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Association of MMP-9 and PAD-4 according to UC extension and severity

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Abstract

Matrix metalloproteinase-9 (MMP-9) and Peptidyl Arginine Deiminase-4, enzymes released mainly from activated neutrophils in the colonic mucosa of patients with Ulcerative colitis (UC) and involved in intestinal barrier dysfunctions. This study aimed to determine the relation of serum and colon biopsies of MMP-9 and PAD-4 in patient with UC to access disease extension and severity. The study group comprised 60 adults newly diagnosed with UC and 20 control groups. Venous blood sample used for both MMP-9 and PAD-4 by ELISA technique while, Real-time PCR was used to study tissue expression of MMP-9 and PAD-4 in colon biopsies obtained during colonoscopy. Both serum and tissue MMP-9 and PAD-4 were significantly elevated in UC patients in compared to control group, although MMP-9 relationship to both extension and severity was significant except when compared control to proctitis and control to mild group. On the other hand, both serum and tissue expression of PAD-4 was significantly correlated to extension and severity indices with better sensitivity and specificity than MMP-9. Combining MMP-9 and PAD-4 in serum and colon biopsies obtained during colonoscopy may be valuable in improving disease severity performance. Matrix metalloproteinase-9 (MMP-9) and Peptidyl Arginine Deiminase-4, enzymes released mainly from activated neutrophils in the colonic mucosa of patients with Ulcerative colitis (UC) and involved in intestinal barrier dysfunctions. Aims: This study aimed to determine the relation of serum and colon biopsies of MMP-9 and PAD-4 in patient with UC to access disease extension and severity. The study group composed of newly diagnosed UC (60 adults) and healthy control group (20 Adults). Venous blood sample used for both MMP-9 and PAD-4 by ELISA technique while, Real-time PCR was used to study tissue expression levels of MMP-9 and PAD-4 in colon mucosal tissue biopsies obtained during colonoscopy. Results: Both serum and tissue enzymes level of MMP-9 and PAD-4 were significantly elevated on UC patients in compared to healthy control group, although MMP-9 relationship to both extension and severity was significant except when compared control to proctitis and control to mild group. On the other hand, both serum and tissue expression of PAD-4 was significantly correlated to extension and severity indices with better sensitivity and specificity than MMP-9. Combining MMP-9 and PAD-4 in serum and colon biopsies obtained during colonoscopy may be valuable in improving disease severity performance.

Keywords: Ulcerative colitis, MMP-9, PAD-4, Mayo score

1.Introduction

Ulcerative colitis disease is characterized by a continuous colonic mucosal inflammation, that may extent from rectum distal colon and in rare case to ileum(backwash ileitis)(1). Annual UC incidence is different around the world with peak in north America and Europe and lowest in Asia the Middle East, although the incidence in these countries has been increased remarkably over past years (2).

UC usually presents with continues diarrhea with blood and mucus, abdominal pain and tenesmus, although in Severe cases may suffer from systemic inflammatory signs (3,57).Although The etiopathogenesis is still largely unknown. Although recent studies proposed that it can by developed due to abnormal immune response against luminal antigens in genetically predispose patients that leads to uncontrolled persistent intestinal inflammation (4).Many inflammatory cells infiltrate damaged intestinal mucosal layer. Among these cells, neutrophiles are first and most invading inflammatory cells.

These cells responsible for recognition, destruction of invading particles , by producing highly reactive oxygen molecules, many peptides with antimicrobial action, and liberating extracellular web-like structures which called neutrophil extracellular traps.(5) . main constitution of neutrophil extracellular traps (NETs) are condensed chromatin, myeloperoxidase, matrix metalloproteinase 9, neutrophil elastase, and other specific antimicrobial molecules (6) .Peptidyl arginine deiminase type-4 (PAD-4) enzyme activation consider as the crucial NETs formation step that leads to ruptures of neutrophiles plasma and nuclear membrane in process called suicidal NETosis (7).

