhood-onset type 1 diabetes in children born by Caesarean section by systematically reviewing the published literature and performing a meta-analysis with adjustment for recognised confounders.
Methods After MEDLINE, Web of Science and EMBASE searches, crude ORs and 95% CIs for type 1 diabetes in children born by Caesarean section were calculated from the data reported in each study. Authors were contacted to

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facilitate adjustments for potential confounders, either by supplying raw data or calculating adjusted estimates. Meta-analysis techniques were then used to derive combined ORs and to investigate heterogeneity between studies.

Results Twenty studies were identified. Overall, there was a significant increase in the risk of type 1 diabetes in children born by Caesarean section (OR 1.23, 95% CI 1.15–1.32, $p<0.001$). There was little evidence of heterogeneity between studies ($p=0.54$). Seventeen authors provided raw data or adjusted estimates to facilitate adjustments for potential confounders. In these studies, there was evidence of an increase in diabetes risk with greater birthweight, shorter gestation and greater maternal age. The increased risk of type 1 diabetes after Caesarean section was little altered after adjustment for gestational age, birth weight, maternal age, birth order, breast-feeding and maternal diabetes (adjusted OR 1.19, 95% CI 1.04–1.36, $p=0.01$).

Conclusions/interpretation This analysis demonstrates a 20% increase in the risk of childhood-onset type 1 diabetes after Caesarean section delivery that cannot be explained by known confounders.

Keywords Caesarean section · Cesarean section · Diabetes mellitus · Epidemiology · Type 1

Introduction

Although type 1 diabetes has an important genetic component [1], the marked increases in incidence rate observed among the under 15 age group in recent decades [2, 3] strongly suggest the role of environmental influences. Various observations have lead to speculation that Caesarean section delivery could be involved. Rapid increases in Caesarean section rates [4] have occurred in parallel with increasing diabetes rates. For example, rates of Caesarean

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section in England, Sweden and the USA have risen from 6%, 8% and 10% in 1975 [5] to 19%, 12% and 22% in 1999 [4], respectively. Animal models suggest a higher risk of diabetes after Caesarean section [6, 7]. Also, children delivered by Caesarean section have been shown to have altered gut microbiotic composition and immune function [8–11], which could increase their risk of type 1 diabetes. Numerous studies have investigated Caesarean section and type 1 diabetes, but findings have been inconsistent, possibly as a result of inadequate size and limited power in some studies. In such a situation, meta-analysis is valuable in synthesising the available evidence [12].

The first aim of this study was to assess the evidence of an association between type 1 diabetes and Caesarean section by performing a meta-analysis. Previous studies have shown that various perinatal and early life factors are associated with type 1 diabetes, such as maternal age, birthweight and breastfeeding [13–15]. As such factors may differ in children born by Caesarean section, the second aim was to adjust the pooled estimate of the association between Caesarean section and type 1 diabetes for the influence of these potential confounders.

Methods

Literature search The main literature search was conducted using MEDLINE, through OVID ONLINE, with the following strategy: ('Cesarean Section' or 'Delivery, Obstetric' or cesarean or caesarean or mode of delivery) and ('Diabetes Mellitus, Type 1' or (diabetes and Type 1) or IDDM), using the terms in inverted commas as MEDLINE subject heading key words. Similar searches were conducted on Web of Science and EMBASE. To identify studies that investigated Caesarean section along with other risk factors, a more general search was conducted on MEDLINE using: ('Diabetes Mellitus, Type 1' and ('Case–Control Studies' or 'Cohort Studies')). The searches were limited to studies on humans, published before September 2007. Abstracts were screened independently by two investigators (C. R. Cardwell and C. C. Patterson) to establish if the studies were likely to provide relevant data based on the following inclusion criteria: (1) they identified a group with type 1 diabetes (containing more than 15 cases) and a group without type 1 diabetes, and (2) they determined the prevalence of delivery by Caesarean section in these groups. Citations generated from the more general MEDLINE search were initially screened to remove obviously irrelevant articles. Finally, the reference lists of all pertinent articles were examined.

