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DIPLOMARBEIT

Titel der Diplomarbeit

Targeting the PI3K Pathway in CLL

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Introduction:

1. Chronic lymphocytic leukemia

1.1. Features of CLL

The chronic lymphocytic leukaemia (CLL) is the most common malignancy in the western hemisphere and has one of the highest prevalences among leukeamias in adults. (15) However, therapy is still restricted to extending survival without being able to cure this type of malignancy. The disease is characterized by the expansion of a clone of cells bearing the common B-cell markers CD19 and CD20 but also the T-cell marker CD5, they exhibit a high expression of CD23 and a weak expression of CD22 while being negative for FMC-7. In addition, CLL cells usually have low surface immunoglobulin. These markers distinguish this leukaemia from other lymphoproliferative disorders like mantle cell lymphoma (CD23-/FMC-7+) or follicular lymphoma (CD5-/CD23-/FMC-7++/CD22++). (14, 15) There is also a very small population of CD5+ B cells in healthy individuals that often produces low affinity auto-antibodies. This feature of auto-antibody production is very prominent in CLL and leads to the autoimmune events that are typical for this disease. (14, 16) The CLL cells are also exhibiting a resistance to apoptosis which results in the expansion of the malignant clones. It is necessary to note that the majority of CLL cells (>98%) are arrested in the G0/G1 phase of the cell cycle which can have important implications for therapy. (13, 16)

Clinically the disease can be divided in stages according to Rai (0-IV) or Binet (A-C). Quite often patients are asymptomatic at diagnosis and not always in need of immediate therapy. Early symptoms include lymphadenopathy, hepatomegaly or splenomegaly and for a definite diagnosis it is necessary to check for the specific CLL markers in the blood by immunophenotyping. (13, 15) Although common in CLL patients, cytogenetic aberrations cannot always be found and are therefore not considered crucial for disease initiation. This assumption is also supported by studies showing that the incidence of CLL does not increase in individuals that were exposed to toxic substances, chemicals or radiation. Even though there are several cytogenetic alterations that often occur in CLL patients, among which are deletions of 17p, 13q and 11q and trisomy of 12q. All of them have an effect on

disease progression and prognosis. In addition there are families with siblings who develop the disease which indicates a certain genetic implication. (15, 16, 17)

Another way to classify CLL cases is through analysis of the immunoglobulin heavy chain (IgVH) genes. CLL cells can be divided according to their mutational status of the IgVH genes as mutated (less than 98% homology to the germline sequence) or unmutated (more that 98% homology to germline sequence). The unmutated status of IgVH gene is associated with poor prognosis. (13)

1.2. Treatment strategies in CLL

The majority of CLL patients has a stable clinical course and is asymptomatic at diagnosis but other patients will develop a progressive disease and require immediate treatment. Therefore it is difficult to decide when to start treating CLL patients. Therefore prognostic markers indicating aggressiveness of the disease and prognosis for the patient are an important tool for the clinician. Among these markers are mutational status, CD38 expression, cytogenetics, soluble CD23 and involvement of the bone marrow all of which are taken in consideration during the selection of therapy. (13, 15, 17) Common therapeutic options include alkylating agents (chlorambucil, bendamustine), purine analogues (fludarabine, cladribine and pentostatin), therapy with monoclonal antibodies (rituximab or alemtuzumab) and also stem cell transplantation. (13, 15)

1.3. Apoptosis resistance of CLL cells and the role of the microenvironment and PI3-K

CLL cells have long life spans and are resistant to apoptosis in vivo. In vitro the situation is quite different. When CLL cells are collected from a patient and placed into culture they die rapidly by apoptosis except if they are exposed to stimulation with cytokines such as IL-4 or supported by stromal cells (7, 16, 19, 27). This suggests a dependence of the CLL cells on external survival signals

which can be delivered, at least in part, by the lymphoid microenvironment. There is increasing evidence that the microenvironment can protect leukaemic cells from apoptosis and might even play an important role in therapy resistance, minimal residual disease and relapse. (6, 7, 18, 27)

The microenvironment, especially in the bone marrow, is a very complex assembly of cells, ECM, adhesion molecules and soluble factors. In CLL patients the marrow is usually infiltrated with CLL cells and the extent of this infiltration has prognostic value which is yet another indication for the importance of the interactions that take place in the microenvironment. In co-culture experiments it could be demonstrated that marrow stromal cells (MSCs) produce factors that attract CLL cells and that they can engage in cell-cell contact through adhesion molecules. CLL cells express a variety of adhesion molecules of the integrin, selectin and immunoglobulin families some of which have been reported to have prognostic relevance. (6, 7)

Since CLL is still an incurable disease and all patients eventually relapse, novel therapeutic approaches are needed. Therefore, targeting the microenvironment and inhibition of the survival signals provided by stromal cells appear to be an attractive therapeutic concept. One of the major anti-apoptotic pathways which could be involved in the prolonged survival of CLL cells is the PI3-K/Akt pathway. (27)

The PI3K pathway is an especially interesting target as it can be activated through many signals that are expected to be offered to the CLL cells by the microenvironment. As we could show in our previous work, the PI3K pathway seems to be highly active in CLL cells and might therefore be a suitable target. (27) Also, PI3K inhibitors have exhibited promising effects in in vitro experiments, making CLL cells more susceptible to apoptosis even in the presence of supporting stromal cells. (6, 27) Another advantage is that the pathway offers several key signaling molecules which could be targeted by available pharmacological compounds: PI3K and its isoforms (α, β, γ and δ), its downstream targets AKT/PKB (protein kinase B) and phosphoinositide dependent protein kinase-1 (PDK1), as well as casein kinase 2 (CK2) which is predominantly responsible for the inactivation by phosphorylation of the main

negative regulator of the pathway, PTEN (phosphatase and tensin homolog on chromosome ten). Therefore it is very important to identify the expression of PI3-K isoforms and their significance to the pathophysiology of CLL.

2. The PI3K Pathway

2.1. The isoforms

Phosphoinositide-3-kinases (PI3K) are a family of enzymes that phosphorylate the 3-hydroxyl group of the inositol ring of several phosphoinositol substrates. Among these substrates are phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns4P) and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). (see Figure 1) Phosphorylation results in generation of 3-phospholipids, like phosphatidylinositol-3,4,5,-trisphosphate (PIP_3). These products can be bound by several effector molecules which are thereby recruited to the plasma membrane. This interacion is accomplished through lipid binding domains in the effectors like pleckstrin homology domain (PH domain), FYVE domain and phox homology domain (PX domain). (4)

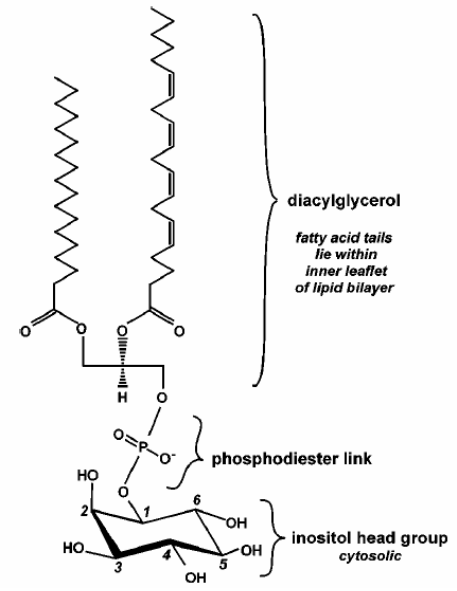


Figure 1: The chemical structure of PtdIns (See Ref 26)

The PI3K family comprises three classes of enzymes with a total of eight different isoforms in mammals. They are divided into class I, II and III based on differences in structure and substrate affinity. (see Figure 2) The class I PI3Ks are the most extensively studied representatives of this family. This class consists of p110α (PIK3CA), p110β (PIK3CB), p110γ (PIK3CG) and p110δ (PIK3CD) and can again be subdivided into class IA (p110α, p110β and p110δ) and class IB (p110γ) according to their preferred binding partners. Class IA enzymes associate with regulatory subunits of the p85 type forming heterodimers. The class IA regulatory subunits comprise five isoforms (p85α, p55α, p50α, p85β and p55γ). The class IB comprises only the p110γ isoform which can associate with either p101 or p87 regulatory subunit. (4, 5)

The p85 subunits can exert a regulatory function through their SH2 domain which enables them to bind phospho-tyrosine residues of thyrosine kinases, thereby connecting them to a wide selection of pathways. In addition the p85 subunits can stabilize the p110 subunit and keep the kinase domain inactive in the basal state. Once p85 binds the phospho-tyrosine residue with its SH2 domain the inactivation is relieved and the p110 subunit can exert its lipid kinase function. In addition to their lipid kinase activity the p110 subunits also possess a protein kinase activity (4)

The class I PI3K enzymes are ubiquitously expressed in mammalian cells but the delta and gamma isoform seem to have a higher expression in leukocytes. Even though they can phosphorylate all three substrates they show a preference for PtdIns(4,5)P_2 in vivo generating PIP_3. (4)

The class II PI3K comprises three isoforms in mammals: PI3K-C2α, PI3K-C2β and PI3K-C2γ. The first two are expressed in a wide variety of tissues whereas the gamma isoform has a less broad expression. The preferred substrates of these kinases are PtdIns and PtdIns4P and they do not require heterodimerization with a regulatory subunit for this process. Their functions and regulation remain unclear. (4)

The class III PI3K consists of just one kinase, the VPS34. Even though it can only convert PtdIns it can aquire other functions through participation in various

multiprotein complexes. VPS34 appears to be mainly an endosomal kinase relevant for processes like endocytosis, phagocytosis and vesicle transport. (4)

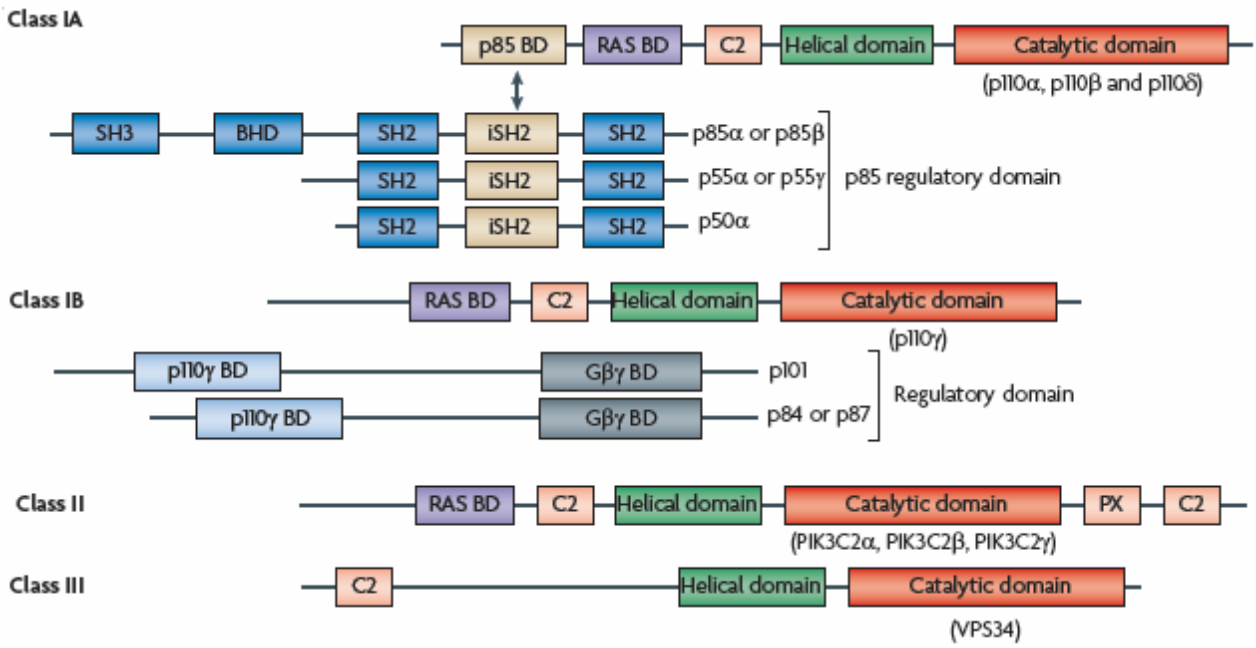


Figure 2: The eight mammalian PI3K isoforms (See Ref 11)

The functions of PI3K enzymes appear to be redundant and overlapping. There are some examples for isotype specific knock out or gene silencing experiments that lead to the assumption that at least some of the isoforms serve a specific, non-redundant purpose. Dissection of distinct isoform functions is complicated by the diverse stimuli activating PI3K enzymes and by the additional diversity added by heterdimerisation with different regulatory subunits. Great advances have been made especially in elucidating the roles of the class I enzymes as for this group pan- and isotype-specific inhibitors are available. (3, 4, 22)

This work will focus on the class I PI3K, their role in CLL and their relevance as a therapeutic target.

2.2. The activation and functions of Class I PI3K

The class I kinases can be activated by very diverse external stimuli and the results of their activation are just as wide spread and range from changes in

motility and adhesion to cell cycle progression and survival. (see Figure 3 and Ref 11) The activation of PI3Ks mainly results from receptor tyrosine kinases (such as the EGFR) but can also be iniciated by G proteins (such as Ras family GTPases) or non-receptor tyrosine kinases (such as JAK). The activation through GTPases is typical for the p110γ isoform. (2, 4)

Although major attention has been directed towards the PIP_3 product of PI3K activation, other lipid entities like PtdIns(4,5)P_2 can interact with a large number of proteins and modulate their activity. (2, 4) The PIP_3 pool is regulated by the interplay of phosphorylation of the 3-hydroxyl group by PI3K and the dephosphorylation at postion 3 or 5 by different phosphatases. The 3-phosphatase PTEN (phosphatase and tensin homolog on chromosome 10) is the most important negative regulator of this pool. PIP_3 is usually bound by proteins possessing a PH domain. Among the most important of its interaction partners are PKB/AKT, PDK1, kinases of the Tec family (such as ITK and BTK) and certain GEFs. (1, 2, 4) Nevertheless, PTEN is considered the main negative regulator of this pathway. The phosphatase activity of PTEN is regulated to a large extent through phosphorylation of several residues (S370, S380, T382, T383 and S385) in its tail domain in the C-terminus which inhibits the recruitment of PTEN to the plasmamembrane and thereby its function as a lipid phosphatase. These phosphorylation events are known to be exerted by CK2. On the other hand, dephosphorylation of this domain allows PTEN to translocate to the membrane and regulate PIP_3 levels but it also reduces protein stability and increases degradation through ubiquitinylation. (28)

AKT/PKB is a PH domain containing serine/threonine kinase that can be relocated to the plasma membrane through binding of PIP_3. Once recruited to the membrane AKT/PKB can be phosphorylated at Ser473 and Thr308, two important regulatory residues. The Thr308 phophorylation is mediated by another PI3K effector: PDK1 (phosphoinositide dependent kinase 1). PDK1 has two PH domains, one in the catalytic N-terminal domain and one in the C-terminal region. Apart from phosphorylation of AKT, PDK1 has several targets that regulate very diverse cellular processes. These targets include: PKC isoforms, S6K1 kinases, GSK3 and AGC family kinases which play into cellular processes like regulation

of cell survival, protein synthesis, cell cycle and glycogen metabolism. The activation of AKT also results in wide spread responses through effectors like mTORC1 or the transcription factors of the Forkhead family that are phosphorylated directly by AKT. AKT activation by phosphorylation is commonly used as an indicator of PI3K activity in experimental settings. (2)

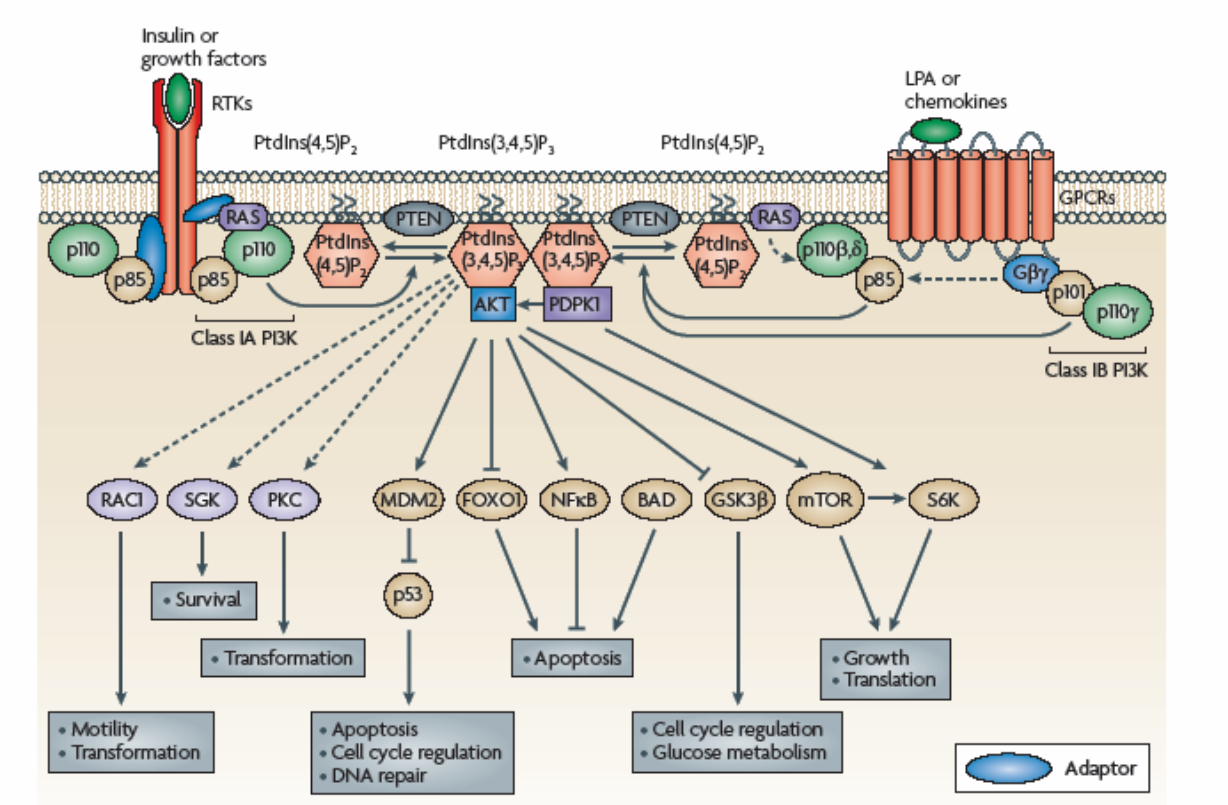


Figure 3: The PI3K signalling network (See Ref 11)

2.3. The role of PI3K in tumourigenesis

As a signalling pathway that can be approached and activated through external and internal stimuli and that also leads to a broad spectrum of pro-survival and pro-proliferation reactions it is a signature pathway to be deregulated in cancer. This disruption of the balance in the PIP_3 pool can be due to either an overproduction by PI3K or to a diminished dephosphorylation of PIP_3 to PIP_2 caused by a lack of phosphatase activity. PTEN which is considered the major

negative regulator of PI3K signalling is a tumour suppressor that is very commonly mutated in various cancers and inherited loss of PTEN function is associated with a predisposition for cancers. In fact, among the tumour suppressors only the p53 gene is more frequently inactivated in human cancers. (9)

The other branch, the PI3K, does not fall short of this record. The gene for the p110α subunit is mutated in 15% of all cancers and might just be one of the most commonly deregulated kinases in human malignancy. (9) As high as the frequency of PI3K mutation is, it is still not the only possibility for intensified PI3K signalling. The various activators of PI3K like receptor tyrosine kinases and GTPases can be the source of the hyperactivation as well as the downstream effectors like PDK1 or AKT. The diversity of this pathway also implies that there are several targets for mutational events and potential deregulation which makes it an interesting potential therapeutic target. (1)

2.4. PI3K pathway inhibitors

Since the PI3K pathway plays an important role in a great multitude of cellular processes, it is also believed to be involved in various diseases and is therefore an interesting target, not only in cancer research. As a consequence, a lot of effort has been made to design new compounds to manipulate this pathway and its key molecules. There are already several clinical phase I and phase II trials in progress applying these drugs. When it comes to the inhibition of PI3K itself a very crucial point is the question if it is better to target the whole class I enzymes with pan-inhibitors or if isotype-specific inhibitors could offer a superior alternative. It could be argued that pan-inhibitors may be too toxic in an in vivo situation. On the other hand, the isotype-specific inhibitors may not be sufficient to block the pathway activation because of the redundancy in isotype functions and the possible compensation if only one isoform is inhibited. (8) The next problem with isotype-specific inhibitors is to pick the right target which requires careful dissection of the pathway and investigation of the distinctive roles the isoforms play in a certain cell type. In addition there are several possibilities to

target the pathway further downstream (AKT, PDK1 and mTOR inhibitors) or to improve the activity of the negative regulator PTEN by targeting CK2 with specific inhibitors. (8)

The following table (8) gives a short overview on existing compounds targeting the PI3K/AKT pathway and the according clinical trials:

Inhibitor	Company	Phase of clinical trial
Dual PI3K and mTOR inhibitors		
BEZ235	Novartis	Phase I/II
BGT226	Novartis	Phase I/II
XL765	Exelixis	Phase I
SF1126	Semafore	Phase I/II
GSK1059615	GSK	Preclinical
PI3K inhibitors		
XL147	Exelixis	Phase I
PX866	Oncothyreon	Phase I
GDC0941	Genentech/Piramed/Roche	Phase I
BKM120	Novartis	Phase I
CAL101 (targets p110δ)	Calistoga Pharmaceuticals	Phase I
Akt inhibitors		
Perifosine	Keryx	Phase I/II
GSK690693	GSK	Phase I
VQD002	Vioquest	Phase I
MK2206	Merck	Phase I
mTOR inhibitors (catalytic site)		
OSI027	OSI Pharmaceuticals	Phase I
AZD8055	AstraZeneca	Phase I/II

Table 1: Inhibitors of the PI3K pathway in clinical trials (See Ref 8)

2.4.1. PI3K Class I pan-inhibitors

2.4.1.1. Wortmannin and LY294002

Among the earliest PI3K inhibitors are LY294002 and Wortmannin whose inhibitory quality was described in 1994 and 1987 respectively. (see Figure 4)

Synthesis of the flavone LY294002 was based on quercetin and Wortmannin was isolated from a fungus (*penecillium wortmanni*) in 1957. (9, 10)

Figure 4: Chemical structure of Wortmannin and LY294002 (See Ref 9)

Both compounds have been used extensively for experimental purposes but as potential drugs they fall short in some important aspects like potency and specificity. LY294002 and Wortmannin demonstrated inhibitory effects on other kinases like CK2, mTOR, MLCK and DNA-PK. The limited solubility and the considerable toxicity prevented the compound from entering clinical application. Nevertheless they both served as molecular templates for drug design leading to a large collection of new compounds. (10, 12)

2.4.1.2. PI-103 and GDC-0941

Due to the increasing interest in PI3K inhibitors other compounds were identified that showed improved specificity and lower IC_{50} values. In 2007 one of these novel inhibitors, called PI-103 (pyridofuropyrimidine PI-103) was published. (23) PI-103 exhibited an elevated selectivity for its main target (class I PI3Ks) and less selectivity and potency for other kinases such as the class II and class III PI3Ks. Even though the p110γ isoform required higher concentrations for effective inhibition all four isoforms could be targeted with IC_{50} values of 2-15nM. Still, the problems with solubility, bioavailability and other pharmacologic issues remained

leading to the development of GDC-0941, another PI3K inhibitor designed to improve these shortcomings of PI-103. (see Figure 5) The overall selectivity for class I PI3Ks was increased in comparison to PI-103 creating an inhibitor that possessed the advantages of PI-103 (selectivity) in addition to an improved bioavailability and better pharmacodynamic characteristics. GDC-0941 can inhibit all four p110 isoforms but shows increased selectivity for p110α and p110δ. (9) As a potential therapeutic compound GDC-0941 has already entered phase I clinical trials (8)

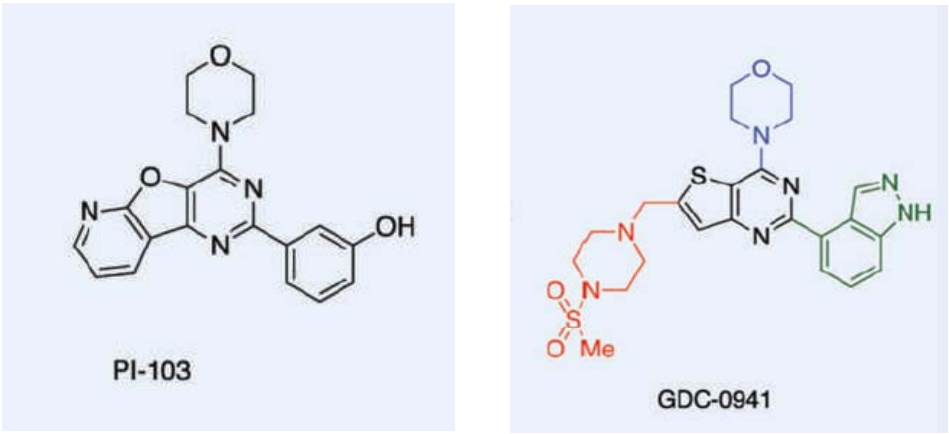


Figure 5: Chemical structure of PI-103 and GDC-0941 (See Ref 9)

2.4.1.3. NVP-BEZ235

This compound is a dual PI3K/mTOR inhibitor that has been developed through modification of a PDK-1 inhibitor by Novartis. It is an imidazoquinoline that can inhibit all four p110 isoforms and mTOR with IC_{50} values in nanomolar range. (see Figure 6) NVP-BEZ235 has also already entered clinical Phase I/II trials for certain malignancies. (10, 12)

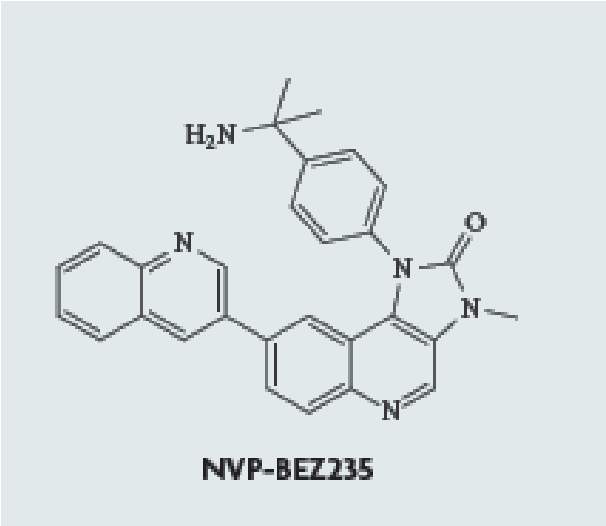


Figure 6: Chemical structure of NVP-BEZ235 (See Ref 11)

2.4.2. Isotype specific PI3K inhibitors

2.4.2.1. PI3K-alpha inhibitor VIII

The PI3K-alpha inhibitor VIII is an imidazopyridine that blocks PI3K-alpha specifically at low concentrations but can block other subtypes at high concentrations.

