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Comparison of RANKL expression, inflammatory markers, and cardiovascular risk in patients with acute coronary syndrome with and without rheumatoid arthritis

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Abstract

The mechanisms responsible for increased cardiovascular risk in patients with rheumatoid arthritis (RA) involve local and systemic inflammatory processes. We aimed to compare inflammatory markers and mortality risk in patients with acute coronary syndrome (ACS) with and without RA. The study involved 95 ACS patients (46 with RA and 49 without RA) and 40 healthy controls. Serum levels of Receptor Activator of Nuclear Factor Kappa B Ligand (sRANKL), Osteoprotegerin (sOPG), high-sensitivity C-reactive protein (hs-CRP) and high-sensitivity Tropinin I (hs-TnI) were tested in all participants. Additionally, ACS patients were assessed on RANKL expression (exRANKL) on coronary arteries and mortality risk on the Global Registry of Acute Coronary Events scale (GRACE). exRANKL was established in 35 (76%) ACS patients with RA, vs. 19 (39%) patients without RA, $p < 0.001$. RA patients had significantly higher levels of sRANKL and sOPG at 24 h and 48 h compared to ACS patients without RA and healthy controls (sRANKL 24 h: 121.33 vs. 51.67 vs. 36.94, $p = 0.019$; sRANKL 48 h: 89.21 vs. 36.95 vs. 36.94, $p = 0.004$; sOPG 24 h: 207.71 vs. 69.39 vs. 111.91, $p < 0.001$; sOPG 48 h: 143.36 vs. 69.38 vs. 111.91, $p < 0.001$). RA patients had significantly higher RANKL:OPG ratio at 48 h (0.062 vs. 0.53 vs. 0.33, $p < 0.001$), hs-CRP (28.82 vs. 23.67 vs. 2.60, $p < 0.001$) and hs-TnI (0.90 vs. 0.76 vs. 0.012). GRACE risk score was significantly higher in RA patients vs. those without RA (140.45 vs. 125.50, $p = 0.030$) and correlated with exRANKL, RANKL:OPG, hs-CRP, and hs-TnI. Our results indicate that exRANKL, inflammatory markers and mortality risk are amplified in ACS patients with RA compared to ACS patients without RA.

Keywords Acute coronary syndrome · Rheumatoid arthritis · Inflammatory biomarkers · Mortality · RANKL · GRACE

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Introduction

Systematic inflammatory joint diseases (IJDs), among which rheumatoid arthritis (RA), are associated with an increased risk and more frequent occurrence of cardiovascular disorders (CVD), subclinical atherosclerosis and earlier endothelial dysfunction leading to pathological remodeling of the arterial wall [1]. Over 50% of premature deaths among patients with RA are due to cardiovascular diseases (CVD) [2].The risk of cardiovascular events in patients with IJD is 1.5 times higher than that of the general population, and their average lifespan is lower by 10 years [3].

The mechanisms responsible for the increased CV risk in patients with IJD are complex, involving local and systemic factors such as: level and extent of systemic inflammation, disease activity, type of treatment, vessel condition, co-morbidity, and others [4, 5]. According to Castellon and Bogdanova chronic inflammation and oxidative stress are

the major etiological factors leading to changes in CV structures [6]. The fundamental role of chronic inflammation in the pathogenesis of atherosclerosis is collaborated by clinical studies of risk factors and assessments of acute-phase parameters in patients with IJD. It has been found that controlling inflammation can prevent an additional event over a period of 10 years for every 400–500 people [7].

Inflammatory biomarkers, such as cytokines, acute-phase proteins and proinflammatory cells, associated with inflammatory joint and cardiovascular diseases have been identified and assessed by a substantial body of related research [8, 9]. The diagnostic and predictive importance of Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) on atheromatous plaque and in serum, Osteoprotegerin (sOPG), RANKL:OPG ratio, high-sensitivity C-reactive protein (hs-CRP), high-sensitivity Tropinin I (hs-TnI) has been extensively studied in RA and CV states; yet separately for each disease, whereas comparative research is rare. This fact served as the main motivation for our study, which aimed to compare patients with acute coronary syndrome (ACS) with and without RA on the following parameters: RANKL expression (exRANKL); serum levels of RANKL (sRANKL) and OPG (sOPG); RANKL:OPG ratio; and acute-phase inflammatory markers hs-CRP and hs-TnI. Additionally, we sought to investigate possible links between mortality risk assessed on the Global Registry of Acute Coronary Events (GRACE) scale and RANKL expression, serum levels of RANKL, OPG, RANKL:OPG ratio, hs-CRP and hs-TnI.

The RANKL–OPG axis

Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL), a member of the tumor necrosis factor alpha (TNFa) family, is considered a trigger of osteoclastogenesis [10]. Osteoprotegerin (OPG) produced by bone-forming osteoblasts acts as an inhibitor of RANKL. Thus, elevated RANKL levels in inflammatory conditions are often linked to insufficient OPG synthesis [10, 11]. The RANKL:OPG ratio plays an important role in regulating bone metabolism, cardiovascular disease, and other conditions. In ACS, RANKL is found in unstable atherosclerotic plaques and is expressed by different cell types [12].

