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ORIGINAL ARTICLE

Age-Related Fecal Calprotectin Concentrations in Healthy Adults

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건강한 성인의 연령별 분변 칼프로텍틴의 농도

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ABSTRACT

Fecal calprotectin (FC) is a marker used for the differential diagnosis of inflammatory bowel disease (IBD). FC is also used to determine the effects of treatment and recurrence prediction because of its non-decomposition by bacteria, relative week stability at room temperature, and its uniform distribution within feces. Healthy male and female adults between the age of 30 and 80 living in Jeju were selected for this study. The FC concentration in the healthy control group (N=45) was distributed widely as 0~545.9 μg/g and showed a significant difference with age in healthy adults. The FC concentration in adults over 70 years old (80.6 years on average) was 160.3 μg/g. The result is approximately 10 times higher than in adults below 50 years (44 years on average), with FC concentrations at 15.88 μg/g. Moreover, adults over 50 years, with an average age of 59.6, had FC concentrations of 35.46 μg/g, which were two times higher than the below 50-year-old group, confirming the significant correlation between age and FC concentration. As the FC test is a non-invasive and cost-effective objective marker in IBD tests, a suitable cut-off value is required for different ages. This study provides the baseline data for differential diagnoses.

INTRODUCTION

Inflammatory bowel disease (IBD) usually affects adults in North America and Northern Europe. However, IBD's prevalence recently expanded to ulcerative colitis and Crohn's disease in Korea, according to recent epidemiology studies [1, 2]. IBD can be characterized by the chronic inflammation of the gastrointestinal system, requiring lifetime management and affecting the patient's quality of life. Diagnosis and bowel inflammation monitoring are vital in IDB diagnosis and follow up. Although diagnosis and follow-up management are closely related to its long term prognosis, the most effective monitoring methods until now are limited to colonoscopy and biopsy, which are invasive, time-consuming, and costly [3, 4]. Thus, a simpler, faster, and less invasive fecal calprotectin (FC) test has been recently reported in various studies. The chronic inflammatory disease, with its cycles of improvement and aggravation, requires a lifetime of disease activity evaluation. As a functional disease of no specific symptom and sign, it is difficult for differential diagnoses from other disorders, especially without standard diagnostic

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criteria. Thus, to monitor and detect IBD, the method of using FC as a biomarker was used for being simple, rapid, sensitive, inexpensive, and noninvasive. FC detection minimizes false-positive results and decreases unnecessary biopsies. Moreover, as the FC level is six times higher compared to the serum level, it also allows a highly sensitive test [5, 6].

Calprotectin was first identified and reported in 1980 as an L1 protein [7]. It was separated and quantified from the stool and reported in 1992 by R ø seth et al [8]. Calprotectin is a calcium- and zinc-bound protein usually found in damaged neutrophil, taking up to 60% of the neutrophil cytoplasm. With bowel inflammation, it is destroyed during the transepithelial migration of the neutrophil through the intestinal mucosa and excreted in stool after secretion within the bowel from cell necrosis [8]. Early studies showed calprotectin to be raised in patients with inflammatory bowel disease3 and to be correlated with endoscopic and histological evidence of inflammation. It has since been shown to be useful sensitive non-invasive biomarker for the diagnosis of inflammatory bowel disease, to predict its severity in adults6 and children, to predict relapse8 and to monitor response to treatment [3, 9]. Calprotectin has one of the most critical roles in gut immunity, which is not only related to IBD but also to obesity. FC levels in obese and overweight adults were observed to be significantly higher compared to obese and overweight children in age-obesity studies [10]. Calprotectin in the stool is not easily decomposed by bacteria and is stable for approximately one week at room temperature. As it is uniformly distributed within the stool, detection is possible with a minimum amount, allowing an active study as a marker for treatment effect determination and recurrence prediction for IBD differential diagnosis [11, 12]. Normal FC levels per age were mostly on infants or children below 12 years old [13-15]. with hardly any studies on healthy adults or elders. The purpose of this study is to measure FC levels in healthy male and female adults between the ages 30 and 80 residing in Jeju, understand the normal range concentration, and to evaluate the usual diagnosis standard on FC tests for intestinal disease patients and IBD patients.

