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Morphological and functional assessment of oesophageal mucosa integrity in children with cystic fibrosis

Morfološka in funkcionalna ocena integritete sluznice požiralnika pri otrocih s cistično fibrozo

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Abbreviations

BMI	Body mass index
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CSS	Combined severity score
DIS	Dilated intercellular spaces
FEV1	Forced expiratory volume in the 1 st second
Fig.	Figure
GOR	Gastro-oesophageal reflux
GORD	Gastro-oesophageal reflux disease
H2-RA(s)	Histamine-2 receptor antagonist(s)
MII	Multichannel oesophageal intraluminal impedance
MII-pH	Combined multichannel oesophageal intraluminal impedance-pH monitoring
No.	Number
PPI(s)	Proton pump inhibitor(s)
Pts.	Patients
SD	Standard deviation

Extended summary

Background

One of the most important functions of gastrointestinal epithelium is the barrier function which prevents the passage of luminal content into deeper tissue layers. Oesophageal epithelium prevents the passage of nutrients, microbes and aggressive chemical substances (gastric acid, enzymes, and bile) which flow into the oesophagus during gastro-oesophageal reflux (GOR) episodes. The integrity of oesophageal epithelium is maintained by normal epithelial cells and intercellular tight junctions. Various oesophageal diseases such as gastro-oesophageal reflux disease (GORD) disrupt the integrity of oesophageal epithelium on the microscopic level seen as a disruption of intercellular tight junctions leading to different histopathological changes and increased distances between epithelial cells which are known as dilated intercellular spaces (DIS). DIS have been reported in erosive and non-erosive GORD, and in other inflammatory diseases of oesophagus. DIS lead to increased permeability of oesophageal epithelium. In clinical conditions it is difficult to measure oesophageal transepithelial permeability directly. Multichannel intraluminal impedance (MII) measures impedance between pairs of electrodes on the catheter placed in the oesophageal lumen. Most of the time oesophagus is empty, hence the mean value of impedance during long duration measurement, also called baseline impedance, reflects the impedance of oesophageal mucosa and can be used as a surrogate marker of the integrity of epithelial barrier.

Patients with cystic fibrosis (CF) are prone to GORD, due to a higher proportion of transient lower oesophageal sphincter relaxations associated with refluxes, and higher pressure difference between thoracic and abdominal cavity because of greater negative inspiratory intrathoracic pressure caused by the lung disease. It has been reported that untreated GORD may lead to more rapid lung function decline, which is a major prognostic factor for worsening the course of CF. Early diagnosis and treatment of GORD in such patients may be critical for prevention of deterioration of CF. However, presence of GORD in CF patients may be easily overlooked, as its symptoms, especially the extra-oesophageal ones such as chronic cough, hoarseness, recurrent pneumonia, loss of appetite, and failure to thrive, may be attributed to the underlying disease. Therefore, it is mandatory to actively search for the presence of GORD in children with CF, employing investigation techniques that may help to

discover also atypical cases, and start effective treatment as soon as possible. In addition to the aforementioned mechanisms, there may be other potential risk factors that render CF patients more susceptible to GORD. Basic pathogenic mechanism of CF is the impairment of a channel for CFTR which is also involved in bicarbonate secretion. The presence and function of CFTR channel in human oesophagus has not yet been clarified. Theoretically however, malfunction of CFTR channels in CF patients could lessen the local defence against the exposure of oesophageal mucosa to harmful agents such as gastric acid, enzymes, and bile acids leading to further epithelial integrity disruption, as it was shown in animal models.

A study which would compare morphological and functional changes of oesophageal epithelium in children with CF has not been published yet. It is not known whether the CFTR channel is also present in the oesophageal glands, and if it is to what extent it influences bicarbonate secretion in the oesophagus. If hypotheses will be confirmed, this would prove the existence of an additional pathogenic mechanism for oesophageal disease in CF patients.

The aim of the present study was to evaluate oesophageal mucosal integrity in children with CF in comparison to a control group regarding both, morphological and functional parameters. The morphology was evaluated with recently proposed histological combined severity score (CSS) and the measurement of intercellular spaces. Epithelial functional integrity was assessed by combined oesophageal multichannel intraluminal impedance-pH monitoring (MII-pH), using baseline impedance measured in Ohms (Ω) as a surrogate marker of the integrity of epithelial barrier. Additionally, we investigated whether the treatment with proton pump inhibitors (PPIs) in CF patients with GORD leads to normalisation of the baseline impedance.

Study hypotheses were:

1. CF patients without GORD have more DIS than children without CF and oesophageal disease.
2. CF patients without GORD have lower baseline oesophageal impedance than a control group of children without CF and oesophageal disease.
3. In CF patients with GORD the baseline impedance will rise after the treatment with PPIs, but will not normalise.

Patients and methods

CF patients aged up to 18 years who underwent upper gastrointestinal endoscopy and MII-pH between 2007 and 2014 were included. The exclusion criteria were oesophageal disease other than GORD (e.g. eosinophilic oesophagitis) and treatment with acid-suppressing drugs. CF patients were initially analysed as a uniform group. Seventeen children with CF (median age 10.4 years, range 4.5 years, 6 males, 11 females) met the criteria for further analyses (group CF_{tot}). Subsequently, they were classified into two groups according to the results of pH-monitoring, the first group with pathologic acid reflux (CF_{gord}; 7 patients) and the second without (CF_{norm}; 10 patients). Pathologic acid reflux was defined as the total acid exposure index of more than 5% of time. A subgroup of five CF patients with co-existent GORD (CF_{gord5}) were treated with PPIs and had another MII-pH after 2 months while still being treated to assess the response to the treatment (CF_{ther5}).

As controls we had to use 2 different groups (one to compare histological examination with DIS measurements, and another one to compare MII-pH measurements) for which we assumed that their results would not be different from normal subjects. As the control for histological changes including DIS (CON_{his}; 12 subjects, median age 6.1 years, range 1.6-16.1 years; 3 males, 9 females) we included the oesophageal biopsy specimens of children who underwent upper gastrointestinal endoscopy for diagnosis of celiac disease during the research period. Celiac disease does not cause changes of oesophageal mucosa. According to the guidelines for gastrointestinal endoscopy in children, this group of children also had at least one biopsy of oesophageal mucosa beside biopsies of duodenum and stomach. Exclusion criteria for this group was the presence of symptoms suspicious for GORD, oesophageal endoscopic changes apart from normal, or signs of oesophageal disease on histological assessment. As the control for MII-pH (CON_{imp}; 16 subjects; median age 8.4 years, range 4.2-14.2 years; 5 males, 11 females) we analysed data of children who underwent MII-pH for chronic cough as a sole disease sign during the study period, but had normal investigation result, so GORD was ruled out as the cause of cough.

Personal data (date of birth, gender, body weight, body height) and medical data (symptoms and signs, indication for investigation) were collected in all subjects. In CF patients, additional data were recorded: forced expiratory volume in the 1st second, CFTR mutation, medications, and GORD symptoms and signs. GORD symptoms and signs (regurgitation,

heartburn, retching, vomiting, dysphagia, odynophagia, cough, hoarseness, recurrent pneumonia, retrosternal pain, epigastric pain, and upper gastrointestinal bleeding) were assessed at the inclusion, and in the group of CF patients with GORD again after 2 months of therapy with PPIs while the patients were still being treated. However, symptoms and signs that could be related directly to the CF rather than GORD, such as cough, hoarseness, and recurrent pneumonia, were omitted from further analyses. Each symptom or sign potentially related to GORD was scored according to its severity from 0 to 3 (0 - absent, 1 - mild, 2 - moderate, 3 - severe), and cumulative symptom score was calculated. The maximal possible score was 27.

Oesophagogastroduodenoscopy was performed in accordance with the guidelines for paediatric gastrointestinal endoscopies in sedated patients with paediatric gastroscopes. Macroscopic changes of oesophageal mucosa were described according to the Los Angeles (LA) classification in which erosive oesophagitis is divided into 4 groups according to the length and extent of oesophageal mucosal breaks. Forceps biopsies of duodenum, antrum, and distal oesophagus approximately 3 cm above the oesophagogastric junction were taken and fixed in 10% formaldehyde, embedded in paraffin, sliced and stained with haematoxylin and eosin. The oesophageal biopsy specimens were analysed for this study according to the Esohisto project protocol which aims to unify oesophageal histology reporting and introduces CSS calculated from: basal cell layer hyperplasia, papillary elongation, DIS, and intraepithelial eosinophils infiltration. The widths of intercellular spaces were measured in the area with the most pronounced changes on light microscopy at 40x magnification and mean value of 10 measurements were used for further analysis.

MII-pH was measured according to the published guidelines. After calibration, the combined antimony catheter was introduced through one of the nostrils and placed into the oesophagus. The position of the catheter was determined under visual endoscopic inspection when upper gastrointestinal endoscopy was performed before the beginning of monitoring so that pH electrode sensor was placed approximately 3 cm above the lower oesophageal sphincter or by fluoroscopy with the pH electrode positioned at the level of the 2nd vertebrae above the diaphragm. After 24 hours the probe was removed and data transferred to the computer. Recordings were analysed. In addition to the routine parameters (total acid exposure index, number of acid refluxes, DeMeester index, total number of acid, weakly-acid, non-acid gastro-oesophageal reflux episodes, and bolus clearance time), the baseline impedance

defined as mean impedance value during the whole period of measurement was calculated for the proximal and distal oesophagus (measured between the most proximal pair and between the most distal pair of oesophageal electrodes).

The study protocol was approved by the Republic of Slovenia National Medical Ethics Committee.

Data are presented using medians and interquartile ranges for continuous variables, and percents for discrete variables. All tests were performed within the whole group and subgroups of CF patients with and without GORD. Means and standard deviation were calculated. Mann-Whitney U test was employed to compare histological and MII-pH parameters between different groups, and Wilcoxon signed rank test to compare MII-pH changes in the group of CF patients with GORD before and after the treatment with PPIs. To compare discrete variables between the groups, χ^2 or Fisher exact test were used according to the sample size. The value $p < 0.05$ was considered statistically significant.

Results

Groups did not differ statistically in the majority of compared parameters: sex, body weight, and height. Only subjects in the group CON_{his} were significantly younger than CF patients in the group CF_{tot}. Another difference was the lower body mass index (BMI) percentile in the group CF_{norm} than in the group CF_{gord}. Lung function in children with CF was good in 57%, 43%, and 71% of children in groups CF_{tot}, CF_{norm}, and CF_{gord}, respectively, and did not differ statistically between the groups. The cumulative symptom score for GORD symptoms and signs in CF patients was higher in the CF_{gord} (7.7 ± 6.8) in comparison to the CF_{norm} (2.8 ± 3.4), approaching the statistical significance ($p = 0.062$). Similarly, the cumulative symptom score in 5 CF patients with GORD after 2 months PPIs treatment lowered (1.0 ± 1.0), although not statistically significantly ($p = 0.125$).

CF patients as a whole (CF_{tot}) had significantly more severe histological changes of oesophageal mucosa when compared with the control group (CON_{his}). The CSS was significantly higher in the group of CF patients (CF_{tot}) (0.75 ± 0.32 vs. 0.27 ± 0.25 ; $p < 0.001$) implying the presence of epithelial impairment. Intercellular spaces were significantly more

dilated in CF_{tot} in comparison to CON_{his} ($2.6 \mu\text{m} \pm 0.6$ vs. $1.9 \mu\text{m} \pm 0.2$; $p = 0.001$). Similar differences were seen with the CSS (0.75 ± 0.20 vs. 0.27 ± 0.25 ; $p = 0.001$) and with DIS ($2.7 \mu\text{m} \pm 0.5$ vs. $1.9 \mu\text{m} \pm 0.2$; $p < 0.001$) between the group CF_{norm} and the control group (CON_{his}).

Comparison of MII-pH parameters between all CF patients (CF_{tot}) and the control group (CON_{imp}) revealed significantly lower baseline impedance value in the CF_{tot} group both between the most proximal ($2876 \Omega \pm 484$ vs. $3703 \Omega \pm 859$; $p = 0.012$) and the most distal pair of electrodes ($2590 \Omega \pm 1013$ vs. $3753 \Omega \pm 1070$; $p = 0.002$). Similarly to morphologic changes described above, CF patients without GORD (CF_{norm}) also had significantly lower baseline impedance between the proximal and the distal pair of electrodes compared to the control group (CON_{imp}) (proximal pair of electrodes baseline impedance in CF_{norm} = $2922 \Omega \pm 363$, in CON_{imp} = $3703 \Omega \pm 859$, $p = 0.027$; distal pair of electrodes baseline impedance in CF_{norm} = $2844 \Omega \pm 457$, in CON_{imp} = $3753 \Omega \pm 1070$, $p = 0.005$). On the same time they did not have any significant differences in total number of acid refluxes ($p = 0.058$), weakly acid refluxes ($p = 0.068$), non-acid refluxes ($p = 0.116$), and total bolus clearance time ($p = 0.958$).

Five of CF patients with GORD (CF_{gord5}) were treated with PPIs and had a second MII-pH monitoring after 2 months (CF_{ther5}). As expected, significant improvement of total acid exposure index (from $9.6\% \pm 5.6$ to $2.2\% \pm 1.5$; $p = 0.043$) and DeMeester score (from 34.0 ± 19.6 to 6.9 ± 4.0 ; $p = 0.043$) was observed. The baseline impedance increased between the proximal pair of electrodes from $2860 \Omega \pm 560$ to $3355 \Omega \pm 750$, $p = 0.068$; and between the distal pair of electrodes from $2178 \Omega \pm 1564$ to $3057 \Omega \pm 594$, $p = 0.225$; but changes did not reach statistical significance. The baseline impedance value in treated CF patients (CF_{ther5}) was lower than in the control group (CON_{imp}) but the differences were not statistically significant. The baseline impedance between proximal pair of electrodes in CF_{ther5} ($3355 \Omega \pm 750$ vs. $3703 \Omega \pm 859$; $p = 0.508$) and the most distal pair of electrodes ($3057 \Omega \pm 594$ vs. $3753 \Omega \pm 1070$; $p = 0.069$) in comparison to CON_{imp} was not statistically significant.

