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Biomarkers involved in evaluation of platelets function in South-Eastern Romanian patients with hematological malignancies subtypes

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Abstract

At present, various researches presented how subtypes of hematological malignancies are related to stages of the immune response, because the activated immune system represents a promising form in cancer treatment. This study explores the relationship between the adaptive immune system (T cells), and the coagulation system (platelets, platelet membrane glycoproteins, platelets derivate microparticles) which seems to play an important role in host immune defense of patients with acute myeloblastic leukemia (AML) or B cell lymphoma (BCL), 2 of the most common hematological malignancies subtypes.

Blood samples (n = 114) obtained from patients with AML or BCL were analyzed for platelet membrane glycoproteins (CD42b, CD61), glycoprotein found on the surface of the T helper cells ($CD4^+$), protein complex-specific antigen for T cells ($CD3^+$), platelet-derived microparticles (CD61 PMP) biomarkers by flow cytometry, and hematological parameters were quantified by usual methods.

In patients with AML, the means of the percentage of the expressions of the molecules on platelet surfaces (CD61 and CD42b, $P < .01$; paired T test) were lower as compared to both control subgroups. The expression of cytoplasmic granules content (CD61 PMP) had a significantly higher value in patients with AML reported to controlling subgroups ($P < .01$; paired T test), which is suggesting an intravascular activation of platelets.

The platelet activation status was presented in patients with low stage BCL because CD61 and CD42b expressions were significantly higher than control subgroups, but the expression of CD 61 PMP had a significantly decreased value reported to control subgroups (all $P < .01$; paired T test). T helper/inducer lineage $CD4^+$ and T lymphoid lineage $CD3^+$ expressions presented significant differences between patients with AML or low stage BCL reported to control subgroups (all $P < .01$; paired T test).

Platelet–lymphocyte interactions are involved in malignant disorders, and CD61, CD42b present on platelet membranes, as functionally active surface receptors mediate the adhesion of active platelets to lymphocytes, endothelial cells, and cancer cells.

Abbreviations: AML = acute myeloblastic leukemia, BCL = B cell lymphoma, $CD3^+$ = protein complex-specific antigen for T cells, $CD4^+$ = glycoprotein found on the surface of the T helper cells, CD42b, CD61 = platelet membrane glycoproteins, CD61 PMP = platelet-derived microparticles.

Keywords: acute myeloblastic leukemia, B cell lymphoma, $CD3^+$, $CD4^+$, CD42b, CD61, CD61 PMP, platelets

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All aspects of ethics approval and consent to participate of patients in this study are made in conformity with the declaration of Helsinki 2000.

All authors made an equal contribution.

The authors have no conflicts of interest to disclose.

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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1. Introduction

Acute myeloblastic leukemia (AML) is characterized by the clonal proliferation of malignant hematopoietic precursor cells in the hematogenous marrow. Malignant cell accumulation in bone marrow results in dissociation of normal hematopoiesis by suppressing the growth and differentiation of normal bone marrow cells and decreasing blood cell production. In consequence, in the peripheral blood appear variable cytopenias associated with clinical manifestations such as anemia, infections, and hemorrhagic syndromes. Leukemic cells can also invade extramedullary tissues such as the meninges, gonad, liver, spleen, and lymph nodes.[1,2]

Malignant lymphoma is part of a heterogeneous tumor group of the lymphoid system, which can involve any organ or tissue. Lymphoma is divided into 2 categories according to World Health Organization (WHO) classification: Hodgkin lymphoma and non-Hodgkin lymphoma.[3] Non-Hodgkin lymphoma is a malignancy characterized by a monoclonal proliferation of B cell or, rarely, of T/NK-cell.[4] Clinically, non-Hodgkin lymphoma is classified as indolent, aggressive, or extremely aggressive malignancy.[5,6]

Leukemia has an incidence rate of 2.1% (1724 new cases/year) in the Romanian adult population. For non-Hodgkin lymphoma, WHO[7] published an incidence rate of 1.7% (1424 new cases/year). AML is the most common type of acute leukemia. The average age at diagnosis is 65 years and the incidence increases with age.[8]

Immunophenotyping by CD-biomarkers expressions with flow cytometry is useful in the diagnoses of the acute myeloblastic leukemia (AML)/B cell lymphoma (BCL). Associated CD antigens of the leukocytes are molecules involved in functions such as cell-to-cell interactions, cytokine receptors, cell signaling, ion channels, transporters, enzymes, immunoglobulins, and adhesion molecules.[9] Effector T lymphocytes exhibit antitumor activity in the tumor, and their intratumoral presence increases the survival rate of these patients. Regulatory T lymphocytes and macrophages generate an immunosuppressive environment that counteracts antitumor immunity, promotes tumor progression, and decreases the patient's survival rate.[10–12]

Bleeding is known to occur during diagnosis and chemotherapy, being another important factor threatening the lives of AML patients. The reduction of platelets number is a determinant to assess the bleeding risk. Patients with a low platelet count, who were diagnosed and treated for a long period of time, presented a small amount of bleeding. The bleeding is associated with platelet dysfunction, not only with the platelet number,[13] and the level of platelet membrane glycoproteins and the expression of the platelet membrane glycoproteins can be analyzed by flow cytometry to assess the platelet function.[14,15] Recently, platelet-derived microparticles (PMPs) are employed as an alternative evaluation of platelet activation. PMPs induce the apoptosis of effector T cells [16–19] and induce T lymphocytes regulatory responses.[20,21]

PMPs that are shed from platelet surface membranes constitute the majority of circulating microparticles, being implicated in procoagulant, pro-inflammatory, and pro-atherosclerotic actions. The diagnostic value of microparticles was investigated in patients with different cancer types, including bladder cancer,[22] prostate cancer,[23] and colorectal cancer.[24]

In our study, the platelets functions in correlation with T lymphocytes count were assessed, by analyzing the platelets membranes glycoproteins expressions (CD61, CD42b), platelets derivate microparticles (CD61 PMP), T lymphoid lineage (protein complex-specific antigen for T cells [$CD3^+$]), and T helper/inducer lineage (glycoprotein found on the surface of the T helper cells [$CD4^+$]) by flow cytometry. Associated parameters such as total platelets, leukocytes, and lymphocytes counts, made by an automated hematologic analyzer, were also analyzed.

2. Materials and methods

2.1. Case selection

All blood samples (n = 114) were obtained from patients (who signed informed consent forms, agreeing to participate in this study), which are treated in the Hematology Department of Sf. Apostol Andrei Clinical Emergency County Hospital in Constanta, Romania. Blood samples were analyzed for CD-biomarkers by flow cytometry and hematological determinations, at the Cell Biology Department of Centre for Research and Development of Morphological and Genetic Studies of Malignant Pathology, *Ovidius* University of Constanta, Romania.

In agreement with WHO classifications, the patients were divided into 2 groups:

1. Patients with AML were, under treatment, divided into 2 subgroups by age (≤65 years, n = 15 and >65 years, n = 14), and a control group of match healthy subjects was assigned for each subgroup (n = 15, ≤65 years and n = 14, >65 years).
2. Patients with BCL, under treatment, divided into 2 subgroups by Ann Arbor stage:
   - patients with Ann Arbor I–II tumoral stages (n = 16), reported to match control healthy patients (n = 16);
   - patients with Ann Arbor III–IV tumoral stages (n = 12), reported to match control healthy patients (n = 12).

2.2. Reagents and equipment

The flow cytometry (Attune, Acoustic focusing cytometer, Life Technologies) was used to measure the expressions of CD61, CD42b, $CD3^+$, and $CD4^+$. Anti-CD42b-PE (HIP1) and anti-CD61-PE (integrin beta 3, Invitrogen, eBioscience) monoclonal antibodies conjugated with phycoerythrin, were used to assess platelet expressions of GPIba and GPIIIa. Alexa Fluor 488 conjugated monoclonal antibodies to $CD3^+$, $CD4^+$ (anti-CD3-Alexa Fluor 488, anti-CD4-Alexa Fluor 488, Invitrogen, Molecular Probes) were used to determine T lymphoid and T helper/inducer cells from blood samples. For negative controls, mouse IgG (Invitrogen, eBioscience) were used.

Before analysis of CD61 PMP, the flow cytometer was first set by using fluorescent beads (Attune performance tracking beads, Labelling and detection, Life technologies), with standard size (4 intensity levels of beads population), and the quantity was established by enumerating PMPs below 1 μm. The number of CD61 PMP was calculated based on the event count from the bead tube collected. PMPs were gated by Forward Scatter and Side Scatter parameters, and then identified with CD61-PE. For data collection, graphics by flow cytometry were used with Attune Cytometric Software v.1.2.5, Applied Biosystems, 2010. Pentra XLR automated blood cell analyzer (Horiba Medical, Diamedix, Romania) was used for determinations of the total number of platelet (PLT), leukocytes, and lymphocytes counts.

2.3. CD61, CD42 B platelet membrane glycoproteins, and $CD3^+$, $CD4^+$ T lymphocytes detections by flow cytometry dual stain

The blood samples were collected into tubes (4 mL) with anticoagulant EDTA-K2. All measurements were performed within 120 minutes after blood withdrawal.

Flow cytometry tubes were divided into 3 categories:

1. samples with CD61-PE and $CD4^+$-Alexa Flour 488 dual stain;
2. samples with CD42b-PE and $CD3^+$-Alexa Fluor 488 dual stain;
3. samples with control negative-IgG stain.

In tubes of flow cytometry were introduced 100 μL blood sample, 5 μL of CD61-PE, and 10 μL of $CD4^+$-Alexa Flour 488. The contents were gently vortexed and incubated into darkness, for 25 minutes at 37°C. 100 μL blood sample, 5 μL of CD42b-PE, and 10 μL of $CD3^+$-Alexa Flour 488 were added into other tubes, vortexed, and incubated into darkness, for 25 minutes at 37°C. For each sample, a control tube with 100 μL blood sample and 5 μL of the negative control (mouse IgG) were made, and the tubes were vortexed and incubated into darkness, for 25 minutes at 37°C. Three milliliters of flow cytometry stain buffer were added into each tube, and vortexed for 1 minute before analysis.

Platelets were identified by flow cytometry based on size and platelet specific CD61 and CD42b surface expressions. T helper/inducer lymphocytes and total T lymphocytes (thymocytes, T lymphocytes, and NK cells) were determined by flow cytometry based on size and lymphocytes specific $CD4^+$ and $CD3^+$ monoclonal antibodies expressions.

2.4. Platelet-derived microparticles-CD61 determination by flow cytometry

Collected blood samples were centrifuged at 10,000× *g* for 10 minutes. From obtained plasma samples, were aspirated 100 μL of plasma and introduced into flow cytometer tubes. Five microliters of CD61-PE were added over the plasma samples, and tubes were vortexed and incubated into darkness, for 25 minutes at 37°C. Two milliliters of flow cytometry stain buffer were added into each tube and were vortexed for 1 minute before analysis at equipment. In parallel, were worked control plasma samples, made from 100 μL plasma and 5 μL of the negative control (mouse IgG), which followed the same steps mentioned before.

2.5. Statistical analysis

Obtained results were presented as mean values with standard errors for CD61, CD42b platelets, $CD3^+$, $CD4^+$ lymphocytes, and CD61 PMP percentages expressions, total platelets, leukocytes, and lymphocytes counts; SPSS v. 23 software, IBM, 2015 were used. Data were analyzed by Levene test for homogeneity of variances of samples, while paired *T* test, ANOVA, were used to establish the differences between samples and controls, and $P < .05$ was considered statistically significant. Pearson correlations were established for AML and BCL subgroups of patients between CD61 PMP and $CD3^+$, $CD4^+$ lymphocytes expressions.

Receiver operating characteristic (ROC) and area under the curve (AUC) made by MedCalc v. 14.8.1, 2014, were used to establish the accuracy of the biomarkers in AML/BCL diagnoses. The sensitivity and specificity of CD-biomarkers are represented by the Youden index which is the optimal cut-off point as the value that maximized the area under the ROC curve.

Figures 1, 2, 6 and 7 were made by the MedCalc program. Figures 3–5 were made with Attune Cytometric Software v.1.2.5, Applied Biosystems, 2010.

3. Results

CD61 expressions (S1-13.30 ± 1.00 vs C1-33.03 ± 3.02, $P < .01$; S2-15.31 ± 0.62 vs C2-56.79 ± 2.70, $P < .01$) and the CD42b percentages (S1-12.83 ± 0.60 vs C1-48.76 ± 0.81, $P < .01$; S2-21.61 ± 0.30 vs C2-49.23 ± 1.22, $P < .01$) were statistically significantly lower in both subgroups of patients with AML compared with the controls (Figs. 1, 3 and 4). T helper/inducer lineage $CD4^+$ (S1-71.32 ± 1.09 vs C1-67.05 ± 0.76, $P < .01$; S2-64.94 ± 0.88 vs C2-59.92 ± 1.33, $P < .01$) and T lymphoid lineage

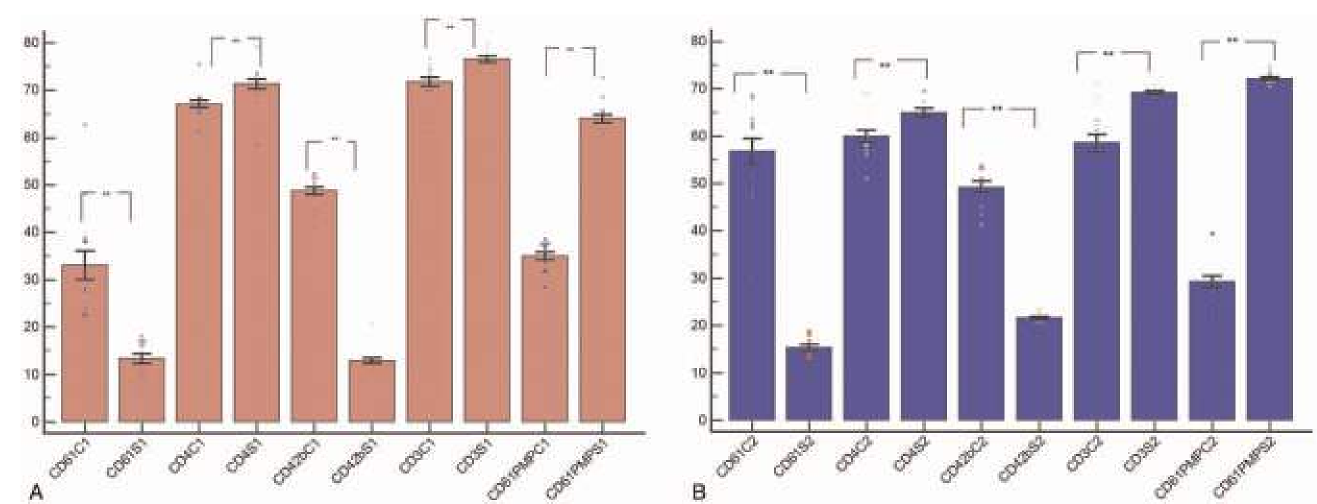

Figure 1. Platelet membrane glycoproteins, T lymphocytes, platelet microparticles-CD61 in patients with acute myeloblastic leukemia reported to controls, in the function of patients' age: (A) ≤65 yrs and (B) >65 yrs. All results were presented as mean values with standard errors. CD61 PMP, platelets derivate microparticles. $^{**}P < .01$ represents significant statistical differences between controls and samples made by paired samples *T* test.

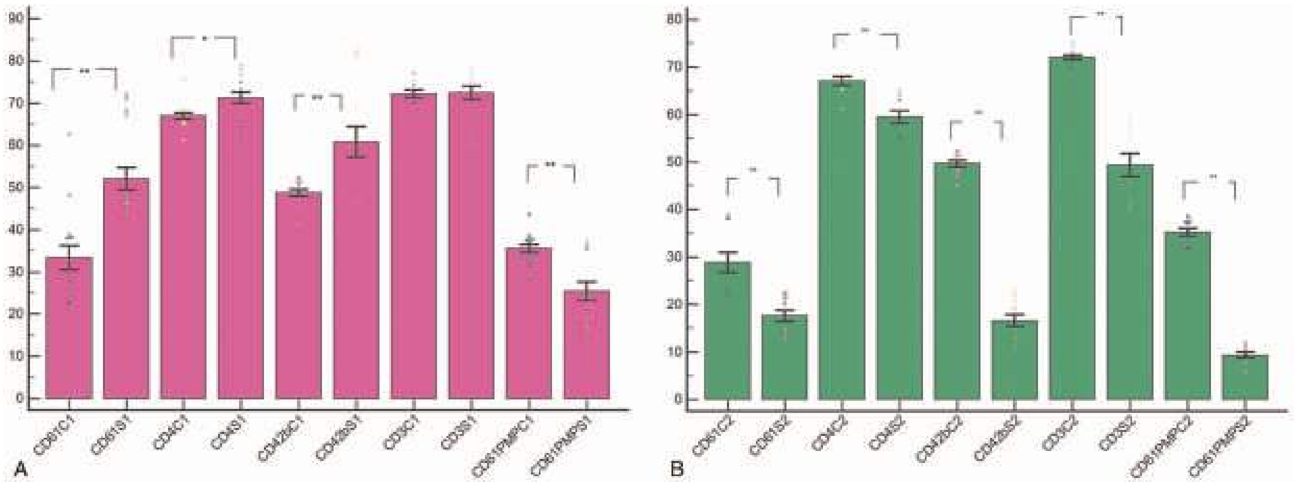

Figure 2. Platelet membrane glycoproteins, T lymphocytes, platelet microparticles-CD61 in patients with B cell lymphoma reported to controls subgroups, in the function of patient's tumoral stages, lymphadenopathies nodes, and splenomegaly: (A) T1–T2 N1–N3 S0–S1; (B) T3–T4 N2–N3 S1–S2. All results were presented as means values with standard errors. T1–T2 N1–N3 S0–S1-Ann Arbor I–II tumoral stages (T1–T2), with lymphadenopathies nodes (N1–N3), and without or with splenomegaly (S0–S1); T3–T4 N2–N3 S1–S2-Ann Arbor III–IV tumoral stages, with lymphadenopathies nodes (N2–N3) and splenomegaly (S1–S2). CD61 PMP, platelets derivate microparticles. $^{**}P<.01$ represents significant statistical differences between controls and samples made by paired samples T test; $^{*}P<.05$ represents significant statistical differences between controls and samples made by paired samples T test.

CD3$^+$ (S1-76.53 ± 0.73 vs C1-71.78 ± 0.91, $P<.01$; S2-69.17 ± 0.35 vs C2-58.53 ± 1.80, $P<.01$) were statistically significantly higher in patients with AML than in controls (Figs. 1, 3 and 4). CD61 PMP had increased values for samples recovered from patients' subgroups with AML in comparison with controls (S1-63.97 ± 0.88 vs C1-34.96 ± 0.82, $P<.01$; S2-72.11 ± 0.32 vs C2-29.26 ± 1.23, $P<.01$, Figs. 1 and 5). The platelets total number presented as associated parameters in Table 1, had lower values with significant statistical differences between AML and controls subgroups of the patients (S1-46,761.00 ± 5749.00 vs C1-238,000.00 ± 13,945.00, $P<.01$; S2-66,159.00 ± 1685.58 vs C2-335,714.28 ± 8939.00, $P<.01$). The leukocytes number from samples of patients with AML (age ≤ 65 years) was statistically significantly higher than in controls (S1-36,303.33 ± 2587.85 vs C1-7018.06 ± 153.93, $P<.01$). Lymphocytes count presented lower values for both subgroups of AML patients in comparison with controls (S1-2683.33 ± 43.00 vs C1-3564.66 ± 152.66, $P<.01$; S2-2152.14 ± 52.70 vs C2-2653.57 ± 135.92, $P<.01$, Table 1).

Because CD61 PMP (S1-64% vs S2-73%) plays an important role in immune response by T regulatory cells activation, these are correlated with estimated percentage of CD3$^+$ lymphocytes (S1-77%, $r=-0.233$, $P>.05$, and S2-72%, $r=0.451$, $P>.05$) and CD4$^+$ lymphocytes count (S1-72%, $r=-0.621$, $P<.05$ and S2-65%, $r=-0.530$, $P>.05$, Table 2) in subgroups of AML patients.

First subgroup of patients with low stage BCL presented statistically significant higher values for CD61, CD4$^+$, and CD42b expressions (S1-51.99 ± 2.65 vs C1-33.35 ± 2.84, $P<.01$; S1-71.26 ± 1.32 vs C1-66.92 ± 0.72, $P<.05$; S1-60.74 ± 3.62 vs C1-48.66 ± 0.76, $P<.01$) than controls (Figs. 2–4). CD61 PMP had statistically lower values for samples recovered from patients with low stage BCL reported to controls (S1-25.45 ± 2.20 vs C1-35.50 ± 0.94, $P<.01$, Figs. 2 and 5).

Second subgroup of patients with high stage BCL had significant lower values for all CD-biomarkers reported to controls (CD61-S2-17.61 ± 1.16 vs C2-28.87 ± 2.09, $P<.01$; CD4$^+$-S2-59.45 ± 1.33 vs C2-67.00 ± 0.94, $P<.01$; CD42b-S2-16.60 ± 1.24 vs C2-49.65 ± 0.65, $P<.01$; CD3$^+$-S2-49.29 ± 8.36 vs C2-72.02 ± 1.76, $P<.01$; CD 61 PMP-S2-9.30 ± 0.60 vs C2-35.16 ± 0.81, $P<.01$, Figs. 2–5).

Platelet count in patients with low stage BCL was significantly higher than in controls (S1-290,937.00 ± 16,261.01 vs C1-238,125.00 ± 13,045.39, $P<.05$), while in patients with high stage BCL, platelet count revealed significantly lower values than in controls (S2-180,000.00 ± 13,595.89 vs C2-222,500.00 ± 11,878.48, $P<.05$). Higher values of leukocytes number were observed in both experimental subgroups of patients with BCL

Table 1

Total numbers of platelets, leukocytes, and lymphocytes divided in the function of patient's age with acute myeloblastic leukemia reported to controls.

	Age ≤ 65 yrs X ± SE		Age > 65 yrs X ± SE	
Parameters	**Control (C1)**	**Sample (S1)**	**Control (C2)**	**Sample (S2)**
Platelets	238,000.00 ± 13,945.00*	46,761.00 ± 5749.00*	335,714.28 ± 8939.00*	66,159.00 ± 1685.58*
Leukocytes	7018.06 ± 153.93*	36,303.33 ± 2587.85*	4912.85 ± 262.38	5328.57 ± 2361.27
Lymphocytes	3564.66 ± 152.66*	2683.33 ± 43.00*	2653.57 ± 135.92*	2152.14 ± 52.70*

SE, standard error; X, obtained results means.
$^{*}P<.01$ represents significant statistical differences between controls and samples made by paired samples T test.

Table 2

Percentage of CD 61 PMP correlated with $CD3^+$, $CD4^+$ lymphocytes expressions at subgroups of patients with acute myeloblastic leukemia.

	$CD\ 3^+$		$CD\ 4^+$	
Pearson correlations (*r*)	**Age ≤65 yrs**	**Age > 65 yrs**	**Age ≤ 65 yrs**	**Age > 65 yrs**
CD 61 PMP	−0.232	0.451	−0.621	−0.530
P	.405	.106	.014*	.857

$CD3^+$, protein complex-specific antigen for T cells; $CD4^+$, glycoprotein found on the surface of the T helper cells; CD61 PMP, platelets derivate microparticles.
* $P < .05$ represents significant statistical differences between parameters.

reported to controls (S1-14,000.00 ± 730.29 vs C1-7036.93 ± 145.22, $P < .01$; S2-12,508.33 ± 1362.00 vs C2-7091.75 ± 101.32, $P < .01$). Lymphocytes number had increased value for patient's subgroup with low stage BCL and decreased value for patient's subgroup with high stage BCL in comparison with control subgroups (S1-3956.25 ± 76.35 vs C1-3604.37 ± 148.02, $P < .05$; S2-3266.66 ± 704.24 vs C2-3547.50 ± 97.53, $P < .05$, Table 3).

Patients with low stage of BCL present the estimated CD61-PMP percentage (26%) positive correlated with estimated percentages of $CD3^+$, $CD4^+$ lymphocytes ($CD3^+$-73%, $r = 0.537$, $P < .05$, $CD4^+$-72%, $r = 0.246$, $P > .05$). Also were observed correlations between $CD3^+$, $CD4^+$ lymphocytes ($CD3^+$-50%, $r = 0.822$, $P < .01$; $CD4^+$-60%, $r = -0.798$, $P < .01$, Table 4), and CD61 PMP quantity (10%) in patients with high stage BCL.

