Alterations of the Chemokine Microenvironment in CLL: Multiplex and ELISA sera analysis in CLL patients with early and advanced disease and healthy individuals

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vorgelegt von Roland Aydin

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Berichterstatter: Prof. Dr. med. Martin Dreyling

Mitberichterstatter: PD Dr. med. Christoph Walz

Prof. Dr. med. Michael Fiegl

Betreuung durch den
promovierten Mitarbeiter: Dr. med. Till Seiler

Dekan: Herr Prof. Dr. med. dent. Reinhard Hickel

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I. Overview of Chronic Lymphocytic Leukemia (CLL)

Introduction to CLL

As a first approximation, CLL in current evident-based medicine is defined as a malignancy of CD5+ B-cells and characterized by neoplastic lymphocytes, which appear at first glance mature, in the blood, marrow and secondary lymphatic tissue (1). It should not be understood merely as an isolated malignancy of B-cells, but rather as a complex dysfunction in the interaction between CLL cells and other, mostly lymphatic cell types such as stromal cells, T-cells and nurse-like cells (1).

While a thorough understanding of the discovery of leukemia begins in the 19th century, the basic understanding of “thick blood” as an illness goes back to ancient Egypt 3000 years BC. In the 19th century, Peter Cullen, Alfred Velpeau, and Elfred Donné separately investigated “milky blood”, “pus in blood vessels”, resulting in the term leucocythemia established by the English physician John Bennet, who based the symptoms on the microscopically visible accumulation of leukocytes. Rudolf Virchow, a German physician, understood leukemia as a reversal of the white and red blood cell balance, resulting in a pathological change in viscosity and a compromisation of the immune system. Leukemia is a compound word whose etymology derives from Greek terms, namely leukos (“white”, λευκός) and haima (“blood”, αἷµα). In 1889, the German physician Wilhelm Ebstein separated a chronic form of leukemia from a rapidly progressive form, such building the basis for the modern distinction. Since then different treatment options were tested, mostly to no avail until in 1962 Emil J. Freireich, Jr. and Emil Frei III initiated the advent of modern leukemia treatment by introducing chemotherapeutic agents (2).

Epidemiology of CLL

From an epidemiological perspective, CLL is the most common form of leukemia in adults, affecting mostly the elderly with a median age of diagnosis of 72 years for male patients and 75 years for female patients (3). In Germany in 2011, 7.4 male and 4.8 female patients per from 100’000, respectively, were diagnosed with CLL, showing a significantly skewed
gender ratio for CLL (M/F=1.7/1). People of African or Asian descent are much less likely to be affected by CLL (4).

In Germany, the yearly mortality amounts to around 1000 men and 850 women, which compared to the German yearly incidence of new cases of 3000 for males and 2000 for females, shows the disease’s status as a chronic condition which in many cases affects quality of life and lifespan, but is not typically immediately acute (5).

![Figure 1: CLL incidence and age of diagnosis separated by gender, 2012-2014. Source: Adapted from (6)](image)

Regarding the CLL’s pathophysiology, the immunophenotype is rather complex (as will be subsequently shown), without a singular underlying mono-cause identified for CLL patients. Correspondingly, the clinical phenotype presents as rather heterogeneous (7).

Corresponding to the large role of genetic features, which in turn are not necessarily strictly de-novo but may also predisposed for by hereditary factors, family history is an important risk factor. Relatives in the first degree of CLL patients have a 8.5 times higher risk of eventually being diagnosed with CLL (8). Regarding de novo mutations, organic solvents such as benzene have been implicated (9).
Most of the patients are first identified for further diagnostic investigation through a routine blood test showing an atypically (e.g. without an accompanying infection) elevated white blood cell count. These blood tests may follow reports of general frailty which given the advanced patient age can often be missed. In other cases the diagnosis is an auxiliary finding when blood tests for a different health issue are conducted. In addition, enlarged lymph nodes may cause further testing, even in the absence of an elevated white blood cell count, although this is comparatively rare. In more advanced stages, the patients may first present with lymphadenopathy, spleno- and/or hepatomegaly, signs of bone marrow deficiency, autoimmune-cytopenia, B-symptoms, infection susceptibility and skin appearances.

Diagnostic criteria for CLL entail a lymphocytosis of at least 5000/µl. In the blood smear, small, mature looking lymphocytes (featuring "Gumpecht Kernschatten" as preparation artefacts, see Figure 2) can be found. In subsequent cytometric analyses of the leukemia cells, antibodies against typical B-cell markers (CD19) as well as CD23 and the T-cell antigen CD5 can typically be found. Also, a small expression of immunoglobins CD20 and CD79b may occur, as well as IgKappa and IgLambda peaks of monoclonal origin (10).

**CLL staging**

In general, two different classifications are commonly applied for staging CLL, Rai and Binet, respectively.

Rai staging includes stages 0 to IV and is based on absolute lymphocytosis as well as clinical symptoms such as splenomegaly (11). Stage 0 is defined as bone marrow and blood lymphocytosis in isolation, stage 1 adds the enlargement of lymphatic nodes, stage II either an enlarged spleen and/or an enlarged liver, stage 3 lymphocytosis with anemia and stage IV with additional thrombocytopenia (11). Gender and age are disregarded as poor survival predictors, and thus poor stage classifiers. Stages 0 is considered low risk, stages I and II as intermediate, and stage III and IV high risk.

In the European health care sector, the Binet classification which has been established in 1981 is commonly preferred (12, 13). In contrast to the Rai classification, which does not include the areas affected by CLL, Binet includes that information and as such offers a level of prediction power superior to that of Rai. There are three stages, differentiated by anemia,
thrombopenia, and the areas involved (the latter providing options of axial, cervical, or inguinal lymph nodes, as well as a distinction between unilateral and bilateral, with options for the spleen and/or liver as well. Patients of group A do not suffer from anemia, nor thrombopenia, and have less than three of the aforementioned areas involved. The survival rate for this group is quite positive, easily surpassing 10 years. The resulting course of treatment is usually a watchful waiting strategy based on the benign outlook contrasted with the (previously) often harsh side effects of treatment (14).

Group B patients are characterized by three or more sites involved in the CLL condition, a hemoglobin level of greater than 100 g/L, with a platelet count still exceeding $10^9$/L. For this group, the median survival is decreased to 7 to 9 years.

Lastly, in stage C the median survival is further lowered to 1.5 to 5 years. For patients in this high-risk group, the criterion for inclusion is not the number of sites involved, but rather the severity of symptoms. A hemoglobin level of less than 100 g/L and/or a platelet count of less than $10^9$/L are inclusion criteria for this stage. A treatment is typically indicated once this stage has been reached.

In comparison to Rai, stage A corresponds to Rai stages 0 to II, stage B corresponds to Rai stages I and II, and stage C corresponds to Rai stages III and IV.

As with most classification systems, prognoses can still vary strongly within a stage. For Binet CLL staging specifically, problems that were eventually revealed include a large disparity in future outlook for stage A patients specifically, with the mutation status of heavy chain genes, and expression of various intracellular markers such as ZAP-70 and extracellular markers such as CD38 leading to an extraordinary heterogeneity of the clinical course (15).

Other markers which were shown to differentiate within stage A Binet patients were levels of serum thymidine kinase (sTK), lymphocytosis, $\beta$2M level, and CD38 expression, all of which could be used as predictors for the clinical outcome in Binet A (16, 17).

The trend in recent years seems to be a movement away from the classical staging of either Rai or Binet, towards an analysis of individual serum levels of certain markers, which seem to yield a prognostic gain superior to that of simple stage classification. This study aims to add to this tendency and further validate markers that can be examined from CLL sera (17).
While many publications have focused on Binet A outcomes, access to large scale CLL trials allows this study to also contrast both healthy subjects and Binet A with later-stage CLL (stages B and C).

**Prognostic factors of CLL**

Due to the unusually large range of possible lifespans for CLL patients, ranging from a few months to upwards of 20 years (18), diagnostic factors are of particular importance in predicting a patient's individual risk and prognosis. Interestingly (see the section on factors influencing therapy on page 13), these prognostic factors in large part have not yet found their way into therapeutic algorithms, i.e., they may offer insight into a disease's probable course, but do not necessarily affect the larger choice of treatment (except for del17p13 and its often correlated TP53 mutation status).

The Binet und Rai stagings do not take into account many factors specific to a patient but rather a small subset of them. Consequently, much research has been focused on identifying and quantifying the pertinent individual markers in order to give patients both a better estimate of their disease trajectory, as well as possibly better individualized treatment options (19).

**Genetic prognostic factors of CLL**

Genetic abnormalities are found in 80% percent of the patients, as discussed in a 2014 review article by Puiggros et al (20). These are typically identified using Fluorescence in situ hybridization (FISH) (19).

The most common of these, with around 50% of CLL patients affected, is the deletion of the 13q14 region. Most of the patients in this group can be considered as low-risk. However, newer research revealed that there may be a high-recurrence rate subgroup within this group, leading to a higher percentage of patients in this group faced with a negative prognosis than originally estimated (21).
Trisomy 12, and 11q23 deletions are the next most common genetic variations of CLL. Depending on different studies, either can seem to be the second-most common after 13q14 deletion.

Trisomy 12 is associated with an intermediate risk assessment (19). Other, more rare trisomies such as trisomies 18 and 19 only co-occur if trisomy 12 is already present (22).

11q23 deletion affects between 5-20% of patients. It has the second-worst outcome after del17p13, with a consistent progression of disease and with worse overall survival compared to normal karyotypes. Clinically, these patients are set apart by their large and extensive lymphadenopathy (19).

Lastly, del17p13, i.e. a deletion in chromosome 17, can be found in just 3-8% of newly diagnosed patients. That prevalence increases to up to 50% in relapsed or treatment-refractory patients, showing the outsize and negative effect size of this genetic abnormality and clonal selection in case of subsequent relapses. Based on its significantly altered disease trajectory, it affects the course of treatment as outlined in the clinical guidelines issues by the German CLL Studygroup. Patients with this mutation are thus seen as a very high risk group, to a degree that their condition may eventually be separated from CLL to constitute its own disease classification.

Their adverse disease progression is deemed to rely in large part on their lacking response to the FCR-regimen of treatment (fludarabine, cyclophosphamide, and rituximab), leading to a special therapeutic category in the guidelines which pivots from to FCR to bendamustine as the first-line therapy, and allogeneic stem-cell transplantation as a potentially curative option, although in a landmark 2008 study only 19 of 44 transplanted patients of this subgroup were alive at last follow up (median observation time 39 months) (23). Regardless of the currently available kind of treatment, overall survival as well as progression free survival time are significantly shortened (24). In the CLL4 trial regarding overall survival, a median survival of 1.5 years after first-line treatment with fludarabine alone, or combined with cyclophosphamide, could be shown in a publication by Stilgenbauer et al (25). Regarding progression free survival, in a UK study, only between 10% to 36% of the 777 patients remained progression free after five years, depending on the treatment group (26).

Patients with a CLL variant characterized by a p53 dysfunction are likewise not responding as well to immunotherapy compared to patients lacking that mutation and therefore are subject to a diminished overall survival rate (27), similar to del17p13 patients. As shown by Zenz et al
(28), TP53 mutation CLL patients suffer a decrease in progression free survival (PFS) of around 40 months (23.3 with a TP53 mutation compared to 62.2 months unmutated, respectively) as well a reduction in overall survival of around 50 months (29.2 for this group compared to 84.6 months for group without a TP53 mutation). In that study (28), the low prevalence of the TP53 mutation in CLL was also made evident; only 28 of 328 CLL patients presented with the TP53 mutation (8.5%), and a TP53 mutation without an accompanying del17p13 status was found in only 4.5%. However, the adverse effect of a TP53 mutation could be shown to be independent from the already strong deleterious effect of the del17p13.

An additional important classification regarding prognosis has been shown to be inherent in the IgVH mutation status (29), that is somatically mutated Ig variable-region heavy chain genes (30, 31). Perhaps counter-intuitively, patients with an unmutated V(H) gene can be considered as high-risk patients with a reduced median overall survival of merely 117 months, compared to 293 months with a mutated V(H) gene (29, 30, 32). The differences in the mimetopes between IgVH-mutated and IgVH-unmutated immunoglobulins may serve as future targets for therapeutic modalities in CLL, as discussed by Seiler et al (33).

Lastly, a study by Schroers et al (34), could demonstrate the validity of the 70-kDa zeta-associated protein (ZAP-70) as a prognostic predictor for CLL patients: "B-CLL cases with a high percentage of ZAP-70\(^+\) (greater than or equal to 20%) leukemic cells are characterized by an unfavorable clinical course and a significantly reduced treatment-free survival as compared to ZAP-70\(^-\) (<20%) patients." (34, 35)

The Nature-published study also showed that ZAP-70\(^+\) CLL patients have a higher risk for autoimmune complications. Different permutations of CD38 and ZAP-70 status were considered, and three distinct outcome groups distilled: ZAP-70 and CD38 both negative which resulted in a generally favorable outcome, patients with one of these two positive who were subject to an intermediate outcome, and ZAP-70\(^+\) as well as CD38\(^+\) group with a poor prognosis. The median treatment-free overall survival for the three aforementioned groups amounted to 130, 43 and 30 months, respectively.

ZAP-70 does not seem to be wholly independent of IgVH status, namely a study by Ertault-Daneshpouy et al could even show that the combination of these ZAP-70 could be used as an easier to determine surrogate parameter in place of the highly correlated IgVH-Status (36-38).
Established serum markers as prognostic factors of CLL

Based on data from the Binet A CLL1 study (39), serum β2-microglobulin, and serum thymidine kinase were among the independent predictors for overall survival, and could be determined from serum alone (40).

Higher levels of serum thymidine kinase were associated with an accelerated disease progression as early as 1984 (41). The same correlation was definitively established for serum β2-microglobulin in 2009 (42).

Amongst the kinetic parameters which could be inferred from serum are the lymphocyte doubling time (LDT), which was amongst the first prognostic tools for CLL, with milestone research publications in 1966 (43) and 1987 (44). The LDT is defined as the time period needed for a doubling of the peripheral blood lymphocyte count to take place (45).

An LDT of under twelve months is associated with an aggressive variant of CLL, while a longer period is correlated with a better (i.e., slower or more moderate) disease progression. However, the search for the best iteration of this simple prognostic metric has been the subject of some debate: While there are many studies indeed exemplifying such an predictive effect of LDT itself (see above, also (46)), others preferred derivative indices such as the lymphocyte accumulation rate (LAR) (47).

Another important category of markers which can be derived simply from testing blood serum, a comparatively cheap and non-invasive diagnostic modality, are cell surface molecules often soluble in the sera, namely clusters of differentiation (CDs).

Among those, CD23 is a protein from the B-cell membrane the concentration of which can be interpreted as a prognostic predictor for overall survival in CLL. Furthermore, monitoring the dynamic progression of CD23 levels may help to identify those patients who progress more rapidly (48). Analogously, CD49d, which is associated with proliferation markers, was identified as an independent risk factors for progressive disease (49).

Lastly, CD38 has been investigated for a number of years. A meta review conducted in 2011 was able to subsume the previous studies into the determination that CD38 expression by CLL cells correspond to a more aggressive clinical behavior requiring earlier intervention and
being affiliated with a lower life expectancy, and that said CD38 expression (‘‘CD38+’’) can be measured in peripheral sera (50).

