Plasma Matrix Gla protein and biochemical parameters in patients with Crohn´s disesase

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UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE

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Love you forever!

1. INTRODUCTION

1.1. Crohn's disease

1.1.1. Definition

Crohn's disease (CD) is a progressive chronic inflammatory disease of the gastrointestinal tract. All parts of the gastrointestinal tract can be affected but is most common in terminal ileum and proximal colon. We can usually find skip lesions, meaning inflamed segments interspersed with healthy segments. The inflammation is asymmetrical and transmural and evolve in a relapsing and remitting manner. The progressive nature of CD leads to complications like strictures, fistulas and abscesses which eventually would need surgery (1-3).

1.1.2. Epidemiology

CD is equally distributed in men and woman. The onset of the disease usually occurs between 10-39 years. We also see a small rise in incidence in 50-60 age group. The incidence and prevalence of CD are highest in developed countries and urban areas (4).

The highest annual incidence:

- Australia 29.3 per 100 000
- Canada 20.2 per 100 000
- New Zealand 16.5 per 100 000
- Northern Europe 10.6 per 100 000

The highest prevalence:

- Europe 322 per 100 000
- Canada 319 per 100 000
- USA 214 per 100 000

In Croatia, the incidence has increased during the last decade, annual incidence rate of CD was 4.1/10000 between 2006-2014 in Split -Dalmatia County (5,6). Rural areas and developing countries with low incidence and prevalence of CD has shown a steady increase in CD rates, almost in parallel with their development. In areas undergoing fast urbanization, they have seen an increase of annual incidence of CD (0.54 per 100 000) (Figure 1) (7). Another interesting observation is seen in populations immigrating from low-incidence to high-incidence regions. The incidence is increased in first or second generation, or if immigration occurred very early in life (8).

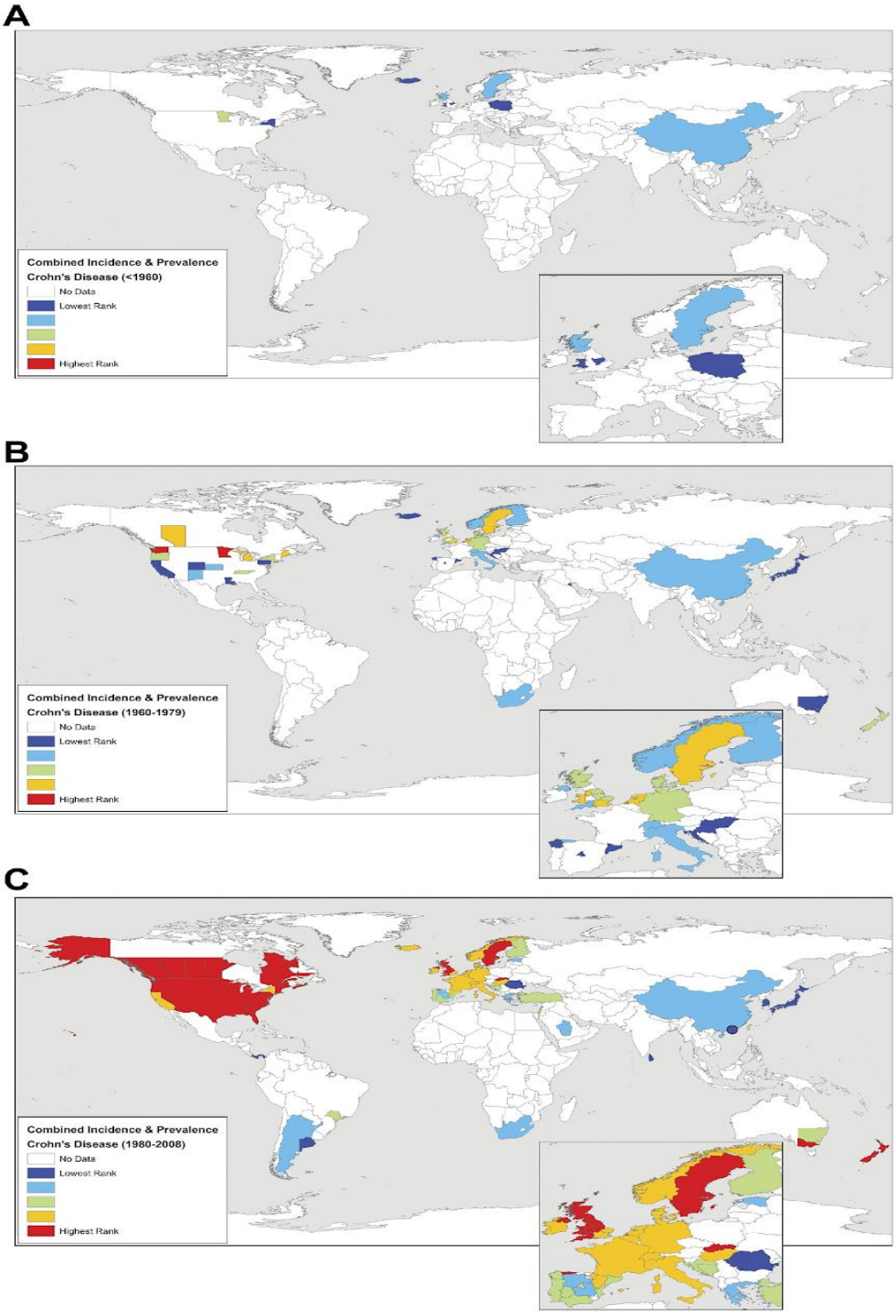

Figure 1. Combined Incidence and Prevalence of CD in three time periods: A < 1960, B 1960-1979, C 1980-2008. (7)

1.1.3. Pathophysiology

The etiology of CD is not known but is believed to be an immune mediated condition in genetically susceptible people. Disease onset is triggered by environmental factors that

disturb both the mucosal barrier and the gut microbiota and starts an abnormal immune reaction (Figure 2) (9).