The RANKL/OPG axis also plays an important role in cardiovascular conditions as shown by a number of studies [13]. Sandberg et al.[14] have observed an extreme expression of RANKL in patients with acute myocardial infarction (AMI) at the 4th hour after AMI and a return to baseline after 24 h [14]. In another study, also involving patients with AMI, OPG levels were significantly higher in patients with AMI at the first h after AMI than those in patients with coronary artery disease and in clinically healthy subjects.

These findings suggest that OPG is increased in response to vascular wall damage, plaque rupture, and ongoing inflammation within the atherosclerotic plaque lesion as part of a complex compensatory mechanism in which RANKL plays a central role [14, 15]. Serum OPG levels may be indicative of a persistent damage to endothelial cells and the activation of vascular smooth muscle cells (VSMCs) in advanced atherosclerotic plaque lesion. Elevated levels of OPG are associated with the proinflammatory base responsible for the progression of atherosclerosis [15].

Hs-CRP and hs-TnI

The serum C-reactive protein (CRP) is an acute-phase protein which has proven a reliable biomarker of systematic inflammation in a variety of disease states. Elevated levels of high-sensitivity CRP test (hs-CRP) are observed in patients with RA as well as in coronary artery disease (CAD) [16]. CRP is considered a robust indicator of the degree of risk in patients with acute coronary syndrome (ACS) and a reliable predictor in the prognosis of cardiovascular disease in healthy individuals [16]. Hs-CRP levels are standardized and show the degree of cardiovascular risk as follows: values < 1 mg/l indicate low risk; values between 1 and 3 mg/l indicate moderate risk; and those > 3 mg/l are associated with higher risk which increases as CRP values rise [19].

Tropinin I (TnI) plays an important role in the diagnosis, risk assessment and treatment of patients with acute coronary syndrome (ACS) [17]. The more recent assay involves high-sensitivity troponin I (hs-TnI) which has an improved ability to detect small amounts of myocardial necrosis. Cullen et al. analyzed 5-year data from patients with ACS, demonstrating a significant relationship between elevated levels of baseline hs-TnI and the risk of CV death, acute myocardial infarction, ischemic stroke, and heart failure [18, 19].

GRACE risk score

Various scales exist for assessment of mortality and morbidity risk in ACS patients, among which the Global Registry of Acute Coronary Events (GRACE) risk score has been found to be more reliable and easier to use than other risk scores [20]. The GRACE score assesses a patient's risk of death and myocardial infarction in short term and 6 months after ACS. The calculation involves clinical data that have been found predictive of adverse events, including age, heart rate/pulse, systolic blood pressure, creatinine, cardiac arrest at admission, ST-segment deviation, abnormal cardiac enzymes, and Killip class [20].

Methods

The study followed a case–control research design. The participants included cardiac patients with and without rheumatoid arthritis (RA) and a group of clinically healthy controls. All participating patients underwent coronary artery bypass (CABG) in the clinic of cardiac surgery at the Medical University of Plovdiv, Bulgaria in the period between April 2014 and December 2016. All procedures performed in this study were in accordance with the World Medical Association Declaration of Helsinki (1964) and its revised version (2000), Edinburgh. Ethical approval was obtained from the Local Ethics Committee at the Medical University of Plovdiv (Date: 05.03 2014). Informed consent in written form was provided by each participant in the study according to the requirements of theWMA Declaration of Helsinki. The cardiac patients provided informed consent at their admission to the hospital. The patients were fully conscious and aware of the voluntary nature of their involvement and their right to decline participation without any reflection on their treatment. They were familiarized with every detail of the study before they were offered to sign a written consent form. Intraoperatively, each patient's condition was further assessed by the surgical team and the material was taken only when the assessment showed no risk for the patient. Informed consent in written form was also obtained from the clinically healthy controls following the ethics requirements of the WMA Declaration of Helsinki (1964) and its latest revisions. The healthy controls were recruited from individuals who paid visits to doctors in the consulting rooms of the Medical University of Plovdiv for prophylactic medical exams or other consultation.

The study included 95 ACS patients of mean age 69.59 ± 7.22, all of whom underwent coronary artery bypass graft (CABG) in the clinic of cardiac surgery at the Medical University of Plovdiv. Among them, 46 were RA seropositive (mean age 68.74 ± 7.0) and 49 were without RA (mean age 70.39 ± 7.40). There was no significant difference between the groups in mean age, $p = 0.268$. Both groups were predominantly male (80% male with RA; 77.5% male without RA), with no significant difference in the proportions of male and female patients in the groups with and without RA, $p = 0.730$.

Our assessment of disease activity states in the RA patients through the Disease Activity Score in 28 joints (DAS28) showed the following distribution of states: 53% ($N = 24$) of the RA patients were in remission or low disease activity DAS $28 \leq 3.2$; 30% ($N = 14$) were in moderate disease activity (DAS28 > 3.2 and ≤ 5.1); and 17% ($N = 8$) showed high disease activity (DAS28 > 5.1).

Among the RA patients, 15 were treated with Methotrexate 20 mg/weekly; 10 with Leflunomide 20 mg/day; 9 with Methylprednisolone 8 mg/day and Methotrexate 20 mg/weekly; 5 with Methylprednisolone 8 mg/day and Leflunomide 20 mg/day; 3 with Methotrexate 20 mg/weekly and Etanercept 50 mg/weekly; 3 with Methotrexate 20 mg/weekly and Infliximab 3 mg/kg/infusion; and 1 with Tocilizunab 162 mg/weekly.

