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THE RISK PROFILE OF THE DIABETIC PATIENT IN ACUTE MYOCARDIAL INFARCTION

ABSTRAT OF DOCTORAL THESIS

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Content

Introduction and premises

Cardiovascular diseases (CVD) are the leading cause of mortality and morbidity worldwide, with a significantly higher prevalence in patients with diabetes [1].They also represent the complications with the highest mortality among patients with diabetes mellitus [2,3]. The relationship between type 2 diabetes (T2D) and CVD is complex and bidirectional and has significant clinical, social, and economic implications. CVD exacerbates insulin resistance (IR) and its effects, perpetuating a vicious cycle of metabolic dysfunction and increased cardiovascular risk. In addition to traditional risk factors such as diabetes, hypertension, dyslipidemia, smoking, and obesity, inflammatory mechanisms contribute to both the etiopathogenesis and progression of CVD [4–6].

Interest in the role of inflammation in patients with coronary atherosclerosis now extends from a chronic to an acute perspective, but research in this field remains in its early stages. Acute ischemic events trigger an intense inflammatory response involved in both the healing process and acute complications. The harmful role of chronic inflammation is well established through extensive preclinical and clinical research such as CANTOS, COLCOT, and LoDoCo [7–9] but it is still challenging to determine the point at which the acute cytokine cascade ceases to be a useful reparative stimulus and becomes a harmful mechanism in the context of acute myocardial infarction (AMI). Diabetes also represents a complex metabolic entity characterized by low-grade chronic inflammation [10]. It is intuitive for a clinician to assume that an acute cardiovascular event superimposed on the diabetic environment could trigger an exaggerated inflammatory response, leading to adverse complications [4,11].

Although the in-hospital mortality rate for patients with ST-segment elevation myocardial infarction (STEMI) has decreased with the introduction of emergency interventional revascularization programs, there remains heterogeneity in the in-hospital outcomes for these patients, even among those with relatively similar clinical profiles and pathological histories. Whether inflammatory mechanisms are at least partially responsible for this remains to be determined. Numerous studies have highlighted the importance of systemic inflammation, showing that it is not just a reactive consequence, but is, in fact, a significant contributing factor and a treatable outcome of the disease [12,13]. In a simplistic point of view, a clinician can easily and cost-effectively observe the inflammatory response in patients with AMI using only routine blood tests, such as measuring leukocytes, neutrophils, and C-reactive protein (CRP). These are elevated in clinical observations, confirmed in cohorts, and predict adverse events. These changes suggest an imbalance of immune cells in correlation with the inflammatory process. Additionally, increasing evidence supports the involvement of the innate immune response in the pathogenesis of AMI, with emerging findings indicating an altered phenotype of immune cells, including neutrophils, in this context [14].

In this context, the present work had the following main objectives: ***the prognostic involvement of inflammatory parameters in patients with AMI during the hospitalization of the index event; comparisons between diabetic and non-diabetic patients regarding the acute inflammatory response; evaluation of the inflammatory profile of neutrophils from patients with AMI.***

The thesis is structured into two parts. The first general section details the basic inflammatory mechanisms, the mechanisms involved in cardiovascular pathology and diabetes, and the points of intersection between them. The second part, dedicated to personal contributions, comprises two studies. The first clinical study evaluates the association between inflammatory parameters and unfavorable post-AMI outcomes during hospitalization , also comparing diabetic and non-diabetic patients. The findings of this study were validated the second in vitro study that investigates the pro-inflammatory phenotype of neutrophils isolated from patients with AMI.

The integration of concepts of cardiology, diabetology, and immunology into the study brings us closer to deciphering the complex pathological mechanisms that are not limited to a single system and begin at the molecular level. Research efforts should focus on identifying and understanding the pathogenic inflammatory mechanisms to develop new prevention and treatment strategies.

I. General part

From this first section, we briefly mention the mechanisms by which neutrophils combat pathogens and their role in acute myocardial infarction.

Neutrophils have various mechanisms to neutralize pathogens, such as phagocytosis, degranulation and release of antimicrobial proteins and peptides (such as elastase, myeloperoxidase, and matrix metalloproteinases), generation of reactive oxygen species (ROS), and the formation of neutrophil extracellular traps (NETs) composed of DNA, histones, and antimicrobial proteins [15], mechanisms summarized in Table 1.

Table 1 Neutrophil Action Mechanisms

Mechanism	**Description**	**Functions**	**Example/ components**
Degranulation - lysosomal enzymes and antimicrobial peptides	Enzymes and small proteins contained in granules	Break down cell membranes and destroys pathogens	Lysozyme, myeloperoxidase (MPO), elastase, matrix metalloproteinases; defensins, cathelicidins
Generation of reactive oxygen species (ROS)	Reactive molecules such as hydrogen peroxide and free radicals	Oxidize and destroy bacterial components	Superoxide, hydrogen peroxide (H2O2
Phagocytosis	Process of ingesting bacteria and other pathogens	Capture and destroy bacteria	Phagolysosome - vacuole where the pathogen is ingested
Formation of neutrophil extracellular traps (NETs)	Structures of extracellular DNA together with antimicrobial proteins	Capture and immobilize bacteria	DNA, histones, elastase, myeloperoxidase (MPO)
Release of Cytokines and Chemokines	Signaling molecules, chemoattractants	Involved in mediating the immune response	Proinflammatory: CXCL8, TNF-alpha, IL-1beta; Anti-inflammatory: IL-4, IL-10
Release of complement proteins	Plasma proteins that form attack complexes on membranes	Lysis of pathogen cells and opsonization	C3, C5, membrane attack complex (MAC)

Mechanism	Description	Functions	Example/ components
Other cytoplasmic proteins	Intracellular proteins of neutrophils present in the cytoplasm but not in granular content. They are released after neutrophil activation, contributing to NET formation	Inflammation mediators. Antimicrobial proprieties. Nutrient sequestration necessary for bacterial growth.	S100 A8/A9

IL - interleukin, NET - neutrophil extracellular traps, TNF - tumor necrosis factor

Recent studies show that neutrophils are more than just pathogen-destroying cells. They exhibit various phenotypes and carry out numerous cellular functions, particularly in inflammation associated with metabolic disorders. When metabolic functions are disrupted, as seen in conditions like diabetes, dyslipidemia, and CVD, neutrophils may be predisposed to stronger pro-inflammatory responses. During AMI, following plaque rupture, activated platelets and neutrophils interact at the site of injury, intensifying NETosis. [16]. Additionally, microvascular obstruction occurs through NET networks and the formation of microthrombi in the microcirculation, which leads to an increase in infarct size [17]. Activated neutrophils are also less deformable, block capillaries, can cause microvascular obstructions, and affect reperfusion. ROS generated by activated neutrophils in atherosclerotic plaques also cause platelet activation. This intensification of prothrombotic processes by neutrophils can significantly influence AMI outcomes [18]. Another mechanism by which neutrophils amplify pro-thrombotic processes is inflammasome-dependent, acting as amplifiers not only for atherogenesis but also for thrombosis [19]. Thus, IL-1β and IL-18 generated by the activation of NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasomes in activated neutrophils enter a self-amplifying loop and also induce IL-6 generation by macrophages. IL-6 stimulates hepatocytes to produce CRP, fibrinogen, and (Plasminogen activator inhibitor-1PAI-1), creating a prothrombotic status.

