Serum Levels of Fibroblast Growth Factor 19 Correlate with the Severity of Diarrhea and Independently from Intestinal Inflammation in Patients with Inflammatory Bowel Disease or Microscopic Colitis

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ABSTRACT

Background: *In chronic diarrhea patients, massive over-reporting symptom-based criteria for functional bowel disorders are pitfalls. There is currently no objective biomarker that may provide a correct correlation with the severity of chronic diarrhea. To clarify the role of fibroblast growth factor-19 (FGF-19) as a biomarker of objective measurements of the severity of diarrhea in comparison with a patient-reported outcome, based on the Bristol Stool Form (BSF) Scale.*

Methods: *Consecutive 100 patients with chronic diarrhea underwent standard investigations with laboratory tests, fecal calprotectin (FC), endoscopy with biopsies, and serum FGF-19. All patients and 14 healthy controls completed a diary recording, BSF, and stool frequency.*

Results: *We found that irritable bowel syndrome with diarrhea (IBS-D) n = 21/23 (91%) reported a high number on BSF ≥6, compared to patients with inflammatory bowel diseases (IBD) 56/77 (72%) with BSF ≥ 6 (P = .011). FGF-19 median serum levels were significantly lower in Microscopic colitis (0.010 pg/mL) and IBD patients (0.009 pg/mL) compare to IBS-D (266.9 pg/mL) and high levels in healthy subjects (463 pg/mL) (P < .001). Strong inverse correlation of FGF-19 with the stool frequency/day and stool index was found (r = −0.800, P < .001; r = −0.739, P < .001), independently from disease activity (r = −0.718, P = .001; r = −0.792, P = .001).*

Conclusion: *Serum FGF-19 can become a new biomarker for evaluating the severity of diarrhea with objectively and independently from intestinal inflammation. FC and FGF-19 are predictive biomarkers for the organic cause of diarrhea.*

Keywords: *Fibroblast growth factor-19, FGF-19, BAM, IBD, MC, IBS-D*

INTRODUCTION

The reported prevalence of chronic diarrhea is 4-5% in the Western population, which is a common disorder in many conditions. The incidents vary a lot and depend on the definition of diarrhea and reported by the patients. A large number of other diseases may cause diarrhea that is not related to the gastrointestinal tract.[1,2] The standard definition of chronic diarrhea is ≥ 3 loose stools per day with a fecal weight ≥ 200 g/day, lasting for at least 4 weeks.[2] The term 'Diarrhea' may be defined as stool frequency, consistency, volume, or weight of the feces, but this differs from patients reporting the number of stools and often focuses around stool consistency.[2,3] The most common causes of diarrhea are functional bowel disorders, such as irritable bowel syndrome (IBS) and functional diarrhea.[4] However, from organic diseases with chronic diarrhea, the most common causes are inflammatory bowel disease (IBD), microscopic colitis (MC), and bile acid diarrhea.[1,4] In chronic diarrhea patients, there is a situation of substantial over-reporting symptoms and more often only symptom-based criteria, but these reports of high number loss of stools per day can lead to exhausting diagnostic procedures and broad differential diagnosis.[5,6]

The reporting precision of the correct number of stools per day has been questioned by fecal water content (FWC) measured on pooled feces by freeze-drying in patients with chronic diarrhea with defined as ≥78% water.[1,7] The degree of dissatisfaction with stool consistency correlated poorly with the FWC and Bristol stool form (BSF) scale changes.[7,8] The measure of fecal weight is 'cumbersome,' and this test is not popular

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among patients and doctors because you have to collect feces for 48 h.[1] The stool frequency is often reported from the patients and only includes the stool frequency, duration, or using the validated BSF scale[1] We need uniform and reproducible biomarkers, that IBS no longer will be distinguished by symptoms alone, but rather with objective measurements such as bile acid diarrhea (BAD) corresponding with the stool frequency, volume, fluidity, and microbial composition.[8]

