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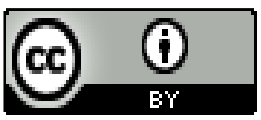

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1-World Medical Association.Declaration of Helsinki: ethical principles for medical research involving human subjects. http://www.wma.net/en/ 30publications/10policies/b3/index.html. Accessed October 14, 2010.

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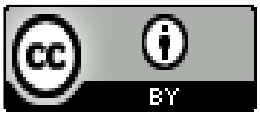

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Experimental Biomedical Research **Original article**

Does the non-union scoring system (NUSS) affect the treatment approach of non-union?

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ABSTRACT

Aim: To investigate the effectiveness of the non-union scoring system **(**NUSS) in predicting the result and in guiding the treatment by comparing the treatment methods applied to non-union patients we treat in our clinic with the treatment methods suggested by the NUSS.

Methods*:* The study included 116 patients, who were diagnosed with long bone (femur, tibia and humerus) non-union and treated in our clinic. Of the 116 patients with non-union, 48 had femur (41.38 %), 39 had tibia (33.62%) and 29 had humerus (25%) non-union. The patient scores were calculated according to the NUSS criteria. The patients were divided into four groups according to their total scores. There were 34 patients in the first group (0-25 points), 49 patients in the second group (26-50 points), 30 patients in the third group (51-75 points) and three patients in the fourth group (76-100 points).

Results: Union that was achieved in 79 (68.10%) of all patients was detected in 97.05% of the patients in the first group, 83.67% in the second group, and 16.66 % in the third group. Amputation, arthroplasty and arthrodesis were applied to three patients in the fourth group. While union rate was 100 % in the femur and tibia in the first group, it was 90% in the humerus. The union rates were 85.71% in the humerus, 75% in the femur and 100% in the tibia in the second group. They were 20 % in the humerus, 15.38% in the femur and 16.66% in the tibia in the third group. The number of patients treated with the treatment proposed by the NUSS: 100% in the group 1, 83.67% in the group 2, 20% in the group 3 and 100% in the group 4. The risk of non-union in those who were not treated according to the NUSS recommendations was 28 times higher than that of others.

Conclusions: The results of our study suggest that more frequent use of the NUSS procedure in non-union treatment planning may increase treatment success. In addition, NUSS can provide information about the treatment process of non-unions.

Key words: Trauma, fracture, pseudoarthrosis, non-union scoring system **(**NUSS), bone defect.

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Introduction

Non-union treatment has always been difficult for orthopedic surgeons. There is no consensus among clinicians, and it was found that there were differences of opinion among clinicians up to 55% on non-unions [1]. According to the US Food and Drug Administration (FDA), the diagnosis of non-union may be established "after 9 months from injury and if the fracture does not show any progressive signs of healing for 3 months", but others state that for long bones like the femur, humerus or tibia this process can be defined as 6 months if no radiological evidence of fracture healing

present [2]. On the other hand the treatment of non-union is a challenge and the treatment failure rate of non-union is approximately %20 [3]. Many classification systems were developed to address this issue. Currently, there are classification systems developed by Weber-Cech, Ilizarov, and Paley [4, 5, 6]. The most commonly used one is the radiography-based classification system developed by Weber-Cech. According to this system, non-unions are classified as atrophic, oligotrophic and hypertrophic. While there is a vascular insufficiency in the fracture zone in atrophic and oligotrophic non-unions, vascularity is sufficient in hypertrophic non-union, but reduction is inadequate. It is inevitable that this classification system based on radiography has deficiencies in the treatment-directing.

The new scoring system, Non-Union Scoring System (NUSS), that is described by Calori et al. in 2008, is the most comprehensive classification system ever developed consisting of many parameters [7]. The NUSS is a system in which many factors related to bone, soft tissue and patient are scored, and the treatment options are specified considering the score obtained (Table 1). The NUSS includes the assessment and scoring in terms of the quality of bone, whether the primary injury is open or closed fracture, the number of previous interventions to ensure healing, invasiveness of previous interventions, adequacy of primary surgery, the Weber-Cech classification, bone alignment, bone defect-gap, soft tissues, the ASA grade, diabetes, blood tests (WBC, ESR, CRP), clinical infection status, drugs and smoking status. The score of the patient is calculated by multiplying the sum of the scores by two. In the light of these scores, patients are treated according to the recommended treatment methods in four groups defined. Scores from 0 to 25 is considered straightforward non-unions that are expected to respond well to the appropriate treatments. The problem in this group is generally mechanical. Scores from 26 to 50 would require more specialized care to be given. The problem is mainly biological and mechanical. For patients with scores from 51 to 75, a specialized care and specialized treatments should be sought. Mechanical and biological failure is more complex and non-union resection and bone defect filling are generally required. Patients with scores over 75 can be candidates for consideration of arthrodesis, arthroplasty or primary amputation [7].

In the present study, the records of patients treated with non-union diagnosis in our clinic were retrospectively reviewed. The results of the treatments were scored according to the NUSS criteria. We investigated the effectiveness of the NUSS in predicting the outcomes and in guiding treatment by comparing the treatment methods applied to non-union patients we treat in our clinic with the treatment methods suggested by the NUSS.

Materials and methods

The study was retrospectively conducted in our clinic. The study protocol was approved by the local Scientific Research Ethics Committee (2019/04) and the study was conducted in accordance with the Declaration of Helsinki. It included 116 patients treated with long bone (femur, tibia, and humerus) non-union. Inclusion criteria were, the presence of the femur, tibia or humerus non-union, over 18 years of age and the patients with adequate follow-up. Exclusion criteria were, pregnancy, pediatric patients, and fractures due to malignancies and autoimmune diseases. The numbers of male and female patients included in the study are 85 (73.3%) and 31 (26.7%), respectively. The average age of the patients

Table 1. Non-union scoring system **(**NUSS).

The bone		**Score**	**Max. score[a]**
Quality of the bone	Good	0	
	Moderate (e.g. mildly osteoporotic)	1	
	Poor (e.g. severe porosis or bone loss)	2	
	Very poor (Necrotic, appears avascular or septic)	3	3
Primary injury –open or closed fracture	Closed	0	
	Open 1º grade	1	
	Open 2–3º A grade	3	
	Open 3º B–C grade	5	5
Number of previous interventions on this bone to procure healing	None	1	
	<2	2	
	<4	3	
	>4	4	4
Invasiveness of previous interventions	Minimally-invasive: Closed surgery (screws, k wires, . . .)	0	
	Internal intra-medullary (nailing)	1	
	Internal extra-medullary	2	
	Any osteosynthesis which includes bone grafting	3	3
Adequacy of primary surgery	Inadequate stability	0	
	Adequate stability	1	1
Weber & Cech group	Hypertrophic	1	
	Oligotrophic	3	
	Atrophic	5	5
Bone alignment	Non-anatomic alignment	0	
	Anatomic alignment	1	1
Bone defect – Gap	0.5–1 cm	2	
	1–3 cm	3	
	>3 cm	5	5
Soft tissues			
Status	Intact	0	
	Previous uneventful surgery, minor scarring	2	
	Previous treatment of soft tissue defect (e.g. skin loss, local flap cover,	3	
	multiple incisions, compartment syndrome, old sinuses)		
	Previous complex treatment of soft tissue defect (e.g. free flap)	4	
	Poor vascularity: absence of distal pulses, poor capillary refill, venous	5	
	insufficiency		
	Presence of actual skin lesion/defect (e.g. ulcer, sinus, exposed bone or	6	6
	plate)		
The patient			
ASA Grade	1 or 2	0	
	3 or 4	1	1
Diabetes	No	0	
	Yes – well controlled (HbA1c < 10)	1	
	Yes – poorly controlled (HbA1c >10)	2	2
Blood tests: FBC, ESR, CRP	FBC: WCC >12	1	
	ESR > 20	1	
	CRP >20	1	3
Clinical infection status	Clean	0	
	Previously infected or suspicion of infection	1	
	Septic	4	4
Drugs	Steroids	1	
	NSAIDs	1	2
Smoking status	No	0	
	Yes	5	5

[a]*Higher score implies more difficult to procure union.*

Table 2. Number of patients treated regarding treatment proposed by non-union scoring system **(**NUSS).

NUSS Score	The number of patients	Union	The number of patients treated regarding treatment proposed by NUSS/ (%)
Group 1 (0-25)	34	33	34 (%100)
Group 2 (26-50)	49	41	41 (%83,67)
Group 3 (51-75)	30	5	6(%20)
Group 4 (76-100)	3	0	3(%100)
Total	116	79	84

was 40.5 (17-86). The mean follow-up period of the patients was 21.79 (11-63) months. Pediatric patients were not included in the study. The patients who underwent non-union treatment were retrospectively evaluated according to the Non-Union Scoring System (NUSS) developed by Calori et al. [1]. The number of patients who developed non-union (116 patients) in the femur, tibia, and humerus was 48 (41.38%), 39 (33.62%) and 29 (25%), respectively. The score of patients was calculated according to the NUSS criteria. The patients were divided into four groups with respect to their total scores. There were 34 patients in the first group (score: 0-25), 49 patients in the second group (score: 26-50), 30 patients in the third group (score: 51-75) and three patients in the fourth group (score: 76-100) (Table 2). All patients were followed-up at 1, 3, 6, 9, 12 months by x-rays or CT-scans and union was assessed. Radiological presence of callus formation (3/4 of cortical) in AP-Lateral x-rays and clinical absence of pain in the fracture side were accepted as healing of the fracture.

Statistical analysis

The normality of distribution of continuous variables was tested by the Shapiro-Wilk test. The Student's t test was used for the comparison of two independent groups of variables with a normal distribution. The relationship between categorical variables was determined by the chi-square test. Univariate logistic regression analysis was used to estimate odds ratio (OR) and 95% confidence interval. Descriptive statistic parameters were presented as frequency, percentage (%) and mean ± standard derivation (mean ± SD). Statistical analysis was performed with SPSS for Windows version 22.0 and a p value < 0.05 was accepted to be statistically significant.

Results

The patients were divided into four groups according to the NUSS score. There were 34 patients in the first, 49 patients in the second, 30 patients in the third and three patients in the fourth group. Union was achieved in 79 (68.10%) of all patients. The union rate was determined to be 97% in the first group, 83.67% in the second group, and 16.67% in the third group. On the other hand, union was not achieved in the fourth group. The mean fracture healing time was 6.8 ± 1.82 months for the first group, 7.1 ± 1.55 months in the second group and 7.82 ± 1.63 months in the third group.

Amputation, arthroplasty and arthrodesis were applied to three patients in the fourth group. While union rates were 100% in the femur and tibia in the first group, it was 90% in humerus. Union rates in the second group were 85.71% in humerus, 75% in the femur and 100% in the

Table 3. Our treatment choices for each bone.

Groups	Treatment choices	The number of patients
Group 1 (NUSS Score 0-25) (n=34)		
Humerus	Fixation system chanced and autogenous bone grafting	10
Femur	Fixation system chanced	3
	Fixation system changed and autogenous bone grafting	6
Tibia	Fixation system chanced	6
	Fixation system changed and autogenous bone grafting	9
Group 2 (NUSS Score 26-50) (n=49)		
Humerus	Fixation system chanced	1
	Fixation system changed and autogenous bone grafting	13
Femur	Autogenous bone grafting	1
	Fixation system chanced	3
	Fixation system changed and autogenous bone grafting	18
	Fixation system changed and vascular bone grafting	2
Tibia	Fixation system chanced	3
	Fixation system changed and autogenous bone grafting	7
	Nonunion resection and segment shifting	1
Group 3 (NUSS Score 51-75) (n=30)		
Humerus	Fixation system changed and autogenous bone grafting	5
Femur	Fixation system changed and autogenous bone grafting	10
	Fixation system changed and vascular bone grafting	2
	Tumor resection arthroplasty	1
Tibia	Fixation system changed and autogenous bone grafting	8
	Fixation system changed and vascular bone grafting	1
	Nonunion resection and segment shifting	3
Group 4 (NUSS Score 76-100) (n=3)		
Humerus	-	
Femur	Arthrodesis	1
	Tumor resection arthroplasty	1
Tibia	Amputation	1

Humerus (n=29); Femur (n=48); Tibia (n=39).

tibia. They were 20% in the humerus, 15.38% in the femur and 16.66% in the tibia in the third group. The fixation system was changed to increase the stability for 34 patients in the first group, and autogenous grafting was performed in addition to the fixation system for 25 patients (Table 3). The fixation system was changed, and autogenous grafting was performed for 38 of the 49 patients in the second group. Seven patients were treated in this group by changing the fixation system used. Two patients underwent vascular bone grafting. The grafting

was applied to only one patient. Non-union was resected, and bone defect was treated by segment shifting method in one patient (Table 3). The fixation system was changed, and autogenous grafting was performed for 23 of the 30 patients in the third group. Vascularized bone grafting was performed by changing the fixation system in three patients. Non-union was treated by non-union resection and segment shifting method in three patients. One patient underwent tumor resection arthroplasty (Table 3). The arthrodesis, tumor resection prosthesis, and amputation were applied for each of the three patients in the fourth group separately (Table 3).

The treatment methods applied to the patients were compared with the treatment recommendations proposed by the NUSS (Table 2). While the treatment methods used in the first and fourth groups were completely compatible with those recommended by the NUSS, it was determined that the treatment methods applied to the second and third group are entirely different from those recommended by the NUSS. The success rate in patients treated with the methods proposed by the NUSS was remarkable.

There was a statistically significant relationship between the treatment proposed by the NUSS and union rate (p=0.001). The risk of non-union in those who were not treated according to the NUSS recommendations was 28 times higher than that of others (Odds Ratio = 28.75% Confidence Interval = 9.66-85.61).

Discussion

In the present study we evaluated the patients who had developed a non-union. And the NUSS was used for scoring the non-unions and to see the process of fracture healing. There are a few studies in the literature investigating the classification systems for non-union [8-9-10]. We used the NUSS for analyzing the non-unions. There are not many studies about this classification system in the literature. Calori et al. have tested the validity of their classification system in the articles published in 2014 [8] and stated that this system might be a valid guideline. In a retrospective study conducted by Abumunaser et al. [11], 40 patients were divided into three groups, asserting that there would be no clear distinction regarding the treatment protocols between the group 2 (Score: 26-50) and the group 3 (Score: 51-75) in this classification. They stated that their treatment protocols were similar to the treatment protocols described in the NUSS, and reported that they achieved similar success rates in the treatment.

All patients in the first group were treated by the methods the NUSS recommended and union rate achieved was 97.05%. In the second group, 83.67% of the treatments were compatible with the methods recommended by the NUSS, and the union rate was 83.67%. On the other hand, only 20% of treatment methods applied to the patients in the third group complied with the treatment recommendations of the NUSS. The treatment success rate in this group was only 16.67%. Those results revealed that the non-union risk was 28 times higher in the patients who were not treated using the methods recommended by the NUSS when compared to patients treated according to the NUSS recommendations. This results were similar the study that published by Calori et al. [8]. In their study they analyzed 300 patients that applied with long bone non-unions.

According to the NUSS, the main problem in the first group is mechanical, and the aim of the treatment is to change the fixation system. In the present study, the fixation system was changed to improve stability in all patients in the first group. Treatment success was achieved

when the mechanical problem was solved. The problem in the second group was generally biological and mechanical. Recovery of fixation and the provision of biological stimulant are suggested for treatment. A more stable fixation was provided by changing the fixation system in 83.67% of the patients we treated in this group and biological support was given with an autogenous iliac bone graft. Union was achieved in these patients. In this group, only the fixation system was changed in seven out of eight patients whose union was not achieved with the treatment, and no biological stimulation was used. Autogenous bone grafting was performed in a patient whose fixation system was not changed. According to the NUSS, the problem is complex in the third group and there is an impairment of both biological and mechanical conditions. Non-union should be resected as the treatment suggestion and the bone defect should be treated. In our series, the treatment protocol suggested by the NUSS was applied only in six patients in this group, and union was achieved in five patients. Thus, the success rate was 83.33% in the treatment. However, union was not achieved in 25 patients in the group. Low success rate in this group may be related to our failure to use more aggressive treatment protocols. The treatment we applied was mostly changing the fixation system and autogenous grafting. Application of more effective methods like segment shifting and vascular grafting after non-union resection could have increased our treatment like our study [12].

The NUSS recommends treatments such as arthrodesis, arthroplasty and amputation for the fourth group. We applied tumor resection arthroplasty, arthrodesis and amputation for our patients in this group.

There are several limitations in the study. The limitations of the study are that the NUSS has yet to be validated [8] and the study was conducted retrospectively. However, it can be thought that as the number of studies on this subject increase, the scoring system will be used more widely.

We think that the more frequent use of the NUSS in non-union treatment planning can increase the success of the treatment and it can be used as a classification guide for the surgeon in the treatment of nonunion.

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Experimental Biomedical Research **Original article**

Prevalence of Helicobacter pylori, gastric atrophy and intestinal metaplasia in gastric biopsy specimens: A retrospective evaluation of 1605 patients

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ABSTRACT

Aim: To evaluate the prevalence of Helicobacter pylori (H. *pylori*) and related histopathological lesions in gastric mucosa samples in this single-center study.

Methods: Esophagogastroduodenoscopy and endoscopic biopsy reports of 1605 elective cases were retrospectively evaluated. Histopathological examination was evaluated according to the Sydney classification. The data were analyzed according to the prevalence of H. pylori, age, gender, gastric atrophy and intestinal metaplasia rates, and the distribution of the study group in the population below 40 years old and over 40 years old.

Results: 584 males (Mean age 51.5 ± 16.5 years) and 1021 females (mean age 49.6 ± 16 years), (p = 0.03), a total of 1605 cases were included in the study. The rate of atrophy, metaplasia and H. pylori positivity in total study population were 0.2%, 16%, 71%, respectively. The rate of atrophy in men and women were 1.2% and 0.8%, respectively *(p = 0.006)*. The rate of metaplasia in men and women were 20.9% and 13.7%, respectively *(p <0.001)*. In the population under 40 years of age, the rates of gastric atrophy and intestinal metaplasia were 0.7% and 2.5%, respectively *(p = 0.02)*, above the age of 40, these rates were determined as 10.8% and 18.4%, respectively. *(p <0.001)*.

Conclusion: According to the data of our center, the prevalence of *H. pylori* is high. In addition, the rate of intestinal metaplasia is relatively high in the male population over the age of 40.

Key words: Helicobacter pylori, Helicobacter infections, gastric atrophy, intestinal metaplasia.

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Introduction

Helicobacter pylori (*H. pylori*) is a common infection that infects nearly half of the world's population [1]. After ingestion orally, *H. pylori* is colonized in the gastric mucosa by creating a micro-alkaline environment thanks to its urease enzyme. It causes damage to the gastric epithelium with the endotoxins it secretes [2]. If it is not eradicated, it may cause many different pictures in a wide spectrum ranging from chronic gastritis to mucosa-related lymphoma and gastric cancer [3].

World health organization acknowledged it as the first-degree agent responsible for malignancy and the detection of a reduction in the risk of gastric cancer with its eradication keep the subject up-to-date [4-6]. While the prevalence of *H. pylori* decreases in parallel with the development level of the society and good sanitation conditions, it has been reported that low socio-economic status, dense family population and poor hygiene conditions are responsible for the increase in its prevalence [7].

There are different data from different geographical regions of our country about the prevalence of *H. pylori* [8]. The aim of this study was to evaluate the prevalence of Helicobacter pylori, atrophic gastritis and intestinal metaplasia in biopsy samples of 1605 patients who underwent esophagogastroduodenoscopy (EGD) in a referral hospital, and to investigate its relationship with gender and age.

Materials and methods

Approval of the study was obtained from Pamukkale University clinical research ethics committee (24.03.2021-2021 / E.35738). Data of 1605 adult cases who applied to Denizli State Hospital Gastroenterology Outpatient Clinic and underwent elective EGD with different indications between October 2015 and May 2016 were retrospectively evaluated. Pentax EPK i-5000 system and EG-2990i model gastroscope were used in EGD procedures. Patients who had gastrointestinal bleeding in the last month, who used antibiotics and bismuth salts, who used proton pump inhibitors in the last two weeks, and those who could not give a clear anamnesis about drug use and whose medical records could not be reached were excluded [9]. In addition, patients who underwent gastric surgery, inpatients, and emergency procedures were excluded. Endoscopic biopsy specimens taken from the two curvatures of the corpus and antrum were transported to pathology in formaldehyde containing tubes, stained with giemsa and toluidine blue, and histopathological examination was evaluated according to the Sydney classification[10]. The prevalence of *H. pylori* in the EGD treated population, its distribution by age and gender, rates of gastric atrophy and intestinal metaplasia, as well as the distribution of the study group in the group under 40 years of age and in the population over 40 years of age were analyzed.

SPSS (Statistical Package for Social Sciences) for Windows 16.0 program was used for the statistical analysis of the findings obtained in the study. Values greater than P <0.05 were considered significant. The normality of data distribution was checked with the Kolmogorov-Smirnov test. T-test was used for statistics of normally distributed data, and Mann-Whitney U test was used to compare data that did not conform to normal distribution. Categorical data were compared using the Chi-square test. The Pearson correlation test was used for the correlation of the data with normal distribution, and the Spearman correlation test was used for the correlation of the data that did not fit the normal distribution.

Results

Total study population was consisted of 1605 subjects (584 men and 1021 women) enrolled to the study. The rate of gastric atrophy, intestinal metaplasia and *H. pylori* positivity in total study population were 0.2%, 16%, 71%, respectively.

Mean age of the men was 51.5 ± 16.5 years, while mean age of the women was 49.6 ± 16 years, respectively *(p=0.03*). The rate of gastric atrophy in men and women were 1.2% and 0.8%, respectively *(p=0.006)*. The rate of intestinal metaplasia in men and women were 20.9% and 13.7%, respectively *(p<0.001)*. The rate of *H. pylori* positivity in men and women were 72.1% and 71.5%, respectively *(p=0.8)*.

