Diploma thesis

Clinical data analysis of aggressive B-Non-Hodgkin lymphomas

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Graz, *, November 5th, 2014* …….. Christina Sabo

Statutory Declaration

I hereby formally declare that I have written the submitted thesis independently and without any outside support except for the quoted literature and other sources mentioned in the paper. I clearly marked and separately listed all of the literature and all of the other sources which I employed when producing this academic work, either literally or in content.

Graz, November 5th, 2014…… *Christina Sabo eh.*

Danksagung

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„So eine Arbeit wird eigentlich nie fertig. Man muss sie für fertig erklären, wenn man nach Zeit und Umständen das Mögliche getan hat!“

Joh. Wolfgang v. Goethe, Italienische Reise 1787

Zusammenfassung

Hintergrund: Diffus großzellige B-Zell-Lymphome (DLBCL) stellen die häufigste Untergruppe aller Non-Hodgkin Lymphome dar. Die Rollen von GGT, Harnsäure und BMI in der Krebsentstehung werden in einigen Studien behandelt; in dieser Entität ist die Datenlage noch unklar. GGT und Harnsäure scheinen einen antioxidativen Effekt zu besitzen. Ein hoher BMI beeinflusst das Immunsystem und die Entzündungsantwort. In dieser Diplomarbeit wird der Einfluss von GGT (>45U/l vs. ≤45 U/l), Harnsäure (>6,5mg/dl vs. ≤ 6,5mg/dl) und BMI (>25 vs. ≤25) in Bezug auf das OS der Patienten mit DLBCL im Rahmen einer retrospektiven Datenanalyse untersucht.

Material und Methoden: Diese retrospektive Studie basiert auf einer Datenbank, welche 382 Patienten mit DLBCL am Universitätsklinikum-LKH Graz umfasst. GGT, Harnsäure und BMI wurden im MEDOC System des Univeristätsklinikum Graz aus den Patientenakten ermittelt und der Datenbank hinzugefügt. Anschließend wurde die statistische Auswertung mittels Statistical Package for Social Sciences version 21.0 und 22.0 (SPSS inc., Chicago, IL, USA) durchgeführt. Zusätzlich wurde Excel (Version 2010) verwendet um Tabellen und Diagramme zu erstellen. Die statistische Analyse erfolgte mittels x2-Test und Kaplan-Meier Kurven inklusive Log Rank Tests um die Überlebenswahrscheinlichkeit zu berechnen. Des Weiteren wurden sowohl univariate als auch multivariate Analysen nach einem multiplen Regressionsmodell nach Cox erstellt.

Ergebnisse: Ein signifikanter Unterschied in der Überlebenswahrscheinlichkeit wurde für Harnsäurewerte mittels Kaplan-Meier Kurven und log rank Test zugunsten von Harnsäurewerten ≤6,5 mg/dl festgestellt *(p=0,002*). Die univariate Cox Analyse ergab, dass höheres Alter (50-75a *p=0,042* und >75a *p=0,002*), ein schlechter R-IPI (*p=0,003*), der Subtyp (GCB vs. NGCB *p<0,001*), erhöhte Harnsäure (>6,5 mg/dl, *p=0,003*), erhöhte LDH (≤243 U/l vs. >243 U/l, *p=0,006*), erhöhtes CRP (≥10 mg/l, *p=0,017*) und vermindertes Hämoglobin (<12 g/dl, *p<0,001*) mit einem schlechteren Gesamtüberleben der Patienten korrelierten. Die Daten der multivariaten Cox Regression wiesen darauf hin, dass der Subtyp (*p=0,005*), die erhöhte Harnsäure (*p=0,004*) sowie vermindertes Hämoglobin (*p=0,003*) unabhängige prognostische Faktoren für das Gesamtüberleben sind.

Conclusio: Unter Berücksichtigung der statistischen Ergebnisse können wir annehmen, dass ein hoher Harnsäurewert zum Zeitpunkt der DLBCL Diagnose als schlechter, unabhängiger Prognosefaktor für das Gesamtüberleben fungiert.

Abstract

Background: Diffuse large B-cell lymphomas (DLBCL) represent the most common group of Non-Hodgkin lymphomas. The roles of GGT, uric acid and BMI as laboratory values in cancer genesis have been discussed in several studies, though in this entity the data situation is still unclear. GGT and uric acid seem to have an antioxidant effect. However a high BMI seems to have an effect on the immune system and the inflammatory response. In this thesis the influence of high or normal GGT (>45U/l vs. ≤45 U/l), uric acid (>6,5mg/dl vs ≤ 6,5mg/dl) and BMI (>25 vs. ≤25) on DLBCL patients' overall survival was analyzed in a retrospective study.

Material and Methods: This retrospective study is based on a database, containing 382 patients diagnosed with DLBCL at the Medical University of Graz. GGT, uric acid and BMI values have been documented using the MEDOC system of the University Hospital Graz. Afterwards, statistical analysis was performed for these parameters by using the Statistical Package for Social Sciences version 21.0 and 22.0 (SPSS inc., Chicago, IL, USA). Additionally, Excel version 2010 was used to create tables and pie charts. For the statistical analysis we used a x^2-test, Kaplan-Meier curves and log rank test were created to show the probability of survival. Univariate and multivariate analysis were performed by using Cox regression.

Results: A significant difference of the probability of survival was found for uric acid values by using Kaplan-Meier curves and log rank test for the benefit of uric acid values ≤6,5 mg/dl (*p=0,002*). The univariate Cox analysis showed that older age (50-75a *p=0,042* and >75a *p=0,002*), a poor R-IPI (*p=0,003*), the subtype (GCB vs NGCB *p<0,001*), elevated uric acid (>6,5 mg/dl, *p=0,003*), elevated LDH (≤243 U/l vs >243 U/l, *p=0,006*), elevated CRP (≥10 mg/l, *p=0,017*) and low hemoglobin (<12 g/dl, *p<0,001*) correlate with a poor OS. The data of the multivariate Cox regression indicated that subtype (*p=0,005*), elevated uric acid (*p=0,004*) as well as low hemoglobin levels (*p=0,003*) are independent prognostic factors for OS.

Conclusion: In conclusion, we demonstrated that high uric acid levels, at the time of DLBCL diagnosis, act as an independent poor prognostic factor for the overall survival.

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List of Abbreviations

IL	Interleukin
CD	cluster of differentiation
HLA	Human Leukocyte Antigen
Fab	Fragment antigen-binding
NHL	Non Hodgkin lymphoma
EBV	Epstein-Barr virus
HCV	Hepatitis C virus
HTLV-1	Human T-cell leukemia virus
HHV	human herpes virus
LN	lymph node
CT	computed tomography
LDH	lactate dehydrogenase
PET	Positron emission tomography
IPI	International Prognostic Index
ECOG	Eastern Cooperative Oncology Group
CR	complete response
OS	overall survival
R-IPI	revised International Prognostic Index
PFS	progression-free survival
NCCN-IPI	National Comprehensive Cancer Network- International Prognostic Index
REAL	Revised European American Lymphoma classification
ILSG	International Lymphoma Study Group
WHO	World Health Organization
CLL	Chronic lymphocytic leukemia
SLL	small lymphocytic lymphoma
LPL	Lymphoplasmocytic lymphoma (LPL)
MALT	Mucosa Associated Lymphoid Tissue
FL	Follicular lymphoma
ML	Mantle cell lymphoma
DLBCL	Diffuse large B-cell lymphoma

BL	Burkitt lymphoma
NOS	not otherwise specified
SOT	solid organ transplantation
HIV	human immunodeficiency virus
GC	germinal center
AID	activation-induced cytidine deaminase
SHM	somatic hypermutation
Ig	Immunoglobulin
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BCL6	B-cell lymphoma 6
FDC	follicular dendritic cells
NF	nuclear factor
IRF	interferon regulatory factor
CSR	class-switch recombination
miRNA	Micro RNA
TNFAIP3	tumor necrosis factor alpha-induced protein 3
GCB	germinal center B-cell like DLBCL
NGCB	non germinal center B-cell like DLBCL
SHIP-1	SH2-domain containing inositol-5-phophatase-1
PI3R1	phosphatidylinositol 3-kinase regulator
AID	activation-induced cytidine deaminase
ABC	activated-cell like or non GCB
PMBCL	primary mediastinal B-cell lymphoma
CHOP	Cyclophosphamid, Hydroxydaunorubicin, Vincristin, Predniso(lo)ne
MUM1	multiple myeloma oncogene 1
FOXP1	forkhead box protein P1
GEP	gene expressing profiling
COO	cell of origin
aaIPI	age-adjusted IPI

GELA	Groupe d'Etude des Lymphomes d'Adulte
DSHNHL	German High-Grade Non-Hodgkin's Lymphoma Study Groupe (Deutsche Studiengruppe Hochmaligne NHL)
HDCT	High-dose-chemotherapy
HSCT	Hematopoietic stem cell transplant
ASCT	Autologous stem cell transplant
CNS	central nervous system
GGT	gamma-glutamyl transferase
GSH	Glutathione
ALP	Alkaline Phosphatase
BMI	Body Mass Index
CI	Confidence intervals
HR	Hazard ratio
CRP	C-reactive protein
EOC	epithelial ovarian cancer
TNF	tumor necrosis factor
IGFBP1	insulin-like growth factor binding protein
IGF	insulin-like growth factor
IR	insulin receptor

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1 Introduction

1.1 Lymphocytes

Lymphocytes are part of the acquired or adaptive immune system and represent about 25 per cent of the total white cell population in an adult body.
A spherical nucleus with a thin rim of cytoplasm is the characteristic histological shape of a lymphocyte and the whole cell measures between 7 to 11 µm. Their count normally ranges from 1500 to 4000 cells/µl (20-40%) (1)(2).
Lymphocytes mature in the primary lymphoid tissue (i.e. bone marrow and thymus) and aggregate in the so called secondary lymphoid tissues such as lymph nodes, spleen, alimentary and respiratory tracts (2).

1.1.1 Maturation of Lymphocytes

Originally all different kinds of white blood cells develop out of hematopoietic stem cells in the bone marrow. Under the influence of IL-7, they convert into lymphoid colony-forming cells. The presence of IL-2, IL-4, IL7 and IL-15 gives rise to the first identifiable progenitor, the lymphoblast. Due to several divisions the lymphoblast gets smaller and forms first the prolymphoblast and finally the lymphoblast (1).
There are two types of lymphocytes; T-cells and B-cells. In the thymus, the lymphocytic stem cell differentiates into the T-cell, while the B-cell maturates in the bone marrow. The percentage of T-cells (70-80%) in the blood is larger than that of B-cells (10-15%) and they can only be distinguished by markers on their cell membranes (e.g. expressed antigens on the surface of the cell) which depend on the type and state of maturity of the cell. According to their reaction with monoclonal antibodies, the cluster of differentiation (CD) nomenclature system tries to sort these antigens (3).
T-cells divide into three different types; cytotoxic T cells, T helper cells and memory T-cells. During an interaction with antigens, the B-cell differentiates into a plasma cell, which produces antibodies against a specific antigen. The next time when the human body gets in contact with the same antigen, the immune response will be faster than the first time (2)(4)(5).

Lymphocytes exist for many years, so they have the longest life of all the white blood cells.

1.1.2 B-Lymphocytes

Once activated by an antigen, the B-lymphocyte proliferates by mitosis to a cell population called a clone, provided that T_H cells give the signal for clonal production (2).

One group of cells continues maturating and ultimately becomes plasma cells which secrete antibodies, while the other group persists as memory cells, in order to accelerate an immune response in case of a second contact with the same antigen (1).

The B-cells have characteristic surface makers such as CD19, CD20 and CD22, as well as CD9 (pre B cells), CD10 (precursor B cells) and HLA class I and II (6).

In contrast to the little cytoplasm of a resting B-lymphocyte (Figure 1), the plasma cell possesses an expanded cytoplasm with a great amount of ribosomes where the antibody production takes place in the well-developed Golgi apparatus (Figure 2) (1).

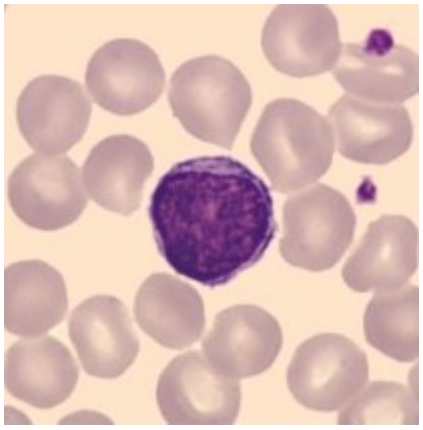

Figure 1: Lymphocyte with little cytoplasm**(7)**

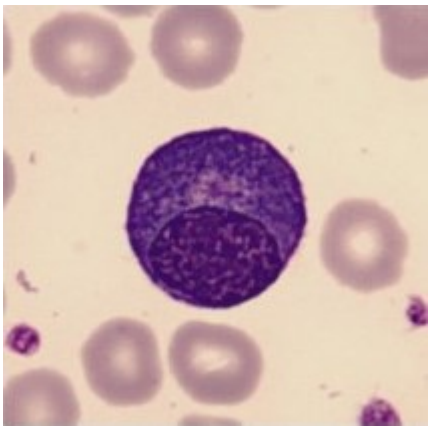

Figure 2: Plasma cell with expanded cytoplasm**(8)**

All antibodies/immunoglobulins have the same Y-shaped form, consisting of two light (κ and λ) and two heavy chains (α,δ,ε,γ and μ), which determine the class of antibody (1).
The Fab region (consisting of both light and heavy chains) is the place where the immunoglobulin binds an antigen in order to destroy it by acting together with the complement system to stimulate the phagocytosis (1).

