


Intestinal involvement in amyloidosis is a sequential process

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Abstract

Background: Gastrointestinal amyloidosis causes dysmotility. A comprehensive histological analysis to explain these symptoms is lacking. Therefore, we systematically examined histological features of intestinal dysmotility in patients with AL and AA amyloidosis, compared to controls.

Methods: Autopsy tissue material from small bowel and colon was used for histological (semiquantitative) evaluation of the mucosa, blood vessels, muscular layers, enteric nervous system (ENS) and the interstitial cells of Cajal (ICC), using hematoxylin and eosin, periodic acid Schiff, Elastic von Gieson and Congo red staining, and immunohistochemistry with α -smooth muscle actin, HuC/D, S100 and CD117 antibodies, according to guidelines of the Gastro 2009 International Working Group.

Key Results: Amyloid deposits were present in the vascular walls of all amyloidosis patients. In the mucosa, amyloid was found in 67% of AA patients. The muscular layers were involved in 64% of amyloidosis patients, most prominent in AA patients, associated with the presence of polyglucosan inclusion bodies, but not with either abnormal α -actin patterns or fibrosis. Amyloid in the muscularis propria surrounding the myenteric plexus was found, but not inside the myenteric plexus. These deposits might be related to loss of the ICC network, but there was no association with decreased neuronal or nerve fiber density.

Conclusions & Inferences: We hypothesize that intestinal dysmotility in amyloidosis patients is a sequential process: amyloid deposition starts in the vasculature, followed by involvement of the muscular layers, ICC loss, and potentially affect the myenteric plexus. This final stage may be accompanied by clinical symptoms of severe intestinal dysmotility.

KEYWORDS

amyloid, amyloidosis, gastrointestinal tract, histology, intestinal dysmotility

Abbreviations: ENS, enteric nervous system; EVG, Elastic von Gieson; GI, gastrointestinal; H&E, hematoxylin and eosin; ICC, interstitial cell of Cajal; PAS, periodic acid Schiff; SMA, smooth muscle actin.

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1 | INTRODUCTION

Amyloidosis is a diverse group of disorders characterized by extracellular abnormal fibrillar protein deposits in various tissues, including the gastrointestinal (GI) tract, resulting in tissue damage caused by several mechanisms.^{1,2}

Amyloid deposits can be found in all layers and structures of the bowel wall, including musculature, vasculature, and neural structures. In the muscles, this leads to pressure atrophy, while in vessel walls amyloid deposits result in ischemia and infarction.³ Different types of amyloidosis show different damage patterns.⁴ AL amyloidosis, that is, light chain amyloid associated with B cell dyscrasias, supposedly shows a preference for the muscularis mucosae and muscularis propria, resulting in myopathy,⁵⁻⁷ which causes constipation, mechanical obstruction or chronic intestinal pseudo-obstruction.⁶⁻⁸ In AA amyloidosis, associated with inflammatory disorders, fine granular amyloid deposits are mainly found in the lamina propria (mucosa), resulting into diarrhea and malabsorption, and in the myenteric plexus, leading to neuropathy.^{5,6,8} Hence, both myopathy and neuropathy may be the underlying cause of intestinal dysmotility. Amyloidosis of the GI tract can therefore be classified as a secondary intestinal motility disorder.⁹ Intestinal dysmotility is present in 10-70% of patients with AL and AA amyloidosis¹⁰; however, the literature is mainly restricted to case reports, rarely larger patient series are described.

Guidelines for studying the intestinal neuromusculature have been presented by the Gastro 2009 International Working Group.⁹ The present study is the first study using these guidelines for histological evaluation of tissues from amyloidosis patients. The aim was to perform a systematic explorative study of the histological characteristics of intestinal dysmotility in amyloidosis patients, in order to develop insights in the mechanisms.

2 | METHODS

2.1 | Subjects

Archived autopsy formalin-fixed paraffin-embedded tissue samples of segments of small bowel (n = 10) and colon (n = 12) were obtained from amyloidosis patients selected for the presence of amyloid deposits in the intestinal wall. Complete autopsy records were available in all patients; and patient dossiers were available in 11 of 14 patients. Amyloidosis patients were included in the AL or AA group, based on the diagnosis described in the autopsy reports and patients

Key Points

- We performed a comprehensive and systematic histological analysis to explain intestinal symptoms of dysmotility in amyloidosis patients, since this is currently lacking.
- Amyloid deposits were found in blood vessels (all patients with AL and AA), the mucosa (AA), and in the muscular layers (AL and AA) around the myenteric plexus, probably related to loss of interstitial cells of Cajal.
- We hypothesize that intestinal dysmotility in amyloidosis patients is a sequential process.

dossiers. Amyloid A antibody (Table 1) was used to confirm the diagnosis AA or non-AA amyloidosis. Control autopsy material of ileum (n = 22) and colon (n = 23) was obtained from patients without intestinal motility problems (according to the pathology reports) and considered as normal.

