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Rizikové faktory vzniku a průběhu léčby zánětlivých střevních onemocnění u dětí

Risk factors of manifestation and course of treatment of inflammatory bowel disease in children

Disertační práce

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Abstrakt

Typická Crohnova choroba (CD), Crohnovská kolitida zasahující pouze tlusté střevo, atypická ulcerózní kolitida (UC) a typická UC s kontinuálně rozloženým zánětlivým postižením tlustého střeva jsou v současné době vnímány jako makroskopicky i mikroskopicky se od sebe lišící formy zánětlivých střevních onemocnění (IBD). Výskyt těchto imunitně podmíněných onemocnění celosvětově narůstá v rámci dospělé i dětské populace. Ačkoliv je do jisté míry znám podíl genetického pozadí a faktorů vnějšího prostřední na vznik těchto onemocnění, přesná příčina rozvoje IBD není stále určena. Komplexní péče vyžaduje precizní a co nejlépe daty podpořený přístup vedoucí k minimalizaci rizika komplikovaného průběhu onemocnění a rozvoji s onemocněním a/nebo léčbou asociovaných komplikací.

Hlavním cílem práce je identifikace nových prediktivních faktorů zasahujících do jednotlivých oblastí péče o dětské pacienty s IBD. Spektrum klinických situací, řešených v rámci této práce zahrnuje možnost predikce diagnózy, obecně komplikovaného průběhu onemocnění, odpovědi na konkrétní terapeutický režim, rozvoj nežádoucích účinků asociovaných se zvoleným terapeutickým postupem a adherence pacienta k léčbě. Část prací byla provedena v retrospektivním designu, část jako prospektivní observační studie, dvě z původních prací vznikly jako multicentrické mezinárodní projekty.

Za nejdůležitější výstup práce lze považovat vytvoření predikčního modelu k individuálnímu odhadu času do relapsu u dětských pacientů s CD na léčbě thiopuriny. Dále jsme vytvořili modely predikující hladinu metabolitů azathioprinu, která odráží vhodnost dávkování léku nebo adherenci pacienta k jeho užívání. Nově byl nalezen optimální cut-off pro hladinu metabolitů azathioprinu, který predikuje dosažení žádoucích hladin infliximabu. Identifikovali jsme infliximab, ve srovnání s adalimumabem, jako prediktor rozvoje kožních nežádoucích účinků u dětských pacientů s IBD. Ukázali jsme, že míra tkáňové exprese CD30+ buněk je silným prediktivním faktorem klasifikace onemocnění do jednoho z typů IBD. Naopak možnost klinického využití nebyla prokázána u stanovení hladin tkáňového kalprotektinu (CPT) a fekálního CPT časně po zahájení indukce výlučnou enterální výživou. Upozornili jsme na limitované klinické využití domácích testů ke stanovení hladin fekálního CPT.

Výsledky jednotlivých studií lze shrnout jako získání souboru nových informací, na základě kterých lze předem odhadnout určitý jev, jehož včasné ovlivnění, nebo lépe jehož předejití přispívá k lepší kontrole onemocnění u dětských pacientů s diagnózou IBD. Díky nově identifikovaným možnostem predikce konkrétních klinických otázek je odpověď na ně informovanější a lze tak předpokládat větší přesnost při rozhodování.

Klíčová slova: predikce, zánětlivá střevní onemocnění, léčba, diagnostika

Abstract

Typical Crohn's disease (CD), Crohn's colitis, typical and atypical ulcerative colitis (UC) are currently perceived as different forms of inflammatory bowel disease (IBD). The incidence of IBD is increasing worldwide in both the adult and paediatric populations. Although the role of genetic background and environmental factors in the development of these diseases is known to some extent, the exact cause of IBD has still not been determined. Comprehensive care requires a precise and data-driven approach to minimize the risk of complicated disease course and the development of disease-related and/or treatment-associated complications.

The main goal of this work is to identify new predictive factors affecting individual areas of care of paediatric patients with IBD. The range of clinical situations addressed in this work includes the possibility of predicting the diagnosis, the generally complicated disease course, the response to a particular therapeutic regimen, the development of side effects associated with the therapeutic procedure and the patient's adherence to the treatment. Part of the original works was done in a retrospective design, part as prospective observational studies and two of the original works were realized as multicentre international projects.

The most important outcome of the work can be considered the creation of a predictive model of estimation of individual time to relapse in paediatric patients with CD on thiopurine treatment. We also developed models predicting the level of azathioprine metabolites, which reflect the suitability of the drug dosage or the patient's adherence to its use. An optimal cut-off of the level of azathioprine metabolites, which predicts the achievement of the effective infliximab levels, has been found. We identified infliximab, compared to adalimumab, as a predictor of the development of skin side effects in paediatric IBD patients. We have shown that the tissue expression level of CD30+ cells is a strong predictive factor of classifying the disease into one of the IBD types. In contrast, the possibility of clinical usefulness has not been demonstrated in the determination of tissue calprotectin (CPT) levels and faecal CPT levels obtained early after initiation of treatment with exclusive enteral nutrition. We indicated the limited clinical use of home tests to determine faecal CPT levels.

The results of individual studies can be summarized as the acquisition of a set of new information, on the basis of which it is possible to predict a certain phenomenon, the early influence or prevention of which, contributes to better disease control. Thanks to the newly identified possibilities of predicting specific clinical questions, the answer to them is more informed and it is possible to assume greater accuracy in decision-making process.

Kye words: prediction, inflammatory bowel disease, treatment, diagnostics

Seznam použitých zkratek

AZA: azathioprin

CD: Crohnova choroba

ELISA: enzyme-linked immuno sorbent assay

ESPGHAN: Evropská společnost pro dětskou gastroenterologii, hepatologii a výživu

fCPT: fekální kalprotektin

GWAS: genome-wide association study

UC: ulcerózní kolitida

IBD: zánětlivé střevní onemocnění

IBD-U: nezařaditelné zánětlivé střevní onemocnění

PCDAI: Pediatric Crohn's Disease Activity Index

PUCAI: Pediatric Ulcerative Colitis Activity Index

ROC: receiver operating characteristic

SES-CD: Simple endoscopic score for Crohn's disease

tCPT: tkáňový kalprotektin

UCEIS: Ulcerative Colitis Endoscopic Index of Severity

wPCDAI: weighted Pediatric Crohn's Disease Activity Index

6-MMP: 6-methylmercaptopurine

6-TG: 6-thioguanine nukleotid

Obsah

1. Úvod

1.1. Zánětlivá střevní onemocnění

Typická Crohnova choroba (CD) s postižením tenkého střeva, Crohnovská kolitida zasahující pouze tlusté střevo, atypická ulcerózní kolitida (UC) a typická UC s kontinuálně rozloženým zánětlivým postižením tlustého střeva jsou v současné době vnímány jako makroskopicky i mikroskopicky od sebe se lišící formy zánětlivých střevních onemocnění (IBD) [1]. Kontinuita IBD a vnímání jednotlivých subtypů spíše s ohledem na odpověď na zvolenou terapii je stále více aktuálním tématem [2]. V případě, kdy nelze spolehlivě určit podtyp IBD, je imunitně podmíněný střevní zánět označen jako nezařaditelné zánětlivé střevní onemocnění (IBD-U) [1].

Klinicky je onemocnění charakteristické především příznaky plynoucími z chronicky probíhajícího zánětlivého postižení gastrointestinálního traktu. Vedle intestinální manifestace se lze u pacientů s IBD setkat i tzv. extraintestinální projevy, mezi které patří např. artritida, kožní projevy (erythema nodosum, pyoderma gangrenosum) nebo uveitida.

Pro samotný průběh onemocnění je příznačné střídání fází remise a tzv. flarů, tedy vzplanutí zánětlivé aktivity. Častá období s vysokou aktivitou onemocnění mohou mít vliv nejen na samotný střevní zánět, jeho průběh a možný rozvoj s onemocněním asociovaných komplikací, ale významně se podílí i na psychosociální stránce života pacienta. Navození a následné udržení remise má tak význam ve více rovinách péče o pacienta s diagnózou IBD. Dobře stanovená diagnóza a pečlivá monitorace aktivity onemocnění hraje důležitou úlohu v léčbě a další prognóze pacienta [3, 4].

1.2. Epidemiologie zánětlivých střevních onemocnění

Narůstající počet pacientů s diagnózou IBD je již dobře známým trendem v zemích tzv. západního světa [5, 6]. Ve Spojených státech Amerických a Evropě je dle recentně provedené meta-analýzy celkový počet nemocných odhadován na více než 1,5 a přes 2 miliony pacientů [7]. V poslední době se začínáme nově setkávat s daty, která indikují nárůst počtu pacientů s diagnózou CD, UC nebo IBD-U i v oblastech pro IBD spíše vzácných jako Jižní Amerika, Asie, Afrika a Východní Evropa [8]. Tento epidemiologický posun v oblastech s recentně narůstající industrializací nápadně připomíná nárůst počtu diagnóz IBD v tzv. západním světě v 50. letech 20. století, pro která je charakteristický velmi rychlý socio-ekonomický rozvoj [7]. Tyto změny v rozložení pacientů s IBD naznačují možnou významnost vlivu faktorů vnějšího prostředí na vznik a průběh onemocnění.

Incidence i prevalence IBD se nicméně v jednotlivých oblastech světa stále výrazně liší [7, 9]. Celosvětová incidence IBD se dle recentně provedené meta-analýzy pohybuje v rozmezí od 0,5 do 23 / 100 000 [9]. Mezi regiony s nejvyšší incidencí IBD stále patří Evropa (12,9 / 100 000) a Severní Amerika (13,9 / 100 000) [9]. Nejvyšší incidence IBD vůbec byla zaznamenána na území Faerských ostrovů. [10] Prevalence je v současné době v evropské populaci odhadována na 0,3 % populace [11].

Nárůst počtu pacientů diagnostikovaných s IBD se významně dotýká i dětské populace. V roce 2014 byla incidence IBD mezi dětskými pacienty uváděna v rozmezí od 2,5 do 11,4 / 100 000 s odhadovanou prevalencí 58 / 100 000 [12]. V rámci dětské populace byl ve vztahu k výskytu IBD zaznamenán tzv. severojižní gradient, tedy nápadně vyšší počet pacientů s IBD v severních zemích. Toto rozložení může naznačovat vliv průměrné roční teploty nebo rozdílnosti ve vyspělosti mezi jižními a severními státy.

Stále se zvyšující počet pacientů s diagnózou IBD s sebou nese i narůstající ekonomické nároky na zajištění péče. Terapie pacientů s IBD stojí v Evropě ročně 4,6 – 5,2 miliardy Euro. [11]

1.3. Patogeneze a etiologie zánětlivých střevních onemocnění

Přesná příčina vzniku imunitně podmíněného střevního zánětu není stále spolehlivě určena. Na rozvoji IBD je předpokládán podíl a následná kombinace více faktorů, konkrétně faktorů vnějšího prostředí a genetické predispozice pacienta. Obecně lze IBD chápat jako multifaktoriální polygenní onemocnění, s výjimkou spíše minoritní skupiny pacientů, u nichž je rozvoj klinické symptomaticky odpovídající IBD podmíněn konkrétním genetickým defektem [13, 14].

1.3.1. Genetické faktory

Účast genetického vlivu na rozvoj IBD lze předpokládat u všech forem tohoto onemocnění, větší podíl genetického rizika byl zjištěn u pacientů s CD [15]. Zvýšené riziko rozvoje IBD bylo popsáno u prvostupňových příbuzných s odhadovaným relativním rizikem 26 u sourozenců a 9 u rodičů [16].

Prvním popsaným genem asociovaným s CD byl gen *NOD2*. Aktuálně jsou ve vztahu k CD popsány tři patogenní varianty. Nejsilnější asociace mezi rozvojem CD u pacientů v České republice byla zjištěna s variantou 1007fs [17], která je dále asociována s nižším věkem prvních projevů onemocnění a postižením ilea [17]. Základní funkce genu *NOD2* spočívá v kódování proteinu, který je intracelulárním receptorem pro část bakteriální stěny

(muramyl-dipeptid) [18], dále se pravděpodobně podílí na indukci stresu endoplazmatického retikula [19] a recentně byla studována i jeho úloha v autofagii vedoucí k eliminaci bakterií [20, 21]. Nicméně, přesný mechanismus, jakým dochází k rozvoji CD pod vlivem patogenních variant *NOD2,* nebyl dosud definitivně určen [22].

Dalším důležitým zjištěním byla asociace mezi CD a variantou genu *IL23R*, tedy genu kódujícím protein IL23 receptoru. V důsledku varianty, záměny Arg381Gln, dochází ke ztrátě funkce výsledného proteinu, a tím k významnému snížení Th17 odpovědi. Přítomnost varianty IL23R je tak ochranným faktorem ve vztahu k rozvoji CD [23].

Dále byly popsány asociace s CD a variantami např. v: *ATG16L1* [21, 24], *IRGM* [25], jejichž výsledné proteiny mají důležitý význam v autofagocytárních drahách nebo *PTPN22*, typicky autoimunitním genu [17].

V průběhu času se značně změnil přístup v metodách aplikovaných při hledání potenciálních genetických variant asociovaných s IBD. Zatímco výše zmíněné geny byly objeveny pomocí vazbových studií s použitím mikrosatelitních markerů k vytipování chromozomálních regionů, kde by se geny podílejícíse na vzniku IBD mohly nacházet, nebo pomocí tzv. metody genome-wide association study (GWAS), dnes je výzkum v této oblasti soustředěn především na metodu sekvenování. Nutnost změny přístupu zdůrazňuje i zjištění, že metoda GWAS dokázala odhalit pouze 13,6 % variability CD a 7,5 % variability UC [26]. Mezi faktory, které nelze pokrýt pomocí metody GWAS a mohou přispět ke heritabilitě IBD, byly zvažovány především epigenetika nebo velmi nízká frekvence výskytu v populaci [27]. V rámci epigenetických změn, které mohou mít vliv na rozvoj IBD, byly postupně popsány DNA metylace, modifikace histonu, RNA interference. Následnou kombinací genetických a epigenetických informací byly zjištěny další genetické faktory asociované s rozvojem IBD [28]. Díky využití nové metody sekvenování byly identifikovány četné monogenní defekty, vedoucí k rozvoji onemocnění klinicky se projevující jako IBD s velmi časným nástupem [29]. Význam znalosti monogenních forem onemocnění spočívá především ve zcela odlišné léčebné strategii. U pacientů s monogenně podmíněným onemocněním léčba směřuje především k alogenní transplantaci hematopoetických kmenových buněk (defekty IL10 signalizace, IPEX, Wiscott-Aldrichův syndrom, chronická granulomatóza, deficience XIAP) nebo podání antagonisty IL-1β receptoru (deficience mevalonát kinázy, chronická granulomatóza).

1.3.2. Faktory zevního prostředí

Znalost vztahu mezi faktory vnějšího prostředí a rozvojem IBD je limitována především faktem, že všechny práce na toto téma, byly provedeny jako observační studie a neumožňují tak hodnotit kauzalitu sledovaného jevu, ale pouze jeho asociaci k IBD.

Jedním z nejvíce studovaných faktorů bylo kouření cigaret, které se prokázalo jako protektivní faktor vzhledem k rozvoji UC, tedy aktivní kouření cigaret bylo asociováno s nižším rizikem rozvinutí UC [30-32]. Ve vztahu kouření cigaret k CD byla naopak popsána asociace se zvýšením rizika rozvoje onemocnění, kdy aktivní kuřáci ve srovnání s jedinci, kteří nikdy nekouřili, měli o 90 % vyšší riziko rozvinutí CD [30]. V případě pasivního kouření nebyl prokázán významný vliv na rozvoj CD nebo UC [33].

Dalším faktorem, u kterého byla popsána asociace se zvýšením rizika CD, je osobní anamnéza appendektomie [34]. V tomto případě je však třeba zmínit pokles zvýšeného rizika (uváděné odhadované relativní riziko je 1,6) zpět na populační pět let po provedení chirurgického výkonu. Tento fakt může v jistém ohledu naznačovat chybnou původní diagnózu.

V rámci dětské populace jsou data, týkající se vlivu faktorů vnějšího prostředí na rozvoj IBD, ještě vzácnější. Jedním z nejsledovanějších jevů u dětských pacientů bylo kojení, jehož uvažovaný protektivní vliv vůči rozvoji IBD nelze z dosud provedených studií přesvědčivě potvrdit nebo vyvrátit [35, 36]. Stejně tak práce, jejichž cílem bylo ověření vlivu hygienické hypotézy na rozvoj IBD v dětském věku, nejsou dostatečné, aby bylo možné na jejich základě tuto otázku spolehlivě uzavřít [37]. Vysokou rozlišností v závěrech se prokázaly i práce zabývající se otázkou indukce IBD u dětských pacientů vlivem psychického stresu [38-40]. Naopak ochranným faktorem ve vztahu k rozvinutí IBD se zdá být pobyt mimo městské prostředí [41, 42].

Dalším intenzivně diskutovaným tématem je zvýšené riziko rozvoje IBD vlivem stravování. Ačkoliv výstupy provedených prací neumožnují vytvoření jednoznačného stanoviska, v rámci jedné ze studií byla prokázána asociace mezi vyšším příjmem vlákniny a nižším rizikem vzniku CD [43]. Další práce spíše naznačují negativní vliv „západního“ způsobu stravování [44, 45]. Jednoznačný vliv na rozvoj IBD nebyl popsán ani ve vztahu k infekcím nebo antibiotické léčbě [11, 37, 46].

1.3.3. Střevní mikrobiom

V současné době je k dispozici celá řada prací prokazující asociaci mezi změnou střevního mikrobiomu a aktivním zánětlivým střevním postižením [47, 48]. Změny ve specifickém zastoupení, ve smyslu chybění i přebývání, a diverzitě kmenů jsou i díky

intenzivně se rozvíjejícím novým technologiím velmi dobře dokumentovány [49]. K dispozici je také recentně publikovaná práce, která zachycuje vztah mezi tíží postižení a zastoupením konkrétních bakteriálních kmenů [50]. Opět však nelze určit, zda je změna střevního mikrobiomu příčinnou nebo důsledkem zánětlivě změněné střevní sliznice.

1.4. Diagnostika

Na základě aktuálních mezinárodních doporučení pro diagnostiku a léčbu dětských pacientů s IBD je definitivní stanovení diagnózy možné až po provedení endoskopického vyšetření, histologického vyšetření bioptických vzorků odebraných ze všech etáží trávicího traktu a zobrazení tenkého střeva, nejčastěji pomocí magneticko-rezonanční enterografie (MRe). Diagnóza je většinou definitivně stanovena po aplikaci tzv. revidovaných Portských kritérií pro diagnostiku IBD v dětském věku [1].

V případě klinického podezření na diagnózu IBD předchází indikaci k provedení endoskopického vyšetření v dětském věku několik kroků. V první řadě je doporučeno vyloučit infekční příčinu klinických příznaků. Po vyloučení infekční etiologické agens a při trvání klinických obtíží je provedeno komplexní laboratorní vyšetření zaměřené na parametry, které mohou naznačovat přítomnost imunitně podmíněného střevního zánětu, jako je sedimentace erytrocytů (ESR), počet trombocytů, hladina hemoglobinu, C-reaktivního proteinu (CRP) a albuminu [51-54]. Negativita těchto parametrů nevylučuje přítomnost zánětlivého střevního postižení a může být zachycena u pacientů s mírným střevním zánětem [53]. Dalšími laboratorními parametry, jejichž asociace s diagnózou CD nebo UC byla popsána v dříve publikovaných studiích, jsou protilátky proti Saccharomyces cerevisiae (ASCA) a protilátky proti cytoplazmě neutrofilů (ANCA) [54]. Dle dostupných dat se nicméně stanovení hladiny těchto imunologických parametrů nejeví jako přesnější s ohledem na určení diagnózy IBD, než využití běžně dostupných hematologických a biochemických parametrů [54]. Biomarkery získané ze stolice byly v tomto ohledu v mnoha předchozích pracích určeny jako nejcitlivější laboratorní parametry [55]. Asociace s přítomností střevního zánětu byla nejvíce studována ve vztahu k fekálnímu kalprotektinu (fCPT), jehož naměřená hladina je mezi laboratorními parametry aktuálně považována za jedno z nejdůležitějších rozhodujících kritérií k provedení endoskopického vyšetření u dětských pacientů [56-58].

Po rozhodnutí o indikaci je dle aktuálních doporučení v rámci endoskopického vyšetření u dětských pacientů s podezřením na IBD nutné provést esofagogastroduodenoskopii (EGDS) a totální kolonoskopii se zobrazením terminálního ilea a adekvátním odběrem

bioptických vzorků k histologickému vyšetření. Podmínkou dokončení diagnostického procesu je doplnění zobrazení tenkého střeva, nejčastěji pomocí MRe [1].

Na základě endoskopického nálezu je tedy v naprosté většině případů definitivně rozhodnuto o přítomnosti nebo nepřítomnosti IBD. Dalším krokem je využití všech získaných informací ke klasifikaci nově diagnostikovaného střevního zánětu do jednoho ze subtypů IBD. Aktuální klasifikace dětských pacientů s IBD je dokumentována v tzv. revidovaných Portských kritériích [1]. Ačkoliv recentně provedené práce poukazují na důležitost vnímat subtyp IBD s ohledem na pravděpodobnost odpovědi na určitý terapeutický režim [2] a tedy zohlednit atypické formy UC nebo CD, aktuálně užívaná Portská kritéria rozdělují dětské pacienty do tří tradičních fenotypů, CD, UC a IBD-U.

K bližšímu určení intenzity a podrobnější klasifikaci je v rámci dětské gastroenterologie užívána tzv. Pařížská modifikace [59] a Montrealské klasifikace [60] (Tabulka 1, 2).

Správné určení diagnózy a tím optimálně zvolená léčebná strategie může podstatně přispět k prevenci rozvoje opakovaných relapsů onemocnění a/nebo s IBD asociovaných komplikací [61]. Identifikace dalších parametrů umožňujících přesnější klasifikaci imunitně podmíněného střevního zánětu, a především predikci odpovědi na konkrétní terapeutický režim, má tak v péči o dětské pacienty s IBD široký význam.

Tabulka 1. Pařížská klasifikace pro pacienty s diagnózou CD [59]

Položka	**Zkratka**	**Popis**
Věk v době diagnózy	A1a	<10let
	A1b	10 – 16let
	A2	17 – 40let
	A3	>40let
Lokalizace onemocnění	L1	Postižení distální 1/3 ilea ± omezené cékální postižení
	L2	Postižení kolon
	L3	Ilekokolické postižení
	L4a	Postižení horního GIT po Treitzovo ligamentum
	L4b	Postižení horního GIT od Treitzova ligamenta po 1/3 ilea
Chování onemocnění	B1	Nestrikturující, nepenetrující

	B2	Strikturující
	B3	Penetrující
	B2B3	Strikturující a penetrující
	p	Perianální fistulující
Růst	G0	Bez známek poruchy růstu
	G1	Porucha růstu

Tabulka 2. Pařížská klasifikace pro pacienty s diagnózou UC [59]

Položka	**Zkratka**	**Popis**
Rozsah onemocnění	E1	Proktitida
	E2	Levostranná kolitida
	E3	Extenzivní kolitida (distálně od lineální flexury)
	E4	Pankolitida (proximálně od hepatální flexury)
Závažnost průběhu	S0	Bez akutní těžké kolitidy
	S1	Minimálně 1 x akutní těžká kolitida

1.5. Léčba

Léčba IBD je téměř vždy rozdělena do dvou částečně se překrývajících fází. Jako první je zahájena indukce remise, která je časně následovaná aplikací dlouhodobé udržovací léčby s cílem navozenou remisi udržet. Volba léčebné strategie se liší jak v závislosti na diagnóze, tedy pro pacienty s CD a UC, tak i v rámci jedné diagnózy, dle rozsahu a chování onemocnění, věku, přítomnosti poruchy růstu, možných nežádoucích účinků asociovaných s léčbu a kvality života pacienta [62, 63]. Význam personalizace léčebné strategie pacientů s diagnózu IBD je v posledních letech stále více zdůrazňován [63].

1.5.1. Crohnova choroba

U většiny dětských pacientů s nově stanovenou diagnózou CD je na základě aktuálních mezinárodních doporučení [63] volena indikce remise cestou tzv. výlučné enterální výživy (EEN), ev. kortikosteroidy při prokázané netoleranci EEN, a to i za využití naso-gastrické sondy k jejímu podání. U pacientů, kteří splňují kritéria mírné až středně těžké choroby, definované pomocí indexu aktivity onemocnění „weighted Pediatric Crohn's Disease Activity

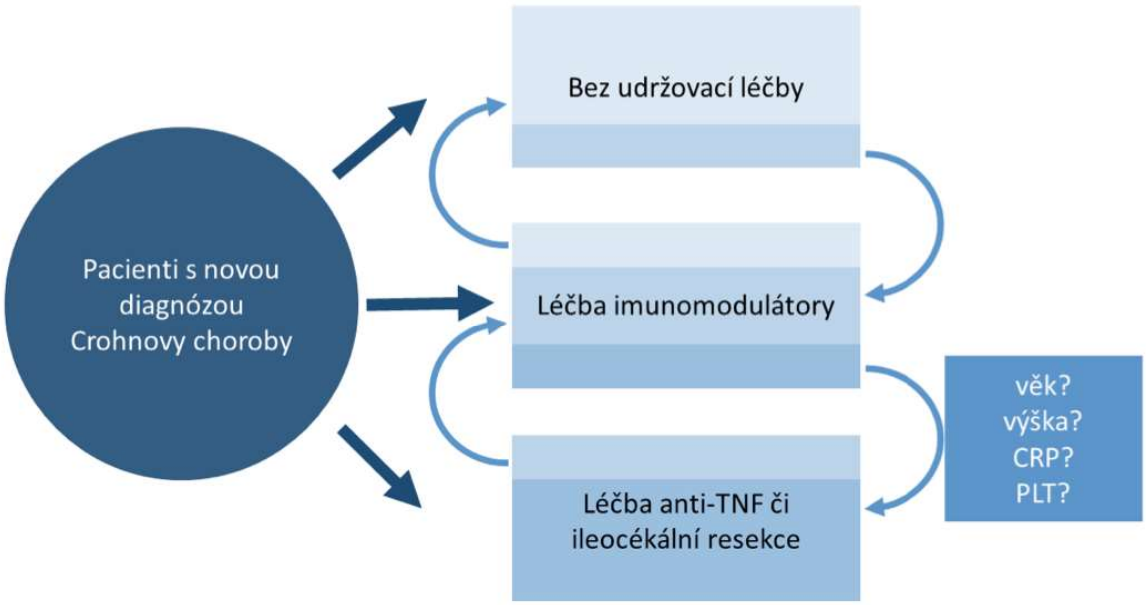
Index“ (wPCDAI) [64] <10 a >40 je dle recentně publikované práce [65] k navození remise možné využít specifického dietního režimu „Crohn’s Disease Exclusion Diet“ (CDED). Při srovnání EEN a CDED byla pacienty prokazatelně lépe tolerována CDED, obě terapeutické metody vedly k navození remise v šestém týdnu, trvající remise byla popsána u signifikantně většího počtu pacientů užívajících CDED. Poslední metodou, u které byl aktuálně prokázán efekt při navození remise CD u dětských pacientů, je kombinace antibiotické léčby [66].

Zdá se, že po úspěšném navození remise nemá již žádná z metod indukční léčby příliš velký efekt na její následné udržení, pokud není současně zahájena dlouhodobá udržovací terapie [67]. I v tomto ohledu lze volit z několika možností. Účinnost na udržení remise u dětských pacientů s diagnózou CD byla prokázána především u thiopurinů [68], které jsou aktuálně v tomto ohledu metodou volby [63]. Dalším lékem, jehož efekt na trvání remise byl popsán v dříve publikovaných pracích, nicméně provedených především na dospělých pacientech, je methotrexát (MTX) [69]. V rámci péče o dětské pacienty s CD je tak ve většině případů volen až při netoleranci nebo neefektivitě azathioprinu (AZA). K léčbě nejrizikovějších dětských pacientů s CD jsou používány preparáty biologické léčby, v první linii anti tumor nekrotizující faktory α (aTNF), infliximab (IFX) a adalimumab (ADA) [63].

Klíčovým krokem pro správně zvolený léčebný postup je identifikace jedinců ve vysokém riziku komplikovaného průběhu onemocnění, jak znázorňuje Obrázek 1. Prediktivní faktory obecně komplikovaného průběhu CD nejsou v dětské populaci dosud spolehlivě definované. Podstatně lépe jsou popsány u pacientů s CD dospělého věku. Z toho důvodu částečně vycházíme z dat získaných na dospělých pacientech i při predikci průběhu onemocnění u dětí [12]. Mezi uvažované prediktivní faktory patří: mladý věk v době diagnózy, extenzivní forma nemoci, postižení horní části gastrointestinálního traktu, lokalizace zánětu v ileu, perianální postižení, strikturující nebo penetrující forma CD, záchyt hlubokých ulcerací při endoskopickém vyšetření, kouření, nutnost užívání systémových kortikosteroidů v době diagnózy a některé varianty genu *NOD2* [61, 70-78].

Jako možné parametry predikující komplikovaný průběh nemoci u dětských pacientů byly zatím zkoumány následující: věk v době diagnózy, pohlaví, růstová retardace, index aktivity onemocnění „Pediatric Crohn´s Disease Activity Index (PCDAI) [64], chování a lokalizace onemocnění, perianální postižení, hladina CRP a fCPT v době diagnózy, míra imunitní reakce a určité formy genotypu. Studie zabývající se tímto tématem nejsou ve svých výsledcích jednotné a parametry, které by spolehlivě predikovaly komplikovaný průběh dětského IBD, nejsou stále jasně vymezené. Dle některých studií byly vyšší věk v době diagnózy, ženské pohlaví, růstová retardace v době diagnózy, lokalizace onemocnění v tenkém

střevě, SES-CD, perianální postižení, zvýšená hladina CRP a fCPT v době diagnózy, zvýšená imunitní reakce a varianty genu *NOD2* hodnoceny jako parametry s možnou prediktivní silou vzhledem k dalšímu průběhu nemoci [79-85].



Obrázek 1.
Drskova T., Hradsky O., Bronsky J., Identifikace prediktivních faktorů časného relapsu onemocnění u dětských pacientů s Crohnovou chorobou léčených thiopuriny, Čes-Slov ped 2017, 72 (5): 282-289

Dalším terapeutickým postupem, který je možné uplatnit u vybrané skupiny dětských pacientů s CD s lokalizovanou formou onemocnění, je ileocékální resekce (ICR). Indikací k provedení chirurgického výkonu je přítomnost striktury v ileocékálním přechodu s prestenotickou dilatací, penetrující forma onemocnění, těžká růstová retardace nebo refrakterita lokalizovaného zánětu k medikamentózní léčbě [63]. Ačkoliv je u malého počtu pacientů ICR kurativním výkonem, s ohledem na vysoké riziko rekurence onemocnění je u těchto pacientů doporučováno pravidelné endoskopické sledování. První postoperační endoskopie by měla být provedena do šesti až dvanácti měsíců od provedeného chirurgického výkonu [63]. V případě záchytu endoskopické rekurence zánětlivého postižení, definovaného pomocí endoskopického skórovacího systému dle Rutgeertse [86], je indikována eskalace terapie. U pacientů dosud neléčených aTNF mohou být ke snížení rizika postoperační rekurence podávány thiopuriny. Eskalace medikace, tedy aTNF preparátů, je doporučena ihned při

zachycení endoskopické rekurence onemocnění. U pacientů, kteří byli krátce před ICR léčeni aTNF preparáty, je doporučeno pokračovat po provedení chirurgického výkonu ve stejné medikaci [63]. Kouření, penetrující forma onemocnění, předchozí resekce střeva a pozitivní endoskopický nález šest měsíců po provedené resekci se zadají být rizikový faktory predikující nutnost další léčby u dospělých pacientů [87]. V rámci dětské populace je v současné době v tomto ohledu k dispozici jen velmi limitované množství evidence.

1.5.2. Ulcerózní kolitida

U dětských pacientů s diagnózou UC je dle aktuálních mezinárodních doporučení možno volit mezi dvěma způsoby indukce remise [62]. Rozhodujícím faktorem je především míra intenzity onemocnění. K objektivnímu hodnocení intenzity nemoci lze u dětských pacientů s UC efektivně využít klinického skórovacího systému „Pediatric Ulcerative Colitis Activity Index (PUCAI) [88] (Tabulka 3), jehož korelace s endoskopickou aktivitou onemocnění je velmi dobře dokumentována v dříve publikovaných pracích [89, 90]. Při PUCAI 10–35 bodů je doporučeno zahájení terapie 5-aminosalycyláty (5-ASA) podanými systémově (perorálně), eventuálně lokálně (rektálně) v případě endoskopického nálezu proktitidy. V případě PUCAI 40–60 bodů je s přihlédnutím na možnou alteraci celkového stavu doporučeno vedle aplikace 5-ASA zvážit podání systémových kortikosteroidů. Pokud je hodnota PUCAI 65 a více bodů, hovoříme o stavu tzv. akutní těžké kolitidy (ASC), která může být život ohrožující situací a jejíž léčba se řídí přesně definovaným protokolem [91]. Terapie je v tomto případě vždy zahájena intravenózně aplikovanými kortikosteroidy se současným přerušením podávání 5-ASA. Pokud nedojde po pěti dnech od zahájení léčby k poklesu PUCAI pod 65 bodů, je indikováno podání tzv. terapie druhé linie, mezi kterou patří IFX, tacrolimus nebo cyklosporin. Druhou alternativou této situace je provedení kolektomie, která pokud není provedena nyní, je indikována kdykoliv v dalším průběhu, nedojde-li ke zlepšení klinického stavu pacienta s poklesem PUCAI pod 65 bodů, nejpozději však čtrnáctý den od zahájení terapeutického postupu.

Tabulka 3. Pediatric ulcerative colitis activity index (PUCAI) [88]

Položka	**Body**
Bolest břicha	
Žádná	0
Může být ignorována	5

Nemůže být ignorována	10
Krev ve stolici	
Žádná	0
Malé množství v méně než 50 % stolic	10
Malé množství ve většině stolic	20
Velké množství krve (více než 50 % objemu stolice)	30
Konzistence většiny stolic	
Formovaná	0
Částečně formovaná	5
Zcela neformovaná	10
Počet stolic za 24 hodin	
0–2	0
3–5	5
6–8	10
>8	15
Noční stolice	
Ne	0
Ano	10
Denní aktivita	
Bez omezení aktivity	0
Občasné omezení aktivity	5
Významné omezení aktivity	10

Dlouhodobá léčebná strategie u dětských pacientů s UC je dle aktuálních doporučení volena s ohledem na odpověď na indukční terapii [62]. U pacientů, u nichž je zaznamenána dobrá reakce, definována jako pokles PUCAI o minimálně 20 bodů, nebo v lepší případě dosažení remise onemocnění (PUCIA 10 a méně bodů), je vhodné pokračovat v udržovací terapii pomocí 5-ASA. V případě trvající aktivity nebo opakovaných relapsů onemocnění na takto zavedené léčbě je doporučeno zahájení podávání thiopurinů. V případě chronicky aktivního nebo kortikodependentního zánětu, který nelze kontrolovat pomocí 5-ASA a/nebo imunomodulátoru, je u dětských pacientů s UC indikováno zahájení biologické léčby. Pokud nedojde k dosažení a udržení remise ani za aplikace preparátů biologické léčby, je třeba zvážit provedení kolektomie [62].

Rozhodování o léčebném postupu u dětských pacientů s UC na základě změn v PUCAI je limitováno především skutečností, že prediktivní hodnota PUCAI vůči průběhu onemocnění byla testována pouze ve vztahu k ASC [89, 90].

1.5.3. Volba terapeutického cíle: Treat-to-Target

Při hodnocení efektu zvolené léčebné strategie je třeba velmi dobře určit podle jakého faktoru je úspěšnost podávané terapie posouzena. Již při zahájení terapie by tak měl být jasně vymezen její cíl, jehož dosažení by mělo s co nejvyšší pravděpodobností predikovat nekomplikovaný průběh onemocnění. Identifikace terapeutického cíle se tak v rámci péče o pacienty s IBD zdá být jedním z nejdůležitějších kroků.

Se záměrem určení optimálních terapeutických cílů byl navržen koncept „Treat-to-Target“, založen na kombinaci informací získaných systematickým prohledáním dříve publikovaných prací a názorů odborníků [92]. Tento koncept, původně omezen pouze na dospělé pacienty s IBD, byl recentně revidován a aktuálně zahrnuje stanovené terapeutické cíle pro obě, dětskou i dospělou populaci [93].

Terapeutické cíle jsou v pojetí konceptu Treat-to-Target časově ohraničeny a vymezeny jako krátkodobé, střednědobé a dlouhodobé, nedosažení každého z nich by mělo vést ke změně a/nebo intenzifikaci terapeutického přístupu. V rámci dětské populace je klinická odpověď, definována jako pokles PCDAI o 12,5 bodu, pokles wPCDAI o 17,5 bodu pro pacienty s CD a pokles PUCAI o 20 bodů u pacientů s UC, zvolena cílem, kterého by mělo být dosaženo krátce po zahájení terapie. Jako střednědobý léčebný cíl autoři práce uvádějí dosažení klinické remise (PCDAI <10 bodů a PUCAI <10 bodů), normalizaci fCPT a CRP. V dlouhodobém časovém horizontu by cílem úspěšné léčby IBD u dětských pacientů měl být normální růst a endoskopicky hodnocené dosažení slizničního hojení.

1.5.4. Nežádoucí účinky a komplikace léčby

1.5.4.1. Thiopuriny

Léčba thiopuriny s sebou nese zvýšené riziko rozvoje gastrointestinální intolerance a reakcí alergického typu, myalgií a artralgií, hepatotoxicity, útlumu kostní dřeně a akutní pankreatitidy [64, 94-96]. V případě podávání thiopurinů je nejobávanějším, ačkoliv velmi vzácným, vedlejším účinkem mírně zvýšené riziko výskytu některých malignit [97]. Imunosupresivní léčba stejně jako chronicky probíhající zánět mají velmi pravděpodobně podíl na rozvoji lymfoproliferativních onemocnění. Ačkoliv odlišit, která ze dvou složek se na vzniku lymfoproliferace podílí více, je komplikované, dle dostupných dat se zdá riziko rozvoje

lymfoproliferativního onemocnění v asociaci s thiopruriny tři až šestkrát pravděpodobnější [98-100]. Na rozvoji dvou typů lymfomů, které byly častěji identifikovány u pacientů užívajících imunosupresivní léčbu, má významný podíl infekce Epstein-Barrové virem (EBV). Monitorace EBV sérologického statusu je doporučována v rámci rutinních vyšetření před zahájením aplikace thiopurinů [63].

1.5.4.2. Biologická léčba

Rozvoj nežádoucích účinků je důležitým faktorem, který může značně limitovat aplikaci aTNF. Nejčastějším projevem jsou v tomto ohledu kožní léze psoriasiformního, méně často atopického charakteru. V retrospektivní práci byl u 84 dětských pacientů s IBD jakýkoliv kožní projev ve vztahu k IFX popsán u 48 % z nich po 2 letech sledování [101]. Podávání aTNF medikace je spojeno s vyšším rizikem závažně probíhajících a oportunních infekcí [102]. Vzhledem k nepostradatelné úloze cytokinu TNFα v potlačení infekce vyvolané mykobakterii je před zahájením aplikace aTNF nezbytné vyloučit latentní formu tuberkulózy. Za tímto účelem je dle aktuálních doporučení hodnocen klinický stav pacienta, prostý rentgenový snímek hrudníku, výsledek kožního tuberkulinového testu Mantoux II a negativita interferon – γ release assay (IGRA) [63]. Závažnou komplikací, která vede k nutnosti ukončení aplikace konkrétního aTNF preparátu, je akutní infuzní reakce, vyvolaná v některých případech přítomností protilátek proti aTNF, které mohou mít za následek i ztrátu odpovědi k podávanému léku. Mezi dětskými pacienty byla v dříve provedené prospektivní studii tato ztráta odpovědi zaznamenána u 39 % pacientů v 60. týdnu od zahájení léčby [103]. Z pohledu ztráty odpovědi k podávanému aTNF, je možné odlišit dvě situace. První z nich je tzv. farmakokinetické selhání, o kterém hovoříme v případě přítomnosti protilátek proti aTNF preparátu a/nebo nedostatečných sérových hladinách léku. Farmakodynamickým selháním rozumíme situaci, kdy nejsou přítomny protilátky a sérová hladina aTNF je současně dostatečná. Farmakokinetiku aTNF léků lze do jisté míry ovlivnit současným podáváním immunomodulátoru [104-106].

2. Cíle práce

Primárním cílem disertační práce je identifikace prediktivních faktorů průběhu IBD u dětských pacientů a jejich následné využití v konkrétních klinických situacích. Spektrum klinických situací, řešených v rámci této práce, zahrnuje možnost predikce diagnózy, obecně komplikovaného průběhu onemocnění, odpovědi na konkrétní terapeutický režim, rozvoj nežádoucích účinků asociovaných se zvoleným terapeutickým postupem a adherence pacienta k léčbě.

Možnost predikovat určitý jev v klinické praxi umožňuje včasně reagovat a optimalizovat péči o pacienta a tím předejít komplikovanému průběhu onemocnění nebo rozvoji s onemocněním asociovaných komplikací, eventuálně podávanou léčbou. V mnoha ohledech lze tak díky znalosti prediktivních faktorů významně přispět k lepší kontrole onemocnění a vyšší kvalitě života pacienta.

3. Metody

3.1. Datové soubory

Získání optimálního datového souboru k účelu identifikace prediktivních faktorů je možné a efektivní jak s využitím retrospektivního, tak prospektivního přístupu. Ačkoliv je v mnoha ohledech design kontrolované randomizované studie (RCT) jistě nejprůkaznějším, v případě identifikace nezávislých prediktivních faktorů může mít důležité limitace. RCT jsou obecně finančně nákladné, obtížně realizovatelné, a především v rámci dětské populace nemusí splnit některé důležité požadavky, jako je dostatečně dlouhá doba sledování a adekvátní množství pacientů ve sledovaném souboru. Jako zcela ideální by se jevilo identifikovat prediktivní faktor a následně pomocí RCT testovat jeho klinický význam. Za nejpříhodnější zdroj vstupních dat pro zisk těchto prediktivních faktorů lze považovat dlouhodobé, mezinárodní, ideálně prospektivní registry.

Z celkem devíti původních prací byly tři provedeny v prospektivním designu jako observační studie [107-109]. V retrospektivním designu bylo provedeno celkem pět prací [110-114]. Poslední práce byla provedena jako retrospektivní analýza prospektivně získaného souboru dětských pacientů s diagnózou CD [115]. Dvě z těchto prací byly realizovány jako multicentrické projekty v rámci mezinárodní spolupráce [114, 115] pod záštitou pediatrické pracovní IBD skupiny „Porto group“ při Evropské společnosti pro pediatrickou gastroenterologii, hepatologii a výživu / The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN). Jedna z těchto prací byla navržena a koordinována cestou Pediatrické kliniky 2. lékařské fakulty Univerzity Karlovy a Fakultní nemocnice Motol (PK 2. LF UK a FN Motol) [115].

Datové soubory pro realizaci všech projektů byly získány jednak od dětských pacientů s diagnózou IBD sledovaných na PK 2. LF UK a FN Motol, tak od pacientů sledovaných v jiných tuzemských i světových centrech specializovaných v péči o dětské pacienty s IBD. Zásadním zdrojem dat jedné ze studií [115] byl mezinárodní incidenční registr EUROKIDS [116] se sídlem v Rotterdamu, Holandsko.

3.2. Určení cíle predikce

V rámci metodického postupu každé práce mělo významnou roli stanovení primárního jevu, který byl cílem predikce, tedy tzv. outcome. Pro každý cíl existuje jiný soubor vhodných prediktorů, jejichž spektrum se tedy významně mění v závislosti na stanoveném outcome. V rámci osmi původních prací jsme vybrali následující cíle: určení diagnózy, předpokládaný charakter průběhu onemocnění, nutnost změny konkrétního terapeutického přístupu, trvající

efekt vybrané léčebné strategie, nutnost aplikace kombinované léčby, endoskopická aktivita onemocnění, rozvoj nežádoucích účinků asociovaných s terapií a adherence pacienta k léčbě.

3.3. Inovativní metodické postupy

Pro realizaci některých z projektů byly využity částečně experimentální postupy, které nejsou aktuálně standardizovanými, plně etablovanými metodami běžné klinické praxe [108-111].

Jednou z takových metod je stanovení hladiny tkáňového kalprotektinu (tCPT). Za tímto účelem byly využity uložené histologické vzorky šesti segmentů kolon po předchozí standardní aplikaci hematoxylinu a eozinu. Tkáňové řezy o tloušťce 1 mikrometr (μm) byly deperafinizovány a obarveny specifickým médiem s obsahem primárního kalprotektinového (CPT) antigenu (Invitrogen v ředění 1:1000). Míra exprese tCPT byla určena jako nejvyšší počet pozitivních buněk na jednotku plochy označovanou jako high power field (HPF).

Druhým inovativní postupem bylo imunohistochemické hodnocení exprese CD30 pozitivních (CD30+) buněk ve střevní sliznici. Podobně jako u předchozího postupu bylo na deparafinizované 1 μm silné řezy tkáně aplikováno médiu s obsahem primárního CD30 antigenu (Cell Marque v ředění 1:50). Exprese byla opět určena jako nejvyšší počet pozitivních buněk na HPF.

Další částečně experimentální metodou bylo využití nově vyvinutého testu určeného ke kvantitativnímu stanovení hladiny fCPT v domácím prostředí (IBDoc, Buhlmann). Set obsahuje extrakční nástroj k odběru přesného množství stolice (Calex Valve), zkumavku s médiem, do nějž je vzorek ihned po odběru aplikován, a destičku určenou pro následné zpracování směsi vzorku stolice a média. Výsledek je odečten pomocí aplikace chytrého mobilního telefonu, kde je zobrazen ihned po provedeném měření a zároveň odeslán do webového úložiště IBDoc Portal. Hladina fCPT je stanovena na základě imunochromatografické analýzy.

