Pharmacological Reports 71 (2019) 42–47

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Enkephalin degradation in serum of patients with inflammatory bowel diseases

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ARTICLE INFO

Article history:
Received 18 April 2018
Received in revised form 10 June 2018
Accepted 1 August 2018
Available online 2 August 2018

Keywords:
Inflammatory bowel diseases
Enkephalins
Degradation in human serum
Mass spectrometry

ABSTRACT

Background: Inflammatory bowel diseases (IBD) are a group of chronic and recurrent gastrointestinal disorders that are difficult to control. Recently, a new IBD therapy based on the targeting of the endogenous opioid system has been proposed. Consequently, due to the fact that endogenous enkephalins have an anti-inflammatory effect, we aimed at investigating the degradation of serum enkephalin (Met- and Leu-enkephalin) in patients with IBD.

Methods: Enkephalin degradation in serum of patients with IBD was characterized using mass spectrometry methods. Calculated half-life ($T_{1/2}$) of enkephalins were compared and correlated with the disease type and gender of the patients. Additionally, statistical analysis was used to examine the dynamics of changes in terms of inhibition of enkephalins degradation within research groups.

Results: Our research indicates that the degree of enkephalins degradation depends on the gender of the patients. The difference is most evident for the rate of Met-enkephalin degradation between men (mean $T_{1/2}$ = 13.61 min) and women (mean $T_{1/2}$ = 21.84 min) with Crohn's disease (CD).

Conclusions: The most significant alternation of enkephalins degradation in serum samples of IBD patients, compared to control group, were observed in both Crohn's disease and ulcerative colitis (UC) female patients. We suggest that the differences observed between the genders in IBD patients may be explained by regulation of enkephalinases activity by estradiol.

Introduction

Inflammatory Bowel Diseases (IBD) constitute a group of chronic and relapsing disorders of the gastrointestinal (GI) tract, which include the most common representatives, Crohn`s disease (CD) and ulcerative colitis (UC) [1]. Clinical management of patients with IBD is very difficult to achieve and currently relies on calming of the inflammation, alleviation of clinical symptoms and upkeeping of the remission as long as possible [2]. Recently, we have proposed several potential therapies targeting the endogenous opioid system in IBD [3–5]. The endogenous opioid system is responsible for regulation of a variety of biological processes such as: stress response, immunity, analgesia, motor activity and inflammation in the bowel [6]. Accordingly, it was shown that endogenous opioid peptides Met- and Leu-enkephalin (Met-Enk and Leu-Enk, respectively) display anti-inflammatory effect in the digestive system and can modify the inflammatory processes through their impact on the synthesis and secretion of pro-inflammatory and anti-inflammatory cytokines [7].

Enkephalins are rapidly metabolized by endogenous enkephalinases such as neutral endopeptidase, aminopeptidase N, dipeptidyl peptidase III and angiotensin-converting enzyme (ACE, dipeptidyl carboxypeptidase I) [2,8]. Aminopeptidase N and ACE appear to be the major peptidases that hydrolyze enkephalins in rat plasma [9]. Detailed studies of the degradation of enkephalins

Abbreviations: BMI, body mass index; CD, Crohn's disease; HPLC, high pressure liquid chromatography; IBD, inflammatory bowel diseases; Leu-Enk, Leu-enkephalin; Met-Enk, Met-enkephalin; MS, mass spectrometry; UC, ulcerative colitis.

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https://doi.org/10.1016/j.pharep.2018.08.001
1734-1140/

indicate that the main cleavage site in the peptide chain is the tyrosine-glycine peptide bond hydrolyzed by aminopeptidase N. In turn, ACE hydrolyzes the glycine-phenylalanine peptide bond and releases tripeptide YGG. The C-terminal dipeptides: phenylalanine-methionine (FM) or phenylalanine-leucine (FL) were found to be the final metabolites in all of these metabolic processes [10].

