Thesis

Genes Associated with Immune-Cell Function are Deregulated in the Pathogenesis of CLL

submitted by

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Graz, 14.05.2025

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Zusammenfassung in Deutsch

Einleitung: Die chronisch lymphatische Leukämie (CLL) ist ein B-Zell-Non-Hodgkin-Lymphom mit leukämischem Verlauf und ist als häufigste Leukämie in der westlichen Welt bekannt. Jedes Jahr entwickeln 0,5-1 % der CLL-Patient*innen eine Richter-Transformation (RT). Es konnte gezeigt werden, dass eine Dysregulation des Immunsystems einen entscheidenden Einfluss auf die Entstehung der CLL spielt. Da das Wissen darüber jedoch begrenzt ist, haben wir uns zum Ziel gesetzt, die Expression von Genen, die mit der Funktion von Immunzellen in Verbindung stehen, in unserer CLL- und Richter-Kohorte umfassend zu untersuchen.

Material und Methoden: Wir untersuchten die mRNA-Expression von insgesamt 31 Genen (19 Immun-Checkpoint-Komponenten, 9 Immunzellmarker, 3 NR4As) in CLL- (n=53) und RT-Lymphknoten (n=14) mittels RQ-PCR. Nicht-neoplastische Tonsillen (n=3) dienten als nicht-maligne Kontrollen.

Ergebnisse: Beim Vergleich von Tonsillen mit CLL- und RT-Lymphknotenproben beobachteten wir eine geringere *PDL2*-Expression in CLL- (2.3-fach, $p = 0.0362$) und eine geringere *GALECTIN9*-Expression in RT-Lymphknoten (4.2-fach, $p = 0.0134$).
Darüber hinaus wurde eine Dysregulation von sechs der 19 Immun-Checkpoint-Komponenten (*CD86* 3.256-fach ($p = 0.0247$), *HVEM* 220.02-fach ($p = 0.0014$), *PTDSS1* 10.10-fach ($p = 0.0146$), *PTDSS2* 2.67-fach ($p = 0.0397$) waren höher und *PDL2* 2.43-fach ($p = 0.0356$), *CEACAM1* 3.83-fach ($p = 0.0121$) niedriger exprimiert) und drei von neun Immunzellmarkern (*IFNG* 3.79-fach ($p = 0.0854$), *EOMES* 5.52-fach ($p = 0.0247$) und *GZMB* 117.53-fach ($p = 0.0072$) waren höher exprimiert) in CLL-Lymphknoten von behandelten im Vergleich zu unbehandelten Patient*innen festgestellt.
Bemerkenswerterweise waren fünf von 19 Immun-Checkpoint-Komponenten (*CD113* 2.83-fach ($p = 0.0258$), *CD86* 3.62-fach ($p < 0.0001$) und *CEACAM1* 3.67-fach ($p = 0.0007$), *CD160* 1114.79-fach ($p < 0.0001$) und *VISTA* 1.5-fach ($p = 0.0347$) waren niedriger exprimiert) und *LSECTIN*, was als Immunzellmarker 4.41-fach ($p = 0.0229$) niedriger war, in RT- im Vergleich zu CLL-Lymphknoten unterschiedlich exprimiert.
Darüber hinaus setzten wir die Expression der 31 Gene explorativ in Beziehung zum Gesamtüberleben und zur Wahrscheinlichkeit einer Therapie in unserer CLL-Kohorte. Wir fanden einen Zusammenhang mit einem schlechteren Gesamtüberleben bei hoher *PDL1*- ($p = 0.031$), hoher *LSECTIN*- ($p = 0.026$) und hoher *CD8b*-Expression ($p = 0.028$). Die

Wahrscheinlichkeit einer Therapie war höher bei niedriger *CD113*- (p = 0.0026), niedriger *CD86*- (p = 0.044), niedriger *VSIG3*- (p = 0.029) und hoher *GALECTIN9*- (p = 0.031), hoher *PTDSS2*- (p = 0.0057), hoher *GZMA*- (p = 0.0095), hoher *LSECTIN*- (p = 0.0054), hoher *EOMES*- (p = 0.035) und hoher *IFNG*-Expression (p = 0.0013). Bei der Durchführung einer Clusteranalyse entdeckten wir ein inflammatorisches Cluster, welches eine Tendenz zu einer geringeren Gesamtüberlebenswahrscheinlichkeit aufwies (p = 0.07).

Schlussfolgerung: Unsere Daten deuten darauf hin, dass das Immunsystem, insbesondere die Immun-Checkpoint-Komponenten, entscheidend für die Entwicklung und das Fortschreiten der CLL sowie für das Gesamtüberleben und die Therapiewahrscheinlichkeit sein könnten. Darüber hinaus scheint es, dass die derzeitigen therapeutischen Interventionen einen signifikanten Einfluss auf die Komponenten der Immunzellen haben könnten.

Abstract in English

Introduction: Chronic lymphocytic leukemia (CLL) is considered being a B-cell non-Hodgkin's lymphoma with leukemic progress and is known as the most common leukemia in the western world. Each year, 0.5-1 % of the CLL-patients develop Richter transformation (RT). Dysregulation of the immune system has been shown to play a crucial role in the pathogenesis of CLL. However, knowledge about this is limited. Thus, we aimed to comprehensively study the expression of genes associated with immune cell function in our CLL- and Richter-cohort.

Material and Methods: We investigated the mRNA expression of a total of 31 genes (19 immune-checkpoints components, 9 immune-cell markers, 3 NR4As) in CLL- (n=53) and RT-lymph nodes (n=14) by RQ-PCR. Non-neoplastic tonsils (n=3) served as non-malignant controls.

Results: Comparing tonsils with CLL- and RT-lymph node samples, we observed a lower *PDL2*-expression in CLL (2.3-fold, $p = 0.0362$) and a lower *GALECTIN9*-expression in RT (4.2-fold, $p = 0.0134$).

Furthermore, we detected a deregulation of six out of 19 immune-checkpoint components (*CD86* 3.256-fold ($p = 0.0247$), *HVEM* 220.02-fold ($p = 0.0014$), *PTDSS1* 10.10-fold ($p = 0.0146$), *PTDSS2* 2.67-fold ($p = 0.0397$) were higher and *PDL2* 2.43-fold ($p = 0.0356$), *CEACAM1* 3.83-fold ($p = 0.0121$) were lower expressed) and three out of nine immune-cell markers (*IFNG* 3.79-fold ($p = 0.0854$), *EOMES* 5.52-fold ($p = 0.0247$), *GZMB* 117.53-fold ($p = 0.0072$) were higher expressed) in CLL-lymph nodes of treated compared to untreated patients.

Remarkably, five out of 19 immune-checkpoint components (*CD113* 2.83-fold ($p = 0.0258$), *CD86* 3.62-fold ($p < 0.0001$) were higher and *CEACAM1* 3.67-fold ($p = 0.0007$), *CD160* 1114.79-fold ($p < 0.0001$) and *VISTA* 1.5-fold ($p = 0.0347$) were lower expressed) and *LSECTIN* being 4.41-fold lower ($p = 0.0229$) as immune cell marker, were differentially expressed in RT- compared to CLL-lymph nodes.

Furthermore, we exploratively set the expression of the 31 genes in relation to overall survival and probability of therapy of our CLL cohort. We found an association with a worse overall survival in high *PDL1* ($p = 0.031$), high *LSECTIN* ($p = 0.026$) and high *CD8b* ($p = 0.028$) expressions. The probability of therapy was higher in low *CD113* ($p = 0.0026$), low CD86 ($p = 0.044$), low *VSIG3* ($p = 0.029$) and high *GALECTIN9* ($p = 0.031$), high *PTDSS2* ($p = 0.0057$), high *GZMA* ($p = 0.0095$), high *LSECTIN* ($p = 0.0054$), high *EOMES*

(p = 0.035) and high *IFNG* (p = 0.0013). When performing a Cluster analysis, we detected an inflamed cluster showing a tendency of lower overall survival probability (p = 0.07).

Conclusion: Our data indicate that the immune system, especially the immune-checkpoint components, might be crucial for the development and the progression of CLL as well as Overall survival and Probability of therapy. Furthermore, it seems that current therapeutic interventions might significantly impact on immune-cell components.

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List of Abbreviations

CLL	chronic lymphocytic leukemia
SLL	small lymphocytic lymphoma
NHL	Non-Hodgkin lymphoma
CD	cluster of differentiation
RT	Richter transformation
DLBCL	Diffuse large B-cell lymphoma
RT-DLBCL	Diffuse large B-cell lymphoma type Richter transformation
SEER	Surveillance, Epidemiology, and End Results (USA)
BCR	B-cell receptor
BCL2	B-cell leukemia/lymphoma 2
TME	tumor microenvironment
MBL	Monoclonal B cell lymphocytosis
FC	flow cytometry
MHC	major histocompatibility complex
IHC	immunohistochemistry
FISH	fluorescence in situ hybridization
miRNA	micro ribonucleic acid
DNA	deoxy ribonucleic acid
IGHV	immunoglobulin heavy variable
M-CLL	mutated CLL
U-CLL	unmutated CLL
PFS	progression free survival
OS	overall survival
iwCLL	International Workshop on Chronic Lymphocytic Leukemia
CK	complex karyotype
^{18}FDG-PET/CT	^{18}Fluorodeoxyglucose positron emission tomography
SUV	standardized uptake value
B-PLL	B-cell prolymphocytic leukemia
Hb	hemoglobin
PC	platelet count
CLL-IPI	International Prognostic Index for CLL
FCCam	Combination of Alemtuzumab, Fludarabine and Cyclophosphamide

FCR	Combination of Fludarabine, Cyclophosphamide and Rituximab
BTKi	Bruton tyrosine kinase-inhibitors
PI3K	Phosphoinositide 3′-kinase
SCT	Stem cell transplantation
CAR T cell	Chimeric Antigen Receptor T cell
BSC	Best supportive care
ITP	Autoimmune thrombocytopenia
AIHA	Autoimmune hemolytic anemia
R-CHOP	rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone
ORR	overall response rate
CR	complete remission
PR	partial remission
PD	progressive disease
SD	stable disease
MRD	minimal residual disease
PCR	polymerase chain reaction
TCR	T cell receptor
APC	Antigen presenting cell
TNF	Tumor necrosis factor
TIM	T cell/transmembrane, immunoglobulin domain, and mucin domain
TH1	T helper 1 cell
COX	cyclooxygenase
IGs	Immunoglobulins
FFPE	Formalin-fixes, paraffin-embedded
RNA	ribonucleic acid
cDNA	complementary deoxyribonucleic acid
RQ-PCR	Semi-quantitative real-time polymerase chaine reaction
PBMC	peripheral blood mononuclear cell
AML	acute myeloid leukemia

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1. Introduction

1.1 Definition of Chronic Lymphocytic Leukemia and Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia (CLL) is a lymphocytic B-Non-Hodgkin-Lymphoma (B-NHL) with leukemic progression. It is defined as a monoclonal, lymphoproliferative disorder with an expansion of mature cluster of differentiation (CD) 5+CD23+ B cells in the peripheral blood, secondary lymphoid tissues and bone marrow (1–3).
The only distinction between CLL and Small Lymphocytic Lymphoma (SLL), is the appearance of a leukemic manifestation. This is only seen in CLL (1,2).

1.2 Definition of Richter Transformation

Richter Transformation (RT) is known as transformation of the existing CLL/SLL mostly into a diffuse large B-cell lymphoma (DLBCL), which is clinically characterized by rapid and aggressive progression (4,5). There are two types of RT. While the majority of transformations lead to a DLBCL, some patients may also develop a classical Hodgkin lymphoma (6).

1.3 Epidemiology

CLL accounts for 25-35 % of all leukemias in the United States of America and is one of the most common types of leukemia in western countries (7). Also, the age-adjusted incidence is 4.6 per 100.000 population (both men and women) per year (7). It is known that CLL occurs 1.9 times more often in men than in women and also more often in elder people, with the median age at diagnosis being 70 years (1). The lifetime risk of developing CLL (at any time during a person's lifetime) is approximately 0.6 % for men and women, based on the Surveillance, Epidemiology, and End Results (SEER) database of the National Cancer Institute of the USA. While 88.5 % survive 5 years after diagnosis, 1.1 per 100.000 men and women die each year. The median age at death is 82 years (7).
According to the National Cancer Institute's SEER database, it is estimated that 1.0 % of all new cancer cases in the United States of America in 2024 will be CLL (7).
There are differences in the incidence of CLL between different ethnic groups. While the above data relates to western countries such as Europe and the United States of America, the

incidence of CLL is much lower in Asian, African and native Latin American ethnicities (7–10). Yang et al. (8) presented data that suggest this difference rather occurs because of genetic than environmental basis. But going into this more detailed exceeds this thesis.
It also has been proven that there is a certain genetic influence on the occurrence of CLL. First-degree relatives of patients with hematological malignancies have a 6-9-fold higher risk than the general population on being contracted with CLL/SLL and also other indolent forms of Non-Hodgkin-Lymphoma (NHL). However, the risk of developing one of these diseases is quite low (11,12).
As a rare, but nonetheless severe complication of CLL/SLL, RT happens in 2-5 % of all patients with CLL/SLL, while the risk of developing RT in CLL/SLL is 0.5-1 % per year (5,13). Despite its rarity, RT is associated with a poor overall survival of 9-12 months from initial diagnosis (6,13).

1.4 Etiology, Pathophysiology and Risk Factors

Yet, the exact etiology and pathophysiology of CLL isn't fully understood. While hereditary genetic factors play a minor role, the dysregulation of B-cell proliferation as well as the inhibition of apoptosis is crucial for its development. An important role plays the B-cell receptor (BCR) as well as the B-Cell leukemia/lymphoma 2 (BCL2) signaling pathway. Both present possible targets for immunotherapy (14).
Furthermore an important role is attributed to the tumor microenvironment (TME) in regard of development and progression of the disease (15).

1.4.1 Monoclonal B Cell Lymphocytosis

The CLL-like Monoclonal B cell lymphocytosis (MBL) is known as a precursor of CLL. It is an asymptomatic augmentation of clonal B cells with a phenotype typical for CLL (16–18). Besides CLL-like MBL being the most common form that occurs in about 75 % of MBL cases, atypical-CLL MBL and non-CLL-phenotype MBL also appear in some individuals (17,18). Depending on the technique of flow cytometry (FC) used in studies to evaluate the spread of MBL, between 3.5-12 % of healthy persons older than 40 years appear to fulfil the diagnostic criteria for MBL (19).
There exist two types of CLL-like MBL: a low-count and a high-count MBL which can be distinguished according to their number of lymphocytes. Though studies had shown that low-count MBL is not associated with a shorter life expectancy than in the general

population as well as being on low risk of progression without needing any follow-up, patients with high-count MBL are at a significantly higher risk of being hospitalized because of serious infections, being at higher risk of hematologic as well as non-hematologic cancers. According to CLL, 1-2 % of subjects with high-count MBL are progressing to a therapy requiring CLL per year (12,18–20).

1.4.2 Richter Transformation

The only clinical factors in association with an elevated risk of transforming to RT are Rai stage III or IV and lymph nodes >3 cm. Of course, there are genetic and biological factors as well as laboratory and clinical parameters being risk factors of transforming to RT. They include an unmutated immunoglobulin heavy chain variable region, a stereotyped BCR subset 8 (prevalently in association with mutations of *NOTCH1*) and a shorter telomere length (21,22). To differentiate between RT and a secondary malignancy de novo DLBCL, a detection of clonal relationship is mandatory (23).

1.5 Symptoms and Diagnostics/Diagnostic Criteria

CLL can present with different symptoms and even without. A great number of diagnoses are incidental findings. Clinical assessment and staging are very important in regard of diagnosis and therapeutic indication.

1.5.1 Symptoms of Chronic Lymphocytic Leukemia and Richter Transformation

Patients with CLL can present with persisting lymphocytosis, lymphadenopathy, splenomegaly, B symptoms including night sweat, weight loss and fever as well as tendency to infection. Especially in early stages, the diagnosis is often an incidental finding (5,24).
Unfortunately, there are no pathognomonic signs nor symptoms to diagnose RT. As it is commonly known that RT occurs with rapid and aggressive progression, symptoms can be a significantly reduced general condition, rapid enlargement of lymph nodes (≥5 cm), high fever, elevated serum lactate dehydrogenase and hypercalcemia. Similar symptoms can appear also with a progression of CLL which is why the diagnosis of RT needs to be confirmed via lymph node biopsy (4,21,25).

1.5.2 Blood Parameter

Mandatory for the diagnosis of CLL is a lymphocytosis of ≥5 G/l for more than three months, also the clonality of these B lymphocytes needs to be confirmed through FC by demonstrating immunoglobulin light chain restriction (2).

The difference between the diagnostic criteria for MBL, SLL as well as CLL are that in MBL there only is clonal B-cell count <5 G/l without any signs for lymphadenopathy/organomegaly and cytopenia while in SLL there is clonal B-cell count <5 G/l combined with lymphadenopathy/organomegaly but without cytopenia. For CLL it exits the condition that clonal B cell count needs to be ≥5 G/l either with lymphadenopathy/organomegaly, cytopenia or without (12).

More precisely, the diagnosis of CLL-like MBL requires a cell count <5 G/l without lymphadenopathy, organomegaly and cytopenia. While there exist two types of MBL, the low-count MBL is characterized by a clonal B-cell count of <0.5 G/l whereas the high-count MBL shows a clonal B cell count of ≥0.5 G/l (12,19). Table 1 shows all requirements mentioned above.

Table 1

Differentiation in diagnostics of CLL-like MBL, SLL and CLL.

	CLL-like MBL		SLL	CLL
B-cell count	<5 G/l		<5 G/l	≥5 G/l
	Low-count MBL	High-count MBL		
	<0.5 G/l	>0.5 G/l		
Lymphadenopathy/organomegaly	-	-	+/-	+/-
Cytopenia	-	-	+/-	+/-

Note. CLL-like MBL (chronic lymphocytic leukemia-like monoclonal B-cell lymphocytosis), SLL (small lymphocytic lymphoma) and CLL (chronic lymphocytic leukemia) can be differentiated in regard of their B-cell count, the appearance of lymphadenopathy/organomegaly and cytopenia. Limit values of the B-cell count are mentioned in G/l (Giga per liter).

Beta2-microblobulin as a serum marker, a component of the major histocompatibility complex (MHC) class I, has been identified as independent prognostic parameter. It is associated with an adverse outcome when being measured >3.5 mg/l (2,26,27)

1.5.3 Pathological Characteristics of Chronic Lymphocytic Leukemia and Small Lymphocytic Lymphoma

The blood shows a medium- to high-graded lymphocytosis with characteristic Gumprecht nuclear shadows. This means darkly stained nucleus with condensed chromatin and indistinct nucleoli showing a narrow rim of basophilic cytoplasm within small, mature lymphocytes. These cells appear to be more fragile than normal lymphocytes (28).

Pathologically CLL and SLL are identical and can only be differentiated by the appearance of a leukemic manifestation (1,2).

CD5+, CD19+, CD20+, CD23+ and surface or cytoplasmatic kappa and lambda light chains are essential markers for the diagnosis of CLL. CD10-, CD43+, CD97b+, CD81, CD200 and ROR1, FMC7, sIgM are additional markers used to differentiate other small B-cell lymphomas/leukemias. These markers can be detected by either FC or immunohistochemistry (IHC) (5,29,30).

A typical expression pattern for CLL is CD5+, CD19+, CD20-/dim, CD23+, CD79b-/dim, FMC7-/dim and sIgM-/dim where -/dim indicates no/weak expression.

Peripheral blood should be used as the sample. Only if the diagnosis cannot be made and the suspicion remains, bone marrow, an enlarged lymph node, and an extra nodal lesion examined by cytological and/or histological methods may also be used (5,28).

1.5.4 Cytogenetics

Unfortunately there is no pathognomonic cytogenetic abnormality for CLL, which means that the genomic setting is heterogenous (31). Peripheral blood can be subjected to interphase fluorescence in situ hybridization (FISH), which detects genetic abnormalities in >80 % of all CLL cases (2,32).

The most important and most common abnormalities are listed below.

Mutations appearing in CLL that are being associated with prognostic value and play a role in both pathogenesis and evolution are deletions of 11q, 13q, 17p and trisomy 12 (33). The most frequent mutation in CLL is 13q14, which occurs in about 50-60 % of all individuals. The 11q22-23 deletion appears to be the second most common mutation with a frequency of 5-20 %, while trisomy 12 is found in 10-20 % and 17p13 deletion in only 3-8 % of CLL patients. All mutations can be acquired during disease progression which is why the appearance is higher at the time of first treatment or relapse or even RT (33–35).

Döhner et al. (32) were able to show that the worst prognosis is associated with 17p deletions, followed by 11q deletions and trisomy 12 while patients with 13q deletions even had a longer survival than those with normal karyotypes. The impact of 13q deletions on the prognosis depends on the size and location of the deletion, as well as on whether the deletion is mono- or biallelic (33,34,36).

As the 17p deletion is associated with a *TP53* mutation (in the remaining *TP53* allele there is often a *TP53* mutation in the 17p13 band), *TP53* encodes for a protein that is very important in the regulation or progression of the cell cycle, apoptosis, and genomic stability. Zenz et al. (37) showed that in patients with a 17p deletion or *TP53* mutation, the response to chemotherapy was significantly lower than in patients without these alterations (36). Progression-free survival and the overall survival were also reported to be shorter. Although this data is quite old, it is still current (37).

While the region of the 13q14 deletion encodes the micro ribonucleic acid (miRNA) miR15A and miRNA16A, which are required for an inhibitory effect on regulators of apoptosis, the loss of these regions leads to a prolonged lifespan of CLL cells. Thus, the aforementioned BCL2,en which is known to code an anti-apoptotic protein, is upregulated in CLL due to the deletion of miR15A/miR16A (33–35).

Loss of *ATM*, with its main function of DNA double-strand break repair, is always associated with deletion of 11q22-23. ATM also induces cell cycle arrest or even apoptosis if the double-strand break cannot be repaired, and this mechanism is impaired in CLL with 11q22—23 deletion (35).

Trisomy 12 is taken into account in being a clonal driver mutation in secondary mutations or chromosomal aberrations like *NOTCH1*, *TP53* and more. But its impact on the patients being categorized as intermediate-/low-risk or high-risk is controversial (33,38–40).

Other mutations in genes such as *SF3B1* and *NOTCH1* are also found in CLL patients, but less frequently. They are also the subject of ongoing research and need to be validated for clinical use beyond clinical trials (35,36).

1.5.5 Immunoglobulin Heavy Variable Gene Mutation Status

According to the immunoglobulin heavy variable (IGHV) mutation status, CLL can be divided into two subgroups called mutated CLL (M-CLL) and unmutated CLL (U-CLL). A cut-off value of 98 % identity with the most proximal germline IGHV genes has been established. While IGHV mutations appear in about half of the CLL cases, M-CLL is

associated with non-progressive disease and longer progression-free survival (PFS), time to first treatment and overall survival (OS) compared to patients with U-CLL (35,36,41–43).
As many prognostic biomarkers have emerged in recent years, it has become very difficult for clinicians to select and interpret the most important factors. A systematic review and meta-analysis conducted by Parikh et al. (43) indicated that in patients with newly diagnosed or previously untreated CLL, IGHV mutation status and FISH analysis for 11q22-23 and 17p13 deletions have a high prognostic value. Therefore, it is recommended that FISH and IGHV status be considered as complementary information are seen as standard clinical tests for all newly diagnosed CLL patients. This has not yet been achieved, according to the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) guidelines published in 2018 (2,5,35,43).
There is much more to be said about prognostic value, the cellular origins of CLL, e.g., and even more to be discovered about IGHV mutation status in CLL. But to explore this vast field would be beyond the scope of this thesis (42,44).

1.5.6 Complex Karyotype

Chromosome banding analysis can be used to detect complex karyotype (CK). If ≥2 metaphases (10-15 need to be examined to distinguish between normal/abnormal) show ≥3 numerical or structural abnormalities in the same clone, this is defined as a CK. CK is reported to occur in approximately 10-20 % of untreated CLL patients and 8 % of patients with MBL. It is even more common in previously treated patients or patients with RT (45,46). According to a multicenter retrospective study of more than 5000 patients with CLL and high-count MBL with available chromosome banding analysis data, Baliakas et al. (31) were able to divide CK into three subgroups based on the number of abnormalities. The group with 3 abnormalities is considered low CK, 4 abnormalities as intermediate CK and ≥5 abnormalities as high CK. While the risk stratification of low and intermediate CK depends on additional factors, i.e. *TP53* mutations, high CK is associated with a poor clinical outcome regardless of clinical stage, *TP53* mutations or the presence of M-/U-CLL, respectively. This results in a shorter overall survival of patients with high CK compared to those with low/intermediate CK (31). Furthermore, Chatzikonstantinou et al. (45) observed that CK status has an impact on the response rate, duration of response, PFS and/or OS of different therapies such as Ibrutinib, Idelalisib, Venetoclax etc. monotherapy or

in combination. Therefore, it may be crucial for prognostic as well as therapeutic value, new prognostic models are about to come, to know the CK status of patients with CLL (31,45,46).