To establish the diagnostic of laboratory, the predictive model (ROC curves) is used to estimate the risk of adverse outcome based on patients, in medical research.[25] ROC curves were used to show accuracies of positive and negative value predictions (PPV and PNV, Table 5) in relationship with TPR (sensitivity) and FPR (1-specificity) for each CD-biomarker. A single test

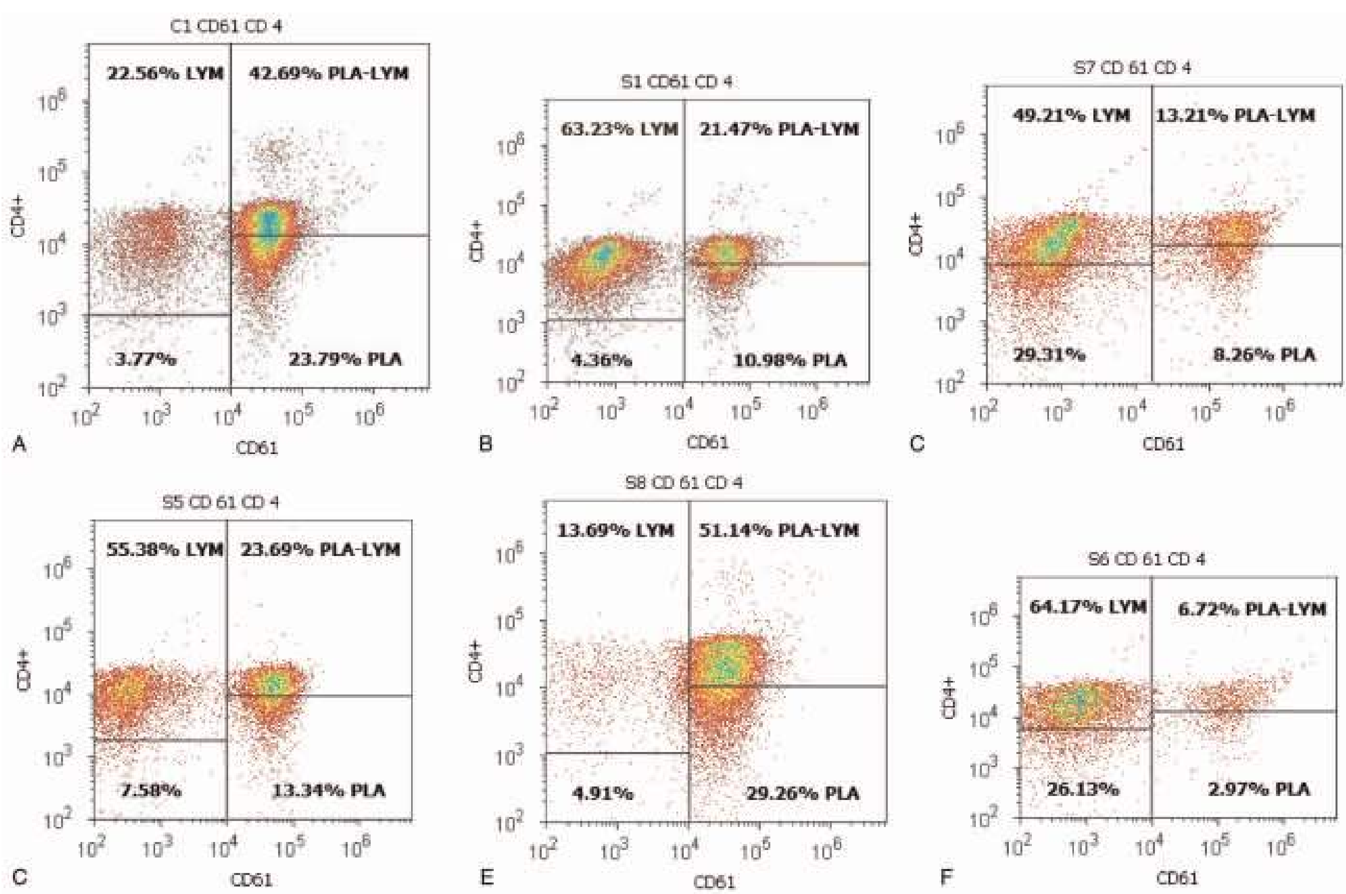

Figure 3. Expressions of the CD61/$CD4^+$ by flow cytometry: (A) healthy patients; (B) patients with AML; (C) BCL patients with Ann Arbor I tumoral stage of BCL with axillary lymphadenopathies nodes (N1); (D) BCL patients with Ann Arbor II tumoral stage with axillary and laterocervical lymphadenopathies nodes (N2); (E) BCL patients with Ann Arbor II tumoral stage with laterocervical, supraclavicular, axillary lymphadenopathies nodes (N3), and splenomegaly (SI); (F) BCL patients with Ann Arbor IV tumoral stage with supraclavicular, axillary, abdominal lymphadenopathies nodes (N3), and splenomegaly (SII). AML, acute myeloblastic leukemia; BCL-B, cell lymphoma; LYM-T, lymphocytes; PLA, platelets; PLA-LYM, platelet–lymphocyte interactions.

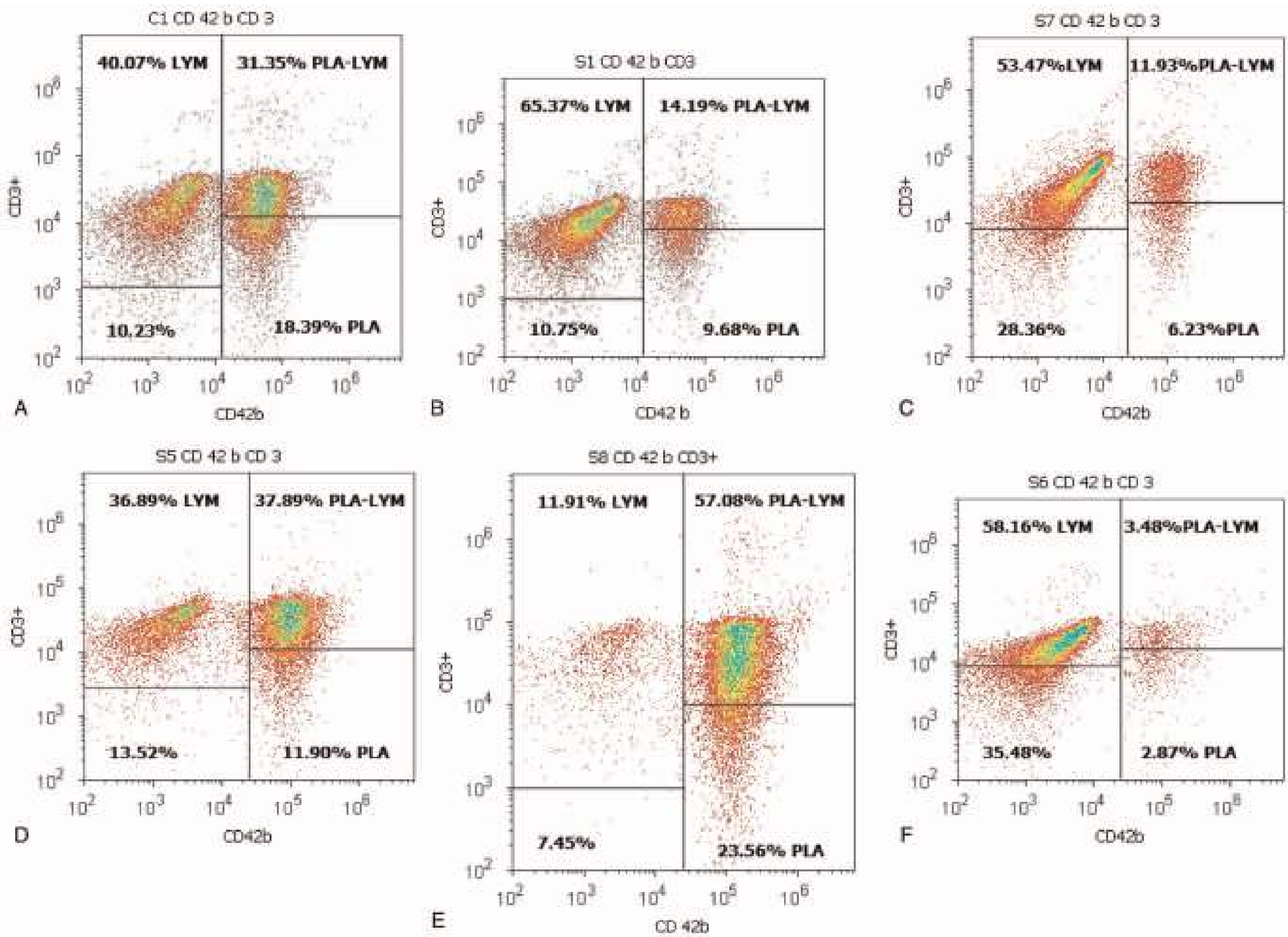

Figure 4. Expressions of the CD42b/CD3+ by flow cytometry: (A) healthy patients; (B) patients with AML; (C) BCL patients with Ann Arbor I tumoral stage of BCL with axillary lymphadenopathies nodes (N1); (D) BCL patients with Ann Arbor II tumoral stage with axillary and laterocervical lymphadenopathies nodes (N2); (E) BCL patients with Ann Arbor II tumoral stage with laterocervical, supraclavicular, axillary lymphadenopathies nodes (N3), and splenomegaly (SI); (F) BCL patients with Ann Arbor IV tumoral stage with supraclavicular, axillary, abdominal lymphadenopathies nodes (N3), and splenomegaly (SII). AML, acute myeloblastic leukemia; BCL, B cell lymphoma; LYM-T, lymphocytes; PLA, platelets; PLA-LYM, platelet–lymphocyte interactions.

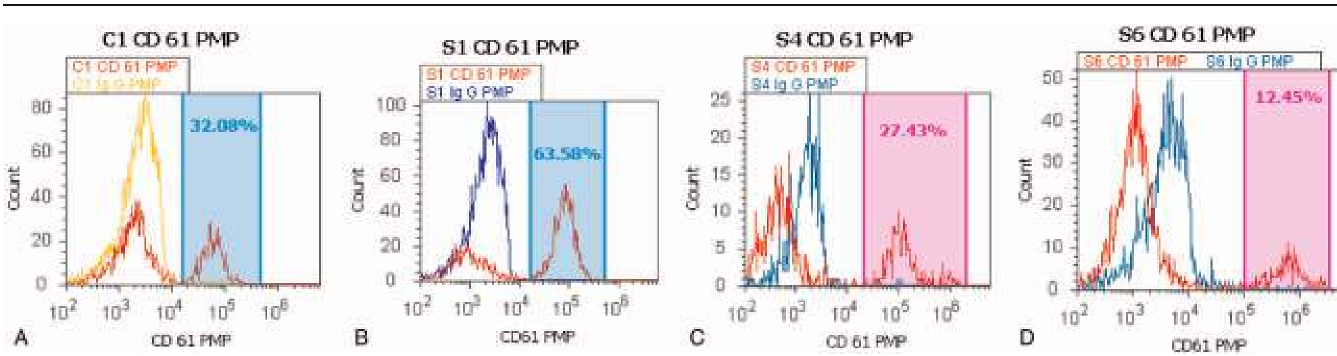

Figure 5. Platelet-derived microparticles-CD61 expressions by flow cytometry in patients with acute myeloblastic leukemia (B) and low and high stages B cell lymphoma (C and D) reported to control (A) C1 CD61 PMP-platelets derivate microparticles in controls; C1 IgG PMP-negative control (IgG) applied to control platelets derivate microparticles; S1 CD 61 PMP-platelets derivate microparticles from samples recovered from patients with acute myeloblastic leukemia; S1 IgG PMP-negative control (IgG) applied to obtained samples from patients with acute myeloblastic leukemia; S4 CD 61 PMP-platelets derivate microparticles from samples recovered from patients with low stage B cell lymphoma; S4 IgG PMP-negative control (IgG) applied to obtained samples from patients with low stage B cell lymphoma; S6 CD 61 PMP-platelets derivate microparticles from samples recovered from patients with high stage B cell lymphoma; S6 IgG PMP-negative control (IgG) applied to obtained samples from patients with high stage B cell lymphoma.

Table 3

Platelet membrane glycoproteins, T lymphocytes, platelet microparticles-CD61, total numbers of platelets, leukocytes, and lymphocytes in correlations with the clinicopathological aspects of B cell lymphoma patient's subgroups.

	X±SE I–II Ann Arbor		X±SE III–IV Ann Arbor	
Parameters	**Control (C1)**	**Sample (S1)**	**Control (C2)**	**Sample (S2)**
Platelets	238,125.00±13,045.39*	290,937.00±16,261.01*	222,500.00±11,878.48*	180,000.00±13,595.89*
Leukocytes	7036.93±145.22†	14,000.00±730.29†	7091.75±101.32†	12,508.33±1362.00†
Lymphocytes	3604.37±148.02*	3956.25±76.35*	3547.50±97.53*	3266.66±704.24*

SE, standard error; X, obtained results means.
* $P<.05$ represents statistical differences between controls and samples made by paired samples *T* test.
† $P<.01$ represents significant statistical differences between controls and samples made by paired samples *T* test.

Table 4

Percentage of CD 61 PMP correlated with CD3⁺, CD4⁺ lymphocytes expressions in subgroups of patients with B cell lymphoma.

	CD 3^+		CD 4^+	
Pearson correlations (*r*)	**I–II Ann Arbor**	**III–IV Ann Arbor**	**I–II Ann Arbor**	**III–IV Ann Arbor**
CD 61 PMP	0.537	0.822	0.246	−0.798
P	.032*	.001†	.359	.002†

$CD3^+$, protein complex-specific antigen for T cells; $CD4^+$, glycoprotein found on the surface of the T helper cells; CD61 PMP, platelets derivate microparticles.
* $P<.05$ represents significant statistical differences between parameters.
† $P<.01$ represents significant statistical differences between parameters.

ROC curve was made for each CD-biomarker with 2 overlapping distributions (control and experimental, Figs. 6 and 7).

True positive values (sensitivity) in patients with AML were increased for CD61 (93.33%), $CD4^+$ (61.29%), CD42b (96.43%), $CD3^+$ (97.07%), and CD61 PMP (96.67%). Specificity (TNV-true negative values) was increased for CD61 (96.43%), $CD4^+$ (96.30%), CD42b (93.33%), $CD3^+$ (68.33%), and CD61 PMP (92.87%, Table 5 and Fig. 6). Sensitivity and specificity of the methods were increased, for patients with BCL: 77.78% and 100.00% of CD61, 60.87% and 100.00% of $CD4^+$, 76.19% and 85.71% of CD42b, 65.79% and 100.00% of $CD3^+$, 75.86% and 100.00% of CD61 PMP (Table 5 and Fig. 7).

4. Discussion

Antigen-specific T cells ($CD4^+$ and $CD3^+$ expressions) play an important role in immune protection toward cancer and represent the cellular basis for specific immunotherapy. Platelets are involved in the hemostasis regulation and interact with leukocytes to avoid substantial blood loss from the circulation.[26] Activated platelets change their shape and size, release cytoplasmic granules contents, and change their glycoproteins expressions. Signals of platelet activation led to biochemical modifications, accompanied by surface glycoproteins changes expressions, which are used in platelet state evaluation.[27–29]

Because flow cytometry is an optimal laboratory method to analyze the platelet function, in this study, we quantify the levels of platelet membrane glycoproteins (CD61 and CD42b) in samples recovered from patients with AML or BCL.

In patients with AML, the means of molecules' expression on platelets' surfaces (CD61 and CD42b) were significant lower than in both control subgroups. Decreased expressions of CD61, were observed in myelodysplastic syndromes (MDS) patients,[30] and the reduced amounts of CD42b were reported to be a common finding in MDS patients.[31]

CD61 and CD42b presence on platelet membranes, as functionally active surface receptors, are factors that mediate the adhesion of active platelets to leukocytes, endothelial cells,

Table 5

Receiver operating characteristic (ROC) analysis of CD-biomarkers in patients with hematological malignancies.

Biomarkers	AUC	95% CI	P	Youden J index	Cut-off value	Sensitivity	Specificity	PPV	PNV
CD61_AML	0.973	0.938–1.000	<.0001*	0.897	≤18.85	93.33	96.43	96.60	93.10
CD61_BCL	0.817	0.697–0.936	<0001*	0.777	>28.14	77.78	100.00	100.00	71.14
$CD4^+$_AML	0.817	0.709–0.926	<.0001*	0.575	>68.50	61.29	96.30	95.00	68.40
CD4+_BCL	0.786	0.651–0.921	<.0001*	0.608	>68.50	60.87	100.00	100.00	78.60
CD42b_AML	0.960	0.894–1.00	<.0001*	0.821	≤23.70	96.43	93.33	93.10	96.60
CD42b_BCL	0.741	0.611–0.872	<.0001*	0.619	≤49.36	76.19	85.71	94.10	54.50
$CD3^+$_AML	0.792	0.671–0.914	<.0001*	0.553	>68.30	97.07	58.33	76.70	93.30
CD3+_BCL	0.733	0.599–0.868	<.0001*	0.657	≤70.12	65.79	100.00	100.00	58.10
CD61 PMP_AML	0.895	0.889–1.000	<.0001*	0.895	>38.54	96.67	92.87	93.50	96.30
CD61 PMP_BCL	0.897	0.817–0.978	<.0001*	0.758	≤24.01	75.86	100.00	100.00	79.40

AUC, area under the curve; AML, acute myeloblastic leukemia; BCL, patients with B cell lymphoma; $CD3^+$, protein complex-specific antigen for T cells; $CD4^+$, glycoprotein found on the surface of the T helper cells; CD42b, CD61, platelet membrane glycoproteins; CD61 PMP, platelets derivate microparticles; CI, confidence interval; PNV, negative value predictions; PPV, positive value predictions; Youden J = sensitivity + specificity − 100.
* Obtained results were significant and statistically different ($P<.0001$).

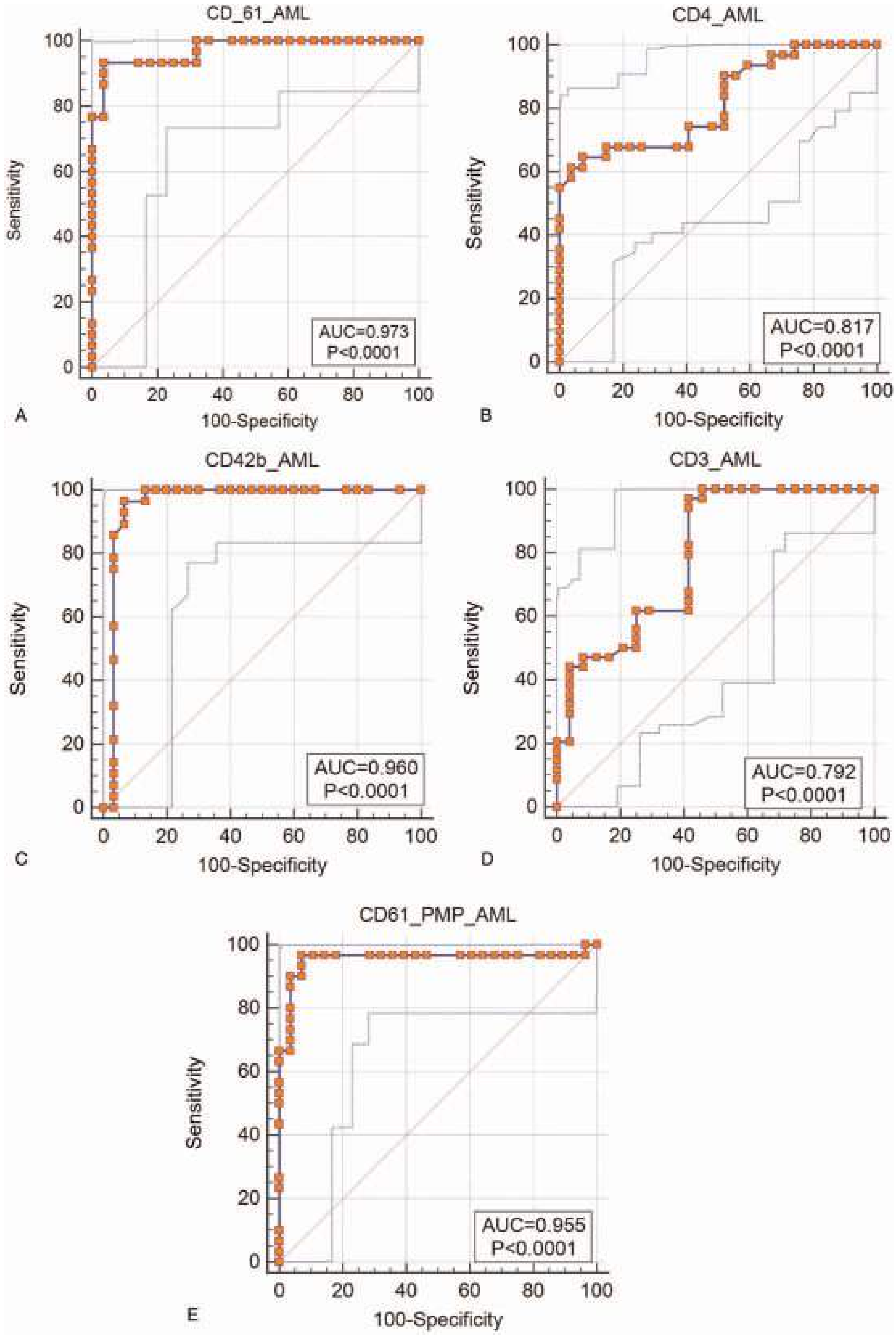

Figure 6. CD-expressions of platelets and lymphocytes, and CD61 platelets derivate microparticles as biomarkers in patients with acute myeloblastic leukemia: (A) platelet CD61 (AUC-0.973); (B) lymphocytes $CD4^+$ (AUC-0.817); (C) platelet CD42b (AUC-0.960); (D) lymphocytes $CD3^+$ (AUC-0.792); (E) CD61 PMP (AUC-0.955).

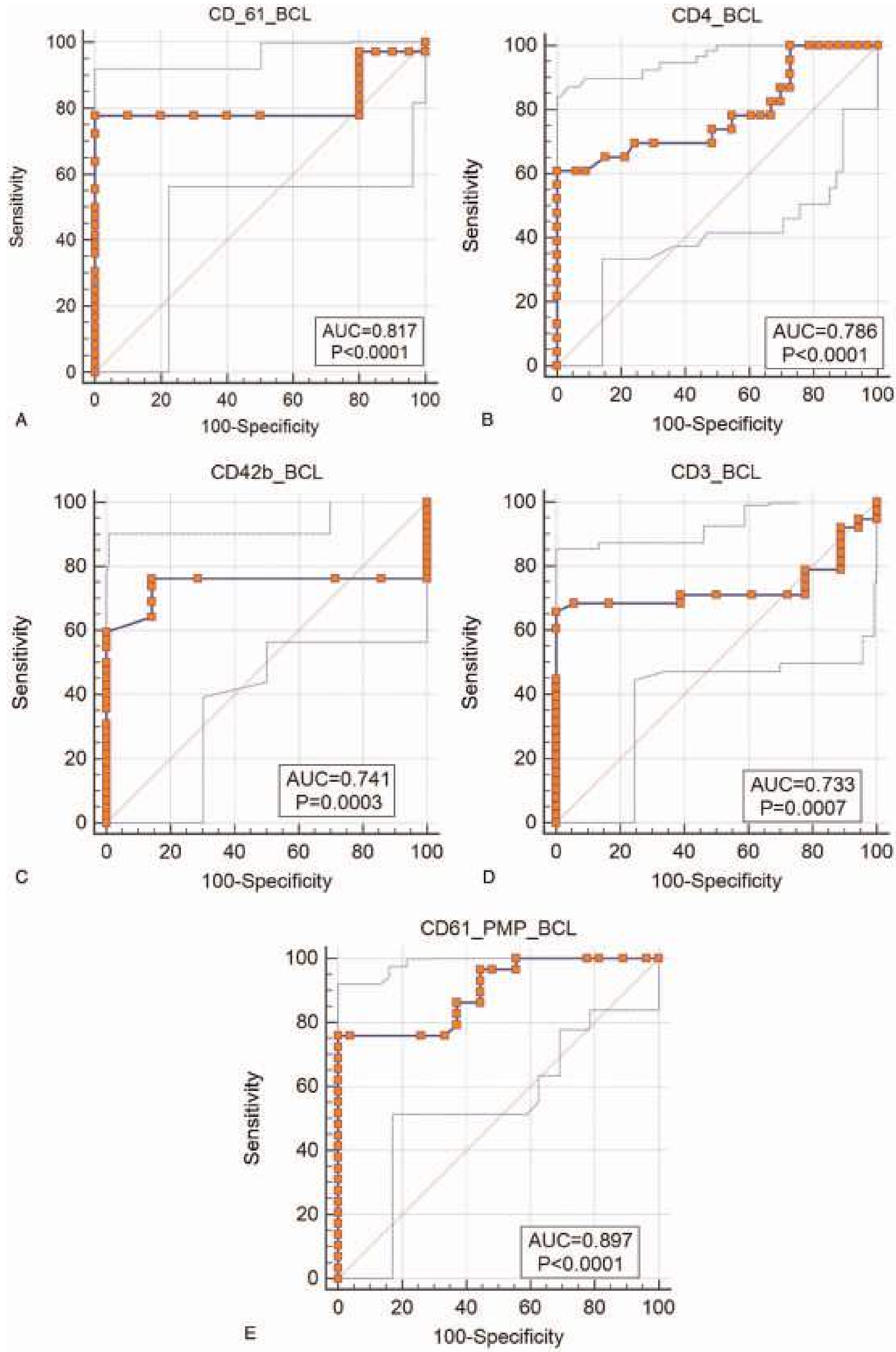

Figure 7. CD-expressions of platelets and lymphocytes, and CD61 platelets derivate microparticles as biomarkers in patients with B cell lymphoma: (A) platelet CD61 (AUC-0.817); (B) lymphocytes CD4+ (AUC-0.786); (C) platelet CD42b (AUC-0.741); (D) lymphocytes CD3+ (AUC-0.733); (E) CD61 PMP (AUC-0.897).

and cancer cells. Platelet activation in patients with low stage BCL manifests itself by increased expressions of CD61 and CD42b antigens, and in patients with AML manifests itself by increased expressions of microparticles derivate platelet-CD61. Increased levels of CD61, CD42b, and CD61 PMP, both in resting state and in in vivo active platelets, intensify the ability of platelets to adhesion and aggregation, increases the risk of thromboembolic events, promotes tumor cells proliferation, angiogenesis, and disease progression, in conformity with scientific literature.[27,29–32]

PMPs have an 18% to 28% contribution to both procoagulant and anticoagulant activities of human platelets stimulated with different platelet agonists. The percentage was higher in unstimulated platelets (28–40%). Leukemia cells can damage vascular endothelial cells, they interconnect with platelets and stimulate platelet activation.[33]

Modified levels of plasma PMPs were associated with bleeding or thromboembolic complications. In normal conditions, the presence of PMPs inhibits coagulation rather than promotes coagulation,[34] but the increased PMPs presence in clinical conditions may be a biomarker of ongoing thrombosis.[35] Increased PMPs count was observed in the blood of patients with thrombotic and inflammatory disorders.[36–39] The relationship between persistent immune system activation and coagulation/inflammation is poorly understood. Interactions between platelets and monocytes are well described by the scientific literature, but little is known about the interaction between platelets and the adaptive immune system. Platelet–T cell complex plays a role in the fast recruitment of antigen-experienced T cells to the inflammation/coagulation place. This mechanism maintains the procoagulation/inflammation status in patients, contributing to the disease pathology.[40]

An increased number of platelets in circulation and their ability to release pro-inflammatory and anti-inflammatory mediators, which are stored in secretory granules, suggest that platelets are critical players in the early phase of the host immune response.[41,42] Platelets are known to be involved in functions beyond hemostasis and were identified as regulators of both innate and adaptive immune systems.[43,44]

Our results agree with the scientific literature presented above because T helper/inducer lineage $CD4^+$ and T lymphoid lineage $CD3^+$ expressions presented significant differences between patients with AML, with low stage BCL, and those in matching control subgroups. In our study activation of the platelets was revealed by a significant increase of CD61 PMP in patients with AML and by increased expressions of CD61 and CD42b in patients with low stage BCL in comparison with control subgroups of patients. Furthermore, total numbers of platelets and leukocytes were significantly higher in patients with low stages BCL compared to controls. In contrast, in patients with high stage BCL, mean values of $CD4^+$ and CD 3^+ levels, CD 61, CD42b expressions, CD61 PMP, the total number of platelets, and leukocytes count were significantly decreased reported to control subgroups of patients.