The study was approved by the local ethics committee (reference number 2014-1256). Samples were obtained in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands.¹¹

2.2 | Tissue preparation

Sections were cut from formalin-fixed paraffin-embedded full-thickness tissue blocks for conventional histology or immunohistochemistry. Sections were deparaffinized, rehydrated in xylene and ethanol series, and rinsed in tap water by standard protocol.

2.3 | Histological staining

Sections of 4 μ m were used for hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining. Elastic von Gieson (EVG) and Congo red staining were performed on 6- μ m sections. Tissues were stained by standard protocols in a Medite TST 30 stainer (Klinipath, Duiven, The Netherlands).

2.4 | Immunohistochemistry

Immunohistochemical staining was performed on 4- μ m sections. Antibodies, suppliers, and dilutions are listed in Table 1.

TABLE 1 Primary antibodies used for immunohistochemistry

Antibody	Clone	Manufacturer	Dilution	Antigen retrieval
Amyloid A	Reu-86.2	Monosan, Sanbio	1:80	None
HuC/D	16A11	Molecular Probes	1:600	Sodium citrate 10 mmol/L (pH 6.0) 30 min at 100°C
S100	POLYCLONAL	DAKO	1:10 000	EDTA pH 9 10 min at 96°C
CD117	YR145	Immunologic	1:200	None
α -smooth muscle actin (α -SMA)	1A4	Sigma	1:7500	None

For HuC/D staining, antigen retrieval was performed in sodium citrate (pH 6) at 100°C for 30 minutes. Subsequently, endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 20 minutes. Sections were then rinsed in PBS and incubated with primary antibody anti-HuC/D at 4°C overnight. After washing in PBS, sections were incubated for 30 minutes with a secondary antibody (Poversion poly-HRP anti-Ms/Rb/Rt IgG, Immunologic, Duiven, The Netherlands) at room temperature. Subsequently, sections were rinsed in PBS and immunoreactivity was developed with PowerDAB (Immunologic) for 7 minutes at room temperature. Finally, sections were rinsed in tap water, counterstained with hematoxylin, rinsed in tap water, dehydrated in 100% ethanol and xylene, and mounted with Permount.

The other immunohistochemical staining reactions were performed in an automated LabVision Autostainer 480 (Klinipath, Duiven, The Netherlands). The method used for antigen retrieval depended on the antibody (Table 1). Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Sections were incubated with primary antibody for 60 minutes. Subsequently, the sections were incubated with Poversion poly-HRP anti-Ms/Rb/Rt for 30 minutes, followed by staining with PowerDAB for 7 minutes and counterstaining with hematoxylin for 1 minute. All incubations were performed at room temperature.

Tissue blocks containing different tissue types were used as controls for the immunohistochemical staining reactions, with known staining patterns for both positive and negative stained tissues.

2.5 | Microscopic analysis

Sections were evaluated blind to diagnosis by two observers. The histology of the bowel wall was examined by H&E. PAS was used to verify the presence or absence of polyglucosan inclusion bodies in the muscularis propria. The presence or absence of fibrosis was assessed in the submucosa, muscularis propria, and myenteric plexus on EVG-stained sections. Congo red stained sections were used to evaluate the presence of amyloid deposits in the mucosa, submucosa, muscularis propria, and vascular walls.

Immunohistochemically stained sections were assessed by semiquantitative scoring using visual analysis to evaluate systematically the smooth muscle layers and neuronal structures, as previously described.¹² α -Smooth muscle actin (α -SMA) staining was used to assess the muscular layers. Staining intensities of circular and longitudinal muscle layers were classified in two grades: 0 (no/weak) and 1 (strong staining intensity).¹² The presence of neurons in ganglia was analyzed on HuC/D-stained sections. The number of neurons in relation to the present plexus was estimated in HuC/D sections as follows: 0 (no neurons), 1 (low neuronal density), and 2 (high neuronal density). S100 was used to assess the distribution of nerve fibers (including nuclei of glial cells) in the submucosa, the myenteric plexus, and both muscle layers of the muscularis propria. The degree of distribution was scored as follows: 0 (no/low density) and 1 (high density of positive fibers).¹² The network of interstitial cells of Cajal (ICCs) surrounding the myenteric plexus was estimated

on CD117-stained sections as described earlier.¹³ The percentage of the circumference which is covered by CD117-positive cells was rated from 0% to 100% in 10% increments. Thus, a percentage of 0% represented no positive cells around the ganglia and in sections estimated as 100% the ganglia were completely surrounded by CD117-positive cells.

