Fecal calprotectin levels are elevated in transthyretin amyloidosis patients with gastrointestinal manifestations

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Abstract
Transthyretin amyloid (ATTR) amyloidosis is a rare systemic disorder characterized by amyloid deposits formed by misfolded monomers of the transthyretin. Gastrointestinal (GI) manifestations are common in ATTR amyloidosis; however, their pathogenesis is not fully elucidated. In the present study, we aim to evaluate the diagnostic role of fecal calprotectin (FC) in ATTR amyloidosis patients with GI manifestations.

We recruited 21 consecutive ATTR amyloidosis patients and 42 sex and age-matched healthy controls. The presentation of GI symptoms and the severity of peripheral neuropathy were evaluated. Colonoscopy and FC assessment were performed in all subjects.

Mean levels of FC in ATTR amyloidosis patients (184 μg/g [30–430]) were significantly higher than those of controls (40 μg/g [30–70]), P < .001. Receiver operating characteristic curve analysis indicated a FC cut-off level of 71 μg/g, which differentiates ATTR amyloidosis with GI manifestations from healthy subjects with 91% sensitivity, 100% specificity, 100% positive predictive value, 95% negative predictive value and 97% overall accuracy. FC values were significantly associated with the presence of neutrophilic granulocytic infiltration in the colonic mucosa (P = .002), with the presence of amyloid deposits in rectal mucosa (P = .007) and the presence of diarrhea (P = .046).

FC levels are elevated in patients with ATTR amyloidosis with GI manifestations, which suggests an inflammatory component in the pathogenesis of the disease. The presence of elevated FC concentrations could help gastroenterologists to include ATTR amyloidosis in their diagnostic work-up.

Abbreviations: ATTR = transthyretin amyloid, CRC = colorectal cancer, FC = fecal calprotectin, GI = gastrointestinal, PND = polyneuropathy disability, POC = point-of-care, TTR = transthyretin.

Keywords: fecal calprotectin, intestinal inflammation, transthyretin amyloidosis

1. Introduction
Transthyretin amyloid (ATTR) amyloidosis is a rare systemic disorder characterized by amyloid deposits formed by misfolded monomers of the transthyretin (TTR); a plasma transport protein for thyroxine and vitamin A that is produced predominantly by the liver.[1] TTR fibrils accumulate in the extracellular spaces of a variety of tissues causing progressive cellular dysfunction that could lead to multi-systemic symptoms and could ultimately result in fatal complications within 10 to 15 years.[2,3]

ATTR amyloidosis is the most common form of hereditary amyloidosis and is caused by mutations that destabilize the TTR protein encoded by the TTR gene on chromosome 18.[4] There are over 120 different mutations described in this gene[5] with p. Val50Met being the most frequent variant.[6]

Based on clinical appearance, ATTR amyloidosis is often classified as a polyneuropathic (presents as a progressive, axonal sensory autonomic and motor neuropathy), a cardiac (infiltrative cardiomyopathy) or a mixed type of disease.[5] Furthermore, gastrointestinal (GI) manifestations are common in ATTR amyloidosis[7,8] and are associated with reduced quality of life.[8] In some cases, GI symptoms are present even before the onset of the polyneuropathy, and initial symptoms are often diarrhea, constipation, unintentional weight loss, or nausea.[8] Furthermore, the disease can present with many different forms with considerable phenotypic variation across individuals and
constipation. Patients with ATTR amyloidosis and an age of ≥50 years during symptom onset were defined as “late-onset cases” following the current clinical praxis. Each patient had 2 sex and age-matched healthy controls that were investigated at the same center and were selected for FC assessment. The FC levels of the controls were compared to those of the ATTR amyloidosis patients.