1.5.7 Diagnosis of Richter Transformation

As mentioned above, there are no pathognomonic signs nor symptoms to diagnose RT which is why the diagnosis needs to be confirmed via lymph node biopsy. As the optimal site of biopsy must be found, a ^{18}fluorodeoxyglucose positron emission tomography (^{18}FDG-PET/CT) may be of use (5,21). Bruzzi et al. (25) were able to show a high sensitivity (91 %) with a high negative predictive value (97 %) by using as a cut-off a standardized uptake value (SUV) threshold in detecting RT-DLBCL. As the positive predictive value of this shows only 53%, the biopsy still is mandatory in the diagnosis of RT.

1.5.8 Differential Diagnosis of Chronic Lymphocytic Leukemia and Small Lymphocytic Lymphoma

The most common differential diagnoses of CLL are MBL, reactive lymphocytosis due to viral infection, different lymphomas like follicular lymphoma, lymphoplasmacytic lymphoma, Mantle cell as well as Marginal zone B-cell lymphoma (16,18). Another important disease to be considered is B-cell prolymphocytic leukemia (B-PLL) which initially can present similar to CLL. B-PLL can be differentiated by the appearance of a great many prolymphocytes in the blood (47). They are only to be mentioned here.

1.6 Clinical Staging

It exists two widespread and accepted clinical staging systems being used in patient care as well as clinical trials. They both refer only to a physical examination and standard laboratory tests, furthermore they do not need imaging studies. Therefore they are a simple and inexpensive, worldwide used method to categorize CLL patients with explicit clinical outcomes (2).

1.6.1 Binet Staging System

For this staging system the amount of affected lymphoid areas with enlarged lymph nodes ≥1 cm diameter/organomegaly and the presence of anemia/thrombocytopenia need to be

considered. According to this data patients get divided in three different groups with different prognosis and clinical outcome (2,5,48).

Binet A comprises <3 involved lymphoid areas, a hemoglobin (Hb) level of ≥10 g/dl as well as a platelet count (PC) of ≥100.0 G/l while Binet B represents an amount of ≥3 involved lymphoid areas, with Hb and PC being the same as Binet A. Binet C on the contrary stands for any rate of involved lymphoid areas whereas Hb level is meant to be lower than 10 g/dl and the PC also needs to be below 100.0 G/l (2,5,48).

There are 5 considered lymphoid areas which must be taken into account for the Binet staging system. These include cervical lymph nodes (also those of the head and Waldeyer ring), axillar nodes, inguinal nodes (including superficial femorals) as well as palpable liver and spleen (2,5).

As the median survival depends on the stage, it is considered decreasing from Binet A to Binet C. While being >10 years for Binet A, 5-7 years for Binet B, for Binet C it is only 2.5—3 years. But it needs to be mentioned that treatment and also supportive therapy have beneficial effects on the median survival (5).

1.6.2 Rai Staging System

The Rai staging system is based upon the presence of lymphadenopathy, organomegaly of liver/spleen as well as anemia/thrombocytopenia. The in 1975 developed system first consisted of five groups, then was revised so now it involves only three groups.

There is defined a low-risk disease (formerly known as Rai 0) where subjects do not have any lymphocytosis, organomegaly and also the Hb level is ≥10 g/dl as well as the PC ≥100.0 G/l. Only lymphocytosis with CLL clonal B cells in the blood and/or bone marrow is indicating on the CLL.

The intermediate-risk disease (formerly known as Rai I and II) comes along with lymphocytosis in peripheral blood, enlarged lymph node areas plus splenomegaly and/or hepatomegaly.

If patients show anemia with Hb level <10 g/dl and/or thrombocytopenia with PC <100.0 G/l they belong to the group of high-risk disease (formerly known as Rai III and IV).

The median survival in the new three Rai stages corresponds to the three Binet stages (2,5,49).

Table 2

Clinical staging of CLL according to Binet and Rai.

	Binet			Rai		
	Binet A	Binet B	Binet C	Low-risk disease (Rai 0)	Intermediate-risk disease (Rai 1-2)	High-risk disease (Rai 3-4)
Comprised lymphoid areas	<3	≥3	Any			
Lymphocytosis/ organo-megaly/ enlarged lymph nodes				-	+	+
Hemoglobin	≥10 g/dl	≥10 g/dl	<10 g/dl	≥10 g/dl	≥10 g/dl	<10 g/dl
Platelet count	≥100.0 G/l	≥100.0 G/l	<100.0 G/l	≥100.0 G/l	≥100.0 G/l	<100.0 G/l

Note. The clinical staging according to Binet includes the comprised lymphoid areas, hemoglobin levels and platelet count. Staging according to Rai includes lymphocytosis/organomegaly/enlarged lymph nodes, hemoglobin levels and platelet count. Hemoglobin is measured in g/dl (gram per deciliter) and platelet count in G/l (Giga per liter).

1.6.3 International Prognostic Index for Chronic Lymphocytic Leukemia

The international working group for the International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI) created an index integrating major prognostic parameters of CLL referring on data from individual patients of eight phase 3 trials of CLL, taking place in different countries, published between 1st January 1950 and 31st December 2010. The aim of this score is a better prediction of the individual development of the disease (5,27).

In their training dataset they identified five different and independent prognostic factors to which the *TP53* status (no abnormalities vs del[17p] or *TP53* mutation or both), the IGHV mutational status (mutated vs unmutated), the serum beta2-microglobulin concentration (≤3.5 mg/l vs >3.5 mg/l), the clinical stage (Binet A or Rai 0 vs Binet B/C or Rai I-IV) as well as the age (≤65 years vs >65 years) count (5,27).

Furthermore, after a weighted grading of these independent factors, a prognostic index was achieved identifying four risk groups (low, intermediate, high and very high risk) presenting an overall survival of five years being significantly different within the used training dataset. Thus the index has been validated and confirmed while using an internal as well as an external-validation dataset (27). Currently the CLL-IPI is known as the most relevant prognostic score whereat it is suggested to use the score only in clinically justified cases or in case of therapeutic indication due to the often lacking clinical consequence of the result (1,5).

1.7 Treatment of Chronic Lymphocytic Leukemia

1.7.1 Management of Newly Diagnosed Patients with Chronic Lymphocytic Leukemia not Requiring Treatment

Most of the patients with newly diagnosed CLL are not in need of a treatment at the time of first diagnosis. It is recommended that these patients obtain a frequent follow-up of 3- 6 months in the first year and 3-12 months afterwards depending on the CLL-IPI risk score. While patients with high or very high risk of progression are being examined more frequently, those with low and intermediate risk get a lower frequency (2,5,12). The regular examinations comprise anamnesis with creating a case history, physical examination with status of peripheral lymph nodes, spleen and liver, blood test (at least Hb, leukocytes, platelets) as well as differential blood count and also clinical staging (5,12).
As different studies have been using different treatments in early-stage and asymptomatic individuals, in newly diagnosed CLL patients has been identified the fact, that an early treatment does not seem to be beneficial to overall survival (2,50,51).

1.7.2 Indications for Treatment

According to the iwCLL guideline, Hallek et al. (2) created a list of criteria for initiation of therapy which has been modified by Kay et al. (12). He named six cases in which therapy is indicated.
The first case is the evidence of progressive marrow failure with general cutoff levels of Hb <11 g/dl or PC <100 G/l. Secondly a massive (≥6 cm below the left costal margin) or progressive or symptomatic splenomegaly and thirdly massive (≥10 cm in longest diameter) nodes or progressive as well as symptomatic lymphadenopathy are an indication for the start

of treatment. The fourth point mentioned is autoimmune complications like anemia or thrombocytopenia that do not sufficiently respond to corticosteroids. The case of symptomatic or functional extra nodal involvement, like skin, kidney etc. is known as his fifth point. Sixth, the occurrence of any disease-related symptoms like weight loss ≥10 % in 6 months, a significant fatigue and fevers ≥38.0 °C for ≥2 weeks without evidence of infection are known to indicate the need of treatment (12).

1.7.3 Different Therapies

While chemotherapy has been the best-known method of treatment for many years, there has been an evolution over the time. Different studies were able to show that the combination of chemo- and immunotherapy outplays the mono and even combined chemotherapy (5,52,53). Until now there have been discovered even more novel agents, some also with the ability of oral application, that have revolutionized the treatment of CLL (12,54).
Due to the vast and always renewing field of treatment options, I will focus on giving just an overview.

1.7.3.1 Chemotherapy.

Over 40 years chemotherapy with alkylating drugs, especially Chlorambucil, were the leading medicament in CLL patients (52). Different combinations of chemotherapeutics have been tried, but until purine analogues came up in the 1990es, none improved the outcome. Then the combination of the alkylating drug Cyclophosphamide and the purine analogue Fludarabine has mostly been used and led to an improvement of response, remission and progression-free survival but none combination led to an improvement in overall survival (52,55–58).
Also to mention is Bendamustine, an in Eastern Europe invented and broadly used double alkylator and antimetabolite (59). Due to better tolerance of Bendamustine in combination with Rituximab, it can still be used in the treatment of older patients or those with comorbidities while it has been shown that this combination still is inferior to Fludarabine, Cyclophosphamide and Rituximab (5,60,61).

1.7.3.2 Immuno- and Chemotherapy.

When Rituximab, a CD20-antibody, came up, Hallek et al. (52) showed in a multinational, multicentric, prospective, randomized, open-labeled, phase 3 study with treatment naïve

CLL patients of Binet stages A-C that the addition of Rituximab to Fludarabine and Cyclophosphamide leads to an improved progression-free survival as well as the overall survival for physically fit patients. Due to cytogenetic analysis of the included patients, they detected that for patients with 17p deletions this combination might not be substantially beneficial. Therefore it is suggested that a molecularly guided treatment approach could be advantageous (52).

Also Fischer et al. (53) were able to detect a better rate of long-term remission as well as a highly improved overall survival for fit patients with specific genetic characteristics, especially for those with mutated IGHV status and all cytogenetic subgroups except for 17p deletions.

While Rituximab is not the only CD20-antibody, over the last years newer, more efficient substances like Ofatumumab, Veltuzumab, Ocrelizumab and Obinutuzumab came up and reached different positions in the treatment of CLL (62,63).

Alemtuzumab, a CD52-antibody, first seemed to be a good therapeutic option for patients with high-risk genetic markers (62,64). Later in a multicenter phase 3 trial Lepretre et al. (65) combined it with Fludarabine and Cyclophosphamide (FCCam) and compared this to the combination of Fludarabine, Cyclophosphamide and Rituximab (FCR). While they discovered the FCCam treatment not being more effective than the FCR regimen, FCCam was associated with an adverse safety profile. As a result, Alemtuzumab now is no longer licensed (5,62,65).

1.7.3.3 Bruton Tyrosine Kinase-Inhibitors/Agents Targeting BCR Signaling.

Ibrutinib is a Bruton tyrosine kinase inhibitor (BTKi) needed to be taken orally once a day. The RESONATE-2 study, a phase 3, open label, multicenter, international, randomized study has investigated ibrutinib vs. chlorambucil in first-line CLL/SLL in patients ≥65 years without 17p deletion (66,67). They have come to the conclusion that ibrutinib is superior to chlorambucil according to a prolonged progression-free-survival and also a good long-term efficacy (67,68). Next-generation BTKi that are newer and also more potent than Ibrutinib are Acalabrutinib and Zanabrutinib, but due to their novelty their range of application is subject of current research (69).

As Idelalisib is a phosphoinositide 3′-kinase (PI3K) delta isoform-selective inhibitor that has many functions all leading to apoptosis, lower survival signals and slower growth in CLL

cells (62). In combination with Rituximab it is registered for treatment of relapsed and refractory CLL patients (69,70).

1.7.3.4 BCL2 Inhibitors.

Venetoclax is a BCL2 inhibitor which, in CLL cells where BCL2 is overexpressed, leads to an activation of proapoptotic proteins so the apoptotic process gets restored (61,69). In combination with other drugs e.g., Obinutuzumab, Ibrutinib or Rituximab, or as monotherapy, different studies were able to show an improved progression-free survival as well as overall survival compared to other therapeutic regimens, some in patients with, others in patients without 17p deletions/*TP53* mutations with newly diagnosed or relapsed and refractory disease (54,69,71–74).
This is just a small overview of different drugs that are used in the treatment of CLL, but there are many more, especially if those tested in studies are also counted.

1.7.3.5 Stem Cell Transplantation.

Stem cell transplantation (SCT) can be divided into an autologous (patient donates his/her own cells) and allogeneic (cells donated by somebody else) form. SCT is a therapy with many risks and therefore it is considered only with high-risk CLL (after failure of several previous therapies) or RT in a certain pre-transplant disease status (5,22). Generally allogeneic SCT is known to be the only potentially curative therapy for CLL (75).

1.7.3.6 Chimeric antigen receptor T cell therapy.

Chimeric antigen receptor T (CAR T) cell therapy is seen as a new therapy with high potential not only in the treatment of hematological cancers. For this treatment cells are drawn from the patient, genetically modified ex vivo (with viral vectors), amplificated and reinfused. The modified, now called CAR T cell, contains genetic material to produce an antigen receptor on its surface with which specific (cancer) cells can be detected and through binding sent to apoptosis. Concerning hematological diseases like CLL, the results in studies with CAR T cells against CD19, CD20, CD30 are quite good so this therapy can be seen as a last attempt when nothing else would work. But still, the CAR T cell therapy is a therapy still developing (76–78).

1.7.4 First-line Therapy

Because CLL is such a heterogeneous disease and there are many ways to best treat it, there is no single first-line therapy that fits all patients. This is illustrated in Figure 1, that can be found below.

If patients need to be treated, they get divided into groups depending on whether they are in fit or unfit condition. Furthermore, it is important to know whether they have a 17p13 deletion, *TP53* mutation, CK or IGHV mutation or they do not. All these factors must be taken into account and depending on those different treatment opportunities and therapeutical groups are recommended. The final decision always needs to be taken individually in a synopsis of all clinical, diagnostical and biopsychosocial characteristics (2,5,12,61).

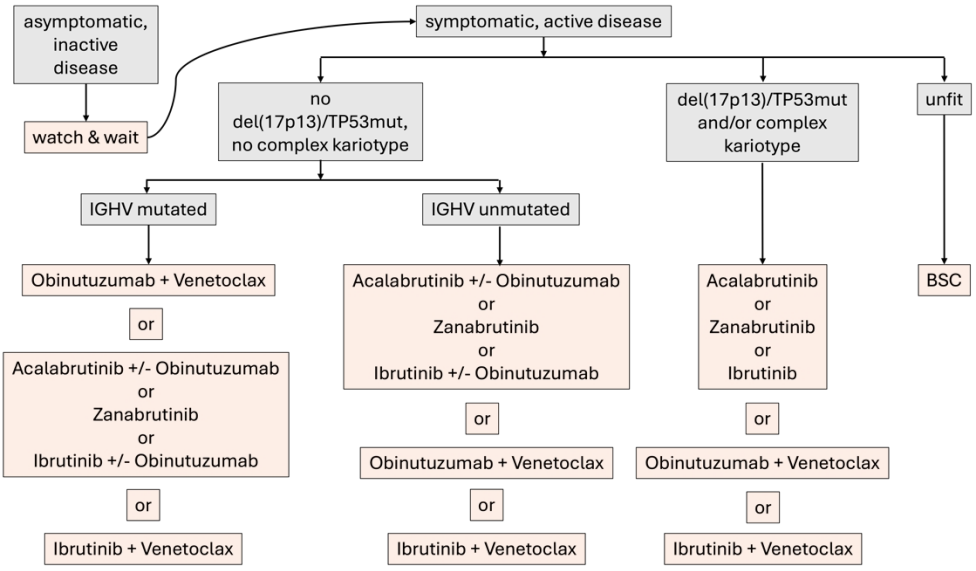
Patients without 17p13 deletion, *TP53* mutation, CK but with IGHV mutations are considered to get first line the combination of Obinutuzumab/Venetoclax, Acalabrutinib ± Obinutuzumab or Zanabrutinib or Ibrutinib ± Obinutuzumab or Ibrutinib/Venetoclax. Patients with the same profile but also without IGHV mutation, the same combinations are recommended, but in a different sequence.

For those with 17p13 deletion, *TP53* mutation, CK and ± IGHV mutation therapy starting with either Acalabrutinib, Zanabrutinib or Ibrutinib, or Obinutuzumab/Venetoclax or Ibrutinib/Venetoclax is recommended.

Unfit patients get best supportive care (BSC). The different options can be found in the graph below inspired by the one from Oncopedia (5,79).

Figure 1

Algorithm of the different first-line therapies of CLL according to Oncopedia (79).



Note. Seen is the Algorithm of the different first-line therapies of CLL including genetic alterations (del(17p13)/TP53mut), the detection of a complex karyotype and IGHV mutation as well as the patient's fitness. As the case may be, different therapeutics is indicated and used. IGHV denotes Immunoglobulin heavy variable gene. BSC denotes best supportive care.

1.7.5 Second-line Therapy

A second-line therapy is indicated when relapse occurs after successful first-line therapy or the CLL is refractory. The choice of a second-line treatment is even more complex than for the first-line therapy. Therefore, we need to know what kind of first line therapy the patient did get, whether it has been chemoimmunotherapy, BTKi or Obinutuzumab/Venetoclax. This is important due to the possible development of a certain resistance profile against a previous therapy why different drug groups are used second-line as first-line (2,5,12,61,79).

1.7.6 Best Supportive Care

The expression best supportive care (BSC) is broadly understood as controlling symptoms and improving quality of life (80). With regard to CLL, that mostly means controlling as preventing infections, other common CLL-associated complications like autoimmune thrombocytopenia (ITP) and autoimmune hemolytic anemia (AIHA) and pain. ITP and AIHA can initially be treated with glucocorticoids as infections can be prevented by vaccinations and controlled by immunoglobulin substitution (2,5,79).

1.7.7 Treatment of Richter Transformation

A traditional treatment of RT consists of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, called R-CHOP. According to Audil et al. (22) an overall response rate (ORR) of 50-60 %, a PFS of 10 months as well as a median OS of 15-21 months could be demonstrated. Nonetheless a treatment related mortality of 3 % was reported as well.

Novel agents like BTKi or immunotherapies were tested as single-agent or in combination with each other. There are many ongoing clinical trials concerning this issue why Audil et al. (22) conclude being hopeful that a treatment might be found improving the outcome of RT patients.

In patients responding to their induction therapy of RT that are fit and present without disqualifying comorbidities, (allo)SCT is considered bringing a durable response (22,81).

1.7.8 Response Assessment

At a minimum, a physical examination and blood and bone marrow tests should be performed to assess response to treatment. Different types of response can be distinguished and, according to the iwCLL guidelines, the definition of response after the treatment of CLL patients is also given in the sections below (2).

A complete remission (CR) is understood when no lymph node is ≥1.5 cm, liver size is normal and spleen size <13 cm, the patient shows no constitutional symptoms, and the circulating lymphocyte count is normal as well. PC needs to be ≥100 G/l, Hb ≥11 g/dl (without transfusion and erythropoietin) and the bone marrow needs to be normocellular without CLL cells and B-lymphoid nodules (2).

For a partial remission (PR) at least two parameters (if initially only one parameter has been abnormal, only one is also needed) of a decrease ≥50 % of the affected lymph nodes as well as liver and/or spleen size, any constitutional symptoms, or a decrease ≥50 % of the circulating lymphocyte count need to be seen in the range. Furthermore, one of the following parameters needs to be applicable as well. A PC ≥100 G/l or an increase of ≥50 %, an Hb of ≥11 g/dl or an increase ≥50 % or a presence of CLL cells or B-lymphoid nodules in the bone marrow, the bone marrow could also not have been examined (2).

A progressive disease (PD) is known as the occurrence of a least one parameter of an increase of ≥50 % of affected lymph nodes, an increase of ≥50 % of liver and/or spleen size, any constitutional symptoms as well as an increase ≥50 % of the circulating lymphocyte count.

Moreover, at least one parameter of decrease ≥50 % of the PC, a ≥2 g/dl decrease of Hb or an increase CLL cells in the bone marrow of ≥50 % on biopsies (2).
The last important category is the stable disease (SD) which is everything in between; the change of affected lymph nodes, the liver and/or spleen size and the circulating lymphocyte count in ranges between -49 % to +49 %, and there are any constitutional symptoms. The change of the PC is also between -49 % to +49 %, Hb decreases <11 g/dl or <50 % or -2 g/dl and no change of marrow infiltrate can be shown. All parameters need to be met here (2).

1.7.9 Minimal Residual Disease

The assessment of minimal residual disease (MRD) becomes more and more important today. The detection of MRD is a well standardized procedure performed by six-color flow cytometry, allele-specific oligonucleotide polymerase chain reaction (PCR), or high-throughput sequencing. These procedures can detect <1 CLL cell per 10000 leukocytes, whereby different markers are used for FC e.g. CD19, CD20, CD5, CD43, CD79b, and CD81. Patients with undetectable MRD (MRD-neg) remission present with <1 CLL cell per 10000 leukocytes in peripheral blood or bone marrow (1,2,12). MRD-neg is associated with better PFS and OS, as Böttcher et al. (82) were the first being able to show in a large multicentric study with patients included in the CLL8 study in 2012. Since then, the evaluation of MRD has become more and more common and important to assess treatment efficacy.

1.8 Tumor Microenvironment and Immune De-/Regulation of Chronic Lymphocytic Leukemia

1.8.1 Structure of the Tumor Microenvironment

The TME consists of many different tumor-infiltrating non-malignant cell types and the extracellular matrix. The composition of the TME depends strongly on the type of tumor. Cells of the innate immune system, such as neutrophils, eosinophils, macrophages etc., as well as cells of the adaptive immune system, such as T and B cells, further, fibroblasts, endothelial cells and many more, are found in the cellular component of the TME, to name just a few. They all interact with each other and with the tumor cells trough molecules such as cytokines, chemokines and more. These interactions are very complex and detailed (83–85).

1.8.2 Immune Regulation and Evasion in Chronic Lymphocytic Leukemia

In general, activation of another cell of the adaptive immune system in the TME requires stimulation of the T cell's T cell receptor (TCR) by peptides presented on an MHC complex on the surface of an antigen presenting cell (APC). In interaction with co-signaling molecules, the immunological synapse is formed and controlled by co-inhibitory molecules, more precisely immune checkpoint molecules. The immunoglobulin-like superfamily and the tumor necrosis factor (TNF) receptor superfamily are the two major co-signaling families. Normally, immune checkpoint molecules regulate the activated T cell response, leading to self-tolerance and limited collateral tissue damage during inflammatory immune responses. However, this mechanism can be used by tumor cells to evade the immune response. A large number of molecules play an important role in this, and the molecules used for this work are discussed in section 1.8.3 (83,84).