![Peripheral blood smear of CLL, using Wright’s stain (marking leukocytes). CLL cells are identified by their low amount of cytoplasm and their stained nuclei. Source: https://commons.wikimedia.org/wiki/File:Chronic_lymphocytic_leukemia.jpg#/media/File:Chronic_lymphocytic_leukemia.jpg (Public Domain)](image)

**Factors currently effecting treatment modality for CLL**

As of today CLL has no definitive curative treatment, either through chemotherapy or application of newer compounds such as antibodies in the course of immunotherapy. The only current curative option, which is not always viable, is allogeneic stem-cell transplantation. With CLL having a much higher incidence for people of an advanced age, given the condition of older patients generally being fraught with more frailty, such a stem-cell transplantation is often not an option. Due to its side effects and the lack of alternatives, mostly high risk patients in good health constitute eligible candidates for stem-cell transplantation.

Figure 3 shows an excerpt of the first-line therapy of the current German clinical guidelines of CLL. In particular, all decision nodes and their dependent variable are depicted. It is thus evident that the current decision for CLL treatment depends first on the status on symptoms, such that for asymptomatic patients in general a watchful waiting approach is first taken. Only after the onset of symptoms is further treatment warranted. That further treatment depends as a first approximation on the general health status of the patient. A patient constitution deemed...
‘fit’ leads to a “go go” approach, while for ‘unfit’ patients a “slow go” approach is preferred. Currently, “frail” patients receive no therapy other than Best Supportive Care (BSC).

For the remaining “go go” and “slow go” groups, the decision rests to some degree on biological age as a proxy for the patient’s resilience regarding treatment (with 65 years of age often used as an indicator, to be modified by a patient’s individual health status), but mainly on the presence of del(17p13) and TP53 mutation status. Thus, it can be stated that even as of 2017, the only markers used for the clinical decision algorithm, other than age, symptomatic status, and overall patient health, are as follows: del(17p13) and TP53 mutation status. While a number of studies, such as CLL7 on the feasibility of watchful waiting compared to an earlier intervention using FCR (51), have been conducted, to the author’s best knowledge no definitive changes have yet been caused by said studies.

For the current clinical guidelines for second-line therapy in the case of a symptomatic disease recurrence, the same criteria apply, with age being substituted for a differentiation between early and late disease recurrence, genetic testing, and tolerability of previous treatments.

In 5-10% of CLL patients a so-called Richter’s transformation, or Richter’s syndrome, can occur (52). In that event, the CLL transforms into a fast-growing B-cell (mostly Diffuse Large B Cell Lymphoma, DLBCL) Non-Hodgkin lymphoma, a non-Hodgkin lymphoma, with poor prognosis and largely resistant to treatment other than possibly allogeneic stem-cell transplantation (52). Typical symptoms in these patients are a rapid clinical deterioration, fever without an infection, enlargement of lymph nodes, and elevated levels of serum LDH. A
possible association and triggering through an EBV infection, as well as an association with unmutated immunoglobulin heavy chains (IgVH) of fewer than 2% and non-del13q cytogenetics, is currently the subject of scientific exploration. The median survival of these patients is reduced to five to eight months (53).

In 1996 the German CLL study group (DCLLSG) under the research auspices of Prof. Dr. M. Hallek was founded in order to optimize the diagnosis and treatment of CLL patients through the large-scale conducting of Phase I-III trials, as well as by establishing a cooperating network of treatment centers, standardizing the diagnostic and therapeutic algorithms (54).

Trials conducted by the DCLLSG were the major source of material used in this study, as leftover sera from the CLL1 and CLL8 studies in particular, along with their clinical phenotypes and outcomes, could be acquired.
The role of the microenvironment in CLL pathophysiology

As a preliminary characterization, B-cell chronic lymphocytic leukemia (CLL) describes the accumulation of CD5+ monoclonal B-cells in secondary lymphoid organs, marrow and blood (55). Such an accumulation generally results from an imbalance between proliferation and apoptosis. For the monoclonal B-cells, both an increased rate of production and a reduced rate of reduction (or a mixture of the two) could in principle result in the accumulation of B-cells. For CLL in particular it has been observed that a majority of leukemia cells are fixed in one of the two gap phases of the cell cycle (G1), as opposed to the S or M phase which would indicate a proclivity to proliferate (56, 57). Consequently, it follows that the accumulation is not due to a heightened proliferation but rather to an inhibition of cell death (58). The pathogenesis of apoptosis inhibition can, however, extend to a deregulated proliferation pattern, mediated through proteins involved in early G1 phase regulation, such as various cyclin-dependent kinases and their universal CDK inhibitor p27 (Kip1) (59). Still, the abundance of B-cells taken in conjunction with their status of non-proliferation (as evident by their stage in the cell cycle) point to the pathology being driven by the disruption of the reductive processes, which for B-cells is mostly apoptosis.

Thus, the question of how exactly the natural induction of apoptosis is perturbed attains major relevance towards furthering the pathophysiological understanding of CLL. In particular, the abundance of B-cells in secondary lymphoid organs and the bone marrow (i.e., specific loci) suggests that the different (micro-)environments may modulate B-cell behavior (60). The importance of micro-environmental complex interactions is underscored by the observation that CLL cells regain their ability to induce spontaneous apoptosis in ex-vivo conditions that resemble culture conditions for B-cells. The contrasting juxtaposition between CLL B-cells in-vivo and in ex-vivo culture conditions suggests that the only differentiating factor, namely the microenvironment, plays a significant role in deregulating the physiological induction of apoptosis.

As it stands to reason that not all of the many complex interactions between B-cells and the various parts of their microenvironment share an equal explanatory weight in explaining the deregulation driving CLL, an important step both in understanding the condition and in finding therapeutic targets consists of identifying those clusters of cytokines whose presence significantly differs between either healthy and ill patients or between low-, medium-, and high-risk patients. While it is already common usage to divide CLL into groups based on
biomarkers of poor prognosis such as ZAP-70 expression (61), cytogenetics ((62, 63)) or IgVH mutation status (30, 32, 64-67), the same is true to the same degree for markers expressed not within the cell but as part of the cell’s microenvironment.

There are two main complicating factors in isolating the individual contributions of chemokines in the CLL microenvironment to the disease’s pathology: On the one hand, the nature of their complex interactions contradicts simple explanatory models in which an isolated chemokine directly affects disease progression without impacting other parts of the microenvironment. While complex interactions do not preclude single cytokines to carry a comparatively greater influence on the deregulation of CLL apoptosis than others, the observation that such an effect may be mediated through complex interactions entails a testing regimen which, rather than isolating individual cytokines, needs to model individual cytokine effects in the context and presence of all other cytokines present in the microenvironment. In particular, rather than individual cytokines, clusters of cytokines could be defined as those functional units of cytokines that are most intimately connected and causally linked (68). On the other hand, the pleiotropy of the gene mutations, i.e. the observation that one gene may influence seemingly unrelated traits and mechanisms, confounds straightforward deductions of cause and effect. For example, when both pro- and anti-apoptotic pathways may be triggered by the same gene mutation, prima facie it is unclear which influence dominates.
As previously outlined, overall, the importance of the cytokine environment has been well established (7). Even though genetic sequencing has increasingly provided the landscape of genetic variations resulting in CLL, the vista such gained is one of a high degree of heterogeneity both intra- and inter-patient (69). Since there is a large gap between identifying a mutation and linking it to a clinically relevant effect in a long causal chain, a better understanding of the cytokines in the CLL microenvironment is not made superfluous by the increasingly better understanding of the CLL genomes, on the contrary, it could well be argued that now that the initial genetic links of the chain have been established, following them through the microenvironment onto their prognostic effects is of all the more importance.

The chemokines chosen in this study will hereafter be outlined in further detail. Note that the list of chemokines which were studied is not exhaustive and in particular does not encompass all candidate cytokines which may play a role in or be elevated with the advent of CLL. Practical factors (such as the availability of cytokines as part of testing kits) did play a role. Nevertheless, the array of cytokines were selected with care, and cytokines already established to play a role or which may arguably play a role due to their central importance in
known B-cell signaling pathways were prioritized. As a next step, the rationale behind the cytokines chosen to be investigated will be presented.

**EGF**

The epidermal growth factor (EGF), a polypeptide ubiquitous throughout the body, can activate a large number of signaling pathways, including among others PI3K/AKT, RAS/ERK, and JAK/STAT (70). Many pathways are related to proteins involved in apoptosis, in both an inhibiting as well as an excitatory/triggering capacity. Due to that pronounced linkage to cell proliferation processes, EGF may be one of the best known cytokines in cancer research, owing to the popularity of its receptor (EGFR), the overexpression of which has been linked to poor prognosis and decreased survival in a variety of types of tumors (71). As such, EGFR inhibitors such as Ibrutinib (which is also used for CLL), Bosutinib, Dasatinib, Nilotinib, Ponatinib (all of which are used for CML), Erlotinib, Gefitinib, and Afatinib have become a mainstay in the therapy of a variety of cancers ranging from colorectal to NSCLC to ZAP70+ CLL. More relevant to this research topic, EGF has been shown to be an independent prognostic factor in CLL patients (70), and has thus been chosen as a cytokine to investigate in differently risk-stratified CLL sera.

**CCL2, CCL3, and CCL4**

The CC family of chemokines plays an important role as pro-inflammatory agents by acting as chemoattractants for macrophages and various types of lymphocytes (72). Many CC family chemokines have been linked to various inflammatory diseases, ranging from CCL1 – CCL5 (73) for encephalomyelitis, to CCL3 and CCL5 for Multiple Sclerosis (74), to CCL2, CCL3, and CCL4 for diabetes (75) to name just a few examples. Some members of the CCL family appear to significantly interact with CLL. CCL2 has been reported to play a dominant role for CLL cells in vitro (76, 77). CCL3 (formerly called the macrophage inflammatory protein-1 alpha, MIP-1α) has also very recently been implicated to be involved in the cross-talk between CLL cells and accessory cells in the lymph node microenvironment (78-80). While this research preceded the aforementioned result, examining the link between levels in the serum microenvironment to the lymph node microenvironment is of interest when inferring properties of one microenvironment that is
hard to access (lymph node) from one that is comparatively much easier to access (serum).

CCL3 and CCL4 (formerly called the macrophage inflammatory protein-1 beta, MIP-1β) are both emitted by CLL cells upon activation by the B cell receptor in the presence of nurse-like cells (81). Some of the major therapeutic agents such as ibrutinib and idelalisib target BCR signaling and as such also influence CCL3 and CCL4 levels (82).

The pathophysiological impact of interrupting the pathways related to CCL3 and CCL4 has been described as interfering with chemokine-mediated adhesion as well as the migration of CLL cells within secondary lymphatic tissue. Driving CLL cells out of their lymphatic microenvironment also seems to deprive them of critical proliferative and anti-apoptosis influences (82). The importance of the BCR pathways as well as CCL3 and CCL4, both of which are linked to the BCR pathways, thus identify CCL3 and CCL4 as good candidates for not only comparing concentrations between different risk-/ and healthy study participants, but also for comparing microenvironmental differences between the microenvironment of secondary lymphatic tissue and that of blood sera.

**Soluble IL-2 receptor alpha chain (sIL-2Ra)**

sIL-2Ra, also known as CD25, is the soluble cell-surface bound receptor on T-cells after it was sheared off the T-cell via proteolysis. sIL-2Ra has been implicated both in the differentiation as well as the proliferation of lymphocytes, and an increase in its concentration has been linked to autoimmune disease, virus induced lymphoproliferation, leukemia (83), and T cell proliferation (84). It has been shown to be a prognostic parameter in a ‘neighboring’ disease, namely for Diffuse Large Cell Lymphoma and T-cell lymphoma. (85).

**TGF-alpha**

Transforming growth factor alpha (TGF-α) is one of the main elements constituting the group of epidermal growth factors (EGF). It is a ligand whose activity is enabled when it binds to a receptor which initiates cellular signaling via protein kinase activation (86). The associated pathway has a pronounced influence on cell proliferation, differentiation, and development,
and is as such critically involved in a variety of cancers (87). The protein is not necessarily transmembrane-bound, but also occurs in a soluble variant (88).

TGF-α is of particularly high potential importance in regards to the CLL microenvironment because it is not only produced and secreted by neurons and astrocytes, as well as keratinocytes, but also macrophages. These are an important constituent of the microenvironment in lymphatic tissue (89, 90). TGF-α and EGF bind to the same receptor, which can be explained by their close relationship as part of the same family. That binding can increase the propensity towards cell proliferation events, which are physiologically appropriate e.g. in the embryonic phase, or for tissue repair. However, naturally such pro-proliferative pathways can also be involved in tumorigenesis. It has thus been implicated for a variety of cancers, such as colon cancer, breast cancer, renal cell carcinoma both papillary and nonpapillary, and non-small-cell lung cancer (91-94), among others, CLL not yet among them. The naming similarity notwithstanding, TGF-α bears no direct relation to TGF-β.

**TGF-α receptor**

The receptor of TFG-α, also called the EGF-receptor, naturally occurs in the same regions in which TFG-α itself is exprimated. It is of potential relevance for measurement in the peripheral blood as it can also be measured in a form disassociated from the cell membrane it typically is bound at (95). Given its role in the signaling chain further downstream from TFG-α, it is unsurprising that its overexpression can, just as with its binding ligand TFG-α, also be associated with poorer survival rates for a host of malignant diseases, such as neck, ovarian, cervical, bladder, esophageal, gastric, breast, endometrial, and colorectal cancers (95). Even though for a host of other conditions based on proliferative dysfunction such as CLL, for which no direct link has yet been established, it is possible that patients could still benefit from anti-EGFR therapies, as speculated in a meta review by Nicholson, 2001 (95). The implication of both EGFR and TFG-α in so many malignancies can be surmised to follow from their position on a rather general proliferative pathway, which can cause and/or amplify proliferation rates and thus disease progression for a host of conditions. In that regard, it is also important to note that the phosphorylation of a protein-tyrosine kinase triggered by the activation of EGFR causes further autophosphorylation to other tyrosine residues, thereby
activating other transduction pathways, and consequently potentially explaining the complex activation patterns that can be influenced by EGFR and its ligand.

**Stem cell factor (SCF)**

Stem cell factor (SCF) is a novel hematopoietic growth factor which constitutes the ligand for the c-kit tyrosine kinase receptor. SCF, is a product of the gene *Sl* (“steel”), “a gene critical to the development of several distinct cell lineages during embryonic life with important effects on hematopoiesis in the adult animal” (96).

**IL16**

Interleukin 16 (IL-16) acts as a chemoattractant factor. It critically modulates T-cell activation. In contrast to most of the other chemokines previously described, IL-16 synthesis and locus of action is centered on lymphatic tissues to the exclusion of most other tissues, emphasizing the profile of IL-16 as an immune regulatory molecule (97).