The inclusion criteria were as follows: (a) ACS diagnosis with evidence for acute ischemic changes and coronary vessel occlusion, established through ECG and SCAG (selective coronary artery graft); (b) hospital admission within the first 24 hours after the CV event; (c) signed informed consent by each patient in full consciousness for all tests and procedures to be performed; (d) no risk for the patient in all procedures and tests that were carried out. In addition, the patients with RA had to have an established RA diagnosis according to the ACR 1987 and ACR/EULAR 2010 classification criteria.

The exclusion criteria for the ACS patients with and without RA included: (a) refusal to sign the informed consent form; (b) high operative risk; (c) intraoperative risk not allowing the material to be taken; (d) the presence of any of the following diseases: renal impairment, hypogonadism, diabetes, and cancer; (e) current treatment with Denosumab.

The study also involved 40 clinically healthy controls, whose mean age (66.93 ± 5.93) was not significantly different from the patients' groups, $p = 0.64$. In the healthy control group, the sex distribution was similar to that of the patients, 75% male vs. 25% female. The inclusion criteria were as follows: (a) signed informed consent form by each participant; (b) clinically healthy individuals with normal levels of ESC, CRP and Fibrinogen. Individuals who declined to sign the informed consent form or had past or present CVD, IJD, cancer, other active inflammation, high levels of the acute-phase markers, and those taking Denosumab were not included in our sample of healthy controls.

All tests were performed in the clinical laboratory of the Clinic of Cardiac Surgery at the Medical University of Plovdiv. The procedures were carried out in compliance with the standards for conducting research with human subjects determined by the WMA Declaration of Helsinki (1964). RANKL expression was assessed from coronary artery samples, taken during traditional CABG or off-pump coronary artery by-pass (OPCAB) surgery. As mentioned in the inclusion criteria, the condition of each patient was assessed intraoperatively, and material was taken only if the procedure was estimated as risk-free.

The obtained material was stabilized in paraformaldehyde for histology and immunohistochemistry according to the instructions and was given for processing and fixation in paraffin blocks, hematoxylin–eosin staining and biomicroscopy. The anti-TNFSF11 antibody kit (RANKL), Rabbit, Polyclonal, IgG, solution 1:100) supplier Bioss-antibodies and the CRF™ anti-Polyvalent HRP Polymer (DAB) Stain Kit ScyTek laboratories were used. The reading of

the corresponding expression in the nucleus, cytoplasm or cell membrane was consistent with the instructions provided in the manufacturer's protocol for the respective antibody. RANKL expression was assessed quantitatively and qualitatively. To increase the reliability of the coding, the staining intensity was determined in comparison to positive and negative controls. The negative control was a 20-year old deceased healthy individual who had died during a car accident. The material was obtained with the written consent of his parents in full compliance with the requirements of the WMA Declaration of Helsinki (1964) and its recent amendments/revisions.

The expression of RANKL was coded in four levels: 0—negative; 1—weak; 2—moderate; 3—strong. The levels were determined semi-quantitatively. We measured the staining intensity (0—no staining; 1—weak; 2—moderate; 3—strong) and calculated the percentage of positively stained cells: 0–10% positively stained cells were coded as 0 (no staining); 10–39% positively stained cells were coded as 1 (weak staining); 40–69% were coded as 2 (moderate staining); and 70–100% were coded as 3 (strong staining). The levels of exRANKL were obtained as the sum of both scales (minimum of 0 and maximum of 6). The combined scores from both scales were used in determining the exRANKL levels as follows: score of 0 from both scales = negative; sum of both scales 1 or 2 = weak expression; sum of 3 or 4 = moderate expression; sum of 5 or 6 = strong expression (Fig. 1).

All patients were admitted within 24 h from the onset of ACS and RANKL and OPG serum levels were measured at the 24th and 48th hour after the onset of ACS.

RANKL serum was analyzed with the commercially available protocol Tumor Necrosis Factor (Ligand) Superfamily, Member 11(TNFSF11), sandwich ELISA, detection range 2.74–2.000 pg/ml, supplier: Cloud-Clone. The inclusion of sRANKL at the 48th hour aimed to assess its role as a biomarker of RA in ACS patients and to examine its relationship with exRANKL, hs-CRP and cardiovascular risk.

OPG serum levels were determined by TRANCE/TNFSF11/RANKL ELISA Tumor Necrosis Factor Receptor Superfamily, Member 11b (TNFRSF11B), sandwich ELISA, detection range 1–900 pg/mL minimum detection 1 pg/ml, supplier: RayBiotech. The preparation of all reagents and samples was done according to the manufacturer's instructions and at room temperature.

