

CAROL DAVILA UNIVERSITY OF MEDICINE AND PHARMACY
BUCHAREST
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**IMMUNOLOGICAL FACTORS INVOLVED IN THE
PROGNOSIS AND PROGRESSION OF MATURE T-CELL
NEOPLASMS**

SUMMARY OF THE DOCTORAL THESIS

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INTRODUCTION

T-cell non-Hodgkin lymphomas (NHLs) comprise a heterogeneous group of neoplasms that are often difficult to treat. Globally, non-Hodgkin lymphoma accounts for 544,322 new cases annually (7.9 cases per 100,000 population) [1,2], with a worldwide prevalence of 4.3 million cases reported in 2015 [3]. Global trends appear to indicate a sustained increase in annual incidence, from 3.1 per 100,000 population to 7.1 per 100,000 in 2021, accompanied by a decline in annual mortality [4].

T-cell NHLs account for approximately 10–15% of all non-Hodgkin lymphomas, with an incidence of 2.1 cases per 100,000 population in the United States [5]. Certain subtypes are more prevalent in endemic regions; for example, adult T-cell leukemia/lymphoma (ATLL) has an incidence of 27 cases per 100,000 population in Japan [6]. Although Romania is an endemic region for ATLL, no data are currently available to establish its annual incidence. T-cell NHLs have been reported to represent 8.2% of all lymphomas in Romania, although these data are derived exclusively from single-center reports [7].

The treatment of T-cell NHL is generally challenging and is frequently associated with inadequate therapeutic responses, either because of primary chemoresistance or rapid adaptation of the neoplasm to standard treatment regimens. These limitations are compounded by the relative scarcity of innovative therapies and clinical trials in recent years compared with B-cell lymphomas.

The present work aims to characterize the population of patients with T-cell lymphomas treated at Fundeni Clinical Institute over the past 10 years and to perform clinical and biological follow-up in a subset of these patients.

Biological assessment was pursued along two main directions:

- assessment of non-neoplastic lymphocyte populations in the peripheral blood of patients with T-cell neoplasms;
- measurement of selected cytokines at disease onset and correlation of their levels with subsequent clinical outcomes.

To this end, we performed a retrospective descriptive analysis of patients with systemic T-cell lymphomas diagnosed and treated in the Department of Hematology at Fundeni Clinical Institute between 2015 and 2024. We evaluated lymphoma subtypes, clinical course, treatment regimens, response to therapy, and potential associations between biological parameters, disease evolution, and prognosis.

A second component of this work focused on factors involved in immune regulation in patients with T-cell neoplasms. We initially assessed lymphocyte subpopulations, with particular emphasis on non-malignant suppressor/regulatory T cells, cytotoxic T cells, and helper T cells. In parallel, plasma interleukin-2 (IL-2), interleukin-10 (IL-10), and interleukin-35 (IL-35) levels were evaluated before the initiation of any form of treatment.

Interleukin-2 is a cytokine with a central role in immune activation, promoting the proliferation of cytotoxic T lymphocytes, natural killer cells, and monocytes [8]. At the same time, IL-2 is essential for the maintenance and expansion of the regulatory T-cell (Treg) compartment. IL-2 is critical for the development of immune responses against infectious agents and tumors; however, high concentrations and prolonged stimulation can promote apoptosis of activated lymphocyte populations through activation-induced cell death (AICD) [9]. Prolonged stimulation also favors expansion of the Treg compartment, thereby attenuating or terminating the immune response [9].

Interleukin-10 is an immunosuppressive cytokine that acts on macrophages and dendritic cells, inhibits the production of pro-inflammatory cytokines, including IL-2 and IFN- γ , and may contribute to immune exhaustion or anergy in chronic disease settings [10].

Interleukin-35 is a relatively recently described member of the IL-12 cytokine family. It is a heterodimer composed of two subunits shared with other cytokines in this family: Epstein-Barr virus-induced gene 3 (EBI3), which is also a component of IL-27, and p35 (the IL-12 alpha chain), which is also a component of IL-12 [11]. IL-35 exerts important immunosuppressive effects on effector T cells and their function and also promotes expansion of the Treg compartment [12]. At the time this doctoral project was initiated, regulatory T cells were considered the principal source of IL-35. Subsequently, regulatory B cells and certain tolerogenic/regulatory dendritic-cell populations have also been shown to produce IL-35.

The aim of this work is to investigate selected immunological mechanisms that may contribute to the unfavorable course of T-cell neoplasms, either through modulation of the immune response or through the development of chemoresistance in the setting of anergic or exhausted immunity. Accordingly, patients with T-cell non-Hodgkin lymphoma were evaluated at diagnosis. Plasma was collected and stored in accordance with international recommendations, including MISEV guidance, with processing designed to minimize platelet activation and the generation of platelet-derived extracellular vesicles before storage at -80°C . Plasma IL-2, IL-10, and IL-35 concentrations were measured by ELISA. Subsequently, extracellular vesicle-enriched fractions were isolated from patient plasma by size-exclusion chromatography (SEC). This approach was based on reports [13] indicating that Treg cells can communicate through exosomal or extracellular-vesicle-mediated signaling and that IL-35 may be transported within extracellular vesicles. The patient-derived exosomal/EV preparations were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), and Western blotting. Samples from three patients were analyzed to identify fractions enriched in exosomes and other extracellular vesicles.

The remaining EV/exosome isolate was incubated with Triton X to disrupt vesicular membranes, and the resulting lysate was subsequently analyzed by ELISA for IL-2, IL-10, and IL-35. These measurements were compared with the corresponding plasma ELISA results.

II. ORIGINAL CONTRIBUTIONS

3. Working Hypothesis and General Objectives

The present work aims to investigate selected immunological factors involved in tumor progression in patients with mature T-cell neoplasms. Romania is an endemic region for human T-cell leukemia virus type 1 (HTLV-1) infection and therefore a region in which aggressive adult T-cell leukemia/lymphoma (ATLL) represents a clinically relevant entity. ATLL frequently follows an unfavorable course under conventional chemotherapy. More broadly, mature T-cell neoplasms are recognized as having substantially poorer outcomes than their B-cell counterparts.

Research progress in these neoplasms remains considerably behind that achieved in comparable B-cell malignancies, including in proteomic characterization, analysis of intracellular signaling pathways, genomic research, and investigation of interactions with the tumor microenvironment. Conversely, major advances have been made in the broader study of the tumor microenvironment and cellular immunity in cancer.

Given the aggressive clinical course and poor prognosis of these neoplasms, their limited responsiveness to conventional therapies, and the particular relevance of studying them in Romania, where ATLL has an increased prevalence, we elected to investigate immunological parameters with potential relevance to disease evolution. At diagnosis, we assessed major lymphocyte subsets and plasma levels of IL-2, IL-10, and IL-35. Some related immunological parameters are already incorporated into prognostic models or have been associated with adverse outcomes as independent risk factors; examples include IL-10 in ATLL and soluble IL-2 receptor levels as an adverse factor in ATLL prognostic scoring systems.

Because these disorders are associated not only with high mortality but also with substantial morbidity and a persistently increased risk of infection throughout the disease course, we considered it relevant to characterize patients using an immunological profile interpreted alongside established clinicobiological factors. The working hypothesis of this thesis is that selected immunological parameters can be quantified in these patients and may provide clinically relevant information. The resulting data were therefore correlated with established clinicobiological prognostic factors with the aim of improving patient assessment and risk stratification.

The information generated by this approach may provide additional support for therapeutic decision-making, both with respect to the underlying T-cell neoplasm and to infectious morbidity related to the disease itself or induced by cytotoxic therapy.

To complement these immunological investigations, a retrospective study was conducted at Fundeni Clinical Institute between 2015 and 2024, including patients with mature T-cell lymphomas. Demographic and clinicobiological parameters conventionally used in the diagnosis, prognostic assessment, and treatment of these disorders were analyzed.

4. General Research Methodology

This doctoral work comprises three studies: two applied research studies and one retrospective statistical analysis.

In the two applied research studies, flow cytometry was used to assess the distribution of lymphocyte subsets at diagnosis in a subset of patients with T-cell lymphoma. Plasma IL-10, IL-2, and IL-35 levels were also measured. Cytokine concentrations were determined by enzyme-linked immunosorbent assay (ELISA) at diagnosis, before the initiation of any cytotoxic treatment (Thermo Fisher Scientific, Waltham, MA, USA; Cat. Nos. BMS221-2 and BMS215-2 for IL-2 and IL-10, respectively, and Cat. No. EEL067 for IL-35).

Because intercellular signaling is mediated not only by direct secretion of soluble interleukins into the extracellular space but also by extracellular vesicles capable of carrying cytokines, particularly IL-35, the second applied research study investigated IL-2, IL-10, and IL-35 in exosome-enriched preparations isolated from patient plasma. We sought to determine the relationship between cytokine concentrations measured in plasma and those measured in the isolated exosomal fraction.