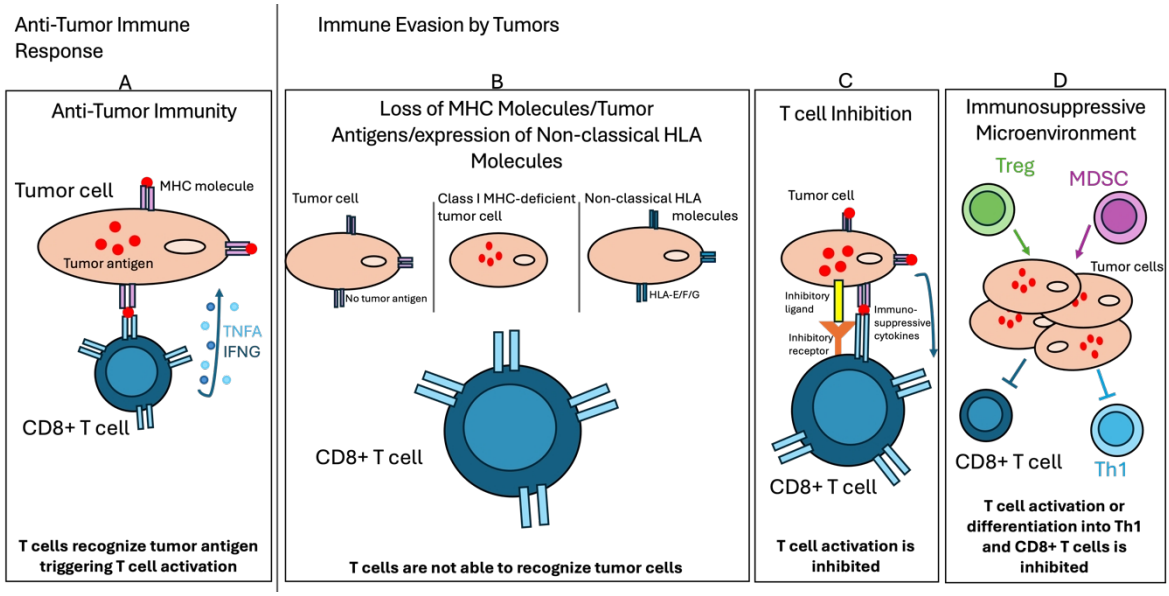
Several studies have shown that the TME is crucial for the development and progression of cancer (83,84,86). There are many different and complex mechanisms by which the microenvironment supports tumor growth, and the tumor evades the immune system, so I will only give a brief overview.

One mechanism is that tumor cells express similar antigens to normal, healthy cells, leading to non-recognition of the tumor cell. These antigens can be used as therapeutic targets in immunotherapy, with the risk that normal cells carrying the targeted antigen will also be affected.

In addition, tumor cells can reduce the expression of MHC class I molecules, beta2-microglobulin and co-stimulatory molecules. Under normal conditions, all these molecules would lead to activation of T cells through complex mechanisms. Other strategies to evade the immune system include the expression of inhibitory tumor-mediated signals by cytotoxic T lymphocytes, the secretion of immunosuppressive molecules that attenuate effector T cells, and the induction of regulatory and/or suppressor cells (84,87,88). This can be seen in Figure 2, adapted from Pansy et al. (83).

Figure 2

Overview of immune response to tumor vs. immune evasion mechanisms by tumors in context of CD8+ T cells, adapted from Pansy et al. (83).



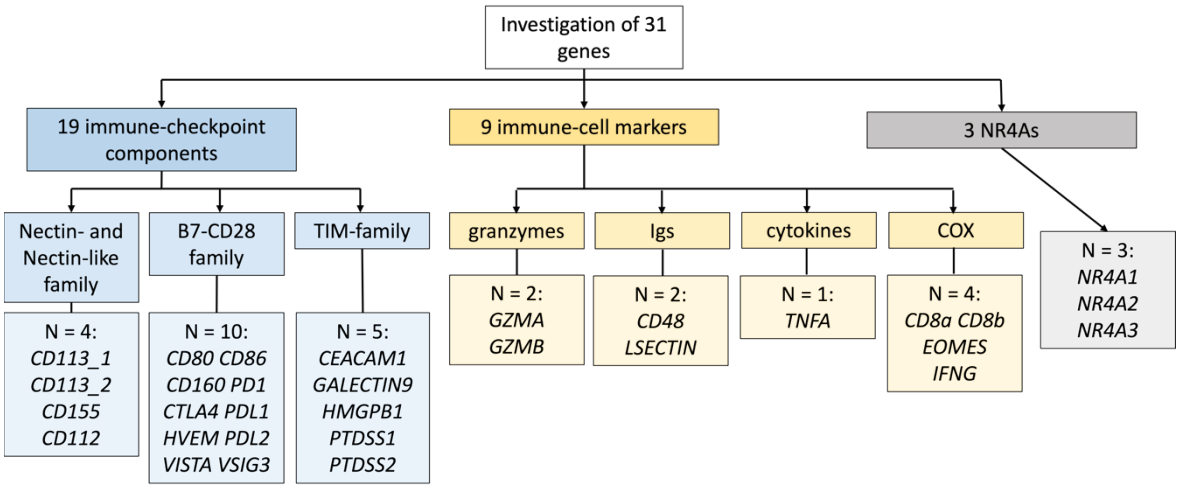
Note. (**A**) Shown is a normal CD8+ T cell response. Tumor cells present their antigens on major histocompatibility complex (MHC) molecules, CD8+ T cells recognize them with their T cell receptor (TCR) and have the ability to kill malignant tumor cells via cytokines like TNFA and IFNG. (**B**) There are different ways how tumor cells can evade the immune system. One way is the loss of MHC molecules, of tumor antigens or the expression of non-classical HLA molecules leading to CD8+ T cells non-recognizing tumor cells. (**C**) Another mechanism is the expression of inhibitory ligands by tumor cells. Effect is an inhibition of CD8+ T cells, resulting in a reduced anti-tumor immune response through inhibitory signals and the secretion of immune-suppressive cytokines by the tumor cell. (**D**) The consistency of the tumor microenvironment has a crucial role in immune regulation. Regulatory T cells (Tregs) are able to suppress the T cell response to tumors, myeloid-derived suppressor cells (MDCSs) accumulate, and both suppress anti-tumor T cell responses by CD8+ T cells and T helper cells 1 (Th1).

1.8.3 Genes

This section lists all the genes we have used in our work, with a brief overview of their function. In the chart below (Figure 3) all groups and subgroups with their genes can be seen.

Figure 3

Overview of the 31 investigated genes.



Note. 19 of our investigated genes belong to the group of immune-checkpoint components, where 4 belong to the Nectin- and Nectin-like family, 10 to the B7-CD28-family and 5 to the TIM-Family. 9 genes belong to the group of immune-cell markers where we had 2 granzymes, 2 Immunoglobulins, one cytokine and 4 belonging to the COX pathway. 3 genes belong to the group of NR4As. TIM denotes human T cell immunoglobulin and mucin domain, Igs denotes Immunoglobulines, COX denotes cyclooxygenases and NR4A stands for orphan nuclear receptors that belong to the larger nuclear receptors (NRs) superfamily.

1.8.3.1 Immune Checkpoint Components.

Immune-checkpoints are essential molecules for immune-cell regulation. In our work we focused on the suppression of anti-tumor immune response in the TME where immune checkpoints play a crucial role. We used the CD-name of genes, synonyms and long names can be found in the chapters below. They will not be mentioned in the list of abbreviations. There are several subgroups of immune checkpoints, but the molecules we investigated just belong to the Nectin- and Nectin-like family, B7-CD28 family and TIM family.

1.8.3.1.1 Nectin- and Nectin-like Family.

Here, we observed ligands of members of the Nectin- and Nectin-like family, namely *CD113*, *CD155* and *CD112*. These ligands can be found on APCs and cancer cells and when their effector molecules bind, it leads to modification of immune response (83,89).

Nectin cell adhesion molecule 3 (*CD113*, also known as *NECTIN3*) encodes a protein working as adhesion molecule at adherens junctions. Besides the interaction with other Nectin-like proteins, it is able to interact with Afadin, a protein playing a role in cell proliferation, survival as well as the regulation of directional motility (90).

PVR cell adhesion molecule (*CD155*) encodes a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. In first place it is useful for poliovirus as a cellular receptor (91).

Nectin cell adhesion molecule 2 (*CD112*, also known as *NECTIN2*) encodes a membrane glycoprotein being part of the plasma membrane components of adherens junctions. It also works for certain herpes simplex viruses and pseudorabies as an entry and helps them spreading cell to cell (92).

1.8.3.1.2 B7-CD28 Family.

Members of the B7-CD28 family are involved in the mediation of the immune invasion of malignant cells, in T cell tolerance and homeostasis. With its different co-stimulatory and co-inhibitory pathways they comprise many molecules of which we observed mRNA expression levels of *CD80*, *CD86*, *CD160*, *PD1*, *CTLA4*, *PDL1*, *PDL2*, *HVEM*, *VISTA* and *VSIG3* (83,93).

Cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) belongs to the immunoglobulin superfamily with the proteins function of transmitting inhibitory signals to T cells. It can only be found on T cells after TCR binding. It is an inhibitory feedback molecule that leads to a down regulation of the amplitude of T cell responses by competing with CD28 for binding to CD80 and CD86. There exist more than one mechanism how the CTLA-4-CD80/CD86 complex achieves the down regulated T cell activation. The protein itself and mutations in it play a role in different autoimmune diseases (84,94).

The protein encoded by the *CD80* gene functions as a membrane receptor. The binding of CD28 or CTLA-4 activates it and leads to T-cell proliferation as well as cytokine production (95).

CD86 encodes a member of the immunoglobulin superfamily, a type I membrane protein being expressed by APCs. It functions as ligand of CD28 and CTLA4, that are both expressed on the T cell surface. Whereat the binding with CD28 activates the T-cell, CTLA4 reduces the immune response as well as the T-cell activation (96).

CD160 encodes a glycoprotein that is expressed mostly on the surface of peripheral blood NK cells and CD8+ T lymphocytes with cytolytic effector activity. Furthermore, its expression could be proved on all intestinal intraepithelial lymphocytes, and it shows a wide specificity as it binds to classical and nonclassical MHC class I molecules. CD160 can be seen as an exhaustion marker being expressed by T cells from CLL patients (84,97).

Furthermore it induces the inhibition of apoptosis, the activation of tumor proliferation and resistance to apoptosis (98).

The programmed cell death protein 1 (*PD1*, also known as *PDCD1*) is only expressed in activated T cells and serves as an immune-inhibitory receptor, being also of use in the regulation of T-cell functions, as well as the differentiation of CD4+ T cells into T regulatory cells. Furthermore, many tumors express PD1 due to its anti-tumor immunity function and PD1 can support the inhibition of effective anti-tumor and anti-microbial immunity. PD1 can also be seen as an exhaustion marker of tumor infiltrating lymphocytes being expressed at high levels due to chronic stimulation by tumor antigens (84,99).

Programmed cell death 1 ligand 1 (*PDL1*, also known as *CD274*) is an immune inhibitory receptor ligand being expressed by both, hematopoietic and non-hematopoietic cells like T cells and B cells. It also is expressed by tumor cells, triggered through stimulation by IFNG. The transmembrane protein ligand inhibits the activation of T cells and cytokine production when interacting with its PD1 receptor. Through cytotoxic T cell inactivation, this mechanism leads to immune escape for tumor cells in TME. In some malignancies *PDL1* is also considered to be of prognostic value. Furthermore, PDL1 is able to interact with CD80 on activated T cells leading to inhibitory signals (84,100).

Programmed cell death 1 ligand 2 (*PDL2*, also known as *PDCD1LG2*) is a membrane bound protein that negatively regulates an activated T cell proliferation, e.g. reduces IFNG or IL10 production, through interaction with its PD1 receptor (101).

TNF receptor superfamily member 14 (*HVEM*, also known as *TNFRST14*) is a protein being involved in signal transduction pathways activating inflammatory and inhibitory T cell immune response (102). It is known as ligand of BTLA with inhibitory function and ligand of CD160 with activating function in T cells (103–105).

V-set immunoregulatory receptor (*VISTA*, also known as *VSIR*) as a membrane bound protein takes part in the negative regulation of cytokine production, positive regulation of macromolecule metabolic process and regulation of T cell activation (106).

Immunoglobulin superfamily member 11 (*VSIG3*, also known as *IGSF11*) is member of the immunoglobulin superfamily being principally expressed in brain and testis. Its structure is similar to the one from coxsackievirus and adenovirus receptor (CXADR) as well as endothelial cell-selective adhesion molecule (ESAM) (107). Lately, a novel VSIG3/VISTA pathway has been described with VSIG3 binding to VISTA leading to T cell inhibition and suppression (106).

1.8.3.1.3 T cell/Transmembrane, Immunoglobulin Domain, and Mucin Domain Family.

The major molecules belonging to the T cell/transmembrane, immunoglobulin domain, and mucin domain (TIM) family are TIM-1, -2, -3, that can be found on the T cell surface, and -4, being found rather on the surface of APCs. But in this study, we investigated rather the mRNA expression levels of distinct ligands *CEACAM1*, *GALECTIN9*, *HMGPB1*, *PTDSS1* and *PTDSS2* of TIM-3. TIM-3 possesses an immune inhibitory function of TH1 and cytotoxic T cell response (83,108).

The protein encoded by CEA cell adhesion molecule 1 (*CEACAM1*) belongs to the gene family of the carcinoembryonic antigen (CEA), which itself is a member of the immunoglobulin superfamily. It exists two subgroups of the CEA family, the pregnancy-specific glycoproteins and the CEA cell adhesion molecules. It is known to be a cell-cell adhesion molecule detected e.g. on leukocytes and other tissues. The encoded protein mediates cell adhesion followed by numerous cellular activities. It plays a role in angiogenesis, apoptosis, tumor suppression and immune responses (109).

GALECTIN9 (also known as *LGALS9*) belongs, as many other galectins do, to the family of beta-galactosidase-binding proteins. They are involved in the modulation of cell-cell and cell-matrix interactions. It is known that GALECTIN9, a S-type lectin, is overexpressed in tissues of Hodgkin's disease and affects cell interaction within this tissue. Therefore, GALECTIN9 might be involved in the pathogenesis of Hodgkin's disease and/or its associated immunodeficiency (110). GALECTIN9 is known to be the key ligand of TIM3. Its binding leads to an exhaustion of CD8+ tumor-infiltrating lymphocytes, but also it has influence on T cell differentiation with induction of immune escape of the tumor (111–113).

High mobility group box 1 (*HMGPB1*, also known as *HMGB1*) is a member of the high mobility group-box superfamily. The encoded protein is a non-histone, nuclear DNA-binding protein. It plays a role in the regulation of transcription, organization of DNA and therefore in multiple cellular processes. They include inflammation, cell differentiation and tumor cell migration (114).

Phosphatidylserine synthase 1 (*PTDSS1*) encodes a protein that catalyzes the formation of phosphatidylserine, being localized in the endoplasmic reticulum, more precisely its mitochondria-associated membrane. There, the proteins role is in structuring but also in signaling (115).

Phosphatidylserine synthase 2 (*PTDSS2*) also encodes a protein catalyzing the formation of phosphatidylserine, but from a different molecule than PTDSS1. It is a structural membrane phospholipid with its purposes in cell signaling, coagulation of the blood and apoptosis (116).

1.8.3.2 immune-Cell Markers.

Granzymes, Immunoglobulins, cytokines, and members of the Cyclooxygenase (COX) pathway are all markers for the presence of immune cells. This is why we investigated several mRNA expression levels of molecules of these groups.

1.8.3.2.1 Granzymes.

Granzymes are cytotoxic serine proteases mediating granule exocytosis-dependent killing. They can be found in secretory vesicles of cytotoxic T cells as well as NK cells and their function is to modify their substrates specifically that they can activate various signaling pathways or inactivate target (117). In this work we investigated Granzyme A and Granzyme B.

Granzyme A (*GZMA*) encodes a protein being specific for a T cell and NK cell serine protease. This might be a necessary component for lysis of target cells by cytotoxic T cells and NK cells that are able to lyse cells presenting a 'nonself' antigen on its surface (118).

Granzyme B (*GZMB*) belongs to the granzyme subfamily that is part of the peptidase S1 family of serine proteases. It is encoded as a preprotein being secreted by NK cells and cytotoxic T cells. The process to its active protease is via proteolyses. The active protease induces apoptosis of target cells, but it also processes cytokines, degrades extracellular matrix proteins. With all these functions it plays a role in chronic inflammation, wound healing and in cell killing in the TME (119,120).

1.8.3.2.2 Immunoglobulins.

Immunoglobulins (IGs) are also known as antibodies that play a crucial role in the immune response and immune cell stimulation and inhibition. We observed mRNA expression levels of *CD48* and *LSECTIN* (83,108).

CD48 belongs to the CD2 subfamily of immunoglobulin-like receptors including SLAM (signaling lymphocyte activation molecules) proteins. The encoded protein plays a role in

activation and differentiation pathways of lymphocytes, other immune cells, dendritic cells and endothelial cells, on which it can be found on the cells surface (121).
C-type domain family 4 member G (*LSECTIN*, also known as *CLEC4G*) encodes a glycan-binding receptor, a member of the C-type lectin family playing a role in the immune response. Being pattern recognition receptors located on immune cells, C-type lectin receptors are important in recognizing and up taking self and non-self-glycoproteins. It also has a function in mediating cell adhesion, glycoprotein clearance and cell signaling. The ability of recognition of activated T cells and negative regulation of TCR-mediated signaling could be shown in mice. In mice furthermore, it could be shown that it works as a liver-specific regulator of NK cell-mediated immunity. If the same could be in human, still needs to be seen (122).

1.8.3.2.3 Cytokines.

Cytokines are small soluble molecules mediating cell-cell communication in the immune system. They are released by immune cells and able to trigger and modify the behavior of surrounding cells that bear corresponding receptors. Different cytokines are released by different cells and affect different cells and it exist a vast variety of molecules and effects (108,123). The only mRNA expression level of a cytokine we observed is Tumor necrosis factor A (*TNFA*).
TNFA encodes a multifunctional proinflammatory cytokine belonging to the TNF superfamily. Macrophages mainly secrete TNFA, and it has multiple functions, e.g. cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation. It also plays a role in many diseases like autoimmune diseases, cancer and many more. As a result of mouse studies, a neuroprotective function of TNFA is suggested (124).

1.8.3.2.4 Cyclooxygenase.

Molecules belonging to this group effect the COX pathway and therefore the TME. The COX pathway results in prostaglandin E2 (PGE2) production that leads to the evasion of IFNG/T cell driven tumor elimination (125,126). We investigated mRNA expression levels of *CD8a*, *CD8b*, *EOMES* and *IFNG*.
CD8 subunit alpha (*CD8a*) and CD8 subunit beta (*CD8b*) both are part of the CD8 antigen, a glycoprotein that is found on the cell surface of most of the cytotoxic T cells and its role is the mediation of efficient cell-cell interactions in the immune system. CD8 as antigen is a

coreceptor with the TCR on the T cell. It recognizes antigens presented by APCs on their MHC class I molecules. The receptor works as a homodimer (two alpha chains) or a heterodimer (one alpha and one beta chain). Alpha as well as beta chains share significant homology to immunoglobulin variable light chains (127,128).

Eomesodermin (*EOMES*) belongs to the T-box brain protein 1 (TBR1) subfamily of T-box genes. The encoded protein is essential for embryonic development, but it also might be important for the differentiation of effector CD8+ T cells being involved in defense against viral infections (129). Furthermore, it is indicative on the exhaustion of CD8+ T cells, together with T-bet and PD1, in the constellation T-bet$^{dim/-}$Eomes^{hi}PD1^{hi}. Both, EOMES and T-bet influence the PD1 expression that leads to regulative functions in CD8+ T cells (130).

Interferon gamma (*IFNG*) encodes a soluble cytokine being a member of the type II interferon class and being secreted by cells of the innate as well as adaptive immune system. The protein triggers as a homodimer, bound to its receptor a cellular response to viral and microbial infections (131). It is the most important cytokine involved in anti-tumor immunity. Nonetheless it also is able to support tumor growth, i.e. through induction of PDL1-expression that leads to immune escape of tumor cells through activation of the PD1 signaling axis (132–134).

1.8.3.3 NR4As.

To the nuclear receptor subfamily 4 group A belongs a group of nuclear receptors being upregulated due to antigen-receptor signaling in T and B cells. They have a wide range of regulating functions in the cell cycle like apoptosis, inflammation, metabolism and DNA repair and are also known as molecules involved in the tumorigenesis of different tumors (135,136).

Nuclear receptor subfamily 4 group A member 1 (*NR4A1*) belongs to the steroid-thyroid hormone-retinoid receptor superfamily and the proteins expression in human lymphocytes is induced by phytohemagglutinin. It acts as a nuclear transcription factor where its translocation to mitochondria induces apoptosis (137).

Nuclear receptor subfamily 4 group A member 2 (*NR4A2*) belongs also to the steroid-thyroid hormone-retinoid receptor superfamily and its encoded protein may also act like a transcription factor. Mutations as well as misregulations in this gene are associated with a large variety of different diseases (138).

Nuclear receptor subfamily 4 group A member 3 (*NR4A3*) belongs also to the steroid-thyroid hormone-retinoid receptor superfamily and its protein may act like a transcriptional activator (139).

1.9 Aim of Deciphering the Interaction of Immune and Tumor Cells in Chronic Lymphocytic Leukemia

As data of immune-checkpoint components, immune-cell markers and members of the NR4A family in tissue is strongly limited, our aim was to decipher the interaction of immune and tumor cells in chronic lymphocytic leukemia. Therefore, we wanted to detect different expression patterns comparing the different subgroups, as analyzing the overall survival probability and probability of therapy, to possibly find new prognostic or predictive marker for CLL/RT.

Our study investigated the mRNA-expression of 31 genes (19 immune-checkpoint components, 9 immune-cell markers and 3 NR4As) in CLL (subgrouped in never treated, previously treated, untreated but with requirement of treatment at a further point) and RT. We were able to show that 11 of 19 immune-checkpoint components and 5 of 9 immune-cell marker were differentially expressed comparing RT, untreated CLL, previously treated CLL and never treated CLL in different ways with each other. Interestingly, no differences were observed in the NR4A group.

1 immune-checkpoint component and 2 immune-cell markers showed a significant difference of overall survival as 5 immune-checkpoint components and 4 immune-cell markers were significantly different in regard of the probability of therapy.

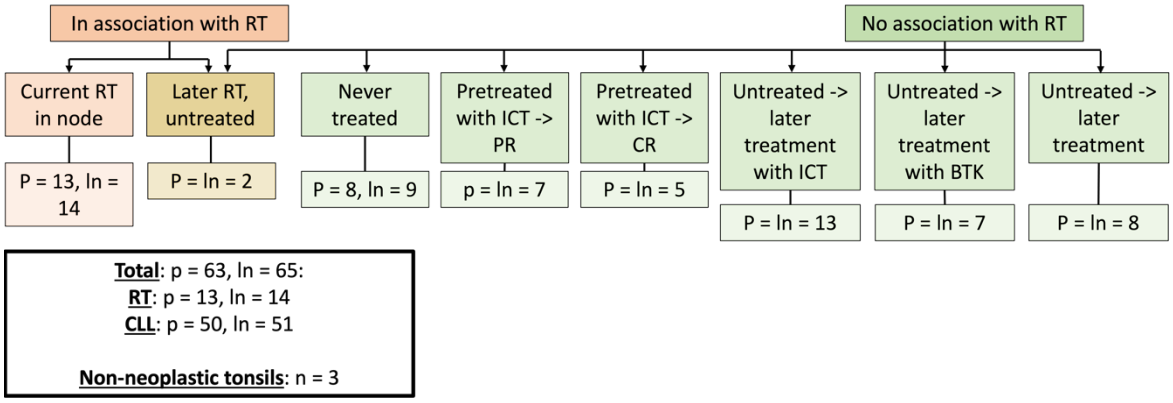
2. Material and Methods

2.1 Patient Population

To comprehensively study the expression of defined genes in CLL and RT, we measured the mRNA expression in our embedded CLL tissue samples (n = 65 from 63 different patients) provided by the Institute of Pathology, Graz. They were manually categorized according to their relation to previous therapy, further therapy, and RT. This is shown in the Figure 4. Our included patients were diagnosed at the Institute of Pathology, Graz between 2011 and 2021. Patients who never received treatment were defined as surviving ≥5 years after the diagnosis of CLL.

Figure 4

Grouping of lymph nodes in our cohort according to their association to RT.



Note. Lymph nodes got grouped into two groups. One in association with RT and one without association to RT. The subgroups of with RT associated lymph nodes were current RT and later RT without previous treatment. The subgroups of without RT associated lymph nodes were never treated ones, pretreated ones with ICT that went into PR, pretreated ones with ICT that went into CR, untreated ones that ended up with ICT later, untreated ones that ended up with BTK later and untreated ones that ended up with any treatment later. For our analyses we combined subgroups so that we hat never treated ones, pretreated ones and previously untreated lymph nodes. Furthermore, we counted nodes that developed RT later to our CLL cohort. In total we hat 65 lymph nodes from 63 patients, subgrouped to 14 lymph nodes from 13 patients with current RT, 51 lymph nodes from 50 patients with CLL and 3 non-neoplastic tonsils serving as non-malignant control. P denotes the number of patients we were able to put in this group. Ln denotes the number of lymph nodes we were able to put in this group. RT denotes Richter specimens, transformed CLL, ICT denotes immunochemo therapy, PR denotes partial remission, CR denotes complete remission, BTK denotes Bruton-Tyrosin Kinase inhibitor.

