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Salivary Calprotectin is Not a Useful Biomarker to Monitor Disease Activity in Patients with Inflammatory Bowel Disease

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ABSTRACT

Background & Aims: Non-invasive biomarkers are gaining interest for monitoring disease activity in patients with inflammatory bowel diseases (IBD). Fecal calprotectin is a reliable biomarker but patients often report the collection of feces being unpleasant and cumbersome. In this study, we aimed to assess if salivary calprotectin could be used as a non-invasive biomarker to determine disease activity instead of fecal calprotectin.
Methods: In this cross-sectional explorative cohort study, stimulated saliva was collected from patients with an established IBD diagnosis and healthy controls. The concentration of calprotectin in saliva was determined by a particle-enhanced turbidimetric immunoassay. Intestinal disease activity was assessed with fecal calprotectin levels and the Harvey-Bradshaw Index (HBI) or Simple Clinical Colitis Activity Index (SCCAI). Missing data were handled using multiple imputation.
Results: Sixty-three patients (41 Crohn's disease and 22 ulcerative colitis) and 11 controls were included. Patients had a mean fecal calprotectin of 138.78 μg/g and a median salivary calprotectin of 1.87 mg/L. No significant correlation was found between salivary calprotectin and fecal calprotectin levels (p=0.495). When patients were stratified in two subgroups based on a fecal calprotectin cut-off value of 250 μg/g, there were no significant differences in salivary calprotectin levels between both patient groups (p=0.641) and between patients and healthy controls (p=0.248). Also, salivary, and fecal calprotectin levels were not significantly different when stratifying patients in two subgroups, active disease and remission, using HBI/SCCAI scores.
Conclusions: Salivary calprotectin does not correlate to fecal calprotectin and disease activity scores in patients, making it unreliable for assessing IBD activity.

Key words: inflammatory bowel disease – calprotectin – saliva – biomarker – ulcerative colitis – Crohn's disease – disease activity.

Abbreviations: CD: Crohn's disease; CP: calprotectin; CRP: C-reactive protein; HBI: Harvey-Bradshaw index; IBD: inflammatory bowel disease; MI: multiple imputation; SCCAI: Simple Clinical Colitis Activity Index; UC: ulcerative colitis.

INTRODUCTION

Treatment of inflammatory bowel disease (IBD) is complex, and monitoring of disease activity is needed to assess its efficacy of reaching disease remission [1-4]. Endoscopy with histologic analysis of collected specimens is considered the gold standard to assess (mucosal) disease activity. However, endoscopy is not without risk, is expensive and uncomfortable for patients. Therefore, a relatively simple, accurate, and easily available test reflecting intestinal disease activity would be beneficial for patients and physicians [5, 6]. Fecal calprotectin (CP) is a biomarker that has been proven to be simple, reliable, and cost-effective in assessing IBD activity [1, 2, 5, 7]. It can be useful in the diagnostic work-up of patients with suspected IBD, but it is also a prognostic marker for upcoming disease activity in patients in remission [8]. Moreover, elevated fecal CP levels are highly correlated with exacerbations of disease [1, 2, 9-11].

Calprotectin is an acute-phase protein and plays a role in the migration, adhesion, and phagocytosis of neutrophils during inflammation. It is a calcium- and zinc-binding protein belonging to the S-100 protein family and accounts for 60% of cytosolic proteins in neutrophils, and, to a lesser extent, of cytosolic proteins in monocytes and macrophages. Besides in

feces, CP can also be detected in plasma, urine, cerebrospinal fluid, saliva, or synovial fluid [9]. Despite the reliability of fecal CP as biomarker in IBD, there have been studies which report barriers to fecal sample collection, including embarrassment and concerns around hygiene [6, 12, 13]. Moreover, problems with transportation of the stool sample impacts the acceptability of fecal CP as a monitoring tool [6]. Saliva on the other hand, is an easily accessible oral biofluid which has potential in the non-invasive diagnostic of several systemic disorders [14]. Since saliva can be easily collected in a non-invasive way at the outpatient clinic and usually, does not cause patient discomfort, measuring CP in saliva could be ideal. So far, only one small study has demonstrated that salivary CP levels are elevated in patients with active IBD, suggesting that intestinal inflammation may be reflected in the oral cavity [15]. By comparing fecal CP with salivary CP, we aimed to explore if salivary CP could be used as a reliable non-invasive biomarker in IBD.