MATERIALS AND METHODS

1. Subjects

A healthy control group was recruited from the hospital staff, families and friends of Cheju Halla General Hospital for this study. From a total of 50 participants, 45 health adults between the age of 40 and 90 without a history of colorectal or systemic inflammation were selected as subjects for fecal sample collection. There were 13 health adults under the age of 50, 16 health adults between 51 and 69 years, and 16 health adults over the age of 70. The healthy control group was classified per age group, gender, and body mass index (BMI; Table 1). The study was approved by the Ethical Committee of Cheju Halla University, Jeju City (1044348-20190304-HR-001-01).

2. Fecal calprotectin

Fecal samples of the normal adult volunteers were collected once using a sterile rod in a sterile box, following the precautions for calprotectin measurement. The provided stool samples were subdivided and stored at −80°C, until use. The measurement used for FC detection followed Park and Kim's [10]. measurements. Afterward, the samples were pulled from refrigerated storage to room temperature for natural defrosting for approximately 30 min. Then, a single 100 mg aliquot was suspended in 1 mL of fecal extraction buffer consisting of 0.1 M Tris-buffered saline with Tween 20, pH 8.0 (MBCell, Kisanbio Tech Co. Ltd, Seoul, Republic of Korea), 0.5% bovine serum albumin, and 0.15 M

Table 1. Demographic characteristics of the study's healthy volunteers (N=45)

Variable	Age (yr)	Sex (M/F)	BMI (kg/m^2)
Value	63.1±15.6	29/16	23.3±3.3

Values are presented as mean±SD.
Abbreviations: BMI, body mass index; M, male; F, female.

NaCl, 10 mM CaCl2. The sample was homogenized for 5 min with a vortex mixer (Scientific Industries, Bohemia, NY, USA). The homogenates were centrifuged for 5 min at 10,000 × g at room temperature. The top portion of the supernatants was removed and kept at −80°C until quantitated by enzyme-linked immunosorbent assay (ELISA).

FC was quantitatively measured using a Legend Max-Human MRP8/14 (calprotectin) ELISA Kit (BioLegend, San Diego, CA, USA). The frozen fecal extracts were defrosted and diluted 1:1000 in an assay buffer. Standards and diluted samples (50 µL) were put into the ELISA plates, which were then shielded and incubated at RT for 1 hr while agitating at 200 rpm. Then, 100 µL of human MRP8/14 detection antibody solution was added to each well after washing the wells 4 times with a wash buffer. The plates were shielded and incubated at RT for 30 min while agitating. After incubation with the detection antibody solution, the wells were washed five times with a wash buffer, and 100 µL of substrate solution was added. Then, 100 µL of stop solution was added, and the absorbance was read at both 450 nm and 570 nm using a PowerWave XS2 Microplate Spectrophotometer (BioTek Winooski, VT, USA). The readings at 570 nm were subtracted from those at 450 nm. Calprotectin values of the fecal sample were expressed as µg/g.

3. Statistical analysis

Data were analyzed using the GraphPad Prism statistical software package (GraphPad Software Inc., La Jolla, CA, USA). Calprotectin values were presented as the mean±standard deviation. A student's t-test was performed to compare FC concentrations according to age and gender.

RESULTS

1. FC concentration per age and BMI

The average age of the healthy control group (N=45) was 64±15.6, with an average FC concentration of 64.98 µg/g, showing a large variation between the age groups. Thus, the age groups were subdivided into 3 groups of below 50, 50~69, and over the age of 70 (Figure 1). The average FC concentration below the age of 50 years was 15.88 µg/g (range, 0.0~86.9 µg/g), then 35.46 µg/g (range, 0.43~166.1 µg/g) between the age of 50~69 years, and 160.3 µg/g (range, 0.93~545.9 µg/g) over the age of 70 years, showing a significant difference between the age groups. There were a total of 13 health adults for the below-50 group, 11 males and 2 females, with an average age of 44 years.

The average age for the 51~69 group was 59.6, with 11 male and 5 female health adults. The average age for the over-70 group was 80.6 years, with a balanced gender ratio of 8 females and 8 males. As the average FC concentration showed a 5~10 times higher concentration in the over-70 age group, significance between the concentration and age could be identified.