We grouped study population according to the age either as younger than 40 years (group I) or 40 years or older (group II). There were 153 (35.2%) men and 282 (64.8%) women in younger than 40 years group while 431 (36.8%) men and 739 (63.2%) women in 40 years and older group *(p=0.54)*. The rate of gastric

Table 1. Distribution of *H. pylori* and associated lesions in study groups.

Parameters	Female	Male	Total	<40 years	>40 years	*P*
***H. pylori* (+)**	730 (71.5%)	421 (72.1%)	1151 (71.7%)	316 (72.6%)	835 (71.4%)	*NS*
***H. pylori* (-)**	291 (27.9%)	163 (28.5%)	454 (28.3%)	119 (27.4%)	335 (28.6%)	*NS*
Gas. Atr. (+)	13 (1.3%)	19 (3.3%)	32 (2%)	3 (0.7%)	29 (2.5%)	*0.01*
Int. Met (+)	140 (13.7%)	122 (20.9%)	262 (16.3%)	47 (10.8%)	215 (18.4%)	*<0.001*

Gas. Atr: Gastric atrophy, Int. Metap: Intestinal metaplasia, NS: Non-significant.

atrophy in groups I and II were 0.7% and 2.5%, respectively *(p=0.02)*. The rate of intestinal metaplasia in groups I and II were 10.8% and 18.4%, respectively *(p<0.001)*. The rate of *H. pylori* positivity in groups I and II were 72.6% and 71.4%, respectively *(p=0.61*). Distribution of *H. pylori* and associated lesions in study groups is summarized in Table 1.

Discussion

The frequency of *H. pylori*, gastric atrophy and intestinal metaplasia in the study population is 71%, 0.2%, 16%, respectively. While there was no significant difference in the prevalence of *H. pylori* in the population aged younger than 40 and 40 or over, gastric atrophy and intestinal metaplasia are significantly higher than the younger group 2.5%, 18.4%, respectively.

Biopsy samples taken from the antrum and corpus walls during EGD performed for various indications, especially dyspeptic complaints, provide valuable information about the gastric mucosa and enable us to detect the presence of *H. pylori* [11, 12]. According to the Sydney classification, histopathological changes in the gastric mucosa guide the clinician about potential precancerous lesions [10]. In the Kyoto classification, endoscopic findings associated with *H. pylori* gastritis and the endoscopic scoring system are recommended to be used to predict the risk of gastric cancer [13]. The sensitivity and specificity of the histopathological method in detecting *H. pylori* is high [14]. Patch colonization of *H. pylori* in the gastric lumen is a disadvantage of biopsy. Despite the high feasibility of urea breathe test in community screenings, its cost poses a significant disadvantage. In our study, only one method [histopathology) was used to detect *H. pylori.* In a study based on the urea breath test conducted throughout Turkey by dividing it into five sub-regions, the prevalence of *H. pylori* was found to be 65.7% in the Aegean region where our center is located, while this rate was relatively high in present study [8]. In this study, there was no data that samples were taken from our city, and there may be a difference depending on the method used in the detection of *H. pylori.* In the reflux study conducted by S. Bor et al. [15] from our region, the prevalence of *H. pylori* was 75.7%. In another study reported from Afyon province, *H. pylori* prevalence was found to be 73% and 85% positive in PCR and antral biopsy results in dyspeptic patients [16]. Serological positivity of *H. pylori* was reported as 79.7% in 2001 from the same province [17]. *H. pylori* positivity was found to be 41.4% according to the results of CLO test performed on 9239 patients in Istanbul [18]. *H. pylori* positivity was found to be 57% and it was reported that there was no difference between the two methods in a study comparing urea breath test and histopathology [19]. *H. pylori* was found to be 40% and intestinal metaplasia was found to be 18.1% as reported in a study conducted in

Konya in 2014 [20]. In a series of 885 cases covering 0-17 years of age, the prevalence of *H. pylori* was found to be 47.2% [21]. Our findings were similar to the literature.

Chronic inflammation and destructions in the gastric mucosa that last for years result in gastric atrophy and metaplasia if *H. pylori* infection initiated in childhood is not eradicated, [3, 22]. The contribution of *H. pylori* to non-cardia gastric cancer is reported to be 74.7% [23] . The rates of gastric atrophy and intestinal metaplasia found in our study in the group above 40 years of age were 2.5% and 18%, respectively which were similar when compared to the results of other centers. Intestinal metaplasia rate was reported as 11.5% from Bursa, while gastric atrophy rate was 16.3% and intestinal metaplasia rate was 13.5% in Thrace region [24, 25]. In two different studies presented from Istanbul, the prevalence of intestinal metaplasia was reported as 13.4% and 17.8% [26, 27]. Intestinal metaplasia occurs as a result of cells migrating from the small or large intestines to the stomach as substitution of the damaged gastric mucosa. Intestinal metaplasia, which is an important step on the way to gastric dysplasia, was found to be significantly higher in the male population above 40 years of age in present report. Undoubtedly, many factors other than *H. pylori* contribute to the development of gastric adenocarcinoma, such as genetic, environmental factors and eating habits [28-30]. Therefore, high *H. pylori* positivity does not always mean a high prevalence of gastric cancer.

This cross-sectional study, which was retrospective in design, based on a single center and a single method, has no claim other than leading large-scale research to be conducted under ideal conditions. Another limitation of the study is that intestinal metaplasia subgroups were not reported in histopathological examinations.

Conclusion

This cross-sectional study, based on data from a single-center referral hospital, showed that the prevalence of *H. pylori* is still high and that the prevalence of gastric atrophy and intestinal metaplasia is similar to data reported in the literature. In addition, gastric atrophy and intestinal metaplasia were found to be significantly higher in the group over 40 years of age. Moreover, multi-centered and ideally up-to-date studies are needed on this subject.

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Ethical statement:

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Experimental Biomedical Research **Original article**

Histopathological distribution of thyroid cancers: A retrospective analysis of 570 patients

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ABSTRACT

Aim: Thyroid cancers are the most commonly encountered endocrine system malignancies. The incidence continues to rise worldwide. Our aim in this study is to investigate the frequency and histopathological subtypes of thyroid cancer in our clinic.

Methods: The present study was conducted with 3614 patients who were followed up in our endocrinology and general surgery clinic and operated with the diagnosis of multinodular and/or nodular goiter between 2015 and 2021. The histopathological types and information of patients diagnosed with thyroid cancer were obtained retrospectively from the pathology reports. Among the patients included in the study, a total of 570 people who were reported to have thyroid cancer due to histopathology were included in the study.

Results: The data of a total of 3614 biopsy reports were examined for the study. Among these patients, 570 (421 females, 149 males) were operated and whose pathology reports were accessed were included in the study. The mean age of the patients was 49.12±10.4 years. As a result of the operations, malign postoperative tissue histopathology was 98.9% (n=564), and uncertain malignancy potential was reported to be 1.0% (n=6). In our study, the histopathological distribution of thyroid cancers was as follows; thyroid papillary cancer 89.4% (n=510), follicular cancer 7.3% (n=42), medullary cancer 2.1% (n=12), and malignancy potential uncertain 1.0% (n=6).

Conclusion: The results of our study suggest that thyroid cancers are more common in women in our country, in parallel with the similar rates reported in the literature, with the increase worldwide.

Key words: Thyroid neoplasms, carcinoma, pathology, histopathological distribution, regional differences.

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Introduction

Thyroid Cancer (TC) incidence is increasing rapidly; however, the mortality rate remains stable. There are five main histological types of TC: papillary, follicular, poorly differentiated, anaplastic (the most aggressive form), and medullary thyroid cancer [1,2]. The prevalence of TC is approximately three times more in women than in men, and it is the fifth most common cancer in women [3]. According to the health data of 2015, TCs represent 12.1% of all cancers in women and 2.7% of all cancers in men in Turkey [4]. TC is the most common endocrine malignancy, constituting 2.9% of all new cancer cases in the USA. These cancers have an excellent prognosis with a 5-year survival rate of 98.3%. Differentiated thyroid cancers originate from follicular epithelial cells and account for more than 90% of all the thyroid malignancies [5,6].

The prevalence of TC is high in areas where nodular goiter is endemic. In this respect, it is important that Turkey is an endemic goiter region. TCs are 3-4 times more common in women than in men [7]. The purpose of the present study was to investigate the prevalence of TCs and histopathological cancer types in our clinic.

Materials and methods

A total of 3614 patients who were followed up in our Endocrinology and General Surgery Clinic diagnosed with multinodular and/or nodular goiter between 2015 and 2021 were selected to include in the present study. Among these patients, 570 people reported TCs in pathology reports and were included in the study. The demographic characteristics, preoperative

Thyroid fine needle biopsy (TFNABx), and postoperative histopathology reports were collected from patient files and electronic records. The Clinical Research Ethics Committee approval of Medicana International Samsun hospital was obtained with the decision at the meeting with the number 7133 - 1 (20.04.2020-08). The study was conducted in line with ethical rules.

Among the patients, those with follicular neoplasia, suspected malignancy, and malignant cytological characteristics were operated on. Some of the patients had benign results in biopsies and were operated on because of aesthetic concerns, compression findings, and other reasons. Among these patients, 570 people reported to have TCs in pathology reports were included in the study.

Statistical analyses

The Chi-Square was used in the categorical data, and the Non-Paired Students ***t***-Test was used to compare the continuous variables. The average number of positive criteria in malignant lesions was compared with the Non-Paired Students ***t***-Test. One-Way ANOVA and Multiple ***t***-Tests were used for the comparison of TC subtypes. Statistical significance level was taken as $p < 0.05$.

Results

The data of patients who underwent a total of 3.614 biopsies were analyzed for the study. Among these patients, 570 (421 women, 149 men), who were operated and whose pathology results were available, were included in the study. The mean age of the patients was 49.12 ± 10.4. The demographic characteristics of the cases are shown in Table 1.

Table 1. Demographic and hematologic characteristics of the cases.

Parameters	**N**
Gender, Female/Male	421/149
Age (years)	49,12±10,4
TSH, mIU/L	2,13±1,5
Anti-TPO, IU/mL	121,6±33,9
Anti-TG, IU/mL	87,2±12,5

Anti-TPO: Anti-thyroid peroxidase antibody, Anti-TG: Anti-thyroglobulin antibody.

Among the 570 patients who were included in the study, the preoperative TFNABx results were benign in 0.1% (n = 1), Follicular, Hurthle cell neoplasia or suspected in 2.8% (n = 16), suspected malignancy in 46.4% (n = 265), and malignant in 50.5% (n = 288) patients (Figure 1A). Postoperative tissue histopathology was reported as malignant in 98.9% (n = 564), and with unclear malignancy potential in 1.0% (n = 6) patients. In our study, the histopathological distribution of the thyroid cancer was thyroid papillary cancer in 89.4% (n = 510) (Figure 1B), follicular cancer in 7.3% (n = 42), and

medullary cancer in 2.1% (n = 12) patients (Figure 1C and D). The TFNABx reports of the cases and the histopathological distribution of the thyroid cancer are shown in Table 2.

We found that thyroid cancers were most common in the 41-50 age group. Half of the cancer prevalence was detected in the 31-50 age group. The distribution of cancers according to age is shown in Table 3. We found that the autoimmunity was 24%, and the frequency of goiter was 3.8% in women with thyroid cancers. The distribution of the prevalence of autoimmunity and goiter in thyroid cancer cases according to gender is shown in Table 4. We found the frequency of autoimmunity to be 30% in our study, and the frequency of goiter was 5.9% in thyroid cancers. The prevalence of autoimmunity, goiter, and histopathological distribution in thyroid cancer is shown in Table 5. We found the multicentricity rate to be 23.2% in thyroid cancer. In thyroid cancer cases, cancer histology shows the distribution of goiter and autoimmunity. Other pathological characteristics are shown in Table 6.

Table 2. Fine needle biopsy reports and histopathological distribution of thyroid cancers.

Parameters	Papillary	Follicular	Medullary	Malignity potential unknown
TIIABx benign, n=1	**-**	**1**	**-**	**-**
TIIABx follicular, Hurthle cell neoplasia or suspicion, n=16	**6**	**9**	**-**	**1**
TIIABx malignity suspicious, n=265	**237**	**17**	**8**	**3**
TIIABx malign, n=288	**267**	**15**	**4**	**2**
Total (F/M)	510 (382/128)	42 (31/11)	12 (7/5)	6 (4/2)

TC: Thyroid cancer, TIIABx: Thyroid fine-needle aspiration biopsy, F: female, M: male.

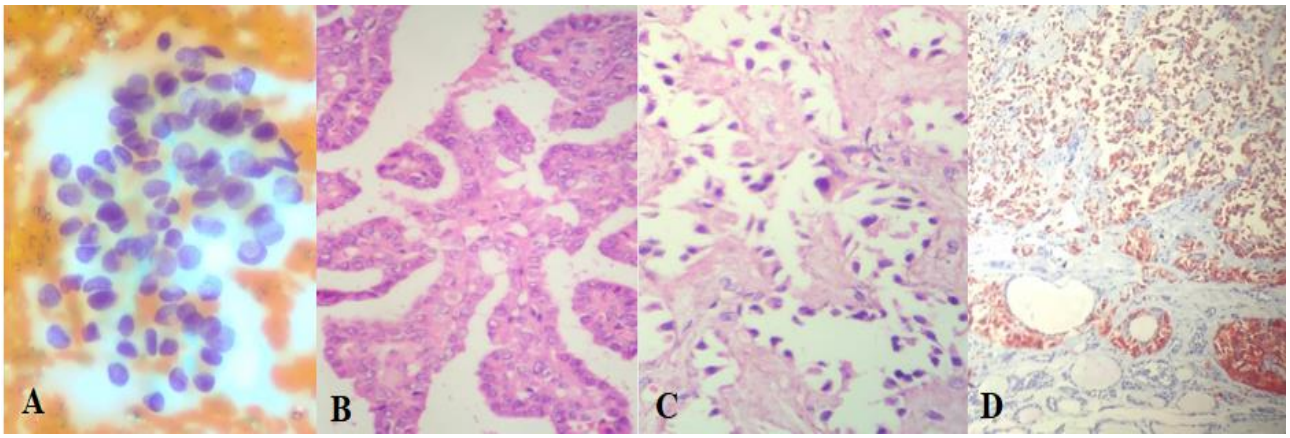

Figure 1. A) Fine needle aspiration biopsy, malignant (consistent with papillary carcinoma). **B)** Tissue histopathology; papillary carcinoma. **C)** Tissue histopathology; follicular carcinoma. **D)** Immunohistochemically examination, chromogranin A (+); medullary carcinoma**.**

Table 3. Distribution of thyroid cancers according to the age range.

Histological types	Age (N/%) 18-30	31-40	41-50	51-60	61-75
Follicular carcinoma	6 (1.0)	11 (1.9)	16 (2.6)	7 (1.2)	2 (0.3)
Papillary carcinoma	52 (9.1)	85 (14.9)	210 (36.8)	113 (19.8)	50 (8.7)
Medullary carcinoma	-	2 (0.3)	3 (0.5)	5 (0.8)	2 (0.3)
Malignity potential obvious thyroid carcinoma	1 (0.1)	1 (0.1)	2 (0.3)	2 (0.3)	-

Table 4. Distribution of autoimmunity and goiter according to gender in thyroid cancer cases.

Histological types	Male	Female	Ratio (Female: Male)
Autoimmune	34	137	4,02:1
Goiter	12	22	1,8:1
Thyroid carcinomas	146	424	2,9:1

Table 5: Distribution and frequency of autoimmunity in goiter and histopathologic thyroid cancers.

Histological types	Frequency (N/%)
Autoimmune	
Hashimoto thyroiditis	84 (14.7)
Lymphocytic thyroiditis	56 (9.2)
Graves' diseases	31 (5.4)
Goiter	
Simple colloid goiter	8 (1.4)
Multinodular goiter	26 (4.5)
Neoplastic, malignant	
Follicular carcinoma	42 (7.3)
Papillary carcinoma	510 (89.4)
Medullary carcinoma	12 (2.1)
Malignity potential obvious thyroid carcinoma	6 (1.0)
Total	775

Table 6. Pathologic features of the patients with thyroid carcinoma.

Pathologic features	Frequency (%)
Multicentricity	23.2
Tumor capsule invasion	5.6
Lymphatic invasion	6.7
Vascular invasion	6.4
Extrathyroidal invasion	3.8

Discussion

Thyroid cancers make up the most common malignancy in the endocrine system and account for 3.4% of all cancers diagnosed annually on a global scale [8]. It has been detected in recent years that the increasing incidence of thyroid cancer and over-diagnosis has begun to slow down [9]. The rapid increase in TC incidence was parallel to the detected thyroid nodules, the more common use of thyroid ultrasonography, and the increase in the incidence of biopsy [10].

Thyroid carcinomas were most frequently detected in the 41-50 age group in our study. We found the prevalence of autoimmunity as 30%, and the frequency of goiter was 5.9% in thyroid cancer cases. The prevalence of autoimmunity in women was 24%, and that of goiter was 3.8%, consistent with the literature data. We also found the multicentricity rate as 23.2% in thyroid carcinomas. These findings are in line with the current literature findings.

Papillary thyroid carcinoma is the most common histological type, which accounts for approximately 85-95% of thyroid malignant neoplasms. The 10-year survival rate is over 90% in these cancers. These tumors generally have silent biological behavior [11,12]. In previous studies, papillary carcinoma was more common in women at a rate of 76.40% and 23.59% in men [13]. In the present study, papillary carcinoma subtype was the most common histologic type at 89.4% (510/570).

We showed in this study that it is more common in women at a rate of 74.9% (382/510). The rate of papillary carcinoma was 37.5% (6/16) in cases with TFNABx follicular, Hürthle cell neoplasia or suspected malignancies, 89.4% (237/265) in patients with suspected malignancies, and 92.7% (267/288) in those with malignancies.

Follicular carcinoma represents approximately 5-10% of thyroid malignancies and can be detected at much higher rates, such as 25-40% in areas with intense iodine deficiency [14,15]. Follicular carcinoma is a more aggressive type of thyroid cancer with an increasing prevalence. Unlike papillary thyroid cancer, which spreads over lymphatics, follicular thyroid cancer is more aggressive because it can metastasize through vascular invasion [16]. In our study, we detected that the Follicular Carcinoma Subtype was at a rate of 8.2% (42/570). We showed that it is more prevalent in women at a rate of 73.8% (31/42). The rate of follicular carcinoma was 56.2% (9/16) in cases with TFNABx follicular carcinoma, Hürthle cell neoplasia, or suspected 6.4% (17/265) in patients with suspected malignancies, and 5.2% (15/288) in those with malignancies.

Medullary thyroid carcinoma is a rare type of tumor that originates from thyroid C cells and constitutes 2-4% of all malignant thyroid tumors. Medullary carcinoma may occur sporadically or be inherited as a part of MEN Type 2 syndrome [17,18]. In our study, the medullary carcinoma subtype was found to be 2.1% (12/570). It was also shown that it is more prevalent in women with a rate of 58.3% (7/12). We found that the rate of medullary carcinoma was 3.0% (8/265) in those with suspected malignancies and 1.3% (4/288) in those with malignancies. In previous studies, the "*well-differentiated tumor with uncertain malignant potential*" term is used for lesions with follicular patterns of the thyroid, which cannot be diagnosed easily as benign or malignant because of the lack of suspicious nuclear changes and capsular or vascular invasion. The basic reason behind this recommendation is that, clinically, such lesions behave benignly with an excellent prognosis [19]. In our study, it was found that the uncertain malignancy potential was 1.0% (n = 6).

This study had several limitations. It was designed retrospectively and must be supported with prospective studies. Also, the anamnesis of the patients and other comorbid diseases could not be evaluated in detail because the study was conducted retrospectively.

As a result, TC covers a wide range of diseases that have varying prognoses. Many patients with this disease have excellent overall survival rates. It is increasing in our country at similar rates in accordance with the literature parallel to the worldwide increase. It was shown in the present study that it is detected more frequently in women with a similar histopathological distribution.

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Ethical statement:

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Experimental Biomedical Research **Original article**

Comparison of eosinophil counts in patients with acute pulmonary embolism: Could it be a predictor factor?

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ABSTRACT

Aim: To investigate whether there is a relationship between both massive and sub-massive pulmonary embolism (PE) and eosinophil counts in order to evaluate it as a predictor factor.
Methods: This retrospective study included 108 patients (64 sub-massive and 44 massive) who received both tomographic and clinical diagnoses of pulmonary embolism, and 75 subjects served as controls. Hemogram parameters were compared between patients with massive and sub-massive pulmonary embolism and those of control groups.
Results: In terms of white blood cell and eosinophil counts, the lowest value was evident in the massive PE group whereas the control group had the highest value. The eosinophil counts increased significantly one week after the treatment when compared to those at the presentation with PE *(0.112 (0.003-0.853) vs. (0.144 (0.011-0.914), p=0.01)*. Spearman correlation test showed a significant positive correlation between right ventricular dysfunction or elevated cardiac troponin level and massive PE *(r=0.54, p <0.001)*, whereas a negative correlation was detected between eosinophil count and the presence of massive PE *(r=-0.36, p<0.001)*.
Conclusion: The results of our study suggest that lower eosinophil counts may lead a physician to suggest a higher probability of acute massive pulmonary embolism rather than sub-massive pulmonary embolism. However, further randomized studies are required to confirm these findings.

Key words: Pulmonary embolism, acute disease, eosinophil count, predictive value.