2.4.2.2. PI3K-beta inhibitor VI (TGX-221)

The PI3K-beta inhibitor VI, also called TGX-221, is a morpholino-pyrimidinone able to block the beta isoform but has also some activity against other isoforms (mainly p110δ) as well as some other kinases. Among its sister molecules (TGX-115 and TGX-286) TGX-221 is probably the most widely used inhibitor to study p110β. (11)

2.4.2.3. PI3K-gamma inhibitor II

The PI3K-gamma inhibitor II is a thiazolidinedione and is quite selective for the isoform with only little activity against other signalling molecules.

The following table gives an overview on the diversity of novel synthetic PI3K inhibitors and their potential clinical use:

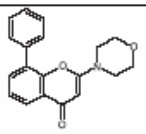
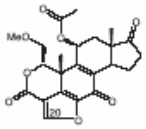
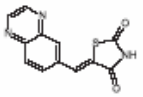
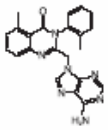
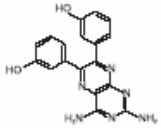
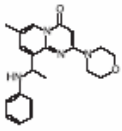
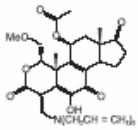
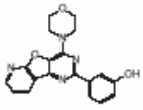
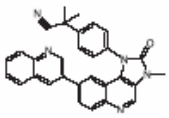
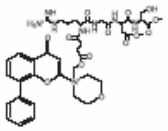
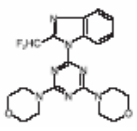
Inhibitor	Structure	IC_{50} (μM)	Selectivity	Targeted disease
LY294002			Pan-PI3K inhibitor,	Cancer
p110α		0.55	mTOR and DNA-PK inhibitor,	
p110β		11	CK2 inhibitor	
p110δ		1.6		
p110γ		12		
Wortmannin			Pan-PI3K inhibitor,	Cancer
p110α		0.004	mTOR and DNA-PK inhibitor,	
p110β		0.001	MLCK inhibitor	
p110δ		0.004		
p110γ		0.009		
AS-605240			PI3Kγ selective	Rheumatoid arthritis, glomerulonephritis
p110α		0.06		
p110β		0.27		
p110δ		0.3		
p110γ		0.008		
IC87114			PI3Kδ selective	Inflammation, allergy, AML
p110α		>100		
p110β		75		
p110δ		0.5		
p110γ		29		
TG100-115			PI3Kγ and δ selective	Myocardial infarction
p110α		1.3		
p110β		1.2		
p110δ		0.235		
p110γ		0.083		
TGX221			PI3Kβ selective	Thrombosis
p110α		5		
p110β		0.005		
p110δ		0.1		
p110γ		>10		
PX-866			Pan-PI3K inhibitor	Cancer
p110α		0.006		
p110β		>300		
p110δ		0.003		
p110γ		0.009		
PI-103			Pan-PI3K inhibitor,	Cancer
p110α		0.002	mTOR and DNA-PK inhibitor	
p110β		0.003		
p110δ		0.003		
p110γ		0.015		
NVP-BEZ235			Pan-PI3K inhibitor,	Cancer
p110α		0.004	mTOR inhibitor	
p110β		0.076		
p110δ		0.005		
p110γ		0.007		
SF1126			Prodrug of pan-PI3K inhibitor,	Cancer
p110α		ND	prodrug of mTOR and DNA-PK inhibitor	
p110β				
p110δ				
p110γ				
ZSTK474			Specific pan-PI3K inhibitor,	Cancer
p110α		0.016	no inhibition against mTOR and DNA-PK	
p110β		0.044		
p110δ		0.005		
p110γ		0.049		

Table 2: Novel PI3K inhibitors (See Ref 10)

2.5. PI3K pathway and CLL

2.5.1. PI3K signalling in normal B-cells

During normal B-cell development the PI3K pathway provides essential pro-survival signals at several crucial stages. It has been shown to be involved in VDJ joining, class switching, somatic hypermutation and selection of B-cells during B-cell receptor formation. The BCR itself can induce PI3K activation and PI3K is also crucial for the interaction of B-cells with the stroma. (24) Among the downstream targets are BTK, AKT and PDK-1 which link the pathway to PLCγ-, survival- and cell cycle signalling networks. It is therefore apparent that enhanced PI3K activation could lead to the expansion of B-cells and prolongation of their survival. There are several data from gene targeted mice demonstrating the importance of PI3K subunits for correct B-cell development. Since p110α and p110β deficient mice are embryonic lethal it will be hard to determine their roles but for p85α and p110δ it could be established that deficits result in a block in B-cell development emphasizing their importance for this cell type. (24)

2.5.2. The PI3K in CLL cells

As it has been established, CLL cells are highly dependent on survival signals provided by the tumour microenvironment. Bone marrow stromal cells can prevent the spontaneous apoptosis of CLL cells in vitro. Some of the pro-survival stimuli of these cells could be channelled through the PI3K pathway. It has been reported that fresh CLL cells exhibit all signs of a highly activated PI3K pathway which might be necessary for receiving the pro-survival stimuli from the microenvironment. (27) The interaction of CLL cells with the cells of the microenvironment is at least in part responsible for minimal residual disease and other resistance phenomena and is therefore an important target for therapy. Novel strategies apply for example a specific PI3Kδ-inhibitior which has already entered clinical phase I trials for potential use in CLL. (20, 25) It is therefore feasible that dissection of the role of PI3K in CLL will not only expand our understanding of the pathology but could also give impulses for therapeutic

applications of new drugs. It is also important to explore the pattern of expression of PI3K isoforms in CLL and their relevance as therapeutic targets.

Aims of this study:

In order to explore the role of the PI3K and its isoforms in the pathophysiology of CLL and its relevance as a therapeutic target, the major aims of this study are as follows:

1. To get insight into the activation status of the PI3-K pathway in CLL cells as compared to normal PBMCs and to analyze the expression of the key molecules in this pathway.

2. To determine whether the activation is regulated at the mRNA level, through protein synthesis or by post-translational modifications.

3. To identify which PI3K isoforms are expressed in CLL cells and which isoforms play a predominant role in the pathogenesis of CLL.

4. To test the effect of PI3K inhibitors which target the catalytic domain of PI3-K (p110 subunit isoforms) on the viability of CLL cells and other cellular events.

5. To identify the most effective PI3-K isoform-specific inhibitors which induce apoptosis in CLL cells and might therefore be important for designing new therapeutic concepts in CLL.

Work plan

1. In order to get insight into the activation status of the PI3-K pathway in CLL cells, the expression of PI3-K isoforms and their downstream target Akt and PTEN will be evaluated at the mRNA level using RT-PCR and at the protein level using western blotting approaches

2. The expression pattern will be compared between CLL patients and healthy individuals

3. The activation state of the downstream targets (Akt and PTEN) will be explored using phosphospecific antibodies against Akt and PTEN

4. To allow a larger scale screening process, FACS analysis using antibodies against PI3-K isoforms, Akt and PTEN will be established

5. To identify the most relevant PI3-K isoforms to the pathogenesis of CLL, pharmacological isoform-specific inhibitors will be tested and cell viability will be evaluated by Annexin V/PI staining

Results:

1. Expression of P110 isoforms on the mRNA level:

The expression of p110 isoform was studied in 15 CLL samples and 7 samples from healthy donors. (see Figure 7 A and B) RT-PCR analysis showed that CLL cells express all four isoforms of p110, the catalytic subunit of PI3K. There was a difference in the expression levels of the different isoforms in CLL cells compared to healthy donor cells. This difference was most prominent for the p110α isoform which was highly expressed in CLL cells at the mRNA level compared to healthy PBMCs. Although CLL cells express mRNA of all four isoforms, the amount of the p110δ isoform appears to be relatively lower in almost all CLL samples compared to the high expression of p110α and the expression of p110β and p110γ which ranges from moderate to high. Another difference between CLL patients and healthy donors was the relative high expression of p110γ in CLL cells. These results further strengthen the assumption that the PI3K pathway is very active in CLL cells and that its activity and regulation might differ from normal cells on several levels.

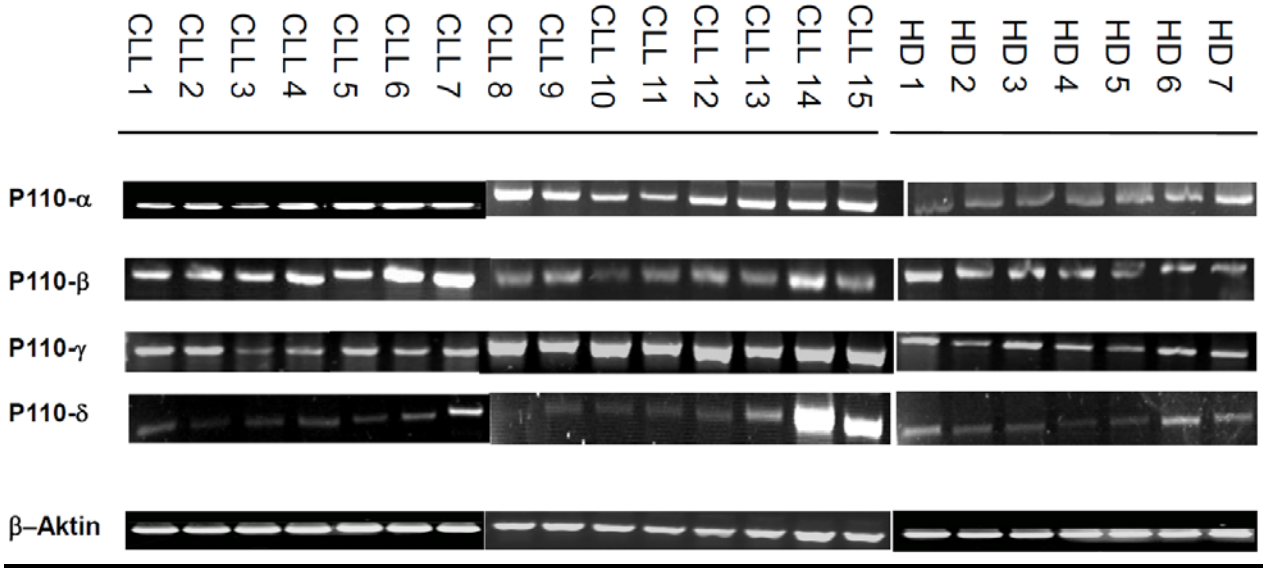


Figure 7A: *The mRNA expression of the four p110 isoforms in CLL and HD samples: RT-PCR analysis shows that all four PI3K-p110 isoforms are expressed in CLL cells. Compared to healthy PBMCs (HD), the expression of p110α and p110γ was significantly higher in CLL cells.*

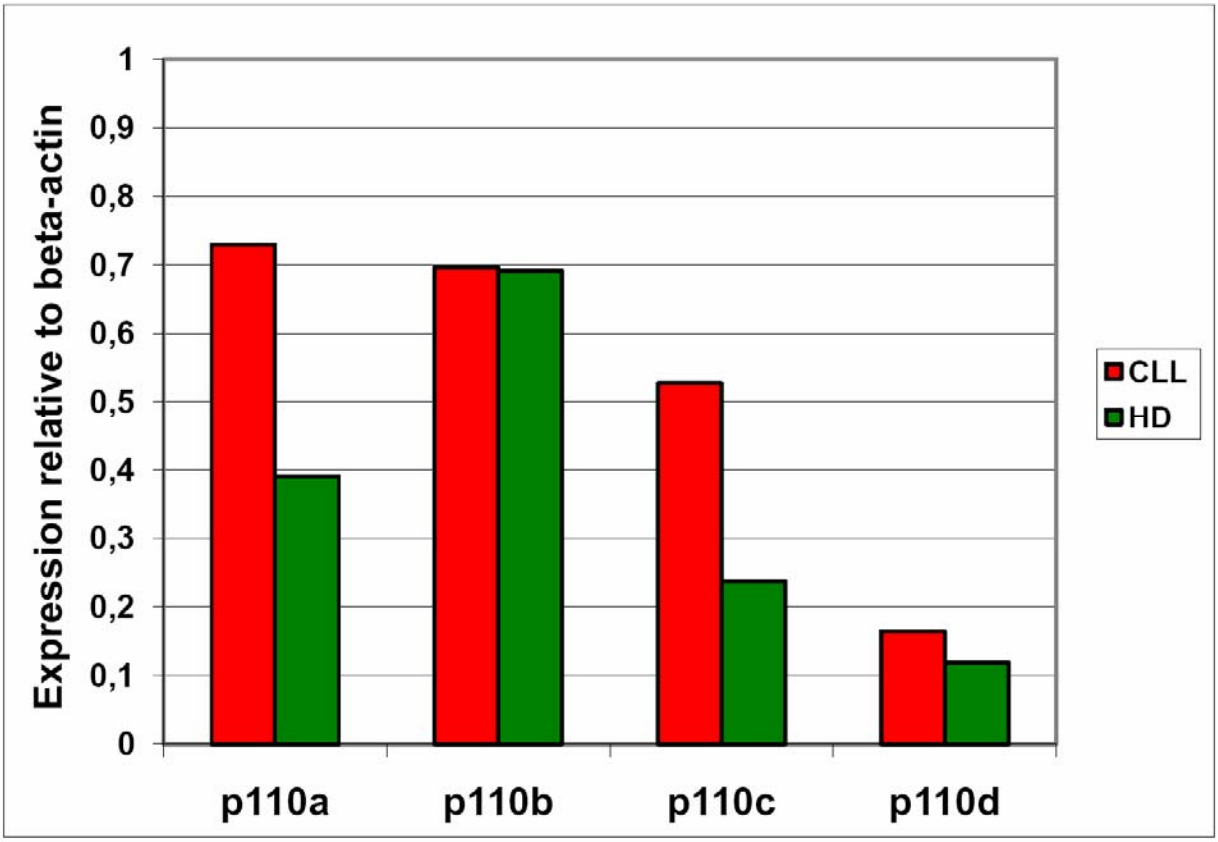


Figure 7B: *Densitometric analysis of the mRNA expression of the four p110 isoforms in CLL (n=15) and HD samples (n=7) compared to beta-actin: Data represent the mean values and confirm the high expression levels of p110α and p110γ in CLL cells (red) compared to healthy PBMCs (green). The isoform with the strongest expression relative to beta-actin was p110α. This isoform also shows the most prominent difference in expression between CLL and HD. The data correspond to the signals obtained in Figure 7A.*

2. Expression of p110 isoforms on the protein level:

Western blot analysis was performed to detect the expression of p110 isoforms on the protein level. As shown in Figure 8, the results support the data obtained from the RT-PCR (Figure 7). Isotype-specific antibodies generally showed increased expression of all p110 isoforms in CLL compared to healthy individuals. In particular, p110β and p110γ isoforms were highly expressed in CLL cells compared to HD cells. Importantly, the data confirm that CLL cells express all p110 isoforms and that these isoforms are highly expressed in CLL compared to HD samples.

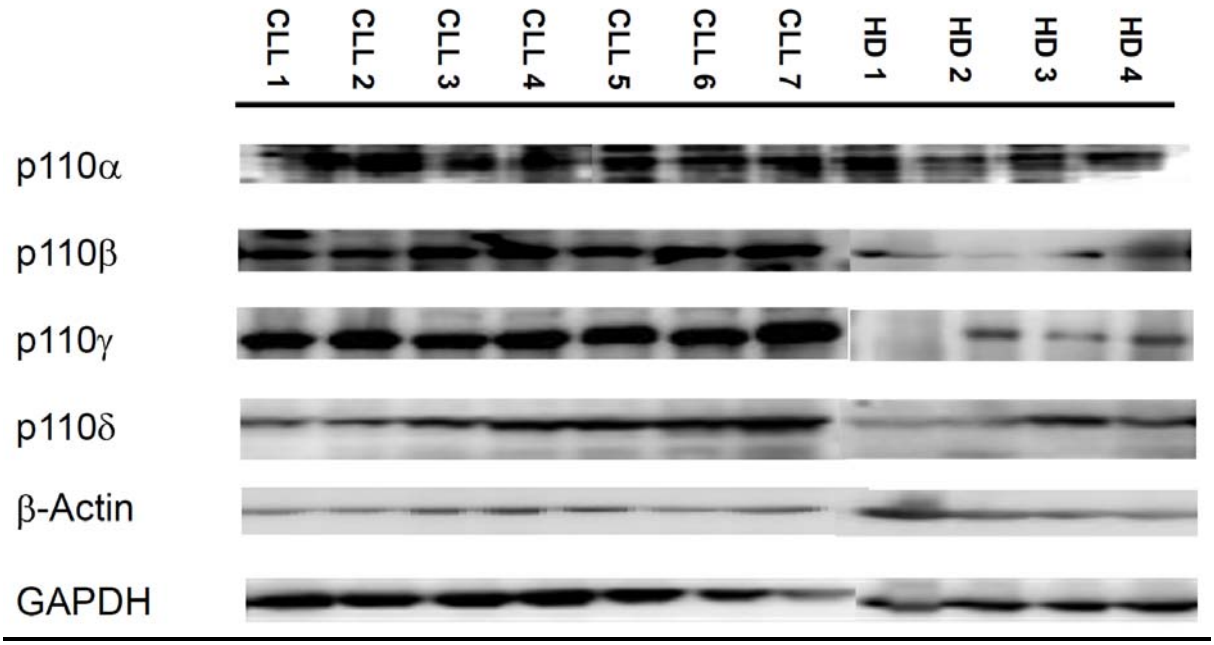


Figure 8: *The protein expression of the four p110 isoforms in CLL and HD samples: The figure shows western blot analsyis with four isotype-specific antibodies. All four isoforms are expressed in CLL cells and p110β and p110γ exhibit the most apparent difference in expression compared to HD samples.*

3. Detection of p110 isoforms by intracellular FACS staining:

In addition to western blot analysis, p110 expression was evaluated by intracellular staining and FACS analysis. The staining was performed using anti-CD19 antibodies to detect the B cell population in CLL and healthy individuals. The combination of anti-CD19 and anti-CD5 was used to detect the leukaemic B cells in CLL patients as well as the minor portion of CD19/CD5 positive cells which are detectable in healthy population. Since the antibodies used in the test were unconjugated we needed to add a second staining step. Negative controls were included by using isotype matched secondary antibody and by omitting the first antibodies. The analysis cut off levels for FL-1 signals were set to exclude any background staining or false positive signals. Gating was performed to focus on the living lymphocyte population. Expression levels were always calculated as percentage of either the total living PBMC population, living CD19 positive population or the CD5/CD19 double positive population.

In the following section, the FACS analysis data are presented as dot plots which depict a representative experiment of the CLL and the HD sample set. This is followed by diagrams which show the percentages of positive cells within the PBMC population in the upper row. The lower row shows a more detailed analysis with focus on the CD19 population and the CD5/CD19 population.

3.1. Total p110 intracellular staining:

The dot plots show a representative case of a staining of one CLL and one HD sample (see Figure 9A).

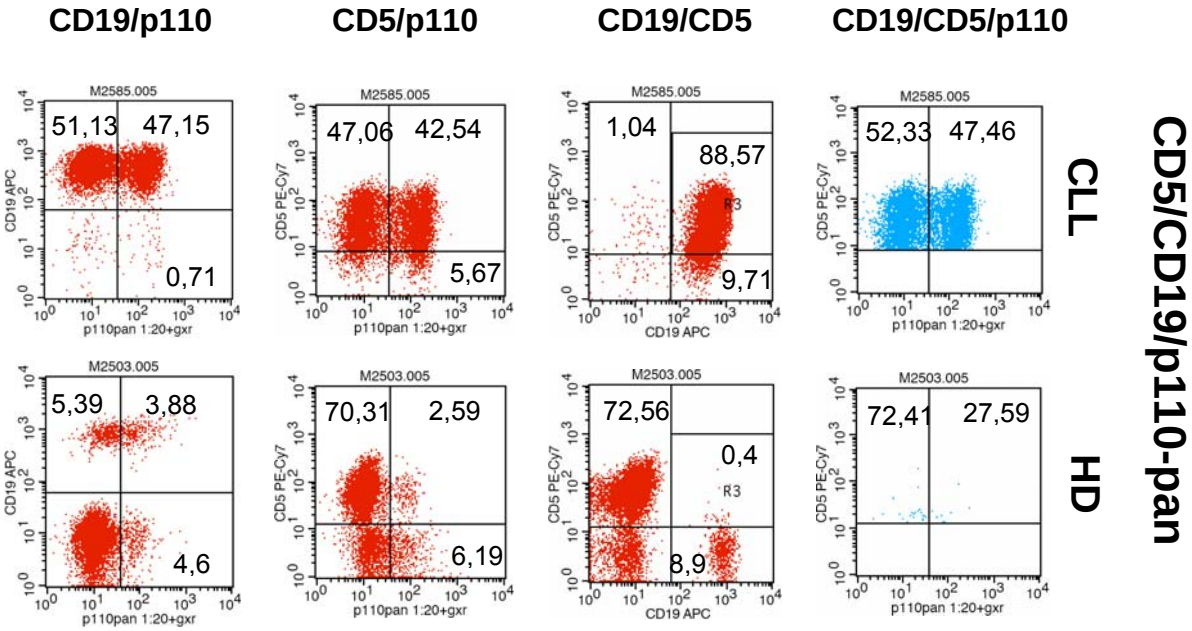


Figure 9A: *Detection of p110 with a pan-p110 antibody in CLL and HD cells: The figure shows a representative CLL and HD sample that were stained for intracellular p110 in combination with surface CD5 and CD19. The first three dot plots from the left side depict the double positive cells and the fourth dot plot demonstrates the triple positive cells (gated on CD5/CD19 in the third dot plot).*

Figure 9B, summarizes the data obtained from 5 CLL patients and 2 healthy individuals using an antibody which recognizes all four isoforms. The expression of p110 was found to be higher in the PBMC population of CLL samples compared to HD samples. In CLL the majority of p110 positive cells were also CD19 positive (45,62% CD19+/p110+) while only only 2,34% of the 5,58% p110 positive cells in HD samples were also positive for CD19. When calculated as percentage of the B-cell population, p110 is expressed in 47,45% of the CD19+ B cells and in 47,98% of the leukemic (CD5+/CD19+) cells in CLL samples. In HD samples 27,49% of the CD19+ cells express p110 pan and 21,2% of the small (0,4% of the PBMCs) CD5/CD19 population. This shows that in PBMCs obtained from CLL patients almost all of the p110 positive cells are leukemic cells (CD5+/CD19+) but not all of the CD5/CD19 double positive cells are positive for

p110 pan indicating that there might be two subpopulations of leukemic cells which differentially express p110.

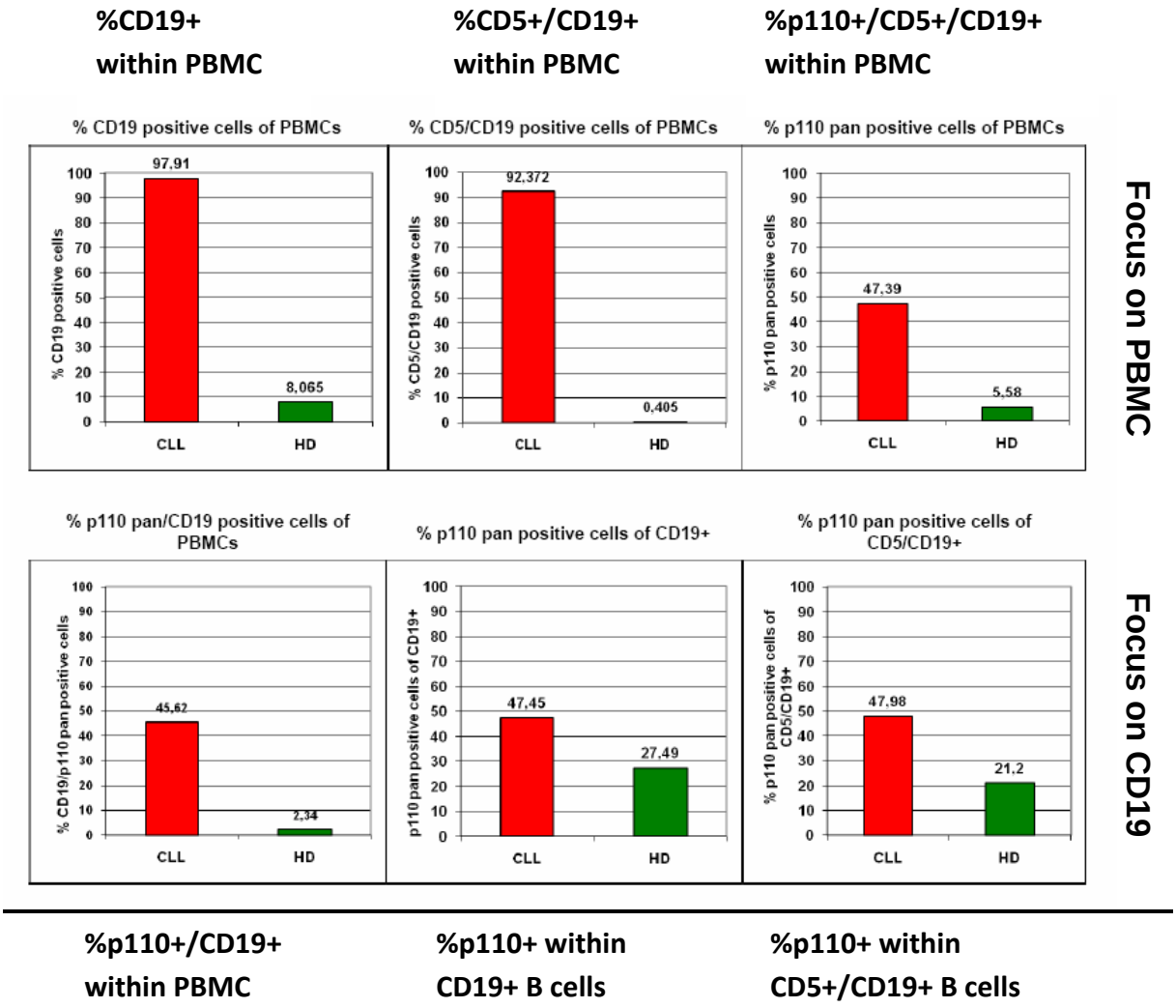


Figure 9B: *Total p110 expression in PBMC and B cell subpopulations in CLL and HD: The diagrams represent the mean values of the data obtained from of 5 CLL and 2 HD samples as evaluated by FACS analysis. The percentage of p110+ cells were first analysed within the total PBMC, within the CD19+ B cells and then within the CD19+/CD5+ leukaemic cells in CLL samples and the corresponding minor CD19+/CD5+ subpopulation within the B cells in the healthy individuals.*

These results show that p110 expression is high in the in PBMCs of CLL samples. However, when calculated as percentage of CD19 positive cells, it became clear that the expression of p110 was high within the leukaemic B-cell population and also within the very small CD5+/CD19+ population (about 0,4%) which was detectable in healthy individuals. The data suggest the significance of

PI3K expression in B cells in general and in the CD19+/CD5+ positive population in particular.