In recent years, increasing evidence supports the notion that platelets participate in immune responses and interactions between platelets and leukocytes contribute to both thrombosis and inflammation.[45,46] Platelet–leukocyte complexes were observed in peripheral blood in patients with myeloproliferative disease.[47]

Because the tumor environment is one of inflammation, the tumor cells release chemokines and cytokines that attract inflammatory cells, which in turn release factors that the tumor cell uses to survive, proliferate, and metastasize.[48] Platelets were implicated in this process, and the platelet count is inversely correlated with survival.[49] Platelets and leukocytes can form complexes with tumor cells that facilitate tumor cell attachment to vessels and invasion of the tissue. P-selectin on both endothelial cells and platelets and the $\alpha 4\beta 1$ integrin on myeloid cells [50] are implicated in metastasis. Platelets and leukocytes release factors that destabilize existing vessels, promote capillary sprout formation, and enhance endothelial cell proliferation.[51]

In this study, because CD61 PMP plays an important role in the immune system by T regulatory cell activation, these are negatively correlated with $CD4^+$ lymphocytes in the subgroup of AML patients (age $\leq$ 65 years). Also, CD61-PMP percentage was presented a positive correlation with the percentage of $CD3^+$ lymphocytes for both subgroups of patients with BCL, and a negative correlation of this with $CD4^+$ lymphocytes in the high stage of BCL patients.

Results of some clinical studies have shown that the leukocytes–platelet interactions are directly correlated with platelet and leukocyte counts, and with the increased expression of leukocyte biomarkers.[52,53] Role of platelet microparticles in cancer development is unknown, but it is well established that tumor cells can activate platelets and induce platelet aggregation. The authors demonstrated that platelet microparticles levels are strongly correlated with aggressive tumors and a poor clinical outcome.[54]

High levels of the T regulatory cells in the tumor microenvironment are associated with poor prognosis in many cancers, such as ovarian, breast, colorectal, ovarian, renal, and pancreatic cancer.[55–60] In some types of cancer the opposite is true, and high levels of T regulatory cells are associated with a positive prognosis, in cancers such as colorectal carcinoma and follicular lymphoma. T regulatory cells can trigger cell proliferation and metastasis and this opposite effect indicate that the T regulatory role in the development of cancer is dependent on both the type and location of the tumor.[56]

Increased levels of CD61 PMP in AML patients and CD61 and CD42 B expressions in BCL patients indicate platelet activation. Since we found that platelet activation was associated with platelet binding to lymphocytes in AML and BCL patients, it is likely that platelet binding modifies the lymphocyte function. Authors showed that platelet bound preferably to memory T lymphocytes.[61,62]

In this study, we observed the accuracy of the potential biomarkers for AML and BCL patients. As expected, flow cytometry represents the most sensitive modality for immunophenotyping. Methods sensitivity average was $80.12 \pm 14.79\%$ and the specificity average of the methods was $92.29 \pm 12.77\%$. Predictive positive values average (94.90 ± 7.02) and predictive negative values average (78.94 ± 15.71) means that the flow cytometry analysis methods had good accuracies to obtain the results. The average of the area under the curve presented by ROC curves (AUC $\pm$ SE) was 0.841 ± 0.085 which means good statistical positive and negative results distribution for both groups of patients and controls. Our obtained results conform with recent studies about the diagnosis of acute leukemia and Non-Hodgkin lymphomas methods.[63–67]

In this study, limitations to develop the utility of these observed biomarkers were the fact that were analyzed only for AML and BCL from all diversity of hematological malignancies, and a small number of samples recovered from treated patients to be able to study the subtypes of AML and NHL. However, we cannot eliminate that medication of AML and BCL patients could affect lymphocytes–platelets interactions. Because this represents a limitation, a study of lymphocyte–platelet complexes in a large cohort of patients before and after taking drugs will be required to study the modulation of these. We show in this study that platelets can bind to lymphocytes in AML and BCL patients, and this interaction is associated with altered immune response. Another limitation is that lymphocyte–platelet complexes were analyzed at a one-time point in each patient during the treatment. Further studies analyzing the same patient before and after taking drugs will be required to validate the potential of lymphocyte–platelet complexes in immune response in patients with AML and BCL. Our observations suggest that controlling these interactions is beneficial for the therapeutic regulation of the patient's immunity.

5. Conclusions

Platelet–leukocyte interactions are involved in tumor diseases, which supports that platelets represent an immune system fundamental part. During these interactions, platelets trigger intracellular signaling in immune cells and they modulate the immune responses.

Platelet activation in patients with low stage B cell lymphoma manifests itself by increased expressions of CD61 and CD42b antigens, and in patients with acute myeloblastic leukemia manifests itself by increased expressions of microparticles derivate platelet-CD 61.

Future directions in this research area will be to study a variety of hematological malignancies subtypes and to establish the valid correlations between observed biomarkers and DNA content and cell apoptosis before and after taking drugs at patients.

Because of the importance of platelet activity in many clinical conditions, some assay of platelet activation will eventually be widely utilized. Another direction to study in correlations with our biomarkers is the DNA content because flow cytometers provide a rapid method to measure altered DNA content, ploidy, and analysis of cell division kinetics (S-phase fraction, mitotic index) has important implications in areas such as clinical tumor prognosis. Also, other future directions correlated with our biomarkers will be the analysis of cell apoptosis, because it is a major research application of flow cytometry and may become a new clinical application for determining the efficacy of chemotherapy in patients with different types of malignancies. Also, our findings suggest that the determination of the levels of circulating lymphocytes with bound platelets in AML and BCL patients may prove to be a useful tool to follow up the activities of the diseases. On the other hand, understanding how platelet microparticles impact different diseases will enable the identification of new cellular pathways that are amenable to therapeutic manipulation. Besides, platelet microparticles show promise as a diagnostic biomarker for diseases and potentially as a delivery system for therapeutics.

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Author contributions

All authors contributed equally to this study and share the first authorship. EM, MA, and MMG designed the research; EM and MM performed the experiments; EM performed the statistical analysis, made the tables and the figures; EM is a certified specialist in biostatistics and computational uncertainty measurement and performance indicators in the laboratory; LP is an expert biostatistician; EM, AM, AC, and ND wrote the manuscript; EM and EG revised and corrected the manuscript.

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Overexpression of Survivin-1, TAG-72 and HERC5 in patients diagnosed with hepatocellular carcinoma in the Black Sea coast geographical area

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Abstract. Epidemiological data regarding hepatocellular carcinoma (HCC) report unsatisfactory morbimortality rates despite the global efforts to decrease the incidence and prolong patient survival. Current guidelines lack diagnostic biomarkers to better characterize patients with HCC. We aimed to validate the overexpression of Survivin-1, tumor-associated glyocoprotein 72 (Tag-72), and HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5) as tissue biomarkers for HCC characterization in patients from our geographical area and to standardize a local biomarker panel to be introduced in the current management guideline. Thirty samples of histologically confirmed HCC were compared to an equal number of samples of benign tumors in terms of Survivin-1, TAG-72, and HERC5 overexpression. Student's t-test, Mann-Whitney U test and Chi-square test were used to find differences between the two studied groups and to compare the categorical variables. The discriminative power of Survivin-1, Tag-72, and HERC5 overexpression was assessed using ROC curves. The multivariate linear regression analysis revealed that Survivin, Tag-72, and HERC5 were significantly overexpressed in older male patients, with α-fetoprotein (AFP) >200 ng/dl, low serum albumin, as well as in patients with imaging features of portal thrombosis and ascites. The diagnostic performance of Survivin-1, Tag-72 and HERC5 tissue biomarkers for HCC characterization was superior to that of the gold-standard AFP. Our study results validate the overexpression of Survivin-1, Tag-72, and HERC5 as tissue biomarkers for HCC characterization in patients from our geographical region and could be standardized in the current HCC management guideline.

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Key words: immunohistochemistry, overexpression, Survivin-1, Tag-72, HERC5, hepatocellular carcinoma

Introduction

Despite the great achievements obtained in the early detection of hepatocellular carcinoma (HCC) through screening programs and application of targeted therapies with protease inhibitors such as sorafenib, the incidence and the poor survival rate reported are still high, especially in endemic areas for hepatotropic viruses (HBV, HCV and HDV) such as southeastern Europe (1-4). Panels of biomarkers are standardized to help clinicians in their efforts to improve knowledge in terms of better HCC characterization for more efficient therapies. A single biomarker still used for HCC diagnosis or follow-up is α-fetoprotein (AFP), which is considered as the gold standard of care. Yet, clinical evidence suggests that it does not help facilitate improvement in HCC progression, prognosis, or survival rates (5). In general, tumor cells present metabolic signatures compared to healthy cells, both at the tissue and bio-humoral levels. The detection of new tumor cell biomarkers, and their validation has presented new research goals for HCC characterization. Viral infections, alcohol abuse, dysmetabolic states [obesity, type 2 diabetes mellitus (T2DM), nonalcoholic steatohepatitis (NASH)], and other rare conditions causing subsequent chronic liver damage promote liver tumorigenesis through different mechanisms and this situation makes it difficult to standardize a panel of predictive biomarkers for HCC progression (6-9). Survivin-1, an anti-apoptotic protein modulated by the p53 gene, presents overexpression in 70% of Asian HCC patients with chronic viral hepatitis in whom mutations of p53-gene are apparent (10). The overexpression of this tissue biomarker has not been studied in other research. It represents an opportunity to study Survivin-1 due to the similarity between risk factors for HCC occurrence in our patients and Asian patients-a vast majority being infected by hepatitis B, C, and D viruses. Tumor-associated glycoprotein 72 (TAG-72) is a mucin-like membrane complex considered to be a feasible biomarker for unfavorable prognosis of adenocarcinomas in general, with potential applicability in HCC (11,12). HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5), a protein with a ligand role, activates the chemotaxis and the local infiltration with T lymphocytes, being considered a biomarker for predicting HCC recurrence, as well as the poor survival rates even for early stage HCC (13,14). In this scenario, we aimed to evaluate and validate the overexpression of Survivin-1, TAG-72, and HERC5 as tissue biomarkers for HCC characterization in patients from our geographical region and to standardize a local biomarker panel to be introduced in the current management guideline.

Materials and methods

Materials. Thirty liver specimens with a histopathological diagnosis of HCC (study group) and a similar number of liver tissue specimens of benign liver tumors (adenomas, HNF, regenerative nodules, and hemangiomas-control group) were selected from the Gastroenterology file database and Pathology Clinic registries from St. Apostle Andrew Emergency Clinical Hospital, Constanta and Fundeni Institute, Bucharest and compared in terms of Survivin-1, Tag-72, and HERC5 overexpression. All cases were registered in our databases in the last 3 years and 6 months (January 2017 to June 2020).

All cases histologically confirmed by pathologists from both clinics, were reinterpreted for the current study during the interval December 2019 to June 2020, and the immunohistochemistry study was conducted at the Research and Development Centre for the Morphologic and Genetic Study of Malignant Pathology (CEDMOG) and founded by 'Ovidius' University. The morphological features of the tumors were noted, establishing the histological type, the grade, and the stage of HCC based on the World Health Organization (WHO) Histological Classification for digestive tumors (15).

Demographic data of all patients (study and control group) providing the liver specimens, including age, sex, provenence, medical history, liver disease background-chronic viral infections with B, C, or D viruses, co-morbidities and laboratory parameters recorded at the time of hospital admission were obtained from the clinical files and are noted in Table I.

The Ethics Committee of Emergency Clinical Hospital St. Apostle Andrew of Constanta approved the study following European and local regulations (no. 32/22.11.2019).

Methods. For the immunohistochemical (IHC) assessment, the representative samples were chosen, and 4-μm sections of formalin-fixed, paraffin-embedded tissue blocks were obtained for each case enrolled. Epitope retrieval was conducted prior to incubation of tissue sections with a panel of three primary antibodies (ready-to-use) from Novus Biological: Survivin-1 (NB100-911 clone), Tag-72 (CC49 clone), and HERC5 (NBP-91985 clone). The immunostaining protocol for each antibody used was provided by the manufacturer. As chromogen, we used 3,3'diaminobenzidine (DAB), and brown staining was obtained. The final step was represented by counterstaining all slides with Mayer's Hematoxylin. Positive control was used for each antibody: Human testis for HERC5 antibody, malign melanoma for Survivin-1 antibody, and human prostate carcinoma for the Tag-72 antibody. Comparisons of the studied biomarker overexpression from HCC tissue samples with a matched non-HCC group of normal liver tissue specimens were made.

Statistical analysis. Quantitative variables such as mean ± standard deviation (SD) and categorical variables are presented as percentages. The Student t-test and Mann-Whitney U test were used to identify differences between the two studied groups. The Chi-square test facilitated the comparison of categorical variables. The correlation between Survivin-1, Tag-72, and HERC5 overexpression and different HCC variables was performed using the Spearman rank correlation test. A multivariate logistic regression analysis detected the independent variables of HCC. The discriminative power of Survivin-1, Tag-72, and HERC5 overexpression was assessed using ROC curves. The predictive performance of biomarker overexpression was classically evaluated by the area under the ROC curve (AUC), sensitivity (Se), specificity (Sp), positive, and negative predictive values (PPV, NPV). SPSS 16.0 software (SPSS, Inc.) was used for statistical analysis. P-values <0.05 were considered statistically significant.

Results

Demographic, clinical and laboratory features of the HCC patients. According to demographics, we noted an evident predominance of male gender in the study group compared to the control group (17 vs. 25 male patients, P=0.023). The age of the HCC patients was significantly older than that of the control group (42.09±9.4 vs. 59.2±5.9 years, P=0.002). Laboratory results were various and without any statistical significance related to cytolysis (30±11.67 vs. 37.3±14.11 UI/ml, P=0.067) but significantly different related to parameters of liver insufficiency such as albumin (4.3±1.2 vs. 2.3±1.4 g/dl, P<0.001), bilirubin (1.9±0.5 vs. 4.3±2.7 mg/dl, P=0.002), and INR (International Normalized Ratio) (1.4±0.8 vs. 1.9±1.0, P=0.026). AFP had values slightly above the normal upper limit in both HCC and controls, but levels >180 ng/ml were more frequently encountered in HCC patients compared to the controls (204.11±17.77 vs. 308.56±44.01 ng/ml, P=0.015). Jaundice was more regularly present in the HCC patients compared with the control (11 vs. 20 patients, P=0.044) (Table I).

Comparison of IHC overexpression of Survivin-1, Tag-72, and HERC5 in liver tissue samples between HCC and controls. Statistical analysis using a multivariate linear regression tool was conducted to correlate the IHC overexpression of Survivin-1, Tag-72, and HERC5 and independent variables as age, sex, laboratory results, imaging and clinical features. The multivariate linear regression analysis revealed that Survivin-1, Tag-72, and HERC5 were significantly overexpressed upon

Table I. Demographic, clinical, imaging and laboratory features of the HCC and control (benign tumor) group.

	Benign tumors (control group) (N=30) n (%)	HCC group (study group) (N=30) n (%)	P-value	r-value
Age, mean ± SD, years	42.09±9.4	59.2±5.9	0.002	0.96
Sex				
Male	17 (56.66)	25 (83.33)	0.023	0.24
Female	13 (43.33)	5 (16.66)	0.033	-0.32
Urban	12 (40.00)	15 (50.00)	0.051	0.70
Risk factors				
Viral infection	3 (10.00)	21 (70.00)	0.001	0.98
Alcohol abuse	15 (30.00)	20 (66.66)	0.048	0.33
NASH/NAFLD	5 (16.66)	11 (36.66)	0.042	0.34
Metabolic disease	2 (6.66)	3 (10.00)	0.7	0.11
Clinical features				
Hepatomegaly	3 (10.00)	22 (73.33)	0.005	0.91
Jaundice	11 (36.66)	20 (66.66)	0.044	0.59
Ascites	1 (3.33)	16 (53.33)	<0.001	0.99
Weight loss	12 (40.00)	24 (80.00)	0.003	0.94
UDB	-	7 (23.33))	-	-
Imaging features				
Multifocal	6 (20.00)	10 (33.33)	0.037	0.28
Portal vein thrombosis	1 (3.33)	6 (20.00)	0.032	0.26
Ascites	1 (3.33)	9 (30.00)	0.027	0.21
Laboratory results				
ALT UI/ml, mean ± SD↑	30±11.67	37.3±14.11	0.067	0.58
	10 (33.33)	23 (76.66)		
GGT UI/ml ↑	34.33±8.22	71.54±6.99	0.029	0.30
	16 (53.33)	24 (80.00)		
Albumin g/dl↓	4.3±1.2	2.3±1.4	<0.001	0.99
	1 (3.33)	22 (73.33)		
Bilirubin mg/dl↑	1.9±0.5	4.3±2.7	0.002	
	11 (16.66)	24 (80.00)		
INR↑	1.4±0.8	1.9±1.0	0.026	0.44
	10 (33.33)	12 (40.00)		
AFP ng/ml ↑	12 (40.00)	23 (76.66)	0.011	0.29
≥6 ng/ml	6.92±2.6	8.22±3.1	0.051	0.70
	11 (18.60)	14 (46.66)		
>180 ng/ml	204.11±17.77	308.56±44.01	0.015	0.18
	1 (4.80)	9 (30.00)		
BCLC classification				
A	-	13	-	-
B	-	11	-	-
C	-	6	-	-

HCC, hepatocellular carcinoma; NASH, nonalcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; ALT, alanine transaminase; GGT, γ-glutamyl transferase; AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; INR, International Normalized Ratio. Laboratory results are expressed as the mean increased/decreased level (top value) and the number of patients (%) with an increased/decreased level (lower data).

IHC analysis in HCC samples in patients older than 50 years (P=0.003, P=0.006, P=0.004, respectively), male gender (P=0.031, P=0.004, P=0.020, respectively) patients with increased AFP over 180 ng/dl (P=0.012, P=0.004, P=0.029,

Table II. Multivariate linear regression analysis to identify the correlation between overexpression of liver biomarkers and clinical, laboratory and imaging variables in HCC.

	Liver tissue biomarker overexpression		
Patient variables	Survivin-1 P-value (r)	Tag-72 P-value (r)	HERC5 P-value (r)
Age, mean ± SD, >50 years	0.003 (0.94)	0.006 (0.92)	0.004 (0.94)
Male gender	0.031 (0.55)	0.004 (0.96)	0.020 (0.70)
Bilirubin	0.076 (0.24)	0.064 (0.41)	0.060 (0.44)
INR	0.055 (0.54)	0.088 (0.27)	0.059 (0.52)
AFP >180 ng/ml	0.012 (0.83)	0.004 (0.96)	0.029 (0.80)
Albumin <3 mg/dl	0.031 (-0.66)	0.021 (0.72)	0.003 (0.94)
GGT	0.059 (0.72)	0.055 (0.51)	0.030 (0.48)
Portal thrombosis	0.004 (0.96)	0.020 (0.70)	0.004 (0.96)
Ascites	0.002 (0.98)	0.004 (0.96)	0.019 (0.82)
Hepatosplenomegaly	0.060 (0.74)	0.003 (0.94)	0.039 (0.59)
BCLC			
A	0.076 (0.33)	0.086 (0.25)	0.055 (0.54)
B	0.045 (0.67)	0.036 (0.24)	0.045 (0.67)
C	0.033 (0.60)	0.001 (0.99)	0.027 (0.78)

HCC, hepatocellular carcinoma; Tag-72, tumor-associated glyocoprotein; HERC5, HECT and RLD domain containing E3 ubiquitin protein ligase 5; GGT, γ-glutamyl transferase; AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; INR, International Normalized Ratio.

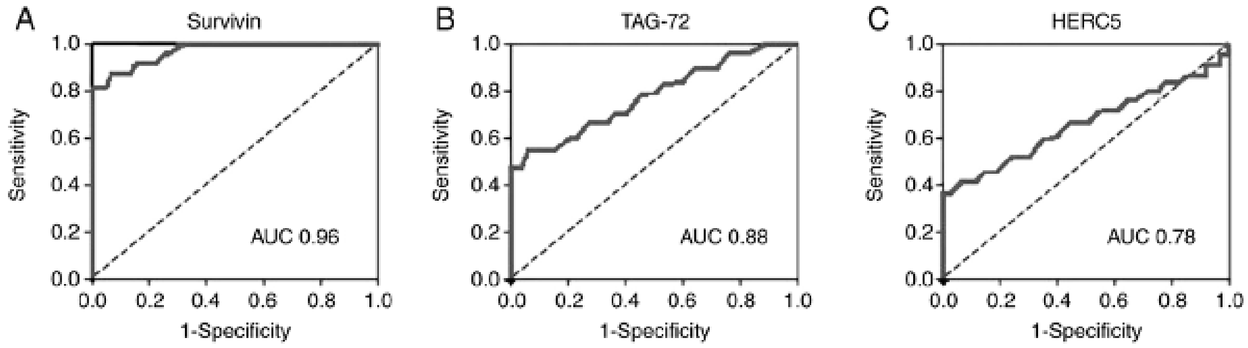

Figure 1. Area under the curve (AUC) for the liver tissue biomarker used for HCC diagnosis: (A) Survivin-1, (B) Tag-72, and (C) HERC5. HCC, hepatocellular carcinoma; Tag-72, tumor-associated glyocoprotein 72; HERC5, HECT and RLD domain containing E3 ubiquitin protein ligase 5.

respectively) and with low serum albumin <3 mg/dl (P=0.031, P=0.021, P=0.003, respectively) as well as in patients with imaging features of portal thrombosis (P=0.004, P=0.020, P=0.004, respectively) and ascites (P=0.002, P=0.004, P=0.019, respectively) and in BCLC B class (P=0.045, P=0.036, P=0.045, respectively) and C (P=0.033, P=0.001, P=0.027, respectively) classified patients (Table II). Overexpression of the studied biomarkers did not correlate positively with cytolysis and other cholestasis tests or with the remaining clinical or imaging features of HCC (Table II).

Diagnostic accuracy of Survivin-1, Tag-72 and HERC5 for HCC. Survivin-1 tissue biomarker had an AUC of 0.96 [95% confidence interval (CI), 0.90-1.00] for the diagnosis of HCC, with 90.0% sensitivity, 100% specificity, 100% PPV, and 94.2% NPV for an optimal cut-off value of 0.3 (Fig. 1A and Table III). Tag-72 tissue biomarker had an AUC of 0.88 (95% CI, 0.78-0.94), with 72.8% sensitivity, 95.6% specificity, 82.2% PPV, and 84% NPV for an optimal cut-off value of 0.3 (Fig. 1B and Table III). HERC5 had an AUC of 0.78 (95% CI, 0.66-0.84), with 65% sensitivity, 86% specificity, 77.4% PPV, and 72.3% NPV (Fig. 1C and Table III) for an optimal cut-off value of 0.3. AFP, still considered the gold standard biomarker used in clinical settings and recommended by the international guidelines for HCC management, had an AUC of 0.34 (95% CI, 0.28-48) for the diagnosis of HCC, with 38.0% sensitivity, 66% specificity, 64% PPV, and 68.8% NPV for an optimal cut-off value of 180 ng/dl (Fig. 2). The diagnostic performance of Survivin-1, Tag-72 and HER-C5 tissue biomarkers for HCC characterization was superior to that of AFP, considered the gold standard biomarker used in clinical guidelines (Survivin-1: Z statistic=2.911, P=0.0039; Tag-72: Z statistic=2.789, P=0.0049, respectively; HERC5: Z statistic=2.844, P=0.0043) and AFP assay alone (Z statistic=5.022, P<0.0001) (Table IV).

Table III. Accuracy parameters of Survivin-1, Tag-72 and HERC5 for HCC diagnosis.

	Survivin-1	Tag-72	HERC5
AUC (95% CI)	0.96 (0.90-1.00)	0.88 (0.78-0.94)	0.78 (0.66-0.84)
Accuracy (%)	97.2	80.4	75
Sn (%)	90	72.8	65
Sp (%)	100.0	96.6	86
PPV (%)	100.0	82.2	77.4
NPV (%)	94.2	84.0	72.3

Tag-72, tumor-associated glyocoprotein; HERC5, HECT and RLD domain containing E3 ubiquitin protein ligase 5; AUC, area under the ROC curve; Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPN, negative predictive value.

Table IV. Diagnostic performance of Survivin-1, Tag-72 and HER-C5 tissue biomarkers for HCC compared to gold standard AFP.

Diagnostic performance	Survivin-1 z statistic (P-value)	Tag-72 z statistic (P-value)	HERC5 z statistic (P-value)
AFP	2.911 (0.0039)	2.789 (0.0049)	2.844 (0.0043)

Tag-72, tumor-associated glyocoprotein; HERC5, HECT and RLD domain containing E3 ubiquitin protein ligase 5; AFP, α-fetoprotein.

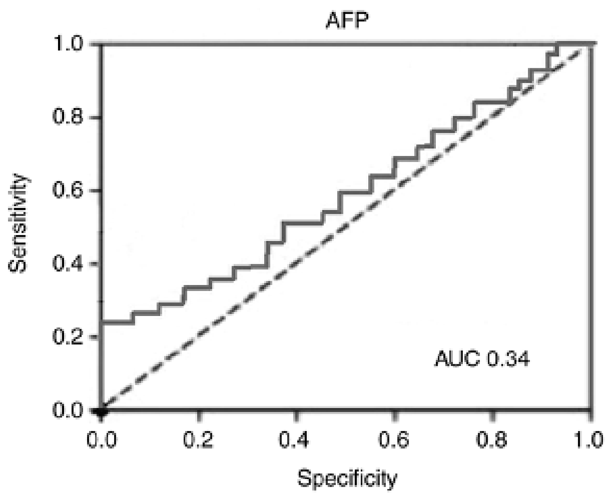

Figure 2. Area under the curve (AUC) for AFP used for HCC diagnosis. AFP, α-fetoprotein; HCC, hepatocellular carcinoma.

