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Alterations of Methylated Arginine Residues and Related Amino Acids During Acute Pancreatic Inflammation

Akut Pankreas İltihabı Süresince Metillenmiş Arginin Rezidüleri ve İlişkili Amino Asitlerdeki Değişimler

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Abstract

Aim: The extent of the spread of inflammation determines the severity of acute pancreatitis (AP). Methylated arginine residues (MAR), a type of inflammatory mediator, reduce nitric oxide levels and cause vasoconstriction-induced endothelial damage. This study aimed to investigate MAR and related amino acids during acute pancreatic inflammation.

Material and Method: This prospective, quasi-experimental study was conducted with patients diagnosed with AP and an age-matched control group. The patient samples were taken during the diagnosis and recovery time, whereas during the study for the control group. Mainly, Asymmetric dimethylarginine (ADMA), Arginine (ARG), Citrulline (CIT), and related chemicals were studied via a mass spectrometer.

Results: A total of 30 patients with AP (mean age=53.3±17.8) and 30 controls (mean age=53.4±18.0) were included in the study. All patients were identified as non-severe (n=8) and severe (n=22). A decrease was detected in the patients' ADMA levels compared to the control group (p=0.01). MAR did not differ concerning disease severity (p > 0.05). However, MAR levels decreased higher in patients with diabetes or chronic kidney disease (CKD). Between the two samplings, the ARG level and ARG to ADMA ratio increased, while the MAR and CIT to ARG ratio decreased.

Conclusion: Our results showed that MAR levels decreased with AP recovery. The start of a decrease in the high-level blood MAR may indicate the healing of pancreatic inflammation. AP inflammation may be more destructive in patients with diabetes or CKD.

Keywords: Acute pancreatitis, arginine metabolism, asymmetric dimethylarginine, nitric oxide

Öz

Amaç: Enflamasyonun yayılma derecesi, akut pankreatitin (AP) şiddetini belirler. Bir tür inflamasyon mediyatörü olan metillenmiş arjinin rezidüleri (MAR), nitrik oksit seviyelerini düşürür ve vazokonstriksiyona bağlı endotel hasarına neden olur. Bu çalışma, akut pankreas iltihabı sırasında MAR ve ilişkili amino asitleri araştırmayı amaçladı.

Gereç ve Yöntem: Bu prospektif, yarı deneysel çalışma, AP tanısı konan hastalar ve yaşca uyumlu bir kontrol grubu ile yürütülmüştür. Hasta örnekleri tanı anı ve iyileşme sırasında, kontrol grubunda ise çalışma sırasında alındı. Başlıca Asimetrik dimetilarjinin (ADMA), Arjinin (ARG), Sitrulin (CIT), ve ilişkili kimyasallar kütle spektrometre ile çalışıldı.

Bulgular: Çalışmaya toplam 30 AP (ortalama yaş=53,3±17,8) ve 30 kontrol (ortalama yaş=53,4±18,0) dahil edildi. Hastalar şiddetli (n=22) ve şiddetli olmayan (n=8) olarak tanımlandılar. Kontrol grubuna göre hastaların ADMA düzeylerinde belirgin azalma saptanıldı (p=0,01). MAR seviyelerinde hastalık şiddeti yönünden farklılık yoktu (p>0,05). Ancak, diyabet ya da kronik böbrek hastalığı (KBH) olan hastalarda MAR seviyeleri daha yüksek oranda düşmüştü. İki kan örneklemesi arasında ARG düzeyi ve ARG/ADMA oranı artarken MAR ve CIT/ARG oranı azaldı.

Sonuç: Sonuçlarımız, AP iyileşmesi ile MAR düzeylerinin düştüğünü gösterdi. Yüksek kan MAR seviyelerinde azalmanın başlaması, pankreas iltihabının iyileşmeye başlamasını gösterebilir. AP inflamasyonu diyabet veya KBH olan hastalarda daha yıkıcı olabilir.

