DISSERTATION

Molecular analysis of aggressive B-cell lymphomas

Submitted by

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For the Academic Degree of

Doctor of Medical Science (Dr. scient. med.)

at the

Medical University of Graz

Department of Hematology

under the Supervision of

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2015

Wer spricht von Siegen? Überstehn ist alles.

Rainer Maria Rilke (1875-1926)

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of Good Scientific Practice.

Graz, 31th of July

Acknowledgment

Ich möchte mich hier an Johann Wolfgang von Goethe halten „Leider läßt sich eine wahrhafte Dankbarkeit mit Worten nicht ausdrücken“. Es gibt jedoch einige Menschen deren maßgebliche Beteiligung an dieser Arbeit nicht unerwähnt bleiben sollte (diese Liste erhebt jedoch keinen Anspruch auf Vollständigkeit):

Zu allererst die Lymphomgruppe der Hämatologie Graz: Unter der Leitung des genialsten und besten Chefs, Prof. Peter Neumeister, DANKE für die Inspiration, die Unterstützung und die besten Laborbesprechungen. DANKE PD Alexander Deutsch, so gut wie alles an Labortechniken habe ich von ihm gelernt, für die Geduld, die unzähligen Erklärungen und den ständigen Beistand. DANKE Kerstin Wenzl, MSc. für die Mitwirkung egal zu welcher Tages- und Nachtzeit und die Beantwortung selbst der dümmsten Fragen. Ich bin stolz ein Mitglied dieser Arbeitsgruppe zu sein und dankbar, diese großartigen Menschen nicht nur im akademischen Umfeld, sondern auch privat zu, so hoffe ich zumindest, Freunden zählen zu können. DANKE auch an PD Martin Pichler, dem nimmermüden Statistik-Experten, der selbst über den großen Teich hinweg immer zur Verfügung stand.

Natürlich möchte ich auch meinen Eltern DANKEN, die mir in jedem Fall die Grundsteine für meinen Lebensweg gelegt haben und deren uneingeschränkte Unterstützung mir stets den Rücken gestärkt hat. Ein großer DANK gilt auch meiner besten Freundin Petra Konrad, die meine Klagen ertragen musste und stets mit Aufmerksamkeit und/oder Prosecco zur Stelle war wenn ich sie brauchte.

Mein besonderer DANK gilt aber vor allem einem, Dr. Wenzel Prochazka, für die unzähligen Stunden des Zuhörens, des immer wieder gut Zuredens, wenn einmal wieder die Phase „Ich schmeiß alles hin und werde Pirat“ kam, und den ständigen Beistand während der letzten sieben Jahre.

DANKE

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I. Abstract

Background: Diffuse large B cell lymphomas (DLBCL) are the most common occurring lymphoma worldwide. Despite recent efforts, their exact pathogenesis has not been elucidated yet. Micro-RNAs are short non-coding single-stranded RNA molecules regulating gene expression at the post-transcriptional level and their expression has been identified to correlate with tumor prognosis.

Methods: For microRNA expression analysis real-time PCR on 81 samples was performed, including 63 DLBCL, and 18 controls, as well as on 4 lymphoma cell lines. Expression levels of a panel of 11 microRNAs that have been previously involved in other types of cancer were measured and correlated with clinical data. Furthermore, cell lines, lacking miR-199a and miR-497 expression, were electroporated with the two respective microRNAs and treated with standard immunochemotherapy routinely used in patients with DLBCL, followed by functional analyses including cell count and apoptosis assays.

In a retrospective data analysis the inflammatory factors derived neutrophil to lymphocyte ratio (dNLR) and the C-reactive protein (CRP) have been evaluated regarding their prognostic explanatory power in a large cohort of DLBCL patients.

Results: Seven microRNAs were statistically significant up-regulated in DLBCL compared to normal germinal center cells. However, high expression of miR-497 or miR-199a was associated with better overall survival ($p=0.042$ and $p=0.007$). Overexpression of miR-199a and miR-497 led to a statistically significant decrease in viable cells in a dose-dependent fashion after exposure to rituximab and various chemotherapeutics relevant in multi-agent lymphoma therapy. Furthermore, we could identify an independent significant association between high dNLR and high CRP levels with poor clinical outcome in multivariate analysis for overall survival (OS), as well as disease free survival (DFS).

Conclusion: Our data indicate that elevated miR-199a and miR-497 levels are associated with improved survival in DLBCL patients most likely by modifying drug sensitivity to immunochemotherapy. This functional impairment may serve as a potential novel therapeutic target in future treatment. We could further show that a high dNLR or a high CRP value at diagnosis of DLBCL represent an independent poor prognostic factor for clinical outcome. Our data encourages the further validation of these easily available parameters in prospective studies and as a potential stratification tool in clinical trials.

This thesis is based on the following publications:

Troppan, K., Deutsch, A., Gerger, A., Stojakovic, T., Beham-Schmid, C., Wenzl, K., Neumeister, P., Pichler, M., (Jan 2014). The derived neutrophil to lymphocyte ratio is an independent prognostic factor in patients with diffuse large B-cell lymphoma. *Br J Cancer,110*(2),369-74.

Troppan, K., Wenzl, K., Deutsch, A., Ling, H., Neumeister, P., Pichler, M., (Feb 2014). MicroRNAs in diffuse large B-cell lymphoma: implications for pathogenesis, diagnosis, prognosis and therapy. *Anticancer Res*, *34*(2),557-64.

Troppan, K., Schlick, K., Deutsch, A., Melchardt, T., Egle, A., Stojakovic, T., Beham-Schmid, C., Weiss, L., Neureiter, D., Wenzl, K., Greil, R., Neumeister, P., Pichler, M., (Jul 2014). C-reactive protein level is a prognostic indicator for survival and improves the predictive ability of the R-IPI score in diffuse large B-cell lymphoma patients. *Br J Cancer, 111*(1),55-60.

Troppan, K., Wenzl, K., Pichler, M., Pursche, B., Schwarzenbacher, D., Feichtinger, J., Thallinger, G.G., Beham-Schmid, C., Neumeister, P., Deutsch, A. (Aug 2015) MiR-199a and miR-497 are associated with better overall survival due to increased chemosensitivity in diffuse large B cell lymphoma patients. *International Journal of Molecular Sciences*, accepted 30th of July.

II. Zusammenfassung

Hintergrund: Diffus großzellige B-Zell Lymphome (DLBCL) zählen zu den häufigsten Lymphomen weltweit. Trotz großer Fortschritte ist ihre exakte Pathogenese bis dato noch nicht vollständig entschlüsselt. MicroRNAs sind kurze, nicht-kodierende Einzelstrang RNA Moleküle, die auf posttranskriptioneller Ebene die Genexpression regulieren. Des Weiteren konnte ihre Expression mit der Prognose verschiedenster Tumoren korreliert werden.

Methode: Zur Analyse der microRNA Expression wurden 81 Proben, darunter 63 DLBCL und 18 Kontrollen, sowie 4 Lymphomzelllinien mittels real-time PCR untersucht. Elf microRNAs, deren onkogenetisches Potential bereits in soliden Tumoren gezeigt werden konnte, wurden untersucht, und diese Ergebnisse mit klinischen Daten korreliert. Weiters wurden 2 Zelllinien, die keine Expression von miR-199a und miR-497 aufwiesen, mit ebendiesen microRNAs elektroporiert, mit der für DLBCL gebräuchlichen Standard-Chemoimmunotherapie behandelt und anschließend mit Hilfe von Zellzahlanalyse und Apoptose Assays funktionell charakterisiert. In einer retrospektiven Datenanalyse untersuchten wir die beiden inflammatorischen Parameter derived neutrophil to lymphocyte ratio (dNLR) und das C-reaktive Protein (CRP) hinsichtlich ihrer prognostischen Aussagekraft in einer großen Kohorte von DLBCL Patienten.

Ergebnisse: Es zeigte sich eine signifikante Erhöhung von sieben microRNAs bei DLBCL im Vergleich zu Keimzentrumszellen. Außerdem konnten wir hohe Expressionsdaten von miR-199a und miR-497 mit einem signifikant besserem Gesamtüberleben assoziieren ($p=0.042$ und $p=0.007$). In den funktionellen Experimenten führte eine Überexpression der beiden microRNAs zu einem statistisch signifikanten Abfall lebender Zellen nach Exposition mit Rituximab oder Zytostatika. Mittels multivariater Analyse konnten wir weiters eine Assoziation zwischen hohen dNLR bzw. hohen CRP Levels mit schlechtem Gesamtüberleben demonstrieren.

Schlussfolgerung: Patienten mit hoher miR-199a und miR-497 Expression zeigen ein signifikant besseres Gesamtüberleben, das in erster Linie einer erhöhten Chemosensitivität zuzuschreiben ist und als neues therapeutisches Ziel genutzt werden könnte. Ein hoher dNLR oder CRP Spiegel zum Zeitpunkt der Diagnosestellung repräsentiert einen unabhängigen prognostischen Faktor hinsichtlich Gesamtüberlebenszeit. Diese einfachen anzuwendenden Parameter bedürfen einer weiteren Evaluierung in prospektiven Studien.

III. Introduction

a. Diffuse large B cell lymphoma

Definition

The diffuse large B cell lymphoma (DLBCL) is a neoplasm of large B lymphoid cells characterized by a diffuse growth pattern and diffuse nodal architectural destruction or extranodal infiltration by large B lymphoid cells.

Incidence

DLBCL is the most common occurring form of lymphoma, counting for more than one third of newly diagnosed patients. The incidence averages out 3-4 cases per 100 000 per year, increasing significantly with age from 0.3/100 000/year in people 35-39 years old to 26.6/100 000/year in people aged over 80 years (Tilly, et al., 2012). It is more common in males than in females (Campo, et al, 2011).

Pathogenesis

As the etiology of the DLBCL still remains unknown, it may arise de novo (primary) or may be a transformation of an indolent lymphoma (secondary) (Gouveia, et al., 2012).

DLBCL is remarkably diverse in both clinical presentation and outcome, reflecting its heterogeneity. Genome-wide expression profiling has identified at least three different groups based on to similarities to the putative cell of origin:

i) the germinal center B-cell like (GCB) DLBCL, originating from centroblasts

ii) the activated B-cell like (ABC) DLBCL, which resembles features of plasmablastic B-cells committed to terminal B cell differentiation

iii) the primary mediastinal large B-cell lymphoma, arising from thymic B-cells (Lenz, et al., 2008).

However, 5-10% of DLBCL cannot be classified into any of the above subgroups. The cell-of-origin based classification has prognostic value, since ABC-DLBCL have a worse overall survival as compared to GCB-DLBCL, and respond less effectively to current therapeutic regimes, with cure rates of around 40% (Schneider, et al., 2011). Immunohistochemical markers have been shown to be able to discriminate the individual subgroups, and several of them –CD10, BCL-6, MUM1, BCL-2 and CYCLIN D2 – have

been shown to be predictive of survival. The combination of CD10, MUM1 and BCL-6 can divide DLBCL in GCB-DLBCL and non-GCB (NGCB) DLBCL with about 80% concordance with the gene expression profile (GEP) (Hans, et al., 2004). Another algorithm using GCET1, CD10, BCL6, MUM1, and FOXP1 was derived that revised the prediction of classification with 93% concordance. This algorithm predicted 3-year overall survival of the subtypes [GCB (87%) versus ABC (44%); $P < 0.001$], simulating the predictive power of the GEP classification (Choi, et al., 2009).

Diagnosis & Staging

Diagnosis of DLBCL is made out of a surgical specimen, excisional lymph node biopsy or biopsy of extralymphatic tissue. Immunohistochemistry with antibodies against at least CD45, CD 19, CD79a, CD20, and CD3 should be performed on the sample. Fresh frozen material for molecular characterization is desirable but not routinely required yet. Histological diagnosis is made according to the WHO classification (Campo, et al., 2011) (Table III-1).

Table III-1: Diffuse large B-cell lymphoma: variants, subgroups and subtypes/entities.

Diffuse large B-cell lymphoma, not otherwise specified
- Common morphologic variants
Centroblastic
Immunoblastic
Anaplastic
- Rare morphologic variants
- Molecular subgroups
Germinal centre B-cell-like (GCB)
Activated B-cell-like (ABC)
- Immunohistochemical subgroups
CD5-positive DLBCL
Germinal centre B-cell-like (GCB)
Non-germinal centre B-cell-like (non-GCB)
Diffuse large B-cell lymphoma subtypes
- T-cell/histiocyte-rich large B-cell lymphoma

- Primary DLBCL of the CNS - Primary cutaneous DLBCL, leg type - EBV positive DLBCL of the elderly
Other lymphomas of large B cells - Primary mediastinal (thymic) large B-cell lymphoma - Intravascular large B-cell lymphoma - DLBCL associated with chronic inflammation - Lymphomatoid granulomatosis - ALK positive LBCL - Plasmablastic lymphoma - Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease - Primary effusion lymphoma
Borderline cases - B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma - B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

The staging of infested lymph nodes is based on the Ann Arbor staging system which considers the anatomic extent in relation to the diaphragm (as shown in Table III-2)

Table III-2: Ann Arbor classification.

Stage	
1	Involvement of a single lymphatic region (I) or localized involvement of single extra lymphatic organ or site (IE)
2	Involvement of two or more lymphatic regions on the same side of the diaphragm (II) or localized involvement of a single extra lymphatic organ or site and of one or more lymphatic regions on the same side of the diaphragm (IIE)
3	Involvement of lymphatic regions on both side of the diaphragm.
4	Diffuse or disseminated involvement of one or more extra lymphatic organs with or without lymphatic involvement.
A, B	Absence or presence of B-symptoms (fever, night sweat, weight loss)
S	Involvement of the spleen

Risk assessment

Historically, clinicians and investigators have relied on prognostic schemes that imply clinical risk factors to predict the risk for disease progression, relapse and death of patients with aggressive Non Hodgkin lymphoma (NHL). One of the most commonly used schemes of rating, the International Prognostic Index (IPI) for lymphomas, developed in the 1990s, remains a robust clinical prognostic index for aggressive lymphomas. It involves 5 features: age, tumor stage, serum lactate dehydrogenase (LDH) concentration, performance status and number of extranodal disease sites. The IPI distinguishes 4 risk groups with different 5-year overall survival, ranging from 26-73% (Shipp, et al., 1993). In the era of Rituximab, a revised IPI (R-IPI) has been introduced, showing superior prediction in outcome of DLBCL patients, treated with standard immunochemotherapy. The R-IPI identifies 3 distinct prognostic groups, with a very good (4-year overall survival (OS) 94%), good (OS 79%), and poor (OS 55%) outcome, respectively (Sehn, et al., 2007). Recently, an advanced IPI (National Comprehemsive cancer Network NCCN-IPI) was published by Zhou et al., established in patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) at 7 NCCN-cancer centers in the United States and in the British Columbia Cancer Agency (Zhou, et al., 2014). This score emphasizes the impact of older age and highly increased LDH levels and clearly propagates extranodal disease relevant for prognosis. Nevertheless, a large group of patients with distinct clinico-pathologic profile and unfavorable outcome remains uncharacterized.

Treatment

First-line therapy

In the 1970s, the discovery of the advantages of a combined chemotherapy with CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) instead of a single agent therapy influenced the treatment of DLBCLs in a significant way (McKelvey, et al., 1976). In 1997 the US Food and Drug Administration homologated Rituximab for the treatment of lymphoma. Rituximab is a chimeric human-mouse anti-CD20 monoclonal antibody with significant anti-lymphoma activity, proven by Coiffier et al. in a prospective randomized phase II trial (Coiffier, et al., 1998). Superiority of R-CHOP to CHOP alone could be demonstrated in all age groups (Coiffier, et al., 2010) (Habermann, et al., 2006) (Pfreundschuh, et al., 2006) (Pfreundschuh, et al., 2011).

For several years, standard treatment for patients with DLBCL is now immunochemotherapy with R-CHOP, given in a 14- or 21-day cycle with rituximab on day one, cyclophosphamide, doxorubicin, and vincristine on day 2, and prednisone on days 1-5 with similar results according to progression free survival (PFS) and OS (Pfreundschuh, et al., 2008). Granulocyte stimulating factor to avoid neutropenia is also recommended. Intensified therapy regimens were not able to show superiority to R-CHOP therapy up to now but are in use as well (Reyes, et al., 2005) (Récher, et al., 2011) (Tilly, et al., 2003) (Pfreundschuh, et al., 2004) (Pfreundschuh, et al., 2004) (Schmitz, et al., 2012). A benefit for OS and PFS for maintenance therapy with rituximab after induction chemotherapy could not be demonstrated and is not standard of care (Habermann, et al., 2006).The role of radiation especially in localized stages of DLBCL is not yet clear and needs to be further evaluated (Held, et al., 2014) (Miller, et al., 1998) (Horning, et al., 2004) (Bonnet, et al., 2007).

After the discovery of different DLBCL subtypes, a special interest focus on the development of therapy regimens, overcoming the poor outcome of patients with NGCB subtype (Ruan, et al., 2011) (Li, et al., 2014).