Along with the phenomena occurring at the vascular level, ischemic injury initiates an inflammatory cascade that induces the activation of leukocytes and endothelial cells. These activated cells secrete pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), IL-1, IL-6, IL-8, chemokines, and adhesion molecules, which exacerbate tissue injury and promote leukocyte recruitment to the affected area. Attracted and activated neutrophils attach to endothelial cells and

penetrate the tissue. Once localized in the tissue, neutrophils secrete ROS, NETs, proteases, and inflammatory agents, thus contributing to oxidative stress, tissue damage, and inflammation [20]. Crucial for ischemia-reperfusion injury is the fact that activated neutrophils represent an important source of ROS, produced through the activity of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex [21]. ROS production by neutrophils intensifies the inflammatory response and worsens tissue injury [18,22].). Moreover, it has been demonstrated that short-term blocking of S100A8/A9 in the first 3 days post-MI with the specific blocker ABR-238901, which inhibits the binding between S100A8/A9 and its receptors, reduces neutrophil and macrophage infiltration into ischemic myocardium and improves cardiac performance [23]. An increase in the neutrophil/lymphocyte ratio (NLR) is correlated with a negative prognosis in AMI, and dynamic changes in NLR precede the clinical state by several hours and can provide rapid prognostic information [24].

II. Personal contributions

Hypotheses and General Objectives

Working Hypotheses

- Inflammation plays an important role in the short-term prognosis of patients with myocardial infarction (MI) and therefore, inflammatory markers could be used in phenotyping high-risk patients in the setting of acute myocardial infarction (AMI).
- The acute inflammatory response is dysfunctional in the presence of diabetes and such a response is associated with a more severe clinical course in hospital.
- Risk stratification should be evaluated differently, using specific prediction scores based on the presence of diabetes
- Neutrophil activation has a prognostic role in the in-hospital evolution of patients with AMI. The neutrophil phenotype of patients with MI and unfavorable outcomes undergoes changes that may be responsible for the unfavorable in-hospital evolution of patients with MI.

General Objectives

- Evaluate the role of inflammatory parameters in the short-term evolution of AMI, highlighting neutrophil activity and its predictive capacity for unfavorable outcomes.

- Evaluate the specific characteristics of diabetic patients during the acute phase of myocardial infarction, particularly regarding systemic inflammation.
- Identify predictors for unfavorable evolution and use them to develop scores for identifying high-risk patients in AMI.
- Identify a specific neutrophil phenotype, defined by certain pro-inflammatory markers, that is responsible for the negative prognosis of patients with MI.

General Methodology

The study is prospective, longitudinal, and consists of 2 stages/2 sub-studies with partially overlapping methodologies, namely:

1. Clinical Study

2. In vitro Study

The **case study** is represented by a cohort that consists of a sample of 229 patients admitted between April 2021 and September 2022 at "Elias" Emergency Hospital in Bucharest, Romania. Of these, 63 were non-diabetic, 78 had prediabetes, and 88 were diabetic. To evaluate clinical factors and biomarkers with prognostic value, patients were divided into a subgroup with unfavorable evolution (with in-hospital complications, N = 77) and a subgroup with favorable evolution (without complications, N = 152).

Negative prognosis and in-hospital complicated evolution is defined by the presence of any of the 5 conditions:

1. Class Killip 3 or 4
2. LVEF at discharge <40%
3. Complex ventricular arrythmias
4. Mechanical complications
5. Death

Inclusion Criteria:

- Patients presenting within the first 24 hours from the onset of AMI -according to the universal definition of acute myocardial infarction[25].

Exclusion Criteria:

- Acute viral/bacterial/fungal infectious syndrome, including acute SARS-CoV-2 infection.
- Pre-existing severe organ dysfunctions – severe hepatic and renal insufficiency that would significantly influence biological tests.
- Inflammatory, autoimmune diseases under treatment (rheumatoid arthritis, psoriatic arthropathy, lupus, inflammatory bowel disease, spondyloarthropathies).
- Neoplasms or other diseases limiting survival for the proposed post-AMI follow-up period.
- LVEF < 30%

Analysis and comparisons were made regarding:

- diabetic/prediabetic/non-diabetic status
- In-hospital evolution (more specifically - analyzing parameters that influence complicated in-hospital evolution)

We decided to limit the enrollment of patients with MI and severely reduced LVEF <30% because severe dysfunction is well-known to affect post-infarction evolution [26,27] and has an independent effect on inflammatory parameters[28,29], regardless of the underlying cause. A severely depressed LVEF was considered a potential source of error that could significantly alter inflammatory markers in our study group. Thus, an LVEF between 30-40% was considered a criterion for acute complications of ACS that does not have a major effect on inflammatory markers despite its prognostic consequences.

Clinical Evaluation

Physical examination was performed daily, starting from the emergency room presentation. Traditional risk factors for diabetes and atherosclerotic disease, such as age, sex, smoking, hypertension, and dyslipidemia, were evaluated. Medical history and previous treatments were documented for each patient.

Investigations

Blood samples were collected and analyzed according to standard care protocols for myocardial infarction upon admission, during hospitalization, and at discharge. Common inflammatory markers, such as C-reactive protein, ferritin, erythrocyte sedimentation rate, and specific diabetes markers,

such as glycated hemoglobin and insulinemia, were also evaluated. Standard biological parameters were measured according to the hospital laboratory procedures. Twelve-lead electrocardiograms were performed daily. Heart rhythm was continuously monitored for the first 72 hours after MI to detect arrhythmias. Transthoracic echocardiography and left ventricular ejection fraction evaluation (LVEF) were performed at admission and discharge or when new symptoms occurred. Angiography and interventional revascularization were performed within the first 60 minutes of admission for all patients. The infarct-related lesion, the number of epicardial coronary arteries with hemodynamically significant stenoses, and the total number of coronary stenoses were documented.

Special blood tests were performed to determine inflammatory biomarkers and the inflammatory profile of neutrophils as follows:

For quantifying various inflammatory markers (IL-1 Beta, IL-18, IL-6, S100A9, NETs):

- Peripheral blood samples were collected in sterile serum tubes within the first 12 to 24 hours after the onset of MI symptoms, and the serum was isolated by centrifugation for 15 minutes at 1500 xg. The serum was stored at -20°C, then processed by ELISA/Sytox Green staining.

For neutrophil isolation:

- Peripheral blood samples from 35 patients with ACS with or without diabetes were collected in Vacutainer EDTA blood collection tubes.
- A group of healthy volunteers (n = 10) was included as a reference for some molecular investigations.

For a better understanding, the special methodology for isolating and evaluating neutrophils in vitro will be detailed in study 2.

Informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the "Elias" Emergency Hospital in Bucharest, Romania, with approval number 3349/06.05.2021.

Statistical Analysis

Data collection was performed using IBM SPSS. Statistical analyses and visualizations were conducted in Python 3, using the pandas package for data manipulation and the matplotlib and seaborn packages for visualizations. For each measured numeric parameter, differences between groups (diabetic, prediabetic, non-diabetic) were checked using one-way ANOVA analysis with

Tukey's post-test for multiple comparisons (statsmodels package) [30].. Alternatively, for comparisons between two groups, student t-tests were used (scipy) [31]. For evaluating correlations between parameters, Pearson's R coefficient and corresponding p-value were calculated individually for each pair of variables using SciPy, after eliminating rows with missing values for either variable. The results were then compiled into a matrix, and columns and rows were grouped using the unweighted pair group method with arithmetic mean (UPGMA) and graphically represented using the seaborn package. A linear logistic regression model (sklearn) was used to evaluate the prognostic value of various parameters. Sensitivity and specificity of biomarkers were evaluated using ROC (receiver operating characteristic) analysis, and the threshold corresponding to the highest Youden J statistic (sensitivity + specificity - 1) was selected. For combinations of markers, a support vector machine (SVM) model with a linear kernel (sklearn package) was used[32].GraphPad Prism 7.0 with data points expressed as mean ± standard deviation (SD) was used for all statistical analyses of in vitro results.

Study 1: Prognostic Implications of Inflammatory Parameters in AMI Patients During Hospitalization

1. Study 1 - Results

Population Characteristics

The clinical data and prevalence of risk factors for the entire study population, as well as for the subgroups of control, pre-diabetes, and diabetes patients, are summarized in **Table 2**. The subgroups (non-diabetic, prediabetic, diabetic) had similar gender distributions, with approximately one-third women and two-thirds men, and no significant differences in age (p = NS) for all intergroup comparisons (ANOVA, Tukey post-test). In our study, there were 63 non-diabetic patients (27%), 78 with prediabetes (34%), and 88 diabetics (38%). The average number of hospital days was the same for all patient groups (4 days). The total in-hospital mortality rate was 5.7%, with most of the deceased patients being non-diabetic. The subgroups had a relatively similar distribution with no other significant differences between groups.