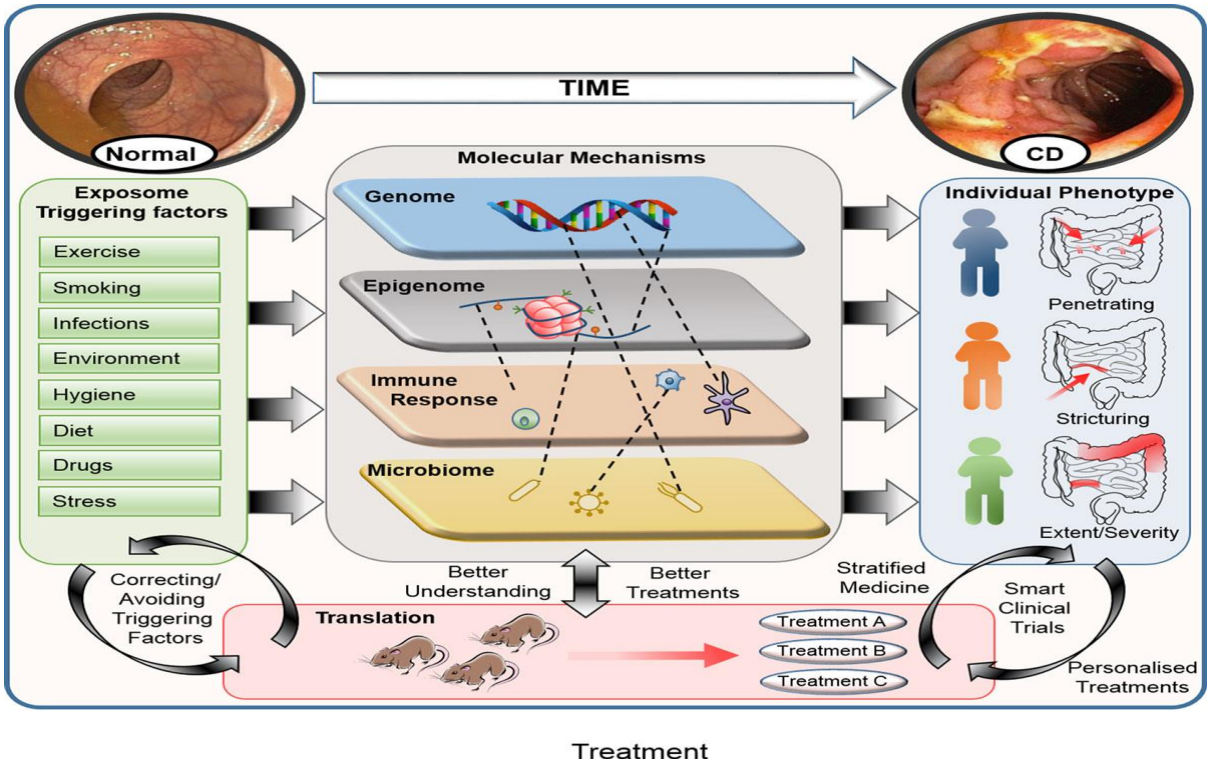

Figure 2. Multi-layer interactions in pathogenesis of Crohn's disease (9).

CD varies in presentation in different patients due to the complex interplay over time between genetic, epigenetic, immunological, and microbiological mechanisms affected by exposure to triggering factors (9).

Many CD susceptibility genes have been identified, with Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) having the strongest association (Figure 3). Decreased diversity of bacteria and changes in ratios of particular species (Microbial dysbiosis). Increased levels of Adherent-invasive *E. coli* (AIEC) that are resistant to phagocytic killing, which leads to release of the cytokine's TNF-α, IL-6 and IL-12 with subsequent inflammation. Both genetic and environmental factors affect microbial dysbiosis which can cause epithelial barrier dysfunction. One theory of how defective NOD2 contributes to CD: a functioning NOD2 senses muramyl dipeptide (MDP), which is a constituent of both gram-positive and gram-negative bacteria (10). This activates a number of innate immune responses that results in bacterial killing; defective NOD2 will not activate an immune response and result in an persistence of intracellular bacteria. Another theory on how NOD2 contributes to CD: activation of a defective NOD2 leads to a suppressive effect on the innate immune system and induces tolerance. IL-23 is a pro- inflammatory cytokine involved in differentiation of Th17

cells especially in the presence of TGF- β and IL-6. Activated Th17 cells produce IL-17A, IL-17F, IL-6, IL-22, TNF- α, and GM-CSF (granulocyte-macrophage colony–stimulating factor). Inflammatory macrophages express IL-23R and are activated by IL-23 to produce IL-1, TNF-α and IL-23. These effects identify IL-23 as a central cytokine in autoimmunity (10). Specific microbes and microbial products can induce particular innate immune responses. Segmental filamentous bacteria (SFB) induces Th17 proinflammatory responses; Clostridium, butyrate and polysaccharide A(PSA) can induce Treg cell responses which suppress inflammation, but they are often reduced in CD. NOD2 recruits' autophagy related 16 like 1 (ATG16L1) protein to the plasma membrane to initiate xenophagy/autophagy.

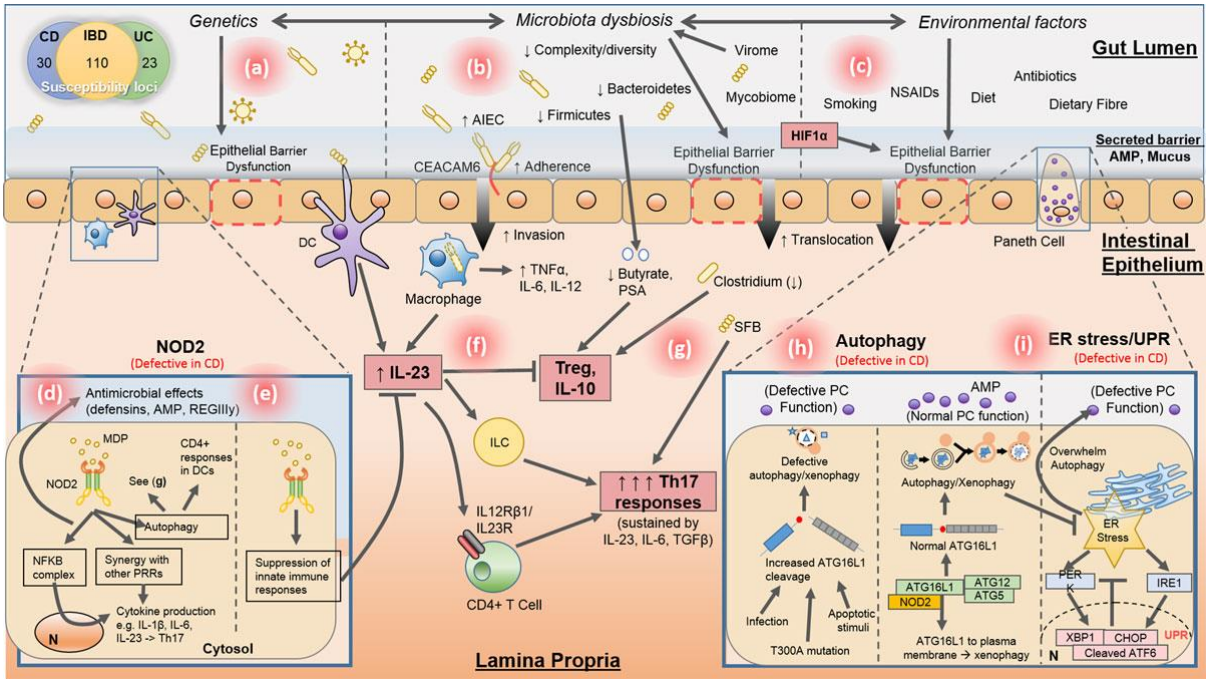

Figure 3. Molecular mechanisms in the pathogenesis of Crohn's disease (CD) (9)