Eligible studies were assessed independently by two reviewers (C. R. Cardwell and C. C. Patterson) to abstract information about the study (country, design and year of publication), participants with type 1 diabetes (source, age at

onset), control participants (source) and mode of delivery (methods of ascertainment).

Attempts were made to contact the corresponding author of all eligible studies to facilitate adjustment for maternal age, birthweight, gestational age, birth order, breast-feeding and maternal diabetes. Authors were requested to provide raw data or to provide adjusted estimates of the association between Caesarean section and type 1 diabetes after conducting specified additional analyses.

Statistical analysis ORs and SEs were calculated for the association between diabetes and Caesarean section for each study. Conditional logistic regression was used to calculate the ORs and SEs for the matched case–control studies. In cohort studies with varying duration of participant follow-up, rate ratios and their SEs were used instead of ORs, which were not directly calculable. As type 1 diabetes is a rare disease, these measures should be approximately equal [16]. Poisson regression was used to adjust these rate ratios for differences in the year of birth between cases and controls, a consequence of this study design [17, 18], by adding a year of birth and age term to the regression model in addition to Caesarean section. Tests for heterogeneity between studies were conducted, and random effects models used to calculate pooled ORs [19]. Random effects models were deemed more appropriate than fixed effects models because it was anticipated that there would be between study heterogeneity due to their observational nature. The I^2 statistic was calculated to quantify the degree of heterogeneity between studies [20]. This statistic measures the percentage of the total variation across studies that is due to heterogeneity. Study-specific weights in the random effects model were calculated and scaled to percentages. Publication/selection bias was investigated by checking for asymmetry in funnel plots of the study ORs against the SE of the logarithm of the ORs [21]. In the absence of publication/selection bias this graph should conform to a funnel shape, as the OR estimates from smaller studies (with larger SEs) show greater variation around the overall estimate than the OR estimates from larger studies (with smaller SEs). An identical approach was adopted to combine ORs for the association between type 1 diabetes and available confounders. To investigate the trend across categories for maternal age and birthweight, an OR (and SE) was calculated per increase in category using regression models appropriate to the design of the study, and then meta-analysis techniques were applied.

A two-stage technique was used to calculate pooled estimates of the association between Caesarean section and diabetes after adjustment for potential confounders [22]. First, adjusted estimates and SEs were calculated within each study using regression models appropriate to the study design (logistic regression for case–control studies, conditional logistic regression for matched case–control studies and Poisson regression for cohort studies) including diabetes as the outcome variable and Caesarean section and the potential confounder(s) of interest as explanatory variables. As explained previously, Poisson regression models additionally included terms to adjust for differences in year of birth between cases and controls in the cohort studies with varying participant follow-up. Meta-analysis techniques were then applied to these adjusted estimates.

Sensitivity analyses were conducted by subdividing studies by quality (whether population-based randomly selected controls were used) and using the Trim and Fill method to calculate pooled estimates after adjustment for any potential publication bias [23]. This method identifies funnel plot asymmetry and imputes study results, which are considered to have been conducted but not published, to create funnel plot symmetry. The overall combined estimate of the association is then based on the observed and imputed study results.

All statistical analyses were performed using STATA 9.0 software (STATA, College Station, TX, USA).

Results

The searches identified nine eligible articles using MEDLINE [15, 17, 24–30]; a further article was identified from Web of Science [31] and another from EMBASE [32]. The more general MEDLINE search identified a further eight articles [33–40], and review of reference lists revealed another two articles [41, 42].

Seven of the identified articles were excluded from further consideration. An earlier study [41] was excluded in favour of a larger study [42] that included all the participants enrolled in the former. Three articles [25, 26, 28] reported the same data. A study [33] was excluded because no raw data were presented in the paper or available from the authors. Another study was excluded as it contained fewer than 15 cases [29]. A meeting abstract [31] was replaced with the subsequently published article [43] and, after contact with authors, an earlier report from a cohort [35] was replaced with a later report [18].