Here, we present for the first time the results of a synthetic enkephalin degradation study in the serum of IBD patients. Met- and Leu-Enk were studied separately to avoid the mutual impact on degradation pathway and hydrolysis rate. The degradation pathway turned to be the same as for enkephalin degradation in rat plasma what suggests that aminopeptidase N and ACE are dominant peptidases in human serum as well.

Serum samples were divided by the type of disease (CD or UC and healthy volunteers as a control group) and further by the patient gender. The samples were analysed by mass spectrometry methods (HPLC–MS). This allowed us to measure the rate of diminishing of parent peptide peaks (Met- or Leu-Enk). Hence, the rate of enkephalin metabolism in the human serum was designated through the estimation of its half-life ($T_{1/2}$). Additionally, apart from parent peaks, we have also observed the formation of characteristic metabolites for enkephalins [10] (Figs. 1 and 2).

Material and methods

Materials

Met-Enk and Leu-Enk were purchased from Abcam Biochemicals (Cambridge, UK). Methanol LCMS quality was provided by Sigma-Aldrich and formic acid was from Merck (Warsaw, Poland) as well as was Z-norleucine. Deionised water (MilliQ) was prepared on Millipore system (Merck, Warsaw, Poland)).

All chromatographic instruments, CBM-20 A controller, two LC-20AD pumps, SIL-20AHT autosampler, CTO-20 A column oven, SPD-M20 A PDA detector and mass spectrometer LCMS-IT-TOF equipped with an electrospray ion source (ESI), ion trap (IT) and time of flight detector (TOF) were manufactured by Shimadzu Corporation (Kyoto, Japan). The Jupiter Proteo C12 column (4 μm, 2.1 mm × 250 mm) was purchased from Phenomenex (Shimpol, Warsaw, Poland).

Patients, control group and blood collection

This prospective study was conducted in patients of Caucasian origin (25 men and 25 women; with mean age ± SEM, 28.5 ± 1.9 years), who were hospitalized from January 2014 to December 2016. The patients were divided into three groups: CD (n = 21), UC (n = 16), and control (n = 13) (Tables 1 and 2). The control group included subjects who were diagnosed as IBD-free during the colonoscopy and the results were confirmed in a histopathological examination.

The inclusion criteria to the study groups were based on the proper diagnosis according to clinical, radiological, endoscopic, and histological criteria recommended by the European Crohn's and Colitis Organization. Current smokers, obese patients (BMI > 25 kg/m^2), patients with a history of cardiovascular disease, pulmonary and kidney disease, allergy, diabetes, lichen planus, psoriasis, atopic dermatitis and other autoimmune skin lesions and those treated with anti-inflammatory drugs (except azathioprine), antioxidants, or statins were excluded from the study.

Concurrently, at the time of admission to Department about 5 mL of blood are drawn from each participant using eight vacutainer tubes. After 30 min, the tube is centrifuged for 10 min at 3000×*g* and the serum is removed, frozen and stored until use.

The protocol was approved by Bioethical Commission.

Degradation of enkephalins in human serum

Solutions of enkephalins (Met-Enk or Leu-Enk) were prepared separately by dissolving them in water solution of internal standard – Z-norleucine. Solution of Z-norleucine was prepared by dissolving it in deionised water (MilliQ). The concentration of enkephalins and internal standard were, respectively, 10 mg/mL and 250 μg/mL.

Aliquots (480 μL) of pooled serum were combined with solution of appropriate enkephalin and internal standard (20 μL). After that the samples were incubated for 0, 5, 10, 15, 30, 60, 120 and 240 min at 37 °C in a final volume of 500 μL. The reaction was stopped at a predetermined time by placing of 50 μL of incubation mixture into eppendorf containing 200 μL of cold methanol. Next, samples were placed in the freezer for 20 min and centrifuged at 14 000 rpm for 20 min in 4 °C. The supernatant (200 μL) was placed in a new eppendorf with 800 μL of deionised water (MilliQ), frozen and freeze-dried. Before HPLC-MS