Table 2: Population Characteristic

	Control (N)	Prediabetes (PD)	Diabetes (D)	Total
Number	63	78	88	229
Sex				
F	19 (30,2%)	27 (34,6%)	29 (33%)	75 (32,8%)
M	44 (69,8%)	51 (65,4%)	59 (67%)	154 (67,2%)
Age	60,9 (40,1 - 82,1)	61,05 (46 - 79,3)	63,5 (43,8 - 79,9)	61,9 (41,5 - 81,4)
AHN	40 (63,5%)	53 (67,9%)	74 (84,1%)	167 (72,9%)
Smoking	48 (76,2%)	47 (60,3%)	55 (62,5%)	150 (65,5%)
Anemia	5 (7,9%)	5 (6,4%)	9 (10,2%)	19 (8,3%)
In-hospital death	6 (9,5%)	3 (3,8%)	4 (4,5%)	13 (5,7%)
No. Days in-hospital	4,0 (2,0 - 13,9)	4,0 (2,0 - 9,3)	4,0 (2,0 - 21,0)	4,0 (2,0 - 18,2)
LDL > 100	50 / 63 (79,4%)	60 / 78 (76,9%)	52 / 85 (61,2%)	162 / 226 (71,7%)
History of MI	2 (3,2%)	5 (6,4%)	18 (20,5%)	25 (10,9%)

	Control (N)	Prediabetes (PD)	Diabetes (D)	Total
Number	63	78	88	229
Sex				
F	19 (30,2%)	27 (34,6%)	29 (33%)	75 (32,8%)
M	44 (69,8%)	51 (65,4%)	59 (67%)	154 (67,2%)
History of	5 / 62 (8,0%)	3 / 77 (3,9%)	9 / 88 (10,2%)	17 / 227 (7,5%)
Unfavorable prognosis	23 (36,5%)	15 (19,2%)	39 (44,3%)	77 (33,6%)

Categorical variables are presented as number (percentage) and numerical variables as median (5%-95% percentile). F - female, M - male, HTA - hypertension, LDL - low-density lipoproteins, MI - myocardial infarction,

For a comprehensive characterization of the population a multinomial logistic regression analysis was performed on clinical data to evaluate independent clinical factors predicting an unfavorable prognosis. In the studied cohort, anemia (HR=3.1, p = 0.045), atrial fibrillation (HR=3.5, p = 0.013), and diabetes (HR=2, p = 0.032) are independent risk factors significantly influencing the early prognosis of MI.

Influence of inflammatory parameters on prognosis

Erythrocyte Sedimentation Rate (ESR), CRP, fibrinogen, IL-1β, IL-6, neutrophil count, NLR, S100A8/A9, and NETs were significantly increased in the group with unfavorable evolution (**Figure 1**). Notably, most of the inflammatory molecules correlated with the unfavorable prognosis group are derived from the neutrophilic response. In addition to the quantitative value of neutrophils (through neutrophil count and NLR), neutrophil activation products NET (P = 0.0297*, **Figure 1C**) and

S100A8/9 (p = 0.0293*, **Figure 1D**) were elevated in patients with unfavorable in-hospital evolution, suggesting that neutrophil activation plays a role in short-term evolution.

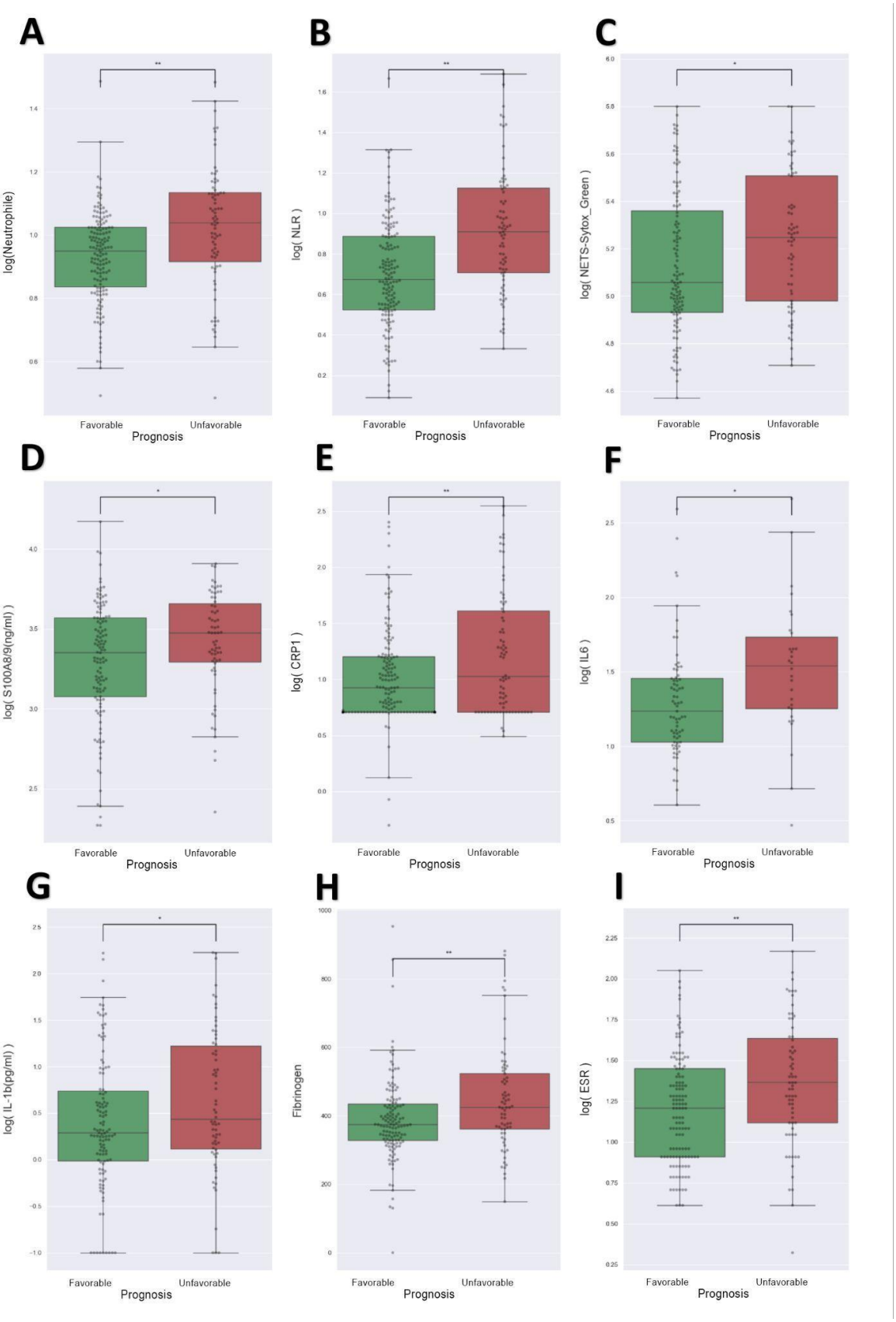

Figure 1. *The influence of inflammatory parameters on prognosis. Red- unfavorable prognosis. Green- Favorable prognosis.*

