A REGIONAL TRANSLATIONAL RESEARCH PROGRAMME FOR MALIGNANT B CELL DISEASES AND ASSOCIATED DISORDERS

"FROM GENOMIC, PROTEOMIC AND CYTOMIC RESEARCH
TO
NOVEL DIAGNOSTIC AND THERAPEUTIC STRATEGIES"

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PROPOSAL SUMMARY

Malignant B cell diseases continue to have a significant impact on the public health, despite advances in diagnosis and treatment and improvements in patient survival. A network of existing experts working in multiple disciplines and institutions created within a formalized infrastructure will overcome fragmented research and thereby achieve basic discoveries of the diseases and development of clinical care in a rapid pace.

Recent improvements in technology are leading to an increased understanding of the molecular and cellular events involved in disease development. However, translation of such knowledge into clinical care requires the establishment of the multidisciplinary network as proposed. The scale of the activity should provide direct international competition and qualify for collaborations with leading research institutions.

Major issues expected to be further clarified are molecular mechanisms related to the pathogenesis of lymphomas, new prognostic variables and models to be used for tailored therapy, new methods to improve the evaluation of treatment response, the role of minimal residual disease in the assessment of prognosis, and new therapeutic approaches improving survival and quality of life of lymphoma patients. Doing this will both accelerate basic insights into the disease process and translate these into the clinical arena rapidly.

The possibility to store and process all such medical knowledge and data has made artificial intelligence of interest in medicine. This bioinformatic based technology seems very attractive to assist or even surpass clinicians in reaching objective diagnosis.

If implemented, the research priorities proposed will dramatically accelerate progress and provide a bold new strategy for rapid translation of basic research into improvement in public health.

The advancements foreseen can be summarised as follows:

- Evidence based definition of standards for disease criteria, response criteria and prognostic variables by integration of basic science into clinical activity.
- Establishment of a network of laboratories that are involved in genomics, proteomics and cytomics in order to develop “array” based patient profiles.
- Development of a coordinated biobank, databank and trial program to validate individualized clinical care rapidly and analyse the potential of artificial intelligence.
- Establishment of a common educational, exchange and training program in molecular medicine.

Putting in place the programme will implement a process, which will drive the research at Aarhus University Hospital forward beyond the end of the programme. In addition we expect it to generate new technologies and to accelerate their development and marketing within the biotechnology area.

0.0 CONTENT

1.0 INTRODUCTION AND OVERALL STRATEGY

Among the most common malignancies in the western world are the broad range of non-Hodgkin's lymphoma (NHL), Hodgkin's disease (HD), chronic and acute lymphoid leukemias (CLL and ALL) and multiple myeloma (MM). This spectrum of B cell malignancies has an incidence of 40 per 100.000 inhabitants, i.e. 2000 new patients per year in Denmark and a prevalence of more that 10.000 patients.

Today's diagnostic tests of these diseases reflect the criteria of the updated WHO classification based on an extraordinary biological, morphological, and clinical heterogeneity (1). The current approaches to the therapeutic management include such morphological and immunophenotypical classification (1). Refinements of the classification are anticipated by recent knowledge in genomic, proteomic and cytomic research. Highly specific and sensitive molecular assays have been developed to detect alterations to be used in the diagnostic and prognostic evaluation, to follow treatment response, and to detect early recurrence or minimal residual disease (2-4).

Further research is needed in order to translate such results into clinical practice as new therapeutic options are available including immunotherapy, radio-immunotherapy, antisense oligonucleotides, vaccines, and clinical trials are a necessity to define the role of these modalities in the future individual care of patients.

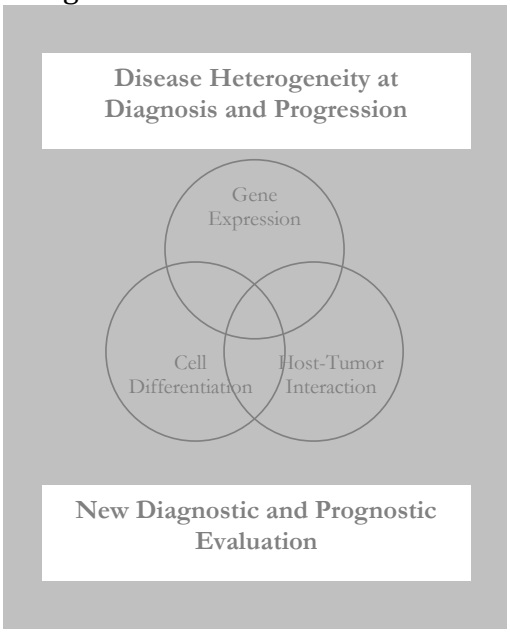
Based on a scientific hypothesis, novel technologies, modern laboratory facilities and a superior clinical research organisation and regional and international collaboration it is the overall goal for this research strategy to translate new knowledge from studies of pathogenesis in B cell malignancies and related disorders into a patient-oriented programme to improve clinical practice and ultimatively public health.

1.1 The background, scientific hypothesis and objectives

The recent upgraded laboratory facilities and clinical research unit with its staff of laboratory scientists and clinicians specialized in haematology will be the fundament for implementation of this strategy. The existing collaboration in a joint regional research and development programme with other clinical departments, the Danish Leukemia, Lymphoma and Myeloma Groups and international co-workers will play a major role in this process. This will overcome fragmentation and be a superior network as one of the international scientific leader in translational science.

One central challenge in medical translational science is to define the disease spectrum in molecular terms. Currently, cell morphology largely determines diagnoses, so that multiple molecularly distinct entities are often lumped together. This underlying molecular heterogeneity means that patients in the same diagnostic category do experience markedly different clinical presentation (heterogeneity) at diagnosis and following disease progression. This

is in accordance with our general understanding of cancer as a step-wise process influenced by oncogenesis, cell hierarchy and host-tumor interaction as illustrated in Figure 1.



It is the *scientific hypothesis* of this programme that molecular characterization of classical medical disorders ideally may include molecular subtypes only found in those patients with a uniform pathogenetic stage at diagnosis. Thus, patients with identical genetic lesions are assumed to have resulting similar pathogenetic characteristics. An optimal molecular diagnosis should therefore identify which normal cell type gave rise to the abnormal process and which molecular mechanisms resulted in progression during differentiation which mechanisms may be responsible for the host defence mechanisms. The pathogenetic model is influenced by the balance of this triad including 1) gene deregulation, 2) abnormal differentiation of the involved cell hierarchy and 3) host-tumour response and interaction which ultimately should be described in a symphony by global analysis of hereditary and acquired genetic and epigenetic alterations, RNA and protein expression, whole cell phenotyping in the broadest sense as well as the biostatistical derived novel molecular pathways necessary for the cell survival, renew, proliferation, differentiation, in light of disease evolution and ultimate progression.