Poslední takovou metodou, která byla použita v rámci této práce, je stanovení hladin metabolitů AZA. Za tímto účelem byla pacientům odebrána jedna zkumavka EDTA (ethylenediaminetetraacetic) plné krve. Ihned po odběru byly za využití centrifugace odděleny leukocyty a plazma. Zbylé erytrocyty byly následně dvakrát promyty, naředěny v poměru 1:1 vodou a zamraženy na minus 20 °C do definitivního odečtení. Zpracování vzorků bylo provedeno dle protokolu Dervieux a Boulieu [117]. Hladiny metabolitů AZA byly odečteny pomocí Agilent 1100 (vysoce účinná kapalinová chromatografie s více vlnovým detektorem).

3.4. Statistická analýza

Všechna data v rámci realizovaných projektů byla analyzována pomocí statistického software R. Přesnost diagnostických metod byla srovnána pomocí DeLong testu ROC (receiver operating characteristic) křivek. V případě sledování pacientů v průběhu času byly použity survival analýzy a Cox regrese, pro opakované měření jednoho pacienta byl aplikován mix model.

4. Výsledky

Obecně lze výsledky jednotlivých studií shrnout jako získání souboru nových informací, na základě kterých lze předem odhadnout určitý jev, jehož včasné ovlivnění nebo lépe jehož předejití vede ke zlepšení péče o dětské pacienty s diagnózou IBD. Prediktory byly vždy hledány ve vztahu ke konkrétní klinické situaci, jako je diagnóza, komplikovaný průběh onemocnění, volba léčebné strategie, odhad jejího efektu nebo bezpečnosti, a tím jsou velmi dobře aplikovatelné v běžné klinické praxi.

4.1. Predikce diagnózy

S cílem zpřesnit diagnostický proces a umožnit predikci další diferenciace zánětlivého střevního onemocnění, které není v době provedení první endoskopie jednoznačně zařaditelné, byla provedena imunohistochemická analýza CD30+ buněk v bioptických vzorcích střevní sliznice [110]. Míra exprese CD30+ buněk byla mezi pacienty s diagnózou CD a UC statisticky významně odlišná při srovnání mediánů, průměrů i maximálních hodnot jak absolutních počtů CD30+ buněk v celé střevní sliznici, tak při hodnocení jednotlivých střevních segmentů (terminální ileum, kolon transversum, kolon descendens a rektum). Nejnápadnější rozdíl v tomto ohledu byl zachycen při srovnání počtů CD30+ buněk ve střevní sliznici z rekta. Rozlišení mezi pacienty s CD a UC pomocí exprese CD30+ nebylo v naší práci závislé na intenzitě střevního zánětu. Na základě nově zjištěných údajů, lze tak imunohistochemické barvení bioptických vzorků střevní sliznice na CD30+ buňky považovat za spolehlivou a jednoduchou metodu, která může jako podpůrný parametr významně přispět k diferenciální diagnostice mezi jednotlivými subtypy IBD u dětských pacientů naivních k léčbě [110].

4.2. Predikce aktivity onemocnění

4.2.1. Endoskopický nález

Cílem další práce bylo rozšířit spektrum spolehlivých možností k určení aktivity onemocnění. K tomuto účelu jsme se rozhodli ověřit, zda lze bezpečně používat u lůžka provedené, telefonem snímané vyšetření fCPT, který je v rámci sledování pacientů s IBD nejdůležitějším laboratorním parametrem. Hladiny fCPT získané pomocí testu navrženého pro domácí použití byly srovnány s hladinami získanými cestou standardizované laboratorní metody ELISA (enzyme-linked immuno sorbent assay). Jako zlatý standard, ke kterému bylo toto srovnání vztaženo, byla zvolena skutečná endoskopická aktivita, definovaná pomocí endoskopických skórovacích systémů. [108].

Statisticky významná asociace s endoskopicky určenou mírou slizničního hojení byla nalezena v obou případech, tedy jak při srovnání s výsledky hladin fCPT získaných metodou ELISA, tak naměřených hodnot pomocí domácího testu. Při srovnání obou metod ke stanovení fCPT byla signifikantně spolehlivějším prediktorem slizničního hojení hladina fCPT získaná pomocí metody ELISA. Domácí test by tak na základě výsledků této práce neměl nahradit rutinní sledování aktivity onemocnění pomocí laboratorně získané hladiny fCPT, s výhodou může být využit pro získání orientačního výsledku v rámci tzv. self-monitoring onemocnění a urychlit tak zjištění o narůstající aktivitě střevního zánětu.

4.2.2. Komplikovaný průběh onemocnění

V další práci jsme se zabývali predikcí obecně komplikovaného průběhu UC. Hlavním cílem bylo posoudit užitečnost imunohistochemicky hodnoceného kalprotektinu získaného ze střevní sliznice (tCPT) [111]. K takovému účelu nebyl dle dostupných informací tCPT nikdy dříve využit.

Korelace mezi tCPT a histopatologickými skórovacími systémy byla hodnocena jako dobá, korelace tCPT s fCPT, PUCAI a UCEIS jako mírná. Hladina tCPT nebyla stejně jako endoskopická aktivita onemocnění (definovaná pomocí UCEIS) nebo histopatologické skórovací systémy (Geboes a Nancy skóre) prokázána jako faktor predikující komplikovaný průběh UC v době stanovení diagnózy. Je tedy třeba shrnout, že význam imunohistopatologického vyšetření v predikci komplikovaného průběhu onemocnění u dětských pacientů s UC zůstává nejistý. Přestože se nám nepodařilo prokázat, že by tCPT predikoval komplikovaný průběh UC, nalezli jsme významnou prediktivní hodnotu vůči rozvoji komplikovaného průběhu onemocnění u hladiny fCPT a aktivity onemocnění definované pomocí indexu PUCAI získaných v době diagnózy.

4.3. Léčba

4.3.1. Predikce individuálního rizika relapsu ve vztahu ke konkrétnímu terapeutickému postupu

Možnost individuální predikce časného selhán léčby thiopuriny u dětských pacientů s diagnózou CD v době stanovení diagnózy, byla cílem velkého mezinárodního projektu, na jehož realizaci se podílelo třináct evropských center a byl koordinován na Pediatrické klinice 2. LF UK a FN Motol [115]. Podařilo se nám identifikovat šest nezávislých prediktivních faktorů časného selhání: BMI Z-skóre v době diagnózy, lokalizace onemocnění v době diagnózy (abnormální nález na zobrazovacích metodách v oblasti kolon sigmoideum,

abnormální endoskopický nález jícnu a ileokolické postižení), hladina CRP v době diagnózy a interakce věku a dávky AZA/6-MP. Kombinací těchto nově zjištěných parametrů byl vytvořen predikční model umožňující stanovit individuální odhad doby do relapsu dětského pacienta s nově stanovenou diagnózou CD při léčbě AZA/6-MP. Za účelem jednoduchého převedení predikčního modelu do klinické praxe jsme vytvořili webovou aplikaci, která po zadání jednotlivých parametrů, z doby před zahájením léčby, vyčíslí individuální riziko časného selhání a pomocí křivky přežití graficky znázorní pravděpodobnost udržení remise při léčbě AZA/6-MP: https://gastroped.shinyapps.io/AZA_time_to_relapse/. S ohledem na další výstup této práce, který prokázal trvající remisi onemocnění při léčbě AZA/6-MP i po 2 letech u 50,3 % pacientů, lze thiopuriny stále považovat za vhodnou léčebnou strategii pro pediatrické pacienty s diagnózou CD. Jejich efektivní aplikaci lze předpokládat především u skupiny CD pacientů vybraných na základě nově vyvinutého predikčního modelu.

4.3.2. Predikce trvající efektu konkrétního léčebného postupu

Otázkou, kterou si kladla další práce, bylo, zda je možné pomocí jednoduše dostupného laboratorního parametru, který reflektuje slizniční hojení, fCPT, predikovat trvající efekt konkrétního léčebného postupu, indukce remise pomocí EEN. Nepodařilo se nám prokázat, že by změna koncentrace fCPT ve druhém týdnu od zahájení EEN (týden 0) predikovala pacienty, kteří nedosáhnou odpovědi na EEN v 6. týdnu. Časně získanou hladinu fCPT po zahájení EEN u dětských pacientů s nově diagnostikovanou CD tedy není možné považovat, za dostatečný parametr umožňující selekci pacientů, kteří neodpoví na EEN ani na jejím konci a tím na základě této informace indukční léčbu změnit [87].

4.3.3. Predikce rozvoje nežádoucích účinků asociovaných s konkrétním terapeutickým postupem

Limitujícím faktorem úspěšnosti medikace je vedle jejího přímého efektu i riziko rozvoje nežádoucích účinků, jejichž přítomnost může do jisté míry ovlivnit i compliance / adherenci pacienta k podávané léčbě. V kohortě dětských pacientů s diagnózou CD léčených aTNF preparátem jsme hledali prediktivní faktory, které by nám pomohly identifikovat pacienty, kteří mají častěji kožní nežádoucí účinky. Zjistili jsme, že takovým faktorem je léčba IFX (ve srovnání s ADA), a to jak pro jakoukoliv neinfekční kožní lézi, tak pro atopickou dermatitidu. Z klinického pohledu je třeba počítat až s dvojnásobným rizikem rozvoje kožních nežádoucích účinků aTNF při volbě IFX a až šesti násobným zvýšením rizika rozvoje atopické dermatitidy. Dalším zajímavým zjištěním byla asociace kožních nežádoucích účinků s nízkými

CRP, která vypovídá o dobré kompenzaci CD. Lze shrnout, že v porovnání s adalimumabem lze IFX považovat za prediktor rozvoje kožních nežádoucích účinků aTNF terapie [112].

4.3.4. Predikce adherence k léčbě

Za využití jednoduše přístupných biochemických a hematologických parametrů (střední objem erytrocytu, šíře distribuce erytrocytu, střední koncentrace hemoglobinu v erytrocytu, hematokrit, počet trombocytů, sérová hladina albuminu, železa a saturace transferinu), věku a pohlaví byly vytvořeny modely umožňující predikovat pacienty s nízkou hladinou metabolitů AZA (6-TGN < 125pmol/8 x 10^8 v erytrocytu a 6-MMP < 5700pmol/8 x 10^8 v erytrocytu), kteří budou pravděpodobně profitovat z eskalace medikace a pacienty s nedetekovatelnou hladinou 6-TGN a hladinou 6-MMP < 240pmol/8 x 10^8 v erytrocytu, tedy velmi pravděpodobně non-adherentní k terapii AZA [113]. S cílem zjednodušit přístup k nově získaných informací v běžné klinické praxi, byly vytvořeny webové aplikace: https://hradskyo.shinyapps.io/6TG_prediction/ and https:// hradskyo.shinyapps.io/Non_adherence/)

4.3.5. Predikce efektu kombinované léčby

Cílem další práce bylo posouzení užitečnosti monitorovat hladiny metabolitů AZA u pacientů na kombinované terapii (AZA a aTNF). Zjistili jsme, že dostatečné hladiny 6-TGN v erytrocytu jsou asociovány se sérovými hladinami aTNF. Hodnota 6-TGN v erytrocytu nad 278 pmol/8 x 10^8 se jeví jako optimální k dosažení cílové sérové koncentrace infliximabu nad 5 μg/ml. Signifikantně vyšší míra ztráty odpovědi k aTNF byla zaznamenána v případě nedetekovatelných hladin 6-TGN. Na základě nově získaných informací lze monitoraci metabolitů AZA považovat za užitečnou při optimalizaci kombinované terapie u dětských pacientů s diagnózou CD [109].

4.4. Mezinárodní observační průzkum

Výsledkem mezinárodní spolupráce je přehled vakcinačního a imunizačního stavu dětských pacientů s diagnózou IBD [48]. Přestože studie svým primárním zaměřením necílí na identifikace prediktivních faktorů, upozorňuje na důležitý aspekt preventivní péče o dětské pacienty s diagnózou IBD. Analýza souboru 430 dětí prokázala nedostatečnou míru serologické odpovědi proti virovým infekcím u dětských pacientů s IBD, který nebyl následován přiměřenou vakcinací. Doporučená vakcinace byla kompletní u 8,8 % sledovaných pacientů. Doporučované rutinní prověření přítomnosti nebo prodělání infekce virem Epstein-Barrové

(EBV) před zahájením aplikace imunosupresivní léčby a vyloučení latentní formy tuberkulózy před první aplikací biologické léčby nebylo dle aktuálních doporučení ESPGHAN možné na základě získaných dat hodnotit jako adekvátní.

5. Diskuze

Ačkoliv, s výjimkou jedné, všechny původní práce spojuje primární záměr přispět ke zlepšení péče o dětské pacienty s IBD díky nově identifikovaným možnostem predikce konkrétního klinického jevu, jednotlivé zvolené klinické situace se od sebe mírně liší a bude tak vhodnější diskutovat výsledky práce postupně.

5.1. Predikace diagnózy: Imunohistochemické hodnocení slizniční exprese CD30+ buněk

Role CD30 antigenu u pacientů s diagnózou IBD nebyla dosud spolehlivě určena. S cílem ověření užitečnosti tohoto parametru v diagnostice IBD byla dosud provedena pouze jedna práce [118]. V rámci této studie byla srovnána míra exprese CD30+ ve sliznici nejvíce postižených úseků střeva u dospělých pacientů s CD a UC. Stejně jako v námi provedené práci [110], byl i v této studii prokázán statisticky signifikantní rozdíl v počtech zachycených CD30+ buněk u pacientů s CD a UC. Nicméně rektální lokalizace a absolutní počet CD30+ v rámci celého kolon byly v námi provedené práci [110] prokázány jako lepší rozlišující faktor mezi CD a UC ve srovnání se vzorky odebranými z nejvíce postiženým úsekem střeva [118].

Další diskutovanou otázkou plynoucí z výsledků námi provedené studie může být využití CD30+ tkáňové exprese pro lepší klasifikaci pacientů s diagnózou IBD-U. Ačkoliv, jak již bylo zmíněno výše, aktuální kritéria [1] pro diagnostiku IBD v dětském věku se kloní k tradičnímu rozdělení pacientů na CD a UC s pojetím IBD-U jako spíše přechodné diagnózy, recentně publikovaná práce [2] naznačuje, že IBD-U může být samostatným subtypem IBD s vlastní charakteristikou a dokonce genetickým profilem. Při srovnání počtů slizničních CD30+ buněk mezi pacienty s IBD-U a CD nebyl prokázán rozdíl, naopak statisticky významný rozdíl byl popsán při porovnání míry exprese slizniční CD30+ u pacientů s IBD-U a UC. Toto zjištění naznačuje možnou větší blízkost diagnózy IBD-U s CD než UC a liší se tak od aktuálních doporučení, na základě kterých je léčba IBD-U shodná s léčbou UC.

5.2. Predikce aktivity onemocnění

5.2.1. Přesnost stanovení hladiny fekálního kalprotektinu pomocí domácího testu

Různá úroveň korelace mezi hladinami fCPT získanými pomocí jednoho z aktuálně tří dostupných testů pro domácí použití a standardní laboratorní metody ELISA byla prokázána ve více dříve provedených pracích realizovaných v rámci dospělé i dětské populace [119-123]. Žádná s těchto studií nicméně nezohledňuje klinická data, ale pouze porovnává jednu metodu vůči druhé. S ohledem na chybějící zlatý standard pro určení skutečné klinické aktivity, je tak obtížné na základě provedených prací hodnotit, která z metod je přesnější. Ze stejného důvodu

nebylo možné v rámci dříve provedených prací určit optimální cut-off hladiny fCPT, která by predikovala slizniční hojení. Hodnoty jednotlivých cut-off spočítané v naší práci pro obě metody měření se od sebe statisticky významně liší a je tak třeba upozornit, že výsledky získané jednou metodou nelze volně srovnat s výsledky druhé metody. V rámci rozsáhlé meta-analýzy byla optimální hladina cut-off predikující slizniční hojení stanovena na 250 ug/g za využití standardní laboratorní metody ELISA [124]. Cut-off 136 ug/g zjištěný v námi provedené práci lze považovat za blížící se této hodnotě [108]. Hladina cut-off zjištěná pro domácí testy se pohybovala v rozmezí od 50 do 150 ug/g [119, 120, 123], v žádné z prací však nebyla počítána ve vztahu k endoskopickému skóre, ale pouze s ohledem na výši fCPT získanou metodou ELISA. Vzhledem k tomu, že v naší práci byla hodnota cut-off hledána ve vztahu k endoskopickému skóre, lze ji považovat za nejlépe určenou a v klinické praxi ji doporučujeme používat (fCPT 48 ug/g) [108].

5.2.2. Limitované využití tkáňového kalprotektinu pro predikci komplikovaného průběhu ulcerózní kolitidy

S přihlédnutím k limitacím, jako jsou velmi nízká specificita, vysoká intraindividuální variabilita nebo přirozeně vyšší hodnoty v nejmladším věku [125-129] jinak spolehlivě zavedeného neinvazivního parametru intenzity střevního postižení, fCPT [130], byla provedena první studie ověřující možnost využití tCPT v péči o dětské pacienty s diagnózou IBD.

Aktuálně jsou k dispozici pouze dvě práce, obě provedené na dospělých pacientech, které se v jistém ohledu zabývaly tCPT. V jedné z těchto prací byla prokázána pouze asociace nízké hladiny tCPT s klinickou, endoskopickou i mikroskopickou remisí u pacientů s UC, a naopak vyšší hladina tCPT byla asociována s horším klinickým průběhem onemocnění [131]. Ve druhé práci byla popsána pouze zvýšená hladina tCPT zachycená u pacientů s IBD [132].

Stejně jako v předchozí studii [131] jsme v námi provedené práci prokázali asociaci mezi hladinou tCPT a histopatologickými skórovacími systémy odrážejícími mikroskopickou aktivitu onemocnění, nicméně prokázat prediktivní hodnotu tCPT vůči komplikovanému průběhu onemocnění u dětských pacientů s diagnózou UC se nepodařilo [111].

5.3. Predikce výsledků léčby

5.3.1. Predikce času do relapsu u dětských pacientů s Crohnovou chorobou léčených thiopuriny

Jedním z výstupů této multicentrické, mezinárodní práce byla observace prvního klinického relapsu onemocnění do dvou let od zahájení terapie thiopuriny u 49, 7 % dětských

pacientů s CD, u nichž byla dosažena remise po indukční léčbě EEN nebo kortikosteroidy [115]. Ačkoliv tato data nejsou natolik optimistická jako výsledek jediné RCT provedené na dětských pacientech s IBD, v rámci které byla remise popsána u 90 % sledované populace po dvou letech užívání thiopurinů [68], jsou téměř shodná s menšími retrospektivními pracemi [67, 133], ukazujícími přibližně 50 % úspěšnost léčby thiopuriny po dvou letech.

Ačkoliv se dle recentně provedené RCT [134] jeví aplikace intenzivního terapeutického režimu, biologické léčby, časně po stanovení diagnózy, jako velmi efektivní způsob dosažení a udržení remise u dětských pacientů s CD, má tento postup své limitace. Mezi tato omezení patří např. vysoká cena léku, limitovaná doba účinku nebo zvýšené riziko komplikovaných a oportunních infekcí [102, 103, 135-138]. Mimo to velmi pravděpodobně existuje skupina dětských pacientů s CD, kteří nevyžadují zahájení intenzivního terapeutického přístupu časně po stanovení diagnózy a pro kterou jsou thiopuriny vhodnou volbou k udržení remise onemocnění. Nicméně na základě dosud publikovaných dat nebylo možné spolehlivě tyto pacienty určit.

Práce, které byly zaměřeny na identifikaci prediktorů obecně komplikovaného průběhu CD v dětském věku [81-85, 139] se od sebe liší v mnoha ohledech, jako je např. definice outcome, zahrnutá populace nebo výběr sledovaných proměnných. Žádná z těchto provedených studií se nezaměřila na posouzení odpovědi na konkrétní terapeutický režim.

Ačkoliv lze do jisté míry využít prediktory obecného průběhu onemocnění, znalost prediktivních faktorů časného selhání specifického terapeutického režimu se zdá být vhodnější. S primárním cílem identifikace prediktorů časného selhání thiopurinů u dětských pacientů s CD byla provedena pouze jedna retrospektivní práce [133] v rámci, které nebyl ze studovaných parametrů (věk, pohlaví, lokalizace a chování onemocnění, přítomnost perianálního fistulujícího onemocnění a extraintestinální manifestace v době diagnózy) nalezen žádný prokazatelný prediktivní faktor. Menší retrospektivní práce, jejímž primárním cílem bylo srovnání indukčních terapeutických režimů (EEN, kortikosteroidy, biologická a chirurgická léčba) u dětských pacientů s CD, popsala věk pod 16 let a postižení horního gastrointestinálního traktu v době diagnózy jako prediktivní vůči časnému selhání thiopurinů, dalšími sledovanými parametry bylo Z-skóre BMI v době diagnózy a elevace trombocytů v době remise [67]. Jiná práce, primárně zaměřená na prediktivní hodnotu histopatologických skórovacích systémů, popsala SES-CD nad 16 bodů v době stanovení diagnózy jako prediktor rozvoje komplikovaného průběhu onemocnění [81]. Nicméně žádný stanovený prediktor nebyl dostatečně silný, aby mohl být spolehlivě využit v klinické praxi. Vhodnou cestou se tak zdá být kombinace více slabších prediktorů.

V rámci dospělé populace pacientů s IBD bylo již několik predikčních modelů odhadující individuální riziko relapsu onemocnění vyvinuto [140, 141]. Avšak srovnání těchto modelů s námi vyvinutým prvním predikčním modelem pro dětské pacienty s CD je s ohledem na odlišnost v mnoha aspektech významně limitováno. Ani jedna z prací nezohledňuje zvolený terapeutický režim u zařazených pacientů, liší se ve studované populaci i stanovených outcome.

5.3.2. Predikce časné odpovědi na zvolený terapeutický režim pomocí fekálního kalprotektinu

Jak již bylo ve výsledcích uvedeno, nepodařilo se nám prokázat, že by hladina fCPT ve druhém týdnu od zahájení EEN byla klinicky užitečným prediktorem trvající odpovědi v době jejího ukončení. Nabízí se tedy otázka, kdy je k hodnocení odpovědi na indukční terapii cestou EEN pomocí fCPT vhodná doba. Jiná studie, která sledovala pokles sérologických parametrů zánětu a fCPT po dobu trvání indukční terapie EEN ukazuje, že zvolený dvoutýdenní interval je možná až příliš krátký, aby během něj mohlo dojít ke změnám takto citlivého parametru [142]. Reakce fCPT byla v této studii prokazatelně nejpomalejší ve srovnání s ostatními sledovanými proměnnými. Další práce prokázala hladinu fCPT získanou až třicátý den od zahájení EEN jako prediktivní faktor trvající odpovědi na konci intervalu EEN [143]. Pro klinické použití je takový časový interval již příliš dlouhý a lépe než odhadovat, zda pacient dosáhne remise např. ve 4. týdnu, se zdá racionálnější počkat do konce EEN, tedy týdne 6. Další práce naopak neprokázala změnu v naměřené hladině fCPT od zahájení EEN do čtvrtého a ani dvanáctého týdne jako prediktor trvající remise po ukončení EEN [144].

Původní otázku, na kterou nelze na základě dosud publikovaných dat zatím spolehlivě odpovědět, podporuje i naše zjištění, že středně zvýšená hladina fCPT v časné fázi aplikace EEN nevylučuje její pokles v době ukončení této indukční terapie [107].

5.3.3. Rizikové faktory rozvoje nežádoucích účinků biologické léčby

Stejně jako v námi provedené práci byl i v jediné studii realizované v rámci dětské populace zahrnující oba aTNF preparáty první linie, IFX ve srovnání s ADA prokázán jako rizikový faktor rozvoje kožních psoriasiformích lézí [145]. Naopak toto zvýšené riziko rozvoje kožních komplikací nebylo opakovaně potvrzeno v rozsáhlých studiích provedených na dospělých pacientech s IBD [146-148], u kterých se obecně incidenci kožních komplikací asociovaných s aTNF zdá být nižší [147]. S ohledem na fakt, že v rámci naší práce byla prokázána silná asociace IFX i k zvýšenému riziku rozvoje atopické dermatitidy, a to i nezávisle na rodinné anamnéze atopie, lze uvažovat o otázce, zda při volbě aTNF v péči o dětské pacienty

s IBD nepreferovat ADA, u kterého byla zaznamenána mírně nižší frekvence nežádoucích účinků kožního typy a jehož bezpečnost a účinnost je srovnatelná s IFX [112]. S přihlédnutím k limitacím a síle datového souboru naší práce není na jejím podkladě možné takto pevný závěr učinit, nicméně v rámci personalizace přístupu lze uvažovat např. o favorizaci ADA u dětských pacientů s rodinnou anamnézou atopie.

5.3.4. Adherence k užívání thiopurinů

Stanovení hladin metabolitů AZA je ideální způsob, jak odhadnout pacienty, u nichž je dávka léku příliš nízká, nebo kteří neužívají léčbu adekvátně. Problematická může být dostupnost tohoto vyšetření v řadě center specializujících se v péči o dětské pacienty s diagnózou IBD. Námi vytvořený model umožňuje predikovat tyto hladiny na základě snadno dostupných laboratorních parametrů. Asociace nízkých hladin 6-MMP nebo 6-TGN a námi identifikovaných faktorů byla zjištěna i dříve [149-154], nicméně nejpravděpodobněji v důsledku častého využití jen jednoho faktoru jako testovaného prediktoru nízkých hladin metabolitů AZA nedosahovaly tyto studie dostatečné síly. Dostatečně silný model kombinující více prediktivních faktorů non-adherence k AZA byl představen v jedné z dříve provedených prací [155]. Mírně nižší přesnost, kterou tento model dosahoval ve srovnáni s námi vyvinutým modelem, může být vysvětlena např. větší heterogenitou populace, nebo nezohledněním některých důležitých aspektů, jako je např. TPMT genotypizace.

S cílem usnadnění dostupnosti nově vytvořeného modelu, byla vyvinuta jednoduchá webová aplikace. Nicméně nelze opomenout, že stanovení hladin metabolitů AZA by mělo být vnímáno jako pouze jeden z více faktorů hodnotících neadekvátní adherenci k medikaci.

5.3.5. Optimalizace kombinované léčby

V rámci námi provedené práce byla poprvé navržena optimální hodnota pro cut-off hladiny 6-TGN predikující dosažení dostatečné hladiny IFX u dětských pacientů s CD. Její srovnání je tak možné pouze s jedinou prací, která byla s tímto cílem provedena u dospělé populace pacientů s IBD [156]. Autoři této studie uvádí jako hladinu pro výše zmíněné cut-off 125 pmol/8 x 10^8 6-TGN v erytrocytu, tedy významně nižší hladinu než námi zjištěnou hodnotu 278 pmol/8 x 10^8 6-TGN v erytrocytu. Z aktuálně dostupných dat není možné spolehlivě vysvětlit, proč se v dětském věku zdá být efektivní hladina 6-TGN vyšší než u dospělých pacientů.

Pokud bychom o pacientech s nedetekovatelnými hladinami 6-TGN uvažovali jako o pacientech, kteří jsou vlastně léčeni pouze IFX, je popsaná asociace mezi nulovými hladinami

6-TGN a zvýšenou mírou ztráty odpovědi k IFX ve shodě s dříve publikovanými pracemi, které prokazují významně vyšší pravděpodobnost udržení remise na IFX při současné aplikaci imunomodulátoru než při podávání IFX v monoterapii [157, 158].

5.4. Imunizační a vakcinační status u dětských pacientů s IBD

Menší pozornost, věnovaná vakcinačnímu serologickému profilu u dětských pacientů s IBD, zjištěná v recentně provedené multicentrické mezinárodní studii, neodporuje výsledkům dalších prací provedených na toto téma. Dle rozsáhlé americké studie otázku týkající se provedených očkování položila svým pacientům pouze polovina ze 178 zúčastněných dětských gastroenterologů. Záznam do dokumentace pak provedlo jen 31 % z nich a jen 9 % vyžadovalo specifické sérologické vyšetření [159].

Z recentní práce vyplývá, že nejčastějším důvodem nepodaného očkování bylo odmítnutí rodičů nebo cena vakcíny. Nutnost akutního podání imunosupresivní terapie jako důvod pro neočkování byla zaznamenána u 27,8 % pacientů. Toto zjištění otevírá myšlenku možného vlivu informovanosti rodičů pacientů a široké veřejnosti. Signifikantní nárůst podaných vakcín byl zaznamenán u 92 dětí s IBD po proběhlé informační akci na téma rizik infekčních onemocnění u dětských pacientů s IBD [160].

I přes doporučení Evropské organizace pro Crohnovu chorobu a ulcerózní kolitidu / The European Society for Crohn‘s and colitis (ECCO) vydané v roce 2014 [161] provést vyšetření na přítomnosti EBV protilátek u všech pacientů s diagnózou IBD před zahájením aplikace thiopurinů bylo u sledovaných pacientů provedeno pouze u 21,9 %.

Dalším zjištěním původní práce byl rozpor mezi aktuálním doporučením vydaným ECCO [162] a reálně uplatňovanou strategií testování latentní formy tuberkulózy před zahájením aplikace aTNF.

6. Závěr

Za jeden z nevýznamnějších výstupů práce lze považovat vybudování predikčního modelu odhadujícího individuální riziko selhání konkrétního terapeutického postupu u dětských pacientů s CD. Díky vytvoření webové aplikace je model snadno dostupný pro běžnou klinickou praxi. Jde o první systematicky vytvořený nástroj, který umožnuje již v době diagnózy odhadnout míru rizika časné ztráty odpovědi ke standardnímu terapeutickému režimu a tím významně přispívá k žádané personalizaci péče o dětské pacienty s CD.

Na základě dalších dvou predikčních modelů, které byly vytvořeny, lze jednoduše získat velmi dobrý odhad dosažené hladiny metabolitů AZA. Díky této informaci, která není standardě dostupnou současní běžných laboratorních vyšetření, lze získat představu o nevhodném dávkování podávaného léku nebo o non-adherenci pacienta k jeho užívání. Taková znalost může značně přispět k rozhodnutí o individuální volbě léčebné strategie.

Identifikace IFX jako rizikového faktoru rozvoje nežádoucích kožních účinků ve srovnání s ADA může mít význam pro pečlivé uvážení aplikace tohoto léku u rizikové populace dětských pacientů s ohledem na osobní a rodinnou anamnézu kožních onemocnění.

Díky nově zjištěnému optimálnímu cut-off metabolitů AZA, které predikuje dosažení vhodné hladiny IFX, byl přiblížen vztah mezi těmito dvěma léky. Výsledek může poskytnout lepší informační základ při rozhodování o individuálním dávkování obou léků v kombinaci.

Nakonec i v rámci diagnostiky dětských IBD práce přináší identifikaci nového parametru, slizniční exprese CD30+, který může být užitečným pomocným faktorem k přesnějšímu určení diagnózy, to nemusí být v některých situacích zcela jednoznačné.

Nebyla potvrzena užitečnost fCPT získaného časně po zahájení léčby jako vhodného parametru pro identifikaci jedinců, kteří na léčbu neodpoví vůbec. Na základě výsledků této práce se při posuzování efektu EEN o fCPT v časných fázích neopíráme a spoléháme jen na klinickou odpověď. Domácího testování fCPT lze využít, ale dle našich poznatků víme, že ELISA vyšetření je třeba považovat za přesnější. Spíše vyvrácena byla i teorie o možném využití tCPT pro predikci dalšího průběhu onemocnění u dětských pacientů s UC.

Nová zjištění plynoucí z této práce přinášejí celou řadu cenných informacích s klinickým dopadem v oblasti péče o dětské pacienty s IBD. Díky nově identifikovaným možnostem predikce konkrétních klinických otázek je odpověď na ně informovanější, a lze tak předpokládat větší přesnost při rozhodování. Jistě ne všechny námi kladené otázky jsou plně zodpovězeny a teprve po ověření těchto prediktorů, např. ověřením modelů na externích kohortách nebo ještě lépe porovnání přístupů za pomocí námi vyvinutých modelů, ukáže definitivní správnost těchto předpovědí.

Souhrn

Typická Crohnova choroba (CD) s postižením terminálního ilea, Crohnovská kolitida, atypická ulcerózní kolitida (UC) a typická UC s kontinuálně rozloženým zánětlivým postižením tlustého střeva jsou v současné době vnímány jako makroskopicky i mikroskopicky se od sebe lišící formy zánětlivých střevních onemocnění (IBD). Z epidemiologického hlediska výskyt těchto imunitně podmíněných onemocnění celosvětově narůstá v rámci dospělé i dětské populace. Pečlivá monitorace onemocnění a co nejlépe daty podpořený přístup v péči o pacienty s IBD mají za cíl minimalizaci rizika komplikovaného průběhu a rozvoje s onemocněním a/nebo léčbou asociovaných komplikací.

Hlavním cílem práce je identifikace nových prediktivních faktorů zasahujících do jednotlivých oblastí péče o dětské pacienty s IBD, díky kterým lze předem odhadnout určitý jev, jehož včasné ovlivnění nebo lépe, jehož předejití, přispívá k lepší kontrole onemocnění.

Za nejdůležitější výstup práce lze považovat vytvoření predikčního modelu k individuálnímu odhadu času do relapsu u dětských pacientů s CD na léčbě thiopuriny. Dále jsme vytvořili modely predikující hladinu metabolitů azathioprinu, která odráží vhodnost dávkování léku nebo adherenci pacienta k jeho užívání. Nově byl nalezen optimální cut-off pro hladinu metabolitů azathioprinu, který predikuje dosažení žádoucích hladin infliximabu. Identifikovali jsme infliximab ve srovnání s adalimumabem, jako prediktor rozvoje kožních nežádoucích účinků u dětských pacientů s IBD. Ukázali jsme, že míra tkáňové exprese CD30+ buněk je silným prediktorem klasifikace onemocnění do jednoho z typů IBD. Naopak možnost klinického využití nebyla prokázána u stanovení hladin tkáňového kalprotektinu (CPT) a fekálního CPT časně po zahájení indukční léčby výlučnou enterální výživou. Upozornili jsme na limitované klinické využití domácích testů ke stanovení hladin fekálního CPT.

Nová zjištění plynoucí z této práce přinášejí celou řadu cenných informacích s klinickým dopadem v oblasti péče o dětské pacienty s IBD. Díky nově identifikovaným možnostem predikce konkrétních klinických otázek je odpověď na ně informovanější, a lze tak předpokládat větší přesnost při rozhodování. Jistě ne všechny námi kladené otázky jsou plně zodpovězeny a teprve po ověření těchto prediktorů, např. ověřením modelů na externích kohortách nebo ještě lépe porovnání přístupů za pomocí námi vyvinutých modelů, ukáže definitivní správnost těchto předpovědí.

Summary

Typical Crohn's disease (CD) with involvement of terminal ileum, Crohn's colitis, typical and atypical ulcerative colitis (UC) are currently perceived as different forms of inflammatory bowel disease (IBD). The incidence of IBD is increasing worldwide in both the adult and paediatric populations. Comprehensive care requires a precise and data-driven approach to minimize the risk of complicated disease course and the development of disease-related and/or treatment-associated complications.

The main goal of this work was to identify new predictive factors affecting individual areas of care of paediatric patients with IBD, on the basis of which it is possible to predict a certain phenomenon, the early influence or prevention of which, contributes to better disease control

The most important outcome of the work can be considered the creation of a predictive model of estimation of individual time to relapse in paediatric patients with CD on thiopurine treatment. We also developed models predicting the level of azathioprine metabolites, which reflect the suitability of the drug dosage or the patient's adherence to its use. An optimal cut-off of the level of azathioprine metabolites, which predicts the achievement of the effective infliximab levels, has been found. We identified infliximab, compared to adalimumab, as a predictor of the development of skin side effects in paediatric IBD patients. We have shown that the tissue expression level of CD30+ cells is a strong predictive factor of classifying the disease into one of the IBD types. In contrast, the possibility of clinical usefulness has not been demonstrated in the determination of tissue calprotectin (CPT) levels and faecal CPT levels obtained early after initiation of treatment with exclusive enteral nutrition. We indicated the limited clinical use of home tests to determine faecal CPT levels.

The new findings of this work provide a wealth of valuable information with a clinical impact in the care of paediatric patients with IBD. Thanks to the newly identified possibilities of predicting specific clinical questions, the answer to them is more informed and it is possible to assume greater accuracy in decision-making process. Certainly not all of the questions we asked, were fully answered and only after verifying these predictors, such as validation of models on external cohorts or even better, comparing strategies using the models in a clinical trial, we can assess the definitive correctness of these predictions.

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ORIGINAL ARTICLE

Immunohistochemical Assessment of CD30+ Lymphocytes in the Intestinal Mucosa Facilitates Diagnosis of Pediatric Ulcerative Colitis

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Abstract

Background Diagnosis of pediatric inflammatory bowel diseases (IBD) remains challenging. We aimed at the value of immunohistochemical assessment of CD30+ lymphocytes in the intestinal mucosa in differential diagnosis between pediatric Crohn's disease (CD) and ulcerative colitis (UC) and its utility as a predictor of future differentiation in patients with IBD unclassified (IBDU).

Methods Seventy-four treatment naive pediatric patients with IBD (33 CD, 30 UC and 11 IBDU) were enrolled into the study. Biopsy samples from six different regions (terminal ileum, cecum, ascending colon, transverse colon, descending colon and rectum) were immunohistochemically stained with anti-CD30 antibody, and the number of positive cells per one high power field was quantified.

Results Significant differences between CD and UC were found when compared total counts of CD30+ cells in median numbers, mean values and maximal numbers and also for separate counts in terminal ileum, transverse colon, descending colon and rectum. The most profound difference between CD and UC was shown for total median values of CD30+ cells and for the values in rectal localization. The difference was independent on the intensity of inflammation. A cutoff value of 2.5 CD30+ cells with sensitivity 83% and specificity 90% was found for the rectum. There was no difference between patients with CD and IBDU, but a marked difference between UC and IBDU patients was revealed.

Conclusion Histopathological assessment of biopsy with rectal CD30+ count is reliable and simple method that could help in differential diagnosis among IBD subtypes in children with IBD.

Keywords Inflammatory bowel disease · Immunohistochemistry · CD30

Introduction

Until recently, the diagnosis of inflammatory bowel disease (IBD) was based on proof of the chronic inflammation of the gastrointestinal tract with typical distribution and exclusion of other causes of inflammation, mainly on clinical grounds [1]. This approach, apparently straightforward and simple, has become complicated and establishing diagnosis of IBD turned into a multidisciplinary process heavily supported by laboratory, radiological, endoscopic and histopathological data [2, 3]. Atypical presentations of IBD have been stressed, and various other diseases manifesting with IBD-like morphology were highlighted [4–6]. Moreover, none of the established criteria is 100% specific for IBD, let alone for Crohn's disease (CD) or ulcerative colitis (UC) [7, 8]. Situation in pediatric population is even more challenging, given the fact that pediatric onset IBD often presents with nonspecific clinical features, it lacks characteristic morphological signs of chronicity and harbors many atypical phenotypes in all three main forms of IBD (CD, UC and IBD unclassified) [1, 9–13]. Porto criteria from 2005 [14] and their revised version from 2014 [1] established diagnostic criteria, integrating the up to date evidence from all diagnostic fields. However, there is up to 30% of pediatric IBD that remain

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further unclassified, in contrast with adult population, where the proportion of such cases remains only 10–15% [15]. This has led to increased demand for new invasive and noninvasive markers that would increase the certainty of the correct diagnosis, since the exact subclassification of pediatric IBD has crucial therapeutic consequences. According the actual recommendations, children with newly diagnosed CD should be treated primarily with exclusive enteral nutrition [16], while treatment of UC should be initiated by corticosteroids or 5-aminosalicylic acid according to severity of the disease [12].

IBD is immune-mediated disease that involves a complex interplay of host genetics, disrupted mucosal integrity and environmental influences [17]. Some T helper lymphocyte subsets in IBD express antigen CD30 at their cytoplasmic membranes. According to a recent study [18], patients with UC show higher amount of CD30+ cells in gut mucosa than those with CD. The aim of our study was to find out, whether the immunohistochemical assessment of CD30 in the intestinal mucosa could facilitate differential diagnosis between pediatric CD and UC an if it could serve as an early predictor of future reclassification into CD and UC in patients with IBD unclassified (IBDU).

Materials and Methods

Between November 2012 and January 2017, all 171 newly diagnosed pediatric patients with IBD were retrospectively screened in our referral center for eligibility to enter this study. First 30 consecutive treatment naïve patients primarily diagnosed as CD and UC and all patients with the primary diagnosis of IBDU were reevaluated by pediatric gastroenterologists, and the diagnosis at the time of the first endoscopic assessment was confirmed or modified following the actual revised Porto criteria for the diagnosis of pediatric IBD. Patients that had already been initiated on IBD treatment, as well as those patients with missing data for the revision of the diagnosis, were excluded from the study. After this revision, 29 patients with CD, 31 with UC and 14 with IBDU entered the study. During the minimum of 18-month follow-up, one patient from UC group developed perianal fistulizing disease and was reclassified to CD. Among 14 patients with IBDU, three cases were reclassified to CD due to the manifestation of the CD traits (all three patients manifested perianal disease). In the end, 33 children with CD, 30 with UC and 11 with IBDU (Fig. 1) were included in the study. Clinical characteristics of the patients are given in Table 1.

Archive histopathological slides stained with hematoxylin and eosin were reviewed by a pathologist blinded to clinical data. To evaluate microscopical findings, six bowel segments from each case of CD, UC and IBDU were analyzed (terminal ileum, cecum, ascending colon, transverse colon, descending colon and rectum). Intensity and activity of the inflammation was objectified using a histopathological scoring system. Given the diversity in scoring systems that are only partially validated and none of them is designed for pediatric population [19], as well as the absence of any scoring system for patients with IBDU, more universal modification of the established systems was used in our study. This modified system evaluates the intensity of chronic and active inflammation and the presence of mucosal defects (Table 2). A similar version of this system has been already used in our previous study [20]. Additional assessed morphological variables included the intensity of the eosinophilic inflammation (graded as none, mild and severe), the presence of the basal plasmocytosis, recently described basal plasmocytosis associated with eosinophilia [21] and the presence of lymphatic follicles. Given the fact that CD30+ cells show predilection to lymphoid tissue, as declared in previous studies [18], the assessment of the follicles was fundamental. Especially in terminal ileum, where physiological presence of the lymphoid tissue (Peyer's patches) can falsely overestimate number of CD30+ cells.

Then, immunohistochemical staining of CD30 antigen was performed on all archive biopsy samples from patients with CD, UC and IBDU. 1-µm tissue sections were deparaffinized, and the anti-CD30 primary antibody (Cell Marque, at a dilution of 1:50) was used. Detection was performed by the PolyDet Dab chromogen (Dako REAL) with phosphate-buffered saline solution. CD30 expression was assessed by counting the highest number of positive cells per one hpf (400×) in the most affected region of each bowel segment.

Ethical Considerations

Legal representatives of the patients signed an informed consent form for inclusion into the study. The study was approved by the Ethics Committee of Motol University Hospital.

Statistical Analysis

We used statistical software R-project (R Core Team, version 3.4.1) for data analysis. Differences among the subgroups of the patients with CD and UC in the median values, maximal numbers and mean values of CD30+ cells calculated from all bowel segments, as well as numbers of positive cells in separate bowel segments, were tested by Welch two sample *t* test. For constructing model of the discrimination between CD and UC, the definitive diagnosis of CD or UC was used. Then, the definitive diagnosis including reclassification of IBDU to CD served as a template to test differences between CD and IBDU, as well as UC and IBDU. After that, the receiver operating characteristics (ROC) curves were used to

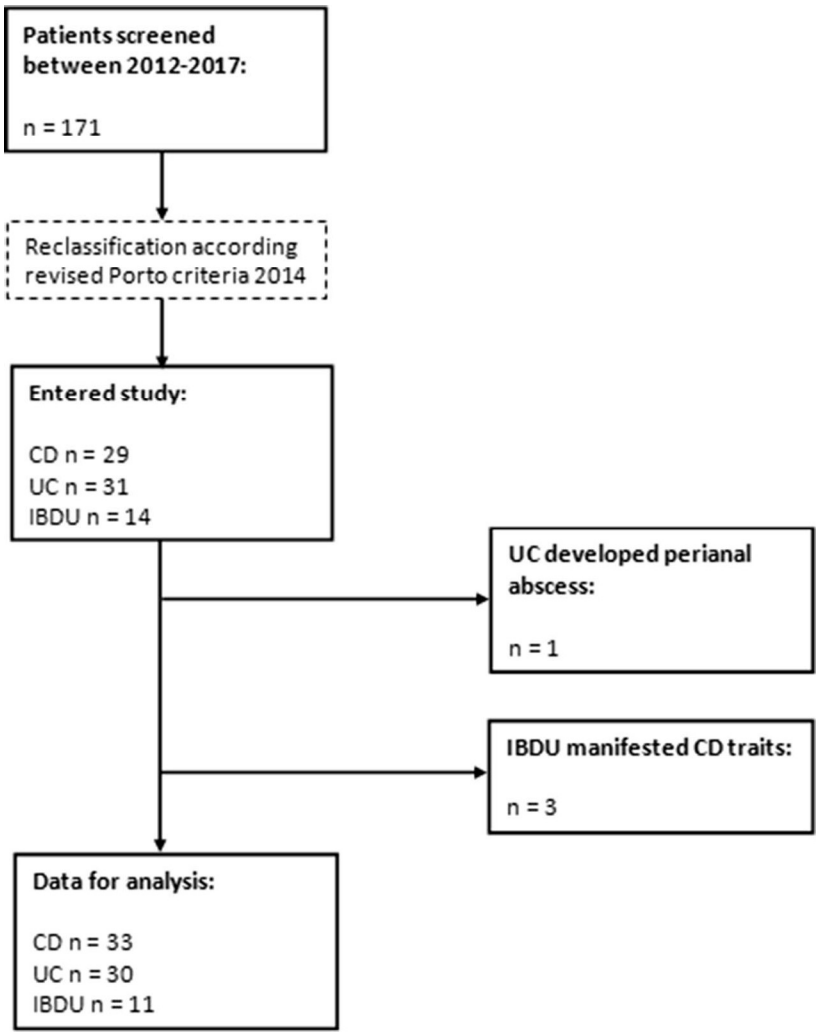

Fig. 1 Flowchart of included and excluded patients. *CD* Crohn's disease, *UC* ulcerative colitis, *IBDU* inflammatory bowel disease unclassified

find the most significant cutoff values for the discrimination between CD and UC (using the software package "pROC"). To compare individual ROC curves, DeLong's test was used. To test, whether the difference in numbers of CD30+ cells in terminal ileum is independent on the presence of lymphatic follicles, a generalized linear regression model was constructed. The same model was applied to find out, if the difference in numbers of CD30+ cells in rectum is dependent on the intensity of histological and endoscopic inflammation. Probability (p) values of <0.05 were considered significant. A 95% confidence interval was used.