1.2 Non-Hodgkin Lymphoma

In the WHO classification (9) a large diversity of histological entities is described, depending on differences in immunophenotype, genetics, clinical appearance and morphology: thus, different localisations (nodal or extranodal), the possibility to stay local or to disseminate throughout the body, and finally the patient's age and additional diseases, account for the heterogeneousity of the Non-Hodgkin's lymphomas (NHLs) (10).

1.2.1 Epidemiology and Pathogenesis

In the United States and Europe lymphomas represent the sixth most common type of cancer and their incidence has been constantly increasing over the past 30 years (10).
Nowadays, it is clinically important to separate high and low-grade lymphomas (11).
Aetiologically, viruses like Epstein-Barr virus (EBV), hepatitis C virus (HCV), human T-cell lymphoma/leukaemia virus (HTLV-1), the human herpes virus (HHV-8) and the human immunodeficiency virus (HIV) are certainly causative factors in the development of lymphomas. Bacteria such as Helicobacter pylori and Borrelia burgdorferi may also have an influence on causing lymphomagenesis. The reason why this illness is widely spread amongst elderly may be explained by age-related immunodeficiency (10).

1.2.2 Clinical Presentation, Staging and Grading

Singular or multiple nodular-expansions, an extra nodal tumour with a typical location or so called B-symptoms are suspicious for the existence of a NHL.

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 1) (12).

Stage	
I	A single LN region (I) is involved or localized involvement of a single extralymphatic organ or site (IE)
II	Two or more separate lymph node regions are diseased on one side of the diaphragm (II) or localized involvement of a single extralymphatic organ or site and of one or more lymphatic regions of the same side of the diaphragm (IIE)
III	Involvement of LN regions on both sides of the diaphragm
IV	One or more extranodal organs are diffuse or disseminated diseased with or without lymphatic involvement.

Table 1: Ann Arbor staging **(12)**

In order to confirm the diagnosis, it is indispensable to characterize the tumour by immunophenotyping in order to determine if CD20 or CD4, CD2 and CD3 antigens are expressed, which is distinctive for a B- or T-cell lymphoma.

Additionally, the patient must be clinically examined and a computed tomography (CT) of the neck, thorax, abdomen and pelvis is necessary. The investigations should comprise detailed laboratory examinations, including LDH and serum β2-microglobulin levels, renal and hepatic function tests, as well as a bone marrow aspiration. Positron emission tomography (PET) is currently no standard- in initial staging but has a great influence in judging the post therapeutic success (11)(10).

The prognostic index used in Non- Hodgkin Lymphoma is the International Prognostic Index (IPI) (Table 2). Both the IPI score and the specific type of lymphoma, are important factors in the therapeutical decision.

Age in years	≤60 vs≥60
Tumour stage	I or II (localized) versus III or IV (advanced)
Number of extranodal sites of involvement	0-1 vs. >1
Patient's performance status	ECOG 0 or 1 vs ≥2
Serum LDH level	Normal or elevated

Table 2: International Prognostic Index (IPI)**(13)**

After checking these parameters, patients can be assigned in one of the four risk groups, based on the number of presenting risk factors; low (0 or 1), low intermediate (2), high intermediate (3) and high (4 or 5) (Table 3). On average, patients in the low-risk groups have an 87% CR (complete response) and an OS (overall survival) rate of 73% at 5 years, whereas the patients in the high risk groups have lower rates with 44%CR and 26% 5 year overall survival (14).

Risk category	IPI score	5-year OS
Low	0 or 1 points	73%
Low/intermediate	2 points	51%
High/intermediate	3 points	43%
High	4 points	26%

Table 3: International Prognostic Index (IPI)-score **(14)**

The revised IPI (R-IPI) (Table 4) is a new arrangement of the IPI factors that identifies 3 groups, according to a very good, good and poor outcome. Nowadays, in times of using Rituximab, the R-IPI is a helpful and easier marker in clinical trials compared to the IPI. A very good R-IPI stands for a 4-year progression-free survival of 94% (with an overall survival of 94%), a good R-IPI for a 4-year progression-free survival of 80% (with an overall survival of 79%) and a poor R-IPI has a 4-year progression-free survival of 53% (with an overall survival of 55%) (15).

Risk group	Number of IPI factors	4-year PFS	4-year OS
Very good	0	94%	94%
Good	1,2	80%	79%
Poor	3,4,5	53%	55%

Table 4: R-IPI **(16)**

There is another International Prognostic Index – the NCCN-IPI – which is very useful for differentiating between low- and high- risk DLBCL patients in the era of rituximab. In this index, clinical data from the National Comprehensive Cancer Network (NCCN) was used to define an outdated IPI with a more refined categorization of age, LDH, sites of involvement, Ann Arbor stage and the ECOG (Eastern Cooperative Oncology Group) performance state. A maximum of 8 points is achievable. Subsequently, a low (0-1 points), low-intermediate (2-3 points), high-intermediate (4-5 points) and a high (6-8 points) risk group are distinguished (17).

1.2.3 Histological Classification

In the history of Non-Hodgkin-Lymphoma classifications, several different systems were used. Until recently, the two main systems were the Kiel Classification, which is based on the knowledge of normal lymphoid cell development and catalogues the Non-Hodgkin-Lymphomas according to their normal cell equivalents, and the Working Formulation for Clinical Usage, which basically draws on clinical survival data.

On the basis of the Revised European American Lymphoma classification (REAL), founded in 1994 by the International Lymphoma Study Group (ILSG) of experienced haematopathologists, the WHO classification was established (Table 5). It links the two methods of approach and gives a clinical pathologic overview (18).

B cell neoplasms: WHO classification
Precursor B-cell neoplasm
Precursor B-cell lymphoblastic leukaemia/lymphoma
Mature B-cell neoplasm
Chronic lymphocytic leukaemia (CLL)/small lymphocytic lymphoma (SLL)
B-cell prolymphocytic leukemia
Lymphoplasmacytic lymphoma (LPL)/Waldenström macroglobulinemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Plasma cell neoplasms: plasma cell myeloma, plasmacytoma, monoclonal immunoglobulin deposition diseases, heavy-chain diseases
Extranodal marginal zone B-cell lymphoma (MALT lymphoma)
Nodal marginal zone B-cell lymphoma
Follicular lymphoma (FL)
Mantle cell lymphoma (ML)
Diffuse large B-cell lymphomas (DLBCL)
Subtypes
Mediastinal large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary effusion lymphoma
Burkitt lymphoma (BL)

Table 5: World Health Organization Classification of Lymphoid Tumours **(10)**

1.3 Diffuse Large B-cell Lymphoma, Not Otherwise Specified (NOS)

Around 30-40 % of adult lymphomas are represented by the diffuse large B-cell lymphoma (DLBCL), which is characterized by either diffuse nodal infiltration or extranodal infiltration by large cells of the B-cell phenotype. Typical for this type of lymphoma is a symptomatic single mass that rapidly increases either at a single nodal or extranodal site.(19)(18)

Up to 40% of the patients initially present with extranodal disease, whereof the most common extranodal site is the gastrointestinal tract. In 11-27% a bone marrow involvement exists. (9)

1.3.1 Epidemiology

DLBCL is the most common group of NHL, with about 25 000 new cases each year in the United States, in developing countries, the percentage is even higher.

Although it also occurs in children and young adults, the median patient's age is in the 7th decade. The male sex is affected slightly more often (9)(20).

1.3.2 Aetiology

The aetiology of the DLBCL is still unknown, it may arise *de novo* (referred to as primary), or may be a transformation of an indolent lymphoma (referred to as secondary) (21).

A malfunctioning immune system is also a significant risk factor. The chance of developing a lymphoma after undergoing a solid organ transplantation (SOT) increases approximately 10-fold in comparison with the general population (22).

In the setting of severe T-cell immunodeficiency, e.g. in an advanced stadium of an HIV infection, the DLBCL can appear as *Immunodeficiency-associated large B-cell lymphoma* (20).

It is also proved that immunodeficient patients with DLBCL, NOS are more often Epstein-Barr virus (EBV)-positive. Only 10 % of the DLBCL patients without a diagnosed immunodeficiency have an EBV infection (9).

Another variant is the large B-cell lymphoma arising in human herpesvirus-8-associated multicentric Castleman disease, which shows a plasmablastic differentiation (23).

1.3.3 Morphology

The common cell size of a DLBCL-cell is four to five times the diameter of a regular lymphocyte. It has large nuclei, open chromatin, and prominent nucleoli (two or three in number). According to the WHO, there are three common morphological variants:

a) *Centroblastic variant (Figure 3):* medium to large lymphoid cells containing oval to round, vesicular nuclei, with fine chromatin and scarce cytoplasm which is amphophillic to basophillic. There are one to three nuclear membrane-bound nucleoli. Only in some cases the tumour is monomorphic, which means that it exists entirely (>90%) of centroblasts. Generally, the tumour is polymorphic and consists of 10% of centroblasts and less than 90% of immunoblasts (21)(9).

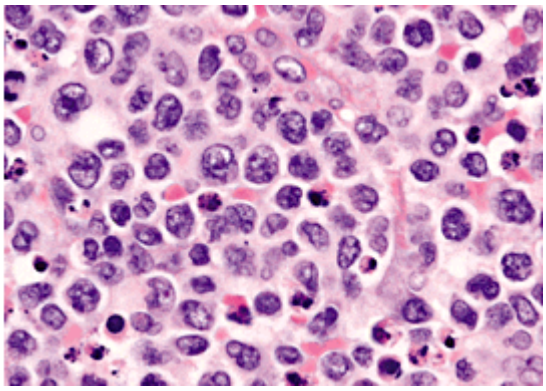

Figure 3: Centroblastic variant of the DLBCL**(24)**

b) *Immunoblastic variant (Figure 4):* In this subtype, the amount of immunoblasts represents more than 90%. The cells have a single, centrally located nucleolus and variable cytoplasm. To differentiate it from the plasmablastic subtype of DLBCL (more common in immunosuppressed, especially HIV patients) immunophenotyping and clinical characteristics are necessary.(21)(9)

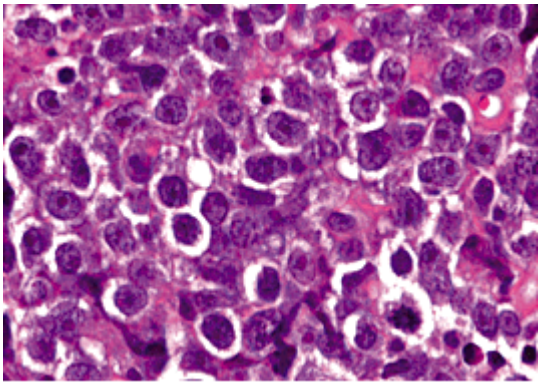

Figure 4: Immunoblastic variant of the DLBCL**(24)**

c) *Anaplastic variant (Figure 5):* Large, round, oval or polygonal cells are characteristic for this type. The bizarre pleomorphic nuclei reminds of Hodgkin and /or Reed-Sternberg cells. As the tumour cells express the CD30 (ki-1) antigen, the main differential diagnoses are Hodgkin's lymphoma and T-cell NHL, which also expresses CD30. (21)(9)

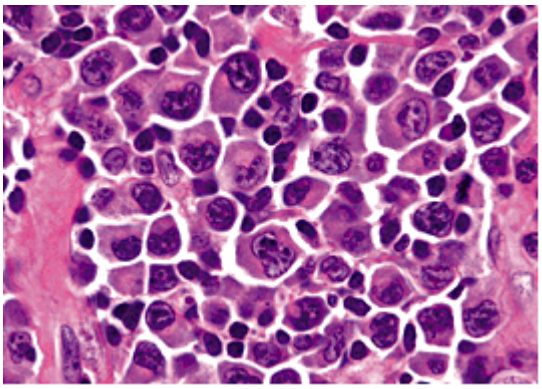

Figure 5: Anaplastic variant of the DLBCL **(24)**

1.3.4 Pathophysiology

As the DLBCL is a cancer of the mature B-cell, molecular alterations can occur at any step of the differentiation and maturation of normal B lymphocytes.

