Detection of Alkaline Sphingomyelinase Activity in Human Stool: Proposed Role as a New Diagnostic and Prognostic Marker of Colorectal Cancer

Luisa Di Marzio,1 Alfredo Di Leo,2,3 Benedetta Cinque,4 Donatella Fanini,4 Alessio Agnifili,5 Pasquale Berloco,2 Michele Linsalata,2 Dionigi Lorusso,2 Michele Barone,3 Claudio De Simone,4 and Maria Grazia Cifone4

1Department of Drug Science, University G. D’Annunzio, Chieti, Italy; 2Laboratory of Biochemistry, Istituto di Ricovero e Cura a Carattere Scientifico “S. de Bellis”; Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy; and Departments of 4Experimental Medicine and 5Surgical Sciences, University of L’Aquila, L’Aquila, Italy

Abstract

Objectives: Intestinal alkaline sphingomyelinase, by exerting a major role in dietary sphingomyelin digestion, is responsible for the generation of messengers able to trigger the rapid turnover and apoptosis in intestinal epithelial cells. Markedly reduced mucosal alkaline sphingomyelinase activity has been associated with human colorectal neoplasms. The aim of this study was to analyze the alkaline sphingomyelinase activity in feces from healthy subjects and colorectal adenocarcinoma patients and to correlate it with the enzyme activity in intestinal tissues.

Materials and Methods: The enzyme activity was measured both in the intestinal samples from 12 healthy controls and 51 patients with colorectal adenocarcinoma (tumoral and paratumoral tissue) and in the fecal samples of 34 healthy subjects and 29 patients with adenocarcinoma. The relation between sphingomyelinase activity and Dukes’ stage, cell differentiation degree, age, and gender was also analyzed.

Results: Alkaline sphingomyelinase was significantly decreased (P < 0.001; mean reduction >90%) in tumoral intestinal mucosa of patients compared with controls independently of Dukes’ stage and tumor differentiation grade. Interestingly, the enzyme activity in histologically normal paratumoral tissues was statistically lower than control samples (P < 0.001). As occurs in neoplastic tissues, a relevant mean reduction (P < 0.0001; almost 90%) of alkaline sphingomyelinase was revealed in stool samples from tumor patients when compared with controls.

Conclusion: These findings may have implications for cancer biology and perhaps also for the design of clinical test, thus suggesting that the fecal sphingomyelinase activity could reflect the human intestinal mucosa enzyme level and could represent a new marker for human colorectal adenocarcinoma, mainly taking into account its early appearance in intestinal neoplasms. (Cancer Epidemiol Biomarkers Prev 2005;14(4):856–62)

Introduction

Sphingomyelinase is a lysosomal acid sphingomyelinase, a secreted enzyme specifically present in the gastrointestinal tract of animals and humans, with high activity in the mid small intestine and low activity in the colon. Recently, Duan et al. (10) have purified alkaline sphingomyelinase from human intestine and characterized its location in the mucosa, expression in colon cancer, and function on colon cancer cells. Its molecular mass is 60 kDa, optimal pH is 8.5, and isoelectric point is 6.6. The biochemical properties of the enzyme are similar to those of rat intestinal alkaline sphingomyelinase but not to those of bacterial neutral sphingomyelinase (11). The same authors have recently identified the cDNA and amino acid sequences of human isofrom that could be inhibited by factors that inhibit nucleotide phosphodiesterase, such as imidazole, orthovanadate, and ATP (12). Intestinal alkaline sphingomyelinase has a role both in the digestion of dietary sphingomyelin (13, 14) and in the hydrolysis of membrane-bound sphingomyelin (15). The generation of ceramide and sphingosine from catabolized sphingomyelin in the intestine has also been suggested to be implicated in gastrointestinal development during gestation and in the neonatal period in the rat (16-18). Sphingomyelin metabolism in the gut may have an effect on cancer development. Indeed, intestinal alkaline sphingomyelinase has been reported to be down-regulated in human colorectal cancer (10). The first report showing the relation between sphingomyelin hydrolysis and colon cancer was provided by Dudeja et al. (19). These authors showed that the administration of 1,2-dimethylhydrazine, a chemical colon
carcinogen, altered the levels of sphingomyelin in the rat colonic plasma membrane. Moreover, Dillehay et al. (20) showed a reduction of aberrant colonic crypt foci (an early stage of colon carcinogenesis) in 1,2-dimethylhydrazine–treated mice fed on a diet of purified bovine sphingomyelin. Similar results were also obtained by Schmelz et al. (21, 22), who also suggested that the sphingomyelin-enriched diet suppresses the conversion of adenomas into adenocarcinomas. The study of Hertervig et al. (23) showed, for the first time, a reduction of activities of all acid, neutral, and alkaline sphingomyelinase in colorectal cancer; the activity of alkaline sphingomyelinase was decreased by 75% in carcinoma tissue compared with adjacent normal mucosa. A significant reduction of alkaline sphingomyelinase was also found in other intestinal diseases including colorectal adenomas and familial adenomatous polyposis, the enzyme activity being decreased by 50% and 90%, respectively (15, 24). Hertervig et al. (25) reported evidence that alkaline sphingomyelinase is decreased by 50% and 90%, respectively (15, 24).