Molecular characterization, we hypothesise, will mediate a shift in diagnostic strategy can be hypothesized and will have significant direct clinical utility in care. Patients with a uniform molecular diagnosis would be likely to have much more homogeneous clinical behaviours and prognoses. A detailed understanding of the molecular abnormalities most likely can be used to guide the patient to the individual treatment modality predicted to be effective. Most important, a molecular diagnosis may reveal novel specific molecular signatures and targets for individual disease monitoring and therapy.

Technologies of particular promise include i) genomic-scale gene expression by DNA chips, ii) protein spectral analysis by chips and iii) cell phenotyping by multiparametric flow cytometry. These technologies, described in details later, are being separately applied to individual disease models in patients, animals and in vitro culture systems, but this programme wants to study the same disease specimens with all of these technologies in parallel and integrate the results to achieve a common molecular portrait of each disorder. An important adjunct to this work will be to fully understand gene and protein expression patterns during normal stages of B cell development so that the normal counterpart of each disease can be identified.

The project will include four interlinked objectives:

1.1.1 *Establish a regional based laboratory and clinical network of scientists in B cell diseases for the exploitation of research and development;*

1.1.2 *Prioritize laboratory based component research on aetiology, gene regulation, cellular hierarchy and host environment and define new clinical useful molecular profiles of the B cell disease spectrum;*

1.1.3 *Support clinical oriented research, particularly prospective clinical validation of laboratory models by highly selected clinical end point; this also includes improved intervention by evaluation of individual therapeutics strategies and identification of individuals and populations at high risk for the disorders;*

1.1.4 *Development of evidence-based guidelines for good clinical practice by accurate, timely, and tailored information on the improved diagnostic and prognostic possibilities as well as access to education and training programs.*

2.0 THE NETWORK AND ITS SCIENTIFIC ACTIONS

Translational requires a connection and collaboration between laboratory research and clinicians (**Objective 1.1.1**). This programme will invite existing experts and organisations across multiple disciplines and institutions in Denmark to participate, within a formalized infrastructure, in which rapid new discoveries of diseases and development of therapies can be effectuated. An important pitfall is the minimal interaction between the clinical departments and other academic institutions, especially in translational research. A concerted effort to integrate these will have to be taken to improve the perspectives of the technological improvements.

Internationally there are already a number of integrated networks translating basic research and advances into the clinical arena, in which the partners participate as individual institutions.

To help ensure the optimal synergistic use of resources in the fight against the spectrum of B cell diseases we have identified scientific priorities and needs. The priority areas are divided into component actions, which may vary in size and structure.

2.1 The laboratory based actions (Objective 1.1.2)

2.1.1 To study the *pathogenesis* by identifying the basic mechanisms responsible for the complex spectrum of diseases. It will require a better understanding of (a) how various types of genes and its products are expressed, processed and regulated in B cells, (b) the impact of various genetic factors on susceptibility, including gene repair capacity and other types of cellular responses and (d) the role of environment in the broadest sense.

2.1.2 To define *the cellular hierarchy and the host biological environment*. The nature of the potential cellular target compartment and interaction with stromal microenvironment are critical determinants of initiation, progression, migration, and response to therapy.

2.1.3 To provide *molecular characterization and profiles* of the diseases, including patterns of genetic and epigenetic alterations and RNA and cell protein expression, as well as characterization of the molecular pathways necessary for self renewal, survival, proliferation, and differentiation of the attached cells.

2.1.4 To develop *normal cell research* as the understanding of the molecular determinants of specific disease characteristics is still limited. Such information may be essential for design of new non-toxic therapies.

2.1.5 Finally, to study the *aetiology* by analysis of interaction among genotype, phenotype, immune functions, infectious agents, environmental factors, and lifestyle factors that can lead to medical disorders. The aetiology is not well understood, yet the development of interventions requires that we know the causes. Prior epidemiological research has focused almost entirely on single conditions. Case-control studies and cohort investigations are needed.

2.2 The clinical based component actions (Objective 1.1.3)

2.2.1 To improve *clinical decision-making and quality of care* during active treatment protocols, clinical trials and follow-up.

2.2.2 To develop *education and training programs* for staff members for diagnosis, treatment, and clinical trials. Certification will lead to significant improvement through optimization of current approaches.

2.2.3 To implement *new therapeutics strategies* by translating "lead" structures and molecules into effective therapeutic agents. Target discovery, validation, and clinical translation will form an important basis.

2.2.4 To foster *partnerships* between cooperative groups and industry to expedite new tests, drug development and availability.

2.2.5 To identify *high-risk individuals and populations* essential for a rational development and testing of intervention and prevention strategies.

In order to ensure an optimal use of the resources, the scientific laboratory and clinical status and needs are described below.

3.0 SCIENTIFIC STATUS AND NEEDS

The field of lymphoma science is an example of how the biotechnology revolution and pathogenetic knowledge has influenced clinical care and defined new needs and possibilities. The description below is based on published peer-reviewed papers regarding the genetics and genomics of malignant B cell diseases.

3.1 B lymphomagenesis and cell of origin

B-cell development takes place in well defined differentiation steps characterized by the specific structure of the B cell receptor (BCR). The BCR is composed of two identical heavy-chain and two identical light-chain immunoglobulin (Ig) polypeptides that are covalently linked by disulphide bridges. The intracellular signalling includes several tyrosine kinases. Depending on the differentiation stage of the B cell that recognizes an antigen and on the activation of other B-cell surface receptors that modulate BCR, the activated B cell might be induced to proliferate and/or undergo further differentiation steps.

Early B-cell development, which occurs in the bone marrow, concludes when a B-cell precursor successfully rearranges Ig heavy- and light-chain genes and is equipped with a functional surface antigen receptor. Cells that express a functional BCR differentiate into mature naive B cells and leave the bone marrow, whereas B-cell precursors that fail to express a BCR undergo apoptosis. In T-cell-dependent immune responses, antigen-activated B cells undergo clonal expansion in the lymph node 'germinal centres' (GCs), where the Ig genes are modified by somatic hypermutation and class-switch recombination.

As distinct stages of B-cell development and differentiation are characterized by the particular structure of the BCR and expression patterns of differentiation markers, and as these processes often take place in specific histological structures, analysis of these features have been used to determine the origin of the various human B-cell malignancies. The rationale for such a classification of B-cell lymphomas is based on the observation that malignant B cells seem to be 'frozen' at a particular differentiation stage, which reflects their origin. One of the main concepts emerging from such studies has been that most types of B-cell lymphoma are derived from GC or post-GC B cells.