The GC Reaction

After the differentiating in the bone marrow, the naïve B cells arrive at the secondary lymphoid tissues (lymph nodes, mucosa and tonsils), where the B lymphocyte ontogeny continues. In the germinal center - a highly specialized microenvironment of the secondary lymphoid tissues - a 'dark' and a 'light' zone (Figure 6) exist. After the T-cell-dependent antigen stimulation, a naïve B-cell enters the 'dark' zone of the GC, differentiates into a centroblast, and undergoes an AID (activation-induced cytidine deaminase) dependent somatic hypermutation (SHM) of the variable region of the immunoglobulin (Ig) gene (IgV) by DNA double strand breaks. In order to produce antibodies with high affinity for the antigen, the SHM produces single-nucleotide substitutions, as well as deletions and duplications in the IgV heavy- and light-chain genes. Not only Ig-genes are concerned by SHM. A huge number of other genes, like the 5'-untranslated region of B-cell lymphoma 6 (BCL6) are affected. The process in the GC is BLC6 dependent which is a transcriptional repressor and centroblasts express BLC6, which is necessary for the rapid proliferation of centroblasts. Afterwards, the cells move into the 'light' zone, where they become centrocytes and get confronted with the antigen by T-cells and follicular dendritic cells (FDC) for a second time. Due to

this antigen provocation, it is possible to distinguish B-cells with high-affinity to the antigen, which undergo another differentiation into plasma- and memory B cells, from those with a low-affinity Ig-receptor, which are removed by apoptosis and represent 90% of the B-cell population. The next step is the differentiation of high-affinity Ig receptor positive B-cells into effector plasma cells, or memory B-cells, which presumes a down regulation of BCL6 in two different ways; first, through activating CD40 by the CD4 ligand (expressed on CD4 positive T-cells), which induces the nuclear factor (NF)-κB-mediated interferon regulatory factor 4 (IRF4; also termed MUM1) and second, through the stimulation of the BCR, which evokes the phosphorylation of BCL6. Usually, B-cells do not express both BCL6 and IRF4/MUM1, this occurs only in the malignant variants. The down regulation of BCL6 is necessary in order to stop proliferation, to repair DNA-damage and to permit the differentiation into plasma cells.

Another critical step, that also takes place in the 'light' zone of the GC, is the AID dependent class-switching recombination (CSR) of the centrocytes, in which the antibodies change their Ig classes from IgD and IgM to IgG, IgA or IgE by an intrachromosomal DNA recombination via non homologous end joining.(25)

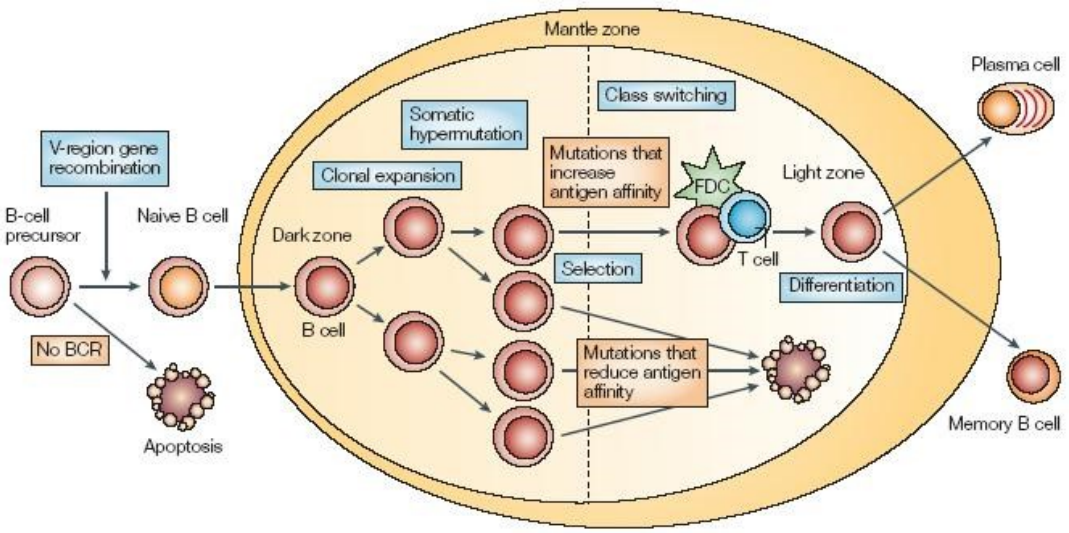

Figure 6: The GC reaction (26)

The Role of MicroRNA as a Critical Regulatory Principle in the Immune System and in the Pathogenesis of DLBCL.

MicroRNAs are endogenous 19-24 –nucleotides-short non-protein-coding which control gene expression at a post-transcriptional level (26)(27)(28).

Some microRNAs and their pathogenic effects in the pathogenesis of DLBCL have been described. One of the most de-regulated pathways in DLBCL is the nuclear factor kappa- light-chain-enhancer of activated B-cells (NF-kB) pathway. This pathway is activated by **miR-125a and miR-125b**, which are often overexpressed in DLBCL and regulate tumour necrosis factor alpha-induced protein 3 (TNFAIP3). TNFAIP3 is a negative regulator of NF-kB and increases NF-kB signalling. The oncogenic task of miR-125a and miR-125b in lymphoma genesis is well shown by ectopic expression and inhibition of these two microRNAs in cell lines.

Another region which is often enhanced in GCB DLBCL is the **miR-17-92 cluster**. This cluster is located at chromosome 13q31-q32 and codes for six variable microRNAs. It is necessary in the survival of early b-cell progenitors and represents a factor in lymphoma genesis by repressing anti apoptotic genes (26).

One of the most studied microRNAs is the **miR-155** that plays a part in haematopoiesis and the immune response, especially in B-cell maturation and lymphocyte activation. The overexpression of SH2-domain, containing inositol-5-phosphatase-1 (SHIP-1) and subsequently leading to the up regulation of miR-155 activates the AKT pathway and promotes the B-cell proliferation (27).

MiR-155 interferes with the effects of IL-6 and has an impact on the cellular motility, the cytoskeleton and the migration in lymphoma. A direct target of miR-155 is phosphatidylinositol 3-kinase (PI3R1), which is a negative regulator of the phosphatidylinositol 3-kinase (PI3K-AKT) pathway (26).

A potential function of the miRNAs is the regulation of the expression of activation-induced cytidine deaminase (AID), which plays an important role in the germinal center reaction by mediating class switch recombination (CSR) and somatic hypermutation (SHM) (29).

It has been shown that BCL6 modulates the transcription of miR-155 and therefore the expression of AID. ABC and PMBCL phenotypes have higher levels of miR-155 in comparison with the GCB type. That is the reason why miR-155 expression is a valuable marker for the diagnosis and prognosis of DLBCL(27).

MiR-21 also plays a major part in lymphoma genesis. Elevated miR-21 caused pre-B malignant lymphoid –like phenotype in a mouse model, while miR-21 inactivation led to tumour regression via apoptosis and cell arrest. This suggests that an oncogenic function is stimulated by elevated miR-21 expression and therefore higher levels of miR-21 are observed in the ABC- subtype than in the

GCB-subtype, identified by microarray analysis. The outcome of a recent study shows that the DLBCL sensitivity to the CHOP chemotherapy regimen is increasing when miR-21 expression is reduced (27).

MiR-34a, however has tumour-suppressor features in DLBCL. The transcription factor FOXP1 (forkhead box protein P1) is a direct target of miR-34a and is a proper oncoprotein. In highly malignant DLBCL, miR-34a is down-regulated. The expression of miR-34a is directly regulated by Myc, which is an oncogenic factor in various DLBCLs. The DLBCL proliferation is inhibited by an ectopic miR-34a expression (30).

In several studies it is shown that the tumour suppressor p53 transcriptionally regulates the gene encoding miR-34a (27).

The B-cell development gets blocked at the pro B cell to pre B cell transition by a constitutive expression of 34a. The result of this block is a reduction of mature B cells. Responsible for this block is the depression of FOXP1. In summary, depression of miR-34a leads to augmented amounts of FOXP1 and mature B cells, whereas miR-34a overexpression provokes a partial inhibition in B cell development (31).

1.3.5 Molecular and Genetic Features

Alizadeh et al. used DNA microarrays to generate a systematic characterization of gene expression of B-cell lymphoma in 2000. Two molecularly forms of DLBCL with gene expression patterns of various B-cell differentiation stages were identified: the GCB (germinal center B-like DLBCL) and the ABC (activated B-cell like or non GCB) (32).

GCB DLCBL derives from a germinal center B-cell, whereas the ABC develops from a further stage of plasmacytic differentiation.(33)

Another subtype, the primary mediastinal B-cell lymphoma (PMBL), was identified in later studies (Figure 7) (34).

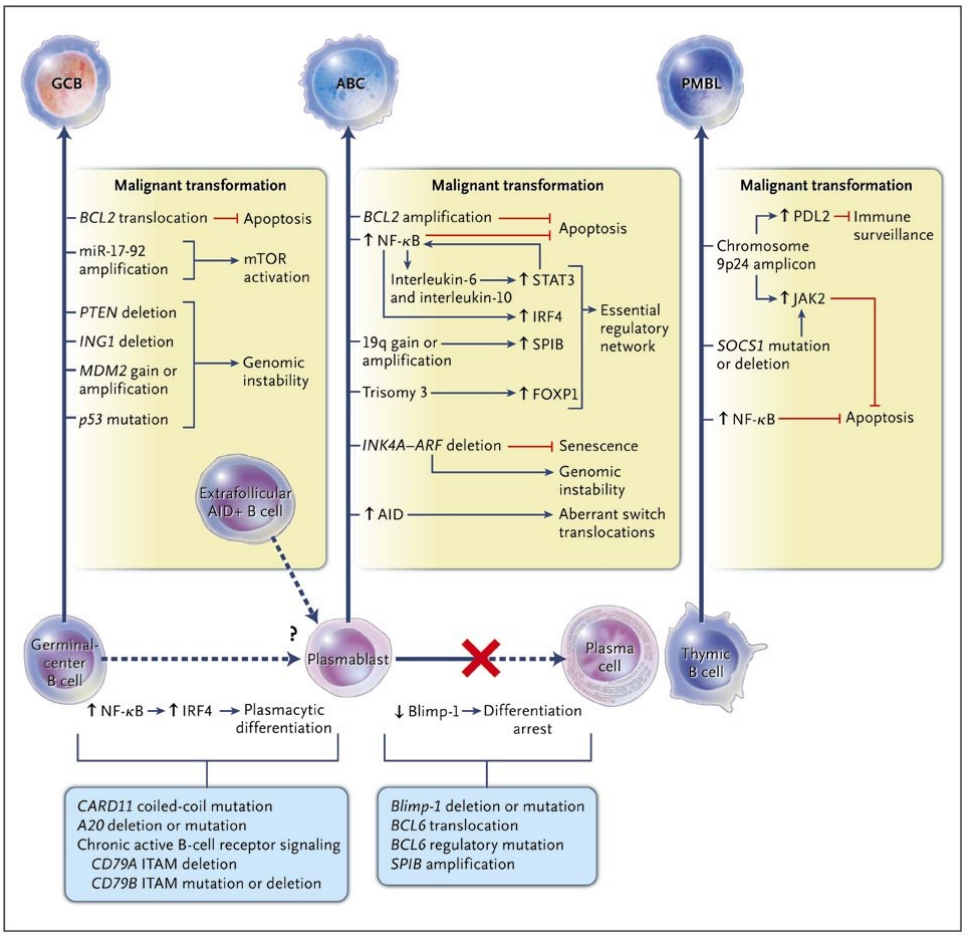

Figure 7: Three subtypes of DLBCL and their possible oncogenic pathways **(33)**

Differences in genetic alterations, the clinical outcome, and the overall survival (in favour of the GCB subtype) were found between the two groups (35).

1.3.5.1 Genetics

- *Antigen receptor genes:* clonal rearrangements of immunoglobulin heavy and light chain genes are characteristic of DLBCL
- *Aberrant somatic hypermutations* in the variable regions in most cases. In more than 50% genetic loci like PIMI, MYC, RHOH/TTF (ARHH) and PAX5 are affected.
- *Chromosome translocations:* the most frequent translocation involves the BCL6 gene and shows abnormalities of the 3q27 region in up to 30% of cases (more often in the ABC subtype). In 20-30 % of cases a translocation of the BCL2 gene occurs (t(14;18)), which is typical of follicular lymphoma. A MYC rearrangement appears in 10% of cases and involves 8q24.

- *Gene expression profiling:* through Gene expressing profiling (GEP) Alizadeh et al. defined the two subgroups GCB and ABC, which are associated with different chromosomal aberrations. A gain of 3q, 18q21-q22 and a loss of 6q21-q22 occurs in the ABC subgroup. In the GCB DLBCL a gain of 12q12 is common. Although the immunoblastic variant and the centroblastic variant are found in some cases of GCB, they prevalently occur in the ABC DLBCL. There is a variable connection between the gene expression subtypes of DLBCL and GC and non GC DLBCL which are identified by immunohistochemistry. (9) (19)

1.3.6 The Cell of Origin (COO) Subtypes

The heterogeneity of 60 cases of DLBCL, NOS was first proven in 2000 by the results of GEP on snap-frozen tissue samples. A customized competitive microarray chip, the Lymphochip, was used. These cases had a different GEP, although they were morphological undistinguishable on the histological level.

1.3.7 Immunophenotype

B-cell associated antigens such as CD19, CD 20, CD22 and CD 79a appear on DLBCL cells, though some of these antigens are missing. In 50-75% of the cases, surface and/or cytoplasmic immunoglobulin (IgM>IgG>IgA) can be evidenced. The expression of CD5 occurs in 10% of the cases and defines a separate variant, the CD5+ DLBCL and represents a *de novo* DLBCL (seldom arising from CLL/SLL and can only be differentiated from the blastoid variant of mantle cell lymphoma because of the absence of cyclin D1). Ki-67 detects the proliferation rate which may be from 40 to more than 90% in some cases (18)(9).