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During UC immunopathogenesis Neutrophil extracellular vesicles which contain PAD-4 are released in to intestinal epithelial cells, within these cells PAD-4 enzymes citrullinates mitochondrial creatine kinase 1 leading to disruption of mitochondrial energy balance and impairs intestinal mucosal barrier integrity, all these factors Amplified intestinal inflammation also increased intestinal cells death in mouse model of colitis (8). Matrix metalloproteinases-9 (MMP-9) is an extracellular Zn-dependent protease (9). And it is belonging to the gelatinase subfamily of Matrix metalloproteinases, and its primary function is to regulate the extracellular matrix protein composition by degradation of denatured collagen (gelatins) and Type 4 collagen, which are the main component of the basement membrane (10).

According to previous study there are a strong correlation between increase MMP-9 over-expression in UC patients with changes in the permeability of intestinal mucosal layers (11). All these molecules participated in chronic inflammatory state and form a bridge between chronic inflammation and cancer(12).The gold standard diagnostic test for the assessment of intestinal mucosal layer damaged is colonoscopy examination. even so, it is consider as invasive costly procedure (13).Therefore, urgency of specific serum serological marker is needed for predicating the severity of UC to specify treatment strategy. Although now there is no definitive medical therapy for UC, most treatment goals focus on controlling the disease activity and improving patient outcomes (14).

2. Materials and Methods

2.1. Subjects and samples

Overall, 90 individuals enrolled in this study, 60 UC patients and 30 healthy controls. The patients were admitted to Gastroenterology unit in Azadi Teaching Hospital in Kirkuk, Iraq, from April 2024, January 2025. The UC was diagnosed by combination of physical examination, abdominal ultrasound, and finally confirmed by endoscopic and histological examination of colon. UC severity index is usually assessed by Mayo Score (15).Based on this score, most patients have mild activity with 37.5%, while only 29.2% of patients have a severe activity. While according to Montreal extension scoring system most patients have Left-Side colitis according to table (3-1). For serum sample, blood was drawn from each group. Then stored at -20˚C until analysis. ELISA was performed for serum biomarkers(16). MMP-9 and PAD-4 (MMP-9/MyBioSource-USA/ MBS2023507, PAD-4/ MyBioSource-USA/ MBS706202). Mucosal biopsies were taken during colonoscopic examination from both inflamed areas in UC group, and normal apparent mucosal layer in healthy control group. MMP-9 and PAD-4 genes expression was identified by qRT-PCR. Macrogen/ Korea company are source of used primers.as described in table (2.1). $2^{-\Delta\Delta CT}$ method used to estimate fold changes

Table (2.1): Primers used in this study

Primer Name		Sequence	AT (ºC)	References
MMP-9	Sense	5′- GAGACCGGTGAGCTGGATAG - 3	57	(17)
	Anti-Sense	5′-TACACGCGAGTGAAGGTGAG-3′		
PAD-4	Sense	5′-TGTGACCCGAAAGCTCTA-3′	59	(18)
	Anti-Sense	5′-CTGCTGGAGTAACCGCTATT-3′		
GAPDH	Sense	5′-GTCCCAGCTTAGGTTCATAG-3′	60	(18)
	Anti-Sense	5′-GATGGCAACAATCTCCACTTTG-3′		

Statistical analysis

The statistical analysis and graph drawing was analyzed by GraphPad Prism. The differences between unrelated two group was analyzed by unpaired t test, on the other hand comparison between more than two group was analyzed by ANOVA with Bonferroni test as post hoc analysis.

Also, correlation analyses were calculated by Pearson's correlation. Receiver-operating characteristics curve was used to determine the diagnostic performance of the parameters.

3.Results

The demographic data of the studied groups show that the mean age of 30± 8.2 years with male/ predominant with ratio 55%. UC extension and severity are demonstrated in table () according to Montreal classification and mayo score.