Taken together, the above observations suggest a role for alkaline sphingomyelinase in the gut pathophysiology. In this context, it would be particularly useful to show that the levels of the alkaline sphingomyelinase present in the feces correlate with those found in the pathologic intestinal mucosa. To this aim, we have examined the alkaline sphingomyelinase activity in both intestinal biopsies and fecal samples of healthy subjects and patients with colorectal adenocarcinoma.

Materials and Methods

Patients and Clinical Study Design. The study was approved by the Technical and Scientific Committee of the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) “Saverio de Bellis” (Castellana Grotte, Bari, Italy; verb. no. 52/99 of March 17, 1999) and conform to the Declaration of Helsinki. Two populations were included in the study: patients with colorectal adenocarcinoma and controls. Participants in the study were from IRCCS “Saverio de Bellis” (Castellana Grotte, Bari, Italy; verb. no. 52/99 of March 17, 1999) and conform to the Declaration of Helsinki. Two populations were included in the study: patients with colorectal adenocarcinoma and controls. Participants in the study were from IRCCS “Saverio de Bellis”. As reported in Table 1, a total of 85 subjects, of which 51 was affected by colorectal adenocarcinoma and controls. Participants in Helsinki. Two populations were included in the study: patients with dysplasia and 60% in patients with dysplasia (26). These findings suggest that a down-regulation of alkaline sphingomyelinase could be a factor involved in bowel carcinogenesis.

Taken together, the above observations suggest a role for alkaline sphingomyelinase in the gut pathophysiology. In this context, it would be particularly useful to show that the levels of the alkaline sphingomyelinase present in the feces correlate with those found in the pathologic intestinal mucosa. To this aim, we have examined the alkaline sphingomyelinase activity in both intestinal biopsies and fecal samples of healthy subjects and patients with colorectal adenocarcinoma.

Table 1. Description of the study population

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Sex

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Fecal sphingomyelinase assay

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Table 2. Characteristics of 51 patients with adenocarcinomas

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NOTE: Duke’s stage A, tumor not penetrating the bowel wall; Duke’s stage B, tumor penetrating the bowel wall without lymph node metastases; Duke’s stage C, tumor penetrating the bowel wall with lymph node metastases in the same region; Duke’s stage D, tumors that have spread to distant sites (such as the liver). Tumor differentiation is classified according to histopathologic criteria: G1, high differentiation; G2, medium differentiation; G3, low differentiation; mucinous, mucin-producing tumor.
buffer (pH 7.4) consisting of 0.25 mol/L sucrose, 5 mmol/L MgCl₂, 0.15 mol/L KCl, 50 mmol/L KH₂PO₄, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L benzamidine at 4°C (27). After mechanical disgregation, the tissues were sonicated for 30 seconds in ice and centrifuged at 10,978 × g for 10 minutes at 4°C. After centrifugation, the supernatant was recovered for determination of enzymatic activity and protein concentration. The protein concentration was determined with the microbicinchoninic acid assay kit (Pierce, Rockford, IL).