The 16 remaining articles corresponded to 20 independent studies, because one study [15] provided data from eight centres, three of which were reported elsewhere [25, 27, 32], and another provided data from two centres [24], one of which was subsequently reported in a larger study [32]. Finally, to ensure two studies [17, 30] provided independent information, authors removed cases from one study [30] that were included in the other [17]. Study characteristics are summarised in Table 1.

The unadjusted association between Caesarean section and childhood-onset type 1 diabetes for all 20 studies, including 9,938 cases, is shown in Fig. 1. Overall, there was a significant increase ($p<0.001$) in the risk of type 1

Table 1 Characteristics of studies investigating the association between Caesarean section and type 1 diabetes, ordered by publication date

First author, year[a] [reference]	Design	Country	Type 1 diabetes Ascertainment method	Age at diagnosis	n	Controls Source (matching criteria)	n	Ascertainment of Caesarean section	GA	MA	BW	BO	BF	MD
Dahlquist, 1992 [42]	C–C	Sweden	Swedish Childhood Diabetes Registry	0–14 years	2,757	Swedish Medical Birth Registry (year of birth, delivery unit)	8,271	Maternity record	✓					
Patterson, 1994 [24]	C–C	Scotland	Hospital admission / childhood diabetes register	0–14 years	271	Scottish maternal discharge records (age, sex and area)	1,355	Maternity record	✓	✓	✓	✓	✓	✓
McKinney, 1997 [25]	C–C	England	Yorkshire Childhood Diabetes Register	0–15 years	220	General practitioner's records (age and sex)	433	Questionnaire	✓	✓	✓	✓	✓	✓
Tai, 1998 [34]	C–C	China	Taipei type 1 Diabetes Registry	0–29 years (mean = 8 years)	117	Classmates or colleagues[c] (age, sex, parental and individual education)	193	Questionnaire	✓					
Rami, 1999 [27]	C–C	Austria	Vienna type 1 diabetes register	0–14 years	114	Schools (age and sex)	495	Maternity record	✓	✓	✓	✓	✓	✓[d]
Bache, 1999 [36]	C–C	Denmark	Hospital admission	0–14 years	839	Medical birth register (age, sex and district)	1,687	Maternity record	✓	✓	✓			
	C–C	Bulgaria	West Bulgaria type 1 diabetes register	0–14 years	176	Schools and policlinics (age)	562	Maternity record	✓	✓	✓	✓	✓	✓[d]
	C–C	Latvia	Latvian type 1 diabetes register	0–14 years	143	Population register (age)	410	Maternity record	✓	✓	✓	✓	✓	✓[d]
EURODIAB, 1999 [15]	C–C	Lithuania	Lithuanian type 1 diabetes register	0–14 years	124	Policlinics (age)	369	Maternity record	✓	✓	✓	✓	✓	✓[d]
	C–C	Luxembourg	Luxembourg type 1 diabetes register	0–14 years	59	Pre-schools and schools (age)	188	Maternity record	✓	✓	✓	✓	✓	✓[d]
	C–C	Romania	Bucharest type 1 diabetes register	0–14 years	111	Pre-schools and schools (age)	342	Maternity record	✓	✓	✓	✓	✓	✓[d]
Visalli, 2003 [43]	C–C	Italy	Lazio type 1 diabetes register	0–14 years	150	Schools (age)	750	Questionnaire	✓	✓	✓	✓	✓	✓
Stene, 2003 [17]	Cohort	Norway	Norwegian Childhood Diabetes Registry	0–14 years	1,824	Norwegian medical birth registry	1,380,778	Maternity record	✓	✓	✓	✓	✓	✓
Stene, 2004 [30]	C–C	Norway	Norwegian Childhood Diabetes Registry	0–14 years	545	Norwegian population registry	1,668	Maternity record	✓	✓	✓	✓	✓	✓[d]
Cardwell, 2005 [32]	Cohort	Northern Ireland	Northern Ireland type 1 diabetes register	0–14 years	991	Northern Ireland Child Health Register	447,633	Maternity record	✓	✓	✓	✓	✓	✓
Šipetić, 2005 [38]	C–C	Serbia	Belgrade Hospital admission	0–16 years	105	Hospital outpatients with skin disease[c] (age, sex and area)	210	Questionnaire	✓	✓	✓	✓	✓	✓[d]