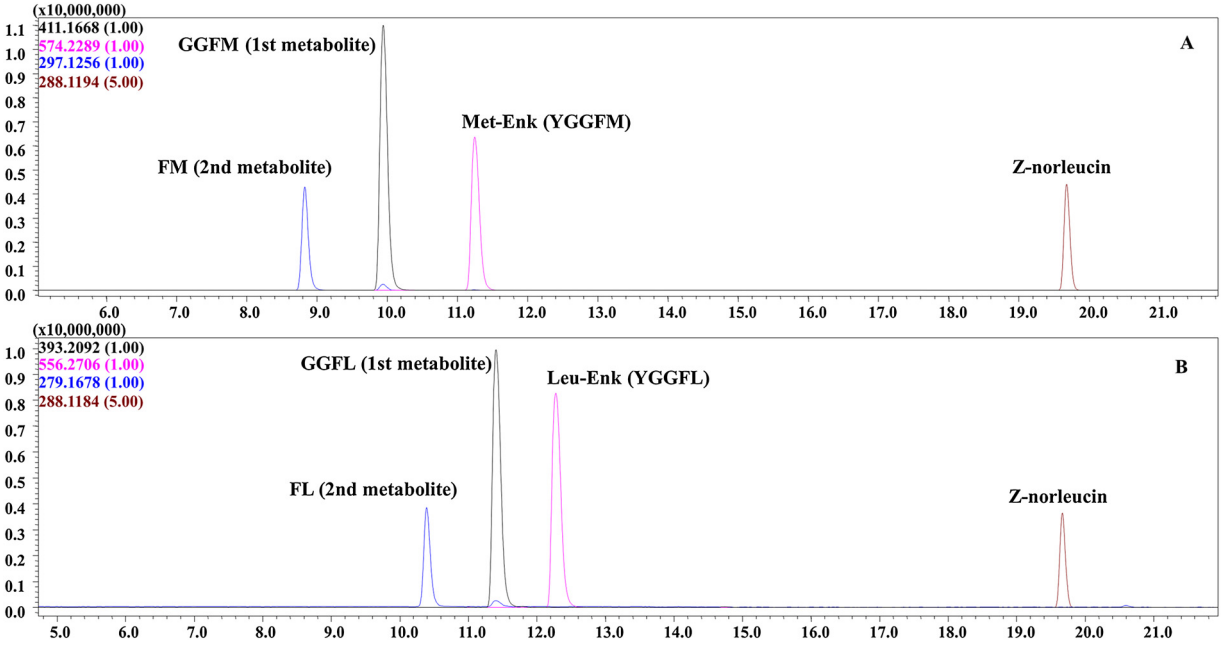

Fig. 1. Examples of extracted mass chromatograms for a) Met-Enk and b) Leu-Enk after 10 min of degradation.