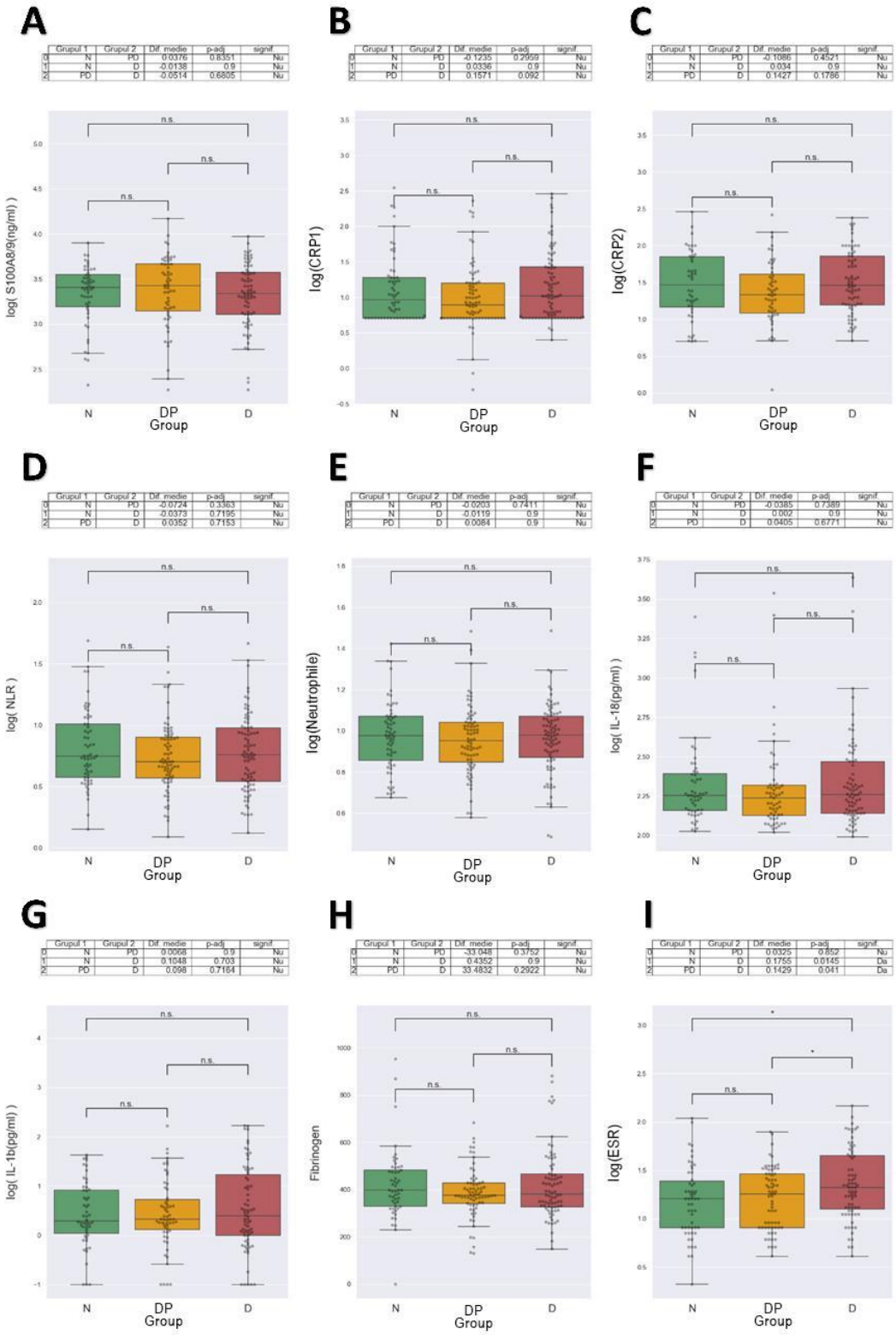

Figure 2. *Comparisons between inflammatory markers by subgroup: A) S100A8/A9 B) CRP1 - C-Reactive Protein at admission C) CRP2 - C-Reactive Protein at discharge D) NLR - Neutrophil-to-Lymphocyte Ratio E) Neutrophils F) IL-18 - Interleukin 18 G) IL-1β - Interleukin 1 beta H)*

*Fibrinogen I) ESR - Erythrocyte Sedimentation Rate D - diabetic, PD - prediabetic, N - normal. (n.s. - nonsignificant, *p < 0.05, **p < 0.001, ***p < 0.001).*

Results of the comparative analysis are presented in box and whisker plots in **Figure 2**. The bars represent the interquartile range (25% - 75%), and the horizontal line inside the bars represents the median. The error bars are 1.5 times the interquartile range. Statistical significance indicated on the graph represents the results of Tukey's analysis for multiple comparisons (n.s. - nonsignificant, $p < 0.05$, $p < 0.01$, $p < 0.001$). The table above the graphs presents the results of the 2-way ANOVA analysis to identify sources of variation between patient groups: weight gain compared to controls, diabetes, and/or the interaction between these factors (**Figure 2)**.

In analyzing inflammatory markers in the acute phase response within the first 12 hours of myocardial infarction symptoms onset, the lack of differences between diabetic, prediabetic, or non-diabetic patients was surprising. The inflammatory response was elevated in the context of acute myocardial infarction (AMI), but without significant enhancement due to the diabetic milieu in terms of quantitative value of inflammatory parameters.

Identifying parameters or combinations of parameters that influence the acute course of myocardial infarction and using them to identify at-risk patients through prediction scores.

Following a detailed of inflammatory parameters and differences between diabetics and non-diabetics, we proceeded to identify biomarkers associated with an unfavorable prognosis.

The results showed that , when the analysis was performed for the entire lot, patients in the unfavorable outcome group had significantly higher values for general parameters that reflect comorbidities :

i) prothrombotic status: D-Dimers ($p = 0.0119$), Fibrinogen ($p = 0.0035$);

ii) inflammation: ESR ($p = 0.0027$), CRP ($p = 0.0021$),

iii) renal dysfunction: creatinine ($p = 0.0024$), eGFR ($p < 0.01$)

iv) glycemic profile: admission glycemia ($p < 0.01$)

v) number of coronary stenoses ($p < 0.01$)

We further evaluated the predictive power of parameters associated with a negative prognosis. For each recorded biomarker, we determined the optimal threshold value to distinguish between favorable and unfavorable prognosis, as well as the sensitivity and specificity of the biomarker at an optimal value. Considering that diabetic patients may have different prognostic factors compared to

non-diabetics, we analyzed the ability of biomarkers to predict outcomes in more homogeneous groups based on the presence of diabetes. It was found that the most efficient predictors for non-diabetic patients did not exhibit the same specificity and sensitivity for diabetic patients **(Figure 3)**. Additionally, it was observed that among the top ten parameters sorted by their prognostic value for diabetic patients, most were inflammatory markers primarily linked to neutrophil activity, unlike non-diabetic patients where inflammatory activity is less indicative of patient outcomes and the main predictive markers are more general, reflecting comorbidities.

Nondiabetic

Biomarker	Threshold	Youden_j	Sensitivity (%)	Specificity (%)
D-Dimers	180	0.604	90	70.37
eGFR	69.84	0.589	73.91	85
IL6	36.27	0.581	63.64	94.44
Creatinina	1	0.571	69.57	87.5
Age	58.9	0.545	86.96	67.5
Potasium	4.5	0.48	60.87	87.18
ESR	18	0.444	72.22	72.22
NLR	5.51	0.439	77.27	66.67
Neutrophile	11.13	0.437	59.09	84.62
Feritine	235	0.422	63.64	78.57

Diabetics

Biomarker	Threshold	Youden_j	Sensitivity (%)	Specificity (%)
IL-18(pg/ml)	206.1	0.398	58.82	80.95
HDL1	46	0.375	50	87.5
Triglicerido1	131	0.375	73.68	63.83
NETS-Sytox_Green	143020	0.362	60.61	75.61
NLR	5.829787	0.325	65.79	66.67
Neutrophile	11.83	0.317	42.11	89.58
Fibrinogen	367	0.301	74.29	55.81
S100A8/9(ng/ml)	2197.97	0.296	67.65	61.9
Limfocite	1.88	0.295	84.62	44.9
IL-1b(pg/ml)	2.28	0.276	66.67	60.98

Figure 3. *Assessment of sensitivity and specificity of markers in predicting prognosis for patients stratified by diabetic status. Results of linear logistic regression analysis show parameters ranked from the best to the worst predictor of prognosis.*

We used the most specific and sensitive parameters separately for diabetics and non-diabetics to construct prognostic scores. To identify the best linear separation between patients with favorable versus unfavorable prognosis, linear support vector machine (SVM) analysis was performed based on the top 8 predictors for each group.