Available confounders[b]

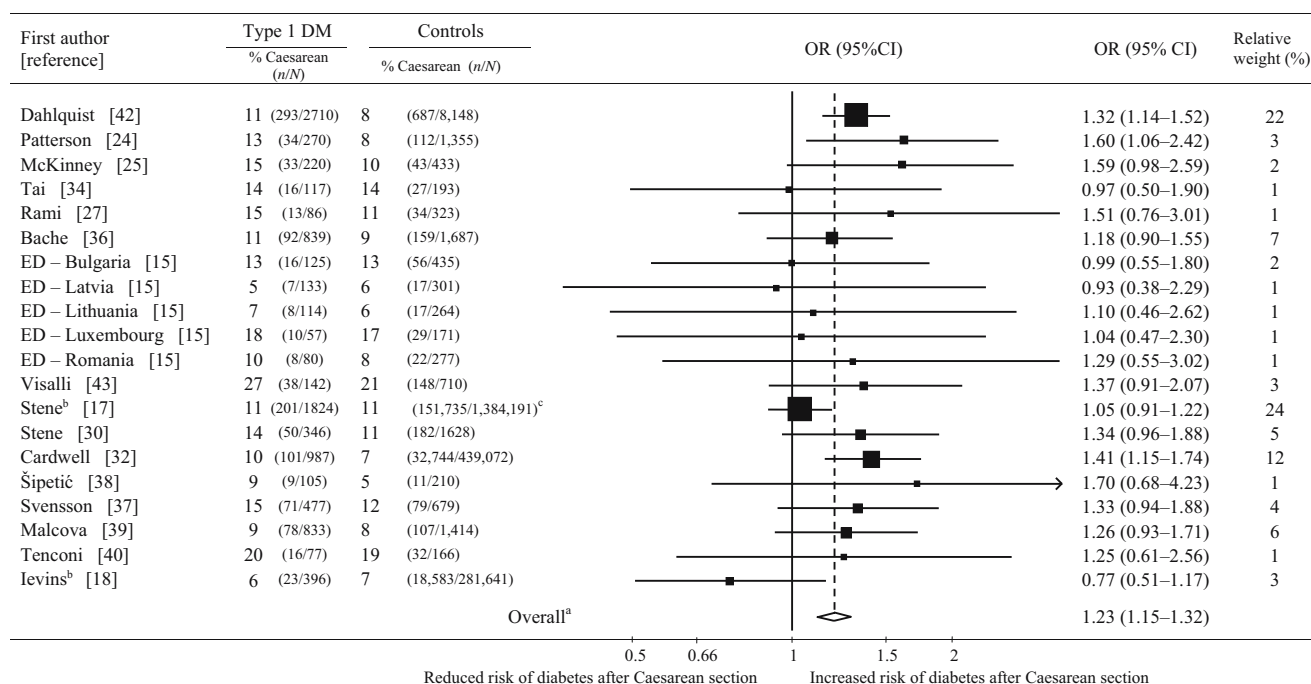| Study	Type	Country	Register	Age range	No.	Control source	No.	Ascertainment							
Svensson, 2005 [37]	C–C	Denmark	Danish register of childhood diabetes	0–14 years	490	Danish population register (age and sex)	696	Questionnaire	✓	✓	✓	✓	✓	✓	✓
Malcova, 2006 [39]	C–C	Czech Republic	Czech Childhood Diabetes Register	0–14 years	868	School friends[c]	1,466	Questionnaire	✓	✓	✓	✓	✓	✓	✓
Tenconi, 2007 [40]	C–C	Italy	Pavia type 1 Diabetes Registry	0–14 years	159	Hospital patients[c] (age and sex)	318	Questionnaire		✓	✓	✓	✓	✓	✓
Ievins, 2007 [18]	Cohort	England	Hospital admission (ICD code for diabetes)	0–14 years	411	Oxfordshire and West Berkshire maternity records	292,845	Maternity record	✓	✓	✓	✓	✓		✓