Discussion

Immunohistochemistry represents a research tool with large applicability of monoclonal and polyclonal antibodies in detecting a specific antigen with a high potential for a positive diagnosis (16). This method is largely used to diagnose malignant tumors, but it presents an area of interest for numerous applications useful not only for positive and differential diagnosis but also to provide a research field in the attempt to identify prognostic markers for cancer evolution, to confirm the positive diagnosis of tumors with uncertain histogenesis, to predict the response to treatment and to identify or confirm certain infections (7,17,18). Such biomarkers facilitate the efforts of researchers for a better understanding of HCC pathogeny, to provide more efficient therapies, and to improve disease prognosis. Epidemiology data confirm the persistent high global morbi-mortality rates for HCC, which are more consistent in endemic areas for chronic viral infections, such as Asia, the Middle East, Mediterranean countries, South America, and Africa (19-22). The current guidelines use a single biomarker for HCC follow-up, this being α-fetoprotein (AFP). The literature reports a low accuracy for AFP, and declines its role as a potent prognostic tool, thus new liver tissue biomarkers are being explored worldwide to improve HCC management (5). The difference between various etiopathogenic mechanisms involved in liver primary carcinogenesis makes it difficult to standardize a global panel of liver tissue biomarkers to simplify disease management. Using this scenario, having literature models from other studies conducted in different populations, we evaluated the expression of three liver tissue markers, aiming to confirm their overexpression in HCC tissue in patients from the Dobrogea area and to provide the background for further research for prognostic predictability or patient classification in risk groups. Our study demonstrated the same pattern of demographic, clinical, and laboratory features as the majority of published data, with a high prevalence of viral infections and alcohol abuse leading the risk factor background, and male gender and middle age, being the main characteristics of our patients. All liver tissue biomarkers explored provided a good accuracy for HCC diagnosis: 97.2% for Survivin-1, 80.4% for Tag-72, and 75% for HERC5, similar to the literature data (10,12,13). AFP over the upper limit did not prove to have diagnostic or predictive value for HCC, similar to other literature articles (23-26). Still, values over 180 ng/dl were highly predictive, and they were found to be positively correlated with all biomarkers studied, Survivin-1, Tag-72, and HERC5 (r=0.83, r=0.96, r=0.80, respectively). Our study results confirm the background hypothesis, indicating the overexpression of Survivin-1, Tag-72, and HERC5 as feasible biomarkers with which to diagnose HCC. Larger IHC studies should sustain the accuracy of the proposed tools before their introduction to international HCC management guidelines. Comparative study with other methods of diagnosis and prognostic evaluation used and other types of carcinomas, such as the study of neoangiogenesis or nuclear morphometry, can bring new useful data both in regards to diagnosis and in the prognosis of the disease (27,28). Despite the importance of our study results, our work had limits due to the increased costs of study materials, a fact that influenced the number of samples evaluated by immunohistochemistry, a problem partially solved by the funds gained through university research grant competition.

In conclusion, our study results validate the overexpression of Survivin-1, Tag-72, and HERC5 as tissue biomarkers for HCC characterization in patients from our geographical region and could be standardized in the current HCC management guideline.

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Availability of data and materials

The data were obtained from the HIPOCRATE file archive of the Emergency Hospital St. 'Apostle Andrew' of Constanta and CEDMOG archive (http://www.spitalulconstanta.ro/). Further information about the present study is available from the corresponding author upon reasonable request.

Authors' contributions

AIS, APS, GIB, VH, CN, LCP, FV, ISM and LM conceived and designed the study; ND, LCP, CB and FB collected the data. LCP, ND, CB, and FV analyzed the data; AIS, APS, CN and ISM validated the results. FB, CN, LCP and ND were responsible for original draft preparation; AIS, GIB, LCP, ND, ISM and VH were responsible for the final manuscript editing. AIS, APS, GIB and CN supervised the manuscript publication. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Emergency Clinical Hospital St Apostle Andrei Constanta approved the study following European and local regulations. Approval no. 32/22.11.2020. Emergency Clinical Hospital St. Apostle Andrew from Constanta is a university hospital and all admitted patients sign an informed consent by which they agree that their data are available for academic and scientific purposes.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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CONFERENCE PROCEEDINGS

BOOK 3
SCIENCE AND SOCIETY
VOLUME III

SGEM

SOCIOLOGY AND HEALTHCARE

GASTROESOPHAGEAL REFLUX DISEASE AND ORAL HYGIENE – RISK FACTORS FOR DENTAL EROSION

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ABSTRACT

Background Dental erosion (DE) is the loss of dental hard tissue due to the action of gastric juice and acidic foods, without the participation of bacterial flora. Gastroesophageal reflux disease (GERD) is the involuntary passage of gastric juice to the esophagus. Patients with GERD have changes in salivary parameters.

Objectives The purpose of this study is to analyze the levels of some salivary parameters and oral hygiene in patients with GERD and DE before and after therapy and to assess their correlations.

Methods Fifteen patients clinically diagnosed with GERD and DE and 15 healthy volunteers (C) were included in this study. Oral hygiene was evaluated by Oral Hygiene Index (OHI). Salivary parameters: pH and Buffer capacity (SBC) were determined on total saliva by using G.C. Saliva Check Buffer kit. The data obtained in this study were statistically processed with MedCalc 14 and for a $p<0.05$ was considered to have statistical significance. The study was approved by the Ethics Committee for medical research from Ovidius University of Constanta and the signed agreement from each subject was obtained.

Results The OHI values for patients with GERD and DE before and after therapy (BT, AT) were statistically significant increased compared with those obtained for healthy individuals: 3.4 BT versus 0.306 C, 0.765 AT versus 0.306 C, $p < 0.001$. Salivary pH levels of patients with GERD and DE were statistically significant lower before and after therapy than healthy persons: 6.127 BT versus 7.08 C, 6.707 AT versus 7.08 C, $p < 0.001$. Patients had significantly lower values of salivary buffer capacity before and after therapy than healthy persons: 5.057 BT versus 10.46 C, 8.8 AT versus 10.46 C, $p < 0.001$. There is a strong negative correlation between OHI with salivary pH and SBC before therapy: $r = -0.951$ pH, $r = -0.933$ SBC, $p < 0.001$. After therapy, there is a moderate negative correlation between OHI with salivary parameters: $r = -0.528$ pH, $r = -0.512$ SBC, $p < 0.001$.

Conclusion The results obtained in this study showed that the increased values of OHI or decreased values of pH and salivary buffer capacity can be a mirror for the amplitude of dental erosion in patients with gastroesophageal disease.

Keywords: gastroesophageal disease, dental erosion, saliva

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INTRODUCTION

Dental erosion (DE) can be defined as progressive dissolution of dental hard tissue by chemical processes without the participation of bacterial flora [1]. The acids responsible for dental erosion come from extrinsic and intrinsic sources [2], [3].

The extrinsic causes are associated with dietary factors, medications, and oral hygiene. The intrinsic causes involved in dental erosion comprise recurrent vomiting and regurgitation events in patients suffering from gastroesophageal reflux disease (GERD), anorexia and bulimia [4].

The mechanism involved in dental erosion starts at the surface of the enamel, where acids or chelating anions destroy the pellicle and organic substrate, followed by the demineralization of the enamel tooth mineral. The critical salivary pH value responsible for erosions changes is around 5.5 [5].

There are different physiological factors among which some protect against erosion and others can increase the degree of dental erosion [6]. These factors include: tooth structure and composition, saliva, dental anatomy.

Saliva is one of the biological factors that provide protection against acid erosion by different ways, [7], [8]:

- contributes to the formation of the pellicle;
- dilutes acid solutions introduced into the oral cavity;
- because of the salivary buffer capacity, the food acids present in the oral cavity are neutralized;
- saliva has in its composition calcium, phosphates and fluoride that are involved in mineralization;
- proteins present in saliva have an important role in dental erosion.

Several studies suggest that salivary metalloproteinases present an increased susceptibility in the progression of the erosion [9], [10].

Gastroesophageal reflux disease is the involuntary passage of gastric juice to the esophagus and oral cavity. GERD is one of the causes involved in dental erosion. Patients with GERD have changes in salivary parameters, such as: pH, buffer capacity. Low pH values and salivary buffer capacity directly influence the severity of dental erosive dental lesions. Oral hygiene is also a risk factor in dental erosion for patients with GERD [11], [12].

The treatment of GERD and the dental erosion as well as lifestyle changes contribute to the rehabilitation of dental health.

The purpose of this study is to analyse the levels of some salivary parameters and oral hygiene in patients with gastroesophageal reflux disease and dental erosions before and after therapy and to assess their correlations.

MATERIALS AND METHODS

Fifteen clinically diagnosed GERD and dental erosion patients aged between 40 to 65 years old and 15 healthy adult volunteers (C) were included in this study. All people included in this study have given their written consent to take part in this research activity.

Oral hygiene was evaluated by Oral Hygiene Index (OHI) before treatment (BT) and after treatment (AT) [13].

Unstimulated saliva for pH and stimulated saliva for buffer capacity was collected from all persons included in study, by using sterile test tubes (Salivette – Starstedt AG&Co. Germany) at 5 minutes after mouth cleansing and before breakfast. Saliva was collected in two steps, for patients with GERD and DE: before treatment and after treatment.

Salivary parameters: pH and buffer capacity (SBC) were determined on total saliva by using G.C. Saliva Check Buffer kit.

The data obtained in this study were statistically processed by using Test – t, One Way Anova test from MedCalc 14.0; $p<0.05$ was considered to have statistical significance.

RESULTS

The results of this current study showed that salivary pH, buffer capacity and OHI values of the patients with GERD and DE before and after treatment were different in relation to the healthy volunteers.

Salivary pH mean levels of patients with GERD and DE were statistically significant lower before and after therapy than healthy persons: 6.12BT and 6.70AT versus 7.08C, $p<0.001< \alpha = 0.05$ (Figure 1).

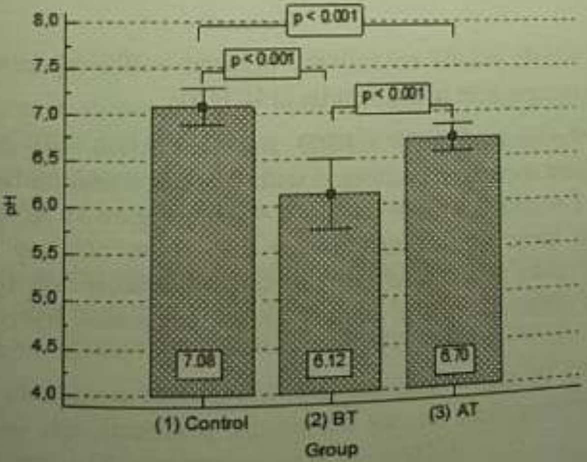

Figure 1 pH mean values and standard deviation in the study groups

The salivary mean values of pH before treatment were significantly decreased compared to values after treatment. Unfortunately, after treatment, the increase of the salivary pH mean value was below the pH mean value of the healthy group. A statistically significant difference ($p <0.05$), suggest that the treatment should be continued.

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The patients had significantly lower values of salivary buffer capacity (SBC) before and after treatment than healthy persons: 5.06BT and 8.80AT versus 10.46C, $p < 0.001 < \alpha = 0.05$ (Figure 2).

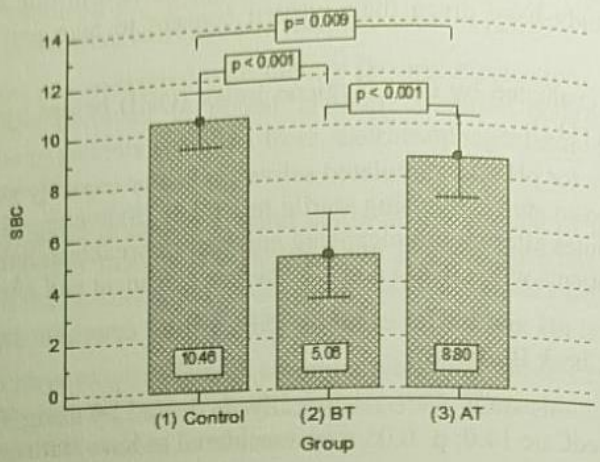

Figure 2. SBC mean values and standard deviation in the study groups

The low values obtained before therapy for pH and the buffer capacity explain the high rate of erosive dental lesions in patients with GERD and DE.

Before therapy, the salivary mean values of buffer capacity were significantly decreased compared to values after treatment. Unfortunately, after treatment, the increase of the salivary SBC mean value was below the SBC mean value of the healthy group. A statistically significant difference ($p < 0.05$), suggest that the treatment should be continued.

There are studies whose results on salivary pH and buffer capacity in patients with GERD and DE are comparable to our results [14], [15].

The OHI values for patients with GERD and DE before and after therapy were statistically significant increased compared with those obtained for healthy individuals: 3.40BT and 0.76AT versus 0.30C, $p < 0.001 < \alpha = 0.05$ (Figure 3). After therapy, the OHI values for patients were significantly lower than those obtained before treatment. Unfortunately, the values obtained after treatment were statistically significantly higher compared to those obtained for the healthy group, suggesting the continuation of the therapy.

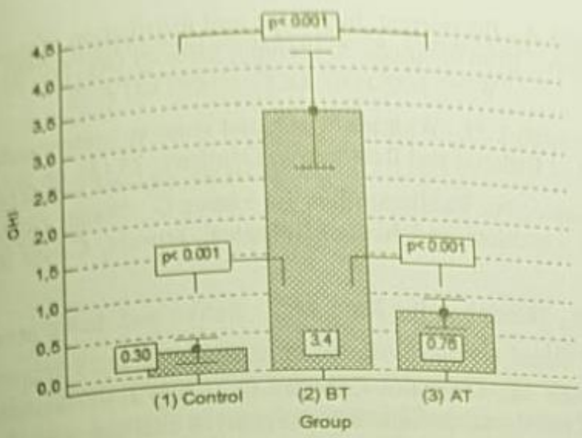

Figure 3. OHI mean values and standard deviation in the study groups

Another objective of this study was to assess the correlations between salivary parameters and the oral hygiene index before and after treatment.

Correlations between the oral hygiene index and salivary pH showed:

- before therapy a high negative coefficient of correlation, $r = -0.951$, $p<0.001< \alpha = 0.05$;
- after therapy a moderate negative correlation, $r = -0.528$, $p = 0.042< \alpha = 0.05$.

Correlations between the oral hygiene index and salivary buffer capacity showed:

- before therapy a high negative coefficient of correlation, $r = 0.933$, $p<0.001< \alpha = 0.05$;
- after therapy a moderate negative correlation, $r = 0.512$, $p = 0.048 < \alpha = 0.05$.

These correlations have shown the link between oral hygiene and salivary parameters for patients with GERD and DE. These correlation indices can be a mirror for the amplitude of the dental erosion rate.

CONCLUSIONS

The results obtained in this study showed that the increased values of OHI or decreased salivary values of pH and buffer capacity can be a mirror for the amplitude of dental erosion in patients with gastroesophageal disease and dental erosion.

The existence of correlation indices between salivary values of OHI, pH and buffer capacity before and after treatment allows the use of these parameters to assess the effect of treatment applied to patients with GERD and DE.

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CHANGES INDUCED BY THE DECREASE OF SALIVARY PH ON THE HARDNESS OF THE DENTAL ENAMEL

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ABSTRACT

Introduction. The objective of this investigation is to pinpoint the influence of the salivary pH on the hardness of the dental enamel. Method. The study was realized using recently extracted 6 non-carious wisdom teeth, that were immersed in solutions of different pH (between 2.5 and 6.5) for 3 minutes, solutions that would simulate the pH of the oral cavity in patients with a potential condition of gastroesophageal reflux disease. The hardness of the enamel was tested using the Vickers method. Results show that there are no statistically significant differences ($p > 0.05$) between the average values of hardness of the specimens immersed in pH 5.5, 6.5 and control group specimens, while among all other specimens immersed in pH = 2.0 to 5.0 compared to the control group sample, there are statistically significant differences ($p < 0.05$).

Conclusion. The study highlighted the fact that lowering the pH value also entails a decrease in the dental enamel hardness, which is significantly depreciating.

Keywords: human enamel hardness, pH variation, gastroesophageal reflux disease, wisdom teeth, Vickers hardness test

INTRODUCTION

The main purpose of the present study is to highlight the changes that appear in the hardness of the dental enamel due to the low level of the salivary pH, situation that can often occur in patients with gastroesophageal reflux disease [1]. This condition most frequently develops in adult patients that over time become invalid, restricting their lives on a daily basis due to the appearance of gastritis, corrosive esophagitis or even gastric mucosa ulcerations and malignant lesions [2].

Therefore, we aim to pinpoint the influence the acid-pH solutions (values between 2.5 – 6.5), similar to the mixture of the gastric acid reflux with the saliva in the oral cavity, on the hardness of the dental enamel of non-carious teeth that were extracted due to orthodontic treatment reasons from adult patients.

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According to the published literature so far, the hardness of the enamel has been reported in the range of 322 to 353 VHN [3,4]. This board variation of hardness values can be produced by various factors, such as specimen preparation, diagonal length reading error [5], variation in chemical composition [6], age and location in the tooth from where the specimens are obtained [7].

METHODS

The hardness of the enamel was tested using the Vickers method [8,9]. The procedure itself requires the impression of each study specimen with a diamond indenter at a specified load and for a specified duration. After load removal, diagonals (d_1 and d_2) of the indentation are measured and, in order to determine the hardness of the specimen, the following equation was used:

$$HV = \frac{2F\sin\left(\frac{136^\circ}{2}\right)}{d^2} \cong 1.854\frac{F}{d^2}$$

Where: F=load in Kgf, d=average between d_1 and d_2.

In order to measure the hardness of a specimen (sample), each one has to be processed accordingly to the study. Therefore, the test surface has to be plain and clean of debris or fat substances and the width of each experimental plate has to be at least 10 times wider than the depth of the indentation mark. Each small sample piece was covered in plastic material that has the corresponding stretch resistance, so it can be fixed in the vise of the microhardness tester.

Six wisdom teeth (M1, ..., M6) recently extracted were used in the study. Each molar was sectioned longitudinally, thus forming 12 specimens (M1A, M1B; ...; M6A, M6B), which were grouped into two lots: Lot_1 (M_{1A}, M_{3A}, M_{5A}, M_{2B}, M_{6B}, M_{4B}) and Lot_2 (M_{2A}, M_{6A}, M_{1B}, M_{4A}, M_{3B}, M_{5B}) with 6 samples each. Six solutions were prepared with the following pH values: 2.5, 3.5, 4.5, 5.0, 5.5, 6.5 . In these solutions, two specimens, one from each lot, were introduced for 3 minutes. In each sample, 15 imprints were produced and the corresponding hardness values were determined for each imprint obtained. The average hardness values obtained were compared within each lot in order to determine what is the effect of the pH of the solution on the dental enamel hardness.

Also, the average hardness values were compared at each pH between Lot1 and Lot2 specimens. The data was processed with the MedCalc 14 program. Descriptive, graphical, parametric statistical tests were used (Test-t for average comparison of two independent samples, One-Way ANOVA Test).

RESULTS AND DISCUSSIONS

Hardness analysis of dental enamel for chemically untreated specimens

The specimens from the control group (M_1, ..., M_6) were subjected to a 100gf load for 10s. In each sample, 10 experimental plates (5 in 2 rows) were used at a distance of at least 2.5 times the diagonal of an indentation.

The diagonals d_1 and d_2 were measured using the *Test Engineer for HV* program. The indentations made in the enamel were carefully analyzed from the point of view of their asymmetry (Figure 1). Thus, a measurement was considered valid only if the absolute difference between diagonals d_1 and d_2 was less than 10%.

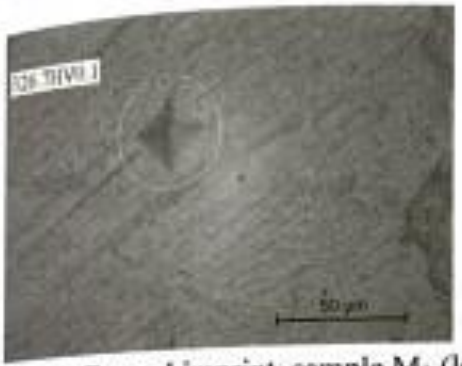

Figure 1. Enamel imprint, sample M_1 (left) and sample M_6 (right), Time = 10s, Load = 100gf

The hardness values of dental enamel for samples (M1, ..., M6) were between 320-334 Kgf/mm2, results that are consistent with literature data.

Table 1 Summary statistics table

	Enamel Hardness [Kgf/mm^2]					
Molar	M1	M2	M3	M4	M5	M6
Mean	327.34	326.19	328.57	329.64	327.31	325.919
SD	3.5127	3.3366	2.4614	2.4598	3.8573	4.1661
Minimum	320.54	322.00	325.00	325.53	322.00	321.00
Maximum	332.00	332.85	333.37	334.00	332.15	332.00

The mean hardness values for the specimens (M1, ..., M6) were compared by one-pass OneWay ANOVA test and also to supplementary PostHoc (Student-Newman-Keuls test) analysis, test that allows the specification of a significant difference between the values analyzed environments if that is the case, and which differs from one medium to another. The significance level chosen for the test was $\alpha = 0.05$. The result of the ANOVA test confirms that there are *no statistically significant differences* ($F = 1.775$, $p = 0.134 > \alpha = 0.05$) between the average hardness values determined at specimens M1, ..., M6, reason for which all the (Figure 2) 60 obtained values were included in the values of the control group.

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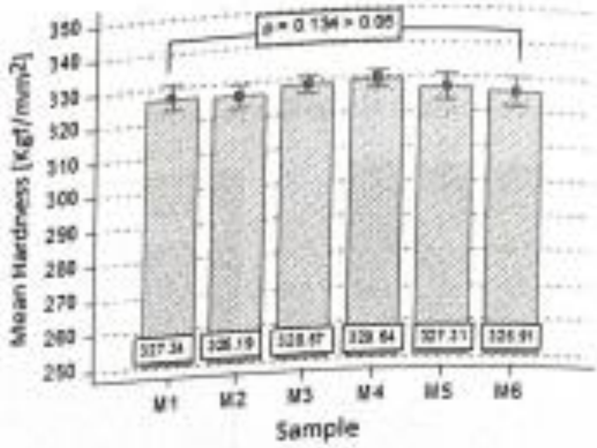


Figure 2 Graphic representation Bar and Error-Bar (Average±SD) for the values of the human enamel hardness for the specimens M1....M6, Time=10 s, Load = 100gf

Hardness analysis of dental enamel for chemically treated specimens

The specimens corresponding to the two lots were subjected to a 100gf load for 10s each. 15 imprints (5 in 3 rows) were placed in each sample at a distance of at least 2.5 times the diagonal of one indentation and only after that the corresponding hardness values were determined (Figure 3). The average hardness values obtained were compared within each lot in order to determine in what way the pH of the solution affects the hardness of the dental enamel. Also, the average hardness values at each pH were compared between specimens from Lot1 and Lot2.

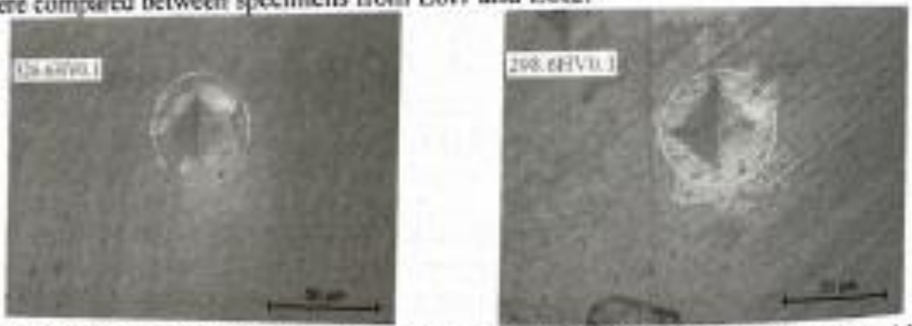


Figure 3. Enamel imprint - Lot_1 - sample M_{1A} at pH = 6.5 (left) and Lot_2 - sample M_{2A} at pH= 2.5 (right), Time=10s, Load = 100gf.

Table 2 **Summary statistics table**

Sample pH Solution	Enamel Hardness [Kgf/mm²] - Lot1						
	2.5pH	3.5pH	4.5pH	5.0pH	5.5pH	6.5pH	Martor
Mean	301,465	311,861	319,994	323,646	326,417	327,578	327,498
SD	2,7255	2,8891	3,2837	4,1989	2,6686	3,8956	3,4706
Minimum	297,000	307,000	315,000	319,000	322,000	322,700	320,540
Maximum	306,000	316,000	325,000	330,500	330,000	333,000	334,000

Sample pH Solution	Enamel Hardness [Kgf/mm²] - Lot2						
	2.5pH	3.5pH	4.5pH	5.0pH	5.5pH	6.5pH	Martor
Mean	299,995	312,585	320,463	322,883	325,802	326,971	327,498
SD	3,4476	1,9796	2,4078	3,7705	2,7511	3,3283	3,4706
Minimum	293,000	309,840	317,920	317,000	321,000	320,500	320,540
Maximum	304,820	317,500	326,000	329,000	330,000	331,010	334,000

The OneWay ANOVA test shows *that there are statistically significant differences* between at least two of the average hardness values of the compared specimens: $F = 153.03$, $p<0.001 < \alpha = 0.05$, for Lot1 (Figure 4); $F = 178.57$, $p < 0.001 < \alpha = 0.05$ for Lot2 (Figure 5).

According to the PostHoc (Student-Newman-Keuls test) analysis, it can be argued that there are *no statistically significant differences* ($p > 0.05$) between the average values of the specimens immersed in pH 5.5, 6.5 and control group specimens, but the other specimens immersed in solutions with pH = 2.0, 3.5, 4.5, 5.0 and specimens immersed in pH = 5.5, 6.5 solutions together with the control group specimens, *there are statistically significant differences* ($p < 0.05$).

The lower the pH value, the more the hardness of the dental enamel will depreciate.