We defined Score N for non-diabetics and Score D for diabetics.

The following prognostic equations were obtained:

$$ScorN = DDimeri/eGFR$$

Score N showed a sensitivity and specificity of 85% for all study patients when a prognostic threshold value of 2.61 was set.

$$ScorD = IL\,18 * \sqrt{(NEETs)}$$

Score D exhibited a sensitivity of 58% and specificity of 78% for all study patients when a prognostic threshold value of 82,864 was set.

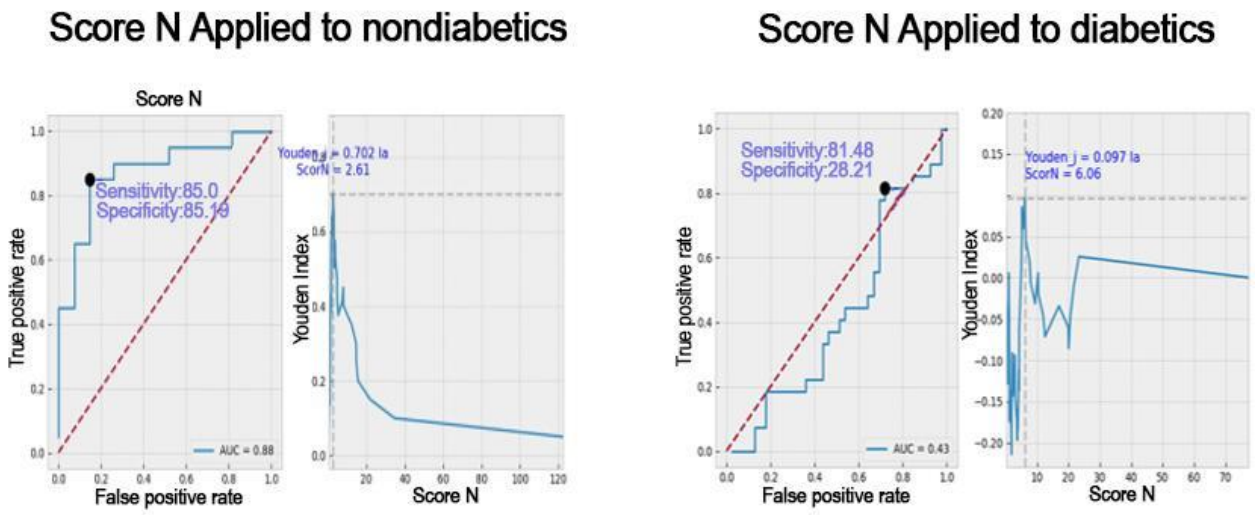

Figure 4. *ROC curve for evaluating applicability for scoreN in non-diabetic patients (left) and diabetic patients (right)*

The non-diabetic score had completely lacked in value for diabetic patients. **(Figure 4)**

2. Discussion of Study 1:

Regarding the ***prognostic implication of inflammatory parameters in patients with acute myocardial infarction (AMI) during hospitalization***, Study 1 showed that inflammation is closely related to in-hospital complications. ESR, CRP, fibrinogen, IL-1β, IL-18, IL-6, neutrophils, NLR, S100A8/A9, and NETs were significantly elevated in the group with in-hospital complications. Among the studied inflammatory molecules, the prognostic value of neutrophil activity biomarkers stands out. In our results, neutrophils are associated with in-hospital progression both quantitatively (by absolute count and NLR) and qualitatively through their products (S100A8/A9 and NETs), and by the inflammatory cascade triggered upon their activation, suggesting a hypothesis of a distinct, more aggressive neutrophil phenotype in patients with complicated progression.

Regarding the ***comparative evaluation of inflammatory parameters between groups (diabetics/prediabetics/nondiabetics)***, no differences were observed among diabetic, prediabetic, or nondiabetic patients. Considering the variety of evaluated inflammatory parameters, the clear

observation from this study is that there are no significant differences in hyperacute inflammatory response between patient groups. This may likely be explained by chronic differences in inflammation between diabetic and nondiabetic environments being masked by the inflammatory cascade triggered during a critical event such as AMI.

When evaluating the ***prognostic value of different markers associated with complicated progression***, we demonstrated that the best predictors for nondiabetic patients did not exhibit the same specificity and sensitivity for diabetic patients. Furthermore, Score N, a prediction score for in-hospital complication in nondiabetics, with a sensitivity and specificity of 85% for these patients, showed no predictive value for diabetic patients.

The most important parameters for diabetic patients were primarily inflammatory markers, particularly neutrophil activation products. The markers that predict unfavorable evolution in nondiabetic patients, were mostly, general parameters, related to comorbidities such as renal function impairment (creatinine/eGFR), coagulation activity (D-dimers), hypokalemia, or age.

Study 2 - In vitro. Evaluation of the inflammatory profile of neutrophils in patients with acute myocardial infarction

1. Materials and Methods

Obtaining and Processing Neutrophils

Peripheral blood samples from 35 patients with ACS, with or without diabetes, were collected in Vacutainer EDTA blood collection tubes. After blood collection, neutrophils were isolated by gradient centrifugation using Polymorphoprep (Proteogenix) or using a human neutrophil isolation kit (Milteni) according to the manufacturer's instructions [33]. After isolation, neutrophils were subjected to:

- Total RNA isolation using the RT-PCR technique
- Protein expression determination by Western Blot
- Measurement of reactive oxygen species
- Evaluation of cellular metabolism using Seahorse
- Detection of NETs by fluorescence microscopy
- Quantification of neutrophil elastase (NE) activity

2. Study Results 2.

Exacerbated inflammatory profile of neutrophils from MI patients and unfavorable evolution

Since clinical data showed that unfavorable post-MI evolution was associated with an increased inflammatory state, we characterized and compared the inflammatory profile of neutrophils isolated from MI patients with or without unfavorable evolution. As seen in **Figure 5**, neutrophils isolated from MI patients with unfavorable evolution (MI_UE) showed a significantly increased gene expression of most of the investigated pro-inflammatory molecules: CCL3, IL-1β, IL-18, S100A9, and the cell adhesion molecule ICAM-1.

Given that the functionality of these molecules is determined by their protein-level expression, some of these molecules were also investigated at the protein expression level.

Protein expression analysis confirmed the gene expression results, with Western Blot data showing that the protein expression of pro-inflammatory molecules S100A9, MCP-1, and IL-1β was significantly higher in neutrophils from patients with negative evolution (**Figure 6**).