Results

Statistically significant differences among patients with CD and UC in median values, maximal numbers and mean values of CD30+ cells were found (Fig. 2a, b). Also, there was a difference between CD and UC in each bowel segment except for cecum and ascending colon. The most significant results were shown for the median values ($p<0.001$), see Fig. 3a, and for the values in rectal localization ($p<0.001$). The box plots of CD30 counts in all bowel segments are given in Fig. 3b–g. Based on the analysis of area under curve, a cutoff value of 4.25 positive cells per 1 HPF was established for median values, with sensitivity 93% and specificity 83%, Fig. 4a. For the values in rectum, the optimal cutoff (2.5 positive cells per 1 HPF) with sensitivity 83% and specificity 90% was found (Fig. 4b). Using Delong's test, the total median values turned out to be more reliable discriminating factor between CD and UC than the values in the most affected bowel segment, but there was no difference between the median values and the values in rectum. Among the patients with CD, 16 of them had microscopical inflammation in rectum, and 13 of them endoscopical signs of rectal involvement. According to the regression analysis, the association between the number of CD30+ cells and diagnosis was independent on the intensity of endoscopic or microscopic inflammation ($p<0.0001$). The basal plasmocytosis was found in 15 cases of UC, five cases of IBDU,

Table 1 Demographic and clinical characteristics of patients

	CD	UC	IBDU
Number of patients	33	30	31
Sex, male (%)	20 (0.61)	14 (0.47)	7 (0.64)
Age at diagnosis, years, median (IQR)	13.1 (11.5–16.2)	11.8 (6.7–15.5)	9.7 (3–12.6)
L1 (%)	10 (0.3)		
L2 (%)	3 (0.09)		
L3 (%)	19 (0.58)		
L4 (%)	21 (0.64)		
B1 (%)	29 (0.88)		
B2 (%)	3 (0.09)		
B3 (%)	1 (0.03)		
p (%)	8 (0.24)		
G1 (%)	4 (0.12)		
E1 (%)		4 (0.13)	
E2 (%)		2 (0.07)	
E3 (%)		2 (0.07)	
E4 (%)		22 (0.73)	
S0 (%)		28 (0.99)	
S1 (%)		2 (0.07)	

L = Localization of the disease (L1—ileal, L2—colonic, L3—ileocolonic); B = Behavior (B1—non-stricturing, non-penetrating, B2—stricturing, B3—penetrating, p—perianal); G1 = Growth delay; E = Extension (E1—proctitis, E2—left side colitis, E3—extensive, E4—pancolitis); S = Severity (S0—never had acute severe colitis, S1—at least one acute severe colitis)

Table 2 Modified histopathological scoring system

Grade	Histopathological change
0	No inflammation
1	Mononuclear cells only
2	Neutrophils in lamina propria
3	Neutrophils in epithelium (superficial epithelium, cryptitis, crypt pseudoabscess)
4	Mucosal defect (erosion or ulceration)

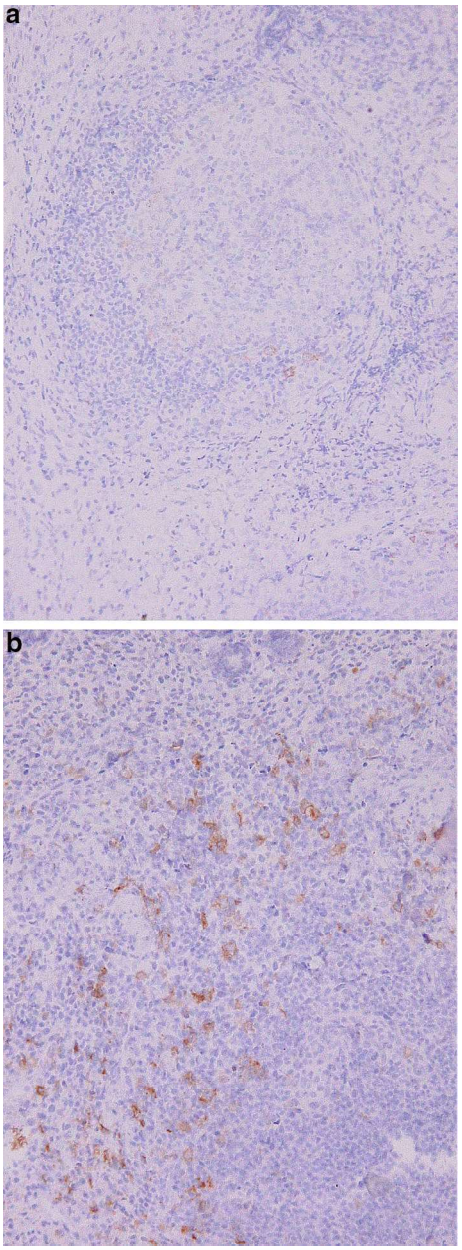

Fig. 2 Photomicrograph showing CD30+ cells by immunohistochemistry (×400). Membrane staining of CD30+ lymphocytes in Crohn's disease (**a**) and ulcerative colitis (**b**) in rectal samples

but only in one case of CD. Also the presence of any eosinophilia (both mild and severe) in rectum was significantly more frequented in patients with UC than CD ($p < 0.0001$). A lymphatic tissue in terminal ileum was not the confounding factor, since the significant difference between CD30+ cells in the ileum was independent on the presence of lymphatic follicles.

Based on the given results, a diagnostic model using calculated cutoff in rectal biopsy was constructed and applied on patients with IBDU. Using the final diagnosis for the median values and the values in rectal localization, there was no difference in CD30+ cells between patients with CD and IBDU ($p = 0.08$), but there was a marked difference between the UC and the IBDU ($p = 0.007$), see Fig. 5. Three patients with IBDU developed CD traits, and two of them had low numbers of CD30+ cells (below the established cutoff). Among the patients without CD traits, there were cases with both low and high numbers (Fig. 5).

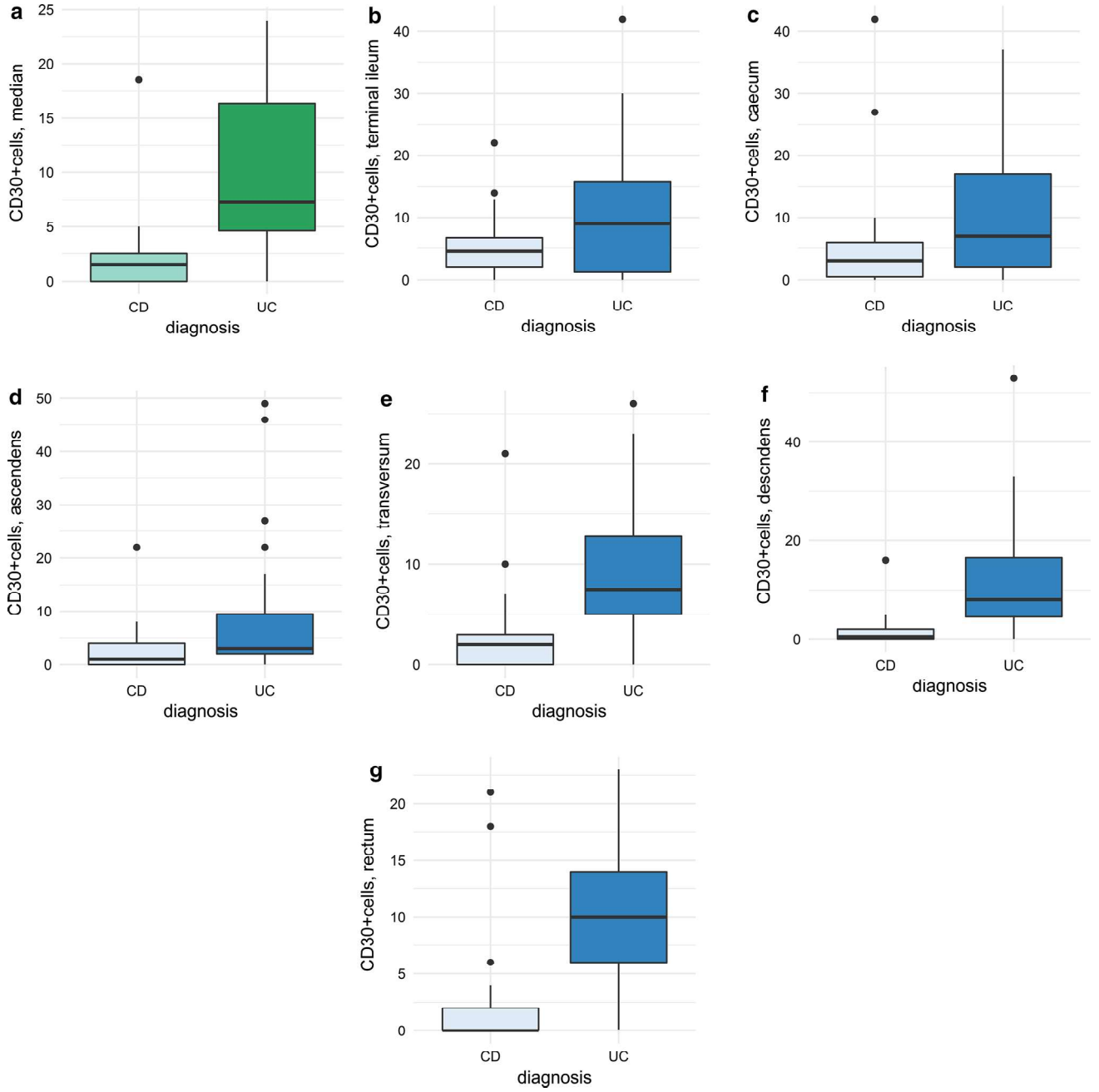

Fig. 3 **a** Box graphs comparing medians values of CD30+ cells, calculated from all bowel segments, in Crohn's disease and ulcerative colitis. **b** Box graphs comparing numbers of CD30+ cells in terminal ileum in patients with Crohn's disease and ulcerative colitis. **c** Box graphs comparing numbers of CD30+ cells in cecum in patients with Crohn's disease and ulcerative colitis. **d** Box graphs comparing numbers of CD30+ cells in ascending colon in patients with Crohn's disease and ulcerative colitis. **e** Box graphs comparing numbers of CD30+ cells in transverse colon in patients with Crohn's disease and ulcerative colitis. **f** Box graphs comparing numbers of CD30+ cells in descending colon in patients with Crohn's disease and ulcerative colitis. **g** Box graphs comparing rectal numbers of CD30+ cells in patients with Crohn's disease and ulcerative colitis

Discussion

The pathophysiological mechanisms of IBD are not fully understood, but are characterized by the immunological imbalance of the intestinal mucosa, associated with cells of the both innate and adaptive immune system. The major role in triggering the repetitive intestinal inflammatory damage in IBD is T cell subpopulations, mainly T helper 1 (Th1), Th17 and regulatory T cells. Th1 lymphocytes are stimulated by certain cytokines, especially interleukin (IL) 12 and

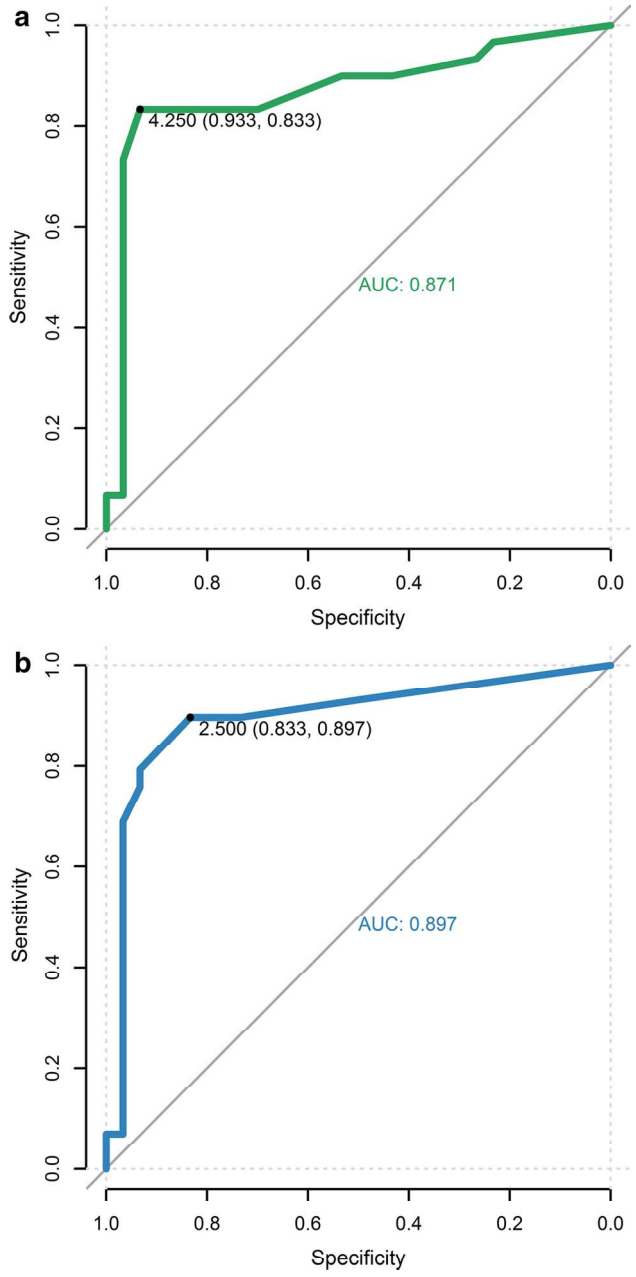

Fig. 4 **a** Receiver operating characteristics curve to determine the CD30 cutoff for ulcerative colitis diagnosis in median. **b** Receiver operating characteristics curve to determine the CD30 cutoff for ulcerative colitis diagnosis in rectal localization

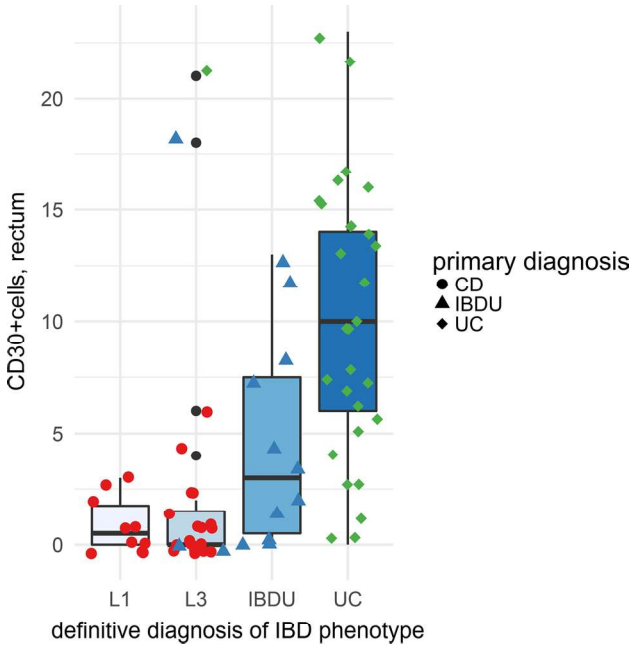

Fig. 5 Box graphs comparing numbers of CD30+ cells in rectum for Crohn's disease, ulcerative colitis and inflammatory bowel disease unclassified, also with highlighted changes in diagnoses after the revision, according the revised Porto criteria

18, inducing production of interferon gamma (IFN-γ). This pathway is more prominent in CD, but increased levels of IFN-γ can be demonstrated also in patients with UC. Th2 lymphocytes play a role in immunopathogenesis of both diseases as well, with oversecretion of IL5, IL10 and IL13. In both CD and UC, there is also a strong influence of Th-17-related cytokines [22].

The role of CD30 antigen in IBD pathogenesis still remains unexplained. It represents a protein with uncertain function, member of tumor necrosis family, firstly described in association with certain malignances as Hodgkin's lymphoma, anaplastic large cell lymphoma or embryonal carcinoma. It was also discovered in normal human tissues, especially some portions of resting and activated T lymphocytes [23]. To the best of our knowledge, only one study [18] has examined the diagnostic utility of CD30 detection in colonic biopsies so far. The authors assessed counts of CD30+ cells in the most inflamed segment of bowel mucosa in adult patients with IBD and demonstrated a significant difference between CD and UC. Our goal was to ascertain, whether we can find a significant difference in numbers of CD30+ cells also in pediatric IBD population and if this difference is independent on localization and the intensity as well as the activity of inflammation. Our study demonstrated a significant difference between CD and UC in median values, mean values and maximal values calculated from all bowel segments. A difference in values from all separate bowel segments was observed as well, but for the values obtained in cecum and ascending colon they were not statistically significant. The most significant difference was found for the median values of CD30+ cell counts and the values obtained in rectal localization. Given the fact that counting of median values would require staining of all bioptic samples for CD30, it seems more practical and economically efficient to assess the CD30+ cell counts in rectum only. Both these variables were proved to be better

discriminating factors than the counts from the most affected bowel segment, as declared in the previously study [18]. The limiting factor of this method could be the fact, that less than half of the CD patients had either macroscopic or microscopic rectal involvement. However, the difference of rectal CD30+ cell counts was independent on the intensity and the activity of the inflammation (both histological and endoscopic); hence, this method is applicable also for the patients with mild rectal involvement only. Neither rectal sparing in UC nor absence of the rectal inflammation in CD should affect the clinical utility of such testing. Interestingly, both patients with UC and rectal sparing had high numbers of CD30+ cells (mean 13.5). Our results are supported by findings of Giacomelli et al. [24], who illustrated elevated levels of serum soluble CD30 antigen in patients with UC. Serum level of CD30 could therefore serve as a simple non-invasive marker of the disease activity and monitoring of the treatment response. Sun et al. [25] even propose a possible therapeutic usefulness of soluble CD30 in case murine IBD models.

Our study demonstrated that other morphological variables can also be independently supportive for the UC diagnosis. The eosinophilia in the rectum was associated with UC. Also the basal plasmocytosis was present in more cases of UC in contrast with CD. These microscopical signs along with rectal localization and the assessment of CD30+ cells may represent reliable and simple methods for the diagnosis of UC.

Eventual benefit of CD30+ cells evaluation is even more important for the patients with IBDU. This subtype of IBD was traditionally regarded as a temporary "immature" form of IBD with the expectation of further differentiation. Even the Porto criteria for IBDU are based rather on the practical clinical experience and do not encompass eventual biological markers or genetic background. However, a recent study by Cleyen et al. [26] indicates that IBDU may represent an independent subtype of IBD with its own characteristic genetic profile. Keeping with this finding, we consider IBDU to be a definite diagnosis in our study, without further shift to CD or UC during the follow-up. We did not find any difference between IBDU and CD, but a marked difference between IBDU and UC was shown. Therefore, CD30+ cell counts should be regarded more likely as a supportive biomarker of UC rather than discriminating factor between CD and UC. These findings diverge from current therapeutic recommendations, where the treatment of IBDU and UC is actually the same [12]. But the difference in CD30+ cell counts may indicate that the biology of those diseases is different and that IBDU stands closer to CD.

On the other hand, if IBDU truly represents an undifferentiated form of IBD with the capacity for further maturation, CD30+ cell counts could be beneficial even in this case. Children with IBDU, that manifest CD traits later, should be reclassified to CD. However, current state of the art in IBD diagnostics does not have a capacity to select IBDU patients that are progressing to UC and re-diagnose them. In other words, children with IBDU will not be able to be changed to UC in the future. Given the fact that our IBDU group contained patients with both high and low numbers of CD30+ cells, their assessment could serve as a marker of possible maturation to UC. Patients with diagnosis of IBDU and high numbers of rectal CD30+ cells that are refractory to current treatment could profit from the switch to the UC therapy. And, as stated above, the serum CD30 could prove useful too, as the progressive elevation of serum levels of CD30 could represent a sign of the progression to UC. However, this hypothesis is based on the limited number of the patients in our IBDU group. Further studies, preferably multicenter, with greater numbers of IBDU patients and their prospective observation are required to confirm such premise.

Conclusion

In conclusion, routine histopathological assessment of biopsy samples with rectal CD30+ cell count represents a supportive biomarker that could improve the differential diagnosis among subtypes of IBD in treatment naïve children with IBD.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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The Accuracy of a Home-performed Faecal Calprotectin Test in Paediatric Patients With Inflammatory Bowel Disease

Tereza Lerchova, Ondrej Hradsky, Ivana Copova, Kristyna Potuznikova, Lucie Gonsorcikova, and Jiri Bronsky

ABSTRACT

Objectives: Owing to the invasiveness of endoscopy, the use of biomarkers, especially faecal calprotectin (FC), has become standard for remission assessment. This study aimed to compare the accuracy for detection of endoscopic activity using recently developed FC home test using smartphone application (FC-IBDoc) against standard enzyme-linked immunosorbent assay (ELISA).

Methods: In all, 102 consecutive observations (89 participants) were included in prospective observational study. FC-IBDoc was performed parallelly with FC-ELISA in paediatric patients with inflammatory bowel disease indicated for endoscopy. Both tests were performed by trained staff. Mucosal healing was defined using Simple Endoscopic Score for Crohn disease (CD) ≤ 2 in patients with CD (n = 44), ulcerative colitis (UC) Endoscopic Index of Severity ≤ 4 in patients with UC (n = 27) and Rutgeerts score i0 and i1 without colon involvement in patients with CD after ileocaecal resection (n = 19).

Results: Out of 102 endoscopic findings 23 were assessed as mucosal healing. We found an association of the mucosal healing scores of the entire group both with FC-ELISA ($P = 0.002$) and FC-IBDoc ($P = 0.001$). The area under the receiver operating characteristic curve for FC-ELISA was 0.883 (95% confidence interval 0.807–0.960), with optimal cut-off at 136.5 μg/g. The area under the receiver operating characteristic curve for FC-IBDoc was 0.792 (95% confidence interval 0.688–0.895) with optimal cut-off at 48 μg/g. The FC-ELISA was more accurate than FC-IBDoc when tested by a Delong test ($P = 0.023$).

Conclusions: Standard FC-ELISA for FC evaluation is more reliable predictor of mucosal healing than the FC-IBDoc in paediatric patients with inflammatory bowel disease. The cut-off values for both tests were incongruous.

Key Words: bedside, biomarkers, endoscopy, point-of-care, smartphones

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What Is Known

- The utility of monitoring faecal calprotectin levels in assessment of inflammatory bowel disease activity was clearly demonstrated.
- The concentration of faecal calprotectin is usually measured by quantitative enzyme-linked immunosorbent assay in laboratory.
- The main disadvantages of enzyme-linked immunosorbent assay are the requirement of a well-equipped laboratory to analyse the samples, high financial cost, and slow evaluation time.

What Is New

- Enzyme-linked immunosorbent assay for faecal calprotectin evaluation is more reliable predictor of mucosal healing than the device for faecal calprotectin home testing in paediatric patients with inflammatory bowel disease.
- The cut-off values for enzyme-linked immunosorbent assay and home faecal calprotectin test were highly incongruous.

Proper diagnosis and precise monitoring play an essential role in treatment of patients diagnosed with inflammatory bowel disease (IBD) (1,2). When assessing disease activity, the results of the whole set of examinations, monitoring various aspects of the disease, must be included. Mucosal healing should, however, be the

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main goal in the treatment of paediatric patients with IBD (3,4). Even though endoscopic examination is the criterion standard in evaluation of mucosal healing (1,3,5,6), noninvasive methods (eg, evaluation of complex clinical status using scales of disease activity (6), radiological examinations (7,8), and laboratory parameters (6,9,10)) have recently gained importance because of disadvantages related to endoscopy, such as invasiveness, financial cost, or limited extent of examination (1,6,11,12). In addition, many of the listed methods allow for assessment of other parameters apart from mucosal healing, such as transmural healing, deep remission, or general clinical conditions of the patient. According to recent data, mentioned parameters are becoming an important part of disease activity monitoring (13,14).

Based on previous data, biomarkers in the stool are considered to be the most sensitive laboratory parameters when assessing the endoscopic and histological activity of inflammation (1,6). It has been shown that the concentration of the neutrophil-derived marker, faecal calprotectin (FC), is associated with endoscopic activity scales and reflects mucosal healing (1,15–19). Moreover, a correlation was demonstrated in the comparison of FC levels and transmural or deep remission (13). The utility of monitoring FC levels in assessment of IBD activity was clearly demonstrated in a randomized controlled trial in 2017 performed in adult patients with Crohn disease (CD) (Cochran-Mantel-Haenszel test adjusted risk difference between the tight control group and the clinical management group of 16.1% in remission rate [95% confidence interval (CI) 3.9–28.3; $P = 0.010$]) (20).

The concentration of FC in stool samples is usually measured by quantitative enzyme-linked immunosorbent assay (FC-ELISA) (6,21). The main disadvantages of this method are the requirement of a well-equipped laboratory to analyse the samples, high financial cost, and slow evaluation time (6,21). To be readily applicable, the test should be simple and the results rapidly available to the physician. A new FC home test based on smartphone application, (FC-IBDoc, Buhlmann Laboratories AG, Switzerland) produces an immediate result and allows FC level monitoring on-site or at home.

The primary aim of the study was to compare the accuracy of detection of endoscopically assessed inflammation using the recently developed FC-IBDoc to a standard FC-ELISA. A secondary objective was to compare the variability of the results from multiple measurements of 1 sample.

METHODS

Participants

The study was designed as a prospective observational study focused on comparing of 2 different FC tests taking endoscopy as a criterion standard. Data from 103 paediatric patients were consecutively collected between October 2016 and March 2018. The stool samples were collected from paediatric patients diagnosed with IBD or with suspicion of IBD indicated for endoscopic examination and hospitalized at the Department of Paediatrics, Motol University Hospital, Prague.

The inclusion criteria were age between 3 and 19 years, diagnosis of IBD based on Porto criteria (22), and indication for endoscopy. Based on study protocol, patients who were unable to provide a stool sample before starting the bowel preparation for endoscopic examination, were taking nonsteroidal anti-inflammatory drugs or anticoagulants, or were diagnosed with IBD unclassified, and patients with diverting stoma, or infectious gastrointestinal disease were excluded. Eligibility of patients is shown in Figure 1. In total, 103 patients, who provided stool sample, were screened to

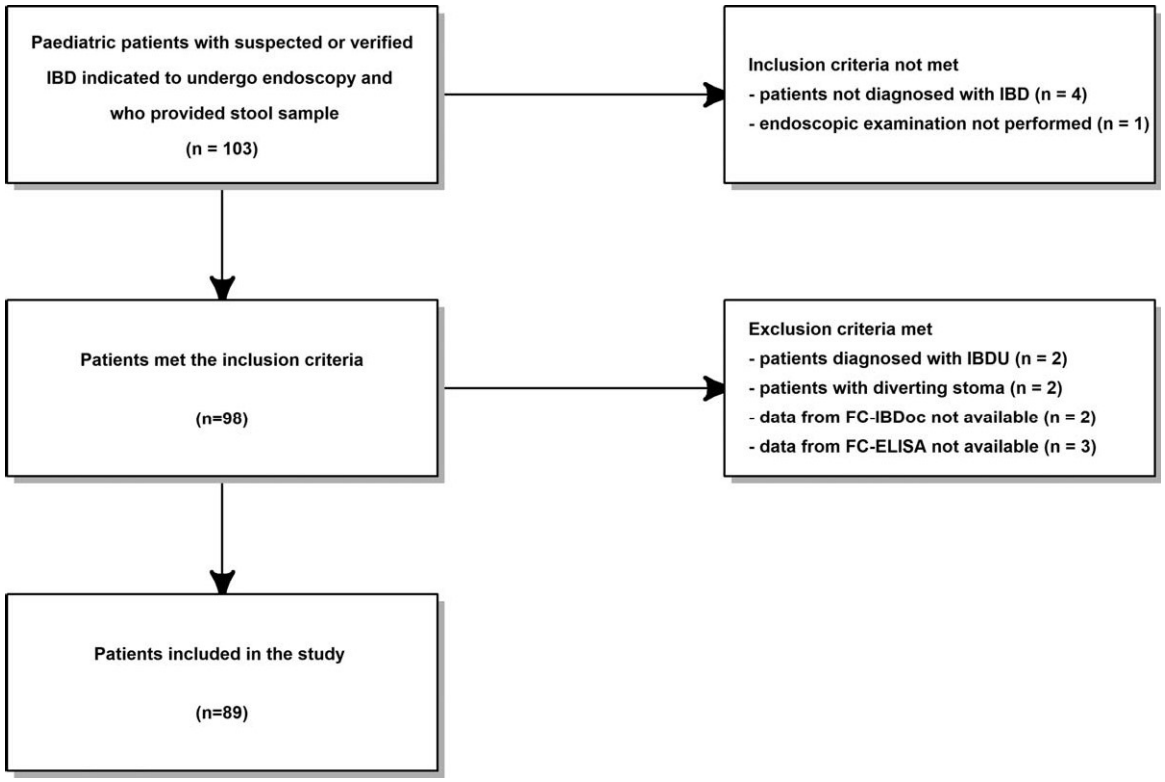

FIGURE 1. Participants selection. Flowchart showing selection of patients based on inclusion and exclusion criteria. FC-ELISA = faecal calprotectin quantitative enzyme-linked immunosorbent assay; FC-IBDoc = faecal calprotectin home test based on smartphone application; IBD = inflammatory bowel disease.

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be enrolled in the study, 5 did not meet inclusion criteria and had to be excluded. Out of the remaining 98 patients, 9 were excluded based on exclusion criteria. Finally, 89 subjects were included in the study, a total of 102 measurements were performed.

Detection of Faecal Calprotectin Concentration

The same sample of stool taken before starting bowel preparation for colonoscopy was analysed using an FC-IBDoc in parallel with a standard FC-ELISA.

A recently developed home test for quantitative determination of FC levels in stool samples is based on an image evaluation from a Lateral Flow device designed for sample processing. Extraction tube called CALEX Valve is used to collect a precise amount of stool sample. In the same tube the stool sample is mixed with an extraction solution and subsequently applied on the lateral flow device. The mixture is then detected by red and gold particles as rising through the test cassette and reaching testing and control strip. The FC levels are measured based on immunochromatographic method. The image is taken by a smartphone and sent for evaluation using an Internet application. The result is displayed within 15 seconds and simultaneously sent to the FC-IBDoc Portal. According to manufacturer's data (23), the test has a range of 30 to 1000 μg/g, results above 1000 μg/g are less accurate. According to the manufacturer's instructions (23), all samples were collected in screw-capped plastic containers and stored in a refrigerator. The time from sample acquisition to processing was no longer than 7 days. All FC-IBDoc analyses were performed by a trained physician, and each sample was tested twice, with the mean of the 2 obtained values used for statistical analysis. In addition, stool samples from 14 patients were measured 5 times to perform intra-assay reliability analysis.

FC-ELISA is a standard laboratory method using a fluorescence immunoassay. Stool samples were collected in the same screw-capped plastic containers and sent to the laboratory to be prepared and analysed using the EliA Calprotectin 2 test (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden). Based on the manufacturer's data, results >6000 μg/g are less precise.

Endoscopic Evaluation

We chose the categorical variable of mucosal healing as a primary goal because it corresponds to the main target of the FC-IBDoc test. Results from both tests were compared with mucosal healing defined by endoscopic scores, in patients with CD, by simple endoscopic score for CD (SES-CD) ≤2 (n = 44) (24,25), in patients with ulcerative colitis (UC), by UC endoscopic index of severity (UCEIS) ≤4 (n = 27) (26,27), and in patients with CD after ileocaecal resection (ICR), by Rutgeerts score i0 and i1 without signs of colon involvement (n = 19) (28).

No information regarding other tests results were available to readers of a particular test.

Statistical Analysis

All data were analysed using R statistical software (version 3.4.4). Continuous variables were described as median and interquartile range (IQR). Categorical variables were described as absolute frequencies and percentages. Values of FC were analysed on a logarithmic scale. Because of multiple observations from 1 subject, the associations between mucosal healing and FC were assessed using a generalized linear mixed model and a linear mixed model (R package "lme4" and "lmerTest"). The accuracy of FC-IBDoc and FC-ELISA for respective scores was analysed using receiver operating characteristic (ROC) curve analysis with crossvalidated area under the ROC curve (AUC) measurements. The AUCs were compared using a DeLong test. Among patients with multiple observations of IBDoc at 1 time, we performed a 2-way mixed-effects model to assess intraclass correlation and, using a bootstrap method, a CI was constructed. To compute Fleiss' kappa, R package "irr" was used. P values <0.05 were considered statistically significant. All P values were 2 sided.

Ethical Considerations

The study was approved by the Ethics Committee of the authors' institution (EK-1491/16). The patients' guardians received written information on the study and signed a consent form. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

RESULTS

Patients Characteristics

We included 102 observations from 89 patients (62 CD and 27 UC, 48 boys [54%], median age 15.36 years [IQR 11.14–17.28]). The patient's demographic and clinical characteristics according to Paris classification (29) are listed in Table 1.

Prediction of Mucosal Healing Based on Faecal Calprotectin Levels

Among 102 performed endoscopic examinations from 89 patients included in the study, 79 did not fulfil the criteria for mucosal healing, 46 SES-CD ≥2 in patients with CD, 22 UCEIS ≥4 in UC, and 11 Rutgeerts score ≥i0 and i1 without signs of colon involvement in CD after ICR (Supplemental Table 1, Supplemental Digital Content, *http://links.lww.com/MPG/B621*). The median measured concentration of FC in all 102 samples was 690 μg/g (IQR 117.5–1924.5) when measured with FC-ELISA and 78.0 μg/g (IQR 30.0–324.0) in FC-IBDoc. Medians measured FC concentration in endoscopically active and inactive patients differ applying both, FC-ELISA and FC-IBDoc, tests (Fig. 2). We found a correlation between the results of both tests (Pearson correlation coefficient of 0.70, 95% CI 0.58–0.78, $P < 0.0001$).

We found an association of the mucosal healing of the entire IBD group both with FC-ELISA ($P = 0.002$) and FC-IBDoc ($P = 0.001$). To determine the best cut-off value of each test to predict mucosal healing, ROC curves were constructed (Fig. 3). The crossvalidated AUC for FC-ELISA was 0.883 (95% CI 0.807–0.960) with optimal cut-off at 136.5 μg/g. The AUC for FC-IBDoc was 0.788 (95% CI 0.674–0.902) with optimal cut-off at 48 μg/g. When a Delong test was applied, the FC-ELISA was more accurate than FC-IBDoc ($P = 0.023$). Similarly, the regression mix-model constructed using FC-ELISA as a predictor, was significantly better than the model using FC-IBDoc. We did not find an association with FC levels, measured with FC-ELISA or with FC-IBDoc, and mucosal healing in subset of patients assessed by SES-CD, UCEIS, or Rutgeerts score.

Prediction of Intensity of Inflammation Based on Faecal Calprotectin Levels

When assessing intensity of inflammation using a linear mixed model for subsets of observations evaluated by SES-CD as the continuous variable, we found an association with FC-ELISA ($\beta = 2.35$, 95% CI 0.87–3.91), but not with FC-IBDoc. We found

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TABLE 1. Patient's demographic and clinical characteristics according to Paris classification (29)

	All	CD*	UC
Number of patients	89	62	27
Sex, male (%)	48 (0.54)	33 (0.53)	15 (0.56)
Age at diagnosis, y, median (IQR)	12.5 (9.5–14.9)	12.7 (9.9–14.9)	11.5 (7.9–14.8)
Ileal involvement, L1 (%)	20 (0.22)	20 (0.32)	
Colonic involvement, L2 (%)	10 (0.11)	10 (0.16)	
Ileocolonic involvement, L3 (%)	31 (0.35)	31 (0.5)	
L4a (%)†	25 (0.28)	25 (0.4)	
L4b (%)‡	3 (0.03)	3 (0.05)	
L4ab (%)§	0 (0.00)	0 (0.00)	
Nonstricturing and nonpenetrating disease, B1 (%)	39 (0.44)	39 (0.63)	
Stricturing disease, B2 (%)	22 (0.25)	22 (0.35)	
Penetrating disease, B3 (%)	0 (0.00)	0 (0.00)	
B2B3 (%)‖	1 (0.01)	1 (0.02)	
Perianal disease (%)	7 (0.08)	7 (0.11)	
Proctitis, E1 (%)	2 (0.02)		2 (0.07)
Left-side colitis, E2 (%)	5 (0.06)		5 (0.19)
Extensive colitis, E3 (%)	1 (0.01)		1 (0.04)
Pancolitis, E4 (%)	19 (0.21)		19 (0.7)
Never acute severe colitis, S0 (%)	24 (0.27)		24 (0.89)
Ever acute severe colitis, S1 (%)	3 (0.03)		3 (0.11)

The Paris classification applies to the time of diagnosis.
CD = Crohn disease; IQR = interquartile range; UC = ulcerative colitis.
*Out of 62 following patients with CD 19 underwent ileocaecal resection since the time of diagnosis.
†Upper gastrointestinal (GI) involvement up to Treitz ligament correspond to L4a, according to Paris classification.
‡Upper GI involvement distal to Treitz ligament correspond to L4b, according to Paris classification.
§Upper GI involvement up and distal to Treitz ligament correspond to L4ab, according to Paris classification.
‖Stricturing and penetrating disease correspond to B2B3, according to Paris classification.

an association of UCEIS with both FC-ELISA and FC-IBDoc ($\beta = 0.62$, 95% CI 0.37–0.86, respectively; $\beta = 0.85$,95% CI 0.44–1.26). Rutgeerts score in a subgroup of patients with CD after ICR was associated with both FC-ELISA and FC-IBDoc ($\beta = 0.46$, 95% CI 0.20–0.69, respectively; $\beta = 0.57$, 95% CI 0.02–0.16).

Intraclass Variability of Faecal Calprotectin Levels Measured by FC-IBDoc

Based on obtained Intraclass correlation coefficient (ICC) in a subset of 70 repeated measurements from 14 patients, computed by a 2-way mixed-effects model, the level of reliability was concluded as good (ICC = 0.79, 95% CI 0.53–0.89). When using Fleiss kappa, the level of reliability was rated as fair to good (Fleiss kappa coefficient = 0.68).

DISCUSSION

To the best of our knowledge this was the first study simultaneously evaluating FC levels measured with the use of a smartphone application, standard ELISA, and endoscopy as a criterion standard. We found that the standard ELISA for FC evaluation is a more reliable predictor of mucosal healing than the FC-IBDoc in paediatric patients with IBD.

Along with the development of a new field of healthcare, e-Health (30), home testing is becoming more accessible. This new concept, the main purpose of which is to use information and communication technology to promote prevention, diagnosis, and treatment of various diseases, intervenes in all branches of medicine including the care of patients with IBD. Home monitoring of FC levels using a smartphone application is now available.

To establish the value of home monitoring of FC concentration in management of care of patients with IBD, it was necessary to compare the results of the home test and standard tests. Several studies comparing the accuracy of FC home testing with the standard test have been recently published. Vinding et al (31) reported in 2016 a moderate agreement between FC levels obtained using ELISA and the smartphone-based home test ($r = 0.685$). The study was conducted on large number of adult patients who performed the test themselves. Elkjaer et al (32) compared 3 methods of FC level measurement including ELISA, the smartphone-based home test, and a lateral flow device–based test linked to a laptop computer. Agreement was found both when comparing FC concentration measured by ELISA with the 2 rapid tests ($r = 0.954$, respectively, $r = 0.939$), and when comparing the smartphone-based home test and lateral flow device–based test to each other ($r = 0.961$). Similarly, a smaller study in 2012 (33) performed in patients with suspicion of IBD (n = 40) or IBD relapse (n = 45) showed a correlation between the quantitative method, time-resolved fluorescence immunoassay (34), and 2 rapid tests, namely Quantum Blue Calprotectin (κ 0.77; 95% CI 0.64–0.90) and Prevent ID CalDetect (κ 0.46; 95% CI 0.32–0.60). Bello et al (11) confirmed a good correlation between the home-based and central ELISA measurements of FC levels in an adult IBD population (ICC = 0.88 with inferior limit of the coefficient 0.82). Similarly, Heida et al (35) has shown that measurements of FC concentrations done with a home-based lateral flow method using a smartphone application were in agreement with measurements made by a hospital-based reader using the Quantum Blue method (Spearman rank correlation coefficient $r = 0.94$, $P < 0.001$) and central ELISA (Spearman rank correlation coefficient $r = 0.85$, $P < 0.001$). In this study, performed in a combined adult (n = 82) and paediatric (n = 19) population, agreement between the home-based lateral flow test and ELISA was found when FC concentrations were <500 μg/g

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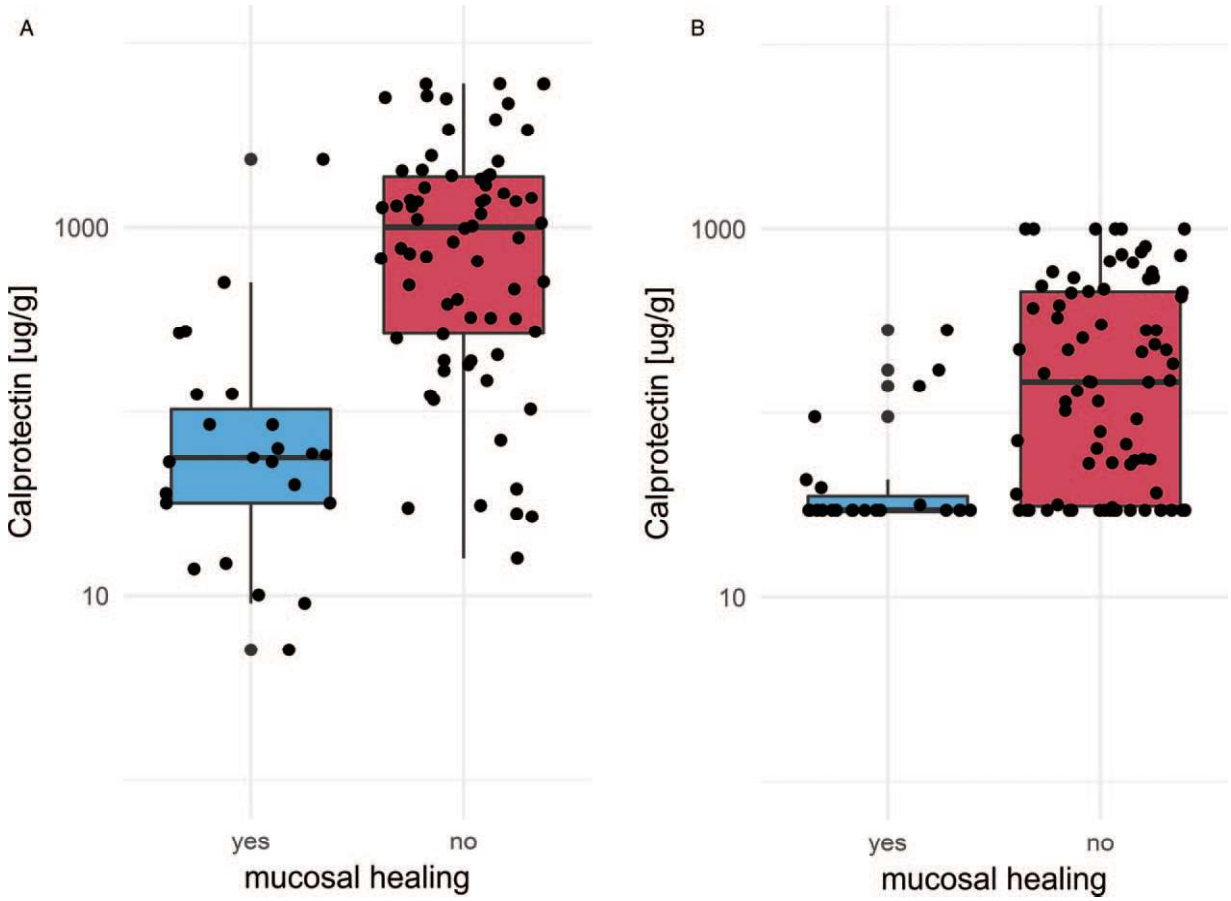

FIGURE 2. Box plots comparing medians FC values measured with FC-ELISA (A) and FC-IBDoc (B) in patients with CD (n = 44), UC (n = 27), and with CD after ileocaecal resection (n = 19) with and without mucosal healing. A, The median value of FC concentration in the stool samples measured by FC-ELISA in endoscopically active (n = 79) and inactive patients (n = 23). B, The median value of FC concentration in the stool sample measured by FC-IBDoc in endoscopically active (n = 79) and inactive patients (n = 23). Mucosal healing was defined as SES-CD ≤2 in patients with CD (n = 44), UCEIS ≤4 in patients with UC (n = 27) and Rutgeerts score i0 and i1 without colon involvement in patients with CD after ileocaecal resection (n = 19). CD = Crohn disease; FC = faecal calprotectin; FC-ELISA = faecal calprotectin quantitative enzyme-linked immunosorbent assay; FC-IBDoc = faecal calprotectin home test based on smartphone application; UC = ulcerative colitis; UCEIS = ulcerative colitis Endoscopic Index of Severity.

(71% of IBDoc results were with agreement with ELISA results). In the latter 2 studies (11,35), the home test was performed by the patients themselves. In these studies, different levels of agreement between ELISA and the home-based method of FC evaluation have been described. None of the studies mentioned above took into account clinical data, but only focused on comparison of measured levels of FC, which were not directly compared with the endoscopic findings, so it is difficult to evaluate which of the 2 methods is more accurate.

Although it is known that FC remains relatively stable in stool samples for at least 3 days at a room temperature (36,37), higher accuracy due to quick and immediate processing with no need for time for transportation, can be assumed. Because FC-ELISA, however, proved to be more accurate than the FC-IBDoc, this speculation was not confirmed in the present study.

It should be noted that results from FC-IBDoc are available in 2½ hours, whereas it takes several days to obtain results from FC-ELISA, which is however approximately 2 times cheaper than FC-IBDoc.

Several point-of-care testing methods for FC detection were available before home-based tests were developed. A good accuracy of these rapid methods has been confirmed in multiple studies (38–42), but only a few compared measured FC concentration with endoscopic findings. Positive correlation between endoscopically assessed activity of inflammation and FC levels measured with point-of-care testing methods was confirmed in several studies performed in adult populations (6,12,43,44). The excellent accuracy of a bedside faecal calprotectin enzyme-linked immunosorbent assay in prediction of histological inflammation was shown in a study performed in paediatric treatment-naive patients (45). The same study confirmed a good accuracy of the method when assessing endoscopic inflammation.