First author [reference]	Type 1 DM % Caesarean (n/N)	Controls % Caesarean (n/N)	OR (95%CI)	OR (95% CI)	Relative weight (%)
Dahlquist [42]	11 (293/2710)	8 (687/8,148)		1.32 (1.14–1.52)	22
Patterson [24]	13 (34/270)	8 (112/1,355)		1.60 (1.06–2.42)	3
McKinney [25]	15 (33/220)	10 (43/433)		1.59 (0.98–2.59)	2
Tai [34]	14 (16/117)	14 (27/193)		0.97 (0.50–1.90)	1
Rami [27]	15 (13/86)	11 (34/323)		1.51 (0.76–3.01)	1
Bache [36]	11 (92/839)	9 (159/1,687)		1.18 (0.90–1.55)	7
ED – Bulgaria [15]	13 (16/125)	13 (56/435)		0.99 (0.55–1.80)	2
ED – Latvia [15]	5 (7/133)	6 (17/301)		0.93 (0.38–2.29)	1
ED – Lithuania [15]	7 (8/114)	6 (17/264)		1.10 (0.46–2.62)	1
ED – Luxembourg [15]	18 (10/57)	17 (29/171)		1.04 (0.47–2.30)	1
ED – Romania [15]	10 (8/80)	8 (22/277)		1.29 (0.55–3.02)	1
Visalli [43]	27 (38/142)	21 (148/710)		1.37 (0.91–2.07)	3
Stene[b] [17]	11 (201/1824)	11 (151,735/1,384,191)[c]		1.05 (0.91–1.22)	24
Stene [30]	14 (50/346)	11 (182/1628)		1.34 (0.96–1.88)	5
Cardwell [32]	10 (101/987)	7 (32,744/439,072)		1.41 (1.15–1.74)	12
Šipetić [38]	9 (9/105)	5 (11/210)		1.70 (0.68–4.23)	1
Svensson [37]	15 (71/477)	12 (79/679)		1.33 (0.94–1.88)	4
Malcova [39]	9 (78/833)	8 (107/1,414)		1.26 (0.93–1.71)	6
Tenconi [40]	20 (16/77)	19 (32/166)		1.25 (0.61–2.56)	1
Ievins[b] [18]	6 (23/396)	7 (18,583/281,641)		0.77 (0.51–1.17)	3
Overall[a]				1.23 (1.15–1.32)	

0.5 0.66   1   1.5   2
Reduced risk of diabetes after Caesarean section   Increased risk of diabetes after Caesarean section

Fig. 1 Meta-analysis of studies of Caesarean section and type 1 diabetes (including 9,938 cases) using the random effects model, studies ordered by publication date. Reference numbers are provided in Table 1. [a]Test fo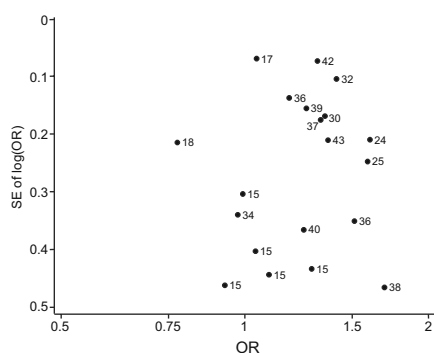r heterogeneity $\chi^2=17.70$, df 19, $p=0.54$; $I^2=0\%$ (95% CI 0–48%); test for overall effect $Z=5.70$, $p\leq0.001$; [b]adjusted for year of birth and age group, as explained in Statistical analysis; [c]approximated from person years. DM, diabetes mellitus; ED, EURODIAB