METHODS

Patient Population

A cross-sectional explorative cohort study was performed with consecutive IBD patients visiting the Gastroenterology and Hepatology outpatient clinic in Amsterdam UMC, location VU University Medical Center. All patients who met the inclusion criteria (i.e. age ≥ 18 years and a diagnosis of IBD according to established criteria), and were due for fecal CP monitoring according to their routine clinical practice were asked to participate from October 2019 up to December 2019. Patients who indicated that they had xerostomia, current periodontal disease, and/or other oral health problems were excluded. In addition, a group of controls, without known IBD, were recruited at the same outpatient clinic. Controls had to be ≥18 years old and were excluded if they had a known diagnosis or complaints of systemic or gastro-intestinal inflammation, periodontal problems, or xerostomia.

Collection of Saliva, Stool, and Plasma Samples

Saliva samples were collected from all subjects. Patients and controls were instructed to refrain from smoking, eating and drinking 30 minutes prior to collecting saliva. Subsequently they were asked to chew on a cotton roll from a Salivette kit (Sarstedt AG & Co, Nümbrecht, Germany) for 90 seconds. Samples were immediately stored at 2-8°C and within 6 hours the saliva was extracted from the Salivette cotton roll by centrifugation for 10 minutes at 3500g. The extracted saliva samples were stored in a freezer until analysis. Previous research had shown no significant difference in fecal CP levels between long-term storage at -20°C or -80 C [16]. Since CP is assumed to be stable at both temperatures, we decided to use -20 °C storage, to enhance the generalizability to daily clinical practice. Patients provided stool samples for fecal CP analysis according to routine clinical practice. In addition, when blood samples were taken as a routine, residual heparin plasma samples were stored within 24 hours at -20 °C until plasma CP analysis. Saliva and plasma samples were stored at -20 °C for a period of 1 to 4 weeks until analysis. Saliva, stool, and plasma samples from each patient had to be collected within a period of maximun two weeks.

Calprotectin Analysis

Calprotectin concentrations in heparin plasma and saliva samples were measured using a particle-enhanced turbidimetric immunoassay (Gentian Diagnostics AS, Moss, Norway) on a Cobas c501 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Measurements were conducted according to the manufacturer's instructions. Salivary CP concentrations in healthy controls were measured immediately after collection and after storage of a sample at -20 °C to investigate the potential effect of a freeze-thaw cycle. Fecal CP concentrations were determined using the EliA Calprotectin 2 assay (Phadia GmbH, Freiburg, Germany), a fluorescence enzyme immunoassay, on the Phadia 250 (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

IBD Activity

Clinical remission was determined during routine clinical practice and defined as a Harvey-Bradshaw index (HBI) score or a Simple Clinical Colitis Activity Index (SCCAI) score <5 [17-19]. Biochemical remission was defined as a fecal CP level <250 μg/g [5, 9, 20, 21].

Statistical Analysis

Data were presented using means with standard deviation for normally distributed data, means with the variable's range for imputed data, median with interquartile range (IQR) for non-normally distributed data and numbers with percentages for categorical data. Normality was tested with a Shapiro-Wilk test. Categorical variables were analyzed using a chi-square test or Fisher exact test. Continuous variables were analyzed using an Independent-Samples T Test for normally distributed variables and a Mann-Whitney U test for non-normally distributed variables. Pearson correlations were used to assess the correlation between normally distributed variables. Spearman's rank correlation coefficients were used to assess the correlation between non-normally distributed variables. The relationship of salivary CP, fecal CP, and plasma CP with HBI and SCCAI scores was determined with a Kendall's tau-b correlation test. A paired sample t-test was used to determine freeze-thaw differences in saliva samples from healthy controls. The correlation between salivary CP levels and the number of storage days was tested with a Spearman's rank correlation test.

Missing C-reactive protein (CRP), fecal CP and plasma CP values were imputed by multiple imputation (MI) and the imputation model consisted of 23 variables [22]. Predictive mean matching was used, with 100 iterations and 20 imputations. Because MI assumes imputed variables follow a normal distribution, CRP, fecal CP and plasma CP were first log transformed before being imputed [23]. All analyses were performed using the imputed datasets. After analysis of the imputed dataset, a complete-case analysis for comparison was performed as sensitivity analyses [24].