Therapy for relapsed and refractory patients

In relapsed or refractory patients, high-dose chemotherapy followed by autologous stem cell transplantation is the therapy of choice if practicable according to patients' age (Philip, et al., 1995) (Gisselbrecht, et al., 2010). Nevertheless, good outcome is only expectable in those patients with at least 12 months response to induction chemotherapy (Gisselbrecht, et al., 2010). Otherwise allogeneic transplantation has to be considered in these patients (van Kampen, et al., 2011).

DLBCL & Inflammation

Inflammation has been identified to be a critical component of tumor progression, highlighting the role of the microenvironment, which is largely orchestrated by inflammatory cells as an indispensable participant in the neoplastic process, fostering proliferation, survival and migration (Coussens & Werb, 2002). For different solid tumors, as well as lymphomas, inflammation parameters, including leukocytes, neutrophils, lymphocytes, and C-reactive protein (CRP), have been associated with higher mortality rates (Mohri, et al., 2010) (Cao, et al., 2012).

One of the most commonly used inflammatory markers in routine diagnostic is the CRP, an acute phase protein, produced by the liver after interleukin-6 (IL-6) and other cytokine stimuli. Its prognostic role in various solid and hematologic cancer types has been demonstrated recently (Allin, et al., 2011) (Toiyama, et al., 2013) (Hall, et al., 2013) (Hong, et al., 2012). In DLBCL, only a few small scale studies that include some of them before the rituximab era, have been reported (Cao, et al., 2012) (Herishanu, et al., 2007) (Elahi, et al., 2005) (Legouffe, et al., 1998).

In addition to absolute counts of inflammation parameters, also the neutrophil-to-lymphocyte ratio (NLR) has been identified as an independent prognostic factor for OS and PFS in various types of cancer, including renal cell carcinoma, colorectal cancer, sarcoma and pancreatic cancer (Pichler, et al., 2013) (Walsh, et al., 2005) (Szkandera, et al., 2013) (Wang, et al., 2012). Recently, the NLR has been suggested to be a simple, inexpensive, standardized prognostic factor to assess clinical outcomes in DLBCL patients treated with R-CHOP (Porrata, et al., 2010). Frequently, the absolute lymphocyte count is not routinely documented in clinical trials despite determining a differential white cell count. To solve this problem, the derived neutrophil to lymphocyte ratio (dNLR) was recently implemented, consisting of neutrophil count divided by (leukocyte count – neutrophil count) (Proctor, et al., 2012). In this study by Proctor et al., they proposed a similar prognostic value of the dNLR compared to the NLR in different solid cancer types but not explicitly for DLBCL patients (Proctor, et al., 2012). However, for hematologic malignancies, the dNLR has not been validated yet.

b. MicroRNAs

MicroRNA Processing

MicroRNAs are 19-24 nucleotides long non-coding RNA strands that regulate gene expression through sequence complementarity with their target, mostly the 3'-untranslated regions of target genes (Bartel, 2004). In the nucleus, microRNAs are transcribed as long primary transcripts, which are then converted into precursor microRNAs by the RNAse III enzyme DROSHA (Fabbri, et al., 2008). The exportin 5 protein arranges the transfer of precursor microRNAs from the nucleus to the cytoplasm. Further processing via cutting of the hairpin-structure of precursor microRNAs by a second RNAse III enzyme, called DICER, leads to a microRNA double strand (Chendrimada, et al., 2005). These double-stranded microRNAs unwind and are loaded onto the RNA induced silencing complex enzyme (RISC). Although both strands may be of functional activity, only one strand is incorporated into the RISC, depending on thermodynamic instability and base-pairing weakness relative to the other strand (Khvorova, et al., 2003). The evolved complex, sometimes referred to as "miRISC", guides the microRNA to its complementary sequence on the appropriate messenger RNA (Zeng, 2006). Dependent on its complementarity, this binding results in either direct RNA degradation or at least inhibition of further protein translation (Pillai, et al., 2007) Figure III-1.

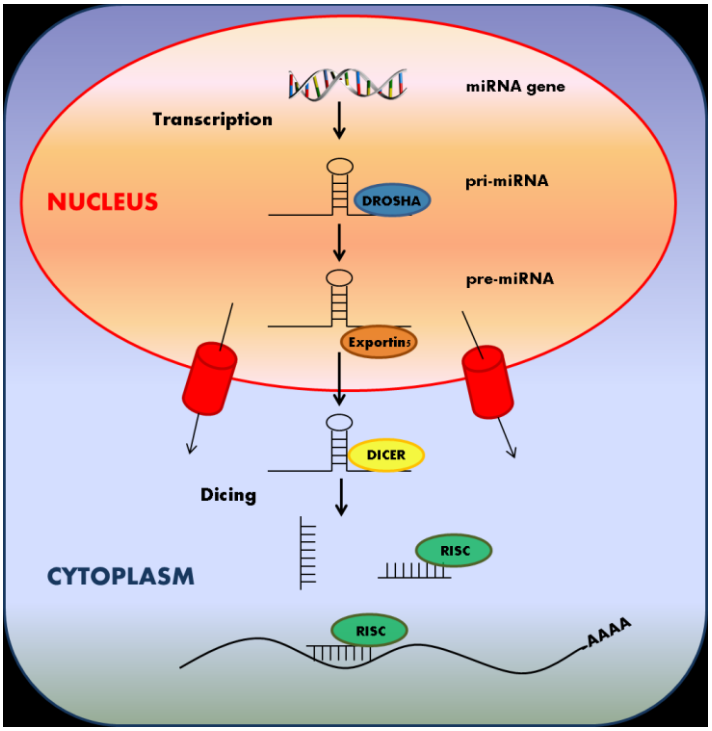

Figure III-1: MicroRNA processing.

MicroRNA Nomenclature

MicroRNAs are named using the "mir" prefix plus a unique identifying number subsequent. The mature microRNA sequences are denoted "miR", whereas the precursor microRNAs are labelled "mir". Abbreviated 3 or 4 letter prefixes indicate the species, which in the microRNA was identified e.g. hsa-miR-101 (for Homo sapiens). The numbers are assigned sequentially, with identical microRNAs having the same number, regardless of the organism (e.g. mmu-miR-101 and hsa-miR-101). Paralogous sequences whose mature microRNAs differ in one or two nucleotides only, are given suffixes (e.g. hsa-miR-199a and hsa-miR-199b). Identical mature microRNAs raised from distinct precursor microRNAs have numbered suffixes (e.g. hsa-mir-497-1 and hsa-mir-497-2).

Two mature microRNAs originating from the opposite arms of one and the same precursor microRNA are denoted with a -3p or -5p suffix. In some cases, the relative expression levels of the microRNAs are known, and those microRNA which is expressed at lower levels compared to the microRNA of the opposite arm of a hairpin, is marked by an asterisk following the name (e.g. miR-27a and miR-27a* share a precursor microRNA hairpin, but in the cell higher levels of miR-27 are found (Ambros, et al., 2003).

MicroRNAs and Cancer

MicroRNAs play a role in various biological processes including cancer development. Genes coding for microRNAs are frequently located at fragile sites or genomic regions, typically associated with cancer (Calin, et al., 2004). In 2002, Calin et al. identified for the first time a direct association of deregulation in miR-15 and miR-16 expression, and development of chronic lymphocytic leukemia (Calin, et al., 2002). Following the initial findings, meanwhile a plethora of microRNAs participating in cancer development have been identified (Calin & Croce, 2006). Additionally, many research groups explored the potential clinical application of microRNAs as diagnostic or therapeutic tools for patients with various cancer subtypes (Pichler, et al., 2014) (Schwarzenbacher, et al., 2013) (Bach, et al., 2013) (Al-Ali, et al., 2012) (Pichler, et al., 2012) (Ling, et al., 2013) (Lawrie, et al., 2009) (Garzon, et al., 2010).

MicroRNAs and the Pathogenesis of DLBCL

In DLBCL, various microRNAs, involved in hematopoiesis and lymphomagenesis, have been identified (Caramuta, et al., 2013) (Di Lisio, et al., 2012) (Lawrie, 2013). Understanding their exact biological function continues to be a huge challenge in this field, as one microRNA can target multiple mRNAs and vice versa multiple microRNAs can influence the same mRNA molecule, making a prediction of the definitive effects of a particular microRNA often difficult (Garzon & Marcucci, 2012). Nevertheless, the pathogenetic effects of some microRNAs in the pathogenesis of DLBCL are well characterized. Kim et al. demonstrated that miR-125a and miR-125b constitutively activate the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) pathway, one of the most deregulated pathways in DLBCL pathogenesis. They showed that miR-125a and miR-125b, frequently gained or overexpressed in DLBCL, target Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), a negative regulator of NF-kB, and consequently enhance NF-kB signaling (Kim, et al., 2012). By ectopic expression and inhibition of these two microRNAs in cell lines, they demonstrated their oncogenic role in lymphomagenesis.

The miR-17-92 cluster, encoding for six different microRNAs, is located at chromosome 13q31-q32, a region frequently amplified in GCB DLBCL (Lenz, et al., 2008). This cluster of microRNAs contributes to improved survival of early B-cell progenitors, and thus adds to lymphomagenesis by repressing antiapoptotic genes (Ventura, et al., 2008).

Hitherto miR-155 is one of the most studied microRNAs, accumulated 10- to 30-fold in several types of B-cell lymphoma, including DLBCL, compared to normal B-cells (Eis, et al., 2005). In addition, miR-155 expression levels are higher in the ABC- compared to the GCB subtype. In mucosa associated lymphoid tissue (MALT) lymphoma, an increase of miR-155 was invariably associated with a suppression of the pro-apoptotic gene Tumor protein p53-inducible nuclear protein 1 (TP53INP1) (Saito, et al., 2012). As a proapoptotic stress-induced p53 target gene, TP53INP1 induces cell cycle arrest and apoptosis when overexpressed. MiR-155 also targets Human Germinal-center Associated Lymphoma (HGAL), a new prognostic biomarker that is indispensable for germinal center formation, immunoglobulin gene class-switch recombination, and somatic hypermutation. By mediating the effects of IL-6, it interacts with the cytoskeleton and cellular motility and migration in the lymphoma (Lu, et al., 2007) (Dagan, et al., 2012). Via quantitative genomics, Huang et al. identified phosphatidylinositol 3-kinase (PIK3R1), a negative

regulator of the phosphatidylinositol 3-kinase (PI3K-AKT) pathway, as a direct target of miR-155 (Huang, et al., 2012). Costinean et al. generated Eμ-mmu-miR-155 transgenic mice, developing a lymphoproliferative disease resembling human acute lymphatic leukemia or high-grade lymphoma when overexpressing miR-155 (Costinean, et al., 2006). This strongly suggests that miR-155 is directly implicated in the initiation and progression of these diseases. Furthermore, because of the disease's polyclonal character, miR-155 could be a downstream target of signal transduction pathways activated in cancer (Costinean, et al., 2006). A further target, bone morphogenetic protein (BMP)-responsive transcriptional factor Mothers against decapentaplegic homolog 5 (SMAD5), was inhibited and disrupted in its activity by miR-155 overexpression (Rai, et al., 2010). Overexpression rendered DLBCL resistant to the growth-inhibitory effects of both transforming growth factor (TGF-β1) and BMPs, via defective induction of p21 and impaired cell cycle arrest. Jiang and Aguiar further dissected the role of miR-155 in modulating this pathway (Jiang & Aguiar, 2014): In DLBCL cell lines and a miR-155 knock-out mouse model, they demonstrated that levels of the transcription factor SMAD5 are elevated in mature B lymphocytes, which display an increased sensitivity to TGFβ1 characterized by suppression of retinoblastoma protein (RB) phosphorylation and more pronounced G 0/G 1 cell cycle arrest (Jiang & Aguiar, 2014).

MiR-34, a pro-apoptotic and growth-suppressive microRNA executes these functions via the TP53 pathway (He, et al., 2007). Another tumor-suppressive effect attributed to miR-34a is the deregulation of its target Forkhead box protein P1 (FOXP1), leading to blocked proliferation of DLBCL (Craig, et al., 2011).

MicroRNAs in the diagnosis of DLBCL

Today, the diagnosis of DLBCL is a histological task, supplemented by immunohistochemistry and, if available, GEP (Tilly, et al., 2012). The addition of a microRNA profile to the diagnosis of lymphoma, especially for subtyping, could represent an important novel future tool and consequently, in uncertain cases microRNA profiling has attended special interest within the diagnostic procedure.

The extensive effort emerging in this research field is well demonstrated by Jima et al., who elucidated the complete small RNA transcriptome of normal and malignant B-cells through deep sequencing of 31 normal and malignant human B-cell samples that comprise the entire spectrum of B-cell differentiation and their common malignant phenotypes

(Jima, et al., 2010). They were able to evaluate the expression of 333 known microRNAs, and to further measure the expression of 286 candidate novel microRNAs in normal and malignant B-cells. Since their study in 2010, every year, hundreds of new microRNAs are identified and added to the analysis (Di Leva, et al., 2014).

Of most important diagnostic relevance is the differentiation between normal lymphatic tissue and the presence of a lymphoid neoplasm. Lawrie et al. identified 40 differential regulated microRNAs in B-cell lymphoma samples, compared to normal B-lymphocyte subsets (Lawrie, et al., 2009). Using the 20 most deregulated microRNAs, they were able to predict the malignant nature of the samples with a success rate of 99%. This microRNA signature also included miR-125b and miR-155, which were significantly up-regulated in DLBCL (Lawrie, et al., 2009).

A further research focus is the distinction between the various lymphoma subtypes. Di Lisio et al. proposed a model of 128 microRNAs enabling the discrimination of various lymphoid malignancies, including Burkitt lymphoma, chronic lymphocytic leukemia (CLL), DLBCL, follicular lymphoma (FL), marginal zone lymphoma, and mantle cell lymphoma. For the distinction between DLBCL and Burkitt lymphoma, often a twilight zone, a signature of 19 microRNAs including miR-155, was sufficient to discriminate these entities with 93% accuracy (Di Lisio, et al., 2012).

Another point of interest is the discrimination between de novo DLBCL and transformed FL, which is indistinguishable in histology, but of eminent importance for prognosis (Kridel, et al., 2012). Lawrie et al. addressed this issue by identifying a cluster of 14 microRNAs, relevant for this distinction, however only in a small cohort of 16 samples of transformed FL (Lawrie, et al., 2009).

By immunohistochemistry and gene expression profiling, the presence of two distinct subtypes of DLBCL, reflecting the cellular origin, has been revealed (Alizadeh, et al., 2000) (Hans, et al., 2004). Several recent reports focused on the differentiation of these subtypes by applying a microRNA signature (Lawrie, et al., 2009) (Caramuta, et al., 2013) (Lawrie, et al., 2007) (Culpin, et al., 2010). In 2007, Lawrie et al. performed a microarray analysis of 225 microRNAs in four different DLBCL cell lines (Lawrie, et al., 2007). They were able to identify miR-155, miR-221, and miR-21 as being more highly expressed in ABC- compared to GCB cell lines. Confirmation was performed in primary 49 DLBCL samples. In a later study, Culpin et al. broadened the sample cohort, as well as the amount of individual microRNAs used for calculation and established a series of nine differential

expressed microRNAs being able to differentiate between the two subtypes (Culpin, et al., 2010). Interestingly, none of the above-mentioned microRNAs detected by Lawrie et al., but four microRNAs of the miR-17-92 cluster were among the nine discriminative microRNAs (Culpin, et al., 2010). In two recent publications, miR-155 was confirmed to be differentially deregulated in DLBCL subtypes, in conjunction with a series of five and eight microRNAs, respectively (Caramuta, et al., 2013) (Roehle, et al., 2008).

In most of the studies mentioned above, the specimens were derived from frozen tumor samples. Formalin-fixed paraffin-embedded tissue (FFPET) is often more widely available in routine clinical practice. Culpin et al. demonstrated the equality of FFPET and frozen samples results with respect to microRNA expression measurements (Culpin, et al., 2013).

Beyond the recognition of novel markers for histopathological diagnosis, some studies suggest the evaluation of serum microRNA expression levels as a noninvasive method for rapid diagnosis or monitoring of minimal residual disease (Heneghan, et al., 2010). The expression level of three DLBCL-associated microRNAs (miR-155, miR-210, and miR-21) was shown to be significantly higher in serum derived from DLBCL patients compared to normal control sera (Lawrie, et al., 2008). In another study, a total of seven microRNAs was analyzed in serum samples from patients with DLBCL and healthy controls (Fang, et al., 2012): The expression levels of miR-15a, miR-16-1, miR-29c, and miR-155 were significantly elevated, whereas miR-34a was significantly down regulated in DLBCL sera samples, suggesting a potential future tool for this non-invasive diagnostic (Fang, et al., 2012). In the diagnosis of primary central nervous system lymphoma (PCNSL), so far diagnosed by brain biopsy, microRNAs have also been shown to play an important role as disease markers in the cerebrospinal fluid (CSF). Three microRNAs, namely miR-21, miR-19, and miR-92a, showed a significant presence in the CSF of patients with PCNSL. Diagnostic accuracy for these microRNAs in diagnosing PCNSL was high, with 95.7% sensitivity and 96.7% specificity (Baraniskin, et al., 2011).

MicroRNAs in the Prognosis of DLBCL

Studies suggest that microRNAs, besides their usefulness in improving diagnosis, also possess a prognostic potential for cancer patients.