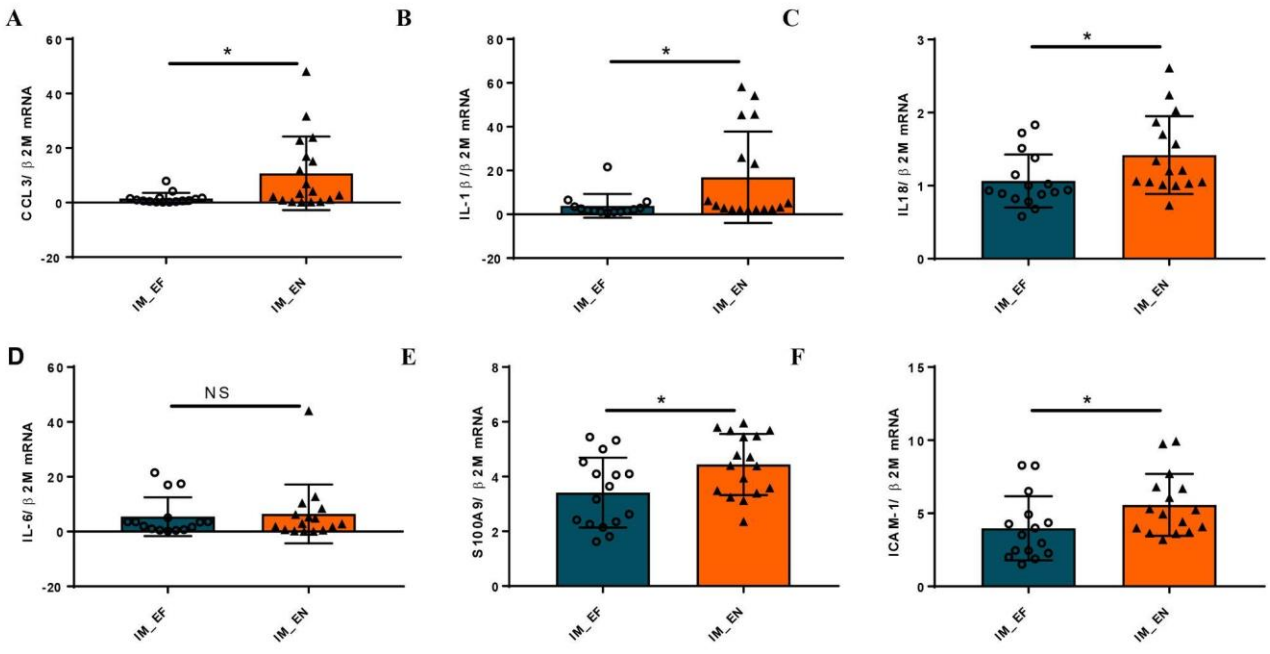

Figure 5. *Neutrophils from ACS patients with a negative prognosis exhibit increased expression of proinflammatory genes. (A-E) Expression of genes associated with an inflammatory phenotype of neutrophils, including CCL3, IL-1β, IL-18, IL-6, S100A9, and ICAM-1, investigated in neutrophils from patients with ACS (MI) or patients with ACS and unfavorable outcome (MI_EN). *p < 0.05, **p < 0.01, (MI_UE vs. MI).*

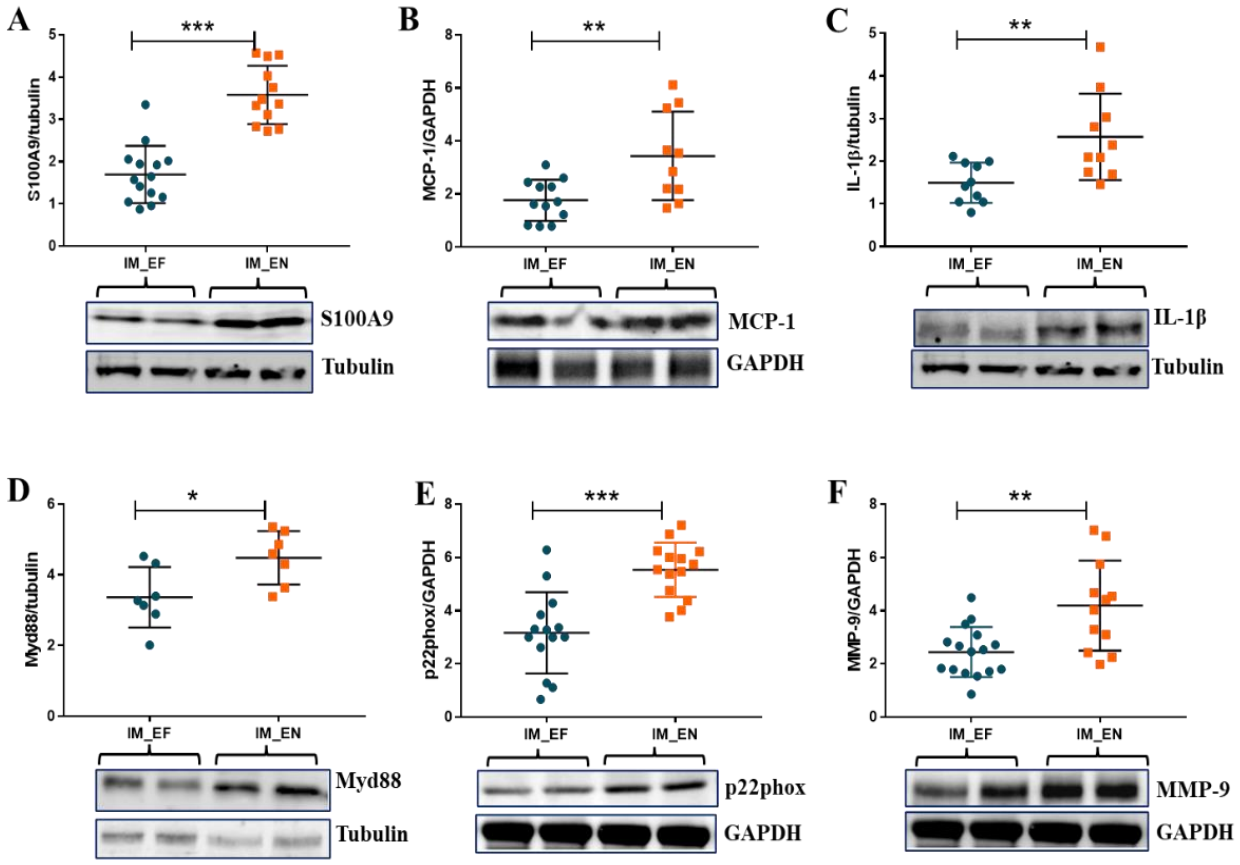

Figure 6. *Protein expression of proinflammatory molecules expressed by neutrophils isolated from ACS patients with or without negative prognosis (A-D). Quantification of protein expression and representative images of proinflammatory molecules S100A9, MCP-1, IL-1β, and Myd88, as determined by Western Blot. (E, F) Quantification of protein expression and representative images of p22phox and MMP-9, as determined by Western Blot. *p < 0.05, ** p < 0.01, *** p < 0.001 (ACS patients versus ACS patients with unfavorable outcome - MI_UE)*

Inflammatory profile of neutrophils in MI patients, with or without diabetes

The results obtained did not show significant differences in the gene expression levels of the pro-inflammatory molecules CCL3, IL-1β, IL-18, IL-6, S100A9, and ICAM-1 **(Figure 7 A-G)**. This results were consistent with the clinical study performed on MI patients with or without diabetes. In our study, although the analysis of inflammatory molecules did not show significant differences between the neutrophils of MI patients with or without diabetes, the molecules p22phox **(Figure 7 H)**, which are part of the active NADPH oxidase complex involved in ROS production, and MMP-9

expression **(Figure 7 E)** were significantly increased in neutrophils from diabetic patients compared to non-diabetic patients.