CD10 (30-60% of the cases), BCL 6 (60-90% of the cases) and IRF4/MUM1 (35-65% of the cases) expression is only variably present. The expression of both, IRF4/MUM1 and BCL6, was found in 50 % of DLBCL. In DLBCL cases that lack the germinal center phenotype, but express IRF4/MUM1 and BCL6 in the absence of t(14;18), a single high expression of FOXP1 was found (18)(9).

1.3.8 GEP Gets Replaced by Immunohistochemical Subgroups

The GEP is not applied in routine clinical practice although it is a scientifically validated method. However, both, the microarray analysis and the requirement for fresh-frozen samples, are too expensive for the clinical setting. Therefore, IHC

algorithms were developed in order to differentiate between GCB and non GCB (36).

The Hans algorithm, has been widely used in clinical trials to determine the molecular subtypes of DLBCL and bases on the immunohistochemical analysis of CD10, BCL6 and MUM1 (35).

DLBCLs which are CD10 positive in more than 30 % of the cells are classified as GCB DLBCLs. In the case of CD10 negativity but BCL6 positivity and also MUM1 negativity, it is also referred to as a GCB DLBCL. Non-GCB cases are CD10 negative and BCL6 negative, or CD10 negative, and BCL6 positive as well as IRF4/MUM1 positive (A, Figure 8).

A similar algorithm to the one by the Hans is the Muris algorithm. However, there exist several more recent ones which aim for an improvement of the Hans algorithm. Example given is the additional use of FOXP1 and GCET1 by Choi (C, Figure 8) (19).

The Tally algorithm, built by Meyer et al implicates CD10 and GCET1 as germinal center markers and FOXP1 and MUM1 as activated B-cell markers. In the case that the number of positive germinal center and activated B-cell markers is equal, the presence of another germinal center B-cell marker, LMO2, is decisive for either a GCB or a non-GCB subtype (D, Figure 8) (37).

Although the immunohistochemical classification is easier than the GEP, its prediction of the overall survival rate remains unclear (38).

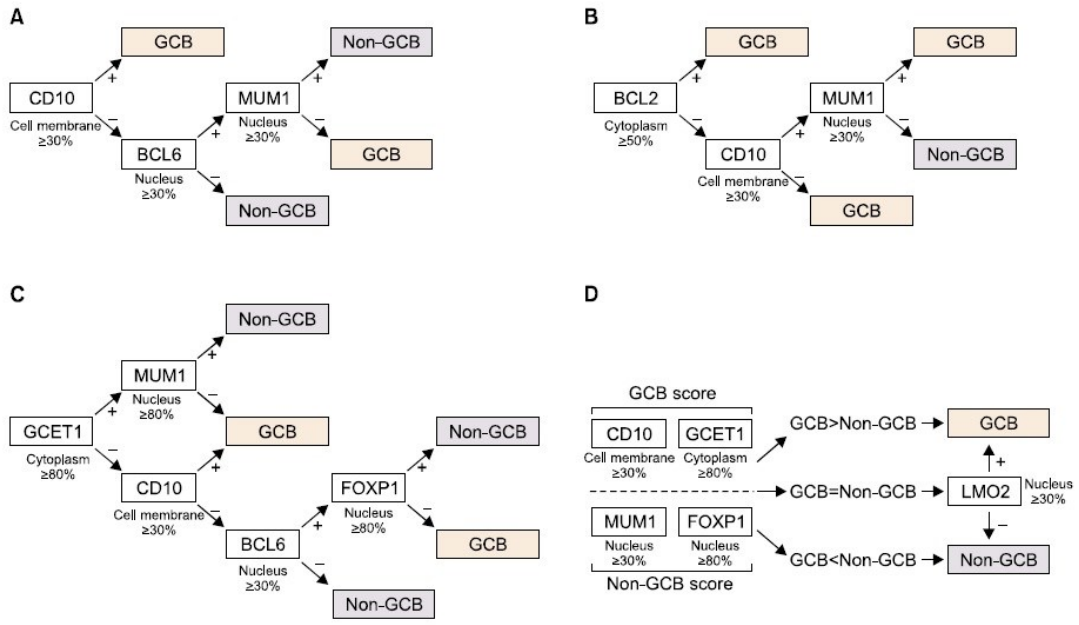

Figure 8: Immunohistochemical subgroups; algorithms of (A) Hans, (B) Muris, (C) Choi and (D) Tally **(38)**

1.3.9 Therapy

History of DLBCL Therapy

In the 1970s, the discovery of the advantages of a combined chemotherapy with CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) or HOP (adriamycin, vincristine and prednisone), instead of a single agent therapy, significantly influenced the treatment of DLBCLs (39).

In 1997, the US Food and Drug Administration approved Rituximab for the treatment of relapsed low grade or follicular lymphoma. Rituximab is a chimeric human-mouse anti-CD20 monoclonal antibody, i.e. a human immunoglobulin (Ig) G1 kappa antibody that contains mouse variable regions which are isolated from a murine anti-CD20 monoclonal antibody (40).

A prospective randomized phase II study emerged the significant anti-lymphoma activity of Rituximab in DLBCL patients in the absence of the toxicity which was usually observed in combination chemotherapy regimens. Consequentially, the standard dose regimen (375mg/m²) in combination with standard chemotherapy in patients with aggressive B-cell lymphoma was established (41).

The Groupe d'Etude des Lymphomes d'Adulte (GELA) presented results of a randomized controlled trial that compared the therapy regimen of advanced stage DLBCL in elderly patients (age ≥60 years). The result was an improved event free survival and an increased overall survival after the addition of rituximab to CHOP. A 19% absolute improvement in event-free survival and a 13% improvement in OS were reported for the chemo immunotherapy with CHOP and rituximab and confirmed in the 2-year analysis. These results were the basis of a new policy of the British Columbia (BC) Cancer Agency, published on March 1, 2001, recommending the combination of CHOP and rituximab for all newly diagnosed advanced DLBCL patients in the province of BC, regardless of age (Figure 9) (42).

As it was shown by randomized trials in 2002 that Rituximab immunotherapy in addition to CHOP improves the survival rates, there was a change in the standard therapy regimen. Patients older than 60 years with advanced stage DLBCL showed an improvement of the complete response rate of 76% in comparison to 63% in the therapy regimen with CHOP (P=0,005). Additionally the 2-year overall

survival (OS) rised from 57% to 70% (P=0,007). These benefits of a combined chemo immunotherapy for DLBCL were confirmed by follow up data from this trial and other randomized, controlled clinical trials (43).

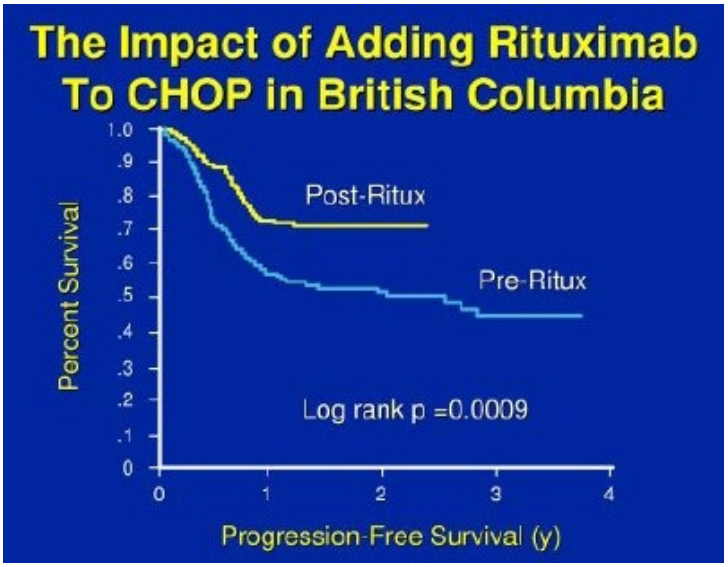

Figure 9: The probability of survival of patients treated in British Columbia with or without Rituximab and the progression free survival (44)

Third Generation Regimen and Development of Further Treatment Strategies

After the introduction of CHOP as the standard therapy regimen in the United States, new treatments options became popular. The M-/m- BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone) was an effective therapy for most of the patients in the long-term follow-up as well as the MACOP-B (methotrexate with leucovorin rescue doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin) and the ProMACE/CytaBOM (cyclophosphamide, doxorubicin, etoposide cytozar, bleomycin, vincristine, methotrexate prednisone)(44)(45)(46).
In 1993, a clinical trial showed that CHOP is the best treatment for patients with advanced-staged, intermediate-grade, or high grade non-Hodgkin lymphoma, in comparison with the so-called third generation regimen (47). However, the research for improved therapy options than CHOP continues (48).
The ACVBP regimen (doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone) introduced by the GELA, consists of an induction phase of 4 cycles of intensified CHOP with CNS prophylaxis, followed by a sequential consolidation

phase. The benefits of adding Rituximab to CHOP gave inspiration for creating a new therapy regimen with Rituximab and ACVBP (R-ABCVBP) for patients <65 years. The ACVBP regimen showed a significant improvement of the event free and overall survival in comparison with CHOP in patients with poor-risk aggressive lymphoma (49).

Another attempt to find a new treatment was made by the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL), which investigated if there is a significant advantage of a reduced treatment interval from 3 to 2 weeks of CHOP (CHOP-14), the additional use of etoposide, an antilymphoma drug, (CHOEP-21) or a combined therapy regimen CHOEP-14 (50).

In young patients, the addition of etoposide seemed to improve results, however, elder patients seemed to benefit from the short chemotherapy CHOP-14 (48). Another study investigated dose-adjusted EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin), which also intended to represent a new treatment regimen of large B-cell lymphomas (51).

Therapy Options in Patients with Recurrence of the Lymphoma after an Initial Remission(48)

For patients with chemotherapy-sensitive non-Hodgkin's lymphoma who relapsed from complete remission, a high-dose chemotherapy followed by autologous stem cell transplantation has been proven to be an effective therapeutic option (52).

The PARMA trial strongly suggested that patients in response to DHAP (dexamethasone, cytarabine and cisplatin) and an IPI>0 benefit from a High-dose-chemotherapy (HDCT) with an improvement of the survival. The same is true for nonresponding patients with an IPI 0-1, and patients with an IPI score of 1 or higher also seemed to benefit from transplantation (53).

There is a large number of non-comparable studies that deals with the validity of implying autologous hematopoietic stem cell transplantation into the primary therapy of patients with DLBCL. In summary, high-risk patients who achieved a complete remission after a complete course of a standard chemotherapy regimen seem to benefit from transplantation (48).

The durable remission in patients with relapsed or refractory DLBCL can be achieved by a Hematopoietic stem cell transplant (HSCT), but it is limited by patient age, comorbidities and a poor performance status (14).

According to an age-adjusted IPI (aaIPI) and the realisability of dose-intensified approaches, the treatment strategies can be scheduled for several groups of patients (54).
For the management, it is necessary to divide them into different groups and each group might require special treatment (e.g. elderly patients, or if the disease spreads into a specific organ (48).

A general treatment recommendation of the DLBCL by K. Herdrich and H. Weinberger complies with the aaIPI:

a) *Young low-risk patients (aaIPI=0) without bulky disease*
   The current standard therapy involves six cycles of combination chemotherapy with CHOP, as well as six additional doses of Rituximab for 21 days. Radiotherapy does not seem to have an additional benefit in this early stage of the disease (54).
b) *Young low/intermediate-risk patients (aaIPI=1) or IPI low-risk (aaIPI=0) patients with bulky disease*
   In addition to the six cycles of R-CHOP-21, the sites of the previous bulky disease get rayed in course of a radiotherapy. An alternative, intensified regimen is the R-ACVBP (rituximab, doxorubicin, vindesine, cyclophosphamide, bleomycin, and prednisolone) for two weeks (54).
c) *Young high and high-intermediate-risk patients (aaIPI≥2)*
   The most frequently applied treatment is six to eight cycles of CHOP with eight doses of rituximab given every 21 days. Changing the number of drugs, increasing dose, alternating the dose intensity (HDC), and performing autologous stem cell transplantation (ASCT) were approved to enhance the outcome. R-CHOEP-14 shows a better outcome in comparison with R-CHOP-14 in young patients with high-risk DLBCL. Another treatment, ER-CHOP, in which a monoclonal antibody (anti CD22) is added, seems to improve the outcome without adding a higher level of toxicity. In first-line therapy, HDC with ASCT is not recommended outside

clinical trials. For selected high risk-patients CNS prophylaxis is discussed (54).

d) *Relapsed/ resistant disease*

Conventionally dosed salvage chemotherapy (e.g. DHAP, ESHAP, EPOCH, ICE with or without Rituximab), a high-dose treatment afterwards, and stem cell support are recommended for suitable patients. Another chemotherapeutic option is BEAM (carmustine, etoposide, cytarabine and melphalan); however, the additional use of the radiolabeled antibody yttrium-90 ibritumomab tiuxetan seems to be more effective than BEAM alone. For GCB and non-GCB, the ASCT has become the standard of care. In selected patients, when ASCT fails, or with very poor risk factors, allogeneic stem cell transplantation in case of relapse is another option. In high-risk NHL patients, who have a HLA identical donor, tandem transplantation represents a potential therapeutic option (54)(55).