MiR-21, already established as differentiator between the GCB and ABC subtype of DLBCL, also plays a role as a prognostic indicator (Lawrie, et al., 2007). High expression levels of this microRNA were found to be associated with longer relapse-free survival in

patients with DLBCL in a multivariate Cox analysis (Lawrie, et al., 2007). The other microRNAs identified in that study, used for predicting cell of origin (COO) subtypes (miR-155 and miR-221) showed no prognostic impact. A confirmatory study by Jung et al. demonstrated no correlation in their cohort of 129 DLBCL patients of expression level miR-155 and OS again (Jung & Aguiar, 2009). Interestingly, a marked trend towards a better survival for patients with ABC subtype with high expression of miR-155 was found.
Alencar et al. identified three (miR-18a, miR-181a, and miR-222) out of 11 microRNAs as being independent predictors of outcome in DLBCL (Alencar, et al., 2011). They integrated these microRNAs in a combined model, including the IPI and a 6-gene mortality predictor score, and demonstrated the predictive power of these microRNAs on the 5-year OS and PFS (Alencar, et al., 2011). Interestingly, miR-155 was also analyzed in this study and again was not associated with prognostic significance.
A further predictive model was created by Montes-Moreno et al. (Montes-Moreno, et al., 2011) for 36 patients by employing a microarray to identify differentially expressed microRNAs, based on OS: 57 microRNAs were correlated positively or negatively with OS, none of them were able to discriminate between the ABC and GCB subtype. A set of nine microRNAs was further evaluated in the entire test group of 240 patients. With these data, a microRNA expression-based model for prediction of OS and PFS was applied. Low expression levels of these nine microRNAs were found to be significantly associated with better OS and PFS. Further accuracy could be improved by the combination of the microRNA-based model and the already existing IPI score (Montes-Moreno, et al., 2011).
Currently, overwhelming evidence suggests that microRNAs are a valuable tool to accurately predict therapy response. Using multi-microRNA expression models instead of a single marker, the predictability can significantly be improved. Further, incorporation into already existing predictive models like the IPI should also be encouraged.

MicroRNAs in (Future) Therapy

Identifying the role of various microRNAs in the pathogenesis of lymphomas has raised the question of their use in therapies. In general, two ways exist of employing microRNA targets for therapeutic use: the down-regulation of oncogenic microRNAs by for instance antagomiRs, or the replacement of suppressed microRNAs, important for normal cell development (Garzon, et al., 2010). In a mouse model, Babar et al. demonstrated a rapid regression of lymphadenopathy in mice with established lymphoma by suppressing miR-

155 (Babar, et al., 2012). They further demonstrated that tumor regression was partly caused by apoptosis and that nanoparticle delivery of anti-miR-155 inhibited miR-155 expression in vitro.

Since microRNAs are down-regulated in the majority of lymphoma cases, microRNA replacement therapy appears to be a promising treatment approach. Although no data about microRNA replacement in DLBCL are available, promising results exist for CLL. Therein, the correction of down-regulated miR-15a and miR-16 induced apoptosis in vitro and led to a restoration of cell cycle control by arresting cells in the G 1 phase (Salerno, et al., 2009).

The paramount importance of microRNAs in the treatment of human disease has been recently demonstrated in patients with hepatitis C (HCV) infection in whom *miravirsen*, an anti-miR-122 component, showed a prolonged dose-dependent reduction in HCV RNA levels (Janssen, et al., 2013). Nevertheless, many questions related to microRNA-based treatment approaches are still unanswered and requires a large amount of continuous research. One challenge is certainly the evaluation of the safety of microRNA therapeutics: The potential immune response, toxicity or unexpected side effects, related to the fact that one microRNA can affect hundreds of target genes is still the focus of research. The successful delivery of the agent to the target tissues or overcoming tissue barriers remains unacknowledged yet (Ling, et al., 2013).

About the impact of the microRNAs investigated within this project in DLBCL, there exists only insufficient knowledge. But their role in solid cancers encouraged us to investigate their role in our cohort of DLBCL samples.

microRNA-199a

Chen et al. identified miR-199a in ovarian cancer cells as a regulator of IkappaB kinase beta (IKKbeta) expression, required for further NF-kappaB activation and therefore a major player in chronic inflammation and cancerogenesis (Chen, et al., 2008). Also a tumor-suppressive function of miR-199a, showed to be pro-apoptotic by targeting the MET proto-oncogene and its downstream effector ERK2 was demonstrated in fibroblasts (Kim, et al., 2008). Decreased miR-199a expression and therefore pro-oncogenic growth of tumors has also been found in hepatocellular carcinoma (Henry, et al., 2010), renal cancer cells (Tsukigi, et al., 2012), ovarian cancer (He, et al., 2013), endometrial cancer (Wu, et

al., 2013), and gastric carcinomas (Peng, et al., 2013). Therein, various target genes of possible relevance in lymphomagenesis like mammalian target of Rapamycin/ mechanistic target of Rapamycin (mTOR) could be identified (Wu, et al., 2013) (Peng, et al., 2013).

microRNA-497

The anti-apoptotic protein B-cell lymphoma 2 (BCL2) has been identified as important target of miR-497, showing significant upregulation in gastric and lung cancer cell lines with downregulated miR-497 expression (Zhu, et al., 2012). Various studies confirmed the insulin-like growth factor 1 receptor as objective of this microRNA with the effect of inhibited cell survival, proliferation and invasion in colorectal carcinoma and cervical cancer (Guo, et al., 2013) (Luo et al., 2013). In addition, miR-497 impairs tumor growth and angiogenesis in non-small cell lung cancer (Zhao, et al., 2013) as well as in hepatocarcinogenesis (Furuta, et al., 2013). In neuroblastoma its role as biomarker showing low expression levels being correlated with worse OS could be demonstrated (Creevey, et al., 2013).

Nevertheless, the role of these two microRNAs in lymphomagenesis has not been elucidated yet. The aim of this thesis was to demonstrate their potential prognostic value in patients with DLBCL as well as identifying their modus operandi.

IV. Material & Methods

a. Material

To investigate microRNA expression levels, 81 different patient samples were analyzed in total (Table IV-1).

Table IV-1: Patient samples used for molecular analysis.

Entity	No
peripheral CD19+ B-cells	9
CD77+ germinal center B-cells	5
lymphadenitides	4
Diffuse large B-cell lymphoma	40
- Germinal center B-cell type - Non-germinal center B-cell type - unclassified	-15 -17 -8
Follicular lymphoma grade III	23

Furthermore, 12 different lymphoma cell lines, representing various lymphoma subtypes, were screened for their microRNA expression levels (Table IV-2).

Table IV-2: Cell lines used for molecular analysis.

Name	Origin
Karpas-422	GCB DLBCL
SuDHL4	GCB DLBCL
SuDHL6	GCB DLBCL
SuDHL10	GCB DLBCL
Ly1	GCB DLBCL
Ly19	GCB DLBCL
NUDUL-1	ABC DLBCL
U2932	ABC DLBCL
RI-1	ABC DLBCL
U937	Histiocytic lymphoma
Raji	Burkitt lymphoma
BL2	Burkitt lymphoma

The cell lines Karpas-422, SuDHL4, RI-1, and U2932 were chosen for further experiments. SuDHL4, RI-1, and U2932 were maintained in Roswell Park Memorial Institute 1640 (RPMI) Medium with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50µg/ml). Karpas-422 was maintained in RPMI-1640 with 20% FBS, penicillin (50 U/ml), and streptomycin (50µg/ml). Cells were periodically checked for mycoplasma by PCR and were found to be negative.

Chemotherapy treatment

After 24 hours, transfected cells were treated with components of R-CHOP: cyclophosphamide (Baxter Oncology GMBH, Halle, Germany), doxorubicin (Pfizer Service Company BVBA, Zaventem, Belgium), vincristin (Pfizer Italia S.R.L. Nerviano, Italy), and rituximab (Roche Austria GMBH, Vienna, Austria) at concentrations ranging from 0.0005 nM to 50 nM (Table IV-3).

Table IV-3: Chemotherapeutics used for treatment of transfected lymphoma cell lines.

Drug	Doses (nM)	Source
Rituximab	0.1-0.25-0.5-1	Roche Austria GMBH, Vienna, Austria
Cyclophosphamide	1-10-25-50	Baxter Oncology GMBH, Halle, Germany
Doxorubicin	0.01-0.03-0.05	Pfizer Service Company BVBA, Zaventem, Belgium
Vincristin	0.0005-0.001-0.0025-0.005	Pfizer Italia S.R.L. Nerviano, Italy

In total, the expression levels of 11 microRNAs were investigated in the above mentioned material (Table IV-4).

Table IV-4: MicroRNAs used in molecular analysis.

Name	sequence	Location
microRNA15b_2	5'uagcagcacaucaugguuuaca	Chromosome 3 (3q25.33)
microRNA16-1*	5'uagcagcacguaaauauuggcg	Chromosome 13 (13q14.2)
microRNA16-2	5'uagcagcacguaaauauuggcg	Chromosome 3 (3q25.33)
microRNA16-2*	5'ccaauauuacugugcugcuuua	Chromosome 3 (3q25.33)
microRNA27a	5'uucacaguggcuaaguuccgc	Chromosome 19 (19p13.13)

microRNA27a*	5’agggcuuagcugcuugugagca	Chromosome 19 (19p13.13)
microRNA98	5’ugagguaguaaguuguauuguu	Chromosome X (Xp11.22)
microRNA103a_1	5’agcagcauuguacagggcuauga	Chromosome 5 (5q34)
microRNA185	5’uggagagaaaggcaguuccuga	Chromosome 22 (22q11.21)
microRNA199a_1	5’cccaguguucagacuaccuguuc	Chromosome 19 (19p13.2)
microRNA497_1	5’cagcagcacacugugguuugu	Chromosome 17 (17p13.1)

Database

This retrospective analysis included data from more than 300 consecutive patients who were diagnosed with DLBCL according to the 2008 World Health Organization (WHO) (Campo, et al., 2011) criteria at the Division of Hematology at the Medical University of Graz between January 2004 and April 2013. All of the clinico-pathological data were retrieved from medical records from the Division of Hematology, as well as from pathology reports from the Institute of Pathology at the same institution. Clinico-pathological parameters included histologically confirmed DLBCL, gender, age, Ann Arbor stage and COO categories (GCB and ABC subtype according to the Hans algorithm (Hans, et al., 2004)). The laboratory data were obtained by pre-diagnosis exploration one to seven days before histological proven diagnosis. Patients were treated by standard R-CHOP regimen every three weeks for 6 to 8 cycles. According to the current European Society for Medical oncology (ESMO) guidelines, early stage lymphoma patients without bulky disease receive 6 cycles of R-CHOP every 3 weeks. Only patients with bulky disease are considered to treat with R-CHOP 21 × 6 with radiotherapy to the sites of previous bulky disease (Tilly, et al., 2012). Post-treatment surveillance included routine clinical and laboratory examination. Regarding imaging methods, computer tomography was predominantly used. Follow-up evaluations were performed every three months during the first five years and annually thereafter. Patients were excluded in case of seropositivity of human immunodeficiency virus (HIV), missing laboratory parameters at diagnosis, lost to follow-up or central nervous system lymphoma. Dates of death were obtained from the central registry of the Austrian Bureau of Statistics or by telephone calls to their relatives. OS was defined as the time (in months) from date of diagnosis until death due to any cause within the follow-up period. PFS was defined as the time (in months) from the date of the diagnosis to the date of demonstration of recurrent disease, confirmed radiologically or histologically. PFS were censored at time of death or at the last follow up if the patients

remained tumor free at that time. The study was approved by the local ethical committee of the Medical University of Graz (No. 25-434 ex 12/13).

For further analysis a broader cohort complemented by 189 patients, diagnosed and treated at the Medical Department at the Paracelsus Medical University Salzburg in Austria was analyzed. Again, Ethics Committee of the provincial government of Salzburg, Austria (415-EP/73/127-2012) approved the study.

b. Methods

MicroRNA expression levels

The above mentioned samples (see table Table IV-1) were analyzed with regard to their microRNA expression levels of the 11 microRNAs (Table IV-4) using the miScript system of Qiagen. First step was conversion of mature RNA into cDNA. For reverse transcription, a total of 250µg of template RNA was mixed with 5x miScript HiFlex Buffer, 10x miScript Nucleics Mix, miScript Reverse Transcriptase Mix, and RNAse free water. After incubation, PCR was performed. The mastermix for real-time PCR contained 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, RNAse free water and template cDNA of the previous step. Expression of the above mentioned microRNAs and 2 additional housekeeping genes (SNORD68_1 and RNU6-2_1) was analyzed in the samples using the Roche® LightCycler® 480 (Roche diagnostics International, Ritkreuz, Switzerland), with an especially designed protocol. Clinical data of the patient samples were collected from SAP Medocs. Two expression subgroups, divided by the median value, were defined and Kaplan-Meier analysis performed.

External validation

To validate our findings in an external cohort, we performed an analysis of a free available patients' data set of DLBCL patients, recently published by Caramuta et al. along our own data set (Caramuta, et al., 2013).

Transfection

Cells were transfected by electroporation using AMAXA® Cell Line Nucleofector® Kit V (Lonza, Cologne, Germany). First, adequate concentrations of siRNA were tested in a range from 400ng to 2500ng using AllStars Hs cell Death Control siRNA (20nmol) (Qiagen, Venlo, The Netherlands). Simultaneously various programs (X001, M013, O017, P005, and G016) of Nucleofector^{TM} 2b Device (Lonza, Cologne, Germany) were tested to determine the perfect adjustments. Program G016 for SuDHL4, X001 for Karpas-422 and U2932, and M013 for RI-1 were found to be suitable for these cell lines showing high viability and successful transfection. Then, required number of cells ($4x10^6$) was centrifuged (800 rpm for 5min), supernatant was removed and the pellet was resuspended

in 100µl Nucleofector® Solution per sample. 2000ng siRNA (AllStars Hs Cell Death Control siRNA) respectively microRNA mimics (Syn-hsa-miR-199a-3p miScript miRNA Mimic, Syn-hsa-miR-497-5p miScript miRNA Mimic; Qiagen) were added and appropriate Nucleofector® program applied. Samples were transferred into 12-well plates containing 1ml culture medium. Until analysis, cells were incubated in humidified 37°C/5% CO_2 incubator. Success of transfection was measured by FACS analysis, using the LSR II (Becton Dickinson, Franklin Lakes, NJ, USA).

Chemotherapy treatment

24 hours after transfection, cell lines (SuDHL4 and RI-1) were treated with R-CHOP therapeutics (rituximab, cyclophosphamide, doxorubicin, and vincristin) in various concentrations and were incubated for 72 hours (Table IV-5).

Table IV-5: Different chemotherapeutics concentration used for treatment of cell lines.

concentrations [nM]	***rituximab***	***cyclophosphamide***	***doxorubicin***	***vincristin***
	5	50	0,1	0,01
	2,5	30	0,05	0,005
	1	20	0,04	0,004
	0,5	10	0,03	0,003
	0,1	7,5	0,02	0,002
	0,05	5	0,01	0,001
	0,01	2,5	0,005	0,0005
	0,005	1	0,003	0,0001
	0,001	0,5	0,001	0,00005
	0,0005	0,1	0,0005	0,00001
		0,05		

RNA-Extraction

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). After addition of 1ml of TRIzol® Reagent to the cell pellet and homogenization, phase separation by adding 0.2 ml chloroform and centrifugation (12.000 x g for 15 minutes at 4°C) was performed. The generated aqueous phase was separated and transferred into a

new tube. 0.5 ml of 100% isopropanol was added and centrifuged (12. 000 x g for 10 minutes at 4°C) again. In the end, washing with 0.5 ml of 75% ethanol, drying and resuspension in 100µl RNAse-free water was performed.

cDNA synthesis

First step was conversion of mature RNA into cDNA using miScript II RT Kit (Qiagen, Carlsbad, CA, USA). For reverse transcription, a total of 250µg of template RNA was mixed with 5x miScript HiSpec Buffer (4µl), 10x miScript Nucleics Mix (2µl), miScript Reverse Transcriptase Mix (2µl), and RNAse free water up to a total volume of 20µl. . After incubation (60min at 37°C, 5min at 95°C), PCR was performed.

Real-time PCR

The miScript SYBR Green PCR Kit (Qiagen, Carlsbad, CA, USA) was used for real-time PCR. The mastermix contained 2x QuantiTect SYBR Green PCR Master Mix (12.5µl), 10x miScript Universal Primer (2.5µl), 10x miScript Primer Assay (2.5µl), RNAse free water and template cDNA of the previous step up to a volume of 25µl. Cycling conditions were as followed:

Initial activation step: 15min 95°C

3-step cycling (40 cycles):

Denaturation: 15s 94°C

Annealing: 30s 55°C

Extension: 30s 70°C

Expression of the above mentioned microRNAs and 2 additional housekeeping genes (SNORD68_1 and RNU6-2_1) was analyzed using the Roche® LightCycler® 480 (Roche Diagnostics International, Rotkreuz, Switzerland).

Table IV-6: MicroRNAs used in quantitative PCR.