The third study was a single-center retrospective statistical analysis conducted at the Center of Hematology and Bone Marrow Transplantation, Fundeni Clinical Institute, and included patients with systemic mature T-cell neoplasms diagnosed between 2015 and 2024. A total of 149 patients were included. Statistical analyses were performed using IBM SPSS Statistics, version 26.0 (IBM Corp., Armonk, NY, USA). Data distribution and normality of continuous variables were assessed using the Shapiro-Wilk test. Normally distributed continuous variables were analyzed using parametric tests (Student's t-test and analysis of variance [ANOVA], with Levene's test for homogeneity of variances and appropriate post hoc testing). Non-Gaussian continuous variables were analyzed using nonparametric tests (Mann-Whitney U and Kruskal-Wallis tests with post hoc analyses). Categorical variables were evaluated using contingency tables and tests of association, including Pearson's chi-square test or Fisher's exact test when expected cell counts were low. Overall survival (OS) was estimated using the Kaplan-Meier method, and survival curves were compared using the log-rank (Mantel-Cox) test.

5. Study I: Determination of Plasma IL-35, IL-10, and IL-2 Levels in Patients With Systemic T-Cell Lymphomas at Diagnosis

5.1. Introduction (Working Hypothesis and Specific Objectives)

Assessment of plasma IL-35, IL-10, and IL-2 levels at diagnosis in patients with mature T-cell non-Hodgkin lymphoma, together with correlation of these levels with other clinicobiological factors, may be relevant for prognostic assessment and estimation of relapse risk. Published studies have reported variable cytokine levels with potential implications for prognosis and the immune response.

For example, in adult T-cell leukemia/lymphoma (ATLL), IL-10 levels increase under the influence of the Tax protein, resulting in suppression of the immune response [102]. Other reports have shown that elevated soluble IL-2 receptor (sIL-2R) levels are associated with a higher International Prognostic Index (IPI), bone marrow involvement, and an unfavorable prognosis [103]. Prognostic scores used in ATLL incorporate soluble IL-2 receptor levels, and elevated sIL-2R represents an independent adverse prognostic factor [104,105].

We therefore aimed to evaluate plasma levels of these cytokines and their correlations with clinicobiological and prognostic parameters, with the objective of refining existing prognostic approaches in T-cell neoplasms. In addition, these interleukins have a substantial impact on the patient's overall immune status and may provide valuable information regarding immune function both at diagnosis and longitudinally during the course of disease.

We also performed quantitative analysis of leukocyte populations at diagnosis and assessed lymphocyte subsets following isolation of peripheral blood mononuclear cells (PBMCs).

5.2. Patients and Methods

Plasma Isolation

Peripheral blood collected into EDTA-containing tubes was centrifuged at $500 \times g$ for 10 minutes at 20 °C with the brake disabled, thereby allowing subsequent isolation of peripheral blood mononuclear cells (PBMCs). Plasma from all collection tubes was pooled into a single 15-mL centrifuge tube and mixed. The pooled plasma was subsequently centrifuged at $2,000 \times g$ for 10 minutes at 4 °C to remove residual cellular debris. After centrifugation, the clarified plasma was aliquoted into 2.0-mL cryovials and stored at -80 °C.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Biosciences; density 1.077 g/mL). The erythrocyte/leukocyte sediment obtained after the first centrifugation step was retained for immediate PBMC isolation, diluted 1:1 with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS; Gibco, Thermo Scientific), and carefully layered over the Ficoll gradient in a 50-mL conical tube. Samples were centrifuged at room temperature at $800 \times g$ for 30 minutes using a swing-bucket rotor with the brake disabled. PBMCs were collected from the interface using a transfer pipette and washed with DPBS. Cells were resuspended in cryopreservation medium containing 90% heat-inactivated

fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) and 10% dimethyl sulfoxide (DMSO; Hybri-Max™, sterile-filtered, hybridoma-tested, ≥99.7%; Sigma-Aldrich).

Cell Viability Assessment

Stem-Kit reagents (Cat. No. IM3630; Beckman Coulter), comprising a murine monoclonal antibody combination (CD45-FITC/isotype control-PE) and the viability dye 7-AAD, were used for flow-cytometric identification and quantification of viable leukocytes (CD45⁺ populations) in fresh or thawed PBMC samples.

Lymphocyte Subset Analysis

For determination of absolute lymphocyte-subset counts, 50 µL of well-mixed anticoagulated whole blood was added to BD Trucount™ tubes and mixed with 20 µL of BD Multitest™ 6-Color TBNK reagent containing fluorochrome-conjugated antibodies against CD3 (FITC), CD16 + CD56 (PE), CD45 (PerCP-Cy5.5), CD4 (PE-Cy7), CD19 (APC), and CD8 (APC-Cy7). Samples were incubated for 10 minutes, followed by erythrocyte lysis with 450 µL of BD FACS™ Lysing Solution. Results were expressed as percentages of total lymphocytes for total T cells (CD3⁺), helper T cells (CD3⁺CD4⁺), cytotoxic T cells (CD3⁺CD8⁺), B lymphocytes (CD19⁺), and natural killer cells (NK; CD3⁻CD16⁺ and/or CD56⁺).

Flow-Cytometric Identification of CD45⁺CD4⁺CD25⁺CD127dim/negFoxP3⁺ Regulatory T Cells

After recovery in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, PBMCs were washed twice with DPBS and centrifuged at 300 × g for 5 minutes. Cell pellets were resuspended and stained with a mixture of fluorochrome-conjugated monoclonal antibodies directed against CD45-VioBlue, CD4-VioGreen, CD25-VioBright515, and CD127-PE. Intracellular FoxP3-Vio667 staining was performed after fixation and permeabilization using the anti-human REAfinity™ Treg phenotyping kit (Miltenyi Biotec; Cat. No. 130-122-994). Samples were briefly vortexed and incubated in the dark at 4 °C for 10 minutes. Cells were then washed with 2 mL of PEB buffer prepared according to the manufacturer's instructions by combining AutoMACS® Rinsing Solution (Cat. No. 130-091-222) with MACS® BSA Stock Solution (Cat. No. 130-091-376; Miltenyi Biotec) in phosphate-buffered saline. Cell suspensions were vortexed and centrifuged at 300 × g for 5 minutes at 4 °C. Fixation and permeabilization were performed by adding 1 mL of freshly prepared solution followed by incubation for 30 minutes at 4 °C in the dark. Cells were then washed sequentially with PEB buffer and permeabilization buffer. For intracellular staining, cells were incubated for 30 minutes at 4 °C in the dark with a mixture containing Tandem Signal Enhancer and anti-FoxP3-Vio667 antibody. After staining, cells were washed with 2 mL of permeabilization buffer, vortexed, and centrifuged at 300 × g for 5 minutes at 4 °C. The supernatant was discarded and the final cell pellet was resuspended in 300 µL of PEB buffer.

Data were acquired using a BD FACSLytic™ flow cytometer, and dot plots were analyzed using FACSuite™ software (BD Biosciences) or Kaluza version 1.8.

5.3. Results

Table 5.1. Lymphocyte subset analysis in the evaluated patients

Sample ID	Treg Abs	Lymph Abs	CD3+ Abs	CD3+C D4+ Abs	CD3+CD4 + excl. dual Abs	CD3+C D8+ Abs	CD3+ CD8+ excl. dual Abs	CD3+ CD4+ CD8+ Abs	CD3+ CD4- CD8- Abs	CD19 + Abs	CD4/CD8 Ratio
AB5 SG	1000	11321	10359	9617	9089	9052	530	493	37	35	136
AB3 SG	3281	2579	742	581	352	346	217	211	6	18	105
AB8 SG	2143	2544	1121	954	575	552	381	358	23	21	86
AB7 SG	1763	2412	1292	980	352	332	568	548	20	80	229
AB4 SG	1000	3063	2803	1910	751	717	1006	973	34	186	808
AB2 SG	2322	2486	1002	716	251	239	383	372	12	94	277
AB9 SG	1197	1496	1244	817	492	458	332	298	34	27	156
AB10 SG	1791	2133	1185	1007	464	447	538	521	17	22	168
AB11 SG	1000	3846	3827	3198	2126	2036	991	900	91	171	107
AB12 SG	1414	2605	1833	1541	902	887	540	525	15	114	61
AB13 SG	4208	2522	596	517	197	190	281	274	6	46	45
AB14 SG	5218	2514	479	334	212	211	94	92	2	30	112
AB15 SG	1001	4968	4938	3982	3312	3251	698	637	61	33	436
AB16 SG	1000	6958	6923	6745	6726	6720	21	15	6	4	138

Table 5.2. Mean lymphocyte-subset values and standard deviations.