In the gastrointestinal tract (GI), the fibroblast growth factor-19 (FGF-19) has a pivoting role in enterohepatic circulation of bile acids (BAs), and it is responsible for a substantial part of the pathogenesis of chronic diarrhea.[9,10] Excessive BA in the bowels activates the farnesoid X receptor (FXR) in the enterocytes, and FXR directly regulates the expression of FGF-19.[11] FGF-19 acts as an enteroendocrine hormone that travels to the liver via the hepatic vein to decrease hepatic BA synthesis by reducing enzyme's rate 7-α-hydroxy-4-cholesten-3-one (C4).[9,10] Serum FGF-19 levels are thus an indirect marker of BA absorption, and reduced FGF-19 levels are associated with ileal dysfunction and correspond with the severity of diarrhea.[12] In animal models, antibodies targeted FGF-19 cause depletion of FGF-19 and produced severe diarrhea in mice and monkeys.[13,14] Using commercially available kits, we can measure FGF-19 easily in serum and may have the utility as a biomarker for functioning ileum in Crohns disease (CD).[11,12,13,15] The aim of this study was to measure serum FGF-19 in patients referred for diagnostic workup of chronic diarrhea and compare with healthy controls to explore the correlation between FGF-19 and the severity of diarrhea.

MATERIALS AND METHODS

Study design: Cross-sectional study

Study period: October 2017 to April 2019

Subjects and Eligibility Criteria for Inclusion or Exclusion

We enrolled 100 consecutive patients referred to our referral clinic (center) to investigate severe chronic diarrhea from primary care and non-gastroenterological hospital clinics. The inclusion criteria of the study were (a) adult patients above > 18 years old and (b) patients with chronic watery diarrhea > 4 weeks with unknown origin. Exclusion criteria: (a) patients who presented incomplete data, (b) a personal history of vagotomy, gastrectomy, or surgery for obesity, (c) other types of enteropathies (including parasitic or acute diarrhea caused from infection); (d) loss to follow-up, (e) studies that included subjects <18 years of age or those conducted in patients with a history of cholecystectomy, (f) radiation enteritis, (g) diverticulitis, (h) Cl. diff infection, (i) infectious colitis, (j) ischemic colitis, (k) neoplastic diseases, (l) neuroendocrine tumors, (m) laxative abuse, (n) bacterial overgrowth (SIBO), (o) immune deficiency syndrome, (p) Carbohydrate malabsorption, (q) endocrinological cause (Hyperthyroidism), (r) pancreatic disorders (chronic pancreatitis, pancreatic cancers and pancreas exocrine deficiency), and (s) patients were restricted from bile acids sequestrants (BAS) during diagnostic workup.

All enrolled patients had a complete history of the patient with a diary for chronic diarrhea was also obtain to assess stool frequency, consistency, and other gastrointestinal symptoms to answer to symptom-based criteria ROME IV.[16,17] All patients underwent standard investigations including laboratory tests for hemoglobin concentration, sedimentation rate, vitamin B12, folic acid, iron saturation, albumin, thyroid hormones, alkaline phosphatase, and serum Anti-tTG (IgA + IgG). Abdominal ultrasound was performed to rule out any other pancreato-biliary disease. Consecutive subjects were undergoing a hydrogen lactulose breath test to exclude SIBO. Stool cultures and stool microscopy for parasites were performed. Fecal calprotectin (FCP) was measured in every patient prior to upper and lower gastrointestinal endoscopy with biopsies from the duodenum and biopsies from every segment of the colon.

Bristol Stool Form (BSF) and Reporting Stool Frequency

All subjects were asked to report stool frequency and consistency using BSF scale[16] and diarrhea was defined as a stool frequency of ≥3/day at least 4 weeks, with a stool form of 6 or 7.[17,18] We calculate the Stool Index (SI) as a daily index (daily stool frequency × BSF) + loperamide use [mg*3].[19]

Assessment the Disease Activity and Different Diagnosis

We defined clinical remission in ulcerative colitis (UC) as a Mayo score (full) of ≤ 2 points and clinical remission in CD as a lack of mucosal lesions (erosions, ulcers, aphthous lesions) on ileocolonoscopy and Crohn's Disease Activity Index (CDAI) < 150.[20,21] The distribution of CD patients was evaluated using standard endoscopic and radiologic techniques. According to the ROME IV criteria for IBS,

patients were diagnosed with IBS-D after performing ileocolonoscopy and laboratory standard tests.[22] After excluding other diseases, history of drug consumption, normal endoscopic findings, and biopsies with specific histopathological findings, MC diagnosis was made. We took 14 healthy subjects as controls to be compared with the other group of patients.