diabetes after Caesarean section delivery, with an OR of 1.23 (95% CI 1.15–1.32). There was little evidence of heterogeneity between the study estimates ($I^2=0\%$, 95% CI 0–48%; $\chi^2=17.70$, df 19, $p=0.54$). A funnel plot, shown in Fig. 2, roughly conformed to the expected funnel shape, providing little evidence of asymmetry and therefore little evidence of publication bias. Similarly, the Trim and Fill method, which attempts to adjust for any publication bias by imputing possible unpublished studies, produced estimates that were unaltered (OR 1.23), suggesting that any effect of publication bias was negligible. Further analysis in the subgroup of 16 studies judged to have used randomly

Fig. 2 Funnel plot of studies of Caesarean section and type 1 diabetes, labelled by reference number

selected population-based controls produced a similar pooled estimate (OR 1.24, 95% CI 1.13–1.35).

Adjustment for potential confounders was possible in 17 studies. Fifteen authors provided raw data, and two calculated adjusted estimates. Raw data from two studies were not available [36, 42] and one author could not be contacted [34].

Table 2 summarises the crude association between childhood-onset type 1 diabetes and potential confounders. Overall, there was an increase in the risk of diabetes with increasing birthweight (combined OR per category increase 1.05, $p=0.006$) and little heterogeneity between studies ($I^2=25$, $p=0.17$). There was evidence ($p=0.02$) of a reduction in risk of diabetes with longer gestation. The pooled risk of diabetes in children born later than 42 weeks was 0.84 times that of children born 38–41 weeks, and was similar across studies ($I^2=10$, $p=0.34$). There was evidence of an increase in diabetes risk with maternal age (combined OR per category increase 1.08, $p=0.001$) but there was considerable heterogeneity between studies ($I^2=50$, $p=0.01$). Overall, there was some evidence that children second born (OR 1.12, $p=0.03$) or third or later born (OR 1.08, $p=0.17$) had a slightly higher risk of type 1 diabetes than first born children, but these associations were also subject to considerable heterogeneity ($I^2=45$, $p=0.03$ and $I^2=25$, $p=0.17$, respectively). Children whose mother had diabetes (OR 4.92, $p<0.001$) or, specifically, type 1 diabetes (OR 4.03, $p=0.001$) had a higher risk of type 1 diabetes, and these associations

were fairly consistent across studies ($I^2=0$, $p=0.49$ and $I^2=0$, $p=0.88$, respectively). Finally, there was some indication that children who were breastfed, or breastfed for a longer duration, had a slightly lower risk of diabetes than children who were not breastfed, or breastfed for a shorter duration, (OR 0.84, $p=0.02$). This association was subject to marked heterogeneity ($I^2=61$, $p=0.001$)—perhaps due in part to the different categorisations used in each study—and should therefore be carefully interpreted.

Table 3 shows the association between Caesarean section and type 1 diabetes after adjustment for confounders. The crude association between Caesarean section delivery and type 1 diabetes was little altered after adjustment for birthweight (OR 1.24, $p<0.001$), gestational age (OR 1.19,

$p<0.001$), maternal age (OR 1.19, $p<0.001$), birth order (OR 1.21, $p<0.001$), maternal diabetes (OR 1.17, $p=0.003$), breast-feeding (OR 1.26, $p<0.001$) or all of these confounders (OR 1.19, $p=0.01$).