Clinicopathologic parameters of our CLL-/RT-cohort are shown in Table 5, in section 3.1.

For our retrospective study, we only used samples received after routine diagnostic procedures. Therefore, written informed consent was not obtained from our patients. The Ethics Committee of the Medical University of Graz approved this study, in which all data were anonymized (ethical application 28-516 ex 15/16).

Non-neoplastic mouse tonsils served as control. They were embedded and processed as the human lymph nodes.

2.2 Collection of Clinical Characteristics

We collected demographic and clinical data, shown in Table 3, such as date of birth, sex, date of diagnosis, diagnosis (CLL/SLL), therapy (which scheme, when, number of cycles, 2nd, 3rd, further line therapies), RT, date of last appointment/contact, date of death, survival, age at diagnosis, time to treatment and time to RT to fully understand our findings. Data collection took place from 15th June 2022 to 31st October 2022 via the local hospital information system (MEDOCS) by a one-person manual search through discharge letters, histopathological, genetical and clinical findings. There are some patients from whom not all information was available.

Table 3

Overview of clinical characteristics.

Demographic data	• date of birth • sex
Clinical data	• date of diagnosis • diagnosis (CLL/SLL other) • Therapy • RT • Date of last appointment/contact • survival

Note. Clinical characteristics were collected via MEDOCS for this thesis. We collected the date of birth, sex, date of diagnosis, kind of diagnosis (CLL/SLL other), kind of therapy, occurance of RT, date of last appointment/contact and survival. RT denotes Richter specimens, transformed CLL.

2.3 RNA Isolation, cDNA Synthesis and Real-Time PCR

The blocks with formalin-fixed, paraffin-embedded (FFPE) lymph nodes containing more than 95% CLL or lymphoma cells were cut by using a rotatory microtome. The blocks were trimmed by 20 μm until the surface was leveled and then 10 cuts of 5 μm were taken and transferred into a 1.5 ml tube (140).

Afterwards the ribonucleic acid (RNA) extraction was performed by using ReliaPrep™ FFPE Total RNA Miniprep System by Promega adapted to our needs (141).
In suite of the RNA-extraction the cDNA-synthesis took place according to the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisherScientific, Waltham, MA, USA).
All of the RNA-/cDNA assays were already tested, validated, and published in previous studies (142).
With the generated complementary deoxyribonucleic acid (cDNA), semi-quantitative real time PCR (RQ-PCR) was performed in duplicates using the Bio-Rad CFX-96 or CFX-384 detection system (Bio-Rad, Hercules, CA, USA). Following, the synthesized cDNA has been compared to housekeeping genes. Therefore GAPDH, PPIA and HPRT1 have been used. All of the RQ-PCR assays were already tested, validated, and published in previous studies (142). Through $2^{-\Delta\Delta CT}$ calculation, results were expressed as relative units. They showed the relative amount of the target gene which is normalized to the endogenous control (geometric mean of GAPDH, PPIA and HPRT1) and relative to non-neoplastic tonsils (142–144). Found undetermined values were set to a maximum of CT 45 (144) and the primers we used can be found in the Supplements, in Table 9.

2.4 Statistics

Following data has been transferred to GraphPad Prism (version 10.2.2) and statistical analysis has been performed separately with every gene.
Data has been grouped for better/clearer comparison, outliers have been identified and removed by GraphPad Prism. We used the Shapiro Wilk test for normal distribution, as most of our data was not normally distributed, then we used the Mann-Whitney test. Then we were able to compare the medians of each gene and each group.
For comparing non-neoplastic tonsils to CLL and RT, we applied Kruskal-Wallis test of Identify outliers, then we performed an ordinary one-way ANOVA.
To analyze demographic data and to perform descriptive statistics, we used SPSS (version 29).

2.5 Heatmap

The cluster analysis has been performed using R (version 3.6.3) (145).
To generate the heatmap we used ΔΔCT-values, comparable to previously described (146–148). The heatmap.2 function of the R package ‘gplots’ (149) (R version 3.6.3) (145) has

been used on scaled data to create the heatmap and hierarchical clustering with Euclidean distance and Ward linkage (143).

To further investigate the CLL cohort we performed a survival analysis using R (version 3.6.3) and proceeded a further cluster analysis, according to the above-mentioned script, to better understand our data (143,145,149).

3. Results

3.1 Demographic Data and Descriptive Analysis of our CLL and RT lymphoma cohort

In total we investigated 65 lymph nodes, isolated from 61 CLL and RT patients, for this thesis. For the 61 patients, I collected clinical and demographic data. Table 4 summarizes gender, stage, relation to treatment, RT and survival.

Table 4

Absolute and relative distribution of our cohort in regard to gender, stage, relation to treatment, RT and survival.

		Absolute frequency (*N*)	Relative frequency (%)
sex	Female	20	30.8
	Male	45	69.2
	Total	65	
Stage according to Rai/Binet prior to lymph node extraction	Low risk Rai/Binet A	7	10.8
	Intermediate risk Rai/Binet B	41	63.0
	High risk Rai/Binet C	13	20.0
	No data available	4	6.2
	Total	65	

Relation to treatment	Never treated	9	13.8
	Previous therapy with ICT -> PR	7	10.8
	Previous therapy with ICT -> CR	5	7.7
	Untreated, later ICT	13	20.0
	Untreated, later BTKi	7	10.8
	Untreated, later any therapy	8	12.3
	In connection with RT	16	24.6
	Total	65	
RT	No RT	49	75.4
	Later RT, untreated	2	3.1
	Current RT	14	21.5
	Total	65	
Survival	Death at any point	40	61.5
	Survived	25	38.5
	Total	65	

Note. Descriptive statistics with absolute and relative frequency of our cohort showing gender, clinical stage according to Rai or Binet, the relation to treatment, RT and survival. RT denotes Richter specimens, transformed CLL. ICT denotes immunochemo therapy, PR denotes partial remission, CR denotes complete remission, BTK denotes Bruton-Tyrosin Kinase inhibitor.

Table 5 summarizes patients ages at different timepoints i.e. age at diagnosis, age at survey and age at death. Furthermore, it shows the time from diagnosis to survey, from diagnosis to death and from diagnosis to treatment.

Table 5

Distribution of Age at diagnosis, Age at survey, Age at death, Time from diagnosis to survey, Time from diagnosis to death, Time to treatment.

	Absolute frequency (*N*)	Minimum	Maximum	Median (*M*)	Standard deviation (*SD*)
Age at diagnosis (years)	65	38	83	65.0	11.237
Age at survey (years)	25	48	94	74.0	11.095
Age at death (years)	40	54	90	76.5	10.614
Time from diagnosis to survey (months)	25	49.0	220.0	116.0	39.15

Time from diagnosis to death (months)	40	77.0	387.0	145.0	70.81
Time to treatment (months)	56	0	261	18.5	51.819

Note. Absolute frequency (N), Minimum, Maximum, Median (M) and Standard deviation (SD) of patients‘ Ages at diagnosis, Age at survey, Age at death, Time from diagnosis to survey, Time from diagnosis to death and Time to treatment. Time of survey is considered June 2022.

Table 6 summarizes clinicopathologic features of our cohort.

Table 6

Distribution of Time to treatment, Time from diagnosis to death, died and survived patients according to their subgroup with relation to RT or treatment.

		Time to treatment (months) (*M, range, SD*)	Time from diagnosis to death (months) (*M, range, SD*)	Dead (*N*)	Survived (*N*)
RT	No RT	35; *R* = 0-261; *SD* = 52	157.88; *R* = 80.0-373.0; *SD* = 66.82	24	25
	Later RT, untreated	60; *R* = 31-89; *SD* = 41	143.5; *R* = 142.0-145.0; *SD* = 2,21	2	0
	Current RT	37; *R* = 0-206; *SD* = 56	187.43; *R* = 77.0-387.0; *SD* = 80.34	14	0
Relation to treatment	RT (later or current)	40; *R* = 0-206; *SD* = 53	181.94; *R* = 77.0-387.0; *SD* = 76.28	16	0

Never treated		123.75; *R* = 117.0-134.0; *SD* = 8.3	4	5
Previous therapy with ICT -> PR	17; *R* = 1-41; *SD* = 16	186.0; *R* = 145.0-210.0; *SD* = 28.53	4	3
Previous therapy with ICT -> CR	3; *R* = 0-6; *SD* = 3	176.5; *R* = 111.0-240.0; *SD* = 56.95	4	1
Untreated, later ICT	49; *R* = 0-261; *SD* = 80	169.88; *R* = 95.0-373.0; *SD* = 101.49	8	5
Untreated, later BTKi	60; *R* = 23-107; *SD* = 31	108.0; *R* = 80.0-136.0; *SD* = 39.6	2	5
Untreated, later any therapy	24; *R* = 1-73; *SD* = 25	134.5; *R* = 124.0-145.0; *SD* = 14.85	2	6

Note. Median (M), Range (R) and Standard Deviation (SD) of Time to treatment and Time from diagnosis to death for the different subgroups of RT and different relations to treatment. Also shown is the absolute frequency (N) of the died and survived patients of each subgroup. RT denotes Richter specimens, transformed CLL, ICT denotes immunochemo therapy, PR denotes partial remission, CR denotes complete remission, BTK denotes Bruton-Tyrosin Kinase inhibitor.

3.2 Expression of Immune Checkpoint Components and Immune cell markers in CLL and RT lymphomas

3.2.1 Expression of Immune-Checkpoint Components

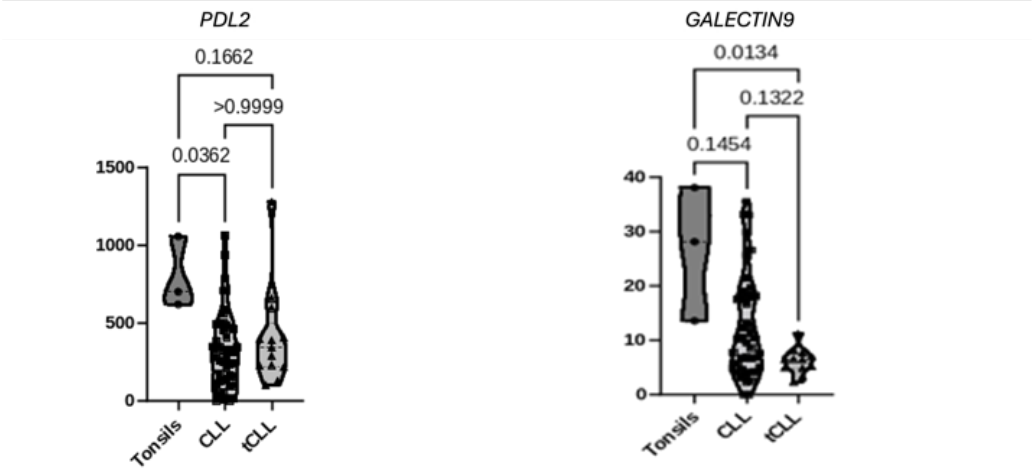
Since knowledge of the expression of immune-checkpoint components in CLL and RT is limited, we studied mRNA expression levels of 19 well-characterized immune-checkpoint components in primary lymphoma tissue samples. They comprise of CLL (*N* = 51), RT (*N* = 14), subgrouped in relation to treatment, as well as non-malignant tonsils (*N* = 3) serving as non-neoplastic controls. To comprehensively understand differences between our

subgroups, we compared them to each other. Non-significant expressions of Immune-Checkpoint-Components can be found in the Supplements, Figure 33-35.

To investigate the differences in non-malignant tonsils, CLL- and RT-lymph node samples, we compared them to one another. We observed a lower *PDL2*-expression in CLL (2.3-fold, $p = 0.0362$, Figure 5) and a lower *GALECTIN9* expression in RT (4.2-fold, $p = 0.0134$, Figure 5) lymph nodes.

Figure 5

Significant differences in PDL2- and GALECTIN9-expression comparing Tonsils with CLL and tCLL/RT.



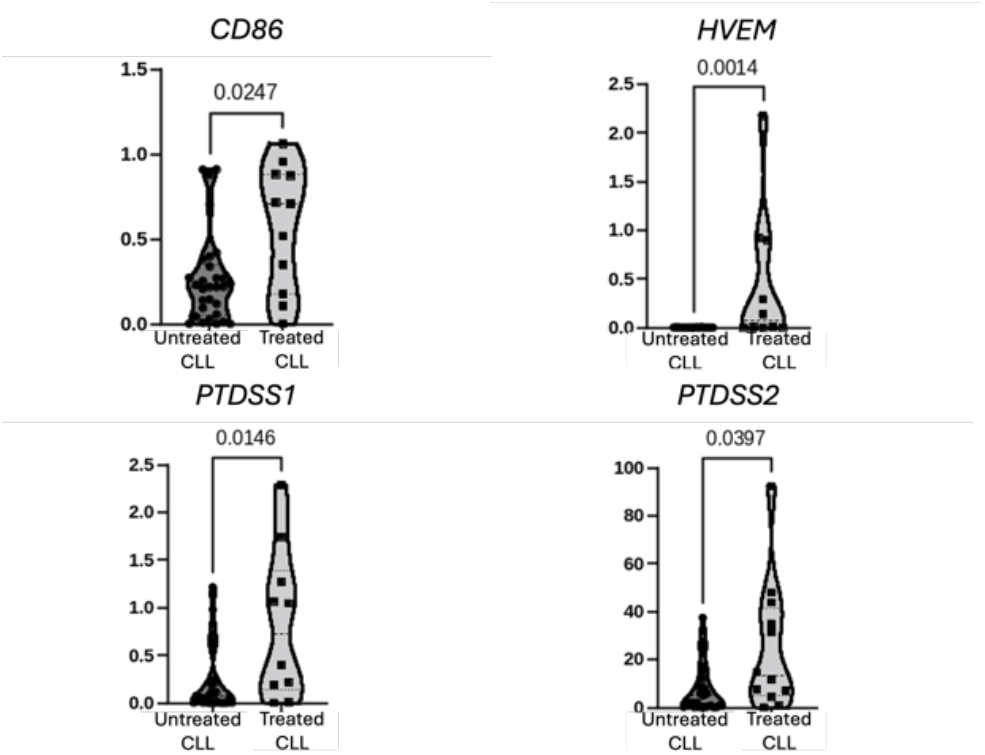
Note. Shown are mRNA expressions of PDL2 (p = 0.0362) and GALECTIN9 (p = 0.0134) in CLL, tCLL, and tonsils, serving as non-neoplastic controls. Values of gene expression are calculated as relative expression. tCLL denotes Richter specimens, transformed CLL.

Furthermore, we wanted to study the differences between CLL-lymph nodes of treated and untreated patients, we compared them and found *CD86* being 3.256-fold higher ($p = 0.0247$), *HVEM* being 220.02-fold higher ($p = 0.0014$), *PTDSS1* being 10.10-fold higher ($p = 0.0146$) and *PTDSS2* being 2.67-fold higher ($p = 0.0397$) in previously treated nodes (Figure 6).

PDL2 was 2.43-fold lower ($p = 0.0356$) and *CEACAM1* was 3.83-fold lower ($p = 0.0121$) in CLL-lymph nodes of treated compared to untreated patients (Figure 7).

Figure 6

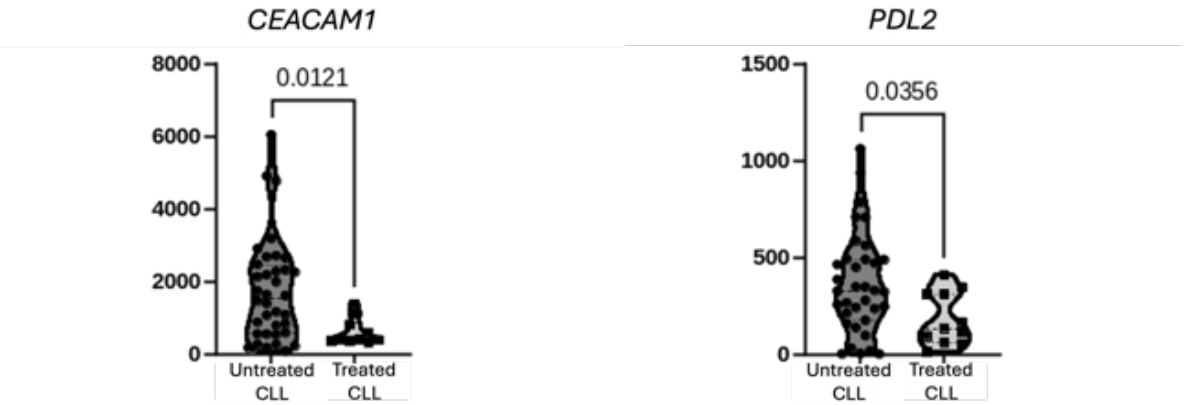
Significant differences in CD86-, HVEM-, PTDSS1- and PTDSS2-expression comparing previously untreated to previously treated CLL.



Note. Shown are mRNA expressions of CD86(p = 0.0247), HVEM (p = 0.0014), PTDSS1 (p = 0.0146) and PTDSS2(p = 0.0397) in untreated and previously treated CLL. Values of gene expression are calculated as relative expression.

Figure 7

Significant differences in CEACAM1- and PDL2-expression comparing previously untreated to previously treated CLL.



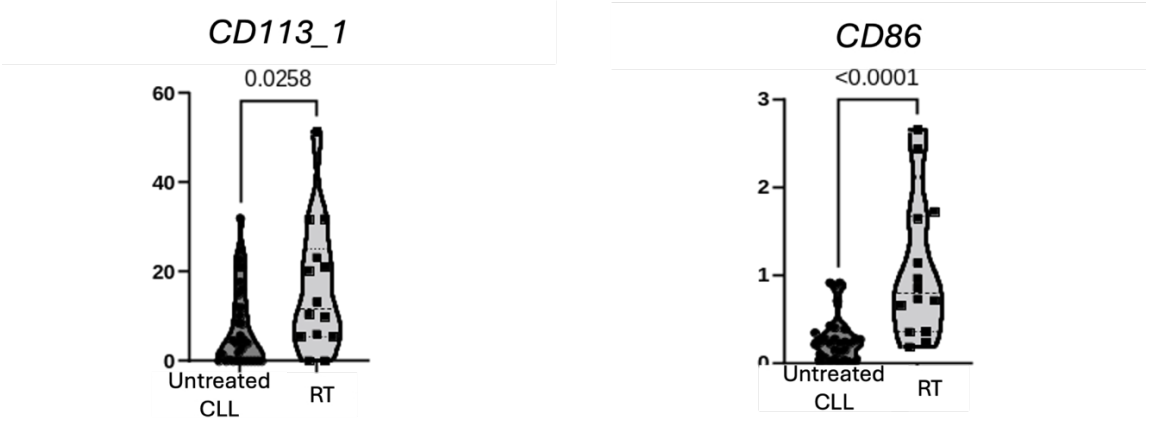
Note. Shown are mRNA expressions of CEACAM1 (p = 0.0121) and PDL2 (p = 0.0356) in untreated and previously treated CLL. Values of gene expression are calculated as relative expression.

Remarkably, we found *CD113_1* being 2.83-fold higher (p = 0.0258) and *CD86* was 3.62-fold higher (p < 0.0001) expressed in RT- compared to untreated CLL-lymph nodes (Figure 8).

Lower expressed were *CEACAM1* with 3.67-fold lower (p = 0.0007), *CD160* 1114.79-fold lower (p < 0.0001) and *VISTA* 1.5-fold lower (p = 0.0347) when comparing RT- to untreated CLL-lymph nodes (Figure 9).

Figure 8

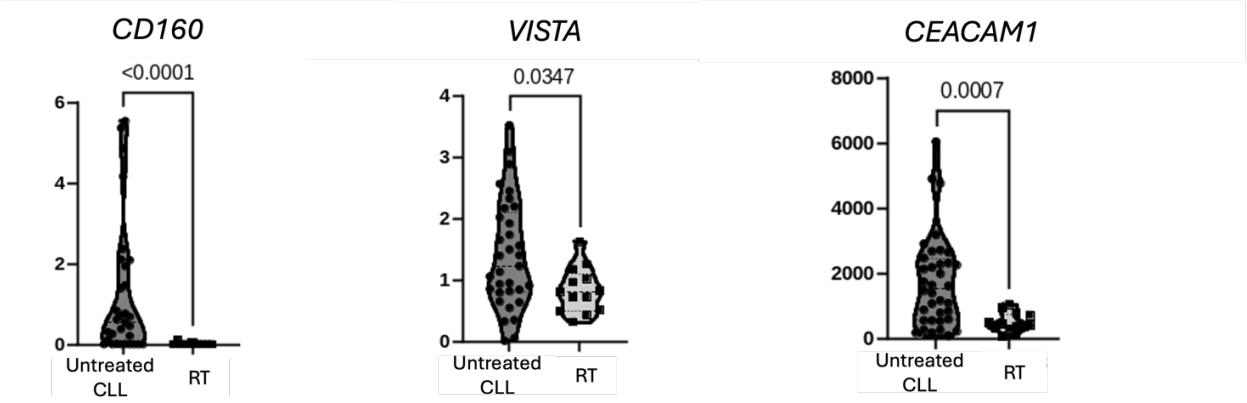
Significant differences in CD113_1 and CD86-expression comparing previously untreated CLL to RT.



Note. Shown are mRNA expressions of CD113_1 (p = 0.0258) and CD86 (p <0.0001) in untreated CLL and RT lymph nodes. Values of gene expression are calculated as relative expression. RT denotes Richter specimens, transformed CLL.

Figure 9

Significant differences in CD160-, VISTA-, and CEACAM1-expression comparing previously untreated CLL to RT.

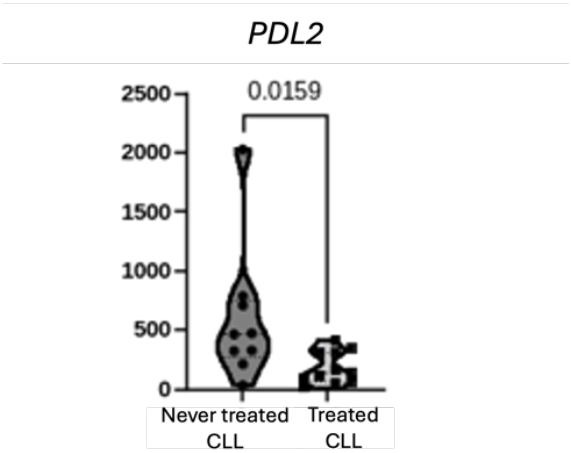


Note. Shown are mRNA expressions of CD160 ($p < 0.0001$), VISTA ($p = 0.0347$) CEACAM1 ($p = 0.0007$) in untreated CLL and RT lymph nodes. Values of gene expression are calculated as relative expression. RT denotes Richter specimens, transformed CLL.

Comparing the nodes that never ended up having therapy (with survival of at least 5 years) to those with previous immunochemotherapy with PR/CR, we discovered *PDL2* being significantly lower expressed in pretreated CLL-lymph nodes. The median of never treated nodes was, compared to the median of treated ones with CLL, 3.45-fold higher ($p = 0.0159$) (Figure 10).

Figure 10

Significant difference in PDL2-expression comparing never treated to previously treated CLL.

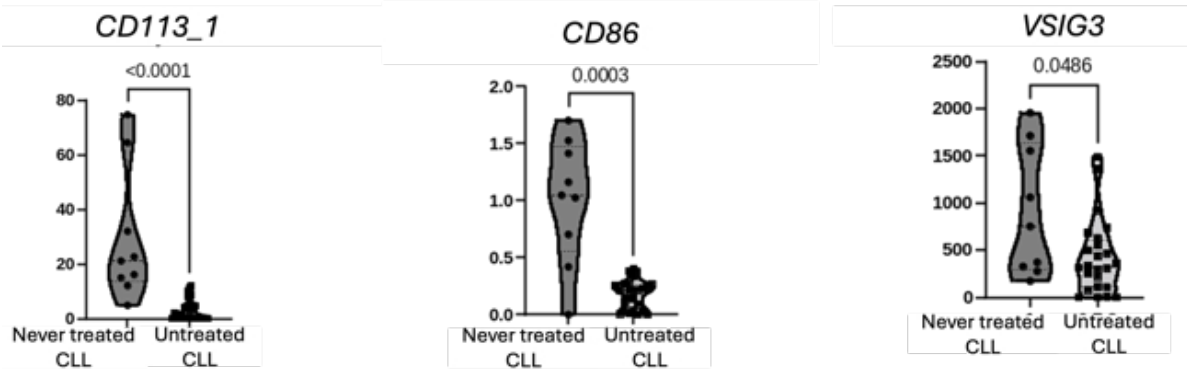


Note. Shown is the mRNA expressions of PDL2 (p = 0.0159) in never treated CLL and and previously treated CLL lymph nodes. Values of gene expression are calculated as relative expression.