2.6 | Statistical analysis

Categorical variables were described by percentages. Differences between categorical variables were assessed by the chi-squared test (likelihood ratio, exact *P*-values compared with the control group). Continuous variables were presented as means \pm standard deviation (SD). The Mann-Whitney test was performed to compare the CD117 scores between groups. A *P*-value of 0.05 was considered significant. Data were analyzed by the IBM SPSS Statistics 22 Software (SPSS Inc., Chicago, IL, USA), GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA), and RStudio version 1.1.419 (RStudio, Inc., Boston, MA, USA).

3 | RESULTS

3.1 | Patients

Tissue material of 8 AL amyloidosis patients (mean age 66 ± 13 years, 88% male), 6 AA amyloidosis patients (mean age 57 ± 20 years, 100% male) and 26 control patients (mean age 65 ± 14 years, 58% male) was evaluated in this study. The AL amyloidosis group ($n = 8$) consisted of 5 small bowel and 7 colon cases, from 4 patients tissues of both locations were analyzed. The AA amyloidosis group ($n = 6$) included 5 small bowel and 5 colon tissues, from 4 patients tissues of both locations were assessed. In the control group ($n = 26$), 22 small bowel and 23 colon cases were included.

Among the patients with amyloidosis, gastrointestinal bleeding was reported in two AL patients and in two AA patients. Ileus was reported in one AL and three AA cases, of which one patient with severe dysmotility of the esophagus. Clinical symptoms of gastrointestinal involvement of the other patients were absent or unknown (Table 2).

3.2 | Amyloid deposition

The presence of amyloid deposits in the mucosa, muscularis mucosae, muscularis propria, myenteric plexus and vascular walls was assessed by Congo red staining (Figure 1). Overall, amyloid deposits were more frequently found in both the mucosa and muscularis mucosae of AA amyloidosis compared to AL amyloidosis, no amyloid was present in the control group. There was no difference between small bowel and colon tissues of the same patients. Amyloid deposits were present in all layers of the intestinal wall, except inside the myenteric plexus. All cases with amyloidosis showed amyloid deposits in the vascular walls, most prominent in the submucosa. In three patients, extravasation of erythrocytes was present in the

TABLE 2 Overview of histological findings, clinical background and dysmotility per amyloidosis patient, small bowel and colon are merged

Amyloidosis type	Patient	Amyloid blood vessels		Amyloid mucosa		Amyloid m. mucosae		Amyloid m. propria		ICC loss ^a		Nerve fiber decrease ^b		Neuronal decrease ^b		Clinical background	Clinical described dysmotility
		Blood vessels	Mucosa	Amyloid mucosa	m. mucosae	M. propria	M. propria	ICC	ICC	Myenteric plexus	Myenteric plexus	Clinical background	Clinical described dysmotility				
AL	1 ^c	+			+	+	+	+	+	+	+	+	+			Unknown	
	2 ^c	+			+	+	+	+	+	+	+	+	+			Plasma cell dyscrasia	
	3	+			+	+	+	+	+	+	+	+	+			Multiple myeloma	
	4	+			+	+	+	+	+	+	+	+	+			Paraproteinemia	
	5	+			+	+	+	+	+	+	+	+	+			Multiple myeloma	
	6	+			+	+	+	+	+	+	+	+	+			Unknown	Ileus
	7 ^c	+			+	+	+	+	+	+	+	+	+			Unknown	
	8	+			+	+	+	+	+	+	+	+	+			Multiple myeloma	
AA	1	+			+	+	+	+	+	+	+	+	+			Crohn	Ileus
	2	+		+	+	+	+	+	+	+	+	+	+			Familiar gout	Ileus, dysmotility esophagus
	3	+		+	+	+	+	+	+	+	+	+	+			Rheumatoid arthritis	
	4	+		+	+	+	+	+	+	+	+	+	+			Bone tuberculosis	Ileus
	5	+		+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.			Rheumatoid arthritis	
	6	+		+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.			Rheumatoid arthritis	

n.a., not assessable, ICC, interstitial cell of Cajal.