Anahtar Kelimeler: Akut pankreatit, arjinin metabolizması, asimetrik dimetilarjinin, nitrik oksit

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INTRODUCTION

Methylated arginine residues (MAR) is an umbrella term encompassing protein transactions involving inflammation as one of the principal manifestations of pathogenesis. Several junctional amino acids and edited-intermediates cast an essential role in the metabolism of MAR.[1] Asymmetric dimethylarginine (ADMA) is a stable MAR formed due to the regulation of methyl groups into arginine (ARG) via protein arginine methyltransferase (PRMT) enzyme subtypes and the degradation of these added proteins.[2] The number and type of methylations also generate symmetrical dimethylarginines (SDMA) and N-monomethyl-L-arginines (L-NMMA). All MAR, nitric oxide synthase (NOS) enzyme inhibitors, were associated with endothelial dysfunction and vasoconstriction in eukaryotes.[3]

Similarly, amino acids such as methionine (MET), homocysteine (HCY), which play a role in the production steps of ADMA, or amino acids such as citrulline (CIT) and ornithine (ORN), which are post-production metabolites, can also be effective on the nitric oxide (NO) impacts on inflammation (**Figure 1**).[2-4] On the other hand, ARG amino acid can work as a substrate at average concentrations and as an inhibitor of NOS at high concentrations (**Figure 1**).[5] These intracellular cycles persist in various cells or organs via cationic amino acid transporters (**Figure 1**).[5]

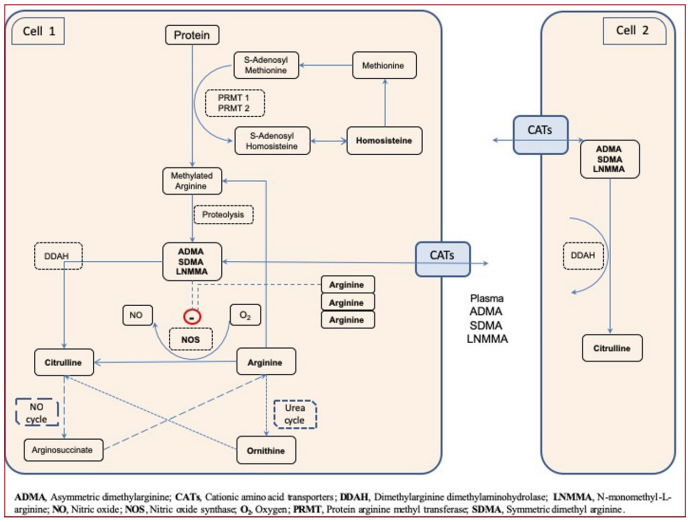

Figure 1: The simplified version of the methylated arginine, homocysteine, and arginine metabolism.

In this context, acute pancreatitis (AP) is an inflammatory process of the pancreatic organ characterized by abdominal pain and elevated levels of pancreatic enzymes detected in the blood.[6] The disease has a broad clinical spectrum, with an overall mortality rate of 3% to 15%.[7,8] Mortality rates stand much higher in subgroups of patients with severe diseases or remarkable comorbidities.[6,9] Radiological confirmation can also be required for diagnostic support and prognostic evaluation, along with patient clinical and laboratory results. The disease severity can be stratified as interstitial edematous or acute necrotizing pancreatitis, depending on the extent of the inflammation.[10]

To date, there has been no agreement on what circumstances about MAR existed on the AP battlefield. We thus aimed to evaluate the prominent MAR level variations during pancreatic inflammation.

MATERIAL AND METHOD

Study Design

This article was a prospective, uni-centered, quasi-experimental study conducted with patients diagnosed with AP between 2020 and 2021. The ethics committee of the Selcuk University Faculty of Medicine approved the study method (2019/374), and an informed consent form was obtained from all participants. This work was also supported by the (Scientific Research Project Found of Selcuk University) under grant number 20401038. In addition, the study was carried out under the Helsinki Declaration.

Patient & Control group

The patient group featured patients over 18 years of age diagnosed with AP, and the control group included age-matched and healthy volunteers over 18 years of age. The exclusion criteria were the presence of other simultaneous infections or active malignancy and medication use that can alter NO levels (angiotensin antagonists, calcium channel blockers, nutritional arginine support for dialysis patients, statins). All patients and the control group's demographic characteristics and laboratory test results were noted simultaneously.