Name	Sequence
Hs_miR-199a_1 miScript Primer Assay (Qiagen)	5'CCCAGUGUUCAGACUACCUGUUC
Hs_miR-497_1 miScript Primer Assay (Qiagen)	5'CAGCAGCACACUGUGGUUUGU

SNORD68_1	Not available
RNU6-2_1	Not available

The results are expressed as relative units based on calculation $2^{-\Delta\Delta CT}$, which gives the relative amount of target gene normalized to the endogenous control (geometric mean of the two housekeeping genes). Control samples, to verify the absence of DNA contamination in the RNA samples were included. Measurements were performed in triplicates at three timepoints (24h, 48h, 72h).

Cell viability and apoptosis assay

Cells were stained with Annexin V-PE/7-AAD with the Annexin V-PE Apoptosis Detection Kit I (Becton Dickinson, San Diego, CA, USA) according to manufacturer's protocol. Briefly, cells were washed and centrifuged in binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl2), and the pellet was resuspended in 5 µL Annexin V-PE, 5 µL 7 AAD (7-amino-actinomycin D) and 200 µL binding buffer, followed by incubation for 15 min at room temperature in the dark. Measurement was performed by flow-cytometer using the LSR II (Becton Dickinson). Percentage of double negative cells was taken to determine viability.

Activity of caspases 3 and 7 was also determined by Caspase-Glo 3/7 assay from Promega (Promega, Madison, WI, USA). 50 µL of the luminogenic substrate of caspases Ac-DEVD-pNA was added to 50 µL cell suspension for 1 h. Luminescence was recorded with the LUMIstar Omega (BMG Labtech, Ortenberg, Germany).

All experiments were performed in duplicates at three time points: 24h, 48h, 72h.

Cell cycle assay

72 hours after transfection, $1x10^6$ cells were incubated for 1 hour at 37°C with 10 µM BrdU solution. BrdU and 7-AAD staining was performed according to the BrdU Flow kit manual from BD. A total of 10 000 events were collected on the LSR II (Becton Dickinson) and the cellular DNA content was analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

Cell growth assay

Transduced Karpas-422, SuDHL4, RI-1, and U2932 lymphoma cells and controls were plated at a density of 10 000/mL and cultured for 72 hours. Three replicates of the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) were done using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). The absorbance was recorded by a BioRad spectrophotometer at 490nm.

Statistical analysis

The primary endpoint was 5-year OS; the secondary endpoint was 5-year PFS. In a first step, the optimal cut-off values for dNLR and CRP to differentiate best between survival and death in cohort 1 from Graz was determined by applying receiver operating curve (ROC) analysis as previously reported (Absenger, et al., 2013). Using these cut off values for the whole cohort, the association between CRP levels and dNLR and clinico-pathological parameters was evaluated by non-parametric tests (chi square test, Mann-Whitney U Test). Kaplan-Meier curves were used to show an association between CRP levels/dNLR with OS and PFS and log-rank test was used for comparison. Backward stepwise multivariate Cox proportional analysis was performed to determine the influence of clinico-pathological variables, meaning whether they are significantly associated with clinical outcome in univariate analysis of 5-year OS and 5-year PFS. Hazard ratios (HRs) estimated from the Cox analysis are reported as relative risks with corresponding 95% confidence intervals (CIs). The patients were categorized according to the R-IPI prognosis risk groups and Harrells concordance index (c-index) was calculated using the individual R-IPI groups followed by the addition of the CRP levels (Harrell, et al., 1982). All statistical analyses were performed using the Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA) or STATA statistical software. A two-sided $p<0.05$ was considered statistically significant.

V. Results

a. Molecular analysis

microRNA identification

Up to now, more than 1 000 microRNAs are already described and partially characterized according to the US National Library of Medicine National Institutes of Health (www.pubmed.org). To gain new information about the role of microRNAs in lymphomagenesis, we conducted a literature search, identifying 11 microRNAs which are indeed described in solid tumors but without further characterization in lymphomas.

microRNA expression analysis

To determine expression levels of these 11 microRNAs in different lymphoma samples, we performed real-time PCR on 81 samples, including 40 DLBCL (15 GCB, 17 NGCB, 8 unclassified), 23 FL grade III, and 15 controls, including 9 peripheral B-cells, 5 germinal-center B-cells, and 4 lymphadenitides.

Analysis of expression levels of 11 microRNAs (miR-15_2, miR-16_1*, miR-16_2, miR-16_2*, miR-27a, miR-27a*, miR-98, miR-103a_1, miR-185, miR-199a, and miR-497) revealed significantly increased expression levels in 7 microRNAs (miR-16_1*, miR-16_2*, miR-27a, miR-103a_1, miR-185, miR-199a, and mi-R-497) comparing germinal center B-cells and lymphoma cells (Figure V-1). Highest overexpression was found in miR-27a (30 fold overexpression), miR-199a (200 fold overexpression) and miR-497 (80 fold overexpression). No statistical significance was found comparing the different lymphoma subtypes; this might be due to low sample number.

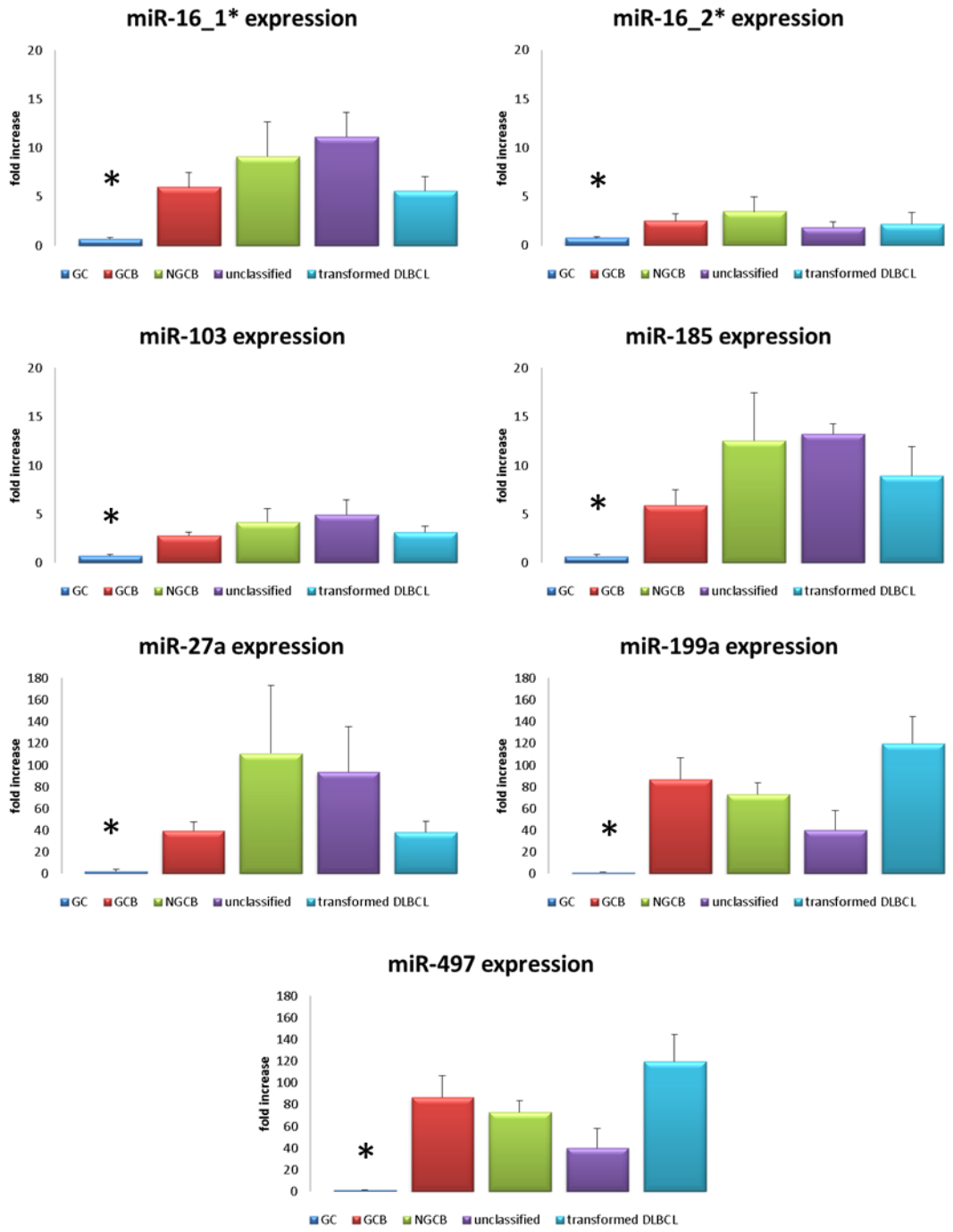

Figure V-1: microRNA expression levels of 7 microRNAs with significantly different expression levels comparing germinal center B-cells and lymphoma samples.

*** indicates reduced expression level of microRNA in germinal center B-cells (GC) compared to lymphoma samples**

Additionally, 12 lymphoma cell lines (Karpas-422, SuDHL-4, SuDHL-6, SuDHL-10, U937, Raji, BL2, Ly19, NUDUL-1, U2932, Ly1, and RI-1) were analyzed respective to their microRNA expression levels of miR-199a and miR-497. None of these cell lines exhibited any microRNA expression but expected expression of the housekeeping genes.

MicroRNAs and survival

Correlation of miR-199a and miR-497 expression levels with survival data of DLBCL patients demonstrated a benefit in overall survival for those patients showing high expression levels of these two microRNA (p=0.002; p=0.012) (Figure V-2).

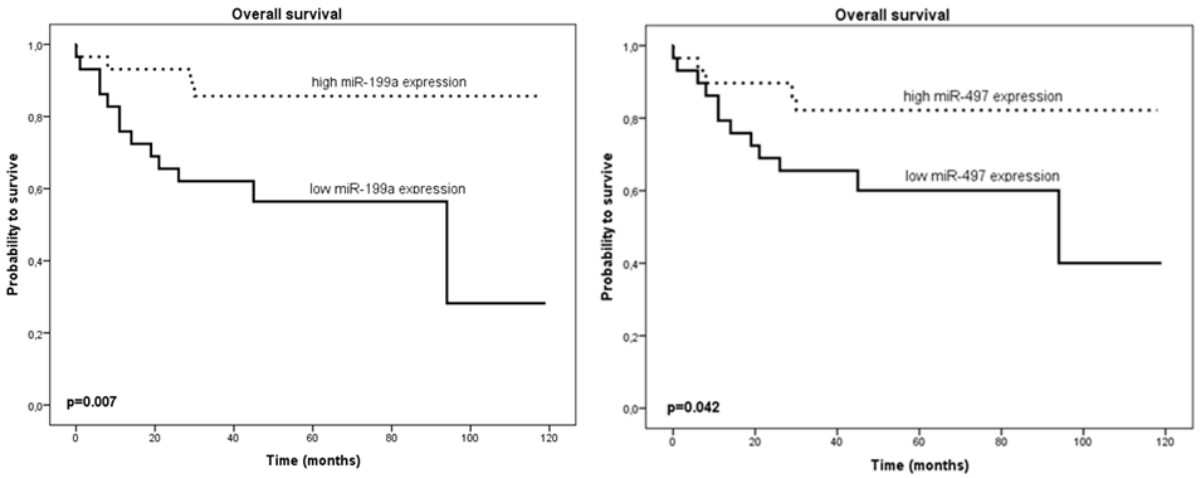

Figure V-2: Kaplan-Meier curves according to microRNA expression levels. High expression levels of miR-199a and miR-497 are correlated with improved OS in DLBCL patients.

In almost all patients (n=60) expression levels of both microRNAs correlated to each other (Pearson correlation coefficient 0.678; p<0.001) and improved survival for patients expressing high levels of both microRNAs could be shown (p=0.004) (Figure V-3).

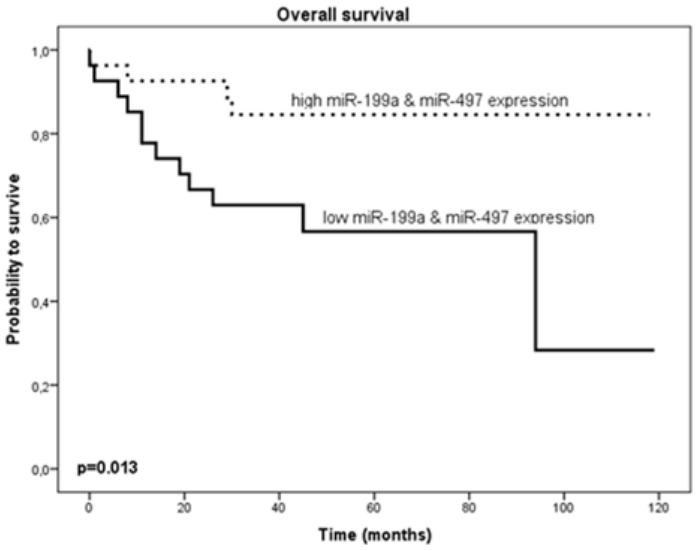

Figure V-3: Kaplan Meier curve showing improved survival for DLBCL patients expressing high levels of both microRNAs.

Survival data analysis of the other nine microRNAs (miR-15_2, miR-16_1*, miR-16_2, miR-16_2*, miR-27a, miR-27a*, miR-98, miR-103a_1, and miR-185) showed no statistical significant differences comparing low and high expression levels.

Validation in an independent data set

To evaluate, if these survival effects are only center specific, we performed *in silico* analysis of an open available data set of DLBCL patients and their microRNA profile, recently published (Caramuta, et al., 2013). Available survival data of 32 patients and their microRNA expression levels confirmed our results of a survival advantage for patients with high expression levels of miR-199a (Figure V-4). No statistical difference was found in this data set for different expression of miR-497 (data not shown).

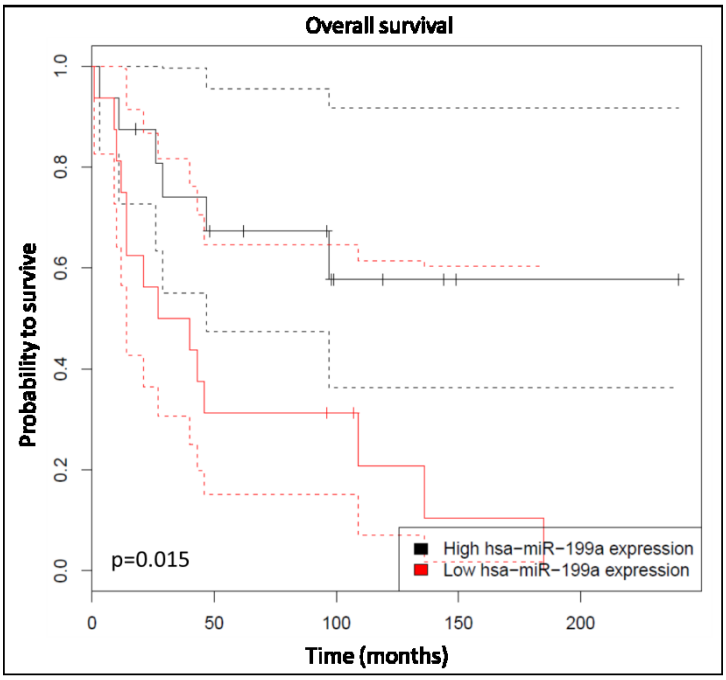

Figure V-4: Kaplan-Meier curves according to microRNA expression levels. High expression level of miR-199a is correlated with improved OS in B-NHL patients in an evaluation set.

Transfection results

Success of electroporation was measured using FACS analysis and could be demonstrated by comparing samples with pulse control only and GFP-labelled siRNA (Figure V-5).

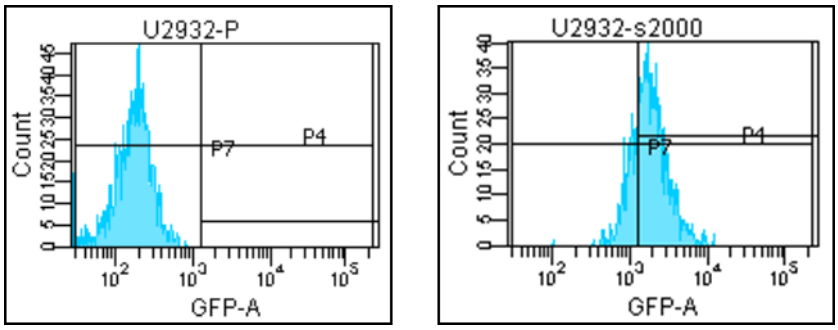

Figure V-5: Transfection of cell line U2932 with GFP-labelled siRNA in contrary to pulse control without any reagent.

Transfection of cell lines with microRNA mimics resulted in an at least 2000-fold increase of microRNA expression in cell lines at different time points (Figure V-6).