All analyses were performed with IBM SPSS 26.0 (SPSS Inc, Chicago, IL) and p values ≤ 0.05 were considered statistically significant.

Ethical Statement

Ethical approval was obtained by the Medical Ethics Review Committee (METC) of the VU University Medical Center (file

number 2019.544) and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

RESULTS

Patients' Characteristics

Demographics and clinical characteristics of the sixty-three included patients with IBD and 11 healthy controls are presented in Table I. In total 63 saliva samples, 54 stool samples, and 41 plasma samples were collected from IBD patients. The differences in sample distribution between patients with CD and patients with UC are presented in Table II. Data of the 9 missing fecal CP, 22 missing plasma CP and 15 missing CRP samples were generated using multiple imputation. All analyses were performed using the imputed datasets. From the control group (7 female and 4 male participants), only saliva samples were collected (Supplementary file).

IBD Activity

Patients with IBD had a mean fecal CP of 127 µg/g (range 9 - 4929) and a mean CRP of 2.36 mg/L (range 0.40 - 13.00). In total, 16 patients had an active CD and 7 patients active UC based on fecal CP cutoff value of 250 µg/g (Table I). Additional disease activity characteristics of all patients and controls are described in Table I. Disease activity scores did not correlate significantly with salivary CP or plasma CP levels (Table III), although there was a significant correlation ($p<0.001$) between fecal CP and SCCAI scores. When patients were stratified in two subgroups, active disease and remission, using HBI/SCCAI scores there were no statistically significant differences in salivary CP, fecal CP, plasma CP levels (Table IV).

Table I. Characteristics of patients and controls

		IBD (n = 63)	Controls (n = 11)	p	CD (n = 42)	UC (n = 21)	p
Age (y)[a]		39 (17)	39 ± 14	0.994	40 ± 14	35 (26)	0.597
Gender F/M[b,c]		36/27	7/4	0.752	22/20	14/7	0.418
Smoking[b], N (%)	Yes	10 (16)	0 (0%)	0.341	7 (17)	3 (14)	1.000
	No	43 (84)	11 (100%)		35 (83)	18 (86)	
Disease duration (y)[a]		7 (11)			9 (11)	5 (15)	0.497
Montreal classification, N (%)	*L1 (ileal)*				19 (45)		
	L2 (colonic)				5 (12)		
	L3 (ileocolonic)				18 (43)		
	+ L4 (upper GI)				0 (0)		
	+ P (perianal disease)				5 (12)		
	+ L4 & P				3 (7)		
	B1 (inflammatory)				34 (81)		
	B2 (stenosing)				8 (19)		
	B3 (penetrating)				0 (0)		
	E1 (proctitis)					2 (10)	
	E2 (left side)					7 (33)	
	E3 (extensive)					10 (47)	
	E missing					2 (10)	
	S0 (remission)					16 (76)	
	S1 (mild)					2 (10)	
	S2 (moderate)					3 (14)	
	S3 (severe)					0	
HBI, N (%)					1 (2.00)		
	< 5				34 (81)		
	≥ 5				5 (12)		
	Missing				3 (7)		
SCCAI, N (%)						2 (2.50)	
	< 5					15 (71)	
	≥ 5					2 (10)	
	Missing					4 (19)	
Fecal CP (µg/g)[d]		138.78 (9 - 4929)			144.34 (9 - 4929)	127.44 (9 - 3651)	0.814
N (%)	<250 µg/L	40 (63)			26 (62)	14 (67)	
Plasma CP (mg/L)[d]		2.95 (0.51 - 4.25)			2.97 (0.51 - 4.03)	2.92 (0.74 - 4.25)	0.961
CRP (mg/L)[d]		2.36 (0.40 - 13.00)			2.27 (0.40 - 7.00)	2.56 (0.50 - 13.00)	0.165
Salivary CP (mg/L)[a]		1.87 (6.27)	3.11 (1.85)	0.248	1.53 (4.78)	3.47 (7.55)	0.641

IBD: inflammatory bowel disease; CD: Crohn's disease; UC: ulcerative colitis; CP: calprotectin; HBI: Harvey-Bradshaw Index; SSCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein; [a] Mann-Whitney U test; [b] Fisher exact test; [c] Chi2 test, CD vs CU; [d] Independent-Samples T Test; Variables reported as: n (%), mean ± SD for normally distributed data, means (range) for imputed data or median (IQR).