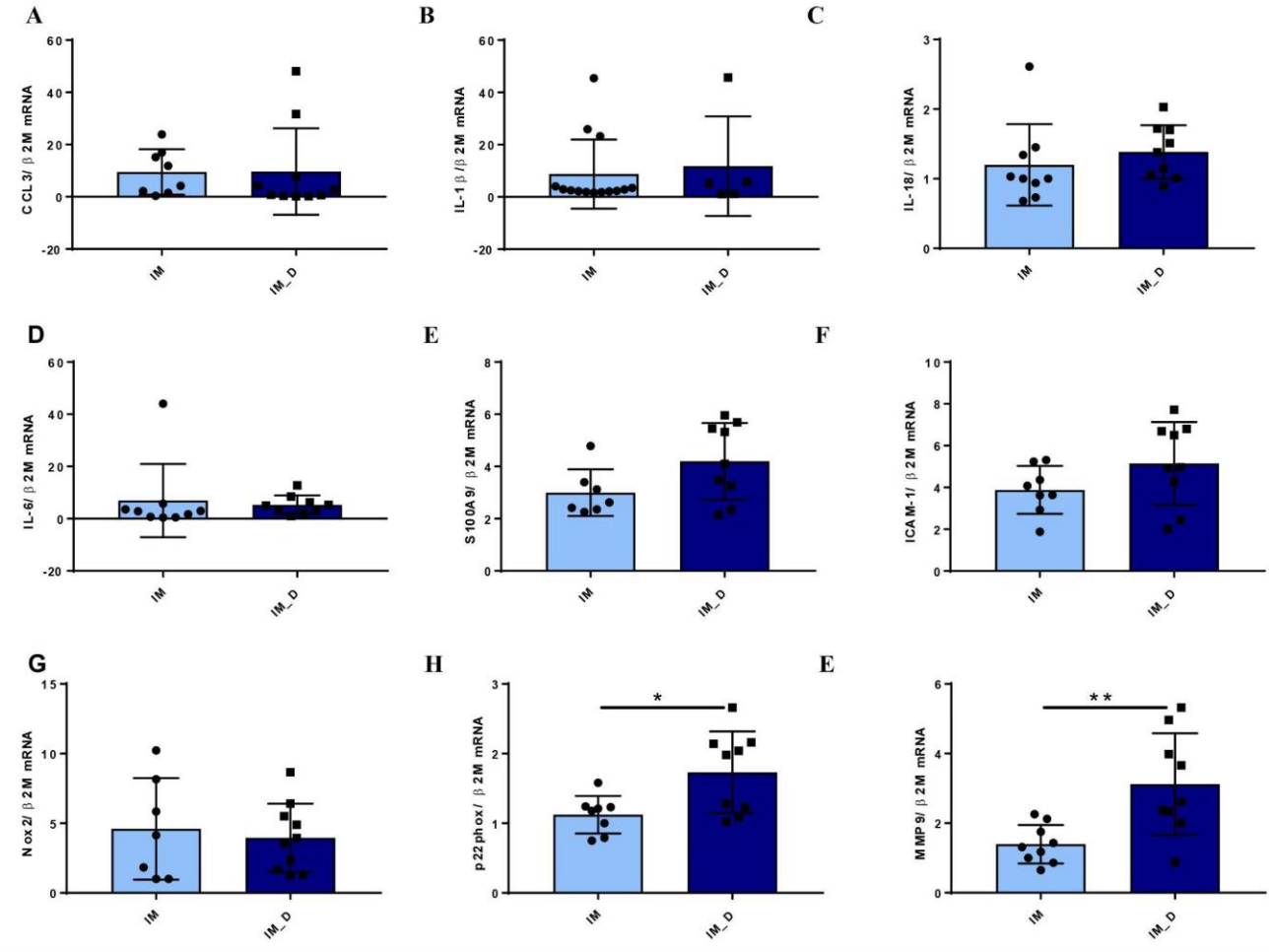

Figure 7. *Gene expression of proinflammatory molecules in neutrophils isolated from myocardial infarction (MI) patients with or without diabetes (D). qPCR analysis (A-E) of pro-inflammatory markers CCL3, IL-1β, IL-18, IL-6, S100A9, and ICAM-1 - NS (non-significant). Expressions of p22phox and MMP-9 molecules are significantly increased in neutrophils isolated from diabetic patients compared to non-diabetics (H-E). *p < 0.05, **p < 0.01 (MI_D versus MI).*

To confirm that the oxidative stress is the one that differentiates between neutrophils of patients with MI with or without diabetes, we further analyzed species of reactive oxygen (ROS) produced by isolated neutrophils from the two groups of patients.

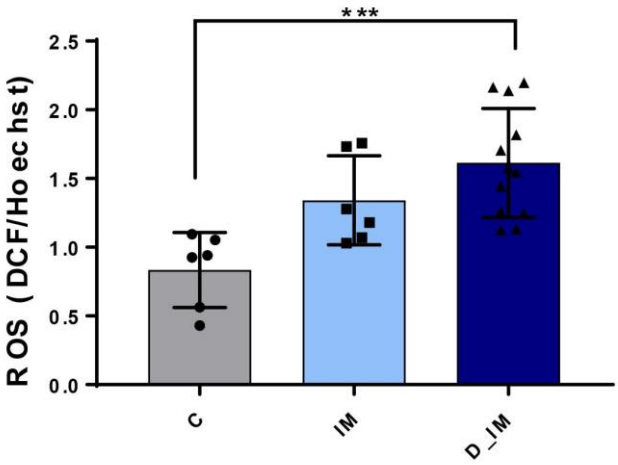

Figure 8. *Intracellular ROS quantification was performed using the DCF-DA fluorophore. Data are presented as fold change compared to control ± SE (standard error). Statistical significance is indicated as ***<0.001*

The results obtained show that indeed, neutrophils from MI patients with diabetes produce a higher level of ROS compared to neutrophils from MI patients without diabetes and a significantly higher level, almost double, compared to healthy subjects' neutrophils (**Figure 8)**.

NET expression in serum and neutrophils of MI patients

In addition to inflammatory molecules, neutrophil activation and degranulation also involve the formation of NETs and the release of various enzymes, including neutrophil elastase (NE) and myeloperoxidase (MPO), which play key roles in host defense and various pathologies [34,35].Therefore, we compared NET levels in the conditioned medium of neutrophils from healthy controls and MI patients who were exposed to 50nM PMA for 1 hour using Sytox green. The results showed that MI patients have increased NET levels **(Figure 9-left)**. This finding was confirmed by fluorescence microscopy, where neutrophils from MI patients released more NETs compared to control neutrophils after 2 hours of culture **(Figure 9-right)**.

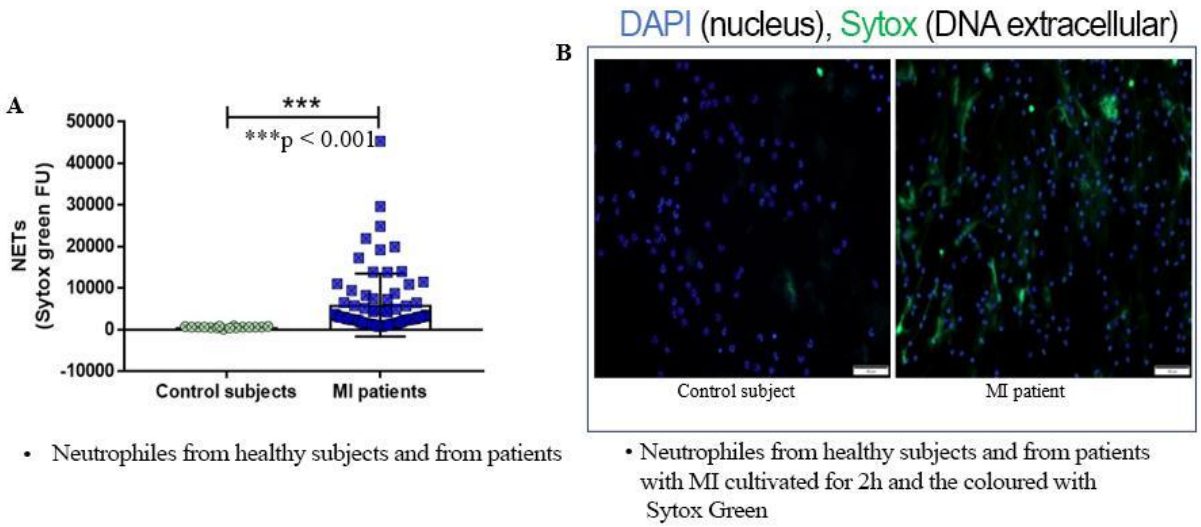

Figure 9 *Evaluation of NETs released by neutrophils from MI patients. (A) NET levels in the conditioned medium of neutrophils from healthy subjects and MI patients, exposed for 1 hour to 50 mM PMA, assessed by Sytox green staining. (B) Fluorescent staining (Sytox Green) of NETs in neutrophils isolated from a healthy subject versus neutrophils isolated from an MI patient after 2 hours in culture (green). Neutrophil nuclei are stained with DAPI (blue). *** p < 0.001 MI patient versus healthy subject.*

Protein expression analysis of these enzymes showed significant differences in NE activity in neutrophils from MI patients with favorable versus unfavorable prognosis **(Figure 10-left)**. Additionally, increased elastase activity levels were observed in the secretome of neutrophils from diabetic patients with MI compared to non-diabetic patients **(Figure 10-right)**.