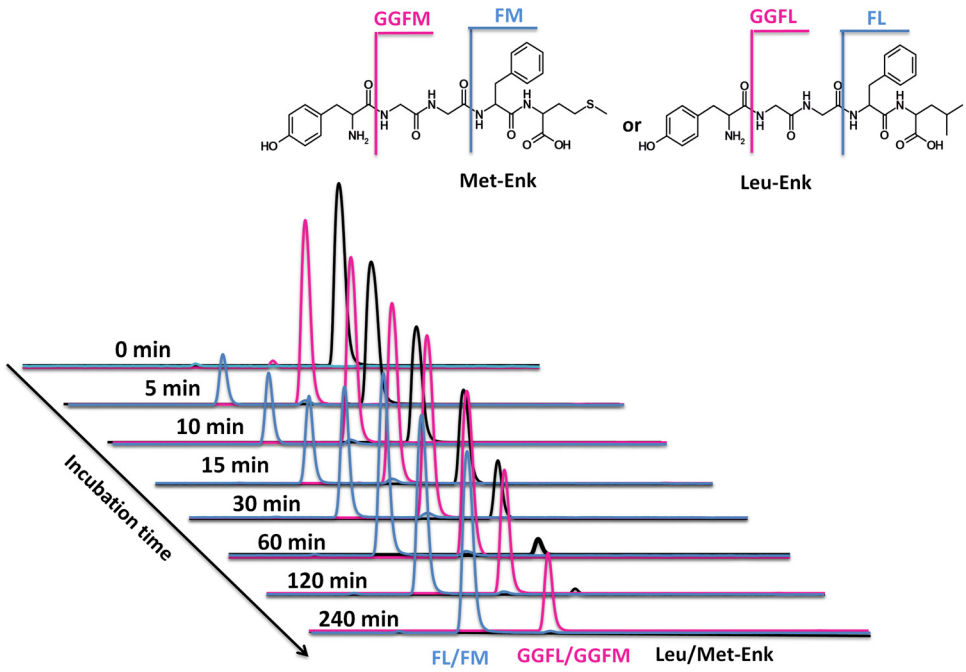

Fig. 2. The progress of Met- and Leu-enkephalins degradation depending on the time of incubation.

Table 1
The mean value of degradation half-life ($T_{1/2}$) of Met-Enk and Leu-Enk without division by gender.

Variable	Group	Mean Age [years]	Mean $T_{1/2}$ [min]	Min-max $T_{1/2}$ [min]	Std. dev.
Met-Enk	C	33.0	17.7	12.48–23.74	4.52
	CD	32.5	17.72	11.57–28.44	6.53
	UC	27.2	17.15	11.49–21.75	3.46
Leu-Enk	C	33.0	19.16	10.16–27.35	5.50
	CD	32.5	22.65	10.71–36.12	8.12
	UC	27.2	26.93	17.16–53.12	10.25

Table 2
The mean value of degradation half-life ($T_{1/2}$) of Met-Enk and Leu-Enk with division by gender: F-female and M-male.

Variable	Group	Mean Age [years]	Mean $T_{1/2}$ [min]	Min-max $T_{1/2}$ [min]	Std. dev.	$T_{1/2}$ males/ $T_{1/2}$ females
Met-Enk	C_M	32.75	15.79	12.48–23.74	5.36	0.80
	C_F	32.2	19.61	16.64–22.92	3,02	
	CD_M	33.4	13.61	11.57–15.67	1.70	0.62
	CD_F	31.5	21.84	11.81–28.44	7.18	
	UC_M	27.8	17.67	14.71–21.75	3.21	1.06
	UC_F	26.6	16.63	11.49–21.62	3.99	
Leu-Enk	C_M	32.75	18.37	14.50–22.78	3.47	0.93
	C_F	33.2	19.78	10.16–27.35	7.10	
	CD_M	33.4	21.67	10.71–36.12	9.17	0.91
	CD_F	31.5	23.85	15.76–34.19	7.76	
	UC_M	27.8	23.56	19.21–28.98	3.52	0.78
	UC_F	26.6	30.29	17.16–53.12	13.99	

analysis lyophilized sample was dissolved in 200 μL of deionised water with 0.1% formic acid, centrifuged at 14 000 rpm for 10 min in 4 °C and introduced to the HPLC-MS analysis.

Chromatography condition and data analysis

Samples after Met-Enk and Leu-Enk degradation were analyzed by RP-HPLC-MS on Phenomenex Jupiter Proteo C12 column using the solvent system of 0.1% formic acid in deionised water (MilliQ) as mobile phase A and methanol without any additives as mobile phase B. During separation a linear gradient of 5–95% B over 19 min at a flow rate of 0.3 mL/min was applied. Parameters of mass spectrometer LCMS-IT-TOF were set as follows: ion accumulation 10 ms, event time 100 ms, CDL and heat block temperature 220 °C, nebulizing gas flow 1.5 L/min and capillary voltage 4.5 kV. All mass spectra were recorded in the positive ion mode with mass range 100–1500 Da. The progress of degradation was determined based on the area under the peaks corresponding to the enkephalins and internal standard (Z-norleucine). Internal standard was applied in order to eliminate a drift in the equipment measurements and to ensure the quality of performed investigations. We chose Z-norleucine because it is an unnatural amino acid and therefore

should not be present in our matrix (human serum). Hence, quotient of area under the peak of enkephalins and area under the peak of internal standard was used to estimate degradation half-life of enkephalins. In turn, the degradation half-lifes ($T_{1/2}$) were calculated using GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA).