3.2. p110 alpha isotype intracellular staining:

Similar to p110 pan the expression of the p110 alpha isotype is higher in the PBMC population of CLL samples than in HD samples. Also within the CD19+ and the CD19+/CD5+ cell compartment the percentage of p110 is higher in CLL samples. About half of the leukemic cells express detectable amounts of p110 alpha. (see Figure 9 C and D)

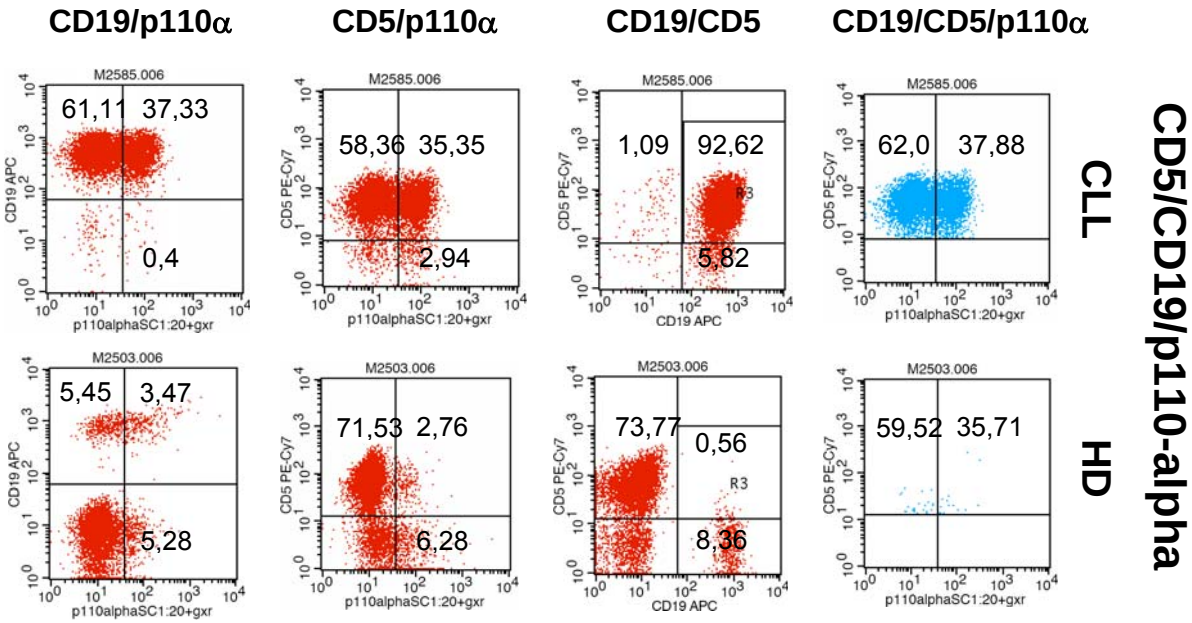


Figure 9C: *Detection of p110 with a α-isotype antibody in CLL and HD cells: The representative dot plots show the differences in expression of the p110α isoform in CLL and HD. The first three dot plots from left side depict the double positive cells and the fourth dot plot demonstrates the triple positive cells (gated on CD5/CD19 in the third dot plot).*

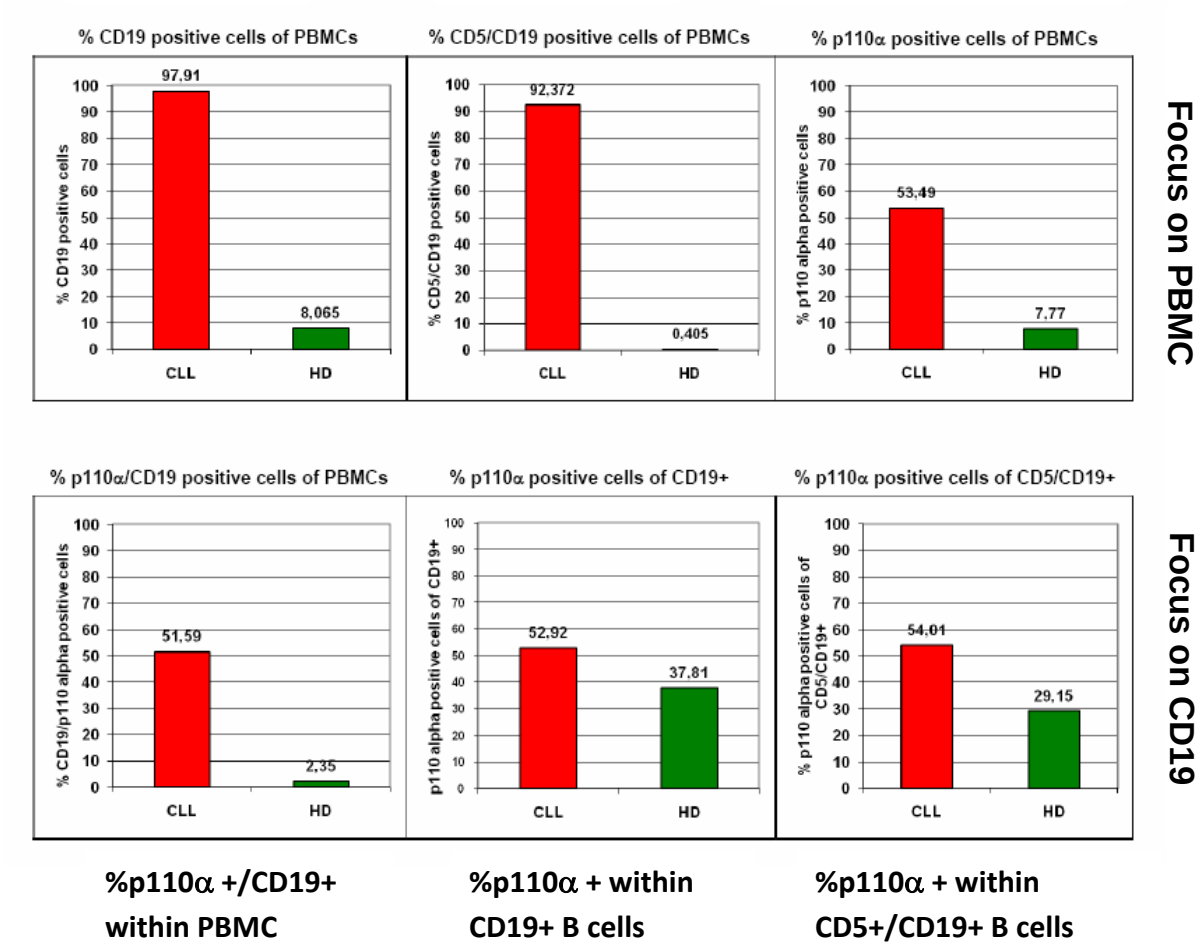


Figure 9D: *Detection of p110α in the different cell populations of CLL and HD cells: The figures show the mean values of p110α expression in the different cell populations (PBMC, B-cells, leukemic cells).*

3.3. p110 beta isotype intracellular staining:

The same observations could be made for the p110 beta isoform. The expression within the total population is much higher in CLL and also in the CD19+ and CD5+/CD19+ subpopulations, even though the difference is less dramatic in these. (see Figure 9 E and F)

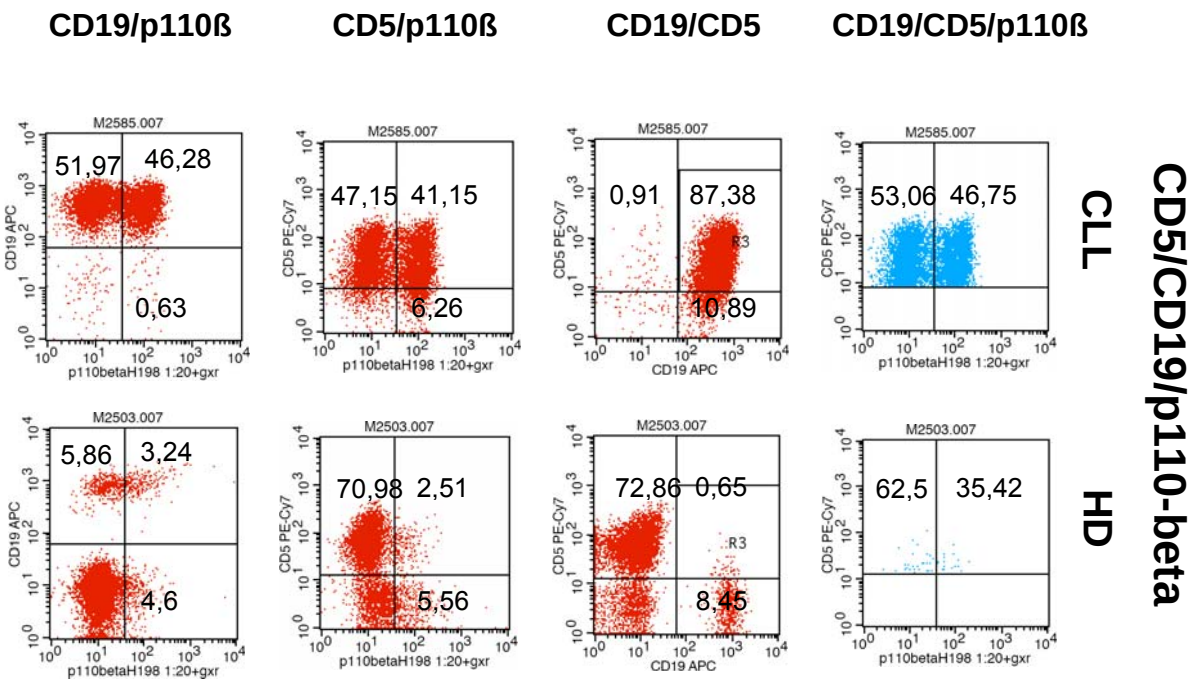
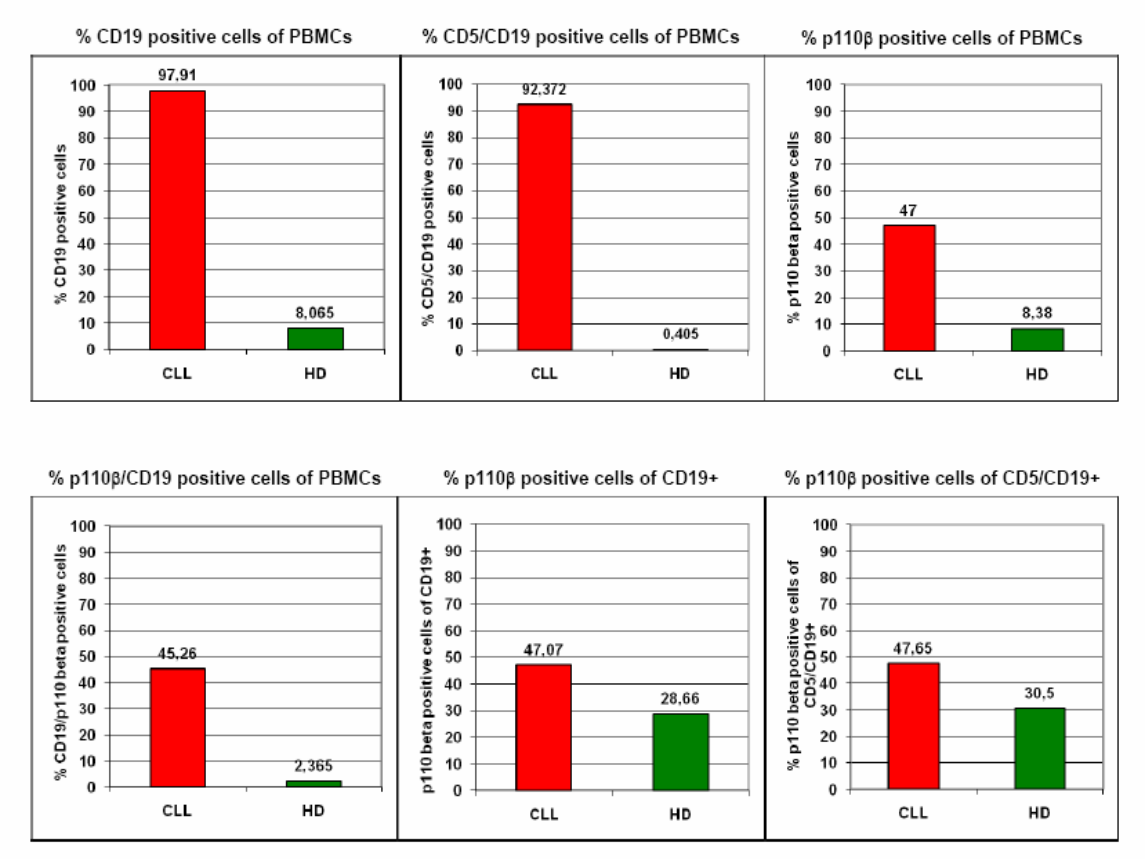


Figure 9E: *Detection of p110 with a β-isotype antibody in CLL and HD cells: Analogue to the previous figures, these plots depict the representative FACS staining of a CLL and a HD sample for p110β and reveal the differences in expression.*

%CD19+ within PBMC | **%CD5+/CD19+ within PBMC** | **%p110ß+/CD5+/CD19+ within PBMC**



Focus on PBMC

Focus on CD19

%p110ß +/CD19+ within PBMC | **%p110ß + within CD19+ B cells** | **%p110ß + within CD5+/CD19+ B cells**

Figure 9F: *Detection of p110β in the different cell populations of CLL and HD cells: The diagrams represent the mean values of the FACS analysis of 5 CLL and HD samples for p110β expression. The lower row shows the detailed analysis of p110β expression within the different cell populations.*

3.4. p110 gamma isotype intracellular staining:

The same analysis was done for the p110 gamma isoform and results looked very similar to the previous ones. P110 gamma expression was higher in PBMCs of CLL cells and also within the two subpopulations the percentage of p110γ positive cells was above the values for healthy samples. (see Figure 9 G and H)

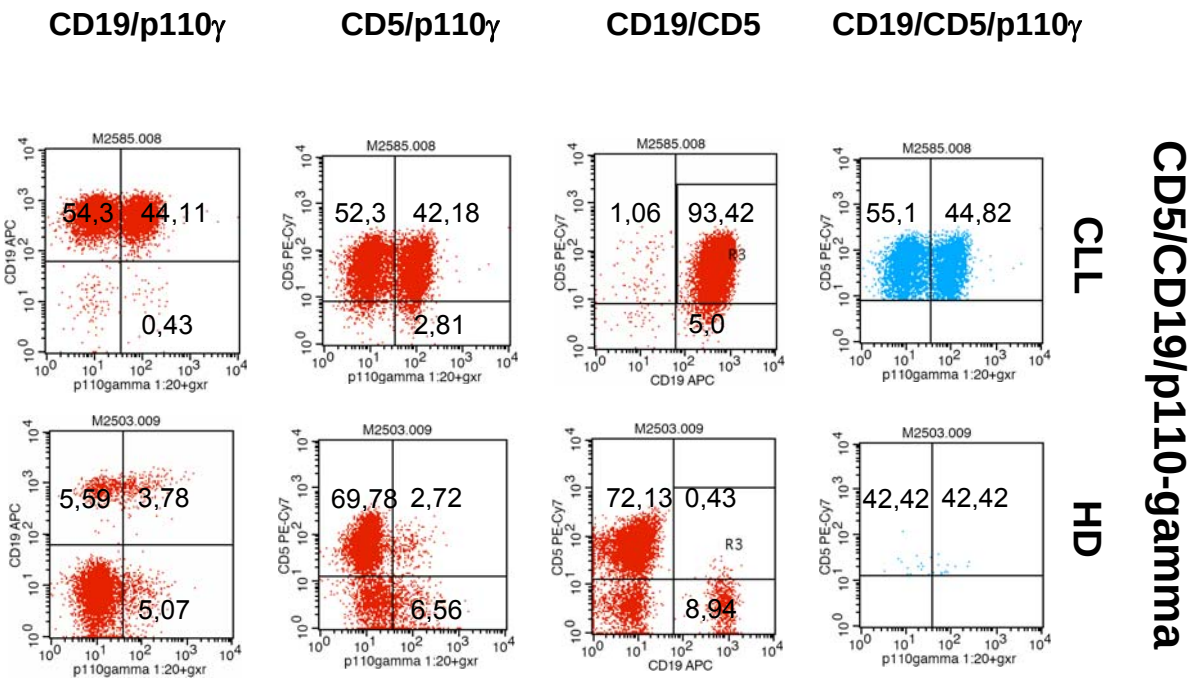
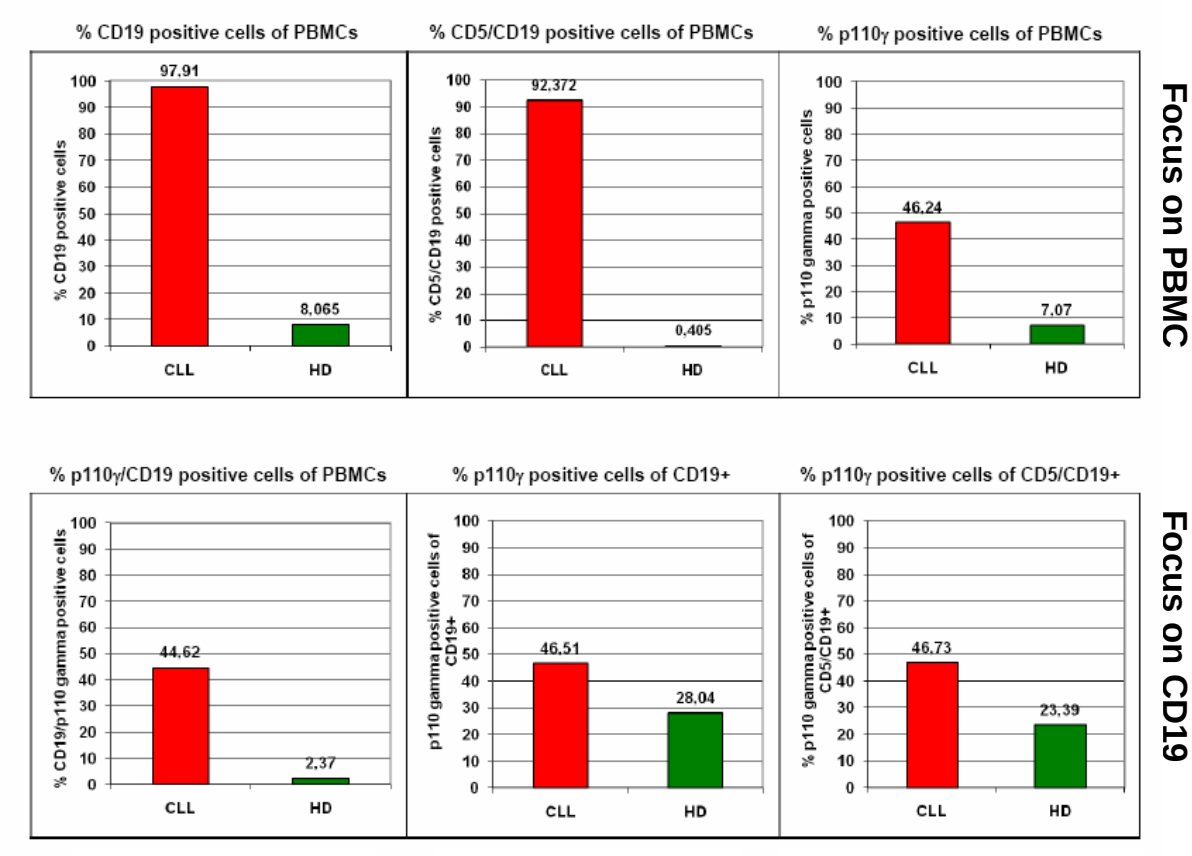


Figure 9G: *Detection of p110 with a γ-isotype antibody in CLL and HD cells: As before, the upper row shows expression of p110γ in a representative CLL sample and the lower row in a HD sample.*

%CD19+ within PBMC | **%CD5+/CD19+ within PBMC** | **%p110γ+/CD5+/CD19+ within PBMC**



%p110 γ +/CD19+ within PBMC | **%p110 γ + within CD19+ B cells** | **%p110 γ + within CD5+/CD19+ B cells**

Figure 9H: *Detection of p110γ in the different cell populations of CLL and HD cells: Percentages of p110γ positive cells within the PBMC, B-cell and leukemic population are depicted for CLL (red) and HD samples (green).*

3.5. p110 delta isotype intracellular staining:

Finally, the results for p110 delta confirmed that all four isoforms are expressed in CLL cells as it has also been shown by RT-PCR and western blotting. Interestingly, p110 delta was the isoform that showed the highest expression within the PBMC population of HD samples (16,24%) compared to the other isoforms (7-8%). Still, expression was higher in CLL cells independent of the cell compartment. (see Figure 9 I and J)

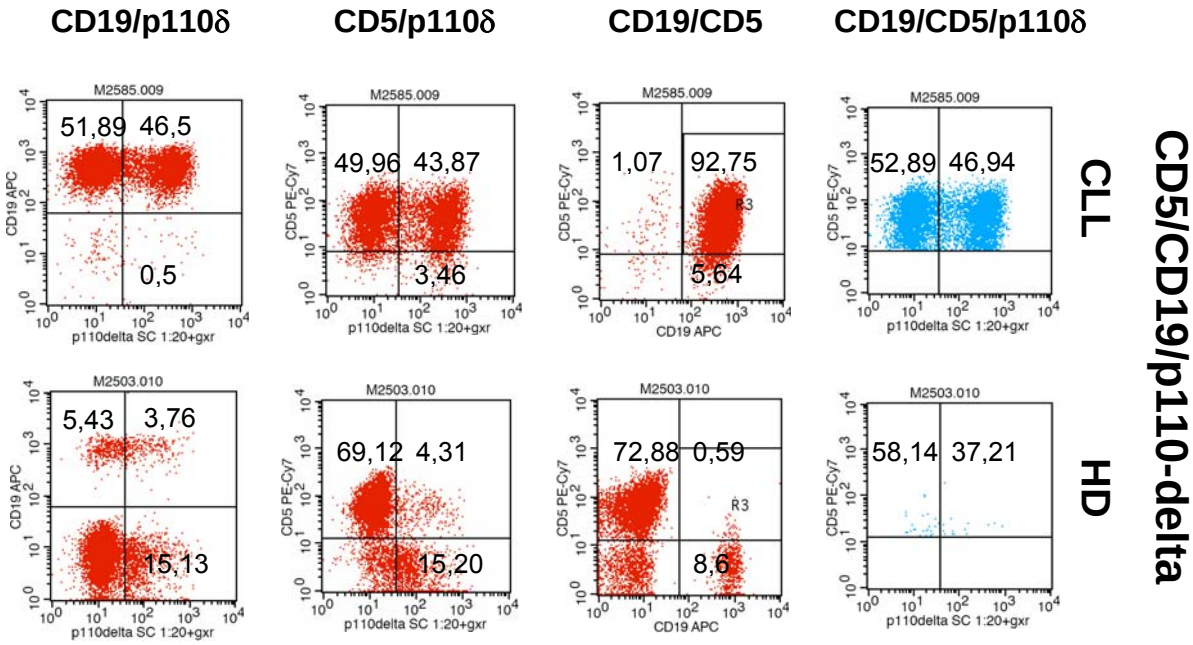


Figure 9I: *Detection of p110 with a δ-isotype antibody in CLL and HD cells: These example CLL and HD cases demonstrate once more the differences in p110 expression, this time for the delta isoform.*

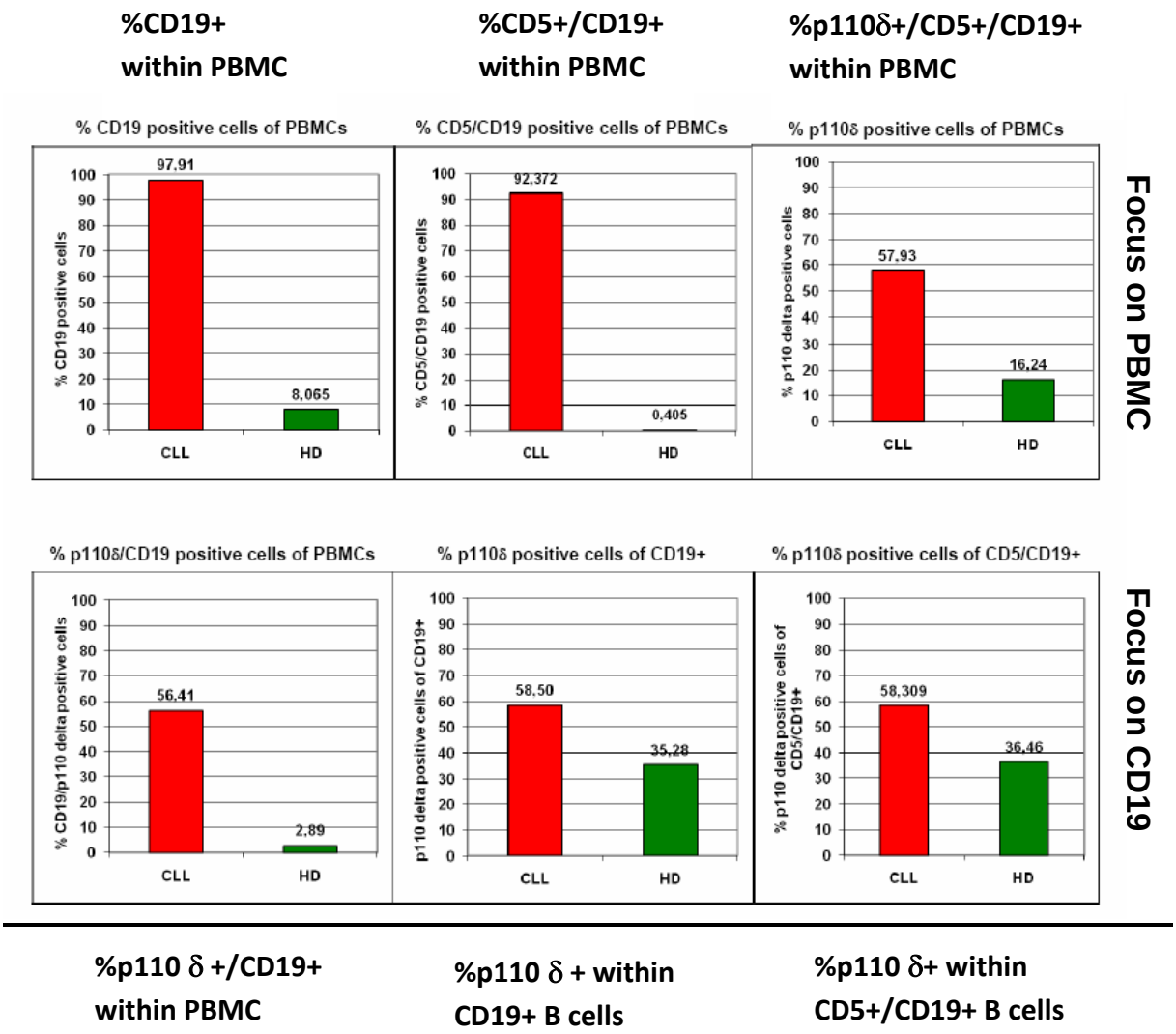


Figure 9J: *Detection of p110δ in the different cell populations of CLL and HD cells: The mean values reveal again the two different expression patterns in CLL and healthy individuals. Of all of the four isoforms, the δ isoform shows the highest expression in the healthy PBMC population.*

Taken together, the data from the mRNA analysis and FACS staining confirmed that CLL cells express all four p110 isoforms and that p110 isoforms could be detected in a large proportion of CD5+/CD19+ cells in CLL samples and also in the CD5+/CD19+ population of healthy PBMCs but to a lesser extent. However, the difference between the expression levels of the isoforms could not be observed as clearly as by western blot or as on the mRNA level. It seemed that expression levels of the four isoforms were more or less comparable. This suggests that all four isoforms may play important roles in the pathophysiology of

CLL which is not surprising since the downstream signalling of the distinct isoforms is believed to be neither completely independent nor completely redundant.