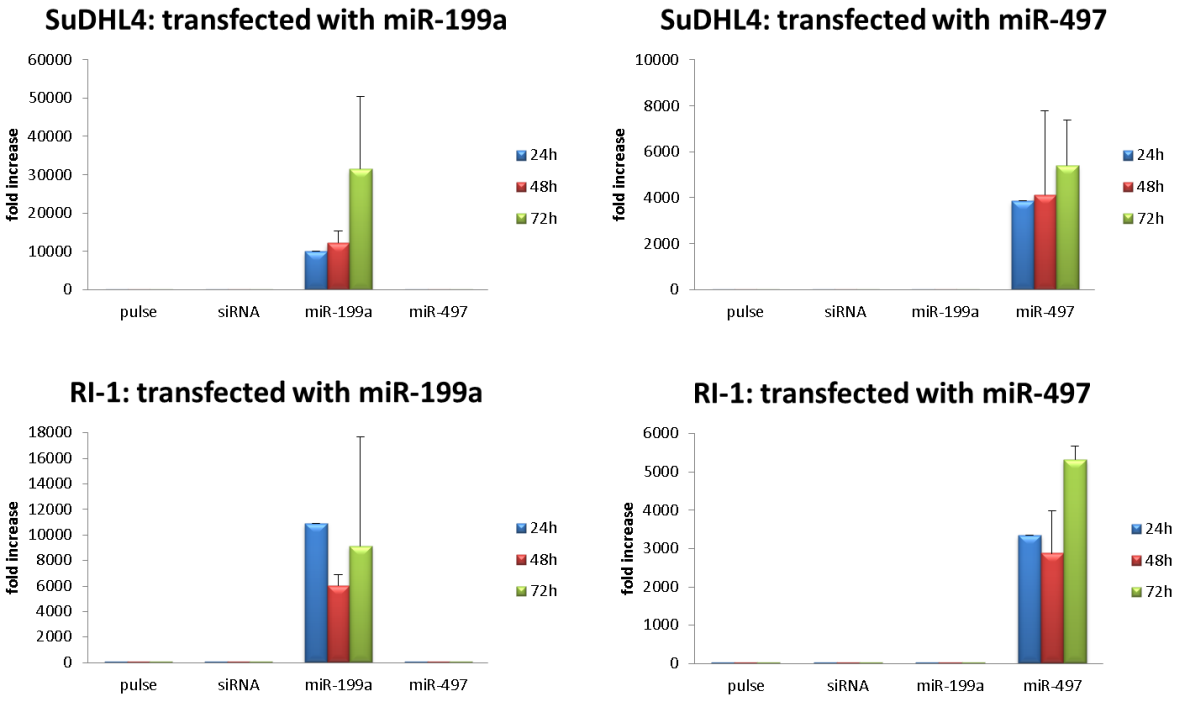

Figure V-6: microRNA expression levels after transfection.

Apoptosis Assays

To determine the viability of cells, overexpressing miR-199a or miR-497, Annexin V and 7AAD staining was performed at time points 24, 48, and 72 hours after electroporation. Flow cytometry was used for analysis and double negative cells were taken for viability determination. No statistical significant differences could be found in viability of cells

comparing those overexpressing the microRNAs of interest or negative controls (siRNAs, pulse control) (Figure V-7).

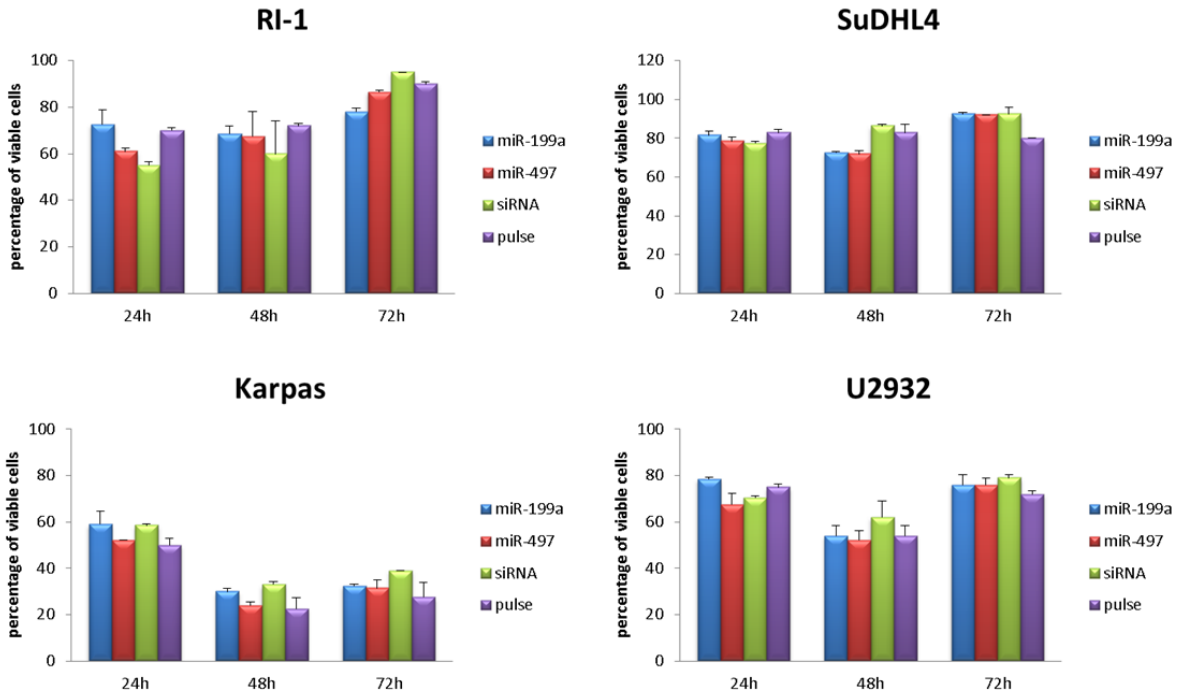

Figure V-7: Percentage of viable cells in 4 cell lines, comparing cells with overexpressed microRNAs and negative controls (pulse control, siRNA).

Another apoptosis assay, using caspase 3/7 activity, showed no statistical significance in apoptosis rate as well (Figure V-8).

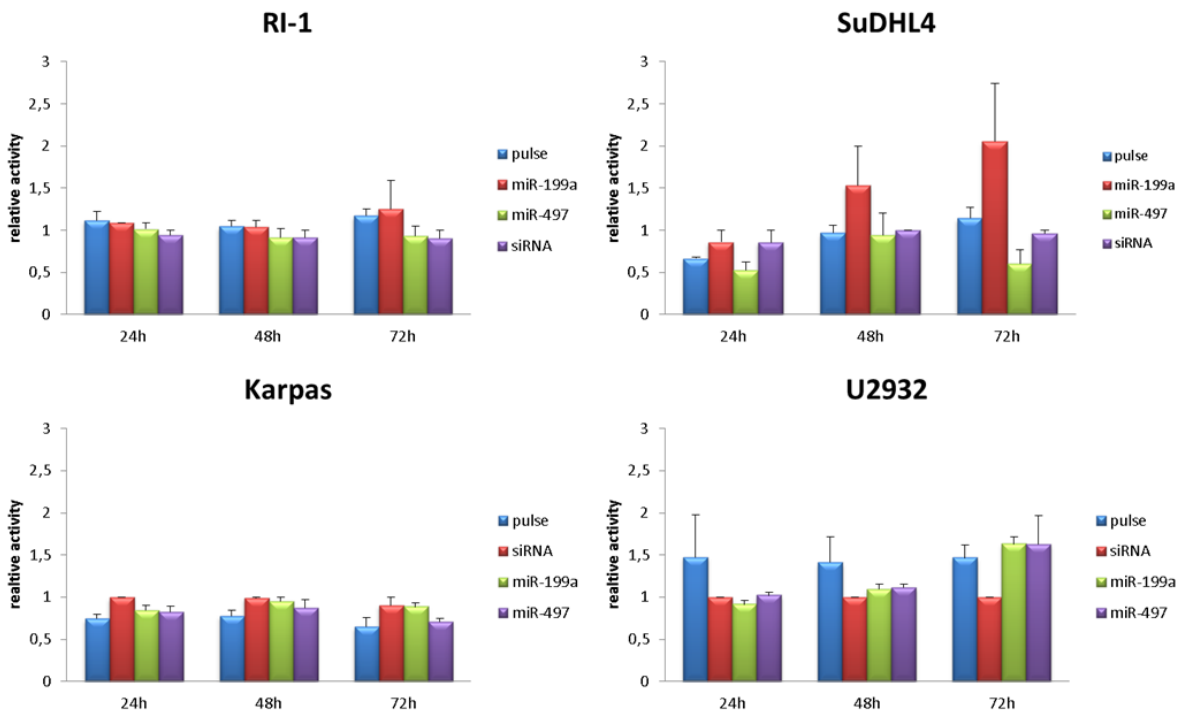

Figure V-8: Caspase 3/7 activity in 4 cell lines comparing cells with overexpressed microRNAs and negative controls (pulse control, siRNA).

Cell cycle analysis

To determine different stages of cell differentiation and may observe any differences in transfected cells compared to negative controls, BrDU cell cycle analysis was performed. No significant differences could be observed in the four tested cell lines (Figure V-9).

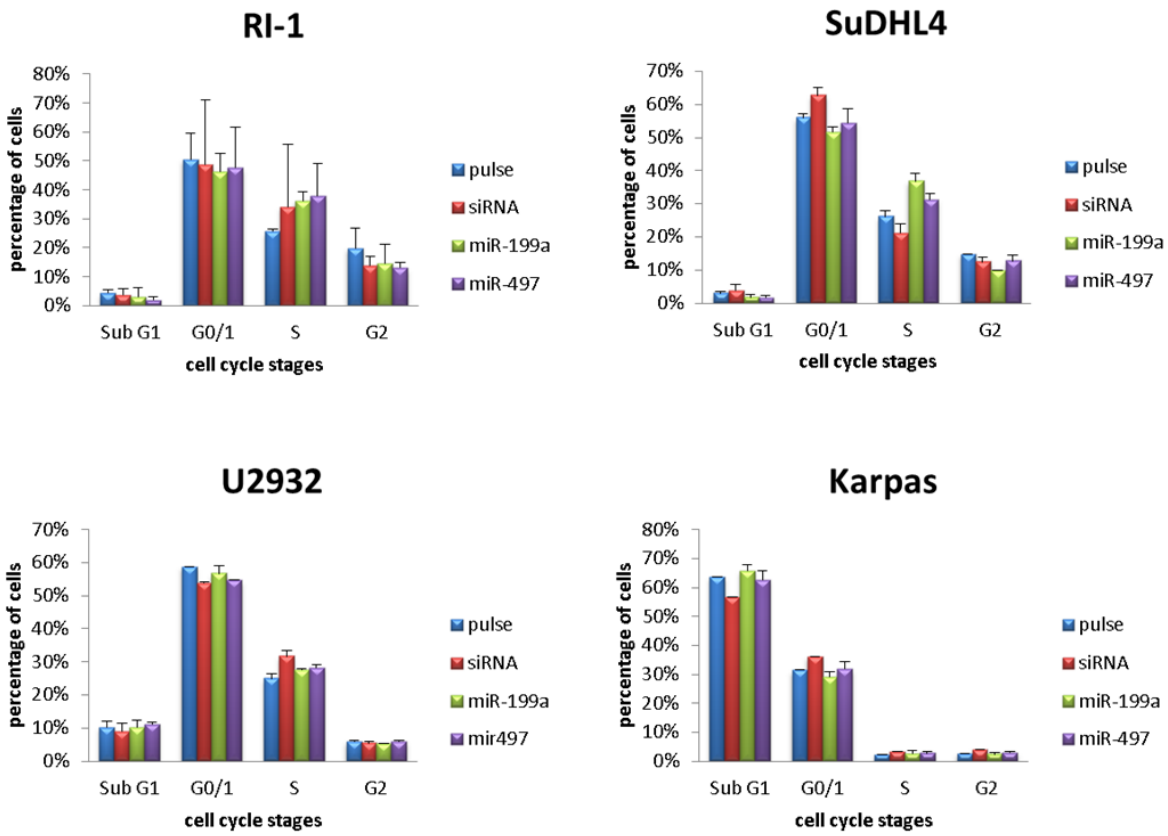

Figure V-9: Cell cycle analysis of 4 cell lines comparing cells with overexpressed microRNAs and negative controls (pulse control, siRNA).

Cell growth assay

To compare the growth rate of transfected cells and negative controls, an MTS assay was performed in four different cell lines. No significant differences have been observed concerning the growth behavior (Figure V-10).

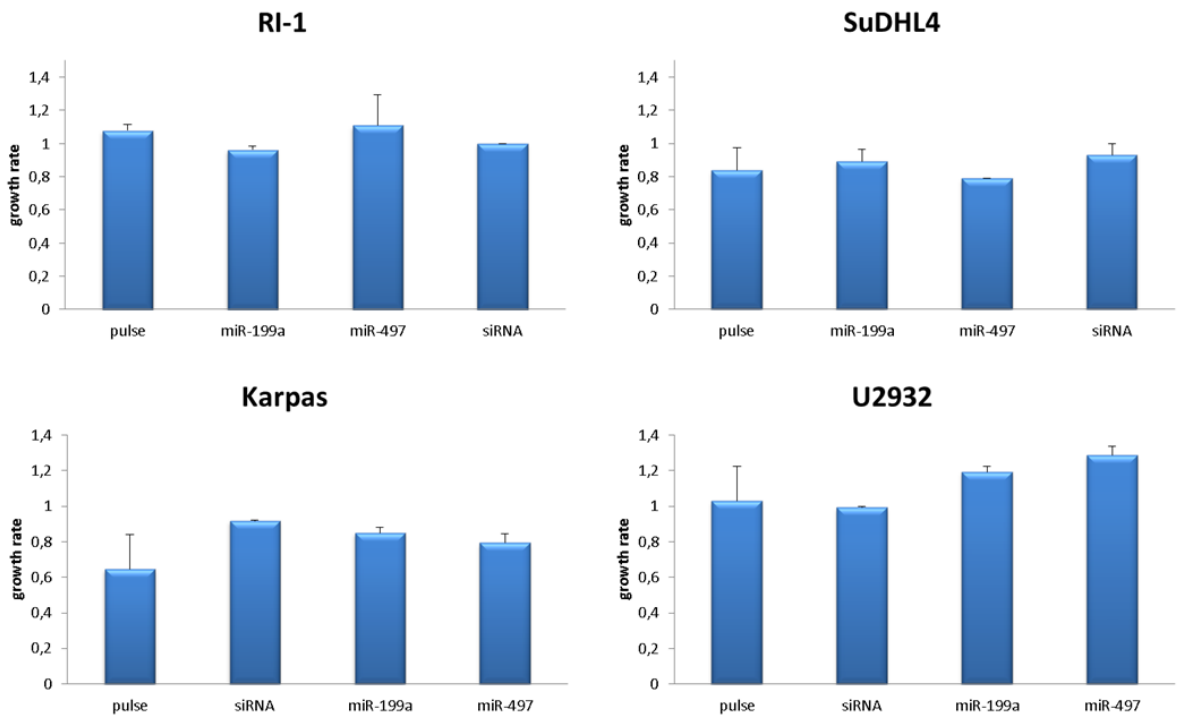

Figure V-10: Growth rate in 4 cell lines comparing cells with overexpressed microRNAs and negative controls (pulse control, siRNA).

Treatment with chemotherapeutics

To identify possible effects in chemotherapy sensitivity, RI-1 lymphoma cells, transfected with either miR-199a or miR-497 and exposed to different concentrations of immunochemotherapy, namely cyclophosphamide, rituximab, vincristin and doxorubicin were investigated and showed significant lower EC50 concentrations calculated by using MTS cell growth assays, compared to controls (Figure V-11, Table V-1). In a second cell line, SuDHL4, EC50 concentrations for doxorubicin and rituximab were also significantly lower in transfected samples, compared to transfection controls (Figure V-11, Table V-1). For vincristin, no statistical significant effect could be found in this cell line. No statistical significant difference was found in both cell lines treated with cyclophosphamide (Table V-1).