**Other cytokines**

Chemokines on recently elevated pathways relating to CXCL12 and CXCL13, targeted by compounds such as Ibrutinib, and relating to B-cell receptor (BCR) signaling and the CXCR4 chemokines, targeted by Idelalisib, both of which have recently been approved for CLL treatment (98), have not been incorporated into the study design due to a combination of reasons. Either the novelty of the discovery of these chemokines being an integral functional part of the CLL microenvironment precluded their integration into the setup of this study, and/or their respective experimental panels were not within reasonable organizational and fiscal constraints easily combinable within one experimental setup, i.e., no panels for multiplex assays incorporating the respective beads for the aforementioned chemokines as well as the others previously expounded upon, and no easily available ELISA assays building on the knowledge extracted from the preliminary multiplex studies. The combination of these factors imposed a limit which was on aggregate deemed acceptable. At this point it has to be
noted that the author knows of no study which incorporates every known chemokine in one experimental setup. As such, with limitations unavoidable, the current setup can be seen as a practical trade-off between choosing amongst the most relevant candidate chemokines for a scientific gain of knowledge, and experimental feasibility.
II. Purpose of, and approach used in this study

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in adults, with a wide spectrum of disease severity and high variability regarding overall survival after diagnosis, and response to treatment (cf. the section on Epidemiology of CLL, page 5).

Dividing this range of outcomes into sensible categories and establishing clinically viable criteria for correctly classifying newly diagnosed patients according to their most likely disease variant is not only important for the patients’ coping with the disease, but also crucial for the treating physician, impacting planning not only for supportive care but also for the choice of first line treatment (cf. the section on Factors currently effecting treatment modality for CLL on page 13). As of 2017, a host of prognostic factors have been established to help to give an accurate prognosis (cf. the section on Prognostic factors of CLL, page 9).

In the identification of prospective predictors, and of new criteria that may lead to sensible categories and subcategories for CLL, this study assigned special importance to the role of the microenvironment of CLL, of many which different candidate markers were considered (cf. the section on The role of the microenvironment in CLL pathophysiology, page 16). Even for chemokines which show promise for such a purpose, their suitability also depends on their concentration in the microenvironment being somewhat linked to / mirrored in the concentration in the peripheral blood. That is because the material used in this study consisted of blood sera available upon conclusion of two large-scale German CLL trials, namely the CLL1 (cf. the section on the CLL1 study, page 26) and CLL8 trials (cf. the section on the CLL8 study, page 28).

The testing paradigm can be interpreted to fall into two broad phases, based on the rationale of first identifying and then confirming the prognostic potential of markers.

At first, a closely considered but rather large set of markers were tested using a high-throughput testing paradigm of multiplex assays (cf. the section on Multiplex testing employed for the main phase of this study, page 35). This permitted the reduction of the large selection of potentially interesting markers to its most promising (regarding our patient collective) analytes.

In a second step, these remaining markers were retested using an established ELISA methodology in order to confirm and validate the results (cf. the section on Conventional ELISA confirmation testing employed for this study, page 40).
Finally, the results are discussed and brought into the clinical perspective, along with possible directions for future research.

### III. Patient selection

#### Pilot studies

Access to the remaining, frozen sera of the large-scale CLL1 and CLL8 trials through Chronic Lymphocytic Leukemia Study Group (CLLSG) was gated by first having to demonstrate a sensible and promising research plan, in order to ensure an appropriate promising research use for the rather valuable sample material. As such, preliminary studies using a multiplex assays were conducted prior to applying for the CLL1 and CLL8 sera.

That initial testing relied on 21 blood sera which were excluded from the CLL1. These were the only excluded samples which were excluded from the CLL1 study for reasons unlikely to compromise any of the preliminary research results. 16 of these staged as Binet A, one both for Binet stages B and C, one without Binet classification. Additional clinical data for these patients was available in the form of Leukocyte counts, IgVH mutation status, and chromosomal deletion- and ZAP70 status. Two samples derived from patients with MCL and one with T-PLL. This results in a total of 21 patients, labelled CLL1 to CLL21.

In addition, five samples could be procured from CLL patients from the University’s oncology department, referred to as Muc1 to Muc5.

Finally, five blood samples from healthy individuals, who were working in the University’s oncology department, were kindly volunteered as well, referred to G1 to G5.

#### Multiplex- and ELISA-based studies

Upon completion of the preliminary multiplex assays, further research was greatly facilitated by access to serum samples both from the CLL1 trial as well as from the CLL8 trial, both conducted by the German CLL study group. An overall 159 patients from the CLL1 trial as well as 49 patients from the CLL8 trial were randomly selected to be included into this study. All serum samples which were investigated were obtained prior to any treatment undergone as
part of the study and stored in a cryogenic freezer at -80°C. The samples were thus obtained just after patients had been included in the respective trial, but before any interventions had taken place. Informed consent was granted in accordance to all relevant guidelines and with the approval of the ethical review boards of the participating institutions. There were also a total of 26 healthy controls used, for a total of 226 subjects.

**CLL1 study**

The CLL1 Study (99, 100) was a prospective, multicentric Phase 3 Study, running from 1997 until 2004, to determine the event-free survival of CLL patients with two therapeutic arms, each with a different treatment option.

Included were 877 patients between the ages 18 to 75 years, all of whom were diagnosed with B-CLL in Binet-stadium A and did not receive treatment at the time they were included in the study. Of the 877 patients, risk stratification was conducted for 788 patients. 99 patients were dropped from the analysis due to trial violations.

The risk-stratified 788 patients were divided in three groups based on their progression risk. The progression risk was determined based on four criteria: S-TK-/ß2-microglobulin testing or elevated thymidine kinase (TK), and lymphocyte doubling time (LDT) or diffuse bone marrow infiltration.

In the first stage the patients were thus divided into subgroups: high risk patients and low risk patients. Low risk patients were summarized in group III (471 patients) and received no treatment, according to a watchful waiting paradigm. The high risk patients (sample size of 218) were randomly divided in two further subgroups: group I (104 patients) was treated with Fludarabin 25 mg/m²/d for 1 to 6 days, with no more than 6 cycles with a break of 28 days, while group II (114 patients) remained untreated, similar to group III.

Group II and III underwent periodic follow-ups after 3, 6, 9, 12, 18 and 24 months, respectively. Group III were reevaluated after 2 cycles and were subsequently restaged.

The study ended after 45 months. The results of the study were published in 3 articles. One of them confirmed a previously published preliminary finding that progranulin could constitute a new diagnostic predictor in CCL patients (101).
In another article it was shown that the treatment with fludarabine in high risk patients prolongs the progression-free survival (PFS), but not the in many respects most important variable, overall survival (OS) (100).

In the third paper, an amalgam of various prognostic parameters was investigated in regards to their relative prognostic value, and their potential value for an aggregate risk predictor. Various clinical and biological factors were chosen for inclusion towards that purpose (TK, LDT, beta-2-MG, absolute lymphocyte count, age, sex), i.e., to help predict the progression in CLL patients. The paper reaffirmed that the underlying paradigm of the CLL1 study, that is separating high-risk and low-risk patients according to the Binet classification, remains an accurate strategy as exemplified by various prognostic parameters also separating in distinct subclasses according the Binet classification (99).

Study patients of the CLL1 study were recruited from Austrian (2 centers) and German (115 centers) study centers, both from clinics as well as private practices (102, 103).

For the purposes of this study, frozen leftover sera of patients from the CLL1 study could be acquired (along with samples from other studies) in order to for new candidate parameters using a new (in regards to this subject domain) multiplexing paradigm.

**Patients included from the CLL1 trial**

For the 151 patients randomly selected from the CLL1 trial, their median age was 61 with a range of 35-76 (this age bracket coinciding with the onset of CLL typically later in life), all of whom (100%, 151/151) were classified as Binet A. Most of the patients had an IgVH status of mutated (74%, 110/151), whereas a quarter of patients (26%, 41/151) were unmutated. With regards to their cytogenetics only 2% (3/151) tested positive for the del17p- deletion, while 4% (6/151) tested positive for the del11q- deletion. In regards to the del17p deletion, that corresponds to a prognostically favorable group composition (the population average would be 7%) (19). Likewise, IgVH status differs significantly from disease averages, as usually up to 50% are IgVH unmutated (104), compared to 26% of the patient collective of the CLL1 trial (105). Lastly, the del11q population is only represent a quarter as often as compared to previously published data (4% to 18%, respectively) (19). Based on these characterizations of
the CLL1 trial, it can overall be stated to represent a particularly benign (in the context of CLL) cohort.

**CCL8 study**

The CCL8 study (106) compared two different treatments of B-CLL patients, classified as either Binet stadium C, or Binet stadium B who also required treatment. Treatment requirement in this context was defined as either being in stadium C, or in Binet stadium B plus one of the following criteria being met: B-symptoms (fever over two weeks with no underlying infection, night sweats, loss of weight, and fatigue), continual progression, signs of progressive failing of the bone marrow system (resulting in anemia and/or thrombocytopenia), splenomegaly which is either pronounced, progressive, or painful, grossly enlarged lymph nodes or cluster clusters thereof, or a symptomatic hyperviscosity with a leukocyte concentration greater than 200 g/l.

The CLL8 study, just as the CLL1 study, was coordinated by the German CLL study group (GCLLSG). The duration of its data gathering started in 2003 and was undergoing until 2008, as a randomized Phase 3 registration study. 760 patients older than 18 years who received no treatment prior to this study were included. They were randomly divided in two groups. The recruitment of patients was conducted multinationaly. A majority of participating institutions were in Germany (121), but there were also contributing centers from Australia (3 centers), Austria (3 centers), Belgium (6 centers), the Czech Republic (5 centers), Denmark (2 Centers), France (6 centers), Israel (6 centers), Italy (5), New Zealand (3), and Spain (2).

The premise of this study was based on the rationale that chemotherapy alone is insufficient in curing CLL at a progressed disease stage, due to the fact that a chemotherapeutic regimen does not eliminate all cancer cells, thus not eliminating the possibility of a future CLL recurrence/relapse. Hence, the aim of this study was to demonstrate whether Rituximab, a chimeric monoclonal CD20 antibody, could contribute towards solving this problem (107).

The Immuno-chemotherapy group was treated with Rituximab, Fludarabin, and Cyclophosphamid (FRC-group), whereas the remaining patients received only a (at that point in time standard) first-line-chemotherapy with Fludarabin and Cyclophosphamide (FC-group)
in the same dosage for six cycles. The primary endpoint of this study was progression free survival (PFS).

The results were published in various papers, some of which published very recently. The main result of this study was that patients who were in the FRC-group had both an improved overall- and progression-free-survival (106).

Furthermore, the study showed that the health-related quality of life (HRQOL) was the same in both groups, with female patients (a minority of CLL patients) showing more treatment related symptoms than male patients (108). The immunochemotherapy resulted in a gain of 1.1 quality adjusted life years in patients with CLL, compared to the chemotherapy without rituximab, while remaining cost effective even considering the higher cost of monoclonal antibodies compared to standard chemotherapeutics (109). The group that was treated with FRC exhibited longer remission times as well as an improvement of overall survival (OS) in specific genetic subgroups as well as the overall patient collective (110). The addition of rituximab to standard chemotherapy for the first time in the history of CLL showed a prolongation of overall survival.

Patients with an increased PTK2 expression were associated with a better outcome when treated with FRC instead of FC (111).

Furthermore, minimal residual disease (MRD) was discovered as a predictor for the overall survival as well as for the progression free survival period (112).

**Patients included from the CLL8 trial**

For the 49 patients randomly selected from the CLL8 trial, their median age was 62 with a range of 43-77 (this age bracket also coinciding with the onset of CLL typically later in life), most of whom (40/49) were male, and of whom 61% (30/49) where clinically staged as Binet B, with 37% (18/49) as Binet C and only 2% (1/49) as Binet A. Their IgVH status was close to balanced between mutated (41%, 20/49) and unmutated (59%, 29/49) which is typical for a cross-section of CLL patients in need of treatment (19, 33), and with regards to their cytogenetics only 7% (3/49) tested positive for the del17p- deletion, while 26% (12/49) tested positive for the del11q- deletion.
These characteristics are partly explained by the selection criteria of the CLL8 trial, which recruited patients with Binet stage C or active disease in stage A or B which had nevertheless never been treated for their CLL. As per inclusion criteria, patients were as yet physically fit at study entry (106, 113).

**Summarized characteristics**

In addition to the available portions of the CLL1 and CLL8 trials as outlined above, as well as the collective for the initial testing, a further group of 26 samples of healthy individuals could be acquired for the multiplex testing, following the preliminary tests. These were required to form a large reference group in order to conduct statistically valid comparisons between healthy and (subgroups of) CLL patients.

Since multiplex assays are a rather sensitive diagnostic modality, in the sense of being prone to amplifying any operational or handling errors into largely unusable data, the decision was made to not include any of the preliminary results into the final analysis such that optimized operating protocols which were implemented upon successful completion of the preliminary stage could be assumed for all further data analysis.

Consequently, the following summary of patients selected for this study encompasses the 26 healthy controls and the cohorts of 151 and 49 patients from the CLL1 and CLL8 trials, respectively, but disregards the patients from the preliminary studies. This is both for the reason of having the same operational multiplex assay protocol for all data which is in direct comparison, and to guarantee that all accompanying clinical data is in the same format and has been taken using the same protocols.

Thus, the collective for all multiplex assays following the preliminary testing consists of a total of 226 blood samples, comprised of 26 healthy individuals, 151 CLL1 patients of Binet stage A, and 49 patients from the CLL8 study of whom 61% were staged Binet B, 18% as Binet C, with just 2% being Binet A.

In terms of prognostic factors, the CLL patients consisted of 130 of 200 patients (65%) with IgVH mutated status and 70 of 200 (35%) unmutated. So in terms of IgVH this cohort exhibits a larger proportion of the prognostically positive mutated status better than a normal cross section of CLL patients, and thus constitutes an overall good cohort in terms of risk
factors (63). Analogously, regarding the cytogenic group makeup, the del17p deletion is underrepresented with only 3% of patients, contrasted with 7% in other published data, as is the case for the del11q (9% in this collective versus 18% in the literature) (63).

These characteristics are also depicted in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Control</th>
<th>CLL1 cohort</th>
<th>CLL8 cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>26</td>
<td>151</td>
<td>49</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>61</td>
<td>62</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>35-76</td>
<td>43-77</td>
</tr>
<tr>
<td>Sex n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Clinical Stage n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binet A NA</td>
<td>151 (100%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>Binet B NA</td>
<td>0</td>
<td>30 (61%)</td>
<td></td>
</tr>
<tr>
<td>Binet C NA</td>
<td>0</td>
<td>18 (37%)</td>
<td></td>
</tr>
<tr>
<td>White Count (G/l)</td>
<td>NA</td>
<td>93,8</td>
<td></td>
</tr>
<tr>
<td>IgHV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutated</td>
<td>110 (74%)</td>
<td>20 (41%)</td>
<td></td>
</tr>
<tr>
<td>unmutated</td>
<td>41 (26%)</td>
<td>29 (59%)</td>
<td></td>
</tr>
<tr>
<td>Cytogenetics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del 17p- (%)</td>
<td>3 (2%)</td>
<td>3 (7%)</td>
<td></td>
</tr>
<tr>
<td>del 11q-</td>
<td>6 (4%)</td>
<td>12 (26%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Characteristics of patients selected for this study, excluding the preliminary test cases
IV. Methodology

Summary of the course of testing

The testing was conducted in three distinct stages. First, a multiplex assay-based analysis including CLL patients from Munich, CLL patients excluded from the CLL1 trial, and a few healthy individuals, were conducted in order to facilitate approval for the larger CLL1 and subsequently CLL8 trials, and to define precisely the correct goals and targets of this study.