Fifteen days after the colonoscopy, stool samples from enrolled subjects were collected. In particular, feces from all the healthy subjects were collected, whereas feces from only 29 of 51 enrolled patients with colonic carcinoma were obtained, always before surgical resection. The remaining 22 neoplastic patients were excluded because they had been resected a few days after colonoscopy. The fecal samples were dried under vacuum and the dry weights were determined. The samples were then resuspended in 10 mL of 0.15 mol/L NaCl and centrifuged at 1,000 g, 4°C, for 10 minutes as previously described (28). The supernatants were collected and analyzed for alkaline sphingomyelinase activity (Fig. 1).

**Sphingomyelinase Assay.** The activity of alkaline sphingomyelinase was determined as previously described (27) with small modifications. Twenty-five–microliter samples (100–200 μg protein) were incubated for 30 minutes at 37°C in a buffer (400 μL final volume) containing 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 2 mmol/L EDTA (pH 9.0) with 3 mmol/L bile salt mixture with a molar ratio of taurocholic acid, taurodeoxycholic acid, glycocholic acid, and glycochenodeoxycholic acid of 3:2:1:8:1. The reaction was started by the addition of 16 μL [N-methyl-14C]sphingomyelin mixture (291 pmol; specific activity, 56.6 mCi/mmol; Amersham, Bucks, United Kingdom), previously dried under nitrogen, resuspended in the assay buffer, and solubilized by short bursts of sonication and vortexed. The reaction was stopped by the addition of 2 mL chloroform/methanol (2:1 by volume). After centrifugation, the aqueous phase was extracted twice more with chloroform. The organic phases, obtained in the different extraction steps, were collected and washed once with 1 mL chloroform/methanol/water (3:48:47 by volume) to remove totally free radioactive phosphorylcholine, the other hydrolysis product of sphingomyelin. The aqueous phases were collected, transferred to scintillation vials, and content was measured by liquid scintillation counting. The counts per minute represented the choline phosphate generated from sphingomyelin hydrolysis. The percentage hydrolysis of substrate was calculated from the ratio of counts of the upper phase to the total counts added in the system × 100. The hydrolysis of sphingomyelin was expressed as pmol sphingomyelin hydrolyzed/h/mg tissue protein or pmol sphingomyelin hydrolyzed/h/mg dry weight of feces.

**Statistical Analysis.** The focus of the analysis was both to evaluate changes in alkaline sphingomyelinase activity in feces from colorectal adenocarcinoma patients compared with control subjects and to study if a correlation existed between the fecal activity of this enzyme with the activity in the intestinal tissue. Associations were also evaluated by tumor site, Dukes’ stage, tumor differentiation grade, age, and gender. Statistical analysis of data was done by using one-way ANOVA followed by the Student’s t test for paired or unpaired data, or by the correlation analysis (Prism 3.0 GraphPad Software, San Diego, CA). Moreover, the two-way ANOVA test was used to determine whether the alkaline sphingomyelinase activity was dependent on gender either in control subjects or patients. Data were expressed as mean ± SE and the significance level was set at P < 0.05.

**Results**

**Alkaline Sphingomyelinase Activity in Intestinal Biopsies from Control and Adenocarcinoma Patients.** The results of ANOVA [F(2,111) = 90.03, P < 0.0001] indicated significant differences between and within groups in the alkaline sphingomyelinase activity measured in intestinal mucosa samples from 12 control subjects and 51 patients. Post hoc comparisons showed a significant reduction (P < 0.0001) of the enzyme activity in tumor samples (mean ± SE, 41.77 ± 5.44; controls, 166.49; range 1.3–166.49) when compared with controls (mean ± SE, 72.02 ± 105.48; range 232.1–1461; Fig. 2A). Control values of the enzyme activity resulted to be significantly higher also when compared with paratumoral samples (mean ± SE, 163.68 ± 25.47; range 3.08–600; P < 0.0001). Moreover, the t test for paired data, used to analyze the results of alkaline sphingomyelinase activity in tumoral and paratumoral mucosa samples, showed a significant difference between the mean values: The enzyme levels in paratumoral tissues resulted to be significantly higher than those from tumoral samples (P < 0.0001). Interestingly, a statistically significant correlation was found when analyzing the alkaline sphingomyelinase activity in relation with the condition of sample (normal, paratumoral, tumoral; R = −0.684, P < 0.001).