2.1. Clinical evaluation of ATTR amyloidosis patients

The presence of GI symptoms and the severity of peripheral neuropathy had been evaluated per protocol by 4 different investigators during a routine physical examination at the Department of Gastroenterology, Tsaritsa Yoanna University Hospital, and the Department of Neurology, Aleksandrovska University Hospital. The different GI symptoms that had been assessed were abdominal pain, nausea, vomiting, unintentional weight loss, constipation, diarrhea, and alternating diarrhea/constipation. Patients’ peripheral neuropathy was assessed by the polyneuropathy disability (PND) score that comprises of 5 stages – I (sensory disturbances but preserved walking capacity), II (impaired walking capacity but ability to walk without a stick or crutches), IIA (walking with the help of 1 stick or crutch), IIB (walking with the help of 2 sticks or crutches or a walker) and IV (confined to a wheelchair or bedridden). All therapies undertaken by the patients before their enrolment was recorded.

Throughout our diagnostic work-up, all the patients’ blood tests, cultures, abdominal ultrasonography, echocardiography, and upper and lower endoscopies with biopsies were performed. The presence of neutrophilic granulocytic infiltration in colonic mucosa was evaluated. Amyloid deposits were identified in rectal biopsies by Congo red staining in combination with polarization microscopy. Fecal samples were collected within 1 to 2 days before the colonoscopy for FC level assessment. The accurate diagnosis was made by genetic testing. TTR sequencing was carried out for the detection of specific amyloidogenic TTR mutations.

2.2. Clinical evaluation of controls

Patients that underwent a colonoscopy for CRC screening with a negative outcome were selected as healthy controls. None of them fulfilled the Rome IV criteria for functional GI disorders. The clinical examination of the controls was performed at the Department of Gastroenterology, Tsaritsa Yoanna University Hospital. FC levels were assessed in all the controls.

2.3. FC assessment

Calprotectin was analyzed in stool samples using the point-of-care desk-top Quantum Blue Reader (POC Reader) method. It is a lateral flow technology based on enzyme-linked immunosorbent assay techniques. The test was performed according to the manufacturer’s instructions (Quantum Blue Calprotectin, Bühlmann Laboratories AG, Switzerland). The POC reader uses internal criteria within a range of 30 to 300 μg/g and sensitivity of <10 μg/g; therefore, guarantying consistency in results. When we received results of >300 μg/g, we performed an additional 1:10 dilution with extraction buffer according to the manufacturer’s instructions, enabling us to receive FC levels of up to 3000 μg/g. FCP levels above the upper limit of the determination ranges were recorded as 3000 μg/g, and FCP levels below the lower limit were recorded as 30 μg/g.

2.4. Statistical analysis

The statistical analysis was performed using SPSS for Windows, Version 25.0. (SPSS Inc., Chicago, IL). Descriptive statistic for tabular and graphical presentation of results was used. Correlation analysis was performed using the Spearman and Pearson correlation coefficient. Mann–Whitney U nonparametric test was used to compare 2 sample means. The receiver operating characteristic (ROC) curves for FC levels were assessed to predict the diagnosis of ATTR amyloidosis. A P-value of <.05 was considered statistically significant.

2.5. Ethics approval

The present study was approved by the Ethics Committee of “Tsaritsa Yoanna” University Hospital in Sofia, Bulgaria. Before initiating this study, written informed consent was obtained from all subjects. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008), as reflected in a priori approval by the Institution’s Human Research Committee.
3. Results

This study enrolled 21 ATTR amyloidosis patients, of which 15 (71%) were males and of the 42 healthy controls, 30 (71%) were males. The mean age of FC assessment was 57 ± 8 (39–77) years. The mean delay in ATTR diagnosis was 3.31 years (0.42–7.25), and it did not correlate to FC levels. An overview of the demographic patient and control characteristics is provided in Table 1.

Fourteen (67%) of the patients carried the p.Glu89Gln mutation and 7 (33%) carried the non-Glu89Gln mutations: 5 patients with the p.Ser77Phe mutation, 1 with the p.Gly47Gln variant and 1 with the p.Val30Met. No significant difference was found between the mutation variant, sex, disease onset, or FC levels.