In a second stage, the 22 most promising markers were tested using multiplex assays on a larger cohort of CLL1 trial patients.

Eventually, further multiplex testing was done on CLL8 patients as well as a larger group of healthy controls, allowing for direct and aggregate comparisons between the largely clinically diverse groups of the CLL1, the CLL8, and the healthy control group.

The third stage consisted of the retesting of selected parameters using a more established conventional ELISA approach, in order to validate the larger group of multiplex assay and to corroborate the most promising prognostic parameters as revealed through the multiplex testing, mainly sIL2-r-alpha.

In the following, the general testing modalities used, as well as the specific tests conducted, are discussed in detail.

xMAP

Multiplex methods such as Luminex assays have been established in a variety of research settings, such as for tissue research (114) and for measuring chemokines in bacteria cultures (115).

The xMAP technology, licensed from Luminex, enables the simultaneous measurement of up to 100 different assays within a single sample. This approach is particularly well suited when examining samples for which only small volumes are available, such that it would not be feasible to obtain enough material to conduct, e.g., 100 different ELISA assays, due to the base material typically being rendered unsuitable for further research during the course of an
ELISA examination. The second use case for xMAP technology is simply to more efficiently expend research time and laboratory resources.

This technique relies on color-coded microspheres, with a number of different groups corresponding to the number of analytes to be tested simultaneously. Each group features a large amount of microspheres with a distinct mix of dyes, coated with a specific antibody.

After this bead-mixture is introduced to the patient serum, a number of incubation and washing steps take place. Beads are incubated using a fluorescence-labeled antibody, which also necessitates that most of the prolonged testing steps need to be conducted in the utter absence of light, and eventually analyzed. During this measuring process, the internal dyes of the remaining microspheres and their bonded antibodies are each differently excited by lasers. The resultant spectrum of excitation allows for the identification of the different proportions of beads, which in turn allows for the simultaneous inferral of the different analyte concentrations. This approach has a potential number of analytes which can be simultaneously tested in the dozens, but due to its involved and intricate underlying principle can be considered to be a comparatively fragile testing process (116).

Analyses were performed according to manufacturer’s instructions. To correct for interassay-variability, control samples were carried on all plates.
Preliminary studies using multiplex assays

In order to establish reliability beyond simply relying on assurances of the manufacturer, a series of validation multiplex measurements were included in the preliminary studies. Not only was intra-assay variability tested by including multiple samples for each analyte. The impact of freezing at different points in time was tested by freezing sera of a healthy volunteer, both immediately, 24 hours, and 48 hours after extraction. This is in addition to the two internal quality controls that are customarily integrated into each measurement.

Two Luminex xMAP compatible plates were used, the first of which was Milliplex Catalog ID.MPXHCYTO-60K-01 Human Chemokine/Cytokine Panel I HCC 109 / HCC 209 Immunology Multiplex Assay (henceforth Panel I), which featured the chemokines seen in Figure 6.

The second panel was the Milliplex Catalog ID.MPXHCYP2-62K-01Human Chemokine/Cytokine Panel II HCYP2 103/203 Immunology Multiplex Assay (henceforth Panel II), with a list of chemokines as listed in Figure 6.

Each plate typically allows for 96 samples to be filled, however, a number of wells is already reserved for the calibration samples in order to compute a valid standard curve, along with blanks and the aforementioned quality controls. With 6 standards, each of which is typically measured twice, two blanks, and two twice-measured internal quality controls, the number of wells remaining for the measurements of freely chosen samples is calculated as 78 (96 – 6•2 – 1•2 – 2•2).
<table>
<thead>
<tr>
<th>Milliplex Catalog ID</th>
<th>Human Chemokine/Cytokine Panel</th>
<th>Immunology Multiplex Assay</th>
<th>Chemokines/Cytokines Included</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPXHCYP2-62K-01</td>
<td>HCYP2 103/203</td>
<td>6Ckine, BCA-1, CTACK, ENA-78, Eotaxin-2, Eotaxin-3, I-309, IL-16, IL-20, IL-21, IL-23, IL-28A, IL-33, LIF, MCP-2, MCP-4, MIP-1d, SCF, SDF-1A+β, TARC, TPO, TRAIL, TSLP</td>
<td></td>
</tr>
<tr>
<td>MPXHCYTO-60K-01</td>
<td>HCC 109 / HCC 209</td>
<td>sCD40L, EGF, Eotaxin/CCL11, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17α, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGF-α, TNF-α, TNF-β, VEGF</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6: Multiplex assay panels used, and chemokines originally included in the preliminary multiplex testing. Source: Product catalog at millipore.com**

**Multiplex testing employed for the main phase of this study**

The same MilliplaxMap (Millipore, USA) panels which were used for the preliminary testing were also applied for the main phase of this study (i.e., testing the CLL1, the CLL8, and the larger host of healthy patient sera). The panels were also each designed in the same 96-well format, allowing for the screening of 96 samples for all specified parameters in one test setting.

However, based on the results of the preliminary testing, only the most prospective and promising parameters were included. Thus, the number of analytes on Panel I was reduced to sIL-2R-alpha, IP-10, EGF, VEGF, MCP-1, MIP-1α, MIP-1β, TNF-α, TGF-α, FLT3 Ligand,
and Fraktalkine, IL12 (p40), while the analytes on Panel II were reduced to MCP-2, MCP-4, SDF-1a+b, I-309, TARC, 6CKine, Eotaxin-2, CTACK, LIF, TRAIL, SCF, BCA-1, IL16, MIP1delta, and TPO.

A total of four kits for Panel I and Panel II each were used for this main phase of the study. The first segment of these main tests was two plates each, sufficient for the testing of the 159 Binet A CLL1 sera as well as the obligatory standard, blank, and quality controls.

Sorted into their functional groups, the overall parameters tested for the CLL1 patients (and also later the CLL8 and healthy collectives) are summarized in

Table 3. An example plate layout is provided in Table 2.

<table>
<thead>
<tr>
<th>blank</th>
<th>S4</th>
<th>S4</th>
<th>Internal validity</th>
<th>CLL8</th>
<th>CLL16</th>
<th>CLL24</th>
<th>CLL32</th>
<th>CLL40</th>
<th>CLL48</th>
<th>CLL56</th>
<th>CLL64</th>
<th>CLL72</th>
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</thead>
<tbody>
<tr>
<td>blank</td>
<td>S4</td>
<td>S4</td>
<td>CLL1</td>
<td>CLL9</td>
<td>CLL17</td>
<td>CLL25</td>
<td>CLL33</td>
<td>CLL41</td>
<td>CLL49</td>
<td>CLL57</td>
<td>CLL65</td>
<td>CLL73</td>
</tr>
<tr>
<td>S1</td>
<td>S5</td>
<td>S5</td>
<td>CLL2</td>
<td>CLL10</td>
<td>CLL18</td>
<td>CLL26</td>
<td>CLL34</td>
<td>CLL42</td>
<td>CLL50</td>
<td>CLL58</td>
<td>CLL66</td>
<td>CLL74</td>
</tr>
<tr>
<td>S1</td>
<td>S5</td>
<td>S5</td>
<td>CLL3</td>
<td>CLL11</td>
<td>CLL19</td>
<td>CLL27</td>
<td>CLL35</td>
<td>CLL43</td>
<td>CLL51</td>
<td>CLL59</td>
<td>CLL67</td>
<td>CLL75</td>
</tr>
<tr>
<td>S2</td>
<td>S6</td>
<td>S6</td>
<td>CLL4</td>
<td>CLL12</td>
<td>CLL20</td>
<td>CLL28</td>
<td>CLL36</td>
<td>CLL44</td>
<td>CLL52</td>
<td>CLL60</td>
<td>CLL68</td>
<td>CLL76</td>
</tr>
<tr>
<td>S2</td>
<td>S6</td>
<td>S6</td>
<td>CLL5</td>
<td>CLL13</td>
<td>CLL21</td>
<td>CLL29</td>
<td>CLL37</td>
<td>CLL45</td>
<td>CLL53</td>
<td>CLL61</td>
<td>CLL69</td>
<td>CLL77</td>
</tr>
<tr>
<td>S3</td>
<td>Q1</td>
<td>Q1</td>
<td>CLL6</td>
<td>CLL14</td>
<td>CLL22</td>
<td>CLL30</td>
<td>CLL38</td>
<td>CLL46</td>
<td>CLL54</td>
<td>CLL62</td>
<td>CLL70</td>
<td>CLL78</td>
</tr>
<tr>
<td>S3</td>
<td>Q2</td>
<td>Q2</td>
<td>CLL7</td>
<td>CLL15</td>
<td>CLL23</td>
<td>CLL31</td>
<td>CLL39</td>
<td>CLL47</td>
<td>CLL55</td>
<td>CLL63</td>
<td>CLL71</td>
<td>CLL79</td>
</tr>
</tbody>
</table>

Table 2: Typical plate layout for the multiplex assays. S1-S6: Different standard concentrations for interpolating the standard curve, Q1 and Q2: Quality controls.
<table>
<thead>
<tr>
<th>Category</th>
<th>Analytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CC-Chemokines</strong></td>
<td>I-309 (CCL1)</td>
</tr>
<tr>
<td></td>
<td>MCP-1 (CCL2)</td>
</tr>
<tr>
<td></td>
<td>MIP-1α (CCL3)</td>
</tr>
<tr>
<td></td>
<td>MIP-1β (CCL4)</td>
</tr>
<tr>
<td></td>
<td>MCP-2 (CCL8)</td>
</tr>
<tr>
<td></td>
<td>MCP4 (CCL13)</td>
</tr>
<tr>
<td></td>
<td>MIP1delta (CCL15)</td>
</tr>
<tr>
<td></td>
<td>TARC (CCL17)</td>
</tr>
<tr>
<td></td>
<td>6CKine (CCL21)</td>
</tr>
<tr>
<td></td>
<td>Eotaxin-2 (CCL24)</td>
</tr>
<tr>
<td></td>
<td>CTACK (CCL27)</td>
</tr>
<tr>
<td><strong>CXC-Chemokines</strong></td>
<td>IP-10 (CXCL10)</td>
</tr>
<tr>
<td></td>
<td>SDF1 (CXCL12)</td>
</tr>
<tr>
<td></td>
<td>BCA-1 (CXCL13)</td>
</tr>
<tr>
<td></td>
<td>Fractalkine (CX3CL1)</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td>IL-6 (LIF)</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
</tr>
<tr>
<td></td>
<td>IL-16</td>
</tr>
<tr>
<td><strong>Cytokine receptors</strong></td>
<td>sIL2-R-alpha</td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td>TPO</td>
</tr>
<tr>
<td></td>
<td>SCF</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
</tr>
<tr>
<td></td>
<td>TGF-α</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td>TRAIL</td>
</tr>
<tr>
<td></td>
<td>FLT3lig</td>
</tr>
</tbody>
</table>

Table 3: Multiplex assay analytes used for the CLL1, CLL8, and healthy collectives. Sorted by their respective category.
ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a method that can identify a specific substance through the utilization of the specific binding of marked antibodies, and a color change through that binding. ELISA is an example of an immunoassay, that is, a biochemical test which uses antibodies to determine the presence or concentration of a molecule in a solution. It has found widespread usage since the development of its predecessor by Rosalyn Sussman Yalow and Solomon Berson in the 1950s. ELISA has become so ubiquitous that in 1977, Yalow was awarded the Nobel Prize for medicine for her work discovering the technique. Her specific immunoassay was used to e.g. identify insulin in plasma in humans (117) and required the use of radioactive labeling, making it a so-called radioimmunoassay (RIA). A variant of the RIA is still in allergy testing, called the radioallergosorbent test (RAST). In this precursor method to the standard ELISA in use today, the antibodies were radioactively labeled, and through their radioactive emissions provided the signal on whether the specific antigen substance is present. Due to the potential health risk of this method, in 1971 two different study groups, namely Peter Perlmann and Eva Engvall at Stockholm University (118) and Anton Schuurs and Bauke van Weemen (119) independently published descriptions of what ended up as the ELISA test as currently conducted, namely replacing radioactive emissions as the signal with the usage of enzymes.

The basic principle of the ELISA test is that for the substance whose presence and/or quantity is to be determined (i.e., the “analyte”), a specific antibody using that substance as an antigen
to bind to is manufactured. Prior to that binding, the antibodies are linked to an enzyme which produces a signal that is then either detected as a binary (present/absence), or quantified (to determine the analyte concentration). In most cases, the signal mediated by the analyte is a change in color if the analyte is present.

Today, ELISA is a standard diagnostic tool not only for research but also in widespread clinical practice. It is employed not only in a medical context, but also as quality control in a variety of industries, with wide distribution specifically in the food industry to check for potential allergens. In medicine, ELISA had its first breakthrough application as a screening tool for HIV. Various other diseases offer antigens suitable for ELISA testing, such as Hepatitis-B, rotavirus, the enterotoxin expressed by E.coli, to name just a few high-profile applications. In 2016, it was shown that an ELISA test can be used even in the diagnostic algorithm of celiac disease (120).

There are a few different subtypes of ELISA in usage today, the main variants of which are direct ELISA, indirect ELISA, the direct sandwich ELISA and the indirect ELISA. The ELISA portion of testing used in this study consists of sandwich ELISAs. Competitive ELISAs are mentioned here only in passing, since they constitute a different paradigm of competitive binding not used in the testing for this study.

**Sandwich ELISA**

The sandwich ELISA uses two antibodies that bind to different antigen binding sites of the substrate. An antibody-antigen-antibody-complex is thus generated. With the antigen thus located in between two antibodies, the method was called sandwich ELISA. After the two antibodies are applied in sequence (the first one typically already bound to the plate), the substrate is applied, which then undergoes a conformational change due to the presence of the enzyme linked to the second antibody and reacts by typically changing color.

A positive result is thus equivalent to a change in color, why no change in color denotes the absence of the antigen, or a failure along the rather complex sequence of steps during the testing. Color changes can be quantified with the help of a spectrometer. Thus, ELISA can be used as either a qualitative test (i.e., providing a positive or a negative result corresponding to presence or absence of an antigen), or a quantitative test, analyzing the strength of the color change.
Sera (in the case of this study) to be tested are added to these wells pre-coated with antibodies, incubated at 37 °C, and then washed such that any sample constituents unbound to the plate’s antibodies are removed. If antibodies remain present, that implies that an antigen-antibody reaction occurred. Likewise, the enzyme-linked antibodies are then introduced, and those that remained free upon their addition then washed off. The more antigen binding sites in the sample, the more Ag-Ab complexes are formed, leading to a higher concentration of enzyme. The marker substance is then added to the wells for a pre-defined amount of time. The degree to which the color-changing reaction is then catalyzed is dependent only on the amount of enzyme available, and since that amount is in turn dependent on the concentration of the original antigens in the sample, a direct link between the degree of color change and the concentration sought after in the sample is established.