Table 3.1. Characteristics of study population

Classification systems	Items	Number / %
Age (Mean ±SD)	30±8.2 years	
Sex (Male/Female)	55%/45%	
Montreal classification	E1	19 (31.6%)
	E2	26 (43.4%)
	E3	15 (25%)
Mayo score	Mild	13 (21.7%)
	Moderate	24 (40%)
	Severe	23 (38.3%)

The mean serum MMP-9 in UC group was (7.34±1.4) while in control group was (4.89±0.6), with (P<0.001). Serum PAD-4 level in UC group was significantly higher than that of the control group, with a mean of (1.51±0.3 and 5.68±0.3, respectively) (P<0.001). Similar to the above results, MMP-9 and PAD-4 tissue expression in UC group was higher than control group with (P<0.001). UC group was classified using Mayo score in to mild, moderate and severe. The mean serum level of MMP-9 in mild, moderate and severe group was (4.89±0.6,7.36±0.7,8.40±0.9, respectively) these results reveled significant differences between each severity groups with (P<0.05), while the relation between control and mild group was not significant (P>0.05). likewise, tissue MMP-9 relative expression was significant in mild, moderate and severe groups when compared in between and with control group. While the results were not significant between control and mild group. Moreover, there was a significant difference(P<0.001) between serum PAD-4 level in all severity index with mean (3.14±0.7,5.31±0.7,7.51±0.5) to mild, moderate and severe respectively. Montreal classification was used for UC extension grouping. The level of serum MMP-9 was elevated in all disease extension with (P<0.001) and mean (5.98±0.9,7.68±0.6,8.58±0.9) to Proctitis, Left-side colitis and Pancolitis respectively. Moreover, Tissue expression of MMP-9 was significantly elevated also in all UC extension groups with (P<0.001). Serum level of PAD-4 in proctitis was (4.00±1.4), Left-side colitis (6.09±1.3) and in Pancolitis (7.11±1.0) with (P<0.001). PAD-4 mRNA expression level in Pancolitis and Left-side colitis was higher than proctitis with (P<0.001).

ROC analyses were done to determine best parameter that could be used on to distinguish UC from normal individuals (Table 3.2). Serum MMP-9 and PAD-4 parameters were able differentiated between UC and control groups with sensitivity, specificity (82%,97% and 100%,100%) receptively with higher AUC for serum PAD-4. CRP level significant level according extension and severity as shown in figure (3.1)

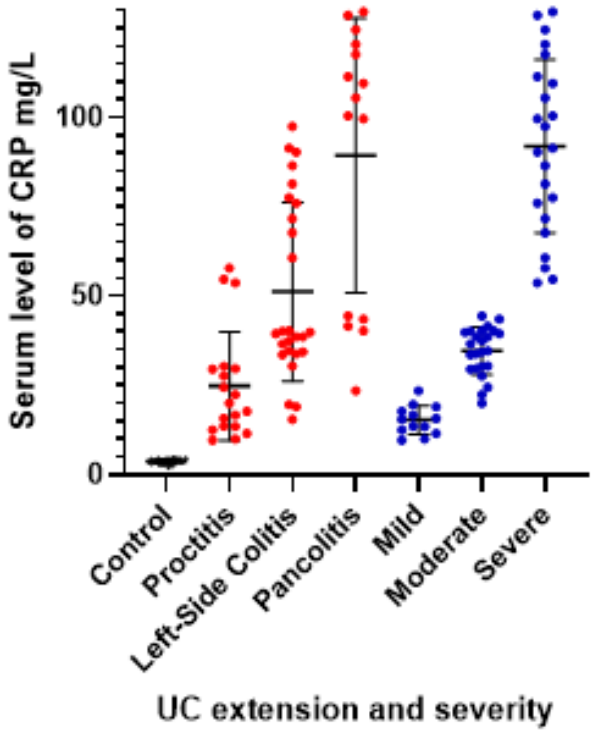

Figure 3.1. The content of CRP was significantly elevated in the UC group compared with that in the control group. P<0.05. CRP: C reactive protein; UC: Ulcerative Colitis

