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Ofatumumab in CLL: Evaluation of in-vitro response mechanisms

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Dedication

To my parents

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List of abbreviations

13 q del	deletion of the long arm of chromosome 13, detected by FISH, prognostic marker for CLL, associated with long survival
11q del	deletion of the long arm of chromosome 11, detected by FISH, prognostic marker for CLL
17p del	deletion of the short arm of chromosome 17, detected by FISH, prognostic marker for CLL, associated with short survival
12+	trisomy of chromosome 12, detected by FISH, prognostic marker for CLL
7-AAD	7-Amino-Acridine, fluorescent dye used for e.g. flow cytometry, late cell death marker used for flow cytometry
ADCC	antibody dependent cytotoxicity
AKT	Protein Kinase B, plays a key role in multiple cellular processes, e.g. apoptosis, cell proliferation
Annexin-V	cellular protein, located on the inner surface of the cell membrane, positive staining of Annexin-V indicates beginning cell death (flow cytometry)
BCL-2	B-cell lymphoma 2, apoptosis regulator protein
BD	Becton Dickinson (company, global player in biotechnology)
BR	Bendamustine (chemotherapeutic agent) + Rituximab
Ca	Calcium
CD	cluster of differentiation, cell surface antigen
CDC	complement dependent cytotoxicity
CLL	chronic lymphocytic leukemia
DMSO	Dimethyl sulfoxide, polar solvent that dissolves both polar and nonpolar compounds
F	Fludarabine, chemotherapeutic agent
FCR	Fludarabine + Cyclophosphamide (both chemotherapeutic agents) + Rituximab
FCS	bovine (fetal) calf serum
FACS	flow cytometry (fluorescence activated cell sorting)
FFP	fresh frozen plasma
FISH	fluorescence in-situ hybridization, specific technique to stain parts of the DNA with a fluorescent dye, read-out e.g. microscopy
FITC	fluorescein isothiocyanate, chemical compound, fluorescent dye used for fluorescent staining of cells before e.g. cytometric read-out
HS5-cells	cell line derived from bone marrow stroma cells
Ig	Immune globuline
IgHV	Immune globuline heavy (chain) variable part:

IgHV m	mutated IgHV (positive prognostic marker for CLL)
IgHV um	unmutated IgHV (negative prognostic marker for CLL)
kDa	kiloDalton (unit for molecular mass of a protein)
mAb	monoclonal antibody
Mg	Magnesium
p53	tumor suppressor protein, regulates cell cycle, encoded by TP53 gene, guardian of the genome (p53 runs as 53 kDa protein on SDS-Page)
PBS	phosphate buffered saline
PCD	programmed cell death
PE	Phycoerythrin, fluorescent dye
Raji-cells	cells derived of a Burkitt-lymphoma derived cell line
RPMI 1640	cell culture medium
SDS-Page	sodium dodecyl sulfate polyacrylamide gel electrophoresis; to separate proteins according to their electrophoretic mobility
SSC	sideward scatter, flow cytometric read-out mode, measures granularity of cells
TP53 gene	encodes for p53
ZAP-70	Zeta-chain-associated protein kinase 70 (70kDa), prognostic marker in CLL

1 Introduction

CLL is the most common adult leukemia in the western world. It is characterized by an expansion of monoclonal, mature B cells, which accumulate in different body compartments. The clinical definition of the disease is based on the characteristic expression of epitopes (positivity for both CD19 and CD5) and a blood count of over 5000 of these malignant B cells. CLL can be further distinguished by dividing the disease into different subgroups. Prognostic factors such as *IGHV* mutational status, β2-microglobulin, ZAP-70 expression, CD38 expression and clinical stage are used to predict disease course. In addition, there are a number of recurrent genomic aberrations occurring in CLL cells, which is relevant for the choice of treatment strategy. CLL cells usually proliferate in lymph nodes and not in the blood compartment, where they mainly accumulate and remain inactive.

Low or intermediate risk CLL patients (patients without a 17p-deletion, without *TP53* mutation, and not refractory to purine analogues) can be effectively treated with chemoimmunotherapy such as FCR (Fludarabine + Cyclophosphamide + Rituximab) or BR (Bendamustine + Rituximab) (Hallek M, 2009, Fischer et al., 2011, Stilgenbauer and Zenz, 2010). It should be noted, that elderly patients, who constitute the majority of CLL patients, are often not regarded as suitable for such aggressive treatment due to comorbidity(Eichhorst et al., 2009). Apart from that, chemotherapeutic agents cause side effects or secondary tumors after years(Morrison et al., 2002). High-risk-patients (defined by p53 mutation) are unlikely to respond to purine analogues such as Fludarabine and F-based therapies. This is because chemotherapeutic approaches often require a functional DNA damage/p53 pathway, which is absent in this patient group (Zenz et al.,2008). There is a need for novel, effective, and well-tolerated treatment options and treatment strategies. A number of substances have been shown to act independently of a functional p53 pathway and these offer theoretic advantages in this patient population, which ideally should be demonstrated in randomized trials. Among those are monoclonal antibodies.

Three different therapeutic antibodies are currently approved and recommended for the therapy of CLL: The CD20-antibodies Rituximab and Ofatumumab as well as the CD52-antibody Alemtuzumab. In Europe, Rituximab is mainly used in com-

binations and rarely as a single agent in CLL. Alemtuzumab is mainly used as a single-agent in Fludarabine-refractory cases and for high-risk CLL patients who are not eligible for other therapeutic options, such as stem cell transplantation. Ofatumumab has been approved for double-refractory CLL or CLL with so-called bulky disease.

CD20 is a 33–37 kDa, non-glycosylated phosphoprotein expressed on the surface of almost all normal and malignant B cells. CD20 has no known natural ligand and CD20 knockout mice display an almost normal phenotype. It probably functions as a store-operated calcium channel following ligation of the B cell receptor for antigen (Walshe et al., 2008, Cragg, 2005). Rituximab and Ofatumumab can be described as type-I-CD20-antibodies. Ofatumumab, Rituximab, and Alemtuzumab are potent inducers of CDC, though each to a different extent and more importantly, depending on the disease and antigen-expression. CLL is known for rather low CD20 expression and even though an antibody such as Rituximab seems highly effective in inducing certain effector mechanisms in other B-cell malignancies, this does not necessarily mean that this accounts for CLL too. We observed a dramatic difference of Rituximab-induced CDC in our control cell-line, Burkitt-lymphoma-derived Raji-cells, which were very susceptible towards it and CLL-patient samples, which didn't seem to be susceptible at all. Other mechanisms such as ADCC, PCD, inducing of a vaccine-like effect or chemosensitization have been described to contribute to the over-all activity of the antibodies in vivo. As multiple factors can influence these mechanisms, it is important to identify the principal one or ones for a certain disease. Combinations of new and different targeted therapeutic options should yield major efficiency and elicit least-possible side effects. Some patients seem to benefit more of antibodies than others. This hints, that intrinsic resistance mechanisms need to be identified. It remains a challenging topic to develop strategies to enhance antibody therapy in CLL.

We tested and compared Rituximab, Ofatumumab, and Alemtuzumab in vitro to assess their biological activity with special regard to possible enhancement of their use in future CLL therapy. Most of the in-vitro antibody assays are usually performed under normal culture conditions. Regarding CLL the disease extent in different body compartments and different disease stages can influence the efficacy of a therapy. We used different culture conditions to mimic the in vivo situation and

to detect possible limitations of the antibodies. We hypothesized different susceptibility to antibody treatment of different CLL subgroups and subdivided our CLL-patient samples according to risk-stratification and antibody-pretreatment. In contrast to Rituximab, relatively little is known about the benefit of Ofatumumab used in combination with other drugs. We tried to discover new strategies to achieve both targeted use of antibodies and potential enhancement of their antitumor activity by combining them with other drugs. We admit that due to the in-vitro character of our assays we cannot guarantee that our results can be fully reproduced in vivo and clinical trials would be needed to determine their significance.

2 Materials and Methods

2.1 Patient samples

CLL cells were isolated from blood taken of CLL patients with high tumor burden in the blood compartment and who have been previously diagnosed according to standard criteria (Hallek et al., 2008). Ficoll gradient centrifugation was used for isolation. Cells were then washed, cryo-preserved in FCS with 5% DMSO for subsequent analysis. Experiments were performed after informed written consent had been obtained from all patients. Age, gender, pretreatment, and risk stratification (interphase-FISH for the detection of chromosomal abnormalities, *TP53*-mutation-analysis, *IgHV*-mutation-status, ZAP-70-expression) of the CLL patients are summarized in tables I to V. (end of "Materials and Methods")

2.2 Data analyses

Error bars represent the SD of experiments which were performed in triplicates. GraphPad Prism 4 was used to perform unpaired t-tests and non-linear regression. Data from successful and valid experiments are summarized.

Assays

2.3 Chemoluminescence based viability assay

CLL-cells were incubated at increasing concentrations of monoclonal antibodies (and partially in combination with either Fludarabine, Alemtuzumab, or AKT-inhibitor) in complete medium with 30 % fresh frozen serum at a cell concentration of 1000 cells /µl. Raji-cells, which showed to be very sensitive to complement dependent cytotoxicity, were used as an internal control. Isotype IgG-antibody and complete medium were used as untreated negative controls. Viability was assessed with Promega Cell Titer Glo Luminescent cell viability assay according to manufacturer's protocol. Read-out was performed after 3, 24, and/or 48 hours. For combination assays Fludarabine and Alemtuzumab, were used at fixed concentrations: 1µmol Fludarabine and 1µg/ml Alemtuzumab. Pan-AKT-Inhibitor GSK 690693 was provided by GlaxoSmithKline and used at different concentrations (maximum 10µmol).

Materials:	
Cell titer Glo luminescent viability assay	Promega GmbH, Mannheim
RPMI 1640 Medium	Biochrom AG, Berlin
BD Pharmigen Purified Mouse IgG1 K isotype control	Becton Dickinson GmbH, Heidelberg
Rituximab	Roche, Hoffmann, La Roche, Grenzach-Whylen
Alemtuzumab	Bayer Schering Pharma AG, Berlin und Genzyme GmbH, Neu-Isenburg
Sera Plus special processed FCS	PAN Biotech GmbH, Aidenbach
Human Serum FFP / off the clot	Sanquin Plasma Products, Amsterdam, The Netherlands
PBS Dulbecco w/o Ca, Mg	Biochrom AG, Berlin
Ofatumumab	Genmab, Utrecht, The Netherlands
Fludarabine	Hospital pharmacy, University of Ulm, Ulm
AKT-Inhibitor GSK 690693	GSK, London, England

2.4 Flow cytometry based cytotoxicity assay

CLL-cells were incubated with therapeutic antibody (10µg/ml) in complete medium with 30 % fresh frozen serum at a cell concentration of 10000 cells /µl. Assay was performed in duplicates. Isotype IgG-antibody and complete medium were used as untreated negative controls. This implies that the amount of apoptotic cells of negative control samples were regarded as spontaneously apoptotic and therefore subtracted of the amount of apoptotic cells of therapeutically treated samples. CLL-cells were stained with both 7-AAD and Annexin-V-PE prior to flow-cytometric measurement. Flow cytometric data were acquired with a FACS Calibur cytometer (BD Biosciences), operated by CellQuest software and 10000 events were collected per sample. Staining with FITC-labelled Anti-CD45-antibody (BD Biosciences) was performed to identify CLL-cells in co-incubation assays with HS5-cells. For these assays, HS5-cells were harvested and redistributed to 48-well-plates 24 hours prior to antibody-incubation at a density of 200cells/µl. Ratio of HS5-cells to CLL-cells was approximately 1 to 5.

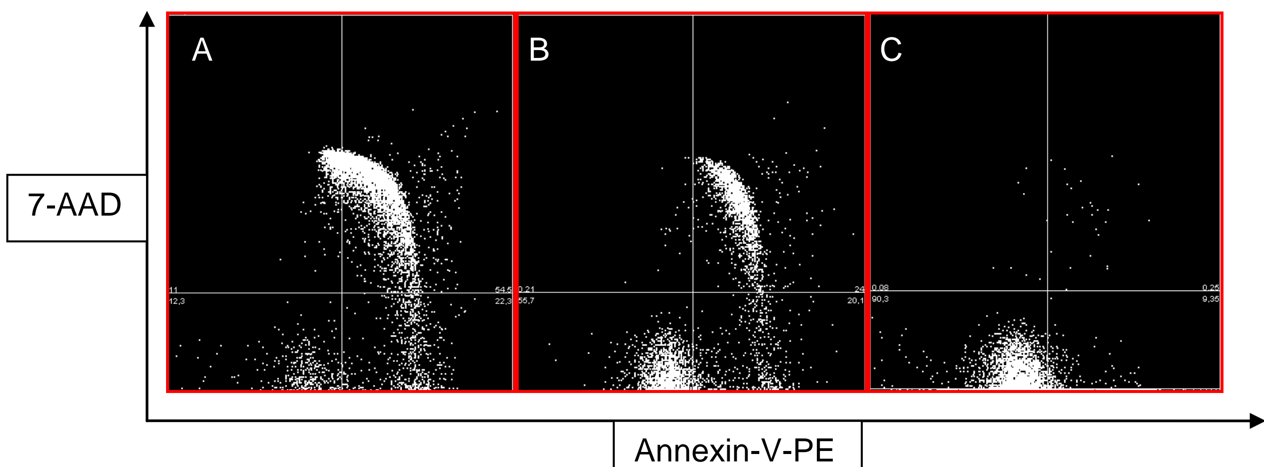


Figure 1 A-C:

Readout after 3 hours, example of cytometry dot plots. A: Alemtuzumab, B: Ofatumumab, C: isotype-antibody: There are three representative dot blots. The x-axis describes the extent of Annexin-V-PE (early cell death marker)-staining in a logarithmic scale, the y-Axis describes the extent of 7-AAD (7-Amino-Acridine, fluorescent dye, late cell death marker) -staining in a logarithmic scale – the dense clouds of white dots represent cells. A: In the case of Alemtuzumab a lot of cells are both positive for Annexin-V-PE and 7-AAD, B: in the case of Ofatumumab there are two major fractions: in the upper right square there is a cloud of cells which are positive for both Annexin-V-PE and 7-AAD and a cloud of cells in the lower left square, which are negative for both Annexin-V-PE and 7-

AAD, which means that there are both apoptotic and viable cells. C: without therapeutic antibody there is only a cloud in the lower left square detectable.)