Normal plasma cell function, including release of antimicrobial peptide (AMP), relies on autophagy; the T300A variant in ATG16L1 which are seen in some CD patients, leads to increased cleavage and defective autophagy. Unfolded protein response (UPR) and autophagy are compensatory mechanisms that help regulate endoplasmic reticulum (ER) stress. Excessive ER stress can overwhelm autophagy, leading to defective plasma cell function (9).

Multiple immune pathways have been found to be dysregulated in CD, including barrier function defects, innate immune defects and adaptive immune defects (3). The intestinal epithelium produces mucus and antimicrobial factors like Regenerating islet-derived protein 3 gamma (REG-3-γ) protein, which makes a protective layer between itself and intraluminal

contents (11). The protective layer can be destroyed by emulsifiers which is very common in a western diet. The protective layer can also be affected by mucin 2 (MUC2) gene mutations that leads to defective mucin 2 production and might promote bacterial translocation (12,13).

Autophagy of unwanted cytoplasmic contents in epithelial cells is important in preventing dissemination of invasive bacterial species (14). Defects in autophagy related genes like ATG16L1 and Immunity-related GTPase family M protein (IRGM) are important risk factors for CD (9). Intestinal tight junction defects are also associated with CD (15).

1.1.3.1. Innate immune defects

NOD-like receptors activate the host defense to intracellular fragments of bacterial peptidoglycan by initiating nuclear factor kappa light chain enhancer of activated B cells (NF-κB) - dependent and mitogen-activated protein kinase (MAPK) - dependent gene transcription producing cytokines. Dendritic cells (antigen presenting cells) are tolerogenic at steady state, but in inflammatory conditions, they contribute to the inflammation by expression of toll-like-receptor (TLR) 2 and TLR4 and production of proinflammatory cytokines (16).

Innate lymphoid cells (ILCs), are involved in the maintenance of barrier integrity and produce cytokines such as TNFα, interleukin 17, interleukin 22, and interferon γ in response to microbial and dietary molecules (17,18). ILCs have been found to be in a disbalance in number and activity in patients with CD, with elevated expression of ILC1 in inflamed ileum and a depressed number of ILC3 in inflamed colon (19-21).

Paneth cells secrete antimicrobial proteins from the base of the crypts of Lieberkühn. Mutations in the genes of NOD2, ATG16L1, LRRK2, XBP1, and IRGM lead to alterations in Paneth cell function, that result in reduced secretion of antimicrobial proteins (22).

1.1.3.2. Adaptive immune cells in Crohn's disease

Th1 and Th17 is found in the inflammatory infiltrate in CD, and their proinflammatory response to bacteria and fungi are thought to be part of the pathogenesis of CD (23). Intestinal Treg cells are important for the regulation of a proper immune response and is found to be impaired in CD (24).

Antimicrobial antibodies produced by B lymphocytes are often found in increased titres in CD as a response to luminal microbes that enters through the defective mucosal barrier, but it is unclear if it contributes to the pathogenesis of CD. There has been seen a decrease in dimeric IgA and increase in IgG and monomeric IgA (25).

1.1.4. Risk factors

1.1.4.1. Genetics

A positive family history of CD is the strongest risk factor for developing CD with higher concordance rates in monozygotic twins and first -degree relatives (26,27). Clinical features of CD such as inflammation pattern and location show a heritable pattern. Offspring of parents with CD often has an earlier onset and more serious disease, indicating the heritable character of CD (28-31). However, its only 12% of patients with CD that has a family history of the disease (32).

Patients with CD and major histocompatibility complex HLA-A2, HLA-DR1 and DQw5 have been linked to extraintestinal manifestations (33,34). CD has also been associated with genetic syndromes, like Turner syndrome, Hermansky Pudlak syndrome and glycogen storage disease type 1b (35).

1.1.4.2. Environmental factors

We have seen a rapid rise in CD in previously low-risk countries such as Japan, China and India after they started to adopt a Western lifestyle (36). Cigarette smoking is associated with a 2-fold increase in risk for CD (37). Antibiotic treatment during childhood has also been found to be a risk factor (38). Some medications like oral contraceptives aspirin and non-steroidal anti-inflammatory drugs have been associated with increased risk of CD (39,40).

Reduced intake in dietary fiber with an increased intake of saturated fat have been associated with increased risk (41). Reduced zinc levels have been proposed to be a risk factor for CD. Zink is very important for innate immunity, where it modulates macrophages, neutrophils and natural killer T cells. It inhibits transcription of inflammatory mediators and inhibits myeloperoxidase activity. Intracellular zinc is also important for autophagy, bactericidal activity and mucosal barrier function. Zink treatment has been shown to reduce the likelihood of relapses (44–45). Reduced vitamin D levels is also believed to be a risk factor for CD. Whereas highest quartile levels of Vitamin D status have been shown to be a protective factor (46).

Lifestyle factors like increased stress, poor sleep and little exercise have also been linked to increased risk for CD (36).

1.1.4.3. Microbiota

Micro dysbiosis with a decrease of *Bacteroides*, *Firmicutes* and *Faecalibacterium prausnitzii* and an increase in *Gammaproteobacteria* and *Actinobacteria* is often seen in CD patients (47,48). A third of patients with CD are found to have increased number of mucosa associated adherent-invasive *Escherichia coli* (AIEC) (49). *Caudovirales* viruses and fungal dysbiosis has also been linked to CD (50).

1.1.5. Clinical presentation

Symptoms of CD can present in many different ways depending on disease location, severity of the inflammation and the course of the disease progression. The most common presentation is a young patient with active CD in distal ileum, that causes chronic watery diarrhoea and steatorrhea, right lower abdominal quadrant pain, low-grade fever, weight loss, malnutrition, fatigue and growth retardation in children. The malnutrition can lead to clotting issues, hypomagnesemia and hypocalcemia (35).