Since the cut-off values for both the FC-ELISA and FC-IBDoc tests were highly incongruous, we have shown that it is not possible to freely compare their results. As none of the previously performed studies considered clinical data, the optimal cut-off values of FC home-based test were calculated with respect to FC levels measured with ELISA. The range of calculated cut-off points was from 50 to 150 μg/g, which tightly meets the optimal cut-off indicated in this study (31,32,35). The optimal cut-off point predicting mucosal healing of ELISA was 250 μg/g in a large meta-analysis conducted in patients with CD and UC (46). The cut-off

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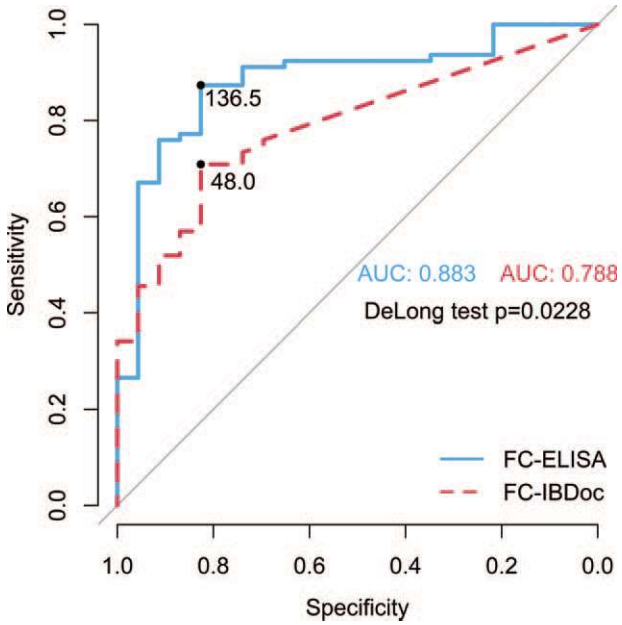

FIGURE 3. Comparison of accuracy in prediction of mucosal healing using FC levels measured by FC-ELISA and FC-IBDoc. FC-ELISA is a more reliable predictor of mucosal healing, defined as SES-CD ≤2 in patients with CD (n = 44), UCEIS ≤4 in patients with UC (n = 27) and Rutgeerts score i0 and i1 without colon involvement in patients with CD after ileocaecal resection (n = 19), than the FC-IBDoc in paediatric patients with IBD based on AUC (Delong test $P = 0.023$). In total, 102 observations were analysed. AUC = area under the curve; CD = Crohn disease; FC-ELISA = faecal calprotectin quantitative enzyme-linked immunosorbent assay; FC-IBDoc = faecal calprotectin home test based on smartphone application; ROC = receiver operating characteristic; SES-CD = simple endoscopic score for Crohn disease; UC = ulcerative colitis; UCEIS = ulcerative colitis Endoscopic Index of Severity.

indicated in the present study can be considered as verging to the value proposed in the meta-analysis.

Comparison of multiple measurements using FC-IBDoc of each sample was performed to reveal the reliability of the test, which was indicated as good. We avoided the potential influence of intraindividual variability of FC concentrations, described in many previously published studies (47,48), by multiple analyses of 1 stool sample. The intra-assay variability was slightly higher in this study than the variability of the FC home test described in recently published studies. Kristensen et al (48) referred the mean coefficient of variation of the duplicated FC level measurements 3.4% (95% CI 2.5%–4.5%). The test of reliability performed in relatively large Scottish study (47) was rated as moderately good (κ 59% [95% CI: 0.27– 0.90] for FC >50 μg/g).

The present study has certain limitations to note. First, although mucosal healing is usually defined using endoscopic scoring systems, there is still no clear consensus on the exact value allowing establishment of mucosal healing. We defined mucosal healing as SES-CD ≤2 in patients with CD, UCEIS ≤4 in patients with UC, and Rutgeerts score i0 and i1 without signs of colon involvement for patients after ICR in agreement with several recently published data (24–27). Second, the sample size was relatively small. Assuming a larger sample size, it is possible that the association between inflammation intensity defined by SES-CD and FC concentration measured with FC-ELISA could be supported. In addition, the FC-IBDoc is designed to be done at home; however, the test was performed in a hospital with participation of trained staff. We chose this approach to eliminate possible measurement failures and errors, whose relatively high number has been described at least in 1 study. Piekkala et al (49) indicated approximately 15% of failures were caused by inappropriate use of the test at home.

CONCLUSIONS

In conclusion, this was the first study comparing the accuracy of FC home test, namely IBDoc, to the standard FC-ELISA using endoscopic scores as a criterion standard. We have shown that in paediatric patients with IBD, the standard FC-ELISA is a more reliable test in predicting endoscopically defined mucosal healing than the FC-IBDoc. Based on our data, the FC-IBDoc should not replace the FC-ELISA in monitoring disease activity. The test may be particularly suited for obtaining an approximate result for monitoring disease activity at home setting. The caution that sensitivity of the FC-IBDoc is lower than the one of ELISA method must be bear in mind.

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Limited clinical significance of tissue calprotectin levels in bowel mucosa for the prediction of complicated course of the disease in children with ulcerative colitis

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ABSTRACT

Background: Fecal calprotectin (F-CPT) represents one of the most widely used biomarkers for intestinal inflammation. However, the levels may be false negative or false positive in some situations.

Aims: To evaluate the usefulness of immunohistochemical (IHC) detection of tissue calprotectin (T-CPT) in bowel mucosa in children with ulcerative colitis (UC). We focused at correlation of T-CPT with levels of F-CPT and endoscopic and microscopic disease activity at the time of diagnosis and tested whether T-CPT could serve as predictor of complicated course of the disease.

Methods: Forty-nine children with newly diagnosed UC between 6/2010-1/2018 entered the study. Endoscopic activity was objectified using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS), clinical activity by Pediatric Ulcerative Colitis Activity Index (PUCAI) and microscopic activity by Geboes and Nancy score. The IHC staining for CPT antigen was performed on bioptic samples from 6 bowel segments and the number of CPT + cells were counted per 1HPF. During the minimal follow-up of 12 months we searched for presence of complications. As outcome for Cox regression model we used composite endpoints: A) Acute Severe Colitis, colectomy, anti-TNF treatment; B) systemic corticotherapy; C) systemic 5-aminosalicylic acid therapy.

Results: Neither levels of T-CPT nor values of UCEIS, Geboes or Nancy score predicted the given complications. We found F-CPT levels (HR 2.42 and 2.52) and PUCAI > 40 points (HR 2.98) as predictors of time to endpoints B and C. Good correlation was found between T-CPT levels and Geboes score (k = 0.65) and Nancy score (k = 0.62) and modest with F-CPT (k = 0.44), UCEIS (k = 0.38) and PUCAI (k = 0.42).

Conclusions: T-CPT correlated well with microscopic scores. F-CPT and PUCAI appear to be better predictors of unfavorable outcome in patients with UC.

1. Introduction

Ulcerative colitis (UC) is a chronic systemic inflammatory disorder with predominant involvement of the gastrointestinal tract [1]. Periodic monitoring evaluating the disease intensity and activity is essential for optimizing the treatment strategy. Even though the disease activity may be assessed by clinical scores, namely Pediatric Ulcerative Colitis Activity Index (PUCAI) [2], the endoscopy is still considered the gold standard for the assessment of the intestinal inflammation and mucosal healing, especially at the time of diagnosis [3,4]. However, this procedure is invasive, time-consuming and burdening, especially for pediatric population, where the general anesthesia is usually required. Therefore, reliable non-invasive markers for monitoring a disease activity are required. Fecal calprotectin (F-CPT) represents one of the most widely used biomarkers for intestinal inflammation with high sensitivity for both adult and pediatric population [5,6]. However, the F-CPT cannot be used to localize the focus of the disease activity and its levels may be false negative or false positive in some situations [7–9].

In our work, we focused at the immunohistochemical assessment of the tissue CPT (T-CPT) in the bowel mucosa of the children with UC. The aim of this work was: 1) to evaluate, whether the immunohistochemical assessment of the T-CPT may serve as an independent predictor of the complicated course of the disease, 2) to establish, whether the numbers of CPT positive cells in the bowel

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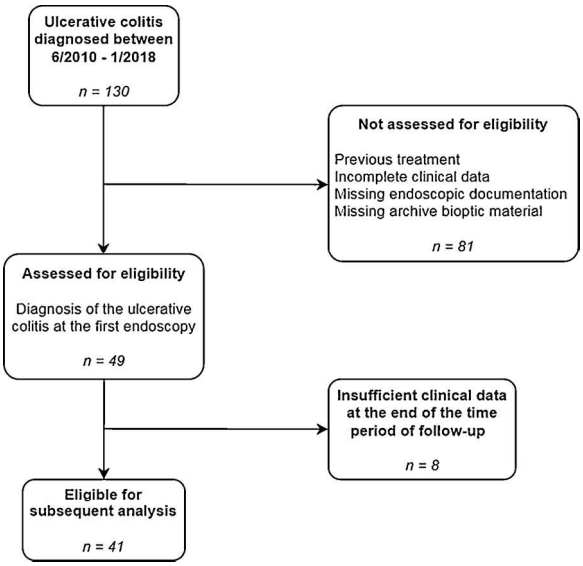

Fig. 1. Flowchart of included and excluded patients.

mucosa correlate with the levels of F-CPT at the time of diagnosis, and 3) to correlate the CPT + cell counts with the microscopic, endoscopic and clinical activity of the disease.

2. Materials and methods

All children (n = 130) with newly diagnosed UC in period between June 2010 and January 2018 were screened for eligibility to enter this retrospective cohort study. Patients were retrospectively re-evaluated by expert pediatric gastroenterologists and the diagnosis at the time of the first endoscopy was confirmed or modified according to revised Porto criteria for the diagnosis of pediatric inflammatory bowel diseases (IBD) [10]. Patients that had already been initiated on UC treatment at the time of biopsy as well as those with missing clinical data, endoscopic documentation or archive bioptic material for the revision of the diagnosis were excluded from the study. After the revision, 49 children with UC entered the study. Eight patients had to be subsequently excluded from the statistical analysis due to insufficient clinical data at the end of the time period of follow-up (Fig. 1). Clinical characteristics of the patients at the end of the minimal follow-up are given in the Table 1. The endoscopic findings at the time of the diagnosis were objectified using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) [11]. This score was chosen for its good reproducibility among the endoscopists and its good correlation with clinical, laboratory and histopathological markers of activity in UC [12,13]. The clinical severity of the disease at the time of the diagnosis was assessed by the PUCAI.

The archive histopathological slides from the bioptic samples obtained at the time of diagnosis stained with hematoxylin and eosin were reviewed by senior pediatric gastrointestinal pathologist blinded to clinical data. To evaluate microscopic findings, six bowel segments from each patient were analyzed (terminal ileum, cecum, ascending colon, transverse colon, descending colon and rectum). The samples

Table 1
Demographic and clinical characteristics of the patients with UC at the end of the minimal follow-up.

Number of the patients	41
Sex, male, n (%)	23 (56.10)
Age at diagnosis, years, median (IQR)	12 (7-15)
Acute Severe colitis, n (%)	2 (4.88)
Anti-TNF, n (%)	11 (26.83)
Colectomy, n (%)	0 (0)
Systemic CS, n (%)	12 (29.27)
Systemic 5ASA, n (%)	3 (7.32)

UC = ulcerative colitis; IQR = interquartile range; anti-TNF = anti-tumor necrosis factor therapy; CS = corticotherapy; 5ASA = 5-aminosalicylic acid treatment.

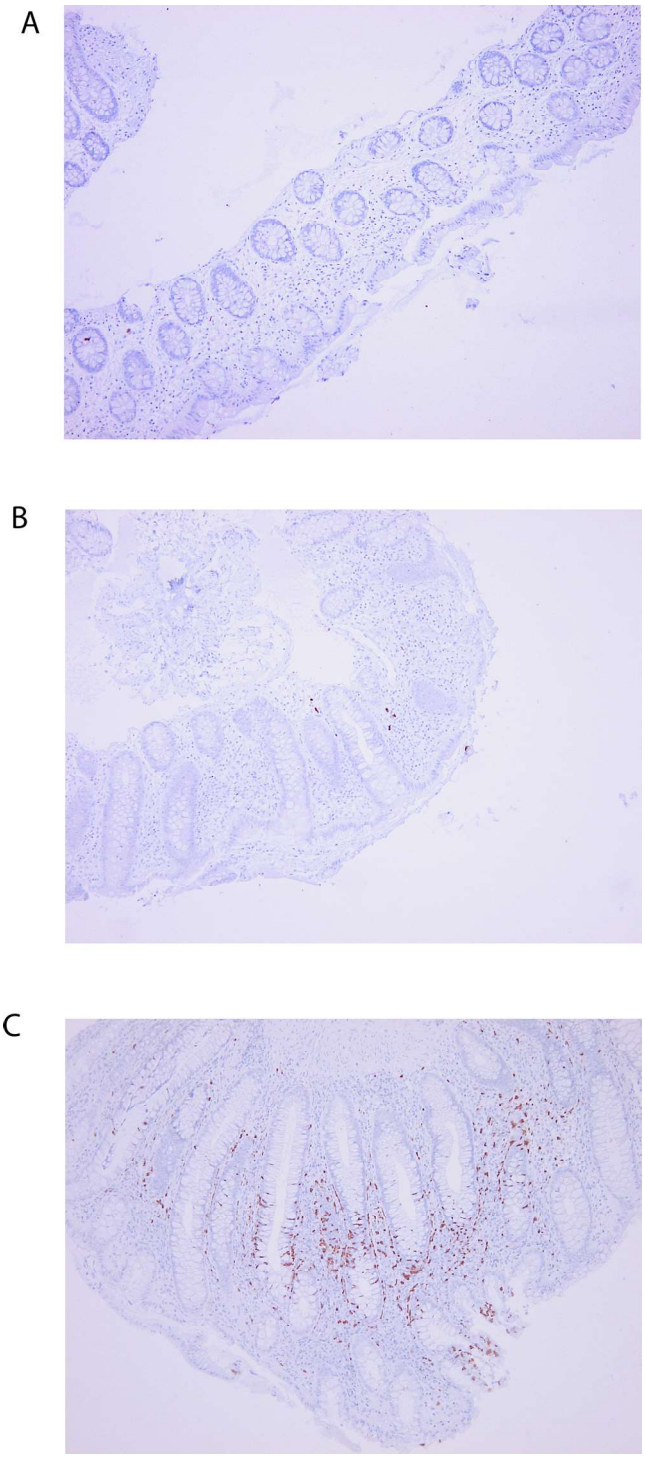

Fig. 2. Photomicrographs showing calprotectin (CPT) positive cells by immunohistochemistry (×400):
A. Bioptic sample of colonic mucosa devoid of any inflammation. No CPT+ cells are present.
B. Bioptic sample of colonic mucosa with mild chronic inflammation. Scarce CPT+ cells in the lamina propria.
C. Bioptic sample of colonic mucosa with marked chronic inflammation. Numerous CPT+ cells in the lamina propria and the epithelium.

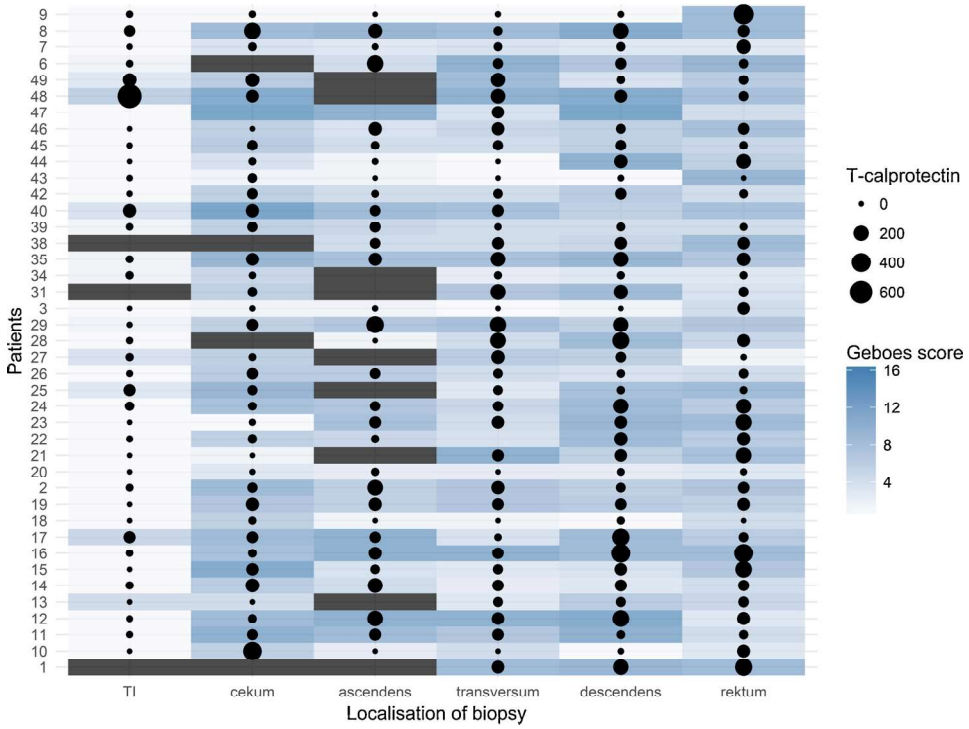

Fig. 3. Heat map reflecting the distribution of the Geboes histopathological score values and numbers of the CPT+ cells in all bowel segments.

were taken from the most affected areas of each segment. Intensity and activity of the inflammation was objectified using the Geboes and Nancy score, two widely used validated histopathological scoring systems for UC with good correlation with endoscopic findings and suitable interobserver reliability [12,14,15].

After that, the immunohistochemical staining of CPT antigen was performed on all archive biopsy samples. One micrometer thin tissue sections were deparaffinized and the anti-CPT primary antibody (Invitrogen, at a dilution of 1:1000) was used. Detection was performed by the PolyDet Dab chromogen (Dako REAL) with phosphate-buffered saline solution. CPT expression was assessed by counting the highest number of positive cells per one high power field (400x magnification) in the most affected region of each microscopic slide. The positive cells were counted separately for lamina propria and the epithelium (Figs. 2, 3 and 4).

During the minimum 12 months of prospective follow-up, we searched for the presence of following composite endpoints, presented as 3 separate substudies based on their severity: A) development of the Acute Severe Colitis (ASC) defined as the PUCAI > 65, necessity of colectomy or initiation of the Infliximab (IFX) treatment; B) first initiation of systemic corticosteroid (CS) therapy; C) first initiation of systemic 5-aminosalicylic acid (5ASA) treatment. The time to endpoint was chosen as outcome. Patients manifesting with ASC at the time of the diagnosis were not included in the study, since the ileocolonoscopy is not recommended in the setting of ASC [16,17] and these patients therefore did not fulfill the inclusion criteria.

3. Ethical considerations

Legal representatives of the patients signed an informed consent form for inclusion into the study. The study was approved by the Ethics Committee of Motol University Hospital on 28.03.2018.

4. Statistical analysis

Statistical software R-project (R Core Team, version 3.4.4) was used for data analysis. For all scoring systems included in the study, the mean values, medians and maximal values from all bowel segments were calculated and used in the subsequent analysis. The CPT + cell counts were assessed separately for lamina propria and epithelium and in total sum for the whole mucosa as well. To test, whether the levels of T-CPT and F-CPT and values of UCEIS, PUCAI and both histopathological scores at the time of diagnosis are independent predictors of the complicated course of the disease, a univariate Cox proportional hazards regression analysis (using the software package "survival") was used. Since the PUCAI > 40 (moderate disease activity) appeared to be one of the predictors (see Results section), a receiver operating curves (ROC) were constructed (using the software package "pROC") to find the cut-off values for both T-CPT and F-CPT, that would be able to select patients on this level of disease activity. Individual ROC curves were compared using the DeLong's test. To verify the correlation between F-CPT and T-CPT levels and values of the histopathological, endoscopic and clinical scores, the Pearson's product-moment correlation was performed. Probability (p) values of < 0.05 were considered significant. A 95% confidence interval was used.

5. Results

5.1. T-CPT as a predictor of the complicated course of the disease

Neither levels of the T-CPT, nor the values of UCEIS, Geboes or Nancy score predicted any complication during minimum 12 months of follow-up since diagnosis. However, the levels of the F-CPT in logarithmic scale and moderate disease activity (defined as PUCAI > 40) were independently associated with the B and C endpoints (initiation of the systemic CS and/or 5ASA therapy) (Table 2). Based on the analysis of the area under curve, no suitable cut-offs for T-CPT or F-CPT levels that could be linked to moderate disease activity at the time of diagnosis was found (AUC = 0.691 for F-CPT and 0.678 for T-CPT). DeLong's test showed no significant difference between those curves.

5.2. Association of T-CPT and F-CPT with microscopic, endoscopic and clinical activity scores

A good level of correlation was found between the median values of

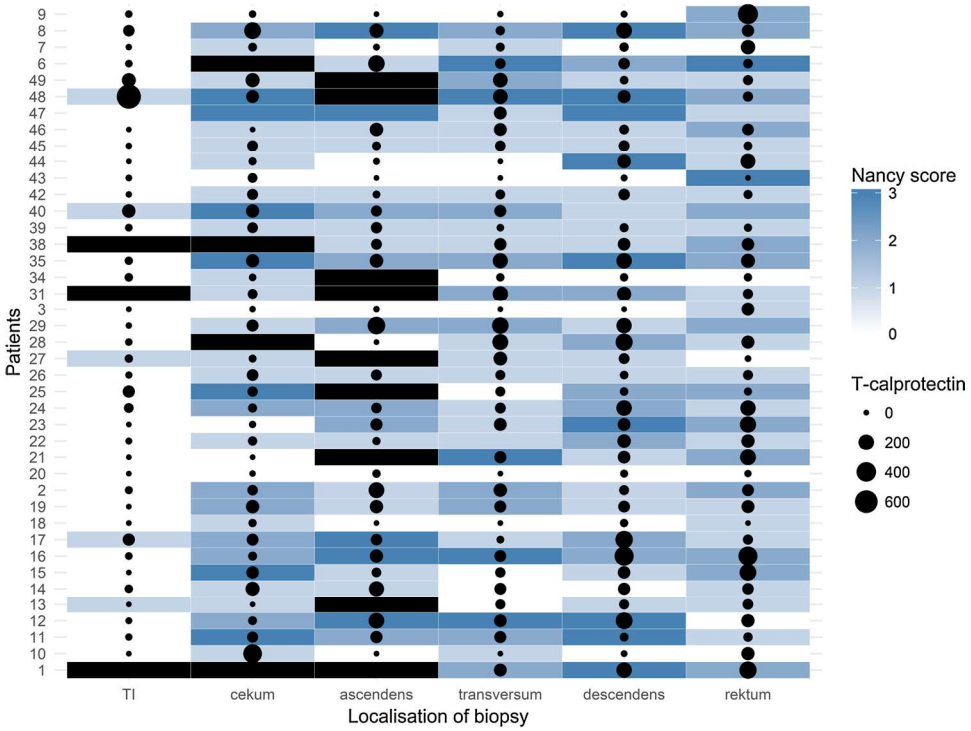

Fig. 4. Heat map reflecting the distribution of the Nancy histopathological score values and numbers of the CPT+ cells in all bowel segments.

Table 2
Univariate Cox proportional hazards regression analysis of PUCAI > 40 and T-CPT as the predictors of complicated course of the disease.

		Risk Ratio (CI)	P
PUCAI > 40	Endpoint A	2.237 (0.539-9.291)	0.268
	Endpoint B	**2.98 (1.011-8.787)**	**0.048**
	Endpoint C	**2.98 (1.011-8.787)**	**0.048**
		Risk Ratio (CI)	P
log(T-CPT)	Endpoint A	2.447 (0.873-6.859)	0.089
	Endpoint B	**2.422 (1.042-5.631)**	**0.04**
	Endpoint C	**2.517 (1.115-5.681)**	**0.026**

T-CPT = tissue calprotectin; CI = confidence interval; PUCAI = Pediatric Ulcerative Colitis Activity Index.
Bold value highlights a statistically significant result.

T-CPT and Geboes score ($k = 0.65$, $p < 0.001$) and median values of T-CPT and Nancy score ($k = 0.62$, $p < 0.001$). There was a weak correlation between T-CPT and UCEIS ($k = 0.38$, $p = 0.02$), PUCAI ($k = 0.42$, $p = 0.01$) and F-CPT ($k = 0.44$, $p = 0.01$).

We found a weak correlation between F-CPT and Geboes score ($k = 0.39$, $p = 0.025$), Nancy score ($k = 0.38$, $p = 0.03$) and UCEIS ($k = 0.36$, $p = 0.039$). There was no significant association between F-CPT and PUCAI ($k = 0.36$, $p = 0.06$).

6. Discussion

To the best of our knowledge, this is the first study exploring the contribution of the immunohistochemical assessment of T-CPT in children with IBD. CPT is a member of the S100 family and contributes to approximately 60% of the protein content in the cytosol of neutrophils [6]. Any active inflammatory process in the bowel mucosa results in leakage of CPT into the lumen and subsequently in the stool, where it can be detected and may serve as a non-invasive marker of the presence of active inflammation in the gut [18]. It appears to be a reliable marker for distinguishing inflammatory bowel disease from irritable bowel syndrome, which may share some common clinical symptoms as abdominal pain, bloating or diarrhea [6]. However, F-CPT yields some significant limitations. It represents a general marker of the active inflammation and is not disease specific, has no informative value in regards to the exact localization of the inflammation in the gut and may be false positive or false negative in some instances. Up to one quarter of healthy adult individuals show abnormal levels of the F-CPT according to some studies (probably due to presence of sporadic large bowel adenomas) [8]. On the other hand, levels of F-CPT can be decreased or even normalized iatrogenicaly by a bowel preparation procedure before colonoscopy [9]. Finally, there are published cases of patients with IBS and high levels of F-CPT, probably due to the inflammatory reaction to bowel dysbiosis [7]. There seems to be an intrapersonal day-to-day variability in F-CPT levels for both healthy persons and patients with IBD as well [19,20]. Moreover, the physiological levels of F-CPT are higher in children with the maximum in the neonatal age and then declines till the adulthood [21]. A metanalysis from Degraeuwe et al. [22] reported a 17% rate of false negative results in children with IBD when using the standard cut-off 50ug/g.

Therefore, we aimed at the immunohistochemical assessment of the T-CPT in the bowel mucosa. We speculated that the direct visualization of the CPT + inflammatory cells could more accurately reflect the actual level of the mucosal disease activity. Of course, the microscopic activity of the inflammation can be adequately assessed on the basis of the presence of neutrophils in the standard hematoxylin and eosin staining. However, in pediatric IBD, there is a lack of data about the predictive value of microscopy for complications development and its correlation with endoscopic or clinical scores of the activity. A study of Ashton et al. [23] demonstrated more extensive disease on the microscopic level compared to the endoscopic appearance in pediatric IBD. However, the study did not clarify, whether this increase in disease extent actually does reflect the real clinical presentation of the disease and whether it provides any predictive value in terms of future clinical course. The T-CPT staining may be beneficial especially for patients with no microscopic signs of inflammation activity in routine haematoxylin and eosin stain. In this setting, the direct visualization of CPT + cells may assess the presence of subtle signs of the active

inflammation more precisely. Therefore, we investigated, whether the T-CPT could improve the exactness of the histopathological assessment of the inflammation activity and better predict the complicated course of the disease. To this day, there are almost no studies regarding this topic. Only two studies engaged in IBD included the T-CPT in their data analysis and both of them were performed on the cohorts of adult patients. Guirgis et al. [24] found a good correlation of the low T-CPT with clinical, endoscopic and microscopic remission in adult patients with UC. On the other hand, elevated T-CPT (median value > 5 cells/HPF) was associated with adverse clinical outcome. A work from Fukunaga et al. [3] focused at the correlation of the F-CPT with the serum CPT and fecal hemoglobin. The immunohistochemical assessment of the T-CPT was a secondary outcome only and the authors showed that the patients with IBD have higher counts of the CPT + cells in the bowel mucosa compared to healthy individuals. No study ever examined the T-CPT as a possible prognostic marker or correlated it with other means of the assessment of the disease activity in children with IBD. In our work, we demonstrated a good correlation between T-CPT and both Geboes and Nancy histopathological scores. The T-CPT thus probably truly reflects the actual microscopic activity of the inflammation. However, we failed to link the levels of the T-CPT and the values of the Geboes and Nancy histopathological scoring systems with the development of the complications.

Regarding the F-CPT and its correlation with the endoscopic findings, the vast majority of the information stem from adult cohorts and the data for pediatric population are sparse. In the study of Fagerberg et al. [E], F-CPT correlated significantly with the endoscopic findings in the cohort of 39 pediatric patients with IBD. Ricciuto A et al. [25] showed that levels of F-CPT were associated with UCEIS values in children with IBD and primary sclerosing cholangitis. Our work confirms a significant, but only a weak correlation between F-CPT and UCEIS. For the clinical scores of the disease activity there is a considerable deal of children in clinical remission (defined as PUCAI < 10) with elevated F-CPT [26]. Our findings are in keeping with these observations, since we failed to prove any correlation between F-CPT and PUCAI. In contrast to previous findings [27], our work showed only a weak correlation of F-CPT with microscopic activity of the disease, neither in Geboes nor Nancy score.

The fundamental question is the ability of the F-CPT to predict the clinical outcome. According to several studies [28,29], level of the F-CPT adequately reflects the actual disease activity with respect to sustained remission or relapse rate. However, there is a lack of data about the F-CPT at the time of diagnosis and its predictive value for the development of subsequent complications. In our study, the levels of the F-CPT at the first endoscopy predicted the necessity of the initiation of the systemic CS and 5ASA therapy. Besides, the moderate clinical disease activity (PUCAI > 40) was the predictor of those complications as well. These findings are in contrast to previous studies, where PUCAI values at the time of diagnosis were not associated with the necessity of immunomodulatory therapy or 5ASA treatment during 1 year of follow-up [30,31].

There are several possible limitations of the study. Only three patients with previous local 5ASA therapy were subsequently initiated with the systemic 5ASA treatment. Therefore, the association of the outcome C with the moderate disease activity and F-CPT levels, although significant, needs to be confirmed by subsequent studies. Another limitation may be the retrospective design of the study. The immunohistochemical staining of anti-CPT required new sections from archive paraffin blocks, which may lead to partial cutting of the material. Moreover, the retrospective assessment of the PUCAI from the electronic documentation could have a limited value. Finally, a large amount of the patients was not enrolled in the study due to strict inclusion criteria. However, since the included sub-cohort shared similar baseline characteristics with the excluded patients, the non-intentional selection bias is unlikely.

7. Conclusions

This is the first study aiming at the immunohistochemical assessment of the T-CPT in pediatric population generally. Despite its good correlation with the histopathological indexes of the disease activity, T-CPT failed to predict the complicated course of the disease. Therefore, the usefulness of the histopathological assessment of the inflammation activity in the prediction of the complications in children with UC, whether in conventional staining or in specialized methods, remains uncertain. On the other hand, F-CPT levels at the time of diagnosis together with moderate disease activity based on the PCUAI score appeared to be independent predictors of the initiation of systemic CS and 5ASA therapy.

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Declaration of Competing Interest

The authors have no conflict of interest.

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Title: Prediction of time to relapse in pediatric patients with Crohn's disease on thiopurine treatment: a multicenter study of the Pediatric IBD Porto group of ESPGHAN

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Short title: Prediction of AZA treatment failure in pediatric CD

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Abbreviations:

ANCA: Anti Neutrophil Cytoplasm Autoantibodies

ASCA: anti-Saccharomyces cerevisiae antibodies

aTNF: anti-tumor necrosis factor-α

AZA: azathioprine

BMI: body mass index

CD: Crohn's disease

CI: confidence interval

CRP: C-reactive protein

CS: corticosteroids

CT: computer tomography

EEN: exclusive enteral nutrition

HR: hazard ratio

IBD: inflammatory bowel disease

IQR: inter quartile range

mg/kg: milligram per kilo

MRE: magnetic resonance enterography

PCDAI: Pediatric Crohn's disease activity index

TPMT: thiopurine methyltransferase

6-MP: 6-mercaptopurine

Authors' contributions:

T.L. worked on background research, participated in work on the conception and design of the study, data collection and its control, statistical analysis of the data and writing of this original article.

O.H. created the conception and design of the study, worked on statistical analysis of the data and revision of the original article, he was highlighting the potential problems and proposing the solutions and revising the work critically for important intellectual content.

M.K. worked on statistical analysis of the data and revision of the original article.

G.V. participated in data collection and their control.

J.A.D. participated in data collection and their control and revision of the original article.

M.S. participated in data collection and their control and revision of the original article.

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K.W. participated in data collection and their control and revision of the original article. T.d.M. participated in data collection and their control and revision of the original article.

J.S. participated in data collection and their control and revision of the original article.

K-L.K. participated in data collection and their control and revision of the original article; she was revising the work critically for important intellectual content.

H.E. was responsible for leading the project team, she was involved in study scheduling, she was revising the work critically for important intellectual content.

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Abstract

Background and Aims: According to current guidelines, most pediatric patients with Crohn's disease (CD) are prescribed long-term immunosuppressive therapy with azathioprine (AZA). This study aimed to identify predictors to develop a model allowing stratification of patients who will not benefit from AZA and who require a more intensive therapeutic approach early after diagnosis.

Methods: This study was designed to develop a clinical prediction rule using retrospective data analysis of patients included in a prospective inception cohort. Out of 1190 CD patients from 13 European centers, 441 were included in the analysis. Clinical relapse was defined as the necessity of re-induction of remission. Sequence of Cox models was fitted to predict the risk of relapse. The final model was fitted on 380 patients with complete data.

Results: Half of the patients did not experience clinical relapse within 2 years of AZA treatment initiation. Median time to relapse was 2.11 (CI 1.59–2.46) years. Of all the tested parameters available at diagnosis, six were significant in multivariate analyses: C-reactive protein (p=0.038), body mass index Z-score >0.8SD (p=0.002), abnormal sigmoid imaging (p=0.039), abnormal esophageal endoscopy (p=0.005), ileocolonic localization of the disease (p=0.023), and AZA dose in a specific age category (p=0.031). A web-based tool for individual prediction was developed (https://gastroped.shinyapps.io/AZA_time_to_relapse/).

Conclusion: Clinical remission was observed in 50.3% of patients after 2 years of AZA treatment. Although the possibility of predicting relapse on AZA treatment appears limited, we developed a predictive model based on six baseline parameters potentially useful and helpful in clinical decision.

Keywords: azathioprine; treatment failure; clinical prediction rule

Introduction

In most newly diagnosed pediatric patients with Crohn's disease (CD), during or shortly after remission induction, a choice of maintenance therapy is required [1]. Currently, this selection is mostly based on experts' opinions [2]. The most recent data [3] suggest that the best choice might be to start treatment with anti-tumor necrosis factor-α (aTNF) drugs in pediatric patients with moderate-to-severe CD. Nevertheless, this top-down approach has its limitations, such as costs and limited duration of action of aTNF [4, 5], making it challenging to apply in daily practice. Moreover, there are patients who do not require aTNF on disease onset and for whom thiopurines are a good option to maintain remission. However, there are yet no available criteria to define this subgroup of patients.

This study primarily aimed to identify baseline predictors of azathioprine/6-mercaptopurine (AZA/6-MP) treatment failure and to develop a predictive model that allows the identification of patients who are unlikely to benefit from long-term AZA/6-MP treatment and who require an intensive therapeutic approach from the time of diagnosis. The secondary goal was to assess the time to relapse on AZA/6-MP treatment. As an ancillary analysis, we intended to briefly describe the side effects associated with this treatment using a suitable subgroup of patients primarily eligible for this study.

Materials and Methods

The study was designed as a multicenter retrospective analysis of prospectively collected data in the inflammatory bowel disease (IBD) incidence registry EUROKIDS [6] to develop a clinical prediction rule. The TRIPOD guidelines for the development of clinical prediction rules (https://www.tripod-statement.org/wp-content/uploads/2020/01/Tripod-Checlist-Prediction-Model-Development.pdf) were used to complete the analysis.

Participants

This multicenter study was conducted in cooperation with European centers specialized in the management and treatment of pediatric patients diagnosed with IBD. Finally, data from 13 centers were included in the analysis.

Based on the inclusion criteria, 1190 newly diagnosed patients with CD were selected from 2241 IBD patients included in the EUROKIDS registry [6] until 2017. They were screened in each center separately and enrolled in this study. The inclusion criteria were as follows: diagnosis of CD up to 18 years of age based on the Porto criteria or revised Porto criteria [7], available data from the EUROKIDS registry, the need for an induction regimen by exclusive enteral nutrition (EEN) or corticosteroids (CS) at the time of diagnosis (including patients who needed a switch between EEN and CS during the induction phase), initiation of AZA or 6-MP treatment within 3 weeks from the time of diagnosis, complete cessation of induction treatment within 12 weeks from diagnosis and subsequent AZA/6-MP therapy at full maintenance dose (empirically or based on thiopurine methyltransferase [TPMT] status), and remission achieved at week 12 and at least 12 months of follow-up since diagnosis. Concomitant 5-aminosalycylates therapy was allowed. Patients who used a top-down strategy (e.g. induction

treatment by aTNF), with active perianal disease, intra-abdominal abscess or fistula, and with missing data on outcome were excluded.

Reported side effects that required AZA/6-MP treatment discontinuation were also considered an exclusion criterion, and patients with such side effects were not included in the primary analysis.

To estimate the frequency of side effects, we used a subgroup of 596 eligible patients in whom side effects of AZA/6-MP could be assessed. The patients were selected from the entire dataset of 1190 pediatric patients diagnosed with CD. All patients included in the primary analysis (n=441) and all patients who were excluded from the primary analysis and for whom information on possible side effects was available were included in the analysis (n=155).

Multicentric data collection

The main part of the data was obtained from the international prospective incidence registry EUROKIDS, the largest European pediatric IBD database that contains detailed data on disease phenotype and patients themselves at the time of diagnosis, date of diagnosis, center, age, gender, nationality, ethnicity, body weight, body height, body mass index (BMI), endoscopic and histological findings, findings on magnetic resonance enterography (MRE) or other imaging methods, extraintestinal manifestations, other CD complications, and any concomitant disease. Some of the variables (age at diagnosis, BMI Z-score) were calculated as both numerical values and factors using calculated cutoffs. To detect changes in therapeutic processes in pediatric CD over time, the years of diagnosis were also classified into the following time periods: 2004–2008, 2009–2011, 2012–2014, and 2015–2017.

Additional parameters that seemed clinically important and were readily accessible but were not recorded in the EUROKIDS registry were collected retrospectively. The selection was partly based on previously published data [8–11] – serum levels of albumin, hemoglobin, C-reactive protein (CRP), blood counts of leukocytes and platelets, fecal calprotectin, TPMT status (if available), and anti-Saccharomyces cerevisiae antibodies (ASCA) IgA and IgG levels – all at the time of diagnosis, final maintenance dose of AZA/6-MP (milligram per kilo [mg/kg]) between the 10th and 14th week, and date of endpoint, along with the reason for treatment change (relapse, complication, drug side effect, type of side effect), or the date of maximum follow-up in cases where the endpoint did not occur.

Potential predictors

We selected the following factors registered in the EUROKIDS registry: BMI Z-score and factorized BMI Z-score at diagnosis, height Z-score at diagnosis, age at the time of diagnosis, years of diagnosis (categorized as 2004–2008, 2009–2011, 2012–2014, and 2015–2017), gender, ethnicity, family history of IBD, extraintestinal manifestation at the time of diagnosis, location of CD at diagnosis, any concomitant disease at diagnosis, findings on small bowel through MRI, abdominal computer tomography (CT), abdominal ultrasound, and capsule endoscopy examinations at diagnosis, to be tested on potential predictive value in the context of early AZA/6-MP treatment failure. These were complemented by nine factors (listed above) obtained via multicentric retrospective data collection.

Definition of remission, relapse, and side effect (clinical evaluation of patients)

The primary outcome of this study was the identification of predictors of AZA/6-MP treatment failure in pediatric patients with CD and creation of a predictive model enabling the differentiation of these patients at the time of diagnosis. For this purpose, the time to relapse,

defined as the need for reinduction of remission (initiation of antibiotics, EEN, special diets, CS, aTNF, other biologicals [e.g., vedolizumab, ustekinumab], thalidomide or surgery [resection or stoma]) or development of CD-related complications (abscess, fistula, clinically significant stricture) or death, was monitored. Assessment of remission was based on the decision of the treating physician, considering the clinical condition and laboratory parameters. The minimum follow-up period without reaching the endpoint was 12 months.

The side effects were assessed by the treating physician and categorized as nausea/vomiting, acute pancreatitis, allergic reactions, liver test abnormalities, and bone marrow suppression.

Statistical analysis

The univariate risk analysis presents grouped empirical hazard estimates. Hazard differences between groups were tested using the log-rank test. In the multivariate analysis, a sequence of Cox models was fitted to predict the risk of relapse across the centers. All models were adjusted for the center. Variables appearing significant on univariate risk analyses were added to the model one by one, interactions among them were tested, and terms that were nonsignificant by partial likelihood ratio tests were dropped. The final model was fitted to 380 patients with complete data, of whom 252 had a relapse observed and the rest were censored.

Ethical considerations

The study was approved by the ethics committee of the authors' institution (EK-1491/16). If applicable, local ethics committee approval was obtained from the participating centers based on local requirements. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institution's human research committee.

Results

Patient characteristics

Among 1190 patients, 441 (251 males [56.9%], median age at diagnosis 13.67 [Inter quartile range (IQR) 11.33–15.25] years) fulfilled the inclusion criteria and did not meet the exclusion criteria. The eligibility of patients is shown in Figure 1. Patients' demographic and clinical characteristics according to the Paris classification are listed in Table 1. Median time of follow-up was 1.47 (IQR 0.76–3.39) years.

Time to relapse on AZA/6-MP treatment

The median time to clinical relapse was 2.11 (95% confidence interval [CI] 1.59–2.46) years. Remission was observed in 50.3% of patients after 2 years of AZA/6MP treatment (Figure 2).

Univariate risk assessment

Of all tested parameters available at the time of diagnosis (Appendix Figure S1), only the following were found significantly associated with early relapse on AZA/6-MP treatment when tested separately: CRP (p=0.0480), Z-score of BMI (p=0.0079) and BMI Z-score >0.8 SD (p=0.0039), Z-score of weight (p=0.043), age at the time of diagnosis (p=0.0052) and at the time of onset of symptoms (p=0.034), year of diagnosis classified into four categories (2004–2008, 2009–2011, 2012–2014, and 2015–2017) (p=0.005), abnormal cecum imaging findings (p=0.044), abnormal esophageal endoscopy findings (p=0.0473), availability of determination of AZA/6-MP metabolites (p=0.0005), TPMT genotyping accessibility (p=0.013), and center (p=0.0005).

In particular, the AZA/6-MP dose did not show any significant effect on relapse in the univariate analysis. The median dose administered was 2.04 mg/kg (IQR 1.77–2.27). Within

AZA dose effect assessment, we were not able to reflect AZA levels, as this information is not available in most centers. Due to the low number of patients with TPMT status tested, it was not possible to analyze whether there was any effect of lower dosing based on genotype.

Multivariate risk assessment

The results are presented in two parts (Table 2a and b). The first part shows the main effects of noninteracting factors: CRP at diagnosis (Hazard ratio [HR]=1.396, 95%CI 1.019–1.912, p=0.038), BMI Z-score >0.8 SD at diagnosis (HR=0.533, 95%CI 0.360–0.789, p=0.002), abnormal sigmoid imaging findings at diagnosis (HR=1.503, 95%CI 1.021–2.212, p=0.039), abnormal esophageal endoscopy findings at diagnosis (HR=1.712, 95%CI 1.176–2.490, p=0.0050), and L3 classification at diagnosis (HR=1.396, 95%CI 1.048–1.860, p=0.023) (Table 2a). The second part presents the interactions between AZA and age (p=0.031). A significant protective effect was observed in the age group of 8–11 years (HR=0.441, 95%CI 0.223–0.870, p=0.0183), with the relapse rate reduced by half per each mg/kg increase in AZA dose (Table 2b). See the appendix for more detailed results.

Based on the aforementioned, a web-based tool that allows the use of this prediction model in clinical practice was developed: https://gastroped.shinyapps.io/AZA_time_to_relapse/.

Using the final predictive model, we were able to identify two subgroups of patients who had either particularly low or particularly high risk of relapse. They were compared with hypothetically “intermediate-risk” patients. Based on the coefficients of the final model, the low-risk group was defined as follows: age at diagnosis 0–7 years with AZA dose <2 mg/kg or age 8–11 years with AZA dose >2 mg/kg or age 15–18 years with AZA dose >2 mg/kg, BMI Z-score >0.8 SD, no abnormal sigmoid imaging findings, and no abnormal endoscopic findings

in the esophagus. In contrast, the high-risk group was defined as follows: age at diagnosis 12–14 years regardless of AZA dose or age 8–11 years with AZA dose <2 mg/kg, BMI Z-score <0.8, abnormal sigmoid radiology findings, or abnormal endoscopic findings in the esophagus. A comparison between the low-risk and high-risk groups is shown in Figure 3.

Side effects

Although this study was not primarily aimed at identifying the side effects of AZA/6-MP, using a group of patients for whom this information was available, we performed an ancillary analysis of this objective. Among all patients with data on the side effects of AZA/6-MP treatment available (n=596), the most common side effect was bone marrow suppression (n=31, 5.7%). Acute pancreatitis (n=14, 2.6%), liver test abnormalities (n=15, 2.8%), nausea/vomiting (n=12, 2.2%), and allergic reactions (n=1, 0.2%) were also recorded. The proportion of all patients treated with AZA/6-MP and all side effects observed in these patients is displayed in Figure 4. AZA/6-MP treatment had to be terminated due to side effects in eight patients (1.34%); five with bone marrow suppression, one with liver test abnormality, one with acute pancreatitis, one with lymphoma. In addition, one lymphoma developed after an outcome with a questionable relationship to the therapy, and one rhabdomyosarcoma was recorded in patients who did not stop AZA therapy before reaching the endpoint. No death was reported. Due to the small number of patients who ceased therapy because of developing side effects, it was not possible to predict these particular cases.