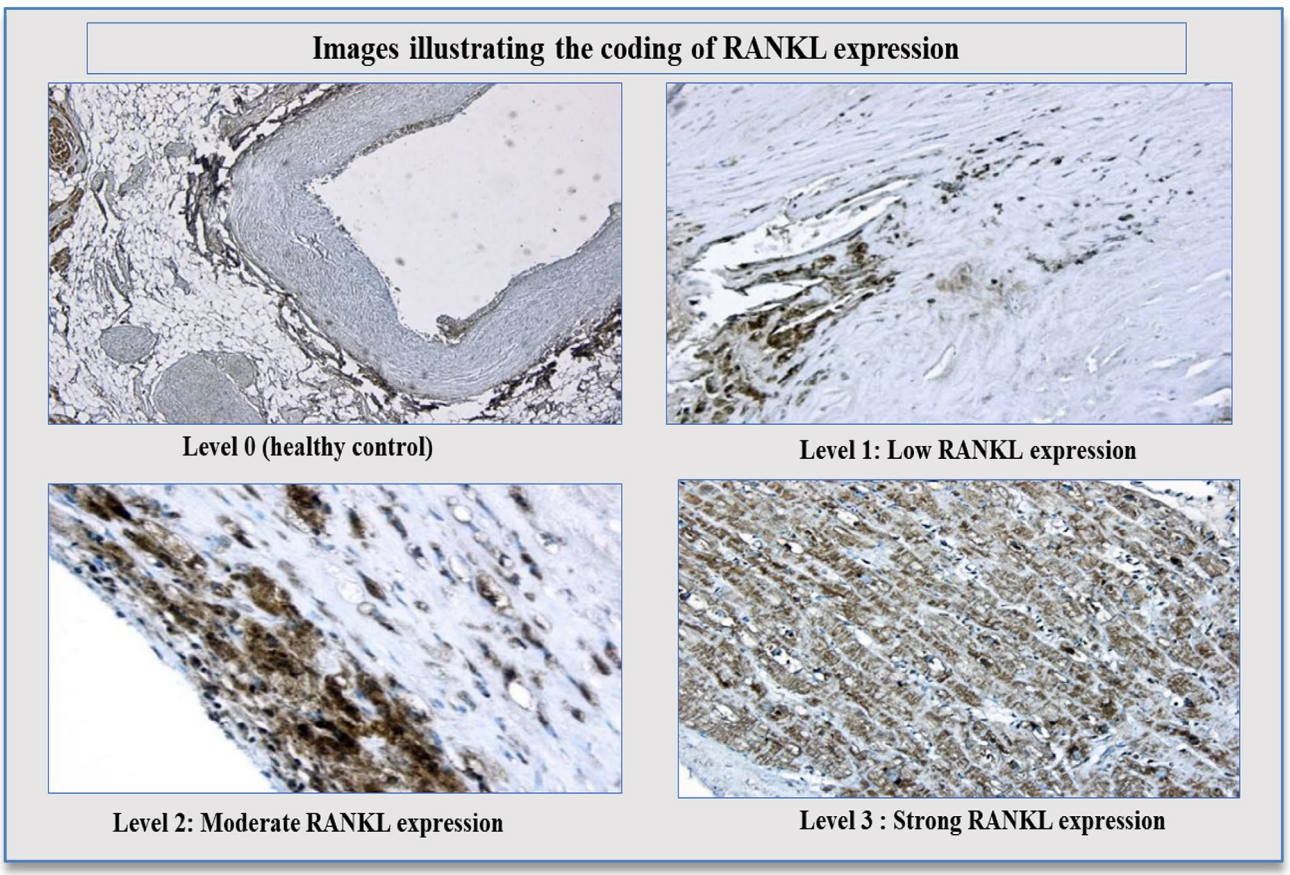

Fig. 1 Images for the four levels of RANKL expression

Hs-CRP was measured with the High-Sensitivity C-Reactive Protein (hs-CRP) kit of AMP Diagnostics BR- 5420-S, with reference value for CV risk > 3 mg/l. High sensitive Troponin I (hs-TnI) was established at the time of patients' admission to hospital and within the 12-h period after admission. The peak value for each patient was included in the data analysis.

GRACE Risk Score was interpreted according to the reference ranges for stratifying risk of mortality determined by the European Society of Cardiology (ESC): (1) in hospital-low risk: GRACE risk score ≤ 108; moderate risk: GRACE risk score 109–140; high risk: GRACE risk score > 140; (2) At 6th months after discharge—low risk: GRACE risk score ≤ 88; moderate risk: GRACE risk score 89–118; high risk: GRACE risk score > 118 [20].

Data analysis

We used the Statistical Package for the Social Sciences (SPSS), version 24 [21] to analyze the data. RANKL expression was measured on an ordinal scale (0–3) and the analysis involved cross- tabulation, Chi square test, and Fisher's exact statistics for comparison of two proportions (e.g., ACS with RA exRANKL positive cases vs. ACS without RA exRANKL positive cases). We found high skewness and lack of homogeneity of variances on most of the other variables, including sRANKL at the 24th and 48th hour, sOPG at the 24th and 48th hour, hs-CRP, and hs-TnI. In such cases, we used the Kruskal–Wallis test to compare more than two groups, which, when significant, was followed by pairwise comparisons through the Mann–Whitney test. RANKL:OPG ratios and GRACE risk score were normally distributed which allowed the use of one-way ANOVAs and Bonferonni post hoc pairwise comparisons. Spearman rank correlation analysis was used to explore associations between ordinal and/or non-normally distributed variables, whereas in the case of normally distributed variables, measured on a continuous scale, Pearson correlations were run. Results were considered significant if p values were < 0.05.