4. Expression of PI3K pathway-associated molecules on the mRNA level:

As demonstrated by Figure 10, mRNA expression of AKT was variable in CLL and healthy samples. Although PTEN mRNA expression was without obvious differences between CLL and HD samples the signal was strong in all samples which is in accordance with reports that PTEN expression is not lost in CLL cells.

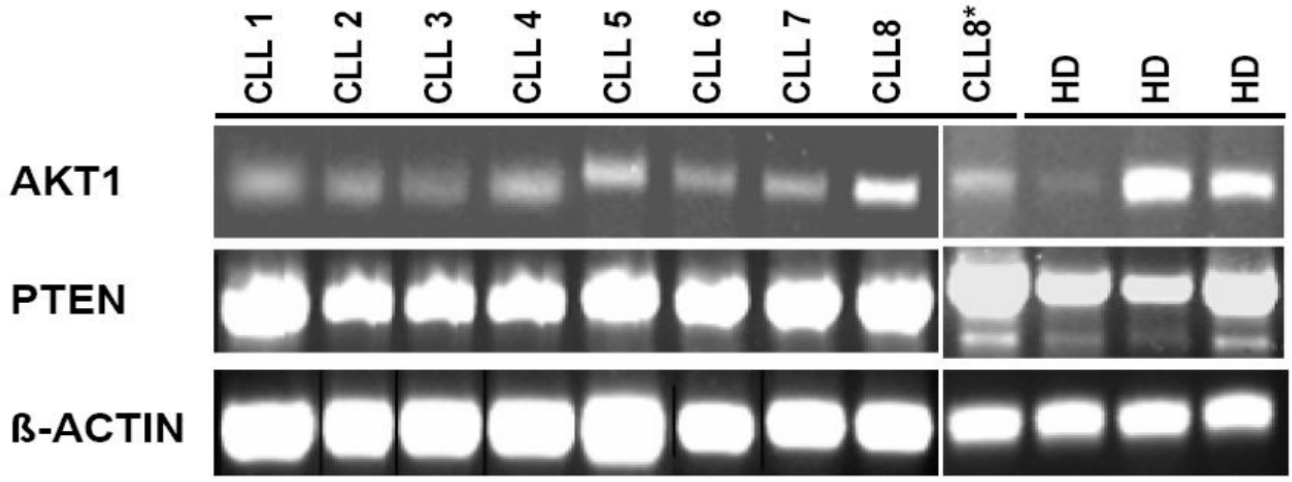


Figure 10: *AKT and PTEN mRNA expression in CLL and HD samples: This figure shows RT-PCR results of 8 CLL and 3 HD samples indicating the variable expression levels of AKT and the comparable expression levels of PTEN.*

5. AKT and PTEN expression and activation on the protein level:

Since Akt is a central downstream target of PI3K, and PTEN is the natural inhibitor of PI3K, the expression of AKT and PTEN at the protein level was determined by western blotting and by intracellular FACS staining. As a first test we analysed 9 CLL cases in comparison to 3 healthy donors (HD). The phosphorylation status of AKT and PTEN was also evaluated. As shown in Figure 11A, total AKT expression was comparable between CLL and HDs. In one CLL and one HD sample AKT was almost undetectable. Interestingly, the pS473Akt-antibody revealed a very strong phosphorylation of this residue. Except for the two cases with very low AKT there is only one CLL case that doesn't seem to have a high level of phosphorylated AKT.

As shown in Figure 11B, there was no significant difference in the total amount of PTEN expression among the CLL group and the HD group but a major difference in the phosphorylation status could be observed. While the pS380 PTEN antibody can detect significant amounts of phosphorylated (inactive) PTEN in the CLL lysates, the HD lysates show a weaker signal.

As the results show, the phosphorylation status of PTEN shows a significant difference in CLL patients and healthy persons which indicates some kind of inactivation of this negative regulator of the PI3K signalling. Accordingly, also the phosphorylation of Akt at Ser473 was significantly increased in CLL samples which points towards an enhanced activation of this downstream target of PI3K. These findings of the western blot analysis could be further confirmed by the intracellular FACS staining performed in the freshly isolated PBMCs from CLL patients and healthy individuals. These data fit our assumption that PI3K plays an important role in the physiology of the CLL cells.

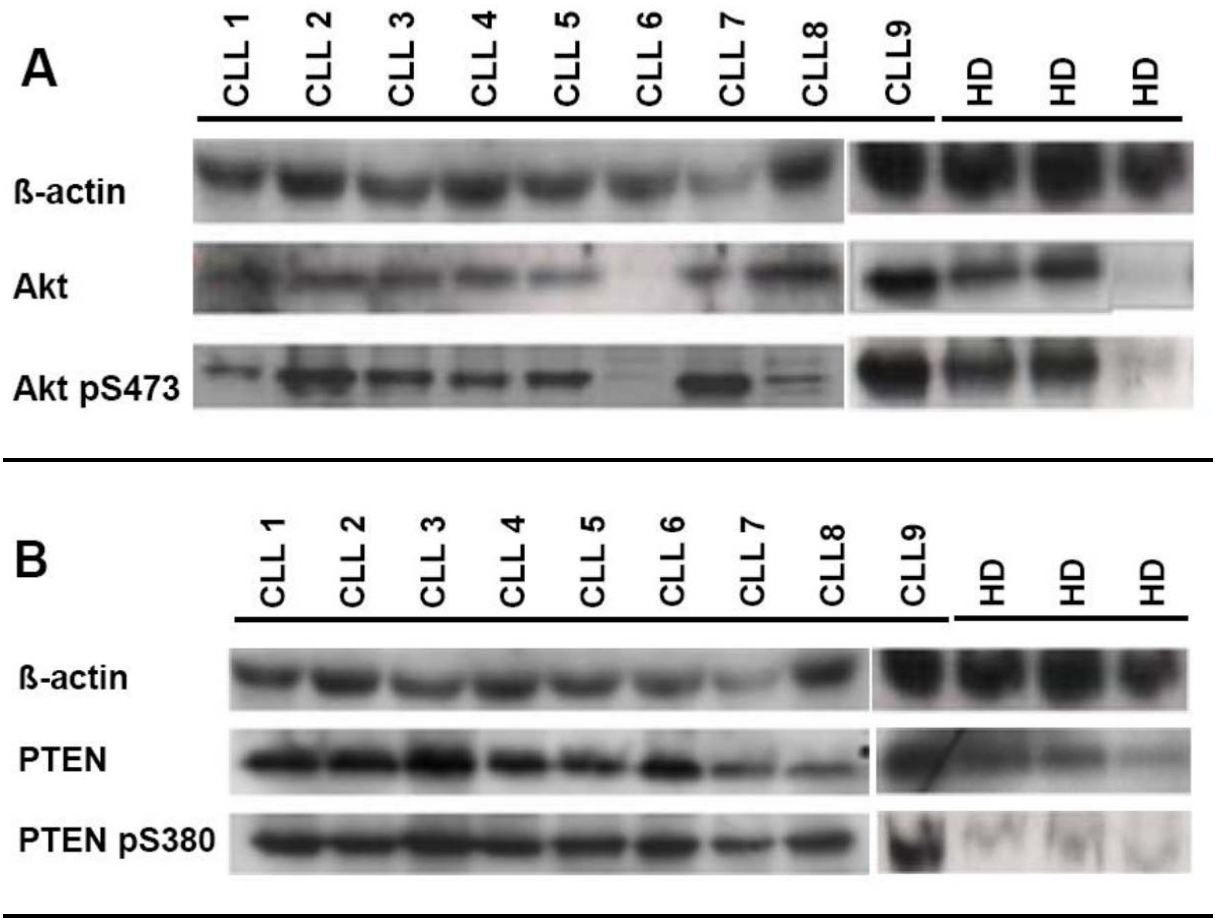


Figure 11: *Phosphorylation status of PTEN and AKT in CLL and healthy cells.*

6. Detection of AKT and PTEN by intracellular FACS staining:

To demonstrate that the pathway components are not only highly expressed in the CLL cells but also highly activated, we performed intracellular FACS staining with phospho-specific antibodies combined with surface staining for the CLL markers CD19/CD5. As the results show all the downstream players of the PI3K pathway that were analysed confirmed the proposed high activation status of that pathway. The dot blots show a representative of several experiments that were conducted to demonstrate the differences in expression of the PI3K pathway components between healthy PBMCs and CLL cells. The diagrams follow a pattern of increasing focus on the CD19 and the CD19/CD5 subpopulation, respectively.

6.1. AKT intracellular staining:

Intracellular FACS analysis was done for AKT (see Figure 12 A and B) and its phosphorylated forms at the residues S473 (see Figure 12 C and D) and T308 (see Figure 12 E and F). Again it seems that there is a significant difference between CLL cells and normal PBMCs concerning the expression of AKT.

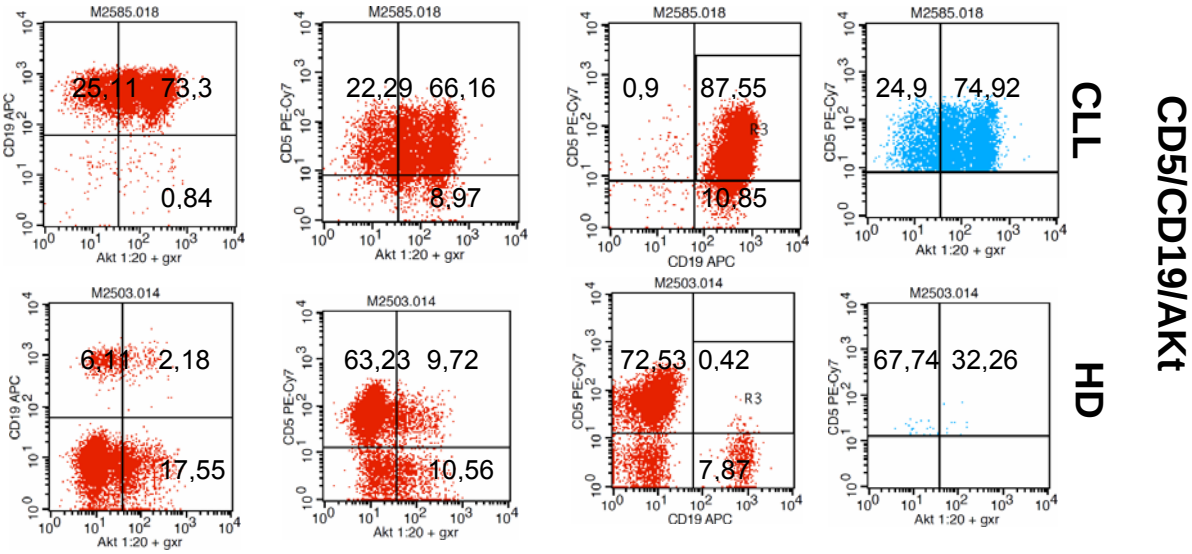


Figure 12A: *Detection of intracellular AKT levels in CLL and HD: These dot plots represent an example CLL case (upper row) and one HD (lower row). Both were analysed by intracellular FACS staining for AKT in combination with surface CD5 and CD19.*

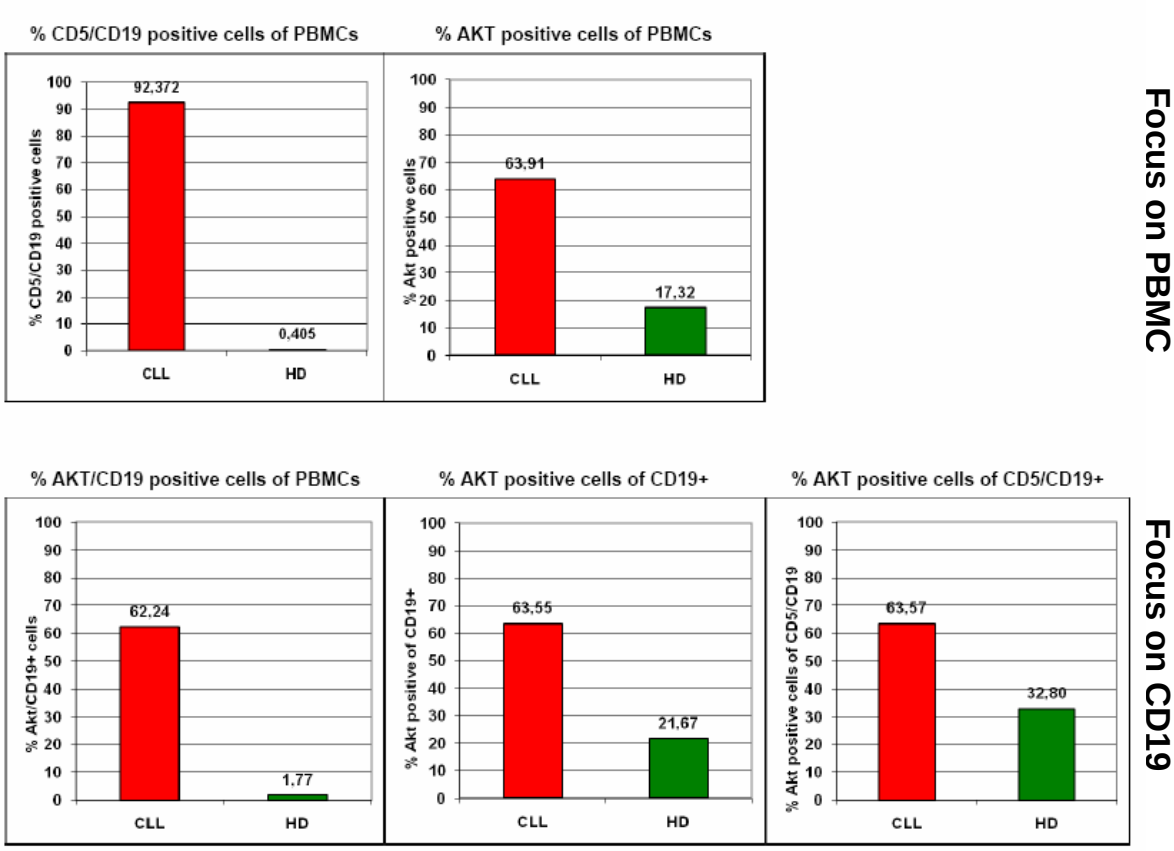


Figure 12B: *Detection of intracellular AKT levels in the different populations of CLL and HD: The diagrams show the mean values of 5 CLL and 2 HD samples that were processed like the example in Figure 12A. The first two upper figures give an impression about the percentage of CD5/CD19+ cells and the expression of AKT in the total PBMC population. The next three figures in the lower row show the co-expression of AKT and CD19 (left) and the expression of AKT within the B-cell (middle) and CD5/CD19-population (right).*

6.2. pAKT intracellular staining:

The CLL cells exhibited high levels of phosphorylated AKT in comparison to the healthy PBMCs. Even though, it should be noted that within the small population of CD5+/CD19+ cells in the healthy individuals the relative percentage of AKT and pAKT positive cells as well as pPTEN positive cells was increased compared to the whole population of PBMCs which could indicate that this population has a higher activation level of the PI3K pathway even in healthy individuals and that this could be a characteristic of this particular population.

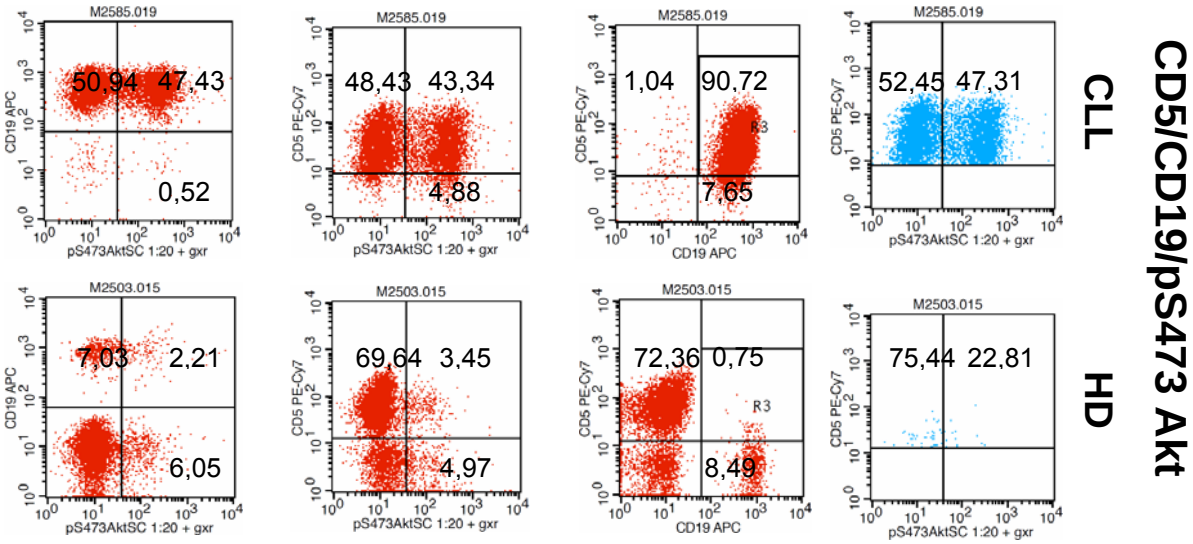


Figure 12C: *Detection of phosphorylation of AKT at S473 in CLL and HD: The representative dot plots show the differences in AKT phosphorylation between CLL and HD cells.*

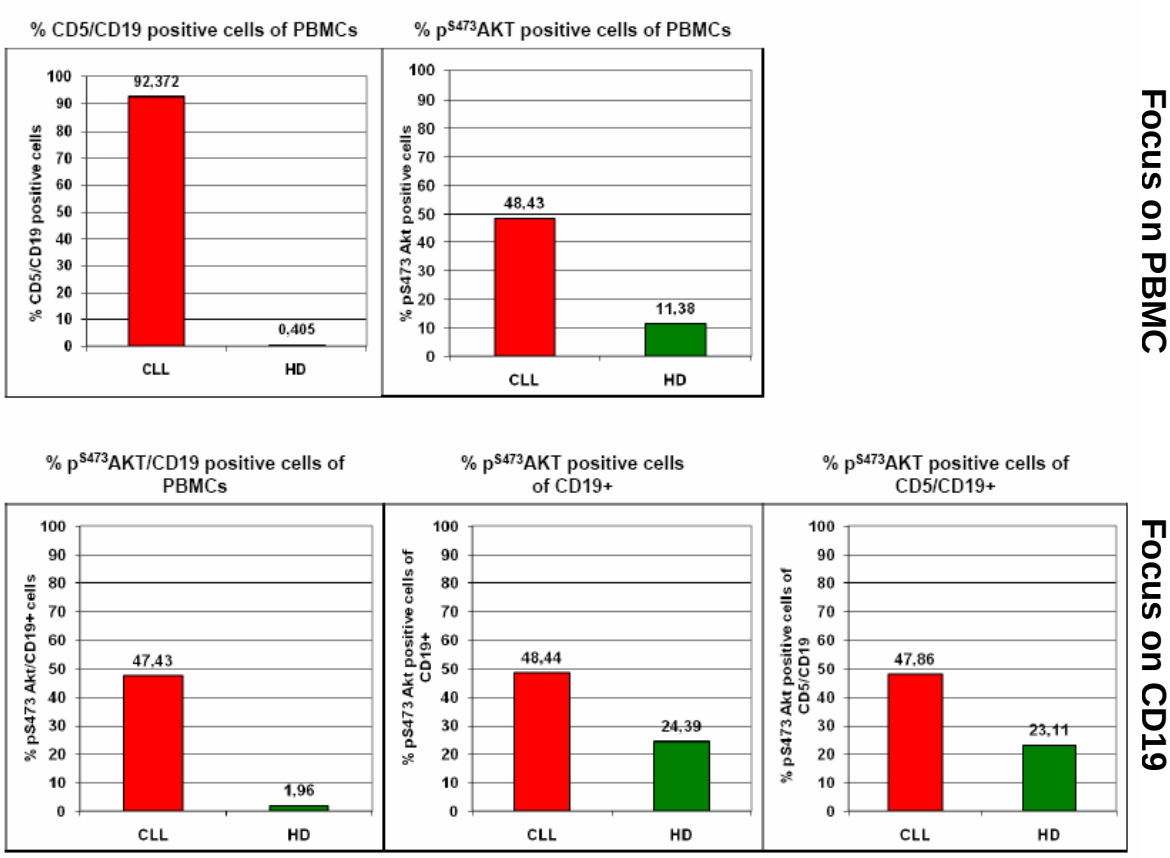


Figure 12D: *Detection of intracellular pS473 AKT levels in the different populations of CLL and HD: Phosphorylation of AKT at site S473 is higher in CLL cells, even within the small CD5/CD19+ cell compartment.*

Figure 12 C and D show the expression of pS473 AKT which is about four times higher in the PBMC population of CLL samples but only twice as high as in HD samples when looking within the CD5+/CD19+ population.

As shown in Figure 12 E and F, AKT is also heavily phosphorylated at T308 which could be demonstrated by two antibodies from different companies. The similar percentages of positive cells obtained from these two stainings underline the specificity of the method.