Enzyme Immunoassay (ELISA)

FGF-19 levels were measured using Thermo Scientific™ Human FGF-19 ELISA Kit (in serum) according to the manufacturers protocol and expressed in pg/ml. Participants provided a fasting blood sample, before 9:00 AM, separated and stored at −80°C until analysis. For total free fecal bile acids (TFFBA) we use enzymatic (ELISA) photometric determination of IDK® Bile Acids Immundiagnostik AG, Germany, and results were expressed in μmol/g. FC was measure using a quantitative immunochromatographic point-of-care test (Quantum Blue®, Bulhmann laboratories AG, Switzerland), and results were expressed in μg/g.

Statistical Analysis

The analysis was performed using descriptive techniques, and non-normal distributed variables were transformed before analysis. Continuous numerical variables are presented as mean ± SD, median, and range. Student's *t*-test and analysis of variance were used to compare continuous variables. Fishers exact test was used to compare means and Pearson correlation between continuous variables. A two-tailed *P*-value of .05 was considered statistically significant. Statistical analysis was performed using SPSS for Windows, Version 23.0 (IBM Corp.; Armonk, NY, USA).

RESULTS

General Characteristics of the Study Cohort

In this prospective study, we enrolled consecutively 100 adult patients with chronic watery diarrhea—40 males and 60 females at average age of 48.2 ± 15 (23-80) years. Fourteen healthy subjects (controls) at an average age of 41 ± 14 (22-61) years were recruited.

After final evaluation with all the investigations, patients were divided into 6 groups: 21 patients with active IBD, 21 patients with IBD in remission and unexplained persistent diarrhea, 21 patients with IBD after surgery [Crohns after ileal resection (IR-CD), UC after J-pouch anastomosis (IPAA)], 23 patients with IBS-D, 14 patients with MC (12 with Collagenous colitis and 2 with Lymphocytic colitis) and 14 healthy control subjects (HS).

Twenty-three of these 100 patients (23%) had no objective findings during the diagnostic workup that could explain their diarrhea. Still, these patients fulfilled the diagnostic criteria for diarrhea-predominant IBS according to ROME IV criteria.[22] The most common organic bowel disease in our group was IBD, followed by MC. We found 21 patients with IBD in endoscopic and biochemical remission (FCP normal), but still, these patients report a higher number of loose stools per day.

Reporting the Severity of Diarrhea in Relation to Final Diagnoses

The mean stool frequency in different groups of patients for IBD active was 7.83/day (range 3-13), patients with IBD in proven endoscopic and biochemical remission reports 5.86/day (scale 2-9), IBD patients after IR-CD or IPAA reports 9.00/day (range 3-15), IBS-D patients reports 4.65/day, MC patients report 9.29 stools/day and healthy subjects has 1.36 stools/day. Of all included patients, 23 patients had median stool form BSF below <6, where the other 77 patients had a median stool form of BSF ≥ 6. We found that a higher proportion of subjects IBS-D $n = 21/23$ (91%) reported a high number on BSF ≥ 6, compared to patients with an organic cause of diarrhea 56/77 (72%) BSF ≥ 6, respectively ($P = .011$). IBS-D reports lower SI and stools per day compare to other groups ($P < .001$). We found that SI in IBD active group was 49 ± 21, in IBD after remission was 33 ± 13, IBD after surgery reports 58 ± 23, IBS-D patients were 31 ± 18, MC patients report 63 ± 25, and healthy controls have 6 ± 3 ($P < .001$) (Table 1). We evaluate the mean duration of diarrhea in all patients, and we found no difference between different groups ($P = .512$). We observe if there are phenomena of diarrhea appearing during fasting, but we not found any statistical difference between the groups ($P = .531$).

Disease Activity Scores

Our study found that the mean CDAI in CD active group was 338 ± 74, which was significantly different from IBD in remission CDAI 107 ± 47 ($P = .043$). There was no difference in disease activity between CD active and CD after surgery ($P = .058$). In UC patients, we found a significant difference in the IBD active group for Mayo score 7.94 ± 2.623 compare to IBD in remission 1.33 ± 0.778 ($P = .004$) and no difference between UC active and UC after surgery ($P = .903$).