Results

RANKL expression

RANKL expression (exRANKL) levels were cross-tabulated between the ACS patients with and without RA. The results revealed a significant difference in the distribution of exRANKL levels in the two groups, $p < 0.001$ (Fig. 2). Put together, the exRANKL-positive cases in the group of ACS patients with RA were 76% ($N = 35$) vs. 39% ($N = 19$) in the ACS patients without RA. The difference of 37% was significant, $p < 0.001$.

Inflammatory markers and GRACE risk score

The serum levels of RANKL, OPG, and RANKL:OPG at the 24th and 48th hour, as well hs-CRP and hs-TnI were compared among the following three groups: ACS patients with RA, ACS patients without RA, and clinically healthy

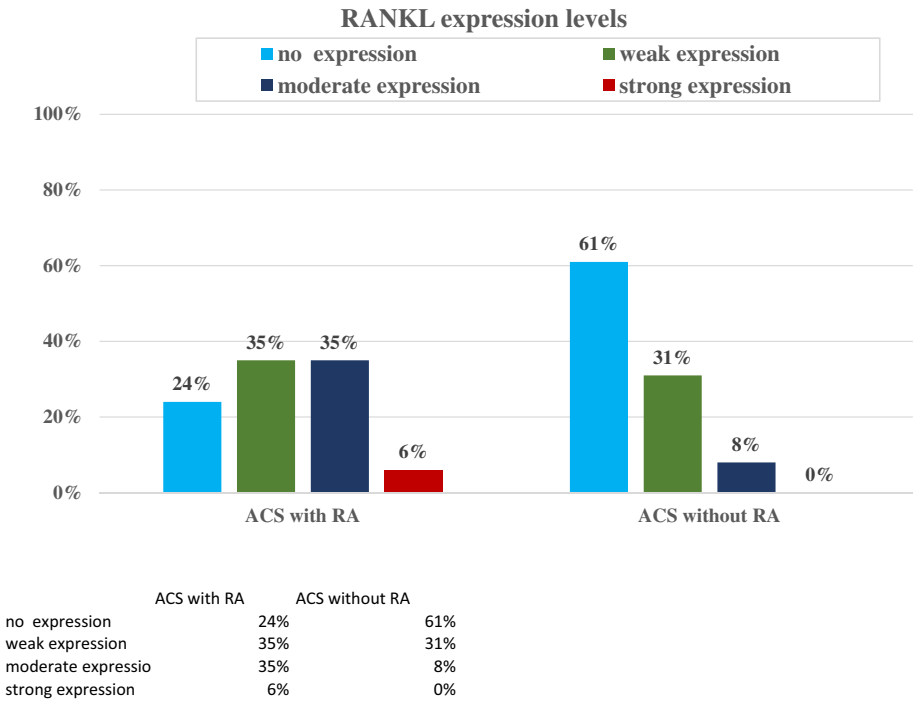

Fig. 2 Distribution of RANKL expression levels in the ACS patients with and without RA

Table 1 Comparisons of inflammatory parameters and GRACE risk score in ACS patients with and without RA and healthy controls

Inflammatory marker	ACS with RA ($N=46$)	ACS without RA ($N=49$)	Healthy controls ($N=40$)	p
sRANKL24hr	121.33 ± 124.52 (59.90)	51.67 ± 41.05 (43.37)	36.94 ± 11.20 (39.80)	0.019[a]
sRANKL48hr	89.21 ± 84.63 (49.30)	36.95 ± 32.85 (39.20)	36.94 ± 11.20 (24.30)	0.004[a]
sOPG24hr	207.71 ± 154.68 (135.23)	99.30 ± 75.33 (72.50)	111.91 ± 27.85 (102.10)	< 0.001[a]
sOPG48hr	143.36 ± 125.2 (103.20)	69.38 ± 59.98 (46.30)	111.91 ± 27.85 (88.20)	< 0.001[a]
RANKL:OPG24hr	0.58 ± 0.21 (0.55)	0.52 ± 0.22 0.67)	0.33 ± 0.07 (0.45)	< 0.001[b]
RANKL:OPG48hr	0.62 ± 0.20 (0.50)	0.53 ± 0.20 (0.49)	0.33 ± 0.07 (0.45)	< 0.001[b]
hs-CRP	28.82 ± 12.75 (29.30)	23.67 ± 16.25 (22.80)	2.60 ± 1.24 (2.30)	< 0.001[a]
hs-TnI	0.90 ± 0.99 (0.90)	0.76 ± 1.61 (0.10)	0.012 ± 0.0103 (0.01)	< 0.001[a]
GRACE risk score	140.45 ± 27.24 (142)	125.50 ± 28.58 (126.50)	NA	0.03[b]

Data are presented as mean ± SD and (median)

sRANKL ng/ml serum receptor activator of nuclear factor kappa B ligand, *OPG ng/ml* osteoprotegerin, *hs-CRP g/ml* high-sensitivity C-reactive protein, *hs-TnI ng/ml* high-sensitivity Tropinin, *GRACE* Global Registry of Acute Coronary Events

[a]Kruskal–Wallis test

[b]One-way analysis of variance (ANOVA)

controls. The results (Table 1) showed statistical significance on all parameters, which prompted multiple pairwise comparisons to identify the groups which were significantly different from each other.