^aPercentage ICC surrounding myenteric plexus 0-30% in small bowel and 0-10% in colon.

^bIn the myenteric plexus, semiquantitative score 0.

^cOnly autopsy reports were available.

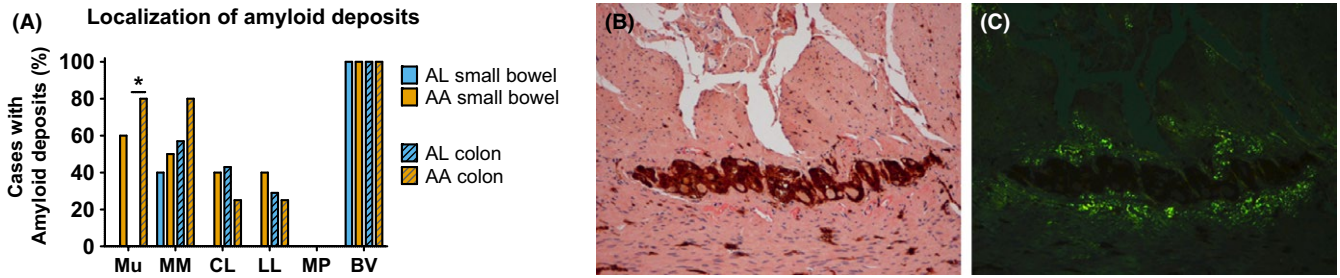


FIGURE 1 Amyloid deposition in the bowel wall. A, Localization of amyloid deposits in various layers of the intestinal wall. AL compared to AA amyloidosis, in small bowel and colon tissues. B and C, Amyloid in the smooth muscle layers surrounding the myenteric ganglion in an AA patient, double staining S100 (brown) and Congo red (clear red in B and apple green in C). Mu mucosa, MM muscularis mucosae, CL circular layer, LL longitudinal layer, MP myenteric plexus, BV blood vessel walls. * $P < 0.05$ vs control

submucosa (one colon AL, two small bowel AA), but there were no signs of chronic blood loss (hemosiderin deposits). Mucosal presence of amyloid was most pronounced in the colon of AA patients (80% vs 0% in AL patients, $P = 0.015$). A comparable trend was found in the small bowel (60% vs 0%, $P = 0.167$). No other differences were shown between the AL and AA groups (Figure 1).

3.3 | Bowel musculature

The presence of amyloid in the musculature of the bowel wall did not result in aberrant staining patterns of α -SMA in small bowel or colon (Figure 2), which would suggest that no significant α -actin filament abnormalities were present in these patients. Polyglucosan inclusion bodies, as a sign of myopathy, were present in the small

bowel ($n = 3$) and colon ($n = 1$) of patients with amyloidosis, but there was no consistent relation with the presence of muscular amyloid depositions (Figure 2). Some control patients also presented with polyglucosan bodies in small bowel ($n = 3$) and colon ($n = 1$). There was no relation between the presence of fibrosis and amyloid (Figure 2).

3.4 | Enteric nervous system

No amyloid deposits were found inside the myenteric plexus, in some cases, amyloid deposits were present in the smooth muscle tissue around this plexus (Figure 1). The presence of neurons relative to nerve fibers was evaluated by HuC/D staining, and the nerve fiber density was assessed by S100 staining. In both the small bowel