The patients recruited from two clinics (gastroenterology and internal medicine) were categorized into two groups based on the mortality predictability of Ranson's criteria.[11] Accordingly, patients with a Ranson score of 0 to 2 were defined as non-severe and three or greater as severe.[12]

Sampling

The first blood samples were gathered upon admission and prior to treatment from the patients and during the study from the control group. For the patient group, the second blood samples were taken during the clinical recovery supported by the amylase, and lipase levels decreased to below three times the normal range (for amylase, 300 U/L; for lipase, 210 U/L). Laboratory evaluations of the control group were conducted in a single session. There was no intervention in treating the patients between the two sampling intervals. A third sample was taken on the day the clinic worsened to indicate the fluctuation of laboratory results in patients with complications.

Laboratory Analysis

The MAR metabolites of the controls and patients with AP were analyzed using an API 3200 LC-MS/MS mass spectrometer (Applied biosystem/MDS SCIEX; CA, USA). Accordingly, ADMA, ARG, CIT, HCY, L-NMMA, SDMA, and ORN chemicals were studied.

Each participant's venous blood sample (10 ml) was taken into serum tubes (BD Vacutainer, USA) and centrifuged in a cooled centrifuge (microfuge R22, Beckman) at 4°C at 3500 rpm for 10 minutes, and then serum samples were separated and stored

at -80°C until analysis. For analysis, samples stored in Eppendorf tubes at -80°C were kept at room temperature to dissolve and then vortexed for 3-5 seconds to ensure homogeneity. All samples were studied with pre-processing steps established and validated in the routine biochemistry laboratory.

ADMA Path Working Procedure: For the pre-processing steps, after adding the internal standard (d7-ADMA) dissolved in 100 µL of methanol to the 200 µL serum sample, the precipitated proteins were removed via centrifugation at 13000 rpm for 10 minutes. The supernatant was taken into a clean tube and evaporated with nitrogen gas at 60°C. For the derivatization process, 200 µL of newly prepared 5% (v v-1) butanol/acetyl chloride solution was added and kept at 60°C for 20 minutes; the solvent was evaporated with nitrogen gas. The dissolution process was carried out with 100 µL of water-methanol (90:10, v:v) containing formic acid 0.1% (%v:v). Serum ADMA, SDMA, L-NMMA, ARG, ORN, and CIT levels were measured in positive mode using electrospray ionization (ESI) on an Applied Biosystems MDS SCIEX (USA) API 3200 brand mass spectrometer (LC-MS/MS) instrument coupled with Shimadzu LC-20AD (Japan) high-performance liquid chromatography. Chromatographic analysis was done with a modified method with a Phenomenex Luna C18 brand column.

Homocysteine Working Procedure

For pre-processing, 25 µL of internal standard (d8-homocysteine) and 100 µL of dithiothreitol (300 mmol/L) were added to the 100 µL sample, incubated for 10 minutes at room temperature, and then 100 µL of trichloroacetic acid precipitating reagent was added and vortexed for 10 seconds. Finally, the samples were centrifuged at 13000 rpm for 10 minutes, and 10 µL of supernatant was injected into the LC-MS/MS device.

According to the manufacturer's instructions, other clinically relevant biochemical parameters were analyzed with Beckman-Coulter AU 5800 (Beckman Coulter, Brea, USA). Additionally, the hematological parameters were measured from complete blood using Beckman Coulter LH 780 analyzer (Beckman Coulter, Miami, FL, USA), and hormone levels were measured using the electrochemiluminescence method (Roche Diagnostics, Cobas 6000 analyzer e601 module, Germany).

Statistical Analysis

Statistical analyses were performed using SPSS version 21 (SPSS Inc., Chicago, IL, USA). The data distribution was determined according to skewness and kurtosis. For the data association, the Pearson correlation was used to compare normally distributed data, and the Spearman was made for non-normally distributed data. An independent t-test was applied to identify MAR significance in normally distributed data between the patient and control groups. In contrast, a Mann–Whitney U-test was used for skewed data. For evaluating MAR and laboratory values in the patient samples on admission and recovery courses, a paired sample t-test was preferred for normally distributed data and a Wilcoxen matched-pairs signed-rank test for those with skewed distribution. For the subgroup analyses, nonparametric tests were chosen due to the decrease in the number of patients. In all intergroup significance evaluations, the chi-square test was utilized. A p-value of less than 0.05 was considered strong evidence for the alternative hypothesis.