Results from Systemic and Intestinal Inflammation (CRP and FCP)

We found a statistically significant difference in the IBD active group with the elevation of mean C-reactive

Table 1. Comparison of FGF-19 with Other Parameters and Patient's Characteristics

	IBD Active	IBD in Remission	IBD After Surgery	IBS-D	MC	Healthy Subjects
Number of patients, *n* (%)	21 (18)	21 (18)	21 (18)	23 (20)	14 (13)	14 (13)
Gender, female (%)	13 (62)	9 (43)	9 (43)	13 (57)	11 (78)	5 (45)
Age, Mean ± SD	45 ± 13	50 ± 15	48 ± 17	46 ± 15	60 ± 17	41 ± 17
CDAI, Mean ± SD, Median (IQR)	338 ± 74 345 (220-440)	107 ± 47 130 (30-150)	257 ± 126 260 (100-410)	-	-	-
Mayo score (full), Mean ± SD, Median (IQR)	7.9 ± 2.6 8.5 (4-11)	1.3 ± 0.7 1.5 (0-2)	7.7 ± 2.7 7.5 (5-11)	-	-	-
Endoscopic Mayo score, Mean ± SD	2.2 ± 0.5	0.4 ± 0.5	2 ± 0.816	-	-	-
Stool Index (SI) Mean ± SD, Median (IQR)	49 ± 21 46.5 (15-84)	33 ± 13 35 (6-54)	58 ± 23 60 (18-98)	31 ± 18 24 (12-84)	63 ± 25 70 (18-91)	6 ± 3 4 (3-12)
Stool frequency per day, mean ± SD, Median (IQR)	7.8 ± 3 8.5 (3-13)	5.8 ± 2 6 (2-9)	9 ± 3 9 (3-15)	4.6 ± 2 4 (3-12)	9.2 ± 3 10 (3-13)	1.3 ± 1 1 (1-3)
Duration of the diarrhea (weeks), Median (25th-75th percent)	5 (4-6)	4 (4-5)	6 (4-7)	5 (5-6)	6 (6-15)	0(0)
Night defecation, *n* (%)	16 (76)	4 (19)	10 (47)	2 (8.7)	9 (64)	0 (0)
Diarrhea appear during fasting, *n* (%)	12 (57)	5 (23)	11 (52)	5 (21)	6 (42.9)	0 (0)
ROME IV, *n* (%)	9.5 (43)	2 (9.5)	6 (28.6)	23 (100)	10 (71.4)	0(0)
CRP, Mean ± SD, Median (IQR), mg/dL	1.2 ± 2.4 0.4 (0.1-12)	0.3 ± 0.2 0.3 (0.2-0.9)	0.4 ± 0.3 0.4 (0.2-1.3)	0.1 ± 0.1 0.1(0.1-0.5)	0.5 ± 0.3 0.3(0.1-1.4)	0.3 ± 0.2 0.2(0.1-0.9)
Fecal calprotectin, Mean ± SD, Median (IQR), µg/g	750.9 ± 465.9 806 (78-1800)	81.3 ± 39.7 85 (10-185)	375 ± 264.5 338 (25-813)	47.5 ± 38.1 30 (5-125)	221 ± 279 115 (44-100)	43 ± 23 30 (18-82)
Total free fecal bile acids, Mean ± SD, Median (IQR), µmol/g	42 ± 19.4 38 (6.7-85.2)	37.5 ± 14.1 37 (5.6-78)	40.1 ± 20.9 38 (6.8-96)	40.8 ± 20.3 37 (12-97)	40.6 ± 25.4 38 (6-96)	44 ± 13.3 38 (32-72)
FGF-19, Median (25-75th percentile) pg/mL	0.009 (0.005-211)	0.009 (0.005-78)	0.005 (0.002-0.012)	266 (78-831)	0.010 (0.004-78)	463 (50-858)

IQR, interquartile ranges; CRP, C-reactive protein; IBD, inflammatory bowel disease; FGF-19, fibroblast growth factor-19; SD, standard deviation.

protein (CRP) levels compare to IBS-D and healthy controls (P = .031) (see Table 1). From the results of FCP, we found that IBD active group was with higher mean levels of 750 ± 465 μg/g (P < .001). IBD in remission was 81 ± 39 μg/g, IBD after surgery was significantly elevated mean levels of FCP 375 ± 264 μg/g compared to healthy controls and those in remission (P < .002). IBS-D had a mean FCP of 47.5 ± 38 μg/g. MC was with elevated mean FCP 221 ± 279 μg/g compare to healthy controls with mean levels of FCP 43 ± 23 μg/g (P < .001) (see Table 1).