When the active inflammation subsides, it can develop into a stricture which causes a new set of symptoms like, postprandial pain, bloating, nausea and vomiting, occlusion or sub-occlusion. If the inflammation is severe enough, it can lead to the development of a transmural sinus or ulcer which can further develop into an abscess or fistula. The symptoms will depend on the location of the fistula. Entero-urinary fistula will present with fecaluria, pneumaturia, and recurrent urinary tract infections. Rectocvaginal fistula will present with dispareunia and stool discharge through the vagina. Enteroenteric fistula can be asymptomatic. Enterocutaneous will lead to leakage of intestinal contents out through the skin. If the patient has colonic involvement, the major symptoms are usually rectal bleeding or bloody diarrhea. If the bacteria enters the circulation, it will present with high fever and can lead to further septic complications (4).

More than one-third of patients suffer from perianal disease which can cause anal fissures, anorectal fistulas and perirectal abscesses (51). Other parts of the gastrointestinal tract can also be affected, but this is much less frequent. Oral manifestations can be aphthous ulcers, esophageal symptoms can be odynophagia or dysphagia. Gastroduodenal symptoms can be epigastric pain, with nausea and vomiting (52). Patients with obstructive intestinal presentation will naturally start to eat less frequently due to the pain and discomfort, which will further add to the weight loss (35).

1.1.5.1. Extraintestinal manifestations

Extraintestinal manifestations (EIM) is seen in up to 40 % patients of patients with inflammatory bowel disease and is more prevalent in CD than in ulcerative colitis. All organ system can be affected, including musculoskeletal system, skin, eyes, hepatobiliary system, lungs, kidneys, immunologic or hematologic system, and cardiovascular system (53-7). Patients often present with extraintestinal symptoms of CD before the gastrointestinal symptoms manifest. And it is most often seen when CD affects the colon. Peripheral arthritis that involve large joints, is the most common manifestation (53).

Other common manifestation is aphthous stomatitis, uveitis, and erythema nodosum. Skin manifestations like erythema nodosum, pyoderma gangrenosum, psoriasis or vitiligo are caused by autoimmune reactions, but other skin lesions like pellagra and cheilitis may also manifest due to underlying nutritional deficiencies. Treatment of CD often involves corticosteroids which can lead to cushingoid features (57).

Erythema nodosum and large joint arthritis is associated with active intestinal disease. While axial arthritis and primary sclerosing cholangitis is found to progress independently from the intestinal CD activity (3). Although rare, the systemic response to chronic inflammation of CD can lead to secondary amyloidosis which can further affect other organ systems (57). The diarrhea and malabsorption in CD can lead to many different problems like; hypercoagulable state with development of venous and arterial thromboembolism; Calcium oxalate and uric acid renal stones; and megaloblastic anemia due to Vitamin B12 deficiency (58-61).

1.1.6. Complications

Serious complications of CD can be toxic megacolon, perforation, obstruction, massive hemorrhage, malabsorption, severe perianal disease and intra-abdominal and pelvic abscesses (35). A long-term complication of CD is colorectal cancer or small bowel carcinoma depending on disease location due to the effect of chronic inflammation in the gastrointestinal tract (62).

1.1.7. Diagnosis

Due to the many different manifestations of CD, we need a big battery of diagnostic interventions. Anamnesis with focus on known risk factors and history of typical symptoms of both gastrointestinal manifestations and extraintestinal manifestations is important. Physical examination should check for signs of acute symptoms like systemic toxicity and dehydration, and chronic symptoms like malabsorption, malnutrition and anemia. A tender mass might be palpated in the right lower quadrant, which can be caused by thickened bowel or mesentery, or

an abscess. In all patients with suspected CD we should examine the perianal region for; skin lesions like ulceration and skin tags; anal canal lesions like stenosis, fissures and ulcers; and fistulas with or without abscesses.

Laboratory tests should include complete blood count, blood glucose, electrolytes, liver enzymes, renal function tests, C-reactive protein (CRP), erythrocyte sedimentation rate, serum vitamin B12, iron and vitamin D (63). Common findings in CD is increased acute phase reactants, hypoalbuminemia, thrombocytosis, vitamin D deficiencies and anemia (3). CRP can be used to monitor disease activity, but it is important to know that it has a poor correlation with endoscopic findings and a third of patients present with a normal level of CRP (53). The stool biomarker fecal calprotectin is used as a screening test and to assess disease activity in IBD. It correlates with neutrophilic infiltrates in the GI-tract and has a high sensitivity and specificity for the diagnosis of IBD (64).

Endoscopy is the gold standard for diagnosis of CD. with colonoscopy
we look for “skip lesions” focal ulcerations next to areas of normal mucosa and “cooblestone” appearance caused by polypoid mucosal changes (3). Even if the mucosa looks normal with endoscopy, we should always obtain biopsies from rectum, right and left colon, because there can still be signs of focal ulceration together with microscopic acute and chronic inflammation. Sometimes we also find granulomas in CD, but we need to rule out infections, lymphoma or rheumatic conditions before we can conclude that it is CD (65).

Upper gastrointestinal series is done with CT, CT-enterography, MRI, MR-Enterography, ultrasound and enteroclysis. Classic findings is “string sign” with a narrow lumen, ulceration and nodularity, fistulas and abscesses, “cobblestone” appearance, and separation of bowel loops caused by thickening of mesentery or bowel wall. Ultrasound can measure small bowel wall thickness, and a thickness more than 3 mm is considered a positive finding that need further investigation (52). Magnetic resonance imaging or endoscopic ultrasound is used to look for perianal fistulas (63). Multispectral optoacoustic tomography is a promising technique to assess active versus inactive disease activity. It detects bowel inflammation by measuring hemoglobin-dependent tissue perfusion (66).

Antibody testing for perinuclear neutrophil antibodies (pANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) help us to differentiate between Ulcerative Colitis and CD, ASCA is often elevated in CD (67).

1.1.8. Differential diagnosis

It is important to differentiate between ulcerative colitis (UC) and CD, since the medical and surgical management are totally different. UC present with rectal bleeding, tenesmus, and fecal urgency. Disease is limited to the mucosa in the colon and usually start in rectum and progress in continuous and symmetrical manner. Histology typically shows crypt architectural distortion, crypt abscesses and ulceration (68).

