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RESEARCH ARTICLE

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Phosphatidylserine and Tyro3-Axl-Mertk Receptor Tyrosine Kinase level detection in plasma and on plasma-derived extracellular vesicle surface in chronic lymphocytic leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is a chronic lymphoproliferative disorder characterized by monoclonal B cell proliferation. Studies carried out in recent years suggest that extracellular vesicles (EVs) may be a potential biomarker in cancer. Tyro3-Axl-Mertk (TAM) Receptor Tyrosine Kinases (RTKs) and Phosphatidylserine (PS) have crucial roles in macrophage-mediated immune response under normal conditions. In the tumor microenvironment, these molecules contribute to immunosuppressive signals and prevent the formation of local and systemic antitumor immune responses. Based on this, we aimed to evaluate the amount of PS and TAM RTK in plasma and on the surface of EVs in CLL patients and healthy volunteers in this study. In this study, 25 CLL (11 F/14 M) patients in the Rai (O-I) stage, newly diagnosed or followed up without treatment, and 15 healthy volunteers (11 F/4 M) as a control group were included. For all samples, PS and TAM RTK levels were examined first in the plasma and then in the EVs obtained from the plasma. We detected a significant decrease in plasma PS, and TAM RTK levels in CLL patients compared to the control. Besides, we determined a significant increase in TAM RTK levels on the EV surface in CLL, except for PS. In conclusion, these receptor levels measured by ELISA in plasma may not be effective for the preliminary detection of CLL. However, especially TAM RTKs on the surface of EVs may be good biomarkers and potential targets for CLL therapies.

KEYWORDS
chronic lymphocytic leukemia (CLL), extracellular vesicles (EVs), Phosphatidylserine (PS), Receptor Tyrosine Kinase (RTK), Tyro3-Axl-MerTK (TAM)

1 | INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a disease characterized by neoplastic B lymphocytes clonal increase and accumulation in the blood, bone marrow (BM), lymph nodes, and spleen.[1,2] Although it is usually diagnosed around the age of 65, only 10–15% of patients are diagnosed under the age of 50.[3]

Extracellular vesicles (EVs) are very small particles produced by all known organisms, and they are in charge of removing cell wastes.[4] Numerous studies conducted in recent years show that EVs are also involved in the communication or interaction of cancer cells in the tumor microenvironment.[5–7]

Phosphatidylserine (PS), in the process of "apoptosis," moves from the inner surface to the outer surface of the membrane, sending

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an "eat me" signal to macrophages. Thus, ensuring the phagocytic engulfment of these cells.[8] In the tumor microenvironment, PS on the surface prevents the formation of antitumor immune responses locally and systemically by contributing to all immunosuppressive signals.[9]

Tyro3-Axl-Mertk (TAM) Receptor Tyrosine Kinases (RTKs) are homologous type 1 receptors that take a critical role in the clearance of apoptotic cells (efferocytosis) in eukaryotes.[10,11] TAM RTKs also have critical roles in inflammation, autoimmunity, cancer progression, and cancer immunology.[12–14] They do not directly affect the proliferation of tumor cells as traditional oncogenes do. However, their ectopic expression in leukemia cells activates signals that stimulate tumor cell survival.[15,16] Moreover, the expression of these receptors is generally associated with poor prognosis in cancer.[17,18]

In this study, we aimed to determine PS and TAM RTK levels in plasma and on the plasma-derived EVs' surface. These molecules, which are known to play an important role in cancer, may contribute to the development of new diagnostic and treatment methods in CLL. Although there are studies on these receptors in various cancers, there are no studies on these receptors in both plasma and EV levels in CLL. That's why we chose to investigate these molecules in CLL. We hope our findings may provide preliminary information for future studies.

Significance statement

In our study, we aimed to compare Phosphatidylserine and Tyro3-Axl-Mertk (TAM) Receptor Tyrosine Kinases (RTKs) levels on extracellular vesicles (EVs) surfaces in chronic lymphocytic leukemia (CLL) patients and healthy volunteers. Our results showed a significant increase in TAM RTK levels on the surface of EV of patients compared to those of healthy volunteers. We believe the data of this study will shed light on future studies. Larger studies involving advanced CLL patients may provide more information on this topic. Thus, the development of new treatments targeting such molecules can be achieved.