Discussion

This meta-analysis demonstrates a consistent increase, of around 20%, in the risk of type 1 diabetes in children delivered by Caesarean section. This observed increase in diabetes risk after Caesarean section delivery could not be explained by the confounding influence of birthweight, gestational age, maternal age, birth order, maternal diabetes or breastfeeding.

Table 2 Pooled analysis of the association between potential confounders and type 1 diabetes

Potential confounder	Number of studies	Heterogeneity			Combined OR (95% CI)	p value
		χ^2	p value	I^2 (95%CI)		
Birthweight (g)	16					
<2,500		21.05	0.14	29 (0–61)	0.87 (0.71–1.07)	0.18
2,500–2,999		11.15	0.74	0 (0–52)	0.93 (0.86–1.02)	0.14
3,000–3,499					1.00 (Ref. cat.)	–
3,500–3,999		10.77	0.77	0 (0–52)	1.03 (0.97–1.10)	0.33
≥4,000		11.24	0.74	0 (0–52)	1.12 (1.02–1.21)	0.01
Trend across categories		20.10	0.17	25 (0–59)	1.05 (1.02–1.09)	0.006
Gestational age (weeks)	16					
≤37		8.56	0.86	0 (0–54)	1.01 (0.91–1.11)	0.87
38–41					1.00 (Ref. cat.)	–
≥42		16.67	0.34	10 (0–47)	0.84 (0.73–0.97)	0.02
Maternal age (years)	17					
<20		29.54	0.02	46 (4–69)	0.84 (0.68–1.04)	0.10
20–24		19.11	0.26	16 (0–52)	0.90 (0.83–0.98)	0.01
25–29					1.00 (Ref. cat.)	–
30–34		17.94	0.32	11 (0–48)	1.03 (0.95–1.13)	0.47
≥35		14.01	0.59	0 (0–51)	1.11 (1.01–1.23)	0.04
Trend across categories		32.22	0.01	50 (13–72)	1.08 (1.03–1.13)	0.001
Birth order	16					
First born					1.00 (Ref. cat.)	–
Second born		27.29	0.03	45 (1–69)	1.12 (1.02–1.24)	0.03
Third or later born		20.05	0.17	25 (0–59)	1.08 (0.97–1.19)	0.17
Maternal diabetes[a]						
No	8				1.00 (Ref. cat.)	–
Yes		6.42	0.49	0 (0–68)	4.92 (3.93–6.16)	<0.001
Maternal type 1 diabetes[a]						
No	8				1.00 (Ref. cat.)	–
Yes		3.05	0.88	0 (0–68)	4.03 (1.76–9.20)	0.001
Breast-feeding[b]						
No or short period	15				1.00 (Ref. cat.)	–
Yes or long period		36.25	0.001	61 (32–78)	0.84 (0.72–0.98)	0.02

[a] Studies recording maternal diabetes and maternal type 1 diabetes are shown in Table 1

[b] Breast-feeding was categorised as breast-feeding at discharge from hospital [18, 32], any breast-feeding [15, 25, 27], breast-feeding for approximately 3 months or more [30, 40, 43] and breast-feeding for approximately 4 months or more [37–39]

Ref. cat., reference category

The main finding was observed consistently across studies, conferring a level of robustness to this result. Importantly, using individual patient data, or adjusted estimates, we were able to demonstrate that the increased risk of diabetes after Caesarean section delivery could not be explained by known confounding factors. However, as this meta-analysis was based upon observational studies, it is impossible to rule out the influence of unrecorded confounders, although any such confounder would have to operate similarly across all studies. Social class is a possibility, as it may be associated with the likelihood of delivery by Caesarean section, but as the association between social class and type 1 diabetes is inconsistent [24, 44–46] it seems unlikely that it could exert the necessary confounding influence. Gestational diabetes is another possibility, but the proportion of mothers with gestational diabetes in these European populations is likely to be small [47], reducing the likelihood of marked confounding, and adjustment for gestational diabetes in seven of the studies [15, 25, 27] revealed little evidence of confounding. A further weakness of this study was that the reason for Caesarean section could not be investigated, as this was not available in the majority of studies, and therefore we were unable to confirm a report suggesting that any increased risk of type 1 diabetes after Caesarean section was most marked after elective procedures [24].