Statistical analysis

The data were analyzed using Statistica 13.1 software (StatSoft, Inc., USA). Continuous data are presented as means ± standard deviation, categorical data were described with absolute frequencies and percentages. An analysis of variance (one-way ANOVA or Kruskal-Wallis test) was used to evaluate differences among groups. Shapiro-Wilk's W test was used to test the distribution of the variables. Comparisons between groups were performed using the Student's *t*-test (or nonparametric Mann-Whitney U-test) and $\chi 2$ test. A value of p values <0.05 was considered statistically significant.

Results

Met-Enk degradation

Statistical analysis of the data from Met-Enk degradation studies indicated that the mean $T_{1/2}$ values obtained for CD and UC patients, without division by gender, were similar to those of healthy volunteers and demonstrated no significant association with the occurrence of the disease (p = 0.96021, Fig. 3A, Table 1). However, when similar analysis was performed for patients with a gender division, some subtle differences (p = 0.16382, Fig. 3B, Table 2) occurred. Namely, the mean value of $T_{1/2}$ of Met-Enk degradation in serum of females suffering from UC was lower in comparison to the females in control group (UC female patients $T_{1/2}$ = 16.63 min, female from control group $T_{1/2}$ = 19.61 min; see Fig. 3B, Table 2), whereas for female patients with CD it was higher (CD female patients with $T_{1/2}$ = 21.84 min; See Fig. 3B, Table 2). In turn, in the case of male patients, the results were opposite to those obtained for the female patients (UC male patients $T_{1/2}$ = 17.67 min, males from control group $T_{1/2}$ = 15.79 min, CD male patients $T_{1/2}$ = 13.61 min; See Fig. 3B, Table 2).

The most interesting results were the differences of the mean value of $T_{1/2}$ of Met-Enk degradation between male and female IBD patients. The mean value of $T_{1/2}$ for male CD patients was lower than for female patients suffering from CD (CD male patients $T_{1/2}$ = 13.61 min, CD female patients $T_{1/2}$ = 21.84 min; Fig. 3B, Table 2). Detailed analysis of the correlation between the mean value of $T_{1/2}$ in serum of male and female patients shows that in the control group the $T_{1/2}$ males/$T_{1/2}$ females ratio was 0.80, whereas in the UC patients 1.06 and in CD patients 0.62 (Table 2).

Within the UC group the mean value of $T_{1/2}$ was similar for both genders (UC males $T_{1/2}$ = 17.67 min, UC females $T_{1/2}$ = 16.63 min, Fig. 3B, Table 2).

Leu-Enk degradation

The relation of Leu-Enk degradation with the type of IBD disease differs from that for Met-Enk. Without division by gender, statistical data suggest that in patients with both, CD and UC the