The level of sRANKL24hr was significantly higher in the ACS patients with RA than those without RA ($p=0.030$) and in comparison to the healthy controls ($p=0.014$). In the ACS patients without RA, we found a higher level of sRANKL at the 24th hour than the healthy controls, but the difference was not significant, $p=0.252$. At the 48th hour, sRANKL remained high in the ACS patients with RA and was still significantly higher compared to those without RA ($p=0.001$) and the healthy controls ($p=0.001$). On the other hand, the level of sRANKL in the ACS patients without RA at the 48th hour decreased to the level observed in the healthy controls ($p=0.998$).

OPG24hr level was significantly higher in the ACS patients with RA in comparison to the ACS patients without RA ($p=0.001$) and the healthy controls ($p=0.020$). The ACS patients without RA and the healthy controls did not differ significantly, $p=0.892$. Regarding OPG48hr, the ACS patients with RA had a significantly higher level than those without RA ($p=0.002$); however, the difference with the healthy controls was not significant ($p=0.513$). No significant difference was found between the ACS patients without RA and the healthy controls, $p=0.06$.

The ratio of RANKL:OPG24hr showed significantly higher values in both patient groups with ACS in comparison to the healthy controls ($p<0.001$ for both comparisons), with no significant difference between them ($p=0.177$).

However, the trend changed regarding RANKL:OPG48hr ratio. Even though both patient groups still had a significantly higher RANKL:OPG ratio vs. the healthy controls ($p<0.001$ for both comparisons), in the ACS patients with RA we recorded a significantly higher RANKL:OPG48hr ratio in comparison to the ACS patients without RA ($p=0.04$).

High sensitivity CRP levels were significantly higher in the ACS patients with RA and without RA in comparison to the healthy controls ($p<0.001$ for both comparisons); however, between the two patient groups, a significantly higher level was detected in the ACS patients with RA ($p=0.012$).

Likewise, hs-TnI was significantly elevated in both patient groups vs. the healthy controls ($p<0.001$ for both comparisons), but the level in the ACS patients with RA was significantly higher in comparison to the ACS patients without RA ($p=0.01$).

The likelihood of death and myocardial infarction 6 months after ACS, calculated by the GRACE risk scale, was compared between the two patient groups: ACS patients with RA and without RA. The results showed a significantly higher mean GRACE risk score in the ACS patients with RA in comparison to those without RA, $p=0.030$. In view of the reference numbers provided by the ESC, the mean GRACE risk score of the RA group (140.45) indicated high in-hospital and at 6th month risk. The mean GRACE risk score of the group without RA (125.50) indicated moderate in-hospital risk and high risk at the 6th month.

Correlations between GRACE risk score, exRANKL and serum inflammatory markers ACS patients

We examined the correlations between mortality risk, assessed by the GRACE risk scale, and exRANKL, sRANKL (24 and 48 h), sOPG (24 and 48 h), RANKL/OPG ratio (24 and 48 h), hs-CRP and hs-TnI, within the whole ACS patient group, including both patients with RA and without RA. GRACE risk score showed a

significant correlation with five out of the ten parameters that we tested, including: exRANKL, $r^s = 0.289$, $p = 0.005$; RANKL:OPG ratio at the 24th hour, $r = 0.339$, $p = 0.001$; RANKL:OPG ratio at the 48th hour, $r = 0.298$, $p = 0.004$; hs-CRP, $r = 0.235$, $p = 0.022$; and hs-TnI, $r = 0.221$, $p = 0.033$. The other five parameters, including sRANKL (24 and 48 h), and sOPG (24 and 48 h) did not show a significant association with GRACE risk score ($p > 0.05$).

To illustrate the association between GRACE risk score and exRANKL, we created an individual value plot of GRACE risk score for each level of exRANKL. The mean GRACE risk scores for the exRANKL levels are also included (Fig. 3). The patients who had negative exRANKL had a mean GRACE risk score of 124. The individual scores are situated below and above the mean value, with a range of 74–149. The patients with weak expression of RANKL had a mean GRACE risk score of 138 and a range of 116–165. In the patients categorized with moderate RANKL expression, the mean GRACE risk score reached 144, with a range of 131–173. There were only three patients with strong RANKL expression, all of them with RA diagnosis. Their mean GRACE score was 149, range 129–176.

In view of the reference numbers, provided by the European Society of Cardiology for in-hospital risk—low risk (GRACE < 108); intermediate risk (GRACE 109–140), and high risk (GRACE > 140)—our results show that the mean GRACE risk scores of the patients with strong and moderate exRANKL fell in the range of high risk. The distribution of individual scores showed that the majority of the patients with moderate or strong exRANKL expression were associated with a high risk of dying; and only a few with moderate risk.

The mean GRACE risk score of the patients with weak expression of RANKL fell in the range of intermediate risk; however, the individual scores showed that this was true for 62% of the patients, whereas 38% were categorized as high risk. The patients with negative exRANKL had a mean GRACE score of 124, associated with intermediate risk. Besides, in this group, there were 5 patients (12.5%) whose GRACE risk scores fell below 108, indicating low risk of dying.

The remaining four significant correlations are illustrated on Fig. 4. They all show a positive association between GRACE risk score and the respective inflammatory marker. Increase in the RANKL:OPG ratio both at the 24th and 48th hour was associated with a higher risk. Likewise, higher hs-CRP and hs-TnI levels were linked to higher mortality risk. We must note, however, that although significant, these correlations are relatively weak.