Furthermore, we exploratively investigated the difference between never treated CLL-and untreated CLL-lymph nodes that ended up with treatment later. In the group of never treated CLL-lymph nodes we showed that the expression of *CD113_1* was 23.73-fold higher ($p < 0.0001$), *CD86* was 4.96-fold higher ($p = 0.0003$) and *VSIG3* was 2.33-fold higher ($p = 0.0486$) in comparison to untreated CLL-lymph nodes receiving treatment later (Figure 11). *CEACAM1* was the only immune-checkpoint component we measured being lower, 4.52-fold lower ($p = 0.0017$), in this group (Figure 12).

Figure 11

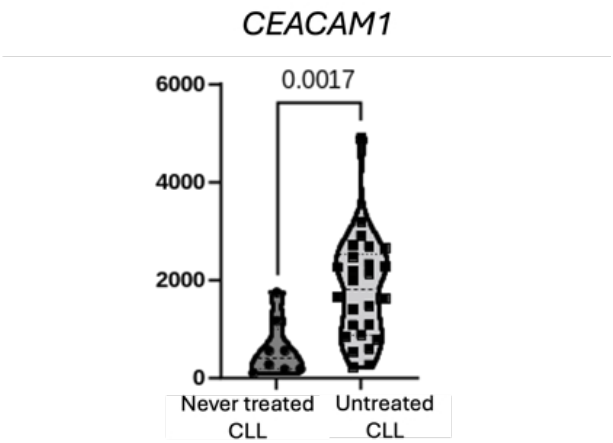
Significant differences in CD113_1-, CD86- and VSIG3-expression comparing never treated to previously untreated CLL.



Note. Shown are mRNA expressions of CD113_1 ($p < 0.0001$), CD86 ($p = 0.0003$) and VSIG3 ($p = 0.0486$) in never treated CLL and previously untreated CLL lymph nodes. Values of gene expression are calculated as relative expression.

Figure 12

Significant difference in CEACAM1-expression comparing never treated to previously untreated CLL.

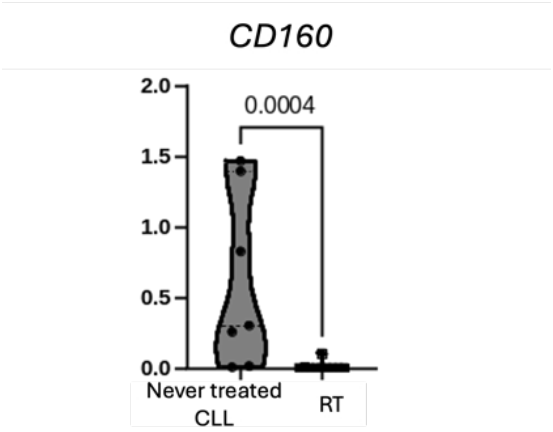


Note. Shown is the mRNA expressions of CEACAM1 ($p = 0.0017$) in never treated CLL and previously untreated CLL lymph nodes. Values of gene expression are calculated as relative expression.

The only significant finding when comparing never treated CLL-lymph nodes to nodes with current RT was *CD160* being 626.04-fold ($p = 0.0004$) lower expressed in RT-lymph nodes (Figure 13).

Figure 13

Significant differences in CD160-expression comparing never treated CLL to RT.



Note. Shown is the mRNA expressions of CD160 (p = 0.0004) in never treated CLL and RT lymph nodes. Values of gene expression are calculated as relative expression. RT denotes Richter specimens, transformed CLL.

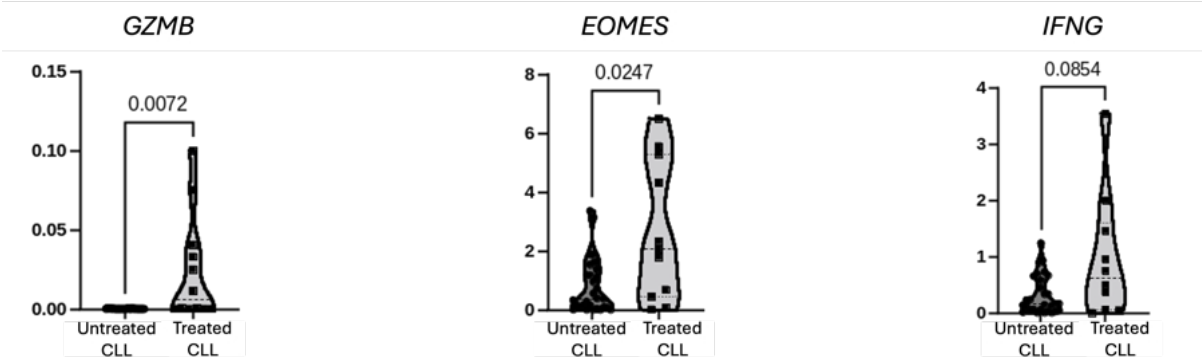
3.2.2 Expression of Immune-Cell Markers

Also, little is known about the expression of immune-cell markers in CLL-lymph node tissue. This is why we investigated mRNA expression levels of 9 well-characterized immune-cell markers in primary lymphoma tissue samples. Therefore, we exploratively studied the differences in the different subgroups of our cohort. Non-significant expressions of Immune-Cell-Markers can be found in the Supplements, Figure 36-39.

When investigating the difference between pretreated CLL-lymph nodes and untreated ones, we found *IFNG* being 3.79-fold higher (p = 0.0854), *EOMES* being 5.52-fold higher (p = 0.0247) and *GZMB* being 117.53-fold higher (p = 0.0072) in pretreated nodes (Figure 14). They all count as cancer inhibitory molecules.

Figure 14

Significant differences in GZMB-, EOMES- and IFNG-expression comparing previously untreated to previously treated CLL.

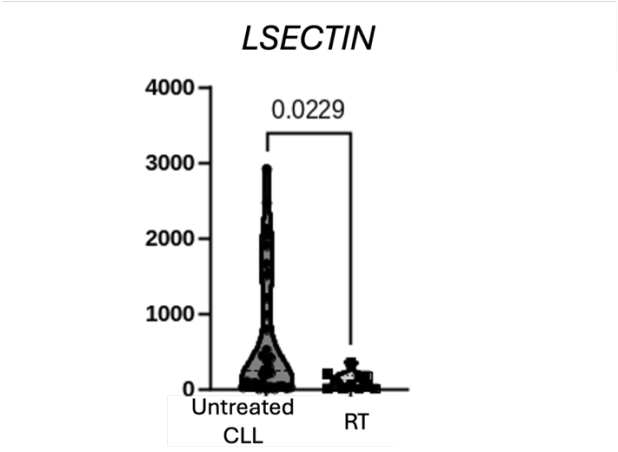


Note. Shown are mRNA expressions of GZMB (p = 0.0072), EOMES (p = 0.00247) and IFNG (p = 0.0854) in untreated CLL and previously treated CLL lymph nodes. Values of gene expression are calculated as relative expression.

We found *LSECTIN* being 4.41-fold lower (p = 0.0229) when comparing RT- to untreated CLL-lymph nodes (Figure 15).

Figure 15

Significant difference in LSECTIN-expression comparing previously untreated CLL to RT.



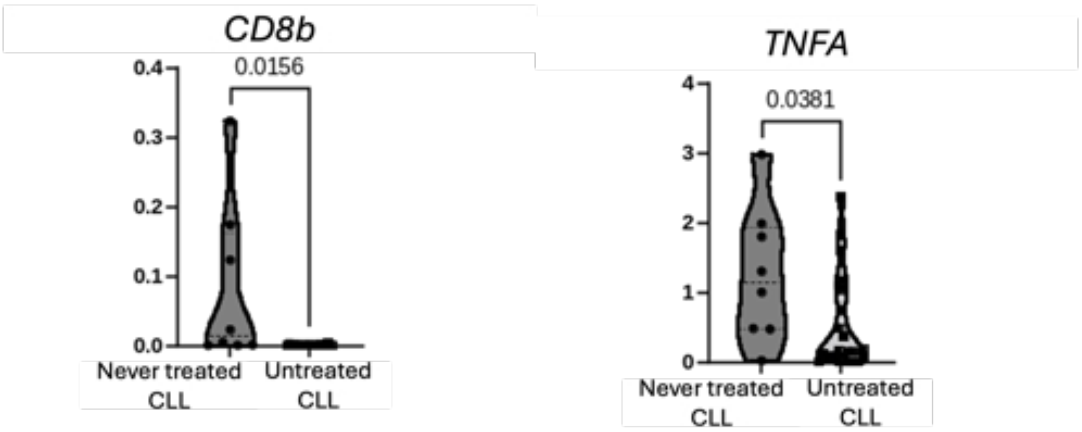
Note. Shown is the mRNA expression of LSECTIN (p = 0.0229) in previously untreated CLL and RT lymph nodes. Values of gene expression are calculated as relative expression. RT denotes Richter specimens, transformed CLL.

In our explorative investigation of differences between untreated CLL-lymph nodes that ended up with therapy later and never treated ones, *CD8b* as cancer inhibitory molecule was 38.34-fold lower (p = 0.0156) and *TNFA* was 7.76-fold lower (p = 0.0381) expressed in

untreated CLL-lymph nodes that ended up with therapy later (Figure 16) compared to never treated ones whereat *LSECTIN* was 5.21-fold higher (p = 0.0215) expressed in untreated nodes that ended up with therapy later (Figure 17).

Figure 16

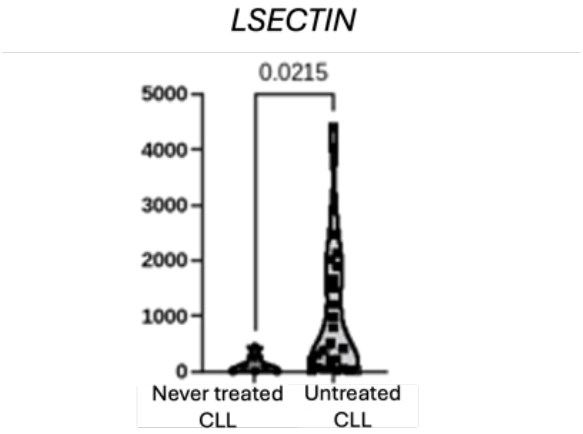
Significant differences in CD8b- and TNFA-expression comparing never treated to previously untreated CLL.



Note. Shown are mRNA expressions of CD8b (p = 0.0156) and TNFA (p = 0.0381) in never treated CLL and previously untreated CLL lymph nodes. Values of gene expression are calculated as relative expression.

Figure 17

Significant difference in LSECTIN-expression comparing never treated to previously untreated CLL.

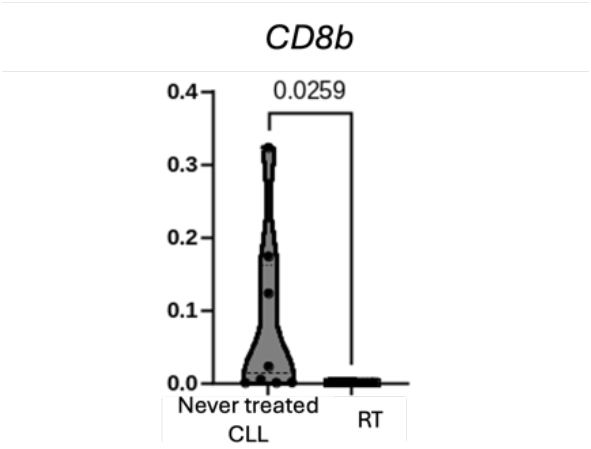


Note. Shown is the mRNA expressions of LSECTIN (p = 0.0215) in never treated CLL and previously untreated CLL lymph nodes. Values of gene expression are calculated as relative expression.

CD8b as cancer inhibitory molecule was 49.06-fold higher (p = 0.0259) expressed in never treated CLL-lymph nodes compared to nodes with current RT (Figure 18).

Figure 18

Significant difference in CD8b-expression comparing never treated CLL to RT.



Note. Shown is the mRNA expression of CD8b (p = 0.0259) in never treated CLL and RT lymph nodes. Values of gene expression are calculated as relative expression. RT denotes Richter specimens, transformed CLL.

3.2.3 NR4As Expression

We performed the same analyses and comparisons to comprehensively study the differences in the expressions of the NR4A-group. Interestingly, no significant difference of any component of the NR4A-group could be found in our research. Graphs of the *NR4A*-expressions can be found in the Supplements, Figure 40.

3.2.4 Overall survival Probability and Probability of Therapy

To investigate the clinical relevance of the generated expression data, we exploratively set the mRNA expression of the 31 genes in relation to overall survival and probability of therapy of our CLL cohort using the first quartile, the median and the third quartile. This is shown in the following graphs (Figure 19-21) and table (Table 7).

Table 7

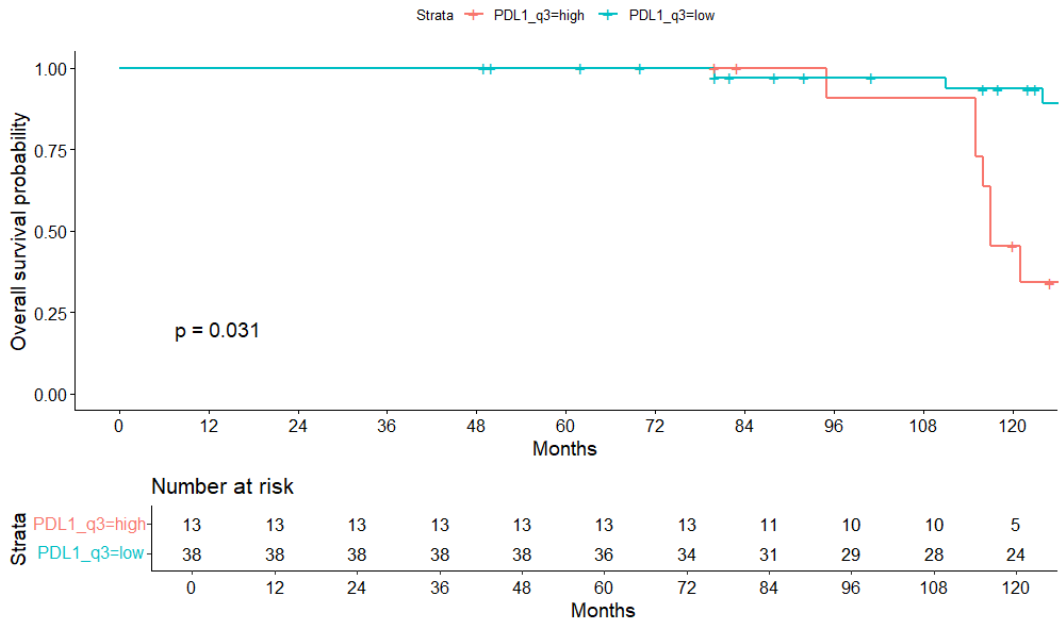
Overall survival probability displayed in relation to gene expression.

		Overall survival probability			
	Genes	Q1	M	Q3	worse survival in:
Immune-checkpoint components – B7-CD28 family	*PD1*	$p > 0.05$	$p > 0.05$	$p = 0.052$	high *PD1*
	PDL1	$p > 0.05$	$p > 0.05$	$\mathbf{p = 0.031}$	**high *PDL1***
Immune-checkpoint components – TIM-family	*GALECTIN 9*	$p = 0.058$	$p > 0.05$	$p > 0.05$	low *GALECTIN9*
Immune-cell markers – Igs	***LSECTIN***	$p > 0.05$	$p > 0.05$	$\mathbf{p = 0.026}$	**high *LSECTIN***
Immune-cell markers – cytokines	*TNFA*	$p > 0.05$	$p > 0.05$	$p = 0.059$	high *TNFA*
Immune-cell markers – COX	***CD8b***	$p > 0.05$	$p = 0.06$	$\mathbf{p = 0.028}$	**high *CD8b***

Note. Comparison of the significance of Q1, M and Q3 in different genes and gene groups according to Overall survival Probability. Q1 denotes the first quartile, M the Median and Q3 the third quartile of mRNA expression levels.

Figure 19

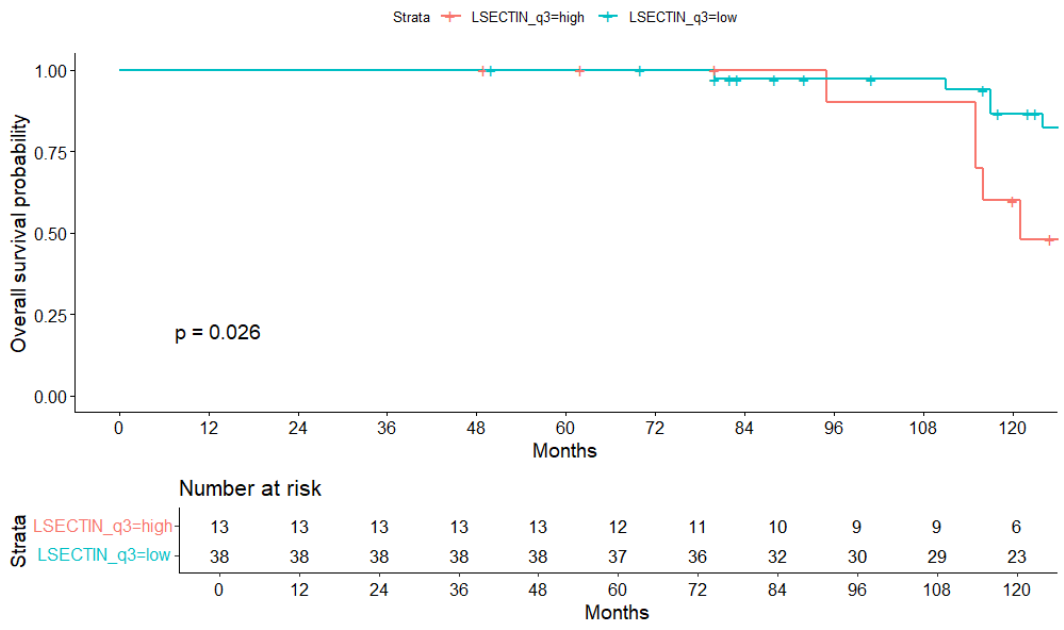
Kaplan-Meier curve of PDL1-expression and Overall survival probability.



Note. Association of lower Overall survival probability in high levels of PDL1 compared to low levels of PDL1 in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 20

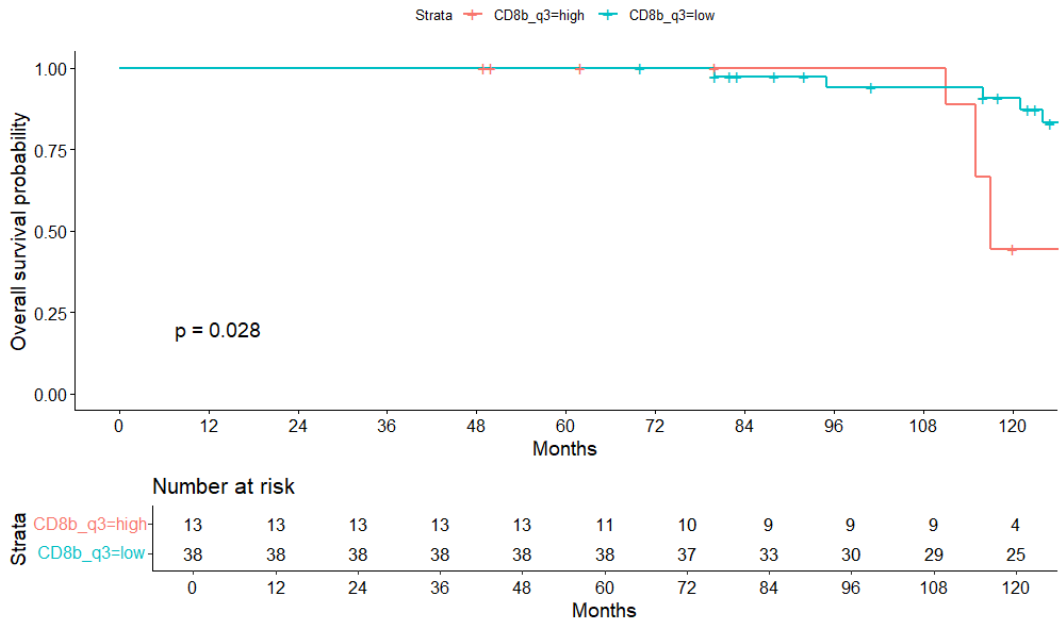
Kaplan-Meier curve of LSECTIN-expression and Overall survival probability.



Note. Association of lower Overall survival probability in high levels of LSECTIN compared to low levels of LSECTIN in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 21

Kaplan-Meier curve of CD8b-expression and Overall survival probability.



Note. Association of lower Overall survival probability in high levels of CD8b compared to low levels of CD8b in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Furthermore, we also observed an association of time to therapy and expression of certain immune checkpoint components and immune cell markers, as shown in Figure 22-30 and Table 8.

Table 8

Probability of therapy displayed in relation to gene expression.

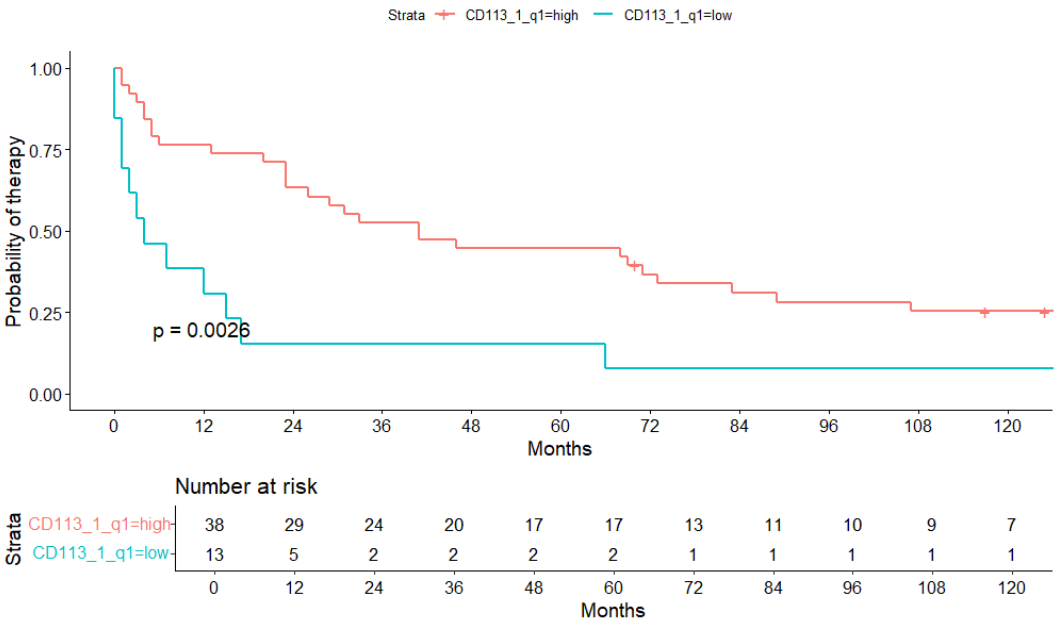
	Probability of Therapy				
	Genes	Q1	M	Q3	Higher probability of therapy in:
Immune-checkpoint components – Nectin- and Nectin-like	***CD113_1***	***p* = 0.0026**	*p* = 0.026	*p* = 0.096	**low *CD113_1***

family					
Immune-checkpoint components – B7-CD28 family	***CD86***	$p > 0.05$	$p = 0.058$	$\mathbf{p = 0.044}$	**low *CD86***
	PD1	$p > 0.05$	$p > 0.05$	$p = 0.059$	high *PD1*
	VSIG3	$\mathbf{p = 0.029}$	$p > 0.05$	$p > 0.05$	**low *VSIG3***
Immune-checkpoint components – TIM-family	*CEACAM1*	$p > 0.05$	$p > 0.05$	$p = 0.074$	high *CEACAM1*
	GALECTIN9	$p > 0.05$	$p > 0.05$	$\mathbf{p = 0.031}$	**high *GALECTIN9***
	PTDSS2	$p > 0.05$	$p > 0.05$	$\mathbf{p = 0.0057}$	**high *PTDSS2***
Immune-cell markers – granzymes	***GZMA***	$p > 0.05$	$p > 0.05$	$\mathbf{p = 0.0095}$	**high *GZMA***
Immune-cell markers – Igs	***LSECTIN***	$p > 0.05$	$\mathbf{p = 0.0054}$	$p > 0.05$	**high *LSECTIN***
Immune-cell markers – COX	***EOMES***	$p > 0.05$	$p > 0.05$	$\mathbf{p = 0.035}$	**high *EOMES***
	IFNG	$p > 0.05$	$\mathbf{p = 0.0013}$	$p = 0.0015$	**high *IFNG***

Note. Comparison of the significance of Q1, M and Q3 in different genes and gene groups according to Probability of Therapy. Q1 denotes the first quartile, M the Median and Q3 the third quartile of mRNA expression levels.