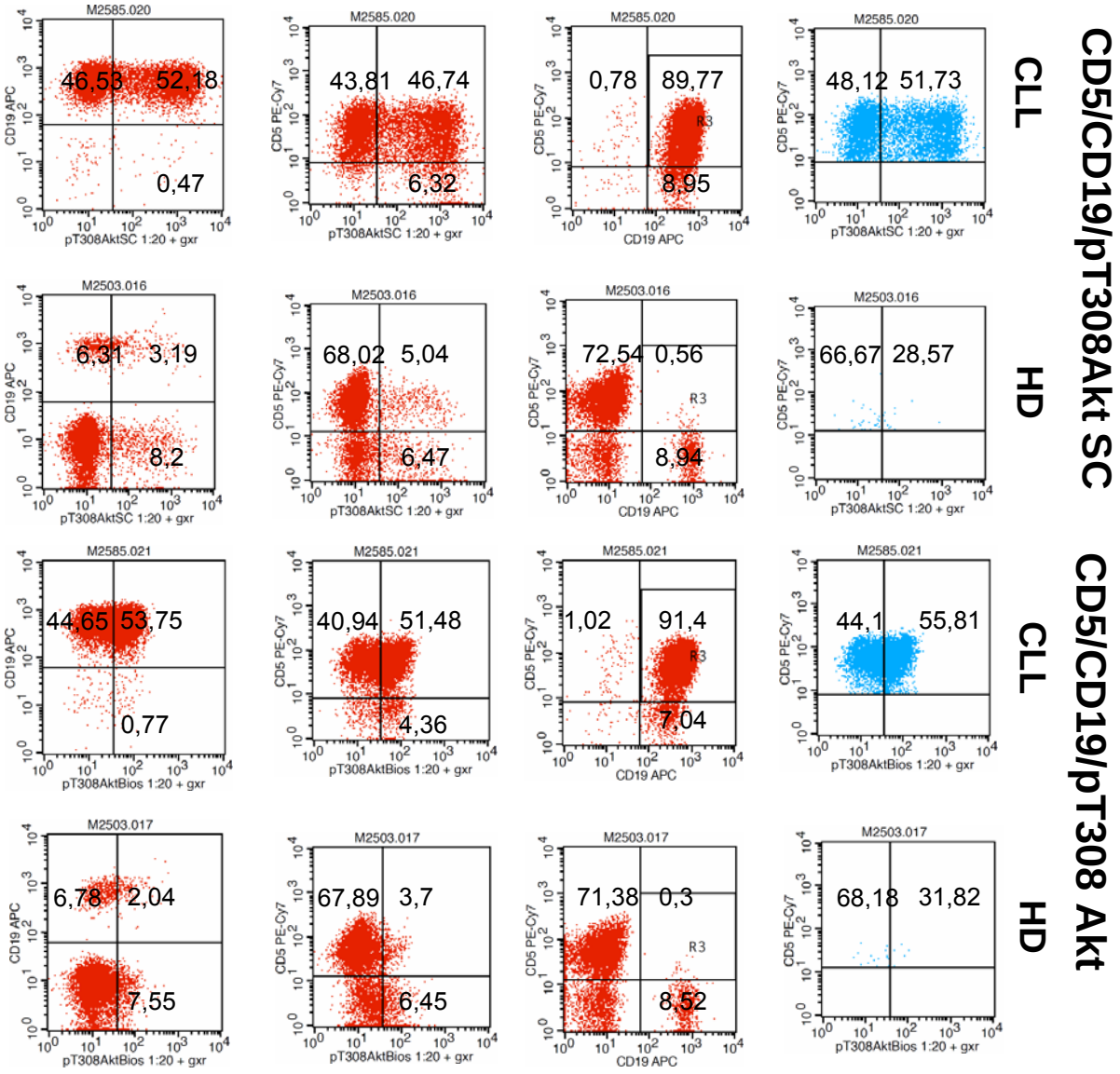


Figure 12E: *Detection of phosphorylation of AKT at T308 in CLL and HD by two different phospho-specific antibodies: Staining with two different pT308 specific AKT antibodies led to similar results and percentages of positive cells. Both showed the increased phosphorylation of AKT at T308 in CLL cells.*

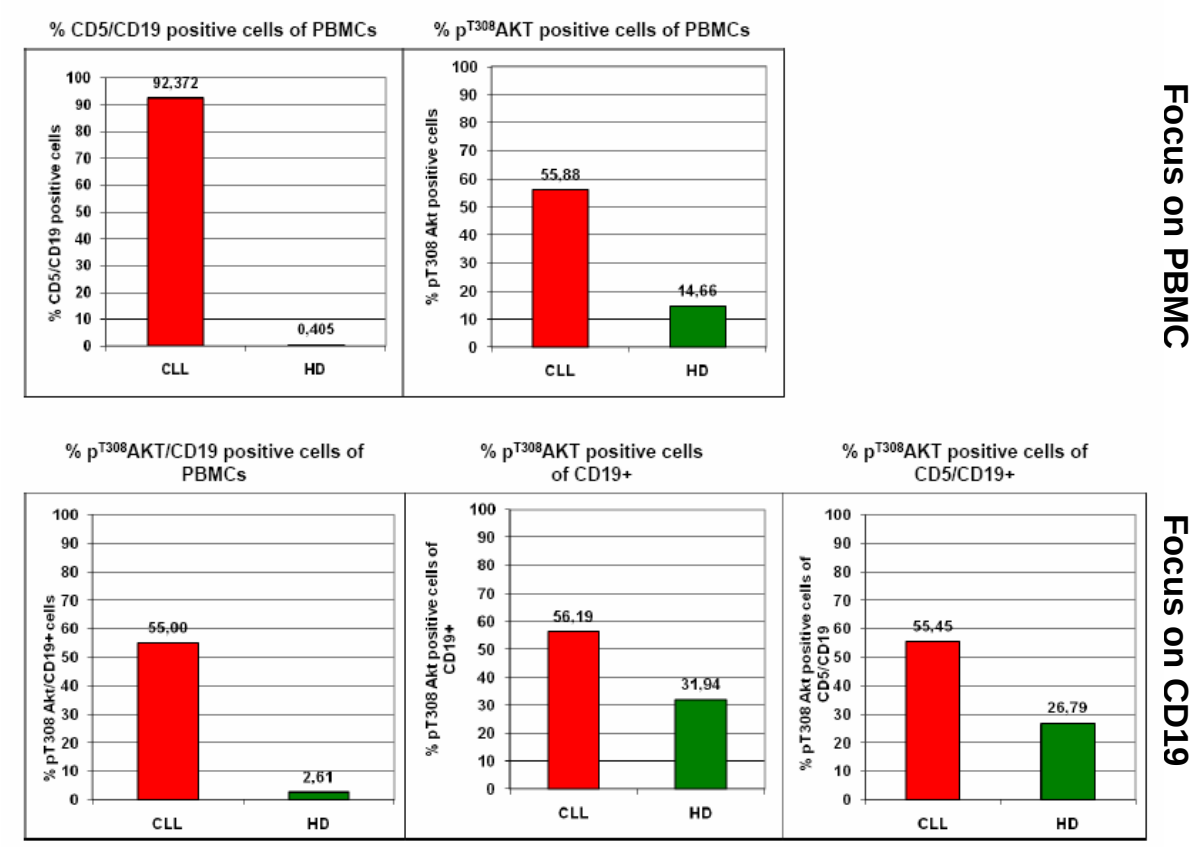


Figure 12F: *Detection of intracellular pT308 AKT levels in the different populations of CLL and HD: Expression of pT308 AKT is two to three-fold increased within the separate cell populations in CLL samples.*

6.3. Total PTEN intracellular staining:

A similar analysis was performed for PTEN and its phosphorylated forms. Interestingly PTEN expression was 2-3 times higher in CLL samples than in HD samples. (see Figure 13 A and B) The rather low percentage of PTEN positive cells in the HD sample that would not be expected for a tumorsuppressor could be explained by our cut off that was determined by the background of the secondary antibody. Therefore the expression of PTEN in these cells should better be considered low or below cut off than absent. The higher expression of PTEN in the CLL sample could be explained by a reactive process that promotes expression of the molecule that counteracts the very active PI3K pathway but becomes inactivated by phosphorylation.

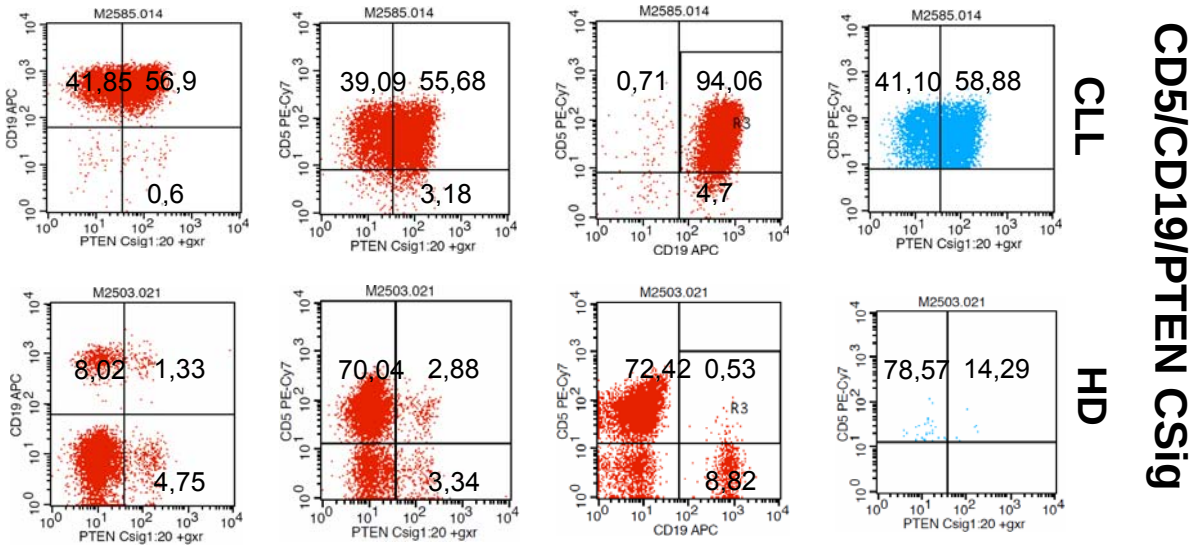


Figure 13 A: *Detection of intracellular PTEN levels in CLL and HD samples: The dot plots show a FACS staining with an antibody directed against total PTEN.*

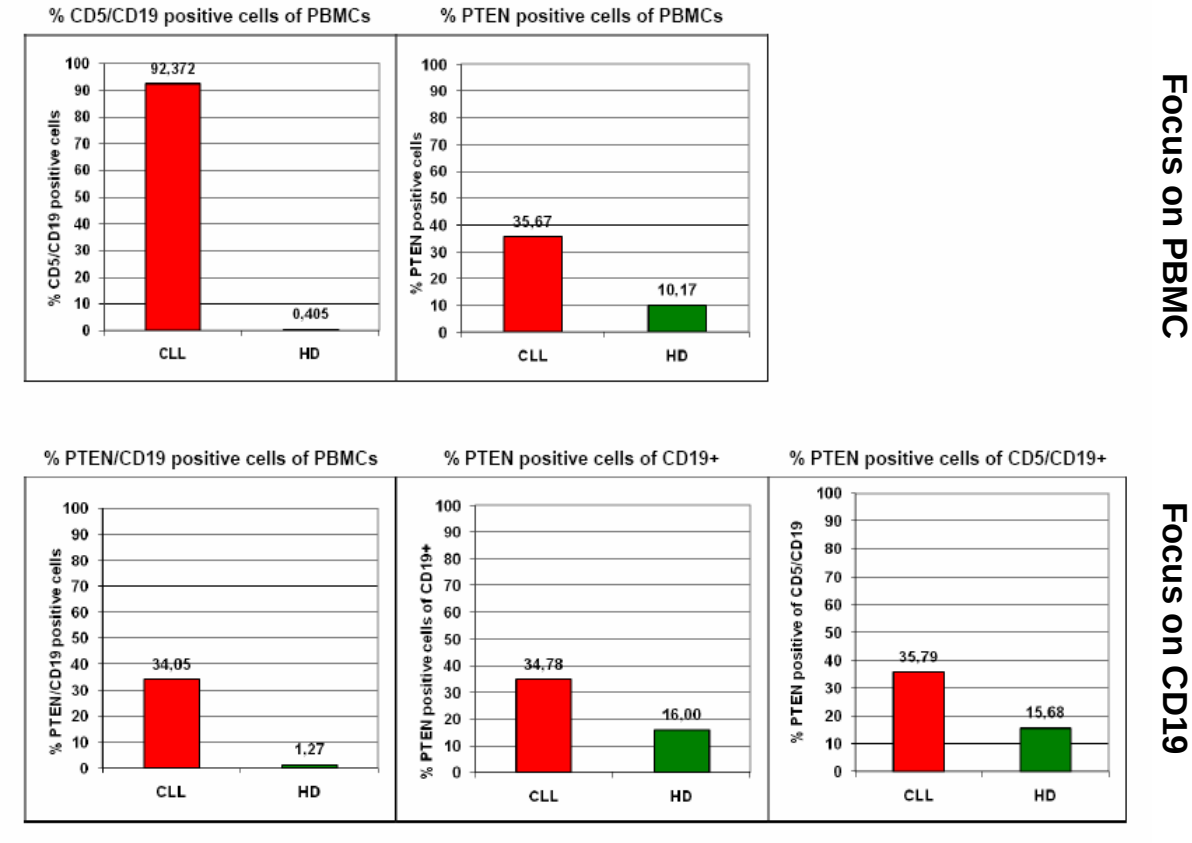


Figure 13 B: *Detection of intracellular PTEN levels in different cell populations of CLL and HD samples: The mean values of PTEN expression within the separate compartments show a paradox effect: PTEN expression is stronger in CLL cells.*

6.4. pPTEN intracellular staining:

The comparison between the CLL samples (n=5) and the healthy donors (n=2) shows that PTEN is heavily phosphorylated at all three residues analysed in the tail domain in CLL cells but not to the same extent in healthy PBMCs. A large proportion of the CD5+/CD19+ cells which can be considered as CLL cells have phosphorylated PTEN. (see Figure 13 C-H) There is usually a very small population of CD5+/CD19+ cells in healthy individuals which could also be detected by this method.

In detail, three different antibodies were used to show heavy phosphorylation of the PTEN tail domain which indicates a decrease of PTEN function as a negative regulator of the PI3K pathway. One detects only phosphorylation at the Ser380 residue (see Figure 13 C and D), one at Ser385 (see Figure 13 E and F) and the third detects phoshporylation at Ser380/Thr383/Ser385 (see Figure 13 G and H). Figure 13 C and D show that PTEN phosphorylation at Ser380 is about twice as strong in PBMCs of CLL cells than in HD cells.

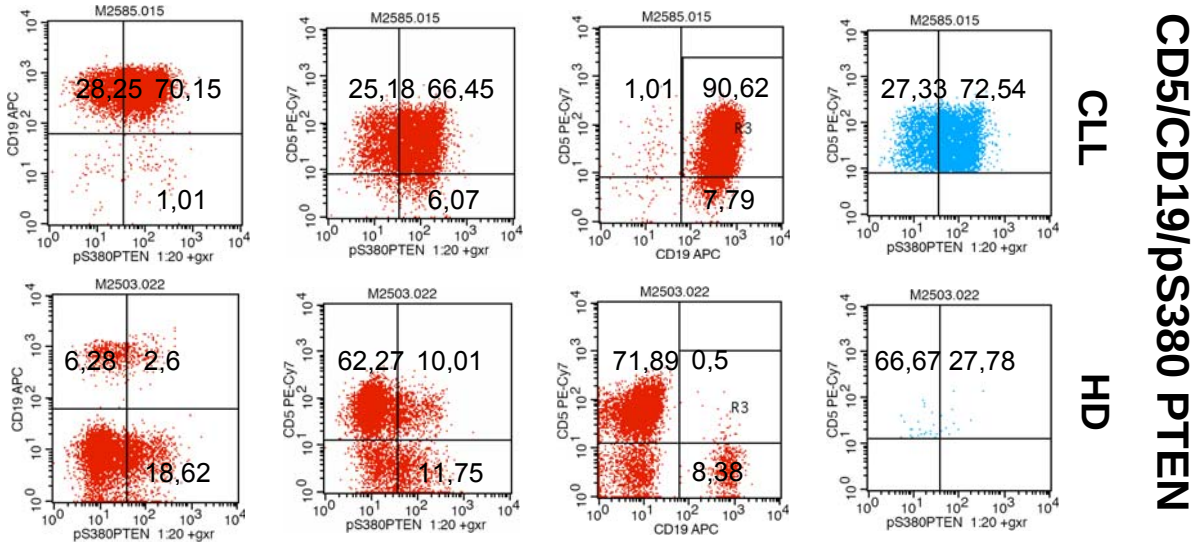


Figure 13 C: *Detection of phosphorylation of PTEN at S380 in CLL and HD samples: FACS analysis with a phospho-specific PTEN antibody directed against S380 shows a higher grade of PTEN phosphorylation in CLL cells.*

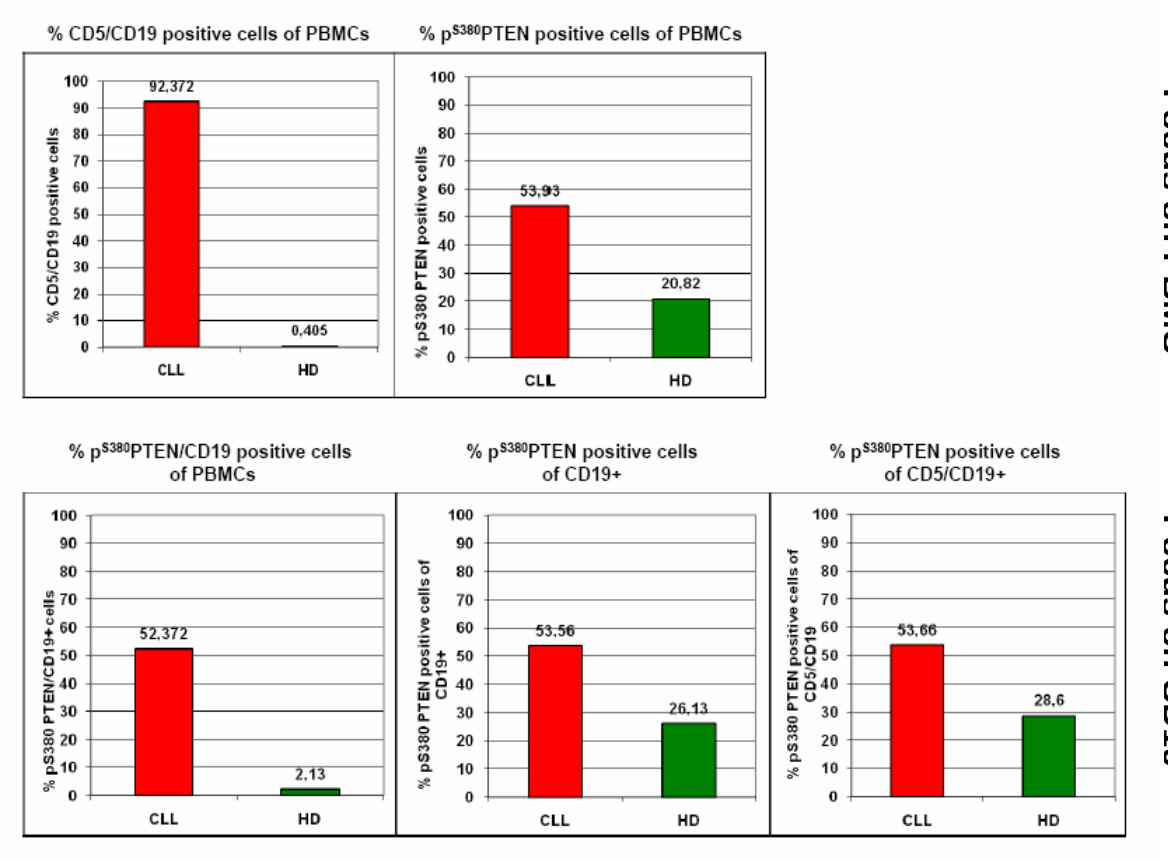


Figure 13 D: *Detection of intracellular pS380 PTEN levels in different cell populations of CLL and HD samples: Within all three cell compartments analysed (PBMC, B-cell and CD5/CD19) phosphorylation of PTEN is about two times higher in CLL samples.*

Figure 13 E and F demonstrate again the heavy phosphorylation of PTEN, this time on the S385 residue. Interestingly, phosphorylation of this residue is comparable within the CD5+/CD19+ population of CLL and HD samples.

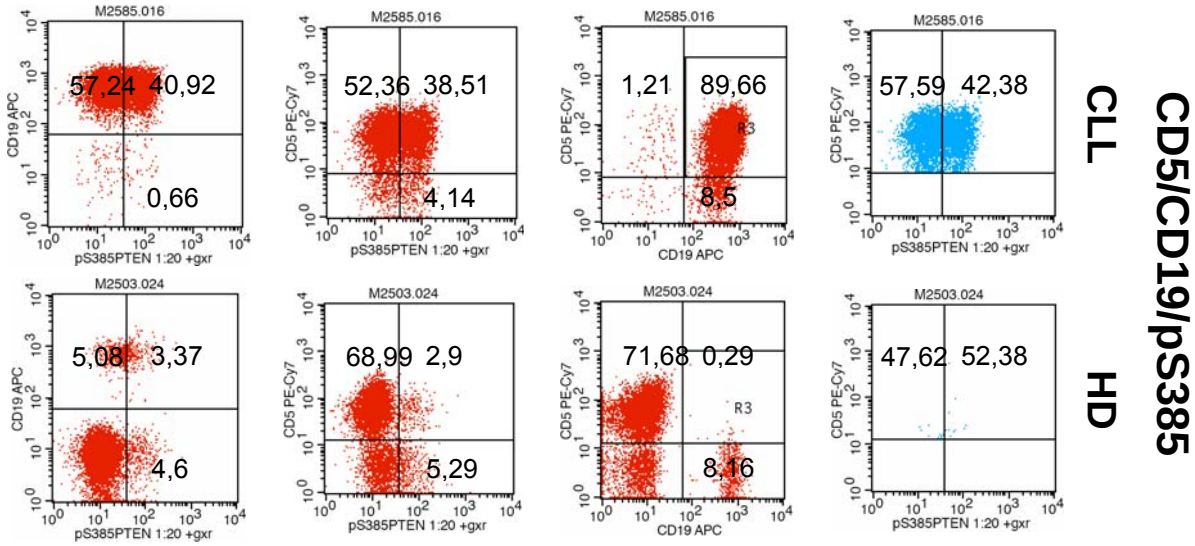


Figure 13 E: *Detection of phosphorylation of PTEN at S385 in CLL and HD samples: These dot plots depict the comparable amounts of pS385 PTEN within the CD5/CD19 population of CLL and HD samples.*

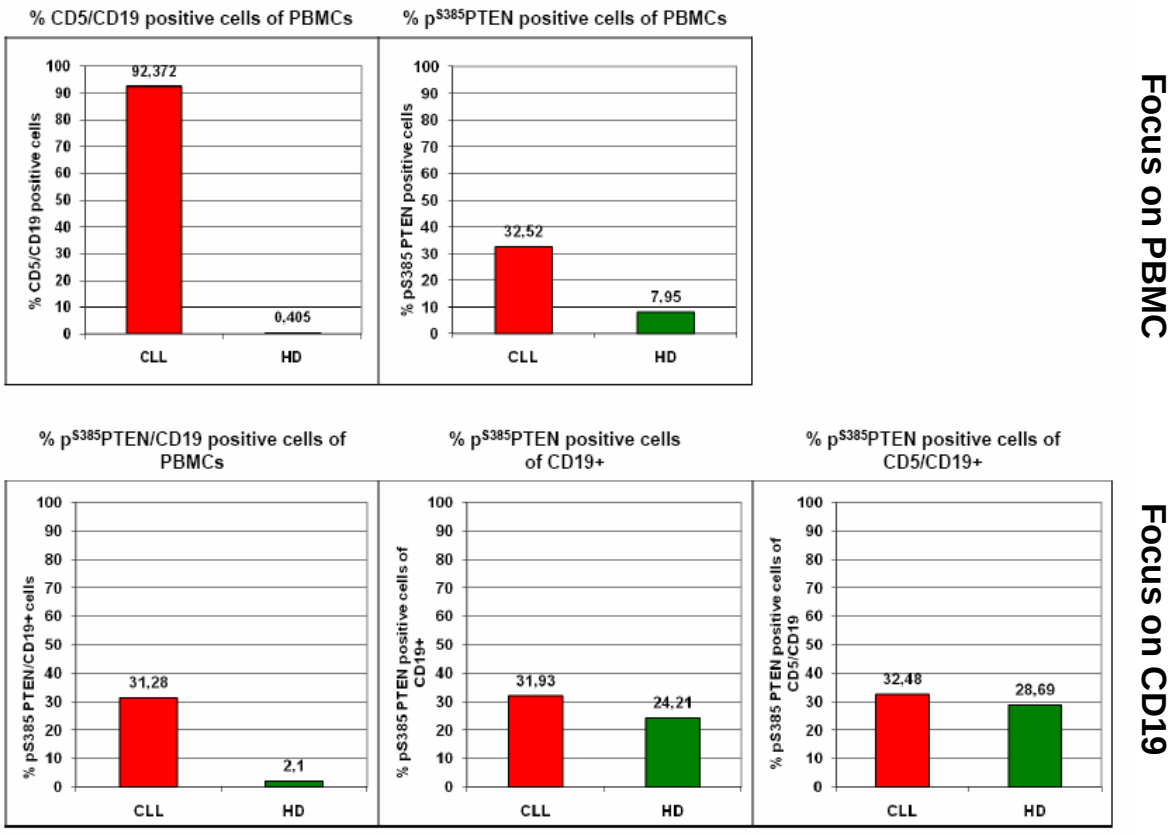


Figure 13 F: *Detection of intracellular pS385 PTEN levels in different cell populations of CLL and HD samples: The mean values of detected pS385 PTEN-positive cells confirm that phosphorylation of this residue is comparable between CLL and HD cells when focusing on the CD5/CD19 population.*

Figure 13 G and H depict the highest amount of phosphorylation in CLL cells at the residues S380, T383 and S385. When comparing this staining with the staining of total PTEN it has to be considered that differences in antibody quality can be responsible for the lower yield in PTEN positive cells as cut off levels were determined by staining with secondary antibody alone and omitting the first antibody. As the dot plots clearly show, MFI values for total PTEN were much lower than MFI values for pSTS380/83/85 PTEN and therefore closer to our cut off.