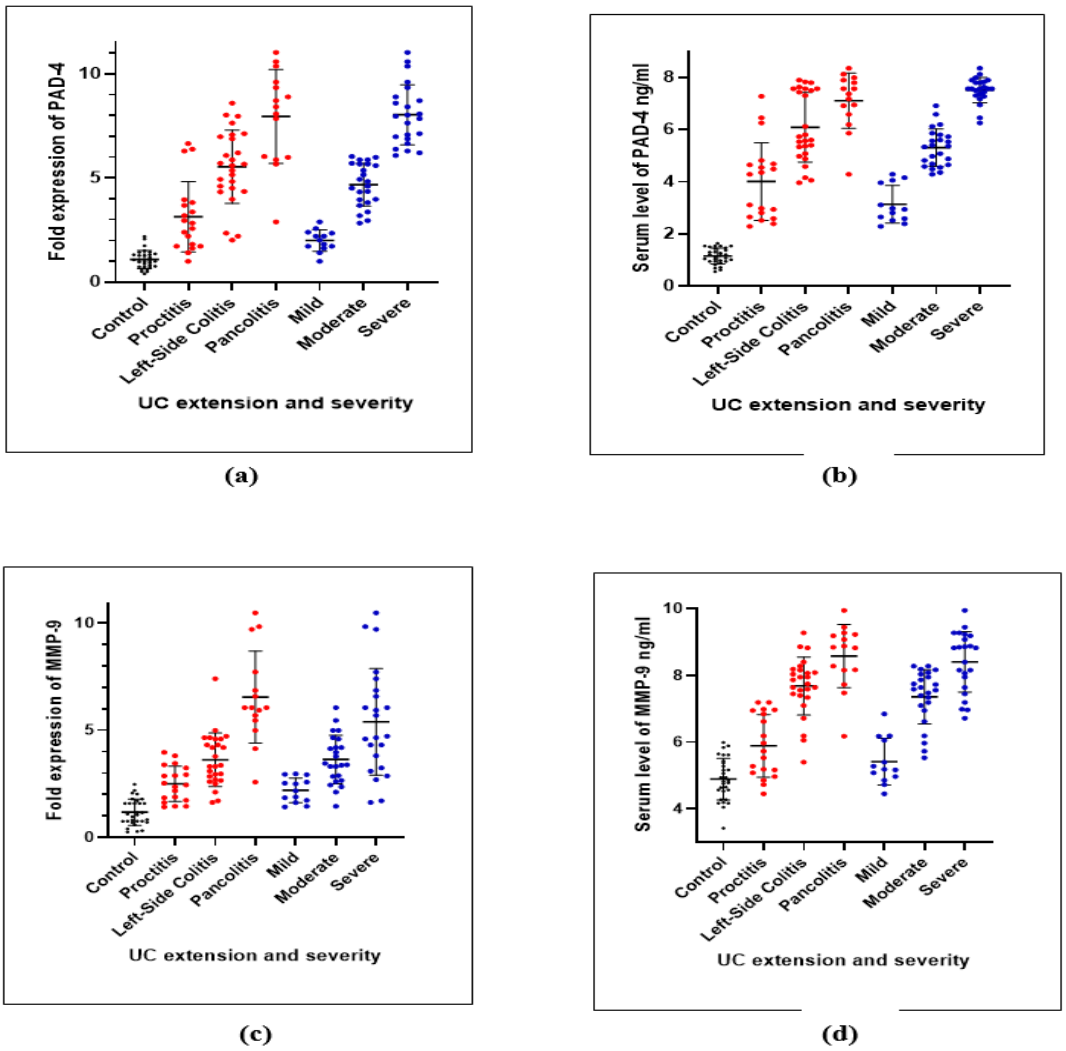

Figure 3.2 Tissue and serum MMP-9, PAD-4 in UC and control group in the control group, UC classified by Montreal extension and Mayo severity indices. (a,b) Significant differences between each group in PAD-4 tissue expression and serum level. (c,d) The differences between each group were significant except the relation between control, proctitis and between control, mild group in both tissue and serum MMP-9. MMP-9: Matrix Metalloproteinase 9; PAD-4: Peptidyl arginine deiminase 4; UC: Ulcerative Colitis