Conclusions

This study showed a previously unpublished observation of important morphological and functional differences in oesophageal mucosal integrity in CF patients in comparison to healthy children even in the absence of GORD.

We proved the first two research hypotheses that CF patients without GORD would have more DIS than children without CF and oesophageal disease, and that CF patients without GORD would have lower baseline oesophageal impedance than a control group of children without CF and oesophageal disease. The third research hypothesis that the baseline impedance in CF patients with GORD after treatment with PPIs will rise, but will not normalise, could not be proven as the differences were not statistically significant, however, the measurements showed a trend which was assumed.

Our study adds a new pathophysiological observation to a complex systemic pathogenesis of CF – dysfunction of oesophageal epithelial integrity even in those CF children without GORD. Hopefully, it would lead to further research revealing exact pathogenetic mechanism and leading to improved treatment options.

Razširjen povzetek

Uvod

Ena ključnih nalog vrhnjice (epitelija) prebavi je, da tvori epiteljsko pregrado, ki preprečuje, da bi snovi iz svetline prehajale v globlje sloje tkiva. Epitelij požiralnika preprečuje vstop molekulam hrane, mikroorganizmom in agresivnim kemičnim snovem (želodčna kislina, encimi in žolč), ki ob epizodah gastroezofagealnega refluksa (angl. *gastro-oesophageal reflux* - GOR) zatekajo v požiralnik. Neokrnjenost epitelija požiralnika zagotavljajo nepoškodovane epiteljske celice in tesni stiki med njimi. Različne bolezni požiralnika, kot je gastroezofagealna refluksna bolezen (angl. *gastro-oesophageal reflux disease* - GORD), porušijo celovitost (integriteto) epitelija požiralnika na mikroskopski ravni, kar se kaže kot oslabljeni tesni stiki med celicami ter vodi v različne patohistološke spremembe in povečan razmik med epiteljskimi celicami, kar imenujemo razširjeni medcelični prostori (angl. *dilated intercellular spaces* - DIS). DIS so opisani tako pri erozivni kot pri neerozivni GORD in tudi pri ostalih vnetnih boleznih požiralnika. Pri kliničnem delu ne moremo neposredno meriti transepiteljske prepustnosti požiralnika. Z večkanalno intraluminalno impedanco (angl. *multichannel intraluminal impedance* - MII) merimo impedanco med pari elektrod na katetru, ki je skozi nosnico vstavljen v svetlino požiralnika. Večino časa je požiralnik prazen, zato povprečna vrednost impedance med dlje časa trajajočimi merjenji odraža impedanco sluznice požiralnika. Imenujemo jo bazalna impedanca in jo lahko uporabljamo kot nadomestni kazalec celovitosti epiteljske pregrade.

Bolniki s cistično fibrozo (CF) so bolj nagnjeni k nastanku GORD predvsem zaradi večjega deleža refluksov med relaksacijami spodnje zapiralke požiralnika. Dodaten vzrok je večja razlika med tlakom v požiralniku in trebuhu, ki nastane zaradi nižjega tlaka v torakalni votlini med vdihom, ki je posledica pljučne bolezni pri CF. Dokazano je, da nezdravljena GORD vodi v hitrejše slabšanje delovanja pljuč, ki je najpomembnejši napovedni dejavnik za slabši potek CF. Z zgodnjo diagnostiko in zdravljenjem GORD preprečujemo zaplete CF. GORD se pri bolnikih s CF zlahka spregleda, saj so ekstraefagealni simptomi in znaki GORD, kot so kronični kašelj, hripavost, ponavljajoče se pljučnice, izguba teka in slabše pridobivanje telesne teže zelo podobni tistim, ki se pojavijo tudi pri sami CF. Zato je pomembno, da pri otrocih s CF aktivno iščemo GORD z uporabo preiskovalnih metod, s katerimi odkrijemo tudi

netipične primere, in čim prej začnemo učinkovito zdravljenje. Poleg omenjenih mehanizmov GOR se pri bolnikih s CF lahko pojavijo dodatni dejavniki, zaradi katerih so bolniki s CF bolj dovzetni za nastanek GORD. Osnovni patogenetski mehanizem CF je okvara kloridnega kanalčka, imenovanega transmembranski regulator prevodnosti pri CF (angl. *cystic fibrosis transmembrane conductance regulator* - CFTR), ki vpliva tudi na izločanje bikarbonata. Pri ljudeh še nista dokazana prisotnost in delovanje kanalčka CFTR v požiralniku. Teoretično pa bi lahko slabše delovanje kanalčka CFTR pri bolnikih s CF zmanjšalo lokalno obrambo sluznice požiralnika pred škodljivimi snovmi, kot so želodčna kislina, encimi in žolčne kisline. To bi lahko vodilo v dodatno okvaro epitelijskega požiralnika, kot je bilo dokazano s poskusi na živalih.

Raziskava, ki bi hkrati primerjala morfološke in funkcionalne spremembe epitelijskega požiralnika pri otrocih s CF, še ni bila objavljena. Ni še jasno, ali je kanalček CFTR prisoten tudi v žlezah sluznice požiralnika pri ljudeh, niti ali vpliva na izločanje bikarbonata v požiralniku. Če bodo raziskovalne hipoteze potrjene, bo to pomenilo, da obstaja še dodaten patogenetski mehanizem bolezni požiralnika pri otrocih s CF.

Namen raziskave je bila morfološka in funkcionalna ocena celovitosti sluznice požiralnika pri otrocih s CF v primerjavi s kontrolno skupino. Morfološke spremembe smo vrednotili z nedavno predlaganim točkovnim sistemom za izračun skupnega rezultata histoloških sprememb. (angl. *combined severity score*). Funkcionalno celovitost požiralnikovega epitelijskega pa smo merili z večkanalno ezofagealno intraluminalno impedanco s pH-metrijo (angl. *combined multichannel intraluminal impedance-pH monitoring* - MII-pH) in uporabili bazalno impedanco merjeno v Ohmih (Ω) kot nadomestni kazalec za merjenje celovitosti epitelijske pregrade. Dodatno smo raziskovali, ali zdravljenje z zaviralci protonske črpalke vpliva na bazalno impedanco požiralnika.

Raziskovalne hipoteze:

1. Bolniki s CF brez GORD imajo bolj razširjene medcelične prostore kot otroci brez CF in brez bolezni požiralnika.
2. Bolniki s CF brez GORD imajo nižjo vrednost bazalne impedance kot kontrolna skupina preiskovancev brez CF in brez bolezni požiralnika.
3. Pri bolnikih s CF in GORD se bo med zdravljenjem z zaviralci protonske črpalke bazalna impedanca zvečala, vendar se ne bo normalizirala.

Bolniki in metode

V raziskavo smo vključili bolnike s CF, stare do 18 let, pri katerih smo med leti 2007 in 2014 opravili gastroskopijo in MII-pH. Izključitveni kriteriji so bile druge bolezni požiralnika (npr. eozinofilni ezofagitis) in zdravljenje z zaviralci protonske črpalke. Najprej smo analizirali vse bolnike s CF. Merila za nadaljnje analiziranje je izpolnjevalo 17 bolnikov (CF_{tot} ; mediana starost 10,4 leta, rang 14,5 leta, 6 moških, 11 žensk). Nato smo jih razdelili v dve skupini glede na izvid pH-metrije: v skupino s patološkim kislim reflukсом (CF_{gord} ; 7 bolnikov) in v skupino brez patološkega kislega refluxa (CF_{norm} ; 10 bolnikov). Patološki kisli refluks smo definirali s skupnim indeksom prisotnosti kisline v požiralniku (angl. *total acid exposure index*), večjim od 5% časa meritve. Podskupino 5 bolnikov s CF (CF_{gord5}) smo zdravili z zaviralci protonske črpalke. Pri njih smo po 2 mesecih, še med zdravljenjem, ponovili snemanje MII-pH, da bi ocenili odgovor na zdravljenje (CF_{ther5}).

Oblikovali smo dve kontrolni skupini (eno za primerjavo histoloških izvidov in meritev DIS ter drugo za primerjavo meritev MII-pH), za kateri smo sklepali, da se rezultati ne bodo razlikovali od skupine zdravih preiskovancev. V kontrolno skupino za primerjavo histoloških sprememb in DIS (CON_{his} ; 12 preiskovancev, mediana starost 6,1 leta, rang 16,1 leta, 3 moški, 9 žensk) smo vključili vzorce sluznice požiralnika otrok, ki so imeli gastroskopijo v sklopu diagnostike celiakije v obdobju, ko je potekala raziskava. Celiakija ne povzroča sprememb sluznice požiralnika. V skladu s smernicami za gastroskopije pri otrocih je bil tudi v tej skupini poleg ostalih biopsijskih vzorcev odvzet vsaj en vzorec sluznice požiralnika. Izključitveni kriteriji za to skupino so bili znaki in simptomi, pri katerih lahko pomislimo na GORD, endoskopske spremembe požiralnika ali znaki bolezni požiralnika, vidni pri histološkem pregledu. V kontrolno skupino za primerjavo MII-pH (CON_{imp} ; 16 preiskovancev, mediana starost 8,4 leta, rang 14,2 leta, 5 moških, 11 žensk) smo vključili podatke in meritve otrok, ki smo jih preiskovali zaradi kroničnega kašlja kot edinega znaka bolezni v obdobju, ko je potekala raziskava, in pri katerih je bila MII-pH normalna, kar je izključilo GORD kot možni vzrok za kašelj.

Za vse preiskovance smo zbrali osebne podatke (rojstni datum, spol, telesna teža, telesna višina) in zdravstvene podatke (simptomi in znaki, indikacije za preiskave). Pri bolnikih s CF smo dodatno zabeležili: forsirani ekspiracijski volumen v prvi sekundi, mutacijo CFTR, zdravila ter simptome in znake GORD. Simptome in znake GORD (regurgitacija, občutek

zgage, spahovanje, bruhanje, disfagija, odinofagija, kašelj, hripavost, ponavljajoče se pljučnice, bolečina za prsnico, bolečina v žlički in krvavitev iz zgornjih prebavil) smo zabeležili ob vključitvi v raziskavo ter v skupini bolnikov s CF in GORD tudi po 2 mesecih, še med zdravljenjem z zaviralci protonske črpalke. Zaradi možnosti, da so nekateri simptomi in znaki posledica CF in ne GORD, smo iz nadaljnje analize izključili naslednje znake bolezni: kašelj, hripavost in ponavljajoče se pljučnice. Vsak simptom ali znak smo točkovali glede na jakost od 0 do 3 (0 - odsoten, 1 - blag, 2 - zmeren, 3 - hud) in izračunali kumulativno vsoto simptomov. Največja možna vsota je bila 27.

Ezofagogastroduodenoskopijo smo izvajali v skladu s smernicami za endoskopije prebavil pri sediranih otrocih. Uporabili smo pediatrične gastroskope. Makroskopske spremembe sluznice požiralnika smo opisovali po Los angeleški (LA) klasifikaciji, ki erozivni ezofagitis razvršča v 4 skupine glede na dolžino in obseg razjed sluznice požiralnika. Med preiskavo smo odvzeli biopsijske vzorce iz dvanajstnika, antruma želodca in spodnjega dela požiralnika približno 3 cm nad ezofagogastričnim spojem. Po odvzemu smo jih fiksirali v 10% formaldehidu, vklopili v parafin, narezali ter barvali s hematoksilinom in eozinom. Za potrebe te raziskave smo jih analizirali v skladu s priporočili projekta Esohisto, katerega cilj je poenotenje histoloških izvidov požiralnika in ki uvaja skupni rezultat histoloških sprememb, ki ga izračunamo iz hiperplazije bazalnega sklada celic, podaljšanja papil lamine proprije, širine medceličnih prostorov in intraepitelijske infiltracije z eozinofilci. Širino medceličnih prostorov smo merili v območju najizrazitejših sprememb s svetlobno mikroskopijo na 40-kratni povečavi. Izračunali smo povprečno vrednost desetih meritev in jo uporabili za nadaljnjo analizo.

MII-pH smo merili v skladu z objavljenimi smernicami. Po umeritvi aparata smo kombiniran antimonijski kateter skozi eno od nosnic vstavili v požiralnik. Kadar smo hkrati opravili tudi ezofagogastroduodenoskopijo, smo ustrezen položaj endoskopsko določili tako, da je bilo pH-metrično tipalo vstavljeno približno 3 cm nad spodnjo požiralnikovo zapiralko. Pri preiskovancih, pri katerih smo opravili samo MII-pH, smo z diaskopijo položaj določili tako, da je bilo pH-metrično tipalo projicirano v področje drugega vretenca nad prepono. Po 24 urah smo odstranili kateter in prenesli podatke iz aparata v računalnik. Posnetke smo analizirali. Poleg rutinskih parametrov, kot so skupni indeks prisotnosti kisline v požiralniku, število kislih refluksov, DeMeestrov index, skupno število kislih, šibko-kislih in nekislih

refluksov ter čas očistka bolusa, smo izmerili še bazalno impedanco med proksimalnim in distalnim parom elektrod v požiralniku.

Raziskavo je odobrila Komisija Republike Slovenije za medicinsko etiko.