Materials:	
RPMI 1640 Medium	Biochrom AG, Berlin
Human Serum FFP / off the clot	Sanquin Plasma Products, Amsterdam, The Netherlands
Sera Plus special processed FCS	PAN Biotech GmbH, Aidenbach
PBS Dulbeco w/o Ca, Mg	Biochrom AG, Berlin
Annexin V-PE	Becton Dickinson Biosciences, Heidel-berg
7-AminoactinomycinD	Sigma, Saint Louis, Missouri, USA
Rituximab	Roche, Hoffmann, La Roche, Grenzach-Whylen
BD Pharmigen Purified Mouse IgG1 K isotype control	Beckton Dickinson GmbH, Heidelberg
Annexin-binding-buffer	Self-production
HEPES Buffer Solution	PAA, Pasching, Austria
Calciumchlorid	Serva Feinbiochemica GmbH & Co., Heidelberg
Natriumchlorid AnalaR Normapur	VWR International Ltd., Leicestershire, England
Alemtuzumab	Bayer Schering Pharma AG, Berlin und Genzyme GmbH, Neu-Isenburg
Ofatumumab	Genmab, Utrecht, The Netherlands
HS5-cells	DSMZ, Braunschweig

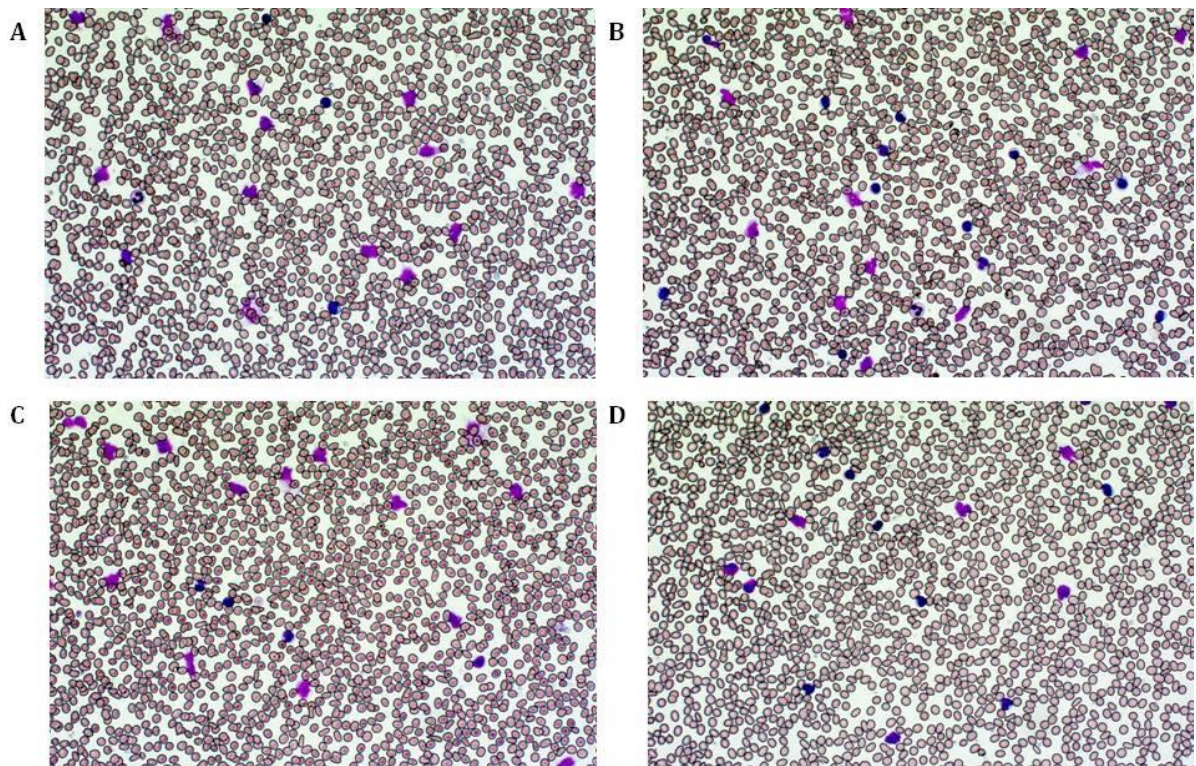
2.5 Whole blood assay (incubation)

Fresh blood from patients with high tumor load (mean CLL-cell concentration, assessed by routine diagnostic, at least 56.89 Giga/L (CD19+/CD5+), mean leukocyte count 73.68 Giga/L) in the blood compartment was directly incubated at room temperature with monoclonal antibodies at a concentration of 10µg/ml on a rotating table.

Materials:	
PBS Dulbecco w/o Ca, Mg	Biochrom AG, Berlin
BD Facs Lysing Solution	Becton Dickinson GmbH, Heidelberg
BD CD19 FITC (4G7)	Becton Dickinson GmbH, Heidelberg
BD Pharmigen Purified Mouse IgG1 K isotype control	Becton Dickinson GmbH, Heidelberg
Aqua Delta Select Spüllösung/ Irrigation Solution	Delta Select GmbH, München
Rituximab	Roche, Hoffmann, La Roche, Grenzach-Whylen
Alemtuzumab	Bayer Schering Pharma AG, Berlin und Genzyme GmbH, Neu-Isenburg
Ofatumumab	Genmab, Utrecht, The Netherlands
ACD (citrate buffer)	Self-production
Blue Heparinate standard syringe blue	Sarstedt , Nümbrecht
Lepirudine Refludan	Celgene, München

2.6 Microscopic readout to assess homotypic adhesion

After 1 hour incubation on a rotating table, blood samples (n=10) were delivered to the diagnostic laboratory of the hematologic department and blood smears were made according to clinical routine procedures. After Giemsa-staining, blood smears were semi-quantitatively assessed for homotypic adhesion. Five randomly picked high power fields were considered and evaluated in a blinded fashion.



A: Ofatumumab B: Rituximab C: Alemtuzumab D: untreated control

Figure 2:

Microscopic readout after 1 hour, 4 different representative high-power fields of the same CLL (chronic lymphocytic leukemia) patient (A-D) are shown: blue cells are mainly CLL cells. The rest of the cells are erythrocytes. No aggregation of CLL cells can be detected in any of the investigated antibodies.

2.7 Flow cytometric readout (True Count method)

This assays had the goal to assess the impact of high cell count on effector consumption as well as the potential contribution of e.g. ADCC under in vivo conditions with special regard to CLL cell lysis. Therefore we chose the BD Biosciences TrueCount cytometric method. After 3 hours of incubation, CD19-FITC stained cells were counted using a bead-based FACS-measurement and cell to bead ratio was compared with 6 negative controls. The assay was performed in triplicates and according to manufacturer's protocol (BD Biosciences) except for the amount of blood used for measurement: in order to obtain a comparable B-cell to bead ratio, a maximum of 10 µl of blood were used instead of 50 µl due to high B-cell-number in CLL patient blood. In order to eliminate possible systematic errors caused by the use of a specific anticoagulant (Heparine interacts with several serum-components including complement factors), whole blood assays were performed with three different anticoagulants: Heparine [n=6], Lepirudine [n=3], and Citrate [n=13]. The incubations of the assays with Lepirudine-anticoagulation were made at the same culture conditions as the other assays (37°C, humidified, 5%-CO_2 incubator).

Materials:	
BD TruCount Tubes	Becton Dickinson GmbH, Heidelberg
ART Aerosol Resistant Tips	Thermo Fisher Scientific-Molecular Bio Products, San Diego, USA
48 Well Cell Culture Plate Cellstar	Greiner Bio-One GmbH, Frickenhausen
Derma Clean Latex gloves	Ansell GmbH, München
Falcon tubes	Falcon, Becton Dickinson, Franklin Lakes, USA
Falcon Serological Pipet	Becton Dickinson GmbH, Heidelberg
Combitips plus/ Spitze Multistep-Pipette	Eppendorf Deutschland GmbH, Wesseling-Berzdorf
Devices:	
BD FACSCalibur/ flow cytometer	Becton Dickinson Bioscience, Heidelberg
Reax 2000	Heidolph Instruments GmbH & Co.KG, Schwabach
GFL-3005 vortex machine	GFL Gesellschaft für Labortechnik GmbH, Burgwedel
Multipette stream	Eppendorf Deutschland GmbH, Wesseling-Berzdorf
SterilGard3 Advance	Baker Company, Sanford, Maine, USA
Julabo SW 20 heater	Julabo Labortechnik GmbH, Seelbach

Coulter Ac T 5diff AL	Beckman Coulter GmbH, Krefeld
Pipetboy acu	INTEGRA Biosciences GmbH, Fern-wald
Eppendorf Research Pipettes	Eppendorf Deutschland GmbH, Wes-seling-Berzdorf
Gilson Pipettes	Gilson Inc., Middleton, USA

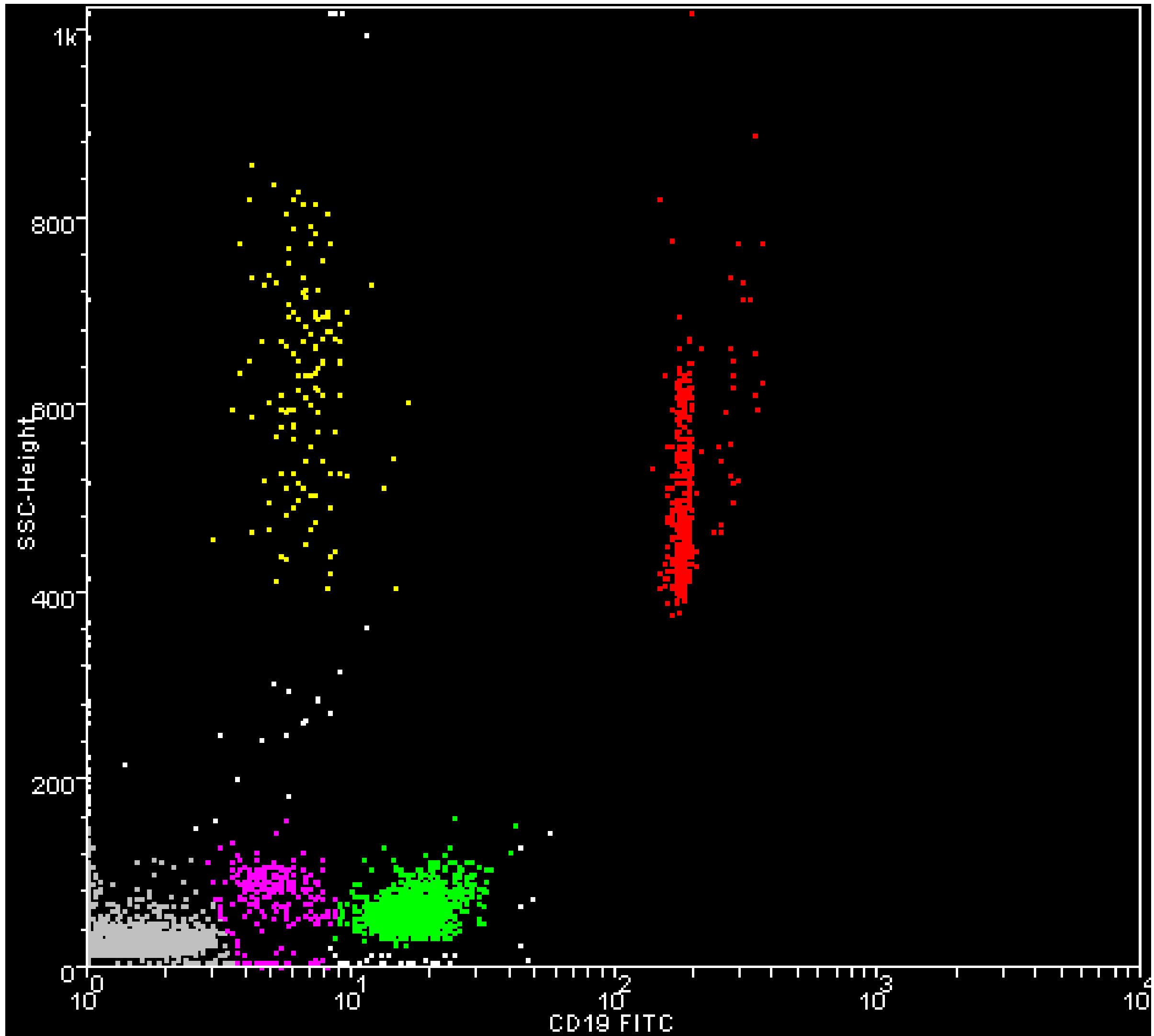


Figure 3:

Readout after 3 hours to assess both the potential cell lysis and the impact of high cell density on CDC (complement dependent cytotoxicity). This CD19-FITC (B-cell-marker, x-axis) / SSC (side-ward scatter, y-axis) dot-plot shows an untreated control: red dots represent beads, lilac dots rep-resent CD19-negative cells (monocytes, T-lymphocytes), green dots represent C19-positive cells (CLL-cells and B-cells), yellow dots represent CD19-negative cells with increased side scattering due to intracellular granula (granulocytes).

Patient samples

Table 1:

CLL (chronic lymphocytic leukemia) samples (taken of CLL patients treated in Ulm during the period of 2000 and 2010, certain prognostic parameters of the CLL cells and clinical data of the patients) used for: Chemo-luminescence-based viability assay, read-out after 3 and 48 hours: The list contains 10 high-risk CLL-cases (they are specified by at least one of the three following characteristics: 17p (short arm of chromosome 17)-deletion, TP53 (encodes for p53)-mutation, Fludarabine-refractory). The *TP53* (tumor suppressor protein 53) -status is a prognostic marker for survival and response to conventional chemotherapy schemes of CLL, the ZAP-70-protein is associated with either long or short survival of CLL patients. FISH (fluorescence in-situ hybridization) is used to further distinguish between CLL subgroups. The IGHV (variable part of the immune globuline heavy chain)-status is another marker, which is associated with survival and response to treatment.

no.	gender	FISH	*TP53*	*IGHV*	ZAP-70	pre-treatment	age
1	F	13q-	WT	M	Negative	yes	64
2	M	11q-, 12q+, t14q32	WT	UM	Positive	no	65
3	F	17p-, 12q+	WT	UM	n/d	yes	87
4	F	17p-14q-	c.584T>C	UM	Positive	yes	65
5	M	11q-, 13q-	c.919-7A>C	UM	n/d	yes	77
6	M	17p, 12q+	WT	n/d	n/d	yes	82
7	M	13q-	WT	M	Negative	no	50
8	M	17p-, 11q-, 13q-	c.499C>T, c.830G>T	UM	n/d	yes	72
9	M	13q-, 11q-	WT	M	Negative	no	49
10	F	13q-	c.847C>T	M	Negative	no	73
11	M	13q-, 11q-	WT	UM	n/d	no	69
12	M	13q-, 11q-	WT	UM	Negative	no	37
13	F	13q-	WT	M	Negative	yes	64
14	F	normal	WT	M	Negative	no	65
15	M	13q-	WT	M	n/d	no	72
16	M	13q-	WT	UM	Positive	no	70
17	M	13q-	WT	UM	Positive	no	69
18	M	normal	WT	UM	Positive	no	71
19	M	13q bidel	WT	M	Negative	no	61
20	M	17p-, 13q-	753-759del7bp	UM	n/d	yes	58
21	M	11q-	WT	UM	Positive	yes	69
22	M	17p-, 12q+, 13q-	WT	UM	n/d	no	68
23	F	normal	n/d	M	n/d	yes	65
24	F	normal	WT	UM	Positive	yes	74
25	F	17p-,13q-,12q+	c.809T>G	UM	n/d	no	73
26	M	17p-, 11q-, 13q-	c.329G>T	UM	Negative	no	69
27	F	normal	WT	UM	Positive	no	38
	10 fema- le 17 male	8 with 17p del	7 with *TP53*- mutation	17 with un- mutated *IGHV*-status		11 with prior treatment	65,78

Table 2:

CLL (chronic lymphocytic leukemia) samples (taken of CLL patients treated in Ulm during the period of 2000 and 2010, certain prognostic parameters of the CLL cells and clinical data of the patients) used for Chemo-luminescence-based viability assay, read-out after 48 hours, combination with Fludarabine: The list contains 11 high-risk CLL-cases (they are specified by at least one of the three following characteristics: 17p (short arm of chromosome 17)-deletion, TP53 (encodes for p53)-mutation, Fludarabine-refractory). The *TP53* (tumor suppressor protein 53) -status is a prognostic marker for survival and response to conventional chemotherapy schemes of CLL, the ZAP-70-protein is associated with either long or short survival of CLL patients. FISH (fluorescence in-situ hybridization) is used to further distinguish between CLL subgroups. The IGHV (variable part of the immune globuline heavy chain)-status is another marker, which is associated with survival and response to treatment.