The cellular origin of B-cell lymphomas has been further clarified, and previously unrecognized distinct lymphoma subtypes been identified, by gene-expression profiling of human B-cell lymphomas and normal B-cell subsets. Such studies identified, for example, a GC B-cell gene-expression signature that is associated with follicular lymphoma, Burkitt's lymphoma and a subset of diffuse large B-cell lymphomas. These findings supported the GC B-cell origin of these tumours. Gene-expression profiling studies of other malignancies also revealed unexpected relationships, in terms of gene-expression patterns. For example, in addition to B-cell chronic lymphocytic leukaemia (B-CLL) cells with mutated Ig variable (V)-region genes, B-CLL cells with unmutated Ig V-region genes showed greatest similarity to memory B cells that had undergone somatic hypermutation, indicating that both subtypes of B-CLL are related to memory B cells. Moreover, a

subset of diffuse large B-cell lymphomas was identified that, among the various B-cell subsets included in the analysis, most closely resembled in-vitro-activated B cells.

However, in these malignant cells, the transformation process might have been associated with an alteration of the gene-expression profile, masking the signature of the cell of origin, as seems to be the case in classical Hodgkin's lymphoma. It is also possible that the normal B-cell counterpart of some cancer types might not have been identified yet. In the activated B-cell type of diffuse large B-cell lymphoma, the normal counterpart could be a poorly defined, small subset of GC B cells that is undergoing plasmacytoid differentiation, or a post-GC immunoblastic population.

This points to the fact that malignancy develops from normal tissues through the accumulation of genetic alterations that act in concert to confer lymphomagenesis. Although we have now identified some of the genes that initiate the process and the mutations that drive the progression, the identity of the cell population(s) susceptible to such transforming events remains undefined for the majority of B lymphoid malignancies.

Recent work, however, indicates that a small population of cells endowed with unique self-renewal properties and a malignant potential – a cancer stem cell - is present in some, and perhaps all B cell malignancies. Incorporating cancer stem cells into our current view of multistep oncogenesis has important implications for defining the specific stepwise events required to achieve malignant potential.

3.2 Stepwise oncogenesis

Reciprocal chromosomal translocations involving one of the Ig loci and a proto-oncogene are a hallmark of many types of B-cell lymphoma. As a consequence of such translocations, the oncogene comes under the control of the active Ig locus, causing a deregulated, constitutive expression of the oncogene. Three types of breakpoints can be distinguished in the Ig loci. Some translocations, such as the BCL2–IgH translocation associated with follicular lymphoma, have breakpoints that are directly adjacent to Ig heavy chain J-region (JH) gene segments. As the breakpoints also often show loss of nucleotides at the end of the JH or DH segments and the addition of non-germline-encoded nucleotides it is likely that these translocations happen as mistakes during V(D)J recombination in early B-cell development in the bone marrow. In other translocations, the breakpoints are found within or adjacent to rearranged V(D)J genes, and these V-region genes are always somatically mutated. These and additional features indicate that such translocations occur as by-products of the somatic hypermutation process, which is associated with DNA strand breaks. The third type of translocation is characterized by breakpoints in the IgH constant region switch regions, in which DNA breaks are introduced during class switching. This indicates that these events occur during class-switch recombination.

Maturation of the antibody repertoire is mediated by three different mechanisms: class-switch recombination (CSR), somatic hyper mutations (SHM), and gene rearrangement. All three mechanisms have been shown to be triggered by

AID, probably by deamination at the Ig locus. The deamination process is potentially mutagenic and an altered AID expression could act as a mutator causing genomic instability that drives oncogenesis. Tumor related activation of AID may play a central role in the development of several human tumors.

The process of somatic hypermutation contributes to lymphoma pathogenesis not only by causing chromosomal translocations, but probably also by targeting non-Ig genes like genes encoding BCL6 and CD95 (also known as FAS) have been found to contain mutations in a considerable fraction of normal GC and memory B cells, indicating that these genes are often targeted by the hypermutation machinery in normal B cells. In diffuse large B-cell lymphomas, aberrant hypermutation of multiple oncogenes has been reported, which might also represent an important mechanism of the progressive or stepwise oncogenesis.

Recently, the SHM process has been shown to be overexpressed in about 50% of DLBCL and MM. These comprise the well-known proto-oncogenes PIM1, PAX5, RhoH/TTF, and c-Myc, all of which have been already implicated in the pathogenesis. Analogous to the physiologic targets (IgV and BCL6), mutations at these loci are scattered through the first approximately 2 kb from the promoter with characteristic hotspot (RGYW/WRCY) motifs and exhibit a bias for transitions over transversions, with higher frequency at G:C pairs. Such distinctive features strongly support the hypothesis that this phenomenon, termed "aberrant somatic hypermutation", results from an aberrant activity, possibly a loss of target specificity, of the physiologic SHM process and therefore of AID itself. Because the mutations affect both regulatory and coding sequences of the affected genes, with several amino acid substitutions predicting a change in the protein structure, this may represent a major contributor to DLBCL pathogenesis. Indeed, for some of the targeted genes, such as c-Myc, the oncogenic effect of some mutations has been demonstrated.

Based on RNA expression data, AID appears selectively expressed in GC B cells and GC-derived malignancies and the pattern of expression of the AID protein has recently been investigated documenting the regulating AID function and its role in lymphomagenesis.

3.3 Role of the lymphoma microenvironment

In many lymphomas, the tumour microenvironment is likely to be important for the survival and/or proliferation of the tumour cells. In follicular lymphoma, the tumour cells reside and proliferate in follicular structures in close association with T-helper cells and follicular dendritic cells, as is typical for normal GC B cells. Some B cells belonging to the lymphoma clone can also be found in the interfollicular areas, but these cells show little proliferative activity. So, the lymphoma cells seem to require the cellular interactions in the GC-like environment for their proliferation. This is supported by studies that showed that follicular lymphoma cells can proliferate in vitro only if they are cultured together with CD4+ T cells, or with stromal cells and an antibody against the CD40 receptor. CD40 is expressed by follicular-lymphoma cells, and its activation is a main survival signal for normal GC

B cells. Notably, it was recently shown that the survival of patients with follicular lymphoma is correlated with characteristic features of non-tumour cells in the lymphoma tissue. So, it seems that follicular lymphoma cells retain key features of normal GC B cells, including the dependency on BCR expression and activation, as well as the interaction with T cells and follicular dendritic cells in the follicular microenvironment.

Histological analyses of classical Hodgkin's lymphoma cells have also indicated an important role of the cellular microenvironment in the pathogenesis of this B-cell malignancy. The HRS cells usually account for less than 1% of cells in the tumour tissue, and most of the cellular infiltrate is composed of T cells, eosinophils, macrophages, B cells, plasma cells and other cells. Although this cellular infiltrate could partly represent an unsuccessful inflammatory response against the HRS cells, there is evidence that at least a large fraction of the non-tumour cells is actively attracted by the HRS cells.