Subset	Mean	Minimum	Maximum	Standard Deviation
Treg Abs (including neoplastic Treg)	2024.143	1000	5218	1332.256
Lymphocytes Abs (including neoplastic lymphocytes)	3674.786	1496	11321	2598.111
CD3+ Abs	2738.857	479	10359	2888.724
CD3+CD4+ Abs	2349.929	334	9617	2739.439
CD3+CD4+ excl dual Abs	1842.929	197	9089	2750.51
CD3+CD8+ Abs	1817	190	9052	2745.253
CD3+CD8+ excl dual Abs	470	21	1006	292.2973
CD3+CD4CD8+ Abs	444.0714	15	973	273.9684
CD3+CD4-CD8- Abs	26	2	91	24.67948
CD19+ Abs	62.92857	4	186	58.14632
NK (CD3-CD16+CD56+) Abs	156.6429	0	578	190.0452
CD4/CD8 Ratio	204.57	45	808	200.863

Flow-cytometric analysis of lymphocyte subsets demonstrated marked interpatient variability. CD4⁺ T-cell subsets showed greater dispersion than CD8⁺ subsets, as reflected by the higher standard deviations, suggesting substantial individual differences in helper/cytotoxic T-cell composition. The CD4/CD8 ratio varied considerably among patients, with extreme values in several samples. Regulatory T cells (Tregs) were detected at low absolute counts with substantial dispersion, suggesting individual differences in immune regulation. CD19⁺ B-cell and CD3⁻CD16⁺CD56⁺ NK-cell populations showed low and relatively stable values. Overall, these findings indicate an imbalanced immunological profile characterized by variability in helper T-cell and Treg subsets, with B-cell and NK-cell populations remaining at low levels.

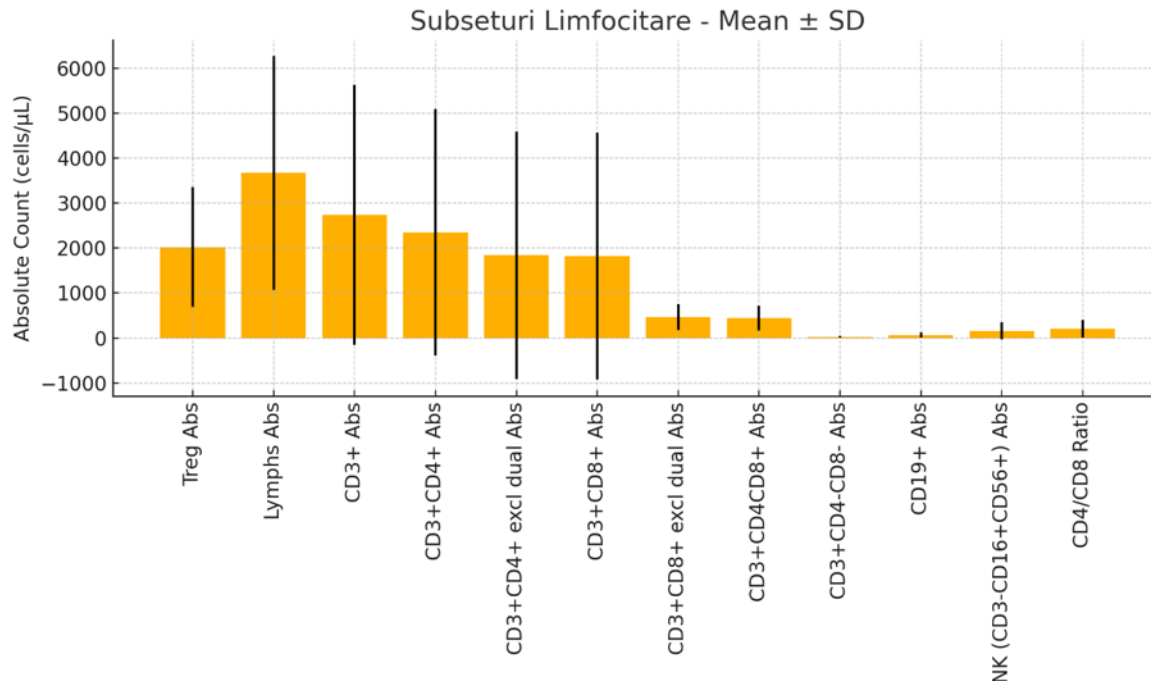


Figure 5.1. Graphical representation of lymphocyte subsets. Columns represent mean values and vertical error bars represent standard deviations. Large standard deviations indicate substantial interpatient variability in the analyzed lymphocyte subsets.

Quantification of Human IL-2, IL-10, and IL-35 by ELISA

Human IL-2, IL-10, and IL-35 levels were measured using commercial ELISA kits according to the manufacturers' instructions. IL-2 and IL-10 kits were obtained from Thermo Fisher Scientific, Waltham, MA, USA (Cat. Nos. BMS221-2 and BMS215-2), and the IL-35 kit was Cat. No. EEL067. Plates were initially washed to remove preservatives; samples and standards were then added to the precoated wells of 96-well plates and incubated with biotin-labeled detection antibodies. Following washing steps, HRP-conjugated streptavidin was applied and colorimetric development was initiated by addition of substrate. The enzymatic reaction was stopped with stop solution, and absorbance was measured at 450 nm using a Sunrise Basic microplate reader (Tecan) and Magellan V6.5 software (Männedorf, Switzerland). Cytokine concentrations were calculated by interpolation from the absorbance standard curve.

Samples from twelve patients were analyzed (twenty-three patients for IL-35), together with samples from two healthy individuals.

Table 5.3. Histological lymphoma subtype among the evaluated patients.

Name	Age	Sex	Lymphoma subtype
P8	40	M	PTCL NOS
P13	64	M	ALCL ALK NEG
P14	57	M	PTCL-TFH
AB06	53	M	MEITL
AB07	69	M	ALCL ALK NEG
P15	49	M	PTCL NOS
AB11	40	F	ATLL, lymphoma type
AB12	56	F	MEITL
AB14	66	M	ALCL ALK NEG
AB15	45	M	ATLL, chronic type
Ab16	45	F	ATLL, unfavorable chronic type
P7	48	F	Nasal T/NK-cell NHL
AB008	60	M	LCT-MF
P10	55	M	AITL
AB013	40	M	ATLL, lymphoma type
P12	78	M	AITL
P2	74	F	PTCL-NOS

Table 5.4. IL-2 ELISA results.

Sample	OD 1	OD 2	Mean OD	Corrected	Calculated	pg/mL
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				OD	value	
P5	0.076	0.037	0.0565	0.0295	-18.4286	-36.8571
P8	0.278	0.087	0.1825	0.1555	41.57143	83.14286
P13	0.059	0.033	0.046	0.019	-23.4286	-46.8571
P14	0.096	0.023	0.0595	0.0325	-17	-34
AB006	0.039	0.025	0.032	0.005	-30.0952	-60.1905
AB007	0.118	0.101	0.1095	0.0825	6.809524	13.61905
P15	0.033	0.021	0.027	0	-32.4762	-64.9524
AB011	0.033	0.03	0.0315	0.0045	-30.3333	-60.6667
AB012	0.028	0.034	0.031	0.004	-30.5714	-61.1429
AB014	0.088	0.114	0.101	0.074	2.761905	5.52381
AB015	0.051	0.046	0.0485	0.0215	-22.2381	-44.4762
AB016	0.034	0.031	0.0325	0.0055	-29.8571	-59.7143
Healthy control 1	0.029	0.026	0.0275	0.0005	-32.2381	-64.4762
Healthy control 1–2	0.069	0.051	0.06	0.033	-16.7619	-33.5238

Table 5.5. IL-10 ELISA results.

Sample	OD 1	OD 2	Mean OD	Corrected OD	Calculated value	pg/mL
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P5	0.065	0.065	0.065	0.022	-0.57391	-1.14783
P8	0.025	0.023	0.024	-0.019	-4.13913	-8.27826
P13	0.03	0.026	0.028	-0.015	-3.7913	-7.58261
P14	0.038	0.034	0.036	-0.007	-3.09565	-6.1913
AB006	0.045	0.041	0.043	0	-2.48696	-4.97391
AB007	0.135	0.118	0.1265	0.0835	4.773913	9.547826
P15	-0.008	0.039	0.0155	-0.0275	-4.87826	-9.75652
AB011	0.06	0.061	0.0605	0.0175	-0.96522	-1.93043
AB012	0.135	0.156	0.1455	0.1025	6.426087	12.85217
AB014	0.213	0.185	0.199	0.156	11.07826	22.15652
AB015	0.051	0.055	0.053	0.01	-1.61739	-3.23478
AB016	0.033	0.037	0.035	-0.008	-3.18261	-6.36522
Healthy control 1	0.12			0.074	3.947826	7.895652
CTR sanatos1 (proba2)	0.073			0.046	1.513043	3.026087

Table 5.6. IL-35 ELISA results.