Conventional ELISA confirmation testing employed for this study

The following Quantikine ELISA kits (R&D Systems GmbH) were used for the ELISA tests in this study (following the MultiPlex pre-studies to determine which cytokines to evaluate): D6C00 (6CKine) with a sensitivity of 33.5 pg/mL and an assay range of 78.1 – 5000 pg/mL
(121), DIP100 (IP10) with a sensitivity of 4.46 pg/mL and an assay range of 7.8 – 500 pg/mL (122), DMA00 (MIP1α) with a sensitivity of 10 pg/mL and an assay range of 31.2 – 1000 pg/mL (123), DR2A00 (sIL2-R-alpha) with a sensitivity of 10 pg/mL and an assay range of 78.0 – 5000 pg/mL (124), DTA00C (TNF-α) with a sensitivity of 5.5 pg/mL and an assay range of 15.6 – 1000 pg/mL (125), DVE00 (VEGF) with a sensitivity of 9 pg/mL and an assay range of 15.6 – 1000 pg/mL (126), and DCK00 (SCF) with a sensitivity of 9 pg/mL and an assay range of 31.2 – 2000 pg/mL (127). These are also listed in Table 4.

All aforementioned ELISAs are solid phase sandwich ELISA assays for 96-well strip plates, with an assay length of between 3.5 to 4.5 hours and viability both for serum and EDTA Plasma, typically for samples of 100-200 µL (with the exception of sIL2-R-alpha, requiring just 13 µL and IP-10 requiring 75 µL).

It should be noted as a caveat that while all kits used for this study are in wide circulation, and have been part of the data acquisition of a host of published data, the assay sensitivity as outlined above as well as the intra- and inter-assay variabilities cannot always be assumed to fall into the ranges as mentioned previously. Instead, the values given should be regarded as the ceiling which can potentially be reached under optimal conditions, rather than the average for typical usage. This is based on experiential impressions gathered during the conducting of this study. Likewise, the majority of possible mistakes during the rather sensitive and complex series of steps during an ELISA test tend to lead to an underestimation rather than an overestimation of the quantity of the analyte. Only failures of washing out unbound antibodies, or of erroneously increasing the proportion of antibodies to antigen, would yield an overestimation of sample. Since even micropipettes have a small amount of error inherent in them, this kind of invariable experimenter-caused inter-assay variability needs to be added to the lower bounds provided by the manufacturer.

<table>
<thead>
<tr>
<th>ELISA parameters for the confirmation testing</th>
<th>6CKine, IP10, MIP-1α, sIL2-R-alpha, TNF-α, VEGF, SCF</th>
</tr>
</thead>
</table>

*Table 4: Parameters re-tested for the ELISA confirmation testing.*
V. Statistical Analysis

Associations between chemokines, groups, and clinical characteristics were assessed using Wilcoxon rank-sum tests, Mann-Whitney U test, Spearman correlation coefficients, and chi-squared tests, as appropriate. Associations between chemokines and time to event endpoints were performed using Cox proportional hazards models and Kaplan Meier curves.

xMAP and multiple hypothesis testing

For screenings of potentially important candidate markers especially, xMAP allows for a larger number of the potentially interesting compounds to be measured within a single sample, and thus moves to an approach that borrows elements from Big Data analysis by generating many more data points per sample. However, this research methodology does not entail only benefits but also necessitates a different approach to interpreting the results. When screening for a large number of potentially related compounds and then comparing to a null hypothesis, which typically states that the distributions between two groups are identical, the absolute number of false positives which are detected will rise. This is a common complication known from Big Data analysis and usually compensated by adjusting the level of significance to require a higher threshold (i.e., lower the p-value from p=0.05 to p=0.00625 when testing 8 different compounds). For biochemical testing kits in particular, a literature review did not yield a universally established methodology to account for false positives as part of the multiple hypotheses testing entailed by multiplexing technology. However, the dominant approach seems to be a variant of Bonferroni correction, in which number of false positives is reduced by increasing the required level of significance for each individual hypothesis to $\alpha/m$, such that $\alpha$ is the desired uncorrected level of significance (typically 0.05) and $m$ is the number of hypotheses (128). It needs to be mentioned that such correction, while filtering out many of the false positives, will also falsely classify many actual results as negatives. For example, in the aforementioned case of 8 hypotheses being tested, the p-level of 0.00625 exceeds many of the p-values of milestone results in clinical research. The question of how to weight false positives versus false negatives is not one that can ultimately be answered by statisticians. For the purposes of this research, in accordance with (129) no explicit multiple hypothesis testing was conducted unless otherwise stated.
On the usefulness of high-$p$ value correlations

Many of the results presented starting on page 45 show strong correlations, both in the sense of distributions of serum level averages and variances being starkly different between different groups of patients, and of serum levels of certain analytes corresponding to an increase in progression free survival, as determined by Student’s t-tests, or ANOVA analysis when comparing more than 2 subgroups. Likewise, Pearson’s correlation coefficients sometimes show a near linear correspondence between serum levels of certain analytes of this study, and clinical markers. In some cases, serum concentrations are so different (see the Results section), that for our large collective of CLL1, CLL8 and healthy samples, allocation to a subgroup (such as Binet A, or advanced disease stage) can be unambiguously enacted at a glance.

However, that is not the case for all analytes, as was expected in the study design. It should be noted that scientific significance, which is usually by convention denoted by a $p$-value of less than 0.05 for the rejection of the null hypothesis (typically: “both groups are chosen i.i.d., i.e., independent and identically distributed, from the same distribution”). Trials which cannot differentiate groups with a lower $p$-value are deemed as not have a clear enough rejection of that null hypothesis to assume an actual difference between the two groups to be compared.

When considering new input parameters for complex risk indices, the important factor to consider is whether including a new parameter introduces additional certainty into the prediction, or is neutral.\footnote{Additional uncertainty by introducing a new parameter does not usually occur and is not usually considered a problem: If a new parameter could consistently “worsen” the prediction, that would imply that it contributes consistently “false” information, i.e., information that is wrong with a higher than random probability. That in turn implies that there is specific domain knowledge included in that parameter, simply in order to be consistently “wrong”. As such, there are statistical methods to in effect “invert” the negative contribution of such a parameter and thus utilizing its specific, formerly misused inherent knowledge to improve the prediction. Consequently, the worst change of adding a “useless” predictor to a prediction algorithm, after optimization, is neutral.} Even a number of weak correlations with, e.g., a Pearson’s correlation coefficient of 0.3, or a Student’s t-test yielding a $p$-value of 0.15, can on aggregate yield correct predictions (cf. Random Decision Forests, (130)), following the principle of many weak predictors combining into a strong predictor. Thus, the usefulness of even weak correlations should not a priori be discounted, instead, the salient distinction needs to be drawn between whether a weak correlation is merely derivative and dependent on an already known, strong correlation (in which case the weak correlation can indeed be disregarded), or whether the weak correlation is independent of previously known predictors, in which case
even weak correlations could be used to significantly improve the overall prognostic power of an aggregate score.
VI. Results

Results of the preliminary multiplex studies

First stage of the preliminary studies

The multiplex assay stability checks, with the same sample measured at different dilutions, different points in time between the freezing of sera and their testing, different number of freezing/thawing cycles, and for different positions within the 96-well multiplex plates, yielded the results that deviations between comparable measurements were generally within 10-20% of the mean, with no stability check being a particular outlier other than one outlier after a twice repeated freezing/thawing cycle.

For the dilution experiments in particular, the deviation between appropriately converted dilution factors ranged under 10%. For example, the calculated concentration of a serum diluted in a 1:1 proportion (and filled with neutral buffering solution) will, once the result is redoubled, fall within 10% of the original, undiluted concentration. The caveat applies that this only holds true as long as the resulting concentration after the dilution is applied still ranges in a region of the standard curve that is appropriately surrounded by valid standard measurements. If the range between the supporting points of the standard curve (i.e., those with a predetermined concentration, e.g. standards 1 to 6) is left, than the necessary extrapolation from the standard curve to the outliers would introduce an unavoidable error in the extrapolatory estimation of the concentration.

The dilution ratios tested were 1:1, 1:2, 1:4, 1:8, and 1:16.
The first series of preliminary tests, using the patients as outlined on page 25 and the analytes outlined for Panel 2 in Table 3, was limited in its statistical power due to the low number of CLL patients, and low number of healthy volunteers. It was, however, feasible to divide them into groups based on

- their risk stratification (Thymdinkinase, Beta2-Microglobulin, Lymphocyte Doubling Time, bone marrow infiltration pattern), yielding a “high risk” subgroup of 7/21 CLL patients, and 14/21 in a “low risk” group.

- leukocyte numbers (>20 versus <20 G/l), resulting in 5/21 in a “high leukocyte count” group, and 16/21 in a “low leukocyte count” group, and

- normal versus elevated LDH, with only 2/21 with an elevated LDH and 19/21 with a normal or lower LDH (unsurprisingly, given that the CLL patients were excluded from the CLL1 study, but still nearly exclusively classified as Binet A).

Even in this low-sample count, various correlations could be depicted with varying statistical significance. Correlations and their p-values are depicted in Table 5. In particular, SDF-1 (CXCL12) and BCA1 (CXCL13) both had different distributions between the leukocyte groups, and the high/low risk groups, and had not yet been the subject of investigation in larger collectives which include cytogenetic characteristics.
Figure 9: MCP-4, 6C-Kine, and SCF distributions for the low risk and high risk subgroups, as previously defined. Includes p-values (p<0.05 for significance), units are in [pg/mL].

Figure 10: MCP-4, 6C-Kine, and SCF distributions for the low leukocytosis and high leukocytosis subgroups, as previously defined. Includes p-values (p<0.05 for significance), units are in [pg/mL].
Table 5: All p-values <0.15 (significance would be <0.05) for the first stage preliminary tests

<table>
<thead>
<tr>
<th></th>
<th>Grouped by risk</th>
<th>Grouped by leukocyte count</th>
<th>Grouped by a LDH&gt;250 threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-2</td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>MCP-4</td>
<td>0.05</td>
<td></td>
<td>0.066</td>
</tr>
<tr>
<td>SDF-1a+b</td>
<td>0.087</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-309</td>
<td>0.096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TARC</td>
<td>0.037</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6Ckine</td>
<td>0.059</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>Eotaxin-2</td>
<td>0.083</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTACK</td>
<td>0.115</td>
<td></td>
<td>0.011</td>
</tr>
<tr>
<td>LIF</td>
<td></td>
<td></td>
<td>0.043</td>
</tr>
<tr>
<td>TRAIL</td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>SCF</td>
<td>0.15</td>
<td>0.18</td>
<td></td>
</tr>
</tbody>
</table>

Second stage of the preliminary studies

The second stage, checking the analytes listed for Panel 1 in Figure 6, likewise yielded significant results. The same subgroups were defined, and two additional subgroups could be added, namely healthy versus CLL patients, and CLL patients grouped by their IgVH status.

A strong difference in means could be observed regarding the mutation status group for EGF, Flt-3, IL-1alpha, IL-5, IL-7, IP-10, TNFalpha, and VEGF, regarding the high risk versus low risk groups for IP-10, MIP1-alpha, sIL-2Ralpha, and TNFalpha. For the leukocyte count groups, strong differences in means were present for MCP-1, TNFalpha, and VEGF. Finally, the healthy versus disease differentiation (at an early stage), was clearly delineable for Fractalkine and TNFalpha.

A more complex difference between groups, as calculated using a Student’s t-test, which simply tests for differences in distribution without requiring differences e.g. in mean or average, was present for the mutation status for EGF, sIL-2Ralpha, TNFalpha, and VEGF. Leukocyte groups varied in their distribution of FGF-2, IFNalpha, MCP-1, MIP-1alpha, MIP-1β, sIL-2Ralpha, TNFalpha, and VEGF. Healthy versus CLL featured different distributions.
for Flt-3, G-CSF, IL-8, IP-10, MCP-1, MIP-1alpha, MIP-1β, sIL2-R-alpha, TGFalpha, and TNFalpha.

Even a significantly different distribution as verified by a Student’s t-test is not as immediately valuable for purposes of prediction and diagnosis as, e.g., a high Pearson’s correlation coefficient which denotes a linear relationship (and as such, can be used to establish cutoffs for use in diagnostic or therapeutic algorithms, or for more complex predictive scores). In contrast, the different correlations regarding groups that are of a more complex kind, i.e., a low Pearson’s correlation coefficient of <0.5 and a significant different as per the Student’s t-test may not be of immediate use. However, these can be used as important indications that the respective marker is involved in both groups in different ways, explaining the different distribution. It follows that even such complex differences can be used as indications for a pathophysiological distribution of said parameter.

<table>
<thead>
<tr>
<th></th>
<th>Healthy versus CLL</th>
<th>Grouped by risk</th>
<th>Grouped by IgVH mutation status</th>
<th>Grouped by leukocyte count</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIL-2R-alpha</td>
<td>0.00002</td>
<td>0.002</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>0.009</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td>0.08</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.03</td>
<td></td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0.001</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.000005</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.000000007</td>
<td>0.004</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: All p-values <0.10 (significance would be <0.05) for the second stage preliminary tests.
The conclusions that could be ascertained through the preceding results include the feasibility of multiplex assay analysis of chemokines and cytokines as an approach in CLL, the robustness of the results regarding preanalytic handling, and the absence of spurious artifacts. Lastly, there were already strong indications for pronounced differences between subgroups, presumably due to pronounced differences in their respective microenvironments (since that is the place of activity for the parameters measured in the peripheral blood). CCL21, CCL13 and SCF levels were significantly higher in high risk CLL and for patients with >20 G/l leukocytes (a surrogate for disease burden) CCL13 and CCL21 levels were significantly elevated.

It is important to note that the preceding results can be considered as somewhat of a statistically low-powered ‘pilot study’. The fact that some of the different groups already had statistically significantly different distributions, both overall and of a linearly correlated kind as depicted in Figure 9 and Figure 10, can be taken as an indication of the strength of the correlation. However, even cytokines which did not reach significance in this ‘pilot study’ can be considered to warrant additional investigation in a larger patient collective, as the lack of immediate significance could be based both on an actual lack of correlation, or on an insufficiently large test group. This should be kept in consideration when considering the choice of parameters for the CLL1 (and later, CLL8) trial testing.
Results of the multiplex assays for the CLL1 trial

Testing proceeded with the cytokines marked for further investigation with the larger CLL1 trial collective, namely those which could be established to a degree sufficient to withstand the Bonferroni correction for multiple hypotheses testing, or to qualify for further testing in a larger collective.