3.4 Laboratory status and needs

Taken together, lymphoid disorders constitute a heterogeneous group of tumours arising in the lymph nodes, extra nodal tissues, and bone marrow with highly variable clinical features, immunophenotypes and cytogenetics. The diagnosis still represents a challenge to the pathologist since morphological criteria do not always help to distinguish between reactive and malignant lymphoproliferations. Clonality assays by means of flow cytometry and molecular genetics are a useful supplement since monoclonal cell proliferation is a strong evidence for malignancy. The polymerase chain reaction (PCR) can be utilized to establish the clonal origin of B- or T-cell lymphocyte populations by amplification of rearranged immunoglobulin and T-cell receptor (TCR) genes.

The histological heterogeneity of lymphomas reflects their complex and combinatorial nature, which remains poorly elucidated at the molecular level. Some patients respond well to current therapy and have prolonged survival, whereas the remainder succumbs to the disease. This variability in natural history most likely reflects unrecognized molecular heterogeneity in the tumours. Today, new techniques allow tackling this diversity by measuring the mRNA expression level of thousands of genes simultaneously in one sample. Recent publications as summarized above - show the potential of molecular genetics to improve the prognostic classification of diffuse large B cell lymphomas, follicular lymphomas and of Hodgkin's lymphoma, by identifying new tumour classes unrecognised by classical factors.

Thus the molecular classification of lymphomas on the basis of genetic lesions can identify previously undetected and clinically significant subtypes of the disease. The molecular and immunophenotypic characterization of lymphomas at presentation seems to be of crucial importance in diagnosis and sub grouping the lymphomas and for optimal treatment decisions and prognostication in the future.

A further issue of laboratory research in lymphomas is the role of minimal residual disease (MRD) in predicting early recurrence of the disease and long-term prognosis. Techniques of molecular genetics may be sensitive markers of remission

status and have a high prognostic predictive value compared with traditional modalities used in the evaluation of lymphomas. The clinical relevance of the small clonal B-cell population in patients without histological undetectable involvement remains an open question and further research is needed to ensure a role of MRD status in the clinical setting.

In summary molecular analysis can, however, aid in the diagnosis of lymphoid malignancies, can establish a "baseline" for detection of imminent recurrence, and is useful in monitoring therapy. Several data also suggest that it is a tool for the pathologist in cases of discordant morphology and should be strongly considered for each site of disease (15).

Some major issues to be considered are –

3.4.1 Normal and malignant B cell development

Immunoglobulin heavy chain gene rearrangement serves as a marker of clonality and cell lineage in B-cell lymphoproliferative disorders. Functional rearrangement of IgH genes (VDJ) in marrow pre-B cells generate an immature CD19+ cell that exits the marrow as a mature virgin B cell homing to lymph nodes. The subsequent interaction with antigen forces the B cell to enter the germinal centre, where it undergoes somatic hypermutation of its IgH genes and antigen selection of high affinity Ig receptors. Germinal centre B cells undergo isotype switch recombination and leave the lymph node to become either memory B cells or lymphoblasts, with potential to differentiate into long-lived end stage lymphoma cells.

Our working hypothesis is that one or more chemo resistant compartments of low-frequent progenitor cells (memory cell or lymphoblasts) are germ cells responsible for disease progression. Application of such information has thus far been relatively limited. However, a significant number of exciting new findings related to oncogenesis and in vitro models for B lymphopoiesis combined with knowledge about the myeloma cell hierarchy is certain to yield greater knowledge into pathogenesis.

3.4.2 Characterisation of the lymphoma clone and its precursors

Although the predominant cell type is the lymphoblast the initial transformation is considered to take place in a more immature B cell. The presence of IgH somatic hypermutation with intraclonal variations leads to the conclusion that the precursor cell could possible be a CD34/19+ pre-B cell or stem cell, and not a mature, antigen selected and hyper mutated memory B cell. The growth and survival are believed to be regulated by the surrounding lymph node and marrow stromal cells in combination with antigens, cytokines and chemoreceptor ligands. Although not conclusive, the above observations support the hypothesis that high grade NHL is a differentiating malignant process initiated in the germinal centre ending with lymphoma end stage cells migrating to the marrow. Further characterisation of the pregerminal cell is needed.

3.4.3 In vitro models for B lymphopoiesis

From animal studies it is concluded that a normal bone marrow environment support the growth of lymphoma cells. In vitro models have established normal mature B cells from pro-B cells and generated lymphoblasts by additional stimuli. The proposed project will focus on improvement based on analysis of the influence of lymph node stromal cells, dendritic cells as well as selected specific peptide mimotopes as BCR binding "antigens".

3.5 Clinical status and needs

In general, prognosis of malignant lymphoma has only been slightly improved in the last decades and the majority of lymphoma patients are not cured. However, the evolution of new treatment options and improved diagnostic and prognostic tests might be of crucial importance. Some major points in the

management of lymphoma patients have to be improved: i) diagnostic techniques, ii) prognostic stratification, and iii) response evaluation reflecting later outcome.

On the encouraging side, the International Prognostic Index (IPI) was developed 10 years ago, and it has proven a useful measuring stick to assess whether patient groups with aggressive lymphoma are truly comparable in different trials. Molecular markers may be useful in conjunction with the IPI to improve risk stratification in NHL. The importance of precision in the diagnosis and classification of malignant lymphoma deserves emphasis. It is even more important today because of the significance of classification in the Revised European-American Lymphoma (REAL) and updated WHO schemes of malignant lymphoma which is the golden standards in diagnostic evaluation including morphology, immunophenotyping, and cytogenetic testing.

Recently, new techniques such as gene and protein profiling, tests for molecular remission and PET-scan have been extensively investigated and may evolve to standard methods in the clinical management of lymphoma patients. In clinical practice, however, it seems difficult to absorb new information on pathogenesis including genetic and epigenetic alterations observed in the laboratory. One reason is the current lack of a common terminology and accepted strategy when a new test needs to be validated by clinical end points.

Some major issues to be considered are –

3.5.1 The need of validation trials

Exciting new tools are emerging at present and we need to develop innovative ways to use these tools and to design sensible trials that put our ideas to the test. In general, new diagnostic or prognostic tests, moving from the laboratory bench to the clinic, have to pass different stages before they are validated by clinically relevant end points. In parallel with therapeutic studies, four different phases has recently been proposed necessary and informative for clinical validation.

In the *first phase*, a novel laboratory technique is established and analysed for specificity, sensitivity, reproducibility and accuracy. The subsequent *second phase* documents clinical influence by retrospective single centre studies. The *third phase* prepare data from retrospective multicenter studies or from prospective single centre evaluation - evolving into the most important *phase four*, a prospective multicenter evaluation by relevant clinical end-points. Ideally, phases I-III document the usefulness, convincing centres to participate in phase IV validation trial, which however, in many cases, has not yet been performed. It is the intention to use this strategy for translational trials of new diagnostic and prognostic tests.