The routine clinical evaluations showed that all ATTR amyloidosis patients reported GI manifestations, most commonly diarrhea in 14 (67%) of cases. Constipation was reported from 4 (19%) patients, alternating diarrhea/constipation from 2 (9.5%), dyspepsia from 8 (38%) and abdominal pain from 5 (24%) patients. Unintentional weight loss was described in 16 (76%) of the ATTR patients. Abdominal ultrasonography described small bowel dilatation, increased intraluminal fluid, and enhanced peristalsis in 9 (43%) patients. Mann–Whitney U test showed that FC levels were significantly different between patients with diarrhea and those without diarrhea; U=22.5, z = -1.980, P=.046, using an exact sampling distribution for U. All the other GI findings were not statistically associated with FC levels.

The neurological examination showed that 12 (57%) of the patients had a PND score of I, whereas 6 (29%) patients had a PND score of II, and 3 patients had a score of IIIa. The PND score showed a moderate correlation with FC levels (r=0.573, P=.007).

The cardiovascular examination revealed infiltrative cardiomyopathy in 16 (76%) patients. Moreover, carpal tunnel syndrome was found in 6 patients (29%). These findings were not related to FC levels.

The mean levels of FC in ATTR amyloidosis patients were 184 μg/g (30–430), which proved to be significantly higher compared to those of the controls, which were 40 μg/g (30–70); U=36, z = -5.955, P<.001 (Fig. 1). ROC curve analysis indicated a FC cut-off level of 71 μg/g (area under the curve, 0.959; 95% confidence interval [CI], 0.883–1.000) for the differentiation of ATTR amyloidosis with GI manifestations from healthy subjects (Fig. 2). The sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy of the cut-off value of 71 μg/g for detecting ATTR amyloidosis with GI manifestations were 0.91 (95% CI, 0.78–1.03), 1.00, 1.00, 0.95 (95% CI, 0.89–1.02), and 0.97 (95% CI, 0.93–1.01), respectively. Furthermore, FC values were significantly associated with the presence of neutrophilic granulocytic infiltration in...

| Variable                  | ATTR amyloidosis patients (n=21) | Healthy controls (n=42) | P-value |
|---------------------------|---------------------------------|-------------------------|---------|
| Proportion of males       | 15 (71%)                        | 30 (71%)                | n.s.    |
| Age at FC assessment      | 57 (39–77)                      | 53 (40–72)              | n.s.    |
| Age at disease onset      | 54 (38–77)                      | N/A                     | N/A     |
| Early onset (n=5)         | 45 (38–40)                      | N/A                     | N/A     |
| Late onset (n=16)         | 57 (50–77)                      | N/A                     | N/A     |
| Delay in diagnosis        | 3.31 (0.42–7.25)                | N/A                     | N/A     |
| Mean FC levels            | 184 (30–430)                    | 40 (30–70)              | <.001   |

ATTR = transthyretin amyloidosis, FC = fecal calprotectin, n.s. = not significant, N/A = not applicable.
the colonic mucosa; \( U = 3, z = -2.781, P = .002 \), using an exact sampling distribution for \( U \) and with the presence of amyloid deposits in rectal mucosa; \( U = 14.5, z = -2.578, P = .007 \), using an exact sampling distribution for \( U \).

4. Discussion

GI manifestations are common in ATTR amyloidosis and are related to poor quality of life and poor prognosis.\(^{[8,18,19]}\) However, the pathogenesis is not entirely understood; it is generally suggested that GI symptoms arise due to motility disturbances of the GI tract caused by autonomic neuropathy.\(^{[20]}\) As a result, the destruction of autonomic nerves and depletion of enteric nerves occurs.\(^{[21,22]}\) Also, a decreased amount of intestinal endocrine cells and interstitial cells of Cajal were described.\(^{[23,24]}\)

This is the first study describing elevated FC levels in ATTR amyloidosis patients with GI manifestations compared to healthy controls, which suggests an inflammatory component in the pathogenesis of GI complications in this disease.