Sample	OD	pg/mL	pg/mL (corrected ×2)
P5	0.059	16.48387097	32.96774194
P8	0.0575	16	32

P13	0.014	1.967741935	3.935483871
P14	0.024	5.193548387	10.38709677
AB006	0.0185	3.419354839	6.838709677
AB007	0.2465	76.96774194	153.9354839
P15	0.0125	1.483870968	2.967741935
AB011	0.012	1.322580645	2.64516129
AB012	0.049	13.25806452	26.51612903
AB014	0.0695	19.87096774	39.74193548
AB015	0.058	16.16129032	32.32258065
AB016	-0.017	-8.032258065	-16.06451613
P7	0.099	1.161016949	
AB013	0.3845	13.25847458	
P6	0.102	1.288135593	
P21	0.8355	32.36864407	
AB008	0.3705	12.66525424	
P10	0.1335	2.622881356	
P20	0.1515	3.38559322	
AB007	0.195	5.228813559	
P12	3.346	138.7457627	
P2	0.0335	-1.61440678	
P3	0.0335	-1.61440678	
Healthy control 1	-0.014	-7.064516129	--14.12903226
Healthy control 1 (sample 2)	-0.015	-7.387096774	--14.77419355

5.4. Discussion

Analysis of the cohort of patients with mature T-cell malignancies demonstrated substantial variation in lymphocyte subsets and cytokine profiles, suggesting complex immune dysregulation. This interpretation is supported by the marked dispersion of the CD4⁺ helper T-cell subset (high standard deviation), indicating considerable interindividual differences in immune status at diagnosis. The CD4/CD8 ratio also varied substantially among patients, with extreme values in some cases, further supporting the presence of complex immune dysregulation in this population.

Treg populations showed low absolute values but high variability, which may partly reflect the influence of lymphoma subtype, pre-existing immune status, and the tumor microenvironment.

Interleukin analysis revealed relevant differences among patients. IL-10 levels were elevated in a subset of patients, consistent with published data associating this cytokine with an immunosuppressive microenvironment and an unfavorable prognosis, particularly in ATLL [104]. IL-2 levels were variable, with low levels in some patients; such values may suggest functional T-cell exhaustion. IL-35 was detectable in most patients, with increased concentrations in selected cases, supporting the initial hypothesis regarding the potential importance of this cytokine in the complex immunosuppression associated with mature T-cell neoplasms.

Overall, these findings, imbalances between helper and cytotoxic T-cell subsets, alteration of the normal CD4/CD8 ratio, and increased levels of the immunosuppressive cytokines IL-10 and IL-35, support the hypothesis that T-cell neoplasms are characterized by a complex and frequently dysregulated immunological profile oriented toward immunosuppression and immune exhaustion. Correlation of these findings with clinicobiological parameters may refine prognostic assessment and help guide therapeutic stratification.

5.5. Conclusions

Patients with mature T-cell neoplasms exhibit substantial variation in lymphocyte subsets, including marked abnormalities of the CD4/CD8 ratio and low Treg values, indicating profound disruption of the normal immunological profile.

Elevated IL-10 and IL-35 levels in a subset of patients support the involvement of these cytokines in the establishment of an immunosuppressive microenvironment, generally associated with an unfavorable prognosis.

IL-2 showed interindividual variability that may be related to T-cell functional status and the degree of immune activation at diagnosis.

Correlation of immunological data with conventional clinicobiological parameters may provide a useful tool for risk stratification at diagnosis and optimization of therapeutic strategies in patients with mature T-cell lymphomas. These findings support continued applied investigation of the immunological profile to identify biomarkers with potential utility for prognosis and therapeutic monitoring.

STUDY II

Assessment of IL-35, IL-10, and IL-2 in Plasma and Plasma-Derived Exosomal/Extracellular-Vesicle Fractions in Patients With Mature T-Cell Neoplasms

6.1. Introduction

This study aimed to assess IL-2, IL-10, and IL-35 levels in patients with mature T-cell lymphoma, both in plasma and in plasma-derived exosomal eluates, and to compare the plasma concentrations of these cytokines with those measured in lysed exosomal fractions. The working hypothesis was that IL-2, IL-10, and IL-35 concentrations differ between whole plasma and lysed exosomal fractions, suggesting the existence of extracellular-vesicle-mediated secretion and transport mechanisms for these cytokines.

In mature T-cell neoplasms, the tumor microenvironment is characterized by a complex network of cytokine interactions in which IL-2, IL-10, and IL-35 have important roles in modulating the immune response. Evidence from other disease settings indicates that these molecules are not restricted to the freely soluble plasma compartment and may also be transported by extracellular vesicles, particularly exosomes.

By comparatively analyzing plasma and exosomal cytokine concentrations, this study investigated whether exosomes may function not only as passive carriers of interleukins but also as active mediators of intercellular signaling by facilitating cytokine transfer to target cells. Accordingly, cytokine signaling in mature T-cell lymphomas was evaluated as a potentially mixed communication system involving both soluble plasma mediators and extracellular vesicles.

The objectives of this study were:

- To determine plasma IL-2, IL-10, and IL-35 levels in patients with mature T-cell neoplasms using ELISA, thereby establishing reference values for the analyzed patient group.
- To isolate and characterize exosomal fractions from patient plasma by size-exclusion chromatography (SEC) and to assess their integrity and composition using dynamic light scattering (DLS) and transmission electron microscopy (TEM).
- To measure IL-2, IL-10, and IL-35 concentrations in lysed exosomal fractions and compare them with the corresponding plasma cytokine levels.
- To evaluate the exosomal-to-plasma ratio for each cytokine as an indirect indicator of the contribution of extracellular-vesicle-mediated transport to immune signaling in mature T-cell lymphomas.

6.2. Patients and Methods

The study included ten patients diagnosed with mature T-cell neoplasms from whom plasma samples were collected according to standardized procedures. The central methodological objective was to compare IL-2, IL-10, and IL-35 concentrations in whole plasma with those obtained from lysed exosomal eluates.

Extracellular vesicles, referred to as exosomes in the original experimental workflow, were isolated by size-exclusion chromatography using Izon qEV Gen2 35 nm columns. Fractions F2–F4, corresponding to the predefined exosome-enriched elution window, were retained for subsequent analyses.

Size-exclusion chromatography separates particles according to their hydrodynamic size. The column matrix consists of porous particles with controlled pore dimensions. Particles larger than the pore diameter are excluded from the pores and therefore migrate more rapidly and elute first, whereas smaller particles enter the pores, are retained for longer periods, and elute later. SEC is a non-denaturing technique and therefore preserves the structural integrity of biological particles such as extracellular vesicles, large proteins, and viral aggregates.

Izon qEV Gen2 35 nm columns are optimized for the isolation of particle fractions with diameters >35 nm while reducing soluble proteins and complexes below this size range. They allow processing of standard volumes of 0.5–1.0 mL of prefiltered plasma or serum and are compatible with downstream ELISA, Western blotting, DLS, and TEM analyses.

Particle presence and integrity were evaluated by DLS and TEM, which demonstrated particle sizes compatible with exosomal/EV populations and relatively homogeneous size distributions.

Hydrodynamic particle size was assessed by DLS using a Vasco Kin system (Cordouan Technologies SAS) equipped with an in situ analysis module. Measurements were performed using a 638-nm laser at 50% power and a detection angle of 170°. Samples were analyzed in mono-acquisition mode and processed using three complementary algorithms: Cumulants, Padé–Laplace, and SBL (Stretched Exponential-Based Laplace). The Cumulants algorithm provided the overall mean particle size (Z-average) and polydispersity index (PDI). Padé–Laplace, which is more sensitive in heterogeneous systems, enabled identification of multiple particle populations from the autocorrelation function. SBL analysis based on intensity, volume, and number provided additional resolution of dominant size populations and facilitated identification of minority populations or aggregates.

Three independent measurements were performed for each fraction, and results were reported as mean $\pm$ standard deviation.

Relative nanoparticle content in each collected fraction was estimated by comparing light-scattering intensity with that of a latex standard of known diameter. A 105-nm latex particle suspension at 1×10^{-4} mg/mL was used as the reference and generated a mean scattering signal of 537 kcps under identical analytical conditions. Relative particle concentration was estimated from the ratio between the mean scattering intensity of each fraction and that of the standard. This

semiquantitative approach permits relative comparison between fractions; however, because scattering intensity is also influenced by particle volume and refractive index, the estimate is applicable primarily to particles of comparable dimensions analyzed under identical conditions.

A bicinchoninic acid (BCA) assay was performed in parallel for each fraction to evaluate protein content. Absorbance values were used to identify protein-rich regions and fractions with minimal soluble-protein contamination, thereby supporting the identification of higher-purity EV-enriched fractions.

For TEM, 5–10 μ L of the exosome/EV suspension was applied to carbon-coated copper grids and allowed to adsorb for one minute. Excess liquid was removed with filter paper. Subsequently, 5–10 μ L of 3% uranyl acetate solution was applied for 30–60 seconds, after which excess stain was removed by lateral blotting. The stained grid was examined at an accelerating voltage of 80–120 kV.

The exosomal eluate was isolated using IZON qEV Gen2 35 nm columns. Based on DLS analysis following loading of 0.5 mL plasma, fractions F2–F4 were retained, yielding a total eluate volume of 1.2 mL. Selected fractions were concentrated by ultrafiltration using Pierce 10 kDa filters to a final volume of 100–200 μ L. Concentrated samples were stored in Thermo Scientific Low Protein Binding Microcentrifuge Tubes at 4 °C for 12–16 hours. Plasma samples were stored at –80 °C, avoiding repeated freeze–thaw cycles.