Table (3.2) ROC analysis performance of MMP-9 and PAD-4 in serum and tissue to differentiating UC from control group

Biomarkers	AUC	SE	95% C.I. (AUC)	P (AUC = 0.5)	Cut-off value	Sensitivity %	Specificity %
Tissue MMP-9	0.9575	0.02	0.923-0.992	<0.000	2.402	80%	97%
Serum MMP-9	0.932	0.03	0.884-0.980	<0.000	5.927 ng/ml	82%	97%
Tissue PAD-4	0.988	0.008	0.972-1.000	<0.000	1.782	92%	93%
Serum PAD-4	1	0	1.000-1.000	<0.000	1.967 ng/ml	100%	100%

Discussion

Inflammatory bowel disease is an autoimmune disorders of the intestine and comprised from ulcerative colitis and Crohn's disease (19). As an important causative factor of colorectal cancer, (20) third most prevalent cancer in the world with high fatality rate. Most initial diagnosis of UC are between (15-30 years), however a second peak usually occurs in occurs between of sixth to seventh decades of life. With slightly predilection for male. Unlike most autoimmune disease in which female is more affected than male another study revealed the most commonly diagnosed UC was in aged between 20- 40 years (21). By using Montreal classification index, most patients had Left-side colitis followed by proctitis then Pancolitis. these results agreed with Burisch et al that demonstrated that pancolitis is presented only in 25% on newly diagnosed UC (22). Sayuri et al who, demonstrated that Left-side colitis was observed in 43% of UC group (23). A report Burri et al mention that nearly forty percent of newly diagnosed UC had distal colitis (24).During UC first diagnosis , most patients have mild to moderate (25). this result similar to our results that show 62% of our patients was in mild to moderate activity .In our study more than half of pancolitis group had severe disease course , Ordás et al proved the same results by his works (26).

The main pathophysiological reason of IBD is the sustained chronic inflammatory reaction in the intestine mucosa (27). The layer which consider as first barrier against bacterial and food allergen (28).one of the most activated and accumulated cells during UC flare up are neutrophiles ,these events usually lead to crypt abscesses accumulation (29).UC activity significantly correlate with neutrophiles cell infiltration (30).The recruitment of neutrophils to the inflammatory area in IBD is mainly mediated by chemoattractant signals (CXCL8, CXCR1/2) which derived from gut epithelial layer after pathogenic or their products invading this layer.

These recruited and activated cells produced MMP-8 and MMP-9 which degrade collagen molecules in to smaller tripeptide fragments, which is known as powerful chemoattractant molecules. All these events leading to a self-maintaining chronic inflammatory situation in IBD (31).Activation of some transcription factors like (NF-κB ,AP-1and SP-1) induced MMP-2 and MMP-9 gene expression(32).Although MMP-9 overexpression during IBD may cause cell-cell adhesion alteration but does not induce intestinal cells apoptosis. Also may induced further attraction of neutrophiles to the gut mucosa .Study by Al-Sadi et al showed that increase expression of MMP-9 usually associated with activation of myosin light-chain kinase and p38 kinase signaling pathway that's leads to intestinal tight-junction permeability (33, 34).In past years invitro study of the effect of chymase enzymes, one of the protease enzymes not only secreted by neutrophiles but also from mucosal mast cells, can convert inactive MMP-9 to active mature form (35),Sullivan et al demonstrated that mucosal macrophages can also produce MMP-9 (36). this reflects complex network of inflammatory cells that playing role in IBD pathophysiology. Our results confirmed previous results toward elevation of MMP-9 usually associated with UC pathophysiology