Correlation of FGF-19 to Stool Index and Stool Frequency

In all the patients with chronic diarrhea, we measure TFFBA, and there is no difference between the patients groups and no difference compared with the healthy controls (P = .315). The median levels and (25th-75th percentile) of FGF-19 were found to be significantly different between organic disorders (IBD and MC) compare to IBS-D and HS (P < .001). The median levels in IBD active were 0.009 pg/mL (0.005-211) and significantly different compared to HS (P < .001). The median levels of IBD in remission were 0.009 pg/mL (0.005-78) and significantly different to HS (P < .001). IBD after surgery were 0.005 pg/mL (0.002-0.012) and different to HS (P < .001). IBS-D were 266.9 pg/mL (78-831). MC were 0.010 pg/mL (0.004-78) and significantly different to HS (P < .001). Median FGF-19 in HS (controls) were 463 pg/mL (50-858). Using univariate Pearsons variation analysis, we found that the serum levels of FGF-19 have a strong inverse correlation with the severity of diarrhea defined by the stool frequency/day and SI (r = –0.800, P < .001; r = –0.739, P < .001). After correction of the selected variables, including inflammatory markers (FCP, CRP, and age of the patients), we confirmed again strong inverse correlation of FGF-19 with stool frequency/day and SI independently from disease activity (intestinal inflammation) (r = –0.718, P = .001; r = –0.792, P = .001). We also found in MC patients that the size of the collagenous band (r = –0.489, P = .002) correlates inversely with FGF-19 and the severity of diarrhea in our patients (see Table 2).

DISCUSSION

In this study, we investigate the role of serum FGF-19 to measure the severity of chronic diarrhea objectively. Our results show that reported stool frequency per day and calculated SI have a strong correlation with the serum levels of FGF-19 in patients with chronic diarrhea from different etiology. Evidence suggests that the most commonly used reporting tool BSF and diary for evaluating the severity of diarrhea are not enough accurate nor sufficient for grading the patients.[14,23]

A higher proportion of subjects with IBS-D reports higher BSF (6 or 7) in our study. This was the reason to consider searching for an easy-to-use serum biomarker, such as

Table 2. Correlation Between lnFGF19 and Stool Index, Stool Frequency, Mayo Score, CDAI, and Endoscopic Mayo Score

FGF-19 Correlation with Other Markers/ Scores	Uncorrected	Corrected Without Intestinal Inflammation (Exclude Age, CRP, and FCP)
Age	r = –0.191, P = .036	-
Gender	r = 0.054, P = .558	r = –0.148. P = .111
Stool index (SI)	r = –0.739, P < .001	r = –0.718, P = .001
Stool frequency/day	r = –0.800, P < .001	r = –0.792, P = .001
Duration of the diarrhea	r = –0.255, P = .005	r = –0.223, P = .016
Mayo score (full)	r = –0.141, P = .427	-
Endoscopic Mayo score	r = –0.120, P = .499	
CDAI	r = 0.063, P = .706	r = 0.262, P = .129
Collagenous band size in μm (n = 38)	r = –0.489, P = .002	r = –0.414, P = .014
Lymphocytes counts in the mucosa	r = –0.208, P = .211	r = –0.124, P = .477
CRP	r = –0.097, P = .292	-
Total free fecal bile acids (μmol/g)	r = –0.107, P = .243	r = –0.146, P = .115
Fecal calprotectin	r = –0.173, P = .058	-

CDAI, Crohns Disease Activity Index; CRP, C-reactive protein; FCP, fecal calprotectin.

FGF-19, that is part of the workup in diagnostic methods of BAD, but can give more information in the mechanisms of diarrhea. Median levels of FGF-19 in IBS-D patients and healthy controls are significantly different compare to IBD and MC. In MC patients, the size of the thickened collagen band correlates with levels of FGF-19, which is another point to consider possible BAD in this group of patients.