Discussion

We identified six independent predictors (BMI Z-score, age, disease localization and CRP) available at diagnosis of early AZA/6-MP treatment failure in patients who achieved remission on EEN or CS and were concomitantly treated with AZA/6-MP. Using these data from an international multicentric registry, we developed a model for individual prediction of time to relapse in pediatric patients with CD. Although none of the predictors was strong enough to be used as a clinical tool, the model based on a combination of multiple variables could be clinically useful. At the moment, we should confess that prospective validation on external cohort is needed before the full introduction of the model to clinical practice.

To the best of our knowledge, we have described the largest group of pediatric patients with CD treated with AZA/6-MP. Relapse was observed in 49.7% of patients within 2 years of AZA/6-MP treatment. This number is not as optimistic as the result published in the only randomized controlled trial in pediatric patients with IBD, where over 90% of patients treated with 6-MP retained remission after 2 years [12]. However, it is almost equal to the findings of recent retrospective studies, which showed an almost 50% failure rate of AZA/6-MP treatment within this time period [8, 9]. We assume that dissimilarities in these findings may be partially caused by a common occurrence where, in general, the results of clinical studies do not achieve such success as a randomized controlled trial. The position of thiopurines in the treatment algorithm for pediatric patients with CD, respectively IBD was also confirmed in a recent prospective study [13]. In this study, thiopurines were safe and effective in 21% of patients with CD and 27% of those with ulcerative colitis. The outcome was defined as CS- and EEN-free remission without treatment escalation for 12 months. However, a subgroup of patients who may benefit from this treatment should be defined.

Many reasons pointing to the importance of choosing an appropriate therapeutic approach for pediatric patients with CD can be identified. The long-term prognosis of CD is significantly influenced by the progression of inflammation or development of complications [14]. Early initiation of an intensive therapeutic regimen, including administration of aTNF drugs, could prevent the progression of inflammation and development of complications if applied correctly [15, 16]. Moreover, the use of monotherapy with aTNF exposes patients to a lower risk of side effects than AZA treatment [17, 18]. Conversely, economic constraints and limited duration of action of the biological treatment [4, 5] along with an increased risk of opportunistic infections and frequent need for combination therapy with immunomodulators [19–22], are the principal disadvantages of aTNF therapy. This emphasizes the need for careful selection of patients suitable for AZA treatment or early initiation of aTNF.

Predictive factors are essential for this selection. The choice of outcome plays a very important role in the search for these predictors. For our purpose, to prevent early failure of the chosen treatment, the first relapse of the disease seems the most appropriate. Many studies describing variables predictive of general outcomes (irrespective of maintenance treatment type) in pediatric patients with CD have been published. They differ in many aspects, such as the definition of the outcome, study population, and examined variables. Kugathasan et al. [23] supported the importance of risk stratification in pediatric patients with CD using a large prospective inception cohort of newly diagnosed children with CD. In a competing-risk model based on clinical and serological variables, only CBir1 seropositivity was significantly associated with stricturing disease behavior and an older age, African-American race, and ASCA IgA and CBir1 seropositivity with penetrating behavior of the disease. Older age and low baseline CRP predicted increased the probability of normal CRP remission (CRP <0.5 mg/dL) at week 12 in a prospective study by Levine et al. [24]. The only predictor for CS-

free remission (defined by Pediatric Crohn's disease activity index [PCDAI] <10 points or <7.5 without the height component) was older age at baseline. Steroid-free remission by itself at week 12 was a predictive factor for remission at week 52. However, all therapeutic regimens (including biological treatment) were allowed in this study. Siegel et al. [25] found that older age at diagnosis, female gender, small bowel location, and perianal fistulizing disease were predictive of a complicated disease course. Older age, female gender, leukocytosis in blood count, elevated serum levels of CRP, hypoalbuminemia, fistulizing and stricturing disease behavior, and growth retardation have been proven to be significantly predictive of the need for surgery in two retrospective studies [26, 27]. None of these studies was designed to assess the response to a specific therapeutic approach in patients with CD.

Although we can use general predictors, predictors of early relapse in relation to a specific therapeutic approach are crucial. We identified only a few studies that focused on predictive factors in thiopurine monotherapy. In contrast to our study, Riello et al. [8] did not identify any predictive factor in the context of early relapse defined as PCDAI >10 with no CS, need for reinduction, or change of immunomodulator. Age, gender, location and behavior of disease, perianal fistulizing disease, and extraintestinal manifestation at baseline were assessed in this retrospective study. Similar to our study, a small retrospective study primarily focusing on induction treatment modality [9] found that age <16 years and involvement of the upper gastrointestinal tract at diagnosis were predictive of early relapse, defined as an increase in disease activity with the need for additional reinduction therapy (CS, EEN, aTNF therapy, or surgery), in patients originally indicated for long-term immunosuppressive treatment of AZA/6-MP. We were not able to confirm other predictors identified in this study (elevated platelet count at remission and lower height Z-score at diagnosis). A simple endoscopic score for CD >16 points at the time of diagnosis was revealed as an independent risk factor for the

development of complications in a retrospective study primarily interested in the predictive value of the histopathological scoring system [28]. None of these studies described a separate predictor strong enough to be applicable in routine clinical practice. A combination of these factors may be needed for the development of clinically useful predictive tools.

A validated web-based tool displaying an individualized predicted outcome for adult patients with CD has already been developed [29]. The final multivariate model in this study included disease location (small bowel, left colonic disease, perianal disease), serologic markers (ASCA, CBir1, Anti Neutrophil Cytoplasm Autoantibodies [ANCA]), the NOD2 frameshift mutation, and an interaction between perianal fistulizing disease and ASCA. Different outcomes (time from diagnosis to first CD-related complication regardless of therapeutic approach) and different study populations (e.g., inclusion of patients with perianal fistulizing disease, who were excluded from our dataset) have to be taken into account when considering that none of the described variables, within those included in our work as well, was found significant in our study. Waljee et al. [30] focused on the prediction of remission, defined as the absence of objective evidence of intestinal inflammation and clinical outcomes in patients on thiopurines. A machine-learning algorithm was created for this purpose. The five most important variables reported in this study were hemoglobin, lymphocytes, hematocrit, neutrophils, and platelets. However, it is also difficult to compare the results from this retrospective study due to different outcomes and study populations [30]. These predictive models, which do not consider a treatment approach, have certain limitations for pediatric patients, especially because they are designed for adult patients.

Even though we were not able to find a universal AZA/6-MP dose effect on relapse, the test for interaction between AZA dose and age was significant. It suggests that AZA may act differently

in different age groups. Small retrospective study [9] found age below 16 years predictive for early relapse in patients treated with AZA/6-MP initially. On the contrary, Riello et al. [8] did not find any predictors at baseline, including age at the time of initiation of AZA/6-MP, as factors influencing the response to AZA/6-MP treatment. In this study mean age in the relapse (11.6) and the non-relapse (12.1) group were comparable (p=0.17). Nguyen et al. 2013 [31] also showed that age does not predict efficacy of AZA treatment in pediatric CD patients (median age 13.8 (10.4–15.8) years). These results correspond to our findings that AZA/6-MP dose probably plays an important role especially in specific age group 8 – 11 years (a non-linear association due to interaction). Since we do not have any evidence explaining why the dose of AZA had an important effect in this age group, we can only speculate about slightly different pharmacokinetics of the drug in younger age groups or adolescents' compliance to the treatment as possible explanations of this occurrence.

Although this study, performed on the largest pediatric cohort, identified useful predictors of AZA/6-MP treatment outcome, it still has some limitations. Validation on an external cohort was not performed and should be done before the full implementation of this model in clinical practice. On the other hand, considering that there are almost no data available, the newly identified predictors of AZA/6-MP effect duration might a useful lead to the clinicians in management of pediatric patients with CD from now on. For the development of the prediction tool, we intended to use as accurate data as possible. Although some data were collected retrospectively, most variables were extracted from a large prospectively recorded Europe-wide database. However, there are many factors that may influence the outcome but cannot be forecasted within our study, such as the effect of diet [32, 33], smoking [34] or other personal habits. Therefore, it is difficult to predict the response to a particular treatment based on the information available at the time of diagnosis. Because relapse was defined as the necessity of

reinduction of remission, different approaches to the management and care of patients with IBD in the participating centers should be considered one of the main limitations of our study. Conversely, collaboration with other centers was the only way to realize this kind of study.

Although this was not the primary aim of the study, we aimed to estimate the number of side effects associated with AZA/6-MP treatment in pediatric patients with CD. However, because of the characteristics of the collected data, it is difficult to consider these numbers as relevant frequencies, as they are collected from a selected population.

Conclusion

In conclusion, we identified BMI Z-score, age, disease localization (abnormal sigmoid imaging findings, abnormal esophageal endoscopy findings and L3 classification) and CRP at the time of diagnosis as independent predictive factors for early AZA/6-MP treatment failure in pediatric patients with CD. The patient's age at diagnosis in interaction with the AZA dose may be a risk or protective factor for early relapse. However, none of these factors alone is strong enough to be used in clinical practice, therefore, using a combination of these factors, a model that is applicable to clinical practice, and thus allows better selection of patients suitable for AZA/6-MP treatment, was created. Given that 50% of patients were in clinical remission after 2 years of AZA/6-MP treatment initiation, thiopurines can still be considered a suitable treatment option for pediatric patients with CD, especially for those selected based on the newly developed web-based application.

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Figure legends

Figure 1. Selection of patients based on inclusion and exclusion criteria.

PIBD: pediatric inflammatory bowel disease, CD: Crohn's disease, AZA: azathioprine

Figure 2. Time to relapse in pediatric patients with Crohn's disease on azathioprine/6-mercaptopurine treatment.

Figure 3. Comparison of risk groups.

Blue: low-risk group (HR=1), median time to relapse 4.98 years, relapse-free probability at 2 years 0.738. Green: intermediate-risk group (HR=2.59), median time to relapse 1.63 years, relapse-free probability at 2 years 0.455. Red: high-risk group (HR=6.54), median time to relapse 0.70 years, relapse-free probability at 2 years 0.137.

Note: These results focus on clinical characteristics and do not consider between-center differences in the risk of relapse. They are all calculated for a patient located in the largest center in terms of participating patients, with medium risk among all centers)

HR: Hazard ratio

Figure 4. Side effects associated to azathioprine/6-mercaptopurine treatment.

The category "other" includes lymphoma, infection, alopecia, headache, tiredness, and skin manifestation (exanthema, pruritus, and warts)

Table 1. Patient's demographic and clinical characteristics according to Paris classification

	CD
Number of patients	441
Sex, male, (%)	251 (56.9)
Age at diagnosis, years, median (IQR)	13.67 (11.33 – 15.25)
Ethnicity, white European, (%)	406 (92.1)
L1, (%)*	68 (17.8)
L2, (%)*	42 (11.0)
L3, (%)*	273 (71.3)
L4a, (%)[#]	85 (36.6)
L4b, (%)[#]	43 (18.5)
L4ab, (%)[#]	21 (9.1)
B1, (%)[+]	372 (85.7)
B2, (%)[+]	62 (14.3)
B3, (%)[+]	0 (0.0)
B2B3, (%)[+]	0 (0.0)
p, (%)	0 (0.0)

*L classification was missing in 58 (13.2%) patients, the values are based on 383 patients with complete data

[#] L4 classification was missing in 209 (52.6%) patients, the values are based on 232 patients with complete data

[+]B classification was missing in 7 (1.6%) patients, the values are based on 434 patients with complete data

Table 2. Parameters of final predictive model

Table 2 a. Main effects of non-interacting factors

	HR	95% CI for HR	p-value
Center	NA	NA	0.0003
CRP (per 100 mg/L)	1.396	1.019 - 1.912	0.0377
BMI Z-score > 0.8	0.533	0.360 - 0.789	0.0016
L3[1] classification	1.396	1.048 - 1.860	0.0226
Abnormal sigmoid radiology	1.503	1.021 - 2.212	0.0388
Abnormal esophagus endoscopy	1.712	1.176 - 2.490	0.0050

Table 2 b. Estimated AZA effects on relapse in different age groups

	HR	95% CI for HR	p-value
Effect of 1 unit of AZA dose in age group 0-7 years	1.667	0.593 - 4.680	0.3323
Effect of 1 unit of AZA dose in age group 8-11 years	0.441	0.223 - 0.870	0.0183
Effect of 1 unit of AZA dose in age group 12-14 years	1.285	0.795 - 2.078	0.3065
Effect of 1 unit of AZA dose in age group 15-18 years	0.722	0.387 - 1.347	0.3056

[1]Ileocolonic involvement

HR: hazard ratio, CI: confidence interval, CRP: C-reactive protein, BMI: body mass index, AZA: azathioprine

Figure 1. Selection of patients based on inclusion and exclusion criteria.

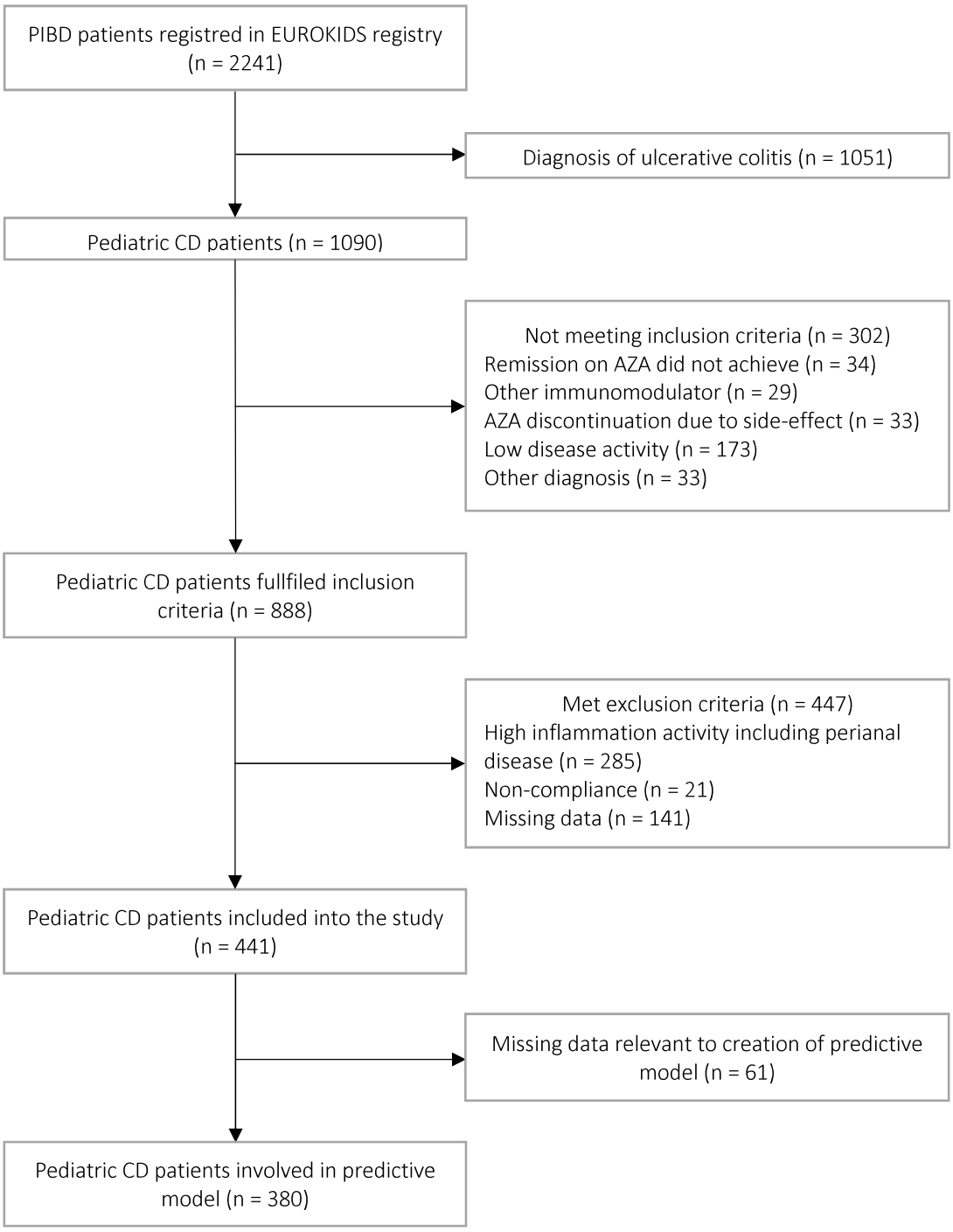


Figure 2. Time to relapse in pediatric patients with Crohn's disease on azathioprine/6-mercaptopurine treatment.

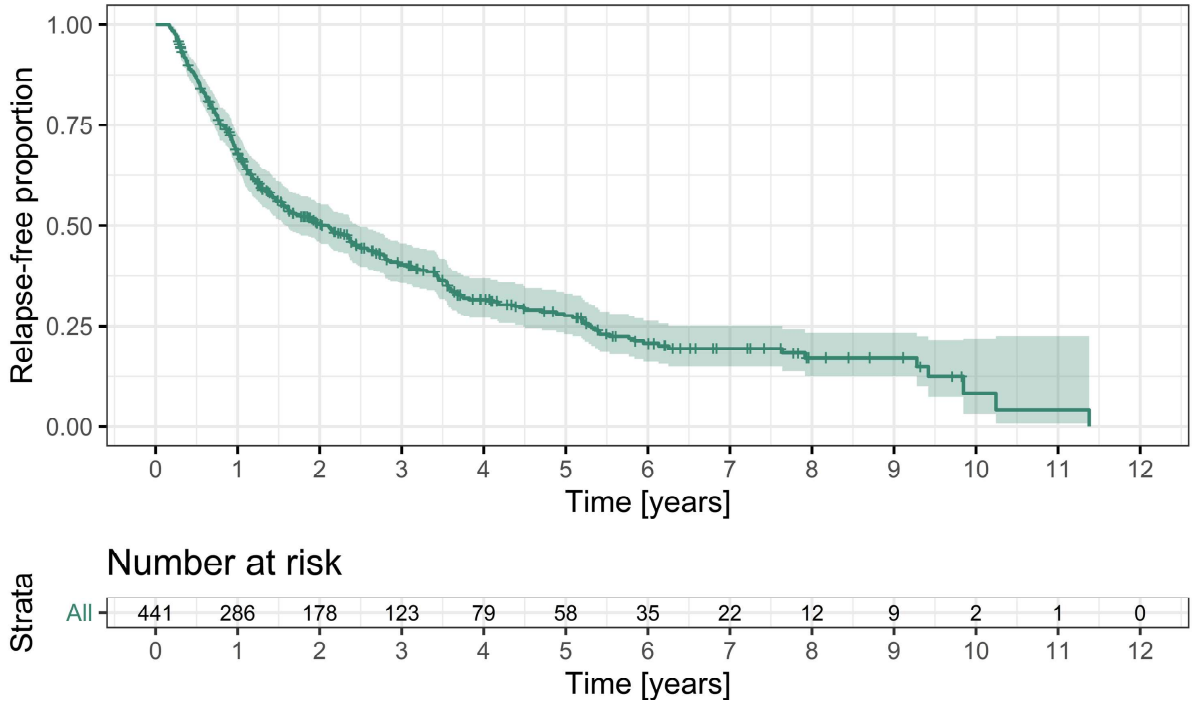


Figure 3. Comparison of risk groups.

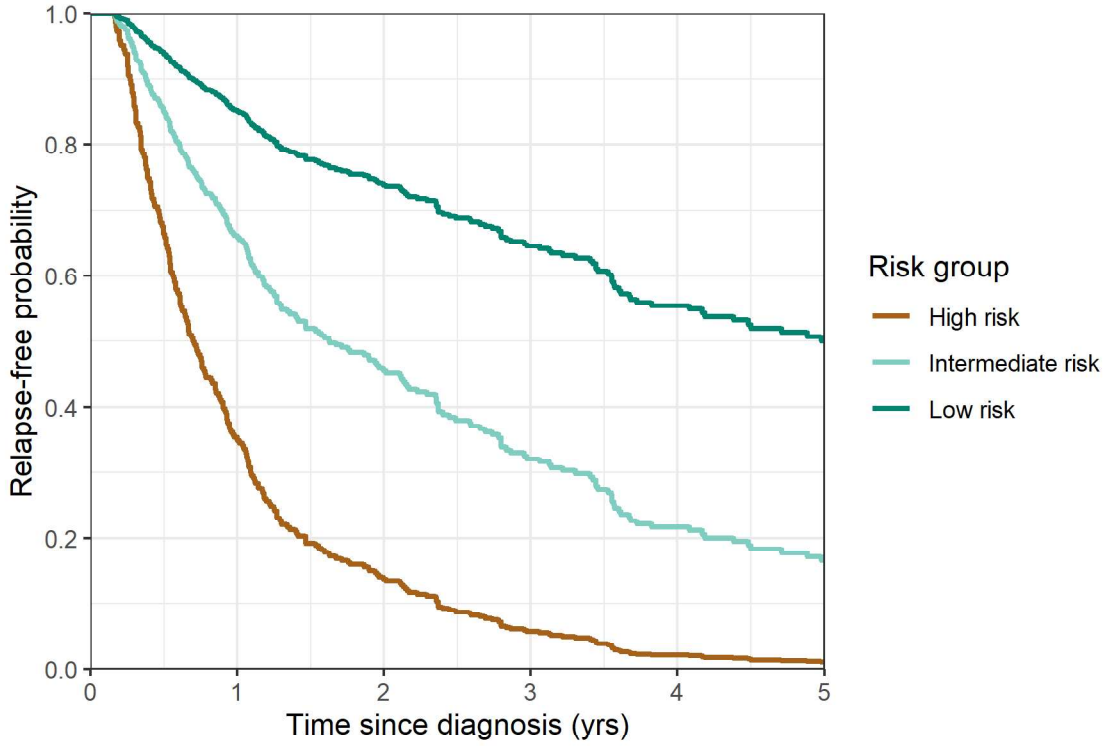
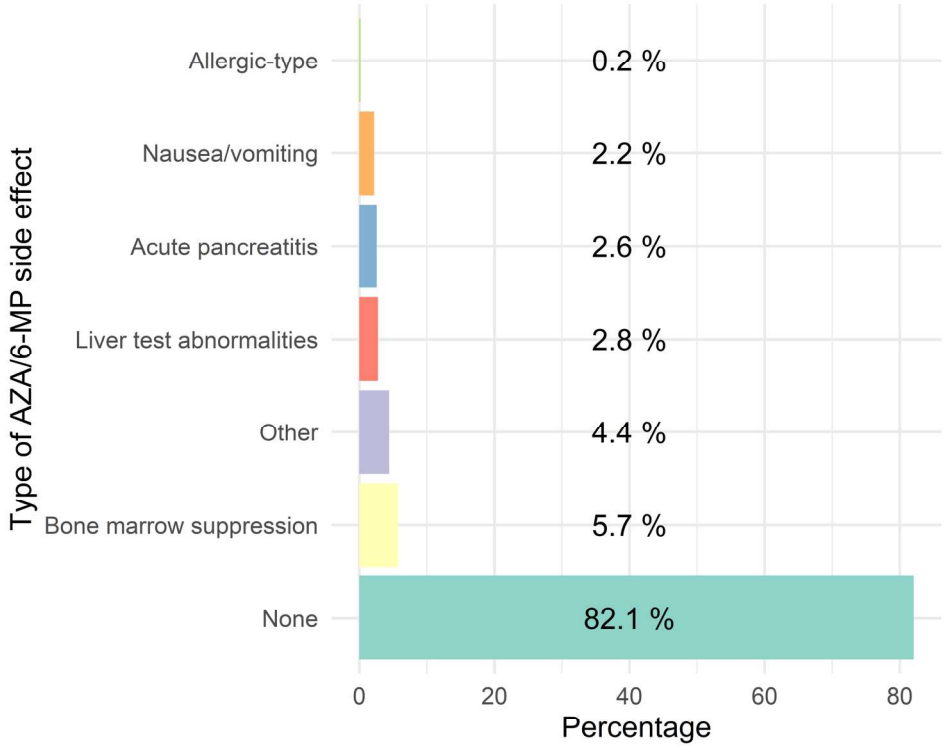


Figure 4. Side effects associated to azathioprine/6-mercaptopurine treatment.



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ORIGINAL ARTICLE

Fecal calprotectin is not a clinically useful marker for the prediction of the early nonresponse to exclusive enteral nutrition in pediatric patients with Crohn disease

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Abstract

Exclusive enteral nutrition (EEN) has been recommended as the first-line therapy in children with active Crohn disease (CD). The primary aim of our study was to determine whether it is possible to use the difference between basal fecal calprotectin (F-CPT) and the value at week 2 of EEN to predict clinical response at week 6. We prospectively collected stool samples for F-CPT analysis and clinical and laboratory parameters during EEN from 38 pediatric patients (28 boys, median age 12.8 years) with newly diagnosed active luminal CD. The difference between F-CPT concentrations before EEN and at week 2 did not predict clinical non-response at week 6 (OR 0.9996 95% CI 0.9989–1.0002, $p = 0.18$); however, it predicted patients who did not achieve clinical remission at week 6 (OR 0.9993, 95% CI 00.9985–0.9998, $p = 0.006$) with sensitivity of 58%, and specificity of 92% for cut-off of F-CPT increase by 486 μg/g.

Conclusions: An early decrease in F-CPT levels in children with newly diagnosed active luminal CD did not predict clinical response at week 6 of EEN induction therapy, and clinical remission was predicted with low accuracy. Therefore, F-CPT cannot be used as a predictor to select the patients in whom EEN should be terminated.

What is Known:

- *The fecal calprotectin (F-CPT) is an important marker of intestinal inflammation.*
- *Approximately 25% of pediatric patients with Crohn disease (CD) do not achieve clinical remission, and there is still no sufficient predictor of response to exclusive enteral nutrition (EEN) treatment.*

What is New:

- *The difference between the F-CPT concentrations before EEN treatment and at week 2 did not predict clinical response to treatment at week 6, even if it predicted clinical remission, however, with low accuracy. F-CPT is not a suitable predictor to select the patients for discontinuing of EEN induction therapy.*

Keywords Calprotectin · Inflammatory bowel disease · Prediction · Remission · Response

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Abbreviations

AUC	Area under the curve
AZA	Azathioprine
CD	Crohn disease
CI	Confidence interval
CRP	C-reactive protein
ECCO	European Crohn and Colitis Organization
EEN	Exclusive enteral nutrition
ELIA	Fluorescence immunoassay
ESR	Erythrocyte sedimentation rate
F-CPT	Fecal calprotectin
IQR	Interquartile ranges
OR	Odds ratio
ROC	Receiver operating characteristic
wPCDAI	Weighted Pediatric Crohn Disease Activity Index

Introduction

Crohn disease (CD) is a chronic inflammatory condition characterized by a relapsing and remitting course. Exclusive enteral nutrition (EEN) has been shown to be as effective as corticosteroids [17], without the side effects, in achieving clinical remission, and superior in achieving mucosal healing in pediatric patients with luminal CD [14]. In addition, EEN promotes optimal nutritional supply to improve malnutrition, linear growth, muscle mass, and bone strength [2]. Based on its multiple benefits, the European guidelines recommend EEN as the first-line therapy in children with active CD [31]. However, approximately 25% of patients do not achieve clinical remission after an EEN course, and the European Crohn and Colitis Organization (ECCO) suggested that an alternative treatment should be considered if EEN does not induce clinical response within 2 weeks [24]. There is still no sufficient predictor of the successful induction of remission in children with CD who are treated with EEN [7, 10].

Over the past few years, fecal calprotectin (F-CPT) has been gaining recognition as an important marker of intestinal inflammation, and data pertaining to its usefulness in monitoring disease activity is becoming evident [13, 15, 20]. The F-CPT value correlates closely with clinical, endoscopic, and most tightly with histological findings in adult and pediatric CD patients, as well as with disease severity and extent [5, 9, 18, 19, 26].

As described above, EEN has many benefits for patients, but the question is how to identify patients in whom the continuation of EEN will probably be ineffective and another induction treatment should be used instead. We hypothesize that early change in F-CPT levels could be used to select the patients in whom continuing in EEN treatment will probably be ineffective. Therefore, the primary aim of our study was to determine whether it is possible to use the difference in the F-CPT value before treatment and in the early phase of treatment with EEN (F-CPTΔ02) to predict the clinical non-response at week 6, as defined by the weighted Pediatric Crohn Disease Activity Index (wPCDAI). The secondary aim was to identify whether F-CPTΔ02 can be used to predict patients who did not achieve clinical remission (as defined by the wPCDAI, F-CPT, or C-reactive protein (CRP)).

Methods

Participants

The study was designed as a prospective-observational study. Between 2015 and 2017, we screened all the pediatric patients with CD followed at our tertiary referral center for eligibility (Fig. 1) to be included in this study. Patients were diagnosed according to the revised Porto criteria [23]. In addition, they had to have a clinically active disease (wPCDAI > 12.5). Enrolled children had to receive EEN for the first time and had a follow-up of at least 6 weeks. Patients could choose a single or combinations of any polymeric enteral formula(s) available at our department ("Fresubin", Fresenius; "Nutridrink", Nutricia; "Ensure", Abbott; "Modulen", Nestlé) according to their preferences. Individuals who could not complete feeds orally were fed via a nasogastric tube. The goal intake for the EEN treatment corresponded to approximately 120% of the daily caloric needs of the patient. The resting energy expenditure was calculated according to the Schofield equation [16] using a web application. All the patients had to be treated with azathioprine (AZA) concomitantly (AZA therapy was initiated along with EEN and continued after EEN withdrawal) [24]. Patients with a history of systemic or local steroid treatment 3 months before the initiation of EEN, and those with a history of receiving anti-TNF-α therapy or having active perianal disease were not included. A responder was defined as a patient with a wPCDAI lower than or equal to 12.5, or a decrease in the wPCDAI by more than 17.5 points at week 6. Remission was defined as wPCDAI ≤ 12.5 points at week 6, as previously published [30]. The remission by CRP (CRP remission) was defined as CRP < 5 mg/kg, and the remission by F-CPT (F-CPT remission) was defined as F-CPT < 150 mg/g [21, 29]. As predictors we used the difference between the baseline level of F-CPT and week 2 (F-CPTΔ02) and percentage change of the F-CPT until week 2 (F-CPT%02). The patients' demographic and clinical characteristics are listed in Table 1.

Detection of F-CPT concentrations

F-CPT was analyzed using fluorescence immunoassay (ELIA) in three consecutive stool samples collected at the time of diagnosis (samples were taken before the start of bowel preparation for colonoscopy) and during treatment with EEN (at

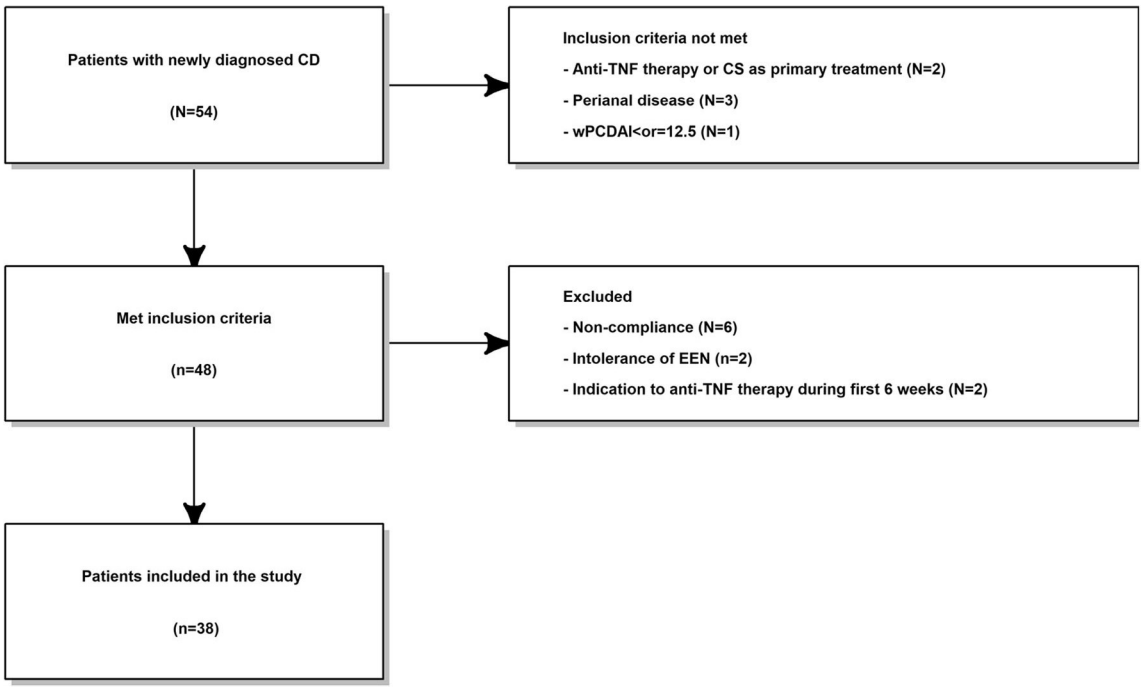

Fig. 1 Participants' selection. Fifty-four pediatric patients, newly diagnosed with Crohn disease (CD), underwent exclusive enteral nutrition (EEN) treatment at our center. In the case of 48 patients, signed consent forms were obtained and EEN treatment was started. Eligible children received EEN treatment for the first time and had a follow-up of at least 6 weeks. During the follow-up, we excluded another ten cases

weeks 0, 2, and 6). Stool samples were collected in screw-capped plastic containers and immediately prepared and analyzed using the EliA Calprotectin 2 test (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Based on the manufacturer's data, the measuring assay with results above 6000 μg of calprotectin per gram of stool (μg/g) was less exact.

Data collection

We prospectively collected F-CPT samples at the time of diagnosis, at week 2 and at week 6 of EEN treatment and clinical, anthropometric, and laboratory data at the time of diagnosis and at week 6 of EEN treatment.

Statistical analysis

All data analysis was performed using the R statistical software (version 3.3.2). Continuous variables were described as medians and interquartile ranges (IQR). Categorical variables were described as absolute frequencies and percentages. Changes in the clinical and systemic markers of disease activity during EEN were assessed with a paired *t* test. To test the association between two numerical variables, a correlation test was used. Differences between the groups of patients were tested using the Welch two sample *t* test. Linear regression and a generalized logistic regression model were used to determine the association between numeric outcomes and numeric predictors and binary outcome numeric or categorical variables, respectively. We used receiver operating characteristic (ROC) curves to define the optimal cut-off values of the inflammatory markers (R package "pROC"). For plot construction, we used R's ggplot2. A p value less than 0.05 was considered statistically significant.

Results

Patients' characteristics

Fifty-four pediatric patients, newly diagnosed with CD, underwent EEN treatment at our center during the study period. Signed consent forms were obtained from 48 patients and these were enrolled in the study. During the study, we excluded another ten children due to non-compliance (stool samples were not provided) of patient or family ($n = 6$), intolerance of EEN (also via nasogastric tube) ($n = 2$) and need for initiation of anti-TNF-α therapy for the development of perianal disease ($n = 2$). Finally, we included 38 pediatric patients (28 boys, median age 12.8 years (IQR 9.7–15.5)) from whom three consecutive stool samples were prospectively collected and tested for F-CPT concentrations at the time of diagnosis and during EEN treatment (at weeks 0, 2, and 6) (Fig. 1). All the patients completed at least 6 weeks of EEN treatment, and all but one child tolerated EEN orally. This child was fed via a nasogastric tube, introduced on the second day of therapy.

Table 1 Characteristics of patients according to the Paris classification and laboratory parameters at the time of diagnosis

	Patients with Crohn disease
Number of patients	38
Gender, female, (%)	10 (26)
Age at diagnosis, years, (median [IQR])	12.8 (9.7–15.5)
Disease location	
L1, (%)	17 (45)
L2, (%)	1 (3)
L3, (%)	20 (53)
Upper GI involvement, (%)[a]	25 (66)
Disease behaviour	
B1, (%)	30 (79)
B2, (%)	8 (21)
B3, (%)	0 (0)
p, (%)[b]	1 (3)
Growth retardation, (%)	2 (1)
F-CPT [μg/g], (median [IQR])[c]	1096 [559, 3814]
CRP [mg/l], (median [IQR])[d]	20.50 [8.00, 34.50]
ESR [mm/h], (median [IQR])[e]	31.00 [19.25, 40.00]
wPCDAI, (median [IQR])[f]	41.00 [28.25, 49.50]
wPCDAI > 12.5, (%)[g]	38 (100)

[a] Upper gastrointestinal (GI) involvement corresponded to L4a, L4b or L4ab

[b] p = perianal disease (the Paris classification)

[c] F-CPT = fecal calprotectin

[d] CRP = C-reactive protein

[e] ESR = erythrocyte sedimentation rate

[f] wPCDAI = weighted Pediatric Crohn Disease Activity Index

[g] Active disease based on the wPCDAI

Development of the wPCDAI, systemic inflammatory markers, and F-CPT levels during EEN treatment

At the beginning of the treatment, 19 (50%) patients had mild disease (wPCDAI > 12.5 < 42.5), 13 (34%) had moderate disease (wPCDAI ≥ 42.5 < 57.5), and 6 (16%) had severe disease (wPCDAI ≥ 57.5) (median wPCDAI 41.0 (IQR: 28.25, 49.5)). At week 6, based on the wPCDAI, 87% responded (assigned as responders), 68% of the patients achieved clinical remission, and the median wPCDAI decreased to 7.0 (IQR 0.0–17.0), Fig. 2a. The development of the laboratory parameters is shown in Fig. 2 b, c.

The basal F-CPT levels in all the patients (100%) were elevated above 150 μg/g (median 1096 μg/g, IQR: 559.2–3814.2) at the time of diagnosis. Until week 2, there was no significant improvement in the F-CPT concentrations, and the median F-CPT dropped to 1055 μg/g (IQR: 535–2003). In addition, in 16 children (42%) the F-CPT levels increased. From the start of treatment to week 6 of EEN treatment, the median F-CPT concentrations decreased by 510 μg/g (IQR: 115–1545) ($p = 0.004$). In only four patients, F-CPT levels ≤ 150 μg/g at week 6 of EEN treatment were achieved, and during the course of the treatment, we found interindividual changes in the F-CPT concentrations, which varied considerably. In summary, while four children (11%) had F-CPT ≤ 150 μg/g, and seven (18%) had concentrations > 150/≤ 300 μg/g, most of the participants ($n = 27$, 71%) continued to have high levels, above 300 μg/g, after 6 weeks of treatment (Fig. 2d). The correlation between the change in F-CPT levels from the time of EEN initiation until weeks 2 and 6 was significant ($r = 0{,}71$) ($p < 0.001$).

Prediction of patients who did not respond to EEN treatment

The F-CPTΔ02 did not predict patients who did not respond to EEN at week 6 (OR 0.9996013, 95% CI 0.9988623–1.000162, $p = 0.18$), even after adjusting for logarithms of base-line F-CPT (OR 0.9991118, 95% CI 0.9979461–1.000076, $p = 0.073$). We did not find an association between F-CPT%02 (OR 1.021935, 95% CI 0.7072691–1.23026, $p = 0.85$) and clinical non-response to EEN treatment at week 6 (Table 2).

Prediction of patients who did not achieve clinical remission

The F-CPTΔ02 predicted patients who did not achieve clinical remission at week 6 (OR 0.9993066, 95% CI 0.9985436–0.9998284, $p = 0.006$). The non-remission was best predicted using the increase of F-CPT by 486 μg/g; this had a sensitivity of 58% and specificity of 92% (AUC 0.753) (Fig. 3a — supplementary material). We found border-line association between F-CPT%02 and non-remission ($p = 0.048$). The association of non-remission expressed as CRP > 5 mg/l or as CPT > 150 μg/g and with F-CPTΔ02 was also found. The CRP > 5 mg/l was not predicted by F-CPT%02. Remission defined as F-CPT > 150 μg/g was predicted by F-CPT%02, please see Table 2.

Using a cut-off of 1.47%, specificity was 79% and sensitivity was 80% (AUC 0.788) to predict patients who did not achieve clinical remission at week 6 of EEN treatment (Fig. 3b — supplementary material). The accuracy of predictors was described in Table 3.

Discussion

This prospective study has shown that the predictive value of F-CPT during the early phase of EEN treatment in children with newly diagnosed luminal CD is not sufficient to select patients who will probably not respond to EEN at the end of treatment.

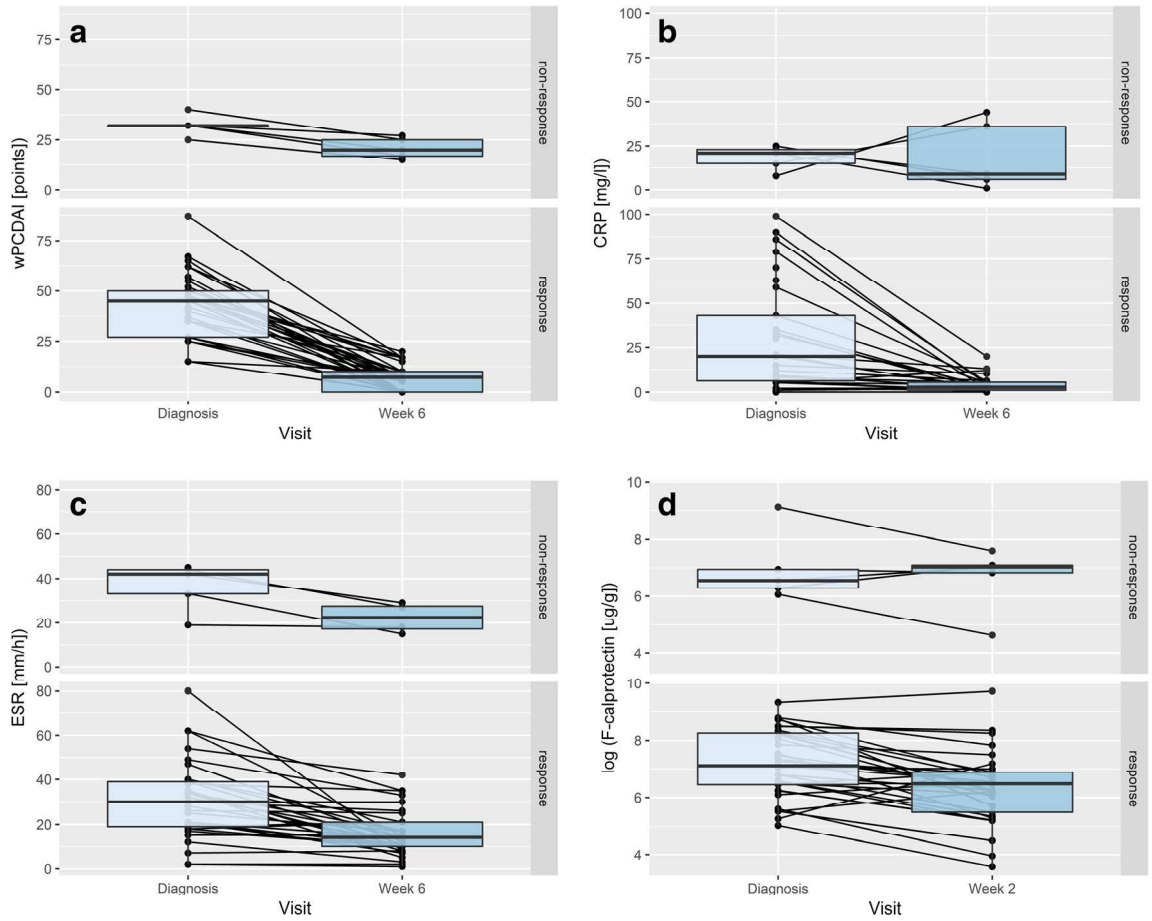

Fig. 2 The development of disease activity from the beginning of EEN treatment till week 6. Expressed as: **a** Weighted Pediatric Crohn Disease Activity Index (wPCDAI): at the beginning of exclusive enteral nutrition (EEN) treatment, all the included children had clinically active disease (wPCDAI > 12.5), and at week 6, 68% of the patients achieved clinical remission and the median wPCDAI decreased to 7.0 (interquartile range (IQR) 0.0, 17.0). **b**, **c** The erythrocyte sedimentation (ESR) and C-reactive protein (CRP) were abnormal in most of the participants (58 and 82%). At week 6 of EEN treatment, the levels of the laboratory markers improved, reaching the normal reference range (median ESR/CRP: 15.0 (IQR: 10.0, 22.0)/4.0 (IQR: 1.0, 6.0)) in majority of the children. **d** The fecal calprotectin (F-CPT) levels in all the patients were elevated above 150 μg/g (median 1096 μg/g, IQR: 559.2, 3814.2) at the time of diagnosis. Till week 2, the median F-CPT dropped to 1055 μg/g (IQR: 535, 2003) and till week 6 it decreased by 510 μg/g (IQR: 115–1545) ($p = 0.004$). In only four patients, F-CPT levels ≤ 150 μg/g at week 6 were achieved

To the best of our knowledge this was the first study trying to predict non-response during the early phase of EEN. Gerasimidis et al. [12] used the F-CPT value at day 30 for the prediction of remission. In this small pilot study, the reduction in baseline F-CPT levels had a sensitivity of 100% (95% CI: 54–100) and specificity of 89% (52 to 100) to predict induction of clinical remission at the end of treatment. However, we also found predictability of remission using F-CPTΔ02 and F-CPT%02; the study differs from the current one in important parameters. The authors chose remission defined by the original version of PCDAI as a main outcome, they used day 30 as the predictor, the duration of EEN was 8 weeks and only patients with colonic involvement were included. We believe that for routine practice the estimate of the patients who should discontinue EEN treatment earlier (non-responders) is an important outcome and that prediction needs to be done in the early stage of induction therapy. For response and remission, we used the wPCDAI to define disease activity based on a modified version of the original PCDAI, which assigns weight for the items of clinical presentation and laboratory parameters and excludes three items (height velocity, abdominal examination, and hematocrit) with insignificant discriminatory power [30]. Although using CPTΔ02, we could predict non-remission assessed by wPCDAI or CRP. The predictive ability was weak (AUC 0.739, 0.787 respectively), and thus clinically not useful. Endoscopic assessment at the end of induction therapy was not part of the protocol and would be ethically problematic; therefore, we chose F-CPT < 150 as approximation of mucosal healing and we were able to predict this using both F-CPTΔ02 and F-CPT%02. However, same as for remission, the predictive parameters were not accurate enough to be useful for clinical practice. Moreover, we found that a transient moderate increase in the F-CPT level in the early phase of

Table 2 Prediction of non-response, and non-remission at week 6 of EEN treatment based on absolute and percentage change of F-CPT till week 2

	F-CPTΔ02[a]	*p*	F-CPT%02[b]	*p*
[d]Non-response	0.9991, (0.9979–1.0001)[c]	0.073	1.0219, (0.7073–1.2303)	0.845
[e]Non-remission	0.9975, (0.9952–0.9989)[c]	<0.001	1.3337, (0.9984–2.2975)	0.048
[f]CRP > 5 mg/l	0.9985, (0.9969–0.9995)[c]	<0.001	1.1541, (0.9297–2.1174)	0.237
[g]F-CPT > 150 μg/g	1.0009, (1.0001–1.002)	0.023	0.7956, (0.5006–0.9838)	0.033

The associations were expressed as odds ratio (OR) with 95% confidence interval (CI). wPCDAI = weighted Pediatric Crohn Disease Activity Index

[a] F-CPTΔ02, difference between baseline level of F-CPT and week 2

[b] F-CPT%02, percentage change of the F-CPT

[c] Adjusted for logarithm of basal F-CPT level

[d] Non-response defined as wPCDAI > 12.5 points, or there is not a decrease in the wPCDAI by more the 17.5 points

[e] Non-remission defined as wPCDAI > 12.5 points

[f] CRP = C-reactive protein

[g] F-CPT = fecal calprotectin, EEN = exclusive enteral nutrition

EEN induction therapy did not exclude its decrease after the completion of treatment. The effect of EEN treatment on mucosal healing and, hence, on F-CPT levels seemed to be gradual, confirming the necessity of a given length of treatment. In a clinical CD trial in which F-CPT and serological inflammatory markers were measured during induction therapy with EEN, F-CPT was the slowest to return to a normal level, implicating its limited responsiveness to change [13]. We measured F-CPT concentration at week 2, which seemed to be too short a period to monitor a significant change in F-CPT levels.