no.	**gender**	**FISH**	***TP53***	***IGHV***	**ZAP-70**	**pre-treatment**	**age**
6	M	17p, 12q+	WT	n/d	n/d	yes	82
7	M	13q-	WT	M	Negative	no	50
8	M	17p-, 11q-, 13q-	c.499C>T, c.830G>T	UM	n/d	yes	72
9	M	13q-, 11q-	WT	M	Negative	no	49
10	F	13q-	c.847C>T	M	Negative	no	73
11	M	13q-, 11q-	WT	UM	n/d	no	69
12	M	13q-, 11q-	WT	UM	Negative	no	37
13	F	13q-	WT	M	Negative	yes	64
14	F	normal	WT	M	Negative	no	65
15	M	13q-	WT	M	n/d	no	72
16	M	13q-	WT	UM	Positive	no	70
17	M	13q-	WT	UM	Positive	no	69
18	M	normal	WT	UM	Positive	no	71
28	M	12q+, t(14:18)	WT	M	Negative	no	81
29	F	17p	c.842A>G	UM	Positive	yes	42
30	M	17p	c.602-603Ins4bp	UM	Negative	yo	69
31	M	17p	WT	UM	n/d	no	73
32	F	normal	c.484A>T	UM	Positive	yes	64
33	M	13q	WT	UM	n/d	no	66
34	M	13q	WT	UM	Negative	yes	77
35	M	normal	WT	UM	Negative	yes	72
36	F	Tris.12, 17p	c.673-2A>T	M	Negative	yes	73
37	M	17p, 13q	c.413C>T	M	Negative	yes	72
38	F	13q	n/d	M	n/d	no	59
39	F	17p, 13q	C.733G>T	UM	Negative	yes	76
	8 female 17 male	8 with 17p del	8 with *TP53*-mutation	14 with un-mutated *IGHV*-status		10 with prior treatment	66,68

Table 3:

This table summarizes the CLL (chronic lymphocytic leukemia) samples (taken of CLL patients treated in Ulm during the period of 2000 and 2010, certain prognostic parameters of the CLL cells and clinical data of the patients) used for: Chemo-luminescence-based viability assay, read-out after 48 hours, combination with either only the AKT (Protein Kinase B)-inhibitor or with both the AKT-inhibitor and Alemtuzumab (these patient sample numbers have a footnote "*"):

The list contains 11 high-risk CLL-cases (they are specified by at least one of the three following characteristics: 17p (short arm of chromosome 17)-deletion, TP53 (encodes for p53)-mutation, Fludarabine-refractory). The Alemtuzumab-cohort consists of 7 high risk CLL-cases and 5 low-risk CLL cases, patient characteristics of patient no.15 is not on the list, as the sample taken from that patient hasn't been investigated in the AKT-cohort , the last row summarizes the table columns. The *TP53* (tumor suppressor protein 53) -status is a prognostic marker for survival and response to conventional chemotherapy schemes of CLL, the ZAP-70-protein is associated with either long or short survival of CLL patients. FISH (fluorescence in-situ hybridization) is used to further distinguish between CLL subgroups. The IGHV (variable part of the immune globuline heavy chain)-status is another marker, which is associated with survival and response to treatment.

no.	gender	FISH	*TP53*	*IGHV*	ZAP-70	pre-treatment	age
6*	M	17p, 12q+	WT	UM	n/d	yes	82
7*	M	13q-	WT	M	Negative	no	50
25	F	17p-,13q-,12q+	c.809T>G	UM	n/d	no	73
27	F	normal	WT	UM	Positive	no	38
26	M	17p-, 11q-, 13q-	c.329G>T	UM	Negative	no	69
28	M	12q+, t(14:18)	WT	M	Negative	no	81
29*	F	17p	c.842A>G	UM	Positive	yes	42
30*	M	17p	c.602-603Ins4bp	UM	Negative	no	69
31*	M	17p	WT	UM	n/d	no	73
32*	F	normal	c.484A>T	UM	Positive	yes	64
33*	M	13q	WT	UM	n/d	no	66
34*	M	13q	WT	UM	Negative	yes	77
35	M	normal	WT	UM	Negative	yes	72
36	F	Tris.12, 17p	c.673-2A>T	M	Negative	yes	73
37*	M	17p, 13q	c.413C>T	M	Negative	yes	72
38*	F	13q	n/d	M	n/d	no	59
39*	F	17p, 13q	C.733G>T	UM	Negative	yes	76
40	M	17p, 13q, 6q	n/d	UM	n/d	yes	66
	7 female 11 male	10 with 17p del	8 with *TP53*-mutation	12 with un-mutated *IGHV*-status		9 with prior treatment	66,24

Table 4:

CLL (chronic lymphocytic leukemia) samples (taken of CLL patients treated in Ulm during the period of 2000 and 2010, certain prognostic parameters of the CLL cells and clinical data of the patients) used for: Whole blood assay, microscopic read-out after 1 h: only in 10 randomly picked patients of the following list we did microscopic assessment of homotypic adhesion, cytometric read-out after 3 and 8 hours. The list contains 6 high-risk CLL-cases (they are specified by at least one of the three following characteristics: 17p (short arm of chromosome 17)-deletion, TP53 (encodes for p53)-mutation, Fludarabine-refractory). The *TP53* (tumor suppressor protein 53) -status is a prognostic marker for survival and response to conventional chemotherapy schemes of CLL, the ZAP-70-protein is associated with either long or short survival of CLL patients. FISH (fluorescence in-situ hybridization) is used to further distinguish between CLL subgroups. The IGHV (variable part of the immune globuline heavy chain)-status is another marker, which is associated with survival and response to treatment.

no.	gender	FISH	*TP53*	*IGHV*	ZAP-70	pre-treatment	leu-kocyte count	age
1	F	13q-	WT	M	Negative	yes	239,8	64
4	F	17p-14q-	c.584T>C	UM	Positive	yes	132,7	65
7	M	13q-	WT	M	Negative	no	102,5	49
11	M	13q-, 11q-	WT	UM	n/d	no	71,9	69
12	M	13q-, 11q-	WT	UM	Negative	no	105,7	36
14	F	normal	WT	M	Negative	no	27,6	65
18	M	normal	WT	UM	Positive	no	95,1	48
20	M	17p-	753-759 del7bp	UM	Positive	yes	46,2	59
27	F	normal	WT	UM	Positive	no	98,8	37
31	M	17p-	WT	UM	Negative	no	47,6	68
34	M	13q	WT	UM	n/d	no	111,4	67
37	F	Tris.12, 17p-	c.673-2A>T	M	Negative	yes	28,4	73
40	F	17p, 13q	n/d	UM	n/d	yes	74,2	76
42	M	normal	WT	M	n/d	no	47,8	39
43	M	t14q32	WT	UM	Negative	yes	59,8	72
44	M	13q-	WT	UM	n/d	yes	61,8	67
45	M	11q-, 13q-, 14q-	WT	UM	Negative	no	141,3	73
46	M	12p+11, t(14;19)	WT	UM	Positive	yes	26,6	49
47	M	13q-	c:716A>6	M	n/d	yes	11,7	76
48	M	13q	WT	M	n/d	no	84,2	57
49	M	11q23	WT	UM	Negative	yes	10,8	58
	6 female 15 male	5 with 17p del	4 with *TP53*-mutation	14 with unmutated *IgHV*-status		10 with prior treatment	77,42	60,3

Table 5:

This table summarizes the CLL (chronic lymphocytic leukemia) samples (taken of CLL patients treated in Ulm during the period of 2000 and 2010, certain prognostic parameters of the CLL cells and clinical data of the patients) used for: Cytometry-based Apoptosis assay / HS5-coculture-assay (*), read-out after 3 hour incubation. The list contains 2 high-risk CLL-cases (they are specified by at least one of the three following characteristics: 17p (short arm of chromosome 17)-deletion, TP53 (encodes for p53)-mutation, Fludarabine-refractory). The *TP53* (tumor suppressor protein 53) -status is a prognostic marker for survival and response to conventional chemotherapy schemes of CLL, the ZAP-70-protein is associated with either long or short survival of CLL patients. FISH (fluorescence in-situ hybridization) is used to further distinguish between CLL subgroups. The IGHV (variable part of the immune globuline heavy chain)-status is another marker, which is associated with survival and response to treatment.

no.	Gender	FISH	*TP53*	*IGHV*	ZAP-70	pre-treatment	age
7*	M	13q-	WT	M	Negative	no	50
13*	F	13q-	WT	M	Negative	yes	64
14*	F	Normal	WT	M	Negative	no	65
16*	M	13q-	WT	UM	Positive	no	70
17*	M	13q-	WT	UM	Positive	no	69
18*	M	Normal	WT	UM	Positive	no	71
20	M	17p-, 13q-	753-759del7bp	UM	n/d	yes	58
21	M	11q-	WT	UM	Positive	yes	69
35	M	Norm	WT	UM	Negative	yes	72
39	F	17p, 13q	C.733G>T	UM	Negative	yes	76
41	F	Norm	WT	M	n/d	no	75
	4 female 7 male	2 with 17p del	2 with *TP53*mutation	7 with unmutated *IgHV*-status		5 with prior treatment	67,18

3 Results

3.1 Assay No. 1: Comparison of CLL-cell-viability after 3 and 48 hour mAb-treatment

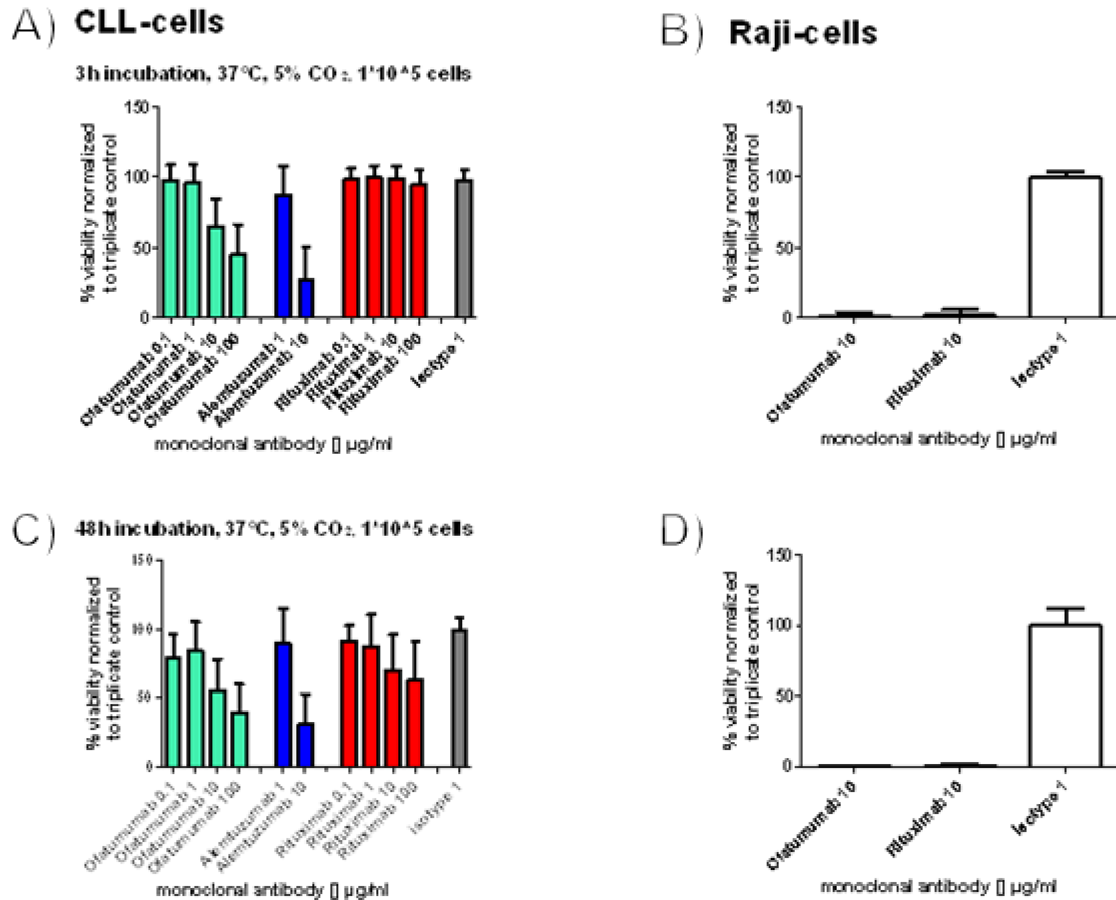


Figure 4 A – D:

Comparison of CLL (chronic lymphocytic leukemia) cell viability after a 3 and a 48 hour treatment. A: viability of CLL cells after a 3 hour incubation with either Ofatumumab (green), Alemtuzumab (blue), Rituximab (red), or isotype antibody (grey) at different concentrations. B: viability of Raji cells after a 3 hour incubation with either Alemtuzumab (first bar), Rituximab (second bar), or isotype antibody (right bar). C: viability of CLL cells after a 48 hour incubation with either Ofatumumab (green), Alemtuzumab (blue), Rituximab (red), or isotype antibody (grey) at different concentrations. D: viability of Raji cells after a 48 hour incubation with either Alemtuzumab (first bar), Rituximab (second bar), or isotype antibody (right bar).