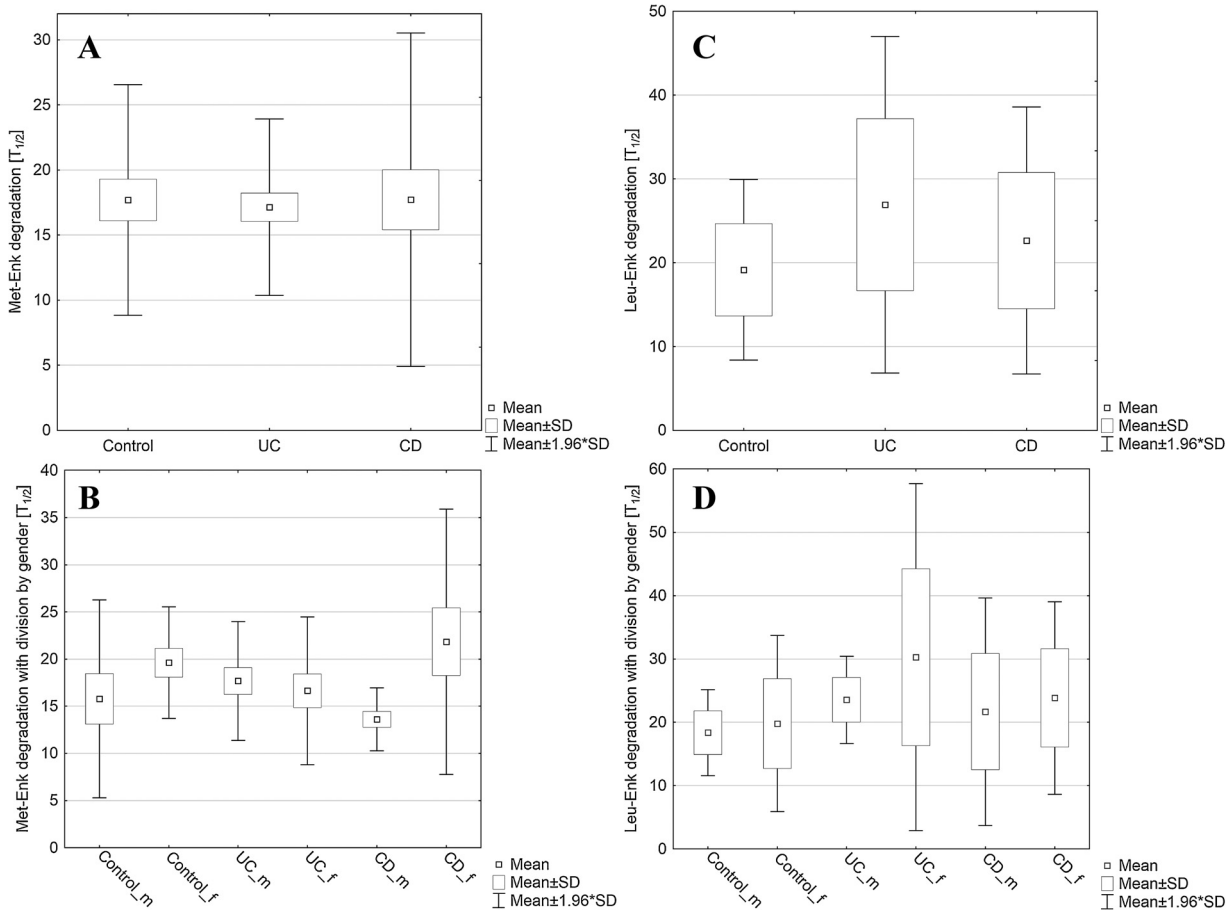

Fig. 3. Mean value of $T_{1/2}$ for Met and Leu-Enk degradation in human serum: (**A**) for Met-Enk without division by gender and (**B**) for Met-Enk with division by gender: female (**f**) or male (**m**); (**C**) for Leu-Enk without division by gender and (**D**) for Leu-Enk with division by gender: female or male (**f** or **m**, respectively).

mean values of $T_{1/2}$ of Leu-Enk degradation in serum were higher than that of control group, with the most significant increase observed for UC patients (control group $T_{1/2}$ = 19.16 min, UC patients $T_{1/2}$ = 26.93 min, CD patients $T_{1/2}$ = 22.65 min, Fig. 3C, Table 1). Stronger inhibition of Leu-Enk degradation in IBD patients was even more evident when groups were divided by gender (males from control group $T_{1/2}$ = 18.37 min, UC male patients $T_{1/2}$ = 23.56 min, CD male patients $T_{1/2}$ = 21.67 min, females from control group $T_{1/2}$ = 19.78 min, UC female patients $T_{1/2}$ = 30.29 min, CD female patients $T_{1/2}$ = 23.85 min, Fig. 3D, Table 2). The correlation between $T_{1/2}$ of Leu-Enk within a given group ($T_{1/2}$ males/$T_{1/2}$ females) also differed from that for Met-Enk. In the control group, the $T_{1/2}$ males/$T_{1/2}$ females value was 0.93, in the patients with UC 0.78, whereas for patients with CD 0.91 (Table 2). This indicates that the inhibition of Leu-Enk for males and females within CD and control groups was similar for both genders. However, in the case of the UC group, a stronger inhibition of Leu-Enk degradation in serum of females was evidenced.