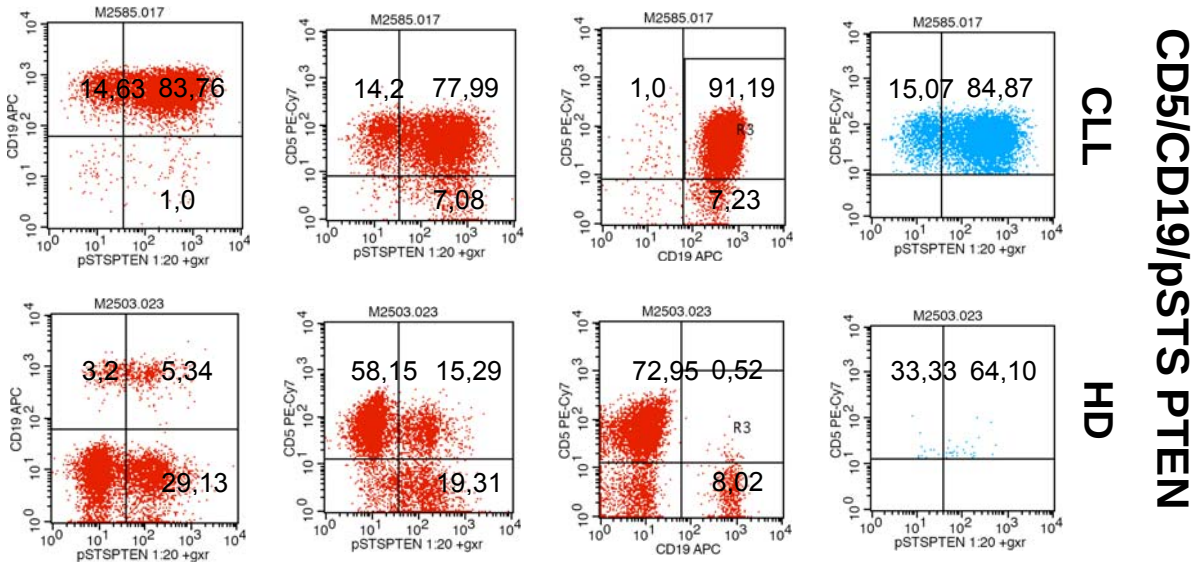


Figure 13 G: *Detection of phosphorylation of PTEN at S380, T383 and S385 in CLL and HD samples: The pS380/T383/S385-specific antibody detects the highest percentage of positive cells in CLL and HD samples. This figure also shows the high MFI values in CLL samples and the moderate MFI in HD samples.*

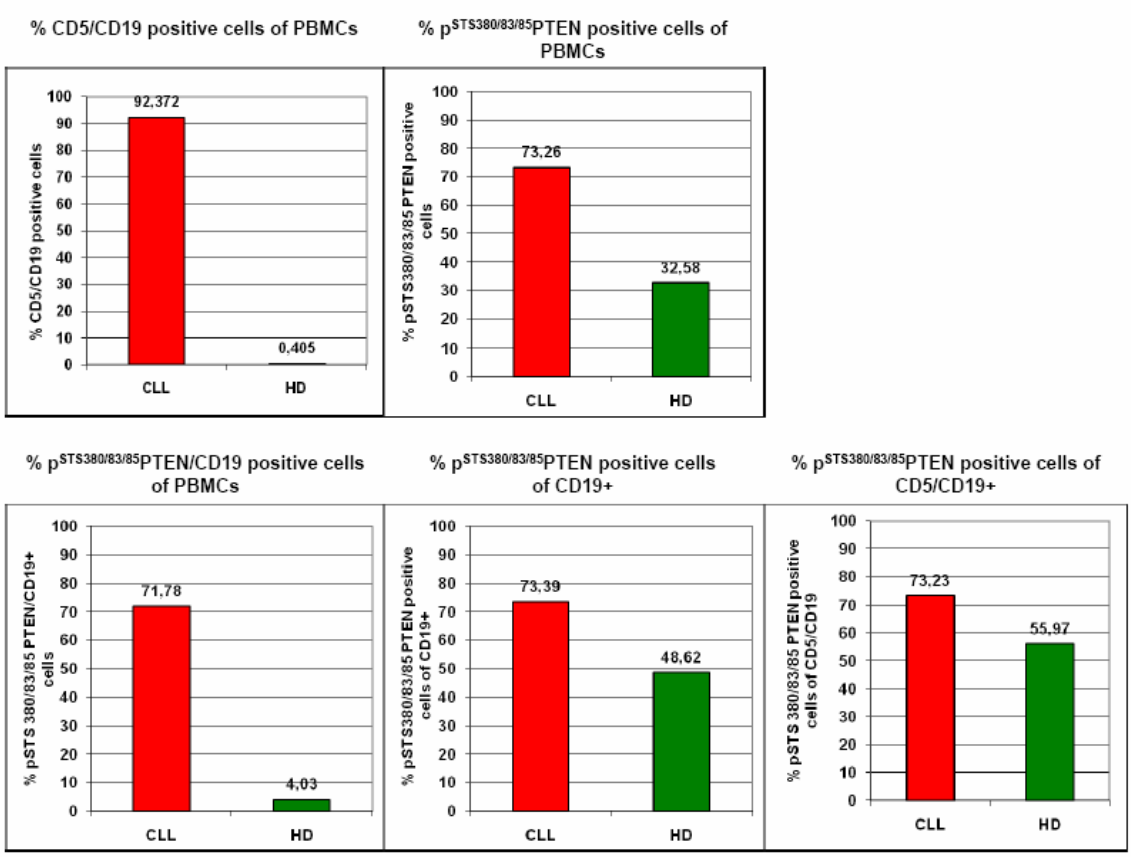


Figure 13 H: *Detection of intracellular pSTS380/83/85 PTEN levels in different cell populations of CLL and HD samples: The diagrams show the major difference in pSTS380/83/85 PTEN expression in the PBMC population (upper right) and the moderate differences in the CD5/CD19 population (lower right).*

7. Evaluation of the potency and IC50 values of PI3-K inhibitors for CLL cells by MTT assays:

First we tried to determine the adequate IC_{50} for the tested compounds and to compare the potency of the different inhibitors using MTT assay. We tested non-isotype specific inhibitors, LY294002 and PI-103 and isotype-selective inhibitors for the alpha, beta and gamma isoform of the PI3K. Since some of these molecules exert an effect on more than one target or lose their specificity at higher concentrations it is essential to use the adequate concentration for specific target inhibition.

For inhibitor testing patient cells were isolated using Ficoll-Hypaque protocol and cells were then distributed in 96 well plates or 6 well plate. On day 0 cells were treated with either pan PI3K inhibitors (LY294002 or PI-103) or with isotype specific inhibitors (PI3K-α, PI3K-β or PI3K-γ). On day 1 cell viability was measured by FACS staining and MTT assay. MTT data was used to calculate the IC_{50} of each compound for CLL cells and to evaluate the most promising candidates.
The viability curves and the IC_{50} values from these experiments demonstrate that the PI3K-alpha inhibitor shows the most promise for inducing cell death in CLL cells at reasonable IC_{50} concentrations that are within a range that should ensure kinase specific effects.

The non-isotype specific PI3K inhibitors LY294002 and PI-103 were also effective in CLL cells. The IC_{50} was calculated as 9,95 µM for LY294002 and 8,45 µM for PI-103. Even though a reduction in cell viability could be achieved with these compounds the effect was moderate and required rather high concentrations of inhibitor. (see Figure 14)

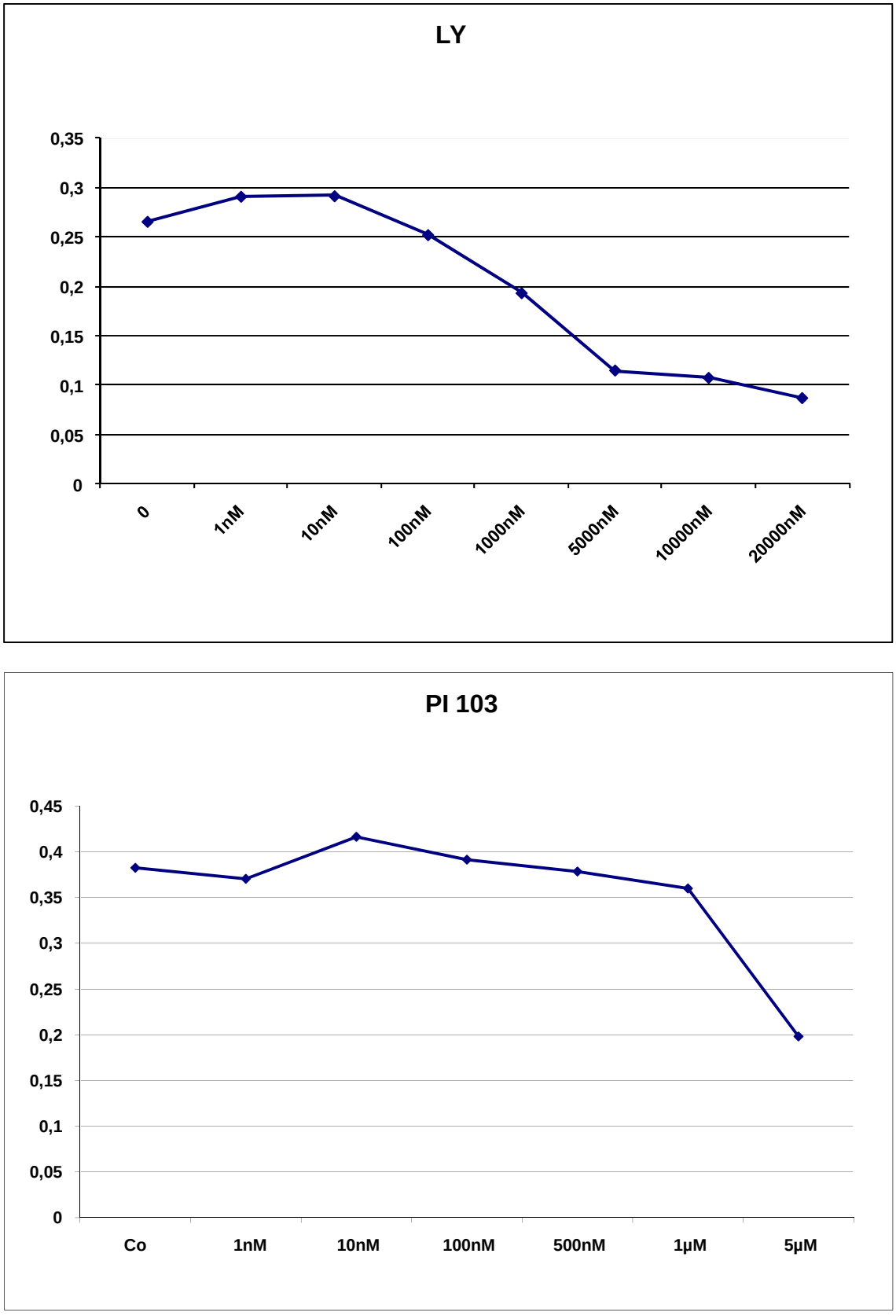
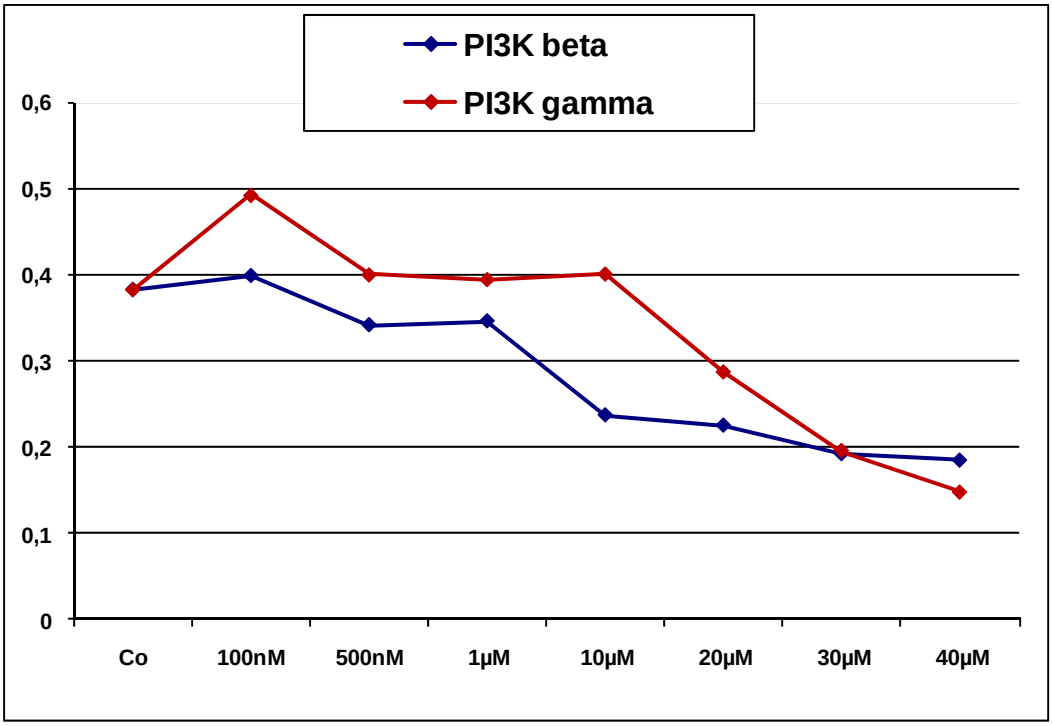


Figure 14: *Inhibitory effects of pan-PI3K inhibitors on CLL cells: Both pan-isotype inhibitors are able to reduce CLL cell viability. Still, the effect is limited and requires rather high concentrations.*



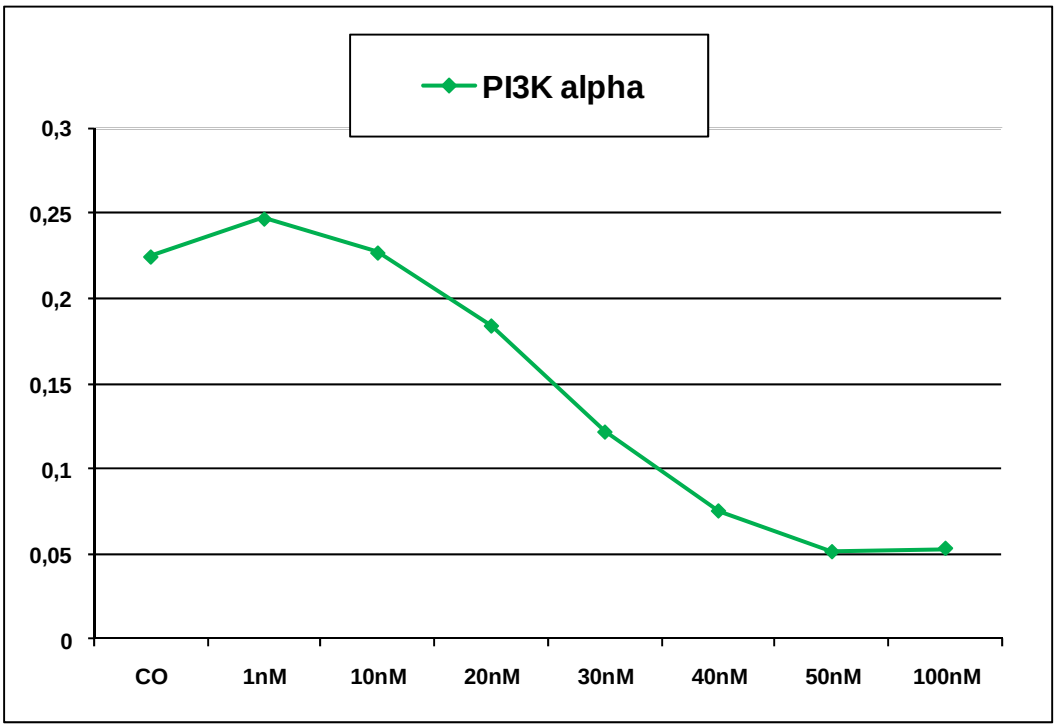


Figure 15: *Inhibition by isotype-specific PI3K inhibitors: Isotype-specific inhibitors for PI3Kβ (blue) and PI3Kγ (red) were able to reduce cell viability but only at high concentrations that do not guarentee specificity. PI3Kα specific inhibitor (green) could achieve a strong reduction of viability at nanomolar concentrations.*

The isotype specific inhibitors for PI3K-β and PI3K-γ were also evaluated by MTT and FACS analysis. There was also a reduction in viability and induction of apoptosis by these compounds but the effect only becomes apparent at concentrations that are reported to be higher than the ones that would ensure isotype-specific induction. (see Figure 15) This means that it has to be assumed that the decrease in viability could also be attributed to the inhibition of off-target kinases, most likely PI3K-α which can be co-inhibited at concentrations of about 5μM by both of these inhibitors. We calculated IC_{50} values for PI3K-β and PI3K-γ inhibitor to be 30,16 μM and 25,55 μM respectively. In contrast to these findings PI3K-α could induce apoptosis in a large proportion of CLL cells and was able to decrease viability extensively within a nanomolar range that ensures selectivity for this isoform. The calculated IC_{50} value was 42,7 nM.

In parallel to the evaluation of the compounds by MTT we made a comparison of the different inhibitors available to us by FACS analysis in order to assess which were the most promising for further testing. Two freshly isolated CLL patients were treated with the respective inhibitor in suspension culture for 24 hours. Then cells were harvested and subjected to FACS analysis. In these preliminary tests we found that the PI3K-α inhibitor showed the most profound effect as demonstrated by Figure 16. The pan-isotype inhibitors could induce apoptosis to some extent which could be attributed to the fact that they also inhibit the alpha isoform. The effect of the p110β and p110γ inhibitor was even lower.

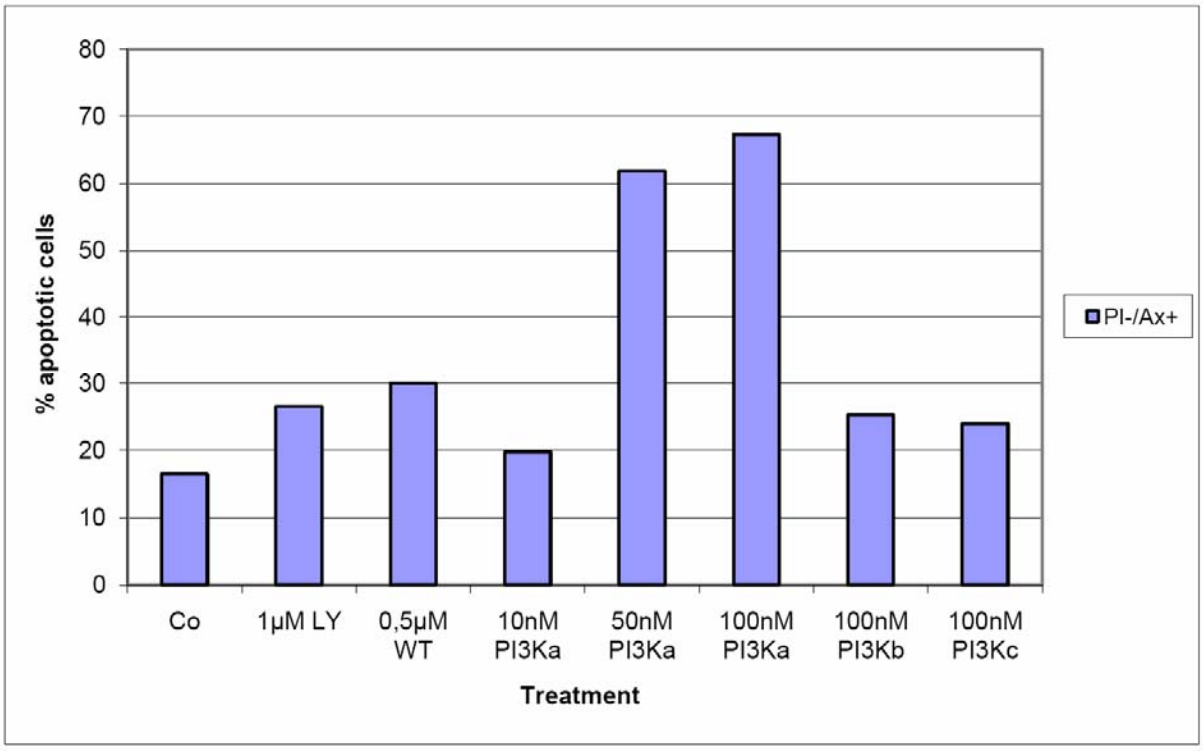
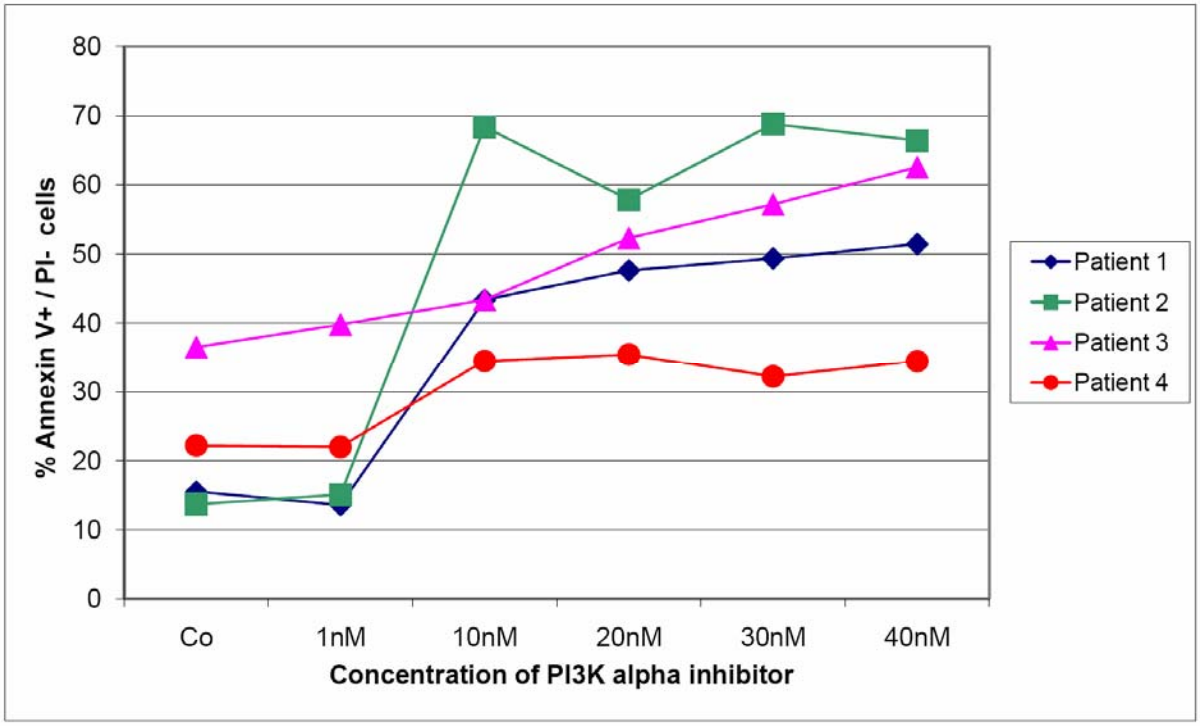


Figure 16: *Induction of apoptosis after 48h assessed by AnnexinV/Pi staining: Viability FACS staining of pre-treated CLL cells shows the moderate effects of the pan-specific inhibitors LY and WT (left), the profound effect of PI3Kα inhibitor (middle) and the low effect of PI3Kβ and PI3Kγ inhibitor (right).*

8. Dose kinetic studies on PI3K-α inhibitor:

As the PI3K alpha inhibitor VIII exhibited an extremely high potency in our first tests it was used for further dose kinetic studies. According to the company specific inhibitory effects on the PI3K-α subunit should start at 0,3 nM concentrations. Only at concentrations of 40 nM or higher other isoforms should be inhibited starting with p110-γ. We used this information for our next experiments. Cells from 4 CLL patients were incubated with increasing concentrations of PI3K-α inhibitor and apoptosis was assessed by AnnexinV/Pi staining.



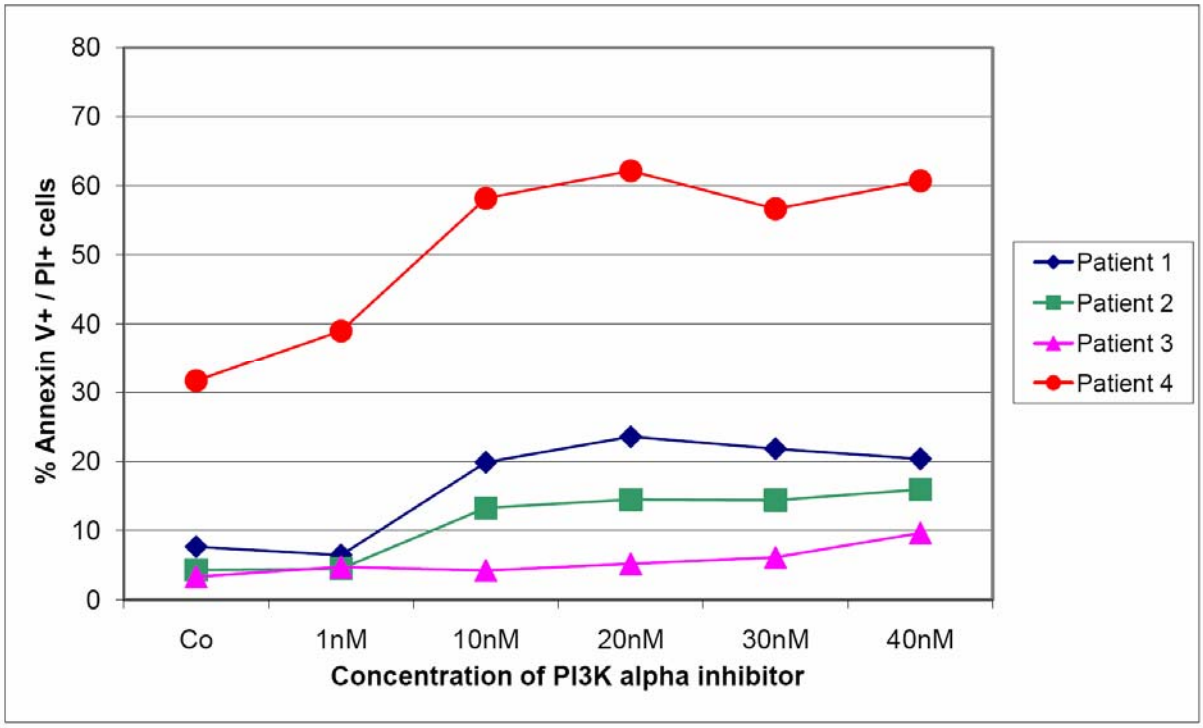


Figure 17A: *Dose dependent induction of apoptosis by PI3K-α inhibitor in 4 CLL patients: The upper figure depicts the percentage of Annexin V positive cells (early apoptosis) in CLL cells treated with increasing doses of PI3Kα inhibitor, the lower figure shows the percentage of Annexin V/Pi positive cells (late apoptosis).*

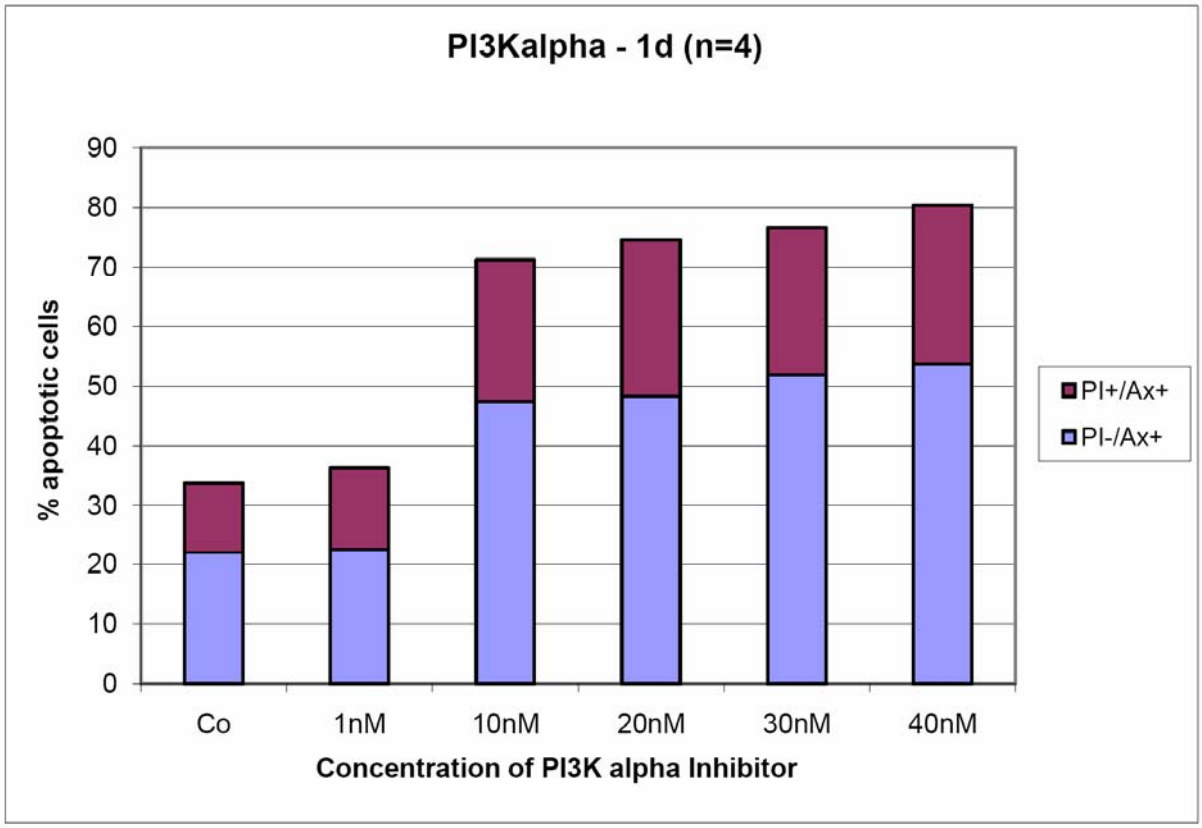


Figure 17B: *Dose dependent induction of apoptosis by PI3K-α inhibitor: The diagram depicts the mean values of the four CLL patients analysed. The induction of apoptosis is dose-dependent and starts at 10nM.*

These data show a strong inhibitory effect after only 24 hours incubation in suspension culture. When analysed separately (see Figure 17A) the four patients show differences in response which may be caused by the different basal viability of the cells in culture. This could result in decreased or delayed apoptosis, especially at low concentrations. The relative high apoptosis rate in the control is quite normal for CLL cells in suspension culture. Still, the increase in apoptosis becomes very apparent at concentrations of 10 nM or more which is in the range of alpha-isoform specific inhibition. (see Figure 17B)

9. PI3K-α inhibitor overcome the supportive effect of bone marrow stromal cells:

The next question we addressed was if the inhibitor could also induce apoptosis in CLL cells that were supported by bone marrow fibroblasts in a co-culture system.
We incubated PBMCs from 4 CLL patients with increasing amounts of PI3K alpha inhibitor in suspension culture or in co-culture with primary bone marrow fibroblasts for 4 days. After 1- 4 days cells were harvested and analysed by FACS. Even though the supportive effects of the co-culture are evident by the reduced apoptosis rate in the control cultured in co-culture compared to the control in suspension, this support can be overcome by inhibiting the PI3K alpha p110 subunit. The effect may be reduced at first but, as demonstrated in Figure 18A, apoptosis levels reach over 50% at concentrations of only 10nM which is well within the range of isotype specificity.