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Introduction

Pulmonary embolism (PE) is characterized by an occlusion of pulmonary arteries. Acute PE is a life-threatening condition that needs immediate treatment to prevent death [1]. The severity and prognosis of PE depend on the involvement degree of the pulmonary arterial bed [2,3]. Identification of predisposing factors and markers for PE is crucial for determining the treatment modality. Eosinophils are the 2nd largest member of the leukocyte family [4]. They have been associated with allergic diseases, parasitic infections, autoimmune diseases, and myelodysplastic syndrome [4]. In addition, evidence has shown that eosinophils are associated with stent thrombosis, vasospastic angina, coronary artery disease, and coronary collateral development [4-6]. Eosinophils are known to comprise many granules facilitating both the formation and growth of thrombus in several diseases [7,8].

However, the literature lacks data from the comparison of eosinophil counts between patients with massive and sub-massive PE. Strong vasoactive and pro-coagulant effects of eosinophils suggest a relationship between eosinophil counts and pulmonary thrombus formation. In this study, to investigate the relationship of eosinophil counts with massive and submassive PE and also to reveal whether it can be used as a predictor.

Materials and methods

In this single-center study, we analyzed retrospectively 108 patients with PE and 75 healthy control subjects who were admitted to Bolu Abant Izzet Baysal University Hospital between January 2018 and July 2019. The study was approved by the Ethics committee of Bolu Abant Izzet Baysal University (Date and Decision no: 2020-168). Patients' demographic data such as age, weight, sex, height, heart rate, blood pressure, medication, comorbidity, smoking, and laboratory parameters were extracted from the electronic medical record. Hemogram values were obtained at initial presentation and after treatment.

Patients who had blood pressure ≥140 / 90 mmHg or use antihypertensive drugs were considered as having hypertension (HT). Diabetes mellitus (DM) was determined by the current use of antidiabetic drugs, fasting blood glucose level > 126 mg/dl, or HbA1c ≥7. Those who had a total cholesterol level ≥200 mg/dL, LDL-c level ≥130 mg/dL, or use of cholesterol-lowering medication were considered as having hyperlipidemia (HL). An individual who was active smoker or had a smoking history of > 10 packs per year was considered a smoker.

For diagnosing and determining the severity of PE, we leveraged current guidelines including symptoms, high D-dimer level, electrocardiogram, computed tomography pulmonary angiogram (CTPA), echocardiography, and positive cardiac enzymes [9]. Radiological data allowed us to distinguish between massive and sub-massive PE. From the radiological point of view, a massive PE is described as a reduction of lung perfusion in one lung (> 90%) or total occlusion of a main pulmonary artery diagnosed with a CTPA. The remaining forms are described as sub-massive PE [10].

Patients were excluded from the study for the following reasons: pregnancy, systemic inflammatory or infectious disease, chronic obstructive pulmonary disease, any known hematological disease, hyper-eosinophilic syndrome, liver failure, and end-stage renal failure (eGFR <15 ml/min/1.73 m^2). The study was approved by the local institutional board.

Samples of peripheral venous blood were gathered from the patients who were admitted with the diagnosis of pulmonary embolism. Levels of fasting plasma glucose, creatinine, high-density lipoprotein cholesterol, triglyceride, and low-density lipoprotein cholesterol were determined using an automatic biochemical analyzer (Architect C8000, USA). We measured complete blood counts using concurrent optical and impedance measurements (Cell Dyn 3700; Abbott Diagnostics, Lake Forest, Illinois, USA). Hematologic measurements on K3EDTA-anticoagulated whole blood were performed using a hematologic analyzer.

Statistical analysis

We analyzed the data using SPSS 18.0 Statistical Package Software for Windows (SPSS Inc., Chicago, Illinois, USA). Quantitative and qualitative variables are expressed as mean ± standard deviation (SD) and numbers and percentages. A one-way analysis of variance (ANOVA) was preferred for parameters with homogenous distribution,

and post-hoc analyses were performed with Tukey's HSD. For parameters with non-uniform distribution or in case of inequality of variances, Kruskal–Wallis test served to compare variables across study subgroups. We used the Bonferroni-corrected Mann-Whitney U test for in-group differences. Variables with normal distribution were compared by T-test and expressed as mean ± SD. The Wilcoxon test was employed to assess the variations in eosinophil counts at the presentation and after the treatment in the pulmonary embolism group. The Spearman correlation analysis was used to assess the correlations between eosinophil counts and the right ventricle (RV) dysfunction on transthoracic echocardiogram or elevated cardiac troponin level with massive PE. A receiver operating curve (ROC) analysis served to find the predictive value of eosinophil count to distinguish between massive PE and sub-massive PE. All p-values of <0.05 were considered significant.

Results

The present study comprised a total of 183 subjects, the control group (n=75), the sub-massive group (n=64), and the massive group (n=44). All of the three groups showed similar baseline clinical characteristics and previous medications (Table 1). Regarding PE etiology, 15 patients (13.9%) had cancer as an underlying disease; 32 (29.6%) had a history of immobilization after an operation and 4 (3.7%) had immobilization after an accident; 5 (4.6%) had genetic predisposition; 3 (2.8%) were in the postpartum period and 49 (45.4%) had no predisposing factor.

Laboratory data other than those of white blood cells and eosinophil counts were similar between the groups (Table 2). The lowest eosinophil count was evident in the massive PE group whereas the control group had the highest value *(p<0.001)*. The massive group showed the highest value of white blood cell counts whereas the lowest value was evident in the

Table 1. General characteristics of the study population.

Baseline characteristics	Control group (n=75)	Submassive group (n=64)	Massive group (n=44)	*p*
Age (mean ±SD) (years))	59 ± 7	59 ± 16	63 ± 16	*0.33*
Body mass index (kg/m2)	31 ± 1	30 ± 2	29 ± 1	*0.24*
Systolic blood pressure	123 ± 14	118 ± 12	110 ± 11	*0.09*
Diastolic blood pressure	74 ± 9	74 ± 9	71 ± 11	*0.85*
Male/female	29/46	22/42	24/20	*0.10*
Hypertension	34 (45%)	31 (48%)	18 (41%)	*0.74*
Smoking	16 (21%)	10 (16%)	10 (23%)	*0.59*
Diabetes mellitus	16 (21%)	10 (16%)	8 (18%)	*0.69*
Acetyl salicylate	23 (31%)	14 (22%)	8 (18%)	*0.26*
Calcium channel blocker	15 (20%)	11 (17%)	7 (16%)	*0.83*
ACE inhibitor	5 (7%)	9 (14%)	8 (18%)	*0.14*
ARB	13 (17%)	11 (17%)	4 (9%)	*0.42*
B- blocker	21 (28%)	15 (23%)	4 (9%)	*0.05*

ACE: angiotensin-converting enzyme, ARB: angiotensin receptor blocker, SD: standard deviation.

control group *(<0.001).*

Kruskal-Wallis and Bonferroni-corrected Mann-Whitney U tests showed significantly different eosinophil counts between the massive PE and control groups *(p <0.001)*, and between the massive and sub-massive PE groups *(p =0.005).* It also tended to vary between the control and sub-massive PE groups (p=0.06).

A CT scanner revealed that mean RV to LV dimension ratios were significantly higher in the massive PE group than those in the sub-massive group *(0.99±0.22 vs. 0.77±0.12, p<0.001, respectively).*

Spearman correlation test indicated that RV dysfunction or elevated cardiac troponin level was significantly correlated with the massive PE *(r=0.54, p <0.001)*; however, eosinophil

Table 2. Laboratory data of the study population.

Laboratory data	Control group (n=75)	Submassive group (n=64)	Massive group (n=44)	p
Median (Min-Max)				
Creatinine (mg/dl)	0.78 (0.58-2.14)	0.81 (0.5-7.6)	0.86 (0.65-1.76)	0.14
Fasting plasma glucose (mg/dl)	102 (76-413)	100 (47-228)	110 (68-280)	0.22
LDL-cholesterol (mg/dl)	119 (60-195)	114 (51-278)	119 (33-210)	0.47
Triglyceride (mg/dl)	133 (37-212)	131 (48-294)	145 (45-338)	0.12
Eosinophil counts (K/uL)	0.174 (0.003-0.853)	0.121 (0.002-0.620)	0.050 (0.003-0.383)	<0.001
Basophil counts (K/uL)	0.065 (0.010-0.580)	0.064 (0.007-0.183)	0.059 (0.008-0.288)	0.68
Mean ± S.D.				
HDL-cholesterol (mg/dl)	45 ± 10	47 ± 12	44 ± 10	0.28
Hemoglobin (g/dL)	13.4 ± 1.5	12.6 ± 1.9	13.0 ± 1.8	0.14
Platelet counts (K/uL)	240 ± 60	230 ± 70	225 ± 75	0.09
MPV (fL)	8.1 ± 1.5	7.7 ± 1.5	7.5 ± 1.2	0.09
PDV (GSD)	17.7 ± 1.2	17.7 ± 1.4	17.9 ± 0.0.9	0.67
RDW (%)	16.5 ± 1.9	16.7 ± 2.8	16.7 ± 2.3	0.79
Monocyte counts (K/uL)	0.52 ± 0.19	0.60 ± 0.30	0.60 ± 0.19	0.53
Lymphocyte counts (K/uL)	2.13 ± 0.79	1.95 ± 0.86	2.05 ± 1.20	0.11
WBC counts(K/uL)	7.35 ± 1.63	8.76 ± 2.64	9.19 ± 3.98	0.001
Total cholesterol (mg/dl)	195 ± 47	200 ± 38	202 ± 51	0.72

LDL: low-density lipoprotein, HDL: High-density lipoprotein, MPV: Mean platelet volume, PDW: Platelet distribution width, RDW: Red cell distribution width, WBC: White blood cell.

count had an inverse correlation with the presence of massive PE *(r=-0.36, p<0.001)*.

A receiver operating curve (ROC) analysis yielded the sensitivity and specificity of eosinophil count for the discrimination of the massive PE group. At a cut-off value of <0.125u/mm^3, the sensitivity and specificity for eosinophil count were 73% and 59%, respectively, for determination of massive PE *(AUC = 0.715, 95% CI, 0.616-0.815)* (Figure 1).

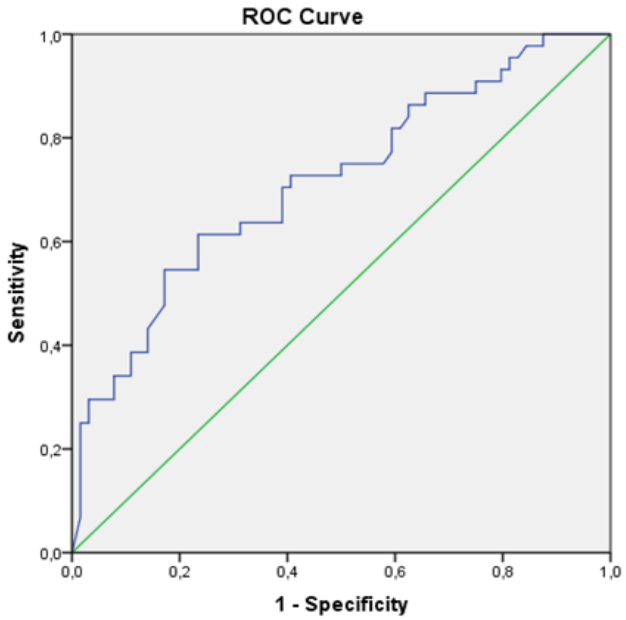

Figure 1. A receiver operating curve (ROC) analysis of eosinophil count for differentiating massive PE from sub-massive PE. At the cut-off value of <0.125 u/mm^3, sensitivity and specificity of eosinophil count were 73 % and 59 % for determination of massive PE respectively. (AUC = 0.715, 95% CI, 0.616-0.815). (AUC: area under the curve; CI: Confidence interval).

All patients in the sub-massive PE group received heparin therapy. As for the massive PE group, heparin treatment was administered in five patients with active cancer, twelve patients in the postoperative period, two patients in the post-accident immobilization period, and five patients who refused thrombolytic consent, and thus the remaining 20 patients received thrombolytic therapy. During six months of the follow-up, four patients (3.6%) died either during the in-hospital stay (two patients) or during the follow-up after hospitalization (two patients).

The eosinophil count increased significantly, regardless of treatment modality, one week after the treatment compared to presentation with PE *(0.112 (0.003-0.853) vs. (0.144 (0.011-0.914), p=0.01, respectively).*

Discussion

The association of decreased eosinophil count with the severity of PE was the principal finding of our study. It was significantly lower in the massive PE than the sub-massive PE and the control groups. As far as we know, this is the first study to show that a lower peripheral eosinophil count may support massive PE rather than sub-massive PE in patients with acute PE. Regarding the clinical aspect of this finding, decreased eosinophil counts should be a new therapeutic target in the massive PE sub-group.

Pulmonary embolism accounts for 10% of all causes of hospital deaths [11]. Patients with pulmonary embolism are clinically classified into massive and sub-massive patients based on blood pressure, right ventricular functions, high cardiac markers, and radiologically affected vascular bed [9].

It remains unclear how eosinophils are involved in the pathogenesis of thrombus formation. Neurotoxins, eosinophil cationic proteins, and major basic proteins released from eosinophils can cause endothelial damage resulting in fibrosis, thrombosis, and infarction [12]. Also, the enzyme peroxidase and the major basic protein released from eosinophilic granules may lead to thrombus formation by directly

activating platelets and inhibiting thrombomodulin [13]. By means of another mechanism, tissue factors released from eosinophils bind to coagulation factor 7 and directly initiate the coagulation cascade [14]. The literature has reported the effects of eosinophils on thrombus formation in patients with the idiopathic hyper-eosinophilic syndrome, and the rare causes of arterial thrombosis and cardio-embolic stroke in childhood [1,15].

Coronary atherosclerotic plaques showed higher eosinophil concentration in the red thrombus, while the number of eosinophils in peripheral blood showed a negative correlation with the troponin count [13]. Furthermore, lower eosinophil counts were reported in myocardial infarction compared to those in unstable angina pectoris [16]. In addition, lower eosinophil counts were associated with a worse prognosis in patients undergoing percutaneous coronary intervention [17]. Mackman et al. investigated whether eosinophils were present in human coronary artery thrombus in patients with acute myocardial infarction of native vessels or stent thrombosis. They revealed that thrombi from female patients with previous stent thrombosis contained significantly elevated numbers of eosinophils. They also discussed which subgroups of patients could benefit most from an eosinophil-inhibition approach [18].

Therefore, a reverse correlation may exist between eosinophil counts and the burden of thrombi. However, eosinophil concentration has not been studied in aspirated PE thrombus. In light of the evidence that, for eosinophils, higher concentrations in coronary thrombi and less number in the systemic circulation are associated with the severity of acute coronary events, the fact that we detected fewer eosinophils in massive PE compared to sub-massive PE can be explained by the accumulation of eosinophils in the thrombus burden and thus their decreased number in the systemic circulation.

The present study showed that lower eosinophil counts may support the diagnosis of massive PE at acute presentation. This study also showed that eosinophil counts increased after treatment regardless of the treatment modality. Due to the retrospective nature of our study, the association of this finding with clinical outcomes is unclear. After conducting further studies on larger populations, these findings may prove useful both in the determination of massive PE and in the preference of treatment modalities including lytic therapy or mechanical interventions.

The main limitations of this study consist in the fact that it is a single-centered study with a retrospective design. Direct evidence was absent on the accumulation of eosinophils in the pulmonary emboli material. Another limitation lies in the fact that measurements were unavailable for the major basic protein, eosinophilic cationic protein, peroxidase, and other cytokine levels that may interfere with thrombosis. The retrospective design of the study did not allow us to determine cardiac enzymes, RV dysfunction data on transthoracic echocardiogram, and arterial blood gas parameters for all patients.

Conclusion

In conclusion, our study demonstrated an inverse relationship between the eosinophil count, a cheap and widespread hemogram parameter in peripheral blood, and the severity of PE. The counts were lower in massive PE than sub-massive PE. Therefore, lower eosinophil counts in an acute PE may lead physicians to suggest a higher probability of massive PE, and taking it as a predictor factor may require initiation of lytic therapy or

mechanical interventions. This finding needs to be supported by larger, prospective, and multicenter studies.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

Ethical statement:

The study was approved by Local Clinical Research Ethics Committee (Date and Decision Number: 2020-168), and written informed consent was obtained from each subject.

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Experimental Biomedical Research **Original article**

The effect of atherogenic plasma index on collateral development in patients with chronic coronary total occlusion

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ABSTRACT

Aim: To demonstrate the correlation between coronary collateral circulation (CCC) and atherogenic plasma index (AIP), one of the factors associated with the formation of collateral vessels.
Methods: Hospital records of patients with chronic total occlusion (100% stenosis) in at least one coronary artery were evaluated. Triglycerides, HDL level, triglyceride/HDL ratio and atherogenic plasma index before coronary angiography were assessed for the 451 patients who met the study criteria.
Results: Comparison of the two groups in terms of laboratory findings showed that triglyceride/HDL ratio *(5.04±3.13 vs 3.56±2.12, p<0.001)* and AIP *(0.63±0.25 vs 0.48±0.25, p<0.001)* were higher with statistical significance in the weak collateral group. The ROC analysis revealed an association between weak collateral formation and atherogenic plasma index with 64.7% sensitivity and 66.2% specificity using a cut-off value of 0.58 for AIP. Accordingly, low AIP was found to be an independent predictor of good collateral artery formation.
Conclusion: This study suggests that a high atherogenic plasma index may be an independent factor associated with poor collateral formation.

Key words: Coronary artery, occlusion, collateral vessels, atherogenic plasma index, triglyceride/HDL ratio.

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Introduction

Atherosclerosis constitutes the base of coronary artery disease and is a progressive process with systemic involvement and is accompanied by inflammation [1]. Chronic total occlusion (CTO) of the coronary arteries is defined as the complete obstruction of the vessel lumen for at least three months. In the coronary angiography series, CTO was found in at least one coronary artery in approximately 1/3 of patients. Revascularisation of CTO lesions by percutaneous coronary intervention has positive impacts on the improvement of left ventricular function. Coronary collateral circulation (CCC), which exists in the normal heart, may be defined as vascular structures formed as a chronic, adaptive response between portions of the same coronary artery, or between different coronary arteries, to maintain the perfusion and viability of myocardial tissue distal to the lesion. This ensures blood flow to the ischemic myocardial tissue when critical stenosis or total occlusion disrupts blood flow [2]. Although it was initially accepted that all coronary collateral vessels develop when

needed, it is currently believed that coronary collateral vascular development occurs both by the formation of new vascular structures (angiogenesis) with the budding of capillaries from existing vascular structures, and by the development of arteriogenesis, which results from the growth and development of anastomotic channels present between the coronary arteries. These two mechanisms occur parallel to each other. When there are cardiovascular risk factors, such as hypercholesterolemia, advanced age, hypertension, diabetes, genetic predisposition, obesity and smoking, the release of angiogenic factors decreases [3]. Therefore, it is considered that the development of CCC is also adversely affected. Collateral arteries often become visible angiographically in cases where more than 90% of the main coronary artery diameter is occluded. Different methods have been used to show and group CCC angiographically. Rentrop classification classified the collateral area by the filling of the vascular structure, while Gibson and Werner classified the area by the size of the collateral vessel. The mechanism of development of coronary collateral circulation is still unclear, and clinical studies on this subject are ongoing. There is a relationship between factors such as hypertension, age, diabetes mellitus, hyperlipidemia, obesity and systemic inflammatory events, which have impacts on the development of coronary collateral and the inflammatory response caused by lipid parameters in metabolic syndrome and ischemic heart disease. Past studies have shown that vascular endothelial cells are an important factor in the development and progression of CCC [4,5]. Myocardial hypoxia may facilitate the development of coronary collateral vessels. Patients with chronic obstructive pulmonary disease (COPD) have chronic hypoxemia. Ramazan Topsakal et al. showed that the development of coronary collateral vessels in 98 COPD patients was better in patients with COPD than in patients without COPD [6]. Jan-Erik Guelker et al. showed that the triglyceride (TG)/high-density lipoprotein (HDL) ratio can be an independent factor for complex CTO lesions [7]. The logarithm of the molar ratio of TG to high-density lipoprotein cholesterol (HDL-C) levels, the atherogenic index of plasma (AIP), is closely related and inversely proportional to the diameter of low-density lipoprotein cholesterol (LDL-C) particles, which indirectly reflects small dense low-density lipoprotein (sdLDL) levels [8]. This value has been established as an index for predicting plasma atherosclerosis and CAD [9,10]. Our study was aimed at investigating the effect of AIP obtained by the logarithmic transformation of the TG/HDL ratio on the development of coronary collateral, regardless of other factors, and to determine the relationship between them in patients in whom CCC was found.

Materials and methods

Study population

Our study was approved by the Ethics Committee of Dicle University Faculty of Medicine on 21.01.2019 with file number 43. Later in our study, the data of the patients were retrospectively reviewed in the coronary angiography series performed between 03/2014-11/2018 at the Department of Cardiology of the Heart Hospital of Dicle University Faculty of Medicine. The inclusion criteria of the study will basically be based on the criterion of having lesion or lesions assessed as chronic total occlusion (100% stenosis) in at least one or more coronary arteries in the coronary angiography. Patients were selected consecutively. The exclusion criteria of the

study include admission to the hospital as myocardial infarction with ST-segment elevation, a history of previous coronary artery bypass, using a statin, being under the age of 18 years, using nonsteroidal or steroid, a history of chronic rheumatological disease, presence of hematological malignancy or any other history of malignancy.

Blood samples and echocardiography

Blood samples were collected from anterior forearm in all patients included in the study in the supine position after hospitalization in our cardiology clinic. Measurements of lipid levels were performed in serum separated by centrifuge at 3000 rpm at room temperature. Total cholesterol (TC), triglyceride (TG), high-density cholesterol (HDL) were assessed by Konelab kits and Konnelab 60i and Thermo Clinical Labsystems devices (Thermo Clinical Labsystems Oy Ratostic 2, Vantae, Finland). The LDL cholesterol level was calculated using the Friedewald formula in patients. Left ventricular ejection fractions were measured by transthoracic two-dimensional echocardiography in the echocardiography laboratory during the admission to the Department of Cardiology (Vivid S6, GE Medical Systems, USA).