Podatki so predstavljeni z medianimi vrednostmi in kvartilnim razponom za zvezne spremenljivke in z odstotki za diskretne spremenljivke. Vse teste smo opravili za celotno skupino in podskupine bolnikov s CF, ki so imeli ali bili brez GORD. Izračunali smo povprečja in standardne deviacije. Histološke in MII-pH parametre smo primerjali s testom Mann-Whitney U, spremembe MII-pH v skupini bolnikov s CF in GORD pred in med zdravljenjem z zaviralci protonске črpalke pa z Wilcoxonovim testom predznačenih rangov. Za primerjavo diskretnih spremenljivk med skupinami smo glede na velikost pričakovanih frekvenc uporabili test χ^2 ali Fisherjev natančni test. Vrednost $p < 0,05$ smo upoštevali kot statistično značilno.

Rezultati

Skupine bolnikov s CF in kontrolna skupina se niso statistično razlikovali v večini parametrov: po spolu, telesni teži in višini. Le preiskovanci v skupini CON_{his} so bili statistično značilno mlajši od bolnikov s CF (CF_{tot}). Druga razlika je bil nižji percentil indeksa telesne mase v skupini CF_{norm} v primerjavi s skupino CF_{gord}. Pljučna funkcija pri otrocih s CF je bila dobra pri 57%, 43%, in 71% otrok v skupinah CF_{tot}, CF_{norm}, in CF_{gord} v tem zaporedju in se statistično ni razlikovala med skupinami. Kumulativna ocena vsote simptomov in znakov GORD pri bolnikih s CF je bila najvišja v skupini CF_{gord} ($7,7 \pm 6,8$) v primerjavi s skupino CF_{norm} ($2,8 \pm 3,4$); rezultat je bil blizu statistične značilnosti ($p = 0,062$), ki smo jo definirali s $p = 0,05$. Podobno je bila kumulativna ocena v skupini 5 bolnikov s CF in GORD po 2 mesecih zdravljenja s PPIs znižana ($z\ 12,2 \pm 9,1$ na $1,0 \pm 1,0$), čeprav razlika ni bila statistično značilna ($p = 0,125$).

Bolniki s CF so imeli kot celota (CF_{tot}) statistično značilno izrazitejša histološke spremembe sluznice požiralnika v primerjavi s kontrolno skupino (CON_{his}). Skupni rezultat histoloških sprememb je bil značilno večji, kar je kazalo na verjetno okvaro epitelija v skupini bolnikov s CF (CF_{tot}) ($0,75 \pm 0,32$ vs. $0,27 \pm 0,25$; $p < 0,001$). Medcelični prostori so bili značilno bolj

razširjeni v skupini CF_{tot} v primerjavi s CON_{his} ($2,6 \mu m \pm 0,6$ vs. $1,9 \mu m \pm 0,2$; $p = 0,001$). Podobne razlike so bile vidne tudi pri skupnem rezultatu histoloških sprememb ($0,75 \pm 0,20$ vs. $0,27 \pm 0,25$; $p = 0,001$) in pri DIS ($2,7 \mu m \pm 0,5$ vs. $1,9 \mu m \pm 0,2$; $p < 0,001$) med skupino CF_{norm} in kontrolno skupino (CON_{his}).

Primerjava med parametri MII-pH med vsemi bolniki s CF (CF_{tot}) in kontrolno skupino (CON_{imp}) je pokazala statistično značilno nižjo vrednost bazalne impedance v skupini CF_{tot} tako med proksimalnima ($2876 \Omega \pm 484$ vs. $3703 \Omega \pm 859$; $p = 0,012$) kot tudi med distalnima elektrodama ($2590 \Omega \pm 1013$ vs. $3753 \Omega \pm 1070$; $p = 0,002$). Podobno kot pri zgoraj opisanih morfoloških spremembah so imeli tudi bolniki s CF brez GORD (CF_{norm}) značilno nižje vrednosti bazalne impedance med proksimalnim in distalnim parom elektrod v primerjavi s kontrolno skupino (CON_{imp}) (bazalna impedance med proksimalnim parom elektrod $CF_{norm} = 2922 \Omega \pm 363$, $CON_{imp} = 3703 \Omega \pm 859$, $p = 0,027$; bazalna impedance med distalnim parom elektrod $CF_{norm} = 2844 \Omega \pm 457$, $CON_{imp} = 3753 \Omega \pm 1070$, $p = 0,005$). Hkrati pa se preiskovanci med tema skupinama niso statistično značilno razlikovali po skupnem številu kislih ($p = 0,058$), šibko-kislih ($p = 0,068$) in nekislih refluksov ($p = 0,116$) ter v celotnem času očistka bolusa ($p = 0,958$).

Petim bolnikom s CF, ki so imeli hkrati tudi GORD (CF_{gord5}) in smo jih zdravili z zaviralci protonske črpalke, smo po 2 mesecih ponovno merili MII-pH (CF_{ther5}). Kot je bilo pričakovano, je bila velika razlika v skupnem indeksu prisotnosti kisline v požiralniku ($9,6\% \pm 5,6$ vs. $2,2\% \pm 1,5$; $p = 0,043$) in točkovanju po DeMeestru ($34,0 \pm 19,6$ vs. $6,9 \pm 4,0$; $p = 0,043$). Bazalna impedance se je med proksimalnim parom elektrod povečala z $2860 \Omega \pm 560$ na $3355 \Omega \pm 750$, $p = 0,068$, med distalnim parom elektrod pa z $2178 \Omega \pm 1564$ na $3057 \Omega \pm 594$, $p = 0,225$, vendar spremembe niso bile statistično značilne. Bazalna impedance pri zdravljenih bolnikih s CF (CF_{ther5}) je bila nižja kot v kontrolni skupini (CON_{imp}), vendar razlike niso bile statistično značilne. Vrednost bazalne impedance med proksimalnim parom elektrod v skupini CF_{ther5} ($3355 \Omega \pm 750$ vs. $3703 \Omega \pm 859$; $p = 0,508$) in distalnim parom elektrod ($3057 \Omega \pm 594$ vs. $3753 \Omega \pm 1070$; $p = 0,069$) v primerjavi s skupino CON_{imp} ni bila statistično značilna.

Zaključki

Raziskava je pokazala pomembne morfološke in funkcionalne spremembe celovitosti sluznice požiralnika pri bolnikih s CF brez GORD v primerjavi z zdravimi preiskovanci, kar do sedaj še ni bilo opisano.

Potrdili smo prvi dve raziskovalni hipotezi, da bodo imeli bolniki s CF in brez GORD bolj razširjene medcelične prostore kot preiskovanci brez CF in brez GORD in da bodo imeli bolniki s CF in brez GORD nižjo vrednost bazalne impedance kot kontrolna skupina preiskovancev brez CF in brez GORD. Tretje raziskovalne hipoteze, da se bo bazalna impedance po zdravljenju z zaviralci protonske črpalke zvečala, a ne normalizirala, nismo potrdili, saj razlike niso bile statistično značilne, čeprav so se meritve približevale pričakovanim vrednostim.

Z našo raziskavo smo ugotovili novo patofiziološko spremembo v že tako kompleksni sistemski patogenezi CF – disfunkcijo epitela požiralnika tudi pri tistih otrocih s CF, ki niso imeli GORD. Upamo, da bo to vodilo v raziskave, s katerimi bi dokazali točen patogenetski mehanizem, kar bo vodilo v izboljšane možnosti zdravljenja.

Introduction

Epithelial integrity and its role in causing or perpetuating disease is an interesting field of research. Epithelia developed into different types according to their function, which is, finally, dependent on its major components, cells and junctions between them. Cells exchange substances with its surroundings with passive diffusion or actively through channels located on the cell membrane. Dysfunction of any epithelial component can lead to disease (1).

Fascinating new field of research and different treatment possibilities pose channelopathies, diseases that are caused by dysfunction of channels mostly located in the cell membranes (2). One of them is CF which is caused by different levels of a chloride channel dysfunction (3). Lately, small molecule drugs which improve functioning of different types of channels are booming, and some of them are already being used in clinical practice for the treatment of CF (4, 5).

Facing new approaches to the treatment of channelopathies, we aimed to explore functioning of oesophageal epithelium with diagnostic means that are available to clinicians for routine diagnosis, upgraded by a few recently introduced, for now experimental parameters in patients with CF and healthy subjects.

Functional anatomy and physiology

Oesophagus is a muscular tube that connects mouth and stomach and serves to transport food. Under normal circumstances, the transport lasts up to 10 seconds for solid food. At rest, oesophagus is relaxed and empty. It is closed off at both ends by the upper and the lower oesophageal sphincter. Relaxations and contractions of both sphincters are precisely regulated to avoid misdirection of the food into the respiratory tract. Oesophagus is located in the low-pressure thorax cavity so sphincters have an important task to prevent backflow of gastric contents into the oesophagus, except during releasing the swallowed air from the stomach (belching) or during vomiting when they relax co-ordinately (6).

The length of oesophagus is 8-10 cm at birth, 19 cm at the age of 15 years, and up to 25 cm in adults. Its wall consists of 2 muscle layers, the inner circular and outer longitudinal. The muscles are striated in the upper third, striated and smooth in the middle third, and only smooth in the distal third. Striated muscles are innervated by somatic nerves originating in the central swallowing centre. Muscle activity is coordinated with other functions, such as respiration and speech. Oesophageal smooth muscles are innervated by autonomic nervous system by the vagal nerve. Oesophagus has also an enteric neurons supply which coordinates local reflexes that supplement central control of swallowing and peristalsis (6).

The primary peristalsis, which starts with swallowing, can be accompanied by secondary peristalsis that is restricted to the smooth muscle portion and is important in clearing a bolus that was not completely expelled during the primary wave, or in clearing the oesophagus of refluxed gastric contents. Secondary peristalsis can be triggered by distension or by acid sensing chemoreceptors. Presence of fluid, especially acid, increases the pressure of the upper oesophageal sphincter, intensity of which is stronger if the upper parts of oesophagus are stimulated. On the other hand, air induces the upper oesophageal sphincter relaxation (6, 7).

Oesophagus is protected from GOR by three lines of defence (6, 8, 9):

- Antireflux barrier at the oesophagogastric junction which consists of the lower oesophageal sphincter, crura of diaphragm, and the angle of His (the angle between the cardia at the entrance to the stomach and the oesophagus)
- Oesophageal clearance performed by peristalsis
- Tissue resistance (mucosal defence) which is further divided into:
 - Preepithelial (protective factors from saliva and oesophageal secretions containing bicarbonate, mucin, prostaglandin E2, and others)
 - Epithelial (tight junctions, intercellular glycoprotein material)
 - Postepithelial factors.

Under resting conditions, the lower oesophageal sphincter is contracted independently from the neural input. Additionally, the contraction is increased by a neurohumoral mechanism involving acetylcholine and gastrin particularly after protein containing meals (6, 7).

The most important pathophysiologic mechanism causing GORD is a phenomenon of transient lower oesophageal sphincter relaxations, a neuronal reflex triggered by distension of

the proximal stomach with efferent and afferent pathways travelling to the brainstem in vagal nerve. Intramural inhibitory neuron becomes activated and releases nitric oxide to relax the lower oesophageal sphincter. The main difference between healthy persons and those with GORD is not a higher number of transient lower oesophageal sphincter relaxations by itself, but the proportion of relaxations accompanied with gastro-oesophageal refluxes (8). Factors involved in the development of GOR are presented in Fig. 1.

Histology

Oesophagus wall is made up of four main layers (from the luminal side): the mucosa, submucosa, muscularis propria, and adventitia. The *mucosa* layer consists of (10):

- A *nonkeratinized stratified squamous epithelium* with an underlying thin extracellular layer of glycoproteins and other components called *basal lamina* which acts as a semipermeable filter
- An underlying *lamina propria* (loose connective tissue rich in blood vessels, lymphatics)
- A *muscularis mucosae* layer of smooth muscles.

The submucosa contains connective tissue with larger blood and lymph vessels. It also contains submucosal (*Meissner*) nerve plexus of autonomic nerves, and small mucus secreting oesophageal glands which are joined by oesophageal cardiac glands at the lower part of oesophagus near stomach. The **muscularis propria** of oesophagus has two layers: the inner circular and the outer longitudinal layer. In the connective tissue between both layers are blood and lymph vessels, and myenteric (*Auerbach*) nerve plexus of autonomic neurons. The outer layer of oesophagus is composed of connective tissue that merges with the surrounding tissues and is named **adventitia** (1, 10).

Epithelial cells adhere to the neighbouring cells and basal lamina with different types of intercellular junctions with different functions (1):

- Tight or occluding junctions (they form a seal between adjacent cells)
- Adherent or anchoring junctions (strong cell adhesion)
- Gap junctions (channels for communication between adjacent cells)

Figure 1. Factors involved in the development of gastro-oesophageal reflux (adapted and modified from (11, 12)).

- Desmosomes (they are bound to intermediate filaments inside the cells and form very strong attachment points for adjacent cells).

Normal histology of the epithelium and histopathological changes in gastro-oesophageal reflux disease

In normal oesophageal epithelium (Fig. 2a) the basal cell layer measures less than 15% of the total epithelial thickness. Papillae measure less than 50% of the total epithelial thickness. Intercellular spaces measure less than one lymphocyte. There are no intraepithelial eosinophils nor neutrophils, and only limited number of mononuclear cells (up to 9 per high power field) (13).

Certain histological findings are more often seen in patients with GORD, but they are not pathognomonic for GORD (13, 14):

- Basal cell layer hyperplasia (Fig. 2b)
- Papillary elongation (Fig. 2c and 2d)
- DIS
- Intraepithelial inflammatory cell infiltration (eosinophils, neutrophils, and mononuclear cells)
- Erosions.

As GORD is a patchy disease, it is better to take more histology samples from different parts of oesophagus (13). In the Table 1, major features are listed together with their quantification.

Dilated intercellular spaces DIS were described for the first time by Hopwood et al. in 1979 who examined oesophageal biopsy samples with electron microscopy (15). Afterwards, the appearance of DIS in isolated rabbit oesophageal mucosa after the exposure to acid solution proved the connection between acid exposure and development of DIS (16). In the same study Tobey et al. also proved increased paracellular permeability to dextran molecules after treating rabbit oesophageal epithelium with acid (16). Farre et al. have shown similar changes after the exposure of rabbit oesophageal mucosa to bile acids in weakly acid and acid conditions (17).