We sought to address the question: when is the best time to evaluate the response to EEN induction therapy using F-CPT? We found an improvement in the levels of F-CPT in a majority of patients; however, the individual concentrations of F-CPT reached high levels in several clinical responders, even after the EEN course. Therefore, both F-CPT values and the trend of the levels (or more accurately, the percentage change of F-CPT in the log scale) during treatment are important. According to our results, the decrease in the F-CPT levels to values of the normal reference range seems not to be necessary in a short time period in patients receiving EEN. As already mentioned above, children with CD achieve clinical remission after the EEN course in approximately 75% [7, 10]; therefore, EEN is still the first choice for induction of remission in children with active CD regardless of the F-CPT levels. In many children with clinical remission/response, F-CPT levels were still high at the end of EEN and we know that not all children with clinical response have mucosal healing already at the end of 6 weeks [14]. For most children, the ability to tolerate EEN is already obvious within the first few days of treatment. In addition, F-CPT has been demonstrated to continue to fall up to 8 weeks of EEN and all the studies that have demonstrated mucosal healing from EEN have been after 8 weeks or more of EEN [3, 12, 28].

Other authors tried to use the predictive value of F-CPT during EEN in a different way. Frivolt et al. did not find changes in the F-CPT level between the baseline and week 4 or 12 of EEN treatment to be predictive of remission duration

Table 3 Prediction of non-response (defined as wPCDAI or CRP or F-CPT level), and non-remission at week 6 of EEN treatment based on absolute and percentage change of F-CPT till week 2

	[e]AUC	Specificity	Sensitivity	Cut-off (μg/g)	AUC	Specificity	Sensitivity	Cut-off (%)
[a]Non-response	0.739	0.848	0.800	−486.0	0.788	0.788	0.800	1.468
[b]Non-remission	0.753	0.923	0.583	−486.0	0.801	0.615	1.000	0.643
[c]CRP > 5 mg/l	0.787	1.000	0.588	246.5	0.721	0.750	0.853	1.821
[d]F-CPT > 150 μg/g	0.787	1.000	0.588	246.5	0.721	0.750	0.853	1.821

wPCDAI = weighted Pediatric Crohn Disease Activity Index

[a] Non-response defined as wPCDAI > 12.5 points, or there is not a decrease in the wPCDAI by more the 17.5 points

[b] Non-remission defined as wPCDAI > 12.5 points

[c] CRP = C-reactive protein

[d] F-CPT = fecal calprotectin

[e] AUC = area under the curve, EEN = exclusive enteral nutrition

[11]. Another study confirmed that a reduction in the F-CPT levels in children with CD undergoing EEN treatment was associated with the induction of clinical remission after EEN completion [12]. The members of the pediatric committee of the ECCO suggested that F-CPT be used only as a secondary outcome measure due to large variability in the results, leading to low precision and ill-defined cut-off levels in the assessment of disease activity [25].

Despite the fact that other clinical and laboratory parameters were previously studied in patients treated with EEN [1, 3, 8], the results in purpose for the prediction of response to EEN treatment are scarce. Day et al. [8] showed an improvement in inflammatory markers (erythrocyte sedimentation rate (ESR), CRP, serum albumin and platelets) after 8 weeks of EEN in patients with newly diagnosed CD who achieved remission. All the improvements in laboratory markers paralleled changes in PCDAI, both seen as early as 4 weeks. Moreover, there are studies which found improvement in ESR and CRP over a much shorter period (1 week) [1, 3]. According to the study by Borrelli et al. [3], the most significant improvement in weight and inflammatory markers is seen in the first 4 weeks of EEN; however, most studies have shown that weight continues to improve up to 8 weeks of treatment [6]. Another study by Gerasimidis et al. [12] performed development of inflammatory markers (serum albumin, CRP, and ESR) in responders and non-responders to EEN treatment. In the responders, systemic markers improved at the end of the EEN course and in the majority of patients they reached values in the normal reference range, contrary to non-responders, in whom none of those markers of disease activity changed significantly.

Another unsolved question regarding EEN treatment is the effect of disease location on the treatment outcome. Based on several studies [6, 27], EEN is not as effective for isolated colonic disease as it is for ileal or ileocolonic disease; however, more recent studies did not find an effect of disease location on EEN treatment outcome [4, 28]. It is very difficult to find a definite conclusion about the predictive role of disease location on treatment outcome according to several differences in published studies, even though it is probably not a strong predictor.

A limitation of the study was the relatively small sample size. However, if a decrease in the F-CPT concentration by week 2 can act as a strong predictor, this can also be proven in a small group of patients. We could not exclude the non-compliance of patients to EEN treatment. However, the patients and their caregivers did not know the results of the F-CPT at week 2, and, thus, this probably did not influence the results of the study. We allowed the patients to choose between different types and tastes of formula in order to decrease non-compliance due to low palatability. Nevertheless, two patients were not able to continue EEN and were excluded. We believe that exclusion of these patients did not fundamentally affect the results. Patients collected the stool samples by themselves, at home; therefore, the impact of this on the F-CPT concentrations is unknown. The less-than-accurate results in the case of high F-CPT levels, as obtained by the assay, is also a potential limitation. According to published studies, the F-CPT concentrations are highest in samples collected when there is a long interval between the defecations, and there is also correlation between stool consistency and the level of the F-CPT [22]. Therefore, the changes in gastrointestinal transit time could affect the F-CPT outcomes in our patients. On the other hand, all mentioned limitations of using F-CPT could be the explanation for its variation; however, it should not affect the main results. The study was not designed to identify patients who will achieve remission or even mucosal healing in the long-term follow-up.

The effect of AZA treatment on induction of remission by EEN is improbable. Based on the published data [25], AZA in monotherapy did not induce remission in active CD; therefore, we did not expect that AZA influenced the induction of remission in our patients treated with EEN.

In conclusion, we found that the difference between the F-CPT concentrations before EEN treatment and at week 2 or the percentage of the baseline value cloud not be used as a single predictor for non-response at the end of induction treatment. Another marker in the early phase should be considered in future studies as a potential predictor since the F-CPT seems to return to a normal level too slowly.

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Authors' contributions Ivana Copova M.D. worked on the conception and design of the study as well as on background research, data collection, and its control and completion of the missing data, statistical analysis of data, and writing of this original article. She was responsible for financial planning and work efficiency within the budget of the study.

Ondrej Hradsky M.D., Assoc. Prof., Ph.D. worked on the conception and design of the study as well as on data collection and its control, statistical analysis of data, and revision of the original article. He critically revised the work for important intellectual content.

Kristyna Zarubova M.D. actively participated in data collection and its control in the context of the research, and revision of the original article.

Lucie Gonsorcikova M.D., Ph.D. participated in data collection and its control.

Kristyna Potuznikova M.D. participated in data collection and its control. She obtained the necessary documents from patients and their guardians, and revised the work.

Tereza Lerchova M.D. participated in data collection and its control. She obtained the necessary documents from patients and their guardians, and revised the work.

Jiri Nevoral M.D., Prof., Ph.D. worked on the conception and design of the study as well as on revision of the original article.

Jiri Bronsky M.D., Assoc. Prof., Ph.D. was responsible for leading the project team, he was involved in study scheduling, obtaining the necessary documents, highlighting potential problems, and proposing solutions. He critically revised the work critically for important intellectual content.

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Compliance with ethical standards

The study was approved by the Ethics Committee of the authors' institution (the Ethics Committee Reference number is 1491/16).

Conflict of interest The authors alone are responsible for the content and writing of the paper. There is no direct conflict of interest in relation to the topic of this study in any of the authors. Jiri Bronsky received honoraria/speaker's fees/coverage of travel expenses from AbbVie, MSD, Nutricia, Nestle, Biocodex, Walmark, and Ferring. Ondrej Hradsky received honoraria/speaker's fees/coverage of travel expenses from AbbVie, MSD, Nutricia, Nestle, Biocodex, Falk, and Ferring.

Informed consent The patients' guardians received written information on the study and signed a consent form.

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ORIGINAL ARTICLE

Risk factors for dermatological complications of anti-TNF therapy in a cohort of children with Crohn's disease

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Abstract

Studies showing a substantial frequency of dermatologic complications in paediatric Crohn's disease (CD) patients on anti-tumour necrosis factor (TNF) therapy preferentially include patients treated with infliximab. We aimed to identify risk factors for the cumulative incidence of skin complications in a paediatric cohort receiving either adalimumab or infliximab and found an association between current skin complications and the patient's current clinical condition. This study retrospectively evaluated dermatologic complications in an inception cohort of 100 paediatric CD patients receiving the first anti-TNF (Motol PIBD cohort). Patient data were collected every 3 months. The lesions were classified as psoriatic, atopic dermatitis, or others. We used Cox regression to evaluate the association between predefined variables and the time to complication and a generalised linear mixed model to assess the association between the patient's current condition and the occurrence of complications. Among the 89 included children, 35 (39%) presented with dermatologic lesions. The only predictor associated with any complication was infliximab (versus adalimumab) therapy (hazard ratio [HR]: 2.07; 95% confidence interval [CI]: 1.03–4.17; $p = 0.04$). Infliximab therapy (HR: 5.5; 95%CI: 1.59–19.06; $p = 0.01$) and a family history of atopy (HR: 3.4; 95%CI 1.35–8.57, $p = 0.002$) were associated with early manifestation of atopic dermatitis. Lower C-reactive protein levels (odds ratio [OR], 0.947; 95% CI, − 0.898 to 0.998; $p = 0.046$) and infliximab (versus adalimumab) were associated with the occurrence of any dermatologic complications (OR, 5.93; 95% CI, 1.59–22.07; $p = 0.008$).

Conclusion: The frequency of skin complications seems high in paediatric CD patients treated with anti-TNF and is even higher in those treated with infliximab.

What is Known:
•*The dermatologic complications occur during treatment with anti-tumour necrosis factor.*
•*The frequency of skin complications in paediatric patients with Crohn's disease is high.*

What is New:
•*Infliximab (vs. adalimumab) was identified as a strong risk factor for the cumulative incidence of skin complications.*
•*Lower C-reactive protein levels were associated with the current occurrence of dermatologic complications.*

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Abbreviation

ADA	Adalimumab
Anti-TNF	Anti-tumour necrosis factor
CI	Confidence interval
CD	Crohn's disease
CRP	C-reactive protein
f-CPT	F-calprotectin
HR	Hazards ratio
IFX	Infliximab
IQR	Interquartile range
OR	Odds ratio
wPCDAI	Crohn's disease activity index

Introduction

Adalimumab (ADA) and infliximab (IFX) are the only two biologics licensed for the treatment of paediatric Crohn's disease (CD) in the European Union and North America. These two anti-tumour necrosis factor (TNF) antibodies have shown similar efficacy as induction and maintenance therapy for CD in randomised control trials [1, 2]. Based mainly on the data from the TISKids study [3], the current European Crohn's and Colitis Organisation and European Society for Paediatric Gastroenterology, Hepatology and Nutrition guidelines recommend anti-TNF as the primary therapy for induction and maintenance in children with a high risk of poor outcome [4]. The safety issues of these drugs are thus becoming even more important owing to their wide use. Several complications of anti-TNF therapy have been described in adults [5, 6]. Recent data have shown that dermatologic complications are the most frequent [7, 8]. Psoriatic lesions, atopic dermatitis, and infections are the most common skin complications in the paediatric population [9–11]. Although most paediatric studies have described these complications preferentially with IFX treatment, data comparing ADA and IFX are scarce. Several risk factors have been proposed, although mostly with conflicting results, and only a few have been described as a risk more than once [7–12].

The aim of this study was to identify risk factors for the cumulative incidence of skin complications in a cohort of paediatric patients treated with either of the anti-TNF drugs - ADA or IFX. We aimed to evaluate the risk factors for all skin complications and separately for psoriatic lesions and atopic dermatitis. Furthermore, we evaluated the association between current skin complications and the current clinical condition of the patient.

Materials and methods

Patient population and data collection

The study was designed as a retrospective evaluation of dermatologic complications in the inception cohort of 100 paediatric patients with CD treated with ADA or IFX as a first-line biological treatment (Motol PIBD cohort). The cohort primarily focused on evaluating the efficacy of anti-TNF treatment, and the follow-up ended when the patient reached the primary endpoint of the cohort (stopping the current anti-TNF therapy, intensification of anti-TNF therapy, abdominal surgery, and complications of the disease, such as newly diagnosed ileocecal stricture, abscess, or perianal disease). The minimum follow-up period without reaching the endpoint was 24 months. The first administration of anti-TNF was administered between 2013 and 2017. Patient data were collected from electronic medical charts using the REDCap [13]. The collected data included demographic characteristics (age, sex, ethnicity); baseline characteristics and classification of the disease (immunopathology, Paris classification, anti-Saccharomyces cerevisiae antibody, anti-neutrophil cytoplasmic antibody), personal and family medical history (dermatologic conditions and inflammatory bowel disease [IBD]); clinical characteristics and basic laboratory data from the time of diagnosis, from the time of first anti-TNF application, and in 2- to 3-month intervals during follow-up (height, weight, weighted paediatric Crohn's disease activity index [wPCDAI], erythrocyte sedimentation rate, C-reactive protein [CRP], albumin, F-calprotectin [f-CPT], simple endoscopic score, and radiological findings). We also collected data on IBD-related medications (induction treatment, immunosuppressants, dose and interval of anti-TNF therapy, and drug levels and drug antibodies). Exclusion criteria for the analysis were lost to follow-up in less than 2 years, patients' non-adherence to anti-TNF treatment, and missing data on the outcome.

Variables as predictors

Based on previously published literature and theoretical discretion, we pre-selected potential risk factors for dermatological complications: sex, upper gastrointestinal tract involvement, penetrating and structuring behaviour, perianal disease, smoking, Z-score of body mass index, age at first application of anti-TNF treatment, type of anti-TNF, concomitant immunopathology, anti-*Saccharomyces cerevisiae* antibody, anti-neutrophil cytoplasmic antibody, family and personal history

Table 1 Demographic and clinical characteristics of patients

	Patients without dermatologic complication N = 54	Patients with dermatologic complication N = 35	p value
Age at first anti-TNF, years	15.03 (12.1–16.86), NA = 0	15 (13.84–16.25), NA = 0	0.28
Sex, man	35 (0.65), NA = 0	20 (0.57), NA = 0	0.47
Smoking	2 (0.04), NA = 0	2 (0.06), NA = 0	0.66
Ethnicity (Caucasians)	51 (0.96), NA = 1	34 (0.97), NA = 0	0.81
Family history of IBD	10 (0.19), NA = 0	2 (0.06), NA = 0	0.07
Immunopathology	2 (0.04), NA = 0	4 (0.11), NA = 0	0.16
Localisation L1[1]	17 (0.31), NA = 0	10 (0.29), NA = 0	0.77
Localisation L2[1]	5 (0.09), NA = 0	1 (0.03), NA = 0	0.21
Localisation L3[1]	32 (0.59), NA = 0	24 (0.69), NA = 0	0.37
L4 (non L4a nor L4b) [1]	19 (0.35), NA = 0	14 (0.40), NA = 0	0.65
Behaviour B1[1]	36 (0.67), NA = 0	28 (0.80), NA = 0	0.17
Behaviour B2[1]	11 (0.20), NA = 0	5 (0.14), NA = 0	0.46
Behaviour B3[1]	4 (0.07), NA = 0	2 (0.06), NA = 0	0.75
Behaviour B2&B3[1]	3 (0.06), NA = 0	0 (NA), NA = 0	0.08
Perianal disease[1]	12 (0.22), NA = 0	8 (0.23), NA = 0	0.94
Growth retardation, G1[1]	10 (0.19), NA = 0	12 (0.35), NA = 1	0.08
ASCA, positivity in IgG or IgA	42 (0.88), NA = 6	26 (0.84), NA = 4	0.65
ANCA, positivity	7 (0.14), NA = 5	6 (0.20), NA = 5	0.51
Number of eosinophils	0.13 (0.07–0.36), NA = 13	0.11 (0.05–0.23), NA = 5	0.06
wPCDAI	22.5 (11.88–32.50), NA = 6	35 (9.38–40.00), NA = 9	0.18
Height, Z-score	− 1.4 (− 4.72 to 0.13), NA = 0	− 1.39 (− 2.90 to 0.04), NA=0	0.20
BMI, Z-score	− 1.07 (− 2.37 to 0.11), NA = 0	− 1.33 (− 1.94 to 0.41), NA=0	0.94
Personal history of atopy	32 (0.59), NA=0	24 (0.69), NA = 0	0.37
Family history of atopy	10 (0.19), NA=0	12 (0.34), NA = 0	0.09
Family history of psoriasis	2 (0.04), NA=0	2 (0.06), NA = 0	0.66
Anti-TNF, infliximab	27 (0.50), NA=0	23 (0.66), NA = 0	0.14
Anti-TNF, adalimumab	27 (0.50), NA=0	12 (0.34), NA = 0	0.14
Follow-up on anti-TNF	2.11 (0.63–3.10), NA=0	2.84 (1.92–3.69), NA = 0	0.02
Time to anti-TNF, years	0.78 (0.38–2.03), NA=0	0.89 (0.31–1.62), NA = 0	0.81
Percentage of time on immunomodulator[2]	1 (0.95–1.00), NA=0	1 (1.00–1.00), NA = 0	0.77

[1] According to the Paris classification, [2] 82 was exposed to azathioprine, one to 6-mercaptopurin and two to methotrexate

Continuous variables are presented as medians (interquartile ranges) and categorical variables as absolute frequencies and percentages, and NA number of missing data

TNF, tumour necrosis factor; ASCA, anti-Saccharomyces cerevisiae antibody; ANCA, anti-neutrophil cytoplasmic antibody; wPCDAI, weighted paediatric Crohn's disease activity index; BMI, body mass index

of atopy or psoriasis, and the number of eosinophils at the time of first administration of anti-TNF therapy. For the secondary outcomes, we tested the associations between current skin manifestations and current parameters of disease activity (wPCDAI, CRP, f-CPT), immunosuppressive therapy, and anti-TNF drug serum levels and antibodies.

Outcome

We collected data on the potential skin complications of anti-TNF therapy at each follow-up visit. All patients who manifested potential skin complications of anti-TNF therapy were instructed to visit a dermatologist. The type of dermatologic complication was assessed by a paediatric gastroenterologist or a paediatric dermatologist. If the skin lesion was determined by a paediatric dermatologist as uncertain, a biopsy was indicated. For the purpose of the incidence cohort, the lesions were classified as psoriatic (including psoriasis, psoriatic eczema, and pustulosis), atopic dermatitis, or others probably related to anti-TNF therapy. Significant worsening of atopic dermatitis was also considered a potential complication of anti-TNF therapy.

Statistical analysis

All data were analysed using R statistical software (version 3.6.0) [14]. Continuous variables were described as medians and interquartile ranges (IQR). Categorical variables were described as absolute frequencies and percentages. Missing data were not imputed. The pre-selected predictors were tested using unadjusted Cox regression for three outcomes (time to the first occurrence of any dermatological compilation, time to first psoriatic lesion, and time to first atopic dermatitis or time to first significant worsening of atopic dermatitis during anti-TNF treatment). To assess the importance of particular variables, we further tested the association of skin complications with the variables using multivariable Cox proportional-hazards models. As the secondary aim was to evaluate the association between current dermatological complications and current patient condition, we used a generalised linear mixed model with dermatological complications as an outcome. All these mixed models were adjusted for the follow-up time. The trough levels were tested as quartiles and antibodies as categorical variables. When the values were missing, we omitted the time points from the current analysis.

Ethical considerations

The study was approved by the local ethics committee, and informed consent was obtained from the parents of all patients.

Table 2 Frequency of dermatologic complications

	Number of patients
Any dermatologic complications	35 (0.39)
Psoriatic lesions	13 (0.15)*
- Psoriasis	4 (0.04)
- Psoriatic eczema	6 (0.07)
- Pustulosis	5 (0.06)
Atopic dermatitis	18 (0.20)
Other, probably associated with anti-TNF	11 (0.12)

Some patients exhibited more than one dermatologic complication. *TNF* tumour necrosis factor. * One patient had psoriatic eczema and pustulosis, and one patient had psoriasis and psoriatic eczema

Results

Among the 100 patients included in the cohort, 11 were excluded (Fig. 1). The characteristics of the 89 included patients are summarised in Table 1 and Supplementary Table S1. The median follow-up period for the patients was 2.49 (IQR: 0.90–3.47) years.

Frequency and cumulative incidence of dermatologic complications

Among the entire cohort, we found 35 (39%) patients with any recorded dermatologic manifestations. The most frequent

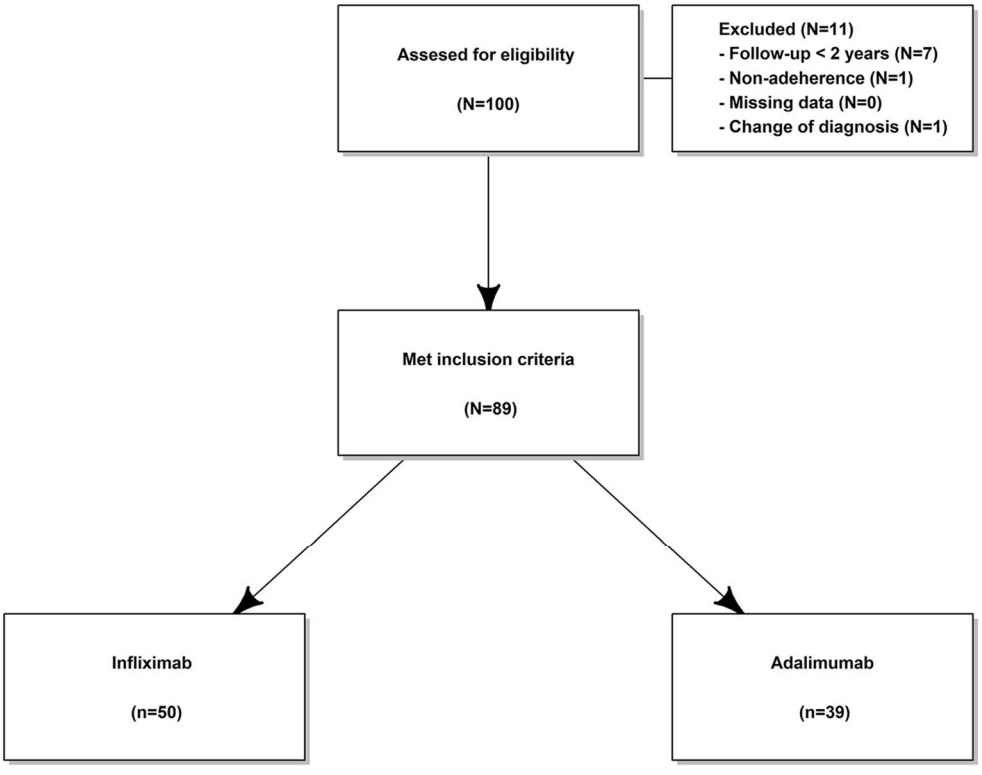

Fig. 1 Flowchart showing exclusions from the cohort

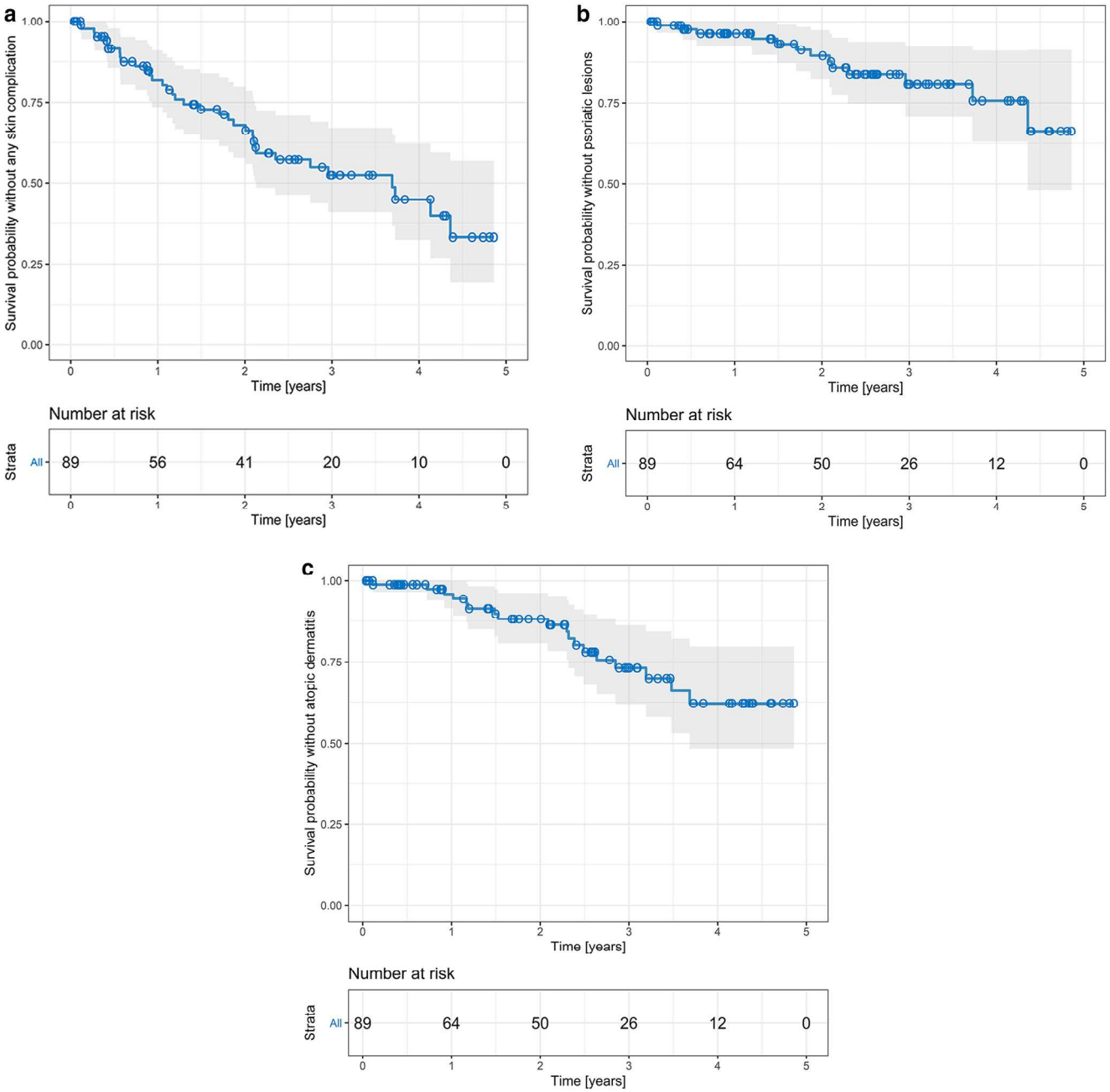

Fig. 2 Cumulative incidence of dermatological complications in paediatric Crohn's disease patients treated with anti-TNF therapy. **a** For any dermatologic complications, **b** for psoriatic complications, **c** for atopic dermatitis. o, censored

symptom was worsening or new-onset atopic dermatitis (n = 18, 20%), followed by psoriatic lesions (n =13, 15%). The occurrence of all skin complications is presented in Table 2. Some patients presented with more than one dermatological complication. Survival curves show the time to first skin complications (Fig. 2). After 2 years of follow-up on anti-TNF therapy, approximately 35% of patients developed at least one of the dermatological complications. During the follow-up, 34 patients stopped anti-TNF treatment; only one patient within the cohort stopped treatment with ADA because of severe psoriasis. All other dermatological complications were managed with topical treatment.

Risk factors for dermatologic complications

In the unadjusted Cox regression analysis with predefined variables (Supplementary Table S2, Fig. 3), the only predictor associated with any complication was IFX therapy (hazard ratio [HR]: 2.07; 95% confidence interval [CI]: 1.03–4.17; p = 0.04). No association was found between the time to the occurrence of psoriatic lesions and the tested predictors. IFX therapy (compared to ADA; HR: 5.5; 95% CI: 1.59–19.06; p = 0.01; Fig. S1A), and a family history of atopy (HR: 3.4; 95% CI: 1.35–8.57; p = 0.002; Fig. S1B) were associated with the

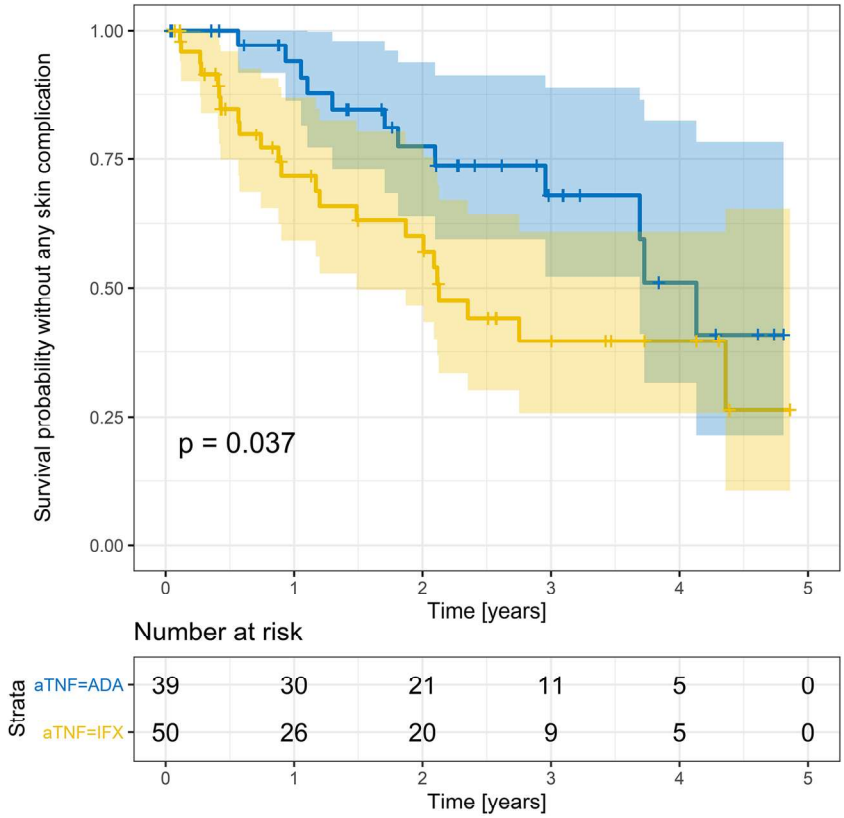

Fig. 3 Difference in cumulative incidence of dermatological complications in paediatric Crohn's disease patients treated with adalimumab or infliximab. o, censored

early manifestation of atopic dermatitis. When both were tested in the multiple Cox regression model, only the association with IFX remained significant (HR, 4.46; 95% CI, 1.26–15.85; $p = 0.021$).

Association between current dermatologic complication and patient's condition

Using a generalised linear mixed model adjusted for follow-up time, we found that lower CRP (odds ratio [OR], 0.947; 95% CI, 0.898–0.998; $p = 0.046$) and the type of anti-TNF were associated with the presence of any dermatologic complications (IFX versus ADA, OR: 5.93; 95% CI: 1.59–22.07; $p = 0.008$). Other factors that expressed patient disease activity (wPCDAI, f-CPT) were not associated with dermatological complications. The associations of both CRP level and type of anti-TNF remained significant in the multiple regression mixed model (Table S3). No association was found with trough levels or antibodies.

Discussion

In the cohort of paediatric CD patients receiving either of the two first-line anti-TNF drugs, after a median of 2.5 years, IFX therapy increased the risk of dermatological complications by two times (HR: 2.07; 95% CI: 1.03–4.17; $p = 0.04$) and by nearly six times when a flare-up of atopic dermatitis was considered the outcome (HR: 5.5; 95% CI: 1.59–19.06; $p = 0.01$).

The only paediatric study, including both drugs (ADA and IFX), found IFX to be a risk factor for psoriasiform lesions [9]. In contrast, large adult studies did not find differences in skin complications with regard to the type of anti-TNF therapy [7, 8, 15]. A recent meta-analysis showed a pooled incidence of eczema in approximately 5.5% (anti-TNF treated) patients in studies that combined 5526 adult patients [16]. Atopic dermatitis was observed in 20% of patients during a median follow-up of 2.5 years, which was slightly higher than that seen in other paediatric studies (12 %–14%) [9, 10]. The type of anti-TNF was strongly associated with atopic dermatitis in our cohort (nearly a six-fold increased risk for IFX) and remained independent of a presumptive association with a family history of atopy on multivariable analysis. Moreover, our data suggested that dermatological complications were associated with decreased systemic disease activity as measured with CRP. It could be hypothesised that effective blocking of TNF accompanied by low CD activity could shift the Th1/Th2 immune response towards Th2, which is the predominant response in atopic dermatitis [17].

The overall incidence of dermatological complications seems to be higher in children than in adults [7]. When prospectively collected, skin complications were found in nearly every other paediatric patient [10]. A total of 39% of non-infectious dermatological complications detected seemed to

be slightly higher than the frequency recognised by retrospective paediatric studies [9, 11].

The true clinical question, assuming the comparable efficacy and safety of these two drugs, is whether this slightly higher frequency of complications can lead to prioritising ADA in paediatric patients (or to give it priority, e.g. in a subgroup of patients). We should admit that even with these new findings, the current data are limited and do not allow us to make a firm generalisable conclusion. It should also be noted that in our cohort, dermatological complications led to anti-TNF withdrawal only in one patient treated with ADA. However, with a personalised approach, one could consider favouring ADA in paediatric patients with a family history of atopy.

This study has some limitations. First, although the cohort was prospectively captured, dermatological complications were collected retrospectively, and it is possible that skin lesions occurred in some patients without documentation in the medical records. Nevertheless, we believe that only a minority of lesions were missed, as the data were collected at intervals of less than 3 months. Moreover, the same technique of data collection was applied for patients treated with both IFX and ADA; thus, the difference should not be explained by that. Importantly, the cohort was homogenous (Table S1). Only the family history of atopy and follow-up period differed between the groups treated with ADA and IFX, and this was statistically managed by adjustment for these covariates in regression mixed models. Second, the cohort sample size was relatively small, and thus generalisation should be performed with caution. Third, although patients were instructed to visit a paediatric dermatologist, some were diagnosed (mainly with minor lesions) by a paediatric gastroenterologist. As the types of dermatologic lesions were not evaluated by one physician alone, lesions could be diagnosed differently.

Conclusion

In conclusion, we found a high frequency of skin complications, especially significant worsening or new-onset atopic dermatitis, in this cohort of paediatric patients with CD treated with anti-TNF therapy. IFX therapy appears to be a predictor of skin complications.

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Data availability N/A

Code availability N/A

Declarations

Ethics approval The study was approved by the local ethics committee.

Consent to participate Informed consent was obtained from the parents of all patients.

Consent for publication N/A

Conflict of interest Ondrej Hradsky: Lectures/congress fees/consultancy (outside the submitted work)—MSD, AbbVie, Nutricia, Nestlé, Ferring, and Falk

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Prediction of Thiopurine Metabolite Levels Based on Haematological and Biochemical Parameters

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ABSTRACT

Objectives: Therapeutic drug monitoring of thiopurine erythrocyte levels is not available in all centers and it usually requires quite a long time to obtain the results. The aims of this study were to build a model predicting low levels of 6-thioguanine and 6-methylmercaptopurine in pediatric inflammatory bowel disease (IBD) patients and to build a model to predict nonadherence in patients treated with azathioprine (AZA).

Methods: The study consisted of 332 observations in 88 pediatric IBD patients. Low AZA dosing was defined as 6-thioguanine levels <125 pmol/8×10^8 erythrocytes and 6-methylmercaptopurine levels <5700 pmol/8×10^8 erythrocytes. Nonadherence was defined as undetectable levels of 6-thioguanine and 6-methylmercaptopurine <240 pmol/8×10^8 erythrocytes. Data were divided into training and testing part. To construct the model predicting low 6-thioguanine levels, nonadherence, and the level of 6-thioguanine, the modification of random forest method with cross-validation and resampling was used.

Results: The final models predicting low 6-thioguanine levels and nonadherence had area under the curve, 0.87 and 0.94; sensitivity, 0.81 and 0.82; specificity, 0.80 and 86; and distance, 0.31 and 0.21, respectively, when applied on the testing part of the dataset. When the final model for prediction of 6-thioguanine values was applied on testing dataset, a root-mean-square error of 110 was obtained.

Conclusions: Using easily obtained laboratory parameters, we constructed a model with sufficient accuracy to predict patients with low 6-thioguanine levels and a model for prediction of AZA treatment nonadherence (web applications: *https://hradskyo.shinyapps.io/6TG_prediction/* and *https://hradskyo.shinyapps.io/Non_adherence/*).

Key Words: 6-mercaptopurine, 6-thioguanine, azathioprine, Crohn disease

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What Is Known

- Therapeutic drug monitoring of thiopurine erythrocyte levels plays an important role in management of treatment in pediatric patients diagnosed with inflammatory bowel disease.
- The monitoring of thiopurine erythrocyte levels is not easily accessible in all centers.

What Is New

- Readily available laboratory parameters were used to build a model to predict low levels of 6-thioguanine along with low 6-mercaptopurine in pediatric patients with inflammatory bowel disease.
- A web application allowing the prediction of low 6-thioguanine erythrocyte levels is now available.

Thiopurines (azathioprine [AZA] or 6-mercaptopurine [6-MP]) are recommended as 1 option for the maintenance of steroid-free remission in children with Crohn disease (CD) (1). The efficacy of thiopurines in children has been shown in 1 randomized placebo-controlled trial (2) and confirmed in a recent meta-analysis in adults (3). Thiopurines are not only used in monotherapy but also in combination with antitumor necrosis factor (aTNF). Despite several controversies that existed, the combined therapy was widely used at least for the first 6 months of treatment (4). The advantage of the combination therapy has been well documented in adults (5), and supporting data from pediatric registry have also been published (6).

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Observational studies show that approximately 50% of pediatric patients loose the response to AZA during first 2 years of treatment (7–9).

The recent meta-analysis enrolling 2049 patients clearly demonstrated an association between 6-thioguanine nucleotides levels and clinical remission rates in patients with inflammatory bowel disease (IBD) (10). When AZA was used in monotherapy, the cutoff value between 235 and 260 pmol/8×10^8 erythrocytes of 6-thioguanine (6-TGN) was found for clinical remission (10). Not all centers have drug levels available, and moreover, if they are available, it usually takes a long time to obtain the results.

Several studies have shown an association between hematological parameters and 6-TGN levels (11–15). Although the mean cell volume and number of lymphocytes were sometimes used in clinical practice (16), the simple linear association was not strong enough to predict 6-TGN levels (13). The first models to predict objective remission, nonadherence, and shunting to 6-methylmercaptopurine (6-MMP) in IBD patients using machine learning algorithms were recently published (17,18).

We aimed first to build a model to predict low levels of 6-TGN along with low 6-MMP in pediatric IBD patients. Second, we tried to build a model to predict nonadherence in patients treated with AZA. Then, we tried to construct a model for the prediction of an exact value of 6-TGN. Finally, we tried to implement all these models into web applications.

MATERIALS AND METHODS

Patient Population and Data Collection

The complete dataset consists of observations from CD patients previously included in 2 observational, not yet published studies (Group A, Group B) and patients with CD not treated with AZA (Group C). The eligibility of patients is described in a flowchart (Fig. 1). After meeting all the inclusion criteria, the entire group consisted of 332 observations. Group A consisted of 270 observations among 50 pediatric CD patients treated with a combination of infliximab (IFX) and AZA as a part of an ongoing study that focused on relationship between IFX levels and levels of thiopurine metabolites. The patients in whom thiopurine metabolites were measured during repeated regular visits were recruited consecutively from the Department of Pediatrics, Motol University Hospital, between February 2016 and June 2017 and Department of Pediatrics, University Hospital Olomouc, between August 2016 and July 2017. Group B involved 39 observations in 19 pediatric CD patients included in the study focusing on the psychological factors and sport activity conducted at the University Hospital Olomouc. Those patients were treated with AZA or AZA in combination with aTNF therapy. Group C encompassed 23 observations from regular blood examinations in 19 CD pediatric patients treated with adalimumab (ADA) or IFX in monotherapy. Observations from patients with at least 1 mutation in thiopurine S-methyltransferase (TPMT), observations with missing TPMT status, 6-MMP, or 6-TGN levels, and observations with missing mean corpuscular volume (MCV) were excluded (Fig. 1). For creating a nonadherence model, we excluded patients with AZA dose >0 and <1 mg/kg/day.

Thiopurine Metabolites and Its Measurement

After sample collection, erythrocytes were washed, diluted in water (1:1), and stored at a maximum of −20°C until analysis. Using a preparation protocol adopted from Dervieux and Boulieu

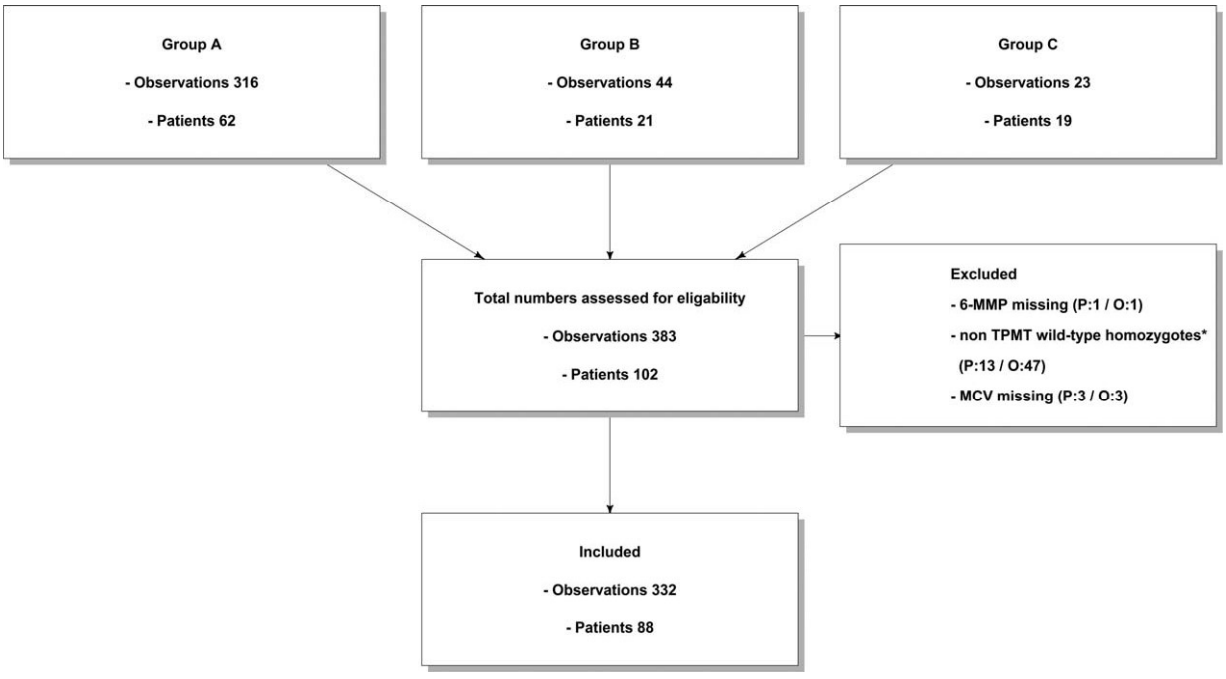

FIGURE 1. Flowchart of pediatric patients with Crohn disease. *TPMT genotype missing in 10 patients, 3 patients were TPMT heterozygotes. Group A consisted of observation of pediatric CD patients treated with combination of infliximab (IFX) and AZA as a part of ongoing study focused on relationship between IFX levels and levels of thiopurine metabolites. Group B involved observations of pediatric CD patients included into the study focused on psychological factors and sport activity conducted at University Hospital Olomouc. Those patients were treated with AZA or AZA in combination with adalimumab or infliximab therapy. Group C encompassed observations from regular blood examinations in pediatric CD patients treated with adalimumab or infliximab in monotherapy. CD = Crohn disease; MCV = mean corpuscular volume; 6-MMP = 6-methylmercaptopurine; O = observations; P = patients; TPMT = thiopurine methyltransferase.

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(19), samples were further prepared and then analyzed using high-performance liquid chromatography with diode array detection (20,21). Calibration curves in the range 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 μmol/L for 6-TGN and 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, and 200.0 μmol/L for 6-MMP prepared from drug-free healthy volunteer erythrocytes and concurrently analyzed were used to determine final concentrations of thiopurine metabolites.

Variables as Predictor

On the basis of previously described association of metabolites, and predictors including their possible co-variables and their routine availability in clinical practice, we selected MCV, red blood cell distribution width (RDW), S-albumin, hematocrit, mean corpuscular hemoglobin concentration (MCHC), platelet count, serum iron, transferrin saturation, actual age, and sex as the predictors for basic regression models.