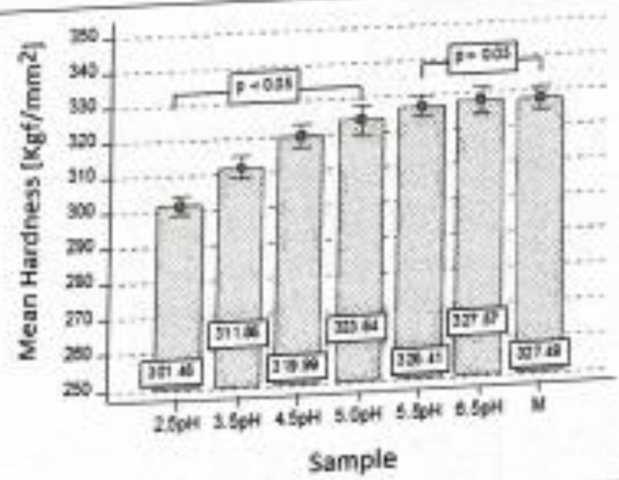


Figure 4 Graphic representation Bar and Error-Bar (Average±SD) for the values of the human enamel hardness for the specimens in Lot 1 and specimens in the control group, Time=10 s, Load = 100gf

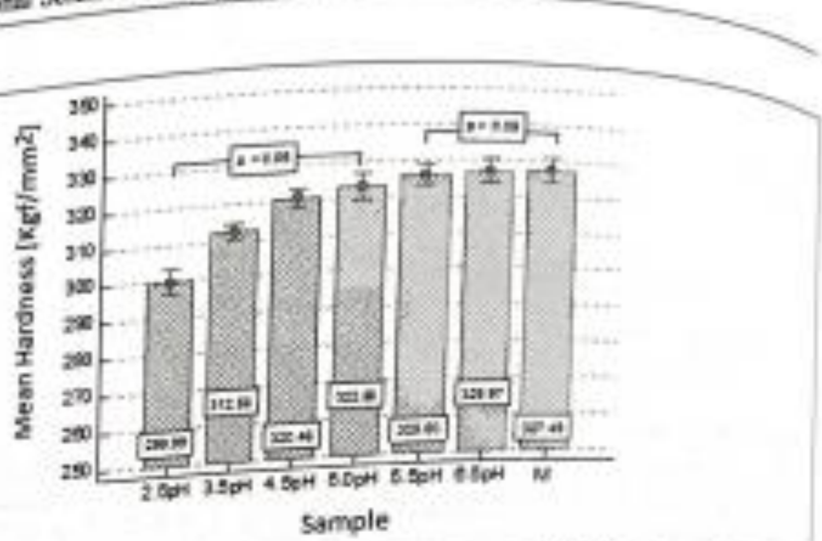


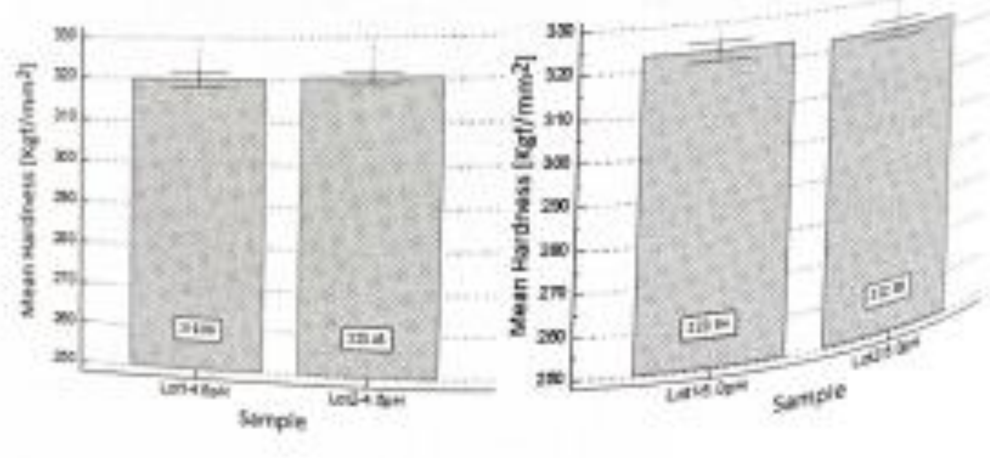
Figure 5 Graphic representation Bar and Error-Bar (Average±SD) for the values of the human enamel hardness for the specimens in Lot 2 and specimens in the control group, Time=10 s, Load = 100gf

Comparative analysis between Lot 1 and Lot 2 specimens according to the pH of the tested solutions

Between the average hardness values of dental enamel calculated at the two lots at each pH value (Figure 6) the results showed that there are *no statistically significant differences* ($p > \alpha = 0.05$ in all cases).

Table 3 **Summary statistics table**

	Duritate Smalţ [Kgf/mm²]					
	2.5pH	3.5pH	4.5pH	5.0pH	5.5pH	6.5pH
Mean Lot1	301,465	311,861	319,994	323,646	326,417	327,578
Mean Lot2	299,995	312,585	320,463	322,883	325,802	326,971
t-stat	-1.295	0.800	0.446	-0.523	-0.622	-0.459
p	0.2059	0.4305	0.6592	0.6048	0.5391	0.6497



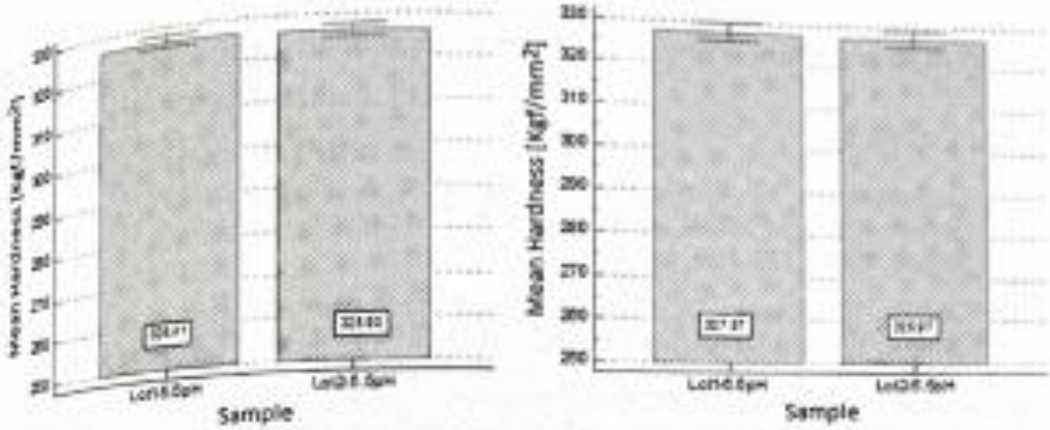


Figure 6 Graphic representation Bar and Error-Bar (Average±SD) for the values of the human enamel hardness for the specimens in Lot 1 and specimens in Lot 2 for comparative analysis, Time=10 s, Load = 100gf

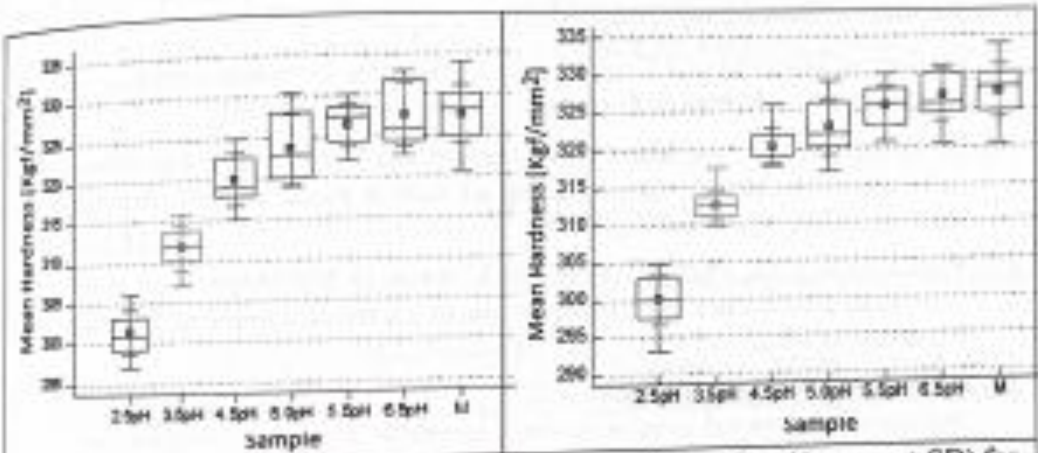


Figure 7 Graphic representation Box-and-whisker and Error-Bar (Average ± SD) for the values of enamel hardness, Lot1 + Control Group (left), Lot2 + Control Group (right), Time = 10s, Load = 100gf.

CONCLUSION

The results obtained in this study show that there are no statistically significant differences ($p > 0.05$) between the average values of hardness of the enamel specimens immersed in pH 5.5, 6.5 and control group enamel specimens, while among all other specimens immersed in pH = 2.0, 3.5 , 4.5, 5.0 compared to the control group sample, there are statistically significant differences ($p < 0.05$).

The study highlighted the fact that lowering the oral pH value also entails a decrease in the dental enamel hardness, which is significantly depreciating (Figure 7). This aspect might put the patients with gastroesophageal reflux disease and salivary pH under 5 value in higher risk for occurrence of dental decay due to the lower pH level of the oral fluids. A proper monitoring of this category of patients by the dentist can prevent future dental loss. As final conclusion, we recommend the existence of a good collaboration between practitioner and dentist, in order to monitor the patients with gastroesophageal reflux disease, to check periodically the oral pH level, to maintain a good oral health in order to prevent enamel demineralization and decay occurrence.

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CARDIOVASCULAR DISEASE AND DIABETES, POTENTIAL RISK FACTORS FOR PERIODONTAL DISEASE

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ABSTRACT

Introduction and aim*: The investigation of potential systemic risk factors, such as cardiovascular disease and diabetes, for periodontal disease.*

Materials and methods*: A cross-sectional sample consisting of 395 dentate adults (mean age 54 ±13), 55.7% female and 44.3% male, were enrolled in the study. Subjects attended periodontal care between 2016-2017 in Constanța, Romania (participation rate was 82%). The self-reported CVD and diabetes were recorded for the study. Clinical parameters such as periodontal probing depth (PPD) and bleeding on probing (BOP) were measured in order to assess the periodontal status. For the purpose of this study and in order to correlate the systemic status to the periodontal status, all patients were categorized according to their maximum PPD: either PPD <4 mm (no periodontitis) or PPD ≥4 (periodontitis). The data was analyzed using IBM SPSS statistics software version 20. Results: Patients with known CVD are more likely to have pockets deeper than 4 mm (periodontitis) OR = 5; 95% confidence interval (CI): 2.41-10.34. Self-reported diabetes was positively associated with the risk for periodontal disease with pockets deeper than 4 mm OR = 3.12; 95% CI: 1.18-8.25. No statistically significant relation was found between BOP and the presence of CVD (p=0.09), as well as between BOP and diabetes (p=0.161).*

Conclusion*: Cardiovascular disease and diabetes act as potential risk factors for periodontal disease, but gingival inflammation assessed by bleeding on probing was not proven to be influenced by CVD or diabetes.*

Keywords: *periodontal disease, diabetes, cardiovascular disease, periodontal pockets, risk factor.*

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Introduction

Periodontal disease is a chronic inflammatory-infectious disease, initiated by the presence of a bacterial biofilm, which affects both the periodontal ligaments and the alveolar bone, with the occurrence of periodontal pockets. As the disease progresses, the periodontal pockets deepen, and the untreated disease leads to tooth loss. Many systemic conditions are proved to interfere with the onset of the periodontal disease and aggravate its evolution. Some of these conditions, such as diabetes and cardiovascular diseases (CVD) could be included among risk factors for periodontal disease, according to recent findings. Some literature studies suggest that periodontal disease (PD) itself can be involved in the etiology of some systemic pathology, especially CVD, due to periodontopathogen bacteria residing in periodontal pockets, bacteria which can enter in systemic blood circulation[(1)].

On the other hand, periodontitis is a very common oral pathology in developed and developing countries[(2,3)]. The identification of risk factors for periodontitis is essential to improve the understanding of the disease pathogenesis and to establish effective health preventive strategies. It has become very important to identify the risk factors that make patients susceptible to PD. Some studies recognize smoking, diabetes and some pathogen bacteria as true risk factors, whereas some background factors have been regarded as risk indicators[(4,5)].

Other studies demonstrate a significant association between periodontal disease and future cardiovascular events. Common risk factors such as diabetes and smoking are shared between cardiovascular and periodontal diseases[(6)].

In diabetic patients, the uncontrolled periodontal infection can make the glycemic level difficult to control. The less diabetes is controlled, the more severe the periodontal destruction becomes[7]. From this point of view, a collaboration between the clinician and the periodontist might help control these pathologies. However, it is important to underline that several recent studies failed to show an association between diabetes and periodontal disease[8]. For this main reason, further investigation is needed to confirm the potential link between these two highly prevalent disorders.

Many epidemiological studies have identified statistically significant associations between periodontitis and cardiovascular diseases[9]. Also, this association is not without dispute[10, 11]. Some previous studies investigated the role of infection in CVD[12]. In fact, periodontal disease has an infectious etiology, which can lead to the assumption that these pathologies can be somehow connected. Pro-inflammatory mediators involved in hyperglycemia have also been associated in some studies with periodontitis, also indicating increased cardiovascular disease risks[13]. The prevalence of coronary heart disease in some cross-sectional studies was greater among individuals with periodontitis than in subjects without periodontitis[14].

In any case, different prevalence rates of periodontitis in populations with different age groups, ethnicity, geographic location, can make it difficult to assess the association between periodontitis, diabetes and cardiovascular disease. There are not many studies on this topic to refer at the Romanian population. Thus, the aim of this paper was to investigate the potential systemic risk factors, such as cardiovascular disease and diabetes, for periodontal disease in a cross-sectional group from Constanta city, Romania.

Materials and Methods: A cross-sectional sample consisting of 395 dentate adults, age >30 years (mean 54±13), 55.7% female, 44.3% male, was used in order to assess the link between periodontitis and CVD, or periodontitis and diabetes. The self-reported systemic condition was used in evaluation. In addition, periodontal probing depth (PPD) and bleeding on probing (BOP) were used in order to assess the periodontal status and inflammation. For the purpose of this study and in order to relate systemic status to existence of periodontal disease, all patients were categorized according to their maximum PPD: either PPD of <4 mm (no periodontitis), or PPD of ≥4 (periodontitis).

The data was analyzed using IBM SPSS statistics software version 20.

Study design. Subjects were selected from patients attending dental care in the Faculty of Dentistry Constanta and Clinical Infectious Diseases Hospital Constanta, Romania. The study was conducted during 2016-2017.

Data collection. All subjects have undergone periodontal examination and have filled in a questionnaire regarding their demographic features, daily oral hygiene, frequency of tooth brushing, lifestyle, cardiovascular pathology, known diabetes condition, as well as smoking habits. An informed consent was obtained both to collect data using a structured questionnaire, and for the dental examination. In order to assess the reliability of the self-reported data, 15 subjects of the study sample were asked to fill in the questionnaire again. This was made 14-20 days after the first appointment. The history of cardiovascular disease (CVD) and diabetes of the participants were established by direct inquiry.

Screening procedure for PD

A previously standardized dentist performed the oral examinations with a periodontal probe, a flat dental mirror, while the patient was sitting on a dental chair. Periodontal probing depth (PPD), bleeding on probing (BOP) and Community Periodontal Index (CPI) were used to assess the periodontal condition. The BOP was recorded for each patient immediately after the pocket depth measurements.

For the purpose of the study the results were dichotomized as follows: (1) no bleeding on gentle probing; (0) bleeding on gentle probing. All permanent fully erupted teeth, excluding third molars and radices, were examined with the manual CPI periodontal probe with a 0.5mm ball tip, black bands at 3.5–5.5 mm, and rings at 8.5 and 11.5 mm from the ball tip. Six sites per tooth were assessed in the mesiobuccal, midbuccal, distobuccal, distolingual, midlingual and mesiolingual sites, in order to determine the maximum PPD per each tooth. Only the depth of the deepest pocket for each tooth was recorded for the study in the chart of the patient. The detailed procedure has been described by Lai and coll.[15].

The maximum CPI score for each patient was recorded according to the following scale: (0) periodontal health; (1) gingival bleeding; (2) calculus detected during probing; (3) pocket 4 to 5mm deep; and (4) pocket 6mm deep and over. The Simplified Oral Hygiene Index (OHI-S) was used to assess the oral hygiene level. The OHI-S has two components, the Plaque Index (PI) and the Calculus Index (CI). For a proper periodontal health, PI and CI should be under 1 value, if possible close to 0[16].

Ethics

Ethical approval for the study was obtained from the local ethics committee. In addition, each patient signed an informed consent for the use of their data in this study, prior to the admission the study. The identity of all subjects was kept anonymous.

Subject eligibility

Subjects qualifying for the study met the following inclusion criteria:

- over 30 years of age;
- more than 10 natural teeth in the mouth;
- regular manual or electric toothbrush users.

Patients were excluded for one of the following conditions:

- severe systemic pathology such as malignancies, leukemia, genetic syndromes.
- pregnancy
- if any antibiotic was taken 4 weeks before the examination (antibiotic intake can improve short-term the level of gingival inflammation assessed by bleeding on probing)
- chronic drugs or alcohol consumers

Statistical analysis

The statistical analysis was performed using IBM SPSS statistics software version 20. Descriptive statistics were performed for all patients as well as within the subgroups of diabetics, non-diabetics, patients with CVD, patients with not known CVD and patients categorized with or without periodontitis (according to maximum PPD). The Odds ratios (OR) and relative risk (RR) were calculated in order to assess the studied systemic diseases risk for the occurrence of periodontal disease.

Results

From the total number 480 of patients invited to participate, 395 agreed to participate in the study, 220 (55.7%) were female and 175 (44.3%) male. The participation rate was 82%. The age interval of participants was 31-85 years old, (mean 54±13).

From the total sample of patients, 12.7% (n=50) reported the existence of cardiac pathology and 5.3% (n=21) are diabetics. There are no patients with both CVD and diabetes (table 1).

	Frequency	Percent	Valid Percent	Cumulative Percent
Healthy	324	82.0	82.0	82.0
CVD	50	12.7	12.7	94.7
Diabetes	21	5.3	5.3	100.0
Total	395	100.0	100.0	

Table 1: Distribution of subjects according to systemic status.

The comparison of periodontal status in terms of oral hygiene and presence of pockets, according to systemic condition is shown in table 2

Systemic status		N	Min	Max	Mean	Std. Deviation
Healthy	PI	324	0.00	3.00	1.4442	.71715
	CI	324	0.00	3.00	1.2590	.77237
	CPI	324	0.00	4.00	2.5679	.85750
	PPDmax (mm)	324	0.00	13.00	3.5432	2.61479
CVD	PI	50	.33	3.00	1.9186	.61112
	CI	50	.33	3.00	1.7730	.71651
	CPI	50	2.00	4.00	3.2000	.72843
	PPDmax (mm)	50	1.00	11.00	5.0600	2.27596
Diabetes	PI	21	.20	3.00	1.4029	.66210
	CI	21	.15	3.00	1.5548	1.01758
	CPI	21	2.00	4.00	3.3333	.85635
	PPDmax (mm)	21	1.00	11.00	5.4048	2.90525

Table 2: Periodontal and systemic status.

The mean of PI and CI are increased in patients with cardiac pathology, as compared to healthy subjects. The CPI scores are higher in patients with diabetes and CVD, as compared to healthy subjects, values of 3 and 4 of CPI suggesting periodontal involvement, with existence of periodontal pockets (table 2).

Also, the medium values for CPI scores calculated (One-Way Anova) for groups of healthy, CVD and diabetic subjects differ significantly ($F = 18.69$; $p < 0.001 < \alpha = 0.05$). The Post-Hoc Multiple Comparations – Tamhane (Leven = 2.534, df1 = 2, df2 = 392, $p = 0.048 < \alpha = 0.05$) shows significant differences between medium values of CPI for groups with no systemic pathology (CPI=2,56) and CVD (CPI=3,20), $p<0.001 < \alpha = 0.05$, as well as groups of no systemic pathology (CPI=2,56) and diabetes (CPI=3,33), $p = 0.002 < \alpha = 0.05$.

Regarding the oral hygiene status, assessed by plaque index (PI) and calculus index (CI), the medium PI scores calculated (One-Way Anova) separately for groups of healthy, CVD and diabetic subjects differ significantly

($F = 10.11$; $p = 0.011 < \alpha = 0.05$). The Post-Hoc Multiple Comparations - Tamhane (Leven =

4.558, df1 = 2, df2 = 392, $p = 0.011 < \alpha = 0.05$) show significant differences between healthy (PI=1,44) and CVD (PI=1,91) groups, $p < 0.001 < \alpha = 0.05$, but between healthy (PI=1,44) and diabetes (PI=1,40) groups there is no significant difference ($p = 0.990 > \alpha = 0.05$).

The medium CI scores calculated (One-Way Anova) separately for groups of healthy, CVD and diabetic subjects differ significantly (F = 10.24; $p < 0.001 < \alpha = 0.05$) and the Post-Hoc Multiple Comparations – Tamhane (Leven = 4.299, df1 = 2, df2 = 392, $p = 0.014 < \alpha = 0.05$) show significant differences between healthy (CI=1,25) and CVD (CI=1,77) groups, $p < 0.001 < \alpha = 0.05$, but between healthy (CI=1,25) and diabetes (CI=1,55) groups there are no statistically significant differences ($p = 0.497 > \alpha = 0.05$).

The maximum periodontal pocket depth (PPD) assessed by probing for every patient was compared within the three subgroups: healthy patients, patients with CVD, and diabetics. The differences between the median values of PPD maxim are statistically significant (F = 11.49; $p < 0.001 < \alpha = 0.05$). The Post-Hoc Multiple Comparations – Tamhane (Leven = 2.505, df1 = 2, df2 = 392, $p = 0.043 < \alpha = 0.05$) show differences between healthy (PPD=3,54) and CVD (PPD=5,06), $p < 0.001 < \alpha = 0.05$, and also between healthy (PPD=3,54) and diabetes (PPD=5,40), $p = 0.027 < \alpha = 0.05$.

Risk analysis found dependent relation between periodontitis with pockets deeper than 4mm and cardiovascular disease (CVD) OR=5, 95% CI for OR: 2.41-10.34 (χ2calc = 21.09, df = 1, $p < 0.001 < \alpha = 0.05$). Those findings suggest that the risk to find pockets deeper than 4 mm in the group with CVD is 5 times higher than in the group of patients without systemic pathology. The bleeding on probing (BOP) was not associated with the CVD (p=0,09). Self-reported diabetes was positively associated with the risk for periodontal disease OR = 3.12; 95% CI: 1.18-8.25, (χ2calc = 5.77, df = 1, $p = 0.016 < \alpha = 0.05$). The risk to find deep pockets in patients with diabetes is 3.12 times higher than in patients without systemic pathology. No statistically significant correlation was found between BOP and diabetes (p=0.161). Sex was not found to be a risk indicator for CVD ($p = 0.186 > \alpha = 0.05$), or for diabetes ($p = 0.482 > \alpha = 0.05$).

Discussion

Using a population-based study to assess periodontal status in adults aged 31-85 years old, we found that the risk of having deep periodontal pockets in subjects with diabetes was 3.12 times higher than in those without diabetes, in both men and women. However, PD is also a systemic disease and may also be affected by other etiological factors, as shown recently by Albandar et al(17). The review of literature studies leads the researchers to the conclusion that diabetes is strongly associated with periodontal disease(18). Tylor suggested a bi-directional relation between diabetes and PD, which means that each disease influences the other(18). Also, their findings show a similar risk rate of 4,2 regarding the periodontal destruction in diabetics as compared to non-diabetics. Several limitations in the present study should be considered. First, the diabetes status was self-reported, we could not correlate the level of glycemia with periodontal status. Second, regarding any causal relationship between diabetes and PD, our study cannot account for the temporal relationship between diabetes and PD. Also, there is evidence that periodontal inflammation may itself contribute to the onset and persistence of hyperglycemia, because inflammation is associated with inadequate glycemic control in individuals with diabetes(19). We could not find correlation between inflammation (expressed by bleeding on probing) and the diabetic status.

The results of our cross-sectional study showed that there was a link between the periodontal involvement (teeth with periodontal pockets >4 mm) and CVD, but bleeding on probing was not positively associated with cardiovascular pathology. The findings of our study agree with an earlier case-control study, where a link between the number of sites with a pocket depth > 4 mm and cardiac pathology was reported(20).

Another study found an association between periodontitis and high systolic blood pressure(21). Because we could not find correlation between BOP and CVD or diabetes, we could speculate that this result occurred since bleeding on probing is primarily influenced by the degree of plaque accumulation. Therefore, it is difficult to separate the local factors such as oral hygiene to the systemic influences when assessing the influence of systemic pathology on bleeding on probing.

Another recent study[22] shows no correlation between the number of teeth with deep periodontal pockets or the number of bleeding sextants and hypertension.

During the last decades, there has been a great interest in the impact of oral health, especially periodontitis, on cardiovascular diseases. The findings of one study resulted in the conclusion that periodontitis and poor oral health overall contribute to the pathogenesis of cardiovascular disease[23]. In our study we also found a poor oral hygiene in patients with CVD compared to healthy subjects. Other meta-analysis reports[24] show that periodontitis may contribute to CVD and stroke, due to systemic bacterial exposure from periodontitis, so the relation between periodontitis and CVD might also be dual.

Our study has some limitations. First, the term "cardiovascular diseases" is a broad term and it should be useful to specifically identified cardiovascular diseases (i.e. stroke, acute coronary syndrome, atherosclerosis) which can be linked to periodontitis. Secondly, the cardiovascular pathology was self-reported, which means that only patients diagnosed were included in statistical analysis. It could be possible that many patients with unknown cardiovascular pathology have been included in our healthy group. In any case, preventive care and proper lifestyle decisions may significantly reduce the risk of diabetes, cardiovascular disease, by maintaining healthy oral conditions.

Conclusion

In conclusion, in this study group, cardiovascular disease and diabetes act as potential risk factors for periodontal disease, in terms of pocket formation, but bleeding on probing was not proven to be influenced by those two systemic pathologies. This data suggests that periodontitis may have overall systemic health consequences in cardiovascular and metabolic pathologies, and a good clinician-periodontist collaboration could prevent future complications of those diseases.

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RISK FACTORS FOR PERIODONTAL DISEASE IN AN ADULT ROMANIAN POPULATION

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ABSTRACT

Aim: To investigate the potential systemic risk factors for periodontal disease in a population from Constanta, Romania.