In the agreement with our results, MMP-9 tissue expression was elevated in colonic tissue than control group (37).Gelatinase B in firstly secreted as pro-MMP-9 then cleaved and activated to form mature MMP-9 by many proteases enzymes(38). In our study we measured active mature form of MMP-9, as both serum forms were elevated in IBD patients in compared to healthy control group (39).Another study by Lakatos et al demonstrate that serum level of MMP-9 can be used to differentiate UC from control group(40).Furthermore our data demonstrate a positive correlation between MMP-9 and disease extension ,as shown that patients with pancolitis have higher MMP-9 gene and protein expression than other extension even control group(36).

On the other hand, in our study, the serum and tissue gene expression of MMP-9 was significantly correlated with the full mayo score severity index, even though mild group not significantly related to control group. The result was similar to study done by Czajkowska et al who found a non-significant correlation between disease activity and MMP-9 concentration (41).In addition MMP-9 were elevated in association with the severity colon tissue damage

(42). Ahmed et al. suggested that MMP-9 activity significantly correlated with the overall Mayo score(43).Additionally, Matusiewicz et al also noted

that MMP-9 were significantly elevated in active than inactive forms of IBD, also can be used to differentiate between active UC and CD (44).

After phagocytic neutrophile activation they expel their interior contents of azurophilic granules such as neutrophil elastase, myeloperoxidase, and PAD-4(45).these products specially PAD-4 can also translocate in to neutrophile nucleus and promote suicidal NETosis and NETs releasing(46). Neutrophil activation during NETosis lead to increase cytosolic Ca^{2+}, a cofactor for PAD-4, then nuclear PAD-4 drives histone citrullination (by post-translational deamination) leading to chromatin decondensation through positive charges losing that necessary for DNA histone interaction. Finally, DNA is released as NETs (47)..Also NETosis is considered a proinflammatory process(48).The NETs have double-edged sword properties, either works as powerful antibacterial or sustained inflammatory stimuli can lead to excessive NETs formation which exacerbating tissue damage during autoimmune disease (49).

Transcriptome analyses done by Leppkes et al and his colleagues showed a significantly elevation of PAD4 expression in active UC than control group(50).

Dinallo et al demonstrate that PAD-4 secretion are the main steps in NETs formation and it is mucosal expression was significantly elevated in UC patients in compared to control group (51).

Immunohistochemical examination of NETs associated PAD-4 was significantly associated with anatomical disease extension and UC activity index by histopathological activity score (47). An exacerbated events of colitis in mice deficient PAD-4 usually associated with elevated rectal bleeding episodes and delay mucosal healing in compared to healthy control (50).

Both adults and pediatric human colonic biopsies has shown a strong staining for PAD-2 and PAD-4 in lamina propria and inflammatory cells in the inflamed tissue with significant correlation with disease activity (52).

One of the most important biological therapies of UC is infliximab (anti-TNF-α). Responder patients with infliximab therapy showed decreased colonic tissue expression of NETs associated proteins specially PAD-4, these results confirm the linkage between TNF-α and NETs formation (53).In mouse model of colitis harmful aspect of PAD-4 elevation are linked to reduction of anti-inflammatory cytokines releasing and powerful effect of pro-inflammatory cytokines (54). Post translation modifications of MMP-9 by PAD-4 yield into increase tissue affinity of MMP-9 to gelatin mucosal gelatin molecules, thus increase disease activity (55-56).

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The study was funded by our charges with no funding from other sources.

Ethical clearance

The ethical committees of the Middle Technical University College of Health and Medical provide the approval for this study accordıng to Ethics Committee number (MEC-49)

Conflicts of interest

The authors declare no conflict of interest.

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