Infectious enterocolitis usually presents with an acute onset of symptoms. Microbiological examination of stool, serology and histology might detect the causative agent. Histology should be normal compared to the findings in CD. The disease is self-limited (3). Microscopic colitis usually affects women more than 50 years old. They present with watery diarrhea and the endoscopy findings appear visually normal. Histological samples by biopsy is essential for diagnosis, where we can find either increased number of intraepithelial lymphocytes, increased chronic inflammatory infiltrate in lamina propria or the presence of abnormal surface subepithelial collagen layer with abnormal thickness. The rest of the wall architecture should be normal.

Intestinal tuberculosis might look like CD on endoscopy and the ileocecal location is the most common site. Chest radiographs reveal suggestive lesions in 50 % of patients with tuberculosis. CT findings may show calcified and necrotic mesenteric lymph nodes. Histological findings will show caseating granulomas. Diagnosis is confirmed with positive Ziehl-Neelsen, culture or PCR (69).

Behçet's disease, can present with both intestinal inflammation and extraintestinal manifestations (EIM). Uveitis and recurrent oral and genital ulceration is common. Vasculitis might be present. Pin prick test with positive pathergy test support the diagnosis (70). NSAIDs associated enteropathy, present with multiple erosions and ulcerations with a history long term use of NSAIDs. NSAID injury can lead to obstructive symptoms caused by small intestine concentric diaphragmatic strictures (71). Other diseases that should be ruled out is, irritable bowel syndrome, ischemic colitis, diverticular colitis, lactose intolerance, carcinoma and lymphoma (35).

1.1.9. Prognosis

Patients with CD in small or large intestine usually have intermittent exacerbation of symptoms followed by periods of remission. After initial presentation, between 10-20% experience a prolonged remission. After 10 years, half of them will have developed stricturing or penetrating disease (53,54).

Predictors of a severe progression include perianal rectal disease, smoking, age below 40, initial requirement for glucocorticoids, and low education level (55,56). Patients with ileal disease have a nine fold increased risk of developing stricturing or penetrating disease compared to patients with colonic disease. If you have ileocolonic disease the risk for developing stricture or penetration is 6-fold (67). Up to 80 % of patients with CD gets hospitalized during the course of their disease (72). Many patients require surgical intervention with intestinal resection because of intractable symptoms caused by obstruction or perforation. And after surgical treatment, the 5-year recurrence rate is 50% (72,73).

The heterogeneity of CD makes it difficult to predict life-expectancy and mortality but seems to be slightly reduced (74).

1.1.10. Assessing disease activity

After the diagnosis is established it is important to assess disease activity, extent, severity, and behavior. We do this with cross sectional imaging. Patients should also be phenotyped (75,76). Clinically, we classify disease activity as mild to moderate, moderate to severe and severe to fulminant. Mild to moderate is when, the patient can tolerate oral nutrition with absence of: dehydration, more than 10% weight loss, painful mass, abdominal tenderness, obstruction and toxicity. Moderate to severe disease is when, the patient has either: weight loss, abdominal pain, nausea and vomiting, fever, anemia, lack of response to therapy. Severe to fulminant disease is when the patient has: High fevers, rebound tenderness, intractable vomiting, intestinal obstruction, presence of an abscess or continued symptoms after glucocorticoids (72).

1.1.11. Treatment

1.1.11.1. Medical treatment

Medical treatment is the pillar of CD therapy. We can use two general approaches, step-up therapy and top-down therapy. Step-up therapy is recommended for low-risk patients. It starts with less potent drugs with fever adverse effects. More potent drugs can be added if the initial therapies are ineffective or if the patient need more than one course of glucocorticoids. With this strategy we spare the patients for unnecessary adverse effects from overly potent drugs (77).

Top-down therapy is recommended for moderate to severe CD. They should be started on biologic or immunomodulator therapy in a top-down approach. The benefit of this strategy

is a faster onset of clinical remission and a more favorable long-term side effect profile compared to patients receiving glucocorticoids (77). Other factors that is important to take into account is, the cost of therapy, patient compliance and the individual susceptibility to drug toxicity (78). The goal is to accomplish histologic, endoscopic and clinical remission by mucosal healing. Medication used includes glucocorticoids, 5-aminosalicylates, immunomodulators and anti-TNF agents.

Dietary interventions is also important, since patients with ileal CD often have malnutrition and increased tendency to be lactose intolerant. Bowel rest and total parenteral nutrition has been shown to be as efficient as glucocorticoids at inducing remission of active disease. Patients who are hospitalized due to complications, should be assessed by fluid and electrolytes replacement, intravenous antibiotics, consultation with gastrointestinal surgeon and possible parenteral nutrition (77).

After clinical remission has been achieved, we use different maintenance therapies, depending on what type of therapy that was necessary to cause remission. It is important to assess patients clinically and with ileocolonoscopy in 6-12 months after clinical remission. We obtain CRP and fecal calprotectin at the time of colonoscopy and correlate them with the mucosal healing (65). Surgical treatment may be necessary in severe disease, where the patient is unresponsive to the medical treatment (51).

1.1.11.2. Surgical treatment

Surgical treatment is unfortunately not curative for CD and recurrence after operative management is the rule. Surgical indications are limited to the complications of the disease and when children experience growth delay due to CD and malnutrition (79). Common procedures include stricture plasty, small bowel resection, endoscopic balloon dilation, segmental colectomy, total colectomy with ileorectal anastomosis, total proctocolectomy with end ileostomy and proctectomy (63). Small bowel resection is the most effective technique for patients with small bowel short-segment strictures or fistulas in order to restore health (80,81). Strictureplasty is a good technique when we need to spare the length of small bowel as much as possible, due to risk of small bowel syndrome (79). If the strictures are less than five centimeters in length, we can try endoscopic balloon dilatation instead if surgery (82).

1.1.12. Quality of life

Health-related quality of life (HRQOL), a state of well-being that comes from being satisfied by his or her physical, psychological and social well-being while performing everyday

activities. Patients with CD have reported a decrease in their quality of life. With higher rates of anxiety, depression and dependency on others, and lower general mental functioning. And as expected the quality of life is further reduced during active disease compared to dormant disease (83-85).

Patients with severe disease, inadequate sleep quality or folic acid deficiency have shown lower HRQOL scores (86). World Health Organization defines CD as a disease that influence physical, psychological, familial and social aspects of life. It can also impair sexual function (87). HRQOL is very important to assess, since therapy greatly can affect the quality of life (86).