Discussion

In our study, patients with ACS and RA were associated with a significantly higher rate of RANKL expression in low, moderate and high form compared to patients with ACS without RA. In fact, the majority of the ACS patients without RA showed no presence of RANKL expression. RANKL expression also showed a significant association with GRACE risk score as the mean GRACE scores for the moderate and strong levels of expression fell in the range

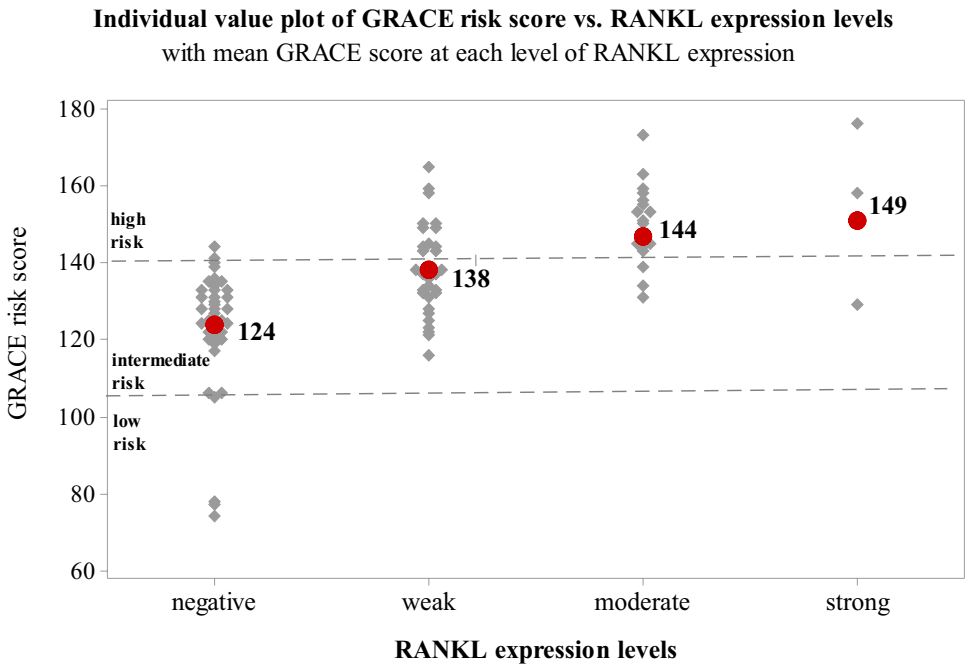

Fig. 3 Individual value plot of GRACE risk score vs. RANKL expression levels with mean GRACE values for each level of exRANKL

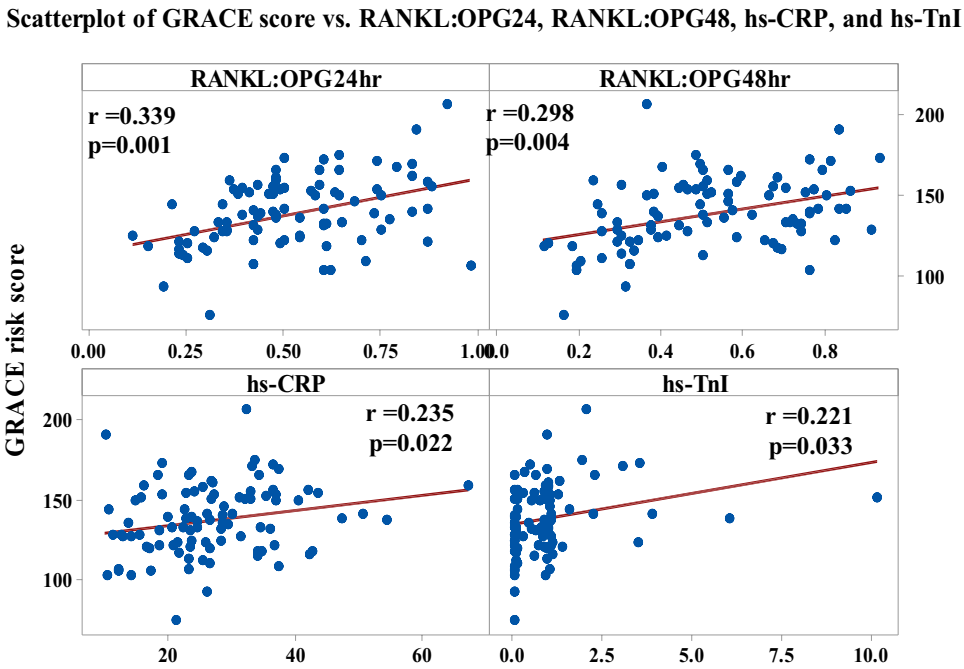

Fig. 4 Significant correlations of GRACE risk score with RANKL:OPG24hr, RANKL:OPG48hr, hs-CRP, and hs-TnI

of high risk of in-hospital death [22, 23]. The individual data revealed that only very few patients with moderate exRANKL had an intermediate risk of dying, and none were associated with low risk.