2 | METHODS

2.1 | Collection of samples

Blood samples were obtained from 25 patients with CLL at the time of diagnosis/followed without treatment. Besides, 15 healthy volunteers were included in this study as the control group, with no history of known acute and chronic inflammatory disease, autoimmune disorder, or cancer. Patients and control group samples were centrifuged at 3000 rpm for 10 min and then plasma was transferred to clean tubes and kept in a freezer at −80°C until the day of this study. This study was approved by the Ankara University Faculty of Medicine Clinical Research Ethics Committee (Decision # 02-132-19). A written informed consent form was obtained from all patients and healthy volunteers under the World Medical Association Declaration of Helsinki.

2.2 | Determination of PS and TAM RTK levels in plasma by ELISA

In this study, the ELISA (Enzyme-Linked Immunosorbent Assay) technique was used to determine the levels of PS and TAM RTK molecules in plasma. For each molecule, antibody-specific ELISA kits were used; PS (BT Lab Cat.No E1419Hu), Mertk (BT Lab Cat.No E4708Hu), Tyro3 (BT Lab Cat.No E6363Hu), and Axl (BT Lab Cat.No E3271Hu). All samples were read colorimetrically at 450 nm on the Biotech Epoch instrument. Results were analyzed in the "Gen5 Mikroplate Reader and Imaginer Software v.3.03" (Biotech) program connected to the device.

2.3 | Isolation and characterization of EVs

Plasma samples, stored before at (−80°C), were centrifuged at 1500*g* for 10 min at 4°C to remove debris. Then, the supernatant was centrifuged at 10,000*g* for 30 min. The supernatant was carefully removed without disturbing the vesicle pellet; afterward, the pellet was suspended in 500 µL of prefiltered (0.22 µm) phosphate buffer saline (PBS w/o Calcium, Magnesium, Capricorn Scientific). Size

TABLE 1 Patients demographics.

Features	Patient (*n* = 25)
Gender	
Female	11 (44%)
Male	14 (56%)
Median age	61 (38–93)
Median lymphocyte (10^9/L)	11 (5,3–294)
Rai stage, (n%)	
0	16 (64%)
I	9 (36%)
β_2–microglobulin (g/l), (n%)	
<3,5	5 (20%)
≥3,5	20 (80%)
CD38, (n%)	
<30%	5 (20%)
≥30%	20 (80%)
CD49d, (n%)	
<30%	12 (48%)
≥30%	13 (52%)
ZAP-70, (n%)	
<20%	10 (40%)
≥20%	15 (60%)

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exclusion chromatography was used to isolate EVs from the obtained samples.[19] This method was applied with qEV isolation columns (qEVoriginalRack, qEVoriginal, 500 µL, IzonScience, New England) according to the manufacturer's instructions. Purified EVs were characterized by three different methods. Firstly, particle size was measured using the qNano (qNano, Izon) device according to the Tunable Resistive Pulse Sensing method.[20] Before starting the measurement of the samples, the device was calibrated by diluting the CPC100B (Izon) calibration beads at the rate of 1/5000. Afterward, the particle count in the samples was performed with the NP100 pore with a measurement range of 50–330 nm and a pore diameter of 100 nm until the number of particles reached 500. The data were analyzed by the "Izon Control Suit v.3.4" program on the computer connected to the device.[21]

TABLE 2 Control group characteristics.

Features	Control group ($n = 15$)
Gender	
Female	11 (73%)
Male	4 (27%)
Median age	37 (22–55)

The other characterization test was performed with CD9 (ELK, cat no ≠ ES1999) and CD81 (Elabscience, cat no ≠ E-A-F1073D) labeling and flow cytometric analysis of them (ACEA NovoCyte® © ACEA Biosciences, Inc.). Due to the size of the EVs, a precoating process was needed for the measurement. At first, special carboxyl latex beads (Biolegend, cat no ≠ 312102) were coated by CD9, and then EVs were attached to this complex. Bead-CD9-EV complex labeled with CD81, then analyzed by flow cytometry.

Additionally, EVs were imaged via Transmission Electron Microscopy (TEM). First, a 5 µL EV sample was loaded onto formvar-coated copper grids (Electron Microscopy Sciences) and incubated for 10 min. Then, the EVs were fixed with 2.5% glutaraldehyde for 5 min and washed with ddH_2O. Images were taken with an Emsis Veleta camera (Münster) mounted on a Jem-100CXII microscope (JEOL) operating at 80 kV.