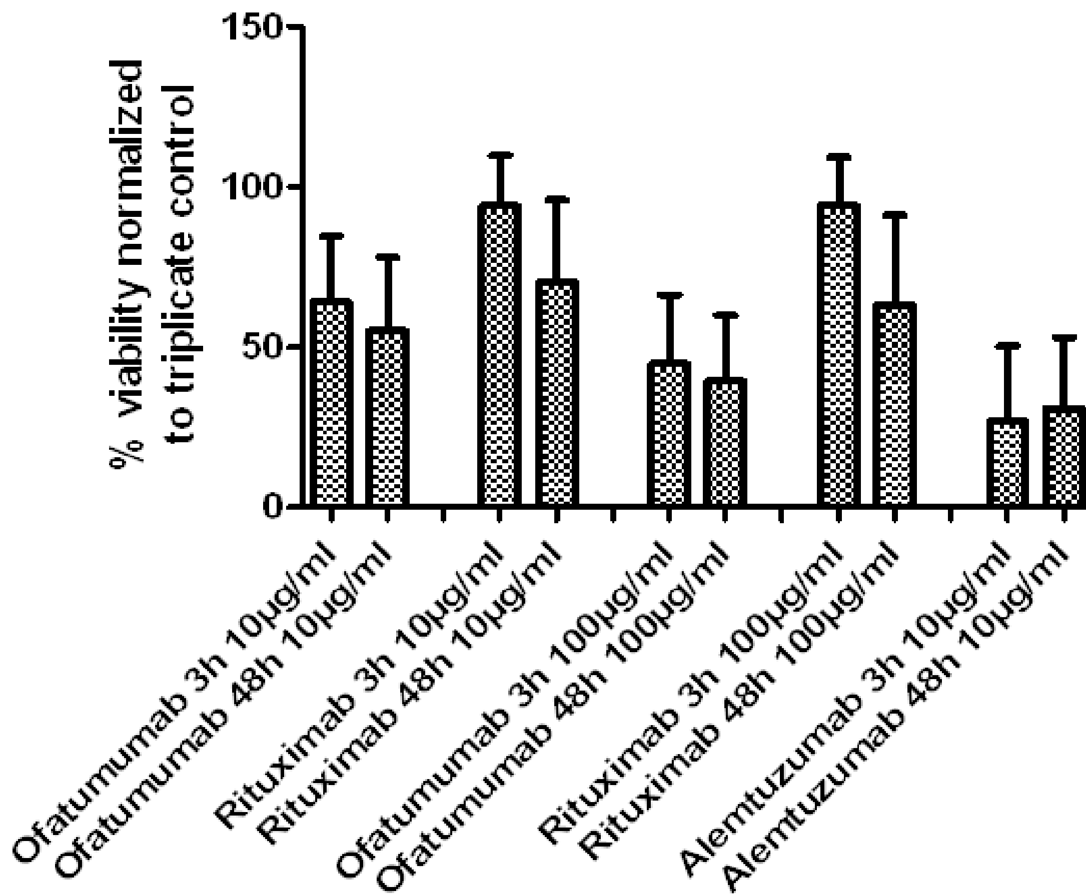


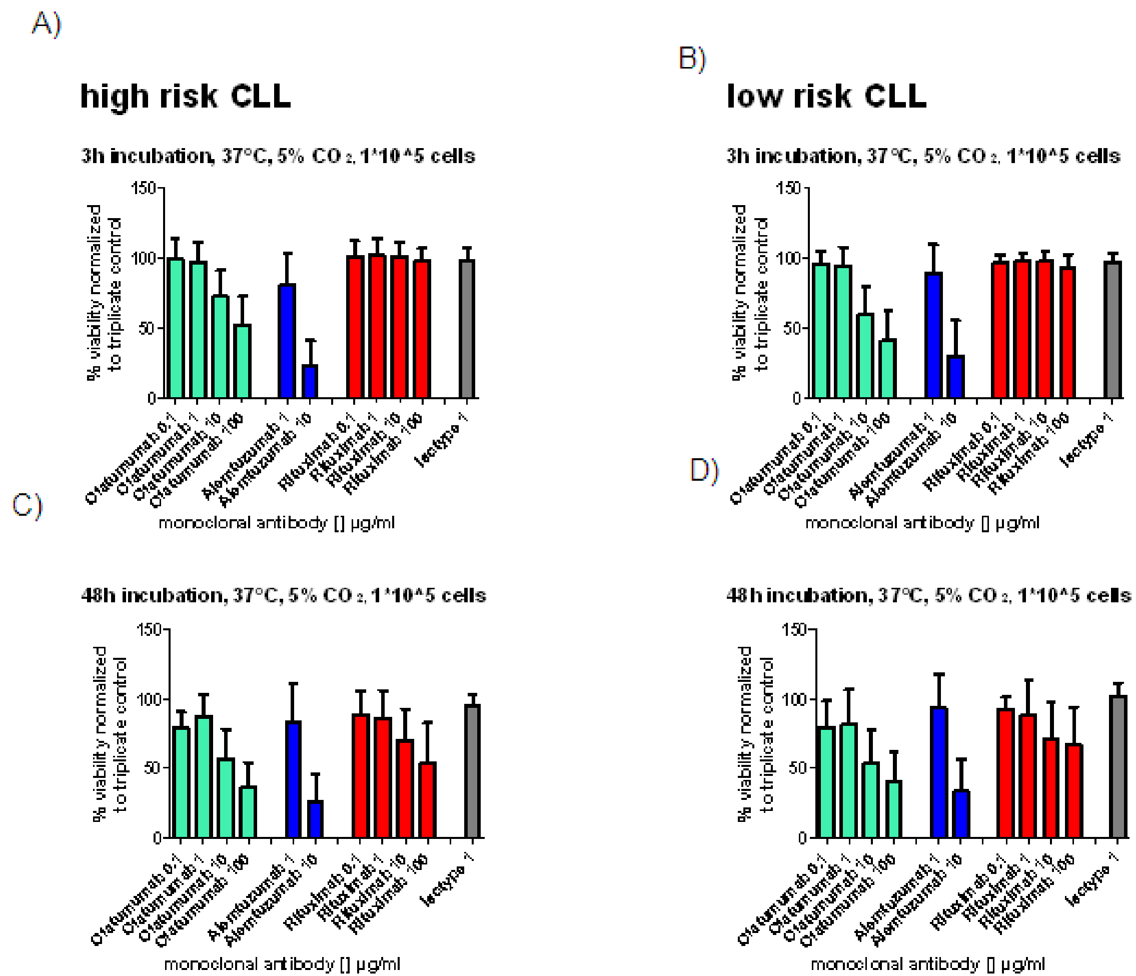
Figure 5:

viability bars of therapeutic antibodies at different concentrations and after different incubation times (3 and 48 hours).

Assay No. 1: Viability measured after 3 and 48 hour incubation represents the means and SD of 27 and 26 experiments, respectively (figure 4 A, C). Both Ofatumumab and Alemtuzumab, but not Rituximab deplete CLL-cells after 3 hours of incubation in the presence of 30% fresh frozen serum (figure 4 A). Mean viability of CLL cells for Ofatumumab is 64.11 % (range: 6.46% - 96.5%) at 10µg/ml and 44.7% (range: 3.83% - 87.3%) at 100µg/ml concentration. Alemtuzumab reduces viability to 26.99% (range: 4.18% - 74.24%) at 10µg/ml. Both Ofatumumab and Rituximab nearly completely deplete Raji-cells after 3 hours of incubation (figure 4 B). Raji-cell-depletion is used to guarantee serum-quality qualitatively (figure 4 B/D). After 48 hours of incubation Ofatumumab, Alemtuzumab, and Rituximab show activity to a different extent. Rituximab leads to a reduction of viability to 72.97% (range: 12.76% – 103.9%) and 63.14% (range: 3.885% - 110.9%) at 10 and 100µg/ml, respectively. Ofatumumab reduces viability to 57.06% (range:

7.79% - 87.82%) and 39.36% (range: 5.28% - 76.5%) at 10 and 100µg/ml viability. Alemtuzumab induces a decrease of viability to 30.65% (range: 4.34% - 77.45%) at 10µg/ml. Comparing viability after 3 and 48 hour incubation shows a delayed effect of the activity of Rituximab in relation to Ofatumumab and Alemtuzumab. Both Rituximab and Ofatumumab show greater reduction of viability after 48 hour incubation compared to 3 hour incubation. (figure 5) Alemtuzumab doesn’t show an increased effect after prolonged incubation.

3.2 Assay No. 2: Comparison of CLL-cell-viability after 3 and 48 hour mab treatment with special regard to genetic CLL-subgroups (high-risk CLL vs. low-risk CLL)



Figures 6 A – D:

A: viability of CLL (chronic lymphocytic leukemia) cells after a 3 hour incubation with either Ofatumumab (green), Alemtuzumab (blue), Rituximab (red), or isotype antibody (grey) at different concentrations. B: viability of Raji cells after a 3 incubation with either Alemtuzumab (first bar), Rituximab (second bar), or isotype antibody (right bar). C: viability of CLL cells after a 48 hour incubation with either Ofatumumab (green), Alemtuzumab (blue), Rituximab (red), or isotype antibody (grey) at different concentrations. D: viability of Raji cells after a 48 hour incubation with either Alemtuzumab (first bar), Rituximab (second bar), or isotype antibody (right bar).

Assay No. 2: Dividing results into subgroups according to clinically relevant risk stratification (see methods) reveals no significant difference of the cytotoxic activity between 10 high-risk and 16 low-risk CLL cases for neither Ofatumumab (p-value: 0.79), nor Rituximab (p-value: 0.97), nor Alemtuzumab (p-value: 0.44) after 48 hours. (figure 6 A-D). CLL cells seem to respond similarly to each of the investigated antibodies, irrespective of genetic background/responsiveness to Fludarabine, which was used to subdivide our samples in accordance with up to date clinical guidelines into different risk-stratified groups.

3.3 Assay No. 3: Comparison of CLL-cell-viability after 3 and 48 hour mab-treatment with special regard to prior Rituximab-treatment of CLL patients (Rituximab-pretreated CLL vs. Rituximab-untreated CLL)

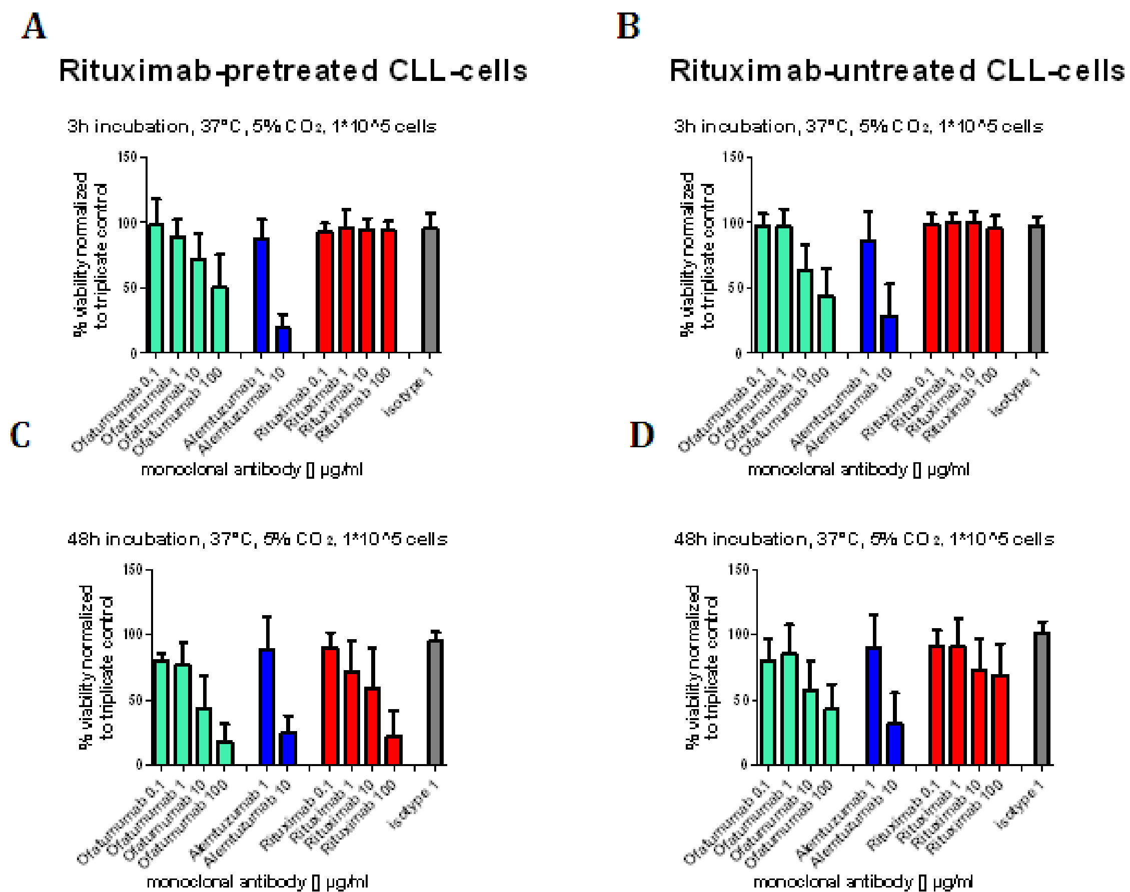


Figure 7 A – D:

A: viability of CLL (chronic lymphocytic leukemia) cells after a 3 hour incubation with either Ofatumumab (green), Alemtuzumab (blue), Rituximab (red), or isotype antibody (grey) at different concentrations. B: viability of Raji cells after a 3 hour incubation with either Alemtuzumab (first bar), Rituximab (second bar), and isotype antibody (right bar). C: viability of CLL cells after a 48 hour incubation with either Ofatumumab (green), Alemtuzumab (blue), Rituximab (red), and isotype antibody (grey) at different concentrations. D: viability of Raji cells after a 48 hour incubation with either Alemtuzumab (first bar), Rituximab (second bar), or isotype antibody (right bar).

Assay no. 3: 4 of the 27 CLL patient samples used were obtained from Rituximab-treated patients. Comparing the viability of this subgroup with the viability of CLL samples, which were obtained of patients who were previously not treated with Rituximab, no different activity of Ofatumumab, Rituximab, and Alemtuzumab can be observed after 48 hours of incubation. However, the group of Rituximab-pretreated CLL samples is too small to obtain definite conclusions.

3.4 Assay No. 4: Comparison of CLL-cell-viability after 48 hour mAb-only, Fludarabine-only, and combined mAb/Fludarabine (high-risk CLL vs. low-risk CLL)

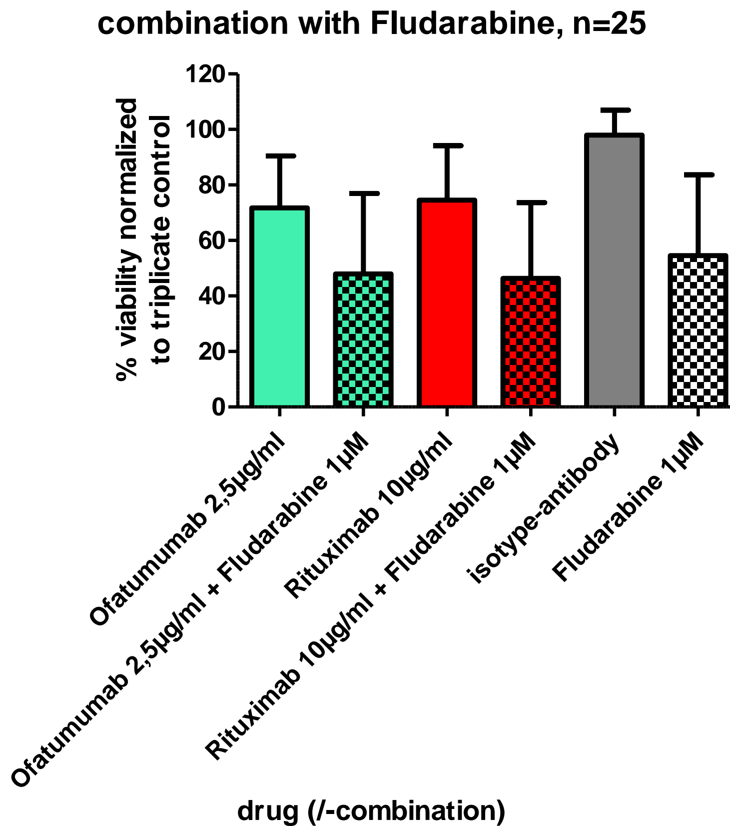


Figure 8:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st bar), Ofatumumab and Fludarabine (2nd bar), Rituximab (3rd bar), Rituximab and Fludarabine (4th bar), isotype-antibody (5th bar) and Fludarabine (6th bar).

Assay No.4: To assess potential synergistic or additive effects, CLL cells were treated with single-antibody at a low concentration, Fludarabine (1µM), or with a combination of both the therapeutic antibody and Fludarabine (figure 8). As mentioned above, Ofatumumab outperforms Rituximab as a single agent by eliciting more immediate cell death (probably via CDC). Four times lower dosed Ofatumumab (mean viability: 47.99%, range: 11.86% - 102.7%, n=25) in combination with 1µM Fludarabine shows equal activity (p-value: 0.84) as the Rituximab-Fludarabine combination (mean: 46.4%, range: 15.13% - 116.0%, n=25). We admit that our assay is a highly simplified in vitro model for the assessment of combined activity.