Pearson coefficient showed significant correlations between FC concentration and age (Pearson r=0.3779, P=0.0096). Linear regression analysis showed a positive relation between FC concentration and age (slope:

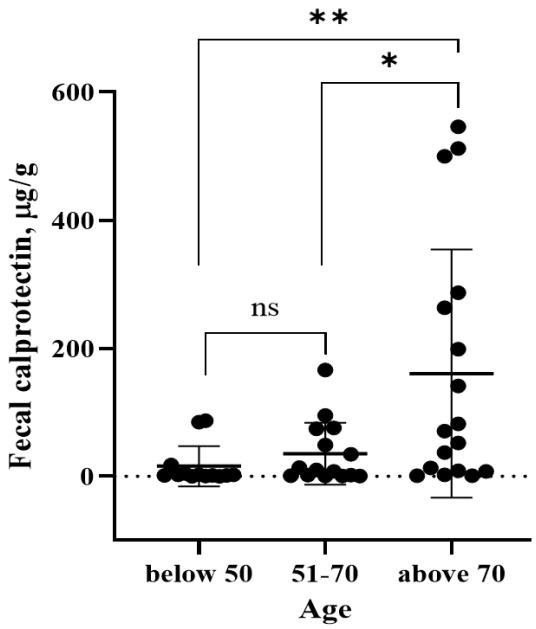

Figure 1. Scatter plots of fecal calprotectin (FC) levels in three age groups. The data were presented as mean±standard deviation of mean. Mann Whitney U-test was used for statistical analysis. Significance level was *P<0.05, **P<0.001.

0.0471, Y intercept: 58.36) (Figure 2).

Unlike the FC concentration, which showed a significant difference between the age groups, no significance was observed between BMI index and FC concentration. Moreover, the average BMI of the total participants were measured at 23.3±3.3, with most participants of the healthy control group showing a normal BMI index.

2. FC concentration per gender

Of the 45 healthy control group participants, 29 were male (64%) and 16 were female (36%). The average FC concentration in the male and female participants were 545.9 and 511.83 each, showing a significant difference between the genders after the analysis, excluding the highest concentration (Figure 3). The average FC concentration in males was 62.43 μg/g (range, 0.14~286.76 μg/g), and 12.21 μg/g (range, 0.25~82.01 μg/g) in females, 6 times lower than males.

DISCUSSION

Despite the active FC-related studies reported abroad, it is a new study area in Korea, with its recent report on infants in 2014 [16]. Most of the reported articles had been about IBD patients, infant diarrhea, and meta-analysis results of the IBD patient group [17] as well as review articles [18-20]. Studies on FC are actively ongoing for use in the differential diagnosis of IBD and as an effective marker that predicts recurrence and determines treatment effects. Moreover, increased FC concentration in children (3~18 years) had been reported to be significant in atopic dermatitis (AD) severity evaluation from the relatedness between FC concentration and AD. The relatedness study between obesity and FC [10, 21] suggested a different pathophysiological mechanism between adult and childhood obesity as a result of significantly higher FC concentration in adults than in children [10]. According to a recent domestic study report on causality between FC and IBD [22], FC had been proven to be a reliable noninvasive differentiating marker in the differential diagnosis for eosinophilic gastrointestinal disorder and functional abdominal pain disorder. As most studies until now had been on the effectiveness of FC as a noninvasive marker for IBD diagnosis, with study subjects composed mainly of IBD patients, there has been a relative lack of data for comparison on healthy adults and elders. Thus, we conducted this study on