2.4 | EVs' surface PS and TAM RTKs levels detection by flow cytometry

The flow cytometry method was also used to determine the PS and TAM RTK levels on the surface of the EVs. At this point, a precoating process, as described in the previous part, was needed. After that, each molecule-specific antibody was bound to this complex (Mertk;

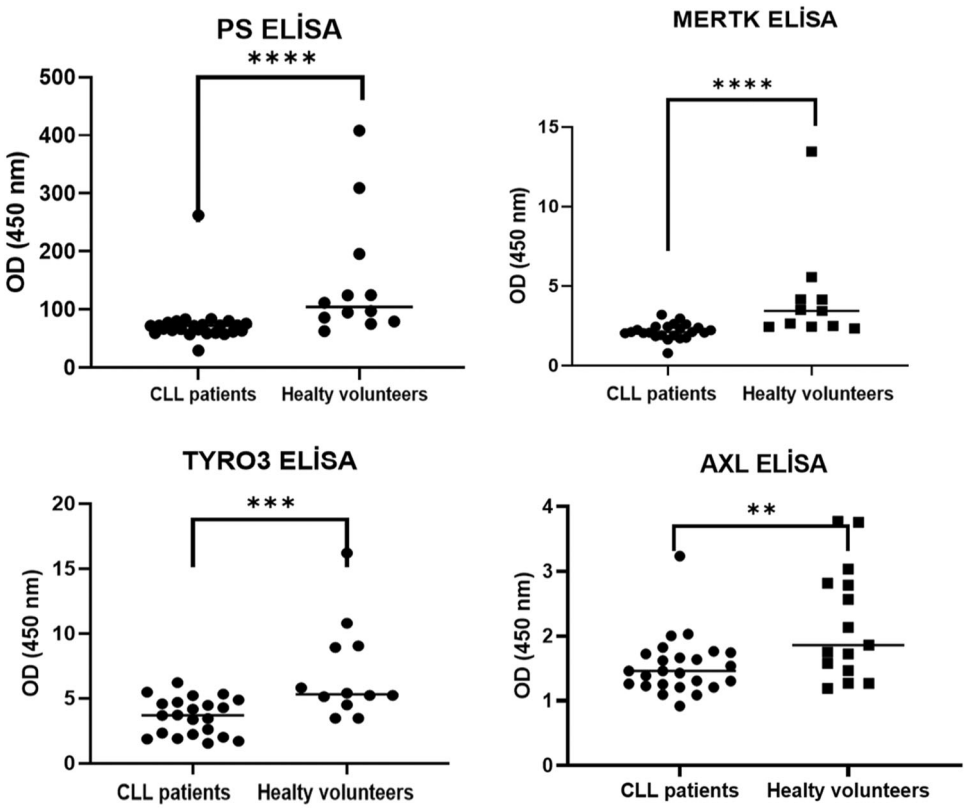


FIGURE 1 CLL patients and control group plasma concentrations of PS, TYRO3, Axl and Mertk plasma via ELISA test. For each reseptor levels were found to be significantly higher ($p < .0001$, $p < .001$, $p < .0096$, $p < .0001$ respectively) in control groups than patients. CLL, chronic lymphocytic leukemia; PS, Phosphatidylserine.