Table II. Collected sample distribution

	CD (n = 42), N (%)	UC (n = 21), N (%)	Control (n = 11), N (%)
Saliva	42 (100)	21 (100)	11 (100%)
Feces	37 (88)	17 (81)	-
Plasma	29 (69)	12 (57)	-

For abbreviations see Table I.

However, CRP levels were found to be significantly higher in patients with active disease (p=0.034).

Calprotectin in Saliva and Plasma

Salivary CP concentrations did not differ significantly between patients and controls (p=0.248) (Table I). Also, there were no significant correlations between salivary CP and fecal CP, salivary CP and plasma CP, and salivary CP and CRP levels (Table III).

Table III. Correlations between disease activity scores, calprotectin levels in feces, plasma and saliva and CRP in patients

	N	r statistic	p value
Salivary CP and Fecal CP[a]	63	-0.092	0.495
Salivary CP and Plasma CP[a]	63	-0.19	0.223
Salivary CP and CRP[a]	63	-0.078	0.602
Salivary CP and HBI[c]	39	0.075	0.652
Salivary CP and SCCAI[c]	17	-0.088	0.742
Fecal CP and Plasma CP[b]	63	0.053	0.755
Fecal CP and CRP[b]	63	0.18	0.300
Fecal CP and HBI[c]	36	0.12	0.503
Fecal CP and SCCAI[c]	13	1.00	0.000
Plasma CP and CRP[b]	63	0.11	0.582
Plasma CP and HBI[c]	25	0.037	0.860
Plasma CP and SCCAI[c]	9	0.00	1.000
CRP and HBI[c]	31	-0.11	0.517
CRP and SCCAI[c]	10	0.48	0.170

For abbreviations see Table I; [a] Spearman rho correlation; [b] Pearson correlation; [c] Kendall's Tau correlation.

Stratifying patients into two subgroups based on the fecal CP cut-off value of 250 µg/g, also revealed no significant differences in salivary CP, plasma CP and CRP levels between patients with active disease or patients in remission (Table V). Moreover, salivary CP levels did not differ significantly between patients with active IBD and controls (p=0.464) or between patients with IBD in remission and controls (p=0.106).

The range of salivary CP levels between controls, patients with active disease and patients in remission are depicted in Fig. 1.

Complete case-analysis led to similar results as multiple imputation analysis.

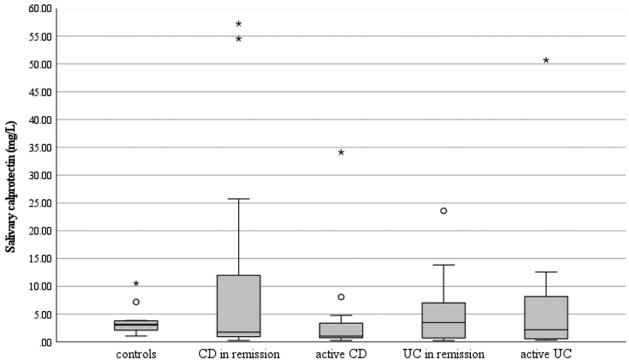

Fig. 1. Salivary calprotectin levels in inflammatory bowel disease (IBD) patients and controls. CD: Crohn's disease; UC: ulcerative colistis; UC/CD in remission: fecal calprotectin < 250 µg/g: active UC/CD: fecal calprotectin ≥ 250 µg/g.

Storage Effects

A mean decrease of 0.456 (± 0.578) mg/L in salivary CP levels (p=0.026) was found after one freeze-thaw cycle in the control group. No significant correlation could be found between salivary CP levels and the number of days a sample was stored in the freezer (rs=0.088, p=0.495).