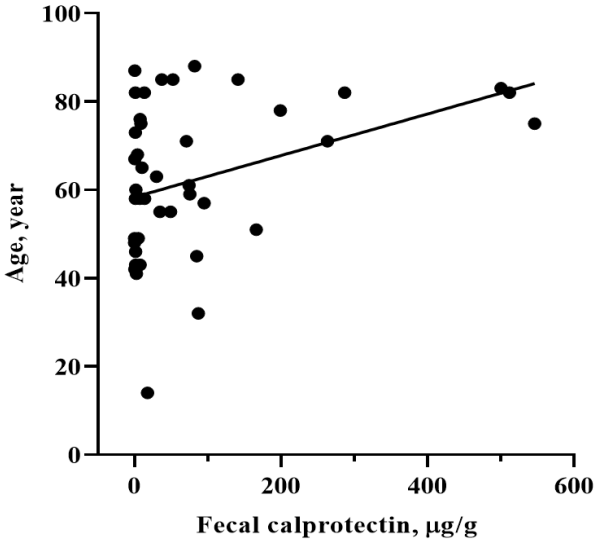

Figure 2. Linear regression analysis of FC concentration by age in healthy volunteers (N=45).

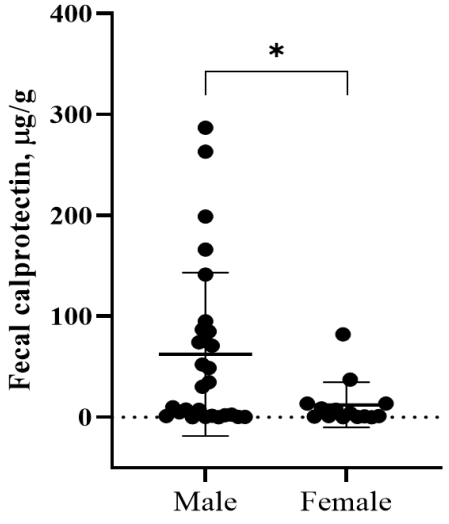

Figure 3. Scatter plots of fecal calprotectin (FC) levels between gender groups. The data were presented as mean±standard deviation of the mean. Mann Whitney U-test was used for statistical analysis. Significance level *P<0.05, **P<0.001.

healthy male and female adults residing in Jeju, between the age of 30 and 90, to understand the normal range of FC concentration.

From meta analysis and large review article, 50 μg/g was the most commonly adopted cut-off FC value both in literature and by commercially available ELISA kits, as a screening cut-off value for further endoscopy examination in clinical practice, with specificity of 60% and pooled sensitivity of 92% [17, 23]. When the cut-off value at 100 μg/g, it showed 91% specificity and 98% sensitivity that indicating the cut-off value increases, sensitivity becomes lower and specificity higher. A negative test result at the lowest cut-off level (30 to 50 μg/g) suggests a diagnosis of a non-inflammatory condition, such as irritable bowel syndrome (IBS). A positive result at the cut-off of 100 μg/g may indicate IBS, with the recommendation to repeat the test in 6 week to confirm the initial result [24].

This study's results show that a significant difference was observed in FC concentration in healthy adults per age group. FC concentration in the over-70 age group (average of 80.6 years) was 160.3 μg/g; approximately 10 times higher compared to the below-50 age group's (average of 44 years) 15.88 μg/g. Moreover, adults over 50 years, with an average of 59.6, had FC concentrations at 35.46 μg/g, two times higher than the below-50 group, confirming a significant correlation between age and FC concentration. For appropriate clinical applications, a standardized cut-off value of FC is important. Zhu et al [25] conducted a study on healthy children below the age of 4, subdividing the age group as 1~2 years, 2~3 years, and 3~4 years for FC concentration, and reported a significant difference between the different age groups as 96.1 μg/g for the 1~2 age group, and 65.36 μg/g for the 3~4 age group. A statistical difference was found between FC in healthy children aged 1~3 months and those aged 3~6 months (375.2 μg/g *vs* 217.9 μg/g, $P<0.001$), as well as between 1~6 months and 6~18 months (median: 282.7 μg/g *vs* 114.9 μg/g; $P<0.001$) [26, 27]. From the present study's results, the age groups suitable for the standardization was below 70 years. As the FC concentration detected over-70 age group (average of 80.6 years) was too high despite the healthy adult selection unrelated to IBD, thus the cut-off concentration setting for each age group is necessary. No previous study has collected samples from healthy over-70 age subjects and provided age-related references ranges determined from statistical principles. Joshi et al [28] also indicated that significant differences between age groups for calprotectin resulted in the following age-related reference ranges: 2~9 years, <166 μg/g, 10~59 years, <51 μg/g, over 60 years, <112 μg/g, which results very similar with our age-related results. Our results from age-related healthy adults suggested that fecal marker in clinical practice has led to their application to a wide spectrum of patients in many age groups and separate reference ranges are required for calprotectin in children aged 2~9 years and over-70 years.