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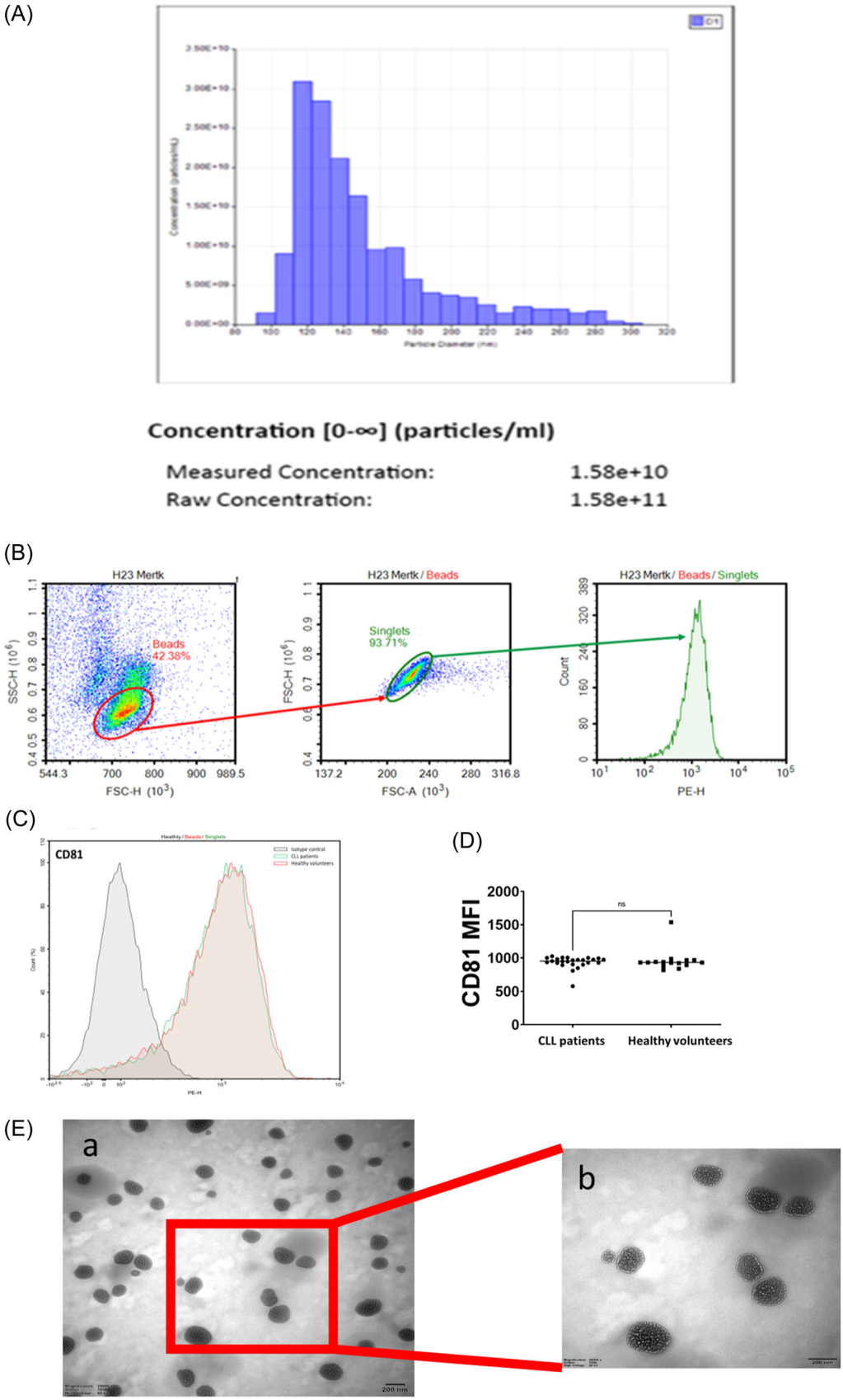


FIGURE 2 (A) This graph shows the EV concentration and size distribution (B) Gating of samples relative to the unstained sample (without CD81) by flow cytometry (C) The green peak, red peak, gray peak represent CLL patient EVs labeled with CD81, EVs of healthy volunteers labeled CD81, and isotype control, respectively (D) This analysis shows the patients and healthy volunteers EVs levels in plasma ($p = .2245$). (E) TEM images of Evs (a) x29000, (b) x58000. CLL, Chronic lymphocytic leukemia; EV, extracellular vesicles; TEM, Transmission Electron Microscopy.

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Biolegend, cat no ≠ 367607, Annexin V; TONBO, Tyro3; R&D, anti-h-Tyro3/DTK cat no ≠ MAB859 and Axl; R&D, anti-h-Axl cat no≠MAB154) and analyzed by flow cytometry. Tyro3 and Axl RTKs were analyzed after secondary antibody (Biolegend, cat ≠ 406607) labeling.

2.5 | Statistical analysis

The GraphPad Prism 9 program was used in the analysis. Due to the different sample numbers, the student's *t*-test nonparametric group was selected. Then, the samples were analyzed by the Mann–Whitney *U* test 2-tailed. A value of $p < .05$ was accepted as a significant difference.