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**Figure 1.** Scheme of the sequence of used procedures.
Alkaline sphingomyelinase of the colon ($n = 31$; mean ± SE, 37.86 ± 4.92 pmol sphingomyelin hydrolyzed/h/mg protein) and rectum ($n = 20$; mean ± SE, 47.82 ± 11.68 pmol sphingomyelin hydrolyzed/h/mg protein) in tumor samples did not differ among them. Similarly, no significant difference in enzyme activity was observed between paratumoral colon ($n = 31$; mean ± SE, 163.68 ± 31.91 pmol sphingomyelin hydrolyzed/h/mg protein) and rectum samples ($n = 20$; mean ± SE, 163.67 ± 43.15 pmol sphingomyelin hydrolyzed/h/mg protein).

No correlation was observed between enzyme activity and age in both tumoral and paratumoral samples ($R = -0.052, R = -0.285$, respectively).

No significant difference was revealed between the levels of alkaline sphingomyelinase in tumor samples from men and women (mean ± SE, 36.62 ± 6.9 and 50.44 ± 8.625 pmol sphingomyelin hydrolyzed/h/mg protein, respectively).

Alkaline Sphingomyelinase Activity in Intestinal Adenocarcinoma and Dukes’ Stage. The changes in alkaline sphingomyelinase activity in relation to Dukes’ stage of adenocarcinoma evidenced by one-way ANOVA [$F(3,58) = 64.66, P < 0.0001$] are shown in Fig. 2A. Dukes’ stage B (47.18 ± 7.36 pmol sphingomyelin hydrolyzed/h/mg protein), stage C (39.75 ± 10.92 pmol sphingomyelin hydrolyzed/h/mg protein), and stage D (29.11 ± 11.51 pmol sphingomyelin hydrolyzed/h/mg protein) were significantly reduced compared with normal control mucosa ($P < 0.0001$, respectively). No statistically significant difference was found when Dukes’ stages B, C, and D were compared among them.

Alkaline Sphingomyelinase Activity in Intestinal Adenocarcinoma and Degree of Tumor Differentiation. The results of the overall ANOVA on the enzyme activity in relation to tumor cell differentiation stage were significant [$F(4,58) = 48.72, P < 0.0001$]. The $t$ test analysis showed no statistical differences between the alkaline sphingomyelinase activity in $G_1$ (mean ± SE, 43.11 ± 12.87 pmol sphingomyelin hydrolyzed/h/mg protein), $G_2$ (mean ± SE, 38.86 ± 9.46 pmol sphingomyelin hydrolyzed/h/mg protein), $G_3$ (mean ± SE, 40.09 ± 7.2 pmol sphingomyelin hydrolyzed/h/mg protein), and mucinous tumors (mean ± SE, 60.71 ± 17.72 pmol sphingomyelin hydrolyzed/h/mg protein), whereas the enzyme levels in tumor samples, at all differentiation stages, were significantly lower when compared with normal mucosa ($G_1$, $G_2$, $G_3$, and mucinous versus controls, $P = 0.0016$, $P < 0.0001$, $P < 0.0001$, $P = 0.0007$, respectively).

Figure 2. A. Alkaline sphingomyelinase activity in intestinal mucosa samples from control subjects and bowel adenocarcinoma patients. ***$P < 0.001$ when compared with healthy subjects’ mucosa. B. Alkaline sphingomyelinase levels in tumor samples in relation to Dukes’ stage. ***$P < 0.001$ when compared with healthy subjects’ mucosa. ***$P < 0.001$ with respect to paratumoral tissue. C. Alkaline sphingomyelinase levels in tumor samples in relation with degree of tumor differentiation. **$P < 0.0016$, ***$P < 0.001$ when compared with normal control mucosa. The enzyme activity from each sample was assessed by radiometric assay and expressed as pmol hydrolyzed sphingomyelin/h/mg protein. Solid lines, mean values.
Alkaline Sphingomyelinase Activity in Feces from Control Subjects and Colon Adenocarcinoma Patients. The analysis of the levels of alkaline sphingomyelinase in stools from healthy subjects (n = 34) and adenocarcinoma patients (n = 29) showed that the enzyme activity in patients' stool was significantly (P < 0.0001) lower than in controls being 32.09 ± 5.47 pmol sphingomyelin hydrolyzed/h/mg dry weight of patients' feces (mean ± SE; range 1.00-96.00) versus 320.9 ± 25.10 pmol sphingomyelin hydrolyzed/h/mg dry weight of control's feces (range 115.00-565.00; Fig. 3A). The results of the overall ANOVA on the fecal enzyme activity in Dukes' stages B, C, and D in patients compared with healthy controls [F(3,58) = 34.16, P < 0.0001] are shown in Fig. 3B.