2.1 Matrix G-carboxyglutamate (Gla) Protein (MGP)

MGP is a small 12 kDa, 84 amino acid Gla protein. It is secreted by chondrocytes and vascular smooth muscle cells (VSMCs). It is expressed in bones, vessels, heart, kidneys and cartilage. It is the most powerful natural inhibitor of calcification in the human body. It can also reverse the calcification process (88). MGP has three different known mechanisms of protection from artery calcification. Firstly, it has a high binding affinity to hydroxyapatite (HA) crystals and thereby nullify their accumulation in the arterial wall. Secondly, after binding calcium and phosphate crystals, it activates macrophages to promote phagocytosis and apoptosis of the MGP-hydroxyapatite complex (88). Thirdly, MGP binds to bone morphogenetic protein-2 (BMP-2), and thereby inhibit its binding to its receptor, consequently down regulating its function (89,90).

BMP-2 is found in endothelial foam cells in atherosclerotic plaques and can induce osteoblast transformation of vascular smooth muscle cells (VSMCs) after binding to its receptor. This will lead to chondrogenesis, osteogenesis and vascular calcification (91-93). MGP requires vitamin K-dependent gamma-carboxylation and phosphorylation to become biologically active. Without carboxylation MGP is not able to bind crystals or BMP-2 (94). Vitamin K deficiency leads to lower levels of active MGP and subsequent progressive vascular calcification. High vitamin K intake can reverse this process (95).

MGP also play a role in bone organization, where MGP transcription is increased by vitamin D. MGP mutation causes Keutel syndrome, which is characterized by peripheral stenosis of the pulmonary artery, abnormal cartilage calcification and midfacial hypoplasia. “Knock out” MGP-Mouse developed to term, but then they will develop progressive arterial calcification, that will lead to deadly blood-vessel rupture within two months (96-99). A

subtype of glioblastoma with overexpression of MGP is linked to worse outcome with shorter survival due to increased migration (100).

MGP derived from mesenchymal stromal cells has showed promise in the therapeutic approach of CD. Higher expression of MGP is linked to better immunoregulatory properties, with suppression of T cell proliferation and cytokine production. It markedly reduced the clinical and histopathological severity of colonic inflammation in mouse experimental colitis models (101).

There is an association between the pathophysiology of vascular calcification and the loss of bone mass in humans. By preventing arterial calcification, MGP has showed the ability to stop the loss of bone (102). MGP also functions as an inhibitor of hydroxyapatite (HA) mineralization and the binding affinity of MGP to HA is enhanced by calcium ions and depressed by phosphate and magnesium ions (103).

Elevated inactive desphospho-uncarboxylated MGP (dp-ucMGP) has been proposed as a marker for estimation of cardiovascular risk, as higher concentrations are associated with both higher cardiovascular and total mortality. Elevated dp-ucMGP is also a circulating biomarker of vitamin K status and vascular calcification and is associated with aortic stiffness (103-105).

2. AIM AND HYPOTHESES

2.1. Aim

The aim of this study was to investigate plasma MGP levels in patients with Crohn's disease in comparison to control group, while additional goal was to investigate association of plasma MGP levels with disease activity and other biochemical parameters.

2.2. Hypothesis

1. Plasma MGP levels will be significantly higher in patients with Crohn's disease in comparison to control group.
2. Plasma MGP levels will have positive correlation with disease activity in patients with Crohn's disease.

3. MATERIALS AND METHODS

3.1 Study design

This cross-sectional study was conducted at University of Split School of Medicine and Department of Gastroenterology at University Hospital of Split, over a period from January to July 2018. Study protocol was approved by the Ethics Committee of the University of Split School of Medicine and Ethics Committee of the University Hospital of Split. All participants provided written informed consent and all procedures were carried in accordance with the Declaration of Helsinki.

3.2 Subjects

This study recruited 35 patients with Crohn's disease evaluated at Department of Gastroenterology, University Hospital of Split and 35 age and sex matched healthy control subjects, aged 18-65 years. Diagnosis of Crohn's disease was established according to the recent guidelines by the European Crohn's and Colitis Organization and the European Society of Gastrointestinal and Abdominal Radiology (106). Patients with history of cardiovascular or metabolic disorders; use of corticosteroids in previous three months; use of psychoactive medications; as well as alcohol and substance abuse were excluded from the study.

3.3 Anthropometric assessment

Body weight (kg) and body height (m) were measured by calibrated stadiometer with integrated weight scale (Seca, Birmingham, UK). Body mass index (BMI) was determined by dividing body mass (kg) with squared body height (m^2). Additional data were gathered from medical documentation.

3.4. Sample collection and laboratory analysis

Venous blood samples were taken through a polyethylene catheter inserted into a forearm vein after fasting for 8 hours. Plasma MGP levels were analyzed by CLIA method using IDS-iSYS InaKtif MGP (Immunodiagnostic Systems, Frankfurt, Germany) with reported limit of detection sensitivity of 200 pmol/L. Intra-assay coefficient of variability (CV) was 4.5% and inter-assay CV of 7.9%. Fasting plasma glucose, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT) were analyzed by using standard laboratory methods (ARCHITECT ci16200, Abbott, Chicago, IL, USA). High-sensitivity C-reactive protein (hsCRP) was determined by the immunoturbidimetric method on Architect c16200 system

(Abbott, Chicago, IL, USA). Complete blood count and differential blood count were determined by using standard flow-cytometry-based hematologic analyses (ADVIA 2120i, Siemens Healthcare, Erlangen, Germany). Fecal calprotectin levels have been measured from stool sample after appropriate collection, following standard laboratory protocols.

3.5. Statistical analysis

Statistical software MedCalc (Ostend, Belgium; version 11.5.1.0) for Windows was used for statistical data analysis. Normality of data distribution has been assessed by Kolmogorov-Smirnov test. Data were presented as means ± standard deviation for continuous variables and as whole numbers and percentage for categorical variables. Student t-test or Mann-Whitney U test were used for analysis of continuous data. Chi-square test has been used for comparison of categorical variables. Pearson's correlation provided assessment of correlation between MGP levels and other parameters. Furthermore, we conducted multiple linear regression analysis adjusted for age, sex and BMI with plasma MGP as dependent variable. The statistical significance was defined as $P<0.05$.

4. RESULTS

Baseline characteristics of subjects included in the study are presented in Table 1. Patients and the controls were sex, age and body mass index (BMI) matched. Furthermore, there was no difference in waist circumference (*P*=0.559).