DISCUSSION

We investigated the potential of salivary CP as a non-invasive biomarker for intestinal disease activity in IBD. We compared the salivary CP level with fecal CP, plasma CP and CRP levels in a cohort of 63 patients with IBD and concluded that salivary CP does not seem to be a reliable biomarker for the determination of disease activity. There was no correlation between disease activity, measured using the well-known biomarker fecal CP and salivary CP in our cohort of 63 patients with IBD. Moreover, we found no significant difference in salivary CP levels between patients, both with normal and elevated fecal CP levels, and controls.

Previous studies investigating the relationship between IBD activity and salivary CP reported contradicting results. One study by Majster et al. [15] reported a significant increase of salivary CP in stimulated and unstimulated saliva in patients with active IBD in comparison to healthy controls and especially

Table IV. IBD patients stratified in two groups based on HBI/SCCAI cut-off value of 5

	Remission (n = 49)	Active disease (n = 7)	p
Fecal CP (µg/g)[b]	332.88 (9 - 4929)	1350.67 (9 - 3599)	0.091
Salivary CP (mg/L)[a]	2.14 (6.02)	4.78 (8.04)	0.512
Plasma CP (mg/L)[b]	1.64 (0.51 - 4.03)	2.08 (0.84 - 3.97)	0.334
CRP (mg/L) [b]	2.83 (0.4 - 10.0)	5.46 (1.9 - 11.0)	0.034

For abbreviations see Table I; [a] Mann-Whitney U test; [b] Independent-Samples T Test; Values reported as: mean (range), median (IQR).

Table V. CD and UC patients stratified in two groups ('remission' and 'active disease') based on fecal CP 250 µg/g cutoff

	CD (n = 42)		p	UC (n = 21)		p
	Remission (n = 26)	Active disease (n = 16)		Remission (n = 14)	Active disease (n = 7)	
FCP (µg/g)[b]	54.93 (9.00 - 230)	844.31 (254 - 4929)	<0.001	34.35 (9 - 202)	1242.22 (410 - 3651)	<0.001
SCP (mg/L)[a]	1.75 (12.33)	1.06 (2.81)	0.294	3.51 (6.94)	2.19 (12.20)	0.856
PCP (mg/L)[b]	2.99 (0.51 - 3.97)	2.92 (0.54 - 4.03)	0.909	2.86 (0.74 - 4.25)	2.11 (1.12 - 2.71)	0.853
CRP (mg/L)[b]	2.18 (0.40 - 6.00)	2.43 (0.90 - 7.00)	0.698	2.31 (0.50 - 11.00)	3.10 (0.80 - 13.00)	0.677

FCP: fecal calprotectin; SCP: salivary calprotectin; PCP: plasma calprotectin; For the rest of abbreviations see Table I. [a] Mann-Whitney U test; [b] Independent-Samples T Test; Values reported as: mean (range), median (IQR).

newly diagnosed treatment naïve CD patients had a significant elevated salivary CP level [15, 25]. Strikingly, after treatment there was a decrease in salivary CP levels in the treatment of naïve CD patients, while in the previously treated group there seemed to be an increase in salivary CP levels [15]. This may indicate that salivary CP could potentially be useful in active and newly diagnosed IBD, but it is less suitable for follow-up, especially in previously treated patients. In our study all patients had an established diagnosis of IBD and received treatment; this could potentially explain the difference. Moreover, we found no difference in salivary CP levels between IBD patients (both with active and inactive disease) and controls. A more recent study which only included IBD patients with an established diagnosis who had already underwent treatment demonstrated that salivary CP levels were significantly lower in CD and UC patients with active disease compared to healthy controls [26]. This finding is more in line with our data in which patients with IBD also had a lower, although not significant, median salivary CP level compared to the control group. They speculated that the reduction in salivary CP concentration could be explained by a decrease in the host's oral defense. A recently published abstract, correlated endoscopic disease activity scores and histologic data with fecal CP and salivary CP in eighteen patients with CD and demonstrated, in line with our findings, no correlation between salivary CP and endoscopic or histologic disease activity scores [27].