Definitions

The low AZA dosing was defined as 6-TGN levels <125 pmol/8×10^8 erythrocytes and 6-MMP levels <5700 pmol/8×10^8 erythrocytes (22,23). Nonadherence was defined as 6-TGN levels $= 0$ pmol/8×10^8 erythrocytes (or undetectable) and 6-MMP levels ≤ 240 pmol/8×10^8 erythrocytes.

Statistical Analysis, Data Imputation, and Model Construction

All data were analyzed using R statistical software (version 3.4.4). Continuous variables were described as median and interquartile range (IQR). Categorical variables were described as absolute frequencies and percentages. Missing data were imputed in 6 steps based on linear regression model after both side elimination using other variables (except outcome) as predictors. Before imputation, the following were the numbers of missing values: hemoglobin, 1; lymphocyte number, 2; RDW, 2; ALB, 20; Fe, 24; and transferrin saturation, 112. To assess the importance of particular variables, we tested the association of 6-TGN with variables in multiple logistic regression model and the final regression models were selected using stepwise model selection by Akaike information criterion. For further analysis, we used selected list of predictors from these models. The prediction models were constructed using R package ''caret'' (24). Data were divided into training (60%) and testing part (40%). For construction of the model predicting lower levels of 6-TGN and nonadherence, the modification of random forest method minimizing the distance to the perfect model with cross-validation (12 number) and resampling (150 repeats) was used. We used modification of random forest method for the construction of the model predicting the value of 6-TGN. The model was judged using root-mean-square error (RMSE) and mean absolute error (MAE). For the model construction, only training part of the dataset was used. The accuracies of these particular models were tested on testing parts of datasets.

No information regarding other tests results were available to readers of a particular test.

ETHICAL CONSIDERATIONS

The study was approved by the ethics committee of the institution, and informed consent was obtained from the parents of all patients.

RESULTS

In all, 88 patients were included into the study (29 girls [33%], median age at the time of diagnosis 12.95 (10,4–15), 63 [72%] treated with IFX, 10 [11%] treated with ADA, 69 [78%] treated with AZA). Table 1 shows the demographic and therapeutic characteristics of observations among particular study groups.

Prediction of ''Need for Azathioprine Escalation''

We found an independent association of ''need for AZA escalation'' (defined as 6-TGN <125 pmol/8×10^8 erythrocytes and 6-MMP <5700 pmol/8×10^8 erythrocytes) with actual age, MCV, and RDW (for all $P < 0.001$) and absolute number of lymphocytes ($P = 0.041$) in multiple logistic regression model using all predictors.

The final model using modified random forest method on training dataset has the following values: area under the curve (AUC), 0.87; sensitivity, 0.81; specificity, 0.80; and distance, 0.31. The importance of each variable is displayed in Figure 2A.

The accuracy of final predictive model applied on testing dataset is displayed in Table 2 and the receiver operating characteristic curve (ROC) of the model in Figure 2B.

Prediction of ''Azathioprine Nonadherence''

We found independent association of ''AZA non-adherence'' (defined as 6-TGN level $= 0$ pmol/8×10^8 erythrocytes

TABLE 1. Demographic and therapeutic characteristics of observations

	Group A (N = 270)	Group B (N = 39)	Group C (N = 23)	All (N = 332)
Sex, female	69 (26%)	8 (21%)	10 (43%)	87 (26%)
Age at diagnosis, years	11.9 (10.2–13.6)	13.2 (10.4–15.1)	13.6 (10.65–15)	12 (10.2–14.1)
Age at visit, years	15.3 (14–16.8)	14.4 (12.95–17.2)	17.5 (16.05–18.1)	15.4 (13.9–17.2)
Infliximab	270 (100%)	5 (13%)	13 (57%)	288 (87%)
Adalimumab	0 (0%)	1 (3%)	10 (43%)	11 (3%)
Azathioprine	270 (100%)	39 (100%)	0 (0%)	309 (93%)
Azathioprine dose, mg/kg/day	1.6 (1.3–1.9)	2 (1.75–2.2)	0 (0–0)	1.6 (1.2–1.9)

Group A consisted of 270 observations among 50 pediatric CD patients treated with combination of infliximab (IFX) and AZA as a part of ongoing study focused on relationship between IFX levels and levels of thiopurine metabolites. Group B involved 39 observations in 19 pediatric CD patients included into the study focused on psychological factors and sport activity conducted at University Hospital Olomouc. Those patients were treated with AZA or AZA in combination with aTNF therapy. Group C encompassed 23 observations from regular blood examinations in 19 CD pediatric patients treated with adalimumab (ADA) or IFX in monotherapy. AZA = azathioprine; CD = Crohn disease.

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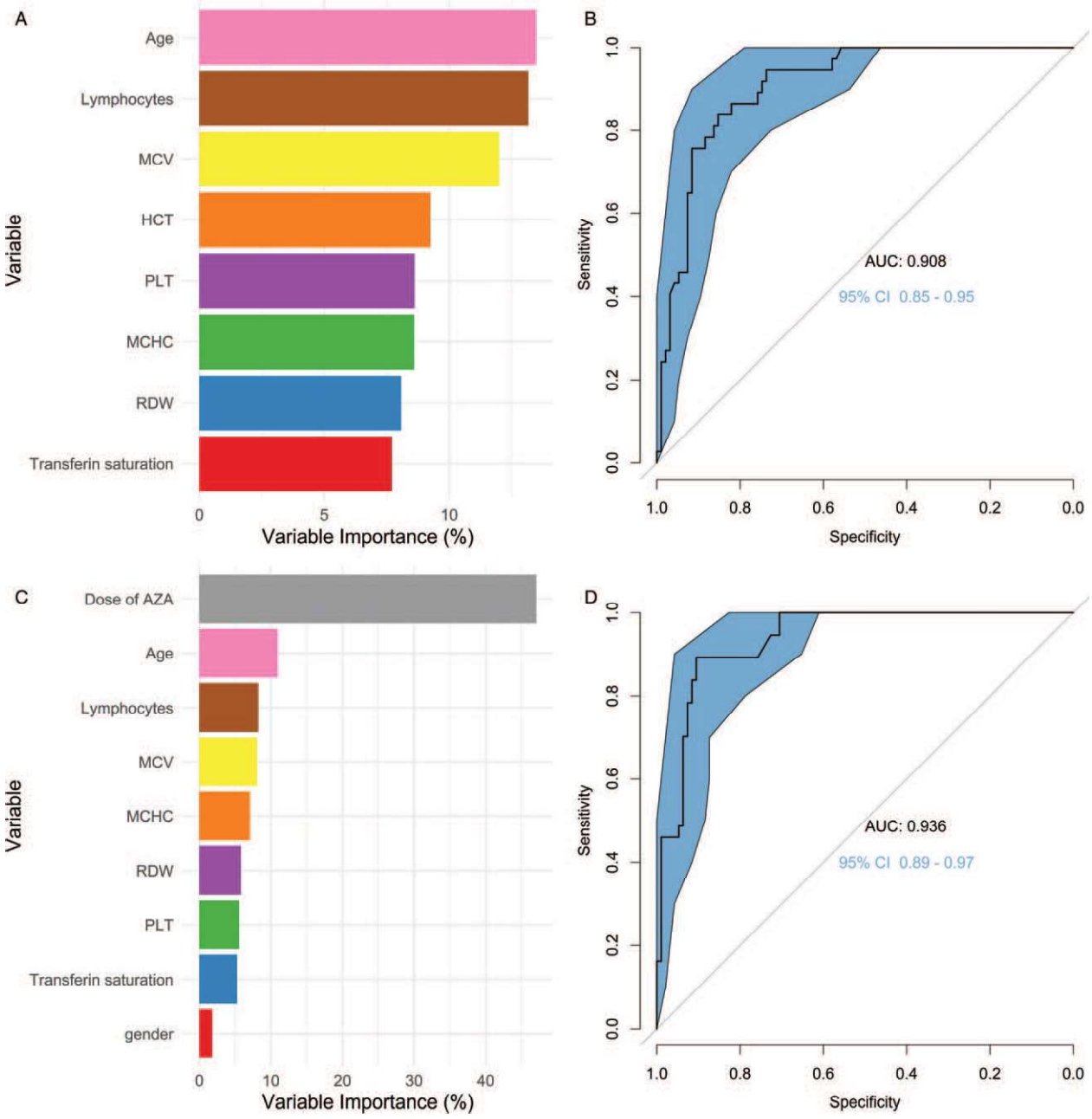

FIGURE 2. Bar plot showing relative importance of predictors in model constructed using training dataset and Receiver operation characteristic curve for final model in testing dataset for prediction of ''need for AZA escalation'' and prediction of ''AZA non-adherence.'' (A) Prediction of ''need for AZA escalation,'' (B) prediction of ''need for AZA escalation,'' (C) prediction of ''AZA non-adherence,'' (D) prediction of ''AZA nonadherence.'' Age = age at the time of observation; AUC = area under curve; AZA = azathioprine; CI = 95% confidence interval-based bootstrap method; HCT = hematocrit; lymphocytes = absolute number of lymphocytes; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; PLT = platelet; RDW = red blood cell distribution width.

and 6-MMP level <240 pmol/8 × 10^8 erythrocytes) with actual age, MCV, absolute number of lymphocytes, and RDW (for all $P < 0.001$), and sex ($P = 0.006$) in multiple logistic regression model using all predictors.

The final model using modified random forest method on training dataset has the following values: AUC, 0.94; sensitivity, 0.82; specificity, 0.86; and distance, 0.25. The importance of each variable is displayed in Figure 2C.

The accuracy of final predictive model applied on testing dataset is displayed in Table 2 and the ROC of the model in Figure 2D.

Prediction of '' 6-Thioguanine Values''

The final model using modified random forest regression on training dataset has RMSE = 110, $R^2 = 0.45$, and MAE = 85. When

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TABLE 2. Parameters of prediction models obtained on testing dataset

	Prediction of ''need for AZA escalation''	Prediction of ''AZA nonadherence''
Accuracy (95% CI)	0.84 (0.77–0.90)	0.87 (0.81–0.94)
Sensitivity	0.85	0.89
Specificity	0.81	0.88
LR+	4.47	7.42
LR−	0.19	0.13

Need for AZA escalation was defined as 6-TGN <125 pmol/8 × 10^8 erythrocytes and 6-MMP <5700 pmol/8 × 10^8 erythrocytes. AZA nonadherence was defined as 6-TGN = 0 pmol/8 × 10^8 erythrocytes and 6-MMP <240 pmol/8 × 10^8 erythrocytes. AZA = azathioprine; CI = confidence interval; LR+ = positive likelihood ratio; LR− = negative likelihood ratio.

applied on testing dataset, we obtained RMSE = 110 and MAE = 78 (IQR 31–97).

DISCUSSION

In this study, we constructed a model with sufficient accuracy for the prediction of patients who have low 6-TGN levels and who will probably profit from AZA dose escalation based on rapidly available standard laboratory parameters.

We found strong association of low thiopurine levels with actual age, MCV, absolute number of lymphocytes, and RDW. These associations have been previously published (11,13–15,25). Some authors, however, attempted to predict the levels of thiopurines solely based on a single predictor, which was not sufficient to reach the required accuracy.

Although the model for prediction of low metabolite level could not fully substitute standard measurement of intracellular metabolites in erythrocytes by high-performance liquid chromatography, it can serve as an approximation until data from testing are available. Moreover, if the method is not available at a center, it could help to shift clinical decision bearing in mind imperfection of the estimation.

Nonadherence

We built a mathematical model for prediction of patients who are probably nonadherent to AZA treatment. Similarly, Waljee et al (17) using machine learning algorithms built the predictive models with AUC of 0.81 and later 0.84 in a mixed group of patients (their age ranged from 5 to 86 years). Some differences between our study and the previously mentioned one exist. We included only patients with CD as we had assumed some singularity between CD and ulcerative colitis in hematological markers (14). Due to the association between RDW and iron deficiency anemia, we added also iron metabolism markers as a predictor (26), and we included only healthy homozygotes of *TPMT* gene. The majority of included patients have been treated with combination therapy (AZA and aTNF therapy), and finally, only pediatric patients were included. These aspects could lead to more consistent group, which may partly explain slightly the better accuracy of our model (AUC 0.91 vs 0.84). On the other hand, part of our study group was recruited from a different center, although levels of metabolites were evaluated in the same laboratory.

The exact proportion of AZA nonadherent IBD patient to monotherapy or even to AZA in combination with biologics is not known. Using thiopurine metabolite monitoring, 17% of adult IBD patients were found to be nonadherent to AZA (27). One may speculate that the frequency of nonadherence could be much higher, when the levels of thiopurine metabolites were not checked. More nonadherence could also be expected in patients on combined therapy. Thus, searching for nonadherence should be part of clinical practice and could significantly improve health outcome. Waljee et al (17). suggested to use MCV/WBC ratio in clinical practice; however, these 2 parameters had worse accuracy in comparison with their complex final model. Using our free web application, clinicians could use our full model. It should be noted, that direct measurement using thiopurine level testing or developed web application can only be considered one of many parts of assessment of nonadherence. The pill count and Adherence Self-report scales (28) should probably be part of the investigation. Prediction models for pediatric IBD patients could have dissimilarities. Age and sex could be more important variables during childhood, for example, because of changes in hematological indices, or because of psychological changes during adolescence. Thus, our models cannot be used for adult IBD patients before validation and even then might not reach the accuracy that they have in pediatric population. Even though we used the data of patients from 2 different centers and tested our models on different subsets of the dataset, it will still be necessary to validate our models on an external validation cohort, preferably using data from a different laboratory.

Limitations

Some limitations of our study should be addressed. Our sample size was relatively small, and thus, generalization of our models should be done with caution. Similar to other authors, (17,18) we used repetitive observation of patients. As our dataset was incomplete, we needed to perform imputation based on regression models blinded to thiopurine metabolites.

CONCLUSIONS

In conclusion, we constructed a model with sufficient accuracy to predict patients with low 6-TGN levels and who will, therefore, probably benefit from AZA dose escalation based on readily available standard laboratory parameters. We also built a mathematical model for the prediction of patients who are probably nonadherent to AZA treatment. These 2 models have been implemented within a web application (*https://hradskyo.shinyapps.io/6TG_prediction/* and *https://hradskyo.shinyapps.io/Non_adherence/*), allowing their use in clinical practice. Using these easily obtained laboratory data, however, we were unable to construct a model to predict exact values of 6-TGN.

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ORIGINAL RESEARCH ARTICLE

Is It Useful to Monitor Thiopurine Metabolites in Pediatric Patients with Crohn's Disease on Combination Therapy? A Multicenter Prospective Observational Study

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Abstract

Background The additional value of azathioprine concomitant treatment on infliximab pharmacokinetics in children is not well described yet.

Aims In the present study, we aimed to describe the relationship between thiopurine metabolite levels, infliximab trough levels, anti-IFX antibody formation, and clinical and laboratory markers of disease activity in pediatric patients with Crohn's disease, and to assess non-adherence.

Methods Data were collected prospectively during repeated visits from pediatric patients followed for Crohn's disease in two Czech pediatric inflammatory bowel disease centers between January 2016 and June 2017. Thiopurine metabolites (6-thioguanine and 6-methylmercaptopurine) were measured by high-performance liquid chromatography. Infliximab trough levels and anti-IFX antibody serum levels were measured routinely by ELISA. The risk of loss of response to infliximab therapy was also assessed.

Results A significant association between infliximab serum levels and 6-thioguanine erythrocyte levels was observed when tested as categorical variables (63 patients, 321 observations). To predict infliximab levels > 5 µg/mL, we propose a 6-thioguanine cutoff of 278 pmol/8 × 10^8 erythrocytes (sensitivity, 0.799; specificity, 0.347). A higher loss-of-response-to-infliximab rate (tested in a subgroup of 51 patients) was observed in patients with undetectable 6-thioguanine levels than in those with detectable levels ($p = 0.026$). Non-adherence to azathioprine therapy was suspected in 20% of patients.

Conclusion Thiopurine metabolite monitoring in pediatric patients with Crohn's disease is useful when optimizing combination therapy. Pediatric patients with undetectable 6-thioguanine levels are more likely to lose response to infliximab therapy. When targeting optimal infliximab levels, the 6-thioguanine cutoff levels in children appear to be higher than in adults.

1 Introduction

In adults, concomitant azathioprine administration during infliximab therapy is associated with lower levels of antibodies against infliximab (anti-IFX) and increased infliximab trough levels, resulting in better clinical outcomes than with infliximab monotherapy [1]. However, after 6 months of combination therapy, the additive effect of long-term

Key Points

We evaluated the association between infliximab and 6-thioguanine (6-TGN) levels in children with Crohn's disease on combination therapy (infliximab and azathioprine).

Patients with undetectable 6-TGN levels are more likely to lose response to infliximab therapy (compared with those with detectable 6-TGN levels).

Thiopurine metabolite monitoring is useful even in combination therapy.

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azathioprine co-administration has been reported to be marginal [2–4].

As 6-thioguanine nucleotides (6-TGN) are active metabolites of azathioprine, they also function as therapy effectors [5]. The simplified azathioprine metabolism is presented in Fig. 1 [6, 7]. Azathioprine in monotherapy has been demonstrated to be more effective when a patient's 6-TGN levels are > 230 pmol/8 × 10^8 red blood cells (RBCs). In combination therapy, 6-TGN levels > 125 pmol/8 × 10^8 RBCs seem to be sufficient to achieve effective trough infliximab levels in adults [8–13]. In children, the dosing of azathioprine in combination therapy may not need to be as high as in monotherapy [13].

Some patients with a *normal* thiopurine *S*-methyltransferase (TPMT) genotype are known to shunt mercaptopurine metabolism in favor of 6-methylmercaptopurine (6-mMP) production (shunters), resulting in higher TPMT activity, low 6-TGN, and high 6-mMP levels [5, 12, 14, 15].

The primary aim of this study was to prospectively observe a group of pediatric patients with Crohn's disease (CD) on combination therapy in order to find suitable 6-TGN cutoff levels in RBCs associated with the optimal serum infliximab trough levels and the absence of anti-IFX. Secondary aims were to investigate azathioprine metabolites as potential predictors of relapse, to reveal non-adherence to azathioprine therapy, to reveal 'shunters', and to evaluate the possible relationship between infliximab, anti-IFX, and 6-TGN levels to clinical and laboratory markers of disease activity.

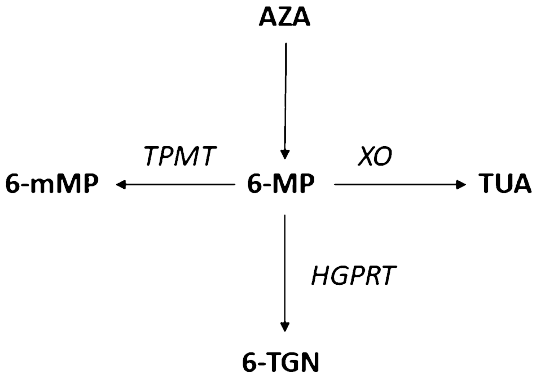

Fig. 1 Simplified scheme of AZA metabolism. AZA, after its absorption from the gut, is quickly converted into 6-MP. 6-MP is then metabolized producing 6-TGN, 6-mMP and TUA. The proportion of end products varies interindividually. 6-TGN is considered to be the therapy effector, whereas 6-mMP and TUA "only" side products. TPMT plays an important role in the final 6-TGN vs 6-mMP ratio, other factors including other enzymes' activity interfere with the final 6-TGN levels [6, 7]. *6-mMP* 6-methyl mercaptopurine, *6-MP* 6-mercaptopurine, *6-TGN* 6-thioguanine nucleotide, *AZA* azathioprine, *HGPRT* hypoxanthine-guanine phosphoribosyltransferase, *TPMT* thiopurine methyltransferase, *TUA* thiouric acid, *XO* xanthine oxidase

2 Material and Methods

2.1 Ethical Clearance

The protocol for this prospective, longitudinal, multicenter, observational study was approved by the Ethics Committees of the University Hospital Motol and the 2nd Medical Faculty of Charles University in Prague, the Czech Republic. Written informed consent was obtained from all patients and guardians before study enrollment.

2.2 Patients and Medication

All eligible patients ($N = 63$) who were treated at the University Hospital Motol between January 2016 and June 2017 and Olomouc University Hospital between August 2016 and July 2017 for CD and who fulfilled the inclusion criteria were included. Inclusion criteria to participate in the present study were as follows: previous diagnosis of CD based on Porto criteria or revised Porto criteria [16]; 2–18.9 years old at time of enrollment; treated with combination therapy of infliximab and azathioprine for a minimum of 3 months; and informed consent of a guardian (or of patient if 18 years of age or older). Exclusion criteria were as follows: unavailable laboratory data on infliximab and azathioprine from at least one timepoint of combination therapy; pregnancy; and unwillingness of patient or guardian to continue in the study. Patients were included as soon as they fulfilled the inclusion criteria. Data and blood samples were collected prospectively at repeated visits during the follow-up period. Only observations reporting both thiopurine metabolite levels and infliximab serum levels were used for data analysis (if either of the data were missing, the observation was not considered). Details on recruitment and patient flow are presented in Fig. 2.

Disease activity was defined both clinically (Weighted Pediatric Crohn's Disease Activity Index [wPCDAI]) [17, 18] and through using laboratory markers (C-reactive protein [CRP], leukocyte count, platelet count, erythrocyte sedimentation rate [ESR], and fecal calprotectin [F-CPT]). Optimization of azathioprine dose as well as infliximab dose and/or application interval (shortened when considered necessary) was allowed during the study period (based on the decision of the treating physician). The infliximab dose was escalated with either patient's weight gain or in case of suspected insufficient effect of therapy (based on clinical and/or laboratory signs). Endoscopy was performed in case of the suspicion of loss of response to therapy (LOR, as defined in section 2.2.1). Patients' infliximab and anti-IFX levels were uncovered to the treating physician only when demanded due to unsatisfactory patient status, thus were not the primary treatment target (no proactive therapeutic drug

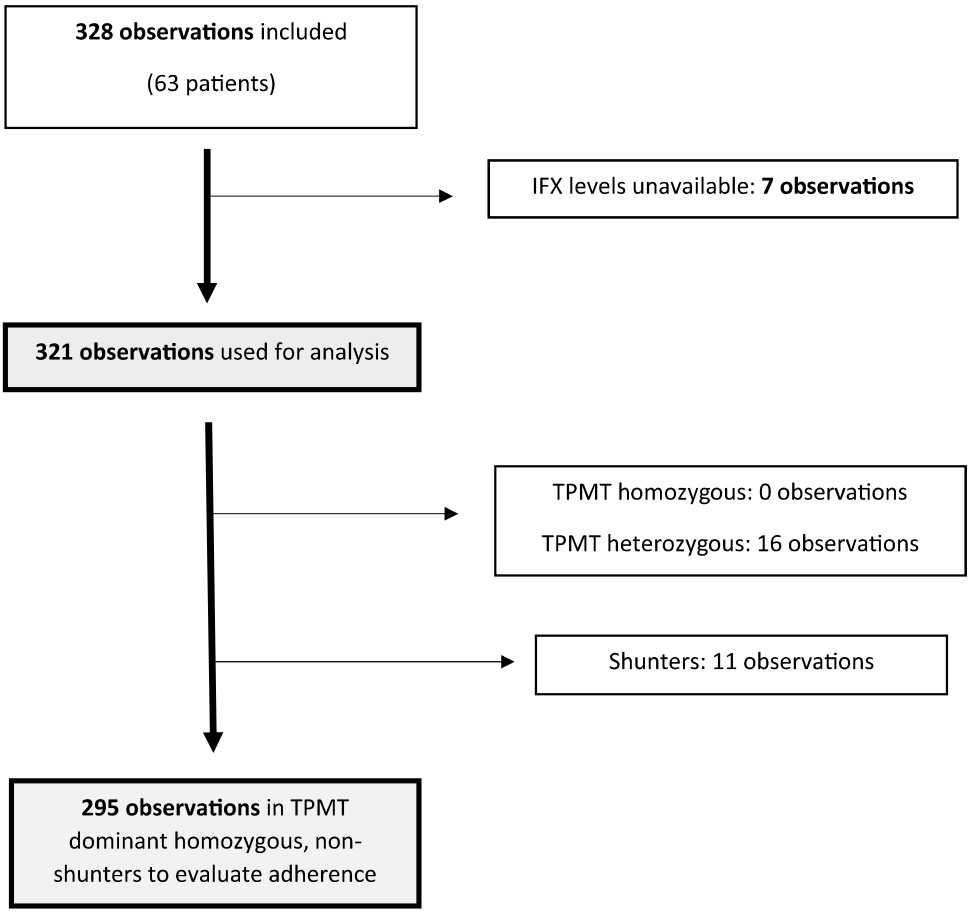

Fig. 2 Recruitment and patient flow. We used 321 observations for data analysis. Evaluation of adherence to AZA therapy was performed on a cluster of 295 observations (patient visits). *AZA* azathioprine, *IFX* infliximab, *TPMT* thiopurine methyltransferase

monitoring was performed). If cessation of infliximab or azathioprine therapy was required, patients were followed up only until the last dose was administered. At 19 years of age, patients were transferred to adult care and were no longer followed up in the present study.

The *TPMT* gene was screened for known polymorphisms. Patients were divided into subgroups according to their *TMPT* genotype. Shunters cannot be spotted by routine screening of the *TPMT* gene, thus patients in our study were considered shunters if the 6-mMP RBC levels were 11 times higher than 6-TGN RBC levels [5]. Patients with TPMT heterozygous genotype and suspected shunters were excluded from the analysis of adherence to thiopurine therapy (Fig. 2).

2.2.1 Survival Study Subpopulation—The Incidence of Loss of Response to Infliximab Therapy Regarding 6-TGN Levels

In patients followed up at University Hospital Motol ($N = 51$), clinical outcome regarding their possible LOR to infliximab therapy was recorded. LOR was defined as the requirement for a major change in therapy (switch or swap to other biologic agents or surgical procedures, such as ileocecal resection) after the ineffectiveness of infliximab was suspected. The decision to change therapy was made by the treating physicians based on clinical, laboratory, and/or endoscopic disease activity. Therefore, LOR was evaluated prospectively. Minor changes in infliximab treatment—dose or interval adjustments—were not classified as LOR. Data obtained from the Motol subpopulation were used to evaluate the LOR by survival analysis (survival study subpopulation).

2.3 Samples and Laboratory Methods

Blood and stool samples and clinical data were prospectively collected at the repeat visits (during which infliximab was administered). The interval between infliximab infusions (and thus visits) ranged from 4 to 8 weeks, based on the decision of the attending physician. Blood samples were taken prior to infliximab administration.

Standard blood tests (blood count parameters—RBC count, leukocyte count and differential, thrombocyte count, hemoglobin; ESR; serum parameters—CRP, lipase, γ-glutamyl transferase, alanine-aminotransferase, aspartate aminotransferase, albumin) were performed routinely. Serum

infliximab trough levels and anti-IFX levels were assessed using the infliximab ELISA test (TANI Medical, Ankara, Turkey). Anti-IFX levels above 5 AU/mL were considered positive. If a stool sample was available, F-CPT was determined using the EliA Calprotectin 2 test (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden).

2.4 Measurement of Thiopurine Metabolites

Whole blood was collected from each patient into ethylenediaminetetraacetic acid (EDTA) tubes, and hematocrit and RBC counts were subsequently determined. Immediately after sampling, the blood was centrifuged, plasma and leukocytes were removed, and RBCs were washed twice. RBCs were then diluted (1:1) with an equal volume of water and frozen at −20 °C until determination of thiopurine metabolites. The sample preparation protocol was adopted from Dervieux and Boulieu [19–21]. Samples were analyzed by Agilent 1100 high-performance liquid chromatography equipped with a multi-wavelength detector.

2.5 Data Analyses

Collected data were analyzed using R statistical software (version 3.5.2, https://cran.r-project.org/bin/windows/base/old/3.5.2/). For repeated measurements, linear mixed model (LMM) and generalized linear mix model (GLMM) analyses were performed using packages *lme4* and *lmerTest*. If not mentioned otherwise, all included results with all included patients were used for analysis.

The TPMT *normal* activity subgroup (patients with two wild-type alleles in the *TPMT* gene according to routine *TPMT* gene screening), was tested separately when assessing TPMT activity and association with metabolite levels. To assess changes in blood count parameters (leukocyte, neutrophil, lymphocyte, and platelet counts), LMMs were performed using 6-TGN RBC levels, infliximab trough levels, CRP, sex, and age as predictors. 6-TGN RBC levels or azathioprine daily dose adjusted to body weight (BW), serum albumin, CRP, wPCDAI, and BW-adjusted infliximab dose and calculated to 8-week dose together with sex, age, and time on infliximab therapy were chosen as predictors for models assessing the association with infliximab and anti-IFX levels. We chose those parameters to primarily evaluate the assumed association between 6-TGN RBC levels and infliximab or anti-IFX levels with potential predictors.

6-TGN cutoff values of 125 pmol/8 × 10^8 RBCs and 230 pmol/8 × 10^8 RBCs were chosen and tested separately in each model using 6-TGN as a categorical variable. After consideration of the selected parameters and sensitivity of the chosen laboratory method, levels of 6-TGN under 60 pmol/8 × 10^8 RBCs were considered *undetectable*. For the adherence assessment, *undetectable* assessments were assigned only to those samples in which both 6-TGN and 6-mMP levels were lower than the detection limit of the method (the 6-mMP detection limit was 500 pmol/8 × 10^8 RBCs). 6-TGN levels above 430–450 pmol/8 × 10^8 RBCs were considered toxic with respect to risk of leukopenia and 6-mMP levels above 5700 pmol/8 × 10^8 RBCs were considered relevant with respect to hepatopathy [12, 22].

When considering disease activity markers as categorical variables, remission was defined as CRP < 5 mg/L, ESR < 20 mm/h, F-CPT <100 µg/g, or wPCDAI < 12.5 points (tested separately if not mentioned otherwise). For the purposes of using infliximab as categorical variables, 3 µg/mL and 5 µg/mL (preferred) cutoffs were used [23–26].

To determine the optimum cutoff values for infliximab trough levels regarding CRP-based remission (CRP <5 mg/L), respective receiver operating characteristic (ROC) curves were plotted (package *pROC*). For repeated measurements (one patient—potentially multiple observations), we calculated the area under the curve using cross-validated area under the ROC curve estimates for pooled repeated measures data (R package "cvAUC").

Survival analysis (presented as a Kaplan-Meier plot) was performed to assess the risk of LOR according to 6-TGN levels (using package *survplot*). Hazard ratio (HR) was also calculated for each of the survival analysis subgroups separately.

All plots were constructed using the R package *ggplot2*.

3 Results

3.1 Study Population

In total, 63 patients were included in the present study (19% females, aged 6.7–18.8 years, 3 patients TPMT heterozygous; Table 1), comprising laboratory measurements from 328 visits. Seven visits were excluded from analysis due to the unavailability of infliximab data; thus, 321 observations were used in the final analyses (Fig. 2). While azathioprine is usually introduced prior to infliximab therapy, in three patients (totaling 16 observations), azathioprine therapy was added after infliximab therapy was initiated. Laboratory results (medians and interquartile ranges) are shown in Table 1. F-CPT was available only in 231 observations.

3.2 Primary Outcome: Optimal 6-TGN Cutoff

An association between serum infliximab levels and 6-TGN RBCs levels was observed when 6-TGN levels were analyzed as categorical variables using a previously suggested cutoff of 230 pmol/8 × 10^8 (LMM, adjusted model; $N = 321$; 95% CI 0.036–3.149; $p = 0.047$) but not when the 6-TGN

Table 1 Characteristics of the study population

	At enrolment/first visit ($N = 63$)	Overall visits ($N = 321$)	Last visit ($N = 63$)
Median number of visits per one patient (range)		5 (1–12)	
Motol/Olomouc hospital	51/12	243/78	51/12
Female gender (%)	19 (33.8)	87 (27.1)	19 (33.8)
Median age (range), y	15.6 (6.7–18.8)	15.6 (6.7–19.0)	16.6 (7.1–19.0)
Median time with IBD (range), mo	28 (5–97)	34 (5–114)	39 (5–114)
Median time on AZA (range), mo	25 (5–97)	32 (5–114)	38 (5–114)
Median time on IFX (range), mo	14 (3–73)	21 (3–84)	26 (3–84)
Receiving mesalamine (%)	6 (9.7)	41 (7.8)	5 (7.9)
History of ileocecal resection (%)	12 (19.3)	64 (19.9)	13 (20.6)
Frequency of TPMT heterozygosity (%)	3 (4.8)	16 (4.9)	3 (4.8)
TPMT heterozygous			
Median AZA dose (range), mg/kg/d	0.8 (0.5–1.2)	0.7 (0.5–1.2)	0.7 (0.5–1.1)
TPMT dominant homozygous			
Median AZA dose (range), mg/kg/d	1.7 (0.4–2.6)	1.6 (0.4–2.6)	1.6 (0.4–2.5)
Median IFX dose (IQR), mg/kg	5.4 (4.9–6.3)	5.4 (4.9–6.3)	5.6 (5.0–5.9)
Median IFX administration interval (IQR), wk	8 (7–8)	8 (6–8)	8 (6–8)
Median IFX dose adjusted to administration interval (IQR), mg/kg/8w	5.6 (4.9–6.7)	5.9 (5.1–7.9)	6.1 (5.3–8.0)

Sixty-three patients were included, from which 321 observations were used for analysis. TPMT heterozygous were rare among included patients

AZA azathioprine, *IBD* inflammatory bowel disease, *IFX* infliximab, *IQR* interquartile range, *TPMT* thiopurine methyltransferase

cutoff of 125 pmol/8 × 10^8 was used (LMM, adjusted model; $N = 321$; 95% CI − 0.161 to 3.402; $p = 0.085$). Using ROC curves, the optimum 6-TGN cutoff for predicting infliximab >5 µg/mL was 278 pmol/8 × 10^8 RBCs (cross-validated ROC; $N = 321$; AUC 0.533; 95% CI 0.469–0.596; sensitivity 0.799, specificity 0.347) (Fig. 3a). For infliximab >3 µg/mL, ROC curves revealed a promising 6-TGN cutoff of 116 pmol/8 × 10^8 RBCs (cross-validated ROC; $N = 321$; AUC 0.535; 95% CI 0.465–0.605; sensitivity 0.819, specificity 0.342) (Fig. 3b). No significant association was observed between anti-IFX antibody levels and 6-TGN RBCs levels, even when previously proposed 6-TGN cutoffs (230 and 125 pmol/8 × 10^8 RBCs) were tested.

3.3 Secondary Outcomes

3.3.1 Loss of Response to Infliximab Therapy

In the survival analysis subgroup ($N = 53$), seven patients experienced relapse during their follow-up in the present study and needed a major change in the therapy (discontinuation of infliximab resulting in switch or swap or surgical procedure) and thus were marked as LOR. Patients were divided into two subgroups regarding 6-TGN levels—patients with 6-TGN RBC levels under the detection limit (*undetectable* levels) and those with *detectable* levels. A significantly higher relapse rate was observed in the subgroup of patients with *undetectable* ($N = 12$, HR 4.71; 95% CI 1.05–21.11) 6-TGN levels, than in patients with *detectable* ($N = 39$, HR 0.212; 95% CI 0.047–0.951) 6-TGN RBC levels (survival analysis; $N = 51$; *undetectable* vs *detectable*; $p = 0.026$). However, for 6-TGN cutoffs of 230 or 125 pmol/8 × 10^8 RBCs, the difference was not significant. The Kaplan–Meier curve and the associated risk table are shown in Fig. 4. Characteristics of this study subgroup are shown in Table 2.

3.3.2 Infliximab Levels and Antibody Formation

No association was found between serum infliximab levels and 6-TGN levels in RBCs (both tested as continuous variables), not even when adjusted for variables selected according to their clinical relevance (infliximab levels adjusted to 8-week intervals, anti-IFX levels, albumin serum levels, age at sample collection, and duration of therapy). Infliximab trough levels were positively associated with patient age at the time of sample collection (LMM; $N = 321$; 95% CI 0.233–0.971; $p = 0.003$), but not with the duration of combination therapy. No significant association was observed between 6-TGN and infliximab trough levels when a subgroup of patients with an 8-week interval of infliximab administration was tested alone (LMM, adjusted model; $N = 192$; 95% CI − 0.0007 to 0.0113; $p = 0.096$). Detailed results are presented in Table 3.

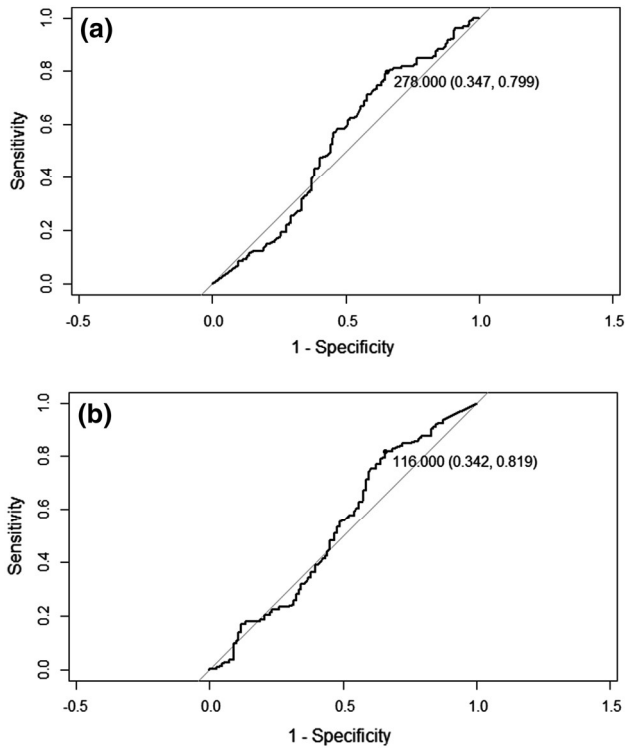

Fig. 3 ROC curves suggesting the best 6-TGN cutoff predicting IFX >5 µg/mL (**a**) and 3 µg/mL (**b**). IFX trough levels 5 µg/mL or higher were previously reported to be optimal for the treatment of inflammatory bowel diseases [23–26]. According to our data analysis, when predicting IFX >5 µg/mL, the best 6-TGN cutoff seemed to be (cross-validated ROC analysis) 278 pmol/8 × 10^8 RBCs. *6-TGN* 6-thioguanine nucleotide, *IFX* infliximab, *RBCs* red blood cells, *ROC* receiver operating characteristic

Positive anti-IFX levels (>5 AU/mL) were detected in seven observations.

3.3.3 Adherence, Thiopurine Metabolite Levels, and Azathioprine-Related Adverse Events

The median levels of thiopurine metabolites, infliximab, and anti-IFX are reported in Table 4 (no TPMT recessive homozygous cases were included). We suspected shunters in 11 observations, which were excluded from the adherence analysis. Thus, adherence to thiopurine therapy was studied in a subgroup of patients from which patients with TPMT heterozygous genotype and suspected shunters were excluded (Fig. 2, $N = 295$ observations). 6-TGN levels <125 pmol/8 × 10^8 RBCs were detected in 76 out of 295 (26%) visits. In 17 out of 76 low 6-TGN observations (6% of total number), azathioprine daily doses < 1 mg/kg were prescribed, resulting in suspected underdosing. In the remaining 59 observations (20% of total number), non-adherence to therapy was considered probable.

A significant association between prescribed azathioprine daily dose adjusted for BW and 6-TGN was recorded in TPMT-dominant homozygous patients (LMM; $N = 305$; 95% CI 67.09–159.19; $p < 0.001$). 6-mMP levels were found to be strongly positively correlated with 6-TGN levels in TPMT dominant homozygous patients (LMM; $N = 305$; 95% CI 2.970–4.439; $p < 0.001$), and to BW-adjusted azathioprine daily dose (LMM; $N = 305$; 95% CI 224.4–853.7; $p = 0.001$). Six patients (comprising 44 visits) received oral mesalamine, but no significant association between mesalamine administration and 6-TGN or 6-mMP levels was observed. No azathioprine-induced adverse events were recorded.

6-mMP levels > 5700 pmol/8 × 10^8 RBCs were detected during only two visits (comprising one patient); normal liver transaminase (AST, ALT, or GGT) levels were observed in both instances. No cases of severe cytopenia were reported. However, a negative association between 6-TGN RBC levels and lymphocyte count was observed (LMM; $N = 321$; 95% CI − 0.0013 to − 0.0006; $p < 0.001$). In addition, the absolute neutrophil count was negatively associated with infliximab serum levels (LMM; $N = 321$; 95% CI − 0.061 to − 0.016; $p = 0.001$). However, absolute neutrophil count was not associated with the RBC levels of 6-TGN or azathioprine daily dose adjusted to BW.

3.3.4 Disease Activity Markers and Association with Thiopurine Metabolites and Infliximab Trough Levels

No association between CRP levels and 6-TGN levels was observed. However, there was a trend observed in association between CRP levels and infliximab levels, but not reaching statistical significance (LMM; $N = 320$; 95% CI − 0.216 to 0.015; $p = 0.09$). When CRP was tested as a categorical variable (less or higher than 5 mg/mL), the significance of the association with infliximab levels slightly increased (LMM; $N = 320$; 95% CI − 0.00007 to 0.015; $p = 0.05$). The significance increased even more when both CRP and infliximab levels (cutoff 5 µg/mL) were tested as categorical variables (LMM; $N = 320$; 95% CI 0.032–0.168; $p = 0.004$). No association was observed between other clinical (wPCDAI) or laboratory (ESR or F-CPT) markers and either infliximab or 6-TGN levels.

4 Discussion

4.1 Optimal 6-TGN Cutoff and Loss of Response to Infliximab Therapy

The optimal 6-TGN cutoff in pediatric patients concomitantly treated with biological agents (e.g., infliximab) for CD remains unknown. To simplify this evaluation for clinicians, we have attempted to determine a 6-TGN cutoff level

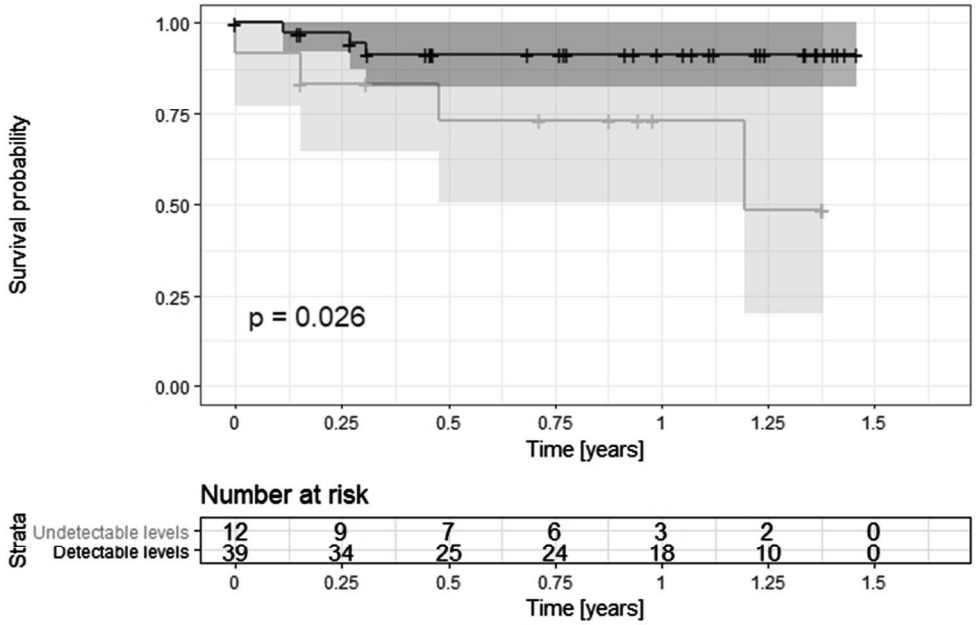

Fig. 4 Survival (Kaplan-Meier) curve showing the risk of LOR according to 6-TGN levels. LOR was observed in seven patients during follow-up. Fifty-one patients (survival study population) were divided into two subgroups using the detection limit of the laboratory method of 6-TGN levels as a cutoff—patients with *undetectable* (light grey) levels and patients with *detectable* levels (dark grey). In the subgroup of patients with *zero* 6-TGN levels, a significantly higher relapse rate was observed than in patients with *detectable* 6-TGN RBC levels (survival; $N = 51$; $p = 0.026$). However, for 6-TGN cutoffs of 230 or 125 pmol/8 × 10^8 RBCs, the difference was not significant. LOR was defined as the requirement for a major change in therapy (switch or swap to other biologic agents or surgical procedures) after the ineffectiveness of IFX was suspected—based on the decision of the attending physician. *6-TGN* 6-thioguanine nucleotide, *LOR* loss of response to therapy, *RBC* red blood cell

Table 2 Characteristics of the survival study subpopulation ($N = 51$)

Female gender (%)	19 (37.3)
Median age on first visit (range), y	15.8 (6.7–18.8)
Median age on last visit (range), y	16.6 (7.1–19.0)
Median length of follow-up (range), m	11 (0–18)
Overall LOR count during follow-up (%)	7
Median 6-TGN RBC levels on last visit (IQR), pmol/8 × 10^8 RBCs	160 (100–224)
Non-LOR ($N = 44$)	166 (115–231)
LOR ($N = 7$)	0 (0–149)[a]
Median IFX trough levels on last visit (IQR), µg/mL	6.2 (3.8–8.6)
Non-LOR ($N = 44$)	6.4 (4.5–9.5)
LOR ($N = 7$)	4.1 (3.0–8.2)

Data obtained from patients followed up at the University Hospital Motol were used to evaluate LOR. For this purpose, LOR was defined as the requirement for a major change in therapy based on the decision of the treating physician. In the subgroup of patients with 6-TGN levels under the detection limit (*undetectable* levels), a significantly higher relapse rate was observed compared with those with *detectable* 6-TGN RBC levels (survival analysis; $N = 51$; $p = 0.026$). The Kaplan–Meier curve and the associated risk table are presented in Fig. 3

6-TGN 6-thioguaninenucleotide, *IFX* infliximab, *LOR* loss of response to therapy, *RBC* red blood cell

[a]Levels under the detection limit of the method are considered as undetectable or 'zero' ('0')

predicting higher infliximab levels (and thus, as expected, better outcomes).