We also found significantly higher mean serum levels of RANKL at the 24th and 48th hour in the ACS group with RA, whereas the levels of the group without RA were very similar to those of the healthy controls. Likewise, the OPG mean serum levels at the 24th and 48th hour after ACS onset were significantly higher in the ACS patients with RA, whereas the ACS patients without RA had similar mean levels to those of the healthy controls. Previous studies (Sandberg et al. [14] have reported that elevated RANKL and OPG levels in patients with acute myocardial infarction decrease 24 h after its onset, but remain higher than those of healthy controls. However, in our study this finding is supported only by the trend observed in the group of ACS patients with RA, who maintained higher levels than both the healthy controls and the ACS patients without RA. On the other hand, the levels of the ACS patients without RA decreased and became almost equal to those of the healthy controls.

Yet, we should note here that taken separately, RANKL and OPG serum levels do not have the same diagnostic power as does the ratio between them due to the compensatory role that OPG plays in response to elevations in RANKL. It has been observed that higher RANKL:OPG ratios are associated with an enhanced risk of CV diseases and vice versa lower RANKL:OPG ratios usually correspond to a lower incidence of coronary disorders [24].

In our study, the RANKL:OPG ratio at the 24th hour after ACS showed no significant difference between ACS patients with and without RA, and in both groups the values were significantly higher than in the healthy controls. At the 24th hour after ACS, both patient groups (with and without RA) had elevated RANKL:OPG ratios and therefore higher risk of future CV events.

However, at the 48th hour after ACS, even though both patient groups with ACS had significantly higher ratios than the healthy controls, the RANKL:OPG48hr ratio in the RA patients was significantly higher than in the ACS patients without RA. All of these findings suggest that systematic inflammation may be the reason for ACS patients with RA to maintain higher RANKL and OPG levels even after the local inflammation has been reduced, whereas in ACS patients without RA the levels subside.

Both RANKL:OPG ratios at the 24th and 48th hour after ACS showed a significant association with the GRACE risk score. Higher ratios were associated with a higher risk of dying following the onset of ACS. In the present context, the results suggest that alterations in the RANKL:OPG balance can be linked to an amplification of cardiovascular risk, especially in patients with rheumatoid arthritis [14, 24].

The acute-phase biomarker hs-CRP showed very high mean levels in both patient groups with ACS (with and without RA), exceeding manifold the lower reference value associated with high cardiovascular risk (> 3 mg/l); however, the ACS patients with RA had a significantly higher hs-CRP level in comparison to the ACS patients without RA and the healthy controls. Hence, our extrapolation supports the conclusions of other studies [25–27] which state that elevated hs- CRP values are not only linked to vascular dysfunction, but also to the extent of the proinflammatory response. A similar tendency

was established regarding hs-TnI, where the highest value was observed in the ACS patients with RA, which significantly exceeded that of the ACS patients without RA and the healthy controls. Both hs-CRP and hs-TnI showed an association with the GRACE risk score, indicating a positive linear relationship and an increased mortality risk in patients with elevated hs-CRP and hs-TnI levels.

Finally, ACS patients with RA had a higher mean GRACE risk score than ACS patients without RA. In view of the reference numbers provided by the ESC, the mean GRACE risk score of the RA group indicated high in-hospital risk, whereas that of the group without RA indicated moderate in-hospital risk [28, 29].

The main strength of our study is that it examined exRANKL and inflammatory serum markers in patients with two disease states, ACS and RA, versus two control groups: patients with ACS and clinically healthy controls. This allowed us to carry out comparative analysis, identify the differences, and extrapolate the role of the target inflammatory markers in ACS and in RA. Another strength is that we traced the dynamics in the serum levels of RANKL, OPG and RANKL:OPG ratio between the 24th and 48th hour after ACS. Hence, we established different developments in the two patient groups: In the ACS patients without RA the levels became almost equal to those of the healthy controls, while remaining significantly elevated in the ACS patients with RA. Based on this finding we made the extrapolation that serum levels of RANKL and OPG may be good biomarkers to diagnose and prognosticate cardiovascular disease. Third, we explored the relation between exRANKL and inflammatory serum markers with mortality risk and from the findings extrapolated an increased risk for ACS patients with RA.

Alongside the strengths, there are also some limitations that need to be recognized. One of them concerns our findings about the dynamics in the RANKL and OPG serum levels between the 24th and 48th hour after ACS. We concluded that in the ACS patients without RA the levels became almost equal to those of the healthy controls, while remaining significantly elevated in the ACS patients with RA. However, this claim will be stronger if corroborated by the inclusion of another control group of RA patients without ACS against which to compare the baseline data and the dynamics after 48 h. We also recognize the need for further research that will help elucidate and validate the role of cytokines and inflammatory serum markers in the diagnosis, monitoring and treatment of patients with acute coronary syndrome with and without RA.

Conclusion

In conclusion, our results suggest that the presence of systematic inflammation in ACS patients with RA maybe responsible for the higher rate and stronger form of RANKL expression on atherosclerotic plaques, elevated levels of inflammatory serum markers, and an amplified risk for in-hospital (short-term) death as compared to ACS patients without RA. Our findings carry some weight for the prognostication and monitoring of CV events in patients with RA.

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Compliance with ethical standards

Ethical approval All procedures were conducted in accordance with the WMA Declaration of Helsinki (1964) and approved by the local research ethics committee at the Medical University in Plovdiv.

Conflict of interest All authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from each participant involved in this research.

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