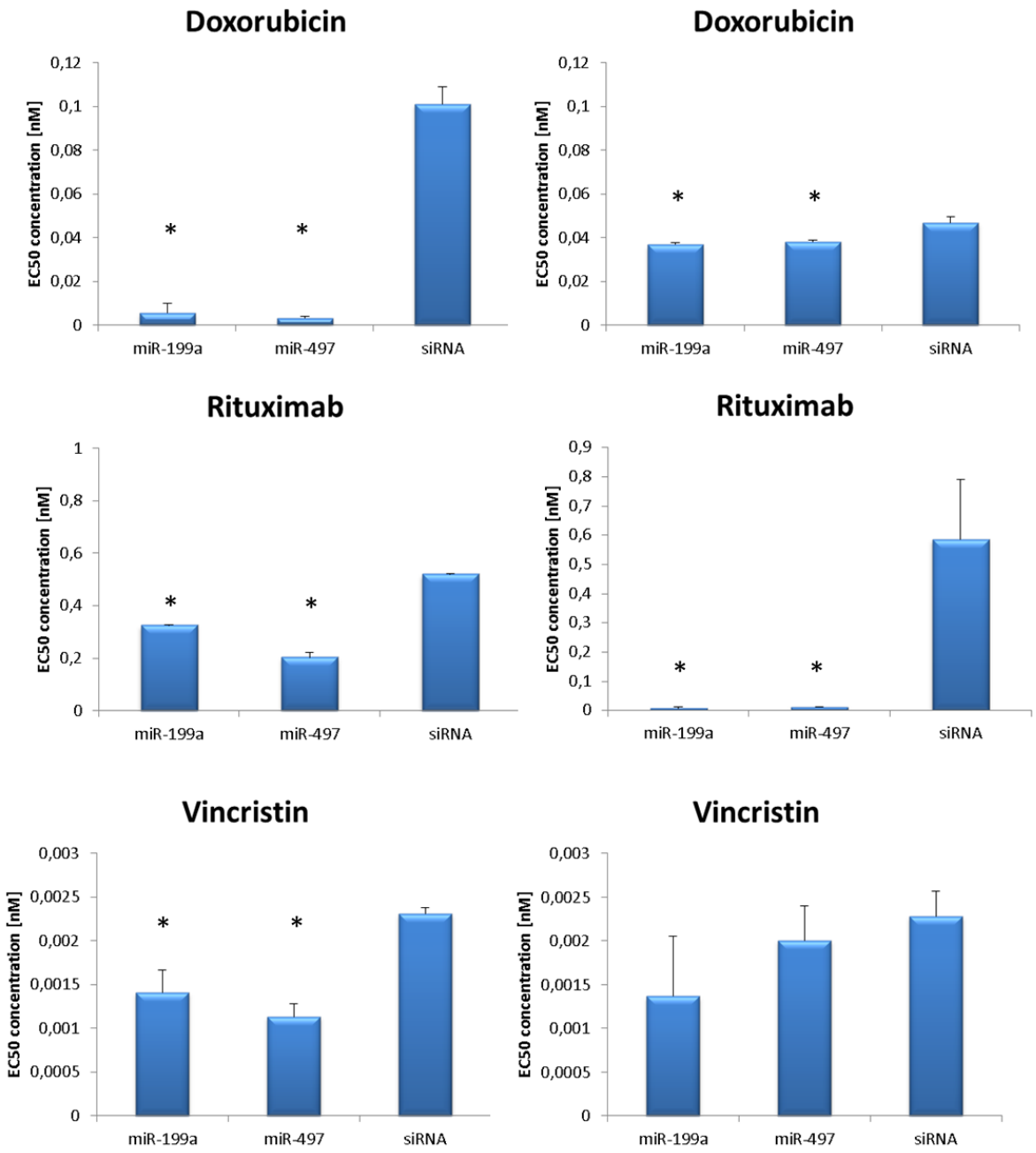

Figure V-11: Screening test with MTS assay. EC50 concentrations of doxorubicin, rituximab and vincristin of transfected RI-1 (left column) and SuDHL4 (right column) cells. Each bar represents the mean values of the EC50 concentration in nM ± standard deviation of either miR-199a or miR-497 transfected cells or siRNA control, respectively. The comparison of the expression levels was performed by using the Mann-Whitney U test.

*** indicates reduced EC50 concentration compared to siRNA ($p<0.05$)**

Table V-1: EC50 concentrations of the cell lines RI-1 and SuDHL4 after immunochemotherapy treatment (cyclophosphamide, doxorubicin, rituximab, or vincristine) in microRNA overexpressing cells and controls, respectively.

EC50 concentrations [nM] (standard deviation)	RI-1				SuDHL4			
	Cyclophosphamide	*Doxorubicin*	*Rituximab*	*Vincristin*	*Cyclophosphamide*	*Doxorubicin*	*Rituximab*	*Vincristin*
miR-199a	9.65 (±0.89)	0.05 (±0.04)	0.33 (±0.002)	0.001 (±0.000)	121.89 (±10.79)	0.03 (±0.000)	0.007 (±0.005)	0.001 (±0.000)
miR-497	30 (±27.4)	0.03 (±0.01)	0.20 (±0.02)	0.001 (±0.000)	9.54 (±0.98)	0.03 (±0.000)	0.011 (±0.002)	0.001 (±0.002)
siRNA	34.85 (±3.63)	1.01 (±0.08)	0.52 (±0.003)	0.002 (±0.000)	2.34 (±18.5)	0.04 (±0.002)	0.585 (±0.21)	0.002 (±0.002)
t-test miR-199a vs. siRNA	***p*=0.01**	***p*=0.004**	***p*<0.001**	***p*=0.04**	*p*=0.33	***p*=0.03**	***p*=0.05**	*p*=0.22
t-test miR-497 vs. siRNA	*p*=0.83	***p*=0.003**	***p*=0.002**	***p*=0.009**	*p*=0.4	***p*=0.04**	***p*=0.05**	*p*=0.50

To confirm this observation, we performed further cell viability testing using RI-1 and SuDHL4 cells, either overexpressing miR-199a, miR-497, or siRNA control and treatment with doxorubicin (0.01nM, 0.03nM and 0.05nM), rituximab (0.1nM, 0.25nM, 0.5nM and 1nM) and vincristin (0.0005nM, 0.001nM, 0.0025nM and 0.005nM) followed by the determination of Annexin V negative and 7-AAD negative cell number (viable cells). For two cell lines, we could confirm a reduced number of viable cells (Annexin V negative and 7-AAD negative) in cells overexpressing either miR-199a or miR-497 compared to negative control siRNA (Figure V-12).

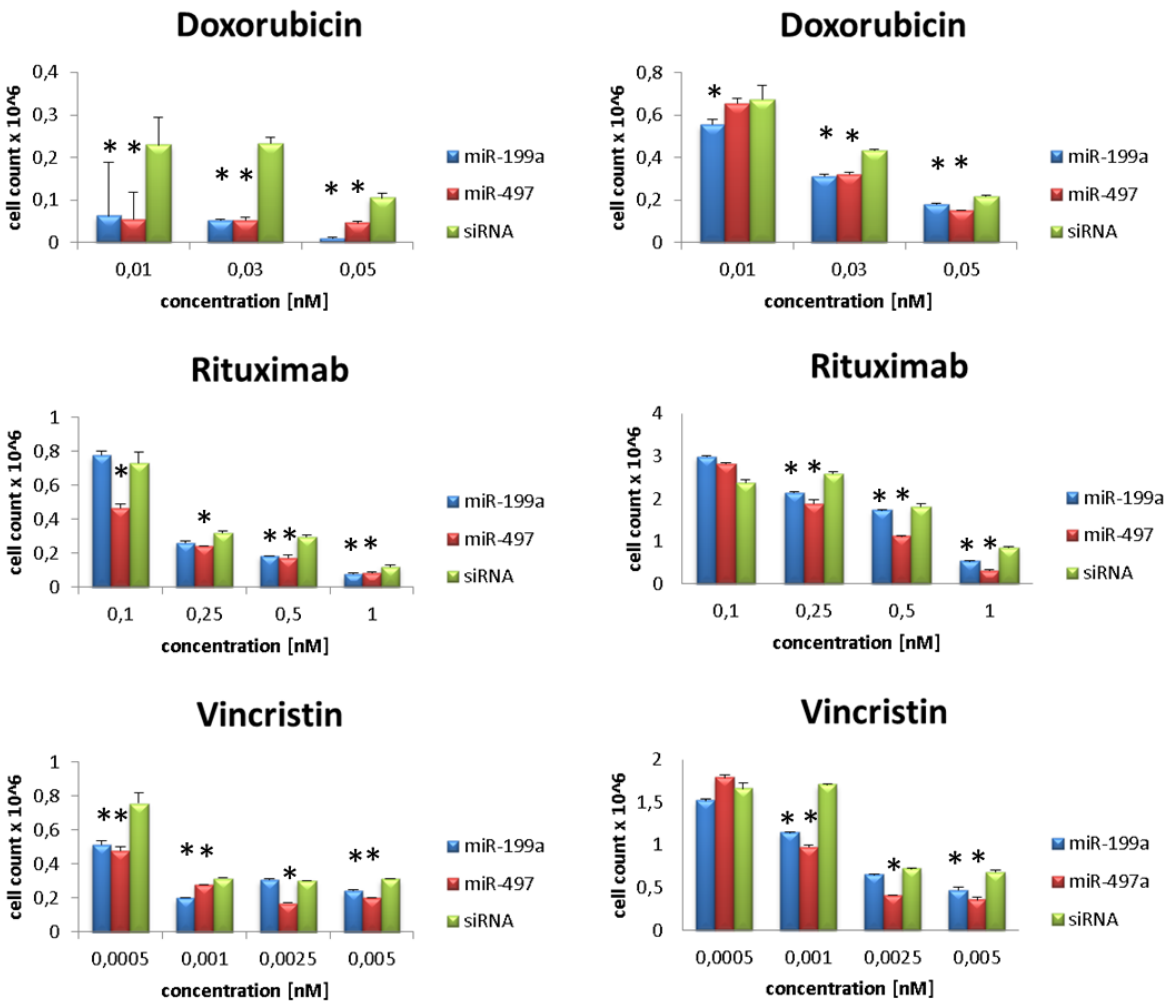

Figure V-12: Cell viability of RI-1 (left column) and SUDHL4 (right column) cells treated with doxorubicin, rituximab, and vincristin. Each bar represents the mean cell viability ± standard deviation of either miR-199a or miR-497 transfected cells or siRNA control, respectively. Plotted against the x-axis are the used concentrations in nM. Percentage of double negative cells was taken to determine viability.

*** indicates reduced cell viability compared to siRNA ($p<0.05$)**

In summary, these data suggest that overexpression of either miR-199a or miR-497 increases the chemosensitivity of aggressive lymphoma cells towards doxorubicin, rituximab and vincristine representing key components of R-CHOP chemotherapy and might explain the observed survival difference.

b. Part II

For clinical data analysis, the data base consisting of more than 300 patients diagnosed with DLBCL at our institution in the last 10 years was set up. This data base includes patient characteristics (name, age, gender, age at diagnosis, Eastern Cooperative Oncology Group Performance Status (ECOG), date of diagnosis, last contact, date of death, therapy), disease characteristics (clinical stage, subtype, IPI, R-IPI, NCCN-IPI) and standard diagnostic values (leukocytes, differential blood count, hemoglobin, thrombocytes, CRP, fibrinogen, gamma-glutamyl transferase, creatinine, bilirubin, uric acid, body mass index (BMI), lactate dehydrogenase, β2-Microglobulin, albumin, plasma total protein).
For the first analysis, recently published dNLR (Proctor, et al., 2012) was analyzed in our cohort. For further studies, cooperation with colleagues from Salzburg was established, enabling to produce higher reliable statistical output with a greater cohort.

Derived neutrophil to lymphocyte ratio

Overall, there were 137 (47.2%) male and 153 (52.8%) female patients diagnosed with DLBCL included in this analysis. The mean age at diagnosis was 65.5 ± 15.5 years. The Ann Arbor tumor stage was defined as stage I in 76 (26.2%) patients, stage II in 78 (26.9%) patients, stage III in 61 (21.0%) patients and stage IV in 75 (25.9%) patients. Extranodal disease was diagnosed in 128 (44.1%) patients. Eighty nine patients featured histologically confirmed GCB subtype, 135 were identified as ABC subtype and 66 were unclassifiable/non-determined. Regarding the R-IPI, 21 (7.2%) had a very good R-IPI, 158 (54.5%) had a good R-IPI and 111 (38.3%) patients were classified having a poor R-IPI. The median LDH was 255 U/l (interquartile range: 191–412 U/l; upper limit of the normal range 200 U/l) and the mean dNLR was 2.94 ± 1.95 and the mean NLR was 5.52 ± 4.78. The Spearman rank correlation between the NLR and dNLR was 0.931 (p<0.001). Median follow-up was 33.5 months (interquartile range 10.7-60 months), 92 (31.7%) patients died and 69 (23.8%) had disease-recurrence by their most recent follow-up visit. Regarding the number of cycles of R-CHOP, we observed a significant lower number of cycles in early stage patients (5.1% of stage I patients received eight R-CHOP cycles versus 34% of stage II-IV patients, p<0.001) and in elderly patients (23.4% of patients <70years received less than 6 cycles versus 45.7% of patients >70 years received less than 6 cycles, p<0.001).

First we evaluated the previously published cut off value (dNLR=2) as the potentially optimal cut-off value for the continuous dNLR by Kaplan-Meier curve analysis (Proctor, et al., 2012). However, we could not find a survival difference between patients with low (<2) and high (≥2) dNLR (p=0.815, data not shown). Therefore, applying the criteria mentioned above, we determined by ROC analysis a cut-off value of 4.0 for the dNLR to be best to discriminate between patients' survival and death in the whole cohort. This cut-off value prompted us to re-evaluate the dNLR as a universally useful prognostic biomarker in our study cohort. Figure V-13 shows the Kaplan-Meier curve for 5-year OS and reveals that a high dNLR (≥4) is a consistent factor for poor prognosis in DLBCL patients (p<0.047, log-rank test).

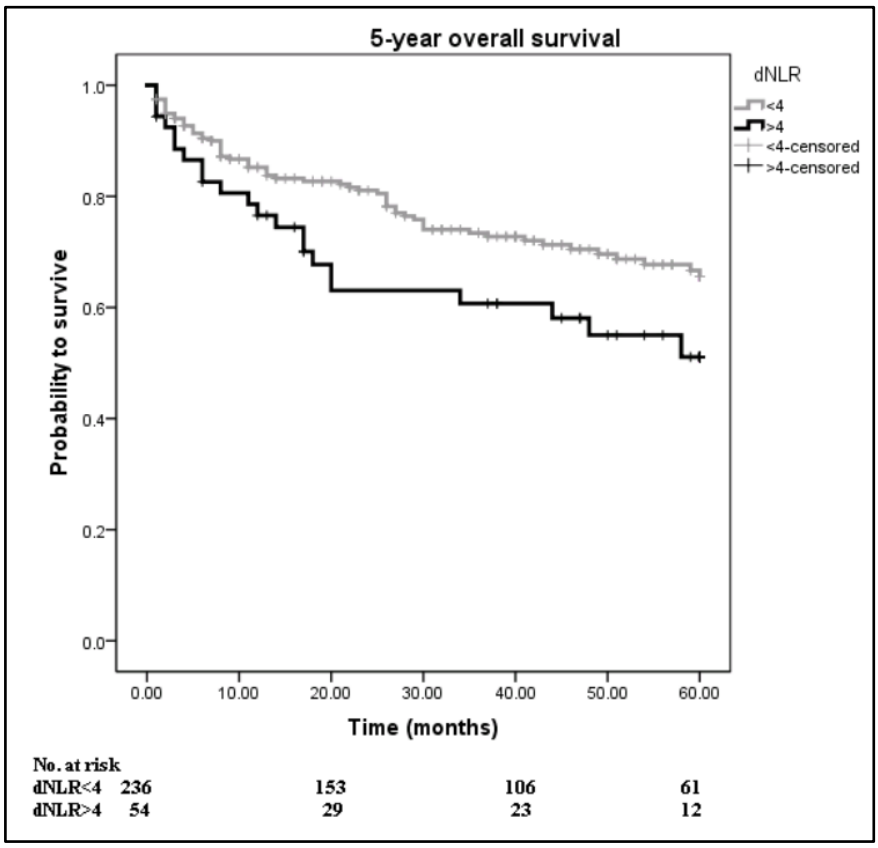

Figure V-13: Kaplan-Meier curves for 5-year OS regarding high (≥4) versus low (<4) dNLR ratio (p<0.043).

A high dNLR was not associated with gender, age, tumor stage, cell of origin or LDH levels (p>0.05, data not shown). Univariate Cox proportional analysis identified older age (<60 versus ≥60, p<0.001), high tumor stage (Stage I+II versus stage III+IV, p<0.001), elevated LDH levels (normal versus >200U/l, p=0.003), cell of origin (GCB versus ABC, p=0.002) and high dNLR (<4 versus ≥4, p=0.047) as prognosticators of poor outcome for

patients' OS, whereas gender and extranodal disease were not statistically significant associated with OS (Table V-2).

To determine the independent prognostic value of the dNLR for OS, a multivariate analysis using a Cox proportional hazard model was performed. In the multivariate analysis, which included all independent parameters significantly associated with clinical outcome in univariate analysis (age, tumor stage, LDH, cell of origin, dNLR), we identified age (p=0.004), tumor stage (p=0.012), cell of origin (p=0.007) and high dNLR (p=0.011) as independent prognostic factors for OS, whereas elevated LDH was not significantly associated with OS (Table V-2).

Table V-2: Univariate and multivariate Cox proportional of clinico-pathological parameters for the prediction of OS in patients with diffuse large B-cell lymphoma (n=290).

5-year OS			**Univariate analysis**		**Multivariate analysis**	
Parameter	No. at risk	No. events	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age at diagnosis (yrs.)						0.004
<60	86	10	1 (referent)	<0.001	1 (referent)	
≥60	204	75	3.82 (1.97-7.4)		3.52 (1.48-8.35)	
Clinical stage (Ann Arbor)						0:012
I & II	154	31	1 (referent)	<0.001	1 (referent)	
III & IV	136	54	2.43 (1.56-3.78)		2.38 (1.21-4.68)	
LDH						
Normal	83	14	1 (referent)	0.003	1 (referent)	0.160
>200 U/l	204	71	2.42 (1.36-4.29)		1.62 (0.83-3.16)	
Cell of origin						
GCB	89	15	1 (referent)	0.002	1 (referent)	0.007
nonGCB	135	50	2.49 (1.4-4.43)		2.25 (1.25-4.04)	
dNLR						
<4	236	63	1 (referent)	0.047	1 (referent)	0.011
≥4	54	22	1.64 (1.01-2.66)		2.03 (1.17-3.5)	
Gender						
Female	153	41	1 (referent)	0.544	n.d.	n.d.
Male	137	44	1.07 (0.86-1.32)			

Regarding PFS, we calculated for the dNLR a cut off value of 1.8 to be optimal to discriminate between PFS and recurrence state. Figure V-14 shows the Kaplan-Meier curves for 5-year PFS and reveals that a dNLR ≥1.8 is a significant factor for shorter 5-year PFS in DLBCL patients (p<0.032, log-rank test).