In previous studies, trough levels of infliximab > 5 µg/mL were reported to be optimal for the treatment of inflammatory bowel diseases [23–26]. In adult patients, effective azathioprine doses appear to be lower in combination therapy than in monotherapy [27, 28]. Furthermore, effective levels of 6-TGN may also be lower in combination therapy [8, 9]. To demonstrate this hypothesis in the pediatric population, we first set and tested a 6-TGN cutoff of 125 pmol/8 × 10^8 RBCs. In our study, the potential association between infliximab levels and 6-TGN observed for this cutoff did not reach statistical significance. However, the significance increased slightly with a 6-TGN cutoff of 230 pmol/8 × 10^8 RBCs. Based on a ROC analysis to predict infliximab levels >5 µg/mL, the best 6-TGN cutoff was 278 pmol/8 × 10^8 RBCs. It is unlikely that this phenomenon is mediated through the formation of anti-IFX as in our cohort the positivity of anti-IFX was rare. Moreover, this is the first study proposing a 6-TGN cutoff for children on combination therapy for CD, and even in adults the data are scarce—the 125-pmol/8 × 10^8 RBCs 6-TGN cutoff has been demonstrated only in one cross-sectional study [9]. The reason why effective 6-TGN levels on combination therapy seem to be higher in the pediatric population than in adults is unclear and needs further

Table 3 Association of IFX trough levels and multiple study variables according to LMM ($N = 321$)

	Estimate (beta)	95% CI		*p* value
↑ 6-TGN levels (as cont. variable)	0.003	− 0.002	0.008	0.29
↓ Anti-IFX levels	− 0.204	− 0.381	− 0.029	**0.029***
↑ IFX dose adjusted to 8-w interval	0.962	0.659	1.254	**< 0.001***
↑ Albumin serum level	0.269	− 0.051	0.488	**0.016***
↑ Age at visit (y)	0.603	0.233	0.971	**0.003***
↑ Time on IFX (m)	0.031	− 0.026	0.087	0.304

No association between 6-TGN and IFX levels was observed when both were tested as continuous variables. IFX trough levels were found to be positively associated with patient age at the time of sample collection, but not with the duration of combination therapy

Bold font indicates statistical significance

6-TGN 6-thioguanine, *IFX* infliximab, *LMM* linear mixed model, *m* months, *w* weeks, *y* years

Table 4 Laboratory results and wPCDAI collected at enrollment, during repetitive visits and on the last visit with respect to administered medication, its effect and possible adverse events and disease activity

	At enrolment/first visit ($N = 63$)	Overall visits ($N = 321$)	Last visit ($N = 63$)
Median 6-TGN levels (IQR), pmol/8 × 10^8 RBCs	229 (138–296)	204 (124–284)	165 (85–224)
6-TGN levels above 125 pmol/8 × 10^8 RBCs (%)	48 (76)	240 (75)	41 (65)
6-TGN levels above 250 pmol/8 × 10^8 RBCs (%)	21 (33)	134 (42)	12 (19)
Median 6-mMP levels (IQR), pmol/8 × 10^8 RBCs	385 (159–814)	357 (139–761)	215 (0–465)
Median IFX serum levels (IQR), µg/mL	4.0 (2.1–6.8)	4.7 (2.4–8.0)	5.6 (2.8–8.5)
Median anti-IFX serum levels (IQR), AU/mL	1.0 (0.9–1.1)	1.0 (0.9–1.1)	1 (0.8–1.1)
Median wPCDAI (IQR)	2.5 (0–5)	0 (0–5)	0 (0–5)
Laboratory results			
Median serum albumin level (IQR), g/L	44.8 (42.7–47.4)	44.8 (43.2–46.6)	45.0 (43.4–46.7)
Median serum CRP level (IQR), mg/L	0.7 (0.5–2.0)	0.7 (0.5–2-4)	0.7 (0.5–2.2)
Median hemoglobin level (IQR), g/L	135 (125–149)	137 (128–146)	137 (127–146)
Median WBC count (IQR), × 10^9/L	6.2 (5.3–7.2)	5.9 (5.1–7.0)	5.9 (5.1–6.9)
Median platelet count (IQR), × 10^{12}/L	339 (279–370)	321 (272–363)	301 (263–349)
Median neutrophil count (IQR), × 10^9/L	3.3 (2.6–4.4)	3.3 (2.7–4.1)	3.3 (2.7–4.0)
Median lymphocyte count (IQR), × 10^9/L	1.6 (1.4–2.1)	1.6 (1.4–2.0)	1.7 (1.4–2.0)
Median ESR (IQR), mm/h	20 (9–34)	18 (9–34)	10 (4–15)
Median F-CPT level (IQR), g/kg	203 (42–716) $N = 44$	189 (50–705) $N = 226$	182 (48–691) $N = 40$

6-TGN levels <125 pmol/8 × 10^8 RBCs were detected in 25% observations. The observed median IFX serum level was 4.7 µg/mL (overall visits). No leukopenia, thrombopenia or anemia (as potential adverse events) were spotted

6-mMP 6-methylmercaptopurine, *6-TGN* 6-thioguaninenucleotide, *anti-IFX* anti-infliximab antibodies, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *F-CPT* fecal calprotectin, *IQR* interquartile range, *RBCs* red blood cells, *WBC* white blood cell, *wPCDAI* weighted Pediatric Crohn's disease activity index

research, going deeper into the pathophysiology of azathioprine metabolism and pharmacokinetics.

Our results did not confirm the previous findings reporting lower optimal levels for combination therapy, when sufficient or higher levels of infliximab were considered the therapy target. However, it is questionable whether we should aim for cutoffs for pharmacokinetics only or base our decisions on clinical outcomes. In clinical practice, predicting LOR might be of even greater importance than predicting 'optimal' infliximab levels alone. According to our survival analysis, 6-TGN levels <60 pmol/8 × 10^8 RBCs (*undetectable*) were found to be positively associated with higher LOR rates in the study cohort. Thus, according to our data, when considering the clinical outcome, target 6-TGN levels appear to be much lower than those previously reported in adults. When we look closer at the results, we can easily conclude that those patients with *undetectable* 6-TGN levels mimic those treated with infliximab in

monotherapy who do not receive azathioprine (or any other immunomodulator) at all (anymore). In our study, LOR is described as a need for major change in therapy (switch or swap of infliximab; thus, infliximab discontinuation or the need for surgery). It has been previously published that children with CD are more likely to remain on infliximab when receiving an immunomodulator (such as azathioprine or others) concomitantly [29, 30]. Thus, it is not surprising that patients with *undetectable* 6-TGN levels are more prone to LOR leading to infliximab discontinuation.

A model for predicting low 6-TGN levels has recently been published. Thus, even clinicians who do not have access to routine thiopurine metabolite measurements may benefit from considering 6-TGN levels as categorical variables [31].

An association was observed between infliximab and CRP only when they were both tested as categorical variables, but no association was revealed between CRP and 6-TGN. We did not prove an association between other disease activity markers (wPCDAI, F-CPT, and ESR) and either infliximab levels or 6-TGN levels. Thus, this may suggest a potential direct effect of azathioprine that is not mediated via infliximab pharmacokinetics. However, the design of our study was not appropriate to evaluate these associations as the majority of patients were in stable remission during the study—there were basically no patients with wPCDAI >12.5 points included in our study, and median CRP was quite low (0.7 mg/L) as well. Some F-CPT results were missing, which made the chance to detect a significant association with infliximab and/or 6-TGN levels even smaller. A larger and possibly more heterogenic study cohort would be needed to confirm (or refute) our observations.

4.2 Infliximab Levels and Antibody Formation

We revealed an association between infliximab levels and patient age (at the time of sample collection), but not with the duration of therapy. These results are in accordance with previously published data suggesting that intensified infliximab dosing is required in younger children to achieve adequate trough levels [32, 33]. At the time of our study, we did not have a proactive strategy in infliximab therapeutic drug monitoring; thus, some of our younger patients may have only achieved lower infliximab levels after standard dosing based on clinical decisions.

The association of infliximab trough levels with infliximab dose adjusted to an 8-week interval is not surprising as it was previously shown that both dosage and interval of infliximab are highly associated with infliximab pharmacokinetics [33–35]. The observed association of infliximab trough levels with albumin serum levels has also been previously well described [35–37].

An advantage of combination therapy, in comparison with infliximab monotherapy, is the added protection against anti-IFX production [1, 38], although this is thought to depend on 6-TGN levels [9]. In contrast to what has been published, we did not find an association between 6-TGN levels and anti-IFX levels. Our results might be (at least partially) biased by the fact that only a few patients developed high anti-IFX levels during follow-up. To confirm (or refute) this hypothesis, a larger pediatric study cohort is required.

4.3 Adherence, Thiopurine Metabolite Levels, and Azathioprine-Related Adverse Events

We suspected nonadherence in 20% of observations, which is lower than what has been previously reported [39]. Despite the supposedly nonadherent patients included in our study, a significant correlation between prescribed azathioprine daily dose adjusted to BW and 6-TGN levels was observed. When azathioprine treatment is initiated, it takes about 55 days to reach stable thiopurine metabolite concentrations in RBCs [40]. Thus, it is highly probable that the amount of time required for the concentration of thiopurine metabolites in RBCs to reach zero (or undetectable levels) in non-adherent patients is not insignificant. We assume that infrequent skipping of azathioprine doses is difficult to observe from an assessment of 6-TGN and 6-mMP levels. It is likely to be even more difficult when the 6-TGN nonadherence cutoff is set to a low value due to the relatively low recommended azathioprine daily dose (1–1.5 mg/kg) used in combination therapy [27]. Moreover, some of our patients are considered ‘underdosed’ with azathioprine daily doses < 1 mg/kg because in our center, we do not usually increase the dose of azathioprine following weight gain in young patients receiving combination therapy if the patient is in clinical and laboratory remission. This effectively complicates the meaning of nonadherence in a subgroup of patients with low doses. Although it is likely that not all of the nonadherent patients were identified, thiopurine metabolite monitoring still seems to be useful in evaluating adherence to thiopurine therapy.

Due to the small number of patients with TPMT heterozygous genotype included in our study, we were not able to study this subgroup separately. The percentage of TPMT heterozygous patients included in our study did not correspond to what would have been expected from a consideration of the whole population [6]. We may have lost to our study some of the potentially suitable TPMT heterozygous patients due to azathioprine intolerance (before the study started). Thus, azathioprine side effects may have led to azathioprine discontinuation months or even years before the study started. In addition, we revealed five supposed shunters (11 observations; Fig. 2). If the present

therapy had proved ineffective in these patients, adding allopurinol into the therapy may have made their treatment more efficient [13, 14, 41–43].

We did not observe any azathioprine-related adverse events. This was probably (at least partially) because all our patients had been on combination therapy for > 3 months before inclusion in the study. Thus, because idiosyncratic adverse events are more likely to occur at the beginning of therapy (and may potentially be responsible for azathioprine discontinuation), patients experiencing azathioprine-related adverse events were likely not included in the study simply because they stopped taking azathioprine before fulfilling the 3-months-or-more criteria. Moreover, dose-dependent adverse events were less likely to be observed because of the relatively low dose of azathioprine administered (as previously discussed).

4.4 Limitations and Strengths of the Study

Some observations had to be excluded because of missing data. Patients were included after different periods of time on combination therapy and this may have affected the outcomes. Finally, some subgroups were under-represented (e.g., shunters, TPMT intermediate metabolizers, 5-ASA co-administrated, history of CD-related surgery).

The main advantages of our study include the comprehensive multicentric cluster of patients, the prospective design, and the use of well-known reliable methodologies for thiopurine metabolite measurements. Because of the great deal of data collected, we were able to study many aspects of combination therapy in children, including predictors.

5 Conclusions

As undetectable levels of 6-TGN seem to be associated with loss of response on combination therapy, thiopurine metabolite monitoring could be useful when optimizing combination therapy and assessing treatment adherence in clinical practice.

Declarations

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Disclosure of potential conflicts of interest Pospisilova K: lectures/congress fees/consultancy (outside submitted work)—MSD, Nutricia, Nestlé and Mead Johnsons; Karaskova E: lectures/congress fees/consultancy (outside submitted work)—MSD, AbbVie, Nutricia, and Nestlé; Hradsky O: lectures/congress fees/consultancy (outside submitted work)—MSD, AbbVie, Nutricia, Nestlé, Ferring, and Falk; Lerchova T: lectures/congress fees/consultancy (outside submitted work)—Nutricia, Ferring and Biocodex; Zarubova K: lectures/congress fees/consultancy (outside submitted work)—Nutricia and Nestlé; Velganova-Veghova M: congress fees/consultancy (outside submitted work)—MSD, AbbVie, Nutricia, and Nestlé; Bronsky J: lectures/congress fees/consultancy (outside submitted work)—MSD, AbbVie, Nutricia, Nestlé, Ferring, Biocodex, and Walmark; Gonsorcikova L, Francova I, Geryk M, Copova I, Mihal V, Siroka J, and Urbanek L report no conflicts of interest.

Ethics approval The protocol for the observational study was approved by the Ethics Committees of the University Hospital Motol and the 2nd Medical Faculty of Charles University in Prague.

Informed consent Written informed consent was obtained from legal guardians before study enrollment.

Consent for publication Non applicable.

Study registration The study has been registered retrospectively in the ENCePP registry (encepp.eu), it can be found under the registration number EUPAS38918. Registered on 12 January 2021.

Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Code availability The code is available from the corresponding author on reasonable request.

Author contributions PK: study design, literature search, data collection, patient recruitment, thiopurine metabolite measurement, data analysis, manuscript writing; SJ: data collection, thiopurine metabolite measurement and laboratory supervision, manuscript critical revision; KE: data collection, patient recruitment, manuscript critical revision; HO: study design, data analysis and interpretation, manuscript critical revision; LT: study design, data collection, patient recruitment, manuscript critical revision, ZK: data collection, patient recruitment, manuscript critical revision; CI: data collection, patient recruitment, manuscript critical revision; GL: data collection, patient recruitment, manuscript critical revision; V-VM: data collection, patient recruitment, manuscript critical revision; FI: data collection, infliximab levels and antibodies measurement supervision, manuscript critical revision; UL: thiopurine metabolite measurement—method optimization, data collection, manuscript critical revision; GM: data collection, patient recruitment, manuscript critical revision; Mihal V: data collection, supervision, manuscript critical revision; BJ: study design and supervision, literature search, manuscript writing. All authors read and approved the final manuscript.

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Vaccinations and Immunization Status in Pediatric Inflammatory Bowel Disease: A Multicenter Study From the Pediatric IBD Porto Group of the ESPGHAN

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Background: Vaccine-preventable diseases and opportunistic infections in pediatric inflammatory bowel disease (IBD) are increasingly recognized issues. The aims of this study were to evaluate vaccinations, immunization status, and consequent therapeutic management in children with IBD and to analyze the differences among patients diagnosed before (Group 1) and after June 2012 (Group 2).

Methods: This was a multicenter, retrospective cohort investigation. Between July 2016 and July 2017, 430 children with IBD were enrolled in 13 centers. Diagnosis, therapeutic history, vaccinations, and immunization status screening at diagnosis and at immunosuppressant (IM)/biologic initiation and reasons for incomplete immunization were retrieved.

Results: Vaccination rates at diagnosis were unsatisfactory for measles, mumps, and rubella (89.3%), *Haemophilus influenzae* (81.9%), meningococcus C (23.5%), chickenpox (18.4%), pneumococcus (18.6%), papillomavirus (5.9%), and rotavirus (1.9%). Complete immunization was recorded in 38/430 (8.8%) children, but specific vaccines were recommended in 79/430 patients (18.6%), without differences between the 2 groups. At IM start, 22% of children were tested for Epstein-Barr virus (EBV) status, with 96.2% of EBV-naïve patients starting azathioprine, without differences between Groups 1 and 2. Screening for latent tuberculosis (TB) before start of biologics was performed in 175/190 (92.1%), with up to 9 different screening strategies and numerous inconsistencies.

Conclusions: We demonstrated a poor immunization status at diagnosis in children with IBD, which was not followed by proper vaccination catch-up. EBV status before IM initiation and latent TB before biologics were not adequately assessed. Thus, the overall impact of the current guidelines seems unsatisfactory.

Key Words: inflammatory bowel disease, pediatrics, vaccinations

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INTRODUCTION

Although the incidence and prevalence of inflammatory bowel diseases (IBDs) differ among countries, the general trend highlights an overall increase over the past few decades, especially in adolescence and young adulthood.[1, 2] It is well known that pediatric IBD is characterized by a more extensive involvement and severe course when compared with adults, including a higher need of immunosuppressive and biological therapies.[3–5] Due to the underlying disease, poor nutritional status, and early aggressive immunomodulatory treatments, the risk of opportunistic infections and their prevention in children with IBD are increasingly recognized issues.[6, 7] Attention to this topic has progressively grown after the widespread use of biologics. The first reported pediatric data on opportunistic infections in IBD came from the REACH study.[8] Of all the infections, 6.8% were classified as severe and included sepsis, pneumonia, herpes zoster and abscesses.[8] In 2009, the European Crohn's and Colitis Organization (ECCO) published the first evidence-based guidelines on the management of opportunistic infections in IBD patients.[9] As these guidelines were not specifically conceived for the pediatric population, in June 2012 the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) published a commentary on the risk and the prevention of infections in children with IBD, with the main objective of adapting the ECCO guidelines to the pediatric scenario.[10] With regards to prevention, the following points were recommended: immunization history should be obtained at the time of IBD diagnosis; children with IBD should receive inactivated vaccines following the routine childhood immunization schedule; attenuated live vaccines are contraindicated in patients treated with immunosuppressive drugs.[10] In addition to these recommendations, the 2014 ECCO guidelines were updated, emphasizing essential topics, such as the need for testing for Epstein-Barr virus (EBV) before starting azathioprine (AZA) and the importance of tuberculosis (TB) screening before initiating biologic treatment.[11] Despite this growing body of literature, few data have been published in children, and pediatric gastroenterologists (GIs) seem not to perceive the importance of this issue, which is frequently overlooked.[12–14] Therefore, the primary aims of this study were to evaluate the vaccinations, immunization status, and management of immunoregulatory therapies in a cohort of children with IBD; the secondary aims were to assess the impact of the ESPGHAN commentary,[10] analyzing the differences among patients diagnosed before and after June 2012.

METHODS

The Vaccinations and Immunization status in Pediatric IBD (VIP-IBD) study was a multicenter, retrospective cohort investigation conducted between July 2016 and July 2017 including 13 different ESPGHAN IBD referral centers. Participating centers were required to select a representative sample of children newly diagnosed with IBD before June 2012 (Group 1) and after June 2012 (Group 2) and to retrospectively collect their data. Each center had to enroll at least the first 5 consecutive patients diagnosed in each of the 3 years before and after June 2012. The inclusion criteria were a confirmed diagnosis of IBD, age at diagnosis ≤18 years, and clinical follow-up of at least 12 months. Diagnosis of IBD was established on the basis of clinical, endoscopic, radiological, and histological criteria according to the Porto criteria.[15] The distribution of the enrolled children among the different centers is shown in Table 1. Each participating center was required to complete a structured 28-item questionnaire form (Supplementary Table 1) for each enrolled patient. The form was designed to measure adherence to the ESPGHAN commentary on the risk and prevention of infections in children with IBD.[10] In addition, some of the topics were derived from the most recent ECCO evidence-based guidelines on the management of opportunistic infections in IBD patients.[11] The questionnaire included all the following items: demographic data (age, sex, parental education); diagnostic characteristics (type, age at diagnosis, disease activity and extent at diagnosis, extra-intestinal manifestations, comorbidities); therapeutic history; evaluation of the recommended routine childhood vaccinations (diphtheria, tetanus, and poliomyelitis [DTP], pertussis, haemophilus influenzae, hepatitis B, pneumococcus, meningococcus C, measles, mumps, and rubella [MMR], chickenpox, papillomavirus, rotavirus); annual influenza vaccination at diagnosis and during follow-up; serological titer assessment for the recommended infectious agents (hepatitis A, B, and C, EBV, rubella, herpes simplex virus [HSV], chickenpox, HIV, TB) at diagnosis, at initiation of immunosuppressant and biological therapies, and at follow-up; tests to detect latent TB (tuberculin skin test [TST], quantiferon TB gold [QFT], Elispot, chest x-ray); reasons for

TABLE 1. Distribution of Enrolled Children Among the 13 Different Centers

Center	Group 1, No. (%) (n = 218)	Group 2, No. (%) (n = 212)
Budapest, Hungary	15 (6.9)	15 (7.1)
Cluj-Napoca, Romania	19 (8.7)	24 (11.3)
Kaunas, Lithuania	6 (2.8)	9 (4.2)
Krakow, Poland	15 (6.9)	15 (7.1)
Málaga, Spain	15 (6.9)	15 (7.1)
Messina, Italy	15 (6.9)	15 (7.1)
Naples, Italy	26 (11.9)	29 (13.7)
Petach Tikva, Israel	15 (6.9)	15 (7.1)
Prague, Czech Republic	15 (6.9)	15 (7.1)
Rome, Italy	15 (6.9)	14 (6.6)
Vilnius, Lithuania	30 (13.8)	15 (7.1)
Warsaw, Poland	17 (7.8)	16 (7.5)
Zagreb, Croatia	15 (6.9)	15 (7.1)

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incomplete immunization and decision-making regarding immunoregulatory therapies on the basis of immunological status. A complete immunization status at diagnosis was defined when a patient was vaccinated and/or showed positive titers for the following pathogens: DTP, poliomyelitis, haemophilus influenzae, hepatitis B, MMR, pneumococcus, meningococcus C, and chickenpox.[10, 11] Disease extent was characterized according to the Paris classification,[16] whereas disease activity was reported using the Pediatric Ulcerative Colitis Activity Index (PUCAI)[17] and the Pediatric Crohn's Disease Activity Index (PCDAI).[18]

Ethical Considerations

The study was approved by the Institutional Review Board (IRB) of the University of Naples "Federico II" with the protocol registration number 175/16. Subsequently, all the remaining enrolling units obtained specific approval from their local IRBs.

Statistical Analysis

Variables were screened for their distribution, and appropriate parametric or nonparametric tests were adopted as necessary. Percentages were rounded to the nearest whole numbers. The Student *t* test and Mann-Whitney test for continuous variables and the χ^2 and Fisher exact tests for categorical variables were used where appropriate. Statistical significance was predetermined as $P < 0.05$. SPSS, version 20 (SPSS Inc, Chicago, IL, USA), was used for all the analyses.

RESULTS

Baseline Characteristics

Four hundred thirty patients met the inclusion criteria and were enrolled between July 2016 and July 2017 (CD: 254, 59.1%; UC: 164, 38.1%; IBD-U: 12, 2.8%; median age at diagnosis [range], 12 [1–18] years; M/F: 236/194). Among the 430 patients, 218 were diagnosed before June 2012 (Group 1: 50.7%) and 212 after June 2012 (Group 2: 49.3%). Baseline characteristics of the 2 groups of children are presented in Table 2.

Vaccinations and Serological Evaluation at Diagnosis

The rates of vaccination at diagnosis were: diphtheria, tetanus, and pertussis (DTP; 427/430, 99.3%), poliomyelitis (426/430, 99.1%), hepatitis B (418/430, 97.2%), MMR (384/430, 89.3%), *Haemophilus influenzae* (352/430, 81.9%), meningococcus C (101/430, 23.5%), chickenpox (79/430, 18.4%), pneumococcus (80/430, 18.6%), papillomavirus (22/430, 5.9%), and rotavirus (8/430, 1.9%). When comparing children diagnosed before and after June 2012, no significant differences were observed for all the vaccines, except for meningococcus C, pneumococcus, chickenpox, and papillomavirus, which were significantly increased in Group 2 (18.8% vs 28.3%; $P = 0.01$; 14.7% vs 22.6%; $P = 0.02$; 14.7% vs 22.2%; $P = 0.03$; 2.8% vs 7.5%; $P = 0.02$, respectively). The rates of vaccination coverage for each pathogen before and after 2012 and the differences among each enrolling country are presented in Figure 1 and Supplementary Figure 1, respectively. The rates of assessment at diagnosis for each pathogen were TB (226/430, 52.5%), EBV (164/430, 38.1%), hepatitis B (161/430, 37.4%), hepatitis C (127/430, 29.5%), HSV (71/430, 16.5%), hepatitis A (54/430, 12.5%), rubella (51/430, 11.8%), chickenpox (70/430, 16.2%), and HIV (53/430, 12.3%). When comparing Group 1 and Group 2, we observed a statistically significant increase in the evaluation of the following pathogens: hepatitis A (10.6% vs 14%; $P = 0.01$), hepatitis B (28% vs 47.2%; $P < 0.001$), hepatitis C (20.2% vs 39.2%; $P < 0.001$), EBV (27.5% vs 47.7%; $P < 0.001$), HIV (9.2% vs 15.1%, $P = 0.04$), rubella (8.3% vs 15.6%; $P = 0.01$), and TB (46.8% vs 58.5%; $P = 0.01$).

Vaccine Catch-up

A complete immunization status was recorded in 38/430 patients (8.8%). An increase in the rate of complete immunization was observed in Group 2, even if this was not statistically significant (Group 1: 16/218, 7.3%; vs Group 2: 22/212, 10.3%; $P = 0.1$). None of the variables were significantly associated with a complete immunization rate. Among the 392 children with incomplete immunization, specific vaccinations were recommended in 79 patients (20.1%), without differences between Group 1 and Group 2 (46/202, 22.7%, vs 33/190, 17.3%; $P = 0.1$). The following vaccines were caught-up: pneumococcus (78/79, 98.7%), meningococcus C (57/79, 72.1%), chickenpox (55/79, 69.6%), hepatitis B (40/79, 50.6%), MMR (29/392, 36.7%), papillomavirus (20/79, 25.3%), rotavirus (5/79, 6.3%), DTP (1/79, 1.2%), and poliomyelitis (1/79, 1.2%). In the remaining children, the reasons for not being vaccinated were need for immediate IM therapies (87/313, 27.8%), parental refusal (24/313, 7.7%), vaccination costs (4/313, 1.6%), and unknown (154/313, 49.2%).

Influenza

One hundred twenty-three children out of 430 (28.6%) underwent yearly influenza vaccination, whereas 276 (64.2%) were not routinely exposed to the vaccine; in 31 (7.2%) cases, the status of influenza vaccination was not known. An increase of influenza vaccination was observed in Group 2 when compared with Group 1 (70/212, 35.4%, vs 53/218, 24.3%; $P = 0.03$). A higher number of children exposed to immunosuppressive and/or biologic therapy underwent annual influenza vaccination when compared with the remaining patients (60/80, 75%, vs 20, 25%; $P < 0.001$).

Screening Before Immunosuppressive Therapy

Two hundred fifty-one (58.3%) out of 430 children started IM (AZA: 238, 94.8%; methotrexate [MTX]: 13, 5.2%). Group 2 children started IM significantly earlier than

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TABLE 2. Baseline Characteristics of the Enrolled Patients

Characteristics	Group 1	Group 2	P^a
	(n = 218)	(n = 212)	
Median age at diagnosis (range), y	12 (1–18)	12.3 (2–18)	0.2
Sex, No. (%)			
Male	113 (51.8)	123 (58)	0.1
Diagnosis, No. (%)			0.8
CD	132 (60.6)	122 (57.5)	
UC	80 (36.7)	84 (39.6)	
IBD-U	6 (2.8)	6 (2.8)	
Median PCDAI at diagnosis (range)	35 (5–70)	32 (7.5–65)	0.7
Median PUCAI at diagnosis (range)	45 (11–85)	45 (15–75)	0.4
Disease location at diagnosis, No. (%)			
CD			
Ileum only (L1)	18 (13.6)	30 (24.5)	0.03
Colon only (L2)	27 (20.4)	26 (21.3)	0.8
Ileum and colon (L3)	83 (62.8)	62 (50.7)	0.06
Upper gastrointestinal tract (L4)	44 (20.1)	50 (23.5)	0.2
Perianal disease	15 (11.2)	13 (10.6)	1
UC			
Ulcerative proctitis (E1)	12 (15)	8 (9.6)	0.3
Left-sided colitis (E2)	12 (15)	16 (19)	0.5
Extensive colitis (E3)	6 (7.5)	11 (13)	0.3
Pancolitis (E4)	50 (62.5)	49 (58.4)	0.6
Induction therapy at diagnosis, No. (%)			
CD			
EEN	36 (27.3)	55 (45.1)	<0.001
Steroids	71 (53.8)	39 (32)	<0.001
Biologics	3 (2.3)	10 (8.2)	0.04
EEN+steroids	7 (5.3)	11 (9)	0.3
Mesalazine	14 (10.6)	6 (4.9)	0.1
Surgery	1 (0.7)	1 (0.8)	1
UC			
Steroids	34 (42.5)	50 (59.5)	0.04
Mesalazine	45 (56.2)	29 (34.5)	<0.001
Biologics	1 (1.3)	5 (6)	0.2
IBD-U			
EEN	1 (16.6)	2 (33.4)	1
Steroids	5 (83.4)	3 (50)	0.5
Mesalazine	0	1 (16.6)	1

Group 1: patients diagnosed before June 2012; Group 2: patients diagnosed after June 2012.
[a]Fisher exact test or Mann-Whitney test was used for categorical and continues variables, respectively.

patients in Group 1 (median time [range], 1 [0–24] months vs 2 [0–108] months; $P < 0.001$). The number of children starting immunosuppressive therapy was significantly decreased in Group 2 when compared with Group 1 (115/212, 54.2%, vs 136/218, 62.3%; $P = 0.03$). Before starting IM, we observed a significant increase in the percentage of subjects tested for HIV (2/136, 1.5%, vs 9/115, 7.8%; $P = 0.01$). There were no statistical differences when comparing the 2 groups with regards to hepatitis A, B, and C (17.9% vs 28.1%; $P = 0.5$; 17.4% vs 19%; $P = 0.2$; 13.9% vs 20.6%; $P = 0.7$, respectively; HSV: 13.9% vs 11.1%; $P = 0.4$; chickenpox: 9% vs 9.6; $P = 0.7$; rubella: 14.6% vs 12.8%; $P = 0.2$), or for the percentage of patients tested for latent TB (19.9% vs 25.2%; $P = 0.2$).

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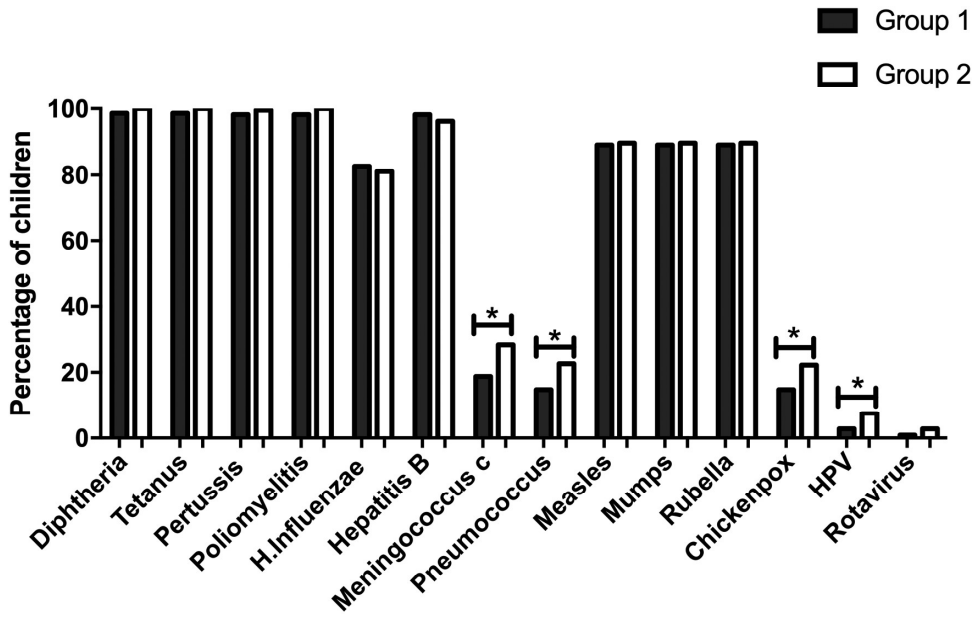

FIGURE 1. Rates of vaccination coverage at diagnosis before (Group 1) and after June 2012 (Group 2). *$P < 0.05$, Fisher exact test.

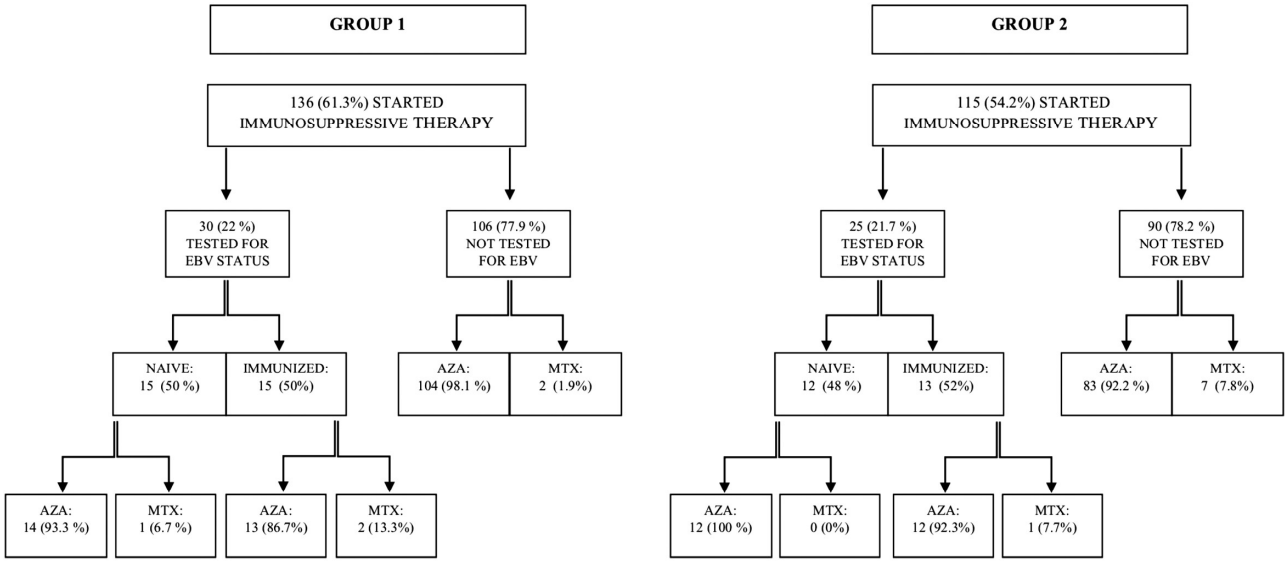

FIGURE 2. Flow diagram of EBV screening before starting immunosuppressive therapy in children diagnosed before (Group 1) and after June 2012 (Group 2).

EBV Status

Fifty-five children (22%) out of 250 children were tested for EBV status before starting IM, with 28 (50.9%) immunized for EBV and 27 (49.1%) EBV naïve. Among those who were EBV naïve, 26/27 started AZA (96.2%), whereas only 1 patient (3.7%) started MTX. There was no statistical difference between Groups 1 and 2 in the percentage of patients tested for EBV before starting AZA ($P = 0.5$); no difference was detected between the 2 groups in the percentage of patients who started AZA being EBV naïve ($P = 1$). The percentage of male sex in EBV-naïve children starting AZA did not differ between Group 1 and Group 2 (8/14, 57.1, vs 5/12, 41.7%; $P = 0.3$). A flow diagram of EBV status and consequent therapeutic management is shown in Figure 2.

Screening Before Biologics

Biologics were started in 190 (44.2%) out of 430 children (infliximab: 152/190, 80.2%; adalimumab [ADA]: 38/190, 20%). The median time to start biologics among the entire population (range) was 12 (0–108) months. Group 2 children

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tended to start biologic therapy significantly earlier than patients in Group 1 (median time [range], 10 [0–82] months vs 18 [0–108] months; $P < 0.001$). In Group 1, biologic therapy was started in 98 out of 218 patients (45%), compared with 92/212 patients (43.4%) in Group 2 ($P = 0.4$). Infliximab was started in a significantly higher percentage of patients in Group 1 compared with Group 2 (87/218, 39.9%, vs 65/212, 30.7%, respectively; $P = 0.03$), whereas ADA therapy was started in a major number of patients in Group 2 compared with Group 1 (27/212, 12.7%, vs 11/218, 5%, respectively; $P = 0.004$). There was no statistically significant difference between the 2 groups in the percentages of patients tested for hepatitis A, B, and C (21.8% vs 21.6%; $P = 1$; 54.6% vs 48.3%; $P = 0.1$; 49.6% vs 48.3%; $P = 0.5$, respectively), EBV (47% vs 47.5%; $P = 1$), HSV (31.1% vs 26.7%; $P = 0.3$), HIV (16% vs 20%; $P = 0.2$), chickenpox (23.5% vs 16.6%; $P = 0.1$), and rubella (11.7% vs 15%; $P = 0.2$). After starting, a significantly higher number of children continued to be monitored for serological titers in Group 2 when compared with Group 1 (78/92, 84.8%, vs 65/98, 66.3%; $P = 0.003$). Serological titers during the course of biologic therapy were checked with the following frequencies: every year (45/65, 69.2%, vs 53/78, 66.7%; $P = 0.4$), every 6 months (2/65, 3.1%, vs 2/78, 2.6%; $P = 0.6$), and every other administration (10/65, 15.4%, vs 7/78, 9%; $P = 0.1$).

Latent Tuberculosis

Screening for latent TB before starting biologics was performed in 175/190 (92.1%), without significant differences when comparing children from both groups (Group 1: 93/98, 94.9%; vs Group 2: 82/92, 89.1%; $P = 0.1$). The 4 available exams—TST, QFT, Elispot, and chest x-ray—were used with 7 and 9 different diagnostic strategies in Group 1 and Group 2, respectively (Fig. 3). No significant differences were observed regarding the use of each specific combination and single test. A huge variation in TB screening modalities was observed among the different enrolling centers (Table 3).

DISCUSSION

To the best of our knowledge, the VIP-IBD study is the largest multicenter survey to evaluate vaccinations, immunization status, and subsequent therapeutic management in pediatric IBD. Our data highlight that children with IBD show insufficient immunization coverage at diagnosis and that vaccination

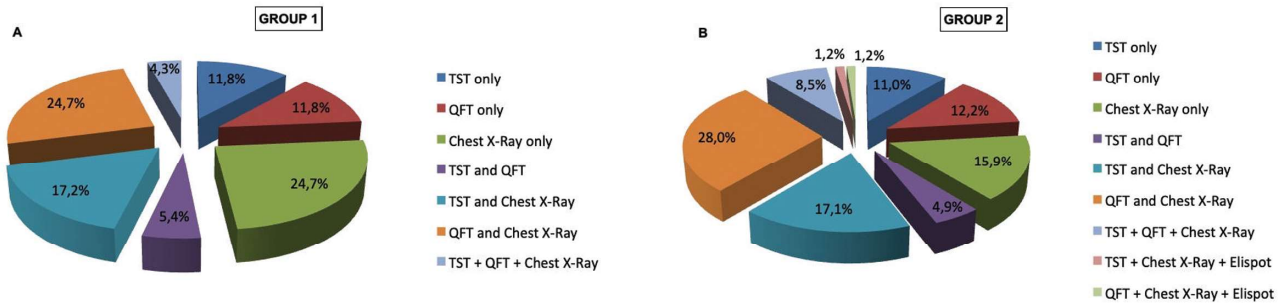

FIGURE 3. Diagnostic strategies to screen latent TB before starting biologics in children diagnosed before (Group 1) (A) and after June 2012 (Group 2) (B).

TABLE 3. Different Diagnostic Strategies to Screen Latent Tuberculosis Before Starting Biologics

Screening Strategy	Croatia (n = 4), No. (%)	Czech Rep. (n = 16), No. (%)	Hungary (n = 27), No. (%)	Israel (n = 18), No. (%)	Italy (n = 53), No. (%)	Lithuania (n = 10), No. (%)	Poland (n = 30), No. (%)	Romania (n = 3), No. (%)	Spain (n = 14), No. (%)
TST only	-	-	-	2 (11.1)	14 (26.4)	-	-	-	4 (28.6)
QFT only	4 (100)	-	-	-	17 (32)	-	-	-	-
X-ray only	-	7 (43.7)	27 (100)		-	2 (20)	-	-	-
TST + QFT	-	-	-	-	1 (1.9)	-	-	-	8 (57.1)
TST + x-ray	-	-	-	16 (88.9)	6 (11.3)	7 (70)	1 (3.3)	-	-
QFT + x-ray		8 (50)	-	-	10 (18.9)	-	28 (93.4)	-	-
TST + QFT + x-ray	-	-	-	-	4 (7.6)	1 (10)	1 (3.3)	3 (100)	2 (14.3)
QFT + Elispot + x-ray	-	1 (6.3)	-	-	-	-	-	-	-
TST + Elispot + x-ray	-	-	-	-	1 (1.9)	-	-	-	-

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catch-up is not adequately performed. We also demonstrated an unsatisfactory awareness of 2 hot topics of IBD preventive care: EBV status at the start of AZA therapy and screening for latent TB before anti–tumor necrosis factor (anti-TNF) agents. Overall, the majority of the analyzed variables were modestly impacted by the ESPGHAN recommendations.

Inflammatory bowel disease is considered a condition at high risk of opportunistic infections, particularly in childhood.[19, 20] Strategies that can be used to decrease the hazard of opportunistic infections include screening, chemoprophylaxis, and vaccination.[7] In line with the literature, which reports nonsatisfactory immunization coverage in Europe and North America,[21, 22] our cohort shows insufficient rates for MMR, *Haemophilus influenzae*, meningococcus C, pneumococcus, chickenpox, papillomavirus, and rotavirus. As a consequence of this overall tendency, only 8.8% of children showed complete immunization at diagnosis. These alarming findings once more point out the essential role of the IBD practitioner, who should promptly test immunization for the most notable vaccine-preventable diseases. Indeed, the period from diagnosis to the initiation of immunosuppressive therapy should be considered a crucial window of opportunity for appropriate vaccination before the initiation of immunosuppressive therapy. In 2015, Lester et al. surveyed 178 North American pediatric GIs, demonstrating that at diagnosis only half of them asked verbally about immunization status, 31% obtained records, and only 9% required specific serologies.[13] More recently, deBruyn and colleagues reported that despite adequate vaccination coverage, a high percentage of the children with IBD showed low serologic protection against the main vaccine-preventable childhood infections.[23] In our cohort, we observed a significant increase of serological titer evaluation after the publication of a paper by Veereman et al.[10] Nevertheless, proving incomplete immunization status did not necessarily translate into active vaccination catch-up, which was performed only in 18.6% of children. When analyzing the reasons for not performing vaccine catch-up, we found that only in about 27.8% of children was there an immediate need to start IM therapy, whereas in the remaining cases parental refusal, vaccination costs, and unknown causes were reported. These factors can be certainly improved with a major commitment of pediatric GIs and with an increase in educational efforts. Actually, different studies demonstrated that specific campaigns are able to revert this tendency.[24–26] Fleurier and colleagues reported a significant increase in vaccination coverage in 92 French children with IBD after an awareness campaign on the risk of infection.[26] Most of these efforts are currently being perpetuated for annual influenza vaccination. In 2015, Huth and colleagues reported the successful results of 2-year prospective study.[27] The authors used 2 different strategies: the administration of an educational module with or without vaccine access in the clinic. Both strategies led to significant increases in the vaccination rate, from 34% at baseline to 75% and 89.5%, respectively.[27] In our multicenter study, we observed a significant increase in the influenza vaccination rate from 24.3% to 33% after 2012. Although significant, this result cannot be considered satisfactory, and it still confirms that the publication of guidelines needs to be followed by specific strategies for their widespread implementation.

Over the last 2 decades, pediatric IBD therapeutic strategies have profoundly evolved, leading to an increasing use of more aggressive "top-down" approaches with the early introduction of biologics.[28, 29] Our study gives a clear overview of this tendency, as demonstrated by the significant rise of biologics as firstline therapy and the decreased use of conventional immunosuppressants in children diagnosed after 2012. Despite this trend, our data show that >50% of children are still exposed to AZA, which remains one of the mainstays of pediatric IBD. This finding is particularly remarkable if we take into account the recent controversial warning on thiopurines' use, due to their potential relationship with EBV infection and the risk of lymphoproliferative disorders.[31, 31] These potential risks led the ECCO to state in 2014 that EBV IgG screening should always be considered before initiation of immunomodulatory treatment and that anti-TNFs are preferred in seronegative children.[11] Despite these recommendations, in our cohort only 21.9% of children starting AZA were checked for EBV status, and among those tested, AZA was started in almost all the cases of seronegative EBV patients, irrespective of sex.

The above-mentioned increase of anti-TNF agents' use, together with the recrudescence of TB-multiresistant strains in Europe, has also raised new concerns regarding biologics' safety.[32] It is well elucidated that anti-TNFs increase the risk of TB, particularly the reactivation of latent TB, and that when TB occurs in children on biologics therapy it is more commonly atypical, extrapulmonary, and disseminated.[33, 34] According to the ECCO guidelines, latent TB should be diagnosed by a combination of patient history, chest x-ray, TST, and interferon-gamma release assays (IGRA) according to local prevalence and national recommendations.[6] Our results demonstrate that a considerable inconsistency in TB screening remains in children with IBD starting biologics. Indeed, we observed a lack of standardization, with 9 different diagnostic strategies, and a huge variation among different countries. In some cases, we also observed a high percentage of inappropriateness: an example is represented by the 30% rate of chest x-ray performed as the only screening test before June 2012, without TST or IGRA. This approach, which obviously lacks the proper sensitivity to exclude latent TB, was decreased in the children in Group 2, but was still performed in 15% of the children.

The present study is not without limitations. The main drawback is obviously related to the retrospective nature, which may have resulted in missing data, especially clinical and microbiological findings. In addition, the concrete possibility of recall biases needs to be taken into account. Otherwise, the main strength of the study lies in the large sample size, well distributed among 13 different ESPGHAN tertiary centers, which

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gives us a very precise picture of the application of the recommendations in Europe and Israel.

In conclusion, the VIP-IBD study demonstrated poor immunization status at diagnosis in children with IBD, which is frequently not followed by proper vaccination catch-up. Moreover, pediatric GIs seem not to perceive the risks of thiopurines in relation to EBV status and frequently do not adequately screen children with IBD for latent TB before starting anti-TNFs. On the basis of these findings, the overall impact of the current guidelines on vaccination and immunization status in children with IBD appears unsatisfactory, highlighting an urgent need for further educational efforts to disseminate the available recommendations and to promote their correct application.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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