3.5.2 The essential patient surveillance

An active diagnostic approach and optimal inclusion into clinical trials are necessary to benefit from treatment improvements. It is the experience that such goals can easily be obtained by "Clinical Research Units" fulfilling the need for surveillance of referred patients. The accurate, timely, and tailored information to patients will improve the decision-making about access to clinical trials and quality of care during treatment and follow-up. Education and training courses for clinicians, nurses, scientists and technicians are necessary to optimize the protocol based treatment including collection of clinical data and monitoring in accordance with Good Clinical Practice.

3.5.3 The demand for clinical recommendations and standard care

Ongoing.

3.5.4 The multidisciplinary cancer group in Haematology (DMCG^{Hæm})

Ongoing.

3.5.5 Focus of a regional strategy

Ongoing.

3.5.6 Bioinformatics

Artificial intelligence (AI) is a computer based science which aims to simulate human brain faculties using a computational system. A brief history of this new science goes from the creation of the first artificial neuron in 1943 to the first artificial neural network application to genetic algorithms. The potential for a similar technology in medicine has immediately been identified by scientists and researchers. The possibility to store and process all medical knowledge has made this technology very attractive to assist or even surpass clinicians in reaching a diagnosis. Applications of AI in medicine include devices applied to clinical diagnosis in the range of medical diseases, as well as the use of expert or knowledge-based systems in routine clinical use for diagnosis, therapeutic management and for prognostic evaluation.

Biological applications include genome sequencing or DNA gene expression microarrays, modelling gene networks, analysis and clustering of gene expression data, pattern recognition in DNA and proteins, protein structure prediction. In the field of haematology the first devices based on AI have been applied to the routine laboratory data management. New tools concern the differential diagnosis in specific diseases such as anaemia, thalassemias and leukemias, based on neural networks trained with data from peripheral blood analysis. A revolution in cancer diagnosis, including the diagnosis of haematological malignancies, has been the introduction of the first microarray based and bioinformatic approach for molecular diagnosis: a systematic approach based on the monitoring of simultaneous expression of thousands of genes, proteins and cells using multiparametric technologies, independently of previous biological knowledge, analysed using AI devices. Using profiling, the traditional specialist dependent diagnostic pathways move from clinical to an objective molecular based diagnostic systems.

The first step to be taken will be to store in a sufficient way all data over a well defined period, use of recommended clinical strategies, information of specific and actual given therapy and outcome before these data can be combined and used for artificial intelligence decision making.

4.0 THE NOVEL TECHNOLOGIES IN USE

Understanding the molecular pathogenesis of B cell diseases will allow clinicians to target specific pathogenic pathways and manipulate tumour cell behaviour. Despite the advances in our understanding of the pathogenesis most current prognostic and staging systems utilise relatively simple clinical data. However, a major criticism of these systems is that they rely on surrogate markers for the behaviour of the tumour. Thus they are not capable of defining distinct biological groups and cannot, therefore, be used to define clinically meaningful subgroups nor can they be used to predict response to specific treatments.

Progress in this area relies on recent technological advances, which have enabled researchers to address changes in DNA structure both at a structural and epigenetic level. Together with these advances it is now possible to examine changes in cellular programming in a global fashion by using *gene expression microarrays*. Lagging a little behind these approaches is the technology for *examining the protein complement* of the cell and global *cellular phenotyping*, but this is now rapidly developing and requires full evaluation in the clinical setting.

4.1 Global analytic strategies by bioinformatics

The ultimate scientific aim, which can now be achieved using these technologies, is the development of cellular 'wiring diagrams' of the abnormalities

associated with disease pattern. Once these pathways are understood it will allow us to manipulate the diseased cell in a therapeutically meaningful fashion. The rapid uptake and evaluation of these technologies requires the use of standardised approaches within clinical networks. One potential bottleneck in the evaluation of the data generated is the utilisation of *statistical and bio-informatics approaches* for the analysis of the complicated data sets generated. The application of these novel technologies has to be within the framework of our current understanding of the pathogenesis. The "Bioconductor project" is an open source and open development software project for such analysis and comprehension of global genomic, proteomic, cytomic, and clinical oriented data. Therefore, there is considerable advantage to bring together clinicians, basic scientist and bioinformatic researchers to translate advanced knowledge into the clinical arena. In this setting it should be possible to develop specific diagnostic and prognostic tools applicable to the clinical individual evaluation of the broad range of B cell diseases.

4.2 Genomics

The development in molecular genetics has opened up a new era in biology and medicine. The centrepiece of molecular genetics is the possibility to map and determine the fine structure of human genes and to define in molecular terms how each gene controls all the enzymes of energy metabolism, structural proteins of cells, the membrane proteins, including transport proteins and receptors, the plasma proteins and those proteins which participate in the synthesis of complex lipids, carbohydrates, lipoproteins and glycoproteins.

The fundamental change of genomic research in medicine started in haematology. No wonder, since the circulating blood cells, the bone marrow, and lymphoid tissue cells are easily available for investigation and most of the haematological diseases are well defined entities.

The technologies in use are –

4.1.1 Classical Cytogenetics

This technology invented in the sixtieth has taught us a great deal about the distinct subtypes of B cell disorders and has allowed us to define clinically significant subgroups suitable for targeted treatment.

The routine application of classical *cytogenetics karyotyping* is hampered by the need to generate tumour specific metaphases. Such approaches have been difficult in B cell malignancies because of problems in reliably obtaining metaphases in an indolent tumour where only 1-2 % of cells are in cycle. An abnormal karyotype is present in 30-90% of patients. Progress in this area is hampered by the use of a variety of methodological approaches, the development of effective probes, automation and development of the technique together with effective sharing of data and application of the technology to well defined clinical case series.

4.1.2 FISH (Fluorescent In Situ Hybridisation)

In order to circumvent the need for metaphase analysis fluorescent techniques applicable to interphase cells have been developed. Many of the results obtained with cytogenetics have now been confirmed using FISH. Particularly chromosome deletions are an important independent adverse prognostic factor with an incidence of approximately 50%. Although cytogenetics, *interphase, and metaphase FISH* have different sensitivities for identifying abnormalities of this area (detection rates range from 15%-50% depending on technique used), abnormalities have prognostic significance regardless of the technique

used. FISH has also been used to determine the prognostic value of aneuploidy and showed association with survival.

4.1.3 Other FISH based approaches

In order to improve the detection rate of chromosomal abnormalities the basic FISH method has been adapted in a number of ways. Chromosomal paints are another useful way of identifying both changes in chromosome number and translocations; however the sensitivity of the technique is poor. *Multicolour Spectral Karyotyping (SKY)* is an adaptation of this technique and offers an exciting way to identify both translocation partners and aneuploidy. Using 24 fluorescently labelled 'chromosome paints' simultaneous visualisation of each of the chromosomes in a different colour is accomplished. This technique is more sensitive than normal *G banding karyotyping*; however it does not overcome the problems of obtaining adequate metaphases from patient cases. As each chromosome is labelled in a unique colour, it offers a way of directly visualising translocation partners without designing specific probes to the regions.