Again, the detailed analysis of the four patients shows that cells that exhibit very good viability and low apoptosis in the control are either more sensitive or react faster to inhibiton of p110 alpha (see Figure 18B, patient 2 and 3). The effect is less dramatic in samples with less basal viability in the control. (see Figure 18B, patient 1 and 4) Neverthless, stromal cell support could be overcome in all 4 patients and an increase in apoptosis is induced at concentrations between 10 nM and 20 nM.

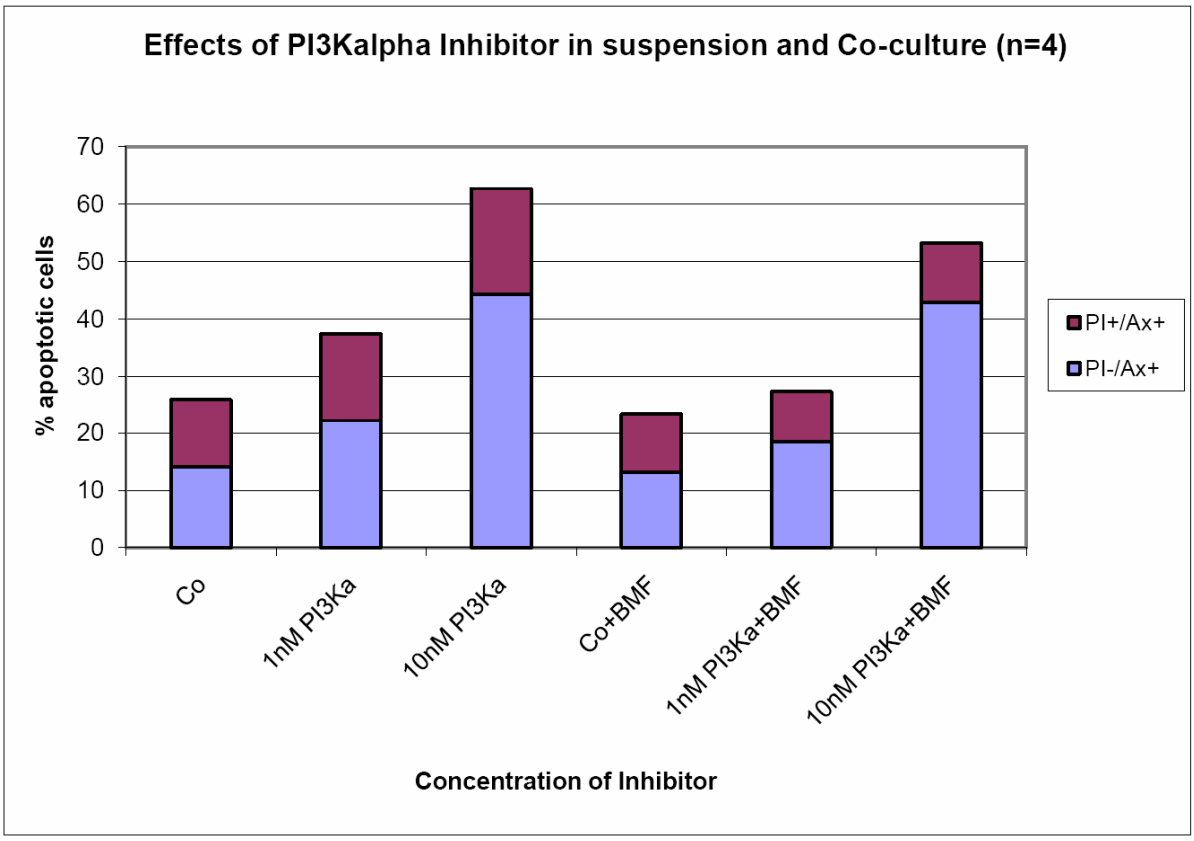


Figure 18A: *Dose dependent induction of apoptosis in suspension (left) and co-culture with bone marrow fibroblasts (right): The induction of apoptosis is dose-dependent. The cells in co-culture (right) show only a slightly reduced apoptosis rate.*

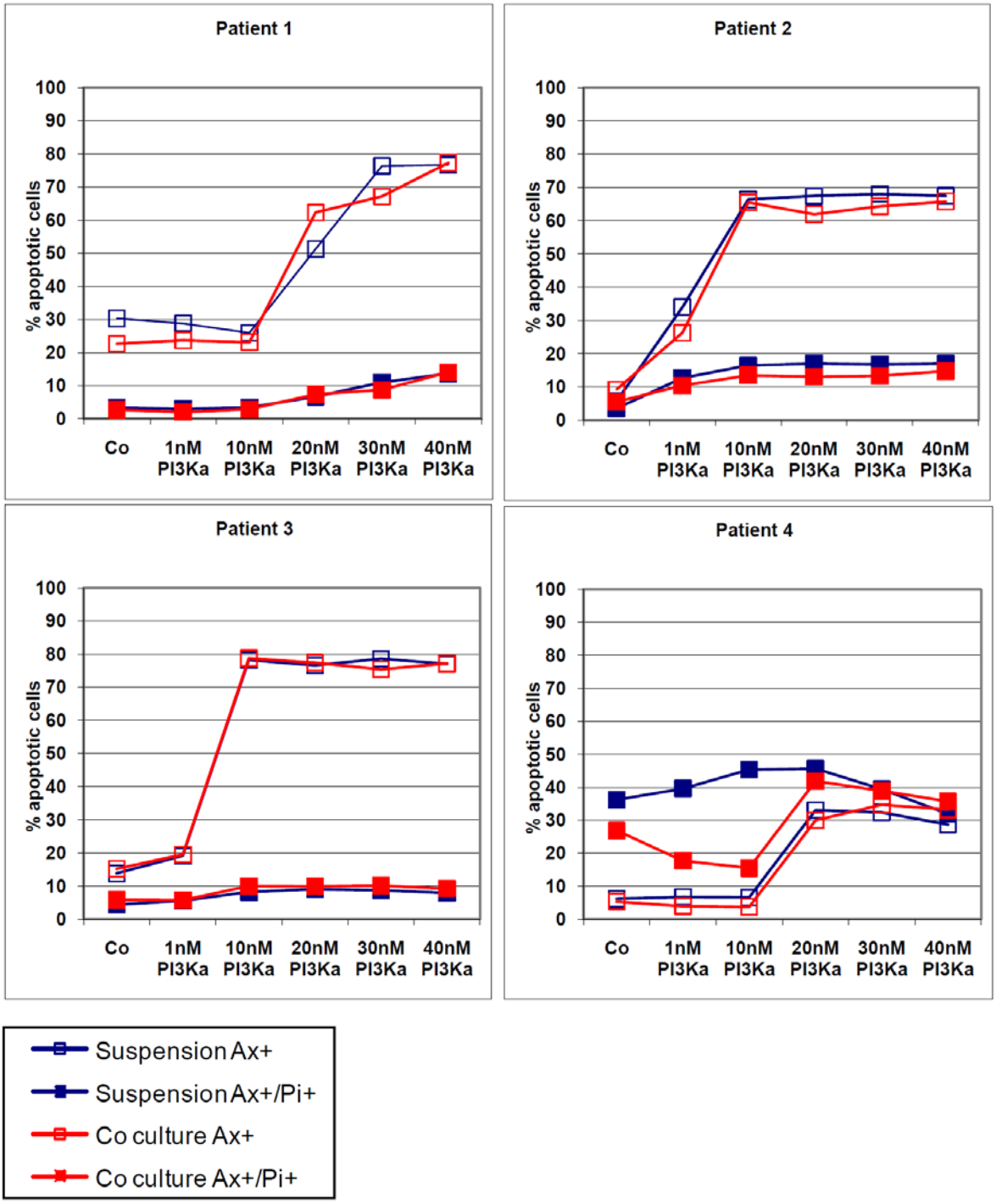


Figure 18B: *Dose dependent induction of apoptosis in suspension (dark blue) and co-culture with bone marrow fibroblasts (red) in 4 different CLL patients.*

10. Effect of PI3K inhibition on the mRNA and protein level:

Analysis of the molecular effects of the tested inhibitors showed that inhibition of PI3K pathway with isotype specific inhibitors had no effect on p110 mRNA expression. This is not surprising as the inhibitor is not expected to directly downregulate p110 transcription. (see Figure 19)

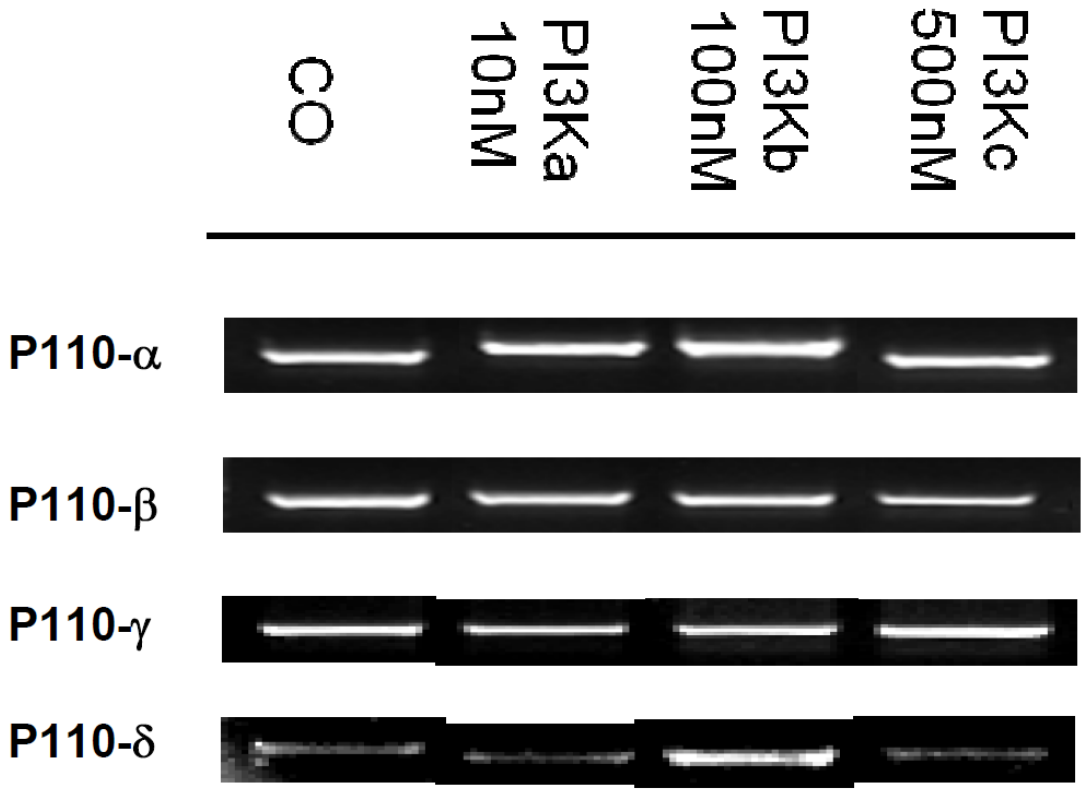


Figure 19: *PI3K inhibitors do not influence p110 mRNA expression: PI3K inhibition does not show major effects on the expression of the four p110 isoforms compared to untreated samples.*

As demonstrated in Figure 20, also mRNA expression of the downstream targets PTEN and AKT was not significantly altered upon incubation with the isotype specific p110 inhibitors indicating that if changes are induced in the downstream signalling, these changes will most likely affect the protein directly, probably through protein modification.

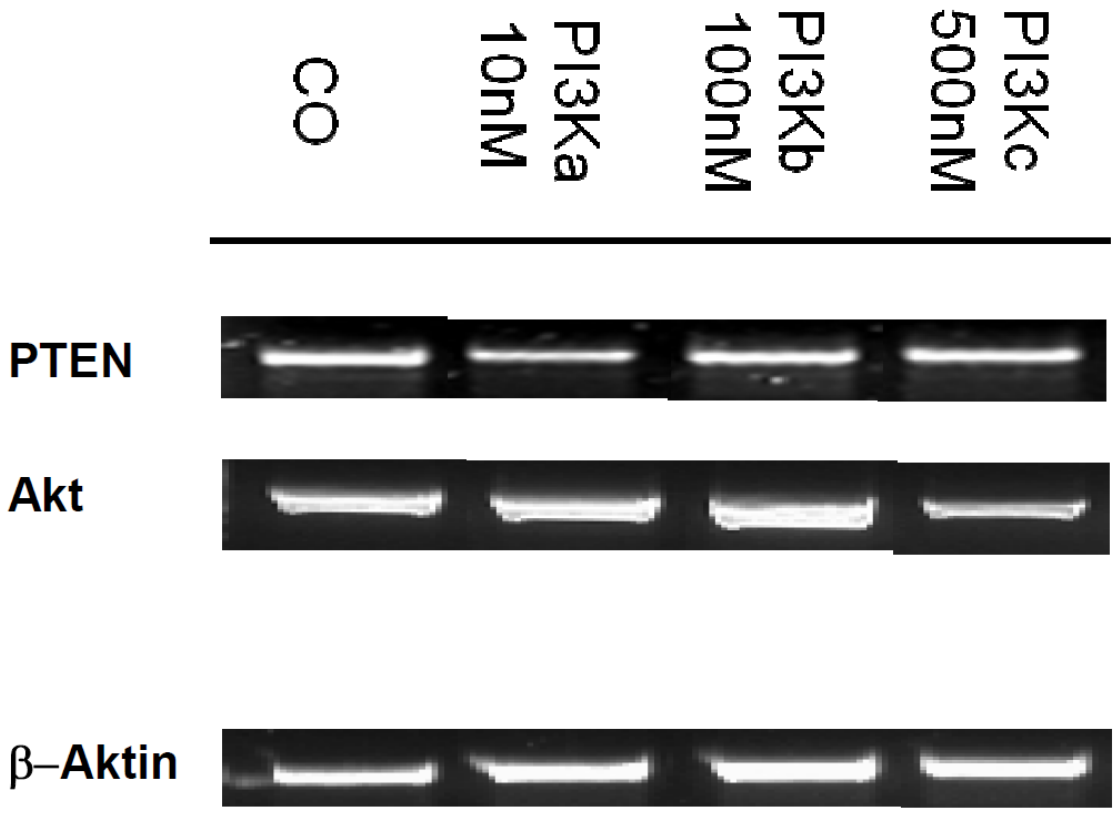


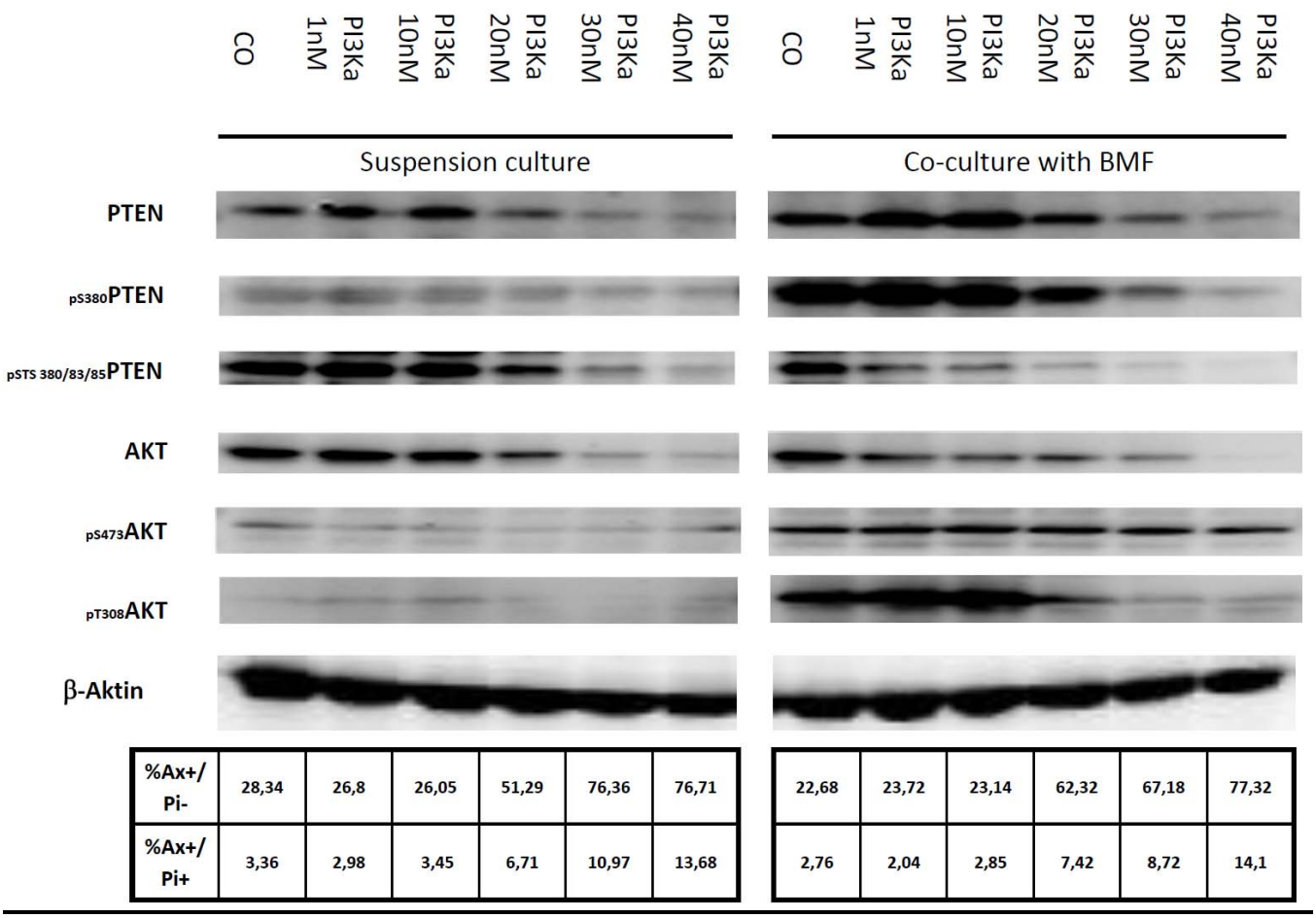
Figure 20: *Effects of PI3K inhibition on the mRNA level: The mRNA expression of the downstream target AKT and the negative regulator PTEN is not significantly modified by PI3K inhibition.*

The analysis of PI3K alpha inhibitor in suspension and co-culture showed that in parallel to the induction of apoptosis, also molecular effects take place under both culture conditions. (see Figure 21) The PI3K alpha inhibitor did not influence the mRNA levels of the downstream targets but could induce changes at the protein level. A decrease in AKT indicates successful inhibition of the PI3K/Akt pathway. The expression of phosphorylated AKT is stronger in the co-culture. This reflects the importance of the role PI3K signalling plays in stromal cell support. The inhibition of p110 alpha leads to a dramatic decrease of phosphorylation at site T308 which is a target of PDK kinase activity. Interestingly, the phosphorylation at site S473 remains unaffected.

The downregulation of PTEN which is accompanied by a dephosphorylation is more difficult to explain. One possibility it that as the PI3K pathway is inhibited, its major negative regulator PTEN will be downregulated as well. Also, we know that PTEN stability decreases upon dephosphorylation. Again, the dephosphorylation

of PTEN is more prominent in the co-culture. This could be explained as the PI3K pathway seems to be more active in the co-culture (reflecting the in vivo situation) and the inhibition of the very active pathway may result in a stronger response than in suspension.

The effects on the two downstream targets are accompanied by a dose-dependent increase in apoptosis under both culture conditions.



%Ax+/ Pi-	28,34	26,8	26,05	51,29	76,36	76,71	22,68	23,72	23,14	62,32	67,18	77,32
%Ax+/ Pi+	3,36	2,98	3,45	6,71	10,97	13,68	2,76	2,04	2,85	7,42	8,72	14,1

Figure 21: *PI3K-α inhibitor induces dephosphorylation of AKT and PTEN: This figure shows the effects of the PI3Kα inhibitor on the protein expression. In suspension culture (left part) the AKT, PTEN and pPTEN expression is influenced in a dose-dependent manner. In suspension culture (right part) the basal expression of pPTEN and pAKT are more severely modified by PI3Kα-inhibition. The lower part shows the corresponding percentages of apoptotic cells determined by FACS analysis.*

Discussion:

It is well established that the PI3K pathway is deregulated in many types of cancer and this is also applicable for CLL. As it could be shown in this diploma work at the mRNA as well as on the protein level, important key molecules of the PI3K/AKT signalling pathway are highly expressed and activated in CLL cells. This could be demonstrated by the RT-PCR, Western Blot and FACS data. It could also be shown that most of the differences in PI3K/AKT pathway activation lie in the altered protein phosphorylation pattern, especially the deactivation of PTEN and the activation of AKT by phosphorylation of crucial sites. It is therefore feasible that this difference to healthy PBMCs generates an opportunity to target the CLL cells. As PI3K inhibitors are now available as a broad range of different compounds with diverse specificity patterns, the potential of PI3K as a therapeutic target becomes more and more apparent. There are already clinical trials in progress that use PI3K inhibitors for several solid tumours. Therefore it would be useful to establish PI3K signalling as a valid target for therapy and to define the most promising ways to interfere with this pathway.

The question whether pan-isotype inhibitors versus isotype specific inhibitors should be preferred for therapeutic application will be of great importance. From this work it is possible to conclude that all four isoforms are expressed in CLL cells. The mRNA data suggested an important role for the p110 alpha isoform and the p110 gamma isoform in CLL cells, while western blotting p110 beta and p110 gamma appeared to be promising targets. FACS data could establish that there is a general increase in p110 expression in CLL cells compared to healthy individuals. Another interesting notion was the enrichment of p110 expression within the B-cell population and especially within the very small CD5/CD19 population of healthy donors. Since this small population shares some features with CLL cells and has been suspected to be somehow related to the origin of CLL cells it could be speculated that these cells may be prone to highly active PI3K signalling.

Because of the differences in the expression patterns on the mRNA and the protein level it is not clear if there is a dominant isoform in CLL cells. In addition

there remains the question if and to what extent the different isoforms can compensate each others function or if there are regulatory feedback mechanism at play that would circumvent the inhibition of a single isoform.