Method: A cross-sectional sample consisting of 740 dentate adults 18-85 years of age (mean 39,9±17,89) were selected from patients attending dental clinics of Constanta Faculty between 2012-2013. The charts of patients were examined to determine patient's self-reported systemic condition: diabetes, cardiovascular pathology. In addition, periodontal probing depth (PPD) and bleeding on probing (BOP) was used to assess the periodontal status. Ethical approval was obtained from the local ethics committee and each patient signed an informed consent. For the purpose of this study and in order to relate systemic status among severity of periodontal disease, all patients were categorized according to their maximum PPD: with PPD of <3.5 mm (no periodontitis), with PPD of ≥3.5 but not exceeding 6 mm (periodontitis with moderate pockets), or ≥6 mm (periodontitis with deep pockets).

Results: It was found dependent relation between BOP and cardiovascular disease (CVD) ($p=0.001$), odds ratio (OR)=2.65, 95% confidence interval (CI) for OR: 1.43-4.91. CVD was also associated with the presence of periodontitis ($p<0.001$), OR=6.82, 95% CI for OR: 3.91-11.91. Self-reported diabetes was associated with the risk for periodontal disease ($p <0.001$), relative risk RR=2.31, 95% CI for RR: 1.94-2.76. The relative risk for deep pockets formation in diabetic patients is 1.43, 95% CI for RR: 1.10-1.87. BOP was also associated with diabetes ($p=0.008$). No significant relation of dependence was found between CVD and deep pocket formation ($p=0.437$).

Conclusion: Cardiovascular disease and diabetes are both risk factors for periodontal disease in terms of bleeding on probing in this adult population. Diabetes increases also the risk for deep pocket formation, which can aggravate the evolution of periodontal disease.

Keywords: risk factors, periodontal disease, diabetes, cardiovascular disease.

INTRODUCTION

Although the principal cause of periodontal diseases (PD) is microbial plaque, not all individuals with plaque develop periodontal disease within the population. Studies in the literature show the involvement of several risk factors in the onset, development and severity of periodontal disease.

Certain factors such as male sex, age, race (ethnicity), socio-economic or educational status, diabetes, smoking, cardiovascular pathology have been identified in more studies as being associated with severe PD [1].

However, few longitudinal studies have been carried out to fully assess the role of these risk factors to periodontal destruction [2, 3, 4].

Some studies have reported associations between periodontitis and cardiovascular disease [5]. The reason for such associations is unknown; the relationship might be weak [6], because periodontitis and cardiovascular disease [6, 7] can share common risk factors (smoking and diabetes) that can confound the association [8].

Furthermore, genetic studies [9] have suggested shared links between periodontal disease and coronary heart disease. Thus, it is of additional interest to evaluate the interaction between family history of myocardial infarction, periodontal disease and future vascular risks.

The association between diabetes and periodontal disease (PD) has been documented in previous studies [10]. Poor glycemic control may lead to increased severity of periodontitis [11]. Despite these findings, the magnitude of relationship between diabetes, cardiovascular disease and PD was not well documented in Romanian population.

The aim of this study was to investigate the potential systemic risk factors for periodontal disease (such as diabetes, cardiac pathology) and other co-factors such as smoking in a population of adults from Constanta, Romania.

METHODS

Study design. A cross-sectional sample consisting of 740 dentate adults 18-85 years of age (mean 39,9±17,89) were selected from patients attending dental clinics of Constanta Faculty of Dentistry between years 2012-2013. Table 1 shows the distribution of study group by age and sex.

Questionnaire and clinical examination. All subjects were clinically examined and filled in a questionnaire regarding demographic features, daily oral hygiene, dietary habits, lifestyle, systemic pathology, as well as smoking habits.

Informed consent was obtained to collect data using a structured questionnaire and dental exam. Pilot studies were carried out in 20 persons (not included in the final study sample) to assess acceptability and clarity of the questionnaire components and to determine the time it would take to complete the questionnaire.

In order to assess the reliability of the self-reported data, 28 subjects of the study sample were re-interviewed a second time. The second interview was made 2–4 days after the first.

Participants' history of cardiovascular disease (CVD), diabetes mellitus (Type 2), and hypertension was established by direct inquiry.

A previously standardized dentist performed the oral examinations with the manual CPITN probe, a flat dental mirror, with the patient sitting on a dental chair. Also, examiners were calibrated during to the study.

Periodontal probing depth (PPD) and bleeding on probing (BOP) was used to assess the periodontal condition. The BOP was recorded for each patient immediately after pocket depth measurements. For the purpose of the study the results were dichotomized as follows: (1) no bleeding on gentle probing; (0) bleeding on gentle probing. All permanent fully erupted teeth, excluding third molars and radices, were examined with the manual CPITN periodontal probe with a 0.5mm ball tip, black bands at 3.5–5.5 mm, and rings at 8.5 and 11.5mm from the ball tip. Six sites per tooth were assessed in the mesiobuccal, midbuccal, distobuccal, distolingual, midlingual and mesiolingual sites, in order to determine the PPD. Only the depth of the deepest pocket for each tooth was recorded.

The examiners were trained in performing the clinical examination and measurements before and during the examinations. For the purpose of this study and in order to relate systemic status among severity of periodontal disease, all patients were categorized according to their maximum PPD: with PPD of <3.5 mm (no periodontitis), with PPD of ≥3.5 but not exceeding 6 mm (periodontitis with moderate pockets), or ≥6 mm (periodontitis with deep pockets). The detailed procedure has been described elsewhere by Lai and co. [12].

Ethics. Ethical approval was obtained from the local ethics committee and each patient signed an informed consent.

Subject eligibility. Subjects qualifying for the study met the following inclusion criteria:

- 18 and over years of age;
- more than 10 natural teeth in the mouth;
- regular manual or electric toothbrush users.

Patients were excluded for one of the following conditions:

- pregnancy
- antibiotic intake status within 4 weeks of the baseline examination

Non-response analysis. In the whole population addressing for dental treatment, including individuals aged 18 years and older, 1036 individuals were eligible for the survey. Of these, 740 (71.4%) subjects agreed to the study.

Statistical analysis

The statistical analysis was performed using IBM SPSS statistics software version 20.

Descriptive statistics were performed for all patients as well as within the subgroups of smokers, non-smokers and patients categorized with no, periodontitis with moderate - and deep pockets. The Odds ratios (OR) and relative risk (RR) were calculated to assess the studied background factors for the occurrence and severity of periodontal disease.

RESULTS

Descriptive results

In total, 740 adults aged 18-85 years old participated to this study between years 2012 and 2013. The mean age (± standard deviation SD) was 39.97 (±17.89 SD), among them, n=395 (53.4%) were female. Table 1 show the age, sex and smoking characteristics of the studied population.

Table 1. Study group according to age, sex and smoking characteristics

Age(years)		Total population n (%)	18-35	36-64	≥65
Subjects	Total	740 (100%)	380 (51.4%)	274 (37%)	86 (11.6%)
	Male	395 (53.4%)	188 (49.5%)	115 (42%)	42 (49%)
	Female	345 (46.6%)	192 (50.5%)	159 (58%)	44 (51%)
Smokers	Total	309 (41.8%)	187 (49.2%)	110 (40.1%)	12 (14%)
Non-smokers	Total	431 (58.2%)	193 (50.85)	164 (59.9%)	74 (86%)

The percentage of smokers in total population (41.8%) is high and can be seen that smoking prevalence increases as age group decreases (49.2% in 18-35 age group, 40.1% in 36-64 age group and only 14% in more than 65 years of age).

The prevalence of diabetes and cardiac pathology self-reported in population is seen in table 2.

Table 2 Study group according to diabetes and cardiovascular pathology

		n	Min. age	Max. age	Mean	SD
No pathology	Total	623	18	83	36.41	16.27
	Male	304	18	83	35.95	16.15
	Female	319	18	80	36.86	16.40
CVD	Total	84	21	78	60.38	12.09
	Male	32	21	78	58.84	16.24
	Female	52	38	78	61.33	8.67
Diabetes	Total	33	21	85	55.18	16.94
	Male	9	45	71	61.78	8.64
	Female	24	21	85	52.71	18.71

From the total of 84 subjects with CVD, female are the majority (n=52). The women are also most affected by diabetes (n=24 from the total of 33 diabetic subjects) as compared to male subjects from the study group.

Table 3 Risk factors associated with PPD

PPD	N (%)	< 3.5 mm	[3.5-6) mm	≥6 mm
Gender	Total	426 (57.6%)	152 (20.5%)	162 (21.9%)
	Male	209 (49.1%)	58 (38.1%)	78 (48.1%)
	Female	217 (50.9%)	94 (61.9%)	84 (51.9%)
Age group	18-35	344 (46.5%)	26 (3.5%)	10 (1.4%)
	36-64	72 (9.7%)	89 (12%)	113 (15.3%)
	≥65	10 (1.4%)	37 (5%)	39 (5.3%)
CVD	Yes	18 (2.4%)	36 (4.9%)	30 (4.1%)
	No	401 (54.2%)	109 (14.7%)	113 (15.3%)
Diabetes	Yes	7 (0.9%)	7 (0.9%)	19 (2.6%)
	No	401 (54.2%)	109 (14.7%)	113 (15.3%)
Smoking	Yes	201 (27.2%)	47 (6.4%)	61 (8.2%)
	No	225 (30.4%)	105 (14.2%)	101 (13.6%)

The maximum periodontal probing depth (PPD) measured for each patient was recorded and compared between gender, age group and systemic status (table 3). Patients were categorized with PPD of <3.5 mm (no periodontitis), with PPD of ≥3.5 but not exceeding 6 mm (periodontitis with moderate pockets), or ≥6 mm (periodontitis with deep pockets).

The percentage of women with medium and deep pockets is slightly high as compared to men (table 3).

Age contributes to occurrence of medium and deep pockets-39 from 86 patients over 65 years has deep pockets and 37 medium pockets. Most of patients with diabetes (19 from the total 33) have deep pockets.

Risk analysis

Risk analysis found dependent relation between BOP and cardiovascular disease (CVD) OR=2.65, 95% CI for OR: 1.43-4.91 (table 4).

CVD was also associated with the presence of periodontitis (OR=6.82, 95% CI for OR: 3.91-11.91), in patients having periodontal pockets deeper than 3.5 mm. Self-reported diabetes was associated with the risk for periodontal disease (OR=9.72, 95% CI for OR: 3.69-25.47 and relative risk RR=2.31, 95% CI for RR: 1.94-2.76).

Diabetes was also associated with the risk for deep pockets formation (OR=2.61, 95%CI for OR: 1.05-6.47). The relative risk for deep pockets formation in diabetic patients is 1.43, 95% CI for RR: 1.10-1.87.

BOP was also associated with diabetes: OR=3.53, 95% CI for OR: 1.22-10.17.

No significant correlation was found between CVD and deep pocket formation (p=0.437), as it is shown in the last row of table 4.

Table 4 Potential risk factors analysis (level of significance $p<\alpha=0.05$)

Variables	X^2 calc	P value	OR	95% CI for OR		RR	95% CI for RR	
				lower	upper		lower	upper
BOP and CDV	10.376	0.001	2.65	1.43	4.91	1.25	1.13	1.39
PD and CDV	56.719	<0.001	6.82	3.91	11.91	2.17	1.87	2.53
BOP and Diabetes	6.156	0.013	3.53	1.22	10.17	1.30	1.13	1.50
PD and Diabetes	30.664	<0.001	9.72	3.69	25.47	2.31	1.94	2.76
PPD and Diabetes	4.597	0.032	2.61	1.05	6.47	1.43	1.10	1.87
PPD and CDV	0.604	0.437	0.804	0.463	1.39	0.893	0.66	1.19

DISCUSSION

Age was associated with deep pockets formation, especially in patients over 65 years old. This finding is not unexpected due to the cumulative effect of the disease.

The literature views the higher prevalence of periodontal destruction in the elderly as a reflection of a lifetime of disease accumulation rather than as an age-specific condition [13]. It is unclear though whether ageing per *se* is a risk factor for severe PD, or if its effect is due to the prolonged exposure of older subjects to other etiologic factors, because from our study age could not be proven to be directly correlated with PD.

An important proportion of the investigated subjects in this urban adult Romanian population have been exposed to cigarette smoking (41.8%). In the smokers population, the young group, aged under 35 years are the most exposed (49.2%) and the last age category, over 65 years are the least exposed (14%) to this habit. Previous studies show that smoking affects periodontal condition especially in young population [14].

Regarding the correlation between diabetes and PD, our findings were consistent with previous findings from Western countries that showed a two- to fourfold increased risk for PD in patients with diabetes [10, 11]. Regarding any causal relationship between diabetes and PD, our study has some limitations: cannot fully account for the temporal relationship between them and did not assess the level of glycemia in the investigated subjects. Maybe more cases from our subjects were left behind not being diagnosed.

In this study CVD was positively associated with PD and with BOP tendency. Similar results were reported by other investigators [15] which confirmed that incident periodontal disease is associated with high vascular event rates; the risk for cardiovascular events was found to be preferentially elevated among women who smoked. No significant difference could be found in our study between sexes and smokers/non-smokers as regarding the risk for CVD. Our study has some limitation related to the type of cardiovascular pathology, the treatment that patient is taking (or not), due to the given the design of the questionnaire. This issue is complex and would require additional research.

However, we could finally believe these data likely to have relevance for the practice of periodontal medicine as they show that cases of periodontal disease put patients at significantly elevated risks for future vascular disease.

CONCLUSION

Cardiovascular disease and diabetes are proven to be both risk factors for periodontal disease in terms of bleeding on probing in this adult population. In addition, diabetes increases the risk for deep pocket formation, which can aggravate the evolution of periodontal disease.

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RELATIONS OF ENVIRONMENTAL FACTORS AND EVOLUTION OF BOUTONNEUSE FEVER IN THE COUNTY OF CONSTANTA – ROMANIA

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Abstract. Boutonneuse fever is an eruptive disease from the group of spotted fever caused by *Rickettsia conorii* having both vector and reservoir a dog tick *Ripicephalus sanguineus*. In Romania, *R. sanguineus* is found during late spring to early autumn, because can develop well at different level of temperature (20–35°C) and relative humidity (35–95%). Although over the studied period (January 2009 – September 2017) there were no major changes in evolution of average environmental temperature and humidity, a decreased number of patients with boutonneuse fever was reported, from 149 patients in 2009 to 31 in 2017. During the analysed period were hospitalised 468 cases of patients with boutonneuse fever. Repartition by groups of age evidenced that the majority of cases were in adults, with 416 cases (88.88%), while in children aged between 1 year old and 18 years old were noticed just 52 cases (11.12%). The majority of patients were female – 249 cases (53.20%), from urban area – 293 cases (62.60%). The decreasing number of patients with boutonneuse fever in Constanta area over 9-year study seems to be related rather with extensive prophylaxis after tick bite, than related with changes in environmental factors.

Keywords: boutonneuse fever, tick, average temperature, average humidity.

AIMS AND BACKGROUND

Boutonneuse fever is an eruptive disease from the group of spotted fever which is endemic in the Mediterranean basin (Marseille fever, Mediterranean fever) and the Black Sea (butonneuse fever), caused by *Rickettsia conorii*, having both vector and source of a dog tick (*Ripicephalus sanguineus*). Boutonneuse (French for spotty) fever was first time described in Tunis in 1910 by Connor and Bruch. Diseases name is related with papular skin rash noticed during evolution of the

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diseases[1]. Carducci in 1920 in Italy and Olmer in Marseilles in 1925 described a mediterranean feverish disease that was called Carducci disease, then Olmer disease and finally in 1932 received the name of Mediterranean boutonneuse fever. Meanwhile, Pieri in 1925 describes the 'tache noir' or black spot or inoculation eschar as characteristic of the disease. Durand and Conseil in 1930 demonstrate the dog tick transmission. In 1932 Brumpt discovers the causal agent: a rickettsia that he will call in honor of Connor: *Rickettsia conorii*[2].

In Romania, this disease was first described, in 1910, by Serafidi in Constanta. Initially, it was considered exanthematic typhus but exanthema was a different type and Weil Felix reaction was negative. Barzanescu and Vasiliu, in 1931, considered that the number of cases in Constanta was higher because there were more innaparent or oligosymptomatic cases. In 1948, the disease was first described in Bucharest area[3]. There is no inter-human transmission of this disease. Receptivity is general and there is a seasonality of disease with a peak during the summer[3]. The prognosis *quo ad vitam* is very good, with a low mortality rate[3]. The prognosis *quo ad sanationem* is very good.

The tick is the main source due to Rickettsial permanent transovariane transmission (hereditary) which ensures permanent survival of germ. The main vector of the disease in Dobrogea is *Ripicephalus sanguineus* (dog tick), *Broophilus anumlatus*, *Dermacentor* and *Haemaphysalis* species. The most important natural source are dogs, but other mammalian species may also be involved: sheep, cattle and only rarely cats[3].

It is already well known that in tropical and subtropical areas *R. sanguineus* is found throughout the year[4,5] whereas in temperate region, like in Romania, this tick is found during late spring to early autumn[6,7]. Also it has been proved that *R. Sanguineus* can develop well at different level of temperature (20–35°C) and relative humidity (35–95%) (Ref. 8).

Although this tick is, a parasite of dog occasionally it can parasitize other hosts like humans. Tick *R. sanguineus* is a vector for different disease agents like: *Coxiella burnetti*, *Ehrlichia canis*, *Rickettsia conorii* and *Rickettsia rickettsia*[9]. It is also very well-known from the literature that the higher is the level of environmental infestation, the higher is the risk of human exposure to *R. sanguineus*[5].

EXPERIMENTAL

We performed a retrospective and prospective study about adults and children cases with butonneuse fever hospitalised in Constanta Clinical Infectious Diseases Hospital over a period of 9 years, between 2009–2017. Over the studied period we analysed 468 cases of patients with boutonneuse fever from demographic and seasonality point of view. It was checked some correlations between number of patients hospitalised with boutonneuse fever and evolution of temperature and humidity in Constanta area.

Material consisted in collecting retrospective data of patients with boutonneuse fever that were hospitalised in Constanta Clinical Infectious Diseases in a period of 9 years. Also, it was analysed evolution of environmental factors as average temperature and humidity in the same studied period.

The selected settings were Constanta Clinical Infectious Diseases Hospital departments for adults and children, cases who required hospitalisation for boutonneuse fever.

Methods consisted in collecting demographic data of patients hospitalised with boutonneuse fever in Constanta County. It was analysed the evolution of yearly cases during the late spring, summer and early autumn. We also checked if there are some changes in environmental factors (temperature or humidity) that can influence the disease evolution (decreasing number of patients that required hospitalisation for boutonneuse fever).

Statistical analysis was performed by MedCalc 14.8.1. For comparison of two shares, we used as nonparametric test Chi-squared test.

In order to publish these scientific data it was obtained the informed consent of patients or guardians and also the agreement of Ethic Committee of Clinical Infectious Diseases Hospital of Constanta.

RESULTS

In a period of 9 years, between 2009–2017, in the Constanta Clinical Infectious Diseases Hospital, which is a tertiary hospital of Infectious Diseases for Dobrogea region, were hospitalised 468 cases of patients with butonneuse fever, during a period of 5 months (May–September). 9 additional cases were registered in April and 3 cases in October which were excluded from statistical analysis due to their discontinuous occurrence. From the total number of cases analysed, the highest number, 149 patients, were hospitalised in 2009 and 27 patients in 2012, respectively. The disease, similarly to other Mediterranean countries, are reported during the last months of spring, summer and the first month of autumn[10]. The repartition of cases by month and years is presented in Fig. 1.

The repartition by groups of age presented in Fig. 2 shows that the majority of cases occurred in adults, with 416 patients (88.88%), while in children aged between 1 year old and 18 years old were noticed just 52 cases (11.12%). The majority of patients were female – 249 cases (53.20%), and from the urban area – 293 cases (62.60%).

The average temperature, measured in Celsius degrees (°C) for the studied period, presented the same trend; environmental temperature increased during the spring and summer, with peaks in July and August and then the temperature decreased during autumn. Trend of temperature during the studied period and months are presented in Fig. 3 (Ref. 11).

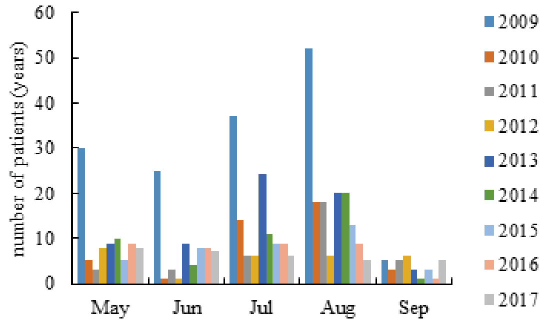

Fig. 1. Repartition of patients by years and months during 2009–2017

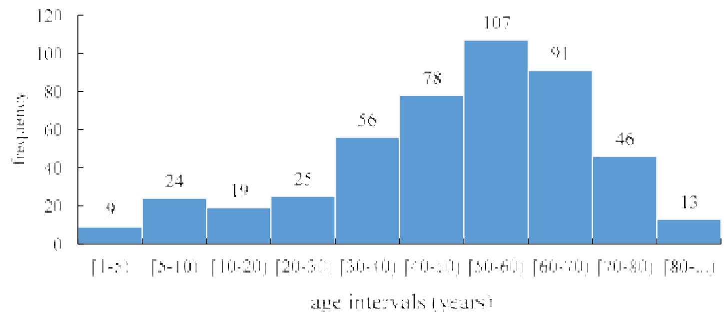

Fig. 2. Frequency by age groups

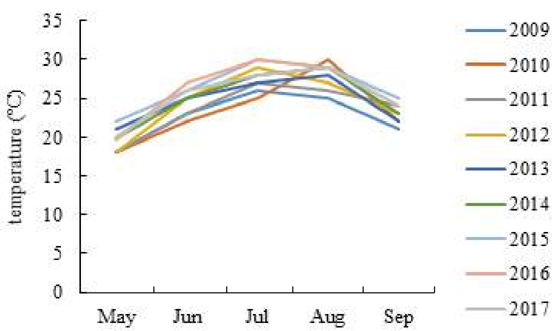

Fig. 3. Evolution of average temperature over the studied period (2009–2017)

Regarding the evolution of average humidity over the studied period, as we can see in Fig. 4, we noticed the same trend in evolution, with exceptions in 2010, 2011 and less in 2014, when, in June and July, the average humidity was higher than in the other years of the studied period.

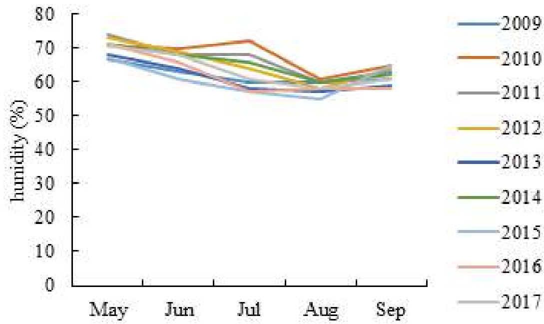

Fig. 4. Evolution of average humidity over the studied period (2009–2017)

Although over the studied years there were no major changes in the evolution of average environmental temperature and humidity, we noticed a lower number of patients that were hospitalised with boutonneuse fever when we compare 2009 with all other years from the studied period.

Table 1. Comparison between the number of cases per month during 2009 and 2017

Month	2009				2017				*p* value
	total	N1	p1	%p1	total	N2	p2	%p2	
May	149	30	0.2013	20.13	31	8	0.2581	25.81	0.481
June	149	25	0.1678	16.78	31	7	0.2258	22.58	0.442
July	149	37	0.2483	24.83	31	6	0.1935	19.35	0.515
August	149	52	0.3490	34.90	31	5	0.1613	16.13	0.041
September	149	5	0.0336	3.36	31	5	0.1613	16.13	0.005

In August 2009, there were diagnosed 52 cases out of a total of 149 patients, and in August 2017, there were 5 cases out of a total of 31 patients, as it is shown in Table 1. Between the share of patients registered in August 2009 p1 = 0.3490 (34.9%) and the share of patients registered in August 2017 p2 = 0.1613 (16.13%), there are considered statistically significant differences $p = 0.041 < \alpha = 0.05$ (Chi-squared test for the comparison of two proportions).

In September 2009, there were registered 5 cases out of the total of 149 patients, and in September 2017 there were 5 cases out of a total of 31 patients. Between the share of patients registered in September 2009 p1 = 0.0336 (3.36%) and the share of patients registered in September 2017 p2 = 0.1613 (16.13%), there are considered statistically significant differences $p = 0.005 < \alpha = 0.05$ (Chi-squared test for the comparison of two proportions).

The same comparison between May, June and July months performed for the first year – 2009 – and the last year – 2017 – of the studied period evidenced that there was no statistical significance regarding the number of cases.

The same comparison performed between 2009 and all other analysed years of the studied period revealed significant differences regarding:

– The share of patients registered in June 2009 ($p1 = 0.1678$–16.78%) and June 2010 ($p2 = 0.2258$–22.58%), with a value of $p = 0.018$;

– The share of patients reported in September 2009 ($p1 = 0.0336$–3.36%) and September 2011 ($p2 = 0.1429$–14.29%), with a value of $p = 0.010$;

– The share of patients hospitalised in September 2009 ($p1 = 0.0336$–3.36%) and September 2012 ($p2 = 0.2222$–22.22%), with $p < 0.0001$;

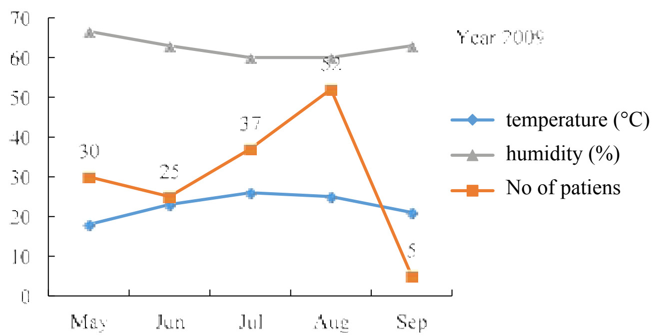

Fig. 5. Evolution of number of cases and average temperature and humidity in 2009

In Fig. 5 are presented the number of patients per month and the average for environmental temperature and humidity, for 2009. The same parameters are presented in Fig. 6 for 2017, respectively. When we analysed these two years, we noticed that, regarding environmental temperature and humidity, there were no difference, but there were differences regarding the number of patients, which is much higher in 2009 compared to 2017.