In general patients in this group should be part of clinical trials, however, elderly, comorbid patients should be assigned to a palliative care unit (54).

1.2 Gamma-Glutamyl Transferase

The gamma-glutamyl transferase (GGT) enzyme catalyses the transfer of the γ-glutamyl group from γ-glutamyl peptides such as glutathione (GSH), to other peptides and to L-amino acids (56).

It represents both, the key enzyme of the glutamate cycle and a part in the regulation of glutathione metabolism. As the endothelial cells and pericytes of the cerebral capillaries hold a strong enrichment of GGT, it seems that this enzyme plays an important detoxifying and protective role at the blood-brain barrier (57).

Being part of the γ –glutamyl cycle, it also plays a role in the transport of amino acids across cell membranes. GGT is found in many tissues such as liver, bile ducts, kidney, pancreas, epididymis, heart, lung, small intestine, bone marrow, salivary glands, thymus, spleen and brain (57).

With a concentration 25 times greater than that of the liver, the kidney implies the highest specific activity of GGT. Yet nephropathies do not show elevated GGT (57).

In the liver, spleen, heart, brain and seminal vesicles there is a lower activity of GGT. Nonetheless, the liver is the predominant source of this enzyme in the serum. The highest GGT activity takes place in the periphery of the liver lobule and it is also found in the membranes of hepatocytes and in bile duct epithelia. After passing from the liver to the bile, it gets eliminated by the kidneys (56)(57).

Usually, the range of the serum values is 0-30 IU/ L and there are normally no value differences between men and women. Elevation of GGT is caused either by a raised *de-novo* synthesis or a damage of the hepatocyte's membrane (57)(56).

Actually, GGT is mainly used to differentiate the source of elevated Alkaline Phosphatase (ALP), for example in the case of elevated ALP with correspondingly high GGT, the source of the elevated ALP is probably the biliary tract. The normal half-value time is about 10 days for GGT.

In 1988, Rajpert-De Meyts reported the success of cloning and identifying the nucleotide sequence of human γ-glutamyl transpeptidase (also called γ-glutamyltransferase). The gene was found in a BCR gene-related region located in band q11-qter of chromosome 22 (58).

Multiple forms of GGT were detected by measuring the amounts of different molecular forms of GGT in the serum of patients with different types of liver diseases. In patients with extrahepatic obstruction and jaundice, a form of GGT with a high molecular mass (>1 000 000 Da) was found. In patients with liver disease, a GGT with an intermediate molecular mass of 250 000 to 500 000 Da is present and can be separated by electrophoresis into multiple bands. One of these bands seems to occur more often in patients with extrahepatic obstructive jaundice, while the others predominate in patients with liver diseases (59).

1.3 Uric Acid

Uric acid is the decomposition product of purine nucleotides and represents a weak acid. The catalyst of this reaction is the xanthine oxidase, which produces uric acid out of hypoxanthine and xanthine. Uric acid acts as a natural antioxidant and is excreted via the kidneys (60).

Under physiological conditions the uric acid concentration in blood is about 6,4 mg/dl (62).

In urine with pH of 5,75 it is only half-ionised; in the alkaline urine, in contrast, a much greater proportion is found in a soluble ionised form. Most of the uric acid precipitates if the urine gets acid. In several situations it is possible that there is an excess, e.g. because of a primary defect of metabolism, a lactate acidosis, or rapid catabolism of protein as a side effect after cancer chemotherapy in subsequence of increased cell decomposition (60)(61).

1.4 BMI (Body Mass Index)

The “Body Mass Index” was introduced to define a patient’s weight in kg/m². It helps to assess weight-related health risks and is applied for the assessment of both, overweight and underweight. It allows a rough estimation of a patient’s body composition; however, the BMI does not differentiate between body fat, muscle mass or body water. Additionally it does not have any expressiveness regarding body fat distribution (63).

The world health organization specified several types of abnormal body weight in adults (Table 6) (64).

Underweight	<18,5 kg/m^2
Normal	18,5-24,99 kg/m^2
Overweight	≥25 kg/m^2
Pre-obese	25-29,99 kg/m^2
Obese	30-34,9 kg/m^2
Highly obese	≥30 kg/m^2

Table 6: BMI classification according to the WHO standards **(64)**

2 Material and Methods

2.1 Parameters of Evaluation

The background for this retrospective study was a database, containing 382 patients with DLBCL, diagnosed according to the 2008 World Health Organization (Campo et al., 2011) criteria between January 2004 and April 2013. Both men and women irrespective of their age participated in this study. The clinic pathological parameters contained histologically confirmed DLBCL, gender, age and R-IPI. Another part of the database was the therapy the patients get (standard R-CHOP regimen, CHOP-like, or third-generation anthracycline-containing regimens). All patients received a combined therapy including the monoclonal antibody rituximab. Another part of the database was whether they respond to the treatment and if relapses were documented. The database overviewed the chronological process from the date of the initial diagnosis to the last contact with the patient and its potential date of death. Additionally it implied the laboratory data, including whole blood counts before and after therapy with monoclonal antibody rituximab as well as CRP (as a marker of inflammation), fibrinogen and LDH.

In case of seropositivity of human immunodeficiency virus (HIV) or missing laboratory parameters at diagnosis, patients were excluded.

The project of this thesis started with the search of the patient's laboratory values of GGT and uric acid in the MEDOC system of the University Hospital Graz. If the BMI existed in the patient's record, it was also added to the database. During this process the date of the last contact and associated data were updated. The overall survival (in months) was defined as the period from the date of diagnosis to the last contact. Dates of death were obtained from medical records. The study was approved by the local ethical committee of the Medical University of Graz.

2.2 Statistical Analysis

The analysis was carried out by using the Statistical Package for Social Sciences version 21.0 and 22.0 (SPSS inc., Chicago, IL, USA). For the creation of the pie charts and tables Excel version 2010 was used.

First the descriptive statistics were performed for the laboratory values.
The next step of the study was to create three new groups based on the values of GGT, uric acid and BMI and if these values were elevated or not. The cut-off value for GGT was 45 U/l. The same system was applied for the uric acid values with the cut off value of 6,5 mg/dl. A BMI higher than 25 was defined as overweight.
Out of the data a new variable was computed: the difference between the date of the last contact and the date of the initial diagnosis by using the date arithmetic. It gave a base for the overall-survival of the patients in months.

2.2.1 Cross Tabulation and Chi Square Test

In order to analyse whether there was a relation between the GGT, uric acid and BMI values and the other parameters like age, RIPI, subtype (GCB and NGCB), leucocytes, LDH and CRP cross tabulation was created. Additionally the Chi square test was carried out for each parameter to infer on the entire population.

2.2.2 Kaplan-Meier Curves and Log Rank Test

Kaplan-Meier curves including the log rank test were generated to show the probability of survival referring to increased or reduced values of GGT, uric acid and the BMI.

2.2.3 Univariate and Multivariate Cox Regression

A multivariate Cox proportional analysis was performed to verify whether clinic-pathological parameters were significantly associated with the clinical outcome in univariate Cox analysis of the overall survival. With 95% confidence intervals (CIs) Hazard ratios (HRs), which were estimated from the Cox analysis, were reported as relative risks.

3 Results

3.1 Characteristics of the Study Population

There was a balanced percentage distribution between the sexes with 49,7 % female and 50,3 % male patients, in total, a number of 382 patients, 192 of them men and 190 women (Figure 10).

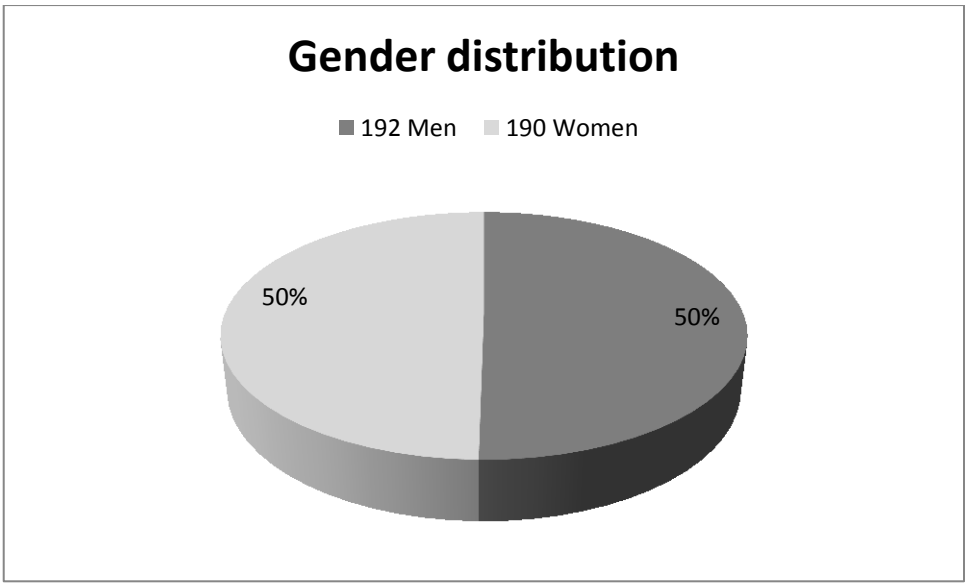

Figure 10: Gender distribution of the study population

At the time of the data collection 259 of the patients were still alive and 120 dead. Expressed as a percentage of 67,8% living patients and 31,4% deceased patients. In 3 cases the documentation of the patients' status (alive or dead) was missing.
The mean age among the patients who were still alive was about 66 years and the median was about 68 years. The youngest patient was 24 years old whereas the oldest had an age of 94 years. So there was a range of approximately 70 years (Figure 11).

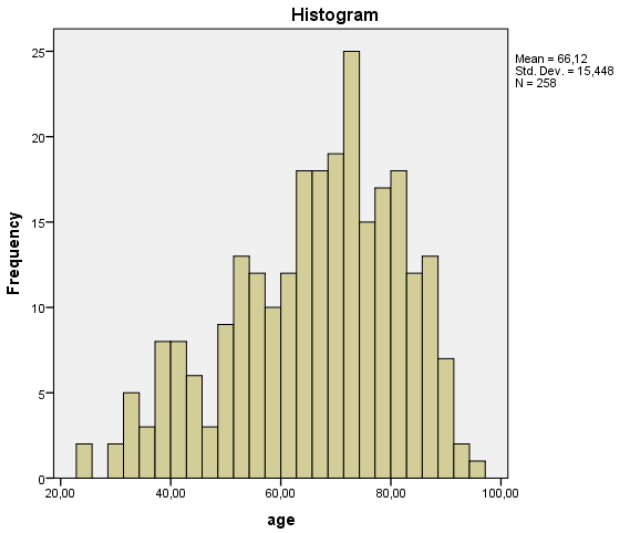

Figure 11: Age distribution of the study population

On average the patients died at the age of 72. The youngest patient died at the age of 33 years and the oldest one was 92 years old. 56,7 % of the people who had died were male (in total 68) and 43,3 % were female (in total 52). This distribution can be seen in Figure 12.

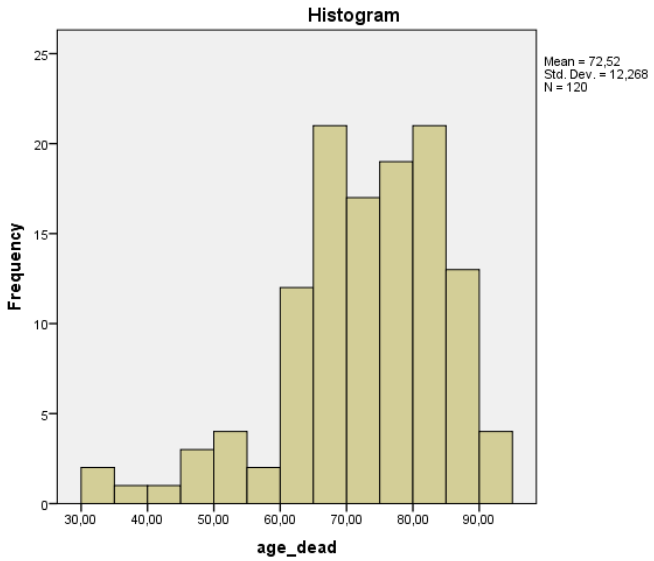

Figure 12: Age distribution of patients who have died in the course of the study

The majority of the diagnosed DLBCL were non-GCB DLBCL with a total number of 172 patients, which is a percentage of 45 %. The GCB subtype represented 28,3% or 108 individuals. 20 patients had a primary mediastinal DLBCL (PM-DLBCL). In 82 patients no subtype could be classified.

A poor R-IPI was determined in 124 patients (32,5%), 209 (54,7%) patients, had a good R-IPI. Only 30 patients (7,9%) had a very good R-IPI. In 19 cases no R-IPI was found (Figure 13).