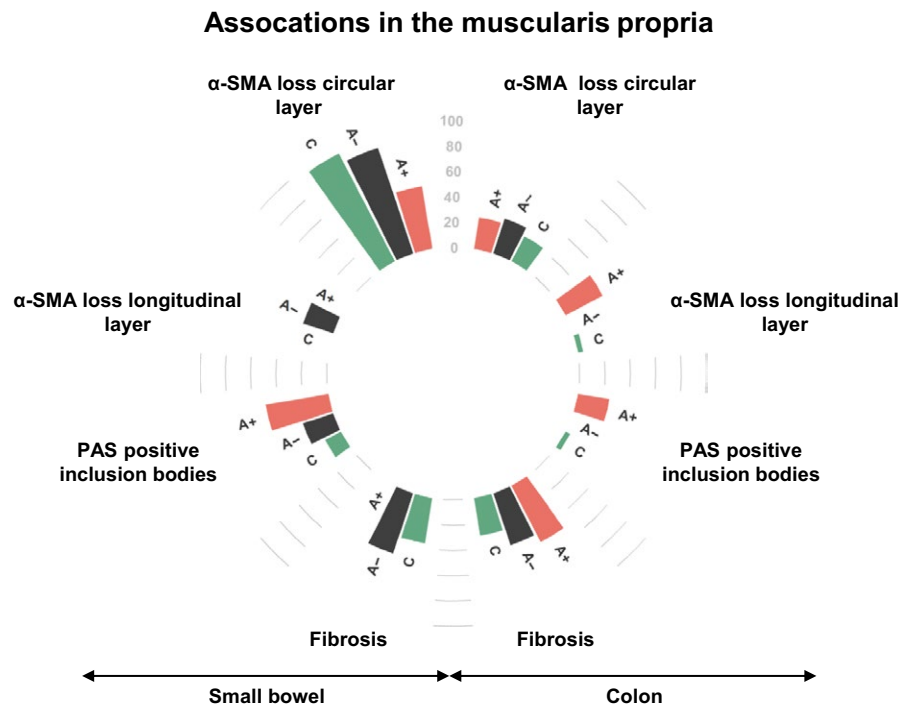


FIGURE 2 Circular plot of aberrations in the musculature of the bowel wall in association with amyloidosis in small bowel (left side) and colon (right side). Amyloidosis patients were divided according to the presence of amyloid deposits in the musculature (A+: present, A-: absent). Percentage of cases are given. C, control; PAS, periodic acid Schiff staining; SMA, smooth muscle actin

and colon, no differences were found between the control, AL and AA groups in the submucosal and myenteric plexus as well as in the muscularis propria (Figure 3A).

3.5 | Myenteric ICC network

Lower percentages of the CD117 positive ICC network surrounding the myenteric plexus were found in the amyloidosis groups compared to the control group, both in the small bowel and colon (Figure 3B,C). In the colon, the density of the ICC network was significantly lower in the AL group compared to controls ($P = 0.019$). Patients with amyloid deposits in the muscularis propria seemed to have a reduced ICC network (red points in Figure 3B). In the small bowel of the AL group, no amyloid in the muscularis propria was present, while in two cases of the AA group amyloid was found in the muscularis propria of the small bowel (percentage ICCs around the myenteric plexus 0% and 30%, red points in Figure 3B). In the colon, amyloid deposits in the muscularis propria were found in three AL patients (percentage ICCs 0%), and in one AA patient (percentage ICCs 10%).

4 | DISCUSSION

The objective of this study was to investigate the mechanisms that cause intestinal dysmotility in amyloidosis patients, by analyzing histological features in a systematic way. Histological findings for individual amyloidosis patients are summarized in Table 2. Amyloid deposits were present in the vascular walls of all amyloidosis patients, regardless of type, most prominent in the submucosa. This may contribute to intestinal dysmotility by causing ischemia and increased permeability.¹⁴ Gastrointestinal bleeding might be caused by disruption of blood vessel walls as a result of amyloid deposition,^{15,16} which was reported in four of our patients.

Endoscopic examination of amyloidosis patients frequently shows a granular and friable appearance of the mucosa,^{8,17,18} which can be correlated with amyloid deposits, as was present in our study in 67% of AA patients. Involvement of the mucosa can lead to diarrhea and malabsorption.^{15,17} It should thus be noted that endoscopic biopsies taken to determine the presence of amyloid, should also include submucosal tissue. When only the mucosa is examined,