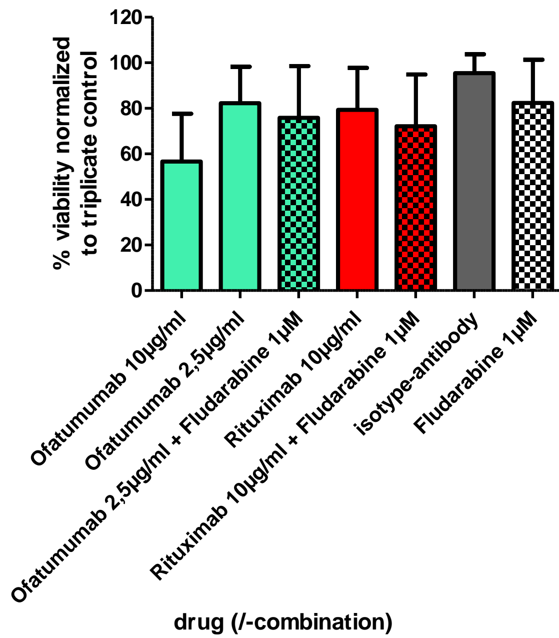


Figure 9:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st and 2nd bar), Ofatumumab and Fludarabine (3rd bar), Rituximab (4th bar), Rituximab and Fludarabine (5th bar), isotype-antibody (6th bar) and Fludarabine (7th bar).

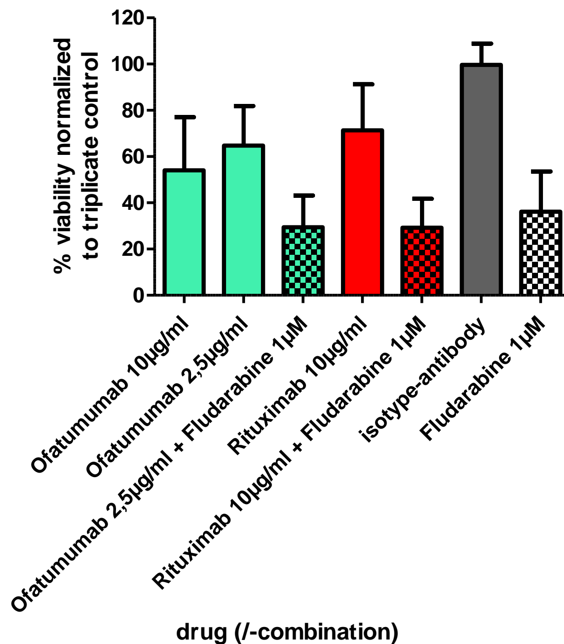


Figure 10:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st and 2nd bar), Ofatumumab and Fludarabine (3rd bar), Rituximab (4th bar), Rituximab and Fludarabine (5th bar), isotype-antibody (6th bar) and Fludarabine (7th bar).

As expected Fludarabine showed almost no activity in high-risk CLL cases and potentially additive or synergistic activity with either Ofatumumab or Rituximab was therefore absent. Single Ofatumumab at a concentration of 10µg/ml was used as an internal control to guarantee the validity of the assay. (figure 9)
Regarding low-risk CLL cases only, the observed antibody-activity was comparable to the observations without clinical risk-stratification and 1µM Fludarabine lead to a considerable reduction of CLL cells (mean viability: 36.1%, range: 13.67% - 64.83%). Four times lower dosed Ofatumumab in combination with 1µM Fludarabine showed comparable activity (p-value: 0.9739) as Rituximab in combination with 1 µM Fludarabine after 48 hour incubation (mean viability Ofa.-F: 29.42%, range 11.86% – 50.83% and Rit.-F: 29.26%, range 15.13% – 57.03%, n=15). (figure 10)

3.5 Assay No. 5: Comparison of CLL-cell-viability after 48 hour Ofatumumab, AKT-inhibitor GSK 690693, and combined Ofatumumab/AKT-inhibitor GSK 690693-treatment

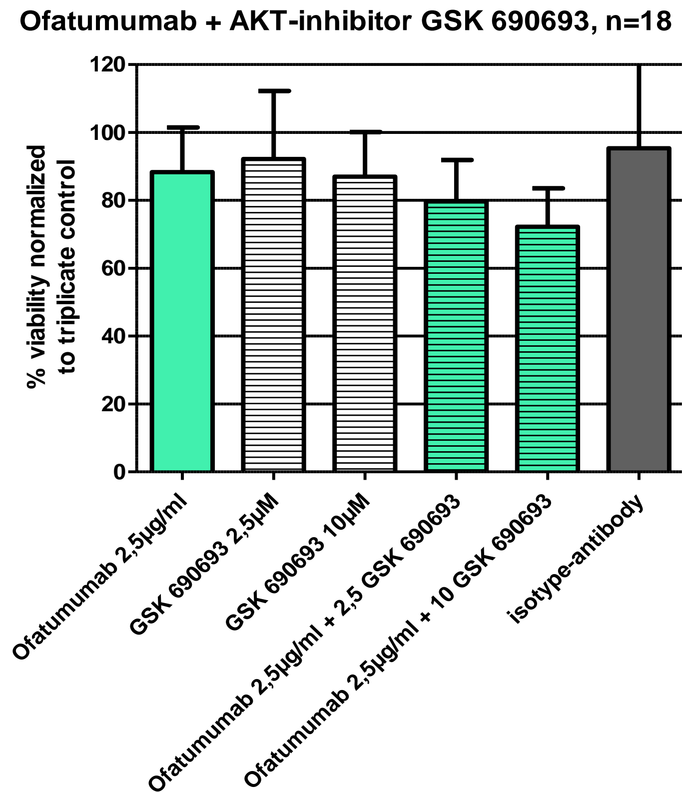


Figure 11:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st bar), AKT (Protein Kinase B)-inhibitor (2nd and 3rd bar), Ofatumumab and AKT-inhibitor (4th and 5th bar), and isotype-antibody (6th bar).

Assay No. 5: To assess potential synergistic or additive effects, CLL cells were treated with single-Ofatumumab, single-AKT-inhibitor GSK 690693, and with a combination of the two. (figure 11)

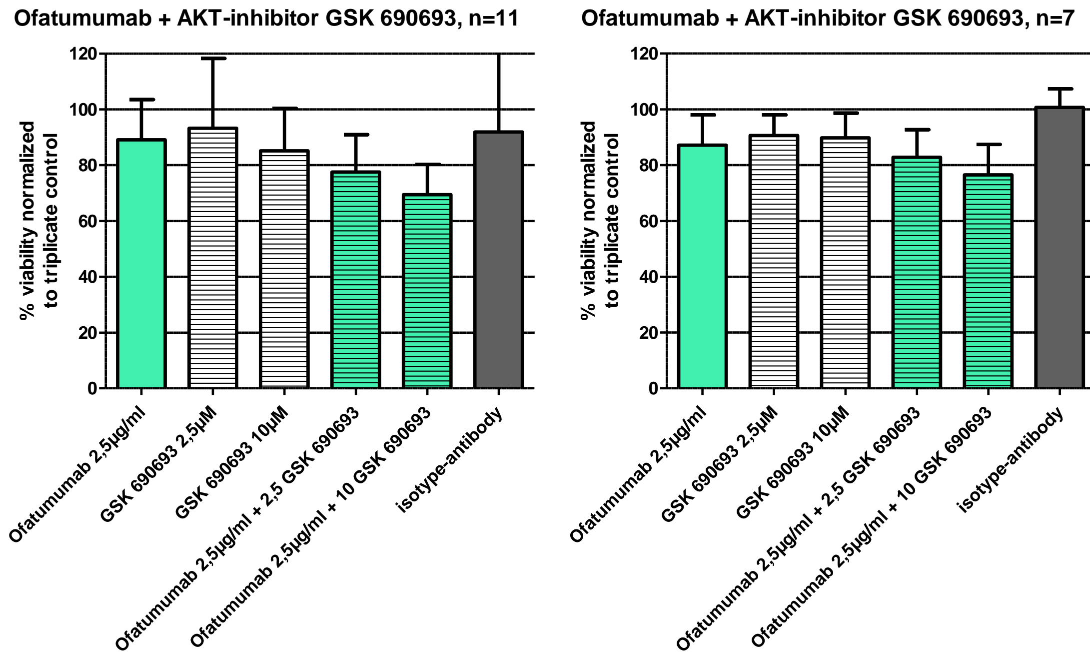


Figure 12:

viability of high- and low-risk CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st bar), AKT (Protein Kinase B)-inhibitor (2nd and 3rd bar), Ofatumumab and AKT-inhibitor (4th and 5th bar), and isotype-antibody (6th bar).

Viability reduction by the combination of Ofatumumab and AKT-inhibitor GSK 690693 was highest after 48 hours (mean viability: 71.75%, range: 45.99% – 88.72%, n=18). The two drugs seem to have at least an additive effect. To further examine the susceptibility of different CLL risk groups, we subdivided results again according to genetic risk stratification. The Ofatumumab/AKT-inhibitor-combination reduced the viability to a higher extent, though not statistically significant, within the high-risk CLL-cohort (p-value: 0.14) compared to the low-risk CLL cohort. (figure 12)

3.6 Assay No. 6: Comparison of CLL-cell-viability after 48 hour incubation with either CD20-antibody-only, Alemtuzumab-only, or combined CD20-antibody/Alemtuzumab treatment (high-risk CLL vs. low-risk CLL)

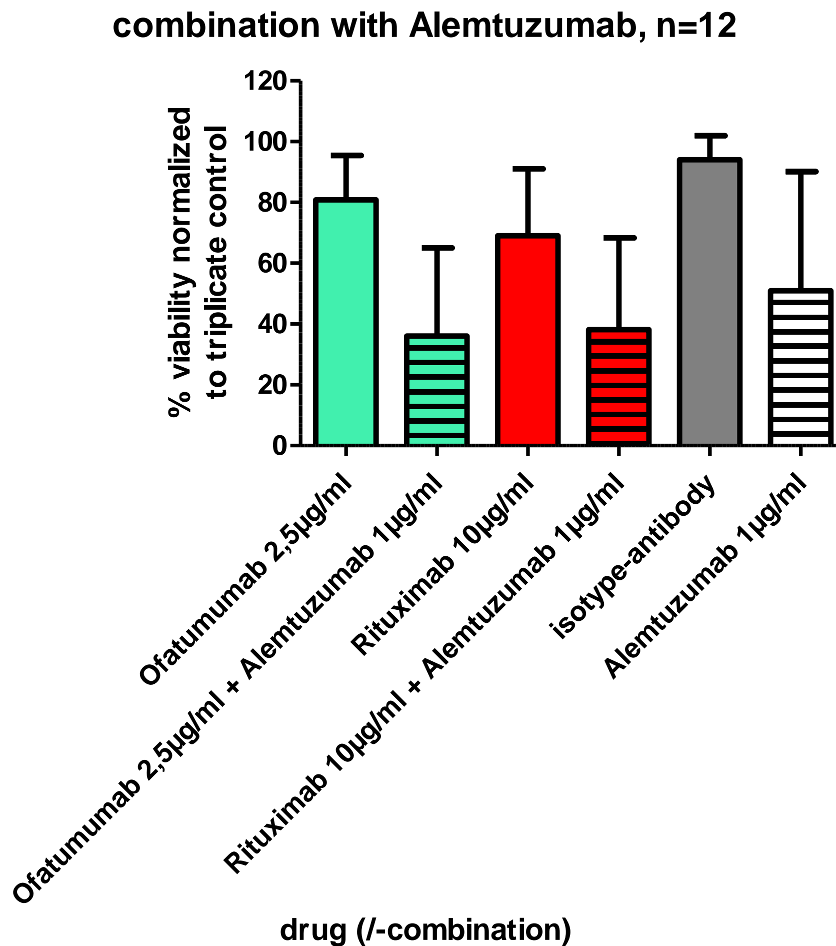


Figure 13:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st bar), Ofatumumab and Alemtuzumab (2nd bar), Rituximab (3rd bar), Rituximab and Alemtuzumab (4th bar), isotype antibody (5th bar) and Alemtuzumab (6th bar).

Assay No. 6: This analysis had the goal to measure potentially additive effects between CD20-antibodies and Alemtuzumab. As Rituximab didn't show its maximum activity until after an incubation of 48 hours, we chose this time-point for measurement. Again, the in vitro assay can only partially mimic the in vivo situation as several effector mechanisms, which could play a relevant role in vivo, are neglected in our model. For example, with regard to pharmakokinetics, much higher antibody concentrations of CD20-antibodies can be achieved and well-tolerated in vivo. CLL samples were treated with either Ofatumumab (2.5µg/m), with Rituximab

(10µg/ml), with single Alemtuzumab (2.5µg/ml), or with a combination of each of the CD20 antibodies and Alemtuzumab, respectively (figure 13). A minor reduction of viability was assessed for single Ofatumumab (2.5µg/ml) compared to single Rituximab (10µg/ml) in this cohort (mean viability Ofatumumab: 80.84%, range: 68.56% - 110.3%; mean viability Rituximab: 69.08%, range: 42.27% - 105.8%). However, Ofatumumab (2.5µg/ml) combined with Alemtuzumab (2.5µg/ml) showed equal mean viability after 48 hour incubation (p-value: 0.8655) compared to Rituximab (10µg/ml) combined with Alemtuzumab (2.5µg/ml).

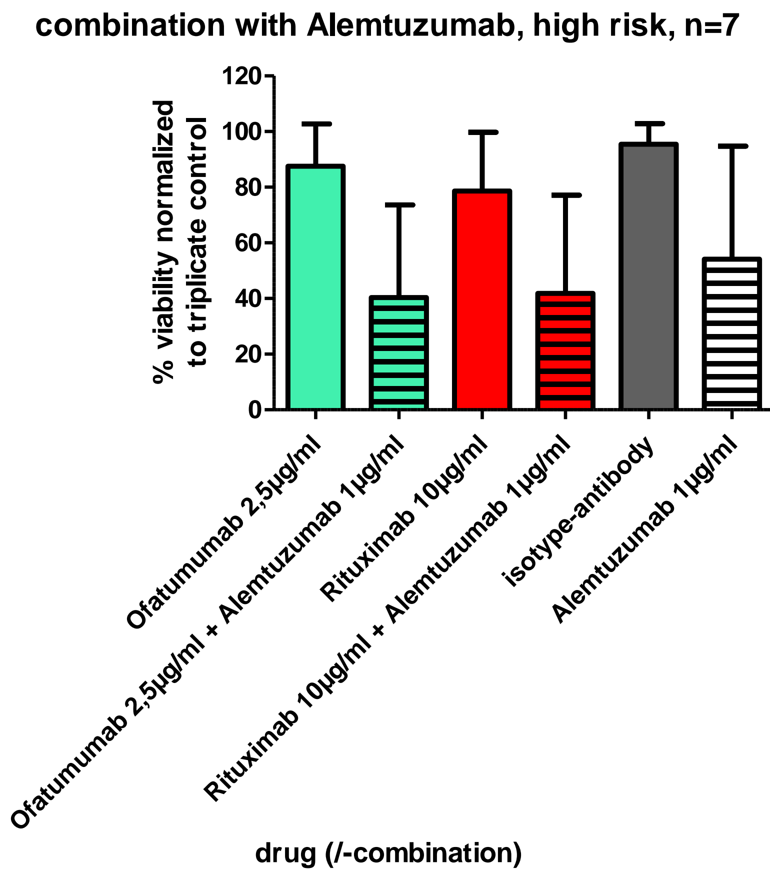


Figure 14:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st bar), Ofatumumab and Alemtuzumab (2nd bar), Rituximab (3rd bar), Rituximab and Alemtuzumab (4th bar), isotype antibody (5th bar) and Alemtuzumab (6th bar) in a cohort of seven high-risk CLL cases.