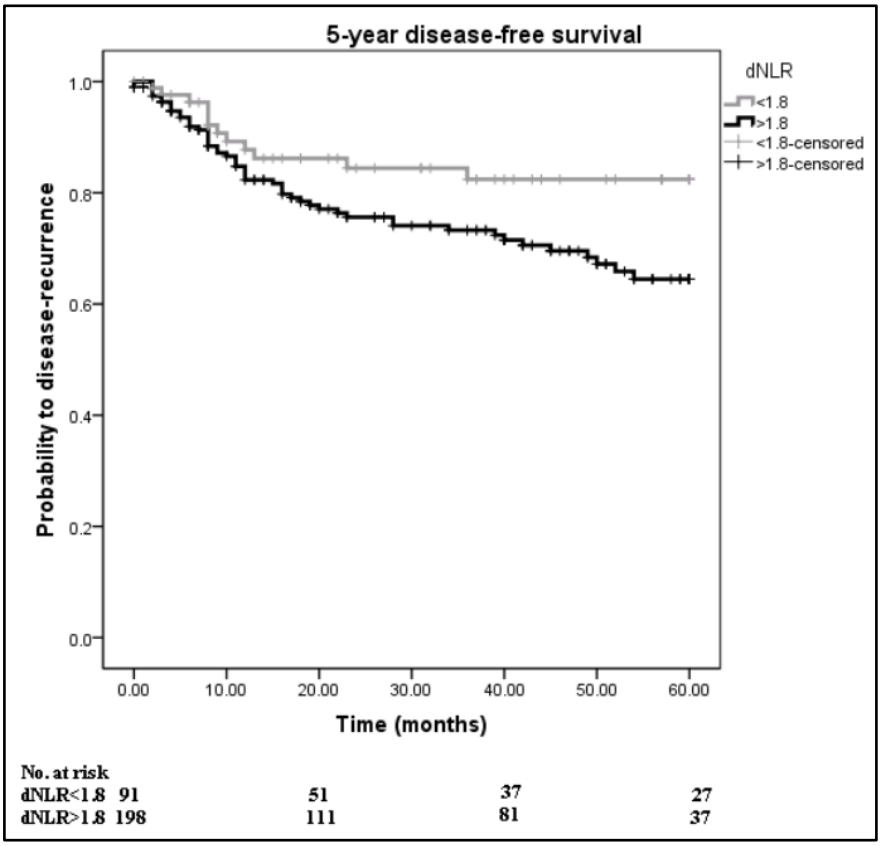

Figure V-14: Kaplan-Meier curves for 5-year PFS regarding high (≥1.8) versus low (<1.8) dNLR ratio (p<0.032).

To determine the independent prognostic significance of the new established cut-off value of dNLR for PFS, a multivariate Cox proportional hazard model including all parameters significantly associated with PFS in univariate analysis (Table V-3) was calculated. In the multivariate analysis, we identified age (p=0.028), tumor stage (p=0.013), cell of origin (p=0.008) and the dNLR (p=0.038) as independent prognostic factors for PFS (Table V-3).

Table V-3: Univariate and multivariate analysis of clinico-pathological parameters for the prediction of PFS in patients with diffuse large B-cell lymphoma (n=290).

5-year PFS			Univariate analysis		Multivariate analysis	
Parameter	No. at risk	No. events	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age at diagnosis (yrs.)						
<60	86	13	1 (referent)	0.02	1 (referent)	0.028
≥60	204	51	2.06 (1.12-3.79)		2.48 (1.11-5.58)	
Clinical stage (Ann Arbor)						
I & II	154	24	1 (referent)	0.001	1 (referent)	0.013
III & IV	136	40	2.42 (1.46-4.02)		2.6 (1.22-5.54)	
LDH						
Normal	83	10	1 (referent)	0.004	1 (referent)	0.083
>200 U/l	204	54	2.67 (1.36-5.25)		2.01 (0.91-4.43)	
Cell of origin						
GCB	89	12	1 (referent)	0.008	1 (referent)	0.008
nonGCB	135	36	2.42 (1.26-4.66)		2.43 (1.26-4.67)	
dNLR						
<1.8	91	12	1 (referent)	0.036	1 (referent)	0.038
≥1.8	198	52	1.96 (1.04-3.67)		2.16 (1.04-4.47)	
Gender						
Female	153	28	1 (referent)	0.183	n.d.	n.d.
Male	137	36	1.18 (0.92-1.51)			

We also found a weak but significantly negative correlation between dNLR and monocyte count (R=-0.136, p=0.021, Spearman correlation). We calculated for the monocyte count a cut off value of 700/mm^3 as optimal for discrimination of OS. This cut off value is very similar to a study by Tadmor et al. (630/mm^3) who demonstrated a prognostic role for monocyte count in DLBCL patients (Tadmor, et al., 2014). To test whether monocyte count is also an independent prognostic value in our cohort, we calculated a multivariate Cox model that also includes the monocyte count as a prognostic variable. Importantly, we found a statistically significant prognostic meaning for both, the dNLR (p=0.004) and the monocyte count (p=0.038, HR=1.75 95%CI=1.03–2.9), indicating that both parameters add independent prognostic information to well-established prognosticators.

CRP

For further analysis, we included patients from Salzburg to broaden our study cohort, so overall, 190 (39.8%) male and 287 (60.2%) female patients with DLBCL were included in the study cohort. The median age at diagnosis was 68 (interquartile range 59 to 77 years).

The Ann Arbor tumor stage was defined as stage I in 127 (26.6%) patients; stage II in 121 (25.4%) patients, stage III in 102 (21.4%) patients and stage IV in 127 (26.6%) patients. Regarding the R-IPI, 44 (9.2%) had a very good R-IPI, 249 (52.2%) had a good R-IPI and 184 (38.6%) patients were classified as a poor R-IPI. Overall, 156 (32.7%) patients died and 184 (38.6%) had disease-recurrence by their most recent follow-up visit. Two-hundred seventy (56.6%) patients received a therapy with R-CHOP, 175 (36.7%) received CHOP-like and 32 (5.2%) received another therapy. The median number of administered therapy was six cycles.

Using ROC analysis, a cut-off value of 15 mg/l for CRP was determined as optimal to discriminate between patients' survival and death in the larger cohort 1. Applied on the whole cohort of 477 patients, a highly significant association (p<0.001, log-rank test) between high CRP levels and poor overall survival as well as decreased 5-year PFS (p<0.001, log-rank test) could be shown (Figure V-15, Figure V-16).

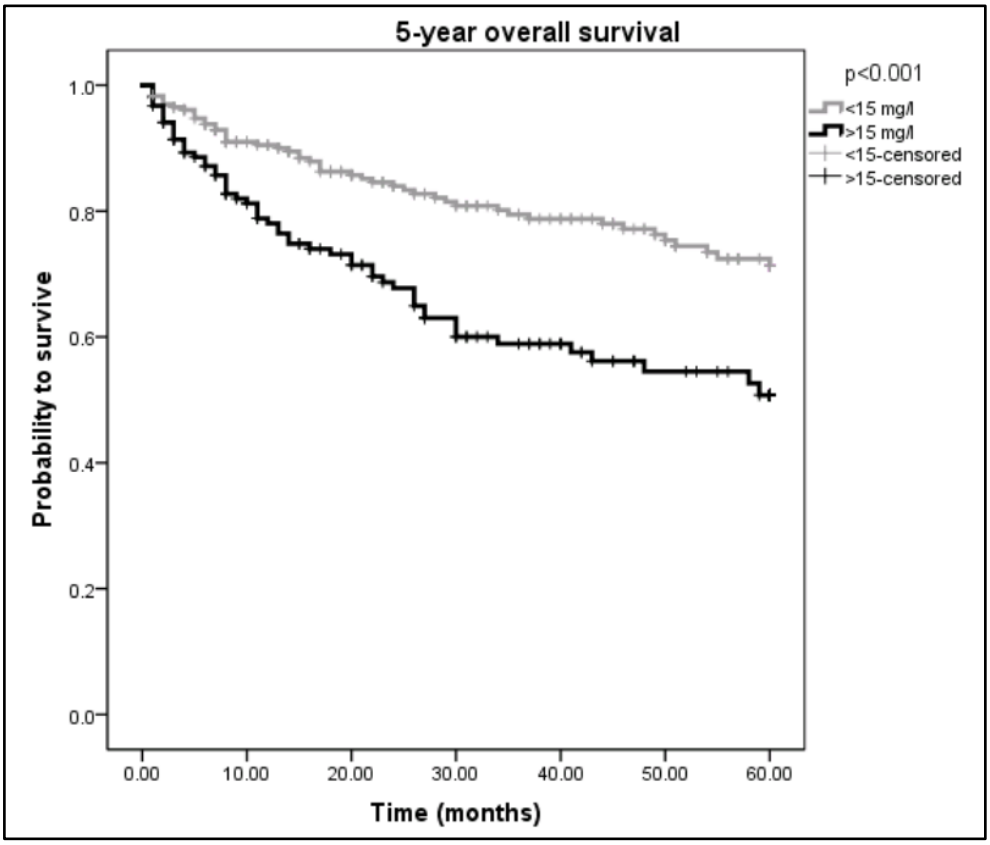

Figure V-15: Kaplan-Meier curves for 5-year OS regarding high (≥15mg/l) versus low (<15mg/l) CRP levels (p<0.001).

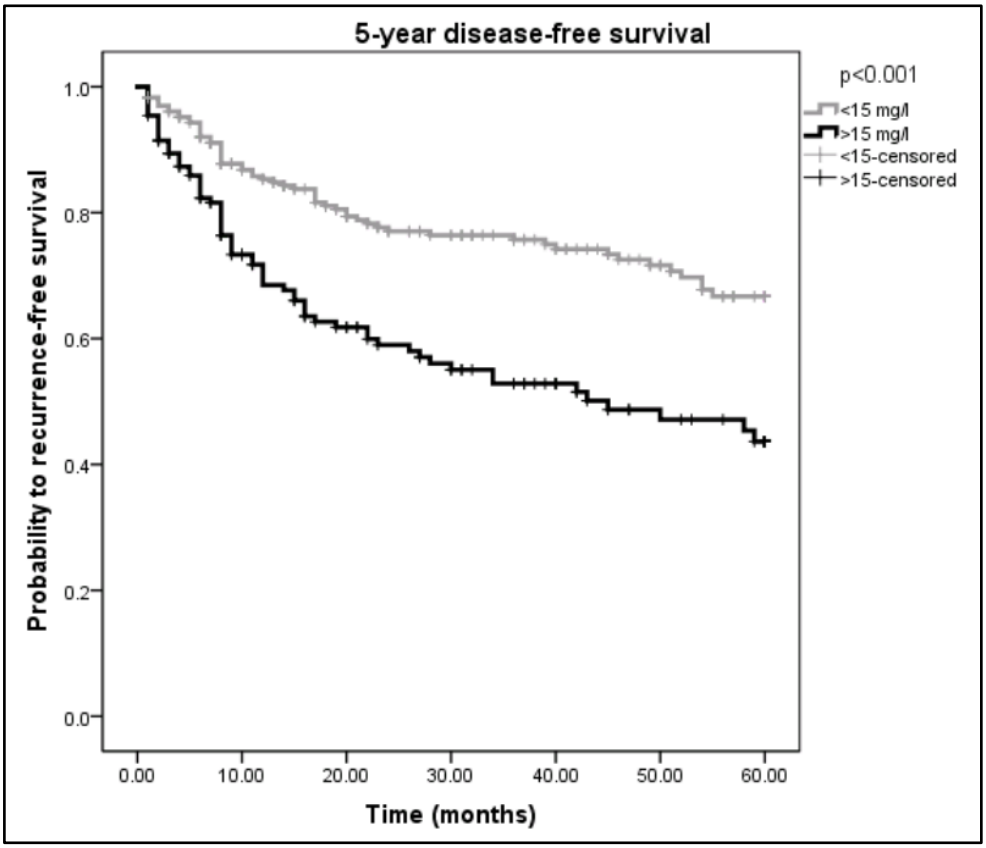

Figure V-16: Kaplan-Meier curves for 5-year PFS regarding high (≥15mg/l) versus low (<15mg/l) CRP levels (*p*<0.001).

Moreover the association between CRP levels and other clinico-pathological factors was tested, and a correlation to advanced tumor stage ($p<0.001$) and a poor R-IPI ($p<0.001$) was observed. No association of CRP with age or gender was found (data not shown). There was neither a correlation of the CRP levels and the number of administered therapy cycles (spearman R=0.33, $p=0.576$), nor a significant association between CRP levels and completed therapy cycles (defined as ≤ 6 cycles, p = 0.754).

Univariate Cox proportional analysis identified gender ($p=0.026$), older age (<60 versus ≥60, $p<0.001$), high tumor stage (Stage I+II versus stage III+IV, $p<0.001$), elevated CRP (<15 mg/l versus >15 mg/l, $p<0.001$), dNLR (<4 versus >4, $p=0.018$) and high R-IPI (very good and good versus poor, $p<0.001$) as prognosticators of poor outcome for patients' 5-year OS (Table V-4). To determine the independent prognostic value of CRP, a multivariate analysis, including gender, age, tumor stage, R-IPI, and CRP levels, was performed. We identified age ($p=0.015$), R-IPI ($p=0.003$) and high CRP ($p=0.031$) as independent prognostic factors for 5-year OS (Table V-4).

Table V-4: Univariate and multivariate Cox proportional of clinic-pathological parameters for the prediction of 5-year OS in patients with diffuse large B cell lymphoma (n=477).

Parameter	Univariate analysis		Multivariate analysis	
	HR (95% CI)	***p*-value**	**HR (95% CI)**	***p*-value**
Gender				
Female (n=287)	1 (referent)	0.026	1 (referent)	0.212
Male (n=190)	0.691 (0.50-0.957)		0.81 (0.58-1.13)	
Age at diagnosis (yrs.)				
<60 (n=180)	1 (referent)	<0.001	1 (referent)	0.015
≥60 (n=297)	2.86 (1.90-4.3)		1.77 (1.12-2.81)	
Clinical stage (Ann Arbor)				
I & II (n=248)	1 (referent)	<0.001	1 (referent)	0.802
III & IV (n=229)	1.86 (1.34-2.6)		1.06 (0.68-1.65)	
R-IPI				
Very good+good (n=293)	1 (referent)	<0.001	1 (referent)	0.003
Poor (n=184)	2.86 (2.06-3.98)		2.02 (1.28-3.21)	
CRP				
<15mg/l (n=266)	1 (referent)	<0.001	1 (referent)	0.031
≥15mg/l (n=211)	2.43 (1.74-3.39)		1.51 (1.04-2.20)	
dNLR*				
<4 (n=367)	1 (referent)	0.018	1 (referent)	0.205
≥4 (n=85)	1.58 (1.08-2.31)		1.28 (0.87-1.89)	

Regarding 5-year PFS, univariate analysis showed statistical significance for older age (<60 versus ≥60, $p<0.001$), high tumor stage (Stage I+II versus stage III+IV, $p<0.001$), elevated CRP (<15mg/l versus >15mg/l, $p<0.001$) and high R-IPI (very good +good versus poor, $p<0.001$) as prognosticators of poor outcome (Table V-5). In multivariate analysis, we identified a high age ($p=0.002$), high tumor stage ($p=0.031$) and high CRP ($p=0.002$) as independent prognostic factors for 5-year PFS (Table V-5).

Table V-5: Univariate and multivariate analysis of clinico-pathological parameters for the prediction of 5-year PFS in patients with diffuse large B cell lymphoma (n=477).

Parameter	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Gender				
Female (n=287)	1 (referent)	0.754	1 (referent)	0.269
Male (n=190)	0.94 (0.64-1.39)		0.80 (0.54-1.19)	
Age at diagnosis (yrs.)				
<60 (n=180)	1 (referent)	<0.001	1 (referent)	0.002
≥60 (n=297)	2.79 (1.63-4.76)		2.51 (1.42-4.44)	
Clinical stage (Ann Arbor)				
I & II (n=248)	1 (referent)	<0.001	1 (referent)	0.031
III & IV (n=229)	2.29 (1.53-3.41)		1.79 (1.05-3.03)	
R-IPI				
Very good+good (n=293)	1 (referent)	<0.001	1 (referent)	0.137
Poor (n=184)	2.80 (1.89-4.16)		1.50 (0.88-2.56)	
CRP				
<15mg/l (n=266)	1 (referent)	<0.001	1 (referent)	0.002
≥15mg/l (n=211)	2.33 (1.58-3.45)		1.91 (1.28-2.85)	
dNLR*				
<1.8 (n=141)	1 (referent)	0.214	1 (referent)	0.434
≥1.8 (n=311)	1.32 (0.85-2.05)		1.20 (0.76-1.87)	

Regarding 5-year OS, the c-index of the R-IPI prognostic model was 0.75 compared to 0.79 when CRP level was supplemented.