Coronary angiography

Selective coronary angiography was performed by the Judkins technique, using 6F or 7F catheters using the right or left femoral or radial approach in patients. Coronary angiography images were assessed by two experienced cardiologists blinded to the laboratory values and clinical characteristics of the patients. The degree of obstruction in the coronary arteries was decided based on the projection with the greatest stenosis. Collateral development was assessed according to the Rentrop grading. In the evaluation made according to the Rentrop grading; Rentrop 0: No collateral filling, Rentrop 1: Barely detectable collateral flow and no filling of epicardial arteries, Rentrop 2: Partial filling, contrast medium enters but fails to completely opacify the epicardial arteries, Rentrop 3: Complete perfusion, contrast medium completely opacifies the epicardial arteries. Rentrop 0 and 1 were evaluated as poorly developed collateral circulation, while Rentrop 2 and 3 were considered to be well-developed collateral circulation.

Measurement of atherogenic index of plasma (AIP)

Triglyceride and HDL values were measured from the blood samples collected before the coronary angiography of the patients in the study and were recorded in mg/dL. The atherogenic index of plasma (AIP) was calculated as the logarithmic transformation of the triglyceride/HDL ratio. AIP of <0.11 was considered low risk, AIP of 0.11-0.21 moderate risk, AIP of > 0.21 increased risk [8,11].

Statistical analysis

All analyzes were performed with SPSS version 25.0 (SPSS Inc., Chicago, Illinois, USA). Whether the distribution of continuous variables is normal was determined by Kolmogorov-Smirnov and Shapiro-Wilk tests. Descriptive statistics were expressed as mean ± standard deviation for continuous variables, and as the number of cases and (%) for the nominal variables. Continuous variables with normal distribution were compared using the Student t-test, and continuous variables with non-normal distribution were compared using the Mann Whitney-U test. Chi-square test and Fisher's Exact test were used to compare categorical variables. Pearson's correlation test was used to evaluate the relationship between normally distributed parameters and Spearman's rho correlation test was used to examine the relationship between parameters that are not normally distributed. In the Pearson

correlation analysis, the correlation coefficient (r) was calculated. Those with a correlation coefficient between 0 and 0.25 were defined as very weakly correlated, those with a correlation coefficient between 0.25 and 0.50 as weakly correlated, those with a correlation coefficient between 0.50 and 0.69 as moderately correlated, those with a correlation coefficient between 0.70 and 0.89 as strongly correlated, and those with a correlation coefficient between 0.90 and 1 as very strongly correlated.

Also, the significance tests of the correlations were performed and p-values, also known as the degree of significance, were given. The statistical significance level of the obtained data was interpreted with the *"p"* value. *p* values of *<0.05* were considered statistically significant.

Multivariate logistic regression analysis was used to evaluate independent determinants influencing collateral development. All significant variables were included in the logistic regression model, and the results were obtained in the 95% confidence interval and expressed as odds ratio (OR).

In addition, ROC analysis and curves (Receiver Operating Characteristics Curve) were used to determine the diagnostic performance and cutoff values of the TG/HDL ratio and the Atherogenic index of plasma (AIP).

Results

A total of 451 patients, 126 women (27.9%) and 325 men (72.1%), aged between 33 and 92 years old, with a mean age of 61.75 ± 11.16

Table 1. Demographic and clinical characteristics of the patients.

Demographic and clinical characteristics		Poor collateral N=232	Good collateral N=219	*p*
Age		60.70±10.85	62.86±11.40	***0.039***
Gender, (N, %)	Female	69 (29.7%)	57 (26.0%)	*0.380*
	Male	163 (70.3%)	162 (74.0%)	
HT, S (N, %)		98 (42.2%)	77 (35.2%)	*0.123*
DM, S (N, %)		105 (45.3%)	55 (25.1%)	***0.000***
Smoking, S (N, %)		127 (54.7%)	57(26.0%)	***0.000***
Family history, S (N, %)		28 (12.1%)	15 (6.8%)	*0.059*
Previous MI, S (N, %)		57 (24.6%)	49 (22.4%)	*0.583*
Beta-blocker, S (N, %)		74 (31.9%)	59 (26.9%)	*0.249*
ACE-I/ARB, S (N, %)		94 (40.5%)	76 (34.7%)	*0.203*
CCB, S (N, %)		47 (20.3%)	33 (15.1%)	*0.143*
Nitrate, S (N, %)		21 (9.1%)	18 (8.2%)	*0.753*
ASA, S (N, %)		39 (16.8%)	40 (18.3%)	*0.685*
Clopidogrel, S (N, %)		10 (4.3%)	5 (2.3%)	*0.230*

HT: Hypertension, DM: diabetes mellitus, MI: myocardial infarction, ACE-I/ARB: Angiotensin-converting enzyme inhibitor/ Angiotensin receptor blockers, CCB: calcium channel blocker, ASA: acetylsalicylic acid.

years, were included in our study. The mean age of the patients was found to be 60.21 ± 10.83 years in men and 65.72 ± 11.05 years in women. 219 patients (48.6%) with Rentrop grade 2 and 3 collaterals angiographically constituted the good collateral group and 232 patients (51.4%) with grade 0 and 1 collateral the poor collateral group. Those with good collaterals and those with poor collaterals were compared regarding gender, age, diabetes mellitus (DM), hypertension (HT), smoking, family history, previous myocardial infarction (Pre. MI), use of Angiotensin-converting enzyme inhibitor (ACE-I), Angiotensin receptor blockers (ARB), acetylsalicylic acid (ASA), clopidogrel, calcium channel blocker (CCB), nitrate, and beta-blocker (Table 1).

Considering the previous histories of the patients, 105 (45.3%) patients of the poor collateral group and 55 (25.1%) patients of the good collateral group had a history of DM. When both groups were compared, there was a statistically significant difference *(p<0.001)*. 127 (54.7%) patients in the poor collateral group and 57 (26.0%) patients in the good collateral group had a history of smoking. Statistically significant difference was found between the two groups *(p<0.001).*

Angiographic findings of patients according to collateral grade after coronary angiography are shown in (Table 2). Comparing the arteries developing poor collaterals angiographically, while chronic total occlusion (CTO≥2 vessels) was found in the LAD in 36%, in the CX in 22.8%, in the RCA in 56%, and in multiple vessels in 14%, chronic total occlusion was found in 32% in the LAD, in 22% in the CX, in 63% in the RCA, in 14% in multiple vessels in the good collateral group. However, there was no statistically significant difference between the two groups *(p>0.05).* Comparing all three coronary arteries, generally more CTOs were detected in the RCA. This result was consistent with previous large-scale studies. Comparing the poor collateral group and the good collateral group in terms of CTOs in the RCA, although good collaterals were seen more, there was no statistically significant difference *(p=0.133).*

Table 2. Angiographic findings of the patients.

Angiographic findings	**Poor collateral N=232**	**Good collateral N=219**	***p***
LAD	84 (36%)	71 (32%)	*0.397*
Cx	53 (23%)	47 (22%)	*0.724*
RCA	129 (56%)	137 (63%)	*0.133*
CTO≥2 vessels	32 (14%)	30 (14%)	*0.977*

LAD: Left anterior descending artery, Cx*: Circumflex artery,* RCA*: Right coronary artery, CTO: Chronic total occlusion.*

Although high-density lipoprotein (HDL) level was higher in the good collateral group, there was no statistically significant difference *(p=0.397).* Fasting glucose level, triglyceride level, and total cholesterol level were significantly higher in the poor collateral group *(p<0.001, p<0.001, p=0.019, respectively).* The sodium level was significantly higher in the good collateral group, incidentally *(p=0.009)* (Table 3).

When two groups were compared in terms of echocardiographic findings, the mean left ventricular ejection fraction (EF) at admission was 48.05 ± 7.96 in the poor collateral group and 51.24 ± 9.77 in the good collateral group and there was a statistically significant difference between the two groups *(p<0.001)* (Table 3).

When two groups were compared regarding the triglyceride/HDL ratio (TGHDL), it was found to be 5.04 ± 3.13 in the poor collateral group

Table 3. Laboratory and echocardiographic findings of the patients.

Laboratory and Echocardiographic Findings	Poor Collateral N=232	Good Collateral N=219	*p*
WBC (x10³ µL)	9.40±2.50	9.13±2.56	*0.262*
RBC (x10⁶ µL)	4.96±0.62	4.96±0.80	*0.976*
HGB (g/dL)	13.84±2.05	13.82±2.18	*0.956*
HCT	42.66±5.25	42.44±5.75	*0.676*
MCV (x10³ µL)	86.20±6.24	86.41±5.94	*0.722*
RDW	12.22±1.96	12.20±1.87	*0.952*
Glucose (mg/dL)	158.5±75.60	132.3±55.23	***0.000***
Urea (mg/dL)	40.97±22.50	42.75±23.23	*0.410*
Kreatinin (mg/dL)	1.03±0.74	1.09±0.89	*0.424*
Total Protein (gr/dL)	7.24±0.82	7.87±0.65	*0.328*
Albumin (gr/dL)	3.71±0.42	3.65±0.42	*0.140*
Total Bilirubin (mg/dL)	0.65±0.35	0.66±0.33	*0.933*
ALT (U/L)	24.96±19.55	25.19±20.93	*0.906*
AST (U/L)	27.50±1.44	30.04±2.05	*0.313*
ALP (U/L)	82.09±28.49	86.23±31.36	*0.175*
GGT (U/L)	37.74±2.63	41.91±4.30	*0.411*
LDH (U/L)	264.6±116.6	274.1±124.4	*0.403*
Triglyceride (mg/dL)	186.7±86.75	143.9±69.16	***0.000***
Total Cholesterol (mg/dL)	183.9±47.95	173.4±46.63	***0.019***
HDL (mg/dL)	41.77±26.73	43.38±8.71	*0.397*
LDL (mg/dL)	102.1±39.12	102.4±38.41	*0.935*
Calcium (mg/dL)	9.19±0.72	9.16±0.55	*0.553*
Sodium (mmol/L)	136.6±2.73	137.3±2.94	***0.009***
Potassium (mmol/L)	4.47±0.41	4.44±0.45	*0.527*
CRP (mg/dL)	0.95±0.12	1.35±0.24	*0.151*
TSH (IU/mL)	1.42±0.15	1.96±0.58	*0.401*
T3 (pg/mL)	4.98±2.88	4.65±1.27	*0.339*
T4 (ng/L)	17.68±5.44	16.99±3.42	*0.318*
TGHDL	5.04±3.13	3.56±2.12	***0.000***
AIP	0.63±0.25	0.48±0.25	***0.000***
EF (%)	48.05±7.96	51.24±9.77	***0.000***

ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alkalen fosfataz, GGT: Gama glutamil transpeptidaz, LDH: Laktat dehidrogenaz, HDL: High-density cholesterol. LDL: Low density lipoprotein, CRP: C-reactive protein, TSH: Thyroid-stimulating hormone, TGHDL: High triglyceride high-density lipoprotein cholesterol ratio, AIP: Atherogenic index of plasma, EF: Ejection fraction.

and 3.56 ± 2.12 in the good collateral group on average. It was statistically significantly higher in the poor collateral group *(p<0.001)* (Table 3). When two groups were compared concerning the atherogenic index of plasma (AIP), it was found to be 0.63 ± 0.25 in the poor collateral group, and 0.48 ± 0.25 in the good collateral group on average. It was statistically significantly higher in the poor collateral group *(p<0.001)* (Table 3).

When correlation analysis was performed between collateral grade and the TG/HDL ratio (TGHDL) *(r_s = -0.299, p <0.001)* and between collateral grade and the atherogenic index of plasma *(r_s = -0.299, p <0.001)*, there was a weak negative correlation which was statistically significant (r_s: Spearman's rho correlation coefficient) (Table 4).

Table 4. Correlation analysis between collateral grade and the TG/HDL ratio (TGHDL) and the atherogenic index of plasma (AIP).

Parameter	r_s	*p*
TG/HDL	-0.299	*0.000*
AIP	-0.299	*0.000*

In the multivariate logistic regression analysis performed to find the factors affecting collateral development, the TG/HDL ratio (TGHDL) and the atherogenic index of plasma (AIP) were found to be statistically significant, independently of other factors. Accordingly, low TG/HDL ratio (TGHDL) and AIP were found to be independent predictors of the development of the good collateral arteries *(p<0.001)* (Table 5).

Table 5. Multivariate Logistic Regression Analysis between collateral development and the TG/HDL Ratio (TGHDL) and the atherogenic index of plasma (AIP).

Parameter	OR	95% CI	*p*
Age	1.008	0.987-1.029	*0.470*
Gender	0.631	0.383-1.038	*0.070*
TG/HDL	0.819	0.741-0.905	*0.000*
AIP	0.083	0.035-0.196	*0.000*

In the ROC analysis of the data, the development of collateral was compared with the TG/HDL ratio (TGHDL) and the atherogenic index of plasma (AIP). There was a statistically significant negative correlation between collateral development and the TG/HDL ratio (TGHDL) and the atherogenic index of plasma (AIP). When the cutoff value was considered 3.81 for the TG/HDL ratio and 0.58 for the atherogenic index of plasma (AIP), the increase in the TG/HDL ratio and the atherogenic index of plasma was found to be associated with the presence of poor collaterals with a sensitivity of 64.7% and specificity of 66.2% (Table 6, Figure 1).

Discussion

The factor affecting coronary collateral development is an inflammatory response. One of the factors that started this inflammatory process is AIP. When starting our study, we aimed to investigate the predictability of a decrease in collateral development when there is an increase in AIP and the relationship between them. In order to reveal this relationship, patients were selected from the groups that showed homogenous distribution in terms of factors other than AIP, which we think is particularly effective on collateral development. Since we aimed to investigate the effect of AIP on CCC in patients with coronary artery disease, we did not include patients taking lipid-lowering drugs in our study. Therefore, in our study, the possible effects of drugs with the ability to reduce lipid levels on HDL cholesterol, CCC and triglyceride levels were not mentioned. In our study, the left ventricular systolic functions were statistically significantly higher in the good collateral group, and a statistically significant correlation was found between AIP, diabetes mellitus, smoking and collateral development. In a study conducted by Abaci et al. on 205 diabetes mellitus (DM) patients, it was shown that collateral vessel development in DM was worse than in non-diabetic patients. We can say that

Table 6: ROC analysis.

Area Under the Curve [AUC]						
	AUC	***p value***	**95% Confidence interval (CI)**	**Cut off value**	**Sensitivity**	**Specificity**
			Lower-Upper Limit			
TG/HDL	673	*0.00*	623-722	3.81	64.7%	66.2%
AIP	673	*0.00*	623-722	0.58	64.7%	66.2%

DM is an important factor among the factors affecting the development of coronary collateral [12].

For visible collateral vessels, there must be total or subtotal occlusion of the coronary arteries [13]. Therefore, we included patients with at least one total occluded coronary artery in our study. Also, collateral development is a time-dependent continuity. The development of coronary collaterals was demonstrated in the first six hours following acute myocardial infarction, and all collaterals developed were found to become visible in the coronary angiography performed within 24 hours after myocardial infarction. To make an accurate assessment of CCC, we did not include patients who underwent coronary angiography in the first 24 hours after acute coronary syndrome. An experimental study showed that HDL-C increases coronary artery blood flow through NO-mediated vasodilation [14]. This finding shows that HDL cholesterol can positively affect collateral development with the increased effect of NO. Rossi et al. have shown that HDL cholesterol is a powerful indicator of the number and function of circulating endothelial progenitor cells (EPCs), which also repair the endothelial layer [15]. Spieker et al. showed that HDL cholesterol is an endothelial protective factor in a hypercholesterolemic man without any risk factors for coronary artery

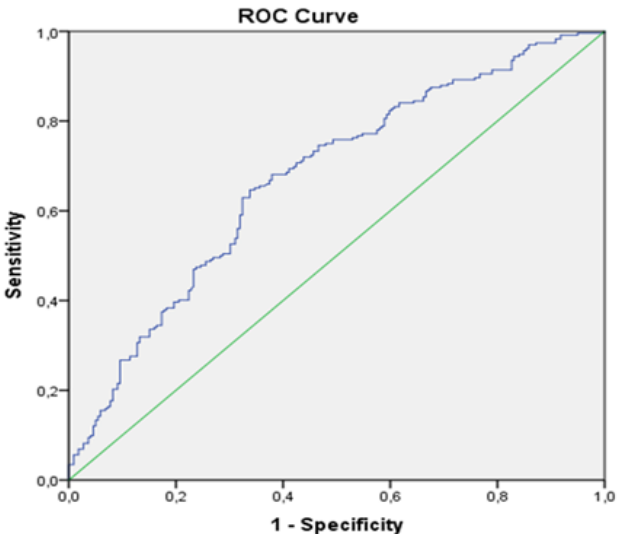

Figure 1: ROC analysis comparison of the relationship between collateral development and the atherogenic index of plasma and the TG/HDL Ratio.

disease [16]. In healthy individuals without an additional risk factor for cardiovascular disease, low HDL-C has been shown to be an independent risk factor for endothelial dysfunction [17].

The results obtained from these studies clearly prove that HDL-C has endothelial layer protection and anti-inflammatory effects. Therefore, patients with weak CCC may have low HDL cholesterol levels. This can be explained by the anti-inflammatory and endothelial protective properties of HDL-C.

However, in our study, although HDL cholesterol levels were found to be higher in the

group developing good collaterals, no statistically significant difference was found *(p = 0.397)*.

First described by Dobiášová and Frohlich in 2001, AIP [18] is a comprehensive lipid index and a powerful marker for predicting CAD risk [19]. While Niroumand et al. suggested that AIP could be used as a regular monitoring index of CAD in daily practice [20], Wan et al. proved an elevated AIP to be a strong independent predictor of all-cause mortality and cardiovascular disease after coronary revascularization [21]. However, several recent studies have proven AIP to be of significant value in assessing the severity of coronary syndrome. Another study showed a significant relationship between AIP and the progression of coronary artery calcification [22]. Furthermore, in a 7–8-year follow-up study of 2,676 middle-aged adults, AIP was confirmed to be a prognostic marker for predicting CAD-related morbidity [23]. AIP is independently associated with the occurrence of CTO and is thought to predict the presence of CTO and disease severity [24].

Nevertheless, a higher AIP may be considered an inflammatory marker that mediates endothelial function and the growth of collateral circulation by affecting a number of inflammatory reactions. Strong evidence in the prediction of the increase in AIP reducing coronary collateral development has provided important data: AIP is a determinant via local inflammatory response.

Our study has some limitations. First, the sample size was relatively small, a multi-center study should be designed to present the result as strong evidence. Secondly, the bias was inevitable as the subjects included as a case-control study were enrolled from a single center. Thirdly, as this study was performed cross-sectionally, not knowing clearly whether the blood sample was collected after proper diet preparation and the condition of other daily events affecting the atherogenic index of plasma related to TG/HDL ratio made it difficult to comment on its relationship with the development of CCC. Fourthly, as HDL cholesterol dysfunction may also occur, serum HDL cholesterol level does not fully demonstrate the function of HDL cholesterol. Fifthly, it is unknown whether the results of our study can be extended to other populations.

Conclusion

We demonstrated that a high atherogenic plasma index is an independent factor associated with weak collateral formation. The atherogenic index of plasma, which is simple, non-invasive, and economic, obtains a fast result and has an important value, should be carefully evaluated in determining individuals at risk for cardiovascular diseases, especially in individuals with a family history of coronary heart diseases. The development of cardiovascular diseases may be decreased by reducing the atherogenic index of plasma.

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Ethical statement:

The study was approved by Local Clinical Research Ethics Committee (Date and Decision Number: 2019.01.21-43), and written informed consent was obtained from each subject.

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Experimental Biomedical Research **Original article**

COVID-19 and comorbidities: Predictors, clinical course, relationship with disease severity, and outcome

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ABSTRACT

Aim: COVID-19 disease has a broad spectrum ranging from asymptomatic course to death. While data show that the prognosis of the disease will be poor in the presence of comorbidity, we witness the death of patients with no comorbidities in our clinical practice. This study aimed to investigate the effect of comorbidity on the clinical course and mortality of COVID-19 pneumonia.

Methods: 155 Rt-PCR (+) adult patients hospitalized at İzzet Baysal State Hospital (Bolu, Turkey) diagnosed with severe and critical pneumonia between August 2020 and February 2021 were included in this single-center, retrospective study. The patients were divided into two groups with and without comorbidity, compared the severity of inflammation parameters, radiological involvement, and oxygen requirement, and evaluated their effects on mortality and hospitalization duration.

Results: There was no significant difference in the severity of the computed tomography (CT) involvement, the oxygen requirement, inflammation markers, and duration of hospitalization in patients with comorbidities compared to those without. When we evaluated the patients with comorbidities in general and their subgroups, the relationship with mortality was not significant. The severity of CT involvement, high oxygen requirement, and inflammation markers such as lymphocyte, lymphocyte ratio, LDH, CRP, troponin, ferritin levels were found to be associated with mortality.

Conclusions: In this study, we found that the presence of comorbidity did not affect mortality and duration of stay and that the severity of radiological involvement, the severity of hypoxemia, and the increase in inflammation markers were the determinants of mortality.

Key words: COVID-19, coronavirus infections, pneumonia, comorbidity, outcome, mortality.