RESULTS

Our study included a total of 30 patients (mean age 53.3±17.8 years) and 30 controls (mean age 53.4±18.0 years). The male and female populations were 11/19 in the patient group and 12/18 in the control group (p=0.791). The gender distribution in the patient groups was nine males in the severe pancreatitis group and two males in the non-severe group, respectively (p=0.424). The overall recovery length for AP was 6 days. Among the patients with comorbidities, 8 had diabetes mellitus (DM), and eight had chronic kidney disease (CKD). The patients' predicted overall mortality rate based on Ranson criteria was 17.5%. This mortality prediction was 23.6% (mean Ranson, 4.04±0.89) in the severe group and 0.9% (mean Ranson, 1.37±0.51) in the non-severe.

In our study, biliary causes were the most common causes of AP etiology. It was accompanied by cholecystitis in 30% and cholangitis in 23%. In evaluating the groups set according to disease severity, mechanical causes such as gallstones or infection were more common in the severe group (63%) than in the non-severe group (25%). In addition, there were eight patients (27%) with elevated HCY in which vitamin B12 is a cofactor, and 12 patients (40%) with B12 deficiency were identified.[13,14] Other notable laboratory results of the patients according to disease severity are summarized in **Table 1**.

We compared the MAR between the groups and found a statistically significant decrease in ADMA (p=0.001, η^2=0.482) and L-NMMA (p=0.001, η^2=0.388); conversely, we found an increase in ORN (p=0.001, η^2=0.255) and HCY (p=0.047, η^2=0.066) levels in patients with AP (**Figure 2**). There was no statistical difference between the groups regarding age, SDMA, ARG, or CIT.

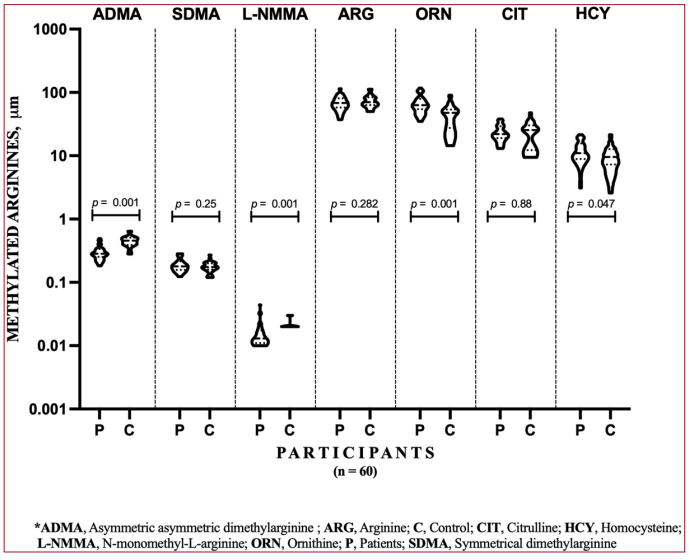

Figure 2: Comparing the characters in methylated arginine metabolism between control and patient groups.