Figure 2. Histological features of the normal oesophageal mucosa (a) and of the oesophageal mucosa in gastro-oesophageal reflux disease: basal cell layer hyperplasia (b), papillary elongation (c) and congestion of capillaries in the papillae (due to the transversal section the whole length of papilla is not shown) with extravasation of erythrocytes (d).

DIS may be assessed by electron or light microscopy (15, 18). Electron microscopy is a more accurate but also a more expensive method and is not routinely used in the diagnosis of oesophageal disease. Several authors have shown that light microscopy of oesophageal biopsy samples after the hematoxylin-eosin staining is more accessible and is a suitable alternative for DIS assessment (18-20).

Table 1. Histologic criteria for the assessment of oesophageal epithelium according to the Esohisto project. Those included in the calculation of the CSS are underlined. The CSS is calculated as the sum of severity scores of listed criteria divided by the number of lesions assessed (four if all criteria were assessed) (adapted from (14)).

Criterion	Definition	Method of assessment (magnification)	Severity score
<u>Basal cell layer hyperplasia</u>	The upper limit of basal cell layer = level below which > 50% of cell nuclei are separated by a distance of < 1 nuclear diameter	Measure basal cell layer in μm and express as a proportion of total epithelial thickness (x10)	0 (absent; < 15%) 1 (15–30%) 2 (> 30%)
<u>Papillary elongation</u>	Papillary length = distance between the upper limit of papillary vessel wall and the base of papilla Base of papilla = lowest adjacent basal membrane or, in broad-based papillae, an ideal line connecting two adjacent basal membranes	Measure papillary length in μm and express as a proportion (%) of total epithelial thickness (x10)	0 (absent; < 50%) 1 (50–75%) 2 (> 75%)
<u>DIS</u>	DIS should be differentiated from artifactual cell separation and from intracellular vacuoles Small intracellular spaces = diameter < 1 lymphocyte Large intracellular spaces = diameter > 1 lymphocyte	Include irregular round dilations and diffuse widening of the intercellular space (x40)	0 (≤ 5 small) 1 (≥ 6 small and ≤ 5 large) 2 (≥ 6 large)
<u>Intraepithelial eosinophils</u>	Only cells clearly identified as eosinophils should be counted. Isolated eosinophilic granules should not be considered.	Count cells in the most affected high-power field (x40)	0 (0 cells in one high-power field) 1 (1–2 cells) 2 (> 2 cells)
Intraepithelial neutrophils	Only cells clearly identified as neutrophils should be counted.	Count cells in the most affected high-power field (x40)	0 (0 cells in one high-power field) 1 (1–2 cells) 2 (> 2 cells)

Table 2. *Con't.*

Criterion	Definition	Method of assessment (magnification)	Severity score
Intraepithelial mononuclear cells	All types of intraepithelial mononuclear cells should be considered. Mononuclear cells within the squamous epithelium should be counted. Mononuclear cells within the fibrovascular core of the papillae should not be counted.	Count cells in the most affected high-power field (x40)	0 (0–9 cells) 1 (10–30 cells) 2 (> 30 cells)
Erosions	Presence of at least one of the following: necrosis, granulation tissue (subepithelial fibrosis and dilated capillaries), or fibrin with neutrophils.	Assess as present or absent (x10)	0 (absent) 1 (present)
Healed erosions	Presence of granulation tissue covered by thinned regenerative epithelium in the absence of necrosis, fibrin, and neutrophils.	Assess as present or absent (x10)	0 (absent) 1 (present)

Acute acid challenge in a group of healthy volunteers resulted in an immediate expansion of the basal layer of oesophageal squamous epithelium and the appearance of DIS (21). In another study with otherwise healthy volunteers, perfusion of distal oesophagus with weakly acid and acid solutions provoked intercellular spaces dilatation also in the proximal non-perfused part (22).

Oesophageal transmucosal electrical potential difference and impedance

The existence of an electrical potential difference across the wall of gastrointestinal tract was first recognised in the 19th century. In the sixties of the 20th century it was suggested that reduced transmucosal potential difference may reflect impaired mucosal integrity (23).

Studies with different approaches to the *in vivo* measurement of transmural electric potential difference soon followed. Geall et al. in 1970 showed that in 11 patients who underwent abdominal operation there was no difference in measurement of electrical potential difference between mucosa and serosa or mucosa and peripheral vein. On the other hand, the measurements of electrical potential differences between mucosa and skin were variable (23).

A comparison of oesophageal transmucosal potential difference in patients with GORD was published by Khamis et al. in 1978. In 19 patients, transmucosal potential difference correlated with mucosal damage, histological changes, and reported symptoms in 9 patients with GORD, but not with macroscopic appearance of oesophageal mucosa on upper gastrointestinal endoscopy. In GORD patients transmucosal potential difference was reduced and polarity reversed (24). A study of Collins et al. proved lower oesophageal transmucosal potential differences in patients with erosive oesophagitis but not in those with reflux symptoms, mild mucosal damage and normal endoscopic appearance (25), which probably resulted in reduced usefulness of this research method and not introducing it in clinical routine.

With the introduction of oesophageal impedance measurement in the nineties of the last century, a renewed interest in the evaluation of mucosal integrity with the means of electrical currents reappeared. At that time the impedance measurement was introduced to assess oesophageal motility and reflux episodes which is still the main use of this investigation, and is in more detail explained below in the section addressing the diagnosis of oesophageal diseases. Further development of this method led to the description of a new parameter named baseline impedance (26-28).

The electrical impedance is expressed in Ω and measures changes in the conductivity to an alternating electrical current by a pair of metallic rings (electrodes) on a catheter. In clinical use catheters with multiple metallic rings are used. The electrical impedance is an equivalent to the resistance to the direct electrical current (26). Measurement of the oesophageal transmucosal potential difference provides useful information about oesophageal mucosal integrity, but it must be taken into consideration that not only tissue integrity but also changes in secretion influence those potential differences (26).

The impedance is inversely proportional to ionic concentrations of luminal content. A bolus with high ionic content, e.g. fluid, will result in lower impedance measurement in contrary to a bolus with low ionic content, e.g. air, which will result in higher impedance measurement ($> 5000 \Omega$). The measurement of impedance when oesophagus is empty are somewhere in between, in the range of few thousand ohms (29). By convention, a liquid reflux is defined as a retrograde drop in impedance of at least 50% of the baseline in at least 2 distal impedance channels. With concurrent measurement of pH, reflux events are classified as acid ($\text{pH} < 4$), weakly acid ($\text{pH} > 4$ and < 7) and non-acid ($\text{pH} > 7$) (Fig. 3 and 4) (28).

Figure 3. Impedance event: liquid reflux: a) non-acid, b) acid.

The term *baseline impedance* was introduced in 2006 (30), and was later defined as an intraluminal oesophageal impedance value when oesophagus is collapsed (between reflux episodes and swallows) and measured impedance level reflexes impedance of the surrounding oesophageal wall (31).

Figure 4. Impedance event: a liquid reflux with preceding air.

Kessing et al. proved that in study groups of adults with and without GORD and with different levels of acid exposure, the baseline impedance was related to the acid exposure. In healthy subjects it was in the range of a few thousand ohms and in GORD patients with pathological acid exposure in the range of hundreds of ohms (31).

Methods of baseline impedance calculation have evolved over time. Kessing et al. (31) calculated the median value of measurements from the lower and the upper impedance channels of a 30-second time interval every 2 hours, and then averaged these values for each channel. Farré et al. (26) calculated baseline impedance values from the 1-hour time interval measured 30 min after the placement of MII-pH catheter at 3, 5, and 7 cm proximally to the lower oesophageal sphincter, excluding swallows and refluxes. Later, the algorithm was developed which filtered out specific impedance values that changed rapidly (swallows and refluxes): all impedance measurement of more than 5000 Ω (gas reflux) were excluded, as well as all rapid changes of impedance according to the special algorithm as described in

more detail by Loots et al. (32). The uncertainty about the best method of the baseline impedance calculation was solved by Ummarino et al. (33) who analysed 10 consecutive multichannel intraluminal impedance recordings in children with non-erosive reflux disease. The baseline impedance was obtained by: including all impedance episodes (reflux, swallows, and gas episodes) or excluding all impedance episodes during the full recording, and also by excluding all impedance episodes and calculating baseline impedance from the data obtained during the first minute every hour, every 2 hours, and every 4 hours. The median baseline impedance values were comparable with all methods of analysis irrespective of impedance episodes. They concluded that in most cases the automatic determination of the median impedance baseline over the total tracing including impedance episodes is appropriate, and that companies should include oesophageal baseline impedance calculation in the software for MII-pH analysis (33).

The connection between baseline impedance and morphological changes in experimental animal setting and in humans was demonstrated by Farré et al. (26). In the first part of the experiment, his group demonstrated that the baseline impedance in a rabbit model *in vivo* correlated with transepithelial resistance assessed *in vitro* and therefore proved the applicability of oesophageal impedance measurement as a surrogate method for transepithelial potential difference measurements. Additionally, they also proved the dilatation of intercellular spaces which was most pronounced when rabbit's oesophageal mucosa was perfused with an acid solution of pH 1. In the second part of the study, five healthy volunteers without upper gastrointestinal symptoms were studied double-blindly by MII-pH during and after the perfusion of oesophagus with different solutions in random sequence: neutral solution, acid solution at pH 2 with added pepsin, and acid solution at pH 1. After oesophageal perfusion with acid solutions lower baseline impedance values were observed. In the third part of the study, patients with oesophagitis and non-erosive reflux disease were compared to healthy subjects. Both patients' groups had lower baseline impedance values than healthy subjects (26). This key study paved the way to many others in this area.

There is also a correlation between GORD symptoms and low baseline impedance. In the study of 20 patients with non-erosive reflux disease and 30 patients with heartburn, Woodland et al. found that patients who were more susceptible to acid had lower baseline impedance and slower recovery after acid challenge, these changes being more pronounced in patients with non-erosive reflux disease (34).

The first study which reported the baseline impedance values in children was published in 2011. Loots et al. (35) measured the baseline impedance before and after the PPI treatment (esomeprazole) in 21 infants with GORD. The baseline impedance rose from 938 Ω (652-1304 Ω) to 1885 Ω (1360-2183 Ω), the difference was statistically significant. A similar study from the same research group in the randomised placebo-controlled setting confirmed the results of the previous one (32).

The baseline impedance as a marker of disrupted mucosal integrity was studied in a retrospective analysis of 285 children and infants who all underwent endoscopy with biopsies and MII-pH. A lower baseline impedance value at the lowest channel was associated with higher grades of oesophagitis. Infants had significantly lower baseline impedance levels than older children (36). An even stronger connection between endoscopic changes due to GORD and baseline impedance was seen in a study of children in which 298 MII-pH tracings and corresponding endoscopies were analysed. Salvatore et al. reported that the factors mostly influencing baseline impedance were: severity of oesophagitis, bolus exposure index, and number of reflux episodes (37).

Baseline impedance is lower in the first 2 years of life, this observation is even more pronounced in very young infants as it was shown in a large study by Salvatore et al. analysing 800 children with symptoms of GORD. There is no clear explanation but different anatomical (e.g. smaller oesophageal diameter) and functional factors (e.g. longer supine and postprandial periods, higher fluid intake) might be involved (38).

Not all studies confirmed the correlation between the low baseline impedance, GORD symptoms, and macroscopic or microscopic oesophagitis. Borrelli et al. investigated oesophageal mucosa in 15 children with non-erosive GORD and in 11 with erosive oesophagitis. They compared baseline impedance at the most distant impedance channel and gastroscopy with biopsies, in which intercellular spaces were measured using transmission electron microscopy. The baseline impedance levels were significantly lower in children with erosive oesophagitis, but there was no correlation with other studied parameters, e.g. with DIS, acid exposure time, and some other MII-pH parameters (39).

Gastro-oesophageal reflux disease

GOR is defined as the involuntary passage of gastric contents into the oesophagus, and is a normal event. GORD in paediatric patients is present when GOR causes troublesome symptoms and/or complications (8, 13, 40). Reflux esophagitis is present in 2-5% of the general population. In children with GOR symptoms, 12.4% had erosive oesophagitis (41, 42).

Pathophysiology

The most important pathophysiological mechanism of GORD is the occurrence of transient lower oesophageal sphincter relaxations, a neural reflex which is triggered mainly by the distension of proximal stomach, and is not followed by a swallow. There are also other important genetic, environmental, anatomical (e.g. hiatal hernia), hormonal, and neurogenic factors predisposing to GORD (13). Harmful factors that damage mucosa can be classified into luminal (e.g. acid, bile acids, pepsin, trypsin, etc.) and endogenous (inflammation of other organs, acute and chronic stress) (43). The GORD develops if harmful factors prevail over protective ones.

Symptoms and signs

Symptoms and signs of GORD are age-dependent and are divided into oesophageal and extra-oesophageal. Children with GORD most frequently present with heartburn, epigastric or retrosternal pain. Regurgitation is not always present. Dysphagia or odynophagia are rarely reported in children. Of extra-intestinal symptoms chronic cough, hoarseness, and sleep disturbances predominate. None of these symptoms is pathognomonic for GORD. Complications in children are Barrett's oesophagus, strictures, and very rarely also adenocarcinoma (13, 40).

Diagnosis

Diagnostic procedures depend on symptom severity, warning signs, and child age. In children older than 8-12 years with typical symptoms and without warning signs, therapeutic trial with PPIs is an option. In children with oesophageal symptoms, upper gastrointestinal endoscopy with histology is in most cases the first investigation. In children with extra-oesophageal symptoms, oesophageal pH-monitoring alone or the combination with impedance (MII-pH) is the investigation to start with. In some cases barium contrast radiography, oesophageal manometry, or other investigations are indicated (8, 13).