3 | RESULTS

Twenty-five patients with CLL (11 F/14E) with Rai stage (0–I) and a median age of 61 years (38–93) were included in this study. Patient demographics are summarized in Table 1.

Fifteen healthy volunteers (11 F/4 M) with a median age of 37 years (22–55) participated in this study as the control group. The characteristics of the control group are given in Table 2.

For the detection of PS and TAM RTK in plasma, after the absorbance levels of all samples were determined, the concentration amounts (ng/mL) were calculated according to the standard curve created for each molecule. According to this, in all parameters, PS, TYRO3, Axl and Mertk plasma concentrations of the control group were significantly higher ($p < .0001$, $p < .001$, $p < .0096$, $p < .0001$, respectively) compared to the patients (Figure 1).

EVs obtained by purification through qEV columns were analyzed on the Q-Nano Gold instrument for size and quantitation. The data for each sample were analyzed with the "Izon Control Suit v.3.4" program on the computer connected to the device. Particle concentration and distribution for only one patient are shown in Figure 2A. For flow cytometry analysis, the bead-CD9-EVs complex (unlabeled) isotype was read as the control, followed by the CD81 added samples that were read and recorded (Figure 2B). According to Figure 2C, both groups were at least one log or farther in the horizontal plane from the isotype control. This situation showed that our patient and healthy control group samples carry the EVs-specific CD81 marker. This confirms the EV content of the samples we obtained. Figure 2D shows the patients' and healthy volunteers' EV levels in plasma. According to CD81 analysis, similar amounts of EVs were detected in both groups ($p = .2245$). Additionally, EV TEM images are shown in Figure 2E.

When the particle size and density were examined in the range of 80–150 nm and 150–300 nm, it was observed that 80–150 nm particles were over 80% (Table 3).

As a result of the analysis performed by flow cytometry, PS and TAM RTK levels of the patients and healthy subjects were evaluated at the EV level according to the unlabeled isotype control tube. Accordingly, the gray curve represents the unstained sample, the green curve represents the molecule levels in patients, and the red colored curve represents the molecule amounts in healthy volunteers. The comparative analysis (overlay) of these three curves for each molecule is shown in Figure 3A.

The level of PS molecule was significantly lower in patients compared to the control group ($p < .0001$). On the other hand, when each of the TAM RTKs was examined separately, a significant increase was found in patients compared to the control group ($p < .0001$). PS and TAM RTK analysis graphs are shown in Figure 3B.

4 | DISCUSSION

CLL is one of the most common adult leukemia.[2] Scientific studies are increasing day by day to develop new diagnosis/treatment methods for this disease, which cannot be fully responded to by current treatments.

EVs were previously known as small cargo carriers in the body, but they were later understood to be involved in many important biological activities such as communication of cancer cells, signal transduction, and suppression of the immune system.[22] Various studies are showing that EV levels increase in the tumor microenvironment. Silva et al. showed that the amount of EV was significantly increased in colorectal cancer patients compared to the control group. They suggest that this is associated with poor

TABLE 3 Qnano particle size 80–150 nm and 150–300 nm values (for six samples).

Sample	Totally (particle/mL) $\times 10^{10}$	80–150 nm (particle/mL) $\times 10^{10}$	80–150 nm (%)	150–300 nm (particle/mL) $\times 10^{10}$	150–300 nm (%)
Sample 1	1.58	1.33	84	0.25	16
Sample 2	0.88	0.71	80	0.17	20
Sample 3	3.80	3.13	82	0.67	18
Sample 4	6.00	5.21	86	0.79	14
Sample 5	1.10	0.88	80	0.22	20
Sample 6	1.83	1.49	81	0.34	19