Figure 22

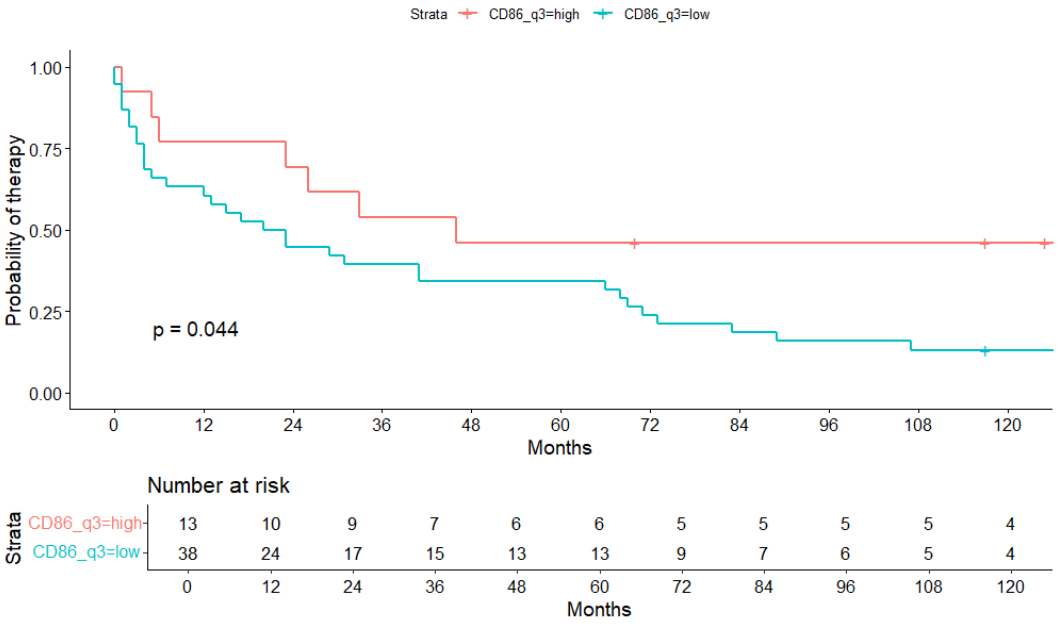
Kaplan-Meier curve of CD113_1-expression and Probability of Therapy.



Note. Association of lower Probability of therapy in high levels of CD113_1 compared to low levels of CD113_1 in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 23

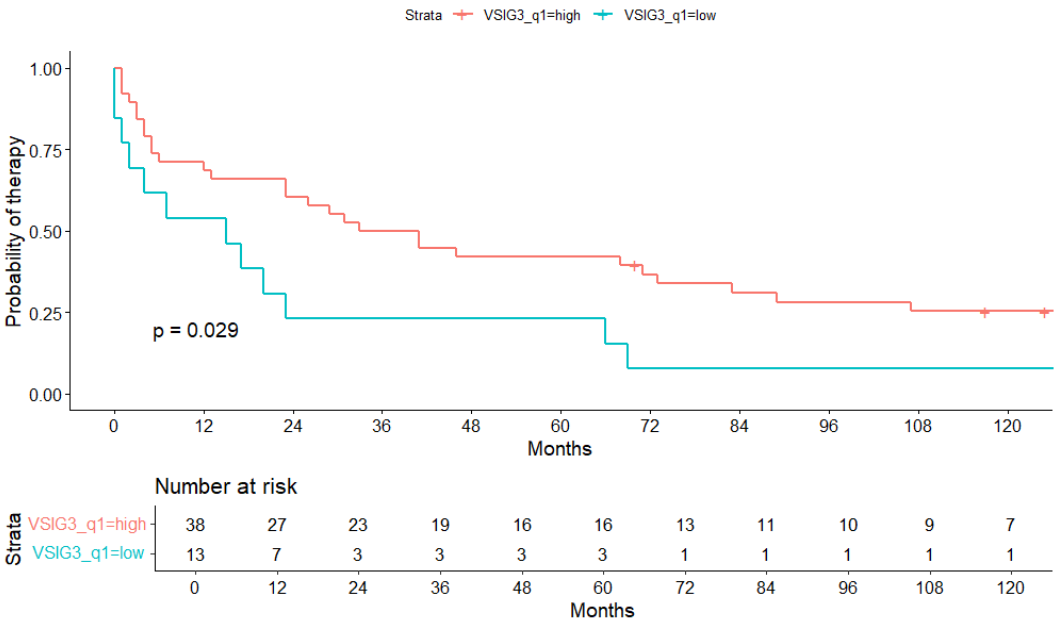
Kaplan-Meier curve of CD86-expression and Probability of Therapy.



Note. Association of lower Probability of therapy in high levels of CD86 compared to low levels of CD86 in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 24

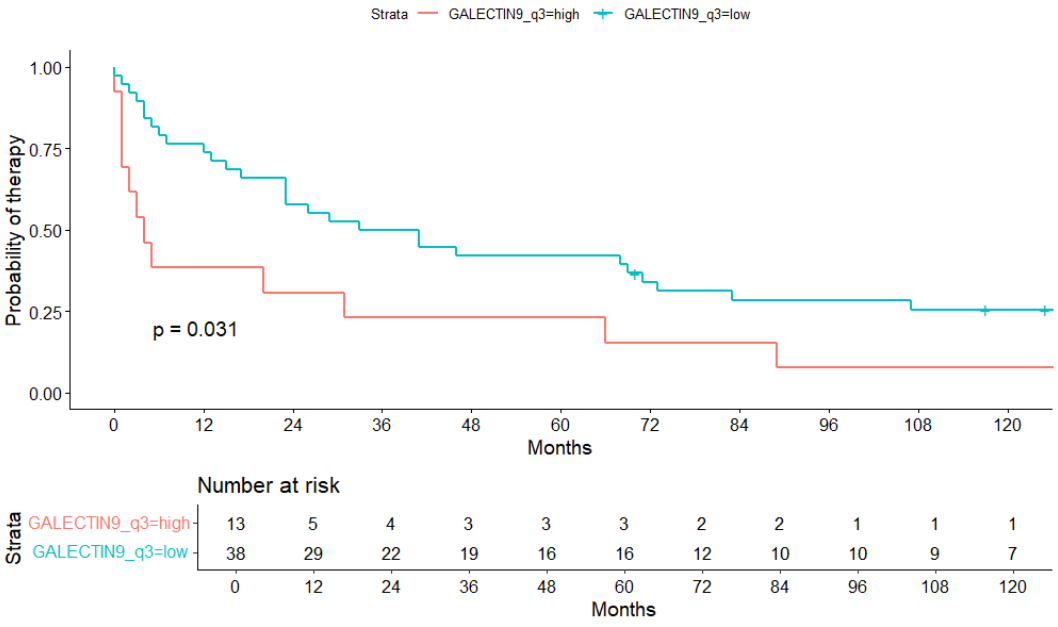
Kaplan-Meier curve of VSIG3-expression and Probability of Therapy.



Note. Association of lower Probability of therapy in high levels of VSIG3 compared to low levels of VSIG3 in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 25

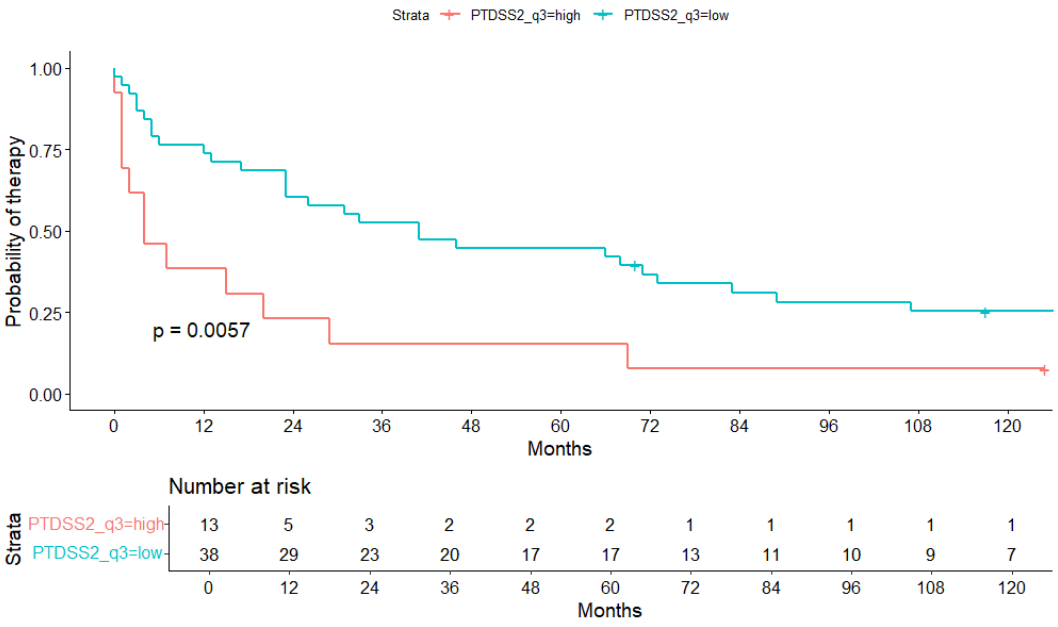
Kaplan-Meier curve of GALECTIN9-expression and Probability of Therapy.



Note. Association of higher Probability of therapy in high levels of GALECTIN9 compared to low levels of GALECTIN9 in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 26

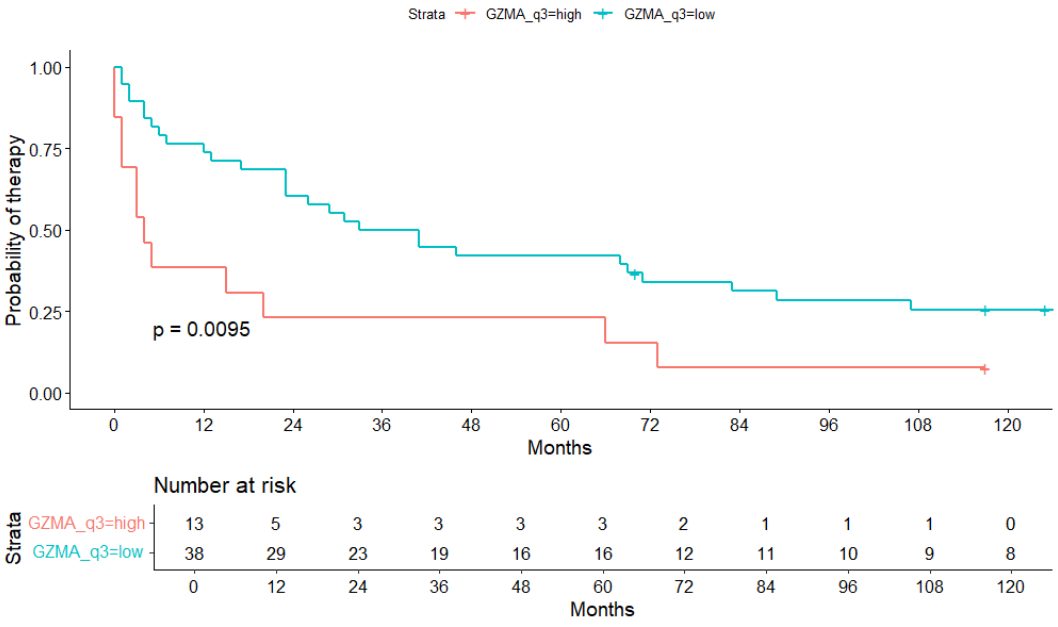
Kaplan-Meier curve of PTDSS2-expression and Probability of Therapy.



Note. Association of higher Probability of therapy in high levels of PTDSS2 compared to low levels of PTDSS2 in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 27

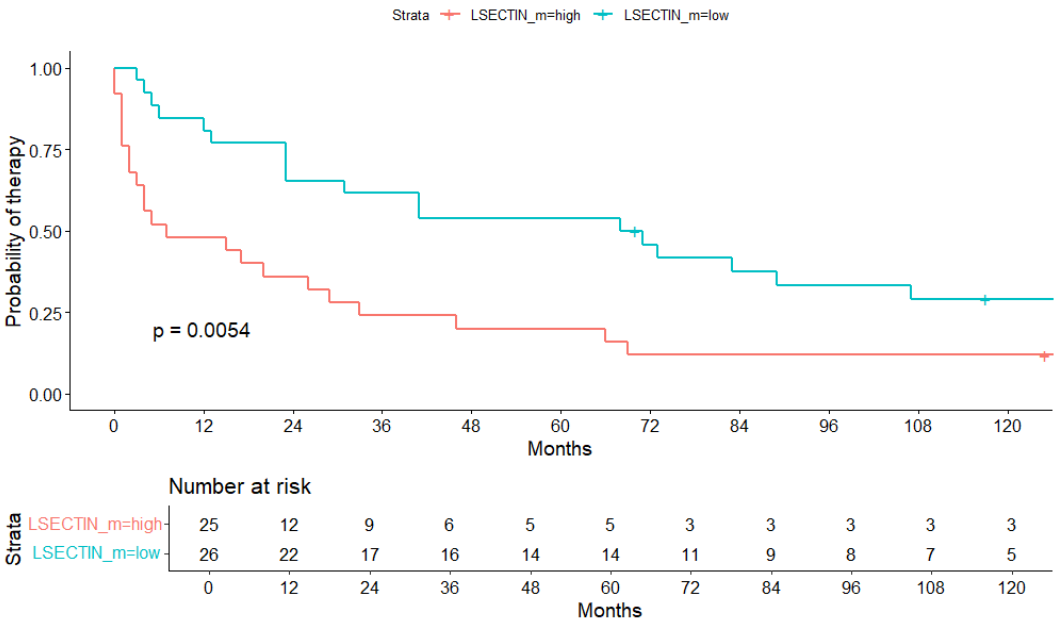
Kaplan-Meier curve of GZMA-expression and Probability of Therapy.



Note. Association of higher Probability of therapy in high levels of GZMA compared to low levels of GZMA in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 28

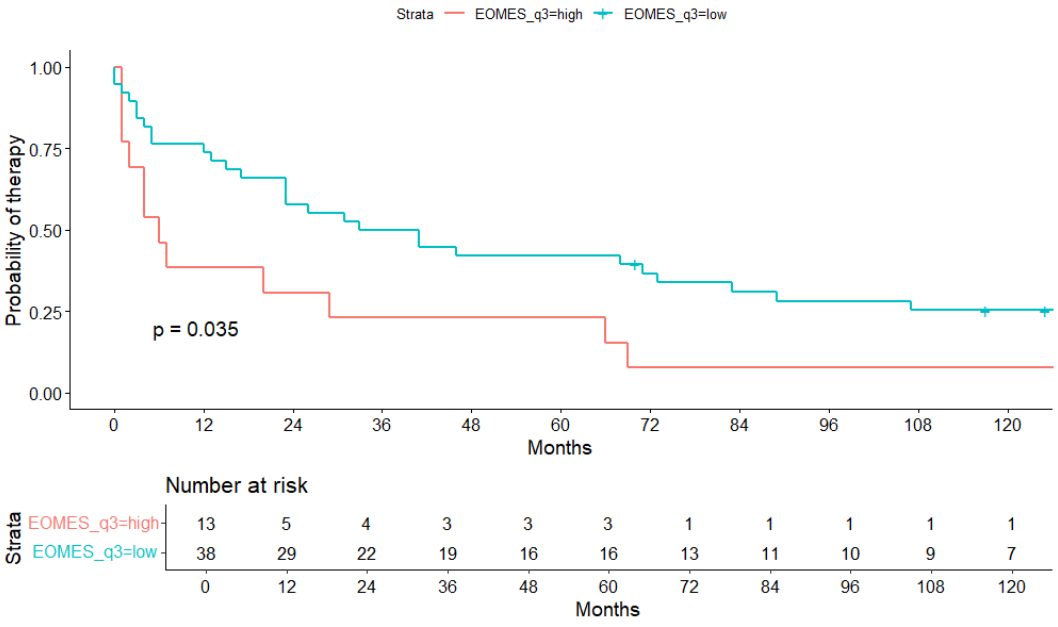
Kaplan-Meier curve of LSECTIN-expression and Probability of Therapy.



Note. Association of higher Probability of therapy in high levels of LSECTIN compared to low levels of LSECTIN in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 29

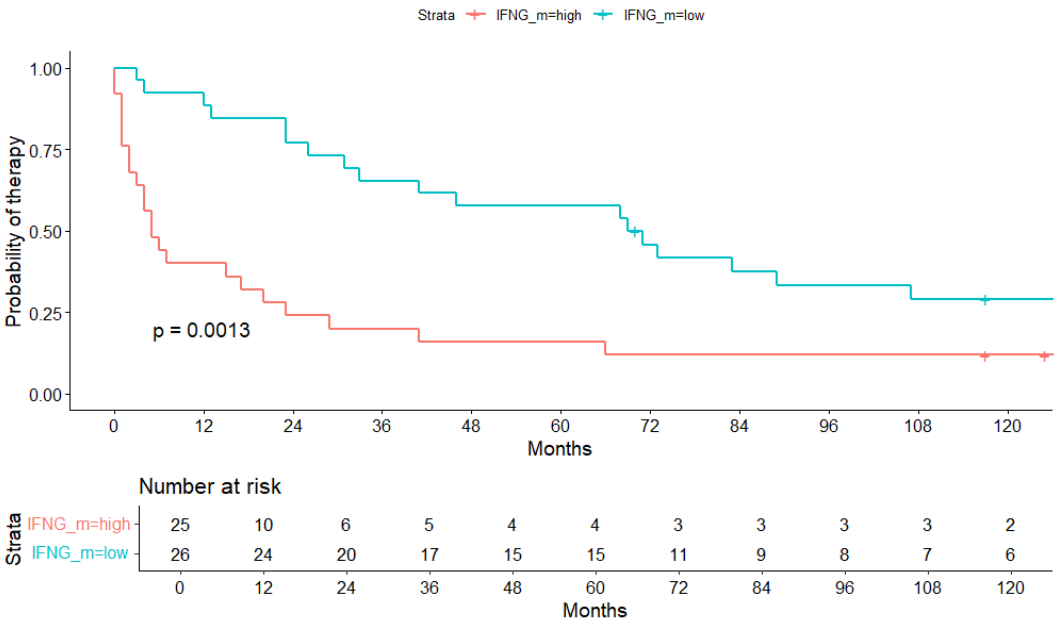
Kaplan-Meier curve of EOMES-expression and Probability of Therapy.



Note. Association of higher Probability of therapy in high levels of EOMES compared to low levels of EOMES in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

Figure 30

Kaplan-Meier curve of IFNG-expression and Probability of Therapy.



Note. Association of higher Probability of therapy in high levels of IFNG compared to low levels of IFNG in CLL lymph nodes. Below the graph the absolute number of patients/lymph nodes can be seen.

3.2.5 Expression pattern of Immune Checkpoint Components and Immune cell markers

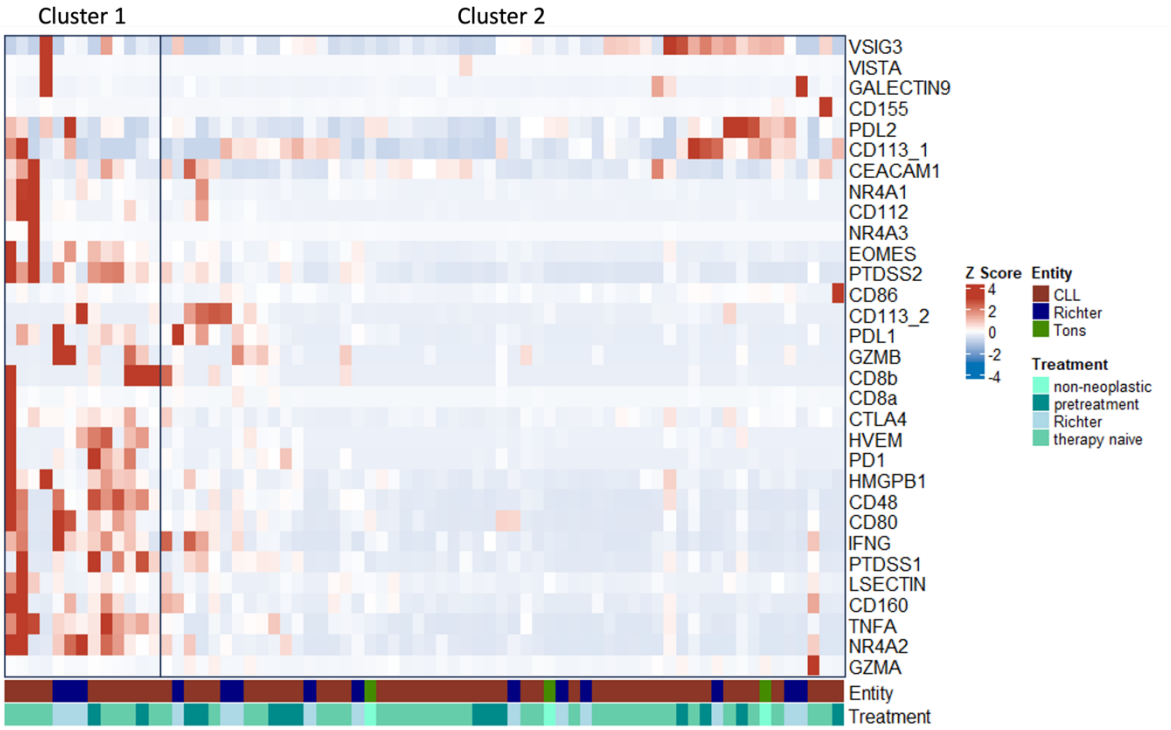
Furthermore, we wanted to explore the mRNA expression pattern, why we performed hierarchical clustering using ΔΔCT-values. Figure 31 depicts two discrete clusters of the samples (n = 70), based on their expression profiles.

Cluster 1, which is associated with an elevated immune response, comprises 10 CLL- and three RT-lymph nodes, whereat Cluster 2 comprises of 43 CLL-, 11 RT- and three non-neoplastic lymph nodes. In regard of relation to treatment, Cluster 1 showed two pretreated CLL-, eight previously untreated CLL- and three RT-lymph nodes. Cluster 2 comprises 12 pretreated CLL-, 34 previously untreated CLL-, 11 RT- and three non-neoplastic lymph nodes.

However, we were able to show that this analysis is not appropriate for dividing the subgroups of our cohort and to assign them to one Cluster. Our heatmap rather represents the heterogeneity of CLL (Figure 31).

Figure 31

Heatmap of CLL, RT and non-neoplastic tonsils.



Note. Richter denotes Richter specimens, transformed CLL. Tons denotes non neoplastic tonsils that served as control. Further displayed is the relation to treatment.

Furthermore, we investigated our CLL-cohort (n = 51) in regard of the relation to treatment more closely and set into relation with clinical survival and time to therapy for our 31 genes. First, we performed another hierarchical cluster using ΔΔCT-values and found two clusters with Cluster 1 highly expressing inflammatory genes (Figure 31). Therefore, we named it inflamed cluster. Cluster 1 comprises three pretreated CLL- and 11 previously untreated CLL-lymph nodes whereat in Cluster 2 10 pretreated CLL- and 27 previously untreated CLL-lymph nodes can be found. Furthermore, we analyzed the Overall survival probability comparing Cluster 1 and Cluster 2 discovering an association of lower overall survival probability (p = 0.07) in our inflamed Cluster 1, compared to Cluster 2 (Figure 32). We didn't find any association of our Clusters with the probability of therapy.

Figure 32

Heatmap of pretreated and therapy naive CLL lymph nodes depicting two clusters with Kaplan-Meier curve of the clusters in regard to the Overall survival probability.

Note. The Heatmap of pretreated and therapy naive CLL lymph nodes depicting two clusters. Below showing the analysis of a lower overall survival probability of Cluster 1 compared to Cluster 2, also showing the absolute number at risk.