Table 1: Laboratory results of the patient groups

	All patients (n=30)	Non-severe (n=8)	Severe (n=22)	P value
WBC*, $\times 10^9$v/L	11.11±4.34	9.76±2.71	11.60±4.75	0.447
Hemoglobin, gr/dL	12.88±1.78	13.17±1.05	12.77±1.98	0.765
Platelet, $\times 10^9$/L	252 (211-305)	260 (238-283)	244 (205-310)	0.765
ANC†, $\times 10^9$/L	8.23±4.22	6.67±2.53	8.80±4.60	0.393
ALC‡, $\times 10^9$/L	1.57±0.69	1.97±0.75	1.42±0.62	0.050
Glucose, mg/dL	127.6±36.8	110.37±25.12	133.86±38.84	0.170
LDH§, U/L	376 (264-728)	210 (180-283)	496 (351-997)	0.001
AST¶, IU/L	63.5 (30.5-431)	32 (21-43)	174 (41-659)	0.006
BUN**, mg/dL	11.5 (8-17)	7.5 (5.5-11.5)	12 (9.75-19)	0.031
Calcium, mg/dL	8.54±0.60	8.60±0.50	8.51±0.64	0.662
Albumin, g/dL	3.84±0.36	3.83±0.41	3.85±0.36	0.909
Total proteine, g/dL	6.67±0.46	6.55±0.50	6.71±0.45	0.597
ALP*†, U/L	119 (81-190)	71 (52-266)	134 (92-183)	0.202
ALT*‡, U/L	66 (26-342)	30 (18-45)	115 (32-392)	0.024
Direct Bilirubine, mg/dL	0.27 (0.12-1.2)	0.14 (0.11-0.73)	0.45 (0.12-1.38)	0.420
Total Bilirubine, mg/dL	0.99 (0.46-2.45)	0.66 (0.42-1.94)	1.34 (0.46-2.77)	0.504
GFR*§, ml/min/1.73 m²	99 (57-118)	126 (85-141)	93 (44-105)	0.013
GGT*¶, IU/L	82 (28-371)	35 (17-209)	150 (47-473)	0.005
Uric acid, mg/dL	5.3 (3.6-6.5)	4.9 (3.1-6.4)	5.5 (3.9-6.6)	0.597
B12, pg/mL	334 (167-498)	228 (130-423)	339 (173-542)	0.258
Ferritin, ng/mL	66 (21-534)	12 (7-59)	102 (38-711)	0.003
CRP†*, mg/L	8 (4.85-16.17)	6.13 (3.6-10.69)	8.43 (5.81-22.3)	0.298
Procalcitonin, ng/mL	0.13 (0.05-0.48)	0.05 (0.05-0.07)	0.23 (0.07-1.48)	0.008
Total cholesterol, mg/dL	192 (156-234)	184 (161-231)	196 (149-238)	0.830
HDL††, mg/dL	45.93±15.04	47.25±18.59	45.42±13.96	0.830
LDL†‡, mg/dL	121.37±51.52	116.00±40.22	123.42±55.98	0.998
Triglyceride, mg/dL	110 (68-182)	127 (56-268)	106 (77-167)	0.830

P values are the comparison of non-severe and severe groups (Mann-Whitney U test); Data are the median, n (%), or n/N (%); * White blood cell; † Absolute neutrophil count; ‡ Absolute lymphocyte count; § Lactate dehydrogenase; ¶ Aspartate aminotransferase; ** Blood urea nitrogen; *† Alkaline phosphatase; *‡ Alanine transaminase; *§ Glomerular filtration rate; *¶ Gamma-glutamyl transpeptidase; †* C-Reactive protein; †† High-density lipoprotein; †‡ Low-density lipoprotein.

In the patient group, statistical significance was present for all MAR pathway products when the results of two samplings taken from each patient at different times were compared, as shown in **Figure 3a** (p<0.05). Notably, the ARG levels increased in the second sampling result, decreasing the others. In addition, the ARG/ADMA ratio (AAR), an index for NO production, increased by 43% between the two sequential measurements; t (29)=-5.709, p=0.001. (**Figure 3b**). Similar results were found for another NO production indicator, CIT/ARG ratio (CAR). The decrease in the CIT level was more significant than the increase in the ARG level; t (29)=6.471, p=0.001 (**Figure 3c**).