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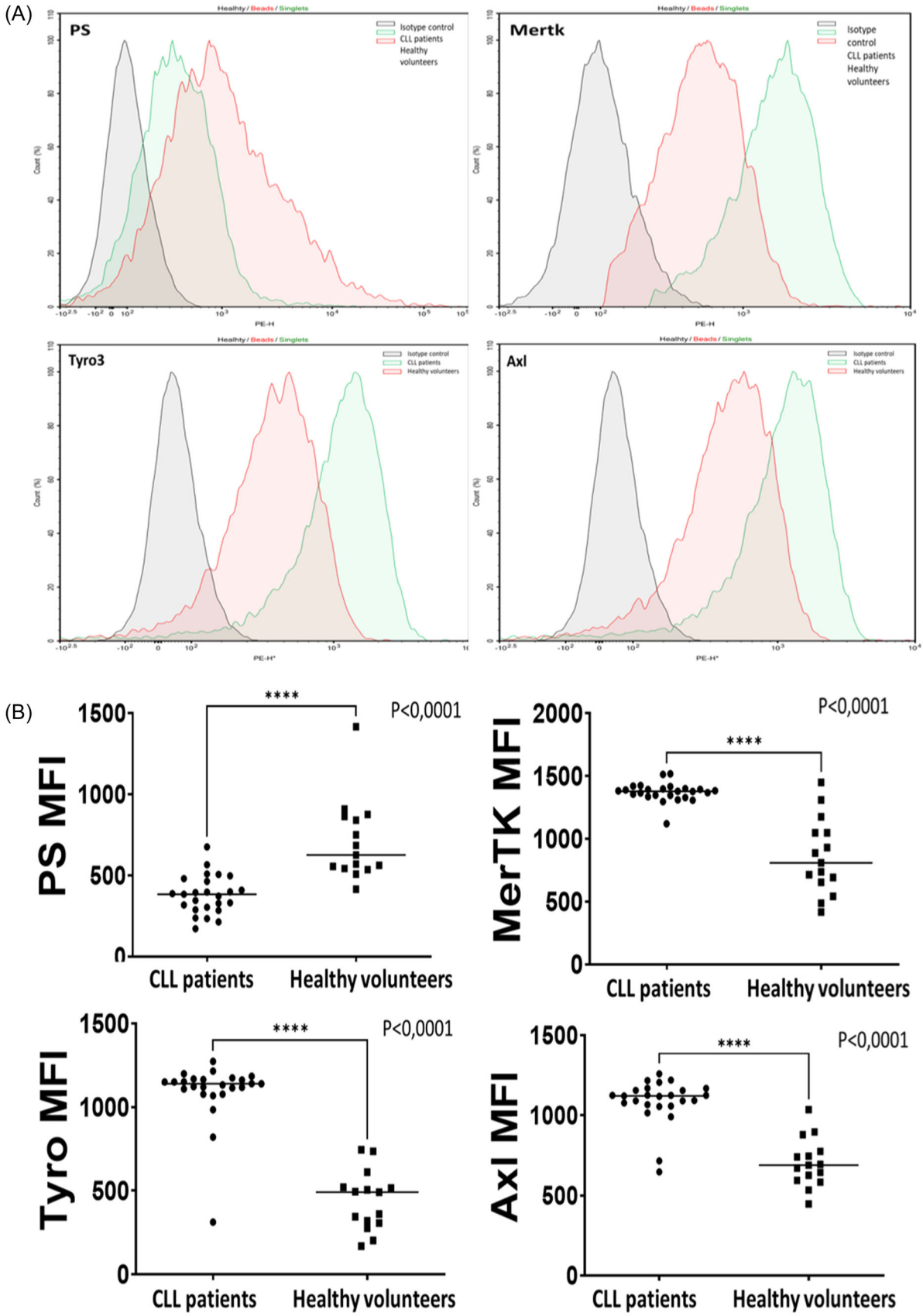


FIGURE 3 (A) Flow Cytometry Analysis of PS and TAM RTK in EVs from CLL patients and healthy volunteers The green curve, red curve, and gray curve represent the PS and TAM RTK levels in CLL patients, healthy volunteers, and the unstained sample, respectively (B) Statistical analysis of plasma-derived EVs PS and TAM RTK levels in CLL patients and healthy volunteers ($p < .0001$). CLL, Chronic lymphocytic leukemia; EV, extracellular vesicles; PS, Phosphatidylserine; RTKs, Receptor Tyrosine Kinases; TAM, Tyro3-Axl-Mertk; TEM, Transmission Electron Microscopy.