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Introduction

The COVID-19 pandemic has been in existence since the end of 2019, and the loss of life continues. The presence of comorbidity may affect the clinical course of COVID-19. Diseases such as type 2 diabetes mellitus (DM), hypertension (HT), atherosclerotic cardiovascular (CVS) diseases, and chronic obstructive pulmonary disease (COPD) increase the risk [1]. In some studies, HT (30%) [2] and in others, diabetes mellitus [3] is the most common comorbidity, causing high mortality rates. Although the prevalence of COPD is low, it exacerbates the clinical course and significantly increases mortality in its presence [4]. It was reported that cancer is not

common comorbidity in COVID-19 patients, and less mechanical ventilation requirement and mortality rates were reported in cancer cases [5]. In light of this information, we aimed to retrospectively investigate the frequency of comorbidities and the relationship of existing comorbidities with mortality and clinical course in Rt-PCR positive severe and critical hospitalized COVID-19 pneumonia patients.

Materials and methods

The single-center, retrospective study was performed with patients hospitalized in the inpatient clinic and intensive care unit of İzzet Baysal State Hospital. This study was approved by Abant İzzet Baysal University Ethics Committee (Date: 2021-10-06 / No:189).

One hundred fifty-five patients with severe and critical pneumonia, according to WHO interim guidance [6], among Rt- PCR (+) COVID-19 patients were included. Patients under the age of 18, pregnant and postpartum patients, patients with missing comorbidity information, CT, and laboratory parameters were excluded. Demographic, radiological (pulmonary involvement less than or above 50% in CT imaging), laboratory data, and treatment outcome of the patients were recorded retrospectively. The severity of oxygen requirement was grouped as mild (up to 5 L/min with the nasal cannula) and heavy (including mask, mask with reservoir, high flow nasal oxygen (HFNO), intubated follow up). Their comorbidities were grouped as HT, DM, DM+HT, chronic pulmonary disease (CPD), cardiovascular system diseases (CVSD), malignancy, connective tissue diseases (CDD), and other (such as goiter, chronic kidney failure, which we could not group).

The analysis of the data obtained as a result of the research was performed in the SPSS 20 statistical package program, and descriptive statistical methods (frequency, arithmetic mean, standard deviation, cross tables) were used. Compliance with normal distribution was evaluated with the Kolmogorov-Smirnov test. The arithmetic mean of the normally distributed groups was evaluated, and the Independent Sample t-test compared two independent groups. The medians of the non-normally distributed groups were determined, and the Mann-Whitney U test compared two independent groups, and the Chi-Square test examined the relationship between categorical variables. A p-value of <0.05 was considered statistically significant.

Results

The mean age of the patients was 68 years (31-95), and the relationship between mortality and age was significant (61 years (31-95) in survivors, 70 years (48-94) in non-survivors, p: 0.002). The group with comorbid diseases was older (69 (35-95) vs. 57.5 (31-94) p: 0.001).

The overall comorbidity rate in the patients was 73%, with HT 49.7%, DM 29%, DM+HT 21.3%, CPD 14.8%, CVSD 9%, malignancy 5.2%, CDD 1.9%, and 18.7% for other diseases (few diseases we did not group, such as goiter, chronic kidney disease). The rate of non-comorbid patients was found to be 27.1%. When the patients with comorbidities were evaluated in general and according to their subgroups, the relationship with mortality was not statistically significant ($p>0.005$, Table 1).

There was no significant difference in the severity of the CT involvement, the severity of the oxygen requirement, inflammation markers, and duration of stay in patients with comorbidities compared to those without ($p>0.05$, Table 2). In terms of duration of hospital stay, a significant difference was found only in the DM and DM+HT subgroups, with a shorter duration of stay.

Table 1. The relationship between the presence of comorbidity and mortality.

Parameters	Survivor (N=113) (N/%)	Non-survivor (N=42) (N/%)	Total (N=155) (N/%)	*p-value*
DM	37/82.2	8/17.8	45/29	*0.095*
Non- DM	76/69.1	34/30.9	110/71	
HT	60/77.9	17/22.1	77/49.7	*0.162*
Non- HT	53/67.9	25/32.1	78/50.3	
DM+HT	27/81.8	6/18.2	33/21.3	*0.194*
Non- (DM+HT)	86/70.5	36/ 29.5	122/78.7	
CVSD	10/71.4	4/28.6	14/9	*0.896*
Non- CVSD	103/73	38/27	141/91	
CPD	17/73.9	6/26.1	23/14.8	*0.906*
Non-CPD	96/72.7	36/27.3	132/85.2	
Malignancy	4/50	4/50	8/5.2	*0.134*
Non- malignancy	109/74.1	38/25.9	147/94.8	
CTD	2/66.7	1/33.3	3/1.9	*0.806*
Non- CT	111/73	40/27	152/98.1	
Other diseases	18/62.1	11/37.9	29/18.7	*0.145*
Non-other diseases	95/75.4	31/24.6	126/81.3	
Non-comorbid	31/73.8	11/26.2	42/27.1	*0.877*

** p<0.05 statistically significant, Chi-Square test. DM: diabetes mellitus, HT: hypertension, CVSD: cardiovascular system disease, CPD: chronic pulmonary disease CTD: connective tissue disease*

Table 2. Baseline demographics, CT involvement, and laboratory parameters of the study population classified by the presence of comorbidity.

Parameters	Comorbidity (+) (N=113) (N/%)	Comorbidity (-) (N=42) (N/%)	All patients (N=155) (N/%)	*p-value*
Gender[1]				
Female	47/74.6	16 (25.4%)	63 (40.6%)	*0.694*
Male	66/71.7	26 (28.2%)	92 (59.3%)	
Oxygen requirement[1]				
Mild	45/71.4	18 (28.6%)	63 (40.6%)	*0.732*
Severe	68/73.9	24 (26.1%)	92 (59.4%)	
CT involvement[1]				
Below 50%	59/73.7	21 (26.3%)	80 (51.6%)	*0.806*
Oover 50%	54/72	21 (28%)	75 (48.4%)	
Mortality[1]				
Exitus	31/73.8	11 (26.2%)	42(27.1%) 113(72.9%)	*0.877*
Alive	82/27.1	31 (27.4%)		
Age[2]	69 (35-95)	57.5 (31-94)	68 (31-95)	***0.001***
Duration of hospitalization[2]	10 (1-55)	7 (3-60)	9 (1-60)	*0.369*
Lymphocyte[2] (0.9-3.4K/uL)	900 (200-3400)	950 (200-2600)	0.90 (0.20-3.40)	*0.957*
Lymphocyte%[2] (25-46 K/uL)	13.1 (1.9-49.3)	18 (1-36.2)	15.0 (1-49.3)	*0.137*
CRP [2] (0-5 mg/L)	92.1 (2.2-139.4)	90.6 (7.40-132)	92.1 (2.20-139.4)	*0.811*
LDH (1-248U/L)	336 (127-1884)	399.5 (183-885)	351 (127-1884)	*0.147*
Troponin[2] (12.6-20.7 ng/L)	9.9 (1.70-2327)	7 (1.6-1654)	9.4 (1.6-2327)	*0.115*
Ferritin[2] (23.9-366.2 ug/L)	233.7 (11.3-1500)	317 (21.4-1500)	245.9 (11.3-1500)	*0.327*
Fibrinogen[2] (170-420 mg/dL)	5.74 (1.47-10.25)	5.82 (2.79-11)	5.75 (1.47-11)	*0.148*
D-Dimer[2] (0-0.5 mg/L)	0.38 (0.03-17.1)	0.29 (0.02-7.70)	0.37 (0.02-17.1)	*0.503*

CT: computed tomography. CRP: C-reactive protein. LDH: Lactate dehydrogenase.

When the survivor and the non-survivor group were compared, the severity of CT involvement, high levels of oxygen requirement, and laboratory parameters such as lymphocyte, lymphocyte ratio, lactate dehydrogenase (LDH), C-reactive protein (CRP), troponin, and ferritin were found to be associated with mortality *(p<0.05)* (Table 3).

Oxygen requirement was high in 88% of those with CT involvement above 50% and 32.5% of those with CT involvement of less than 50% *(p<0.05)*. A decrease in the lymphocytes and lymphocyte ratios and an increase in LDH, CRP, troponin, ferritin, D-dimer, and fibrinogen values were found to be significant in the group with severe CT involvement group compared to the other group *(p<0.05)*, Table 4).

Table 3. Baseline demographics, CT involvement, and laboratory parameters of the study population classified by the overall mortality.

Parameters	**Survivor (N=113) N/%**	**Non-survivor (N=42) N/%**	**All patients (N=155) N/%**	***p-value***
Gender[1]				
Female	51/81	12/19	63/40.6	*0.062*
Male	62/67.4	30/32.6	92/59.3	
Oxygen requirement[1]				
Mild	62/98.4	1/1.6	63/40.6	***<0.001***
Severe	51/55.4	41/44.6	92/59.4	
CT involvement[1]				
Below 50%	75/93.7	5/6.3	80/51.6	***<0.001***
Over 50%	38/50.7	37/49.3	75/48.4	
Median(min-max)				
Age[2]	61(31-95)	70(48-94)	68 (31-95)	***0.002***
Duration of hospitalization[2]	7 (3-55)	22.5(1-60)	9(1-60)	***<0.001***
Lymphocyte[2] **(0.9-3.4K/uL)**	1000 (200-3400)	600(200-2000)	0.90(0,20-3.40)	***<0.001***
Lymphocyte%[2] **(25-46 K/uL)**	18.3(1,9-49.3)	9.45(1-30.9)	15.0 (1-49.3)	***<0.001***
CRP[2] **(0-5 mg/L)**	68.1 (2.2- 139.4)	123(3.5- 132.4)	92.1(2.2-139.4)	***<0.001***
LDH[2] **(1-248U/L)**	337 (127- 1884)	422 (192-1375)	351(127-1884)	***0.001***
Troponin[2] **(12.6-20.7 ng/L)**	7.7 (1.6-1654.4)	18.4 (2.2-2327	9.4 (1.6-2327)	***<0.001***
Ferritin[2] **(23.9-366.2 ug/L)**	214.3 (14.8-1500)	493.9(11.3-1500)	245.9(11.3-1500)	***<0.001***
Fibrinogen[2] **(170-420 g/dL)**	5.67 (2.67-10.25)	5.95 (1.47-11)	5.75(1.47-11)	*0.148*
D-dimer[2] **(0-0.5 mg/L)**	0.39 (0.02-7.7)	0.33(0.03-17.1)	0.37(0.02-17.1)	*0.123*

** p<0.05 statistically significant, [1]Chi-Square test [2]Mann-Whitney U test. CRP: C-reactive protein. LDH: Lactate dehydrogenase.*

Table 4. Oxygen requirement and laboratory parameters of the study population classified by the severity of CT involvement.

Parameters	CT involvement mild (n= 80) N/%	CT involvement severe (n=75) N/%	All patients (n=155)	*p-value*
Oxygen requirement[1]				
Mild	54/85.7	9/14.3	63/40.6	***<0.001***
Severe	26/28.3	66/71.7	92/59.4	
Median (min-max)				
Duration of hospitalization[2]	7 (3-31)	16 (1-60)	9 (1-60)	***<0.001***
Lymphocyte[2] (0.9-3.4K/uL)	1.30 (0.20-3.40)	0.70 (0.20-2.60)	0.90 (0,20-3.40)	***<0.001***
Lymphocyte%[2] (25-46 K/uL)	19.6 (1.90-49.3)	9.3 (1-30.9)	15.0 (1-49.3)	***<0.001***
CRP[2] (0-5 mg/L)	51.4 (2.20-139.4)	120.8 (3.5-133.8)	92.1 (2.20-139.4)	***<0.001***
LDH[2] (1-248U/L)	291 (172-868)	440 (127-1884)	351 (127-1884)	***<0.001***
Troponin[2] (12.6-20.7 ng/L)	7.2 (1.6-203)	12.7 (1.7-2327)	9.4 (1.6-2327)	***< 0.001***
Ferritin[2] (23.9-366.2 ug/L)	196.2(21.4-1500)	455.7 (11.3-1500)	245.9 (11.3-1500)	***<0.001***
Fibrinogen[2] (170-420 mg/dL)	5.22 (1.47-9.9)	6.4 (2.02-11)	5.75 (1.47-11)	***< 0.001***
D-Dimer[2] (0-0.5 mg/L)	0.31 (0.04-4.95)	0.46 (0.02-17.1)	0.37 (0.02-17.1)	***0.014***

** p<0.05 statistically significant.* [1]*Chi-Square test.* [2]*Mann-Whitney U test.*

Discussion

In a meta-analysis including ten studies in China, the mortality rate in COVID-19 was 5% [7], while in a Chinese Center for Disease Control and Prevention report containing 72314 cases of COVID-19, the mortality was reported as 2.3%, 14.8% in patients over 80 years old, and 49% in critical cases [8]. In another study, the mortality rate in inpatient COVID-19 patients reached 28.3% [2]. The overall mortality was 27% in our study.

While the most common comorbidities in COVID-19 patients were HT (30%), DM (19%), and coronary heart disease (8%) [2], in patients with ARDS, they were HT (27%), DM (19%), and cardiovascular diseases (6%) [9]. Uçan et al. reported in their study that 62.1% of the patients had comorbidity, and hypertension was the most common [10]. In our study, comorbidity was observed in 73% of our patients, and in order of frequency, HT was 49.7%, DM 29%, DM+HT 21.3%, CPD 14.8%, CVSD 9%, malignancy 5.2%, CDD 1.9%, other diseases (a few diseases that we did not group, such as goiter, chronic kidney failure) 18.7%, and the non-comorbid patient rate was 27.1%.

However, when we grouped the diseases as presence/absence of comorbid diseases and grouped them individually, no relation was found with mortality *(p>0.05).*

Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) increase ACE2, and virus binding also increases [11,12]. The increased soluble ACE2 in the circulation can bind SARS-CoV-2, reducing its ability to damage the lungs and other ACE2-bearing organs [13]. Therefore, ACE inhibitors and ARBs may reduce the potential for patients with COVID19 to develop acute respiratory distress syndrome, myocarditis, or acute kidney injury. ARBs have been proposed as a treatment for COVID-19 and its complications [14]. There is no evidence yet that hypertension is associated with the consequences of COVID-19 or that the use of ACE inhibitors or ARBs during the COVID-19 pandemic is harmful or, therefore, beneficial [15]. In our study, no increase was found in mortality in patients with HT. No prolongation of hospitalization was observed (8 days (1-55) in the group with HT vs. 9.5 days (3-60) in the non-HT group, p:0.310).

In the study of Mithal et al., 47.1% of the hospitalized COVID-19 patients had Diabetes, and it was found to be associated with severe illness and mortality. HT was reported as the most common comorbidity in patients with DM [16]. In another study, DM was reported as the most common comorbidity in COVID-19 and was associated with mortality [3]. Mortality rates of 7.3% [8] have been reported in diabetic patients in China and over 30% in Europe [17]. Although the mechanism underlying the relationship between diabetes and COVID-19 severity remains unclear, Yang et al.[18] indicated that ACE2 could be strongly expressed in pancreatic islet cells, suggesting that these cells may be targeted by SARS-CoV-2 [19]. Metabolic disorders may impair macrophage and lymphocyte function, resulting in poor immune function [20] and may make individuals more susceptible to disease complications [21]. Diabetes COVID-19 has also been associated with increased severity of complications and symptoms [22]. This may be partly due to increased risk of endothelial dysfunction, increased systemic inflammatory response, micro-and macrovascular disease, and diabetic cardiomyopathy [23,24,35,26]. Despite the presence of DM in 31-80% of those who died due to COVID-19 in studies [16,17], DM was associated with ICU hospitalization and duration of hospitalization in the multivariate analysis, but no correlation with mortality was demonstrated [28].

In the study of Guo et al, higher CRP, ferritin, interleukin-6 (IL-6), sedimentation levels were found, and Zhu et al. found an increase in the incidence of lymphopenia, higher CRP, and procalcitonin levels in diabetic patients [29,30]. In our study, we did not determine increased mortality in patients with diabetes. At the same time, oxygen requirement was not high, and CT involvement was not more severe in this group. Also, we did not detect increased inflammation parameters. For these possible reasons, the duration of hospitalization was also found to be shorter (8 days (1-31) in the diabetes group vs. 10 days (3-60) in the non-diabetes group, p: 0.052).

In their study, which included 1590 hospitalized COVID-19 patients, Guan et al. reported that the most common comorbidities among infected individuals were hypertension (16.9%) and diabetes (8.2%), and patients with two or more comorbidities (8.2%) [31]. In another study, 149 of 1138 patients (13.09%) had two comorbidities, and hypertension+DM was the most commonly observed comorbid disease. The co-existence of more than one

comorbidity significantly increased the risk of death. In multivariate analysis, more than four times mortality risk was observed in patients with more than three comorbidities. Age-classified analysis revealed that the effect of comorbidities on death decreased with increasing age [32]. In our study, the association of DM + HT was 21.3%, and its relationship with mortality was not significant, but the length of hospital stay was found to be significantly shorter (DM+HT co-existence 7 days (1-31) vs. non-DM+HT 10 days (3-60), p: 0.031). While the expected issue was the prolonged duration of hospitalization, the fact that the oxygen requirement was not high, the CT involvements were not severe, and the inflammation markers were not high in these comorbid groups may explain this result.

Although the prevalence of COPD is low in COVID-19, these patients' mortality and severe disease rates have been reported to be high [4]. Viral infections cause an increase in systemic inflammation in COPD patients [33,34]. Comorbid conditions, which are usually associated with COPD patients, may also be associated with increased hospitalization [35,36].

Questions remain about the effects of inhaled (ICS) and systemic corticosteroids, short- and long-acting β2-agonists, and short- and long-acting muscarinic antagonists in alleviating or exacerbating COVID-19. ACE-2 expression in airway epithelial cells from patients with asthma is reduced in those receiving ICS compared to those not using ICS, raising the possibility that ICS exposure may reduce viral entry. It is not clear that the same relationship is valid in the COPD airway, which increases susceptibility to pneumonia following ICS use [37]. In our study, no significant relationship was found between the presence of COPD and mortality.

Asthma comorbidity was found to be more common in non-severe cases of COVID-19. Compared with patients with asthma, COPD patients were found to have increased mortality and a higher risk of developing severe disease and ARDS. In this study, downregulation of ACE2 protein expression in the lower airways was found in asthmatics compared to controls and patients with COPD. Besides, asthmatics with higher peripheral blood eosinophilia show lower ACE2 expression, indicating potential regulation of ACE2 expression by type 2 inflammation in asthmatics [38]. Our study created a chronic pulmonary disease category, including interstitial lung disease without distinction between asthma and COPD. In this group, no increase in mortality and n prolonged hospitalization (8 days (4-31) in the group with CPD vs. 10 days (1-60) in the group without CPD) were observed ($p>0.005$).

When patients with CVSD have COVID-19, the rate of complications such as myocardial infarction, malignant arrhythmia, mesenteric infarction, cardiovascular death, heart failure, myocardial suppression increases [39]. COVID-19 is associated with endothelitis and myocarditis [40,41]. It is believed that SARS CoV-2 can elicit an intense systemic inflammatory response, and the atherosclerotic plaque may become unstable and more prone to rupture, leading to higher myocardial infarction rates and faster systolic dysfunction and heart failure [42]. However, in our ASCVD group, which consisted of 14 patients, no increase in mortality and no prolonged hospitalization (8 days (4-19) in patients with ASCVD vs. 9 days (1-60) in patients without ASCVD) were determined ($p>0.05$).

In the study of Jarahzadeh et al., cancer was not among the most common comorbidities in confirmed COVID-19 patients [5]. A lower rate of mechanical ventilation or death has been

reported in patients with cancer infected with COVID-19 than in patients without cancer [5]. In a nationwide study including 1590 hospitalized COVID-19 patients, Guan et al. reported that 18 cases (1.1%) had a history of malignancy [31]. In another study, those with a history of malignancy were found to be 6% [43]. A meta-analysis showed that 2% of COVID-19 patients had a history of malignancy, with a higher risk of severe symptoms in the cancer group with a history of chemotherapy or surgery within one month (75% vs. 43%) [44].

In COVID-19 patients with lung cancer, higher disease severity and higher rates of intensive care admission and mechanical ventilation requirement have been reported compared to other malignancies and the general population [45]. Multicenter studies have reported a high mortality rate of 33% in thoracic malignancies [46]. In our study, patients with cancer were few, and there was no significant difference in terms of mortality and hospitalization (22.5 (3-45) days in the cancer group, 9 (1-60) days in the non-cancer group) (p>0.05). Although the number of our patients was low in terms of connective tissue diseases, we did not find any effect on mortality and duration of stay (9 days (1-60) in non-CDD patients vs. 24 days (14-27) in patients with CDD) (p>0.05).

In the study conducted by Uçan et al., the mean age was reported as 61.9±20.1 years [10]. In our study, the mean age of the patients was 68 years (31-95), and the relationship between mortality and age was statistically significant (61 years (31-95) in survivors vs. 70 years (48-94) in non-survivors, p: 0.002). The group with comorbid diseases was older. (69 years (35-95) vs. 57.5 years (31-94), p: 0.001). An analysis of 77,932 patients (41510 men) infected with COVID-19 indicated that men had 1.71 times higher mortality rate than women [47]. The fact that genetic factors or risk-increasing factors such as diabetes, hypertension, and coronary artery disease are more common in men may explain this increase [1]. The increased smoking rate in men may also be associated with increased mortality [48]. Also, the stronger immune system of women and the location of most of the genes that regulate immunity on the X chromosome may have turned this ratio in favor of women [49]. Biological gender is also related to T cells and autoimmune response; for example, women secrete higher amounts of interferon than men [50] while testosterone suppresses immunity, estrogens activate it [51]. Similar to the male dominance in the literature[8], 59.3% of all patients in our study consisted of men. Comorbid diseases were not more common in males, and no gender difference was found in mortality rates *(p>0.05).*

In the study of Uçan et al., Ferritin, CRP, troponin, d-dimer, LDH, and neutrophil/lymphocyte ratio were found to be high in the mortal group [10]. In our study, lymphocyte, lymphocyte ratio, LDH, CRP, troponin, ferritin were also associated with mortality (p>0.005), but no significant correlation was found with fibrinogen and d-dimer (p>0.05).