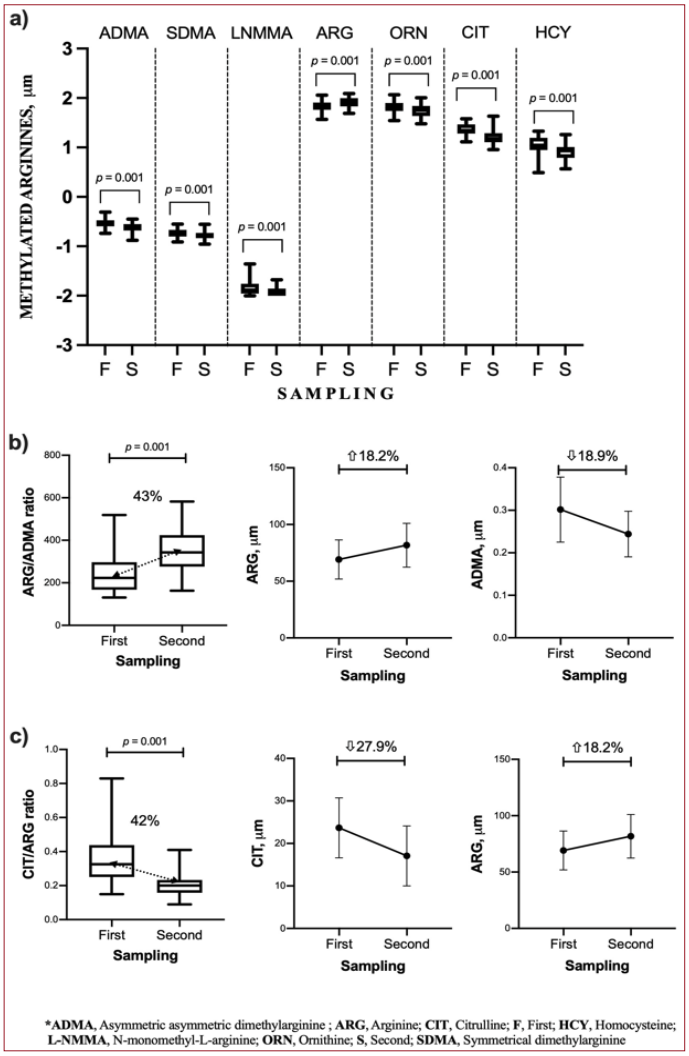

Figure 3: a) Variation of methylated arginine residues and related amino acids between the samples taken at admission and recovery; b) The increase of the Arginine to Asymmetric dimethylarginine (ADMA) ratio between the samples taken at admission and recovery, increase in Arginine, whereas a decrease in ADMA; c) The decrease of the Citrulline to Arginine ratio between the samples taken at admission and recovery, decrease in Citrulline, whereas an increase in Arginine.

Considering the comparison of subgroups, all MAR levels did not differ between the non-severe and severe groups (p>0.05). However, in patients with higher HCY levels, ADMA levels were higher (p=0.04, η^2=0.143), and AAR levels were lower (p=0.013, η^2=0.205).[14] Due to the negative effects of ADMA metabolites on the vessels, we also planned subgroupings based on two comorbidities (DM and CKD) with a high prevalence of vascular complications. As a result, there was a statistically significant decrease between the two samplings in all MAR in non-diabetic patients; on the contrary, the reduction did not persisted in diabetic patients, except for ARG, ORN, and CIT (**Figure 4a**). Likewise, MAR in patients with and without CKD (except for ORG, ADMA, SDMA, L-NMMA, and HCY in CKD patients) did differ between the two samplings (**Figure 4b**). In addition, inter-sample AAR results were statistically decreased in patients with acute pancreatitis without CKD (**Figure 5a**) or DM (**Figure 5b**) (p<0.05).

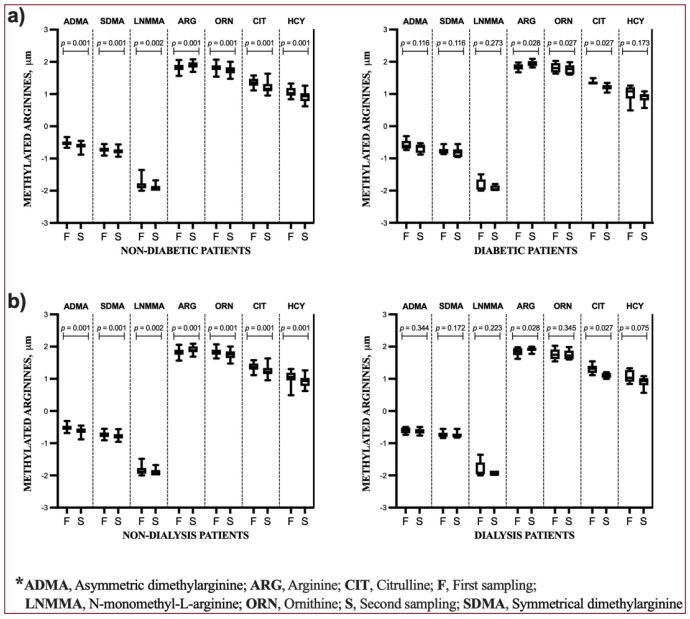

Figure 4: Alterations of MAR and related amino acids; a) In diabetic and non-diabetic patients; b) In chronic kidney disease patients with or without receiving dialysis.