4.1.4 Matrix CGH (Comparative Genomic Hybridisation)

Hybridisation of whole chromosome preparations as performed by *comparative genomic hybridisation (CGH)* has proved to be a useful tool for the detection of quantitative genomic alterations. A number of 'hot spots' have been identified including chromosomal gain and loss. The use of *array based solid phase* approaches to detect chromosomal losses or gains have recently been developed *(arrayCGH/aCGH*), with the aim of improving the resolution and definition of the boundaries of the abnormal loci compared to traditional CGH and simplifying the analysis procedure. This is achieved by substituting the target chromosomes with nucleic acid preparations consisting of defined sequences, such as sequence pools representative of whole chromosomes or chromosome arms down to the relevant fragment cloned in a YAC, BAC, and PAC, cosmid or other vectors. The resulting platform consists of a glass slide with immobilised target DNA arrayed in small spots. Using this new aCGH method both direct mapping of aberrations to genomic sequence and low and high copy number gains and losses are readily identified with the minimal size of deletions detectable being approx 100kb. The introduction of methods like these provide the basics for the development of automated diagnostic procedures with biochips with the ultimate aim of developing a CHIP-based technique to detect all of the genetic changes associated with malignancy.

4.1.5 PCR approaches for characterising chromosomal translocations

While the majority of the work characterising translocations involving chromosome 14 has used Southern blotting, given the large amount of DNA required for this test it is not readily applicable to patient material. More practical is *RT-PCR methodology* which can also be used to look for the range of specific known translocations occurring relatively frequently in B cell malignancies.

4.1.6 Epigenetic changes

In addition to gene deletion and mutation as a mechanism for tumour suppressor gene *inactivation by methylation* is also a relevant clinical mechanism. Approximately 40-60% of patients show methylation of p16 at diagnosis, whereas methylation of p15 is less frequent. The presence of p16 hypermethylation correlates with other known poor prognostic factors and with a short progression free and overall survival. The use of methylation sensitive PCR and CHIP based strategies for the detection of gene inactivation, need to be developed specifically for disease subtypes and evaluated within the context of well characterised clinical material.

4.1.7 Gene expression analysis

Perhaps the most important new approach for the sub classification currently under development is the microarray based approach. Expression microarrays produce high density, ordered arrangements of immobilised nucleic acid spots on a solid substrate. These immobile probes are then exposed to the cDNA target that is hybridised to the array, following its synthesis by reverse transcribing tumour mRNA. The data is analysed using sophisticated statistical packages algorithms able to handle the complex data generated. One of the important uses of microarray data is to define global patterns of expression

relevant to the biology and cellular differentiation of normal and malignant plasma cells which may be useful clinically. Although there are several different methods for producing *DNA microarrays*, most researchers use one of two platforms, cDNA or oligonucleotide microarrays.

With cDNA arrays, polymerase chain reaction products of cDNA libraries are robotically spotted onto glass slides. Oligonucleotide microarrays differ as although the oligonucleotide probes can be spotted onto the chip they can also be synthesized directly on the surface of the chip using photolithographic chemistry. Fluorescence analysis of oligonucleotide microarrays uses only one target sample the tumour cDNA as each microarray contains housekeeping genes, allowing comparison following normalization. The most commonly used system (Affymetrix) uses a single colour fluorescent label, where experimental mRNA is enzymatically amplified, biotin-labelled for detection, hybridised to the wafer, and detected through the binding of a fluorescent compound (streptavidin-phycoerythrin).

Microarray experiments require between 10 and 40 microgram of high-quality total RNA. Hence obtaining sufficient RNA for gene microarray analysis from patient samples can be challenging. This has led to the development of methods for amplification of starting RNA. The amplification method needs to be linear and representative, which can be a problem, as some methods tend to over express the smaller segments. Newer methods including the SMART technique and Eisens in-vitro transcription (IVT) method overcome some of these concerns and are now being widely used. Tumour cell heterogeneity can also complicate the interpretation of microarray data and e.g. in myeloma where plasma cells may only constitute 10% of the total nucleated cells, tumour cell selection is required. This can be done in two ways, the first utilises flow sorting and the second magnetic micro-beads selection.

4.1.8 Single Nucleotide polymorphisms

The characterisation of inherited genetic variation and its effect on tumour response and the development of side effects of chemotherapy have become more relevant since the completion of the human genome project. *Single nucleotide polymorphisms (SNPs)* within important genes can alter the function of the gene, particularly if they occur within promotor or functional coding regions. Until recently the detection and characterisation of SNPs was laborious usually involving restriction fragment length polymorphism techniques (RFLP). Real time PCR has facilitated the rapid and accurate detection of SNPs. TaqMan and minor groove binding (MGB) probes are the current optimum approach but other high throughput platforms are being developed. These techniques include single nucleotide extension combined with mass spectrometry detection and microarray based approaches. The exploitation of this technology requires the development of a large clinical resource of characterised clinical material with the power to detect relevant changes, a standardised approach to SNP detection and effective analysis of the data generated

A number of studies have explored the use of these techniques to look at the aetiology and outcome of B cell malignancies. Genetic variants within TNF can affect the pro-inflammatory response and it has been suggested that they may influence the risk of developing cancer and the outcome after treatment. Variants at the GSTP1 locus are however clinically relevant. This is a xenophobic metabolizer gene which metabolizes environmentally encountered alkylating agents and many of the therapeutic agents used in myeloma. Underactive variants of this gene are associated with increased risks of developing secondary leukaemia and can also affect the response to treatment. Although in its infancy this area of pharmacogenetics is likely to be an area of intense activity in the next years.

Progress in this area will depend upon the availability of large banks of DNA from patients on known treatment regimes.

4.2 Proteomics

Proteomics, the investigation of the expressed protein complement of a cell, is expanding rapidly into many aspects of post-genomic biology and medicine. The global qualitative and quantitative analysis of the pattern of proteins expressed at any particular time in any system provides important information about the effects of environmental factors on a genome. Proteins displaying changes in expression levels can be implicated in and linked to changes in cellular state, including alterations in signal transduction pathways, metabolic changes, effects of disease or drug intervention. Proteomics effectively combines the well-established procedures of high-resolution protein separation by 2-dimensional electrophoresis (2D-PAGE) with high-throughput protein identification involving mass spectrometry (MS).

4.2.1 2-dimensional electrophoresis (2D-PAGE)

Typically complex protein mixtures can be resolved by 2D-PAGE to give patterns of up to 1000-2000 protein spots. By utilising various physical or chemical methods to sub-fractionate the source material and a variety of conditions for 2D-PAGE, a much greater total number of proteins can be resolved over a number of gels. Following visualisation of gel spots, by staining with silver or Sypro RUBY, the gel is scanned (using laser densitometer or fluorimager) and a digital image is created and archived. Sophisticated image analysis software can then be used to analyse and compare multiple images to establish a "basic" protein expression profile and identify changes that occur in different situations. Protein spots identified as being of interest due to a change in expression level are then excised from the 2D gels, subjected to proteolysis using trypsin and the peptide digests are analysed by MS.