4. Discussion

Our study's aim was the explorative investigation of the mRNA expression pattern of 19 immune-checkpoint components, 9 immune-cell markers and 3 NR4A members in CLL and RT lymph nodes. Several of these genes have been proven to play a crucial role in lymphomagenesis, immune evasion and regulation (83), but most of them have not yet been investigated intensively in CLL and RT lymph node samples. Even though our cohort was quite small we discovered interesting associations in the mRNA expression in the different subgroups, in the overall survival and time to therapy. To the best of our knowledge, we were one of the first to use CLL- and RT-lymph node samples and to measure the mRNA expression instead of measuring the effector molecule in peripheral blood.

11 genes (*PDL2*, *GALECTIN9*, *CD86*, *HVEM*, *PTDSS1*, *PTDSS2*, *CEACAM1*, *CD113_1*, *CD160*, *VISTA*, *VSIG3*) belonging to the group of immune-checkpoint components were differently expressed comparing the different subgroups of CLL and RT.

We found lower *PDL2* expression in CLLs compared to our non-neoplastic controls. Furthermore, *PDL2* was higher expressed in treatment-naive CLL lymph nodes compared to previously treated ones. Notably, in our cohort we did not find any association of *PDL2* with survival, but a higher *PDL1* expression was associated with poorer survival. Data on PDL2 in CLL is very limited. Korkmaz et al. (150) investigated expression of *PDL2* in CLL, but they did not observe any association of higher *PDL2* with poorer survival due to a very small cohort.

GALECTIN9 was lower expressed in RT compared to our non-neoplastic tonsils. For de novo DLBCL, it was shown that TIM3/GALECTIN9-axis causes immune evasive processes in DLBCL (111). This finding contrasts with our findings. This might be caused by different method of analyses, small cohort, different non-neoplastic control, and the fact that we investigated RT-DLBCL instead of de novo DLBC. Furthermore, we showed that low *GALECTIN9* expression was associated with a lower probability of therapy in CLL lymph nodes. As several recent researchers published GALECTIN9 as a new potential biomarker of disease prognosis, our findings are concordant with theirs (112,113,151–153). They also pointed out the relevance of GALECTIN9 in immune escape of the tumors. Several studies stated the Tim3/GALECTIN9-axis as new additional therapeutic target in combination with PD1/PDL1 targeted therapies (112,113). But still, the exact biological role of GALECTIN9 is not fully understood and needs more research.

CD86 is lower expressed in therapy naïve compared to previously treated CLL, untreated CLL and RT. Furthermore, we found a higher *CD86* expression in RT compared to all of our CLL cases. Our finding is concordant with their suggestion of patients with higher *CD86* levels presenting with higher proliferation rate (154,155). We found an association between a high *CD86* expression and a lower probability of therapy in contrast to the study of Huemer et al. (154) and Takács et al. (155). This might be caused by the different types of samples, we were using (lymph node vs. peripheral blood).

Furthermore, we found lower *HVEM* expression in therapy naive CLL lymph nodes compared to treated ones. HVEM is ligand of BTLA with inhibitory function, but also of CD160 with activating function. Binding to BTLA, HVEM negatively regulates T cell functions (103–105). The BTLA/HVEM-axis has been found dysbalanced in several solid and hematological tumors as well as in CLL. This leads to a possible impairment of immunosurveillance (104). Data of HVEM/BTLA expression in CLL is very limited.

PTDSS1 was found lower expressed in untreated compared to previously treated lymph nodes. No literature for *PTDSS1* expression in CLL was found, but in other cancers. Therefore, PTDSS1 might also play a role in the pathogenesis and tumor promotion of CLL.

Interestingly, *PTDSS2* was lower expressed in untreated compared to previously treated lymph nodes and we were able to show an association with lower probability of therapy in patients with lower *PTDSS2* expression levels. Our findings in CLL are comparable, but further investigation is needed.

CEACAM1 showed a lower expression in RT compared to CLL, also it was lower expressed in RT- and never treated CLL- compared to previously untreated CLL-lymph nodes. We did not find a significant difference between RT and untreated CLL-lymph nodes. CEACAM1 is known to play a role in different cancers. De novo expression of CEACAM1 was found to be associated with progression and reduced disease-free survival in different tumors with an important role in tumor progression, invasion and metastatic potential (156–158). Further investigation of the role of CEACAM1 in CLL still needs to be performed.

We found *CD113* being lower expressed in previously untreated CLL compared to RT and untreated CLL. We also found a positive association between a high *CD113* expression and a lower probability of therapy. Data on CD113 in CLL is very limited. Studies have pointed out the role of CD113 being associated with poor clinical outcome in ovarian cancer cells and in breast cancer (159,160). Even though these findings are with different tumor entities, they are analogous to our finding of a positive association between high *CD113* expression and lower probability of therapy in CLL.

CD160 is not expressed in normal B cells, but abnormally in CLL cells. During the last years studies showed, that it is a new prognostic marker in CLL and elevated levels indicate enhanced CLL-cell survival and cellular activation, but also T-cell exhaustion (98,161,162). Our findings of elevated *CD160* mRNA expression levels in CLL lymph nodes in association with lower OS allows the conclusion supporting the findings of these studies. Moreover, we found higher *CD160* expression in therapy naive CLL lymph nodes, compared to RT nodes. Additionally, *CD160* is higher expressed in all our CLL lymph nodes compared to RT. These findings cannot be explained by the results reported in literature of higher CD160 expression levels in patients with higher RAI-stages (161). This might be caused by different cohorts and methods used. Of course, further studies need to investigate the exact role and mechanism of CD160 in CLL.

VISTA was higher expressed in previously untreated CLL- compared to RT-lymph nodes. No data of VISTA expression in CLL is available to date, but VISTA is suspected as a potential therapeutic target in acute myeloid leukemia (AML) (163). In cervical cancer the elevated expression of VISTA was associated with poor survival indicating its use as a biomarker (164).

Our analysis for *VSIG3* showed that it was higher expressed in therapy naive lymph nodes, and we were able to show an association with lower probability of therapy in patients with higher *VSIG3* expression. Data is very limited concerning the expression of *VSIG3* generally in tumors. As VSIG3 is a receptor of VISTA, Olbromski et al. (165) recently investigated both expression levels in cancer cells in invasive ductal breast carcinoma. They found an association with a higher tumor aggressiveness. Elevated levels of VSIG3 together with VISTA might show also influence on the modulation of immune responses to cancer (165). The exact levels and functions of VSIG3 in CLL remain to be uncovered to set our findings in relation to.

PDL1, as the most studied ligand of PD1, was found in higher levels in advanced clinical stages of CLL (112). It induces immune escape and evasion of tumor cells which leads Taghiloo et al. (112) to conclude that elevated PDL1 levels lead to a worse prognosis in CLL patients. This is consistent with our finding of a better OS in CLL patients with lower *PDL1* expression levels in their lymph nodes. But this still needs to be studied more precisely as well.

CD80 is a ligand of CTLA4. CTLA4 leads to a down-modulation of CD80 which leads through complex mechanisms to a negative regulation of T cell immune response (166,167). We found high *CD80* expression levels being associated with lower OS. According to the

above mentioned, high *CD80* levels indicate a positive regulation of T cell immune response. That would fit into our understanding of an inflamed cluster.

Six immune cell molecules (*GZMB*, *EOMES*, *IFNG*, *LSECTIN*, *CD8b*, *TNFA*) were found to be differentially expressed in the CLL- and RT-subgroups.

GZMB was lower expressed in CLL- compared to RT-lymph nodes and in previously untreated compared to previously treated ones. Usually, GZMB is expressed in immune cells, and it shows cell killing ability in the TME (119,120). At least two studies (120,168) described a lower expression of GZMB in colorectal carcinoma is associated with poor clinical outcome. However, the exact biological function and effect of different GZMB expression levels in CLL have not been in investigated so far.

EOMES was lower expressed in therapy naive compared to previously treated CLL-lymph nodes. Furthermore, we observed an association between lower expression of *EOMES* and lower likelihood of therapy. The work of Jiménez et al. (130) points out the importance of EOMES in the detection of exhausted CD8+ cells. Additionally, an association between increased levels of these exhausted CD8+ cells only in progressing CLL patients was shown (130). Comparing these findings to ours, we can speculate that similar effects might occur in our cohort.

IFNG was lower expressed in untreated CLL-lymph nodes compared to treated ones and low expression was associated with lower probability of therapy need. IFNG is the most important cytokine involved in anti-tumor immunity. Despite this effect, IFNG is also able to support tumor growth, i.e. through induction of PDL1-expression leading to immune escape of tumor cells through activation of the PD1 signaling axis (132–134). Studies have shown an association of low IFNG levels with poor outcome of cervical cancer and high expression levels indicating a significantly longer progression-free and overall survival in patients with ovarian cancer, respectively (169,170). Though this data is from different solid tumor entities, our findings are consistent with previous observations. Therefore, further investigation of IFNG levels in CLL needs to be done. As PD1 checkpoint blockade is not mentioned in current guidelines as a first-line therapeutic option in CLL (5,24), it is known as potential agent in relapsed CLL and even RT, rather in combination. But this needs to be evaluated further (5,171).

We found higher *LSECTIN* expression in therapy naive lymph nodes compared to previously treated or in untreated ones. Additionally, *LSECTIN* was also higher expressed in CLL compared to RT-lymph nodes. Furthermore, we found an association between low *LSECTIN* expression and better OS and lower probability of therapy. In several solid

tumors the expression of LSECTIN has already been investigated but not in CLL. Lower LSECTIN expression in hepatocellular carcinoma has been linked to poor prognosis, immune cell infiltration, and higher tumor grade, making it a potential therapeutic target (172,173). Wegscheider et al. (174) also identified LSECTIN as a prognostic marker in breast cancer, correlating with inferior disease-free survival, while in gastric cancer, it may contribute to lymphatic metastasis (173). Our findings contrast with existing literature due to differing tumor types, yet the association between lower LSECTIN expression and better OS with reduced therapy probability remains similar.

CD8b was higher expressed in never treated CLL lymph nodes compared to previously untreated and to RT lymph nodes. Additionally, we observed that low *CD8b* expression was associated with better OS in our cohort. CD8 is a marker for T cells, and it is speculated that its expression might be associated with the anti-tumor immune response. It could be that CD8+ T cells have anti CLL properties (83,111).

We found higher *TNFA* expression in never treated compared to previously untreated CLL-lymph nodes. Ferrajoli et al. (175) linked elevated TNFA in CLL to advanced disease stages, higher β2-microglobulin, lower Hb and PC, chromosomal abnormalities, and shorter survival, suggesting its prognostic and therapeutic relevance. Our findings differ, possibly due to sample origin and methodology. Further studies highlight TNFA and its receptor's role in CLL progression (176–178).

When investigating Clusters, in our heatmap Cluster 1, the inflamed cluster with association to a lower OS, containing high expression levels of inflammatory genes, i.e. *PTDSS1*, *PTDSS2*, *HMGPB1*, *CEACAM1*, *PDL1*, *CD160* and *CD80*, we were able to detect elevated expression levels. The first four genes code for TIM3-ligands. According to the above mentioned influence of this pathway on the immune system, more precisely on the immune escape of tumor and the development of a T cell disbalance (113), our findings of elevated expression levels of *PTDSS1*, *PTDSS2*, *HMGPB1* and *CEACAM1* in association with a lower OS might be explained.

In our Cluster 1, the inflamed cluster with association to a lower OS, we were also able to detect elevated expression levels i.e. of *IFNG*, *CD8b*, *CD48* and *TNFA*. They all lead to inflammation, immune response and invasion/activation of immune cells in the tumor environment (83). Possibly in the future a mRNA expression panel as Ayers et al. (179) detected, might be of use in the prediction of response to therapy and OS in CLL patients.

Furthermore, we found higher expression *NR4A1* and *NR4A2* in Cluster 1. Even though Deutsch et al. (135) investigated the expression of NR4A1 in aggressive B-cell lymphomas

and not in CLL, they were able to show an association of lower NR4A1 expression with poor cancer-specific survival and higher expression of NR4A1 with tumor suppressive function. In this study, NR4A2 was not reduced. NR4A2 is the least studied member of the NR4A-family, especially in hematologic malignancies. Its role in different solid cancer types has been described in various studies and in a recent review by Wan et al. (180). High NR4A2 expression in breast cancer correlates with better OS and lower tumor grade (181), though Llopis et al. (182) noted its dual role—essential for normal breast epithelial differentiation but tumor-promoting at low levels. In gastric cancer, high NR4A2 predicts worse postoperative survival, influenced by age, TNM stage, and therapy type (183). Its role varies across cancers, necessitating further investigation in CLL.

4.1 Limitations

Our monocentric study had a small, subdivided cohort and assessed only mRNA expression. FACS and IHC could have clarified cell-specific expression in lymph nodes. We couldn't determine if mRNA levels were from tumor or immune cells. Rai and Binet stages were inconsistently updated, limiting reliability. The cohort's heterogeneity in age, therapies, and comorbidities, along with evolving treatment guidelines over 10 years, complicated case comparability.

4.2 Conclusion

Our data suggest that the immune system, particularly immune checkpoint components, plays a crucial role in CLL development and progression. Additionally, current therapies may significantly influence immune-cell dynamics.

Our approach is novel, as few studies have examined mRNA expression in CLL lymph node tissues. We identified new genes with potential as therapeutic and prognostic targets, warranting further investigation. Moreover, we detected an inflamed CLL subtype associated with reduced OS. Its potential as a prognostic tool in clinical practice requires further comprehensive evaluation.

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Supplements

Nucleotide sequences of the used Primers

Table 9

Used Primers (forward and reverse) for RQ-PCR.

Gene	DNA-Sequence 5’ -> 3’	Length (bp)
hu_RQ_CTLA4_fw	CAT GAT GGG GAA TGA GTT GAC C	22
hu_RQ_CTLA4_rev	TCA GTC CTT GGA TAG TGA GGT TC	23
hu_RQ_PD1_fw	CCA GGA TGG TTC TTA GAC TCC C	22
hu_RQ_PD1_rev	TTT AGC ACG AAG CTC TCC GAT	21
hu_RQ_CD8a_fw	CTT ACC AGT GAC CGC CTT G	19
hu_RQ_CD8a_rev	AGG TTC CAG GTC CGA TCC	18
hu_RQ_EOMES_fw	CGC CAC CAA ACT GAG ATG AT	20
hu_RQ_EOMES_rev	CAG TGG GAT TGA GTC CGT TT	20
hu_RQ_GZMB_fw	TAC CAT TGA GTT GTG CGT GGG	21
hu_RQ_GZMB_rev	GCC ATT GTT TCG TCC ATA GGA GA	23
hu_RQ_IFNG_fw	TTT GGG TTC TCT TGG CTG TT	20
hu_RQ_IFNG_rev	TCC GCT ACA TCT GAA TGA CCT	21
hu_RQ_CD8b_fw	TCA ATC TCA CAA GCG TGA AGC	21
hu_RQ_CD8b_rev	GGT AAC CGG CAC ACT CTC TT	20
hu_RQ_PDL1_fw	TGG CAT TTG CTG AAC GCA TTT	21
hu_RQ_PDL1_rev	TGC AGC CAG GTC TAA TTG TTT T	22
hu_RQ_GZMA_fw	ATT CTT GGG GCT CAC TCA ATA AC	23
hu_RQ_GZMA_rev	GGG TCA TAG CAT GGA TAG GGA AA	23
hu_RQ_CD86_fw	CTG CTC ATC TAT ACA CGG TTA CC	23
hu_RQ_CD86_rev	GGA AAC GTC GTA CAG TTC TGT G	22
hu_RQ_CD80_fw	TGG AGT CTT ACC CTG AAA TCA AA	23
hu_RQ_CD80_rev	GTT CCT GGG TCT CCA AAG GT	20
hu_RQ_PDL2_fw	ACC GTG AAA GAG CCA CTT TG	20
hu_RQ_PDL2_rev	GCG ACC CCA TAG ATG ATT ATG C	22
hu_RQ_VISTA_fw	ACG CCG TAT TCC CTG TAT GTC	21
hu_RQ_VISTA_rev	TTG TAG AAG GTC ACA TCG TGC	21
hu_RQ_VSIG3_fw	CCA CGG TAG GGT AGG ATT TAC A	22
hu_RQ_VSIG3_rev	CTA TGT CTG GAA GGT TGT TGA CC	23
hu_RQ_HVEM_fw	ACC GAG AGT CAG GAC ACC C	19
hu_RQ_HVEM_rev	AGC AAA CAA TGA CGA TGA CGA	21
hu_RQ_CEACAM1_fw	TGC TCT GAT AGC AGT AGC CCT	21
hu_RQ_CEACAM1_rev	TGC CGG TCT TCC CGA AAT G	19
hu_RQ_GALECTIN9_fw	TCT GGG ACT ATT CAA GGA GGT C	22
hu_RQ_GALECTIN9_rev	CCA CTG GAG CTG AGA ACG G	19
hu_RQ_HMGPB1_fw	GAA GTG CTC AGA GAG GTG GAA	21
hu_RQ_HMGPB1_rev	TCC GCT TTT GCC ATA TCT TC	20
hu_RQ_PTDSS1_fw	ATC ACC CTG CTC AGC TTC AC	20
hu_RQ_PTDSS1_rev	GGA TGC CTC TCC AGA TGT TG	20
hu_RQ_PTDSS2_fw	CTC AGG ACA CGG CCT ACA AC	20
hu_RQ_PTDSS2_rev	AAA TGG CCC GTC TTT AGC TT	20

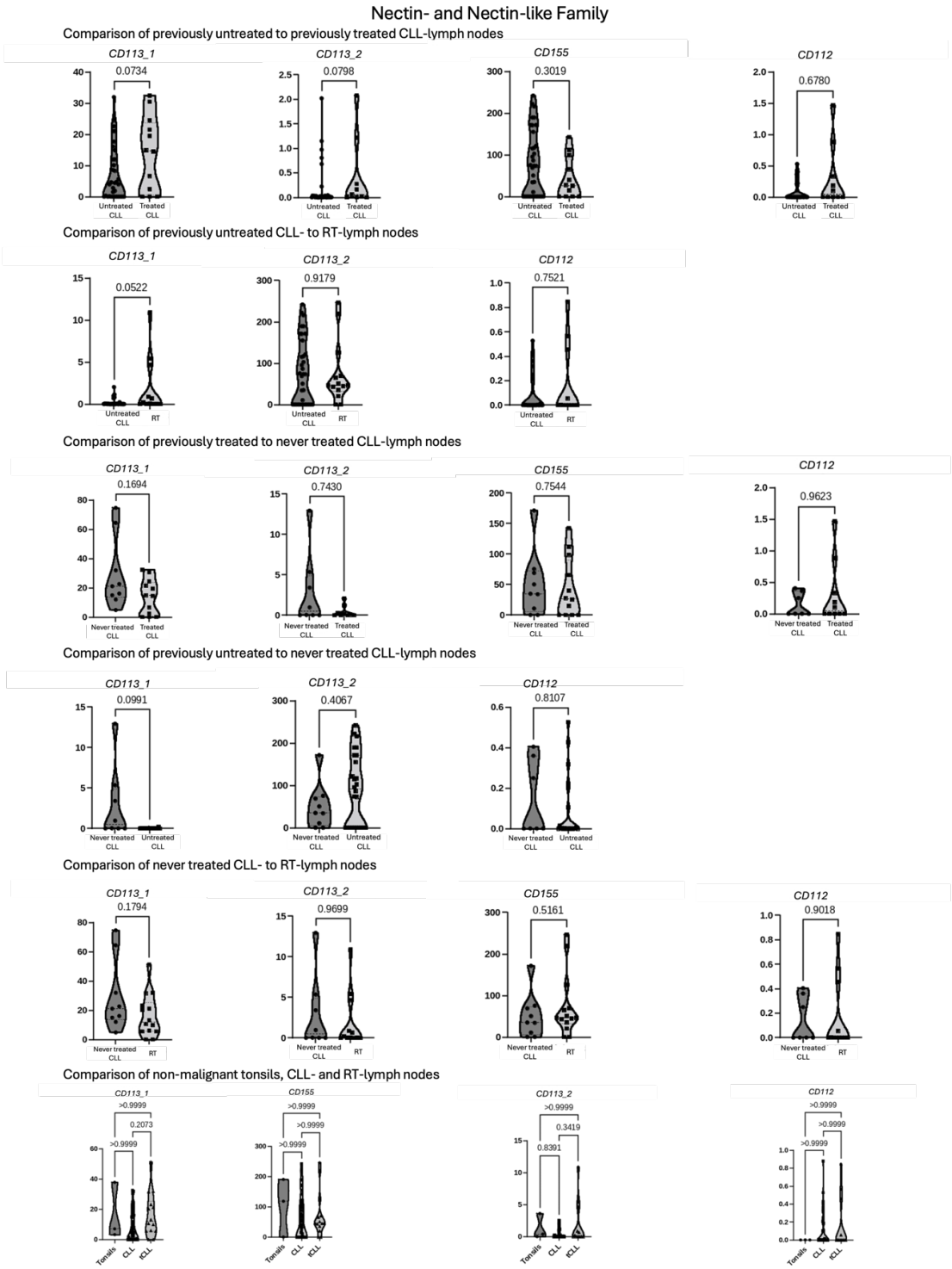
hu_RQ_LSECTIN_fw	AGT CCT TTG GGC TGT GAT TCT	21
hu_RQ_LSECTIN_rev	AGG CGT TTG TCC TCA GCA G	19
hu_RQ_CD48_fw	AGC TGC AAG TGC TTG ACC C	19
hu_RQ_CD48_rev	CAG ACT CGC CAG GTA TCA CAC	21
hu_RQ_CD112_fw	CAC TTG CGA GTT TGC CAC C	19
hu_RQ_CD112_rev	GCC ACT GTC GTA GGG TCC T	19
hu_RQ_CD155_fw	TGG AGG TGA CGC ATG TGT C	19
hu_RQ_CD155_rev	GTT TGG ACT CCG AAT AGC TGG	21
hu_RQ_CD113_1_fw	GCA GTT CAC CAT CCC CAA TAT G	22
hu_RQ_CD113_1_rev	TCC AAG CGG GAA TGT AAC AGC	21
hu_RQ_CD113_2_fw	TGC TCT TCT CCA GGC TCT GT	20
hu_RQ_CD113_2_rev	TCC CCA TAC TGC TGT GAC AT	20
hu_RQ_CD160_fw	CCC CCA GTC TGA GAA CAA GA	20
hu_RQ_CD160_rev	ACT CTG GCC TGG TTG ACA GT	20
hu_RQ_GAPDH3428_fw	AAG GTC GGA GTC AAC GGA TTT	21
hu_RQ_GAPDH3428_rev	ACC AGA GTT AAA AGC AGC CCT G	22
hu_RQ_HPRT1_fw	ATG GGA GGC CAT CAC ATT	18
hu_RQ_HPRT1_rev	ATG TAA TCC AGC AGG TCA GCA A	22
hu_RQ_NR4A1_DBD_ISOA_fw	AGC ATT ATG GTG TCC GCA CAT	21
hu_RQ_NR4A1_DBD_ISOA_rev	TGC ACT GTG CGC TTG AAG A	19
hu_RQ_NR4A2_fw	CGA TTT CAG AAG TGC CTG G	19
hu_RQ_NR4A2_rev	TAA ACT GTC TGT GCG AAC CAC	21
hu_RQ_NR4A3_fw	CTC AGT GTT GGA ATG GTA AAA GAA G	25
hu_RQ_NR4A3_rev	CCC TTT CAG ACT ATC TGT ACG GAC	24
hu_RQ_PPIA_fw	CTC CTT TGA GCT GTT TGC AG	20
hu_RQ_PPIA_rev	CAC CAC ATG CTT GCC ATC C	19
hu_RQ_TNFA_fw	CTC TTC TGC CTG CTG CAC TT	20
hu_RQ_TNFA_rev	GCC AGA GGG CTG ATT AGA GA	20

Note. Listed are 34 primers that were used for our RQ-PCR. 3 Housekeeping genes (GAPDH, HPRT1 and PPIA) and 31 target genes were investigated. Fw denotes forward, rev denotes reverse, bp denotes base pare.