Upper gastrointestinal endoscopy

Upper gastrointestinal endoscopy (13, 44) is a routinely performed investigation in children allowing direct mucosa visualisation. The presence of endoscopically normal oesophageal mucosa does not exclude a diagnosis of non-erosive reflux disease or oesophagitis of other aetiologies. Macroscopic lesions seen in GORD patients are:

- Erosions
- Exudate
- Ulcers
- Strictures
- Areas of possible oesophageal metaplasia
- Polyps.

Hiatal hernia, which predisposes to GORD, might also be seen.

An important part of the endoscopy is tissue sampling for histological analysis. According to the current guidelines (13), histology by itself is not enough to diagnose GORD. The main role of oesophageal histology examination is to rule out other oesophageal diseases, such as eosinophilic esophagitis, infection, and others (40). Normal histology and abnormalities seen in GORD are discussed above in the section Histology. Newer developments in histological diagnosis, including the research from this study, may possibly give histology a more important role in diagnosing GORD.

Oesophageal pH-monitoring and combined oesophageal multichannel intraluminal impedance-pH monitoring

Oesophageal pH-monitoring measures pH at the electrode(s) located at the end of a nasally inserted catheter (13). A drop of pH < 4.0 is considered an acid reflux episode. The parameters obtained from the investigation are:

- The total number of reflux episodes
- The number of reflux episodes lasting > 5 minutes
- The duration of the longest reflux episode
- Total acid exposure index (formerly called reflux index: percent of time with pH < 4).

In adults, *DeMeester score* is automatically calculated from 6 parameters by a built-in software:

- Total acid exposure index
- Acid exposure index for supine and prone positions
- Number of acid refluxes
- Number of acid refluxes longer than 5 min
- The duration of the longest reflux.

Score results greater than 14.7 mean pathological GOR in adults. DeMeester score is not validated in paediatric patients but is nevertheless commonly reported in paediatric studies (45-47).

Other investigations

Other investigations are sometimes indicated: oesophageal manometry, radiologic contrast studies, nuclear scintigraphy, and others (8). They are rarely performed and will not be further discussed here.

Differential diagnosis

The differential diagnosis includes anatomical, infectious, and inflammatory disorders (48). An important differential diagnosis of GORD is eosinophilic oesophagitis, which is defined by eosinophilic oesophageal inflammation with more than 15 eosinophils present at histological examination per high power field. To exclude PPI responsive eosinophilia, eosinophilic infiltration has to persist after 2 months of treatment with high dose of PPIs (49). Eosinophilic inflammation has an important impact on epithelial integrity as it was shown by the dilatation of intercellular spaces and their normalisation after an appropriate treatment (50).

Treatment

Proton pump inhibitors (PPIs) are the mainstream of pharmacological treatment of GORD although the evidence of their efficacy in children is less well documented comparing to adults (51, 52). PPIs are superior to histamine-2 receptor antagonists (H2-RAs) (53). It has been shown by Xue et al. that the width of intercellular spaces assessed by transmission electron microscopy is significantly decreased in patients with GORD after the PPI treatment (54). Antacids and H2-RAs are more suitable for on-demand treatment. Prokinetic therapy, not well supported by the evidence, is rarely used, as potential adverse effects outweigh the potential benefit. Alginate and sucralfate showed some benefit but are not widely available (8, 13).

PPIs are generally considered as safe, but some concerns for their long-term use exist, and there are not enough safety data for their use in children (51-53). The most common side effects reported in children are headache, abdominal pain, and diarrhoea (55). Additional concerns are the higher incidence of acute gastroenteritis and pneumonia, and potential risk of reduced absorption of nutrients including vitamin B₁₂ and iron due to reduced gastric acidity. Other possible concerns are their effects on calcium absorption and consequent reduced bone density, and impact on bowel microbiota, especially if the PPI treatment was introduced during the first year of life (56). However, a study of 228 children with CF showed that a subgroup of patients who were treated with PPIs (40%) had fewer declines in lung function.

Authors also did not find higher rates of colonisation with *Pseudomonas aeruginosa* or *Staphylococcus aureus* (57).

An antireflux surgery is indicated in children with chronic pulmonary aspirations of refluxate, dependence on long-term medical therapy, and for prevention of further progress of complications, such as Barrett oesophagus or oesophageal stenosis due to GORD after successful dilatation (8, 13). Rinsma et al. investigated the influence of endoscopic fundoplication (32 pts.) or PPI treatment (15 pts.) on baseline impedance before and 6 months after the treatment compared with the control group of healthy adults (29 subjects), and found significant increase of baseline impedance, however it did not reach the levels of healthy controls (58).

Certain conditions predispose patients to severe, chronic GORD through different mechanisms seen in patients with some neurological diseases, anatomical abnormalities, and also in patients with CF (13). CF and its influence on oesophagus is discussed below.

Cystic fibrosis

Cystic fibrosis (CF) is caused by a mutation in a gene for CFTR protein, the chloride channel that is expressed in many epithelial cells. CF leads to chronic suppurative lung disease, exocrine pancreatic dysfunction, hepatobiliary disease, intestinal disease, and many other clinical manifestations (59). CF is an autosomal recessive genetic disease with more than 1500 mutations identified which are grouped in 5 different classes according to the functional CFTR present. The most frequent mutation worldwide which is present in about two-thirds of European and North American population is F508del (according to the new nomenclature: c.1521_1523delCTT), a class II mutation (defect of protein trafficking and inability to reach the plasma membrane) (3). According to the latest European Cystic Fibrosis Society Registry report published in 2014 with the data from 2010, in Slovenian population 58% of patients are homozygous for F508del, 35% are heterozygous with one F508del mutation, and the rest have other less frequent mutations (60).

CFTR functions mainly as a chloride channel; in addition, it has many other regulatory functions (e.g., inhibition of sodium transport through the epithelial sodium channel) and is

also involved in bicarbonate-chloride exchange. A deficiency in bicarbonate secretion which leads to poor solubility and aggregations of luminal mucins is recognised as an important pathogenic mechanism of CF (3, 61).

Disease development in CF can be described with several hypotheses or most likely by their combination (3). They are mostly focused on airway disease but some mechanisms explain also a possible pathogenic mechanism in disease development in other organs:

- The low volume hypothesis explains increased thickness of mucus with the loss of inhibition of epithelial sodium channels which leads to excess sodium and water resorption. In combination with the loss of chloride efflux it lowers airway surface water content. Thick mucus harbours bacteria, particularly *Pseudomonas aeruginosa*.
- The high salt hypothesis explains the colonisation of bacteria by impaired activity of innate antibiotic molecules (e.g. human β -defensin 1) induced by higher concentrations of sodium and chloride in lung mucus.
- Dysregulation of the host inflammatory response is the basis of hypothesis which is supported by high concentrations of proinflammatory molecules in the absence of infection determined in CF cell cultures and lung lavage fluid from 4 weeks old children.
- Primary predisposition to infection by inability of CFTR to initiate an innate immune response when *Pseudomonas aeruginosa*, *Staphylococcus aureus* or other bacteria bind to the airway epithelium.

Cystic fibrosis and gastro-oesophageal reflux disease

CF patients are prone to GORD. The prevalence of GORD in children with CF is approximately 50% (46, 62). The study of Pauwels et al. (12) showed that at the level of antireflux barrier at the oesophagogastric junction, the most important mechanisms of GOR in CF patients are:

- Higher proportion of transient lower oesophageal sphincter relaxations associated with reflux
- Higher pressure difference between thoracic and abdominal cavity due to greater inspiratory negative intrathoracic pressure.

At the level of tissue resistance (mucosal defence), the neutralisation of acid reflux by bicarbonate from oesophageal submucosal glands which secrete bicarbonate may be impaired. Abdulnaour-Nakhoul et al. (63) for the first time localised CFTR channel in oesophagus in pigs with immunofluorescence, reverse transcriptase polymerase chain reaction, and *in situ* hybridization. Afterwards they measured bicarbonate secretion from oesophageal submucosal glands in isolated perfused pig oesophagus under different conditions: genistein, an activator of CFTR stimulated bicarbonate secretion, glibenclamide, a chloride channel blocker, and a specific CFTR blocker named CFTR_{inh}-172 blocker decreased and inhibited bicarbonate secretion, respectively. CFTR expression has not yet been proven in humans (63). Indirect evidence from the studies of GORD in CF patients (64-66) exists that CFTR expression is probable in human oesophageal submucosal glands, too, and that CFTR dysfunction leads to the epithelial damage (63).

GOR has an important impact on the lung function in patients with CF as it was shown by Van der Doef et al. in a study of 218 children. Five percent of children, who had GORD diagnosed by pH-monitoring or upper gastrointestinal endoscopy, had bigger decline in lung function and higher colonisation rate with *Pseudomonas aeruginosa* or *Staphylococcus aureus* at the age of 10 years (57). Multichannel oesophageal intraluminal impedance with pH-measurement (MII-pH) showed high prevalence of acid and non-acid refluxes in patients with CF, sometimes associated with more cough (46).

Research challenges in cystic fibrosis related oesophageal disease

Despite extensive knowledge of oesophageal structure and function, there are still many unanswered questions, especially in specific diseases such as CF. There is no published evidence of the characteristics of intercellular spaces between oesophageal epithelial cells in CF. Neither has anybody yet published the results of a novel parameter measured by oesophageal impedance - baseline impedance which reflects epithelial integrity. This information may add to the knowledge of the pathophysiology of oesophageal disease in patients with CF and may pave the way for new discoveries, such as the proof of CFTR channels existence in oesophageal mucosa. Besides better understanding of oesophageal disease in CF patients new knowledge might lead to new diagnostic and treatment approaches.

Purposes of the study

The purpose of the study was to measure and compare morphological (assessed by histology) and functional parameters (assessed by MII-pH) of oesophageal mucosa in patients with CF, with or without GORD. Measurements were compared to control groups of healthy children to determine if there are any additional pathophysiological mechanisms intrinsic to CF irrespective of the GORD. In addition, we studied the functional response of patients with CF and GORD to the treatment with PPIs.

Aims of the study

1. Specification of the characteristics of the study group

- 1.1. To compare the genetic background regarding CFTR mutation between the study group and the data from the overall Slovenian CF population.
- 1.2. To compare the lung function in subgroups of CF patients.
- 1.3. To evaluate the cumulative symptom score for GORD symptoms and signs in CF patients and compare it between the study subgroups.
- 1.4. To describe the endoscopic findings in the CF group.

2. Histological evaluation of oesophageal biopsy samples

- 2.1. To compare the histological characteristics including the width of intercellular spaces (DIS) between the CF group (CF_{tot}) and the control groups (CON_{his}).
- 2.2. To compare the histological characteristics including the width of intercellular spaces (DIS) between the subgroup of CF patients without GORD (CF_{norm}) and the control group (CON_{his}).
- 2.3. To compare the histological characteristics including the width of intercellular spaces (DIS) between the subgroups of CF patients with and without GORD (CF_{gord} vs. CF_{norm}).

3. Functional evaluation of oesophageal epithelium with combined oesophageal multichannel intraluminal impedance-pH monitoring

3.1. To compare the overall MII-pH parameters and the baseline impedance between the CF (CF_{tot}) and the control group (CON_{imp}).

3.2. To compare the overall MII-pH parameters and the baseline impedance between the subgroup of CF patients without GORD (CF_{norm}) and the control group (CON_{imp}).

3.3. To compare the overall MII-pH parameters and the baseline impedance between the subgroup of CF patients with (CF_{gord}) and without GORD (CF_{norm}).

4. The influence of proton pump inhibitor treatment in a subgroup of cystic fibrosis patients with gastro-oesophageal reflux disease

4.1. To assess effects of the PPI treatment on the cumulative symptom score for GORD symptoms and signs in a subgroup of CF patients with GORD before and during the PPI treatment.

4.2. To compare the overall MII-pH parameters between the subgroup of CF patients with GORD before and during the PPI treatment (CF_{ther5}).

4.2. To compare the baseline impedance between the subgroup of CF patients with GORD before and during the PPI treatment (CF_{ther5}).

Study hypotheses

1. CF patients without GORD have more DIS than children without CF and oesophageal disease.

2. CF patients without GORD have lower baseline oesophageal impedance than a control group of children without CF and oesophageal disease.

3. In CF patients with GORD the baseline impedance after the treatment with PPIs will rise, but will not normalise.

Subjects and methods

Subjects

Cystic fibrosis patients

From 2007 to 2014, 23 children with CF underwent MII-pH at our institution. Nineteen patients also had upper gastrointestinal endoscopy and were therefore eligible for further analysis. In accordance with the exclusion criteria, which were oesophageal disease other than GORD (e.g. eosinophilic oesophagitis) and treatment with acid-suppressing drugs, 2 patients were excluded from further analysis: one patient had MII-pH monitoring while being treated with PPIs, and another patient had eosinophilic oesophagitis, which is known to have an important additional impact on mucosal integrity (50).

Seventeen children with CF (median age 10.4 years, interquartile range 14.5 years) met the criteria for further analyses (group CF_{tot}). Six were males and 11 females. All patients had pancreatic insufficiency and were receiving supplementation with pancreatic enzymes.

CF patients were initially analysed as a uniform group (CF_{tot}). Subsequently, they were classified into two groups according to the pH-monitoring parameters, the first group with pathologic acid reflux (CF_{gord}; 7 pts., median age 12.0 years, interquartile range 6.2 years, 3 male and 4 female) and the second without (CF_{norm}; 10 pts., median age 10.2 years, interquartile range 10.2 years, 3 male and 7 female). Pathologic acid reflux was defined as the total acid exposure index of more than 5%, and DeMeester score > 14.7 (both parameters are described more in detail below in the Study Protocol and Methods).