4.2.2 Mass Spectrometry (MS)

Usually this involves a first pass screen using MALDI/TOF-MS, which measures the mass of each peptide in the mixture and generates a "mass map" unique to the parent protein. This mass map is used to search protein sequence databases, in which each sequence has been digested in-silico to produce theoretical mass maps. Comparison of observed and theoretical mass maps produces a hit list of potential matches for the parent protein. Relating probability scores to the biology then enables confident assignment of protein identity. This approach works very well for organisms that have been subjected to genome sequencing projects, which have led to the deposition of complete sequence information in public domain databases. In other cases, or where the mass maps produced are inadequate (*e.g*. few peptides, heavy post-translational modification.) further MS is required. The second stage, which typically involves QTof-MS/MS, aims to generate partial sequences for each of the peptides in the mixture. These "sequence tags" provide additional information to enhance database searches and enable protein identification, or where still unsuccessful provide de-novo sequence information for future use in homology searches and cloning.

Currently this technology is time consuming, cumbersome and extremely expensive. New high-throughput technology is becoming available with robotics facilities for sample manipulation. In conjunction with this bioinformatic techniques are also improving including sophisticated image analysis software allowing for quicker interrogation of the peptide databases. Another approach in its infancy is protein chips.

4.2.3 Protein microarrays

Proteins can be arrayed either on flat solid phases or in capillary systems and binding can be covalent (via chemical linkers) or non-covalent (via hydrophobic, ionic or other interactions). As with traditional proteomic techniques charge, viscosity, membrane pore size, pH and binding capacity play an essential role. Detection is similar to cDNA array methods using fluorescent intensity. Although in its infancy these methods build on the

DNA microarray technology and represent the next level of diagnostic tools allowing direct analysis of gene function.

Protein microarray technology is still in its infancy and only a few systems have been described. Since protein chips technology is relatively new, they have not generated the attention bestowed upon conventional proteome approaches. The surface-enhanced laser desorption ionization (SELDI) ProteinChip array (Ciphergen, Fremont, CA, USA) is one approach to array-based proteomics. The protein sample is introduced onto chips that selectively bind hydrophobic, hydrophilic, basic, and acidic proteins. Non-specifically bound species are removed and the remaining proteins detected by laser desorption/ionization time-of-flight mass analysis. Proteome samples from normal and diseased cells have been analyzed using the same type of chip and differences in the mass spectra identified.

Differential mass patterns have been observed between samples, which require additional MS analyses for protein identification. Specific proteins (such as an antibody) have been bound to the surface of the SELDI chip and used to capture interacting proteins based on their binding properties

In non–SELDI-based chip based approaches, more than 10,000 protein spots can be arrayed on a single slide through covalent attachment by primary amine derivitation. By fixing one protein to the slide and probing with its fluorescently labelled partner, binding or interacting proteins can be detected and quantified. The shelf life of the array and the percentage of proteins that retain their native structure on an array are under investigation. A global proteomic study utilizing this method requires a massive protein expression and purification effort, which is underway in commercial and academic institutions.

4.3 Cytomics

Immunophenotyping by multiparametric flow cytometry (MFC) is a still evolving technology, which allows the collection of multiple parameter data on a large number of individual cells. Multicolour labelling of haematopoietic lymphoid cells allows a complex analysis of cellular compartments and provides an accurate image of the cellular stages of differentiation. The method is complicated by the difficulty in comprehending the complete numerical description of the results. Usually, flow cytometry results are simplified and expressed for diagnosis in a qualitative or semi-quantitative manner.

However, in large series of patients it is possible to construct and organize the results in a way that allow bio-statistical data analysis according to a clustering strategy based on ascendant ordering. Such analysis allows recognition of molecules (CD markers) that are co-expressed on distinct subsets, as well as groups of patients with similar immunophenotypic profiles. Furthermore, the analysis may define up- and down-regulated markers. It is expected that such results generated and extended to cover the hierarchy model of differentiation may identify patients in a manner, which will open new perspectives for MFC diagnosis, staging and prognostic classification.

4.3.1 Diagnosis by immunophenotyping of lymph node biopsies.

Only the most recent lymphoma classification systems (REAL/WHO) incorporate immunophenotypic features in the diagnostic criteria for lymphoma subgroups. Studies published before 1997 are therefore not included in the summary below.

Evaluation of the diagnostic performance of MFC immunophenotyping in B cell malignancies is hampered by the variability in antibody combinations and diagnostic criteria A minimum number of 10-20 antibodies is recommended, and only one study seems to have exceeded that number, probably due to small sample sizes (few cells) and the

limited use of 4-color combinations. Few studies use gating strategies beyond FSC/SSC lymphocyte gating, which obviously limits the ability to detect small populations of abnormal cells and the cut-off limits for kappa/lambda ratio vary by 300% among recent studies.

For adequate samples, the diagnostic sensitivity for ML versus a benign state varies from ca. 77% to 99% (for MFC and cytology combined). Correct sub classification is reported in 50-60% of cases, based on panels of 14 antibodies. An excellent introduction to the application of flow cytometry in the differential diagnosis can be found in a review by Weisberger et al (2000). A description of subgroup-specific patterns of phenotypic aberrations in chronic B-cell malignancies has been published by Sanchez (2002) and in T-cell lymphomas by Jamal (2001).

4.3.2 Detection of lymphoma infiltration in bone marrow

Flow cytometry immunophenotyping of BM aspirate is a more sensitive method for detecting lymphoma infiltration than histopathological examination of BM core biopsy material (excepting tumours with a focal or paratrabecular growth pattern, such as FL), mainly because light chain restriction in a small population is easier to detect. However, the clinical significance of BM minimal disease is still unclear, and staging and risk stratification remain based on microscopic BMI until the prognostic impact of FC BMI is clarified.

4.3.3 Diagnostic criteria for monoclonality in B-lymphocytes

Mature B-cell lymphomas usually express surface immunoglobulins and exhibit light chain restriction. The monoclonal cells uniformly express either kappa or lambda chains, or none of these. The presence of a monoclonal population is diagnosed when the kappa/lambda ratio is skewed in either the entire B-cell population, in an abnormal B-cell subpopulation, or when a major subpopulation completely lacks sIg-expression. The cut-off limits for kappa/lambda ratio given in the literature are widely diverging, from 6-0 to 3-0.5, the latter being most frequently cited in recent studies. Most malignant samples exceed these limits by at least factor, but even small populations of malignant cells can be reliably detected without false positives when cut-off limits are not too strict.