The EV/exosomal eluate was incubated with RIPA buffer at a 1:1 ratio for 30 minutes at 4 °C in the presence of a protease-inhibitor cocktail. RIPA detergent buffer was used to lyse samples intended for Western blotting, whereas Triton X-100 (0.1%) was used for samples intended for ELISA.

Exosome/EV preparations that were not analyzed on the day of separation were cryopreserved in PBS supplemented with protease inhibitor and trehalose as cryoprotectant (0.1 M; 15 mg per 400 μ L of exosomal eluate).

IL-2, IL-10, and IL-35 were subsequently measured by ELISA in both plasma and lysed exosomal eluates. Thermo Fisher Scientific kits BMS221-2 and BMS215-2 were used for IL-2 and IL-10, respectively, and kit EEL067 was used for IL-35. Assays were performed according to the manufacturers' instructions. The enzymatic reaction was stopped by addition of stop solution, and absorbance was measured at 450 nm using a Sunrise Basic microplate reader (Tecan) with Magellan v6.5 software (Männedorf, Switzerland). Cytokine concentrations were calculated by interpolation from the corresponding absorbance standard curves.

6.3. Results

Size analysis of SEC-separated fractions demonstrated marked differences in hydrodynamic diameter, population distribution, and scattering-signal intensity. Integration of the DLS and BCA results allowed definition of an optimal EV/exosome-enriched elution window and identification of protein-contaminated or lipoprotein-rich fractions.

Fraction F1 showed a very large hydrodynamic diameter (380 ± 25 nm) and a high PDI (0.39), indicating a heterogeneous population consisting predominantly of aggregates or apoptotic-vesicle-like structures. Padé–Laplace and SBL analyses confirmed highly variable particle populations, including modes at 164 nm and >900 nm, which were not characteristic of exosomes. The relatively low scattering intensity (322 ± 176 kcps) corresponded to a low estimated relative concentration ($0.60 \pm 0.33 \times 10^{-5}$ mg/mL). F1 was therefore excluded from the EV pool.

Fractions F2 and F3 represented the main region of interest for EV/exosome isolation. Z-average values were 179 ± 6 nm for F2 and 137 ± 5 nm for F3, with PDI values below 0.28. Signal intensity reached 1038 ± 234 kcps and 1173 ± 282 kcps, respectively. Padé–Laplace and SBL analyses identified a dominant population at approximately 60–70 nm, compatible with exosome-sized particles, together with a secondary population at approximately 240–260 nm, potentially corresponding to microvesicles. BCA protein content was below the detection limit, supporting the relatively high purity of these fractions. Estimated relative concentrations were 1.93 ± 0.44 and $2.18 \pm 0.52 \times 10^{-5}$ mg/mL for F2 and F3, respectively.

Table 6.1. DLS parameters and estimated relative particle concentration in EV fractions (Patient 9).

Fraction	Z-average (nm)	PDI	Size populations (nm)	Signal (kcps)	Crel (10 ⁻⁵ mg/mL)	Summary
F1	380 ± 25	0.39	164; ~900	322 ± 176	0.60 ± 0.33	Large aggregates; marked heterogeneity
F2	179 ± 6	0.28	≈100; 330–400	1038 ± 234	1.93 ± 0.44	Beginning of EV window; high concentration
F3	137 ± 5	0.27	63–66; 240–260	1173 ± 282	2.18 ± 0.52	EV peak; high purity; exosomes + microvesicles
F4	95 ± 5	0.31	45; 230–280	614 ± 103	1.14 ± 0.19	EV transition toward lipoproteins
F5	50 ± 4	0.22	35–37; ~130	387 ± 57	0.72 ± 0.11	Increasing LDL and protein content
F6	41 ± 1	0.15	29–33; 75–97	374 ± 48	0.70 ± 0.09	HDL-like profile; EVs absent
F7	45 ± 3	0.18	27–31; 90–100	453 ± 76	0.84 ± 0.14	Lipoproteins and plasma proteins
F8	38 ± 3	0.19	24–28; 74–82	462 ± 58	0.86 ± 0.11	Predominantly lipoproteins
F9	42 ± 6	0.22	22–30; 75–120	577 ± 166	1.07 ± 0.31	Protein/lipoprotein tailing
F10	33 ± 1	0.18	20–33	424 ± 50	0.79 ± 0.09	High protein content; EVs absent
F11	35 ± 8	0.22	10–30	533 ± 191	0.99 ± 0.35	Albumin and HDL
F12	28 ± 4	0.28	8–30	416 ± 78	0.77 ± 0.14	Soluble protein; BCA-confirmed

Fraction F4 had a Z-average of 95 ± 5 nm and a higher PDI of 0.31, reflecting increased heterogeneity. Particle populations at 45 nm and 230–280 nm suggested co-elution with low-density lipoprotein particles. Scattering intensity decreased relative to F2–F3 (614 ± 103 kcps), and

the estimated relative concentration was $1.14 \pm 0.19 \times 10^{-5}$ mg/mL. Although F4 still contained particles of interest, its purity was lower.

Fractions F5–F9 were dominated by smaller particles, with Z-average values of 41–50 nm and size modes characteristic of lipoproteins (24–37 nm), indicating transition toward LDL- and HDL-rich fractions. F6 and F7 in particular showed particle sizes around 27–33 nm, consistent with HDL-sized particles. Protein content increased progressively, and these fractions were not considered suitable for exosomal pooling because of biochemical contamination.

Fractions F10–F12 showed Z-average values below 35–40 nm and were dominated by soluble-protein-sized particles. BCA testing demonstrated substantial protein content, particularly in F10, and the EV contribution was considered negligible. Albumin and other plasma proteins were supported by the observed size profiles and positive BCA reaction.

The second patient (Patient 21) showed a broadly reproducible elution profile. The strongest signal was observed in F3 (1260 ± 271 kcps). Fractions F2 and F3 again constituted the principal EV/exosome-enriched region, with Z-average values of 175 ± 7 nm and 133 ± 6 nm, low PDI values, and SBL peaks centered around approximately 65 nm. F4–F5 showed greater heterogeneity and transition toward lipoprotein-rich fractions.

Table 6.2. DLS parameters and estimated relative particle concentration in EV fractions (Patient 21).

Fraction	Z-average	PDI	Size populations	Signal (kcps)	Crel (10^{-5})	Summary
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	(nm)		(nm)		mg/mL)	
F1	365 ± 20	0.37	170; ~800	410 ± 179	0.76 ± 0.33	Large aggregates; heterogeneous particles
F2	175 ± 7	0.27	100; 330–400	1021 ± 222	1.90 ± 0.41	Transition zone toward EVs; strong signal
F3	133 ± 6	0.26	63–68; 240–250	1260 ± 271	2.35 ± 0.50	EV peak; clear distribution and maximum intensity
F4	94 ± 6	0.30	45; 230–280	738 ± 135	1.37 ± 0.25	EVs + lipoproteins; increased heterogeneity
F5	51 ± 3	0.21	35–38; ~130	478 ± 76	0.89 ± 0.14	LDL + small particles; moderate signal
F6	43 ± 1	0.16	29–32; 75–90	453 ± 61	0.84 ± 0.11	HDL profile; uniform sizes
F7	46 ± 2	0.18	27–31; ~100	513 ± 62	0.96 ± 0.12	Lipoproteins + proteins; particles <50 nm
F8	40 ± 2	0.17	24–28; 74–82	488 ± 56	0.91 ± 0.10	Predominantly lipoproteins
F9	44 ± 6	0.21	26–33; 80–130	618 ± 147	1.15 ± 0.27	Protein tailing; mixed sizes
F10	34 ± 2	0.19	20–33	486 ± 55	0.90 ± 0.10	Protein fraction with small particles
F11	38 ± 10	0.24	10–35	581 ± 178	1.08 ± 0.33	Albumin and HDL; heterogeneous
F12	30 ± 5	0.27	8–30	450 ± 78	0.84 ± 0.15	Soluble protein; low signal

DLS Conclusions

Physicochemical characterization of SEC fractions obtained from the plasma of two patients demonstrated a reproducible elution profile with a well-defined region corresponding to extracellular vesicles. Integration of DLS, multiple size-distribution algorithms (Cumulants, Padé–Laplace, and SBL), and BCA testing identified F2–F3 as the principal exosome/EV-enriched window, characterized by:

- hydrodynamic diameters of approximately 130–180 nm by Z-average;
- PDI values <0.28, indicating relatively homogeneous distributions;
- high DLS signal intensities (>1000 kcps);
- unimodal or mildly bimodal distributions with peaks in the 60–100 nm range;
- absence of a detectable BCA protein signal, supporting relatively high purity.