In our study, just one-third of the ATTR patients had amyloid inclusions detected in the rectal mucosa, which is unsurprising. In a retrospective observational study, Freudenthaler et al.\(^{[10]}\) showed that amyloid deposits were not commonly found in the mucosa of ATTR amyloidosis patients but were described in the submucosa of all the patients.\(^{[10,21]}\) Thus, ATTR amyloidosis carries the highest risk of a sampling error when the submucosal layers are not enclosed in the biopsy specimen.\(^{[10]}\)

The amyloid deposits in the submucosa could be a potential trigger for increased recruitment of polymorphonuclear neutrophils from the circulation to the intestinal mucosa and submucosa, consequently leading to intestinal inflammation. Similar to other inflammatory diseases of the colon, because of leukocyte shedding in the intestinal lumen, calprotectin can be detected and evaluated in feces.\(^{[13]}\) In our study, we show that the concentration of FC is directly proportional to the presence of neutrophilic infiltration in the gut mucosa.

As we excluded all other possible factors that could lead to elevated FC levels in our patients and we compared the FC levels of ATTR amyloidosis patients to sex and age-matched healthy controls, we believe that our results are reliable. The onset of the disease, the genetic variant, the sex, and the delay in diagnosis were not related to the level of FC concentrations. However, FC levels were higher in more advanced stages of the polyneuropathy of the patients, suggesting an increased inflammatory response within the intestines throughout the later stages of the disease. The most probable explanation of this is due to more intense amyloid deposits in the GI tract.

Most of the ATTR amyloidosis patients were referred to our center for accurate diagnosis and determination due to longstanding diarrhea. When the patient develops continuous diarrhea, the condition is challenging to treat due to several factors such as severe malabsorption of fat and bile acids as well as the abiding presence of bacterial overgrowth of the small bowel.\(^{[19]}\)

Nonetheless, we showed that ATTR amyloidosis patients with diarrhea had higher FC levels than those without diarrhea, which could mean that inflammation also plays a role in the multifactorial pathogenesis of diarrhea in ATTR amyloidosis. Consequently, additional treatment strategies could be discussed.

ATTR diagnosis is a real challenge for the modern clinician, especially the gastroenterologist, due to the relatively rare GI onset of the disease and the broad differential diagnosis. The disease can easily be misdiagnosed with many other diseases of the GI tract. There has never been a blood or stool marker described that could help us with the diagnosis of ATTR amyloidosis with GI manifestations. In this study, we show that FC levels above 71 μg/g and the presence of specific clinical settings (diarrhea/constipation and cardiomyopathy and/or polyneuropathy; unintentional weight loss in a patient with neuropathy and/or cardiomyopathy and familial history) could lead the gastroenterologist to the correct diagnosis and help him decide whom to send for genetic testing.

One of the limitations of the study is the small sample size due to the difficulties of enrolling a significant cohort of patients with a rare disease. Another limitation is the lack of immunohistochemistry of TTR deposits in the study as this method is not available in our country. Future international studies on bigger cohorts are needed to evaluate the role of FC in the diagnosis of ATTR amyloidosis with GI manifestations. Furthermore, it would be valuable to compare the FC concentrations in ATTR patients with those of patients with other inflammatory GI diseases.

In conclusion, FC levels are elevated in patients with ATTR amyloidosis with GI manifestations, which suggests an inflammatory component in the pathogenesis of the disease. ATTR amyloidosis is frequently misdiagnosed with many other diseases and leads to diagnostic challenges in GI practice. The presence of elevated FC concentrations could help gastroenterologists to include ATTR amyloidosis in their diagnostic work-up.

Author contributions

Radislav Nakov, Stayko Sarafov and Ventsislav Nakov designed the study, enrolled and followed up the patients, performed statistical analyses, collected and analyzed the data and wrote the manuscript. Mariana Gospodinova, Tihomir Todorov, Gianluca Ianiro, and Albena Todorova were responsible for patient inclusion and evaluation in the study, enrolled and followed up the patients, performed the histopathological evaluation and wrote the manuscript. Radislav Nakov orcid: 0000-0002-5056-8417.

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