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prognosis and short survival and that these molecules can be used as tumor markers.[23]

In our study, we did not detect a significant difference in EV levels between the control group and the patients ($p = .2245$). However, this may be due to our small number of patients and advanced-stage CLL patients not being included. This is the limitation of our study. There are also studies showing that the amount of EVs increases in CLL. Gosh et al. reported that the number of EVs increased in CLL patients compared to healthy volunteers. However, they could not detect a significant difference between patients in different stages (Rai 0-IV).[24,25] In another study, Luca et al. reported that serum EV amounts increased in CLL patients compared to healthy volunteers. In contrast to Gosh's data, Luca et al. found that the amount of EVs increased significantly in advanced-stage (Rai III–IV) CLL patients, whose survival rate was lower and treatment duration was longer than in early-stage patients.[26]

Many receptors or molecules have been associated with cancer and many studies have been conducted. PS on the surface of cancer cells can enable these cells to be differentiated from other cells.[27,28] Based on this feature, monoclonal antibodies, antibody-drug conjugations, liposomal carriers, and natural products have been developed. However, the mechanism of PS-targeted therapies is still not fully understood.[29] There is no study this way in CLL and/or other leukemias.

Lea et al.[30] showed that the amount of PS on the surface of exosomes obtained from plasma increased significantly in patients with ovarian cancer compared to healthy volunteers. They also found that PS levels were higher in the malignant group than in the benign group.[30] On the other hand, there are many preclinical and clinical studies showing the use of PS as a target molecule in destroying cancer cells in various solid tumors[9] However, studies on this molecule in hematological cancers are limited. There are two published articles about PS levels in plasma in CLL.[31,32] Besides, there are no studies about PS levels on the surface of the EVs in CLL.

Based on this, we aimed to investigate plasma and EV levels of PS in CLL. TAM RTKs, under normal physiological conditions, bind to PS receptors on the surface of cells undergoing apoptosis through their ligands (GAS6 and ProS) and ensure their engulfment by macrophages. These receptors can cause cancer cells to escape the immune response by survival, metastasis, angiogenesis, and resistance to chemo-radiotherapy in the tumor microenvironment.[9,11,14] Vouri et al.[33] mention that in their review, these molecules may have important effects on chemotherapy resistance and can be used in targeted therapies.[33]

Sinha et al.[34] report that a high-affinity Axl inhibitor, as a single agent or in combination with Bruton's tyrosine kinase inhibitors, can be used to treat patients with CLL. They also report treatments targeting the Axl receptor may be successful in CLL[34] Mendez, et al. suggest that expression/activation of MER may potentiate CLL cell survival via crosstalk with the BCR signal. In addition, they mention if MER receptors could be targeted, CLL progression could be blocked.[35] But, even though a few studies in CLL about TAM RTK, there is not enough study about all three receptors (Tyro3, Axl, and Mertk) in plasma and on the surface of EVs. For this reason, we aimed to investigate of the PS and TAM RTK levels in plasma and on the surface of the EVs in CLL.

In this study, we detected the PS and TAM RTK levels were significantly decreased ($p < .0001$, $p < .001$, $p < .0096$, $p < .0001$, respectively) in CLL patients versus to control group in plasma. But except for the PS levels, all of the TAM RTK levels were found to significantly increase in patients with CLL compared to healthy volunteers ($p < .0001$) on the surface of EVs. In conclusion, detecting plasma levels of these molecules by ELISA may not be effective because plasma includes many kinds of proteins in it. Thus, on the surface of EVs TAM RTK levels detect and target may be more effective. We believe that our findings may shed light on further studies in the future to develop new techniques for CLL diagnosis and therapeutic approaches.

STUDY LIMITATIONS

To evaluate the prognostic impact of EVs on patients with CLL, we included early-stage/no-treatment follow-up patients instead of patients with treatment indication, with the idea that various treatment regimens' effects unknown on these molecules.

FUTURE PERSPECTIVE

For further studies, comprehensive studies that include advanced-stage (Rai III-IV) CLL patients may be more useful in understanding the mechanism of CLL. Additionally, TAM RTKs may be used as a diagnostic marker or a potential targeting of plasma-derived EVs in patients with CLL. On the other hand, to better understand the disease mechanism are needed also molecular studies. The effects of these molecules on signaling pathways and EMT mechanisms and their ectopic/overexpression and molecular differences may be investigated.

AUTHOR CONTRIBUTIONS

Study design, sample collection, test steps, data collection, and statistical analysis were conducted by Meltem Bay, Guldane Cengiz Seval, and Oznur Coskun. Data reporting and evaluation of study results were performed by Meltem Bay, Gunhan Gurman, and Nesrin Ozsoy Erdas. All authors received this paper and Meltem Bay submitted the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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