Comparing mean viability after dividing samples according to genetic CLL subgroups, no significant difference between high and low risk CLL cohorts can be observed between each of the CD20-antibody/Alemtuzumab-combinations (Ofa-

tumumab/Alemtuzumab mean viability high risk: 40.35%, range: 0.4809% - 87.97%, low risk: 30.00%, range: 2.299% - 58.64%, p-value = 0.5783; Rituximab/Alemtuzumab mean viability high risk: 41.83%, range: 0.6237% - 84.68%, low risk: 32.91%, range: 9.9% - 62.21%, p-value = 0.62).

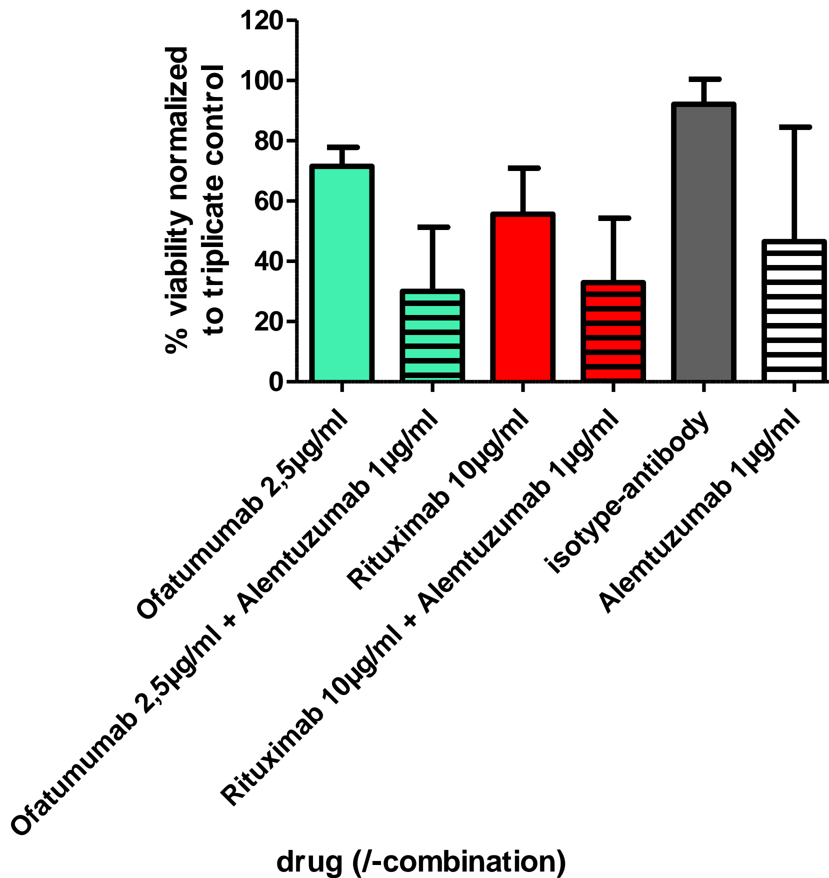


Figure 15:

viability of CLL (chronic lymphocytic leukemia) cells after a 48 hour incubation with Ofatumumab (1st bar), Ofatumumab and Alemtuzumab (2nd bar), Rituximab (3rd bar), Rituximab and Alemtuzumab (4th bar), isotype antibody (5th bar) and Alemtuzumab (6th bar) in a cohort of five low-risk CLL cases.

3.7 Assay No. 7: Flow cytometry based cytotoxicity assay

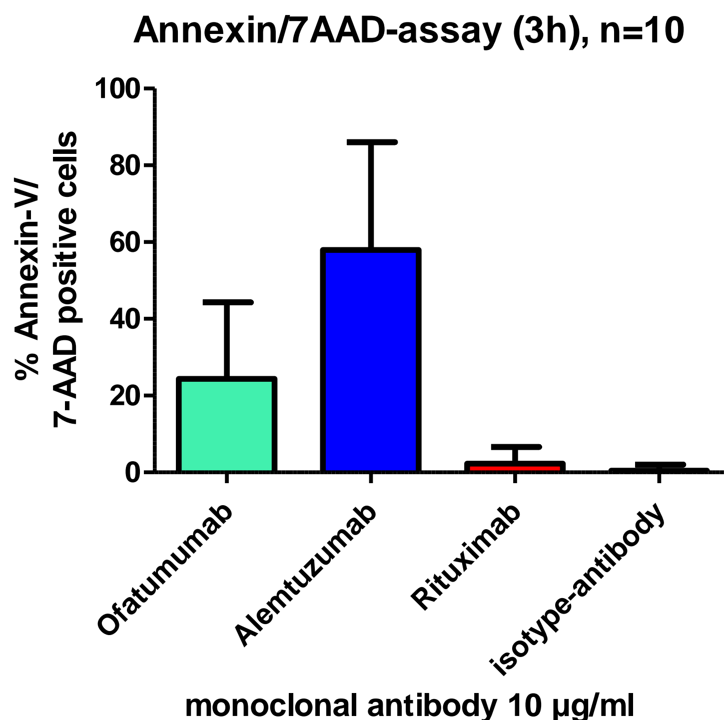


Figure 16:

percentage of “Apoptosis-marker-stained” CLL (chronic lympocytic leukemia) cells in relation to untreated CLL cells after a short incubation time with either therapeutic or isotype-antibody. Spontaneous background apoptosis has already been subtracted.

Assay No.7: Early (Annexin-V-PE) and late (7-AAD) Apoptosis markers were used for the identification of antibody induced killing mechanism. After a 3 hour lasting incubation at a cell density of 10000 cells/µl and in the presence of 30% human serum, both Ofatumumab (mean of Annexin-V-PE/7-AAD-positive cells: 24.37%, range: 5.38% – 69.49%, n=11) and Alemtuzumab (mean of Annexin-V-PE/7-AAD-positive cells: 57.97%, range: 15.36% - 83.46%, n=10) display rapid cell death induction, while Rituximab (mean of Annexin-V-PE/7-AAD-positive cells: 2.31, range: -1.46% - 10.03%, n=11) doesn’t. This is in accordance with the results in the assays described above. However, cell death elicited by Ofatumumab and Alemtuzumab was less when compared with the viability assays no.1 to 3, which were performed at a concentration of 1000cells/µl. Another possibility for slightly reduced activity compared to the viability assays could be cell lysis, which wouldn’t be detected by this readout.

3.8 Assay No. 8: Flow cytometry based cytotoxicity assay after coincubation of cells derived of the fibroblastoid bone marrow derived cell line HS5 with CLL cells

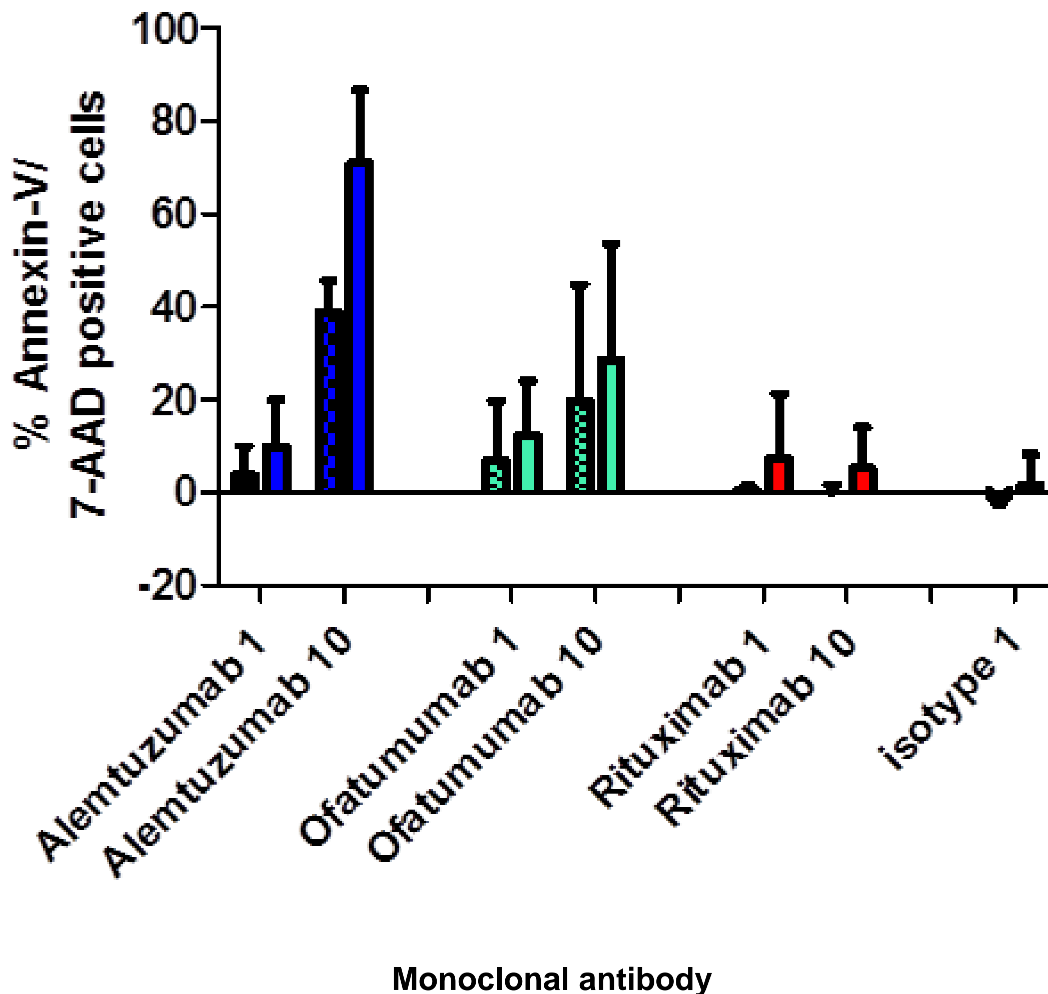


Figure 17:

percentage of apoptotic CLL (chronic lymphocytic leukemia) cells after 48 hours of incubation. The left bar represents the coincubated CLL cells, the right bar represents the CLL cells, which were not coincubated. Cells were treated with either therapeutic antibodies (first four columns: Alemtuzumab, next four columns Ofatumumab, next four columns Rituximab) at different antibody concentrations (1st concentration 1µg/ml, 2nd concentration 10µg/ml) or isotype-antibody (last 2 columns). Spontaneous background apoptosis has already been subtracted. An additional staining (CD45) was used to differ between HS5 (fibroblastoid bone marrow stroma) cells and CLL cells.

Assay No.8: Comparing antibody-mediated cell death in the presence or absence of fibroblastoid bone marrow stroma cell line HS5 (CLL coculture model) reveals a significant reduction of induced cell death by the antibody Alemtuzumab (Mean

apoptotic cells in the absence of HS5-cells: 71.33%, range: 46.7% - 83.46%, mean apoptotic cells in the presence of HS5-cells: 37.71%, range: 26.35% - 47.71%, p-value: 0.0022), and only a slight reduction of cytotoxicity of Ofatumumab (Mean apoptotic cells in the absence of HS5-cells: 28.66%, range: 2.26% - 69.49%, mean apoptotic cells in the presence of HS5-cells: 18.10%, range: -0.8% - 67.07%, p-value: 0.5282).

As bone-marrow stroma cells seem to provide protection of CLL-cells against antibody-induced apoptotic-like cell death, further research to determine the underlying mechanisms of protection are required to enhance antibody activity (Kurtova et al., 2009). It is known, that SYK-inhibition can reduce resistance to chemotherapy of CLL-cells, which are co-incubated with nurse-like cells (Buchner et al., 2010). Apart from that, bone marrow derived fibroblastoid cells induce expression of PI3-Kinase, AKT, NF-KB-pathway genes and a pro-angiogenic phenotype (Edelmann et al., 2008). Drugs targeting the SYK, IP3, AKT, NF-kB pathway or drugs as Lenalidomide may act synergistic to antibodies by possibly decreasing the protective effect of cells like HS5-cells.

3.9 Assay No. 9: Whole blood assay, microscopic read-out to assess homotypic adhesion

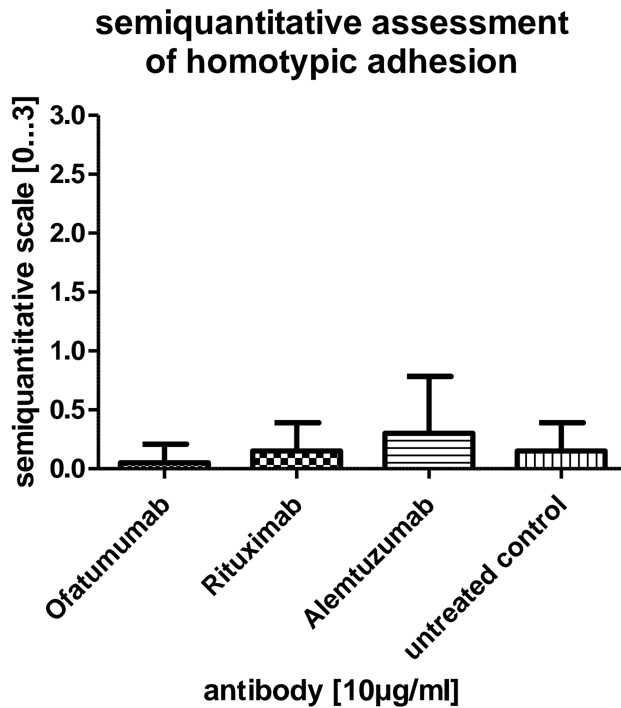


Figure 18:

extent of homotypic adhesion of CLL (chronic lymphocytic leukemia) cells caused by the different antibodies (1st bar: Ofatumumab, 2nd bar: Rituximab, 3rd bar: Alemtuzumab, each at 10µg/ml) and a negative (untreated) control after 1 hour incubation at room temperature.