Table 1. Baseline characteristics of Crohn's disease patients and control group

Parameter	CD group (N=35)	Control group (N=35)	P*
Age (years)	37.9±14.4	38.5±11.9	0.842
Men (N, %)	25 (71.4)	22 (62.9)	0.448
Women (N, %)	10 (28.6)	13 (37.1)	
Body weight (kg)	73.5±15.4	79.2±14.0	0.106
Body height (cm)	177.5±9.7	180.8±9.7	0.159
BMI (kg/m^2)	23.1±3.7	24.1±2.8	0.253
Waist circumference (cm)	85.2±11.9	87.0±12.9	0.559

Data is presented as mean±standard deviation or number (percentage)
*t-test for independent samples or chi-square test
CD – Crohn's disease; BMI – body mass index

The measured plasma MGP level was significantly higher in the Crohn's disease group in comparison to control group as presented in Figure 1 (641.1±141.6 vs. 532.6±98.8 pmol/L, P<0.001).

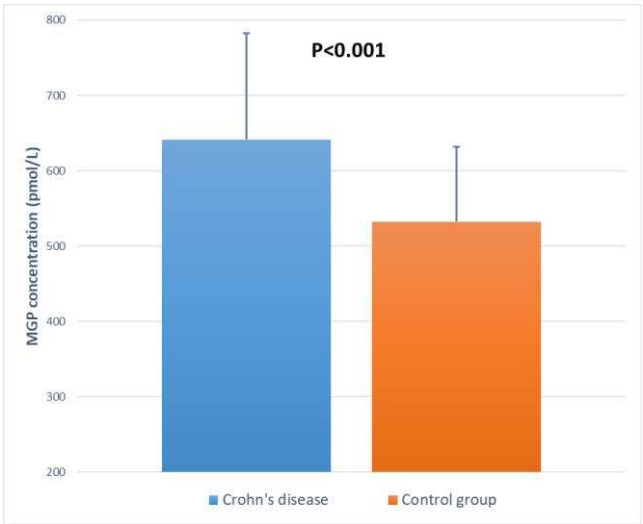

Figure 1. Plasma MGP levels in Crohn's disease and control group

Significant positive correlation was found between plasma MGP concentrations and waist circumference and hsCRP (P=0.028 and 0.024, respectively). Furthermore, MGP levels positively correlated with liver function tests. Additionally, significant negative correlation was found between MGP concentrations and HDL level (r=-0.242, P=0.043). 4. There were no significant correlations between MGP levels and disease duration or fecal calprotectin level. The data is presented in Table 2.

Table 2. Correlations between MGP concentrations and selected parameters

Parameter	**r***	**P**
FCal	0.131	0.454
hsCRP	0.268	0.024
BMI	0.129	0.284
Waist circumference	0.261	0.028
HDL	-0.242	0.043
LDL	-0.145	0.231
TG	0.111	0.360
Cholesterol	-0.129	0.285
GGT	0.302	0.011
ALT	0.263	0.027
AST	0.258	0.031
Duration of the disease	0.034	0.846

* Pearson's correlation coefficient
Fcal – fecal calprotectin; hsCRP – high sensitive C-reactive protein; BMI – body mass index; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; GGT – gamma glutamyltransferase; AST – aspartate aminotransferase; ALT – alanine aminotransferase.

The differences in biochemical parameters between patient's with Crohn's disease and control group are presented in Table 3. Patients with Crohn's disease had significantly lower haemoglobin (P=0.012), total cholesterol (P=0.004) and low-density lipoprotein levels (P=0.004), but higher hsCRP levels (19.6±44.2 vs. 1.28±1.32 mg/L; P=0.019).

Table 3. Biochemical parameters of Crohn's disease and control group

Parameter	CD group (N=35)	Control group (N=35)	P*
Hemoglobin (g/L)	138.9±16.8	148.6±14.7	0.012
FPG (mmol/L)	5.04±0.79	5.1±0.79	0.753
AST (mmol/L)	29.6±56.8	20.4±7.1	0.346
ALT (mmol/L)	29.8±46.7	26.2±14.9	0.661
GGT (mmol/L)	26.9±29.7	21.0±9.1	0.265
hsCRP (mg/L)	19.6±44.2	1.28±1.32	0.019
Cholesterol (mmol/L)	4.48±1.29	5.41±1.29	0.004
TG (mmol/L)	1.58±1.45	1.30±0.65	0.302
LDL (mmol/L)	2.48±0.9	3.42±1.17	0.004
HDL (mmol/L)	1.28±0.39	1.40±0.32	0.172

Data is presented as mean±standard deviation
*t-test for independent samples
CD – Crohn's disease; hsCRP – high sensitive C-reactive protein; BMI – body mass index; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; FPG – fasting plasma glucose; GGT – gamma glutamyltransferase; AST – aspartate aminotransferase; ALT – alanine aminotransferase.

Table 4 shows a multiple linear regression analysis adjusted for age, sex and BMI with plasma MGP as dependent variable. Age was the only statistically significant independent variable (β =5.01, SE=2.01; P=0.018).

Table 4. Multiple linear regression of MGP and selected parameters

Variable	β*	SE**	P
FCal	0.033	0.049	0.502
hsCRP	0.840	0.542	0.131
BMI (kg/m^2)	-2.99	8.81	0.736
Age (years)	5.01	2.01	0.018
Sex	3.32	57.71	0.954

* unstandardized coefficient β
** standard error
hsCRP – high sensitive C-reactive protein; Fcal – fecal calprotectin; BMI – body mass index

5. DISCUSSION

Inflammatory bowel disease has been known to potentially cause venous thromboembolism, but recently it has also been linked to arterial stiffness, atherosclerosis, ischemic heart disease and myocardial infarction. The hypothesis is that due to the compromised intestinal mucosal barrier in IBD patients, endotoxins and bacterial lipopolysaccharides can enter into circulation and activate a systemic inflammatory response that leads to oxidative stress and elevated levels of inflammatory cytokines, which further activate phenotypic changes in smooth muscle cells and development of cardiovascular diseases (107).

Our cross-sectional study, as far as we know, is the first which investigated inactive plasma MGP levels in patients with CD. We showed that plasma inactive MGP levels are significantly higher in patients with CD in comparison to control group. It has been known that MGP is dependent on vitamin K to be biologically active. Mineralization of endothelial extracellular matrix induces an increased expression of MGP as a negative feedback mechanism. This causes a relative deficiency in vitamin K, which if not replenished will lead to inactive MGP and vascular calcification (108). One possible mechanism of the increased inactive MGP in CD is the malnutrition deficiency caused by CD that can lead to vitamin K deficiency and further vascular calcification.