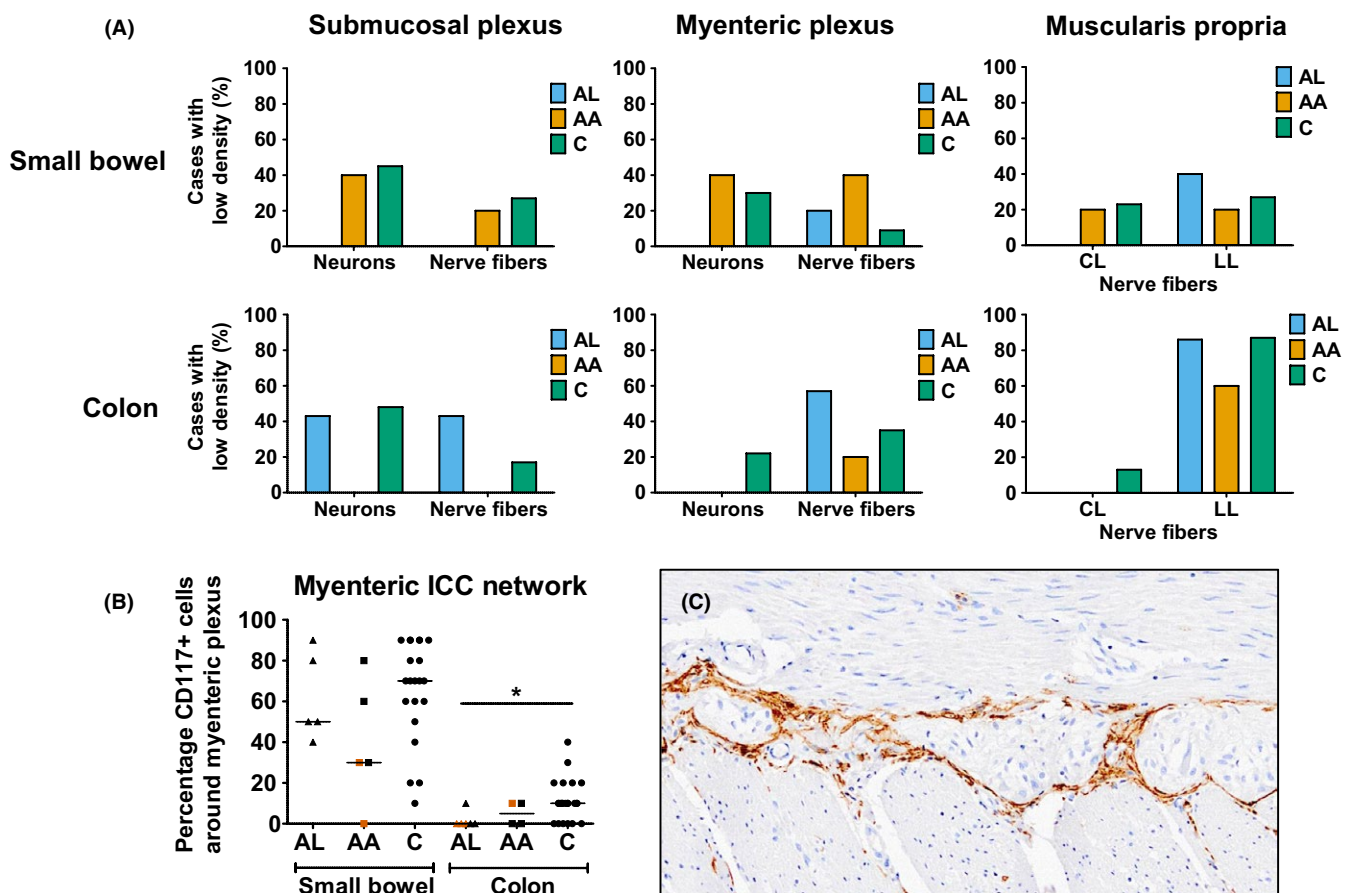


FIGURE 3 Histological findings in the enteric nervous system and the ICC network. A, The density of neurons and nerve fibers was estimated by HuC/D and S100 immunohistochemical staining, respectively. Results for small bowel and colon showed no differences between the AL, AA and control groups. B, Percentage of CD117 positive cells (ICCs) around the myenteric plexus. The black points represent cases without amyloid in the muscularis propria, the red points cases with amyloid in muscularis propria. Horizontal lines show the medians. C, Picture of the positive stained ICC network around the myenteric plexus shows control tissue of small bowel. The percentage in this picture was scored as 100%. AL, AL amyloidosis, AA, AA amyloidosis, C, control group, CL, circular layer of muscularis propria, LL, longitudinal layer of muscularis propria, ICC, interstitial cells of Cajal. * $P < 0.05$ vs control

particularly in AL cases, the results are unreliable. Muscular involvement was present in 64% of amyloidosis patients, with deposits in all muscular layers, again most pronounced in AA patients. The presence of amyloid deposits in the muscularis propria tended to be associated with the presence of polyglucosan inclusion bodies, which may be related to symptoms of intestinal dysmotility and constipation,¹⁹ although this could not be confirmed in our study. The presence of amyloid deposits was not related to either abnormal α -actin patterns or fibrosis, suggesting that amyloid-induced muscular failure is not because of primary myopathy, but more likely due to mechanical failure.