Assay No.9: Inspection of five randomly picked high power fields after 1 hour whole-blood-incubation at room-temperature with therapeutic antibody (10µg/ml) or RPMI medium as negative (untreated) control displayed normal CLL blood smear appearance. No effector cells could be detected in close proximity to CLL cells. The Type-I-CD20 mAb-property (absent induction of homotypic adhesion) of both Ofatumumab and Rituximab could be confirmed in 10 out of 10 tested patient samples. (figure 18)

3.10 Assay No. 10: Whole blood assay, cytometric read-out, assessment of the impact of CLL-typical blood cell count on antibody-induced anti-CLL effect

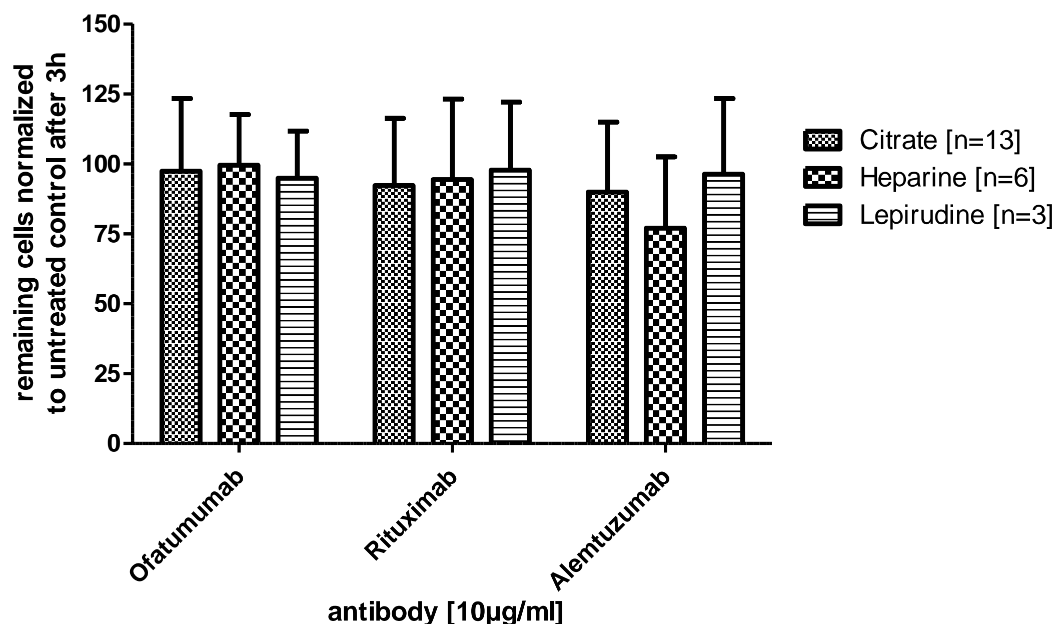


Figure 19:

relative numbers of leukocytes after a 3 hour incubation with either Ofatumumab, (first three bars), Rituximab (next three bars), or Alemtuzumab (last three bars) using different anticoagulants (left bar: Citrate, middle bar Heparine, right bar: Lepirudine) at a constant antibody concentration of 10 µg/ml.

Assay No. 10: This assay had the goal to assess the impact of high cell count on effector consumption under in vivo conditions as well as to assess other possible antibody induced mechanisms which were neglected in the above assays such as ADCC. We chose the BD Biosciences True Count cytometric method to exclude the possibility of undetected cell lysis and stained CLL cells with CD19-FITC. Three different anticoagulation methods had no impact on the results. Results were assembled according to used anticoagulation: Heparine [n=6], Lepirudine [n=3], and Citrate [n=13]. Almost no antibody activity was detectable for all antibodies at high cell concentrations. (figure 19)

CLL cells reside predominantly and at high concentrations in the blood compartment, where effector- to target cell ratio is rather unfavorable with respect to ADCC. We tried to prevent erythrocyte sedimentation and mimic the continuous in vivo blood flow by incubating the blood samples on a rotating table to imitate in vivo conditions. It is possible that reduced antibody activity was simply caused by overwhelming CLL cell density and consecutive effector consumption resulting in failure of antibody-induced activity.

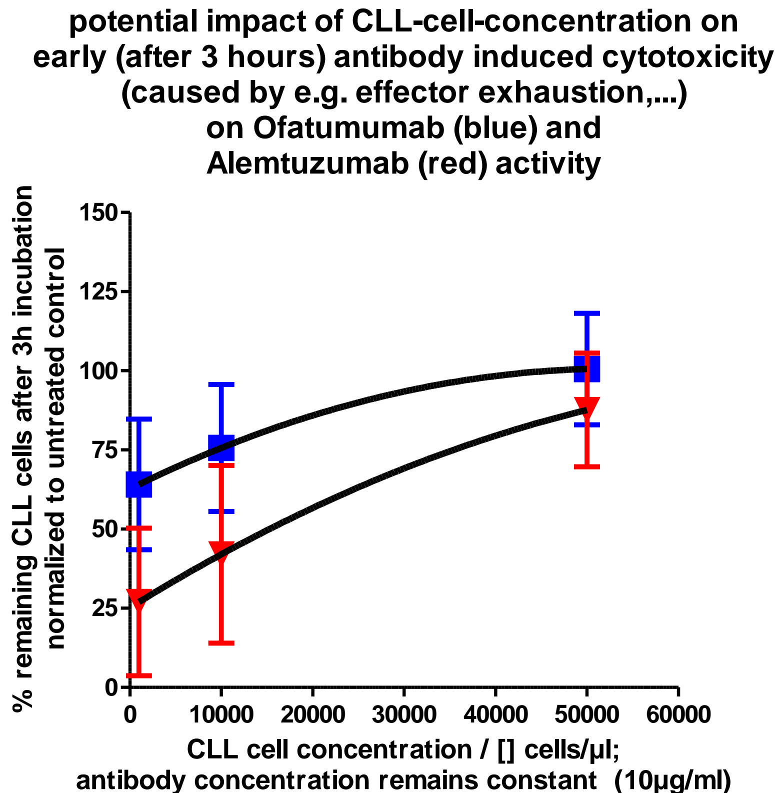


Figure 20:

assembly of results taken of several different assays (no.1, 7, and 10) with special focus on the CLL (chronic lymphocytic leukemia) cell concentration.

Non-linear regression of the values (viable cell number) leads to saturation curves of both Ofatumumab and Alemtuzumab with regard to quickly induced cytotoxic antibody activity. This points to the possibility of effector exhaustion, as it was described recently by Beurskens et al. (Beurskens et al., 2012). Methods to circumvent this failure include the additional application of fresh frozen plasma or even combined immunotherapy and plasmapheresis to guarantee complement sources.

4 Discussion

Ofatumumab seems an active CD-20-antibody, which could enhance CLL-therapy in various ways. Ofatumumab seems effective in its immediate depleting activity in low- and high-risk CLL and induces complement dependent cell death. Comparing the single-agent activity of the three monoclonal antibodies at a concentration of 10µg/ml after 48 hours of incubation, Alemtuzumab has a higher activity than Ofatumumab, while Ofatumumab has a higher activity than Rituximab. However, it should be noted, that much higher doses of CD20-antibodies can be administered in vivo compared to Alemtuzumab. Alemtuzumab has a wide spectrum of severe side effects (Lundin et al., 2002). Comparing the viability after 3 and 48 hours of antibody-incubation, we detected a difference especially in Rituximab-treated CLL-cells, but only a minor difference in the Ofatumumab-treated CLL-cells: Rituximab showed almost no effect after 3 hours and considerably more activity after 48 hours, suggesting a different mode of action. Ofatumumab-treated CLL cells were depleted mostly after 3 hours by complement-dependent cytotoxicity and a small additional depleted fraction could be detected after 48 hours of incubation. The activity of Alemtuzumab remained constant comparing 3 and 48 hour results. (figure 1E) We were especially interested in the activity of all antibodies in high-risk CLL and Rituximab-pretreated CLL. We defined high-risk CLL as the presence of at least one of the following: 17p-deletion detected by FISH -, TP53-mutation detected by wave and confirmed by direct sequencing, or Fludarabine-refractory disease (Stilgenbauer and Zenz, 2010) . No difference in their activity was observed for all three antibodies if results were divided into subgroups neither according to genetic risk-stratification nor Rituximab pretreatment. All antibodies seem to act independently of genetic aberrations in CLL.

Ofatumumab seems to possess at least equal potential and intrinsic activity for immunochemotherapeutic combination as Rituximab (antibody + Fludarabine). This option seems suitable especially for low-risk patients.

In order to assess potential chemo-sensitizing activity and potential use in immunochemotherapeutic combinations, we used a lower Ofatumumab-concentration to prevent rapid cell depletion. Regarding Fludarabine combinations, comparable depletion was induced by the two CD20-antibodies. The combined activity of the CD20-antibodies and Fludarabine could only be observed in low-risk CLL. As ex-

pected, high-risk CLL did not respond to Fludarabine, which is in accordance with previous findings (Zenz et al., 2008) .If Rituximab can sensitize lymphoid cells to Fludarabine treatment by down-regulating BCL-2, the same mechanism could be induced by Ofatumumab.

Ofatumumab can be effectively combined with Alemtuzumab or the AKT-inhibitor GSK 690693 and possibly other/similar-acting small-molecule inhibitors, which target the same pro-apoptotic pathway. This seems to be an option rather for high-risk patients.

Both Ofatumumab and Alemtuzumab seem to elicit their activity in a similarly fast and complement-dependent way. The results showed a greater CLL cell depletion not only for the combination of Alemtuzumab and Ofatumumab, but as well for the combination of Alemtuzumab and Rituximab. However, as the time course of cell death induction is rather different between the latter ones (Alemtuzumab and Rituximab) it is debatable if a synergistic effect at least on an immunologic basis (CDC) takes place in vivo. Apart from that it needs to be noted that not only for the Rituximab but as well for the Ofatumumab combination, our in vitro models cannot reflect the in vivo situation. It is possible that complement components, especially in compartments, where CLL cells are proliferating and residing in high concentration, become rapidly consumed and this consumption could be even accelerated if antibodies are used in combination. However, the solution of this possible problem could simply be the substitution of complement components by intravenous application of fresh frozen plasma as described previously (Klepfish et al., 2009).

Type-I-antibodies (such as Rituximab) can inhibit the constitutively activated PI3Kinase/AKT pathway (Baritaki et al., 2011, Suzuki et al., 2007, Bonavida, 2007).We used a 4-fold lower antibody-concentration of Ofatumumab compared to Rituximab for the combination not only with Fludarabine or Alemtuzumab (see above), but also for the combination with the AKT-inhibitor GSK 690693. Interestingly we could detect a slightly, though statistically not significant, higher activity of this combination in high-risk CLL compared to the activity in low-risk CLL. Taken together Ofatumumab seems a more potent antibody than Rituximab. There seem to be several combination options for Ofatumumab and especially high-risk patients might benefit of those. This is in accordance with recently published findings (Bologna et al., 2013).

Ofatumumab elicits its activity through so-far not fully understood mechanisms. In contrast to Rituximab, Ofatumumab is able to induce a quick cell death induction in CLL, which is complement-dependent. However, the observed saturation of its fast activity points to complement consumption (Boross et al., 2011). As the ATP-dependent luciferase-based assay, which we used in the assays no. 1-6 is restricted to low cell concentrations (maximum: 50000 cells/well), we used a cytometric read-out for assays with higher cell concentrations (assay no. 7 – 10). We used double-staining with 7-AAD (late-apoptosis marker) and AnnexinV-PE (early apoptosis marker) to differentiate between apoptotic and viable cells. The cells were incubated at a concentration of 10000cells/µl in this assay, which reflects the upper boarder of physiological leukocyte counts in healthy humans. Ofatumumab-induced CDC was decreased by approximately one third compared to the assays performed at a concentration of 1000cells/µl. It should be noted that a high frequency of complement deficiency in CLL has been described (Klepfish et al., 2009). Taken together with our results, this points again to the potential benefit of the substitution of complement components.

Ofatumumab seems to possess higher activitiy in assays with low leukocyte concentrations. This observation is in accordance with recently published results of Beurskens et al.(Beurskens et al., 2012) and could be due to the dependency of Ofatumumab on effector mechanisms which are not regenerating, refreshing, that is to say consumed during in vitro incubation or due to unknown reasons. Internalization and shaving of CD20/antibody complexes lead to CD20-loss on the surface of CLL cells. It has been recently published, that shaving/trogocytosis happens even faster than internalization and could constitute a major obstacle of antibody-mediated therapeutic effects (Beum et al., 2011, Pedersen et al., 2011). In theory shaving should happen less if the antibody molecules are bound by complement, which would point again to the combined application of ofatumumab and fresh frozen plasma.

To better mimic the in vivo microenvironment of CLL cells and create survival-inducing culture-conditions, we co-incubated them with HS5 cells (Schulz et al., 2011). Under these circumstances, we could detect a significantly reduced activity of Alemtuzumab and a slight reduction of Ofatumumab-induced activity.

A new treatment strategy could be to combine Alemtuzumab and Ofatumumab with fresh frozen plasma in order to tackle high-risk CLL with an extended therapeutic arsenal. This has been recently proposed by Baig et al. (Baig et al., 2012). The results of the currently recruiting study which investigates the combination of Alemtuzumab, Ofatumumab, and high-dose glucocorticoids will hopefully provide an insight whether or not at least the combination of the two antibodies might be an effective new therapeutic option (Jennifer R. Brown, 2011 - present).

Overlapping effector mechanisms elicited by a single antibody have been described previously (Zent et al., 2008, Boross et al., 2011). We varied the culture conditions to assess relative contribution of different mechanisms of action. Our whole blood assay was designed to mimic the in vivo situation in the blood compartment. A significant contribution to antibody activity by effector cells couldn't be detected. This, however, might not reflect the in vivo situation where blood CLL cells are often in close contact to cells of the reticulo-endothelial system: Effector cells, which often remain rather stationary in liver, spleen, lymphatic nodes and so on, and which can influence antibody activity (Gong et al., 2005) via ADCC, ADCP (phagocytosis), or reduce antigen expression via shaving are probably much higher concentrated and active under in vivo circumstances than in our obtained blood samples (Beum et al., 2006). Development of SIRS-like immune cascades cannot be entirely mimicked on incubation plates. In-vitro assays can't detect the long-lasting antitumor protection by anti-CD20 antibody through cellular immune response as described by Abès et al. (Abes et al., 2010). Finally, blood samples with very high leukocyte counts were used for the whole blood assays. Again, effector consumption (this time, consumption of effector cells) could be a reason for low antibody activity. Taken together, we couldn't detect a significant contribution to antibody activity of effector cells in whole blood assays. It remains an enigma whether Rituximab uses effector cells and ADCC to elicit its activity.

5 Summary

Introduction:

The application of monoclonal antibodies has become standard of care in the treatment of Chronic lymphocytic leukemia (CLL). We compared the single-agent-activity of three currently approved therapeutic antibodies for CLL, namely Rituximab, Alemtuzumab, and Ofatumumab.

Question:

Our goal was to identify underlying mechanisms of action of monoclonal antibodies in CLL in order to recognize potential limitations of their use and to develop strategies to overcome these limitations or at least to use the antibodies in the most beneficial way. We had our major focus on the impact of genetic subgroups of CLL and on Ofatumumab, a relatively new CD20-antibody in comparison with Rituximab and Alemtuzumab.

Method:

Patient samples were analysed by flow cytometry, fluorescence in-situ hybridiziation, liquid chromatography, and classic sequencing. Antibody-treatment was performed in both cell culture and whole blood assays. We assessed CLL-cell-viability, homotypic adhesion, potential synergism with other substances, and the impact of the cellular micro-environment by co-culturing CLL cells with fibroblastoid stroma cells. In parallel, CLL-cell-concentration, and effector consumption were assessed. Read out methods comprised microscopy, multi-color flow cytometry and luminometry,

Results:

Rituximab, Alemtuzumab, and Ofatumumab showed varying activity and different mechanisms of action. Regarding each antibody individually, no considerable difference could be detected among different risk-stratified CLL subgroups. In a subgroup of patients, we mimicked in vivo micro-environment by coincubation of CLL cells with HS5-cells and discovered a decrease of Alemtuzumab-activity but not Ofatumumab-activity. Rituximab is most beneficial if used in combination with other agents such as Fludarabine, Cyclophospamide (FCR) or Bendamustine (BR). We hypothesized a potential synergistic activity of Ofatumumab: submaximal concentrations of Ofatumumab combined with each Fludarabine, Alemtuzumab, and

the nanomolecular AKT-inhibitor GSK 690693, respectively, lead to promising results.
Summary:
Ofatumumab seems highly suitable for various combinations and high-risk CLL patients might benefit of those. Based on our in vitro findings, we suggest several strategies to enhance in vivo Ofatumumab activity.