F4 represented a transition toward greater heterogeneity, with bimodal distributions suggesting co-elution of exosomes, microvesicles, and aggregates together with the beginning of lipoprotein contamination. F5–F9 were dominated by particles <50 nm with LDL/HDL-compatible size modes and progressively increasing protein content. F10–F12 consisted predominantly of soluble proteins, particularly albumin, with no convincing evidence of an exosomal contribution. Overall, the combined characterization strategy provided a practical basis for selecting EV-enriched fractions for subsequent functional and molecular analyses.

ELISA Results

Table 6.3. Plasma IL-2 versus exosomal IL-2 (dilution factor 2.4).

Patient	Plasma mean (pg/mL)	Plasma SD (pg/mL)	Exosome mean (pg/mL)	Exosome SD (pg/mL)	Corrected exosome mean (pg/mL)	Corrected Exo/Plasma ratio
ab09	56.56	0	56.5557	2.1947	135.7337	2.3998
ab11	86.8	13.0909	147.1292		353.1102	4.0679
ab12	122.89	37.9522	178.0156		427.2374	3.4764
ab14	116.06	40.2415	205.7791	12.4166	493.8698	4.2552
ab15	NA		NA			
ab16	NA		NA			
p14	41.32	3.5249	58.5669	18.5498	140.5605	3.4014
p15	42.52	39.1477	96.7547	2.9597	232.2114	5.4611
p8	88.97	10.0214	97.456		233.8943	2.6288
p13			34.44	8.58	82.67	

Table 6.4. Plasma IL-35 versus exosomal IL-35 (dilution factor 2.4).

Patient	Plasma mean	Exosome mean	SD	CV%	Corrected exosome mean	Corrected Exo/Plasma
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					(pg/mL)	ratio
ab09	337.96	300.02	21.86	7.29	720.05	2.13
ab11	345.35	302.70	31.89	10.54	726.48	2.10
ab12	556.58	319.53	13.03	4.08	766.87	1.38
ab14	372.78	317.26			761.42	2.04
p14	341.69	303.73	7.64	2.52	728.95	2.13
p15	339.50	293.28	12.33	4.20	703.88	2.07
p8	356.58	299.28	1.35	0.45	718.28	2.01
ab15	NA	314.53			754.88	
ab16	NA	274.40			658.56	
p13	NA	298.33			715.98	

Figure 6.27. Plasma IL-35 level versus exosomal IL-35 level, by patient.

6.4. Discussion

Comparative analysis of plasma and exosomal IL-2, IL-10, and IL-35 in patients with mature T-cell neoplasms supports the hypothesis that a relevant proportion of these cytokines may be associated with the extracellular-vesicle compartment. After correction for the initial sample volume, ELISA measurements showed higher cytokine concentrations in lysed exosomal eluates than in plasma. IL-10 results are not presented because the assay did not generate an acceptable standard curve. Because of technical constraints and limited biological material, the IL-10 ELISA could not be repeated.

By contrast, interpretable data were obtained for IL-2 and IL-35. Exosomal-to-plasma ratios above 1 suggest, for these two cytokines, either selective transfer or preferential incorporation into extracellular vesicles. Vesicular transport may provide protection from enzymatic degradation through the lipid membrane and may facilitate targeted delivery to responsive cells within the tumor microenvironment, mechanisms previously described in the literature [201–203]. For IL-35 in particular, the relatively consistent ratio around 2 suggests a reproducible pattern of vesicular association, with potential implications for maintenance of a localized immunosuppressive microenvironment.

Interpatient differences in these ratios may reflect biological variability related to disease subtype and stage, the degree of regulatory T-cell activation, and systemic inflammatory status. IL-2 ratios occasionally exceeded 4–5. Given the established role of IL-2 in T-cell proliferation and maintenance, its association with extracellular vesicles may represent a mechanism regulating local cytokine availability while limiting rapid consumption from the freely soluble plasma compartment.

These findings are consistent with the broader concept that extracellular vesicles participate in the transport of immunological mediators in T-cell lymphomas and support a complex communication network involving both soluble and vesicle-associated signaling. They further suggest that combined assessment of plasma and EV-associated cytokines may provide information regarding disease biology and potentially the response to targeted or immunological therapies.

6.5. Conclusions

This study demonstrates differences between plasma and exosomal concentrations of IL-2 and IL-35 in patients with mature T-cell neoplasms and supports the involvement of extracellular vesicles as carriers that may transport and protect these cytokines.

IL-35 showed a relatively consistent exosomal-to-plasma ratio, suggesting stable or recurrent incorporation into extracellular vesicles and a potential role in maintaining a localized immunosuppressive microenvironment.

IL-2 showed more variable and frequently higher exosomal-to-plasma ratios, potentially indicating an important mechanism regulating cytokine availability through the extracellular-vesicle compartment.

These findings support the hypothesis that simultaneous assessment of soluble plasma and EV-associated cytokine levels may provide additional information on disease biology, clinical evolution, and potential prognostic stratification.

Evaluation in larger patient cohorts will be required to determine whether plasma/exosomal cytokine ratios can be validated as biomarkers of disease activity and treatment response in mature T-cell lymphomas.

STUDY III

Clinical and Biological Characteristics and Prognosis of Patients With Systemic Mature T-Cell Lymphomas: A Single-Center Retrospective Analysis (2015–2024)

7.1. Introduction

Mature T-cell lymphomas comprise a heterogeneous group of relatively uncommon malignancies characterized by aggressive biological behavior and generally unfavorable outcomes. In Romania, these entities are estimated to account for approximately 8.2% of all non-Hodgkin lymphomas [8]; however, available data remain limited and are derived predominantly from single-center reports.

In the absence of standardized national registries, as is currently the case in Romania, single-center retrospective studies can provide valuable information regarding the epidemiological, clinical, and biological characteristics of these lymphomas.

The primary objective of this retrospective study was to characterize patients diagnosed and treated for mature T-cell lymphomas at Fundeni Clinical Institute between 2015 and 2024. The study aimed to describe the demographic and clinicobiological distribution of the cohort, correlate histological subtypes with clinical characteristics, laboratory parameters, and stage at diagnosis, evaluate overall survival (OS), and identify factors associated with prognosis.

The study was intended to improve understanding of these diseases in Romania and to provide a basis for refining prognostic and therapeutic strategies.

7.2. Patients and Methods

This single-center retrospective, descriptive, and analytical study was conducted between January 2015 and December 2024 at the Center of Hematology and Bone Marrow Transplantation, Fundeni Clinical Institute. A total of 149 consecutive patients with a histopathological and immunohistochemical diagnosis of systemic mature T-cell lymphoma were included. No exclusion criteria based on treatment or disease stage were applied.

The following data were collected:

- demographic parameters (age and sex);
- histological subtype according to the WHO classification;
- disease stage according to Ann Arbor and Lugano classifications;
- laboratory parameters at diagnosis, including complete blood count, LDH, C-reactive protein (CRP), fibrinogen, liver enzymes, and ferritin;
- bone marrow and extranodal involvement;
- administered treatment and response;
- overall survival (OS).

Continuous-variable distributions were assessed using the Shapiro–Wilk test. Normally distributed continuous variables were analyzed using parametric tests (Student's t-test and ANOVA, with Levene's test and post hoc comparisons), whereas non-Gaussian variables were analyzed using Mann–Whitney U and Kruskal–Wallis tests with post hoc analyses. Categorical variables were analyzed using contingency tables and Pearson's chi-square or Fisher's exact test. Survival was estimated using the Kaplan–Meier method and compared with the log-rank (Mantel–Cox) test. Statistical significance was defined as $p < 0.05$. Analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA).

7.3. Results

Baseline demographic and disease characteristics

The mean age at diagnosis was 57.2 ± 13.8 years, with a broad age range. Most patients were in the fifth and sixth decades of life. Male patients predominated, accounting for approximately 59.9% of cases, compared with 40.1% female patients.

Histological distribution was heterogeneous. The most frequent entities were anaplastic large cell lymphoma (ALCL), including both ALK-positive and ALK-negative disease (28%); T-follicular helper-cell lymphoma (TFH lymphoma; 21%); peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS; 30%); and adult T-cell leukemia/lymphoma (ATLL; 20%).

Advanced Ann Arbor stage (III–IV) was present in 78.3% of cases and was significantly more frequent in ATLL, AITL/TFH lymphoma, and PTCL-NOS than in ALCL, in which limited-stage disease was more common ($p < 0.001$). Lugano staging showed the same pattern: advanced disease predominated in ATLL, AITL/TFH lymphoma, and PTCL-NOS, whereas localized disease was more frequent in ALCL ($p < 0.001$).

Bone marrow involvement at diagnosis was significantly more frequent in ATLL (59.3%), TFH lymphoma (44.4%), and PTCL-NOS (39.4%) than in ALCL (8.8%) ($p < 0.001$). Extranodal involvement differed between subtypes and was reported in 56.8% of ALCL, 46.2% of ATLL, 30.8% of PTCL-NOS, and 21.4% of TFH lymphoma cases ($p = 0.017$). Splenomegaly occurred in 51.9% of ATLL, 53.6% of TFH lymphoma, and 43.6% of PTCL-NOS cases, compared with 8.1% of ALCL cases ($p < 0.001$).