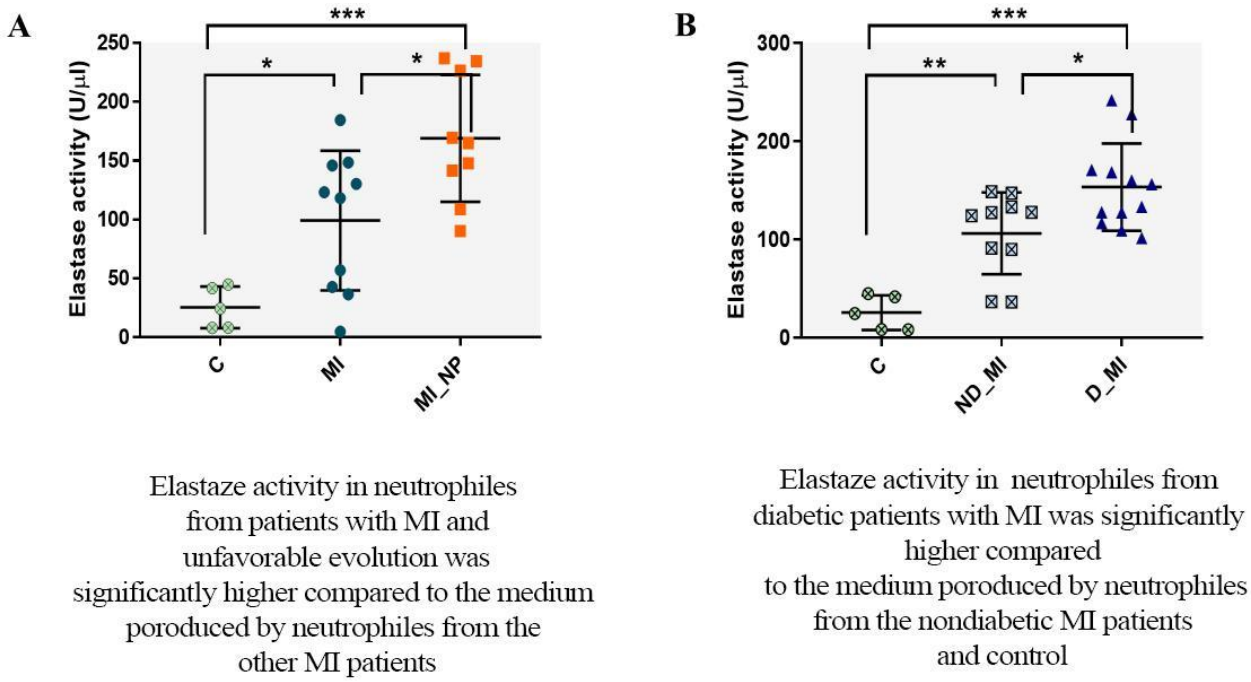

Figure 10 *Neutrophil elastase activity by group.*

3. Study 2 Discussions

In this chapter's study, we took a step forward and investigated the phenotype of neutrophils in MI patients, with or without diabetes, with favorable or unfavorable prognosis, to identify the neutrophil phenotype responsible for worsening post-MI outcomes. Detailed in vitro analysis of neutrophils from MI patients yielded interesting results that support clinical data, showing an altered, more aggressive neutrophil phenotype in MI patients with unfavorable outcomes. When neutrophils from healthy subjects were compared with those from MI patients, all inflammatory molecules were significantly increased in the neutrophils from MI patients. This altered neutrophil profile was also accompanied by an exacerbated level of NETs found in both serum and neutrophils from MI patients compared to healthy controls.

Comparative analysis of neutrophils from MI patients with unfavorable versus favorable outcomes highlighted a significant increase in inflammatory cytokines such as CCL3, IL-1β, IL-18, and the alarmin S100A9.

When the **gene expression of the same molecules was compared between neutrophils from MI patients with or without diabetes**, these significant differences disappeared, a result that may again suggest, as in the clinical study, that acute post-MI inflammation masks the specific effects induced by chronic hyperglycemia, making them undetectable in this context. Although inflammatory molecules did not show significantly different expression between diabetic and non-diabetic patients,

components of the NADPH oxidase enzyme complex, the main source of reactive oxygen species, were significantly increased in neutrophils from diabetic patients compared to non-diabetic patients. Additionally, oxidative stress-associated molecules, such as MMP-9, were significantly higher in neutrophils from diabetic MI patients compared to non-diabetic ones. MMP-9 is a potential biomarker for cardiac remodeling, as previously demonstrated in both animal models and clinical studies [36]. Quantification of ROS showed that neutrophils from MI patients with diabetes indeed produce higher levels of ROS compared to those from non-diabetic MI patients.

Elastase and myeloperoxidase are pro-inflammatory enzymes involved in tissue damage in various pathologies, including ischemic cardiovascular diseases [37,38]. The protein expression analysis of these granular neutrophilic molecules in the present study showed significantly increased NE levels in neutrophils from MI patients with unfavorable outcomes compared to those with favorable outcomes. Moreover, NE activity was found to be elevated in the serum of these patients, as well as in MI patients with diabetes, indicating the activation state of neutrophils. The increased expression and activity of NE in neutrophils from MI patients with unfavorable outcomes or diabetes could represent a contributing factor to adverse post-MI progression in these individuals. Study 2 confirmed our previous clinical findings, showing no significant quantitative differences in inflammatory parameters between diabetic and non-diabetic patients. However, the first biomarkers with predictive value for diabetic patients turned out to be inflammatory in study 1 , suggesting a more aggressive profile of inflammatory molecules, with neutrophil activity standing out. In vitro analysis showed indeed increased activity of ROS and NE in neutrophils in diabetics. Furthermore, MMP-9, a potential biomarker for cardiac remodeling, was significantly higher in diabetic MI patients.

Together, the data from this chapter provide evidence that the neutrophil phenotype in MI patients undergoes transformations that may be responsible for the unfavorable in-hospital evolution of MI patients. Notable differences in the inflammatory profiles observed in neutrophils from MI patients with complicated in-hospital evolution, which involve potential alterations in the innate immune response in this specific patient group, may partially explain the role of altered neutrophil phenotypes in disease progression.

Final Conclusions and Personal Contributions

In conclusion, our data indicate that unfavorable in-hospital evolution of MI patients is associated with systemic inflammatory markers and that neutrophil biomarkers are more predictive of short-term prognosis in diabetics.

Circulating neutrophils in MI patients exhibit a modified pro-inflammatory phenotype even before reaching the infarcted area, a phenotype that is more pronounced in patients with a negative prognosis. Significant differences in the inflammatory profiles of neutrophils from MI patients with complicated in-hospital evolution suggest potential changes in the innate immune response in this specific patient group, with potential adverse effects on disease progression. The study introduces novel elements by evaluating MI risk parameters through the lens of more homogeneous groups, separating patients based on diabetic status, given the different known prognosis for diabetic patients.

Our study, integrating concepts from cardiology, diabetology, and immunology, provides a clearer perspective on pathological mechanisms that are not limited to a single system or organ and originate at molecular level.

The main limitations of this study stem from the short-term evaluation of patients during the index event. These limitations arise from the study's complex design, aiming to profile MI risk and conduct detailed in vitro analysis of the main clinical observations and the fact that patient enrollment and evaluations were conducted during the SARS-CoV-2 pandemic restrictions.

It is also important to note that the overall results generated by this doctoral thesis represent a collective effort of the entire team from the Cardiology Clinic of Elias University Emergency Hospital, particularly the Laboratory of Biopathology and Inflammation Therapy at the "Nicolae Simionescu" Institute of Cellular Biology and Pathology.

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