Xie et al. stated that dyspnea and hypoxia despite oxygen support at admission were strong independent predictors of mortality. 99% of patients with an oxygen saturation level of 90% or more survived, while 69% of those with oxygen saturation of 90% or less despite oxygen support died [52]. In our study, it was the increase in oxygen requirement that affected mortality (p>0.005). Chatterjee et al. also found higher mortality in hospitalized COVID-19 patients with oxygen saturation of <92% or respiratory rate of >22/min [53].

Higher CRP values were found in severe pneumonia with diffuse involvement. In these

patients, the duration of stay in the intensive care unit, ventilator support, and mortality rates was also found to be higher [54]. In our study, CT involvement of over 50% was found to be associated with mortality (p>0.005). Oxygen requirement was high in 88% of those with CT involvement above 50% and 32.5% of those with CT involvement of less than 50% (p<0.05). A decrease in lymphocytes and lymphocyte ratios and an increase in LDH, CRP, troponin, ferritin, D-dimer, and fibrinogen values were significant in the group with severe CT involvement compared to the other group (p<0.05).

Conclusions

Most of our patients were patients followed up with critical pneumonia (92 out of 155 patients (59.4%) had severe oxygen requirement, 75 (48.4%) had more than 50% CT involvement). We found that the presence of comorbidity did not affect mortality, while CT involvement and oxygen requirement were associated with mortality. Among the laboratory parameters, we found that lymphocyte, lymphocyte ratio, LDH, CRP, troponin, and ferritin were associated with mortality. Patients with severe CT involvement had higher oxygen requirements and higher inflammation markers. Limitations of our study are that results should be replicated with larger and more representative samples. In particular, the number of our malignancy and CDD group was low, and more extensive studies are required. Another limitation is the study population consisting of inpatients only. In terms of hospitalization, advanced age and the presence of comorbidity should be taken as criteria without hypoxemia, the severity of CT involvement, and abnormal laboratory parameters.

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Ethical statement:

The study was approved by Local Clinical Research Ethics Committee (Date and Decision Number: 10.06.2021-189), and written informed consent was obtained from each subject.

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Experimental Biomedical Research **Original article**

The relationship of serum asymmetric dimethylarginine concentrations and lung involvement in patients with COVID-19 infection

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ABSTRACT

Aim: COVID-19 infections the tissue through angiotensin converting enzyme 2 receptor, which is also expressed on endothelial cells. Endothelial dysfunction may be associated with lung involvement. Asymmetric dimethylarginine (ADMA) is an indirect marker of endothelial dysfunction. The aim of our study was to evaluate ADMA concentrations and to identify its association with lung involvement in patients with COVID-19 disease.

Methods: We included 42 patients with COVID-19 infection and lung involvement (Group 1). Forty-two age and sex matched patients without pneumonia acted as the control group (Group 2). All patients gave blood samples for ADMA at the 1st month control visit after discharge. We compared C-reactive protein (CRP) and ADMA concentrations in addition to routine biochemical parameters between groups.

Results: Patients with lung involvement had higher admission glucose, CRP, and ADMA concentrations, and displayed lower hemoglobin concentration and lymphocyte count compared to patients without lung involvement. Although patients with lung involvement had higher ADMA concentrations with respect to those without; plasma ADMA levels were also higher than normal values in control group. Multivariate analysis identified log CRP concentration *(OR= 3.047, 95% CI=1.881-5.023, p<0.001)* as the independent predictor for lung involvement. And, there was a correlation between ADMA and CRP *(r: 0.318, p: 0.003).*

Conclusion: We revealed elevated ADMA concentrations as the surrogate of endothelial dysfunction in COVID-19 patients whether they have pneumonia or not.

Key words: COVID-19, pneumonia, endothelial dysfunction, asymmetric dimethylarginine, CRP.

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Introduction

SARS-CoV-2 infection, also nominated as Coronaviral Disease 2019 (COVID-19), was characterized as a pandemic since March 2020 by World Heart Organization [1]. The number of cases and mortality has increased gravely ever since [2]. Although the majority of patients tend to be well or asymptomatic, 14% experience severe, and 5% suffer critical disease with lung involvement [3].

Interestingly several cardiovascular co-morbidities have been identified to be

associated with a higher rate of pulmonary complications.

COVID-19 infects the tissue thorough angiotensin converting enzyme 2 receptor. This receptor is abundantly expressed in endothelial cells besides lung, heart, kidney and bowel tissue [4]. The infection of endothelial lining may induce endothelial dysfunction, which may be in part responsible for multi-systemic involvement. Endothelial dysfunction can be assessed by several invasive or non-invasive methods [5]. Nitric oxide (NO), synthesized by the endothelial nitric oxide synthase, is a multifunctional molecule involved in vasodilation, and the immune system. NO has additional activity in cytokine secretion, non-specific immunity, inhibition of viral replication, and transplant rejection [6,7]. Asymmetric dimethylarginine (ADMA) is an inhibitor of endogenous nitric oxide synthase. Since serum ADMA concentration is inversely related with nitric oxide synthesis, ADMA concentration may be an indirect marker of endothelial dysfunction. Moreover, ADMA is non-invasive, cheap and a practical measure for detecting endothelial dysfunction.

As we have witnessed a global pandemic in the past months, identification co-morbidities and predictors associated with more frequent lung involvement and higher mortality is essential. We hypothesized that ADMA concentrations reflecting endothelial dysfunction might be related to lung involvement in this specific patient group. So, this study planned to investigate ADMA concentrations in patients with COVID-19 infection

Materials and methods

Study Design & Demographics

Our study, prospective, and observational cross-sectional cohort in-design, included consecutive 42 patients with COVID-19 infection and lung involvement (Group 1). Age and sex matched consecutive 42 COVID-19 patients without pneumonia acted as the control group (Group 2). Assuming an alpha of 0.05, a power of 0.80, and 30% potential change from baseline value in terms of pulmonary involvement consistent with previous reports, the estimated sample size was at least 40 patients in total.

All patients were informed about the study and their written consent was given. The research was realized in accordance with the principles set by the Declaration of Helsinki, the International Good Clinical Practice guidelines and all applicable legal requirements. The study protocol was approved with registration number of 2020/164 by Recep Tayyip Erdoğan University School of Medicine Ethics Committee.

Patients who did not have any lung pathology at onset and got lung involvement in the follow-up period were included in the first group. Diagnosis of COVID-19 was proven using polymerase chain reaction (PCR) method and/or computed tomography (CT). Medication history including previous and novel medications throughout hospitalization were recorded.

We excluded patients with immunosuppressive treatment, renal or hepatic failure, malignancy, idiopathic pulmonary fibrosis, and patients who passed away through follow-up.

Following diagnosis of COVID-19 infection by an infectious disease expert or a pulmonologist, complaints, demographic and laboratory parameters, and CT images of the patients were reviewed and recorded. Diabetes mellitus was acknowledged as the fasting blood glucose concentration > 126 mg/dL or any blood glucose measurement >200 mg/dL or use of anti-diabetic medication. Hypertension was accepted as blood pressure above 130/85

mmHg or use of anti-hypertensive drugs. Those who smoked regularly in the last 6 months were denoted as smokers.

Biochemical analysis

Blood samples were taken for ADMA analysis using antecubital veins at the first month control of recovered patients and stored in a -80^{0}C refrigerator after centrifugation. Human ADMA Elisa kit (Cloud-Clone Corp., China) was used to detect serum ADMA levels. While assay range is indicated between 12.35 ng/mL and 1000 ng/mL, the intraassay and interassay coefficient of variations (CV) were<10%, <12% respectively, according to the manufacturer. The sensitivity of the assay was stated as 4.99 ng/mL by manufacturer.

C reactive protein (CRP) levels were quantitated using immunoturbidimetric assay on Beckman Coulter AU5800 autoanalyzer (Beckman Coulter Diagnostics, USA). While the assay range for CRP was below 5 mg/L, the within run and total precision are less than 5% CV, according to the manufacturer.

Computed tomography

Thorax CT acquisitions were performed by Alexion 16 detector CT (Toshiba Medical Systems, Japan) machine in the supine position during breath-holding following deep inspiration from lung apices to umbilicus without non-ionic contrast, using the parameters of 120 kV, 125 mA, 16x1.5 mm collimation and 3 mm thickness, 512x512 matrix. A specialized thoracic radiologist assessed axial views at parenchyma window (1500 HU, -600 HU). The images were transferred to a workstation to evaluate typical or atypical COVID-19 disease signs. The patients were staged according to Radiological Society North America (RSNA) criteria using CT images of patients during hospitalization. *Typical appearance* includes highly specific imaging features, *indeterminate appearance* denotes non-specific features and *atypical appearance* implies uncommonly or not reported features of COVID-19 pneumonia. *Negative for pneumonia* shows no features of pneumonia. The group 1 patient mainly included patients with typical appearance, and some with indeterminate appearance. Control subjects were mostly negative for pneumonia with a few atypical appearances.

Statistical analysis

The Statistical Package for the Social Sciences 20.0 statistical software program (SSPS Inc, Chicago, Illinois) was used for the statistical analysis. Kolmogorov Smirnov was used to check normality of continuous variables. Continuous variables were expressed as the mean ± standard deviation. Categorical variables were presented as percentages. The normally distributed variables were compared with the Student's t-test and the variables that did not conform to a normal distribution were compared with Mann–Whitney U- test. Categorical variables were compared with chi-square or Fisher exact test. Continuous variables without normal distribution were log-transformed for univariate and multi-variate analysis. Linear and logistic regression analyses were used for the multivariate analysis of independent variables which were included if they were significantly different in the univariate analyses. All tests of significance were two-tailed. Statistical significance was defined as $p < 0.05$.

Results

We included age and gender matched 42 COVID-19 patients to each group according to pneumonic involvement on CT or not. Our study included 39 female and 45 male patients with a median age of 55 (46-69). Demographic characteristics of the study group are presented in Table 1. The patients with lung involvement

Table 1. Demographical and clinical findings of the study population.

Variable	Lung Involvement (-) (n=42)	Lung Involvement (+) (n=42)	*p*
Age (Year)	54.2±12.8	54.1±11.7	*0.965*
Gender (n/%) Female Male	 19 (45.2) 23 (54.8)	 20 (47.6) 22 (52.4)	 *0.829* *0.741*
BMI (kg/m^2)	28.7±4.02	27.6±2.9	*0.163*
SBP (mmHg)	124.6±12.5	122.1±25.9	*0.558*
DBP (mmHg)	76.4±8.9	73.3±15.1	*0.261*
Heart rate (bpm/min)	69.8±15.2	74.1±21.8	*0.292*
LVEF (%)	60.4±2.7	59.6±3.2	*0.267*
HT n%	21 (50)	22 (52.4)	*0.830*
Current smoking (n/%)	6 (14.3)	6 (14.3)	*1.000*
Fever (C)	14 (33.3)	26 (61.9)	***0.008***
Asymptomatic (n/%)	13 (31.0)	0 (0)	***<0.001***
HPL (n/%)	9 (21.4)	11826.4)	*0.614*
COPD (n/%)	1 (2.4)	1 (22.4)	*1.000*
Previous CAD (n/%)	3 (7.1)	6 (14.3)	*0.296*
B. Blocker (Adm) (n/%)	6 (14.3)	12 (28.6)	*0.113*
CCB (Adm) (n/%)	5 (11.9)	7 (16.7)	*0.539*
ACEI (Adm) (n/%)	4 (9.5)	8 (19.0)	*0.217*
ARB (Adm) (n/%)	10 (23.8)	8819)	*0.600*
Statin therapy (Adm) (n/%)	8 (19)	10(23.8)	*0.600*
Glucose (Adm) (mg/dL)	107.4±22.5	133.6±60	***0.011***
Se Cr (mg/dL)	0.86±0.33	0.81±0.18	*0.352*
CRP (mg/dL)	1.72±1.39	3.85±1.22	***<0.001***
DDimer ng/mL	380 (315-675)	585 (250-880)	*0.149*
Troponin I ng/mL	3.2 (1.7-4.8)	5.8 (3.9-8)	***0.026***
ADMA (10^3 ng/mL)	213±118	386±297	***0.003***
WBC 10^3/ µL	5.8±2.5	6.1±2.8	*0.595*
Hemoglobin (g/dl)	13.5±1.6	12.7±1.6	***0.019***
Neutrophil 10^3/ µL	3.6±1.6	3.9±1.8	*0.359*
Eosinophil ∗ 1/ µL	60 (20-187)	10 (20-70)	***0.033***
Lymphocyte 10^3/ µL	1.917±0.821	1.429±0.674	***0.005***
Monosite 10^3/ µL	0.47±0.18	0.38±0.20	*0.062*
Favipiravir (n/%)	9 (21.4)	33 (80.5)	***<0.001***
Oseltamivir (n/%)	25 (59.5)	24 (58.5)	*0.928*
Azithromycin (n/%)	24 (57.1)	27 (65.9)	*0.421*
Chloroquine (n/%)	39 (92.9)	32 (78)	*0.056*
Vitamin C (n/%)	2 (4.8)	18 (43.9)	***<0.001***
Dexamethasone (or potentially other glucocorticoids) (n/%)	1 (2.4)	7 (17.1)	***0.023***
Tocilizumab (n/%)	0 (0)	3 (7.3)	*0.076*
Convalescent plasma (n/%)	0 (0)	3 (7.3)	*0.076*
Enoxaparin (n/%)	18 (42.9)	30 (73.2)	***0.005***

Continuous variables are given as mean ± SD. BMI: body mass index. HT: Hypertension, HPL: Hyperlipidemia, DM: Diabetes Mellitus, BMI: Body Mass Index; SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, LVEF: Left Ventricular Ejection Fraction; CCB: Calcium Channel Blocker. ACEI: Angiotensin-converting enzyme inhibitors. ARB: Angiotensin II receptor blockers. ADMA: Asymmetric Dimethylarginine, WBC: White blood cell; CRP: C Reactive Protein.

Table 2. Logistic regression analyze of ADMA with lung involvement and CRP.

Parameters	Univariate			Multivariate		
Variables	OR	95%CI	*p*	OR	95%CI	*p*
Lung Inv. (+)	3.333	1.284-8.653	*0.013*	2.070	0.571-7.502	*0.268*
CRP (Log)	1.633	1.182-2.255	*0.003*	1.633	1.182-2.255	*0.003*

ADMA: Asymmetric Dimethylarginine, CRP: C Reactive Protein.

had fever more frequently *(61.9% vs. 33.3, p=0.008)* compared to patients without lung involvement. The remaining characteristics including previous drugs and the presence of hypertension and coronary artery disease were not different between groups.

Patients with lung involvement had higher admission glucose *(133.6±60 vs. 107.4±22.5 mg/dl, p=0.011)*, C-Reactive Protein (CRP) *(3.85±1.22 vs. 1.72±1.39, p<0.001)*, and ADMA concentrations *(386±297 vs 213±118, p=0.003)*, and had lower hemoglobin concentration *(12.7±1.6 g/dl vs. 13.5±1.6, p=0.019)* and lymphocyte count *(1.429±0.674 vs. 1.917±0.821 10^3/µl, p=0.005)* compared to patients without lung involvement. Moreover, we observed a trend for decreased monocyte count in group 1, which did not reach statistical significance *(p=0.062)*. Interestingly, only 11 (13%) patients had normal ADMA values (12.35 ng/mL and 1000 ng/mL), 90.5% of group 1, and 83.3% of group subjects had very high ADMA concentrations. We observed a good correlation between ADMA and CRP (Figure-1). As expected, patients in group 1 received favipiravir, vitamin C, intravenous steroids, and anti-coagulants more frequently than the control subjects. Multivariate analysis including presence of fever, hemoglobin value, glucose concentration, lymphocyte count, and log ADMA identified log CRP concentration *(OR= 3.047, 95% CI=1.881-5.023, p<0.001)* as the single independent predictor for lung involvement (Table 2).

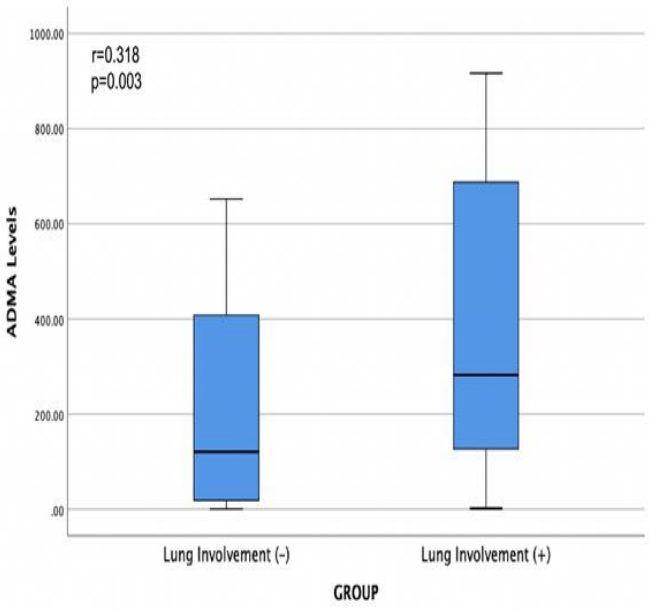

Figure 1. Relation of ADMA with lung involvement.

Discussion

The principal finding of this study is that the patients with lung involvement had higher ADMA concentrations with respect to those without lung involvement, plasma ADMA levels were also considerably higher than normal values in control group.

Vascular endothelium is an active paracrine, endocrine and autocrine organ invariably involved in regulation of vascular tonus and vascular homeostasis [5]. Autopsy series of deceased COVID-19 patients reported direct viral invasion of endothelial cells and widespread endothelial inflammation [8]. In addition to endothelial dysfunction, an imbalance towards vasoconstriction and prominent microvascular pro-coagulant activity is frequently encountered [9]. Correspondingly,

Micro CLOTS is defined as a complication of severe COVID-19 infection with obstructive thrombo-inflammatory involvement of lung capillaries, which possibly reflects an atypical form of acute respiratory distress syndrome (ARDS) [10].

NO is a critical molecule that has a role in maintenance of organ perfusion and vascular balance [11,12]. ADMA is a protein breakdown product that inhibits endogenous NOS enzyme and thus lowers NO activity. A study involving critically ill patients in intensive care units revealed ADMA as the strongest independent predictor of mortality. Moreover, patients at the highest quartile had 17-fold increased mortality compared with the lowest tertile [13]. Therefore, ADMA accumulation has been proposed to be an etiologic factor that is involved in multiorgan failure pathogenesis due to decreased NO production [14].

In this study, the patients with lung involvement had higher ADMA concentrations, majority of the study patients had considerably higher plasma ADMA levels than normal values. We think that endothelial dysfunction is invariably present in COVID-19 disease whether there is an accompanying pneumonia or not. Interestingly, in the Cardiovascular Risk in Young Finns Study, investigators followed 1043 infection-related hospitalization in early childhood and documented adverse adulthood atherosclerotic changes 33 years later. Childhood infection-related hospitalization was closely associated with increased ADMA concentrations in the adulthood [15]. Although we don't have a long-term follow-up data regarding our patient population, we may hypothesize that elevated ADMA levels reflect inflammatory response severity in our study at the 1st month of follow-up.

Inflammatory response has a central role with increasing severity of COVID-19 disease, especially when cytokine storm is present. CRP is a non-specific acute phase reactant synthesized by the liver, and a biological marker of inflammation, infection and tissue damage [15]. Computed tomography is the main imaging method to determine the severity of COVID-19 pneumonia. A recent study revealed increased CRP concentrations in the early stage of COVID-19 disease and CRP was an independent predictor of severe lung lesion [16]. Similarly, CRP in addition to interleukin 6, was highly predictive of mechanical ventilation requirement [17]. We also identified CRP as the sole independent predictor of lung involvement similar to these studies.

Increased CRP activity may cause endothelial dysfunction due to inhibition of NO synthesis and bioactivity [18]. High ADMA levels were closely associated with CRP, cardiovascular risk factors, and cardiovascular death in patients with newly diagnosed diabetes mellitus [19]. Data suggests that ADMA and CRP are involved in endothelial dysfunction [20,21]. We identified CRP as the sole determinant of ADMA concentration and revealed a correlation between these parameters in our study, which are in accordance with the aforementioned studies.

Our study has several limitations that must be considered when interpreting these results, most importantly the small population size. We only have ADMA concentrations at the 1st month of follow-up after discharge. We lack serial ADMA measurement and a healthy control group. So, there is need for larger and multi-center studies which powered for improvement in clinical outcome (particularly thrombogenic events). Beyond these limitations, this is a pioneering study for further research that will focus on modalities to detect patients with pulmonary involvement at an early stage.

Conclusion

The findings of this study emphasize that elevated ADMA concentrations as the surrogate of endothelial dysfunction in COVID-19 patients whether they have pneumonia or not. And, It deserves consideration as a laboratory parameter for our better understanding of COVID-19 physiopathology.

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Ethical statement:

The study was approved by Local Clinical Research Ethics Committee (Date and Decision Number: 2020/164*), and written informed consent was obtained from each subject.*

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Experimental Biomedical Research **Original article**

The effect of intestinal ischemia on plasma thiol/disulphide homeostasis in an experimental study

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ABSTRACT

Aim: To investigate the effects of acute intestinal ischemia on plasma thiol/disulphide homeostasis (TDSH), which has been investigated in a limited number of studies in the related literature.