Upon analysis, this first multiplex testing for the larger CLL1 trial cohort (n=157) yielded aggregate results as summarized in Table 7, comparing CLL samples to healthy controls.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>CLL1 cohort (pg/mL)</th>
<th>Healthy control (pg/mL)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>IL16</td>
<td>90</td>
<td>0.5 – 3750</td>
<td>not detected</td>
</tr>
<tr>
<td>sIL2-R-alpha</td>
<td>12</td>
<td>2 – 4007</td>
<td>not detected</td>
</tr>
<tr>
<td>VEGF</td>
<td>354</td>
<td>0.7 – 7400</td>
<td>221</td>
</tr>
<tr>
<td>CCL2</td>
<td>740</td>
<td>21 – 12097</td>
<td>430</td>
</tr>
<tr>
<td>CCL15</td>
<td>2127</td>
<td>6 – 52512</td>
<td>1066</td>
</tr>
<tr>
<td>CCL3</td>
<td>13</td>
<td>7 – 1454</td>
<td>57</td>
</tr>
<tr>
<td>CCL21</td>
<td>89</td>
<td>3 – 1720</td>
<td>124</td>
</tr>
<tr>
<td>CXCL12</td>
<td>1651</td>
<td>12 – 15759</td>
<td>2486</td>
</tr>
<tr>
<td>TRAIL</td>
<td>77</td>
<td>1 – 42401</td>
<td>43</td>
</tr>
</tbody>
</table>

Table 7: Multiplex results for the first CLL1 trial cohort, comparing CLL patients to healthy samples
Inter-assay variability between the CLL1 and CLL8 studies was higher than expected, and exceeded the manufacturer’s specification of being at most 10-20%. In comparison, while the inter-assay variability within the first CLL1 multiplex testing, as well within the later CLL8 multiplex testing was low, inter-assay variability comparing the CLL1 and CLL8 plates was high. This determination could be made not only based on averages of the various concentrations (which may differ naturally given that the CLL1 and CLL8 patient characteristics are significantly different), but on a number of samples which were tested on both CLL1 and the later CLL8 panels, in order to assess inter-assay variability. It bears noting that while the suppliers for both rounds of testing (with several panels each) were identical (aside from the charge numbers being different), the testing apparatus used were not identical, but in fact based in different laboratories. While both performed according to their specifications, there is potential variability inherent both in the often large accepted range of the quality control samples (which validated both test setups successfully), and to the technical setup itself. This was also part of the manufacturer’s possible explanation upon inquiry.

To solve this complication, and in order to draw valid conclusions in comparing both large groups, both of which were individually validated, the samples which were measured on both setups were used to re-calibrate the values, taking into account previous testing on how measured concentrations may diverge over time. The recalibrated values were considered to be quite stable and plausible, and in any case could be point-wise validated using the conventional ELISA assays, whose use was prompted partly to validate the approach as outlined above.

In CLL1 patients, serum levels of EGF, CCL2, CCL3, CCL4, TNF-α, IL-16, sIL2-R-alpha and SCF were significantly higher (p<0.05) than in healthy control subjects. Serum level of EGF, CCL2, CCL3, CCL4, TNF-α, IL-16, sIL2-R-alpha and SCF were significantly elevated in CLL1, compared to healthy individuals. Similar results are obtained when only newly diagnosed CLL patients in stage Binet A were compared to healthy controls; again, EGF, CCL2, CCL3, CCL4, IL-16, sIL2-R-alpha and SCF were significantly higher (p<0.05) than in

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2 Prof. Markus Schwarz’ laboratory, now at the Institute for Laboratory Medicine, Hospital Campus Großhadern, and the laboratory of the Hämatologikum (HelmholtzZentrum München), at the Hospital Campus Großhadern.
healthy control subjects, indicating that these changes either drive or are associated with a very early disease stage.

When comparing Binet A patients with advanced disease patients, significantly higher values were observed for EGF, CCL4, CCL8, CCL21, CCL27, CXCL12, IL-16 and TNF-α, in the CLL8 subcohort, indicating a role of these factors in disease progression.

Serum levels of CCL3 and CCL4 were elevated compared to healthy controls. These are cytokines secreted by CLL cells upon BCR engagement. High CCL3 levels in particular strongly correlated with high CCL4 levels (p<0.001) and tended to be associated with unmutated IgVH status (p=0.06), further supporting the interpretation of BCR triggering in selected CLL patients. No significant difference in progression free survival was noted. Serum levels of CCL2 and CCL17, both of which bind the chemokine receptor CCR4, were found to be high within the same patient (p<0.001), suggesting a possible role in chemotaxis of CCR4+ T cells towards these chemokines.

CXCL12, CCL21 and CXCL10, chemokines associated with chemotaxis of CLL cells as discussed in the section on The role of the microenvironment in CLL pathophysiology, are also simultaneously elevated in a fraction of patients:

Patients with higher than median serum levels of CCL21 also have higher levels of CXCL10 (p<0.001) and CXCL12 (p=0.02). In addition, high levels of CCL21 significantly correlated with progression free survival (72 months and 35 months, p=0.03). Since CLL patients have been found to have abnormal neovascularization in the bone marrow and lymph nodes, VEGF levels were closely considered. In our cohort, elevated VEGF level were strongly associated with elevated EGF level (p<0.001). In addition, high VEGF level correlated with a high white blood cell count (p=0.008) and showed a trend towards association with del 11q- (p=0.07) and lymphadenopathy (p=0.165). No significant difference could be found in PFS between patients with high or low VEGF serum levels. High levels of sIL2-R-alpha correlated with PFS. When confirmed by conventional ELISA, median PFS in patients within the highest quartile of sIL2-R-alpha levels were 22 months and 72 months in the lower three quartiles (p<0.001). In addition, high sIL2-R-alpha level were strongly associated with unmutated IgVH genes (p=0.003).³

³ The content of the preceding paragraphs were adapted from Dr. med. Till Seiler’s presentation of our work at the “wissenschaftliches Symposium in Herrsching”.

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<table>
<thead>
<tr>
<th></th>
<th>Healthy control</th>
<th>CL (all patients)</th>
<th>CLL</th>
<th>CLL8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>106.2 ± 71.4</td>
<td>269.8 ± 341.5</td>
<td>&lt;0.001</td>
<td>294.2 ± 371.3</td>
</tr>
<tr>
<td>CCL2</td>
<td>1168.2 ± 351.7</td>
<td>1779.3 ± 1792.5</td>
<td>&lt;0.001</td>
<td>1777.3 ± 2011.4</td>
</tr>
<tr>
<td>CCL3</td>
<td>10.0 ± 10.1</td>
<td>86.5 ± 204.6</td>
<td>&lt;0.001</td>
<td>83.9 ± 229.5</td>
</tr>
<tr>
<td>CCL4</td>
<td>36.1 ± 23.8</td>
<td>88.0 ± 94.0</td>
<td>&lt;0.001</td>
<td>80.3 ± 91.8</td>
</tr>
<tr>
<td>sIL2Rα</td>
<td>2.1 ± 4.6</td>
<td>159.2 ± 283.2</td>
<td>&lt;0.001</td>
<td>139.3 ± 309.7</td>
</tr>
<tr>
<td>TGFα</td>
<td>11.8 ± 6.8</td>
<td>10.1 ± 20.6</td>
<td>0.68</td>
<td>10.7 ± 23.2</td>
</tr>
<tr>
<td>TNFα</td>
<td>21.4 ± 7.2</td>
<td>59.2 ± 94.4</td>
<td>&lt;0.001</td>
<td>34.6 ± 82.5</td>
</tr>
<tr>
<td>VEGF</td>
<td>554.8 ± 410.0</td>
<td>691.6 ± 776.4</td>
<td>0.38</td>
<td>689.7 ± 798.8</td>
</tr>
<tr>
<td>CCL8</td>
<td>106.9 ± 24.9</td>
<td>86.0 ± 46.9</td>
<td>&lt;0.001</td>
<td>81.6 ± 48.2</td>
</tr>
<tr>
<td>CCL13</td>
<td>122.8 ± 88.9</td>
<td>172.0 ± 184.8</td>
<td>0.18</td>
<td>174.8 ± 180.3</td>
</tr>
<tr>
<td>CXCL12</td>
<td>2771.1 ± 822.9</td>
<td>2394.1 ± 1371.9</td>
<td>0.17</td>
<td>2266.0 ± 1427.7</td>
</tr>
<tr>
<td>II6</td>
<td>113.5 ± 57.3</td>
<td>408.1 ± 746.2</td>
<td>&lt;0.001</td>
<td>264.6 ± 580.2</td>
</tr>
<tr>
<td>CCL15</td>
<td>3337.4 ± 1192.1</td>
<td>3752.2 ± 4481.2</td>
<td>0.64</td>
<td>3587.2 ± 5108.3</td>
</tr>
<tr>
<td>CCL17</td>
<td>116.1 ± 68.4</td>
<td>162.5 ± 485.3</td>
<td>0.63</td>
<td>126.8 ± 112.0</td>
</tr>
<tr>
<td>CCL21</td>
<td>383.4 ± 187.1</td>
<td>482.6 ± 494.5</td>
<td>0.05</td>
<td>345.7 ± 369.3</td>
</tr>
<tr>
<td>CCL24</td>
<td>1275.2 ± 987.7</td>
<td>2903.8 ± 4928.0</td>
<td>0.10</td>
<td>2872.6 ± 5599.6</td>
</tr>
<tr>
<td>CCL27</td>
<td>912.7 ± 117.9</td>
<td>90.1 ± 323.0</td>
<td>0.72</td>
<td>871.0 ± 361.5</td>
</tr>
<tr>
<td>TPO</td>
<td>1254.9 ± 508.0</td>
<td>3145.5 ± 16833.5</td>
<td>0.11</td>
<td>3495.6 ± 19288.4</td>
</tr>
<tr>
<td>SCF</td>
<td>8.7 ± 14.4</td>
<td>38.3 ± 38.6</td>
<td>&lt;0.001</td>
<td>35.7 ± 39.3</td>
</tr>
</tbody>
</table>

* in comparison with healthy controls

Table 8: Chemokine levels as measured for the different groups of patients, using normalized data

Figure 11: Chemokine levels of CCL2, CCL3, CCL4, and TNF-α compared between different subgroups. Units are [pg/mL].
As shown in Figure 11, CCL2 levels share a similar average for healthy individuals and patients staged as Binet A, differing mainly in higher variability for the CLL population. Higher average levels are seen in advanced cases, still partially overlapping with levels for Binet A. As previously discussed, one drawback for the Binet staging is the lack of further sub-differentiation within the Binet A group. Differences such as these may help filter patients that have erroneously been sorted as Binet A, or who are about to graduate towards Binet B and higher.

For CCL3, normal levels fall within a small spectrum, overlapping only the smallest quintile of Binet A patients (cf. Figure 11). While serum levels for both Binet A and advanced disease stages overlap to some degree, the difference between either of them and the healthy control group is quite pronounced. This may lead to CCL3 as a potentially useful component of a CLL screening, although such a screening is currently generally unlikely given the clinical characteristics and the typical epidemiology. Serum concentrations of above 100 pg/mL can be considered to occur near-exclusively in advanced disease-stage patients.

CCL4 has a profile resembling that of CCL3, with the same implications, albeit its prognostic value for distinguishing healthy/CLL status is comparatively diminished in comparison to CCL3 (cf. Figure 11).

TNF-α exhibits a clear and strong distinction between the advanced disease group, and both the healthy control and Binet A group. A value of over 90 pg/mL is practically pathognomonic for the advanced disease stage, compared to healthy controls or Binet A (cf. Figure 11).

As such, TNF-α could be used to help distinguish Binet A from advanced disease stage CLL patients for edge cases, or in situations where the usual staging fails for other reasons.

For SCF (cf. Figure 12), averages for all three groups are widely different, however due to the rather large variance, the only clear prediction that is viable from a single SCF serum concentration in this context would be that concentrations higher than 50 pg/mL are only compatible with a CLL diagnosis (versus group membership in the healthy group). Very high levels are slightly more congruent with the advanced disease stage.

For EGF, the results are similar to SCF, as seen in Figure 12, without the possibility of further differentiating between Binet A and advanced disease stage.
IL-16 (cf. Figure 12), similarly to TNF-α (cf. Figure 11), exhibits a clear demarcation of 500 pg/mL, above which a sample can be assuredly classified as of a patient with advanced diseases. For concentration levels in the peripheral blood of between 250 and 500 pg/mL, classification as a CLL patient can be confidently effected, however, at those concentration levels no clear choice between Binet A and advanced disease is possible. This predisposes IL-16 as a serum marker potential useful for determining the disease stage.

The strongest predictor, not only to distinguish one subgroup from the other two, but also to uniquely identify each subgroup with only some remaining amount of uncertainty, is sIL2-R-alpha. In healthy individuals sIL2-R-alpha is not found, to a degree that it is practically absent. In Binet A, measurable serum levels in the peripheral blood occur, but in much lower proportions compared to the advanced disease stage. Only the upper quartile of Binet A serum concentrations overlaps with the values within one standard deviation of the advanced disease subgroup.

Figure 12: Chemokine levels of SCF, EGF, sIL2Ralpha, and IL16 compared between different subgroups. Units are [pg/mL].
In summarizing the above observations, TNF-α, IL-16, and sIL2-R-alpha demonstrated the best result in dividing at least 2 of the subgroups from the remaining one:

- TNF-α showed an excellent differentiation of the advanced disease group, versus the composite two other groups,
- IL-16 did likewise, although with more overlap and thus reduced predictive power, and
- sIL2-R-alpha, respectively its serum concentration in the peripheral blood, best divided each of the three major groups from each other, hinting not only at its possible prognostic value, but also in its potential intricate involvement in CLL pathophysiology.

The observation that chemokine homeostasis differs so strongly between the groups, and that the chemokines are specifically involved in the migration of B- and T-cell, as well as their homing and recruitment, in conjunction with overexpression of the pro-inflammatory and proliferative factors, point toward the specific microenvironment being more than a passive envelope in which the B-cell clones proliferate, but rather as enabling pathways that are integral for the disease formation. Such pathways may constitute targets for future therapeutic strategies.

For reference, median serum levels of the aforementioned cytokines for each of the group are listed in Figure 13. It should be noted that median levels alone are not enough to infer good prognostic power, for which the previous boxplots Figure 11 and Figure 12 give a better graphical overview.

<table>
<thead>
<tr>
<th>Median serum level (pg/ml)</th>
<th>Healthy control</th>
<th>CLL1</th>
<th>CLL8</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-1β</td>
<td>116</td>
<td>266</td>
<td>196</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>sIL2-R-alpha</td>
<td>21</td>
<td>504</td>
<td>2255</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>12</td>
<td>40</td>
<td>80</td>
<td>p=0.008</td>
</tr>
<tr>
<td>IL-10</td>
<td>3</td>
<td>19</td>
<td></td>
<td>p=0.001</td>
</tr>
<tr>
<td>IL-16</td>
<td>55</td>
<td>203</td>
<td>425</td>
<td>p=0.013</td>
</tr>
<tr>
<td>6CKine</td>
<td>319</td>
<td>307</td>
<td>764</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>SCF</td>
<td>10</td>
<td>79</td>
<td>55</td>
<td>p=0.001</td>
</tr>
</tbody>
</table>

*Figure 13: Median chemokine serum levels in healthy volunteers compared to Binet A (CLL1 cohort) and advanced CLL (CLL8 cohort)*
For progression free survival, 6C-Kine (CCL21) and sIL2-R-alpha were also strongly associated with progression free survival (PFS), as shown in Figure 14 and Figure 15, respectively.

Figure 14: Progression free survival in months, tracking 6C-Kine concentrations. (1) 6C-Kine levels separated by median (2) 6Kine levels, upper quartile versus quartiles 1-3. Adapted from Seiler, Aydin et al’s presentation for the “12. Wissenschaftliches Symposium der Med. III”, 2010.

Figure 15: Progression free survival in months, tracking IL2-R-alpha concentrations. (1) IL2-R-alpha levels separated by median (2) IL2-R-alpha levels, upper quartile versus quartiles 1-3. Adapted from Seiler, Aydin et al’s presentation for the “12. Wissenschaftliches Symposium der Med. III”, 2010.
Results of the conventional ELISA confirmation studies

ELISA CLL 1

The results, depicted for sIL2-R-alpha in Figure 16 largely confirm the multiplex assay results as described in the previous sections.