CF patients with co-existent GORD were treated with a PPI omeprazole and 5 of them (CF_{ther5}) subsequently had another MII-pH after 2 months while still being treated to assess the response to the treatment; they did not have upper gastrointestinal endoscopy at the same time.

Control groups

For controls we had to use 2 different groups (one to compare histological examination with DIS measurements and another to compare MII-pH measurements) for which we assumed that their results would not be different from normal subjects. As upper gastrointestinal endoscopy and MII-pH are invasive investigations, it would be unethical to investigate healthy children without any symptoms.

As the control group for histological changes including DIS (CON_{his}) we included the oesophageal biopsy specimens of children who underwent upper gastrointestinal endoscopy for diagnosis of celiac disease during the research period. Celiac disease does not cause changes of oesophageal mucosa (67). According to the guidelines for gastrointestinal endoscopy in children (44), this group of children also had at least one biopsy of oesophageal mucosa taken beside biopsies of duodenum and stomach. Exclusion criteria for this group was the presence of symptoms suspicious for GORD, oesophageal endoscopic changes apart from normal, or signs of oesophageal disease on histological assessment. We identified 12 patients who fulfilled criteria described above, and were further analysed as the control group for histological analyses (CON_{his}; median age 6.1 years, interquartile range 16.1 years; 3 boys, 9 girls).

In the control group for MII-pH (CON_{imp}) we analysed the measurements of children who underwent MII-pH for the diagnosis of chronic cough during the study period, but had normal investigation result, so GORD was ruled out as a cause of cough. Sixteen patients fulfilled criteria described above and had normal MII-pH (CON_{imp}; median age 8.4 years, interquartile range 14.2 years; 5 boys, 11 girls).

Study protocol and methods

A search of the databases and extraction of data

Personal data for all studied subjects: date of birth, gender, body weight, and body height were extracted from the hospital information system (Think!Med).

Additional data were acquired:

- In the group of patients with CF (CF_{tot}): degree of lung impairment (forced expiratory volume in 1 s: FEV1, measured only in children older than 6 years), pancreatic (in)sufficiency, CFTR mutation, GORD symptoms and signs, medication.
- In the control groups (CON_{his} and CON_{imp}): indication for upper gastrointestinal endoscopy or MII-pH, check-up for exclusion criteria (signs and symptoms).

GORD symptoms and signs (regurgitation, heartburn, retching, vomiting, dysphagia, odynophagia, cough, hoarseness, recurrent pneumonias, retrosternal pain, epigastric pain, and upper gastrointestinal bleeding) were assessed at the inclusion and in the group of CF patients with GORD again after 2 months of therapy with PPIs while the patients were still treated. However, patients with symptoms and signs that could be related directly to the CF rather than GORD, such as cough, hoarseness, and recurrent pneumonia, were omitted from further analyses. Each symptom or sign potentially related to GORD was scored according to its severity from 0 to 3 (0 - absent, 1 - mild, 2 - moderate, 3 - severe) and *cumulative symptom score* was calculated. The maximal possible score was 27.

Upper gastrointestinal endoscopy

Upper gastrointestinal endoscopy was performed in accordance with the guidelines for paediatric gastrointestinal endoscopies (44) in sedated patients (68) with paediatric endoscopes (Olympus and Pentax, Tokyo, Japan). Macroscopic changes of oesophageal mucosa were described according to the LA classification in which erosive oesophagitis is divided into 4 groups (LA-A: mucosal break(s) shorter than 5 mm that do(es) not extend between the tops of two mucosal folds; LA-B: the same as in LA-A, only mucosal break(s) is (are) longer than 5 mm; LA-C: mucosal break(s) of less than 75% of the oesophageal circumference, and in LA-D mucosal break(s) of more than 75% of the oesophageal circumference) (69). Forceps biopsies of duodenum, antrum, and distal oesophagus approximately 3 cm above the oesophagogastric junction were taken.

Histological examination and analysis of oesophageal mucosal biopsies in the group of cystic fibrosis patients and in the control group

The biopsy samples of oesophageal mucosa of CF patients and those obtained during upper gastrointestinal endoscopy in the control group (CON_{his}) were assessed. The biopsy samples were obtained for routine diagnostics described above, fixed in 10% formaldehyde, embedded in paraffin, sliced and stained with haematoxylin and eosin. The oesophageal biopsy specimens were analysed according to the Esohisto project protocol (14) which aims to unify oesophageal histology reporting: basal cell layer hyperplasia, papillary elongation, DIS, intraepithelial eosinophils, neutrophils and mononuclear cells, erosions, and healed erosions. Esohisto project also introduces the CSS calculated from 4 of the above histological characteristics: basal cell layer hyperplasia, papillary elongation, DIS, and intraepithelial eosinophils. The evaluation and calculation of the CSS is described in the Table 1. CSS of 0-0.25 represents normal mucosa, 0.5-0.75 mild oesophagitis, and more than 1 severe oesophagitis (14).

The morphometrical analysis was performed with NIS Element Microscope Imaging Software for Microscopy (Nikon, Tokyo, Japan) software. The widths of intercellular spaces were measured in the area with the most pronounced changes on light microscopy at 40x magnification and 10 largest values were used for further analysis (Figure 5).

Figure 5. Examples of normal oesophageal mucosa (a), mildly (b) and severely dilated intercellular spaces (c). Haematoxylin and eosin.

Analysis of combined oesophageal multichannel intraluminal impedance-pH monitoring in the group of cystic fibrosis patients and the control group

MII-pH was performed by Ohmega measurement system (MMS, Enschede, the Netherlands) according to the published guidelines (13, 28). After the calibration the combined antimony catheter (VersaFlex Z, Given Imaging, Israel) was introduced through one of the nostrils and placed into the oesophagus. The position of the catheter was determined under visual endoscopic inspection when upper gastrointestinal endoscopy was performed before the beginning of monitoring so that pH electrode sensor was placed approximately 3 cm above the lower oesophageal sphincter or by fluoroscopy with the pH electrode positioned at the 2nd vertebrae above the diaphragm. After 24 hours the probe was removed and data transferred to the computer. Recordings were analysed with MMS software (version 9.1). In addition to the routinely analyzed and calculated parameters listed and described below, the baseline impedance defined as the mean impedance value during the whole period of measurement (70) was calculated for the proximal and distal oesophagus (measured between the most proximal pair and between the most distal pair of oesophageal electrodes) (Figure 2). Analysed data were:

- For the pH-measurement (71):
 - Total acid exposure index (formerly called reflux index: percent of time with pH < 4)
 - DeMeester score (calculated automatically from 6 parameters: total acid exposure index, acid exposure index for supine and prone position, number of acid refluxes, number of acid refluxes longer than 5 min, the duration of the longest reflux). Generally, if the score is more than 14.7 this proves the presence of pathological GOR in adults.

DeMeester score is not validated in paediatric patients but is commonly reported in paediatric studies (45-47), therefore we added it to make the study more comparable to the similar studies in children.

- For the MII-pH (28):
 - Number of acid refluxes
 - Number of weakly-acid refluxes
 - Number of non-acid refluxes

- Number of liquid refluxes
- Number of mixed liquid-gas refluxes
- **Baseline impedance value between the upper and the lower electrodes.**

Figure 6. Schematic drawing of a inserted MII-pH catheter.

Statistical analyses

Data were analysed using the IBM SPSS Statistics 19 software (SPSS Inc, USA). Data are presented using medians and interquartile ranges for continuous variables, and percents for discrete variables. All tests were performed within the whole group and the subgroup of CF patients without GORD. Means and standard deviation were calculated. Mann-Whitney U test was employed to compare histological and MII-pH parameters between different groups, and Wilcoxon signed rank test to compare MII-pH changes in the group of CF patients with GORD before and after the treatment with PPIs. To compare discrete variables between the

groups, χ^2 or Fisher exact test were used depending on the sample size. The value $p < 0.05$ was considered statistically significant.

Ethical committee approval

The study protocol was approved by Republic of Slovenia National Medical Ethics Committee (No. 35/02/15).

Results

Seventeen children with CF (CF_{tot}) fulfilled the criteria for further analysis as already presented above in the Methods section. Seven of them (CF_{gord}) were diagnosed with GORD according to the MII-pH criteria (total acid exposure index > 5% and DeMeester score > 14.7) and 10 had no pathological GOR (CF_{norm}). Descriptive and some clinical data of these groups are presented in the Table 2.

The two control groups consisted of 12 patients with normal upper gastrointestinal endoscopy (CON_{his}) and 16 patients with normal MII-pH (CON_{imp}). Their descriptive data are presented in the Table 3.

Table 3. Characteristics of all studied CF patients (CF_{tot}), and subgroups without (CF_{norm}) and with (CF_{gord}) GORD.

Group	CF_{tot}	CF_{norm}	CF_{gord}
Number	17	10	7
Male : female	6:11	3:7	3:4
Median age [25 th ; 75 th percentile]	10.7 [8.4; 14.5]	10.2 [3.5; 13.7]	12.0 [8.6; 14.8]
Median BMI percentile [25 th ; 75 th percentile]	31.0 [14.5; 75.5]	23.5 [6.8; 42.0]	74 [31.0; 94.0]
Number and the proportion of patients homozygous/heterozygous for F508del mutation	12 / 4 71% / 24%	8 / 1* 80% / 10%	4 / 3 57% / 43%
Lung function – median FEV1% [25 th ; 75 th percentile] ‡	78.2 [58.6; 92.2]	63.9 [54.6 ; 85.9]	91.6 [60.0; 95.0]
The proportion of patients with good lung function (FEV1>70%) [%]	57%	43%	71%

Abbreviations: CF – cystic fibrosis, SD – standard deviation, FEV1% - the proportion of forced expiratory volume in 1s as the percentage of age adjusted normal subjects.

Note: * one patient did not have any F508del mutation, ‡ measured only in CF patients older than 6 years.

Table 4. Characteristics of the two control groups for histological (CON_{his}) and functional evaluation (CON_{imp}).

Group	CON _{his}	CON _{imp}
Number	12	16
Male : female	3:9	5:11
Median age [25 th ; 75 th percentile]	6.2 [2.2; 9.8]	8.5 [4.9; 11.5]
Median BMI percentile [25 th ; 75 th percentile]	25.5 [7.7; 38.2]	60.5 [5.0; 89.2]

Abbreviation: BMI – body mass index.

Characteristics of the study groups

The groups did not differ statistically in the majority of compared parameters: sex, body weight, and height. Only the subjects in the group CON_{his} were significantly younger than CF patients in the group CF_{tot} ($p = 0.03$). Another difference was lower BMI percentile in the group CF_{norm} than in the group CF_{gord} ($p = 0.04$).

CFTR mutations

Patients with CF were homozygous for the most common mutation in our population F508del in 71% and heterozygous in 24%, while one patient did not have any F508del mutation (Table 2). The prevalence of F508del mutation in studied groups was higher than in the overall Slovenian CF population (60). Although the prevalence of F508del mutation was higher in the CF_{norm} than in CF_{gord} group, the difference was not statistically significant.

Lung function in subgroups of patients with cystic fibrosis

Good lung function was defined as FEV1 of more than 70% for age matched healthy children (72). In most of the patients it cannot be measured before the age of 6 years as children must actively participate during the measurement and rapidly exhale in a peak flow meter. In study groups CF_{tot}, CF_{norm}, and CF_{gord}, the lung function was good in 57%, 43%, and 71% of children respectively, and did not differ statistically.

The cumulative symptom score for gastro-oesophageal reflux disease symptoms and signs in patients with cystic fibrosis

The cumulative symptom score for CF patients was modified and cough, hoarseness, and recurrent pneumonia were excluded from further analysis, as these symptoms and signs are very often part of CF itself and cannot serve as a marker for GORD in this specific population. The following symptoms and signs were scored and analysed: regurgitation, heartburn, retching, vomiting, dysphagia, odynophagia, retrosternal pain, epigastric pain, and upper gastrointestinal bleeding, as described in more detail in the section *Study Protocol and Methods*.

As expected, the cumulative symptom score for GORD symptoms and signs in CF patients was higher in the CF_{gord} (7.7 ± 6.8) in comparison to the CF_{norm} (2.8 ± 3.4), approaching the statistical significance ($p = 0.062$).

Endoscopic findings in patients with cystic fibrosis

In accordance with the Los Angeles classification which divides erosive oesophagitis into 4 groups (69) (LA-A being the mildest and LA-D the most severe; in details described in the *Upper gastrointestinal endoscopy* section of *Study Protocol and Methods*), we did not find oesophageal mucosal erosions in any of the patients from the CF_{norm} group. In the CF_{gord} group, mild oesophagitis was seen in 4 out of 7 patients (LA-A in 3 (43%) and LA-B in 1 (14%)).

Histological evaluation of oesophageal biopsy samples

A summary of analysed data for histological analysis is presented in the Table 4.

Table 5. Aggregated data of the main CF group (CF_{tot}), and CF subgroups according to the presence (CF_{gord}) or absence of GORD (CF_{norm}), and the control group (CON_{his}) for morphological analysis.

	CF_{tot}	CF_{norm}	CF_{gord}	CON_{his}
Basal cell layer hyperplasia [%]	27.0 ± 11.7	24.7 ± 8.5	30.1 ± 15.4	17.7 ± 8.3
Papillary elongation [%]	72.3 ± 18.5	76.4 ± 18.7	66.4 ± 18.0	42.8 ± 18.3
Intraepithelial eosinophils [No. per high power field]	1.2 ± 4.1	0.1 ± 0.32	2.7 ± 6.3	0.08 ± 0.29
Intraepithelial mononuclear cells [No. per high power field]	8.9 ± 15.9	6.6 ± 11.8	12.3 ± 21.1	4.0 ± 2.4
CSS	0.75 ± 0.32	0.75 ± 0.20	0.75 ± 0.46	0.27 ± 0.25
DIS [average of 10 measurements, $\mu m \pm SD$]	2.6 ± 0.6	2.7 ± 0.5	2.34 ± 0.64	1.9 ± 0.2

Abbreviations: No. – number, SD – standard deviation, CSS – combined severity score.