Methods: Twenty-four rats were randomized into control (operation without ischemia, GIS), and ischemia groups (GII-60, GIII-180). For ischemia, the superior mesenteric artery was sutured and the rats were exposed to 60 and 180 minutes of intestinal ischemia, respectively. Plasma TDSH was measured in blood samples collected at the end of the ischemia, and the pathology of ileum segments resected was evaluated.

Results: The experimental ischemic conditions provided were confirmed by the total histopathological scoring system statistically. The levels of serum human albumin and ischemia modified albumin (IMA) in groups were detected in quite a close range of each other. There was no found a statistically significant difference for IMA between groups *(p>0.05)*. The alternations on the levels of plasma TDSH parameters were observed in the study. According to ischemic conditions, the thiol/disulfide ratio fluctuations were detected in the plasma TDSH. The native thiol and total thiol levels seem to have decreased according to ischemia; no statistical difference was detected. In addition, the disulfide levels increasing according to ischemia either was not found significant statistically *(p>0.05)*.

Conclusion: Although this study showed the oxidative balance in intestinal ischemia had affected plasma TDSH, also it revealed that intestinal ischemia didn't create a statistically significant difference between plasma TDSH components.

Key words: Intestinal ischemia, oxidative stress, injury, thiol/disulphide homeostasis, rat.

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Introduction

Intestinal ischemia occurs by reduced intestinal blood flow due to several clinical situations, such as strangulated hernia, intestinal volvulus, necrotizing enterocolitis, small bowel and multi-visceral transplantation, hemorrhagic shock, sepsis, severe burns, trauma, and mesenteric thrombosis/embolism. It results in dysfunction of the gut barrier, which leads to an increase in permeability of the epithelium and infiltration of the intestinal wall by inflammatory cells. These cells cause a systemic inflammatory response by releasing pro-inflammatory cytokines, and finally the delay in diagnosis and treatment may be fatal for the patient is associated with high mortality

and morbidity [1]. The role of oxidative stress (OS) and reactive oxygen species (ROS) have been shown to contribute to the pathogenesis of intestinal ischemic diseases [2]. The main mechanism causing OS is considered to be lipid peroxidation of mitochondria and cell membranes in ischemic tissue [2,3].

Thiols in the organisms are components that involve the sulfur group and are the essential antioxidant buffers that interact with almost all physiologic oxidants. The majority of the plasma thiol pool consists mainly of human serum albumin (HSA), the other proteins, and the small part consists of thiols with low-molecular-weight such as cysteine, cysteinyl glycine, glutathione, homocysteine, and c-glutamylcysteine [4]. The thiol groups of proteins are transferred to reversible disulphide bond structures by the oxidization of oxidant molecules in the environment. The disulphide bond structures may reduce back to thiol groups, and thus, the thiol-disulphide balance is maintained [4]. In the dynamic plasma thiol/disulphide homeostasis (TDSH) that consists of the native thiol, total thiol, and disulphides, the shift from balance towards disulphides has been shown in various diseases previously [5-7].

The plasma compartment is characterized by having relatively low concentrations of thiols and by the presence of serum albumin as the most abundant one [8]. Ischemia, hypoxia, increased free radicals, and acidosis leads to changes in the molecular structure of serum albumin by decreasing metal binding capacity at N- terminal. This new isoform of albumin is called ischemic modified albumin (IMA), and its serum levels can be measured [9]. Although studies have recognized IMA as a marker of cardiac ischemia, several investigations of IMA have also shown increased levels in ischemic diseases [10].

In line with the aforementioned theories and literature, the plasma dynamic TDSH of the organism is expected to be affected by intestinal ischemia. However, the studies investigating the effects of intestinal ischemia on plasma TDSH is not found in the related literature. In the present study, we aimed to investigate the effect of intestinal ischemia on the plasma TDSH, and IMA levels, biochemically and histopathologically, and whether it can be used as a predictor of intestinal ischemia.

Materials and methods

Each experiment was performed in accordance with the National Guidelines for The Use and Care of Laboratory Animals after obtaining the approval of the Animal Experiments Ethics Committee of Uludag University (number: 2017/13/04). Twenty-four Wistar albino rats weighing 300-400 g were included in the study. All animals were kept on a 12 h light: 12 h darkness cycle at 22–24 °C with free access to food and fasted for 24 h before the operation, but were allowed free access to water. Rats were divided randomly into three groups, 8 in each: in Group sham (GIS), rat intestines were eviscerated by laparotomy and then put back; the rats in Group II-60 and Group III-180 were exposed to intestinal ischemia for 60 and 180 minutes, respectively. The applied intestinal ischemia model in the current study was described by Megison in 1990 [11]. Briefly, the rats were anesthetized by using 3% isoflurane; laparotomy was performed by median incision, and the intestines were eviscerated. In ischemic groups, the base of the superior mesenteric artery was identified and ligated by 3/0 silk suture, and intestines were placed back into the abdominal cavity. After 60 minutes of ischemic period for both GIS and GII-60, and 180 minutes for GIII-180, relaparotomy was performed for gathering blood and intestine

tissue samples without reperfusion. The animals were sacrificed after operation using an intracardiac injection of pentobarbital (45 mg/kg) and open pneumothorax.

Biochemical analysis

Blood samples were centrifuged to separate the plasma and serum. The serum samples were stored at -80°C until the time of evaluation. Serum TDSH that consists of the native thiol, total thiol, and disulphide was investigated by the novel and automatic spectrophotometric measurement method developed by Erel and Neselioglu; and the disulphide/native thiol ratio, the disulphide/total thiol ratio, and the native thiol/total thiol ratio were calculated [4]. Also, serum IMA levels were measured using the method described by Bar-Or et al. [9]. Serum albumin levels were analyzed using a chemical auto analyzer (Beckman Coulter Chemistry Analyzer AU480, Brea, CA, USA).

Histopathologic examination

The resected intestinal segments were fixed with formaldehyde (10%) solution. All samples were embedded in paraffin. Tissues were sectioned in 4-5 μm pieces and stained with hematoxylin-eosin (HE), then analyzed under a light microscope by the same pathologist who was blind to the study. Histopathological findings were graded from 0 to 5 in accordance with the total histopathologic score (THS) defined by Chiu et al. [12].

The grades of the THS are as follows: Grade 0: Normal mucosa, Grade 1: Villous subepithelial detachment formation accompanied by capillary congestion, Grade 2: Subepithelial detachments exerted and a moderate amount of upward push on the mucosa epithelium, Grade 3: Large subepithelial detachments exerted a massive amount of upward push on the mucosa epithelium along the villi, and few denuded villus tips were observed, Grade 4: The villi were denuded to the level of lamina propria and dilated capillaries, Grade 5: Presence of ulceration, the disintegration of lamina propria, and hemorrhage.

Statistical analysis

Statistical analyses were performed with IBM SPSS ver.23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). The data were examined by the Shapiro Wilk test whether or not it presents normal distribution. The results were presented as mean ± standard deviation or median (minimum-maximum) for continuous variables including plasma TDSH (native thiol, total thiol, disulphide, disulphide /native thiol ratio, disulphide /total thiol ratio, and native thiol /total thiol ratio) and IMA levels. As the variables were distributed normally, one-way ANOVA was employed to analyze the data. The THS of groups were analyzed as quantitative levels by the Kruskal-Wallis test. The p-value <0.05 was considered as statistically significant.

Results

Histological examination revealed normal small intestines in GIS, while ischemic changes were confirmed in the other experimental groups (Figure 1). The histopathological results are displayed as a boxplot graphic in Figure 1. The THS of ischemia were found significantly higher in the GII-60 and G III-180 groups when compared with GIS *($p<0.05$).*

The results of total plasma TDSH (native thiol, total thiol, disulphide, disulphide/ total thiol ratio, and native thiol /total thiol ratio), HSA, and IMA levels were summarized in Table 1.

The mean HSA and IMA levels in the groups were detected quite close to each other. The mean levels of IMA were displayed in Figure 2A. Although the highest value of IMA within groups was expected in GIII-180, they were observed to course in close range of each other.

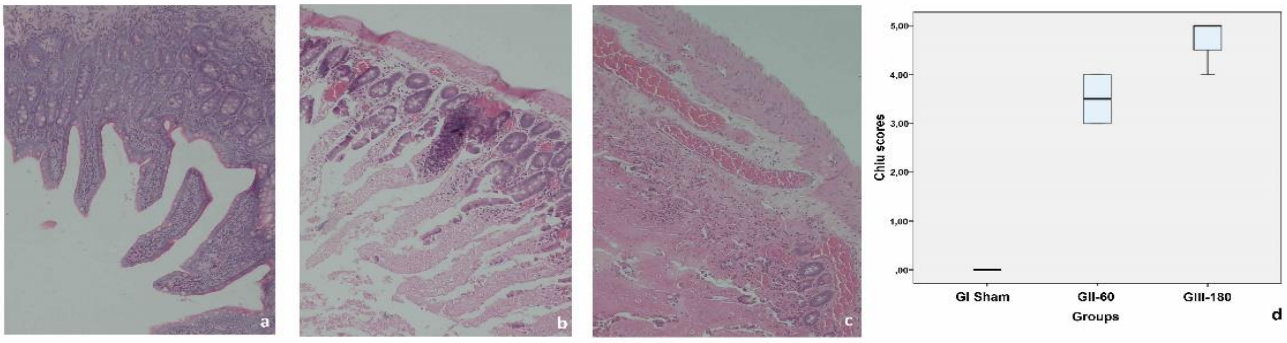

Figure 1. Representative light microscopy (Hematoxylin & Eosin staining) images of the intestinal epithelium and histopathological scoring. **A:** Non-ischemic group (GIS) with normal mucosa (Grade 0). **B:** Ischemia for 60 minutes (GI-60). The denied villi with lamina propria by dilated capillaries (Grade 4). **C:** Ischemia for 180 minutes (GII-180). The intestinal wall damage with ulceration, the disintegration of lamina propria, and hemorrhage (Grade 5). **D:** Histopathologic intestinal damage induced by intestinal ischemia was studied using the Chiu score. Each point depicts a sample evaluation (n=8 per group).

Table 1. The comparison analysis of plasma thiol pool parameters (ischemia-modified albumin, serum albümin, native thiol, total thiol, disulphide, and their ratios).

Thiol pool parameters	**GIS**	**GII-60**	**GIII-180**	***P value***
HSA (g/dl)	3,49±0,57	3,298±0,38	3,423±0,323	*0,684*
IMA (U/ml)	1,289±0,08	1,300±0,09	1,209±0,085	*0,097*
Native thiol (µmol/l)	83,00±49,67	82,60±34,10	62,91±21,23	*0,468*
Total thiol (µmol/l)	114,91±52,45	112,91±34,60	97,76±20,69	*0,620*
Native thiol / total thiol (%)	68,19±10,92	71,50±12,06	63,38±11,83	*0,392*
Disulphide (µmol/l)	15,957±2,74	15,156±5,024	17,425±4,877	*0,589*
Disulphide / native thiol (%)	24,955±11,953	21,811±12,882	31,825±18,097	*0,396*
Disulphide / total thiol (%)	15,902±5,463	14,247±6,031	18,308±5,916	*0,392*

Values: Mean±SD. HSA: Human serum albumin; IMA: Ischemia-modified albumin; SD: Standart deviation

There was no significant statistical difference between experimental groups for IMA levels (Table 1) *(p>0.05).*

The native thiol (62.91 µmol/l) and native/total thiol ratio (63.38 %) were significantly lower in GIII-180 and GII-60 than in GIS (Figure 2B). However, these values were not statistically significantly different between groups *(p=0,468, p=0,392)* (Table 1). As seen in Figure 2C, the disulphide levels were different among the three groups. Both GIS and GII-60 groups had lower disulphide values compared with the GIII-180 *(p=15,957 and p=15,156, respectively)* (Table 1). Nevertheless, no statistically significant difference in disulphide levels was found between groups. The disulfide/native thiol ratio was significantly higher in the GIII-180 group than in others (31.82%). Although the increasing ratio was seen in Figure 2D, there was no statistically

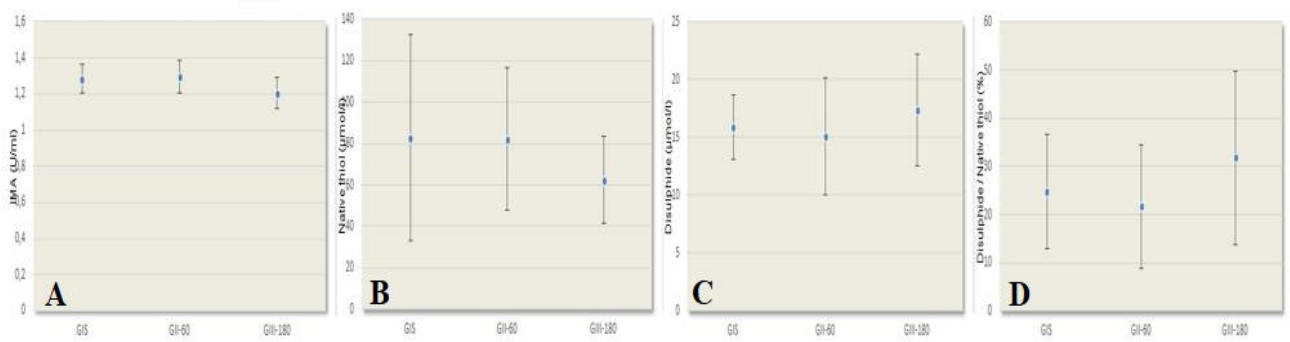

Figure 2. A: The course of serum IMA levels in groups. **B:** Native thiol levels by groups. GIS had higher native thiol values than both GII-60 and GIII-180. **C:** Disulphide levels by groups. GIII had higher serum disulphide levels than both groups. **D:** Disulphide/native thiol ratios by groups. The disulphide/native thiol ratio was higher in the GIII-180 than in others.

significant difference between groups *(p=0,396)* (Table 1).

Discussion

Intestinal ischemia progresses rapidly and leads to irreversible damage leading to high morbidity and mortality [13]. This severe condition is associated with a progressive inflammatory response causing OS, cellular dysfunction, hemorrhage, and even necrosis [14]. Various biomarkers have recently been reported as a facilitative factor in the process of diagnosing intestinal ischemia, such as acidosis, elevated amylase, D-dimer, intestinal fatty acid-binding protein (I-FABP), serum alpha-glutathione, S-Transferase (alpha-GST), cobalt-albumin binding assay (CABA), procalcitonin, etc. [15]. However, there are no specific laboratory biomarkers in a blood sample for diagnosing intestinal ischemia in daily practice.

IMA is an altered HSA that forms under the conditions of OS and is considered a predictor [9,14,15]. In acute ischemic situations, the metal binding capacity of albumin for transition metals such as copper, nickel, and cobalt decreases, resulting in a metabolic variant of the protein commonly known as IMA [9,10]. In almost all ischemic or hypoxic clinical studies, IMA and serum albumin levels were found to be high [10,16,17]. Gunduz et al. found that preoperative IMA levels were significantly higher in patients with intestinal ischemia [10]. In the study of Acar et al. they found that IMA levels were high in rats that underwent renal ischemia and reperfusion, although it was not statistically significant [16]. In our study, the IMA levels were not statistically significantly different between ischemic groups. It was considered that the changes of the ischemic tissues may not affect systemic circulation yet, and hence the levels of IMA in ischemic groups are not increased. We also think that the binding affinity of albumin to other metals was not changed since our model produces complete ischemia, not reperfusion.

In 2014, Dr. Erel and Neselioglu developed a novel and automated assay for the organism's antioxidant - oxidant balance, which determined plasma TDSH [4]. Thiols, as a major antioxidant, play an important role in the eradication of ROS via non-enzymatic pathways. The thiol groups of proteins are transferred to reversible disulphide bond structures by the oxidization of oxidant molecules in the environment. In comparison with other parameters, the authors provide an easy, relatively cost-efficient, practical, fully

automated spectrophotometric assay for the determination of plasma dynamic TDSH. The role of plasma TDSH in various clinical and experimental ischemic studies such as testicular and ovarian torsions, cardiac and renal ischemia, and pulmonary embolism, although most studies are conditions that cause ischemia-reperfusion or chronic OS and its importance has been investigated [5,16,18-20]. In the present study, the plasma TDSH was not investigated for ischemia-reperfusion injury. The main purpose was to evaluate whether intestinal ischemia affected plasma levels of thiol and disulphide or not.

The dynamic TDSH has been studied in many different clinical situations and experimental organ ischemia models, and results are inconsistent with each other. Kundi et al. in a study conducted in patients with acute myocardial infarction with chest pain lasting at least 20 minutes, thiol and disulphide levels were found to be significantly lower, but disulphide/thiol levels were found to be higher compared to controls [5]. Similarly to our study, Topuz et al. also found that thiol levels were lower, disulphide levels high, and disulphide/thiol ratios significantly higher in patients who were diagnosed with pulmonary embolism and had high pulmonary embolism severity index compared to controls [18]. Urkmez et al. found that natural, thiol, total thiol and disulphide levels were significantly lower in detorsion after 4 and 8 hours of torsion in the testicular torsion model [19]. They reported that prolongation of ischemia leads to a greater decrease in thiol/ disulphide levels and testicular damage may indicate the prognosis. Avci et al. investigated thiol/disulphide levels in experimental ovarian torsion and found that thiol and disulphide levels increased in the 3-hour torsion group, decreased in the detorsion group compared to the sham group, and the ratio of disulphide and thiol groups increased to the thiol groups [20]. Finally, Acar et al. found that the thiol group increased significantly in the reperfusion group in their study in which they performed renal ischemia reperfusion [16]. Considering all previous studies, it is seen that different organs and experimental models have different homeostasis results in ischemic conditions. In the current study, by the literature, as the intestinal ischemia increased; the native thiol and total thiol values decreased and the disulphide and disulphide /native thiol values increased. Although it was shown that while thiol levels were decreased the disulfide values were increased (Figure 3,4,5), no statistically significant difference was found (Table 1).

In addition, we think that the proved intestinal ischemia by the high consistent THS in our study does not reveal a statistically significant difference between TDSH components in plasma. Similarly, Dumlu et al. also conducted a study with hypothesizes that both OS and lipid peroxidation contribute to the pathophysiology of intestinal ischemia [21]. They observed that OS and lipid peroxidation play no significant role in intestinal ischemia and did not provide the expected effects on plasma oxidant and antioxidant capacity accompanied by histopathologic signs of ischemia.

It's well known that ROS, an unstable and cytotoxic reduction product of molecular oxygen, is responsible for producing the increased capillary permeability associated with intestinal ischemia and an increase of OS parameters is expected [22]. In fact, in our study, the thiol /disulphide balance was expected to shift towards disulphides as well, which could be considered as an indicator of OS due to an inflammatory process. Although the shift was observed in the values of the TDSH components in groups per the degree of

intestinal ischemia, it was anticipated that more considerable differences. Thus, the findings of the current study did not completely result in parallel outcomes with the literature statistically.

We think that these results could have been affected by steady-state concentration due to the plasma kinetic barrier. Because of the plasma kinetic barrier, thiol and disulphides do not stay at equilibrium in plasma. Turell et al. have proposed that the steady-state concentrations are the result of several concurrent actions that are affecting not only total concentrations but also oxidized ratios against reduced thiols [8].

Furthermore, a detailed examination of plasma TDSH by several studies pointed out that plasma concentrations of thiols and oxidized derivatives are affected in such conditions, including the ratios of thiol-disulfide exchanger actions, the rates of thiol oxidation with ROS, and the breakthrough rates of thiol-containing molecules from liver or kidney [23-25]. Hereby, detected thiol and disulphide concentrations in our study are not fully consistent with the expected spectrophotometric measurement for thiol oxidation to disulphide. Within the scope of the aforementioned papers, it is possible that our results might be related to the dynamic process of the plasma thiol pool.

In plasma, total thiols are at a lower concentration than in cells, and the predominant thiol is serum albumin. The serum albumin thiol reacts with a wide variety of oxidant species. These reactions are evidenced by the detection of the thiol oxidized forms of albumin in the circulation that increase in the circumstances related to OS [8,24,25]. As it is emphasized by Turell, the nature of oxidant species cannot be elucidated exactly because of the kinetic factors depending on the overlapping of the final products, the concentration of both serum albumin, and potentially competing for targets together with diffusion and compartmentalization aspects [8]. According to these pieces of information, it is considered that the HSA levels of our study groups might have been affected by the mentioned overlapping factor.

As for the limitations of the study, the plasma TDSH is an unstable configuration, and its effects and states in metabolic reactions are unpredictable. Despite the experimentally performed intestinal ischemia in groups, whether or not the results of the parameters of plasma TDSH are real affected indicators or random states may never be known because of the limited number of samples. In addition, it might be stated that as a result of being a relatively novel assay procedure, susceptible to reversible and irreversible modifications. It was not possible to control all of the variables that might potentially affect plasma TDSH. Finally, we were unable to compare the basal plasma thiol/disulphide parameters into our study data because these were not investigated pre-intervention.

Conclusion

As a consequence, the mesenteric occlusion caused histopathological and thiol- disulphide balance changes in the current intestinal ischemia model. The parameters of plasma TDSH may have affected by cellular redox signalling in tissue destruction but is insufficient to demonstrate the reflection of acute intestinal ischemia on plasma.

Nevertheless, this is the first study to evaluate the effect of intestinal ischemia on plasma TDSH in a small intestine ischemia model. Therefore, further studies are needed to clarify the interaction between intestinal ischemia and plasma thiol/disulfide pool.

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Experimental Biomedical Research **Original article**

The effect of early tranexamic acid administration on hemoglobin levels after unstable pelvic fracture: An experimental study in rats

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ABSTRACT

Aim: To investigate the effect of early systemic tranexamic acid (TRA) administration on hemoglobin (Hb) levels in rats with pelvic fracture.