The explanation for the observed increase in the risk of type 1 diabetes in children born by Caesarean section is unknown, but various theories are plausible. The gut microbiota are thought to play an important role in stimulating the development of the immune system [48]. Recent studies have shown that the gut microbiotic composition differ in children born by Caesarean section compared with vaginally born children [8–11], perhaps because such children

are first exposed postpartum to bacteria originating from the hospital environment rather than to maternal bacteria [11]. This difference in gut microbiotic composition could increase the risk of type 1 diabetes. Similarly, the hygiene hypothesis suggests that children with reduced or delayed exposure to infection in early life may have an increased risk of type 1 diabetes [49]. According to this hypothesis, as children born by Caesarean section may have a reduced exposure to infections compared with children born vaginally, this could increase their diabetes risk. Alternatively, a previous study [42] speculated that any increased risk of diabetes after Caesarean section could be caused by non-specific perinatal stress.

Our study also allowed the documentation of pooled estimates of the crude risk associated with various perinatal factors. Although not the result of a systematic review of the literature for each perinatal factor, there is no obvious reason why this selection of studies would not be representative. To our knowledge, this is the largest selection of studies that have been combined to investigate associations with birthweight, gestational age, maternal age, birth order and maternal diabetes. These analyses indicated that children who are heavier at birth, have a shorter gestation and whose mother has diabetes have a greater risk of type 1 diabetes. Although there was also evidence of an increased risk of type 1 diabetes with greater maternal age and later birth order, these associations varied considerably between studies and should be interpreted more cautiously. The findings for breast-feeding, of a slight reduction in type 1 diabetes risk, although broadly similar to that observed in two previous meta-analyses [50, 51], were subject to considerable heterogeneity, perhaps reflecting differences in the recording of breast-feeding in the individual studies.

Table 3 Pooled analysis of the association between Caesarean section and type 1 diabetes after adjustment for various potential confounders

Adjusted potential confounder(s)[a]	No. of studies	No. of cases	Heterogeneity			Adjusted combined OR (95% CI)	p value
			χ^2	p value	I^2 (95%CI)		
None	20	9,938	17.70	0.54	0 (0–48)	1.23 (1.15–1.32)	<0.001
Birthweight	16	6,138	13.55	0.56	0 (0–52)	1.24 (1.13–1.35)	<0.001
Gestational age	16	6,005	14.10	0.52	0 (0–52)	1.19 (1.09–1.31)	<0.001
Maternal age	17	6,246	16.04	0.45	0 (0–51)	1.19 (1.09–1.30)	<0.001
Birth order	16	6,029	16.19	0.37	7 (0–43)	1.21 (1.10–1.34)	<0.001
Maternal diabetes	16	6,150	16.79	0.33	11 (0–48)	1.17 (1.05–1.29)	0.003
Breastfeeding	15	3,874	9.00	0.83	0 (0–54)	1.26 (1.12–1.42)	<0.001
Birthweight, gestational age, maternal age and birth order	15	5,791	11.30	0.66	0 (0–54)	1.17 (1.06–1.28)	0.001
Birthweight, gestational age, maternal age, birth order and breastfeeding	13	3,444	7.86	0.80	0 (0–57)	1.21 (1.06–1.38)	0.005
Birthweight, gestational age, maternal age, birth order, breastfeeding and maternal diabetes	13	3,424	9.16	0.69	0 (0–57)	1.19 (1.04–1.36)	0.01

[a] Adjustments were made for potential confounders using broadly the categories shown in Table 2

In conclusion, our study detected a small but significant and consistent increase in the risk of type 1 diabetes after Caesarean section, which could reflect differences in exposure to bacteria in early life.

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