![Graph showing survival functions for sIL2-R-alpha](image)

As with the multiplex assay testing, the ELISA confirmation studies could also identify sIL2-R-alpha to be an independent prognostic variable for progression free survival for the Binet A cohort of CLL1, i.e. in the early CLL disease stage. Concordantly with our expectations, the determination of sIL2-R-alpha serum levels (along with the other ELISA-retested parameters) was cost effective, and is broadly available.
VII. Discussion

From a cellular perspective towards a holistic view encompassing microenvironments

The objective of this study was to illuminate aspects of the CLL microenvironment as an integral part of CLL pathophysiology. CLL cells have originally been viewed in isolation. Prima facie, such a focus seems sensible, not least because that is the locus of the mutation, i.e., the pathological change originates in the cell, not in its environment.

In addition, the disease burden in part correlates with the concentration of CLL cells as measured in blood samples, often drawn peripherally. As both the “damaging agent” (the preponderance of CLL cells which displace and supplant functional B-cells), and the proximate cause of the change (mutations in the CLL precursor cells’ genomes) are revolving around CLL cells, at first no external modulators seem necessary, as both the “starting point” and the “end point” which characterize the disease are centered within the same cells. As such, treatment strategies as well as strategies for analyzing the pathogenesis have had a focus on cell biochemistry.

Such a predilection and focus on a cell-centric view was further helped by soft factors within the scientific community, namely that the complexity of thoroughly understanding just the changes within CLL cells is of high enough complexity as to reach – if not surpass – the edges of scientific understanding, even without further complicating the picture by introducing interacting factors external to the cell.

However, a more dynamic-system-oriented view is increasingly necessary and in circulation. Similarly to DNA which by itself is inert, it is through the dynamic interplay with environmental factors that macro-behaviors manifest themselves. In the example of DNA, it is the transcriptional infrastructure and other external factors that determine and modulate how genetic imperatives (such as proliferative or anti-apoptotic SNPs in CLL genomes) are expressed. Whether one takes the increased vascularization in tumors being caused by microenvironmental changes, or tumors metastasizing via durotaxis along stiffness gradients (131), examples abound of the importance of looking at specific microenvironments. Consequently, the role of complex microenvironments on cell behavior is increasingly becoming an important field of study. The handling and burden of modeling such additional complexity is facilitated by the development of more powerful computational methods (such
as multi-physics models) and resources (such as parallelization) for conducting simulations, and the general increase in research interest on subcellular-level dynamics.

That CLL cells do not proliferate “regardless” of their environment can be demonstrated simply by observing that CLL cells die when cultured in vitro. Support by a feeder layer increases viability of CLL cells, supporting the idea of external stimuli being necessary for CLL survival (132). Such stimuli are putatively antigens (33, 133). In addition, the main locus of proliferation for CLL is not just in any tissue, but in lymphatic tissues in particular. Taken in conjunction, it follows that there must be differences between microenvironments that cause / mediate CLL proliferation, and normal tissues which do not: Specific interactions which take place in lymphatic microenvironments, but not in other tissues.

These differences must originate from within the CLL cells (as the disease is triggered by cell-based DNA changes), then interact with specific elements of the microenvironment, after which depending on those interactions proliferation is induced back within the cell. In other terms, the organizational structure of a CLL cell and its microenvironment constitute a dynamic system and a kind of control loop in which changes within the CLL cell change their local microenvironment, which in turn factors into and modulates the highly changeable dynamics within the cell (134).

The highly dynamic nature of such CLL kinetics, which has been shown to vary from day to day (134), underscores the importance of the continuous changes within that dynamic system, and the importance of dysregulations that do not merely accompany the disease, but which are disease-aggravating perturbations of the healthy equilibrium by themselves, throwing the system further out of balance, and inducing even more changes similar to a vicious circle reinforcing itself. Mechanisms for such a pathological dynamic overload are explored in the section Methodologically: Pathway analysis and clustering.

As such, even though both the cause of the disease, as well as the damage caused by the disease, center on the CLL cell, the pathway between the cause of the disease and its ultimate damaging effects leads from the cell to its microenvironment, and from there back into the cell.

Understanding that dynamic enables a new approach, namely focusing on the traces the microenvironmental changes leave e.g. in patient blood samples (specifically the serum component). While not every change in microenvironmental dynamics leads to peripherally measurable changes in chemokine concentrations, the compositional aspect at least can leave
such traces (i.e., changing concentration gradients). This is especially plausible because the microenvironment in lymphatic tissues, hosting CLL cells, may be (and has to be, to some degree) compartmentalized from other surrounding microenvironments, but cannot be hermetically sealed: In the end, even in the physiological case B-cells need to be able to migrate and leave their lymphatic tissue reservoirs to fulfill their physiological function. It thus stands to reason that traces of aberrational chemokine concentrations likewise follow the flow of cells into the peripheral blood, where they can then be measured. This approach validated the search for correlations due to disease-driven microenvironmental changes, as undertaken in this study, and its potential relevance to staging, by indirectly classifying microenvironments based on the traces they leave in easily obtainable (comparatively to other material) patient sera.

**Evaluating the results of this study in the context of their methodology**

**Multiple testing as a controversial paradigm**

While the previous section discussed the validity of looking for microenvironmentally-driven correlations in sera, and for assuming their potential impact on CLL disease outcomes, this study also employed high-throughput methods for screening for such correlations. These have not been without controversy, as will be discussed in this section.

As a first approximation, the advent of multiplex assays seems to allow for a much broader and easier search for correlations. However, multiple testing concerns constitute a major caveat to such broad-band searches for previously unknown correlations, and may if used excessively, contribute to the so-called “replication crises” (135). The problem arises from the paradigm of regarding samples taken from two unknown distributions as drawn from distinct (different) distributions if there is a less than 5% probability of the same distribution generating both groups of samples (the usual null-hypothesis), with p=0.05 being a value that is due more to scientific tradition than to statistical arguments.

If only a single hypothesis is carefully chosen and then tested, this approach can be well argued for. Complications are revealed, however, when a large number of hypotheses are tested, which would lead to an average of 1 out of every 20 tested hypotheses generating a
false result of statistically significant differences, even if all pairs of tested groups had been taken from the same distribution.

Not least due to such concerns did this study use the approach of using multiplex testing only as a first stage, and then follow up with more established ELISA confirmation testing.

Moreover, p-values themselves are not exempt from controversy, not least due to their common misinterpretation as probabilities (136), which has culminated in a peer-reviewed journal banning the usual use of p-values in the articles they publish (137). While the p-values yielded by this study well cross the customary 0.05 threshold for significance, and even the stricter Bonferroni-corrected thresholds for significance in a context of multiple hypothesis testing, significance on its own does not necessarily translate into good predictors. Even if two samples (e.g., different clinical risk groups with different outcomes as in this study) can be shown to stem from different distributions, that does not automatically entail an effect size sufficient for clinical decisions. Generally as a broad approximation, even small differences in the distribution of a variable between two groups may result in highly significant p-values as the number of samples increases. Conversely, large differences in the distribution may lead to highly significant p-values even with few samples. The observation that p-values in this study are passing not only the customary 0.05 threshold, but rather the strict Bonferroni threshold hint at the differences in the chemokines being of enough import as to possibly allow for incorporation into clinical risk indices.

Additional steps for correcting and screening potential false positives based on multiple hypothesis testing were taken, in the form of varying methodologies for the confirmation testing (multiplex assays followed up by ELISA testing).

Nevertheless, ELISA confirmation testing on its own but using the same samples may also not have been sufficient to plausibly and convincingly alleviate multiple testing concerns. This is because even with highly significant p-values, which denote the probability that the observed results would be generated by a test if the null hypothesis is true, repeat experiments would not be able to check p-values for being spurious results if they were conducted on the same samples. They would contribute additional information if they used other testing methods, and as such confidence in the actual significance of a given p-value could in fact increase, however it is only by using confirmation tests on additional samples, with a different methodology, that confidence in correlations initially yielded by multiple hypothesis testing can be best increased.
In summary, this study corrects for multiple hypothesis testing generating false positives by using a different methodology, on an expanded base of samples, complemented by Bonferroni correction of the required p-values for significance. Taken together, this composite approach leads to a high degree of confidence in the results of this study not being merely spurious, or coincidental correlations.

However, even having taken these steps, it is only the confidence in the presence of correlations that can be relied upon. Eventually, correlations have to be matched to causal explanations and pathways in order to gauge not their statistical, but their clinical significance. Otherwise correlations could simply be caused by the presence of a confounder, an unknown third variable, and be for example merely byproducts of already known, other correlations.

**Serum samples as surrogates for the microenvironment**

Ideally, if one wanted to study alterations in the microenvironment in CLL, the subject that is then experimentally tested would be precisely that: the microenvironment, harvested i.e. from lymphadenectomies. Even material from needle biopsies would not be sufficient, as the tissue geometry and structure would be perturbed if not fully disrupted, thus invalidating observations about the microenvironment.

Gaining enough viable material to test for the direct biochemical composition of the microenvironment would thus entail pronounced and invasive deviations from the established diagnostic and therapeutic algorithms used in clinical practice. In short, only in very narrow scenarios (e.g. debulking procedures) is direct material gained in large quantities such that the microenvironment could be presumed to be undisturbed.

There lies the crux of the problem: In order to use the microenvironment in actual clinical practice, a method needs to be devised that allows for inferences about the microenvironment based on a suitable proxy, which needs to be obtainable from patients as non-invasively as possible.

The patient sera used in this study constitute such a proxy. Since many of chemokine concentrations measured in this study can only plausibly be an effect of the microenvironmental composition within the lymph nodes (since that is the main point of divergence between the different CLL groups tested in this study, causing the divergence in
serum concentrations), the sera evidently carry information about the otherwise inaccessible CLL microenvironment.

It needs to be noted, however, that as promising as the presence of such a convenient and clinically viable proxy may be, by necessity not all microenvironmental changes can be deduced from differences in serum concentrations. While differences affecting the relative and absolute concentrations of the microenvironment constituents may translate into corresponding differences in serum concentrations, not least because there is some interchange and transference of substrate via the circulating CLL cells, microenvironmental changes could be present that affect mostly the dynamic interactions, without strongly affecting concentration gradients (i.e. concentrations in absolute numbers). Such changes would not be detectable through the use of patient sera as proxies.

One further important caveat that should be mentioned both to qualify the research done in this study as well as to outline future directions is to note that the concentrations as observed in the serum samples will deviate from the microenvironmental concentrations found in e.g. the marrow, or the secondary lymphatic tissue – i.e., in the loci of CLL. If there were e.g. a kind of conversion factor, denoting how to calculate the concentration of an analyte in the lymphatic tissue based on the analyte’s concentration in the peripheral, then such a factor could differ from analyte to analyte. Even though evidently microenvironmental constituents “leak” into the serum to some degree, such leakage (or transference) need not be uniform across all analytes, i.e., not all analytes share the CLL cells’ circulating behavior between lymphatic reservoir and blood in equal measure.

Overall, the use of sera offers both important restrictions for deducing the causal roles of the clusters and chemokines identified (since their concentrations may differ between the serum and the other CLL loci), as well as substantial practical benefits in allowing potential tests based on the relative and absolute abundance of certain chemokines, to be conducted on nothing more than a patient’s serum, without any bone marrow extractions, or lymphadenectomies.

As shown in the section on Prognostic factors of CLL, previous results already strongly suggested chemokine dysregulation as an effect strongly correlated with CLL cell proliferation and survival. In particular, in previous work on CLL microenvironments as inferred via serum levels, three clusters of highly correlated cytokines had been identified, namely (CXCL9, CXCL10, CXCL11, CCL3, CCL4, CCL19, IL-5, IL-12, and IFNγ), (TNFα,
IL-6, IL-8, and GM-CSF), and (IL-1β, IL-2, IL-4, IL-15, IL-17) (68). Different relations between these clusters correlated with time-to-first treatment and with overall survival (68). The existence of such correlations suggests common pathways, and different relations and ratios between such clusters may suggest the associated pathways behaving either synergistically or antagonistically. It has been stated that the discovery of such relations may present new opportunities for therapeutic strategies. Such statements, while certainly true in a general sense, should however be further qualified:

While the correlation of high expressions of different chemokines with disease progression shown in this dissertation suggests a causal role (especially since many of those chemokines have been known to affect CLL signaling), the causative direction remains tentative. If some of the alterations of the microenvironment which seem to correlate with high-risk substrata of the patients were indeed to provide a causative link, then new therapeutic targets could be deduced based on such a deeper pathophysiological understanding of CLL. Conversely, if the aforementioned correlation proved to be just that, namely a non-causative artefact coinciding with high-risk patients, then that too could prove to be a future boon, namely in providing an additional tool to sieve the high-risk patients from the standard population. Even given that other methods have already been established in order to stratify the population of CLL patients, by necessity each prognostic factor is associated with a certain probability of being in error. Adding additional – and ideally independent – predictors such as the elevated chemokine levels found in this study can only alleviate that error by adding an additional layer of confidence based on such predictors. Pathway and cluster analysis, as proposed in the section Methodologically: Pathway analysis and clustering provides an orthogonal axis of information and can help further qualify which correlations may rise to the level of therapeutic target, and which remain valuable mainly as diagnostic proxies.
Next steps

Experimentally: Different patient collectives and longer follow-up periods

It is advisable to try to generalize the results from this study using a different patient collective, which could be a sensible next step of investigation. Similarly, results could potentially be repeated for the same cohort after a longer period of follow-up, depending on the availability of remaining serum aliquots.

These two strategies (validate with more collectives, longer follow-up periods) are generally promising perspectives for probably nearly every clinical study. Both of them can be considered to be advisable for this study, although their added value may not amount to as much as with many other studies: The collectives used in this study were carefully and broadly chosen by the DCLLSG, as previously discussed. If they had been chosen from a single treatment center, or from a special subclass of patients, then further experiments would increase the data’s external validity, i.e., its ability to generalize to other cases. However, owing to the excellent recruitment as performed by the CLL1 and CLL8 trials, this study’s results are unlikely not to generalize, at least not for reasons of using an insufficiently varied and representative base of patients. Nevertheless, as with the meta-study paradigm which pools studies, if the experiments conducted in this study were to be repeated for other patient collectives, doing so could only benefit the validity of this study’s results.

Regarding repeating the observations using longer follow-up periods, it should be considered that the endpoints, i.e., the target classes (clinical outcomes) by which the data was divided, were already correctly identified without any need for further follow-up. As with the intent-to-treat paradigm, the initial allocation should be considered final. Thus, the division of the patients into different risk-classes would not have yielded a different outcome however long the follow-up would be extended, since those risk-classes were already defined for those patients at the time this study was conducted. However, a case for longer follow-ups can be made in order to allow for new testing metrics, such as more extended comparisons regarding overall survival / progression free survival with the analytes considered in this study. Also, if follow-ups were to make available new sera taken from the same collective, then not only could the dynamic course of chemokines be tracked, but also the development and persistence of cytokine/chemokine clusters (cf. the section on clustering).
Towards increasing relevance of complex risk indices in clinical practice

Future insights into lymphatic pathways in which the analytes investigated in this study are implicated could also be useful to better interpret which correlations in this study are located on different pathways, and which of the cytokine alterations are antecedent, and which are descendants of each other (i.e., which alterations are causally “upstream” and therefore may carry additional information). It remains to be seen whether any simple causations actually emerge, seen from a dynamics system perspective there remains the possibility that there is no single small set of “drivers” of the pathological process, but rather a general “skewing” of the microenvironmental infrastructure as a whole, causing dysfunction in the form of overmodulated cell proliferation. Investigating the relative weight and contribution of different components of the microenvironment for the CLL pathogenesis is of major importance in converting the correlations found in this and other studies into therapeutic interventions.