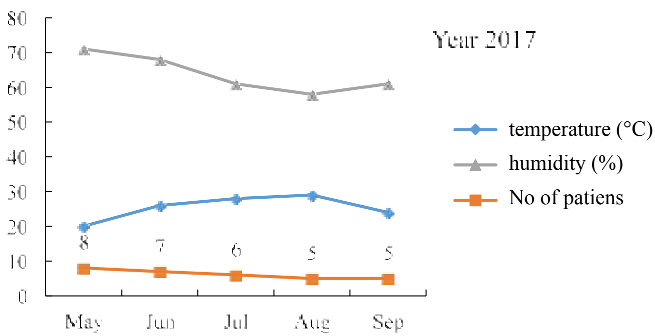

Fig. 6. Evolution of number of cases and average temperature and humidity in 2017

DISCUSSION

The risk of human parasitism by *R. sanguineus* could increase in areas with warmer or longer summers, and consequently can increase the risk of transmission of zoonotic agents like *R. conorii* or *R. rickettsii*[5].

The parasitism by *R. sanguineus* on other hosts than dogs is unusual in some areas. The human parasitism by *R. sanguineus* is relatively common in Europe, especially during summer time[12]. It was took into consideration that the prevalence and mean intensity of dog infestation by *R. sanguineus* vary widely geographically and seasonally. It is already well known that younger dogs are more infested than older ones[13], free dogs (untreated against ectoparasites) were more infested than domiciled dogs[14], and tick burden is often higher among urban dogs in comparison with rural ones[5,15].

This human parasitism by *R. sanguineus* is more often associated with the presence of heavily infested dogs and highly infested environments (presence of ticks in grassy yards).

It is considered that tick biology and ecology were directly influenced by climate factors like temperature and humidity. The global warming seems not to affect ticks like *R. sanguineus* that are more resistant to desiccating conditions, and less dependent of high humidity. Contrarily, it is speculated that an increase of about 2–3°C in average temperature from April to September could establish a population of *R. sanguineus* in regions of northern temperate Europe[16].

In our study it was noticed that during April for the reference years 2012, 2014, 2016, the average temperature was around 15–17°C and patients hospitalised with boutonneuse fever were registered. For the same period in years 2009 and 2017, the average temperature was around 11°C, and we noticed no case of butonneuse fever in April. Nevertheless, the impact of global warming on *R. sanguineus* remains uncertain[5].

For a long period of time, *R. sanguineus* was considered one of the most frequent ticks of the dog in south-temperate European countries including Romania. In a study performed by Sandor et al. they found that in wetlands of Danube Delta *R. rossicus* had a dominant occurrence in dogs from the area[17]. *R. rossicus* is reported occasionally from humans, but it seems to present a vectorial role for zoonotic pathogens like *Coxiella burnetii*, *Francisella tularensis*, or West Nile viruses. According with this study, further analyses are necessary in order to establish its role as a disease vectors in the region for the above mentioned pathogens or for others like *R. conorii*[17].

Although the environmental factors and air, water or soil pollution seem to influence human pathology[18–22], in our study we noticed that evolution of boutonneuse fever is not influenced by environmental factors. The environmental factors that can influence the presence of *R. sanguineus* ticks were moderate temperature and humidity. These environmental factors were essential in the 1910s for adapting

Rickettsia conorii and *Ripicephalus sanguineus* to our geographic area. Initially, the main source of *Rickettsia conorii* were sheep, and in time dogs became the most important cause. Another aspect that can influence decreasing number of cases is prophylaxy after tick bite. In the last 5 years, during late spring, summer and early autumn, we recommend a dose of Doxycicline (200 mg) in adults and children over 8 years old and a dose of Azitromycine (10–15 mg/body weight) in children less than 8 years old after tick bite. Despite this prophylaxis is controversial, we had to recommend it at the pressure of the media and public opinion. Over the years, this prophylaxis can influence the sensibility of *R. connorii* to these two drugs. In the future, this aspect will require to be monitored carefully.

CONCLUSIONS

Environmental factors were essential when the boutonneuse fever was introduced in the Southeast part of Romania in the 1910s and expanded into our geographical area during commercial trade between the riverine countries to the Mediterranean Sea and Constanta Harbor.

In our region, the level of temperature and relative humidity from late spring to early autumn are favourable conditions to the spread of *R. sanguineus*, the vector for *R. conorii*, which, accidentally, can parasitise humans and cause boutonneuse fever.

The decreasing number of patients with boutonneuse fever in Constanta area over the 9 year study seems to be related rather with extensive prophylaxis after tick bite, than related with changes in environmental factors.

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EVOLUTION OF SALMONELLOSIS IN CONSTANTA AREA IN CORRELATION WITH ENVIRONMENTAL FACTORS

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Abstract. Climate change and global warming have been reported to increase spread of foodborne pathogens. To better understand the effects of *Salmonellosis* infections, the objective of this study was to analyse the demographic data of the patients and environmental features from Constanta area, localised on the Black Sea Coast. A retrospective study was conducted on 377 patients with *Salmonella spp.* disease over a 10 years period, between 1st January 2009 and 31st December 2018 at Constanta Clinical Infectious Diseases Hospital from Romania. Demographic (i.e. sex and location) and meteorological (i.e. atmospheric pressure, humidity, atmospheric temperature, and rainfall) data were collected. The results showed that the risk of appearance of *Salmonella* spp. cases during the period June–September comparative with October–May of 2.629 more in condition of increased humidity, and 2.264 more in condition of increased atmospheric temperature. Therefore, our study shows that bacterial pathogens is positively correlated with atmospheric temperature and humidity in Constanta area, as warmer temperatures enable more rapid replication of the pathogen.

Keywords: *Salmonella* spp., environmental factors, temperature, climate.

AIMS AND BACKGROUND

Salmonella represents a major public health concern especially in low socioeconomic areas. *Salmonella* genus is composed of two species, *S. enterica* and *S. bongori*. Further, *S. enterica* is split into six subgroups (i.e. *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica*, and *houtenae*) being responsible of more than 95% of human infections. In contrast, *S. bongori* usually infect cold-blooded hosts[1,2].

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Most of the infections are being developed by serovars of *S. enterica*. The infections namely nontyphoidal *Salmonella* (NTS) can lead often to gastroenteritis. The most spread NTS serovars are represented by Typhimurium and Enteritidis[3].

One recent study shows that each year there are more than 93.8 million human infection of gastroenteritis around the world[4,5]. In this regard, many infections were shown to be influenced by different environmental factors such as climate or land[6]. Many geographical features like climate, weather, and hydrology could influence the frequency transfer of pathogenic microorganisms from environmental medium[7].

The climate change associated with the diseases appearance showed already to comprise approximately 4.6% of all environmental hazards. Furthermore, it has been shown that in 2000 year, the changes in climate produce 2.4% of all diarrhea in the world and 7% of dengue fever episodes in many industrialised cities[8]. The changes in weather patterns showed to be the reason for leafy vegetables and herbs contamination. Dry climate can lead to dust storms that leave dust particles on vegetables. Interestingly, higher degrees of temperatures can increase the percentage of microbial growth[9,10]. At the same time, it could negatively influence the insects and pests around farms which could transfer human pathogens to vegetables. Moreover, relative humidity can have a strong effect on human pathogens survival rate[11]. Numerous infections agents and rate of pathogen replication are being more sensitive to climate changes. In this respect, *Salmonella* spp. proliferates more rapidly at higher temperature, especially in animal gut and food. In the areas with lower temperatures and low rainfall restrict transmission of diseases, and climate changes could give the ecological habitat and trigger epidemics[12]. Therefore, changes of climate and environmental factors could contribute to spreading of many foodborne pathogens leading to different infections disease[13].

The present study was undertaken to investigate the effects of *Salmonella* spp. infections and the correlation with environmental factors like atmospheric pressure, humidity, atmospheric temperature and rainfall from the Constanta city area.

EXPERIMENTAL

A retrospective study was conducted on 377 patients with *Salmonella* spp. disease over a period of 10 years, between 1st January 2009 and 31st December 2018. All these patients were hospitalised at Constanta Clinical Infectious Diseases Hospital from Romania.

Demographic data (i.e. age, sex, and location) including environmental factors like atmospheric pressure, humidity, atmospheric temperature, and rainfall were collected in the same period. We also analysed if changes in environmental factors influenced evolution of *Salmonella* spp. during the course. Agreement of Ethic Committee from Constanta Clinical Infectious Diseases Hospital from Romania and informed consent of patients and parents were obtained.

During the year, there are four seasons. Spring months were between March and May with an average atmospheric temperature of 15°C. Summer, starting from June to August, has an atmospheric temperature average of 27°C. Autumn starts from September until November, in which the temperatures could be also raised around 18.5°C. In order to control seasonal effects, data were collected from two time periods, the first being based on June till September period, and the other from October till May period. For comparison in the summer period was included the first month of the autumn. June and September months although there are months in different seasons they presented the same average temperature and case number. Average temperature (°C), atmospheric pressure (mb), average humidity (%), and average rainfall amount (mm) on days were measured according with Constanta Monthly Climate Averages[14].

Statistical analysis. For statistical analysis we performed a logistic regression (method Backward Stepwise – Wald) using SPSS Statistics 23. For temperatures, pressure, humidity, and rainfall, the averages of values were calculated for each month over a 10 years period, grouped into two classes: values above and below the monthly average. The new variables obtained and demographic data of the patients were introduced into logistic regression model, and they were considered variable of interest.

The logistic regression equation is:

$$\text{logit}\,(p) = -0.932 + 0.966\ \text{humidity} + 0.816\ \text{temperature} - 0.380\ \text{sex},$$

where p is the probability of presence of the characteristic of interest. Logit (p) can be back-transformed to p by the following formula:

$$p = 1/(1 + e^{-\text{logit}(p)}).$$

RESULTS AND DISCUSSION

In a study of over 10 years period were admitted in the hospital 377 cases of *Salmonella* spp. aged between 9 months and 86 years. Children less than 5 years old represent 38.99% from the total cases.

The highest number of cases were noticed in year 2015 (45 cases, 11.93%) in the October-May period and the lowest incidence was seen for the June-September period in 2009 year (9 cases, 2.38%) (Fig. 1).

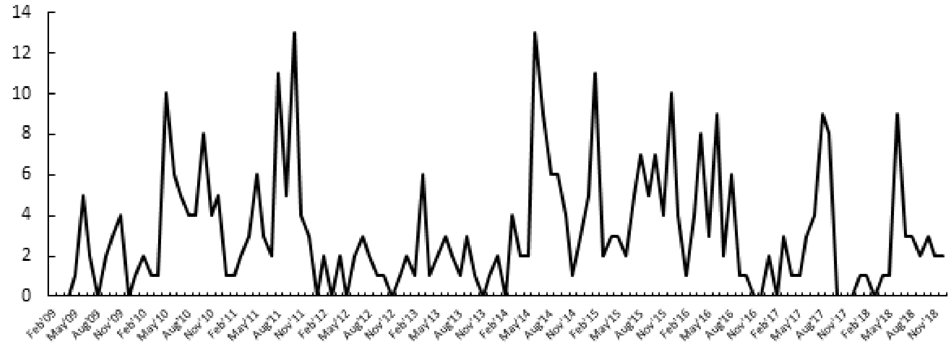

Fig. 1. Evolution of *Salmonella* spp. in Constanta area over 10 years studied period

Moreover, from the total of the patients, 196 (51.98%) were males and 181 (48.01%) females (Fig. 2).

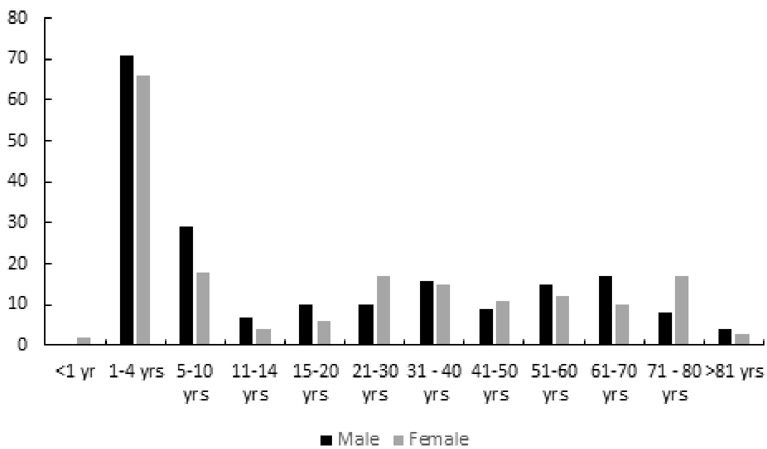

Fig. 2. Distribution between males and females diagnosed with *Salmonellosis*

The higher incidence of locations shows the higher number of patients (231 cases, 61.27%) from urban area comparing with rural area with only 146 cases (38.72%) from all years taken into the study (Fig. 3).

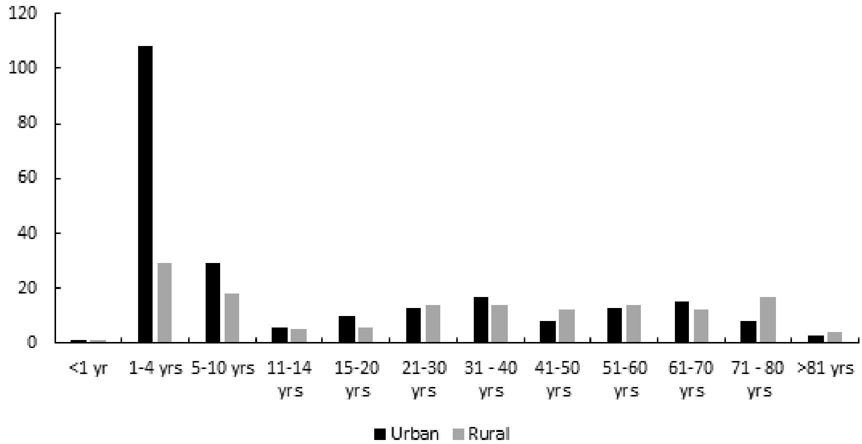

Fig. 3. Distribution of *Salmonella* spp. by local area and group of age

After the distribution by sex and year, and sex and month it was shown that the higher prevalence was for male (196 cases, 51.98%). In June–September were noticed 87 cases of male patients from the total of 178 registered in all years in the mentioned months of the studied years (Figs 4 and 5).

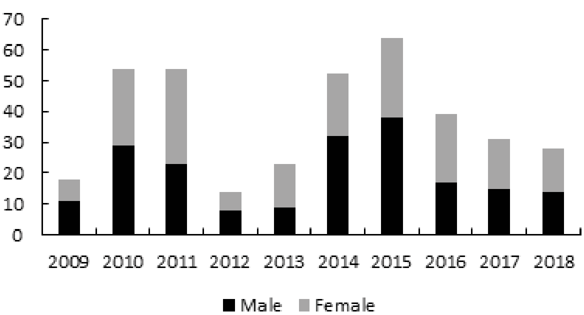

Fig. 4. Repartition by sex and year

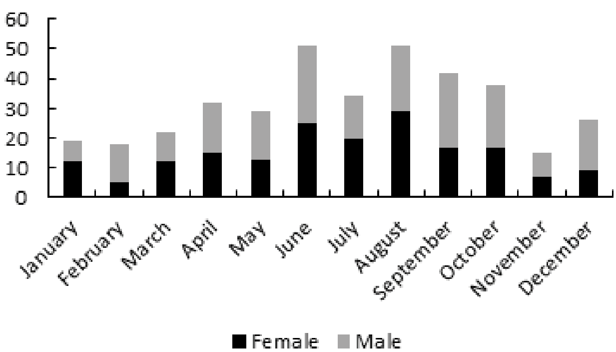

Fig. 5. Repartition by sex and month

From the total cases of Salmonella infections, sepsis was registered in 17 cases while in the other 360 cases were noticed enterocolitis with Salmonella. All the cases from our study were with *Salmonella enterica*, except one case with *Salmonella typhi* in a seafarer.

Over the ten years studied, the medium atmospheric pressure for October–May period was 1017.55 mb, and for June–September period was 1012.86 mb.

Regarding medium temperature over the studied period, we noticed a value of 9.85°C for October–May period and 24.67°C for June–September period.

After measuring the rainfall values, we noticed that medium value for October–May period was 56.66 mm, while medium value for June–September period was 39.89 mm.

The medium value for humidity, over the 10 years studied, was for October–May period of 75.2%, while the medium value for June–September period was 62.3%.

When we performed statistical analysis of the prediction variables originally included in the regression model, the following variables were removed: atmospheric pressuse ($p = 0.388$), rainfall ($p = 0.956$), location ($p = 0.867$), and sex ($p = 0.08$) being considered variables that do not make a significant contribution ($p > 0.05$).

The variable that were included in the model were humidity ($p < 0.001$) and atmospheric temperature ($p < 0.001$). The results showed that those 2 predictor variables favourise the risk of appearance of *Salmonella* spp. cases during the period June–September comparative with October–May of 2.629 more in condition of increased humidity, and 2.264 more in condition of increased atmospheric temperature. Increased values were considered when they were high comparative with average value of each month (Table 1).

Table 1.Variables in the equation for logistic regression

Independent variable	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for exp (B)	
							lower	upper
Humidity (over average)	0.966	0.224	18.547	1	0.000	2.629	0.447	1.047
Temperature (over average)	0.817	0.232	12.447	1	0.000	2.264	1.438	3.566
Sex	–0.380	0.217	3.060	1	0.080	0.684	0.447	1.047
Constant	–0.932	0.248	14.156	1	0.000	0.394		

In the current study, the effects of climate variation on *Salmonella* spp. infections were examined. Results indicated that atmospheric temperature and humidity are positively correlated with *Salmonella* spp. infections.

One study found no other significant changes in temperature and precipitation values. The highest temperature was recorded in 2007 having the average of 64.95 F with the lowest precipitation (42.33 inches), and in 2009 the highest precipitation of 68.64 inches[15].

The higher prevalence of *Salmonella* spp. infection is found in countries such as Africa, being the most common bacterial isolates in adults and children. The infection was associated with fever together with a 20–25% fatality[16]. However, in developing countries the surveillance of the bacteria is less than 1% (Refs 17 and 18).

Therefore, the higher temperature leads to higher concentrations of bacteria in food industry and can be found especially in warmer months alongside with inadequate cooking of the food. The transmission of the bacteria is made by different pathways like direct effect on proliferation or indirect by ingesting food in warm days[19], including the opportunistic infections which are frequently seen in immunosuppressant patients[20,21].

Environmental impact on human health and quality of life is usually related to occupational exposure to chemical substances, dust or particles[22,23]. Environmental contacts and environmental factors like high temperature, high relative humidity and light rainfall are factors that can have a positive influence on Salmonella infections and hospitalisations[24,25].

CONCLUSIONS

Salmonella spp. transmission to humans represents a complex process. Higher temperatures combining with eating behaviour could contribute to *Salmonella* spp. infection. However, the environmental factors that influence *Salmonellosis* in the studied period were atmospheric temperature and humidity. Although *Salmonella* spp. has better adapted at different environmental conditions, the solution for reducing the prevalence of infection will not be easily found. Therefore, increasing the knowledge of environmental transmission can help to minimise the worldwide spreading.

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INFLUENCE OF ENVIRONMENTAL FACTORS ABOUT EVOLUTION OF SHIGELLOSIS IN CONSTANTA COUNTY OF ROMANIA

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Abstract. Bacillary dysentery caused by *Shigella* spp. bacteria represents a significant public health problem especially in developing countries. The objective of this study was to analyse the demographic and environmental factors of patients from Constanta city, localised on the Black Sea Coast. A retrospective study was conducted on 376 patients with *Shigella* spp. disease over a 10 years period, between January 2009 and December 2018 at Constanta Clinical Infectious Diseases Hospital from Romania. Demographic data (i.e. age, sex, location and *Shigella* spp. types) including environmental factors like atmospheric pressure, humidity, atmospheric temperature, and rainfall were collected. The results showed a higher prevalence of patients less than 5 years (38.03%) from which 15.42% were included in September-November period. Moreover, from the total of the patients, 209 (55.58%) were women, and 167 (44.72%) were men. Regarding the Shigellosis type, the higher incidence was seen for *S. flexneri* in about 44.41% cases. The environmental factors that influence Shygellosis in the studied period were atmospheric temperature ($p = 0.0023$), humidity ($p = 0.003$) and rainfall ($p < 0.001$). The results show a strong support for public health experts in order to easily evaluate the control strategies for Shigellosis spreading based more on environmental factors.

Keywords: *Shigella* spp., atmospheric temperature, humidity, rainfall.

AIMS AND BACKGROUND

Shigella infection is an acute infectious inflammatory colitis caused by a family of bacteria known as *Shigella*[1]. There are several types of *Shigella*, member of the family Enterobacteriaceae: *S. dyzenteriae*, *S. flexner*, *S. boydii*, and *S. sonney*. From all Shigella spp., *S. sonnei* shows to have the higher incidence and *S. flexneri* especially in developing countries[2]. Over the last decade, antimicrobial resistance

* For correspondence.

has been increased among *Shigella* spp., limiting the pragmatic treatments[3], like in other diseases [4].

People infected with bacteria remove the stool and contaminate ground, water, or food, and subsequently transmitted to others by fecal-oral route[1,5]. Signs and symptoms of shigellosis usually begin a day or two after contact with *Shigella* spp., but sometimes may take up to a week to develop. Common symptoms of bacillary dysentery are abdominal pain, fever, watery diarrhea with mucus and blood, rectal tenesmus, nausea and vomiting[1,5].

The disease is more common in children less than 5 years, travelers, refugees who live in camps and in developing countries. Usually the infection is mild and self-limited. Another important epidemiological aspect is that although some people have no symptoms after they have been infected with Shigella, their feces may still be contagious up to a few weeks[6].

Although *Shigella i*nfection usually clears up without complications, sometimes complications like dehydration, seizures, hemolytic-uremic syndrome, rectal prolapse, toxic megacolon or reactive arthritis can be present[6].

Studies performed over the last years evidenced the effect of climate variation on shigellosis. These studies highlighted that temperature, rainfall and humidity influenced replication rate of bacterial and protozoan pathogens involved in diarrheic diseases. All these climate changes present an impact also on the pathogens reservoirs in environment[7–10]. Therefore, in this study, we analysed the demographic and environmental factors of bacillary dysentery, evaluation of the high-risk populations and climate changes that is of great importance in last years regarding disease control and prevention.

EXPERIMENTAL

A retrospective study was conducted on 376 patients with *Shigella* spp. disease over a period of 10 years, between 1st January 2009 and 31st December 2018. The patients were evaluated at Constanta Clinical Infectious Diseases Hospital from Romania with age between less than 1 year and 82 years. The selected settings were all departments of Constanta Clinical Infectious Diseases Hospital, and all cases that required hospitalisation for different infectious with *Shigella* spp.

Demographic data (i.e. age, sex, location and *Shigella* spp. infection types) including environmental factors like atmospheric pressure, humidity, atmospheric temperature, and rainfall were collected in the same period. We also analysed if changes in environmental factors influenced evolution of Shigellosis during the course. Agreement of Ethic Committee from Constanta Clinical Infectious Diseases Hospital from Romania and informed consent of patients and parents were obtained.

Constanta is a city located in the Dobrogea region of Romania, on the Black Sea coast, being one of the warmest cities from Romania. Constanta city has a humid subtropical climate with oceanic and semi-arid parts. During the year, there are four seasons: winter (December–February), spring (March–May), summer (June–August) and autumn (September–November). In order to control seasonal effects, data were collected from two periods, the first being based on September until November period, and the other from December until August period. Average atmospheric pressure (mb), average cloud and humidity (%), temperature (^{o}C), and average rainfall amount (mm) were analysed using Constanta Monthly Climate Averages[11].

Statistical analysis. For statistical analysis we performed a logistic regression (method Backward Stepwise – Wald) using SPSS Statistics 23. For temperatures, pressure, humidity, rainfall, the averages of values were calculated for each month over a 10 years period, grouped into two classes: values above and below the monthly average. The new variables obtained were introduced into the logistic regression model along with the demographic data of the patients.

RESULTS AND DISCUSSION

In a study of over 10 years period were admitted in the hospital 376 cases of Shigellosis (Fig. 1) aged between 8 months and 82 years. The highest number of cases were noticed in year 2009 (111 cases – 29.52%) and then we noticed a slow and continuous decreased of cases each year, except a second peak in year 2012 (64 cases – 17.02%). From the total cases, patients that were hospitalised between September–November were 189 cases, which represents 50.26%.

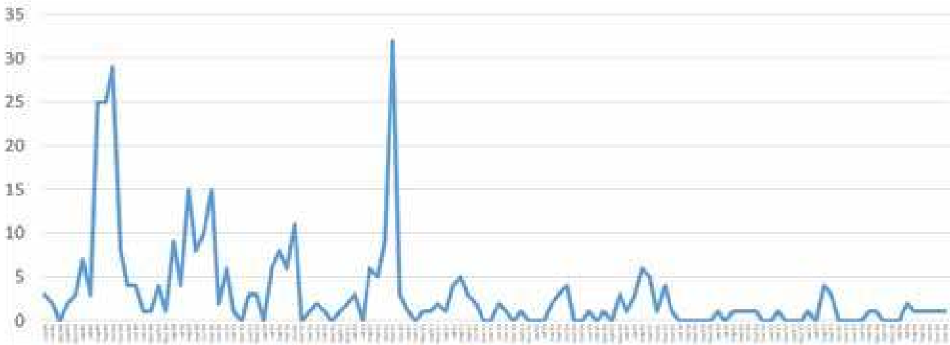

Fig. 1. Evolution of Shigellosis over the 10 years

The higher frequency was noticed in patients aged less than 5 years (143 cases, 38.03%) who were included between September–November period (58 patients, 15.42%) and December–August period (85 patients, 22.6%) (Fig. 2). Moreover, from the total of the patients, 209 (55.58%) were women, with preponderance in September–November period of 109 (29.22%) cases and in December–August period with 100 (26.59%) cases. The rest of 167 (44.72%) patients were men, with a higher incidence in December–August (87 cases, 23.13%) comparing with September–November period (80 cases, 21.27%).

The higher incidence of locations shows the higher number of patients in December–August period (114 cases, 30.31%) from urban area comparing with rural area with only 79 cases (21.01%) in September–November period and 73 cases (19.41%) in December–August period from all years taken into the study.