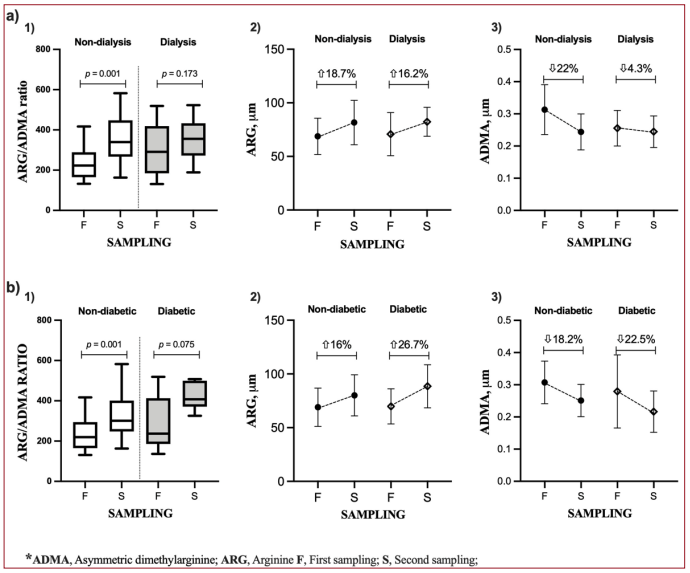

Figure 5: a) 1) Arginine to Asymmetric dimethylarginine (ADMA) ratio comparisons in the two samplings between dialytic or non-dialytic patients, 2) The distribution of Arginine increase between dialysis or non-dialysis patients, 3) The distribution of ADMA decrease between dialysis or non-dialysis patients; b) 1) Arginine to Asymmetric dimethylarginine (ADMA) ratio comparisons in the two samplings between diabetic or non-diabetic patients, 2) The distribution of Arginine increase between diabetic or non-diabetic patients, 3) The distribution of ADMA decrease between diabetic or non-diabetic patients.

Amid the detected correlations, age was positively correlated with Ranson grade (r=0.741, p=0.001), mortality (r=0.707, p=0.001) and ARG levels (r=0.372, p=0.043). Triglyceride level was positively correlated with ADMA (r=0.392, p=0.036), HCT (r=0.442, p=0.016). ADMA was positively correlated with HCT (r=0.371, p=0.044) and Ca^{+2} (r=0.463, p=0.01), and negatively correlated with ferritin level (r=-0.405, p=0.027) and age (r=-0.375, p=0.041). ARG level was negatively correlated with HCT (r=-0.470, p=0.009).

DISCUSSION

This study evaluated methylated arginine variations in patients with AP. According the results, there was a decrease in ADMA and L-NMMA levels, whereas an increase in ORN and HCY levels in the patient group compared to the control group. In comparing the MAR at the diagnosis and recovery period in the pancreatitis arm, only an increase in the ARG level was observed; conversely, a decrease was detected in all other MARs. When the MAR distribution was evaluated alongside disease severity, there were reductions in all MAR results that were not statistically significant. In addition, AAR variations were compatible with disease recovery. Finally, there was no difference in MAR outcomes throughout the disease duration in diabetic or dialysis patients.

As a clinical determination, inflammation may be more rapid in pancreatic tissue, as does mortality.[8,15] It would be reasonable to expect MAR shifts directly to pancreas inflammation, which can also be a catabolic process of systemic proteins.[16] Another important consideration is minimizing oral feeding during AP treatment; therefore, a semi-essential amino acid such as ARG is caused to be significantly reduced in the earlier stages of AP. However, the ARG will return to acceptable levels with the initiation of the oral feeding of the patient.