The fecal sphingomyelinase levels in patients at Dukes' stages B, C, and D (mean ± SE, 27.13 ± 6.79 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces, P < 0.0001; 52.51 ± 11.68 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces, P < 0.0001; 14.53 ± 5.93 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces, P = 0.0010; respectively) resulted significantly lower when compared with controls (mean ± SE, 320.9 ± 25.10 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces), but no statistical differences were found when Dukes' stages B, C, and D were compared among themselves. The G1 grade (mean ± SE, 320.9 ± 27.13 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces; female: mean ± SE, 36.39 ± 8.77 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces; female: mean ± SE, 29.62 ± 9.12 pmol sphingomyelin hydrolyzed/h/mg dry weight of feces; P = 0.63).

Strengths and Limitations of the Study. Taken together, these preliminary results add another dimension or a possible step-forward in the quest to search a noninvasive and sensitive marker for early detection of colorectal adenocarcinomas. We report that alkaline sphingomyelinase was significantly decreased in tumoral intestinal mucosa of patients compared with controls and this decrease was independent of Dukes' stage and tumor differentiation grade, thus strengthening the hypothesized validity of this assay to be used as fecal test for the early detection of colonic neoplasia. Indeed, a correlation exists between the fecal activities of this enzyme with the activity in intestinal tissues. The findings presented, although very intriguing, should be considered as preliminary and need to be validated and confirmed by other studies.

Discussion

The sphingomyelin pathway is considered one of the most important intracellular mechanisms in regulating cell growth, differentiation, and apoptosis (5-9, 29, 30). At the intestinal level in this pathway, the first step, which leads to ceramide and phosphorylcholine generation, is catalyzed mainly by alkaline sphingomyelinase, an enzyme with a high activity not only in the mucosa but also in the lumen, because it may originate from the sloughing of epithelial cells and from the dissociation of the enzyme from the mucosal surface caused by luminal factors, primarily the bile salts (31). Alkaline sphingomyelinase, by exerting a major role in dietary sphingomyelin digestion, is responsible for the generation of anti-proliferative and/or apoptosis sphingolipid messengers, mainly ceramide, which are able to trigger the rapid turnover and apoptosis in intestinal and colon epithelial cells (13, 32-36). Markedly reduced mucosal alkaline sphingomyelinase activity has been associated with colorectal carcinoma (23), colorectal adenomas (15), familial adenomatous polyposis (24), and

Figure 3. A. Fecal alkaline sphingomyelinase activity in healthy subjects and patients. ***P < 0.001 with respect to control feces. B. Alkaline sphingomyelinase levels in stool of subjects with adenocarcinoma in relation with Dukes’ stage. **P = 0.001 and ***P < 0.001 when compared with control subjects’ stool. C. Stool alkaline sphingomyelinase levels in tumor samples in relation with degree of tumor differentiation. **P = 0.0087 and ***P < 0.001 when compared with control subjects’ stool. The stool alkaline sphingomyelinase was analyzed with a radiometric assay and expressed as pmol hydrolyzed sphingomyelin/h/mg dry weight of feces. Solid lines, mean value.

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inflammatory bowel disease (26). The luminal localization of alkaline sphingomyelinase presumably leads to its excretion and, consequently, to its presence in stool in human, as previously shown in rat model (28).