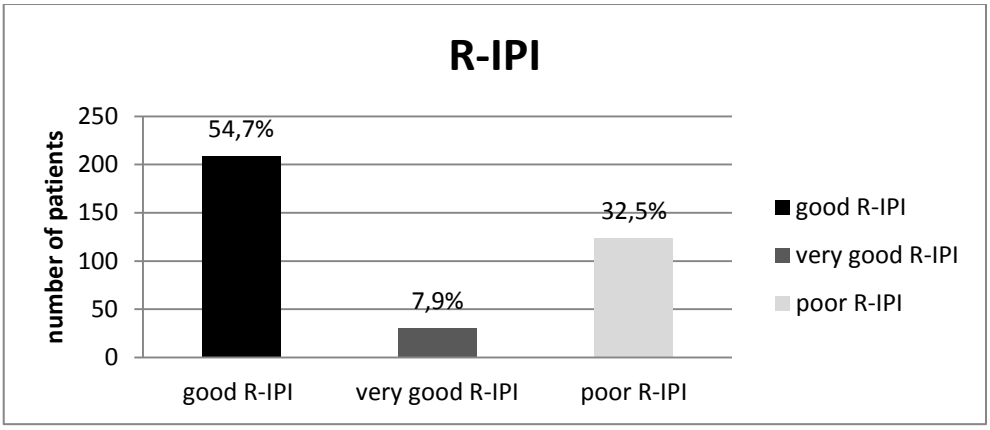

Figure 13: R-IPI distribution in percentage

3.2 GGT

283 sets of valid GGT values were retrieved from the system, representing 74,1 % of the total amount of patients. In 99 patients' records, 25,9%, no valid GGT value could be found. The smallest value amounted to 3,2 U/l and the maximum value ran up to 448 U/l. This resulted in a range of 444,8 U/l. A mean of 55,75 was calculated and the analysis reports a median of 31. Both the Kolmogorov-Smirnov and the Shapiro-Wilk tests of Normality showed a high significance with $p \leq 0,0001$, which meant that the values of GGT were not normal distributed. The distribution of the GGT is pictured in Figure 14.

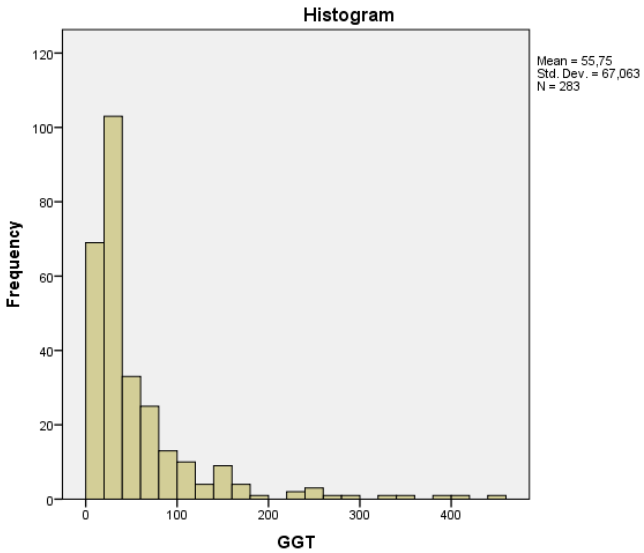

Figure 14: Distribution of the GGT values in U/l including mean, standard deviation and total number

186 patients had GGT values beyond 45 U/l. This represented a percentage of 65,7%. In 34,3% of the cases, which was a number of 97 people, GGT values higher than 45 U/l were found. This can be seen in Figure 15.

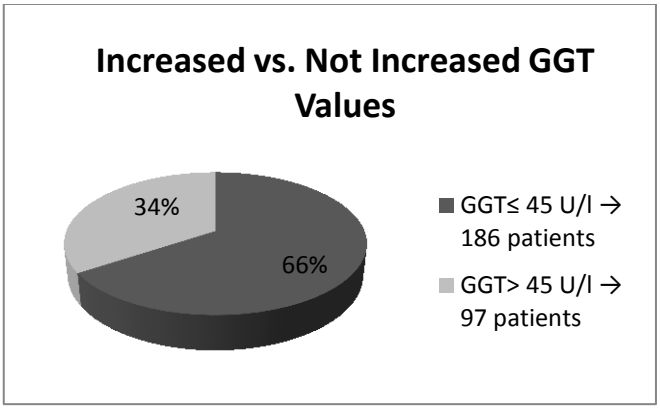

Figure 15: Distribution of increased and normal GGT values

Cross tabulation was made in order to analyse whether there was a correlation between GGT and diverse parameters like gender, age, RIPI, subtype, LDH and CRP (as acute phase protein). Afterwards a Chi-Square test was made to prove if there really was a relationship between the parameters.

Taking into account the statistical data seen in Table 7, a connection between the gender and the GGT values can be surmised. By using the Chi-Square test the initial suspicion is substantiated (Pearson Chi-Square *p<0,001*). According to Table 7 it can be seen, that more male patients (69,1%) had increased GGT values than female (30,9%).

			GGT		Total
			≤45 U/l	>45 U/l	
Gender	male	Count	82	67	149
		Expected Count	97,9	51,1	149,0
		% within GGT	44,1%	69,1%	52,7%
	female	Count	104	30	134
		Expected Count	88,1	45,9	134,0
		% within GGT	55,9%	30,9%	47,3%
Total		Count	186	97	283
		Expected Count	186,0	97,0	283,0
		% within GGT	100,0%	100,0%	100,0%

Table 7: Cross tabulation of GGT and gender

However no statistical correlation was found between GGT, age, RIPI, subtype, LDH and CRP (Pearson Chi-Square *p>0,05*).

3.3 Uric Acid

In 268 patient records suitable uric acid values were found. This represented a data completeness of 70,2%.

The mean was 5,894 mg/dl and the median 5,5 mg/dl with a variance of 5,822 and a standard deviation of 2,413. It ranged from 1,4 mg/dl to 19 mg/dl with a spread of 17,60 mg/dl (Figure 16).

Both tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk) showed a high significance with p≤0,0001.

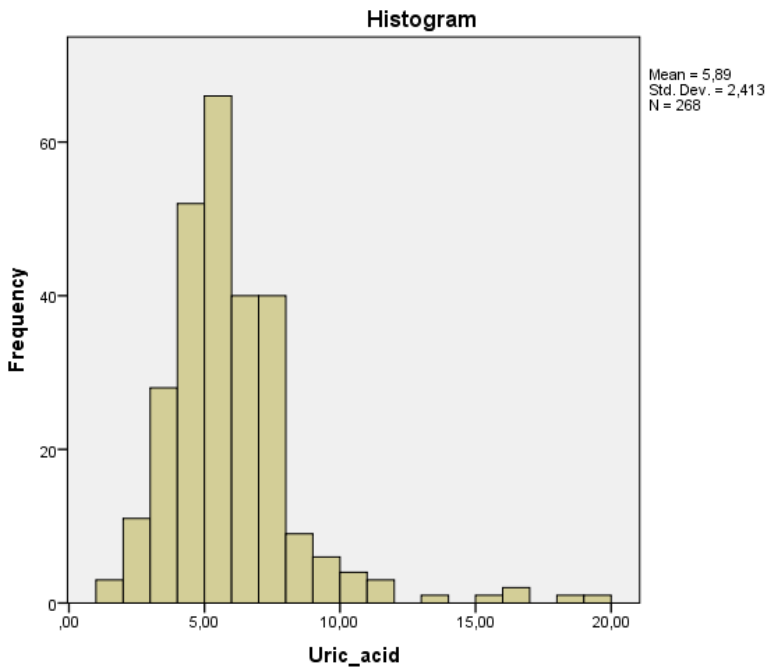

Figure 16: Distribution of the uric acid values in mg/dl including mean, standard deviation and total number

Of the total 268 patients in whom valid uric acid values were found, 188 people had uric acid values less than or equal 6,5 mg/dl, which represented a percentage of 70,1% (Figure 17). Those patients with uric acid values greater than 6,5 mg/dl constituted a percentage of 29,9%, so 80 people had increased uric acid values.

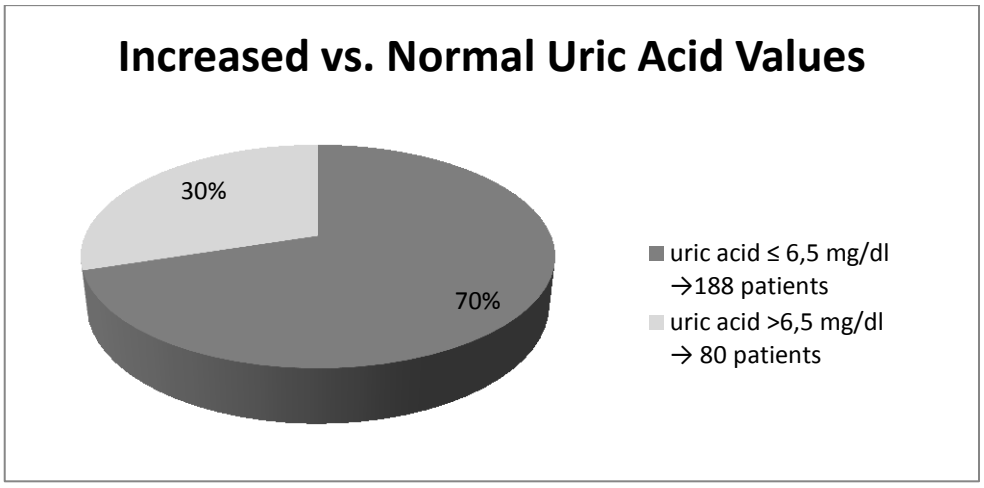

Figure 17: Distribution of increased and normal uric acid values

Cross tabulations between uric acid and the same parameters that had been studied for the GGT (gender, age, RIRI, subtype, LDH and CRP) were created. Additionally the correlation between GGT and the BMI and uric acid was analyzed. There seemed to be a significant correlation between the age and the uric acid values (Pearson Chi Square *p=0,001*). The majority of the patients (60%) was in the group of 50- to 75-year-olds and has elevated uric acid values (Table 8).

			Uric acid_		Total
			<=6,5	>6,5	
age	<=50a	Count	36	2	38
		Expected Count	26,6	11,4	38,0
		% within uric acid_	19,3%	2,5%	14,2%
	50-75a	Count	97	48	145
		Expected Count	101,6	43,4	145,0
		% within uric acid	51,9%	60,0%	54,3%
	>75a	Count	54	30	84
		Expected Count	58,8	25,2	84,0
		% within uric acid	28,9%	37,5%	31,5%
Total		Count	187	80	267
		Expected Count	187,0	80,0	267,0
		% within uric acid	100,0%	100,0%	100,0%

Table 8: Cross tabulation uric acid and age

A significant correlation was found between uric acid and CRP as well (Pearson Chi-Square *p=0,036*). One can also read from Table 9 that with a percentage of 88,8%, patients had both elevated CRP and uric acid values.

			uric acid coded		Total
			<=6,5	>6,5	
CRP erhöht	1,00	Count	41	9	50
		Expected Count	34,8	15,2	50,0
		% within uric acid coded	22,3%	11,3%	18,9%
	2,00	Count	143	71	214
		Expected Count	149,2	64,8	214,0
		% within uric acid coded	77,7%	88,8%	81,1%
Total		Count	184	80	264
		Expected Count	184,0	80,0	264,0
		% within uric acid coded	100,0%	100,0%	100,0%

Table 9: Cross tabulation between uric acid and CRP

Actually the Chi-Square test between uric acid and BMI indicated a significant relationship between the two parameters (Pearson Chi-Square *p<0,001*) (Table 10).

			uric acid coded		Total
			<=6,5	>6,5	
BMI overweight	1,00	Count	55	10	65
		Expected Count	43,8	21,2	65,0
		% within Uric acid coded	57,9%	21,7%	46,1%
	2,00	Count	40	36	76
		Expected Count	51,2	24,8	76,0
		% within Uric acid coded	42,1%	78,3%	53,9%
Total		Count	95	46	141
		Expected Count	95,0	46,0	141,0
		% within Uric acid coded	100,0%	100,0%	100,0%

Table 10: Cross tabulation of uric acid and BMI

No significant correlation was found between uric acid and RIPI, gender, LDH, subtype and GGT (Pearson Chi-Square *p>0,05*).

3.4 BMI

The BMI was only recorded in 40,3% of the cases (154 patients). The mean was 25,95 and the median 25,44. The standard deviation was 4,377. It showed a range of 23,69 from the minimum of 15,52 to the maximum of 39,21 (Figure 18). Both the

Kolmogorov-Smirnov and the Shapiro-Wilk tests of normality showed a significance with *p=0,022* and *p=0,011*.

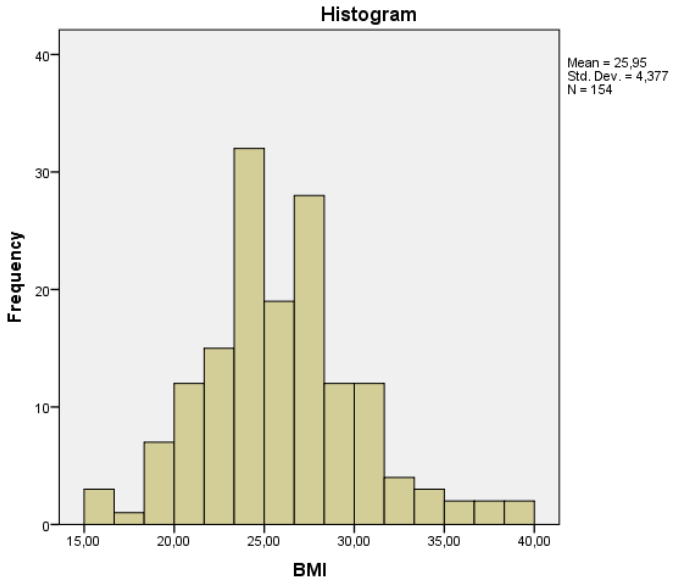

Figure 18: Distribution of the BMI values in mg/dl including mean, standard deviation and total number

A BMI greater than 25 was detected in 85 patients (55,2%), so the majority of the study participants was overweight. In 69 cases (44,8%) a BMI less than or equal 25 was found.