6 References

1. ABES, R., GELIZE, E., FRIDMAN, W. H. & TEILLAUD, J. L. Long-lasting antitumor protection by anti-CD20 antibody through cellular immune response. Blood, Volume 116 Issue 6, p. 926-934. (2010)
2. BAIG, N. A., TAYLOR, R. P., LINDORFER, M. A., CHURCH, A. K., LAPLANT, B. R., PAVEY, E. S., NOWAKOWSKI, G. S. & ZENT, C. S. Complement dependent cytotoxicity in chronic lymphocytic leukemia: ofatumumab enhances alemtuzumab complement dependent cytotoxicity and reveals cells resistant to activated complement. Leuk Lymphoma, Volume 53 Issue 11, p. 2218-2227. (2012)
3. BARITAKI, S., MILITELLO, L., MALAPONTE, G., SPANDIDOS, D. A., SALCEDO, M. & BONAVIDA, B. The anti-CD20 mAb LFB-R603 interrupts the dysregulated NF-kappaB/Snail/RKIP/PTEN resistance loop in B-NHL cells: role in sensitization to TRAIL apoptosis. Int J Oncol, Volume 38, Issue 6, p. 1683-1694. (2011)
4. BEUM, P. V., KENNEDY, A. D., WILLIAMS, M. E., LINDORFER, M. A. & TAYLOR, R. P. The shaving reaction: rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes. J Immunol, Volume 176, Issue 4, p. 2600-2609. (2006)
5. BEUM, P. V., PEEK, E. M., LINDORFER, M. A., BEURSKENS, F. J., ENGELBERTS, P. J., PARREN, P. W., VAN DE WINKEL, J. G. & TAYLOR, R. P. Loss of CD20 and bound CD20 antibody from opsonized B cells occurs more rapidly because of trogocytosis mediated by Fc receptor-expressing effector cells than direct internalization by the B cells. J Immunol, Volume 187, Issue 6, p. 3438-3447. (2011)
6. BEURSKENS, F. J., LINDORFER, M. A., FAROOQUI, M., BEUM, P. V., ENGELBERTS, P., MACKUS, W. J., PARREN, P. W., WIESTNER, A. & TAYLOR, R. P. Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. J Immunol, Volume 188, Issue 7, p. 3532-3541. (2012)
7. BOLOGNA, L., GOTTI, E., DA ROIT, F., INTERMESOLI, T., RAMBALDI, A., INTRONA, M. & GOLAY, J. Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy. J Immunol, Volume 190, Issue 1, p. 231-239. (2013)
8. BONAVIDA, B. Rituximab-induced inhibition of antiapoptotic cell survival pathways: implications in chemo/immunoresistance, rituximab unresponsiveness, prognostic and novel therapeutic interventions. Oncogene, Volume 26, Issue 25, p. 3629-3636. (2007)
9. BOROSS, P., JANSEN, J. H., DE HAIJ, S., BEURSKENS, F. J., VAN DER POEL, C. E., BEVAART, L., NEDEREND, M., GOLAY, J., VAN DE WINKEL, J. G., PARREN, P. W. & LEUSEN, J. H. The in vivo mechanism of action of CD20 monoclonal antibodies depends on local tumor burden. Haematologica, Volume 96, Issue 12, p. 1822-1830. (2011)
10. BUCHNER, M., BAER, C., PRINZ, G., DIERKS, C., BURGER, M., ZENZ, T., STILGENBAUER, S., JUMAA, H., VEELKEN, H. & ZIRLIK, K. Spleen tyrosine kinase inhibition prevents chemokine- and integrin-mediated stromal

protective effects in chronic lymphocytic leukemia. Blood, Volume 115, Issue 22, p. 4497-4506. (2010)

11. CRAGG, M. S., WALSHE, C. A., IVANOV, A. O., GLENNIE, M. J. The Biology of CD20 and Its Potential as a Target for mAb Therapy. Curr Dir Autoimmun., Volume 8, p. 140–174. (2005)
12. EDELMANN, J., KLEIN-HITPASS, L., CARPINTEIRO, A., FÜHRER, A., SELLMANN, L., STILGENBAUER, S., DÜHRSEN, U. & DÜRIG, J. Bone marrow fibroblasts induce expression of PI3K/NF-κB pathway genes and a pro-angiogenic phenotype in CLL cells. Leukemia research, Volume 32, Issue 10, p. 1565-1572. (2008)
13. EICHHORST, B., GOEDE, V., HALLEK, M. Treatment of elderly patients with chronic lymphocytic leukemia. Leuk Lymphoma, Volume 50, Issue 2, p. 171-178 (2009)
14. FISCHER, K., CRAMER, P., BUSCH, R., STILGENBAUER, S., BAHLO, J., SCHWEIGHOFER, C. D., BOTTCHER, S., STAIB, P., KIEHL, M., ECKART, M. J., KRANZ, G., GOEDE, V., ELTER, T., BUHLER, A., WINKLER, D., KNEBA, M., DOHNER, H., EICHHORST, B. F., HALLEK, M. & WENDTNER, C. M. Bendamustine combined with rituximab in patients with relapsed and/or refractory chronic lymphocytic leukemia: a multicenter phase II trial of the German Chronic Lymphocytic Leukemia Study Group. J Clin Oncol, Volume 29, Issue 26, p. 3559-3566. (2011)
15. GONG, Q., OU, Q., YE, S., LEE, W., CORNELIUS, J., DIEHL,L., LIN, W., HU, Z., LU, Y., CHEN, Y., WU, Y., MENG, Y., GRIBLING, P., LIN, Z., NGUYEN, K., TRAN, T., ZHANG, Y., ROSEN, H., MARTIN, F., CHAN, A. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. J Immunol, Volume 174, Issue 2, p. 817-826. (2005)
16. HALLEK, M., CHESON, B. D., CATOVSKY, D., CALIGARIS-CAPPIO, F., DIGHIERO, G., DOHNER, H., HILLMEN, P., KEATING, M. J., MONTSERRAT, E., RAI, K. R. & KIPPS, T. J. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood, Volume 111, Issue 12, p. 5446-5456. (2008)
17. HALLEK M, FINGERLE-ROWSON G, FINK AM, BUSCH R, MAYER J, HENSEL M, HOPFINGER G, HESS G, VON GRUENHAGEN U, BERGMANN M, CATALANO J, ZINZANO P, CAPPIO F, SEYMOUR J, BERREBI A, JAEGER U, CAZIN B, TRNENY M, WESTERMANN A, WENDTNER C, EICHHORST B, STAIB P, BOETTCHER S, RITGEN M, MENDILA M, KNEBA M, DOEHNER H, STILGENBAUER S, FISCHER K. 2009. First-line treatment with fludarabine (F), cyclophosphamide (C), and rituximab (R) (FCR) improves overall survival (OS) in previously untreated patients (pts) with advanced chronic lymphocyticleukemia (CLL): Results of a randomized phase III trial on behalf of an international group of investigators and the German CLL Study Group. ASH congress: Abstract 535, https://ash.confex.com/ash/2009/webprogram/Paper16896.html, Download 12/2012
18. JENNIFER R. BROWN, M., PHD http://clinicaltrials.gov/ct2/show/NCT01465334. Dana-Farber Cancer Institute. 2011 - present, Download 12/2012

19. KLEPFISH, A., GILLES, L., IOANNIS, K., RACHMILEWITZ, E. A. & SCHATTNER, A. Enhancing the action of rituximab in chronic lymphocytic leukemia by adding fresh frozen plasma: complement/rituximab interactions & clinical results in refractory CLL. Ann N Y Acad Sci, Volume 1173, p. 865-873. (2009)
20. KURTOVA, A. V., BALAKRISHNAN, K., CHEN, R., DING, W., SCHNABL, S., QUIROGA, M. P., SIVINA, M., WIERDA, W. G., ESTROV, Z., KEATING, M. J., SHEHATA, M., JAGER, U., GANDHI, V., KAY, N. E., PLUNKETT, W. & BURGER, J. A. Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. Blood, Volume 114, Issue 20, p. 4441-4450. (2009)
21. LUNDIN, J., KIMBY, E., BJORKHOLM, M., BROLIDEN, P. A., CELSING, F., HJALMAR, V., MOLLGARD, L., REBELLO, P., HALE, G., WALDMANN, H., MELLSTEDT, H. & OSTERBORG, A. Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia (B-CLL). Blood, Volume 100, Issue 3, p. 768-773. (2002)
22. MORRISON, V. A., RAI, K. R., PETERSON, B. L., KOLITZ, J. E., ELIAS, L., APPELBAUM, F. R., HINES, J. D., SHEPHERD, L., LARSON, R. A. & SCHIFFER, C. A. Therapy-related myeloid leukemias are observed in patients with chronic lymphocytic leukemia after treatment with fludarabine and chlorambucil: results of an intergroup study, cancer and leukemia group B 9011. J Clin Oncol, Volume 20, Issue 18, p. 3878-3884. (2002)
23. PEDERSEN, A. E., JUNGERSEN, M. B. & PEDERSEN, C. D. Monocytes mediate shaving of B-cell-bound anti-CD20 antibodies. Immunology, Volume 133, Issue 2, p. 239-245. (2011)
24. SCHULZ, A., TOEDT, G., ZENZ, T., STILGENBAUER, S., LICHTER, P. & SEIFFERT, M. Inflammatory cytokines and signaling pathways are associated with survival of primary chronic lymphocytic leukemia cells in vitro: a dominant role of CCL2. Haematologica, Volume 96, Issue 3, p. 408-416. (2011)
25. STILGENBAUER, S. & ZENZ, T. Understanding and managing ultra high-risk chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program, Volume 2010, p. 481-488. (2010)
26. SUZUKI, E., UMEZAWA, K. & BONAVIDA, B. Rituximab inhibits the constitutively activated PI3K-Akt pathway in B-NHL cell lines: involvement in chemosensitization to drug-induced apoptosis. Oncogene, Volume 26, Issue 42, p. 6184-6193. (2007)
27. WALSHE, C. A., BEERS, S. A., FRENCH, R. R., CHAN, C. H., JOHNSON, P. W., PACKHAM, G. K., GLENNIE, M. J. & CRAGG, M. S. Induction of cytosolic calcium flux by CD20 is dependent upon B Cell antigen receptor signaling. J Biol Chem, Volume 283, Issue 25, p. 16971-16984. (2008)
28. ZENT, C. S., SECRETO, C. R., LAPLANT, B. R., BONE, N. D., CALL, T. G., SHANAFELT, T. D., JELINEK, D. F., TSCHUMPER, R. C. & KAY, N. E. Direct and complement dependent cytotoxicity in CLL cells from patients with high-risk early-intermediate stage chronic lymphocytic leukemia (CLL) treated with alemtuzumab and rituximab. Leuk Res, Volume 32, Issue 12, p. 1849-1856. (2008)

29. ZENZ, T., BENNER, A., DOHNER, H. & STILGENBAUER, S. Chronic lymphocytic leukemia and treatment resistance in cancer: the role of the p53 pathway. Cell Cycle, Volume 7, Issue 24, p. 3810-3814. (2008)

7 Appendix

Table 6:

Summary of the CLL samples taken of CLL patients treated in Ulm during the period of 2000 and 2010, certain prognostic parameters of the CLL cells and clinical data of the patients used for: All assays (all patients). The list contains 18 high-risk CLL-cases (they are specified by at least one of the three following characteristics: 17p (short arm of chromosome 17)-deletion, TP53 (encodes for p53)-mutation, Fludarabine-refractory). The *TP53* (tumor suppressor protein 53) -status is a prognostic marker for survival and response to conventional chemotherapy schemes of CLL, the ZAP-70-protein is associated with either long or short survival of CLL patients. FISH (fluorescence in-situ hybridization) is used to further distinguish between CLL subgroups. The IGHV (variable part of the immune globuline heavy chain)-status is another marker, which is associated with survival and response to treatment.

no.	gender	FISH	*TP53*	*IGHV*	*ZAP-70*	pre-treatment	age
1	F	13q-	WT	M	Negative	yes	64
2	M	11q-, 12q+, t14q32	WT	UM	Positive	no	65
3	F	17p-, 12q+	WT	UM	n/d	yes	87
4	F	17p-14q-	c.584T>C	UM	Positive	yes	65
5	M	11q-, 13q-	c.919-7A>C	UM	n/d	yes	77
6	M	17p, 12q+	WT	n/d	n/d	yes	82
7	M	13q-	WT	M	Negative	no	50
8	M	17p-, 11q-, 13q-	c.499C>T, c.830G>T	UM	n/d	yes	72
9	M	13q-, 11q-	WT	M	Negative	no	49
10	F	13q-	c.847C>T	M	Negative	no	73
11	M	13q-, 11q-	WT	UM	n/d	no	69
12	M	13q-, 11q-	WT	UM	Negative	no	37
13	F	13q-	WT	M	Negative	yes	64
14	F	normal	WT	M	Negative	no	65
15	M	13q-	WT	M	n/d	no	72
16	M	13q-	WT	UM	Positive	no	70
17	M	13q-	WT	UM	Positive	no	69
18	M	normal	WT	UM	Positive	no	71
19	M	13q bidel	WT	M	Negative	no	61
20	M	17p-, 13q-	753-759del7bp	UM	n/d	yes	58
21	M	11q-	WT	UM	Positive	yes	69
22	M	17p-, 12q+, 13q-	WT	UM	n/d	no	68
23	F	normal	n/d	M	n/d	yes	65
24	F	normal	WT	UM	Positive	yes	74
25	F	17p-,13q-,12q+	c.809T>G	UM	n/d	no	73

26	M	17p-, 11q-, 13q-	c.329G>T	UM	Negative	no	69
27	F	normal	WT	UM	Positive	no	38
28	M	12q+, t(14:18)	WT	M	Negative	no	81
29	F	17p	c.842A>G	UM	Positive	yes	42
30	M	17p	c.602-603Ins4bp	UM	Negative	no	69
31	M	17p	WT	UM	n/d	no	73
32	F	Normal	c.484A>T	UM	Positive	yes	64
33	M	13q	WT	UM	n/d	no	66
34	M	13q	WT	UM	Negative	yes	77
35	M	Norm	WT	UM	Negative	yes	72
36	F	Tris.12, 17p	c.673-2A>T	M	Negative	yes	73
37	M	17p, 13q	c.413C>T	M	Negative	yes	72
38	F	13q	n/d	M	n/d	no	59
39	F	17p, 13q	C.733G>T	UM	Negative	yes	76
40	M	17p, 13q, 6q	n/d	UM	n/d	yes	66
41	F	Norm	WT	M	n/d	no	75
42	M	normal	WT	M	n/d	no	39
43	M	t14q32	WT	UM	Negative	yes	72
44	M	13q-	WT	UM	n/d	yes	67
45	M	11q-, 13q-, 14q-	WT	UM	Negative	no	73
46	M	12p+11, t(14;19)	WT	UM	Positive	yes	49
47	M	13q-	c:716A>6	M	n/d	yes	76
48	M	13q	WT	M	n/d	no	57
49	M	11q23	WT	UM	Negative	yes	58

8 Curriculum Vitae

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