The analysis of alkaline sphingomyelinase activity in intestinal biopsies from control and colorectal adenocarcinoma patients showed a significant decrease between the enzyme activity in tumor samples and controls with a mean reduction of ~90%, thus confirming previously reported results (24, 26). Of note, the difference was statistically significant also when paratumoral samples were compared with normal values with a mean reduction of almost 80%. Moreover, alkaline sphingomyelinase activity was significantly different in the same patient when paratumoral and tumoral tissue samples were analyzed. The alkaline sphingomyelinase activity was reduced by ~70% in adenocarcinoma samples compared with tumor surrounding mucosa. These results lead us to consider that, in general, even if the paratumoral samples are “normal” at histologic assessment, several authors questioned whether the histologic normal tissue adjacent to adenocarcinoma represent truly normal epithelium (37). For example, Moss et al. (38) showed considerable differences in the degree of apoptosis between histologically normal adjacent epithelium in comparison with the truly normal tissue, with degree of apoptosis being generally lower in the latter. Such alterations in normal tissue adjacent to colorectal neoplasms may reflect an early stage in the process of colorectal carcinogenesis or a response to the adjacent tumor. Our data strongly suggest that a similar condition could also occur with regard to the levels of alkaline sphingomyelinase.

In relation to Dukes’ stage of adenocarcinoma and tumor cell differentiation, the alkaline sphingomyelinase activity in tumor samples was shown to be always significantly reduced when compared with normal mucosa, whereas no significant difference was observed when tumor samples, at all Dukes’ stages or differentiation grade, were compared among them. No significant difference among enzymatic levels was found in both tumoral and paratumoral samples from colon or rectum. These results suggest that the decreasing activity gradient from ascending colon to rectum previously reported by Duan et al. (39) could be totally abrogated in adenocarcinoma patients.

The previous observation of an age-related physiologic decrease of alkaline sphingomyelinase activity (26) prompted us to analyze whether the intestinal enzyme levels could also be age dependent in colorectal adenocarcinoma patients. The results indicated that no correlation was found between alkaline sphingomyelinase activity in tumor samples and age of patients, thus suggesting that in the presence of a neoplastic transformation the relation between the levels of alkaline sphingomyelinase and age could be lost. No significant gender-dependent difference was revealed between the levels of enzyme in tumor samples. To assess the possible relation between intestinal alkaline sphingomyelinase in tissue samples and stools, the levels of fecal enzyme activity were first compared between control subjects and adenocarcinoma patients. Also, in this context, a relevant difference was found between stool alkaline sphingomyelinase activity and the subject’s condition (normal or tumoral), with enzyme levels being significantly reduced in tumor patients compared with controls. The results showed that, independently on Dukes’ stage and differentiation grade, alkaline sphingomyelinase activity of patients’ stool was even significantly lower than controls, thus reflecting the results obtained when enzyme activity was analyzed in tissue samples. Indeed, the mean reduction of the alkaline sphingomyelinase activity in stool samples from patients compared with control values is ~90%, comparable with the mean reduction of the enzyme activity in cancer tissues when compared with normal samples (~90%). No significant differences were observed between stool alkaline sphingomyelinase activity and both age and gender either in healthy subjects or patients.

Altogether, our results are consistent with previously reported observations showing that a significant deficit of intestinal mucosa alkaline sphingomyelinase, at least from the enzymatic activity point of view, is a consistent and reproducible finding in patients with bowel adenocarcinoma. Of interest, a comparable difference between healthy and tumor patients was also revealed when the level of stool alkaline sphingomyelinase activity was measured, thus supporting our first hypothesis that the fecal sphingomyelinase activity could really reflect the human intestinal mucosa enzyme level. Thus, a decrease in tissue and/or functional deficit of alkaline sphingomyelinase activity in the feces, besides representing further confirmation of the crucial role played by alkaline sphingomyelinase in the pathophysiology of gut, could strongly indicate that a low fecal alkaline sphingomyelinase activity level could be considered as a useful index of intestinal dysfunction and as a diagnostic screening marker of intestinal cancer, particularly taking into account its early appearance in the intestinal tissue of adenocarcinoma patients.

In conclusion, the results of this study, although still need to be confirmed with a properly designed case-control study, suggest that the assessment of fecal alkaline sphingomyelinase activity could represent an innovative, simple, and sensitive approach to the early diagnosis of intestinal malignant transformation. Furthermore, the similar decrease found in the levels of alkaline sphingomyelinase, both in the mucosa and the stools, potentially provides an easy, noninvasive, and practical tool for the routine screening of intestinal disease risk in large population groups. In this context, to better verify the possibility to consider stool alkaline sphingomyelinase level as an early diagnostic marker of intestinal disorders, a screening study on numerically relevant group of enrolled healthy subjects (n ~ 500) is actually in progress.

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References


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