The ageing and the "inflammaging" act at different levels of complexity involving several tissues and organs as well as the immune system and our associated ecosystems (gutmicrobiota). The aging process is accompanied by a chronic, smoldering background of inflammation that researchers call "inflammaging". All of these factors are thought to contribute to the systemic inflammatory state, through the imbalance of pro-inflammatory and/or anti-inflammatory mediators [29]. Poullos et al [30] reported that FC levels are associated with lifestyle risk factors for colorectal cancer. Low-level asymptomatic bowel inflammation may be the link between lifestyle and the pathogenesis of circulating proinflammatory cytokines (CRC), which may be part of the mechanism for this link. Thus, a study on the various reasons for high FC concentrations in IBD patients and elders, as well as confirming the difference in FC concentration between genders, is necessary. Furthermore, various lifestyle factors such as drinking, smoking, exercise, and stress score, need to be included as well for a more detailed study on its relatedness with hematological factors.

Another notable finding in the present study's results

is that FC showed a statistically significant difference between males and females. The average FC concentration in males was 62.43 μg/g (range, 0.14~286.76 μg/g) and 12.21 μg/g (range, 0.25~82.01 μg/g) in females, 5 times higher than females. To understand the higher FC concentration observed in males than in females, the average age and average BMI were studies. The average age in male and female subjects was 57±13.9 and 69±14.8, respectively. Meanwhile, the average BMI in male and female subjects were 23.9 ±3.4 and 22.4±3.11, respectively. The results showed no other significant factors other than the females have a slightly lower BMI by 1.6. Other than several international study reports on no significant difference in FC concentration per gender in healthy children under the age of 12, there are hardly any study reports on gender difference on FC concentration in healthy adults [24]. Most of the study reports on FC concentration in healthy persons were mostly for infants below 1 year old or on children below 12 years old, with a lack of results on adults, especially in elders over 70. The vast amount of studies on infants under the age of 1 is assumed from the fact that feces sample collection in infants is easier [27].

In conclusion, calprotectin, a relatively stable protein secreted from damaged neutrophil, had been presented in various study reports to be a useful test method for IBD diagnosis. However, as no definite cut-off value has been defined for health adults, especially over-70 years, the fact that the cut-off value could be an important factor for different age groups was presented in this study.

요 약

분변 칼프로텍틴(fecal calprotectin, FC)은 염증성 장질환의(inflammatory bowl disease, IBD)의 감별진단을 위한 표지자(marker)로 이용되고 있다. 또한 FC는 세균에 의한 분해가 어려워 실온에서도 1주일 정도 안정적으로 유지되며 대변 내에 균일하게 분포한다는 장점으로 치료효과의 판정과 재발을 예측하는 표지자로 활발히 연구되고 있다. 본 연구는 30~80대까지의 제주 거주 건강한 성인 남녀를 대상으로 하였다. 전체 건강대상군(N=45)의 FC 농도 범위는 0~545.9 μg/g로 아주 넓게 분포하였다. 건강한 성인들의 FC농도는 연령에 따라 유의한 차이를 보임을 알 수 있었으며 3개의 연령군 중에서 평균연령이 80.6세인 70세 이상의 연령군의 FC농도는 평균연령이 44세인 50세 미만의 연령군의 FC농도인 15.88 μg/g에 비해 약 10배 이상의 농도인 160.3 μg/g을 보였고 평균연령이 59.6세의 경우에도 50세 미만 연령층에 비해 2배 이상 농도인 35.46 μg/g 보여 연령과 FC 농도 간에는 유의한 상관관계를 보였다. 결론적으로 FC검사가 비침습적이며 비용 효과적이고 객관적인 IBD검사의 marker로 이용되기 위해서는 연령에 따른 정교한 cut-off 값이 필요하며 본 연구 결과가 IBD의 감별진단을 위한 기초자료를 제공할 수 있을 것으로 생각된다.

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Conflict of interest: None

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