In a study that inspired our research, patients with AP were staged according to Atlanta criteria.[17] The ADMA reduction was more pronounced between the two sequential samplings in moderately severe patients. Moreover, they found impaired glucose tolerance to be higher in the moderately severe group after recovery and correlated this with ADMA levels. We preferred staging as stated by the Ranson Criteria since the clinical validity was still retained.[12] We decided on the second sampling time based on patient recovery rather than a typical day to fully reconcile with the clinic. Since the ADMA reduction at recovery was determined in the current study, we preferred to study other factors and cofactors in MAR and MAR metabolism that caused this decrease. Consistent with their investigation, our research found further interactions between MAR deactivation and patient profiles.

ADMA and L-NMMA can directly, and SDMA can competitively decrease NO levels, induce oxidative stress lead to apoptosis.[18,19] Regarding the harmful effects of MAR at the intracellular level, we detected a significant decrease in MAR levels at the recovery of pancreatitis compared to samples taken at admission. At this point, the contribution of the ARG level, which we found to be increased, is essential. The ARG boost may have achieved this in several ways. First, the initiation of oral feeding will provide an adequate ARG supply. Second, ADMA formation can be inhibited at high ARG levels,[5] thereby indirectly increasing NOS activity. Third, the amount of ARG from the NOS cycle increases due to NOS activity (**Figure 1**).

Continue to stand at the micro level; the AAR and CAR acting on NOS will also be of substantial help in supporting the results. NOS activity inhibition can be overcome by increased extracellular AAR due to excessive substrates.[20] In the AAR, which we found increased by half, the increase in ARG was almost equal to the decrease in ADMA. In contrast, we found that the balance between the ARG substrate and the CIT product, which can also be considered an NOS enzyme indicator, decreased against the CIT. The reduction in CAR may also mean that a decrease in CIT, despite an increase in ARG, can cause a relative increase in NO. All these consequences will contribute to NO increase, blocking the effects of MAR, thus finalizing vasoconstriction and endothelial damage, and improving pancreatic inflammation.[21]

Clinically, the harmful effects of MAR on the human body are more destructive to endothelial structures.[22,24] Therefore, MAR can be more damaging in diseases such as DM and CKD, which are prone to vascular complications.[25] The current study compared ADMA levels in diabetic patients with or without atherosclerosis and found higher ADMA levels in the arm with macro complications. Our study found no difference in the MAR and HCY variations in patients with DM, while a decrease was found in patients without DM in all MAR, ORN, CIT, and HCY evaluations. The good tidings were that the ARG elevation was redemptive in the diabetic and non-diabetic groups. Likewise, there were similar determinations in the CKD group.

The increases in ARR outcomes in patients with DM and CKD were also similar. The lack of difference in the ARR results in those with DM or CKD may indicate a poor prognosis. The following can be stated for CAR, another indicator of the MAR pathway: a statistically significant decrease was detected between all subgroups with comorbidities. Thus, comorbidities did not seem to have a dominant impact on NOS enzyme activity. All of these evaluations indicate that pancreatitis prognosis is more destructive in patients with DM or CKD.

This study was primarily limited by the absence of patients with necrotic pancreatitis. Next, if we could measure NO levels, we could be more precise about the NOS pathway. Finally, all MAR measurements reflected blood levels; therefore, we did not provide any information about MAR levels in the pancreatic gland.

CONCLUSION

Our study was designed to examine invivo MAR metabolism in the acute pancreatitis stages. Reductions toward the end of pancreatic inflammation in all MAR and increased ARG levels were notable. MAR reductions were lower in the course of acute pancreatitis in diabetic or dialytic patients. This research supports the idea that providing oral nutrition earlier and adequately will provide arginine support and accelerate MAR clearance. Moreover, an ARG test can be performed without detecting low blood albumin levels. As our study was the first to focus on the variation of MAR and HCY in pancreas inflammation, further inspections regarding the roles of MAR and ARG would be beneficial.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Selcuk University School of Medicine Ethics Committee (Date: 25.12.2019, Decision No: 2019/374).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: This study was supported by Selcuk University Research Fund (Project Number: 20401038).

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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