Additionally to the general parameters (gender, age, RIPI, subtype, LDH and CRP) the correlation between BMI and GGT as well as Leucocytes was analysed.
In cross tabulation a significant correlation between BMI and RIPI (Pearson Chi-Square *p=0,018*) could be detected. In this database the majority of patients with a BMI > 25 had a good RIPI, they account for 68,3% (Table 11).

			BMI overweight		Total
			1,00	2,00	
RIPI coded	poor	Count	29	21	50
		Expected Count	23,0	27,0	50,0
		% within BMI overweight	41,4%	25,6%	32,9%
	good	Count	32	56	88
		Expected Count	40,5	47,5	88,0
		% within BMI overweight	45,7%	68,3%	57,9%
	very good	Count	9	5	14
		Expected Count	6,4	7,6	14,0
		% within BMI overweight	12,9%	6,1%	9,2%
Total		Count	70	82	152
		Expected Count	70,0	82,0	152,0
		% within BMI overweight	100,0%	100,0%	100,0%

Table 11: Cross tabulation of BMI and RIPI

According to the statistics no significant correlation was found between BMI and age, gender subtype, LDH, CRP, GGT and Leucocytes (Pearson Chi-Square *p>0,05*).

3.5 Overall- Survival

Only 100 patients (26,2%) survived more than five years. 279 patients (73%) did not (yet) achieve the 5-year survival rate.

The mean overall survival after the initial diagnosis date was approximately 3,5 years. The median overall survival was almost 3 years. The shortest overall survival was less than a year and the longest was 21,5 years.

3.5.1 Kaplan-Meier Curves and Log-Rank Test

In order to compute the probability of survival, Kaplan-Meier curves were made for GGT, uric acid and the BMI. The difference of increased and reduced values of GGT, uric acid and BMI were analysed in connection with the probability of survival. Additionally the log-rank test was made to compare the survival distribution between the increased and reduced values.

Fehler! Verweisquelle konnte nicht gefunden werden. represents the survival urve of Kaplan-Meier analysed by GGT values lower or equal 45 U/l and GGT values higher than 45 U/l. As the significance of the log rank test was *p=0,663* there was no significant difference related to the survival between increased and reduced GGT values.

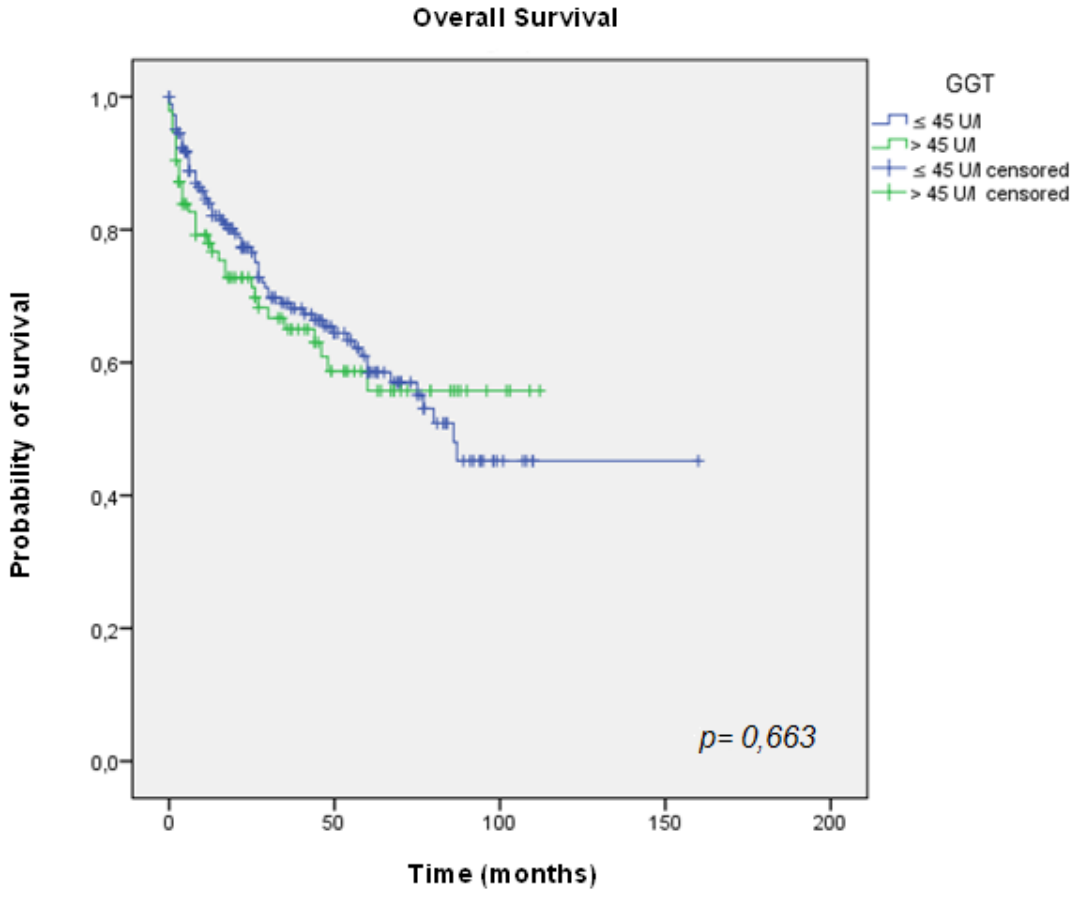

Figure 19: Kaplan–Meier curves for overall survival regarding high (>45 U/l) vs low (≤45 U/l) GGT levels (p=0,663)

There was a significant difference between uric acid values lower or equal 6,5 mg/dl and values higher than 6,5 mg/dl pertaining to the survival. This was proven by the significance of *p=0,002* which was calculated by the log rank test.

As can be seen from the Kaplan-Meier curve in Figure 20 the five-year survival probability for patients with reduced uric acid values was 82,4% whereas the five-year survival probability for patients with increased uric acid values was only 52%.

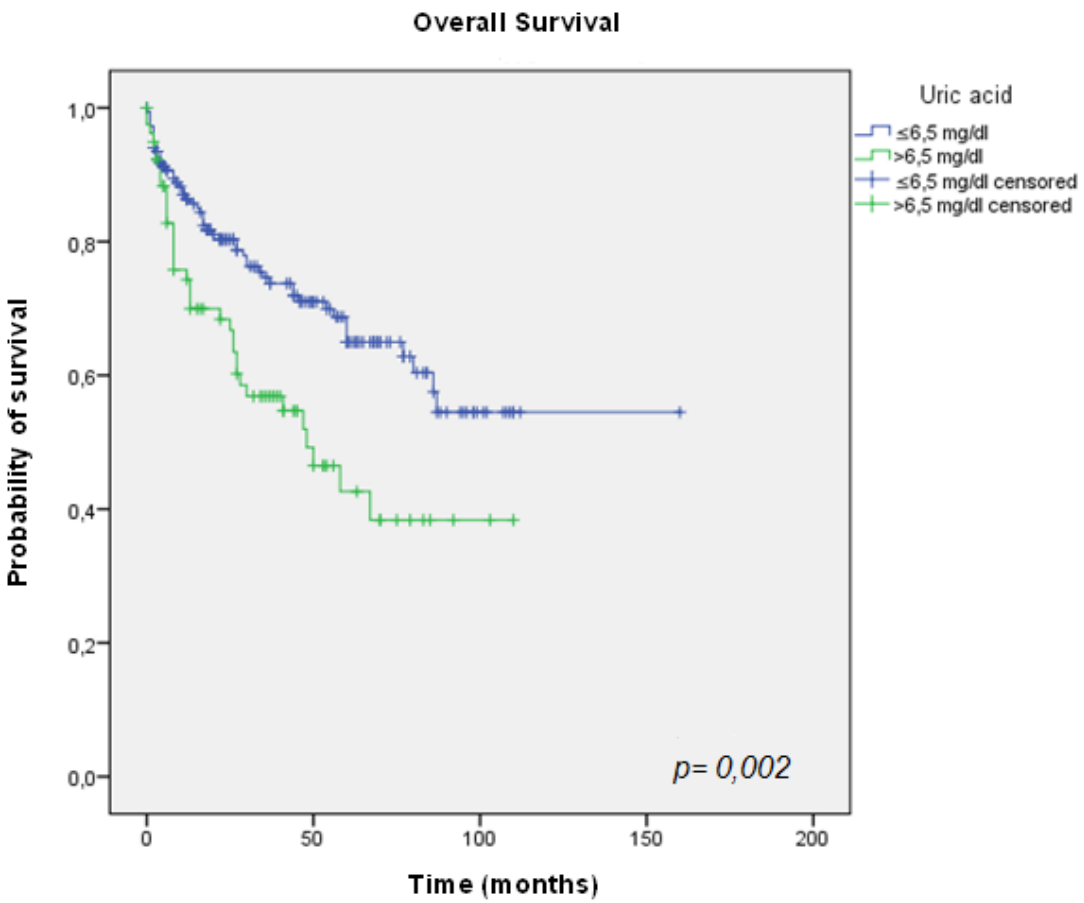

Figure 20: Kaplan–Meier curves for overall survival regarding high (>6,5 mg/dl) vs. low (≤ 6,5 mg/dl) Uric acid levels (*p=0,002*)

According to the implementation of the log rank test with a significance of *p=0,271* there was no significant difference concerning the survival of overweight individuals and normal weight ones.

Figure 21 shows the Kaplan-Meier curve of the survival of people with a BMI lower or equal 25 and patients with a BMI higher than 25.

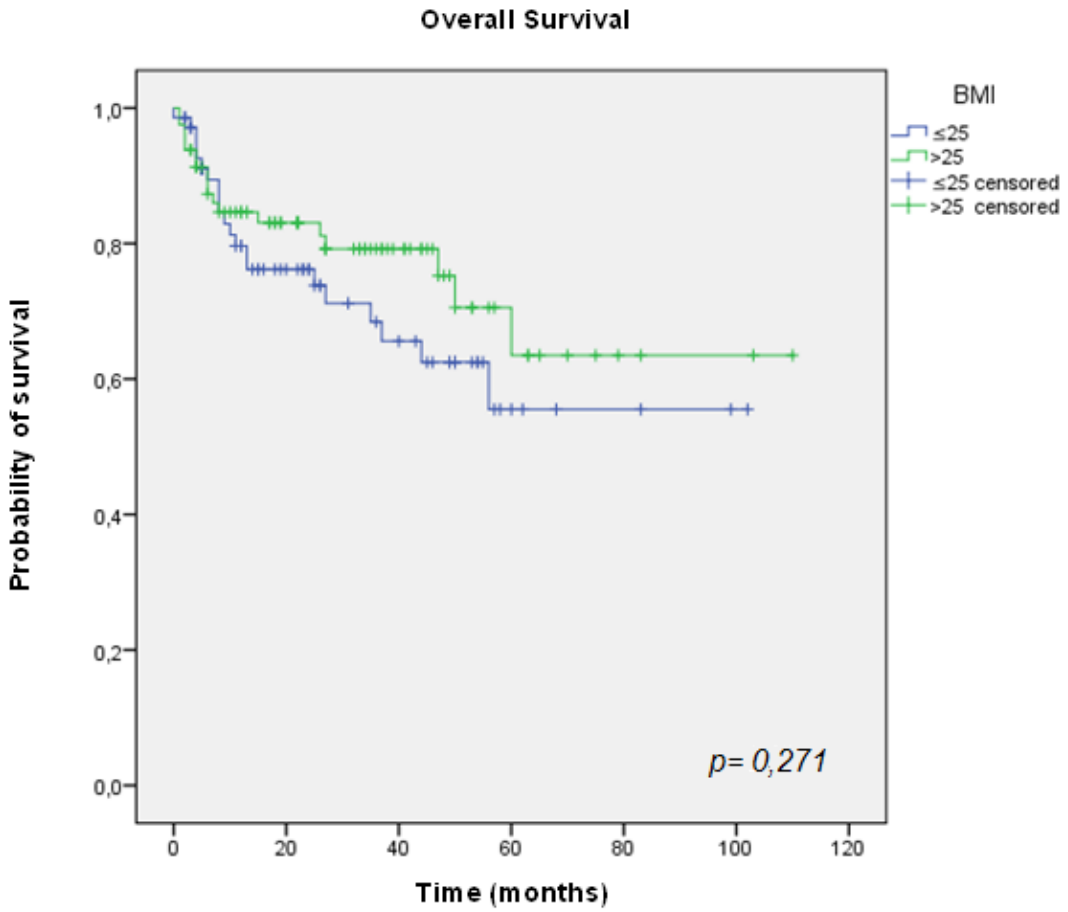

Figure 21: Kaplan–Meier curves for overall survival regarding overweight (BMI>25) vs normal weight (BMI≤25) patients (*p=0,271*)