Comparison of histological characteristics between the whole cystic fibrosis group and the control group

CF patients as a whole (CF_{tot}) had significantly more severe histological changes of oesophageal mucosa when compared with the control group (CON_{his}). The CSS according to the Esohisto protocol (14) revealed significantly higher score and therefore the presence of epithelial impairment in the group of CF patients (CF_{tot}) (0.75 ± 0.32 vs. 0.27 ± 0.25 ; $p < 0.001$).

Similar changes were seen when comparing intercellular spaces, which were significantly more dilated in CF_{tot} in comparison to CON_{his} ($2.6 \mu m \pm 0.6$ vs. $1.9 \mu m \pm 0.2$; $p = 0.001$).

Comparison of histological characteristics between the subgroup of patients with cystic fibrosis without gastro-oesophageal reflux disease and the control group

Changes described in the paragraph above persisted also when the subgroup of CF without GORD was compared to the control group. CSS was significantly higher in the CF_{norm} group

(0.75 ± 0.20 vs. 0.27 ± 0.25 ; $p = 0.001$) and a similar statistically significant change was observed also when measuring DIS ($2.7 \mu\text{m} \pm 0.5$ vs. $1.9 \mu\text{m} \pm 0.2$; $p < 0.001$) (Fig. 7).

Figure 7. Comparison of mean dilatation of intercellular spaces (DIS) and CSS of all patients with CF (CF_{tot}), those without acid reflux (CF_{norm}) and the control group of healthy children (CON_{his}).

Comparison of histological characteristics between the subgroup of patients with cystic fibrosis with and without gastro-oesophageal reflux disease

Unexpectedly, neither DIS nor CSS differed significantly between the groups CF_{gord} and CF_{norm} .

Evaluation of oesophageal epithelium with combined oesophageal multichannel intraluminal impedance-pH monitoring

A summary of analysed data of MII-pH is presented in the Table 5.

Table 6. Aggregated data of the whole CF group (CF_{tot}), the CF subgroups according to the presence (CF_{gord}) or absence of GORD (CF_{norm}), and the control group (CON_{imp}) for functional analysis.

	CF_{tot}	CF_{norm}	CF_{gord}	CON_{imp}
Total acid exposure index [%]	5.4 ± 4.6	2.6 ± 1.4	9.3 ± 4.8	1.9 ± 1.0
DeMeester score	18.4 ± 16.2	8.3 ± 3.1	32.7 ± 16.8	6.9 ± 3.2
Acid refluxes [No.]	22.0 ± 14.8	13.2 ± 8.2	34.5 ± 13.1	7.3 ± 4.3
Weakly-acid refluxes [No.]	7.8 ± 5.7	7.9 ± 6.9	7.6 ± 5.2	4.5 ± 4.9
Non-acid refluxes [No.]	10.8 ± 40.3	17.4 ± 52.5	1.4 ± 3.7	0.8 ± 2.7
Liquid refluxes [No.]	15.7 ± 18.8	13.8 ± 23.3	18.4 ± 10.9	3.7 ± 3.5
Mixed liquid gas refluxes [No.]	24.6 ± 25.5	24.7 ± 32.2	24.4 ± 13.5	8.9 ± 9.6
Baseline impedance value between the upper electrodes [Ω]	2876 ± 484	2922 ± 363	2810 ± 646	3703 ± 859
Baseline impedance value between the lower electrodes [Ω]	2590 ± 1013	2844 ± 457	2226 ± 1470	3753 ± 1070

Abbreviations: No. - number, Ω - Ohm.

Comparison of combined oesophageal multichannel intraluminal impedance-pH monitoring parameters between the whole cystic fibrosis group and the control group

Comparison of MII-pH parameters between all CF patients (CF_{tot}) and the control group (CON_{imp}) revealed significantly lower baseline impedance value in the CF_{tot} group both between the most proximal ($2876 \Omega \pm 484$ vs. $3703 \Omega \pm 859$; $p = 0.012$) and the most distal pair of electrodes ($2590 \Omega \pm 1013$ vs. $3753 \Omega \pm 1070$; $p = 0.002$).

Comparison of combined oesophageal multichannel intraluminal impedance-pH monitoring parameters between the subgroup of cystic fibrosis patients without gastro-oesophageal reflux disease and the control group

Similar to already described morphologic changes, even CF patients without GORD (CF_{norm}) had significantly lower baseline impedance between the proximal and the distal pair of electrodes compared to the control group (CON_{imp}) (proximal pair of electrodes baseline impedance $CF_{norm} = 2922 \Omega \pm 363$, $CON_{imp} = 3703 \Omega \pm 859$, $p = 0.027$; distal pair of electrodes baseline impedance $CF_{norm} = 2844 \Omega \pm 457$, $CON_{imp} = 3753 \Omega \pm 1070$, $p = 0.005$). At the same time there were no significant differences in the total number of acid refluxes ($p = 0.058$), weakly acid refluxes ($p = 0.068$), non-acid refluxes ($p = 0.116$), and total bolus clearance time ($p = 0.958$) (Fig. 8).

Comparison of combined oesophageal multichannel intraluminal impedance-pH monitoring parameters between the subgroup of cystic fibrosis patients with and without gastro-oesophageal reflux disease

The changes of pH-monitoring parameters between the subgroups of CF patients without (CF_{norm}) and with GORD (CF_{gord}) were expected as the groups were defined by these parameters (total acid exposure index in CF_{norm} was: 2.61 ± 1.40 , and in CF_{gord} 9.31 ± 4.81 ; $p = 0.001$); DeMeester score in CF_{norm} was 8.2 ± 3.1 , and in CF_{gord} 32.7 ± 16.8 ; $p = 0.001$). The number of acid refluxes measured with the impedance differed significantly as well (CF_{norm} vs. CF_{gord} : 13.2 ± 8.1 vs. 34.5 ± 13.1 , $p = 0.002$). On the other hand, the baseline impedance between the upper pair of electrodes did not differ at all (CF_{norm} vs. CF_{gord} : 2922 ± 363 vs. 2810 ± 646 , $p = 1.0$). The difference between the lower pair of electrodes was present, but not statistically significant (CF_{norm} vs. CF_{gord} : 2845 ± 457 vs. 2225 ± 1470 , $p = 0.118$).

The baseline impedance between the proximal and distal pair of electrodes in different CF groups (CF_{tot} , CF_{norm} , CF_{gord}) in comparison to the control group (CON_{imp}) is shown in Fig. 8.

Figure 8. Comparison of baseline impedance between the proximal and distal pair of electrodes in the following groups: all studied CF patients (CF_{tot}), CF patients without acid reflux (CF_{norm}), CF patients with acid reflux (CF_{gord}), and the control group (CON_{imp}).

Influence of proton pump inhibitor treatment in a subgroup of cystic fibrosis patients with gastro-oesophageal reflux disease

CF patients with GORD were treated with PPIs, and 5 underwent another MII-pH monitoring (without upper gastrointestinal endoscopy) scheduled after at least 2 months while still being treated with PPIs. On average, the second MII-pH was performed after 12.2 ± 6.8 weeks. For this analysis only those CF patients with GORD (CF_{gord5}) who had another MII-pH while treated with PPIs (CF_{ther5}) were further analysed. The results are shown in Table 6.

Table 7. Aggregated data of the CF subgroup of 5 patients with GORD (CF_{gord5}), who had a repeated MII-pH before and while they were treated with PPIs (CF_{ther5}), and the control group CON_{imp} .

	CF_{gord5}	CF_{ther5}	CON_{imp}
Total acid exposure index	9.6 ± 5.6	2.2 ± 1.5	1.9 ± 1.0
DeMeester score	34.0 ± 19.6	6.9 ± 4.0	6.9 ± 3.2
Acid refluxes [No.]	29.6 ± 8.6	6.0 ± 9.6	7.3 ± 4.3
Weakly-acid refluxes [No.]	5.2 ± 3.9	29.0 ± 15.6	4.5 ± 4.9
Non-acid refluxes [No.]	2.0 ± 4.4	1.1 ± 2.4	0.8 ± 2.7
Liquid refluxes [No.]	14.5 ± 6.3	6.3 ± 1.1	3.7 ± 3.5
Mixed liquid gas refluxes [No.]	22.3 ± 15.9	29.8 ± 23.3	8.9 ± 9.6
Baseline impedance value between the upper electrodes [Ω]	2860 ± 560	3355 ± 750	3703 ± 859
Baseline impedance value between the lower electrodes [Ω]	2178 ± 1564	3057 ± 594	3753 ± 1070

Abbreviation: No. – number, Ω - Ohm.

Effects of the proton pump inhibitor treatment on the cumulative symptom score

The cumulative symptom score in 5 CF patients with GORD after 2-months PPI treatment lowered (from 12.2 ± 9.1 to 1.0 ± 1.0), although the change was not statistically significant ($p = 0.125$).

Comparison of combined oesophageal multichannel intraluminal impedance-pH monitoring parameters between the subgroup of cystic fibrosis patients with gastro-oesophageal reflux disease and the same group after the proton pump inhibitors treatment

As expected, significant improvement of total acid exposure index (from $9.6\% \pm 5.6$ to $2.2\% \pm 1.5$; $p = 0.043$) and DeMeester score (from 34.0 ± 19.6 to 6.9 ± 4.0 ; $p = 0.043$) was observed. All results in this paragraph are reported for the CF_{gord5} and CF_{ther5} group, respectively. As for other MII parameters, the number of acid (29.5 ± 8.6 vs. 6.04 ± 9.6 ; $p = 0.043$) and liquid refluxes (14.5 ± 6.2 vs. 6.3 ± 1.15 ; $p = 0.043$) significantly improved.

The difference between non-acid refluxes in both groups was insignificant (1.98 ± 4.4 vs. 1.1 ± 2.4 ; $p = 0.655$). Expectedly, the number of weakly-acid refluxes (5.2 ± 3.9 vs. 29.0 ± 15.6 ; $p = 0.043$) significantly increased.

The baseline impedance increased, between the proximal pair of electrodes from $2860 \Omega \pm 560$ to $3355 \Omega \pm 750$, $p = 0.068$; and between the distal pair of electrodes from $2178 \Omega \pm 1564$ to $3057 \Omega \pm 594$, $p = 0.225$; but changes did not reach statistical significance (Fig. 8).

Figure 9. Comparison of baseline impedance between the proximal and distal pair of electrodes in the subgroup of CF patients with GORD (CF_{gord5}) who were treated with PPIs and had another MII-pH approximately 2 months later while still being treated (CF_{ther5}), in comparison to the control group (CON_{imp}).

Moreover, the baseline impedance values in treated CF patients (CF_{ther5}) were still lower than the ones in the control group (CON_{imp}), but the differences were not statistically significant. The baseline impedance between the proximal pair of electrodes in CF_{ther5} was $3355 \Omega \pm 750$

and in CON_{imp} $3703 \Omega \pm 859$ ($p = 0.508$). Corresponding values for the distal pair of electrodes were $3057 \Omega \pm 594$ in CF_{ther5} and $3753 \Omega \pm 1070$ in CON_{imp} ($p = 0.069$) (Fig. 9).

Discussion

To the best of our knowledge, this study is the first to assess oesophageal mucosal integrity in children with CF simultaneously with morphological (histological) assessment, including measurements of intercellular spaces, and a functional (MII-pH) method, including measurement of baseline impedance.

Diminished epithelial integrity with DIS and increased epithelial permeability can be observed in many different oesophageal diseases, such as GORD, eosinophilic oesophagitis, etc. (50, 73). In this study we found significant differences in histological parameters compound severity score and DIS between CF patients and the control group. In a similar way the baseline impedance value between the upper and lower pair of electrodes was significantly lower in CF patients in comparison to the control group. It is not surprising that CF patients with simultaneous GORD, either with erosive oesophagitis or non-erosive GORD defined by pathological pH-monitoring results, had significant changes in both areas of evaluation. However, significant differences were found also in children with CF who did not have GORD. Moreover, although basal impedance values improved in CF children with GORD with treatment with PPIs, they did not reach normal values of healthy controls.

Morphological assessment by histology

Present study confirms the first study hypothesis that CF by itself causes histopathology changes of oesophageal epithelium as assessed by the Esohisto protocol criteria (14) and also by the measurement of intercellular spaces when they are analysed separately.

We found significantly more DIS and higher CSS in all CF patients, including those without GORD, when compared with healthy controls. Unexpectedly, intercellular spaces in the subgroup of CF patients with GORD (CF_{gord}) and without it (CF_{norm}) did not differ importantly. The cause for this might be technical, as the measurement of DIS with light microscopy is not as precise as it is with electron microscopy (18). Other possible explanation is that the pathogenic mechanism of CF has a higher impact on the epithelial integrity than GOR, at least that of medium to low level. Patients in the group of CF with GORD (CF_{gord})

clearly fulfilled the criteria of pathological GOR but the pH-monitoring parameters were moderately and not extremely pathological. Furthermore, endoscopically only approximately half of them had classifiable erosive esophagitis, however, even these of low to moderate grade.

Improved evaluation and reproducibility of histopathology in GORD as it is attempted with Esohisto protocol might renew the importance of histological diagnosis in GORD, not only as a means of exclusion of other oesophageal diseases, but also as a better quantitative assessment tool for GORD. In the study of Schneider et al. performed in 1071 adults who underwent gastroscopy for different reasons, 27.6% had severe, 39.5% had mild, and 32.9% had no oesophagitis according to Esohisto protocol. Microscopic oesophagitis was highly significantly associated with patients' symptoms, endoscopic diagnosis of oesophagitis, and a history of PPIs intake. Histologic diagnosis had a higher sensitivity than the upper gastrointestinal endoscopy for GORD (74). In the present study, Esohisto protocol (14) has been found suitable for the quantitative histological assessment of oesophageal epithelium. However, a comparison with conventionally used histologic criteria introduced by the ESPGHAN Working Group on Gastro-oesophageal Reflux Disease in 1994 (75-77) has not been performed.