However, one needs to distinguish between necessary information for finding new therapeutic interventions, versus information with a potential use in diagnostic algorithms: Not knowing causal pathways but instead “only” the correlations of analytes to clinical outcomes does not diminish the diagnostic value of such correlations, notwithstanding the possibility that they may only be peripherally involved in driving the disease and/or be a kind of metabolical bystander / side-effect. In that sense, there exists a strong dichotomy between the value of a correlation in identifying a new therapeutic target (for which identifying a correlation is only a first step) versus the value of a correlation as a predictor of risk (for which a correlation all on its own may already suffice). For the first purpose, further studies are certainly necessary, while for the latter the causal relationships are of secondary concern.

It follows, then, that the diagnostic utility of a gradient in concentrations between clinical groups does not rely on an exhaustive understanding of its causal underpinnings, but rather on a strong effect size, clearly delineating different groups according to a target variable (e.g., overall survival, Binet stage, etc.).

The strategy of combining such measurements into finely calibrated risk measures aims to allow for the best possible categorization of patients into risk groups, while minimizing the patient burden (invasiveness) in gaining the required information components.

This approach of aggregating prognostic factors is gaining acceptance in clinical practice. One of the main previous obstacles in adopting more complex formulae consisted in the
interruption of the clinical workflow, and the practical difficulties of combining a potentially large number of measurements in potentially complex mathematical relationship. With the advent and increasing penetration of electronic devices into everyday clinical practice and the ubiquity of computers and software which can support more sophisticated risk indices, incorporating more complex measures into clinical practice is increasingly viable and less prone to human error, as well as in line with the increasing reliance on guidelines and evidence-based medicine.

The CLL-IPI (17) may be the most promising such prognostic index for CLL patients, able to yield e.g. 5 year survival percentages with 3 significant digits, based on large amounts of clinical data. In order to calculate both survival and time-to-first-treatment, it uses clinical stage, age, TP53, del(17p), as well as IgHV mutational status and \( \beta \)-microglobulin. A number of validation studies have recently been published (138-140).

In the case of CLL in particular, stratifying by risk is of particular importance given that one of the hallmark therapeutic approaches to CLL is that of „watchful waiting“; i.e. waiting for the burden of disease to become so problematic as to justify a more active therapeutic intervention (57). This approach, while serving low- to medium risk CLL patients well, carries the burden of potentially missing appropriate therapeutic windows of opportunity to positively influence future aggressive disease progressions, as would be more common with high risk patients (who on average have a much diminished progression free survival). As such, diagnostic criteria which do not serve for an initial diagnosis of the disease (which would mostly lead into a watchful waiting strategy) but rather to differentiate low-risk from high-risk carriers are potentially more relevant in clinical practice. The CLL7 DCLLSG study which dealt precisely with the early intervention for high-risk patients will yield further important results in this respect. In the context of the CLL7 trial, the relevant result of this study would be that compared to patients at diagnosis, serum levels of CCL21, CCL27, TNF-\( \alpha \), IL-16 (p<0.001), CXCL12, EGF (p=0.02), and CCL4 (p=0.04) were significantly elevated in patients at advanced disease.
**Methodologically: Pathway analysis and clustering**

The previous discussions have shown the importance of placing significant chemokine differences into the context of not only their statistical power in contributing to diagnostics, but also as starting points for reaching a better pathophysiological understanding of CLL. Hopefully, doing so can culminate in better treatment choices, and more informed and targeted follow-up research. In particular, as previously explained, clusters of chemokines differing between groups can hint at the pathways involved.

The clustering observations as presented in this study, i.e. identifying correlations not only on the level of individual analytes, but on the level of clusters of chemokines, based on this study’s experimental data, were conducted mainly by Dr. med. Till Seiler and Dr. med. Tobias Herold. These yielded the pronounced presence of clusters of analytes, which to some degree separate healthy, CLL1, and CLL8 patients. Moreover, those CLL1 patients which fit into the CLL8-overlapping cluster had worse clinical outcomes than their CLL1 cohort. A heat map visualizing this clustering is provided in Figure 17.

![Figure 17: Hierarchical cluster analysis shows a differential chemokine chemokine serum pattern in healthy individuals, asymptomatic patients at diagnosis and in advanced disease. Adapted from cluster analysis conducted on this study’s data with the support of Dr. med. Tobias Herold, 2014. Each colored line represents the concentration of a distinct cytokine, each row represents one sample.](image)

The use of such clusters will be an important step in transcending the use of singular concentrations in clinical assessments towards more dynamic profiles, which rely not only on absolute values, but on more complex relative relations between analytes. While some relations have already found usage in clinical practice, such as the De Ritis ratio for differentiating causes of liver disease⁴, generally many diagnostic algorithms still predominantly rely on absolute concentration values. Identifying clusters is a first step not only in gaining a more nuanced and detailed understanding of CLL pathophysiology, but also

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⁴ The De Ritis ratio is defined by the relation of AST to ALT, such a fraction being the easiest relational measure.
in identifying which of the chemokines may be “independent” of each other (or weakly causally linked). Clusters may to some degree already approximate quantitative relationships which can then optimally be distilled into quantitative risk measurements (cf. the section Towards increasing relevance of complex risk indices in clinical practice).

Preliminary cluster analysis\(^5\) yielded that CLL patients and healthy individuals clustered separately: Healthy controls had low levels of SCF, sII2-Ra, CCL3 and CCL4, while none of the assessed chemokines were overexpressed in healthy controls. Among CLL patients, a subgroup of patients clustered separately based on high expression of sII2-Ra, CCL3, CCL4, II16 and TNF alpha. In particular CCL3 and CCL4 are indicative of the involvement of BCR signalling, to which they are closely related. Since, as previously explained, BCR signalling is of vital importance for the survival of both healthy B-cells as well as CLL cells, the increased serum level of CCL3 and CCL4 in patients with more aggressive disease may be well explained by this phenomenon which fits well with prior knowledge concerning the role of the BCR pathway (141, 142). The elevation of sII2-Ra – the shedded CD25 antigen – correlates with activation of the immune system in autoimmune disorders, inflammatory disorders (HLH) and T cell lymphoma (143-145). With a disease such as CLL which features inflammation as a central component (146, 147), this observation generalizes well from other conditions which likewise focus on inflammation as a driving force, such as autoimmune disorders. II16, already well known as a T-cell chemoattractant fits well into this pattern of triggering, facilitating, or sustaining inflammation (148, 149). These newly attracted T-cells may also act as an important source of TNF alpha, which sustains CLL proliferation (149), forming a causal chain from II16 to TNF alpha to CLL stage progression. The role of SCF is less clear, although its role as a growth factor (even if mostly for hematopoiesis, as previously mentioned (96)) seems to in principle explain its involvement (or rather, the lack thereof in healthy controls). Ideally, if SCF was to represent a pathway orthogonal to the inflammatory processes in which the other chemokines are involved, that could mean that it contributes additional and independent information, as well as potentially implicating novel pathways. As yet, SCF has not been a focal research interest in the context of CLL.

Patients in the aforementioned CLL group which clustered separately were mainly subjects with advanced disease. However, some patients in Binet stage A clustered together with these patients (n=64/159, 32.8%), whereas the majority of early stage patients (n=95, 67.2%)
displayed an expression pattern resembling the healthy control population. Interestingly, patients with an “advanced stage” pattern did not significantly differ in age, white blood count or IgHV mutational status from the other early stage patients. However, the former patient subset showed a significantly higher level of beta2-microglobulin (p=0.003), suggesting a higher proliferative activity. Both sII2-Ra and beta2-microglobulin are proliferation markers in the context of the immune system.

Patients with the aforementioned “advanced stage” chemokine expression profile showed a worse clinical outcome with a median progression free survival of 60.6 months, whereas the median PFS was not reached in the subcohort of the other early stage patients (median PFS not reached after 72 months follow up, p=0.006) (cf. Figure 17) No difference in overall survival was observed between the two subgroups.

Notwithstanding that result, this study has also established that serum levels of EGF, CCL2, CCL3, CCL4, TNF-α, IL-16, sII2-Ra and SCF differed significantly between healthy controls and CLL patients as well (p<0.001), allowing for differentiating all three groups from each other. The similarities between these two clusters point at the nature of the distinction which sets apart CLL microenvironments from healthy microenvironments, as well as high-risk from low-risk CLL trajectories: There is a mechanical dimension to the chemokine dysregulation. Rather than CLL affecting the microenvironment purely by changing biochemical balances driven by biochemical reactions, thereby shifting chemokine concentrations and reaction equilibria, instead there is also a component of physically rearranging the constituents of the microenvironment including the proportion of cellular actors to their interstitial embedding. This rearrangement of which actors are present to be involved in shaping the microenvironment can all by itself create and magnify further changes of the microenvironment, and help explain the large discrepancies between healthy and dysregulated microenvironments such as found in this study.

Evidently, this process of rearranging the actors which then influence the microenvironment is initially triggered and driven only by different biochemical reactions of the original microenvironmental actors (namely, the initial mutated cells with which CLL started). However, as the rearrangement progresses and the system is thrown out of balance, the BCR pathway which is strongly associated with the cluster found within this study is strengthened, and a pro-inflammatory environment together with an influx of chemotactically attracted lymphocytes (and CLL cells) gains dominance. The resulting new microenvironment is from that point onwards not only driven by the initial mutation, or by the biochemical shift towards
an inflamed microenvironment alone, but also by being generated through cells attracted by this altered pro-inflammatory microenvironment, which further throws the microenvironment out of balance, which may in turn drive further activation of the BCR pathway and a continued influx of cells, potentially ad infinitum. In reality, some boundaries to such a runaway dysregulation remain, and it could be speculated that the individual robustness and functionality of such boundaries in slowing down such self-reinforcing feedback loops play a role regarding individual disease progression and risk group classification. For example, given the evident mechanical component (cells migrating into the lymphatic tissue), eventually the tissue will be inundated with cells, without further room for cells to enter nor to proliferate. To some degree, disease progression is dependent on circumventing this lack of space, both by expelling superfluous cells (such as newly generated CLL cells) into adjacent blood vessels, and by triggering tissue growth (enlarging lymph nodes). This growth may also to an extent be driven by the mechanical stress induced by the pathological cellular accumulation and constant inflammation within the lymph nodes, which is strongly implicated in stimulating tissue growth (150). As the lymphatic tissue enlarges, driven by the mechanical stress, the spatial impediment to further microenvironmental and pro-inflammatory dysregulation disappears as new space is created for B-cell trafficking, T-cell chemotaxis, and the BCR-pathway in general.

In particular, the plausibility of the aforementioned correlations and explanations is strengthened by considerations about how the chemokines in question are functionally linked together, as explained in the section on The role of the microenvironment in CLL pathophysiology: CCL3 and CCL4 are described to be secreted by CLL cells in vitro after BCR engagement. CCL2 and CCL17 both bind CCR4, are expressed on T cells, and facilitate chemotaxis for T cells. CXCL12, CCL21 and CXCL10 are all chemokines involved in the B cell trafficking previously mentioned.

The central conclusion, building on previous results, may be stated such that chemokine homeostasis is altered in CLL, possibly reflecting events in the tumor microenvironment. sIL2-R-alpha is an independent prognostic factor (PFS). It has been implicated in a host of diseases centered on lymphatic tissue and may potentially be a general marker for tumor mass (bulkiness) (151, 152), or its leukemic equivalents (CLL-cell count). Still, it is not exclusively linked to malignancies, and has also been implicated in autoimmune disorders (153), in which dysregulations in the microenvironment may likewise be central components. The observation that the determination of sIL2-R-alpha serum level by ELISA is cost effective and broadly
available may allow for sIL2-R-alpha to be investigated further as a potential component or contributor to new quantitative diagnostic risk assessments, up to potential inclusion in future iterations of commonly used risk indices, owing to its apparently (as shown in this study) strong differences in concentration between different clinical groups. These differences were shown to be robust both after multiple hypothesis corrections, and over different experimental methodologies.

Future developments both of indices such as CLL-IPI, as well as further research into CLL microenvironments, remain a highly prospective and promising field of research, to which this study may hopefully have contributed to some degree.
VIII. Summary

Having established CLL as a chronic disease with a wide-ranging spectrum of outcomes regarding overall survival and response to treatment, prognostic factors to classify patients in regards to their outcome category are not only important for the patient’s individual quality of life, but also affects therapeutic decisions. As of today there a several such prognostic factors, such as various genetic mutations and serum markers to help achieve that goal. The aim of this analysis was to identify prospective markers in test sera from previous large-scale CLL-trials using multiplex testing, and then to conduct confirmation tests with a more established methodology such as ELISA. The markers were chosen with a particular pathophysiological rationale, namely their import on the CLL microenvironment which is understood as an important factor in modulating the disease.

This study was therefore separated into different stages: In the first stage, a rather novel form of testing with high throughput, the multiplex method, was used to test for various previously scientifically underexplored markers in order to identify good candidate markers.

In the second stage, the confirmation ELISA assays were conducted to reproduce the correlations revealed through the multiplex method. Amongst some others, sIL2-R-alpha could be identified as a strong predictor of progression free survival (PFS). The statistically significant results withstood Bonferroni correction for multiple hypothesis testing.
IX. Zusammenfassung

Bei der CLL handelt es sich wie ausgeführt um eine chronische Erkrankung mit einem breiten Spektrum in Bezug auf Lebenserwartung und dem Ansprechen auf verschiedene Therapien. In diesem Kontext sind Prognosefaktoren nicht nur für die individuelle Lebensqualität und Planung der Patienten wichtig, sondern auch essentiell für die korrekte Zuordnung zu CLL-Untergruppen und damit einhergehend für die unterschiedlichen Therapieentscheidungen. Dem heutigen Wissensstand folgend gibt es eine Anzahl an erforschten Prognosefaktoren, unter diesen verschiedenste genetische Mutationen als auch Blutwerte, die zu einer solchen Zuordnung einen Anteil zu leisten vermögen.

Die Zielsetzung der vorliegenden Studie war es, weitere Prognosefaktoren aus den verbliebenen eingefrorenen Sera aus bereits abgeschlossenen CLL-Studien größeren Umfangs zu eruieren.

Zum Einsatz kamen hierfür Hochdurchsatzverfahren nach dem Multiplex-Prinzip als eine Art Screening, und danach Bestätigungstests nach dem etablierten ELISA-Verfahren, welches dann auch die Daten für die vorliegenden Korrelationen lieferte. Die zu testenden Parameter wurden insbesondere anhand ihres definierten pathophysiologischen Hintergrundes, nämlich im Hinblick auf ihre Rolle im sog. CLL microenvironment, einem wichtigen Faktor für die Ausprägung der Erkrankung, ausgewählt.


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Ich erkläre hiermit an Eides statt,

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Multiplex and ELISA sera analysis in CLL patients
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