Histological signs of direct neuropathy could not be found in this study. No amyloid was found inside the myenteric plexus. The presence of amyloid in the smooth muscle surrounding the plexus, which has been suggested to cause secondary degeneration or loss of neurons,⁵ was not associated with decreased neuronal or nerve fiber density in our study. Based on our observations, we reject the hypothesis that that intestinal pseudo-obstruction is caused by myopathy in AL amyloidosis and neuropathy in AA amyloidosis.^{5,20} This hypothesis is based on the study of Tada et al, where all patients were on total parenteral nutrition because of severe intestinal pseudo-obstruction.⁵ These patients are very likely in a late stage of amyloidosis induced motility disorder, where distinction of different mechanisms might be hard. In our study, only one AA patient received total parenteral nutrition, which was given because of dysmotility of the esophagus. This patient showed amyloid deposits in

the mucosa and muscle layers of the intestines. Although no amyloid was found in the myenteric plexus, the ICC network and nerve fiber network were reduced.

We propose an alternative hypothesis about intestinal dysmotility in patients with amyloidosis (Figure 4). Firstly, amyloid deposition occurs in the vascular walls, this was shown in all our amyloidosis patients and is commonly found in literature.^{14,21,22} Secondly, amyloid may accumulate in the mucosa and muscular layers of the bowel, decreasing motility by infiltration the smooth muscle tissue.²³⁻²⁵ Thirdly, involvement of the muscular layers may result in loss of the myenteric ICC network, which may lead to dysmotility.²⁶ A study into transthyretin amyloidosis patients (ie, a third form of amyloidosis, non-AA, non-AL) reported loss of the ICC network in the muscularis propria of the stomach without degeneration of the enteric nervous system (ENS).²⁷ By our knowledge, this is the only study into the ICC network in amyloidosis patients. Neither degeneration nor loss of the ENS was found in our study, suggesting that loss of ICCs may precede damage of the ENS in amyloidosis patients. Therefore, neurodegeneration and neuronal loss may occur in the final stage of intestinal dysmotility, although this might well be a secondary phenomenon. This sequence would explain intestinal dysmotility in both AL and AA amyloidosis, but there are obvious differences between the subtypes. In AL amyloidosis, endoscopic findings seem to be related to muscular involvement resulting in constipation and intestinal pseudo-obstruction, while in AA amyloidosis a fine granular appearance and