Discussion

IBD are composed of CD and UC. It is assumed that 1–2% of the human population suffers from IBD and it is more prevalent in women than men [1]. It is believed that IBD are responsible for a dysregulation of the immune response in the intestine and it is well established that CD and UC have distinct immune profiles that are linked to their specific pathogenesis [11]. The resulting infiltration of the bowel by granulocytes and macrophages leads to the release of enzymes, reactive oxygen intermediates, and proinflammatory cytokines, all of which cause extensive damage to the local tissues [12]. UC is an ulcerating inflammation of the mucosal layer generally restricted to the colon, whereas CD is a deep transmural inflammation that mostly occurs in the terminal ileum but can also occur anywhere in the GI [13]. UC and CD have a lot of common features like an increased cytokine secretion and accumulation of mononuclear cells in the affected areas. However, CD is mainly associated with a Th1 type inflammatory process, with greater expression of interferon (IFN)-γ and interleukin (IL)-2, as well as the Th1-inducing cytokine IL-12. In the case of UC an atypical cytokine profile can be observed. Therefore there is a greater expression of IL-5 and IL-13, cytokines more commonly bound to a Th2 response [12].

The diagnosis of IBD should rely on a number of factors including clinical and endoscopic evaluation as well as histologic, serologic and radiologic assessments [14]. Unfortunately, many of the current methods of IBD diagnosis have disadvantages, for example endoscopic evaluation can be related to significant risks such as perforation [15]. The most appropriate diagnostic tool could be a blood test, in which characteristic biomarkers for IBD would be determined. A biomarker is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to an intervention [16]. In case of IBD, there is no single sensitive or enough specific biomarker which could indicate inflammation with high confidence. Moreover, the use of biomarkers for the diagnosis of IBD is still a subject of scientific studies, and many of the compounds found to be indicative of systemic inflammation have limitations in their use. For example, C-reactive protein (CRP) is produced in response to inflammation, stimulated by certain cytokines like tumour necrosis factor-alpha (TNF-α), IL-6 and IL-1β [17]. CRP level may rise significantly in an active IBD but also in the case of infection, autoimmune conditions, other inflammatory conditions, and malignancy as well as cell necrosis [18]. That is why finding new and specific biomarkers for IBD is still a considerable challenge for modern science.

Recently, great hopes for finding new characteristic biomarkers of CD and UC are placed in the opioid system, which may be involved in the regulation of inflammatory processes [1]. Opioids act both in the central nervous system and in the periphery and this has encouraged their use for diagnostic and therapeutic purposes also in the GI tract. Particular attention is paid to the opioids produced by leukocytes, for example Met-Enk [7]. They can act as analgesics and immunomodulators, affecting leukocyte migration, their bactericidal activity and apoptosis. Enkephalin action can also be manifested through their impact on the synthesis and secretion of proinflammatory and anti-inflammatory cytokines [19]. Hence, Met- and Leu-Enk in serum of IBD patients can be possible biomarkers of the disease activity. However, the short half-life of enkephalins in the serum and other tissues is a serious limitation for their application as biological indicators of active inflammation which need further investigation.