Post hoc comparisons further demonstrated that ALCL differed significantly from the other major subtypes. Limited Ann Arbor stage I–II was observed in 45.9% of ALCL versus 6.7% of ATLL ($p = 0.007$), while advanced stage III–IV occurred in 54.1% of ALCL versus 89.3% of AITL/TFH lymphoma ($p = 0.002$). Limited-stage disease was also more frequent in ALCL than PTCL-NOS (45.9% vs 12.5%; $p = 0.001$). Using Lugano staging, localized disease was present in 45.9% of ALCL versus 3.7% of ATLL ($p < 0.001$), 14.3% of AITL/TFH lymphoma ($p = 0.007$), and 12.5% of PTCL-NOS ($p = 0.001$). Bone marrow involvement occurred in 8.8% of ALCL versus 59.3% of ATLL ($p < 0.001$), 44.4% of TFH lymphoma ($p = 0.001$), and 39.4% of PTCL-NOS ($p = 0.003$). Splenomegaly occurred in 8.1% of ALCL versus 51.9% of ATLL, 53.6% of TFH lymphoma, and 43.6% of PTCL-NOS (all $p < 0.001$).

Laboratory parameters

For the analysis of demographic, hematological, and biochemical parameters, the source document reports a cohort of 132 patients. Missing data were limited for most variables; notable missingness was reported for albumin (17.4%), ferritin (40.9%), IgG (75%), and D-dimer (50%). This 132-patient analytical cohort is retained as stated in the source and is not reconciled with the 149 patients reported for the overall retrospective cohort.

Mean age was 57.2 ± 13.8 years (range 28–87 years). Mean hemoglobin was 12.2 ± 2.4 g/dL. Leukocyte, lymphocyte, neutrophil, monocyte, and eosinophil counts showed marked dispersion, with large standard deviations and positive skewness. Mean LDH was approximately 652 U/L, with maximum values >5000 U/L. Mean CRP was 38.6 mg/L (median 13.8 mg/L), and mean ferritin was approximately 954 ng/mL (median 436 ng/mL; maximum 16,500 ng/mL). Liver and renal function variables also showed positively skewed distributions.

Table 7.1. Distribution of hematological and biochemical parameters.

Parameter	Distribution	Central value	Unit
Age	Normal	57.2 ± 13.8	years
Hemoglobin	Normal	12.2 ± 2.4	g/dL
Leukocytes	Non-normal	8365 (6888)	/mm ³
Lymphocytes	Non-normal	1460 (1395)	/mm ³
Neutrophils	Non-normal	5415 (4418)	/mm ³
LDH	Non-normal	373 (419)	U/L
CRP	Non-normal	13.8 (51.2)	mg/L
Ferritin	Non-normal	436.6 (648)	ng/mL
Albumin	Normal	3.8 ± 0.8	g/dL
Platelets	Normal	$253,000 \pm 147,893$	/mm ³
Creatinine	Non-normal	0.93 (0.43)	mg/dL
ALT	Non-normal	27 (28)	U/L
AST	Non-normal	27.5 (32.8)	U/L
Total bilirubin	Non-normal	0.6 (0.4)	mg/dL
Fibrinogen	Non-normal	373.5 (203)	mg/dL
D-dimer	Non-normal	1200 (1531.5)	μg/mL

Kruskal–Wallis testing with Bonferroni-corrected post hoc comparisons was used for non-normally distributed continuous variables. ATLL showed significantly higher median leukocyte counts than ALCL, TFH lymphoma, and PTCL-NOS; higher neutrophil counts than ALCL and PTCL-NOS; higher monocyte counts than all other subtypes; and substantially higher LDH than all

other subtypes. ALCL showed higher fibrinogen than ATLL and TFH lymphoma. AST was higher in ATLL than ALCL, whereas IgG was higher in TFH lymphoma than in ALCL and ATLL.

Table 7.4. Median values of statistically significant variables according to lymphoma subtype.

Parameter	ALCL median [IQR]	ATLL median [IQR]	TFH median [IQR]	PTCL-NOS median [IQR]
Leukocytes (/mm ³)	8770 [6665]	12780 [11460]	8365 [6245]	7350 [4633]
Neutrophils (/mm ³)	6080 [5820]	6530 [7430]	5820 [4273]	4755 [2785]
Monocytes (/mm ³)	640 [505]	1130 [4060]	630 [598]	625 [523]
LDH (U/L)	311 [273]	924 [1230.8]	375 [213.5]	339 [508.8]
Fibrinogen (mg/dL)	493.5 [204]	323 [153.7]	360 [128]	361 [178]
AST (U/L)	20.5 [11.8]	38 [52.8]	25 [23.5]	30.5 [43]
IgG (g/L)	8.7 [3.2]	8.1 [6.8]	14.8 [13.1]	12.4 [11.5]

Table 7.5. Statistically significant differences in continuous variables according to lymphoma subtype.

Parameter	Kruskal–Wallis p	Significant post hoc differences
Leukocytes (/mm ³)	<0.05	ATLL > ALCL, TFH, NOS
Neutrophils (/mm ³)	<0.05	ATLL > ALCL, NOS
Monocytes (/mm ³)	<0.01	ATLL > ALCL, TFH, NOS
LDH (U/L)	<0.001	ATLL > all other subtypes
Fibrinogen (mg/dL)	0.003	ALCL > ATLL, TFH
AST (U/L)	<0.001	ATLL > ALCL
IgG (g/L)	<0.05	TFH > ALCL, ATLL

No significant between-subtype differences were reported for eosinophils, platelets, calcium, albumin, CRP, ferritin, D-dimer, creatinine, total bilirubin, or ALT. Overall, ATLL was distinguished by leukocytosis, monocytosis, increased LDH, and increased liver enzymes; ALCL by higher fibrinogen; and TFH lymphoma by hypergammaglobulinemia. PTCL-NOS showed intermediate values for most parameters.

Overall survival

Overall survival differed significantly by histological subtype ($\chi^2=22.4$; $p<0.001$). ALCL had the most favorable survival, with median OS not reached and mean OS of approximately 72 months. ATLL had the poorest outcome, with median OS of 6.5 months. Median OS was 26.5 months for TFH lymphoma and 16.6 months for PTCL-NOS.

No significant OS difference was observed according to sex ($\chi^2=1.42$; $p=0.233$): median OS was 22.5 months in women and 13.1 months in men. Age <60 versus ≥ 60 years was likewise not statistically significant ($\chi^2=0.92$; $p=0.338$), with median OS of 26.5 and 14.6 months, respectively.

ECOG performance status ≥ 2 was associated with significantly inferior prognosis ($\chi^2=14.47$; $p<0.001$): median OS was 46.4 months for ECOG 0–1 versus 9.0 months for ECOG ≥ 2 . B symptoms were associated with shorter survival (median OS 59.3 months without B symptoms vs 11.5 months with B symptoms; $p=0.006$).

Patients with Ann Arbor stage I–II had median OS not reached (mean OS 76.5 months), compared with median OS of 14.3 months for stage III–IV ($p=0.002$). Localized Lugano-stage disease had median OS not reached (mean OS 77.4 months), whereas advanced disease had median OS of 11.5 months ($p<0.001$).

Patients without bone marrow involvement had median OS not reached (mean OS 65.0 months), compared with 5.9 months in those with marrow involvement ($p<0.001$). Patients without splenomegaly had median OS not reached (mean OS 53.9 months), compared with 9.1 months in those with splenomegaly ($\chi^2=12.1$; $p<0.001$).

Hematological and biochemical prognostic parameters

Thrombocytopenia was associated with median OS of 5.4 months, compared with 41.7 months in patients with normal platelet counts; median OS was not estimable in patients with thrombocytosis ($p<0.001$). Eosinophilia was associated with median OS of 9.4 months versus 22.6 months in its absence ($p=0.021$).

Patients with hypercalcemia had median OS of approximately 1.5 months versus 7.6 months in patients with normal serum calcium. Although the direction of effect suggested an adverse prognosis, the difference did not reach statistical significance ($p=0.206$), most likely because of the small number of cases.

Patients with LDH $<2\times$ the upper limit of normal (ULN) had median OS not estimable (mean OS 62.1 months), whereas those with LDH $\geq 2\times$ ULN had median OS of 6.9 months ($p<0.001$). Hypoalbuminemia was associated with reduced survival ($\chi^2=22.6$; $p<0.001$), with median OS of 6 months versus median OS not reached in patients with normal albumin.

Elevated fibrinogen was associated with favorable OS, whereas low fibrinogen was associated with very poor survival ($\chi^2=46.5$; $p<0.001$). Patients with normal CRP had median OS

not reached (mean OS 69.0 months), compared with 13.1 months in patients with elevated CRP ($p=0.004$).