Method: In our study, 30 Wistar Albino rats were randomly divided into 3 groups in equal numbers and their hemoglobin levels were measured by taking blood samples from each. No trauma was applied to the first group and it was taken as the main control group of the study. Closed bilateral unstable pelvic fractures were created in all rats in groups 2 and 3. Fracture creation time is considered as minute 0. 10 minutes after the fracture was formed, TRA was given to the 1st group, TRA to the 2nd group and saline solution to the 3rd group systemically. Hemoglobin levels were measured by taking blood samples from all rats at 30th minute and 24th hour. The initial Hb values obtained were normalized to 100 and the percentages of 30th minute and 24th hour values were calculated. The initial, 30th minute and 24th hour values of all groups were compared statistically with each other. The 30th minute and 24th hour values were compared statistically between the groups.

Results: No death was observed within 24 hours in all three groups. When the first Hb values of each group are normalized to 100, the mean Hb percentages were calculated in the first group as 99.54 and 99.84 at 30 minutes and 24 hour, respectively; 92.95 and 87.73 in the second group; and 87.95 and 73.16 in the third group. When these values obtained were compared statistically within the groups (initial, 30th minute, 24th hour Hb percentages), there was no significant difference between the initial, 30th minute and 24th hour values in group 1. However, a statistically significant difference was found between the initial, 30th minute and 24th hour values in group 2 and 3 *($p<0.01$)*. In the comparison between the groups, a statistically significant difference was found between group 1-2, group 1-3 and group 2-3 between both 30th minute and 24th hour values *($p<0.01$)*.

Conclusion: In rats with bilateral unstable pelvic fractures due to blunt pelvis trauma, early administration of TRA after trauma significantly reduced the first 24-hour decrease in Hb value. Our study supports the early and prehospital use of TRA in traumas that are predicted to progress with acute bleeding, such as unstable pelvic fractures.

Key words: Blunt pelvic fracture, hemorrhage, hemoglobin level, tranexamic acid, rat.

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Introduction

One in ten deaths worldwide are caused by traumatic injuries, and uncontrolled bleeding is responsible for almost half of these traumatic deaths [1]. Pelvic ring fractures occur as a result of high-energy trauma and constitute 1.5 to 3 percent of all fractures [2]. As is known, pelvic ring fractures are fractures with high mortality and morbidity. Although accompanying additional injuries such as head trauma, chest trauma, and abdominal injuries are among the main factors that highlight mortality in patients with pelvic fractures, it has been stated that the main reason that increases mortality in isolated pelvic fractures is massive hemorrhage [3-7]. Considering all types of pelvic fractures, mortality is seen at a rate of 5-10%. It has been reported that this rate reaches up to 60% when it is accompanied by hemodynamic instability [8,9]. Despite the advancement and development of innovative multidisciplinary approaches in trauma management, posttraumatic bleeding emerges as the main cause of death in the first 24 hours after trauma [10].

Tranexamic acid (TRA) is the synthetic analogue of lysine amino acid. It shows antifibrinolytic effect by binding to plasminogen or plasmin through lysine receptors. With this effect, it prevents plasmin from binding to fibrin and preserves the matrix structure of fibrin [11]. The article on the first clinical use of TRA was published in 1968 regarding its use in the treatment of severe menstrual bleeding [12]. In the course of time, its use in dental, urological, cardiac and transplant surgeries has become widespread, and its use in arthroplasty and spinal surgeries in the orthopedic field has been accepted. Its use in patients with polytrauma and uncontrolled bleeding has been investigated, and studies on its use to reduce the amount of bleeding in patients with polytrauma in both prehospital and emergency services gained popularity. In this animal experiment we conducted, we aimed to shed light on the use of TRA in the early post-traumatic period in patients with life-threatening pelvic fractures due to severe bleeding by observing the effect of early TRA administration on hemoglobin levels in rats with pelvic fracture created by blunt pelvis trauma.

Materials and methods

This study was conducted with the ethical approval given by Animal Experiments Local Ethics Committee at Bolu Abant Izzet Baysal University (approval number 2018/19). Thirty female Wistar Albino rats, 2-4 months old and weighing 200-250 g, were used in our study. Rats were randomly divided into 3 groups with 10 rats in each group and the rats were numbered from 1 to 30. Blood samples were taken from all rats under general anesthesia to measure the hemoglobin levels of the rats. For general anesthesia, ketamine/xylazine was administered to each rat at 90/10 mg/kg intramuscularly (im). 0.25-0.5 ml blood sample was taken from the right periorbital venous bed of anesthetized rats with the help of a glass capillary tube into blood collection tubes containing ethylenediaminetetraacetic acid (Mini Collect Complete K2EDTA Greiner Bio-one) and sent to the biochemistry laboratory for complete blood count. After the first blood was taken, no pelvis trauma was applied to the rats in group 1 (n=10) which were designated as the control group. In group 2 (n=10) and group 3 (n=10), a closed bilateral unstable pelvic fracture was created under general anesthesia (ketamine/xylazine, 90/10 mg/kg im). In order to create a pelvic fracture and to expose the rats to a standard trauma as possible, a *weight dropping device* was used, which allows the

Figure 1. **A)** Weight dropping device that allows the metal cylindrical block weighing 100 g to fall upon the desired point from a height of 1 meter. The distance between the lower and upper table is 1 meter. The diameters of the upper and lower round trays are equal to each other. **B)** Image of the anesthetized rat was placed on the lower round metal plate in the left lateral decubitus position and the pelvis in the middle of the lower round table. **C)** The image of the 2 cm diameter cylindrical channel in the center of the upper plate through which the cylindrical metal block with a diameter of 2 cm can pass. **D)** Demonstrative view of the target. We took care to match the falling cylinder base between the most proximal point of the iliac wing and the trochanteric protrusion (red target symbol) which can be felt by palpation.

metal cylindrical block weighing 100 g to fall upon the desired point on rat from a height of 1 meter (Figure 1A).

The anesthetized rat was placed on the lower round metal plate in the left lateral decubitus position and the pelvis in the middle of the lower round table, as seen in Figure 1B. A metal cylinder weighing 100 g was placed in the channel in the middle of the upper table and the metal cylinder was released from a distance of 1 meter and dropped onto the pelvis of the rat placed in the lateral decubitus position on the lower plate (Figure 1C). It was taken care to match the falling cylinder base between the most proximal point of the iliac wing and the trochanteric protrusion which can be felt by palpation (Figure 1D).

The presence of instability in both hemipelvis was confirmed by the movement of the hemipelvis in the axial, coronal and sagittal planes with pelvic examination. In 4 rats (2 in group 2 and 2 in group 3) in which complete instability could not be felt in the pelvic examination after weight release from height method, the fracture was displaced and instability was achieved by the same researcher with slight manipulation to the hemipelvis, where instability could not be felt completely, and pathological movement and crepitation were made noticeable by physical examination. Pelvic fractures were demonstrated by anteroposterior (AP) pelvis radiographs (Figure 2A). In cases where the fracture could not be seen with AP pelvis radiographs, the iliac oblique pelvis radiographs were taken to reveal the fractures (Figure 2B, C).

This sequence of methods used to create fractures was repeated in the same way in all rats in groups 2 and 3. In the rats in groups 2 and 3, the fracture moment was accepted as 0 minutes. At the 10th minute, rats in groups 1 and 2 were administered intraperitoneally by diluting 30 mg/kg TRA in 1 cc of saline solution. Rats in group 3 were given only 1 cc

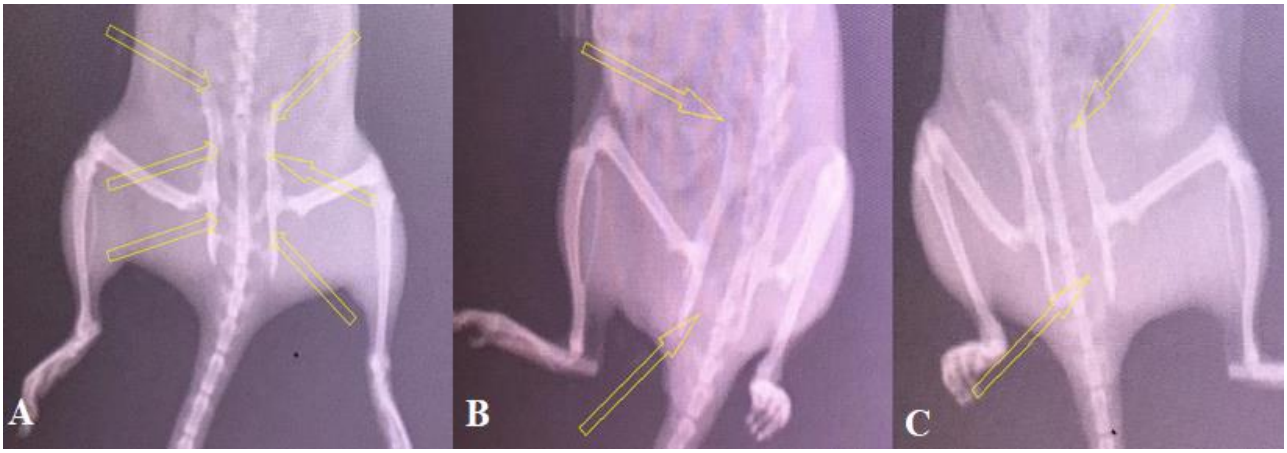

Figure 2. A) AP pelvis radiograph of a rat after trauma. **B, C)** Iliac oblique radiographies of a rat after trauma.

of saline solution intraperitoneally. All rats in all three groups were administered metamizole sodium 50 mg/kg intramuscularly as a pain reliever.

At the 30th minute, blood samples were collected from all rats under general anesthesia (ketamine/xylazine, 90/10 mg/kg i.m.), this time from the left periorbital venous beds with the same method for complete blood count. The survival of all rats was monitored until the 24th hour. All rats were fed ad libitum for 24 hours. At the 24th hour, under general anesthesia (ketamine/xylazine, 90/10 mg/kg i.m.), thoracotomy was made and ventricular blood discharge was performed on all rats and the rats were sacrificed. With this method, the last blood samples were taken for complete blood count. All of the blood samples taken were studied on the Cell Dyn 3700 (Abbott, IL, USA) device on the same day. The hemoglobin values obtained were recorded.

The first hemoglobin value of each rat was normalized to 100 and the percentages of 30th minute and 24th hour values were calculated. With the one-way Anova and Tukey HSD tests, the initial, 30th minute and 24th hour values of each group were statistically compared with each other within the group itself. The 30th minute and 24th hour values were compared between the groups using the same statistical methods.

Statistical analyses

Analysis of all data groups are performed in a sequence as one way analysis of variance ANOVA and Tukey multiple comparisons to test if any significant differences between the observed group of samples in 0 min, 30 min and 24 hours which is A, B and C. The p-value corresponding to the F-statistic of one-way ANOVA is expected to be <0.05 (5%) for indication significant difference of means, and Q-statistic - value for Tukey test is expected to be <0.01 (1%) for indication of significant difference between the par of groups (A vs B, A vs C, B vs C). All analysis are performed using JASP *(JASP Team (2020). JASP (Version 0.14.1) Computer software).*

Results

No death was observed within 24 hours in all three groups. When the initial hemoglobin values of each group are normalized to 100 (initial value), the average hemoglobin percentages in the first group at 30th minute and 24th hour were respectively 99.54 and 99.84; 92.95 and 87.73 in the second group; in the 3rd group, it was calculated as 87.95 and 73.16

Table 1. Mean values of Hb levels (initial, 30th minute and 24th hour) and ratio of 30th minute and 24th hour values to initial values.

Groups	Initial Hb (g/dl)	Normalized value (to 100)	30th minute Hb (g/dl)	30th minute % ratio	24th hour Hb (g/dl)	24th hour % ratio	*P*
Group 1	15.20	100	15.13	99.54	15.17	99.84	***<0.01*****
Group 2	14.79	100	13.75	92.95	12.98	87.73	***<0.01*****
Group 3	15.28	100	13.44	87.95	11.19	73.16	***<0.01*****
p				***<0.01****		***<0.01****	

**shows the difference between group 1, 2 and 3 at 30th minute and 24th hour*

***shows the difference between initial, 30th minute and 24th hour values in group 1, 2 and 3.*

(Table 1). When these values were compared statistically with the one-way Anova test and Tukey HSD test within the groups (initial, 30th minute and 24th hour hemoglobin percentages), no significant difference was found between the initial, 30th minute and 24th hour values in group 1 *(p> 0.01)*. A statistically significant difference was found between the initial, 30th minute and 24th hour values in group 2 (initial> 30th minute> 24th hour values, *p <0.01)*.

A statistically significant difference was found between the initial, 30th minute and 24th hour values in group 3 (initial> 30th minute> 24th hour values, *p <0.01*). When 30th minute values were compared between the groups, a statistically significant difference was found between group 1, group 2 and group 3 (group 1> group 2> group 3, *p <0.01)*.

When the 24th hour values were compared between the groups, a statistically significant difference was found between group 1, group 2 and group 3 (group 1> group 2> group 3, *p <0.01)*. When the numerical similarity between the 24th hour values of the 2nd group and the 30th minute values of the 3rd group attracted attention, these values were compared statistically with the one-way ANOVA method and it was concluded that there was no statistically significant difference between them *(p> 0.05)*.

Discussion

In pelvic fractures, 85% of bleeding is caused by disruption of the integrity of the presacral venous plexus and bone [13,14]. Looking at the methods used to control bleeding in patients with pelvic fractures, we see non-invasive methods such as circumferential sheet wrapping, circumferential compression sling / binder device that allow temporary mechanical stabilization and reduce pelvic volume. These are the most common ones in the literature and can be easily applied by emergency teams at the time of diagnosis or suspicion of pelvic fracture [15-17].

Among the invasive methods of controlling bleeding, various pelvic external fixator applications are among the main methods. Bleeding control is aimed by temporarily providing pelvic stabilization with external fixator applications. It should be kept in mind that pelvic external fixator application is effective on bleeding control, but these applications should be performed by experienced and expert teams who know which technique to use in which fracture type [18]. Another invasive method, preperitoneal pelvic packing can be life-saving in cases with bleeding that cannot be controlled, however, it has been associated with deep tissue and wound infections as a complication afterwards [19].

Arterial injuries are seen at a rate of 10-15% in pelvis traumas. Transarterial embolization, recommended by the Eastern Association for the Surgery of Trauma guidelines, is an effective method that can be used in the control of arterial bleeding in patients with hemodynamically unstable pelvic fractures. Resuscitative Endovascular Balloon Occlusion of the Aorta (REBOA) technique is another arterial intervention that can be used in cases with uncontrolled bleeding originating from the arterial. It should be kept in mind that both methods have their own vascular and visceral complications [20,21].

The frequency of use of TRA in many surgical areas has increased, apart from orthopedic surgeries, because it reduces the amount of bleeding and the need for transfusion. Although it was first used in arthroplasty surgeries and elective spinal surgeries in orthopedic surgery, recently its use has come to the fore in femoral fractures, hip fractures, pelvis-acetabulum fractures and surgeries, which are known to be more bleeding from the fracture itself or due to the surgery to be performed. Although the literature gives information that the use of TRA in hip fracture surgeries will decrease the amount of bleeding and the need for transfusion, this situation has not been fully clarified in pelvis and acetabulum traumas. However, there is a consensus in these studies that the risk of thromboembolism does not change with the use of TRA [22].

In addition to elective surgeries in orthopedics, the frequency of use of TRA in fracture surgeries has increased in recent years. Studies revealing that the need for blood transfusion decreased with the use of TRA are in majority. Shaoyun Zhang et al. in their review of the literature to summarize the recent studies on TRA administration in traumatic orthopedic surgery, they concluded that topically applied TRA in hip fracture surgery reduces blood loss without increasing the risk of thromboembolism and reduces the need for transfusion. In their review of the literature, they concluded that its effectiveness in other orthopedic surgeries such as pelvic and acetabular fractures is not clear [22]. In a meta-analysis of the use of TRA in patients with femur fracture, Pei Zhang et al. reported that both intravenous and topical TRA administration decreased the transfusion rate in femoral fracture surgery [23]. In their study to investigate the role of antifibrinolytics in pelvic and acetabular fracture surgery, Piggott RP et al. emphasized that in elective spinal and joint arthroplasty surgeries, antifibrinolytics provided benefits by reducing the amount of bleeding, but their role in trauma and fracture surgeries was not yet clear [24]. Spitler CA et al. conducted a randomized controlled trial to evaluate the safety and effectiveness of TRA use in pelvic ring, acetabulum and proximal femur fractures. They reported that the use of TRA in high-energy fractures of the pelvis, acetabulum, and femur significantly reduced the amount of bleeding, but did not decrease overall transfusion rates and also that the frequency of venous thromboembolism did not increase [25]. Watts CD et al. reported in the randomized clinical trial they performed on patients who underwent hemi or total hip arthroplasty due to femoral neck fracture, the amount of blood loss and blood product transfusion rates in the TRA applied group were significantly decreased compared to the control group without TRA and that there was no difference between the two groups in terms of adverse effects within the first 30 days and 90 days after surgery [26].

Because unstable pelvic fractures can be fatal with massive bleeding, the intervention to be performed at the first encounter of patients with

a pre-diagnosis of pelvic fracture in the area of trauma, such as pelvic binders and circumferential wrapping, which also allows temporary stabilization of the pelvis and can be easily applied by emergency teams, almost routinely started to be applied. In addition, prehospital application of TRA has come to the fore in order to reduce blood loss. Although there is no specific clinical study about the administration of TRA in patients with a pre-diagnosis of pelvic fracture in the literature, this issue has been included in the prehospital management of polytraumatized patients with acute bleeding, and studies suggesting early TRA administration in patients with uncontrolled bleeding have come to the fore. In this study, we created an acute bleeding trauma patient model by creating an unstable pelvic fracture in rats.

We aimed to see the effect of early TRA administration on hemoglobin value, without applying other measures that can be taken to reduce the amount of bleeding. When we look at the literature, in the Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage 2, (CRASH-2) study, which is one of the most prominent studies on the subject, Shakur H et al. reported that administration of TRA within 1 hour significantly reduced the amount of bleeding and mortality [27]. Morrison JJ et al. retrospectively examined 896 patients who were injured during the war and had blood transfusion in Afghanistan, reported that although the mean injurity severity score was higher in 293 patients treated with TRA, the mortality was significantly lower [28]. In their retrospective study, El-Menyar A et al. compared 102 trauma patients who underwent prehospital TRA with the same number of trauma patients without TRA, they associated prehospital TRA with less need for blood transfusion. In their study, they demonstrated that there was no significant change in mortality rates and also thromboembolic events [29]. Levi M evaluated the use of TRA in patients with severe trauma-related blood loss in a large international randomized controlled study, concluded that TRA administration reduced bleeding-related mortality, and that TRA did not cause thromboembolism and other serious adverse effects [30]. Neeki MM et al. identified injured patients who were exposed to penetrating or blunt trauma, with signs of hemorrhagic shock in prehospital evaluation, and who underwent prehospital TRA as the study group (362 patients in TRA group), and comprised the control group of injured patients who were exposed to penetrating or blunt trauma, with signs of hemorrhagic shock but did not receive TRA in the same center (362 patient in control group). When they compared the control group and the TRA group, they reported that mortality was 3.6% in the TRA group in the first 28 days of follow-up and 8.3% in the control group. When severely injured patients with ISS> 15 are compared, they reported that this difference is higher (6% vs 14.5% for TRA and control, respectively). When they evaluated in terms of blood transfusion, they revealed that this need was significantly less in the TRA group. Similar to the literature, they also reported that there was no increase in side effects related to TRA [31].

In this experimental study we conducted, when the percent hemoglobin values at the beginning, 30th minute and 24th hour of group 3 were compared with each other, a statistically significant difference was found between these values, and this, first of all, showed us experimentally, that the decrease in hemoglobin values with the pelvis trauma continued significantly for 24 hours. This result is consistent with the information in the literature that there will be significant bleeding

in unstable pelvic fractures. In our study, the 30th minute values of group 2 (TRA after pelvic fracture) were found to be significantly higher than group 3 (no TRA after pelvic fracture). And also the percentage values of group 2 at the 24th hour after the trauma were found to be significantly higher than the percentage values of group 3. This result shows us that TRA application significantly reduces the amount of bleeding due to pelvic fracture. This result is consistent with the studies in the literature reporting that using TRA especially in the first hour after trauma reduces the amount of bleeding. In this context, our study supports studies stating that TRA can be used in the earliest period after trauma and even in the prehospital period, in addition to other interventions that can be applied to reduce bleeding, especially in traumas with acute bleeding such as pelvic fractures that are unstable and tend to bleed seriously. When we look back on the literature, we see that the side effects that may arise in the use of this easy-to-apply, cost-effective agent in traumas with bleeding that may threaten the life of the patient are at a level that can be ignored depending on the gain to be achieved.

Another result that draws our attention in our study is that there is no statistically significant difference between the percentage values of the 24th hour of the second group in which TRA was applied after fracture, and the percentage values at the 30th minute of the 3rd group, who did not receive TRA after the fracture. The fact that the decrease observed in the 24-hour period in the TRA applied group occurred in only 30 minutes in the group without TRA, experimentally demonstrated a strong negative effect of TRA on bleeding. Whether it has the same strong effect in the clinic can only be determined by clinical studies.

Conclusion

In rats with bilateral unstable pelvic fractures due to blunt pelvis trauma, early administration of TRA after trauma significantly reduced the first 24-hour decrease in hemoglobin value. This experimental study supports the early and prehospital use of TRA in traumas that are predicted to progress with acute bleeding, such as unstable pelvic fractures. However, we think that more comprehensive clinical studies are needed to verify this and draw its boundaries.

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Ethical statement:

The study was approved by Local Clinical Research Ethics Committee (Date and Decision Number: 2018/19), and written informed consent was obtained from each subject.

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