There was a significant positive correlation of high sensitive C-reactive values and MGP values indicating that MGP, potentially may be used as a marker of disease activity. However, our multiple regression analysis did not confirm fecal calprotectin or high sensitive C-reactive protein as independent predictors of MGP-values. Interestingly, age was the only significant independent variable which predicted proportional increase in MGP.

Furthermore, we found a statistically significant positive linear relationship with inactive MGP and waist circumference, GGT, ALT and AST. A significant negative linear relationship with MGP was observed for HDL levels what is consistent with previously published studies on patients with asymptomatic aortic stenosis in example (109). Same study, also showed significant positive correlation of high sensitive C-reactive values and MGP values, which is consistent with our findings (109). Study of Liu et al. investigated inactive MGP levels related to adverse health outcomes found a positive linear relationship between inactive MGP and cholesterol (110).

One other study on the mouse experimental colitis model, showed that mesenchymal stromal cells (MSC) with higher expression of MGP is linked to better immune regulatory properties, with suppression of T cell proliferation and cytokine production. It markedly reduced the clinical and histopathological severity of colonic inflammation. Their results

indicated that MGP might be a novel important mediator of MSCs-mediated immunomodulation in treating CD (101).

Hemoglobin, cholesterol and LDL were all significantly lower in the Crohn's disease group compared to the control group, while hsCRP level was significantly higher in the Crohn's disease patients. Low cholesterol and LDL could be a result of either malabsorption or hepatic damage. One interesting side note is that vitamin K is transported in LDL, so a low LDL could indirectly promote arterial calcification via MGP. LDL has also been found to have immunological properties. It can attach itself to pathogens and promote clearance by immune cells. It can also bind to LPS and lower the systemic inflammation as a result (111). The high levels of hsCRP is to be expected as it is an acute phase protein secreted by the liver, as a response to increased IL-6 secreted by T cells and macrophages (111).

Even though our study has some limitations like small sample size, cross sectional design in a single-center, this is the first time as far as we know that a data study on MGP levels in CD has been done. Further research is needed to clarify MGP role in the complex pathophysiology of CD and its complications on a larger number of patients.

6. CONCLUSIONS

1. Plasma MGP level was significantly higher in patients with Crohn's disease in comparison to control group.
2. Plasma MGP level showed positive correlation with disease activity measured with hsCRP in patients with Crohn's disease.
3. Significant negative correlation was found between MGP and HDL level.
4. There were no significant correlations between MGP levels and disease duration or fecal calprotectin level.
5. Hemoglobin, total cholesterol and LDL were all significantly lower in the Crohn's disease patients compared to the control group.

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8. SUMMARY

Objectives: The aim of this study was to investigate plasma Matrix Gla protein (MGP) levels in patients with Crohn's disease in comparison to control group, while additional goal was to investigate association of plasma MGP levels with disease activity and other biochemical parameters.

Materials and methods: A total of 35 patients with Crohn's disease and 35 sex, age and BMI matched control subjects underwent anthropometric assessment which were measured by calibrated stadiometer with integrated weight scale. Venous blood samples were taken and plasma MGP levels were analyzed by Chemiluminescence immunoassay (CLIA) method using IDS-iSYS InaKtif MGP (Immunodiagnostic Systems, Frankfurt, Germany). Other biochemical parameters were determined by standard laboratory procedures.

Results: The plasma MGP levels were significantly higher in the Crohn's disease patients in comparison to control group (641.1±141.6 vs. 532.6±98.8 pmol/L, $P<0.001$). Significant positive correlation was found between plasma MGP concentrations and waist circumference and hsCRP (P=0.028 and 0.024, respectively). Furthermore, MGP levels positively correlated with liver function tests. Additionally, significant negative correlation was found between MGP levels and HDL level (r=-0.242, P=0.043). There were no significant correlations between MGP levels and disease duration or fecal calprotectin level.

Conclusion: In conclusion, this study confirmed that plasma MGP levels are significantly higher in patients with Crohn's disease in comparison to control group. Furthermore, plasma MGP levels can be related with disease activity in patients with Crohn's disease. Further studies are needed to clarify this connection.

9. CROATIAN SUMMARY

Naslov: Plazmatski Matrix Gla protein i biokemijski parametri u bolesnika s Crohnovom bolesti

Cilj: Cilj ovog istraživanja bio je ispitati koncentraciju MGP-a u plazmi u bolesnika s Crohnovom bolesti u usporedbi s kontrolnom skupinom, dok je dodatni cilj bio istražiti povezanost koncentracija MGP-a u plazmi s aktivnošću bolesti i drugim biokemijskim parametrima.

Materijal i metode: Ukupno 35 bolesnika s Crohnovom bolesti i 35 zdravih ispitanika upareno prema dobi, spolu i indeksu tjelesne mase podvrgnuto je antropometrijskoj procjeni koja je mjerena kalibriranim visinomjerom s integriranom težinskom skalom. Uzorkovana je venska krv, a koncentracija MGP-a u plazmi određena je kemiluminiscencijskom imunoesej (CLIA) metodom koristeći IDS-iSYS InaKtif MGP (Immunodiagnostic Systems, Frankfurt, Njemačka). Ostali biokemijski parametri određeni su standardnim laboratorijskim postupcima.

Rezultati: Plazmatske koncentracije MGP bile su statistički značajno veće u skupini bolesnika s Crohnovom bolesti u usporedbi s kontrolnom skupinom (641,1 ± 141,6 naprema 532,6 ± 98,8 pmol / L, P <0,001). Utvrđena je značajna pozitivna korelacija između koncentracija MGP u plazmi i opsega struka i hsCRP-a (P = 0,028 i 0,024). Nadalje, koncentracija MGP-a pozitivno korelira s jetrenim enzimima. Osim toga, utvrđena je značajna negativna korelacija između koncentracije MGP-a i HDL-a (r = -0,242, P = 0,043). Nije pronađena značajna korelacija između koncentracije MGP-a i trajanja bolesti i razina fekalnog kalprotektina.

Zaključak: Ova studija je potvrdila da su koncentracije MGP-a u plazmi značajno veće u bolesnika s Crohnovom bolesti u usporedbi s kontrolnom skupinom. Nadalje, koncentracija MGP-a u plazmi može biti povezana s aktivnošću bolesti u bolesnika s Crohnovom bolesti. Potrebne su daljnje studije kako bi se pojasnila ta povezanost.

10. CURRICULUM VITAE

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