Functional assessment with combined oesophageal multichannel intraluminal impedance-pH monitoring

Our study confirmed that baseline impedance value between the upper and lower electrodes were lower even in the CF patient group without GORD when comparing the measurements to the control group. After the treatment with PPIs in a subgroup of CF patients with GORD, the baseline impedance values increased, but did not reach those in the control group.

It is well established that patients with CF have an increased risk for GORD because of increased number of transient lower oesophageal sphincter relaxations associated with reflux events, and bigger difference between the pressure in thoracic and abdominal cavity due to a greater negative inspiratory intrathoracic pressure (12). Even in the absence of pathologic acid exposure, other constituents of the refluxate can damage oesophageal epithelium. For

example, experiments in animal models revealed that proteolytic enzymes and bile acids from duodenal juice when refluxed into the oesophagus could result in mucosal injury (43, 78). In our previous studies we found that children with isolated pathologic duodenogastro-oesophageal reflux developed reflux oesophagitis, although of milder grade than the ones with combined pathologic acid and bile exposure (79, 80). However, when MII-pH-monitoring results of CF children without acid GORD were compared with those of their healthy controls, we could not find any significant difference in the number of reflux episodes, not only acid but also weakly acid and non-acid, and neither in the bolus clearance time. Observed changes of the oesophageal baseline impedance values in CF children can therefore not be attributed to the reflux. We can hypothesise that they are presumably the consequence of CF itself.

The absence of significant changes in MII-pH and histopathological parameters of CF patients with and without GORD could be the consequence of small samples size, but could also mean that in our study cohort the influence of acid reflux was less important than epithelial disruption caused by CF.

Among 9 published studies of oesophageal disease assessment with MII-pH in patients with CF, in none of them the baseline impedance was determined. Five were performed in children with CF (46, 47, 66, 81, 82). The common denominator of all studies is a high prevalence of GORD in children with CF. Blondeau et al. proved that GOR was a primary phenomenon and not just a consequence of CF itself, as was the cough a consequence of GOR in 72%. Reflux followed cough in only 27% (66). Another study in 20 young children with CF (aged 8-34 months) did not find more GOR during physiotherapy with the head-down position (82).

As reported by other authors, treatment of GORD patients with PPIs significantly increased the baseline impedance values (32, 58). In a randomised double-blind study by Loots et al. performed in 40 infants, the impedance values rose significantly in those treated with PPIs, but not in those who received placebo (32). The same effect was confirmed in a randomised study in adult population which assessed baseline impedance values in 15 patients with GORD before and after 6 months of PPI treatment, and in 32 patients with GORD before and after the anti-reflux operation. The baseline impedance values rose significantly with both treatments, but were not correlated with the symptom (heartburn) resolution (58).

Application of baseline impedance value introduced by this study adds additional information to oesophageal epithelial characteristics. However, before the introduction of the baseline impedance concept in the routine analysis in patients with CF or other diseases, more data on normal values and larger studies are needed. For the time being, the clinical relevance of the baseline impedance value could be for the follow-up of patients comparing measurements before and after specific treatments.

Possible explanations for oesophageal epithelial dysfunction in children with cystic fibrosis

One presumption is that a normal CFTR channel function is important for oesophageal epithelial integrity maintenance. It has been shown that beside chloride and sodium, the secretion of bicarbonate is dependent on the CFTR channel as well (63, 83). Impaired CFTR dependent bicarbonate secretion by oesophageal submucosal glands may be a plausible mechanism for an increased epithelial vulnerability in CF patients.

Possible evidence for this hypothesis is provided in a study of Woodley et al., who compared MII-pH in 16 children with CF and 16 children with GORD symptoms. Acid GOR events were recorded with the same frequency in both groups, however, acid GORs were longer, acid GORD index, and DeMeester score were higher in CF group. Chemical clearance which follows the volume clearance was significantly slower in CF patients than in the control group (47). Prolonged chemical reflux points toward less efficacious local mucosal defence (pre-epithelial tissue resistance) which is in accordance with our study hypothesis and indicates a possible mechanism of lower secretion of bicarbonate by oesophageal submucosal glands.

Our results were in accordance with the results of the earlier studies that showed increased intestinal permeability in CF patients by studying intestinal absorption of sugar molecules with different molecular weights, and their secretion in urine (84-86). Although permeation of both, small and large molecular weight sugars were found to be increased in CF patients, when compared to healthy individuals, this was especially pronounced for large molecules, such as lactulose, which pass through the epithelial barrier via a paracellular route (87).

Different explanations for the increased intestinal permeability have been proposed, such as:

- A defect in the tight junctions (86)
- Abnormal mucus secretion (88)
- Acid-related damage of intestinal epithelium due to the lack of acid neutralisation by bicarbonate secondary to pancreatic insufficiency (89).

Hallberg et al. found that increased intestinal permeability was related to the patient's genotype; those patients who were homozygous or heterozygous for F508del had a significantly increased permeability compared with patients with unidentified genotypes who had permeability values within the normal range (84). The increase in intestinal permeability was partially but not completely corrected with PPI treatment (90).

The genetic background regarding CFTR mutation of our patients was homogenous, the majority being homozygous for F508del mutation, one quarter being heterozygous and only 1 patient having no F508del mutation. This was not enough to perform the analysis of a baseline impedance values in relation to the CFTR mutation.

Alternatively, disruption of the normal oesophageal epithelium structure and barrier function does not necessarily indicate that the injurious agents act from the luminal side. In eosinophilic oesophagitis, for example, epithelial changes including DIS are the consequence of tide of inflammatory cytokines originating from allergic reactions to environmental and food antigens (91). Moreover, it has been shown that in GORD mucosal infiltration with inflammatory cells and cytokine secretion precede the development of epithelial changes and not vice versa as could be mechanistically expected (92). One of the characteristics of CF is a recurrent nature of respiratory tract infections resulting in a systemic chronic inflammatory state. It has been shown that cells of the mucosal immune system (T_H2 lymphocytes) may circulate between different mucosal tissues such as respiratory, and gastrointestinal tracts (91, 93). Another site of inflammation in CF is the gut. Even in the absence of gastrointestinal symptoms, there is evidence of chronic intestinal inflammation and abnormal balance of microbiota (94). Oesophageal epithelial changes may therefore be a consequence of minor intrinsic inflammatory events that echo a much more pronounced inflammation in distant parts of the body, such as respiratory tract.

Regardless of the mechanisms of their emergence, the observed epithelial structural and functional changes in CF patients result in a decrease of effectiveness of epithelial barrier

function, enabling easier penetration of luminal contents, such as acid, enzymes, bile salts, and food antigens into deeper layers, that may possibly contribute to the deterioration or even development of oesophageal pathology, such as GORD and eosinophilic oesophagitis. In addition to the aforementioned risk factors this may render CF patients even more prone to the development of severe oesophageal GORD lesions.

In our study, only approximately half of CF patients with GORD had a low grade erosive oesophagitis, another half had non-erosive GORD. This is in contrast to the more intense and refractory GORD known in CF patients. A possible explanation is that we studied a paediatric population and that the same patients might develop higher grades of GORD with time. This was shown by Sheikh et al. who reports 75% of patients having GORD and 12% underwent surgical treatment (Nissen fundoplication) in a cohort of 600 children and adult CF patients (95). GORD is an even bigger problem after lung transplantation (96, 97).

Impact of the study results on cystic fibrosis management

Data on the importance of the diagnosis and treatment of GORD in CF patients exist, and should be routinely implemented in all CF patients with worsening of lung function disproportionately to the decline provoked by lung infection or natural course of the disease, lung disease poorly responsive to the treatment, after lung transplantation, and with even minor symptoms of GOR (46, 47, 58, 62, 97). Probably, the best strategy would be periodical screening of all CF patients for GORD with oesophagogastroduodenoscopy and MII-pH implementing all studied parameters. This approach has to be studied in prospective trials.

The best treatment depends on the clinical picture of GORD. In patients with erosive esophagitis aggressive treatment with PPIs is the most effective (53). In patients with lung aspiration of refluxate and in those with intractable GORD surgical treatment with fundoplication is at present the best choice (58, 95, 98).

The role of a new class of CFTR modifiers has not been evaluated in oesophageal disease in CF patients yet. Two medications are available at present, a potentiator ivacaftor for the treatment of patients with the specific mutation G551D (4), and a combination of activator lumacaftor and aforementioned ivacaftor for the treatment of patients homozygous for

F508del mutation, with varying results of treatment in different organs (5). There are no reports on their effectiveness in GORD in CF patients, but the reports showing (mild) overall improvement, including an increase in body weight are encouraging, specially for ivacaftor in G551D mutation (4). The only report proving the concept of better bicarbonate secretion is a study of 11 patients with CF and G551D mutation (of 151 patients included) which, among other parameters reported significantly improvement in early ability to neutralize gastric acid and better clearance after gastric emptying after 1 month treatment with ivacaftor (99).

Our study adds a new pathophysiological observation to a complex systemic pathogenesis of CF – dysfunction of oesophageal epithelial integrity even in those CF children without GORD. Hopefully, it would lead to further research revealing exact pathogenetic mechanism and leading to improved treatment options.

Contribution to the science

This is an original multidisciplinary study of oesophageal morphology and function in a group of children with CF. For the first time histological assessment of intercellular spaces and impedance measurement of a new modality baseline impedance were assessed simultaneously. Results confirmed our hypotheses that oesophageal epithelium in children with CF without GORD has different properties and shows morphological and functional signs of dysfunction in comparison to healthy subjects. This study may lead to further more detailed morphological research looking for the presence of the CFTR protein in human oesophagus and therapeutic studies researching the influence of new and upcoming medications enabling and improving CFTR function.

Study limitations

There are several limitations to our study. The most important one is related to the low number of included patients. CF is a rare disease and in addition to the standard medical care which is very intense, it is quite difficult to recruit patients for invasive and time consuming investigations. An important limitation is also a relatively small size of biopsy samples of the

oesophageal mucosa and the assessment of DIS with light microscopy. Therefore, in contrast to the established and reproducible Esohisto protocol (74) the measurements of DIS in this study must be interpreted with caution. In addition, basal impedance values in patients with CF may be influenced by factors other than increased epithelial permeability. They may reflect changes of the homeostatic milieu as transepithelial potentials created by epithelial ion fluxes at the mucosal surface may be altered by ion transport defects through CFTR channel in CF patients. This pathophysiological phenomenon which influences impedance is well described in the nasal and rectal mucosa, and can be used to study the function of CFTR channel (100). As oesophageal stratified squamous epithelium significantly differs from other epithelia of gastrointestinal tract due to mostly protective and less active secretory and absorptive function, we speculate that in the oesophagus of CF patients the effect of potential difference and therefore impedance might be less pronounced than the influence of disrupted epithelial barrier. The study from Orlando et al. who measured the potential difference of oesophageal and rectal mucosa in CF patients and healthy persons did not find any differences in oesophagus as compared to rectum, thus supporting this view (101).

Study conclusions

1. Characteristics of the study group

1.1. The prevalence of F508del mutation in studied groups was higher than in the overall Slovenian CF population, but the difference was not statistically significant.

1.2. The lung function in subgroups of CF patients did not differ statistically.

1.3. Cumulative symptom score for GORD symptoms and signs in CF patients was higher in the CF_{gord} in comparison to the CF_{norm} group, approaching the statistical significance.

1.4. The patients from CF_{norm} group did not have any oesophageal erosions, in the CF_{gord} group erosions were seen in 4 patients: 3 having oesophagitis grade LA-A, and 1 LA-B, both grades signifying mild oesophagitis.

2. Histological evaluation

2.1. The whole group of children with CF (CF_{tot}) had significantly higher CSS. Intercellular spaces were significantly more dilated in CF_{tot} in comparison to the control group (CON_{his}).

2.2. The subgroup of CF patients without GORD (CF_{norm}) had significantly higher CSS score and more DIS than the control group (CON_{his}).

2.3. The histological characteristics including the width of intercellular spaces (DIS) between the subgroups of CF patients with and without GORD (CF_{gord} vs. CF_{norm}) were not significantly different.

3. Evaluation with combined oesophageal multichannel intraluminal impedance-pH monitoring

3.1. Baseline impedance values in the CF_{tot} group both between the most proximal and the most distal pair of electrodes were significantly lower than in the control group CON_{imp}.

3.2. Comparison of baseline impedance values between the subgroup of CF patients without GORD (CF_{norm}) and the control group (CON_{imp}) also revealed significantly lower levels in the CF_{norm} group between the upper and the lower pair of electrodes.

3.3. There was no difference between the upper pair of electrodes between the groups CF_{norm} and CF_{gord}. A difference between the lower pair of electrodes was present, but not statistically significant.

4. Influence of proton pump inhibitor treatment in a subgroup of cystic fibrosis patients with gastro-oesophageal reflux disease

4.1. The cumulative symptom score for GORD symptoms and signs in a subgroup of CF patients with GORD lowered during the treatment with PPIs, but the difference was not statistically significant.

4.2. Significant improvement of total acid exposure index, DeMeester, and the majority of MII-pH parameters was observed.

4.2. The baseline impedance values between the upper and the lower pair of electrodes rose after the PPI treatment but did not reach the control group values. However, the differences in impedance values between CF patients during treatment with PPIs and controls were not statistically significant.

Answers to research hypotheses

1. CF patients without GORD had significantly more DIS than children without CF and oesophageal disease.
2. CF patients without GORD had significantly lower baseline oesophageal impedance than the control group of children without CF and oesophageal disease.
3. In CF patients with GORD the baseline impedance after the treatment with PPIs rose and, expectedly did not normalise, but the differences were not statistically significant.

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