In order to analyze the overall survival precisely, the BMI was subdivided into 4 groups and a Kaplan-Meier Curve was generated once more (Figure 22). However no significant value was found (Log Rank *p=0,619*)

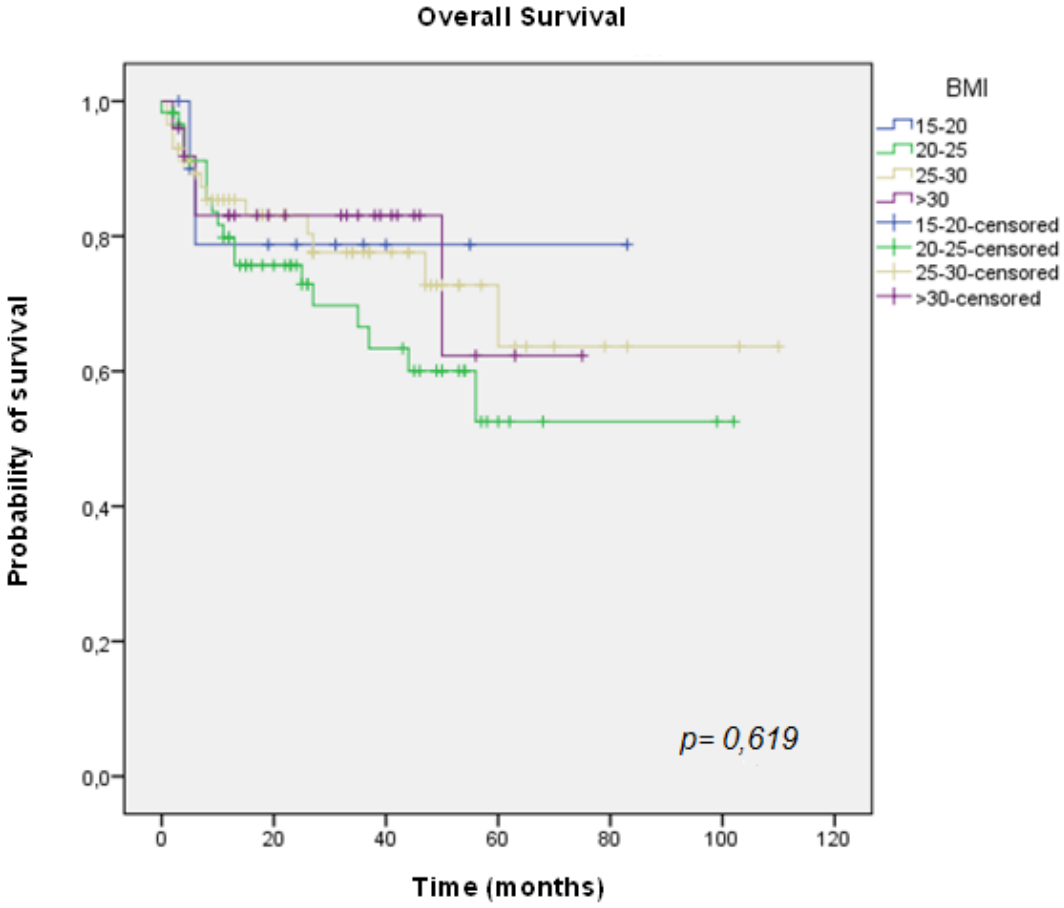

Figure 22: Kaplan–Meier curves for overall survival regarding the BMI precisely ((BMI 15-20), (BMI 20-25), (BMI 25-30) and (BMI >30)), (*p=0,619*)

3.5.2 Cox Regression

Five-year OS		Univariate analysis		Multivariate analysis	
Parameter	Frequency	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age					
≤ 50	54	1 (Referent)		1 (Referent)	
50-75	197	2,154 (1,028-4,511)	**0,042**	0,959 (0,380-2,421)	0,929
>75	127	3,306 (1,571-6,957)	**0,002**	1,413 (,0556-3,587)	0,467
Clinical stage (RIPI)					
very good	122	1 (Referent)		1 (Referent)	
good	208	0,433 (0,298-0,629)	**0,000**	0,681 (0,390-1,188)	0,176
poor	30	0,051 (0,007-0,366)	**0,003**	0,470 (0,058-3,828)	0,481
Subtype					
GCB	169	1 (Referent)	**0,000**	1 (Referent)	**0,005**
NGCB	108	0,333 (0,201-0,553)		0,427 (0,236-0,773)	
Gender					
Male	190	1 (Referent)	0,209	n.d	n.d
Female	188	0,793 (0,553-1,138)			
GGT					
≤ 45	185	1 (Referent)	0,666	n.d.	n.d.
> 45	94	1,097 (0,721-1,668)			
Uric acid					
≤ 6,5	185	1 (Referent)	**0,003**	1 (Referent)	**0,004**
> 6,5	79	1,922 (1,255-2,942)		2,160 (1,282-3,640)	
BMI					
≤ 25	70	1 (Referent)	0,276	n.d.	n.d.
> 25	81	0,705 (0,375-1,374)			
Leucocytes					
< 4000	21	1 (Referent)		n.d.	n.d.
4000 - 10 000	264	1,897 (0,694-5,181)	0,212		
>10 000	68	2,691 (0,935-7,746)	0,067		
Fibrinogen					
≤ 200 mg/dl	5	1 (Referent)		n.d.	n.d.
200-400 mg/dl	144	1,958 (0,477-8,046)	0,351		
> 400 mg/dl	166	0,832 (0,552-1,253)	0,378		
LDH					
≤ 243 U/l	173	1 (Referent)	**0,006**	1 (Referent)	0,829
>243 U/l	176	1,715 (1,166-2,523)		1,063 (0,612-1,847)	
CRP					
normal	75	1 (Referent)	**0,017**	1 (Referent)	0,250
increased	274	2,026 (1,135-3,618)		1,658 (0,701-3,923)	
HB					
reduced Hb	184	1 (Referent)		1 (Referent)	
normal Hb	164	0,399 (0,263-0,603)	**0,000**	0,395 (0,216-0,722)	**0,003**

Table 12: Univariate and multivariate Cox regression of clinico-pathological parameters (significant values are marked in bold)

The results of the univariate Cox proportional analysis (Table 12) demonstrated that age, RIPI, subtype, uric acid, LDH, CRP and reduced haemoglobin values were significant factors for the OS of the patients. Older age (50-75a *p=0,042* and >75a *p=0,002*), R-IPI (good vs. poor *p=0,003*), subtype (GCB vs NGCB, *p<0,001*), uric acid (normal vs increased values*, p=0,003*), LDH (≤ 243 U/l vs >243 U/l, *p=0,006*), CRP (normal vs increased, *p=0,017*) and haemoglobin (reduced vs. normal haemoglobin, *p<0,001*) correlated with a poor outcome of the patients' OS. However gender, GGT, BMI, count of leucocytes, fibrinogen and increased Hb values were not statistically significant associated with the OS.

A multivariate analysis using a Cox proportional hazard model was compiled in order to determine the influence of clinico-pathological variables which were significantly associated with the clinical outcome in univariate analysis of OS (age, RIPI, subtype, uric acid, LDH, CRP and Hb). The data would seem to suggest that subtype (*p=0,005*), uric acid (*p=0,004*) and decreased haemoglobin (*p=0,003*) were independent prognostic factors for the OS.

4 Discussion

The DLBCL represents the most frequent Non-Hodgkin lymphoma. Many studies deal with the influence of clinical parameters on the DLBCL development and prognosis. This study though is the first one which analyzes the parameters GGT, uric acid and BMI and their influence on the OS of patients with DLBCL.

In this retrospective study a significant difference between elevated and normal values concerning the overall survival was only found for uric acid values (log rank $p=0,002$). Both the univariate and multivariate Cox analysis proved that uric acid acts as an independent prognostic factor for overall survival.
A correlation by using the Chi-Square test was found between GGT and gender ($p<0,001$), uric acid and age ($p=0,001$), CRP ($p=0,036$) and the BMI ($p<0,001$).

In general GGT, uric acid and BMI and their role in cancer genesis is discussed in several studies.
In the last few years the antioxidant effect of serum GGT was investigated in detail (65).
By breaking down extracellular glutathione it makes its components approachable to the cells (65).
As GGT-positive tumor cells are able to use the serum glutathione they have a selective growth advantage in vivo in contrast to GGT-negative tumor cells (66).
In a large prospective, long-term study the correlation between GGT and risk of cancer incidence was analyzed in more than 92 000 healthy Austrian women of all ages. The results of this study showed that increased GGT significantly correlates with an increased risk of developing cancer (67).
In 79 279 men of all ages, the results of a similar study showed that increased GGT is associated with an elevated risk of developing cancer especially among men, younger than 65 years (68).
GGT has also been the subject of a recent study in which a relationship between GGT and the severity of disease and prognosis of ovarian cancer at diagnosis was investigated. An association between high pre-therapeutic GGT serum levels with advanced tumor stage was found. So in patients with epithelial ovarian cancer

(EOC) it can be used as an independent prognostic marker for poor overall survival (69).

Another multicenter trial dealt with the prognostic significance of GGT in patients with endometrial cancer, it has been found that for patients with endometrial cancer, GGT is an independent prognostic parameter for the progression-free survival (70).

A further aim of research is to determine the antioxidant effect of uric acid. Several studies deal with the antioxidant properties of serum uric acid and its protecting role against cancer genesis (71).

The hypothesis that uric acid inhibits cancer growth was rejected in a study, which comprises a cohort of 7889 Japanese men in Hawaii (72).

A slight, though significant association between elevated serum urate concentration and total mortality, which was entirely explained by neoplastic deaths ($p<0,01$), was found. These findings counter the assumption of the cancer protective antioxidant effect of uric acid (73).

An epidemiological study of Petersson and Trell was the reason for another prospective population study on hyperuricaemia and cancer mortality conducted by H. Takkunen et al (73)(74).

The cohort of a recent study is a rather large one with more than 28 000 elderly Austrian women (75).

In contrast to the proposed antioxidant effect of serum uric acid against cancer high serum uric acid seems to be associated independently with outcome and is not necessarily linked to cancer mortality (75).

An association between obesity and the risk of several cancer types as well as a poorer treatment and increased mortality was found in several studies (76).

As there is a link between obesity, altered inflammatory and immune response it is assumed that a high BMI could influence the risk of non-Hodgkin's lymphoma (77).

Adipose tissue secretes proteins, called adipokines, which take part in the regulation of inflammation (TNFα, interleukin-6, serum amyloid A) and immune response (leptin) ((78).

The local elevated TNFα, due to an increased production in adipose tissue, interacts with insulin signal transduction and leads to a local desensitization to insulin signaling. Obesity is also associated with a systemic rise of inflammatory

markers such as CRP. Elevated CRP and the additional increase of other acute phase reactants are associated with insulin resistance (79).

The insulin resistance and the associated hyperinsulinaemia leads to a reduction of synthesis and blood levels of insulin-like growth factor binding protein 1 (IGFBP1), as well as insulin-like growth factor binding protein 2 (IGFBP2). Consequently the levels of bioavailable IGF1 increases. The cellular proliferation and apoptosis inhibition are controlled through insulin and IGF1 signal by binding on the insulin receptors (IRs) and IGF1 receptors. This process may support tumor genesis(Figure 23) (78)

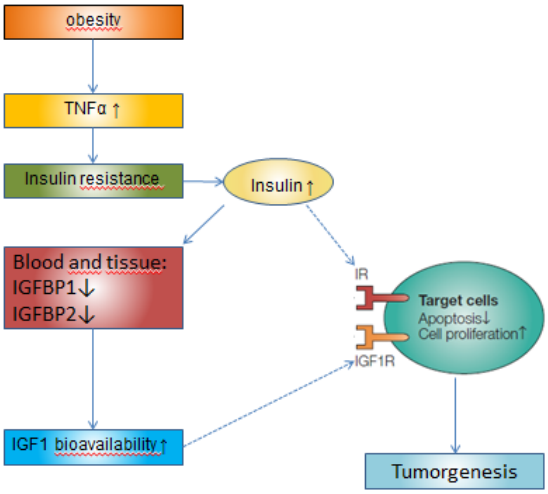

Figure 23: Influence of obesity on tumour development (78)

A multiethnic cohort study showed that a pre-diagnostic BMI could be an appropriate prognostic marker for NHL patients (80).

One of the largest studies was performed in Denmark and Sweden which included 3055 patients with NHL, 618 patients with HL and 3187 population-based control subjects. However no association between BMI and the risk of neither NHL nor HL could be detected (81).

Also the findings of Chang et al. assume that a high BMI does not seem to influence the development neither of overall NHL, most of the NHL subtypes nor HL. They suggest though, that high BMI may play a role in the development of DLBCL (64).

In conclusion a high uric acid value at the time of diagnosis seems to be an independent predictor for patients' OS. However, these results have to be confirmed in a larger study comprising an independent cohort of patients.

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