Elevated creatinine was associated with median OS of 9.8 months versus 28.4 months with normal creatinine ($p=0.004$). Elevated bilirubin was associated with median OS of 4.2 months versus 22.6 months with normal bilirubin ($p=0.036$). D-dimer $<2\times$ ULN was associated with median OS of 41.7 months versus 5.6 months for D-dimer $\geq 2\times$ ULN ($p=0.006$).

Elevated AST was reported to be associated with median OS of 8.3 months versus 31.1 months for normal AST ($p=0.001$). The source text immediately thereafter also states that AST elevations did not show a statistically significant difference in OS. This internal inconsistency has been retained rather than silently corrected.

7.4. Discussion

The retrospective analysis of the 132-patient analytical cohort demonstrated clinically, biologically, and prognostically relevant differences among histological subtypes, with direct implications for therapeutic management and risk stratification.

Consistent with published observations, ALCL was more frequently diagnosed at limited stage and showed less systemic dissemination, including lower rates of bone marrow involvement and splenomegaly. This was reflected in significantly better OS, with median survival not reached during follow-up. In contrast, ATLL and PTCL-NOS were frequently associated with advanced-stage disease, bone marrow involvement, and splenomegaly, corresponding to markedly shorter median OS of 6.5 and 16.6 months, respectively.

TFH lymphomas showed a distinct biological profile characterized by hypergammaglobulinemia and frequent bone marrow involvement, with intermediate median OS of 26.5 months and heterogeneous responses to standard therapy.

Laboratory analysis identified a characteristic ATLL profile consisting of leukocytosis, monocytosis, elevated LDH, and elevated AST, compatible with higher tumor burden and proliferative activity. ALCL was associated with higher fibrinogen, whereas TFH lymphoma was associated with hypergammaglobulinemia. PTCL-NOS generally showed intermediate values, consistent with biological heterogeneity.

Clinicobiological variables significantly associated with adverse prognosis included ECOG ≥ 2 , B symptoms, advanced disease, bone marrow involvement, splenomegaly, LDH $\geq 2\times$ ULN, hypoalbuminemia, low fibrinogen, elevated CRP, elevated creatinine, and elevated bilirubin. These findings support integrated clinicobiological risk assessment and overlap with parameters incorporated into established prognostic systems such as the IPI and PIT.

Elevated fibrinogen was paradoxically associated with favorable prognosis in this cohort, potentially reflecting a reactive inflammatory state rather than tumor aggressiveness. Hypercalcemia was associated with very poor survival numerically but did not reach statistical significance, likely because of the limited number of affected patients.

Overall, the findings emphasize the marked biological and clinical heterogeneity of mature T-cell lymphomas and support the development of prognostic models adapted to individual histological subtypes, potentially incorporating proteomic and molecular-biological variables.

7.5. Conclusions

Histological heterogeneity was associated with major clinical and prognostic differences. ALCL showed a more favorable profile, with more localized disease and significantly better survival, whereas ATLL and PTCL-NOS were associated with advanced disease and markedly reduced OS.

ATLL was characterized by leukocytosis, monocytosis, elevated LDH, and elevated AST; TFH lymphoma by hypergammaglobulinemia; and ALCL by higher fibrinogen levels.

Major adverse prognostic factors included ECOG ≥ 2 , advanced stage, bone marrow involvement, splenomegaly, LDH $\geq 2 \times$ ULN, hypoalbuminemia, and elevated CRP, all of which were associated with significantly reduced OS.

Elevated fibrinogen emerged as a potential favorable prognostic marker and requires validation in prospective studies.

The results support histology-adapted prognostic stratification and the inclusion of relevant biological parameters in therapeutic decision-making algorithms for T-cell lymphomas.

CONCLUSIONS AND ORIGINAL CONTRIBUTIONS

Achievement of the Research Objectives

The proposed research objectives were fully achieved. Evaluation of the immunological profile, including lymphocyte subsets and IL-2, IL-10, and IL-35 levels in plasma and exosomal fractions, demonstrated substantial immune dysregulation. These abnormalities could be correlated with established clinicobiological and prognostic factors in the patients analyzed.

We identified imbalances in CD4/CD8 lymphocyte subsets together with reduced regulatory T-cell (Treg) populations, supporting the presence of systemic immune dysfunction in mature T-cell neoplasms.

We demonstrated increased IL-10 and IL-35 levels in these neoplasms, findings associated with an immunosuppressive tumor microenvironment and an unfavorable prognosis.

Analysis of exosomal-to-plasma cytokine ratios indicated that extracellular vesicles/exosomes may act as important vectors for IL-35 and IL-2 transport, supporting the existence of a complex mechanism of intercellular signaling.

Technical and Scientific Strengths and Limitations

Correlation of immunological data with clinicobiological parameters provides a more refined approach to risk stratification that can be adapted to individual histological subtypes. Beyond histological classification, this approach may contribute to refinement of therapeutic strategies at diagnosis and during treatment, as well as to strategies aimed at reducing infectious or hyperinflammatory morbidity and mortality.

Exosomal analysis enables detection of immunosuppressive mechanisms that may be difficult to identify from plasma measurements alone and therefore provides an additional perspective on the patient's immune status beyond conventional assessment of circulating lymphocyte subsets and plasma interleukins.

Study Limitations

The principal limitations include the incomplete analysis of exosomal IL-10 because of technical problems and the limited amount of available biological material. These findings require subsequent validation. The number of analyzed samples should also be increased, and plasma/exosomal correlations should be evaluated in a larger cohort.

Extracellular-vesicle characterization should be complemented by additional methods, including Western blotting and quantitative particle-analysis techniques such as nanoparticle tracking analysis (NTA).

Unresolved Issues

One of the main unresolved issues is the need to validate the proposed markers—plasma IL-2, IL-10, and IL-35 levels and the exosomal-to-plasma ratios of IL-35, IL-2, and IL-10—in a larger cohort capable of providing adequate statistical power. A larger cohort would also allow more robust analysis of individual lymphoma subtypes. In the present study, the mature T-cell lymphoma group includes multiple entities with different cells of origin, biological aggressiveness, and clinical courses.

At the time of completion of this thesis, no validated immunological prognostic score was available for T-cell lymphomas comparable to the Immunoscore used in solid tumors. The present data may provide a basis for development of such an approach, with the practical advantage that the required analyses can be performed using peripheral blood and plasma. The principal challenge remains the marked immunological and biological heterogeneity of these lymphomas, including differences in cell of origin, aggressiveness, mutational profile, and treatment.

Validation of these findings and of potential prognostic, stratification, and therapeutic approaches will require longitudinal correlation of the immunological profile with treatment response, both during conventional chemotherapy and during non-chemotherapeutic approaches such as immunotherapy and molecularly targeted therapies.

Future Research Directions

Future work will focus on validation of the plasma concentrations of the investigated interleukins, particularly IL-35, and of exosomal-to-plasma cytokine ratios as prognostic and predictive biomarkers. This will require inclusion of a larger patient cohort and longitudinal assessment of these parameters.

Expansion of immunophenotypic analysis to the single-cell level, for example using spectral flow cytometry, may allow more precise identification of lymphocyte subsets and differentiation between reactive immune populations and malignant cells. Integration with transcriptomic and gene-expression data may provide a more comprehensive biological characterization of these populations.

Future studies will also evaluate the impact of therapy on the dynamics of extracellular vesicles and cytokines over time. This approach may facilitate identification of predictive markers of treatment response and biomarkers associated with depth of response.

Original Contributions

Definition of the Immunological Profile in Patients With Mature T-Cell Neoplasms

A comprehensive characterization of lymphocyte subsets, including Treg populations and the CD4/CD8 compartment, was performed and demonstrated significant imbalances with potential

prognostic relevance. Plasma levels of the investigated interleukins were quantified at diagnosis, and IL-35 was shown to be detectable in most patients before treatment compared with samples obtained from healthy individuals (Sections 5.3 and 5.4).

Determination of IL-2, IL-10, and IL-35 Levels in Plasma and Exosomal Fractions

Plasma and exosomal cytokine concentrations were quantified and compared. An exosomal-to-plasma ratio >2 for IL-35 and IL-2 was identified in most evaluable cases (Sections 6.3 and 6.4).

Correlation of Immunological Data With Clinicobiological Parameters

Associations were demonstrated between immunological parameters and established prognostic factors, including ECOG performance status, LDH, CRP, fibrinogen, and disease stage, supporting the potential integration of immunological variables into risk-stratification algorithms (Sections 7.3 and 7.4).

Identification of Extracellular Vesicles/Exosomes as Cytokine Vectors

The study demonstrated the involvement of extracellular vesicles/exosomes in the transport of IL-35 and IL-2 and highlighted their potential contribution to the development of a localized immunosuppressive microenvironment (Sections 6.4 and 6.5).

Proposal of Potential Prognostic Biomarkers

The exosomal-to-plasma IL-35 and IL-2 ratios were proposed as potential prognostic indicators for risk assessment and monitoring of treatment response (Section 7.5).

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