The typical clinical scenario for considering BAD in IBD patients with unexplained chronic diarrhea is Crohns disease with previous ileal resection (IR-CD). Our data confirm that IR-CD patients have more severe BAD with clinically significant diarrhea. Our findings are similar to Nolan et al.,[12] where they were measured FGF-19 levels in 58 patients and found an inverse correlation with ileal resection length in IR-CD patients ($r = -0.54$, $P = .02$). The authors concluded that reduced FGF-19 levels are associated with ileal resection, diarrhea, disease activity, and FGF-19 may have utility as a biomarker for functioning ileum in CD.[12]

However, measured FGF-19 in IBD without resection (NR-CD) in our group was not consistent with the level of mucosal inflammation. No correlation was found between FGF-19 with Mayo score and CDAI ($P = .427$, $P = .706$) (see Table 2). With or without correction of FCP and CRP, we still found a relationship between FGF-19 and stool frequency. Literature is conflicting on whether ileitis causes lower FGF-19 concentrations, and some studies have demonstrated lower FGF-19 when there is an ileal inflammation,[12] but other studies show that ileal inflammation actually increases levels of FGF-19.[24,25] Therefore, IBD patients in clinical and endoscopic remission with unexplained chronic diarrhea have to be investigated further because BAD can be one of the causes of diarrhea independently of the intestinal inflammation.

Colonic BAs stimulate epithelial Cl^- secretion and play the role of colonic 'osmosignals' for defining the severity of chronic watery diarrhea.[26] A potential drug that can target FXR and FGF-19 is obeticholic acid, a semisynthetic FXR agonist. This medication has previously demonstrated anti-fibrotic and anti-inflammation qualities in the liver.[27] FXR and FGF-19 can play an essential role in revealing the different new therapeutic targets in patients with chronic diarrhea.[28] Based on our current hypothesis that FGF-19 deficiency is associated with BAD and the proof-of-concept is the trial with obeticholic acid by Walters et al.[19]

We found a strong inverse correlation between fasting serum FGF-19 and daily liquid bowel movements of the patients with chronic diarrhea, suggesting that FGF-19 is a potential biomarker for measuring the severity of diarrhea independently from disease activity. In IBD patients with endoscopic and biochemical remission, who have unexplained diarrhea, we found a strong correlation with FGF-19 ($r = -0.800$, $P < .001$). Therefore, bile acid malabsorption (BAM/BAD) has a crucial part in the pathogenesis of chronic diarrhea in IBD and MC patients.

The limitations of our study are a small sample size, and we need further enriching the study cohort with more patients with different types of diarrhea. Our study cohort is diverse from the normal population distribution of chronic diarrhea, but this is because we are a referral center for severe IBD patients, and this may also have altered the proportion between functional and organic causes for chronic diarrhea. FGF-19 was not compared with either the 75-Selenium homotaurocholic acid test (SeHCAT), C4, or a therapeutic trial with sequestrants. The lack of screening colon cancer program in Bulgaria is why the lack of colon cancer patients in our study. At this point, patients with constipation were not included in our study, but this will be a future aim to investigate the role of FGF-19 in constipated patients.

CONCLUSION

None of the investigated tools nowadays for chronic diarrhea are perfect. However, we still depend on the correct definition of diarrhea as watery stools according to the BSF scale, and we evaluate stool frequency by what patients report to us. We found that FGF-19 in patients with chronic diarrhea can be proposed not only for detecting BAD/BAM but as a new biomarker for evaluating the severity of diarrhea with objectively and independently from their intestinal inflammation. A combination of elevated FC and low levels of FGF-19 seem to be good predictors of having an organic cause of diarrhea. Future studies are needed to establish the efficacy of FGF-19 on chronic diarrhea.

What are the significant and/or new findings of this study?

(1) We found a strong inverse correlation between fasting serum FGF-19 and daily liquid bowel movements of the patients with chronic diarrhea.
(2) FGF-19 can be proposed not only for detecting bile acid malabsorption but as a new biomarker for

evaluating the severity of diarrhea with objectively and independently from intestinal inflammation.

(3) FGF-19 in IBD was not consistent with the level of mucosal inflammation because there is no correlation with FC, Mayo score, and CDAI.

(4) Combination of fecal calprotectin and serum FGF-19 seem to be good predictors of having an organic cause of diarrhea.

Ethics Committee Approval: The study was approved by the local Ethics Committee of University Hospital "Tsaritsa Yoanna - ISUL" from Project No 8510/12.12.2016, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Informed Consent: Before initiating this study, written informed consent was obtained from all subjects participating in Project No 8510/12.12.2016.

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