Due to the fact that half-life of enkephalins in tissue samples is really short (counted in minutes), in our study we have decided to degrade exogenous Met- and Leu-Enk in serum samples derived from patients suffering from IBD. For this purpose Met- and Leu-Enk were added to a serum sample (to provide constant level at the beginning of each assay); moreover, both enkephalins were measured separately to avoid competition between them for degrading enzymes. As a result of our research we have observed a number of interesting correlations and trends. In the case of Met-Enk degradation in the serum samples, we showed that some correlations appeared only when the studied cohort was divided by gender. A stronger inhibition of Met-Enk degradation occurred in serum samples of females suffering from CD than in the other investigated groups, whereas the lower inhibition was observed in the serum samples of males with CD. Perhaps this high inhibition was caused by higher concentration of endogenous inhibitors (e.g. sialorphin [20]) in serum of females with CD or by presence of female hormones like estradiol, which are suspected of regulation of the expression of an important enkephalin-degrading enzyme, aminopeptidase N [21]. In turn, the inhibition decrease in the case of CD male patients may be caused by the absence of estradiol and additionally by the presence of some endogenous compounds, which can compete with endogenous inhibitors of enkephalinases. All of these suggestions should be examined in further studies on enkephalin degradation in serum of patients with IBD. Notably, no such correlation was observed in the case of Met-Enk in patients with UC. Consequently, the half-life of Met-Enk in the serum samples of female and male patients with UC was similar, hence an equal correlation of 1.06 ($T_{1/2}$ males/$T_{1/2}$ females). These results suggest that the activity of enzymes degrading Met-Enk could play a role as a biomarker specific for CD.

Based on our data we can indicate that the activity of enzymes degrading Leu-Enk could in turn play a role as a biomarker specific for UC. In contrast to the results of Met-Enk degradation, we observed a higher half-life of Leu-Enk in the serum samples of patients with UC not only when the results were divided by gender, but in the whole cohort. This higher inhibition in the whole cohort of patients with UC was mainly caused by a stronger inhibition of Leu-Enk degradation in serum samples of females than of males. It suggests again that estradiol might play an important role in the regulation of the enkephalinases activity. Importantly, such a correlation between genders was not observed in the case of other investigated groups of patients (CD and control). For that reason the activity of the enzyme degrading of Leu-Enk could be a specific biomarker for UC. Summarizing, our results demonstrate that the faster Met-Enk metabolism in the serum of patients with IBD is mainly associated with CD, whereas that of Leu-Enk with UC. It still needs to be determined whether different rates of degradation of exogenous enkephalins in the serum of patients with CD and UC *vs.* control group depend on the concentration of endogenous ligands or enzyme inhibitors, or the concentration and activity of

endogenous enzymes. Noteworthy, in both CD and UC, the most interesting results were obtained for female patients. This observation may be explained by the regulation of enkephalinase activity by estradiol. The importance of estradiol action in IBD is very likely, especially since the prevalence of menstrual abnormalities including menorrhagia and irregular menses has been observed in 60% of women suffering from CD and 53% of women suffering from UC [22]. Moreover, Pierdominci et al. demonstrated a significant reduction of estrogen receptor (ER)β expression in peripheral blood T lymphocytes from CD/UC patients with active disease as compared to those in remission and healthy controls [23]. Additionally, studies from Armstrong laboratory demonstrated that estradiol protects against inflammation-associated colon tumor formation [24]. All of these documented positive aspects of anti-inflammatory actions of estradiol in the bowel diseases suggest that this hormone also influences the serum level of endogenous opioids as well as enzymes activity in patients with IBD. However, the mechanisms of estradiol action on IBD activity have not been as of yet established and for that reason this phenomenon should be studied more extensively, which we are planning to do in the near future.

In conclusion, our results demonstrate a tendency in inhibition of degradation of Met-Enk in CD and Leu-Enk in UC patients. This suggests that inhibition of enkephalins degradation may be associated, depending on the IBD disease type, with different activity of enzymes, enkephalin type and gender and could be groundwork for a new IBD diagnostic test development. Moreover, our research indicated the estradiol influence on enkephalinases activity should also be considered when a new diagnostic test is designed.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgments

This work was supported by grants from the Medical University of Lodz (503/1-156-04/503-11-001 to JF) and from National Science Center (#UMO-2013/11/B/NZ7/01301 and #UMO-2014/13/B/NZ4/01179 to JF). Project carried out with the use of CePT infrastructure financed by the Operational Program Innovative Economy 2007–2013 Priority Axis 2. -R&D Infrastructure, 2.2 Support for development of research infrastructure of scientific entities.

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