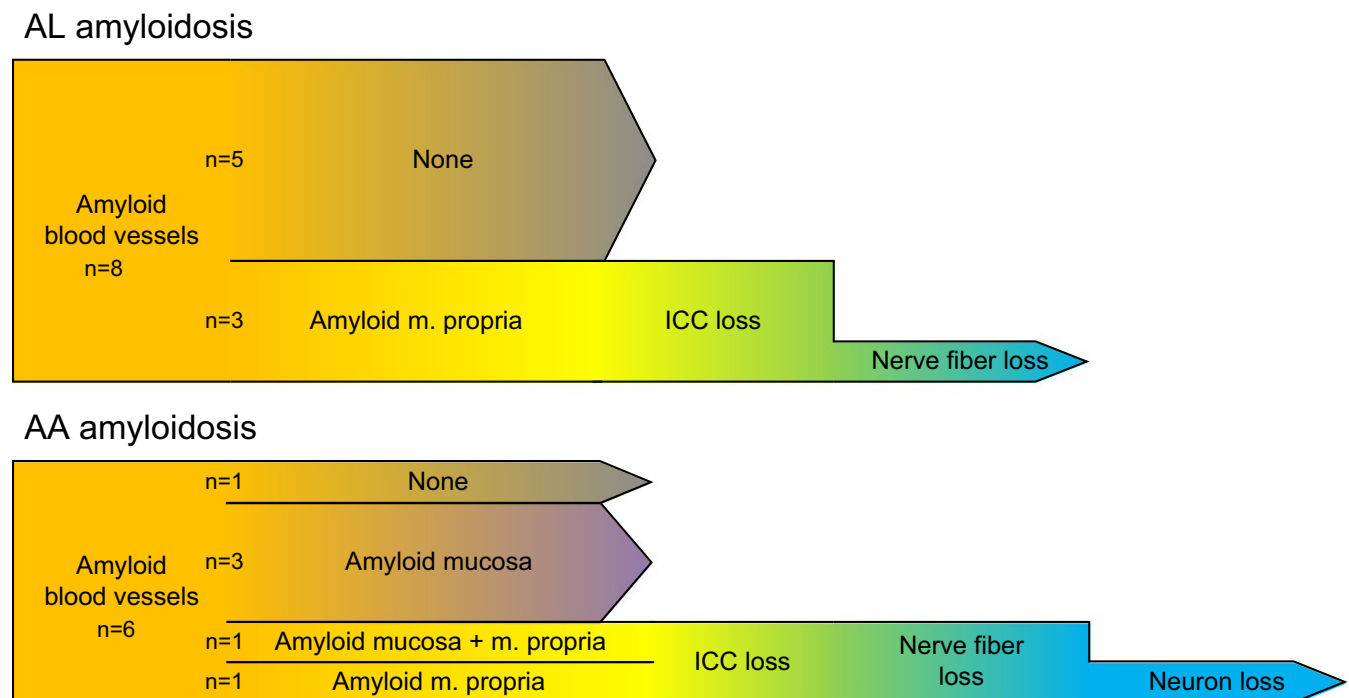


FIGURE 4 Hypothesis about the sequential events that cause intestinal dysmotility in amyloidosis patients. The process may start with amyloid deposition in the blood vessel walls, which was found in all AL and AA amyloidosis patients, followed by mechanical disruption of the smooth muscles, loss of ICC, and potential involvement of the myenteric plexus (reflected in nerve fiber or neuronal loss). Amyloid deposits were found in the mucosa of AA patients, which may cause symptoms of diarrhea and malabsorption, but is not directly related to bowel dysmotility. "None" represents cases without amyloid in both mucosa and muscularis propria. ICC, interstitial cells of Cajal

	AL	AA
Precursor protein	Immunoglobulin light chain	Serum amyloid A
Organs involved	Kidneys, heart, digestive system, nervous system	Kidneys, gastrointestinal tract, liver, heart (rare)
Clinical presentation	Constipation, mechanical obstruction, chronic intestinal pseudo-obstruction, bleeding	Diarrhea, malabsorption, weight loss, bleeding
Endoscopic findings	Polypoid protrusions and thickening of the folds	Fine granular appearance and mucosal friability

TABLE 3 Comparison of AL and AA amyloidosis^{4,8,17}

friability of the mucosa might be related to diarrhea and malabsorption (Table 3).

Our study had several limitations. The study was performed on autopsy material. Hence, the quality of the material was not optimal in some cases, for instance as a result of autolysis of the mucosal layer or imperfect embedding. Therefore, only tissues with all layers of the bowel wall present were included. Scoring of clinical dysmotility was difficult due to limited information in the medical dossier about symptoms related to intestinal dysmotility. Severe dysmotility was described in one AA patient (dysmotility esophagus, ileus) and may be related to extensive amyloid deposition, ICC loss, and reduction of nerve fibers. Loss of nerve fibers in the myenteric plexus may have contributed to ileus in three other patients, although in two of these, the ileus could have been a result from earlier bowel resections. The available endoscopic information was insufficient to conclude anything about the relation between microscopic and macroscopic changes. Our study was aimed on exploration of sequential events and forms the basis for future research: larger patients groups and more tissue per patients are required. In those studies, histological examination should be combined with physiological and endoscopic data and accurate knowledge of clinical symptoms of intestinal dysmotility. In this way, the proposed sequential pathogenesis of intestinal involvement in amyloidosis can be investigated more precisely. The AL type of amyloidosis could not be confirmed in three patients in the AL group, but these patients were non-AA (negative amyloid A staining) and non-hereditary transthyretin amyloidosis (Dutch study population²⁸). Excluding these three cases from analysis did not affect the results. The degree of amyloid deposition in AA amyloidosis may be affected by treatment of the underlying disease (in all but one patient), which could have suppressed the acute phase response resulting in reduction of the production and deposition of serum amyloid A protein.

In conclusion, the main initial histological finding in patients with amyloidosis was deposition of amyloid in the vasculature. The most important sign of muscular damage was the presence of amyloid deposits in the musculature, no primary myopathy or neuropathy was detected. We hypothesize that the involvement of the intestines is a sequential process: deposition of amyloid in the vasculature, followed by mechanical disruption of the smooth muscles, loss of ICC, and potential involvement of the myenteric plexus. This final stage may be accompanied by clinical symptoms of severe intestinal dysmotility.

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COMPETING INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

MBY collected the tissue sections; MBY and IDN designed the study; MBY and SH performed the microscopic assessments; SH collected information from the patient dossiers; MBY analyzed the data and wrote the manuscript; all authors participated in discussing the content of the manuscript, and read and approved the final manuscript.

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