Expression of Immune-Checkpoint Components

Figure 33

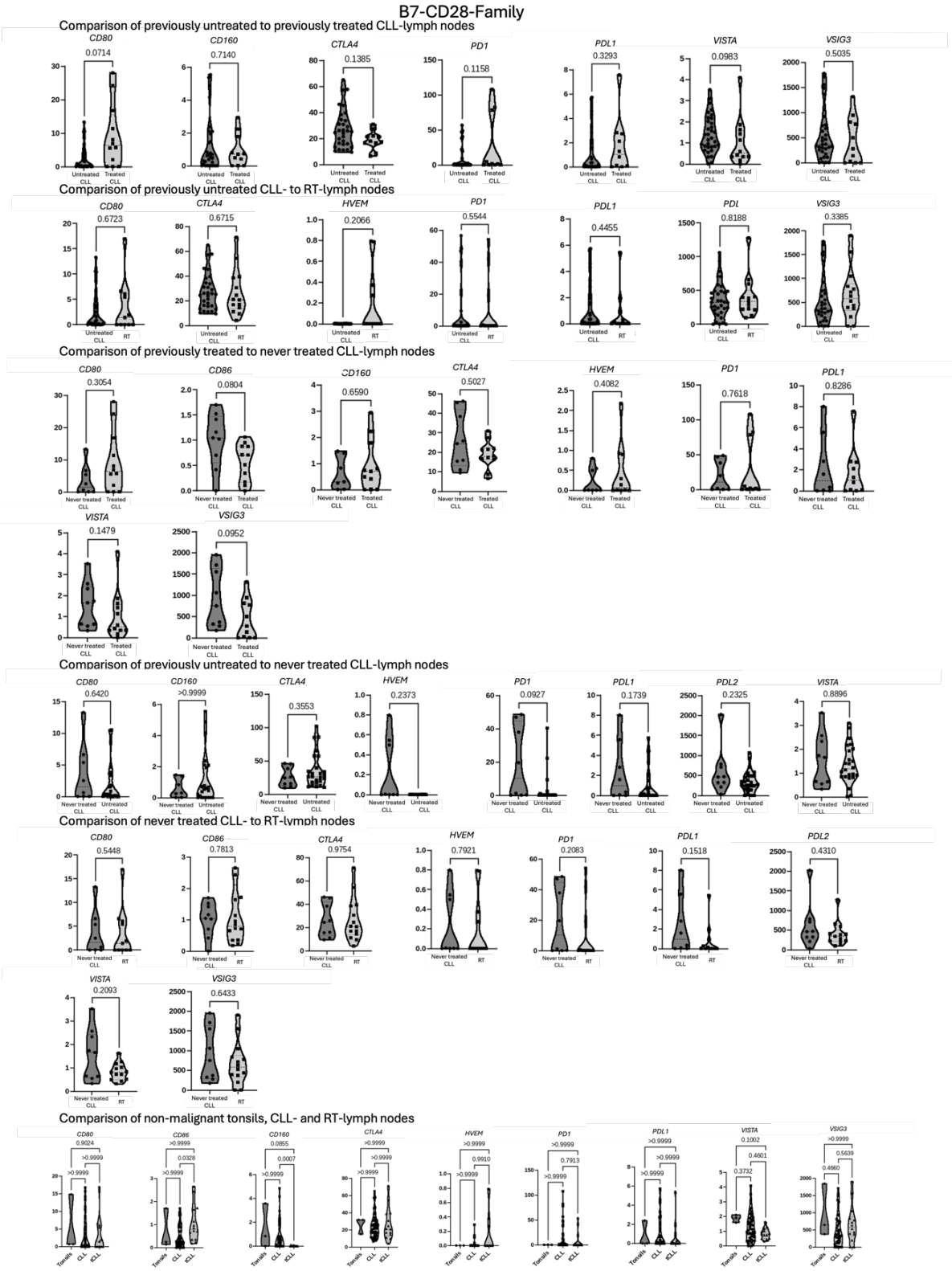
Non-significant results in the Nectin- and Nectin-like Family.



Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to the Nectin- and Nectin-like Family in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Figure 34

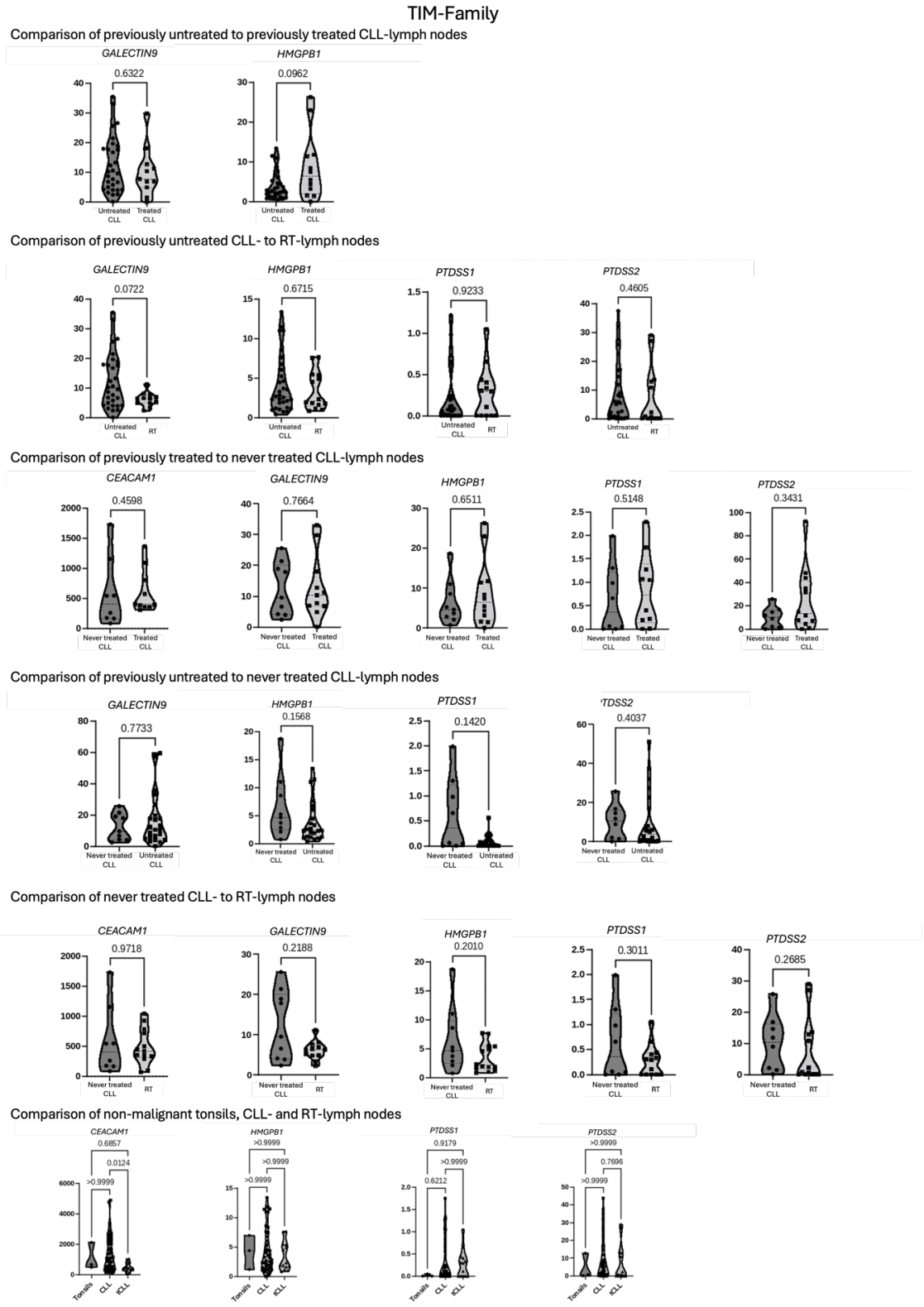
Non-significant results in the B7-CD28-Family.



Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to the B7-CD28-Family in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Figure 35

Non-significant results in the TIM-Family.

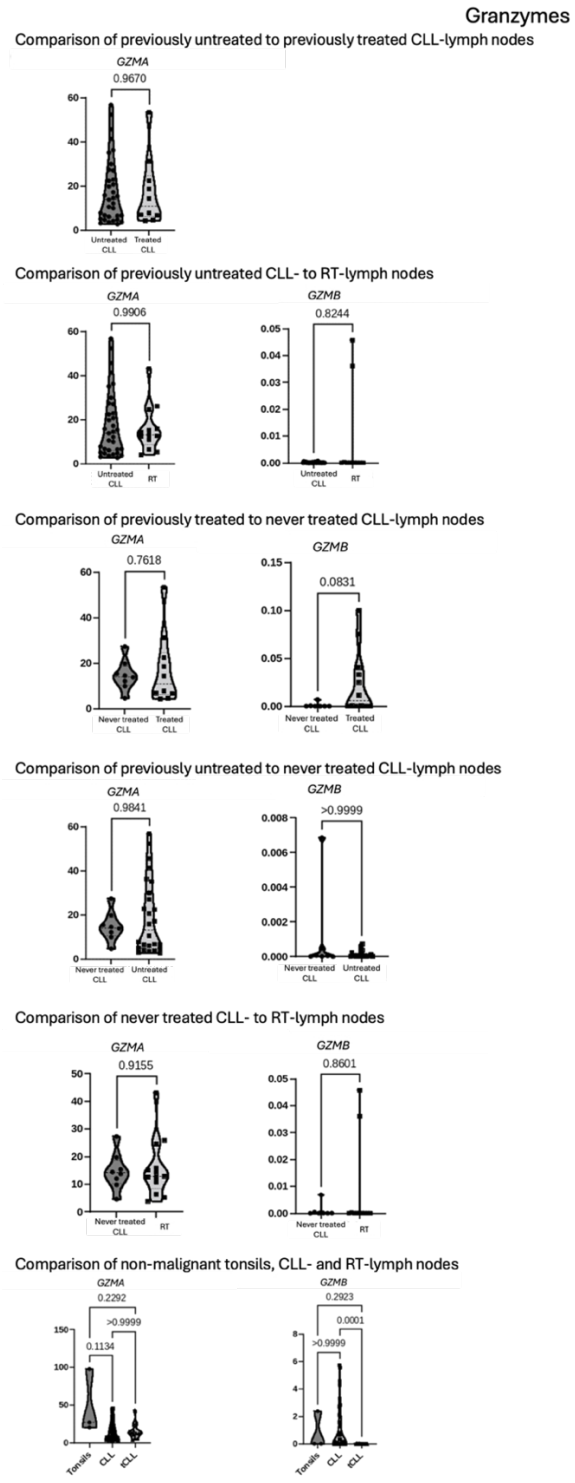


Note. Shown are the non-significant results ($p > 0.05$) of gene espressions belonging to the TIM-Family in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Expression of Immune-Cell Markers

Figure 36

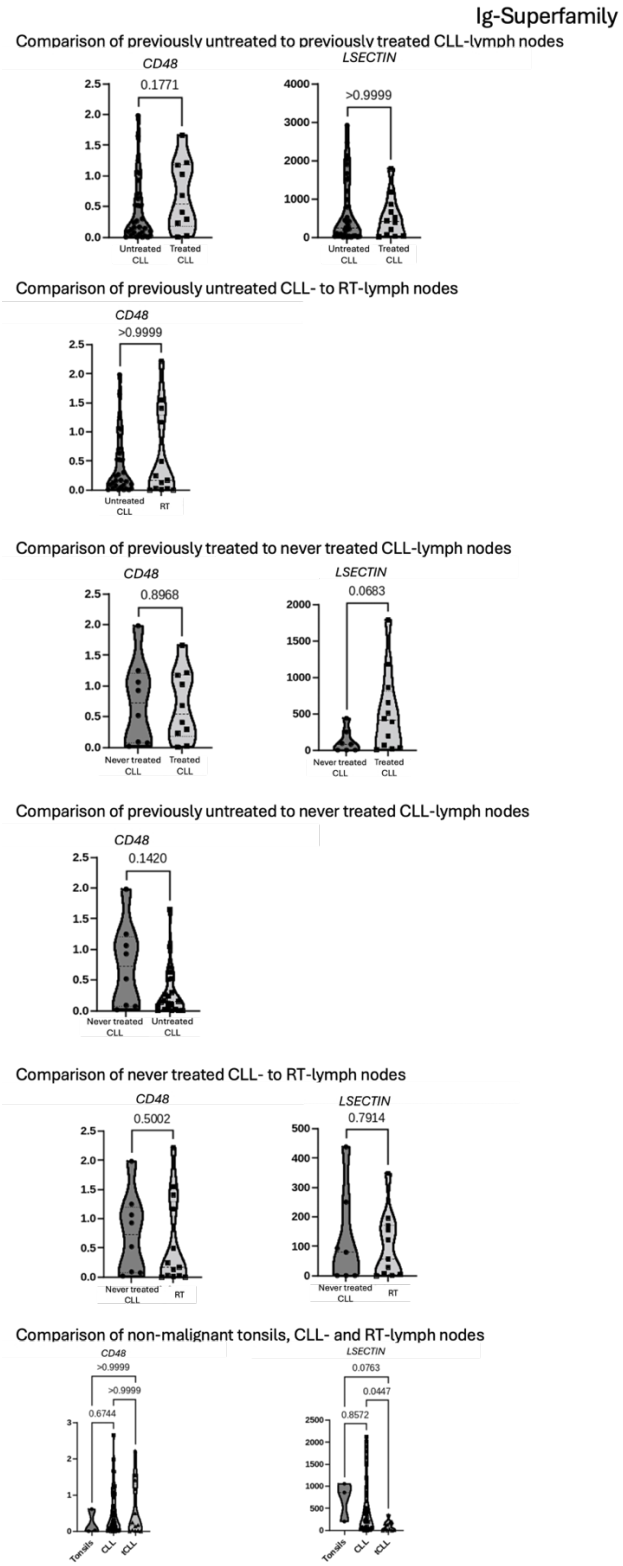
Non-significant results of Granzymes.



Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to Granzymes in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Figure 37

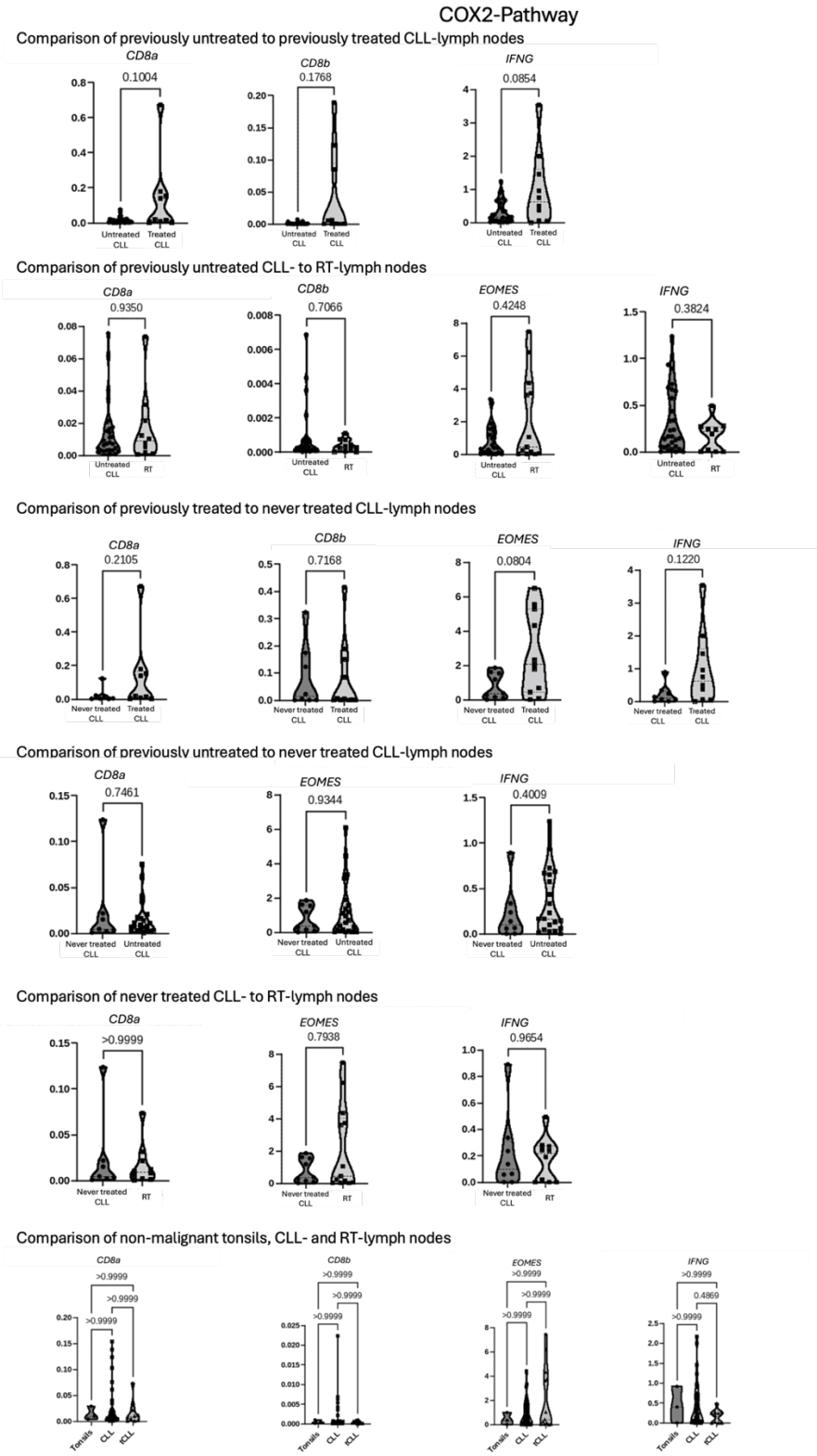
Non-significant results in the Ig-Superfamily.



Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to the Ig-Superfamily in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Figure 38

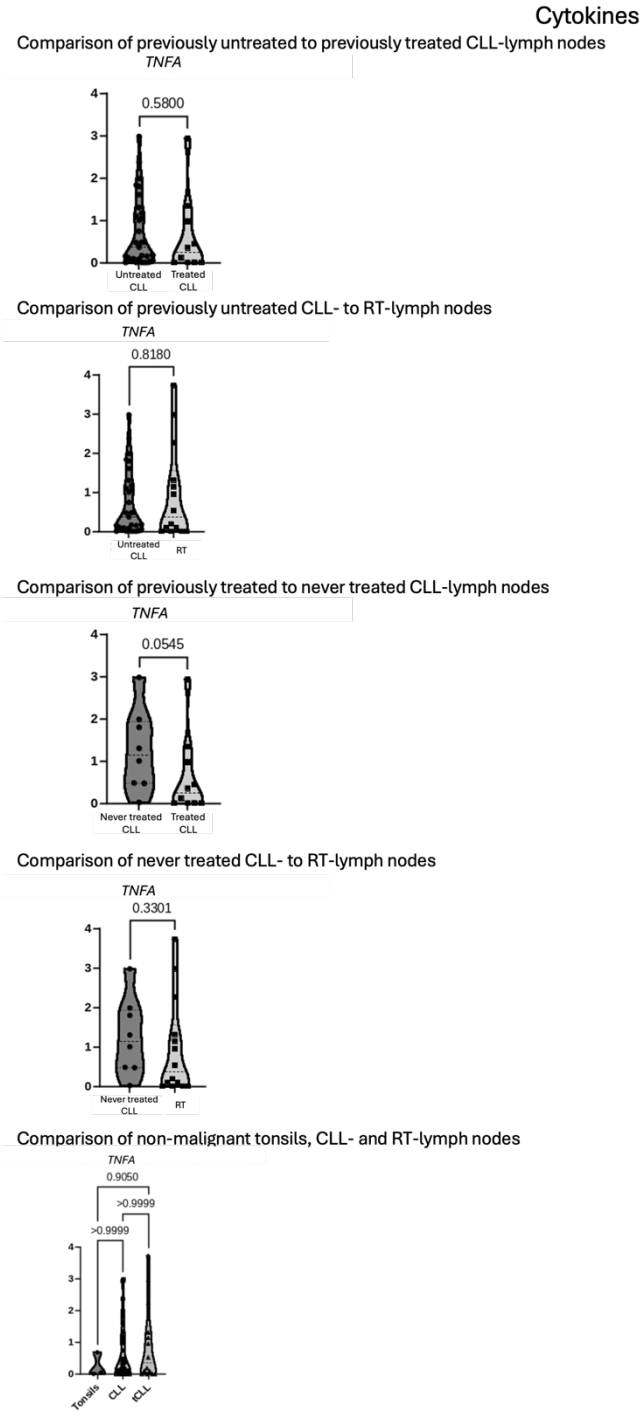
Non-significant results in the COX2-Pathway.



Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to the COX2-Pathway in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Figure 39

Non-significant results in the Cytokines.

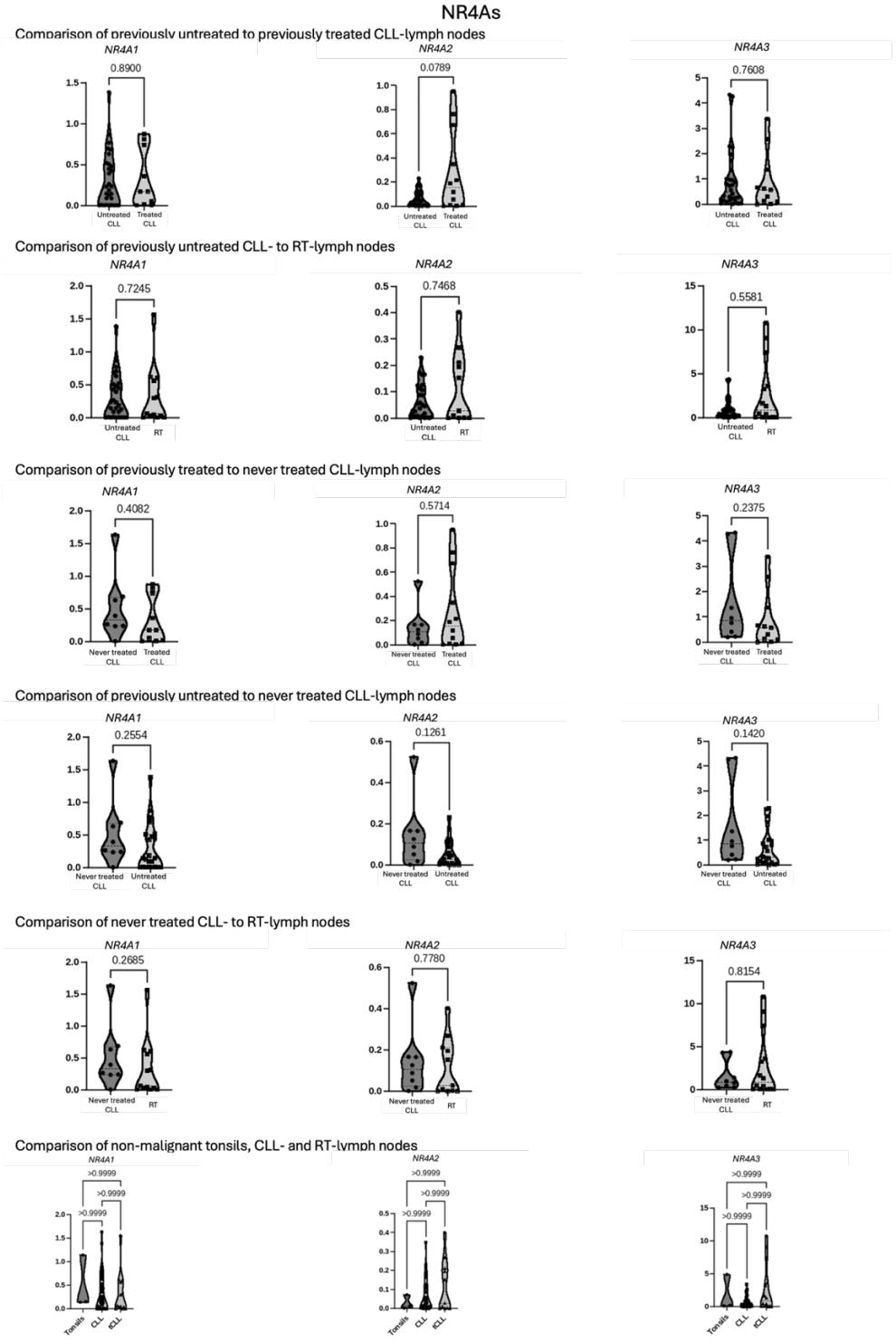


Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to the Cytokines in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.

Expression of NR4As

Figure 40

Non-significant results in the NR4A group.



Note. Shown are the non-significant results ($p > 0.05$) of gene expressions belonging to the NR4As in the comparisons between our different subgroups. CLL denotes chronic lymphocytic leukemia, RT and tCLL denotes Richter specimens, transformed CLL.