Regarding the repartition of Shigellosis by type, from the total patients, about 44.41% cases have presented *S. flexneri*, 43.35% presented *S. sonney*, 7.97% cases presented *S. dysenteriae*, and other 4.25% cases presented *S. boydii*. From out data, we noticed that *S. boydii* was present in the least analysed cases (Fig. 3).

Therefore, the higher prevalence for the September–November period was seen for *S. sonne* (120 cases, 31.91%) and for the December–August period for the *S. flexneri* (116 cases, 30.85%) from all 10 years studied. Interestingly, the lowest prevalence was seen for *S. boydii* with the same cases (i.e. 3 cases, 0.79%) in both period of the each analysed year.

After the atmospheric pressure measuring, it was seen a higher prevalence of 1020.34 mb in December with a less prevalence in 1012.06 mb in July (Fig. 4). For the September–November period it was seen an average of 1018.40 mb and for the December–August period an average of 1015.59 mb.

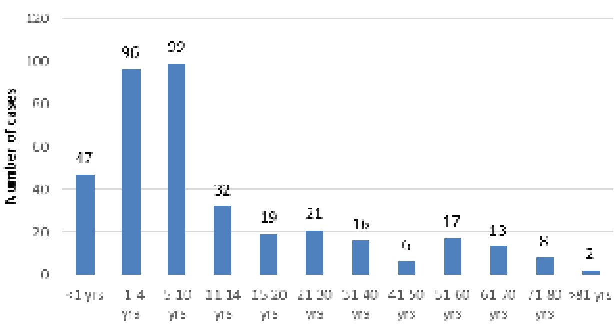

Fig. 2. Repartition of cases by groups of age

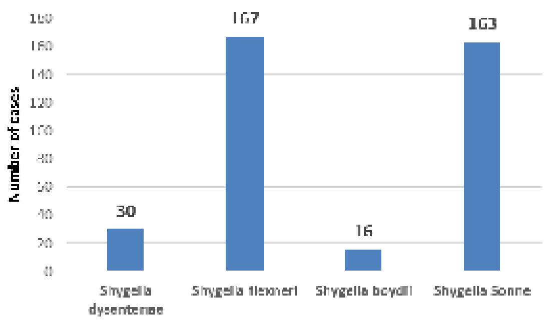

Fig. 3. Repartition of cases by type of Shigella

The higher value of atmospheric humidity was 83.2% in January with a less value in August with 58.2% (Fig. 5). For the December–August period it was seen a value above 71.57% comparing with 68.97% for the September–November period.

The higher value of atmospheric temperature of 26.6^{o}C was seen in August with a less value of 2.6^{o}C in January (Fig. 6). For the September–November period it was seen an average of 16.3^{o}C and for the December–August period an average of 14.28^{o}C.

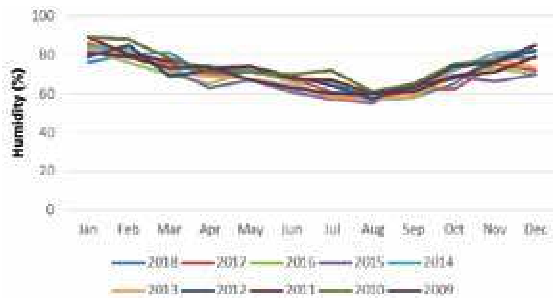

Fig. 4. Atmospheric pressure

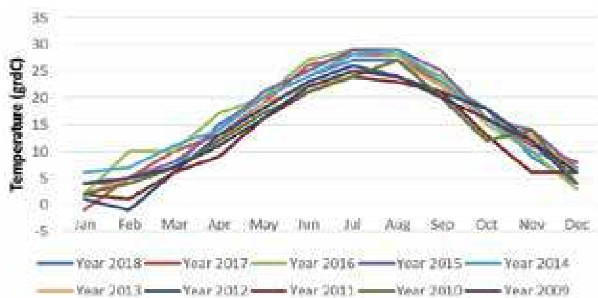

Fig. 5. Humidity

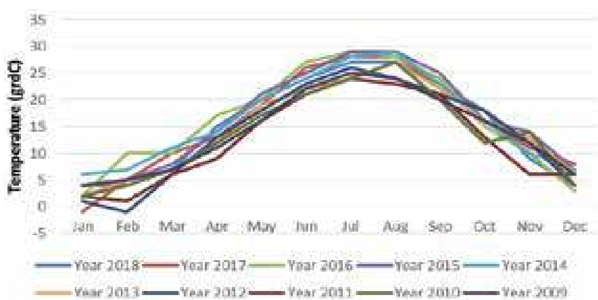

Fig. 6. Atmospheric temperature

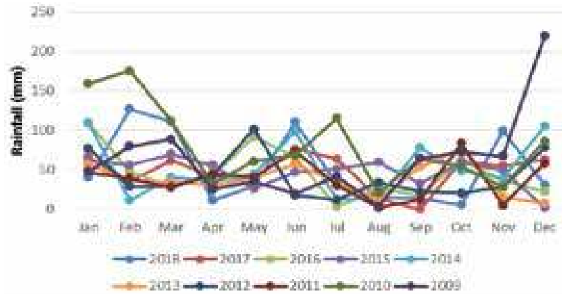

Fig. 7. Rainfall

Regarding atmospheric rainfall, it was seen a higher value of 67.35 mm in December with a less value of 20.39 mm in August (Fig. 7). For the September–November period it was seen an average of 54.73 mm and for the December–August it was seen an average of 53.35 mm.

In the present study, we divided the environmental factors into four categories, namely atmospheric pressure, relative humidity, temperature and rainfall. Here, it was shown a similar atmospheric pressure for both periods (1018.40 mb for the September–November period and 1015.59 mb for the December–August). The same considerations were also for humidity (71.57% in the December–August period and 68.97% in the September–November period) and rainfall (i.e. 54.73 mm for the September–November period and 53.35 mm for the December–August period).

When we performed statistical analysis of the prediction variables originally included in the regression model, the following variables were removed: age (p = 0.569), atmospheric pressuse (p = 0.597), sex (p = 0.209), location (p = 0.642), being considered variables that do not make a significant contribution ($p > 0.05$).

The variable that were included in the model were humidity (p = 0.003), atmospheric temperature (p = 0.0023) and rainfall ($p < 0.001$). The results showed that those 3 predictor variables favorise the risk of appearance of Shygella cases during the period September–November comparative with December–August of 2.135 more in condition of increased humidity, 2.2021 more in condition of increased atmospheric temperature and 3.328 more in condition of increased rainfall. Increased values were considered when they were high comparative with average value of each month.

In the present study, bacillary dysentery was seen in all individual categories, but patients aged less than 5 years were majority (143 cases) followed by those aged 5 to 10 years with 99 cases, which represents a rate of bacillary dysentery, similar with other countries[12–15]. These features suggest that additional studies are required in order to evaluate risk factors of bacillary dysentery.

No person is immune to shigellosis, but the incidence was showed to be highest among children less than five years of age, although it has been observed that during epidemics all age groups are affected[16]. Moreover, the prevalence has been reported to increase steadily after the age of 40, along with bacterial load. Therefore, young children and old people could share the highest bacterial load[17]. In children with poor nutrition or growth retardation, *Shigella* could lead to a vicious cycle[16]. Furthermore, the difference in proportions between analysed male and female individuals, especially between September–November period (i.e. 209 women and 109 men) could be due because some individuals are generally more active, gaining also much opportunities to be exposed at different environmental factors containing bacteria[18]. Therefore, our findings show that preventive actions should better control bacillary dysentery, especially at individuals less than 5 years and those between 5 and 10 years.

In our study, it was shown a higher prevalence in the September–November period for *S. sonne* (120 cases, 31.91%) and in December–August period, for *S. flexneri* (116 cases, 30.85%) with the lowest prevalence for *S. boydii* (i.e. 3 cases, 0.79%). A study made on eight Asian countries found that *S. flexneri* (50%) and *S. sonnei* (45%) were predominantly identified among 98 *Shigella* isolates[19].

Although there were inconsistent results, the studied environmental factors could affect the evolution of different disease[20,21], specially the way of food-borne disease transmission by influencing the pathogens[22–24]. Another study sustains the fact that environmental factors could be used as predictors of the bacillary dysentery, and more precisely the temperature[25]. Therefore, it was considered that a rising with 1°C in maximum or minimum temperature could be associated with higher increase (until 12%) of the bacteria[25]. However, public health interventions should be gaining more attention regarding the spread of Shigellosis in order to prevent the result of weather changing considering the local climatic conditions.

CONCLUSIONS

In the present study, we noticed a peak of Shigellosis cases in year 2009 and then a constant decrease in number of studied cases. The highest incidence in our area was registered in autumn months (September–November) being common in urban area. Furthermore, from all Shigellosis types analysed, *S. flexneri* was seen to be very spread.

The environmental factors that influence Shygellosis in the studied period were atmospheric temperature, humidity and rainfall. Therefore, priority interventions should be offered at population target group, Shigellosis being an important public health problem in respect with isolates profile before starting the treatment.

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CORRELATION OF AN ACUTE VIRAL HEPATITIS TYPE A OUTBREAK IN THE CONSTANTA COUNTY WITH ENVIRONMENTAL RISK FACTORS

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Abstract. Outbreaks of acute viral hepatitis type A (VHA) occurs mainly in collectivities, related with poor hygiene. Hepatitis virus type A is transmitted by contaminated water, food or objects (toys). The aim of the research was to assess the environmental risk factors of acute viral hepatitis type A among children younger than 16-year old hospitalised in Children Infectious Diseases Department over year 2013, with a peak of hospitalised patients of 85.08% in autumn–winter, in the Constanta county. The experimental study consisted in collecting epidemiological and clinical data from medical files of 181 patients admitted in our Department, for VHA in year 2013. Statistical analysis was performed with IBM SPSS Statistics 20 software. It was found that the mean age of studied population was 6.72 years (min =1 year, max = 16 years, SD = 3.32). None of the 181 inpatients (95 boys and 86 girls) had been vaccinated for VHA. Repartition by rural-urban area noticed that the most frequent cases were from urban area (n= 157/181– 86.74%). From the ethnic point of view, majority of children were Gypsy (136/181– 75.13%). Epidemiological pattern reveals 19 Gypsy family foci, with a total of 98 patients (54.14%). The identified environmental risks factors for VHA were urban settings, and difficulties in the supply of running water.

Keywords: acute viral hepatitis type A, environmental risk factors, outbreaks, children.

AIMS AND BACKGROUND

The main purpose of the study was to assess the environmental risk factors that can play a role in an outbreak of acute viral hepatitis type A (VHA) among children younger than 16-year old hospitalised in Children Infectious Diseases Department over year 2013.

Acute viral hepatitis type A (VHA) is transmitted in children by contaminated water, food or objects (usually toys) or from child to child by fecal – oral route. Like in other diseases transmitted by water or food the simple rule 'boil it, cook it, peel it of forget it' always worked. Outbreaks of acute VHA occur mainly in

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collectivities, related with poor hygiene. Therefore, VHA can be avoided by using bottled water in area of high risk outbreaks, by cooking food, or no eating shellfish from sewerage contaminated seawater. Another two important measures of diseases prevention were vaccination or regular and careful hand washing.

EXPERIMENTAL

Retrospective study carried out among children with acute VHA hospitalised between January 1st, and December 24th, 2013, in the Constanta county, a south-eastern part of Romania.

Material consisted in collecting epidemiological and clinical data from medical files of in-patients, from 0 to 16-year old, admitted in the Children Infectious Diseases Department of Constanta Clinical Infectious Diseases Hospital, Romania, for VHA in year 2013. Cases were represented by 181 patients with families inform consented to assessing data for medical research. The highest numbers of cases were registered in a period of 5 months (August–December) with a total numbers of 154 cases.

The selected settings were children from the Constanta county who required hospitalisation in our department for VHA. Children Infectious Diseases Department is the only one in the Constanta county where are hospitalised children with different infectious diseases that require isolation in hospital and specific treatment. The main affected area was Medgidia town, and its countryside.

Statistical analysis was performed by IBM SPSS Statistics 20 software. Different demographic, clinical and epidemiological data were compared using chi squared (χ^2_{calc}) tests for associations. We used also the contingency coefficient (C), which is a measure of the degree of relationship of the classifications in the frequency table. As the value of this coefficient is higher the higher is the degree of association. The maximum value of the coefficient is never greater than 1.

We performed also a logistic regression for which we used the Backward Stepwise method (Wald) following the next model:

$$p = P(y = 1|x_1, x_2, ..., x_k) = \frac{\exp(B_0 + B_1x_1 + B_2x_2 + ... + B_kx_k)}{1 + \exp(B_0 + B_1x_1 + B_2x_2 + ... + B_kx_k)},$$

where $p = P(y = 1|x_1, x_2, ..., x_k)$ is the probability of achieving value $y = 1$ (probability of occurrence of the event concerned) conditioned by value $x_1, ..., x_k$.

$$\mathrm{OR} = \frac{p}{1-p} = \exp(B_0) \times \exp(B_1x_1) \times \exp(B_2x_2) \times ... \times \exp(B_kx_k),$$

where Exp (B) is the odds ratio values for each independent variable separately, and OR (odds report) – overall hazard ratio.

Dependent variable was chosen as membership outbreak (foci = 1, isolated cases = 0), and predictor variables were: month, gender, rural/urban area, ethnicity, age, water source, and the initial suspicion of diagnosis based on clinical or laboratory data.

Methods consisted in collecting demographic, clinic and epidemiologic data about any child hospitalised with acute VHA. The studied children population was divided in 2 groups, the first one included children from foci and the second one with isolated cases of VHA. Because the most of cases were grouped in foci, we try to find the environmental risk factors involved in diseases transmission.

All parents of hospitalised children with acute VHA agreed and signed informed consent to processing data for scientific point of view. Agreement of the hospital ethics committee was taken.

RESULTS AND DISCUSSION

Over the year 2013 were hospitalised 181 children aged 0–16 years. Mean age of studied population was 6.72 years (min = 1 year, max = 16 years, SD = 3.32). None of the 181 in-patients (95 boys and 86 girls) had been vaccinated for acute viral hepatitis type A (VHA). Most cases of VHA are occurring between August–December (n = 154/181; 85.08%). Comparative with the same period of year 2012 was noticed an increased number of cases (154 cases in year 2013 versus 64 cases in year 2012). Regarding repartition by rural-urban area we noticed that the most frequent cases were from urban area (n= 157/181 – 86.74%). The highest number of VHA cases was registered in kindergarden children (n= 69/181 – 38.12%), and in primary school children (n= 66/181 – 36.4%). From the ethnic point of view we noticed that the most of patients were Gypsys (136/181 – 75.13%). Epidemiological pattern of contamination reveals 19 Gypsy family foci with a total of 98 patients (54.14%). Even there were reported cases from all over the county the most affected area is Medgidia (n= 121/181 – 66.85%). In this city and its countryside surroundings over the years periodically appear outbreaks of different infectious diseases due to difficulties in water supply and poor hygiene in large families with many children.

Even there were suspicions that water was contaminated we could not prove this. Water from the four reservoirs that supply the city with water was tested and was negative for VHA. On the other hand, we considered that there are problems in water transmission network, because in some area of the Medgidia town (especially countryside areas) the water supplying are not constantly.

Statistical analysis performed found that in year 2013, in children hospitalised for VHA, there is an association between ethnicity and urban/rural area (χ^2_{calc} = 31.302, df= 1, p<0.001 < α = 0.05, C = 0.384), between ethnicity and foci (χ^2_{calc} = 10.935, df= 1, p = 0.004 < α = 0.05, C = 0.210), and between ethnicity and water source (χ^2_{calc} = 12.551, d*f* = 1, $p < 0.001 < \alpha = 0.05$, $C = 0.225$), as we can see in Figs 1, 2 and 3).

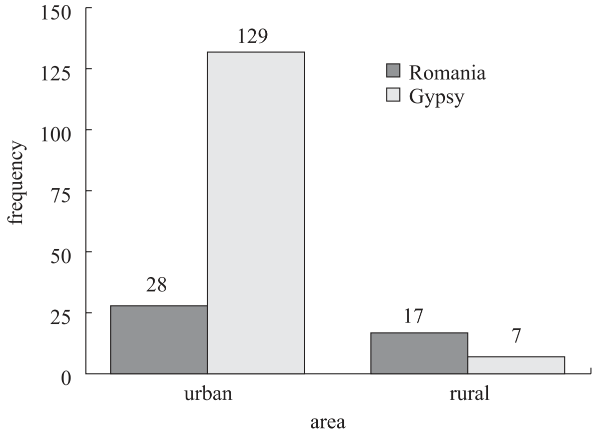

Fig. 1. Frequency of children by ethnicity and urban/rural area

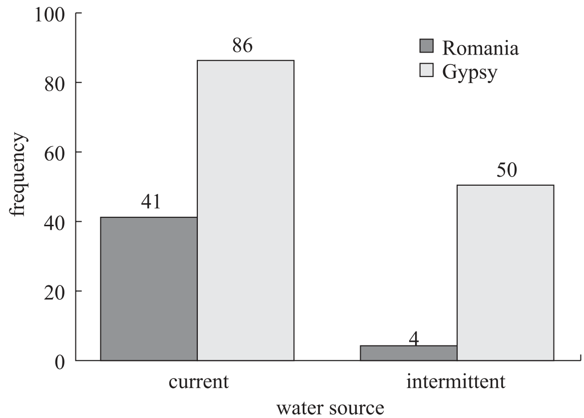

Fig. 2. Frequency of children by ethnicity and water source

Regarding association between ethnicity and distribution by months of the year we noticed that there is no association (χ^2_{calc} = 3.232, d*f* = 3, $p = 0.357 > \alpha = 0.05$, $C = 0.132$).

Also we noticed that in studied population we found an association between foci and symptoms suggestive for acute hepatitis (χ^2_{calc} = 11.872, d*f* = 1, p=0.001 < $\alpha = 0.05$, $C = 0.248$), as we can see in Fig. 4, and between foci and increased level of aminotransferases before hospitalisation (χ^2_{calc} = 10.935, d*f* = 1, p= 0.001 <α = 0.05, $C = 0.239$), as we can see in Fig. 5. Statistical processing results sustain an outbreak, because in such cases susceptible contacts were periodically checked by laboratory (level of aminotransferases), and often they were previously modified the emergence of symptoms; on the other hand, in outbreaks of acute VHA we can diagnosed more asymptomatic cases. Statistical evaluation also sustain that in this outbreak there are more Gypsy children from urban area and from area where were presented problems with water supply.

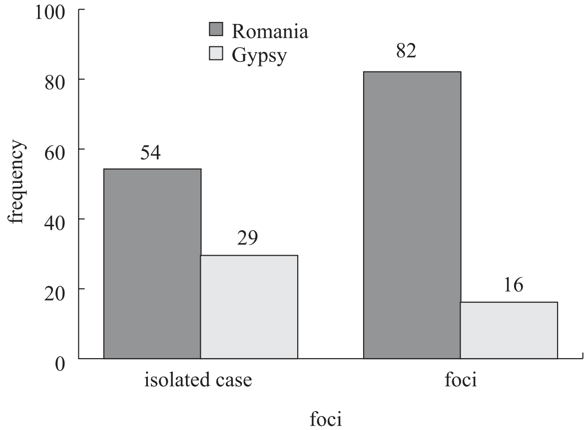

Fig. 3. Frequency of children by ethnicity and foci

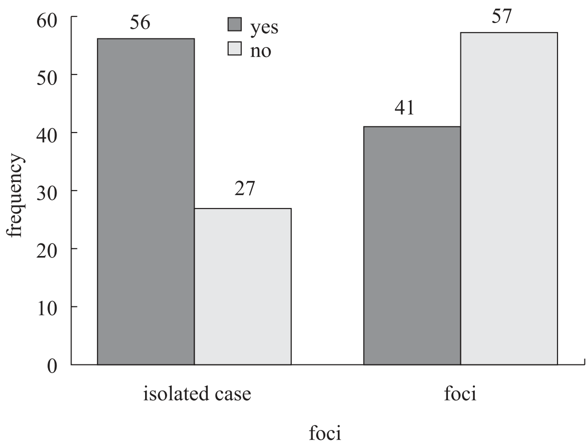

Fig. 4. Frequency of children by symptoms and foci

Logistic regression show that variables like area, ethnicity, age, water source, specific symptoms, and increased level of aminotransferases (laboratory), influence the possible belonging to an outbreak of VHA, and month (p = 0.167) and sex (p = 0.647) are variable that can not have any influence.

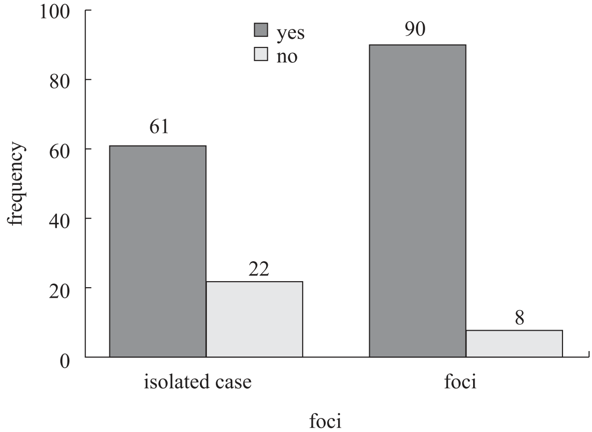

Fig. 5. Frequency of children by laboratory findings and foci

Calculated coefficient for area is $B_1 = 1.506$ which is considered significant: $\text{Wald}_1 = 6.104$, d$f = 1$, $p = 0.013 < \alpha = 0.05$. Exp ($B_1$) is 4.508, and IC95% for Exp (B_1) is (1.365, 14.884). Therefore, the risk of being part of outbreak is 4.508 times higher for patients in rural areas than urban ones. Ethnicity calculated coefficient is $B_2 = 1.083$ and is considered with significant contribution: $\text{Wald}_2 = 4.810$, d$f = 1$, $p = 0.028 < \alpha = 0.05$. Exp ($B_2$) is 2.954, and IC95% for Exp (B_2) is (1.122, 7.776). Hence, the risk of being part of outbreak is 2.954 times higher for Gypsy than for Romanian patients. Age calculated coefficient is $B_3 = -0.184$ is considered significant: $\text{Wald}_3 = 8.014$, d$f = 1$, $p = 0.005 < \alpha = 0.05$. Exp ($B_3$) is 0.832, and IC95% for Exp (B_3) is (0.733, 0.945) Since $B_3 < 0$, the presence of factor Age reduce the probability of the event $y = 1$ (belonging to the outbreak). Calculated coefficient for water source is $B_4 = 2.382$ is considered significant: $\text{Wald}_4 = 22.07$, d$f = 1$, $p < 0.001 < \alpha = 0.05$. Exp ($B_4$) is 10.830, and IC95% for Exp (B_4) is (4.008, 29.260). Consequently, the risk of being part of the outbreak is 10.830 times higher for patients who do not have access to the current source of water than for those who have access. If we take into consideration calculated coefficient for symptoms before hospitalisation, this is $B_5 = -0.969$ which is considered significant: $\text{Wald}_5 = 5.164$, d$f = 1$, $p < 0.023 < \alpha = 0.05$. Exp ($B_5$) is 0.379, and IC95% for Exp (B_5) is (0.164, 0.875). Then, the risk of being part of the outbreak is 0.379 less for asymptomatic patients who are presenting to hospital than for those who presented specific symptoms. Finally, calculated coefficient for increased level of aminotransferases in the moment of presentation in hospital is $B_6 = 1.586$, which is considered significant: $\text{Wald}_6 = 6.765$, d$f = 1$, $p < 0.009 < \alpha = 0.05$. Exp ($B_6$) is 4.885, and IC95% for Exp (B_6) is (1.478, 16.144). Accordingly, the risk of being part of the outbreak is 4.885 higher for patients who present increased level of aminotransferases before presenting to hospital than for those who did not.

Correspondence between the observed values of the dependent variable and those predicted from the regression model used is 77.3%. Effect size estimated by logistic regression expressed by R^2 coefficient is 0.434 (i.e. only 43.4% of the variation in the dependent variable are explained by variation in the independent variable used; the rest up to 100% is due to other causes).

For logistic regression models considered we obtain an overall hazard ratio/odds ratio (OR) = 83.08, for being part of the outbreak.

We know that more frequent outbreaks of acute VHA were related with contaminated food or water. Even the epidemiological link between water supply and this outbreak of VHA could not been completely demonstrate like in other studies[1] our statistical analysis and some epidemiological data sustain that beginning of outbreak was related with water supply network. There are also studies, like Mbithi et al.[2] which concluded that virus of VHA survives better in relative humidity area. In literature there were also many studies in which outbreaks of acute viral hepatitis were associated with different food as mussel[3], onion[4,5], potato salad[6], hot dogs[6], or ice[6,7], but in our case we could not involve any food. In order to prevent spreading diseases by food the researcher noticed that is better to keep food very cold or very hot, but this measure will prevent just some types of food poisoning. Such measures will not prevent illness caused by viruses (hepatitis, or noroviruses) and some types of bacteria such as pathogenic *Escherichia coli*, *Staphylococcus*, *Salmonella* or *Lysteria*[8].

In another study performed by Salahei et al.[9], in two different regions from Iran, they found discrepancy in morbidity pattern of acute hepatitis which is consequence of differences in socio-economic status and environmental risk factors. According with results of this study, VHA is more related with poor socio-economic status and environmental risk factors.

Constanta is a county in south-east of Romania which presents a good economic status, but less social and ecologic status than other region of Romania[10,11]. These socio-economic data are in favour of outbreaks of acute viral hepatitis A, in our geographic area. In our case there are two major measures that were important to implement. The first one is economical and can suppress environmental risk factors, and aims to solve for all county safe water supply sources and networks. The second measure is an epidemiological and medical one which targeting routine vaccination for acute VHA in whole county population. This measure was practiced sporadically in the county and stopped the outbreaks of acute VHA related to water sources in different areas of the county especially in times of natural disasters – floods.

CONCLUSIONS

The identified environmental risks factors of acute VHA in this outbreak were urban area, and problems with network water supply.

Taking into consideration our logistic regression model we can consider that the probability of a child to be part of outbreak of acute VHA in the Constanta county is higher among Gypsy ethnics, in rural areas where patients have less access to current water supply.

In order to prevent such outbreaks of acute VHA is important to implement some economic measures, like current water supply in all rural areas of the county that can suppress intervention of environmental risk factors and also some medical measures like routine vaccination, especially in children.

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