Nevertheless, this work tested several commercially available inhibitors to determine their potency in an in vitro setting. In our in vitro model the non-specific PI3K inhibitors LY294002 and PI-103 had some effect on CLL cells, even more than some of the isotype-specific inhibitors. Actually, the PI3Kβ inhibitor and the PI3Kγ inhibitor showed less effect than pan-specifc inhibitors. The weak effect of the PI3Kγ inhibitor was unexpected as there was a significant difference in protein expression between CLL cells and healthy PBMCs. As we had no access to a specific PI3Kδ inhibitor we could not determine how effective it would be against CLL cells. The most profound effect could be observed with the PI3Kα inhibitor VIII which exhibited this effect within hours and at a concentration that was well within a range that is reported to ensure specificity for this single isoform. Considering the high levels of p110α mRNA which were considerably lower in healthy controls and the high percentage of CD5/CD19/p110α triple positive cells detected by FACS analysis these results seem compatible. The fact that the PI3K alpha inhibitor could also induce apoptosis in CLL cells that were co-incubated with supporting stromal cells gives reason to assume that the inhibitory effect can overcome the positive signals supplied by the microenvironment. We could also show that the induction of apoptosis is accompanied by a decrease of AKT expression on the protein level and a decrease of AKT phosphorylation at Thr 308. This residue is critical for AKT function. Interestingly, both phoshporylation sites (Thr308 and Ser473) were highly phosphorylated in co-culture as well as PTEN. It can be assumed that this reflects the important part that PI3K signalling plays in the communication between CLL cells and the microenvironment and the exchange of pro-survival signals. It is also possible to speculate that cells that depend on these signals in vivo might be more sensitive to the disruption of this pathway than in an experimental setting in suspension culture.
The second effect of the PI3Kα inhibitor VIII was the induction of dephosphorylation of PTEN which leads to its re-activation. The expression of total PTEN was comparable in the suspension and co-culture but treatment with

the inhibitor decreased expression to some extent but more importantly phosphorylation at residue S380, T383 and S385 was decreased dramatically in the co-culture and also in the suspension culture.
This two-branched reaction on the molecular level mirrors the inhibitory effect on the PI3K pathway and all of these molecular effects on downstream targets of PI3K suggest that the specific inhibition of p110α subunit can accomplish not only induction of apoptosis but also a reasonable molecular response that reflects the inhibition of this pathway.

Essentially, the most promising inhibitor, PI 3-Kα inhibitor VIII, could prevent the stromal cell support and overcome apoptosis resistance in CLL cells. As there is a need for effective therapy for CLL, combination of conventional therapy with agents that also target the tumour microenvironment will gain importance. These drugs are also of great interest for therapies that aim at eradicating the residual pools of CLL cells that sooner or later lead to relapses which still seem to be inevitable in this disease.

Taken together, this work provides evidence that targeting the PI3K pathway could be a very valuable strategy in fighting CLL and in further elucidating the molecular mechanisms involved in the development and progression of the disease. The possibility to target this pathway at various levels and the growing number of small molecule inhibitors with improved pharmacologic properties will provide new concepts for therapy of CLL.

Conclusion:

In agreement with our recent publication and the available data in the literature (21, 27) which demonstrate the important role of the PI3K pathway in the inhibition of apoptosis in CLL cells, this diploma work provides further evidence on the activation state of the PI3K pathway in CLL cells. This work also shows the high expression of all four PI3K-p110 isoforms (α, β, γ and δ) in CLL cells compared to healthy individual PBMCs and B cells as demonstrated at the mRNA and protein level and by different methods including RT-PCR, western blotting and flow cytometric analysis. Due to the fact that all four isoforms are expressed in CLL cells, it might be reasonable to consider pan-PI3K inhibitors as a suitable tool for therapy of CLL. This assumption is supported by the data presented in this work which show that PI3K inhibition might even work under co-culture supportive conditions which mimics the situation in vivo, thus supporting the notion that this approach would be also effective in vivo in CLL patients. This work also points to the significant role of the PI3K-p110α isoform in the pathophysiology of CLL and the potential role of p110α isoform-specific inhibitors in therapy of CLL. Importantly, the ability of the PI3K inhibitors to overcome stromal cell support is crucial and makes them attractive for combinational therapy with cytotoxic compounds or other therapeutics. Finally, the translational research concept of this diploma work demonstrates the feasibility of applying combined approaches to target the PI3K pathway at several key points such as PI3K, AKT and PDK1 which may thus provide new concepts for therapy of CLL and other hematological malignancies.

Materials and Methods:

Protein isolation:

Cells were collected as a pellet and resuspended in RIPA-buffer (50mM Tris-HCL (pH7,4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% deoxycholic acid sodium salt, 0,1% SDS, 1mM PMSF, 1mM Na_2OV_4 and Complete Protease Inhibitor Cocktail™ (Roche)). During 30 min. of incubation on ice the samples were vortexed vigorously several times. After centrifugation at 14000rpm at 4°C for 10min. the supernatants were collected and protein concentration was determined by Bradford assay using a BSA standard curve. The lysates were then mixed with equal volumes 2xSDS-sample buffer (100mM Tris-HCL (pH6,8), 25% gycerol, 2% SDS, 0,01% bromphenol blue, 10% 2-mercaptoethanol), incubated for 5min. at 99°C and stored at –20°C.

SDS-PAGE:

For SDS-PAGE 20µg of total protein were loaded on polyacrylamide gels (7,5-15% acrylamide/bisacrylamide). Gels were run in electrophoresis buffer (25mM Tris base, 192mM glycine, 0,1% SDS) at 200V using Mini-Protean II® system (Bio-Rad).

Western Blot:

After electrophoresis proteins were transferred to nitrocellulose (Amersham) or PVDF (Millipore) membranes using MiniTrans-Blot Electrophoretic Transfer Cells (Bio-Rad) and blotting buffer (25mM Tris base, 192mM glycine, 20% methanol). Transfer was done at 100V and transfer efficiency was determined after blotting by Poinceau S staining of the membranes. The membranes were then equilibrated in TBS/T for 10min. and blocked in blocking buffer (3%BSA/0,1%Tween20/TBS) for one hour at room temperature. After blocking the blots were incubated with primary antibody (dilutions between 1:100 - 1:5000 in TBS/T) for 1h at RT or over night at 4°C. Then blots were washed three times with TBS/T and incubated with a 1:25000 – 1:100000 dilution of HRP-conjugated secondary antibody in TBS/T for 1h. Afterwards membranes were washed three times with TBS/T. For detection we used Immun-Star™Western C™ (BioRad).

Antibodies:

For analysis of the PI3K-pathway and PI3K-pathway-associated molecules we used the following antibodies for western blotting:

1) β-actin (A2066) antibody (Sigma) was used 1:5000 in combination with goat-anti-rabbit HRP antibody (Amersham)
2) PTEN antibody (Cell Signaling) was used 1:750 in combination with goat-anti-rabbit HRP antibody (Amersham)
3) pS^{380}PTEN (Cell Signaling) was used 1:750 in combination with goat-anti-rabbit HRP antibody (Amersham)
4) Akt (Cell Signaling) was used 1:1000 in combination with goat-anti-rabbit HRP antibody (Amersham)
5) pS473Akt (Santa Cruz) was used 1:500 in combination with goat-anti-rabbit HRP antibody (Amersham)
6) pT308Akt (Santa Cruz) was used 1:500 in combination with goat-anti-rabbit HRP antibody (Amersham)
7) p110alpha (C17) (Santa Cruz) was used 1:50 in combination with donkey-anti-goat HRP antibody (Santa Cruz)
8) p110beta (D4) (Santa Cruz) was used 1:200 in combination with goat-anti-mouse HRP antibody (Amersham)
9) p110 gamma (H199) (Santa Cruz) was used 1:100 in in combination goat-anti-rabbit HRP antibody (Amersham)
10) p110 delta (H219) (Santa Cruz) was used 1:200 in in combination goat-anti-rabbit HRP antibody (Amersham)
11) GAPDH (Santa Cruz) was used 1:5000 in combination with goat-anti-rabbit HRP antibody (Amersham)
12) pSTS380/83/85 PTEN (Invitrogen) was used 1:500 in combination with goat-anti-rabbit HRP antibody (Amersham)

For all antibodies optimal dilution and membrane type were determined.

RNA Isolation :

10-50 million cells were lysed in approx. 1mL Trizol (Invitrogen) and stored at –80°C. For RNA isolation tubes were thawed and incubated at room temperature

for 5min. After homogenisation 200µL $CHCl_3$ were added and mixed thoroughly by shaking. Following 2min of incubation at room temperature the lysates were centrifuged at 12000rpm/4°C for 15min. The aqueous phase was collected and transferred to a new tube. 500µL isopropanol were added. Samples were kept at –20°C for at least 30min for precipitation. Tubes were centrifuged at 12000rpm/4°C for 10min. The pellets were washed with cold 75% ethanol and centrifugation at 7500rpm/4°C for 5min. Pellets were dried and dissolved in 20-50µL sterile deionized water. Concentration and quality were determined photometrically. For further usage the RNA had to show a minimal ratio (260nm:280nm) of 1,7.

cDNA Synthesis

RNA was isolated using the Trizol protocol and was measured. For cDNA synthesis 2-2,5µg RNA were mixed with 0,5µL oligo(dT)15 primer (Promega) and incubated at 72°C for 5min. Then the samples were immediately chilled on ice. Afterwards 5µL 5xBuffer (Promega), 0,625µL RNasin (Promega), 1,25µL dNTP mixture (10mM), and 1µL M-MLV reverse transcriptase (Promega) were added and incubated for 1h at 37°C.

RT-PCR:

All PCRs were performed using GoTaq® PCR system (Promega). 1µL of cDNA was mixed with GoTaq® PCR Mix (1,5mM $MgCl_2$ (GoTaq® Reaction Buffer), 0,2mM dATP, 0,2mM dCTP, 0,2mM dGTP, 0,2mM dTTP, 1,25U GoTaq® DNA Polymerase), 0,5nM forward-primer and 0,5nM reverse-primer. Pre-PCR was performed with 3min/94°C, 40s/52°C and 1min/72°C followed by variable cycles of 30s/94°C, 45s/ 54°C and 1min/72°C with a final elongation of 10min/72°C. All PCRs were performed with NTCs. PCR products were separated on 1% agarose gels containing GelRed (Biotium) and gels were photographed under UV light for documentation.

Primers:

For analysis of the PI3K-pathway and PI3K-pathway-associated molecules we used the following primers for real time and RT-PCR:

1) PTEN: primers for PTEN were obtained from VBC Genomics. PCR was performed with 28 cycles.

PTEN-s: 5’ – CAG AAA GAC TTG AAG GCG TAT – 3’

PTEN-as: 5’- AAC GGC TGA GGG AAC TC – 3’

2) β-actin: primers for β-actin were obtained from VBC Genomics. PCR was performed with 25 cycles.

KL-6 (actin-s): 5’- GTG GGA ATT GTC AGA AGG ACT CCT ATG TG-3’

KL-7 (actin-as): 5’- GAA GTC TAG AGC AAC ATA GCA CAG CTT CTC-3’

3) P110-alpha: primers for p110 alpha were obtained from VBC Genomics. PCR was performed with 35 cycles.

P110α-s: 5’- GAC TTA TTG AGG TGG TG -3’

P110α-as: 5’- GGC ATG CTG TCG AAT AG – 3’

4) P110-beta: primers for p110 beta were obtained from VBC Genomics. PCR was performed with 38 cycles.

P110β-s: 5’- GCT AAT GTG TCA AGT CG -3’

P110β-as: 5’ - CCG ATT ACC AAGTGC TC -3’

5) P110-gamma: primers for p110 gamma were obtained from VBC Genomics. PCR was performed with 38 cycles.

P110γ-s: 5’- CCT GCA GAA TTC TCA AC -3’

P110γ-as: 5’ - CAG AAT CTC GAT CAT TC -3’

6) P110-delta: primers for p110 delta were obtained from VBC Genomics. PCR was performed with 38 cycles.

P110δ-s: 5’- TCA TGT ACA GCA ACG AGG AG-3’

P110δ-as: 5’ –CTT TAG GAC CAA TGT GCT TG-3’

7) GAPDH: primers for GAPDH were obtained from VBC Genomics. PCR was performed with 28 cycles.

GAPDH-s: 5’-GTC AGT GGT GGA CCT GAC CT-3’

GAPDH-as: 5’-TGT GAG GAG GGG AGA TTC AG – 3’

8) AKT: primers for AKT were obtained from VBC Genomics. PCR was performed with 35 cycles.

Akt-s: 5’- CTACCTGCACTCGGAGA-3’

Akt-as: 5’- GTCAGTCTCCGACGTGA– 3’

FACS Surface Staining:

1-2x10^6 cells were washed with PBS and blocked in 50µL 20%BSA for at least 20min. Then the blocked cells were incubated with fluorochrome-conjugated antibody or unconjugated primary antibody and conjugated secondary antibody. After each antibody incubation cells were washed with PBS/0,3%BSA/0,1%NaN_3. For analysis the cells were resuspended in 250µL PBS/0,3%BSA/0,1%NaN_3. For all stainings where secondary antibodies were used, isotype controls and/or controls with secondary antibody alone were included.

CLL Phenotyping :

PBMCs from all patient samples were characterized by FACS analysis using CD19-APC and CD5-PE-Cy7 as markers for CLL cells.
All antibodies for this panel were obtained from BDBiosciences.

FACS Intracellular Staining:

For flow cytometry analysis several protocols were tested (Fix and Perm (Calatag), Staining from whole blood with BD FACS Lysing Solution and staining with saponine after Ficoll isolation of PBMCs). Finally we selected a protocol using formaldehyde for fixation and saponine for permeabilization. We used two different concentrations of saponine. The very low concentration of saponine still led to a significant signal for most of the antibodies and seemed to increase specificity.

Intracellular Staining of PBMCs using Formaldehyde/Saponine:

Cells were isolated by Ficoll-Hypaque protocol and then incubated in PBS/2%formaldehyde for 15min. for fixation. Then cells were washed with PBS/0,3%BSA/0,1% NaN_3, blocked in 20% human AB serum for 15min. and washed with PBS/0,3%BSA/0,1% NaN_3. Then cells were incubated with unconjugated antibody diluted in PBS/0,3%BSA/0,1% NaN_3/0,1‰ saponine to permeabilize the cells. After 15min cells were washed again with PBS/0,3%BSA/0,1% NaN_3 and were then incubated with secondary antibody (either goat-anti-mouse-Alexa488 (Molecular Probes) or goat-anti-rabbit-Alexa488 (Molecular Probes)) diluted 1:400 in PBS/0,3%BSA/0,1% NaN_3/0,1‰ saponine for 15min. Then cells were washed in PBS/0,3%BSA/0,1% NaN_3 and

incubated with CD5PE-Cy7 and CD19-APC diluted 1:50 in 20% human AB serum for 15min. Finally cells were washed once more in PBS/0,3%BSA/0,1% NaN_3, resuspended in 250µL PBS/0,3%BSA/0,1% NaN_3 and analyzed on a FACS Calibur using CellQuestPro software.

Viability Staining:

Viability and apoptosis rates were assessed by AnnexinV-FITC/PI staining or by 7-AAD/CD3/CD14/CD19 staining. For AnnexinV/PI staining cells were collected and resuspended in 200µL Annexin-binding buffer containing 5µL AnnexinV-FITC and incubated for 10min. at RT in the dark. Then cells were washed with PBS and resuspended in 200µL
Annexin-binding buffer containg 10µL PI. Samples were then analysed on a FACSCalibur. We used Annexin V kits (BenderMed Systems).

MTT Assay:

For determination of inhibitory effects EZ4U proliferation assays (Biomedica) were performed. 4x10^5 CLL cells/well were placed in 96 well culture plates in RPMI containing 1-5% FCS. Cells were treated with inhibitors or controls for 24-48 hours and then incubated with substrate according to the manufacturer's instructions and conversion of the tetrazolium substrate to the formazan derivate was measured by microplate reader (Dynatech) at 450nm wavelenghth.

Antibodies:

For analysis of the PI3K-pathway and PI3K-pathway-associated molecules we used the following antibodies for FACS analysis:

Antibody	*Dilution*	*Company*
Aktin	1:20	Sigma
PTEN	1:20	Cell Signaling
GAPDH	1:20	Santa Cruz
pS380PTEN	1:20	Cell Signaling
pS385PTEN	1:20	Invitrogen
pSTSPTEN	1:20	Invitrogen
Akt	1:20	Cell Signaling
pS473Akt	1:20	Santa Cruz
pT308Akt	1:20	Santa Cruz, Biosource
P110 alpha	1:20	Santa Cruz
P110 beta	1:20	Santa Cruz
P110 gamma	1:20	Santa Cruz
P110 delta	1:20	Santa Cruz

Isolation of PBMCs from peripheral blood:

Peripheral blood was collected and mixed with PBS. Then 15mL Ficoll-Hypaque® (Amersham) were pipetted into 50mL tubes and the peripheral blood was slowly transferred onto the Ficoll layer. Afterwards the tubes were centrifuged at 1850rpm at 16°C for 30min with weak or without centrifuge break. The PBMC layer was collected and the obtained cells were washed twice with PBS. PBMCs were counted and used for protein or RNA isolation, FACS analysis, cell culture experiments or were frozen in RPMI/10%DMSO and stored at -80°C or in liquid nitrogen.

Isolation of primary BMFs from bone marrow:

Bone marrow samples were slowly transferred onto a Ficoll layer. Afterwards the tubes were centrifuged at 1850rpm at 16°C for 30min with weak or without centrifuge break. The BMMC layer was collected and the obtained cells were washed with PBS and then put into culture in αMEM/20%FSC. Medium was changed at least two times a week until fibroblasts could be obtained for expansion.

Inhibitor Testing:

All inhibitors were dissolved in DMSO and diluted in either DMSO or RPMI. For inhibitor testing cells were cultured in RPMI with FCS content ranging from 1%-5% in a density of 5-10 x 10^6 cells per mL medium. In experiments using a co-culture setting primary bone marrow fibroblasts were cultured until they reached confluency and then medium was exchanged to RPMI/1-5% FCS and CLL cells were put into culture onto the monolayer.

For the commercial inhibitors we tested the following data on their specificity and IC_{50} has been provided.

PI 3-Kα inhibitor VIII inhibits the following kinases at the specified concentration:

P110α	P110γ	PI 3-K C2β	P110β
0,3nM	40nM	100nM	850nM

PI 3-Kβ inhibitor VI inhibits the following kinases at the specified concentration:

P110β	P110δ	P110α	P110γ
5nM	100nM	5µM	3,5µM

PI 3-Kγ inhibitor II inhibits the following kinases at the specified concentration:

P110γ	P110α	P110β
250nM	4,5µM	20µM

LY294002 inhibits the following kinases at the specified concentration:

P110α	P110β	P110δ	P110γ
183nM	98nM	1227nM	1967nM

PI103 inhibits the following kinases at the specified concentration:

P110α	P110β	P110δ	P110γ
8nM	88nM	48nM	150nM

PI 3-Kα inhibitor VIII, PI 3-Kβ inhibitor VI, PI 3-Kγ inhibitor II LY294002and PI 103 were purchased from Calbiochem.

Culture of cell lines:

Several hematologic cell lines and some cell lines derived from non-haematological malignancies were used as references for western blotting, PCR and FACS experiments.

Hematologic cell lines (Jurkat, HL-60, Ramos, RL-7, K562) were cultured in RPMI/10%FCS at 37°C/5%CO_2 fully humidified atmosphere. Harvested cells were used for protein or RNA isolation or were frozen in RPMI/10%FCS/10%DMSO and stored at –80°C or in liquid nitrogen.

Culture of adherent cells:

Adherent cell lines (HepG2, MNK, MCF-7, A-431) were cultured in RPMI/10%FCS at 37°C/5%CO_2 fully humidified atmosphere. For splitting the medium was removed, the flasks were washed with PBS and cells were detached by adding trypsine/EDTA. Cells were collected in RPMI and washed with RPMI by centrifugation at 800rpm for 7-10min. Harvested cells were used for protein or RNA isolation or frozen and stored at –80°C or in liquid nitrogen.

This project has been approved by the ethical committee of the Medical University of Vienna (MedUni Wien Ethikvotum: 036/2007).

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List of Figures and Tables:

List of Abbreviations:

PI3K *phosphoinositide-3-kinases*

PIP3 *phosphatidylinositol-3,4,5-triphosphate*

PIP2 *phosphatidylinositol-4,5-bisphosphate*

GEF g*uanine nucleotide exchange factor*

BMF b*one marrow fibroblast*

MSC *marrow stromal cell*

HD *healthy donor*

CLL *chronic lymphocytic leukemia*

TPA *tetra-decanoyl phorbol acetate*

ECM *extracellular matrix*

CK2 *casein kinase 2*

PTEN (MMAC1, TEP-1) *phosphatase and tensin homolog*

AKT/PKB *protein kinase B*

PDK (PHP3) *3-phosphoinositide-dependent protein kinase 1*

PtdIns *phosphatidyl inositol*

SH2 *src homology domain 2*

BCR *B-cell recpetor*

PBMC *peripheral blood mononuclear cells*

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(1) 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.

Kurtova AV, Balakrishnan K, Chen R, Ding W, **Schnabl S**, Quiroga MP, Sivina M, Wierda WG, Estrov Z, Keating MJ, Shehata M, Jäger U, Gandhi V, Kay NE, Plunkett W, Burger JA.; *Blood. 2009 Nov 12;114(20):4441-50. Epub 2009 Sep 17.*

(2) NOTCH2 links protein kinase C delta to the expression of CD23 in chronic lymphocytic leukaemia (CLL) cells.

Hubmann R, Düchler M, **Schnabl S**, Hilgarth M, Demirtas D, Mitteregger D, Hölbl A, Vanura K, Le T, Look T, Schwarzmeier JD, Valent P, Jäger U, Shehata M.; *Br J Haematol. 2010 Mar;148(6):868-78. Epub 2009 Dec 8.*

(3) Reconstitution of PTEN activity by CK2 inhibitors and interference with the PI3-K/Akt cascade counteract the antiapoptotic effect of human stromal cells in chronic lymphocytic leukemia.

Shehata M, **Schnabl S**, Demirtas D, Hilgarth M, Hubmann R, Ponath E, Badrnya S, Lehner C, Hoelbl A, Duechler M, Gaiger A, Zielinski C, Schwarzmeier JD, Jaeger U.; *Blood. 2010 Oct 7;116(14):2513-21. Epub 2010 Jun 24.*

Weitere Kompetenzen:

Sprachkompetenzen (Deutsch, Englisch, Spanisch, Französisch, Latein)

Computerkompetenzen (Office inkl. Access, Bild-, Audio- und Videobearbeitung)

Summary:

This work was performed in the tumor microenvironment laboratory at the Department of Internal Medicine I of the General Hospital Vienna under the supervision of PD. Dr. Medhat Shehata.

Based on the accumulating evidence on the involvement of PI3-K/Akt pathway in the pathogenesis of CLL, this work addresses the question which isoforms of the PI3K are expressed in chronic lymphocytic leukaemia (CLL) cells and which of these isoforms could be therapeutic targets in CLL. Therefore, the expression of the four isoforms of the p110 subunit of the PI3K (α, β, γ and δ) was investigated in CLL cells and compared to normal blood cells. The analysis was performed at the mRNA and protein level using RT-PCR, western blotting and FACS. These investigations were followed by in vitro targeting of the PI3K using isoform-specific pharmacological inhibitors and the evaluation of the effect of these inhibitors on the viability of CLL cells.

The results showed that CLL cells have a highly activated PI3K pathway and express all four isoforms of the p110 subunit of PI3-K. It was also possible to induce apoptosis in CLL cells by inhibiting the PI3K pathway with specific inhibitors as demonstrated by MTT assays and Annexin V/PI staining and flowcytometric analysis. CLL cells exhibited variable degrees of sensitivity to the different inhibitors of PI3-K isoforms. Importantly, a PI3Kα-specific inhibitor was most effective in inducing apoptosis in CLL cells. It was also possible to demonstrate that the PI3-K inhibitors are able to overcome the pro-survival effect of bone marrow stromal cells and induce apoptosis in CLL cells.

This work thus further demonstrates the important role of the PI3K pathway in the pathogenesis of CLL and provides evidence on the significance of the p110-α isoform in particular for the survival of CLL cells. The data suggest that targeting this pathway might be therapeutically relevant and deserves further investigation and clinical evaluation in CLL.

Zusammenfassung:

Diese Arbeit wurde im Labor der Tumormicroenvironment Group an der Klinik für Innere Medizin I des AKH Wien unter der Leitung von Dr. Medhat Shehata durchgeführt. Basierend auf der Feststellung, dass der PI3K/AKT Signaltransduktionsweg in der Pathogenese der CLL eine Rolle spielt, beschäftigt sich diese Arbeit mit der Fragestellung welche Isoformen der PI3K in der chronisch lymphatischen Leukämie (CLL) exprimiert werden und welche dieser Isoformen als therapeutisches Target dienen könnten. Im Besonderen sollte im Rahmen dieser Arbeit analysiert werden, welche der vier Isoformen der p110 Untereinheit der PI3K (α, β, γ und δ) in CLL Zellen exprimiert werden und ob es hierbei Unterschiede zu gesunden Blutzellen gibt. Diese Analyse wurde auf mRNA- und Proteinebene und mittels RT-PCR, Western Blot und FACS durchgeführt. Ein weiterer Schritt in den Untersuchungen war das in vitro-Targeting der PI3K mittels Isotyp-spezifischer, pharmakologischer Inhibitoren und die Evaluierung ihres Effekts auf CLL Zellen. Die Ergebnisse zeigen, dass CLL-Zellen einen hochaktiven PI3K Pathway haben und alle vier Isoformen der p110 Untereinheit der PI3K exprimieren. Es war weiters möglich, mittels spezifischer Inhibitoren in CLL Zellen Apoptose zu induzieren, was mittels MTT Assay, Annexin V/PI Färbungen und Durchflusszytometrie gezeigt werden konnte. Die Zellen reagierten unterschiedlich stark auf die verschiedenen Inhibitoren der PI3K-Isoformen, wobei der PI3Kα-spezifische Inhibitor die stärkste Apoptose-induzierende Wirkung in CLL Zellen zeigte. Außerdem konnte festgestellt werden, dass die PI3K-Inhibitoren trotz des Effekts von stromalen Zellen aus dem Knochenmark, die sich positiv auf das Überleben der CLL Zellen auswirken, Apoptose in den CLL Zellen auslösen können.

Weiters zeigt diese Arbeit, welche wichtige Rolle der PI3K Signaltransduktionsweg in der Pathogenese der CLL spielt und wie bedeutend im Besonderen die p110α-Isoform für das Überleben der CLL Zellen ist. Die Daten erlauben die Annahme, dass dieser Signalweg als therapeutisches Target relevant sein könnte und seine Untersuchung und klinische Evaluierung in der CLL weiterhin von Bedeutung sein werden.