Besides CP, saliva contains a wide range of other molecular components and molecules, including growth factors, cytokines, hormones, enzymes and antibodies and is already used to diagnose several systemic diseases such as Cushing's syndrome and human immunodeficiency virus, and can also be used in the follow-up of pathologies [25, 28-30]. Although our study does not show this for CP, several studies have reported that saliva, in some cases, can be used as a surrogate for plasma drug concentrations, inflammatory markers, and hormones [25, 31-35]. A recent abstract by Kamp et al. [27] demonstrates a low degree of correlation between plasma CP and endoscopic outcomes, and a relatively high degree of correlation between plasma CP and histological outcomes. This might suggest that plasma CP could be a potential biomarker for histologic disease activity; however the sample size was limited and a low number of patients had active CD [27].

This study is one of the first where the relationships between salivary CP, fecal CP, and plasma CP were assessed in a cohort of well-defined IBD patients using standardized methods for sample collection. We used a pragmatic study design which could easily be replicated in daily clinical practice. Although we did not determine intestinal disease severity endoscopically, we demonstrated with our study design that salivary CP does not seem to correlate with the well-established and reliable non-invasive biomarker fecal CP or with disease activity scores [5, 7].

Although we did not perform oral examinations, we did exclude patients who indicated that they had xerostomia or current periodontal disease, since it is known that this could, independently, lead to elevated salivary CP levels [36-38]. Excluding these patients could, however, also be a limitation since De Vries et al. [39] demonstrated that IBD activity is related to oral complaints and experienced xerostomia. Excluding these patients could potentially have led to the exclusion of patients with active disease in the present study. Despite the low number of patients with active disease in our study, we think this does not hamper the ability to answer our question if salivary CP could be a reliable biomarker. Since IBD is associated with alternating periods of remission and disease activity, a reliable biomarker should be able to distinguish these periods and needs to be reliable in both. In our study there was no difference in salivary CP levels in patients with active disease based on an elevated fecal CP or patients in remission, moreover, salivary CP levels did not even differ between patients with active disease and healthy controls. However, our control group was relatively small and not matched by gender, and even though we assume that this has a minor influence on our results, given that there seems to be no difference in salivary CP levels between male and female subjects, our small and non-matching control group remains a limitation [40]. Another limitation of our study is the freeze-thaw cycle all samples underwent, since after one cycle, the salivary CP concentrations in our control samples decreased significantly. This seems to contradict previous research which demonstrated that undergoing several freeze-thaw cycles increased calprotectin concentrations [15]. The increased concentration after freeze-thaw cycles found in the other studies might be explained by the release of intracellular CP from destructed neutrophils in saliva leading to an increase in salivary CP. Since we collected our saliva samples using a Salivette kit and centrifuged them before freezing, this should have resulted in a limited number of neutrophils in the frozen samples, preventing such an increase [41]. Since every sample in our study went through a similar cycle and was stored similarly at -20 °C, suggests that the effect on the comparative analyses and the conclusions drawn will be limited.

CONCLUSION

Salivary CP does not correlate with the well-established and reliable non-invasive biomarker fecal CP and disease activity scores. This study gives more insight into the potential use of salivary CP as a non-invasive biomarker and suggest that salivary CP is not a reliable non-invasive biomarker for assessing disease activity in patients with IBD. However, larger studies with more patients with severe IBD activity are required.

Conflicts of interest: G.B. has received consultancy fees from Roche and Takeda. M.D. received advisory fees from Echo Pharma and Robarts Clinical Trials, Inc., speaker fees from Janssen, Merck & Co., Inc., Pfizer, Takeda and Tillotts Pharma, and nonfinancial support from Dr. Falk Pharm. N.K.d.B. has served as a speaker for AbbVie and MSD and has served as consultant and principal investigator for TEVA Pharma BV and Takeda. He has received a (unrestricted) research grant from Dr. Falk, TEVA Pharma BV, MLDS and Takeda. For the remaining authors no conflict of interest was declared.

Authors' contributions: V.B, F.C, H.H, N.K.d.B. conceived and designed the study. V.B, F.C, P.W, G.B, M.D. and N.K.d.B. recruited patients and controls. F.C, H.H. and V.B. did the data-extraction. F.C. and V.B. drafted the first version of the manuscript. P.W, G.B, M.D, H.B, H.H. and N.K.d.B. critically revised the manuscript. All authors approved the final version of the article, including the authorship list.

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Supplementary material: To access the supplementary material visit the online version of the *J Gastrointestin Liver Dis* at http://dx.doi.org/10.15403/jgld-4215

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