VI. Discussion

a. Part I

MicroRNAs are crucial players in many pathophysiological processes and with their promising potential as novel diagnostic, as well as predictive markers for therapy, our knowledge regarding their role in the development of solid tumors and in hematologic malignancies is rapidly expanding (Iorio & Croce, 2012). The majority of known and characterized microRNAs may function as tumor suppressors or oncogenes and their respective function depends on the cell type- or tissue specific context (Fabbri, et al., 2008). For instance a tumor suppressive role for miR-16 was identified in breast cancer (Mobarra, et al., 2015). On the other hand miR-27a seems to act oncogenic in gastric cancer by promoting metastasis (Zhang, et al., 2011), as well as miR-103 which promotes multidrug resistance (Zhang, et al., 2014). In colorectal-, as well as in prostate cancer, miR-185 has been identified as a tumor suppressor (Liu, et al., 2011) (Akcakaya, et al., 2011) (Li, et al., 2013). In our study, we identified two (miR-199a and miR-497) of eleven microRNAs as prognostic relevant in DLBCL patients uniformly treated with immunochemotherapy. MiR-199a and miR-497 are mostly found to act tumor suppressive in most solid cancers (Callegari, et al., 2013) (Minna, et al., 2014) (Duan, et al., 2011) (Xu, et al., 2014) (Furuta, et al., 2013) (Guo, et al., 2013). In hepatocellular carcinoma, miR-199a has been found to be down-regulated in nearly all cases investigated, and their tumor suppressive function is exerted partly by their anti-proliferative and anti-growth potential by regulating HIF-1α and PAK4, as well as by modulating effects via mTOR pathway (Callegari, et al., 2013) (Jia, et al., 2014) (Hou, et al., 2011) (Fornari, et al., 2010). Restoring attenuated levels of miR-199a also increased sensitivity to doxorubicin induced apoptosis (Fornari, et al., 2010). The correlation of miR-199a and the mTOR pathway is also confirmed in human osteosarcoma, in which transfection of precursor miR-199a acts tumor suppressive (Duan, et al., 2011). In papillary thyroid carcinoma, miR-199a induces lethality via a non-apoptotic form of cell death (Minna, et al., 2014). On the other hand, NF-ƙB, one of the key transcription factors in proinflammatory response, could be identified as a crucial target of miR-199a in ovarian cancer cells, in which IKKβ, the direct upstream activator of NF-ƙB is tightly regulated by this microRNA (Chen, et al., 2008). Also a tumor-suppressive function of miR-199a, being pro-apoptotic by targeting the MET proto-oncogene and its downstream effector ERK2, was demonstrated in fibroblasts (Kim,

et al., 2008). The same effect of decreased miR-199a expression and therefore pro-oncogenic growth of tumors has also been found in hepatocellular carcinoma (Henry, et al., 2010), renal cancer cells (Tsukigi, et al., 2012), ovarian cancer (He, et al., 2013), endometrial cancer (Wu, et al., 2013), and gastric carcinomas (Peng, et al., 2013). In hematologic malignancies, a regulatory role of miR-199a by negatively affecting cell migration has been identified in multiple myeloma so far (Raimondi, et al., 2014). Of possible relevance in lymphomagenesis again the activated mTOR pathway could be identified (Wu, et al., 2013) (Peng, et al., 2013). Furthermore, in comparison of two forms of primary CNS lymphoma, different expression levels of miR-199a were found (Fischer, et al., 2011).

For miR-497, down-regulation was correlated with breast cancer progression, demonstrating a tumor suppressive role (Wang, et al., 2013). As direct target, the key cell cycle regulator WEE1 has been identified in neuroblastoma, again associated with impaired survival in miR-497 down-regulated samples (Creevey, et al., 2013). Another focus of interest described by Guo et al. (Guo, et al., 2013) was the observation of a significant correlation between upregulated insulin-like growth factor 1 receptor and down-regulated miR-497 expression levels in patients with colorectal cancer. In hepatocellular carcinoma, miR-497 appears to act over cell cycle regulation, demonstrating a growth-suppressive activity with induction of G1 arrest (Furuta, et al., 2013).

These reports demonstrate the crucial role of miR-199a and miR-497 in development and progression of cancer. However, since there are currently no systematic studies in aggressive lymphoma patients, we evaluated the role and detailed function of miR-199a and miR-497 and could demonstrate that high expression levels of these two microRNAs are correlated with a better overall survival. Furthermore, we were able to confirm this survival benefit in an independent patient cohort, thus substantiating this novel observation. To identify the causative pathomechanisms behind this phenomenon, we performed functional analysis in microRNA transfected lymphoma cell lines. Neither of these cell lines showed a different behavior in cell viability or apoptosis by solely overexpression of the respective microRNAs. Nevertheless, after chemotherapy treatment of transfected cells involving the standard immunochemotherapeutical compounds of R-CHOP, we could demonstrate a significant decrease in cell viability in transfected cells, compared to normal controls. First, in a screening test, using MTS assays, we could prove a higher chemosensitivity against doxorubicin, rituximab, and vincristin in cells with

upregulated microRNAs, possibly explaining the prolonged survival of these patients. We validated these results in a subsequent experiment, using Annexin V/7-AAD cell viability testing and found again statistical significant reduced cell viability in cells transfected with microRNAs and subsequent chemotherapeutical treatment. This demonstrates a higher chemosensitivity induced by microRNA up-regulation following therapeutic exposure and would explain their tumor suppressive role in treated DLBCL patients. This has already been demonstrated by Fornari et al. (Fornari, et al., 2010) in hepatocellular carcinoma (HCC) cells. By restoring attenuated levels of miR-199a in HCC cells they detected an increased sensitivity to doxorubicin-induced apoptosis. In colorectal cancer, Guo et al identified miR-497 overexpression as an inhibitor of cell survival, as well as responsible for increased sensitivity to apoptosis induced by chemotherapeutics (Guo, et al., 2013). Re-sensitization to chemotherapy by miR-497 overexpression could also be demonstrated in pancreatic cancer, in which low expression levels showed an adverse prognostic factor (Xu, et al., 2014). However, such functional characteristic has not been performed in DLBCL yet.

As summary, our findings imply that miR-199a or miR-497 up-regulation significantly increases the probability of patients' survival by augmenting the chemosensitivity of lymphoma cells. These findings strengthen the understanding of the role of microRNAs in DLBCL development and might eventually lead to a more refined characterization of these microRNAS for future diagnostic and therapeutic application.

b. Part II

In general, inflammatory processes have been identified as critical components of tumor progression (Coussens & Werb, 2002). Inflammatory cells can release growth and survival factors, promoting angiogenesis and lymphangiogenesis, stimulate DNA damage and promote tumor evasion of the host defense mechanisms (de Visser & Coussens, 2005) (de Visser, et al., 2005). Although the inflammatory response can be expected to have tumor suppressive actions, cancer patients often lack sufficient inflammatory response (Finn, 2012). In various types of cancers, for example breast cancer, melanoma and lymphoma, innate immune cells like granulocytes, macrophages and mast cells correlate with increased angiogenesis and/or poor prognosis, which is in part explained by up-regulation of cyclooxygenase-2 or suppression of anti-tumor adaptive immune response (Leek, et al., 1996) (Schoppmann, et al., 2002) (Dannenberg & Howe, 2003) (Ribatti, et al., 2003). On

the other hand, infiltrating lymphocytes are associated with favorable prognosis which was recently shown in non-small cell lung cancer and ovarian cancer (Horne, et al., 2011) (Sato, et al., 2005). The adaptive immune cells such as B-lymphocytes, CD4+ helper T-lymphocytes and CD8+ cytotoxic T-lymphocytes, modulate cancer development via cytokine-mediated lysis of tumor cells or establishing a pro-inflammatory state in the tumor microenvironment, revealing the paradoxical role of adaptive and innate leukocytes as crucial opposing regulators in cancer development (Ishigami, et al., 2000) (Zou, 2005).

The critical role of B-lymphocytes in initiating chronic inflammation during pre-malignancy has already been demonstrated by De Visser et al. In a tumor-prone mouse model deficient in B and T cells, adoptive transfer of B-lymphocytes restores innate immune cell infiltration into pre-malignant tissue and reinstates necessary parameters for full malignancy (de Visser, et al., 2005). These findings support the hypothesis in which B-lymphocytes are required for establishing chronic inflammatory states that promote de novo carcinogenesis. Further, in a murine model a subset of regulatory B cells was recently found to inhibit anti-CD20 immunotherapy mediated lymphoma depletion through the production of interleukin-10, a potent regulator of inflammation and autoimmunity. Even if present in small amount, they negatively influence effector functions of monocytes and in consequence, the lymphoma response to antibody targeted therapy (Horikawa, et al., 2011). However, despite the substantial progress and novel insights into lymphomagenesis during the past years, clinicians also require fast and easily measurable tools as indicators for patient survival. Within the last recent years, the systemic inflammatory response has been identified as an important driver of cancer progression in different types of cancer (Proctor, et al., 2011).

Different laboratory parameters including the modified Glasgow prognosis score (Proctor, et al., 2011), the NLR, platelet-lymphocyte ratio, CRP or fibrinogen levels (Pichler, et al., 2013) have been proposed as prognostic parameters that adequately reflect this systemic inflammatory response. However, there is plenty of clinical trial data, where only leukocyte and lymphocyte counts have been recorded. To overcome the lacking data, Proctor et al. developed the dNLR and demonstrated the non-inferiority to the NLR in a large cohort of patients with different types of cancer (Proctor, et al., 2012). As the dNLR is mainly derived from the count of neutrophils and lymphocytes, our study also supports the potential of widespread use of this biomarker as a surrogate for inflammatory response.

Importantly, the variation over time and factors that might influence the dNLR have to be discussed. In general, the NLR (and also the dNLR) is supposed to reflect the systemic inflammatory response that accompanies chronic diseases, but might also be influenced by many different factors, including systemic infections, atherosclerosis, hypertension, chronic renal diseases and diabetes and can be even affected by drug treatment (Szkandera, et al., 2013). In our study, the previously published cut-off value of 2, as proposed in the study of Proctor et al, showed no prognostic information in our cohort. The reasons for this discordance might be explained by the missing information in their study (Proctor, et al., 2012). In their original report about the dNLR, Proctor et al. reported data from the Scottish cancer registry including hematological cancers. However, they did not separately analyze different hematological cancer entities. Moreover, they have no data for important prognostic variables like stage or others, which were included in the multivariate model in our study cohort. However, our study was not without limitations. Although we used a strategy to separately determine the optimal cut off value for each endpoint as previously reported (Absenger, et al., 2013), these cut off values have to be externally validated in independent cohorts, most preferable in a prospective manner.

CRP has been identified as a prognostic variable in various solid as well as hematologic malignancies yet. Increased CRP concentrations have been found to be associated with poor OS in many types of cancer, including colorectal carcinoma, prostate cancer, soft tissue sarcoma, pancreatic cancer and small cell lung cancer (Toiyama, et al., 2013) (Hall, et al., 2013) (Szkandera, et al., 2013) (Szkandera, et al., 2013) (Hong, et al., 2012).

The role of CRP in the prognosis of NHL patients had also been well studied in the pre-Rituximab era (Legouffe, et al., 1998) (Pavlidis, et al., 1993). More recently, the correlation between CRP, IPI and long-term survival in DLBCL was investigated, but only in a small group of 94 Chinese patients (Cao, et al., 2012).

Hence prognostic significance for increased CRP in DLBCL has been indicated, however, a validation in large middle European cohort with different genetic background is still missing. In our study, a significant association between elevated CRP and poor outcome of 477 DLBCL patients of two Austrian centers could be confirmed. Univariate analysis, as well as multivariate analysis identified the pre-treatment CRP level as useful prognostic marker of 5-year OS and 5-year-PFS. Furthermore, by integrating the CRP level in the well-established R-IPI score, the predictive ability of this score was improved by 4%. Beyond the results of our study that focuses on the CRP levels before treatment initiation,

Herishanu et al. identified the early-mid treatment CRP level as a reliable marker in NHL patients to predict PFS and OS (Herishanu, et al., 2007). Patients who did not achieve low early-mid treatment CRP levels, had earlier disease progression or earlier relapses and also appear to have an inferior OS. However, less than hundred patients were included in this study and further confirmation in larger series is needed.

Our findings are in line with previously reported data also demonstrating a prognostic value for CRP levels in various other hematological disorders. For instance, in extranodal natural killer/T-cell lymphoma, a heterogeneous histopathologic subtype of NHL with increased incidence in Asia, high CRP levels were found to impair the natural course of disease (Li, et al., 2013). In this study, beside lower rates of complete remissions, inferior PFS and inferior OS were found in patients with high pretreatment CRP levels. Therefore the authors constructed a new prognostic model, including CRP, age, hypoalbuminemia and elevated LDH levels, to identify different prognostic subgroups (Li, et al., 2013).

The reason for the general poor prognosis associated with higher CRP levels remains elusive. Recently, Yang and colleges demonstrated that CRP enhances cell proliferation under stressed conditions and protected myeloma cells from chemotherapy drug-induced apoptosis by binding to activating Fcγ receptors, activating PI3K/Akt, ERK, and NF-kappaB pathways and inhibiting caspase cascade activation induced by chemotherapy drugs (Yang, et al., 2007). Whether these or other mechanisms may play a role in DLBCL has to be clarified.

Apart from CRP and dNLR, other inflammatory markers have been found to be involved in the development and progression of lymphoma. Purdue et al. conducted a prospective study of 67 serum immune and inflammatory markers to identify possible predictors of NHL (Purdue, et al., 2013). A significant association between elevated levels of BCA-1, sTNFR2 and sVEGFR2 and increased risk of lymphoma development, several years after blood collection, could be shown.

Nevertheless, comparing the costs and availability of these markers, dNLR and CRP seem to be more convenient for routine use. Several other blood-based inflammatory parameters have been suggested to play an important role in NHL cancer progression and prognosis (Purdue, et al., 2013) (Troppan, et al., 2014) (Porrata, et al., 2010) (Porrata, et al., 2014). These include the Glasgow prognostic score, the fibrinogen levels, the neutrophil-lymphocyte ratio and the lymphocyte-monocyte ratio. All these studies have been performed on retrospective datasets, including in part several hundreds of patients. Future

prospective trials should assemble a panel of these markers and evaluate the prognostic value of these parameters for differentiating patients with varying clinical outcome. Each of these parameters can add additional prognostic value and supplement established risk scores like the R-IPI or the recently proposed NCCN-IPI to improve prediction of individual patients' outcome (Sehn, et al., 2007) (Zhou, et al., 2014).

Together with the whole blood count and differential leukocyte count, dNLR and CRP are routinely measured in most medical laboratories, which make an implementation for clinical routine more easily. Taken together, the role for pretreatment dNLR and CRP levels in risk-stratification should be strongly considered. In our opinion, the integration in preexisting prognostic risk models for a refined new risk stratification score should be prospectively evaluated. In the end, an improvement of existing risk stratification tools could results in better treatment choices for DLBCL patients.

VII. Bibliography

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VIII. Abbreviations

ABC	activated B-cell like subtype
BCL-2	B cell lymphoma 2 protein
BMI	body mass index
BMP	bone morphogenetic protein
BrdU	Bromodeoxyuridine
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CI	confidence intervall
CLL	chronic lymphocytic leukemia
COO	cell of origin
CRP	C-reactive protein
CS	clinical stage
CSF	cerebrospinal fluid
DLBCL	diffuse large B-cell lymphoma
dNLR	derived neutrophil to lymphocyte ratio
DNA	deoxyribonucleic acid
EC 50	half maximal effective concentration
ECOG	Eastern Cooperative Oncology Group Performance status
ESMO	European Society for Medical Oncology
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FFPET	formalin-fixed paraffin-embedded tissue
FL	follicular lymphoma
FOXP1	Forkhead box protein P1
GCB	germinal center B-cell subtype
GEP	gene expression profile
GFP	green fluorescent protein
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGAL	Human Germinal-center Associated Lymphoma
HIV	human immunodeficiency virus

HR	hazard ratio
IKKbeta	inhibitor of nuclear factor kappa-B kinase subunit beta
IL-6	interleukin 6
IPI	international prognostic index
LDH	lactate dehydrogenase
MALT	mucosa associated lymphoid tissue
mRNA	messenger ribonucleic acid
mTOR	mammalian target of Rapamycin/ mechanistic target of Rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	sodium chloride
NaOH	sodium hydroxide
NCCN	National Comprehensive cancer Network
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGCB	non-germinal center B-cell subtype
NHL	Non Hodgkin lymphoma
NLR	neutrophil to lymphocyte ratio
OS	overall survival
PCNSL	primary central nervous system lymphoma
PCR	polymerase chain reaction
PE	Phycoerythrin
PFS	progression free survival
PIK3R1	phosphatidylinositol 3-kinase
RB	retinoblastoma protein
R-CHOP	rituximab, cyclophosphamide, doxorubicin, oncovin, prednisolone
R-IPI	revised international prognostic index
RISC	RNA induced silencing complex
RNA	ribonucleic acid
ROC	receiver Operating Characteristic
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
siRNA	small interfering ribonucleic acid
SMAD5	Mothers against decapentaplegic homolog 5

TGF-β1	transforming growth factor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TP53INP1	Tumor protein p53-inducible nuclear protein 1
WHO	World Health Organization
7-AAD	7-amino-actinomycin D

IX. List of Figures

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