4.3.4 Cellular interactions in lymphatic germinal centres.

All studies cited in this paragraph are based on immunohistochemical analysis of biopsy material as no flowcytometri studies of stromal elements in lymph node or bone marrow have been identified. It is therefore uncertain to which extent stromal cells can be identified by flow cytometry within the CD45 negative compartment in aspirated material from LN or BM. A number of membrane-bound molecules involved in chemotaxis, apoptosis and other cell-to-cell interactions have been studied in FL. The commercial availability of fluorochrome-conjugated antibodies against these antigens is a limiting factor for the application of flow cytometry. Results of potential interest in the context of this study are outlined below.

Like in normal germinal centres, the malignant B-cells, at least in early FL, maintain a close association with intrafollicular T-cells and specialized antigen-presenting follicular dendritic cells (FDC) which mediate proliferation and prolonged survival. Fully differentiated FDC are characterized by membrane expression of CD21, CD23, CD35, and the chemokine and growth factor receptors, CXCR13 and LNFGR. It is unclear whether these cells are adventitial reticular cells of bone marrow origin, partially differentiated into nodal-type FDC or rather represent recruitment of nodal or circulating FDC.

Interestingly, the presence of dispersed neoplastic B-cells in the extra follicular areas of FL is associated with an increased expression of LNGFR in adjacent fibroblastic stroma, but without signs of FDC differentiation. These cells, that are not found in areas with diffuse growth but dispersed between follicles, are clonally related to the intrafollicular malignant cells, but have a resting PGC memory B-cell phenotype with down regulation of CD10, CD38, CD95, CD80, and CD86 compared to IF cells, and a low proliferation activity. They were found to recirculate between the EF and IF compartment. Searching for a subpopulation of chemo resistant malignant cells, these cells would seem to be a likely candidate.

4.3.5 Immunophenotyping profile in diffuse large B cell lymphoma

Immunophenotyping profiles can be distinguished according to the pattern of differentiation: germinal center-CD10$^+$ (GC-CD10$^+$; CD10$^+$/Bcl-6$^+$/MUM1$^-$/CD138$^-$), germinal center-CD10$^-$ (GC-CD10$^-$; CD10$^-$/Bcl-6$^+$/ MUM1$^-$/CD138$^-$), post-germinal center (pGC; CD10$^-$/bcl-6$^\pm$/ MUM1$^+$/CD138$^-$), and plasmablastic (CD10$^-$/bcl-6$^-$/MUM1$^+$/CD138$^+$) (28). The pGC profile has been associated with primary nodal presentation and immunoblastic morphology, whereas GC-CD10$^+$ tumours often presents with disseminated disease, centroblastic morphology, bcl-2 rearrangement, and lower Ki-67 proliferative index. GC-CD10$^-$ patients more often present with primary extra nodal origin, early stage, normal lactic acid dehydrogenase (LDH) levels, and low or low/intermediate International Prognostic Index (IPI) scores. However, no significant difference has been found in terms of response or survival (OS) according to these profiles.

5.0 MATERIAL, METHODOLOGY AND PROTOCOLS

At diagnosis and following therapy, patient samples are collected from lymph node, other tumour biopsies, bone marrow and blood aspirates for storage of frozen cells, DNA/RNA and plasma.

The statistical analysis of clinical and analytic data will be performed as described below and include descriptive statistics, survival analysis by Kaplan Meyer estimates and regression analysis.

5.1 The methodologies and competences

The methods used in the laboratory research programme are described in references and includes:

5.1.1 Storage of cells, DNA, RNA and tissue fluids in the haematology biobank
Coordinator: L Pilgaard, TL Andersson and HE Johnsen

5.1.2 Multiparametric flow cytometry for identification and sorting of lymphoid subsets inclusive single cell.
Coordinator: A Schmitz, L Pilgaard and HE Johnsen

5.1.3 Single whole cell RT-PCR & limiting dilution for estimation of low frequent cells and single cell cDNA archives.
Coordinator: L Pilgaard, KBH Fogd; KD Sorensen and HE Johnsen

5.1.4 Identification of clonal IgH gene rearrangement and design of ASO primers. Real time Q-PCR based on a patient specific probe and primers.
Coordinator: HE Johnsen and KD Sorensen

5.1.5 Gene expression analysis by cDNA chip array (Affymetrix platform).
Coordinator: M Nyegaard and KD Sorensen

5.1.6 Protein expression analysis by Protein chip array (Ciphergen Biosystems).
Coordinator: TL Andersson and HE Johnsen

5.1.7 Transfection of cell lines by pCMS-EGFP mammalian expression vectors.
Coordinator: KD Sorensen

5.1.8 M component phage panning and mimotope design.
Coordinator: HE Johnsen and KBH Fogd

5.1.9 Cell line and in vitro B cell differentiation model.
Coordinator: KBH Fogd and HE Johnsen

5.1.10 Lymphoma mice model.
Coordinator: KD Sorensen

5.2 Translational protocol status (Project descriptions=protocols are available)

Recently granted translational projects:

5.2.1 The impact of stepwise oncogenesis and the cellular hierarchy in pathogenesis and the disease heterogeneity of malignant lymphoma.
Coordinator: HE Johnsen & Anne Bukh. Granted by KE Jensen Foundation 2005

5.2.2 From mice to man.
i) Analysis of retroviral induced murine lymphoma and immunocytoma as a model for identification of new pathogenetic pathways.
ii) MiRNA in B cell malignancies with focus on disease progression.
iii) Analysis of oncogenesis in T cell lymphomas.
Coordinator: K Dybkaer. Granted by KE Jensen Foundation 2006

5.2.3 Normal and malignant B cell profiling:
i) Analysis of mRNA and miRNA expression profiles in biopsies from DLBCL;
ii) Retrospective analysis of chemosensitivity profile in DLBCL and MM;
iii) Study of hereditary and acquired GEP variables in DLBCL;
Coordinator: K dybkaer. Granted by KE Jensen Foundation 2006

5.2.4 A translational programme identifying and targeting the myeloma stem cell. Myeloma Stem Cell Network (MSCNET).
Coordinator: HE Johnsen. Granted by EU 2006-09

5.2.5 New prognostic evaluation and intervention in monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). The Danish Myeloma Study Group (DMSG)
Coordinator: HE Johnsen. Granted by Danish Research Agency 2006-09

5.2.6 Implementation of routine molecular assays for individual therapeutic strategies in malignant B cell diseases.
Coordinator: HE Johnsen & P Hokland. Granted by ISM 2006-09

5.2.7 Blast cell heterogeneity detected by multiparametric flow cytometry and gene expression profiling is an adverse prognostic factor in intermediate-risk AML (2002-2006)
Coordinator HE Johnsen & MH Hoffmann, Granted by KE Jensen Foundation 2009

5.2.8 Identification and quantitation of the progenitor/stem cell pool in AML by Flow Cytometry at diagnosis and following therapy: Implication for Tailored Therapy and Graft Quality.
Coordinator: HE Johnsen& OJ Bergmann: Granted by the Danish Cancer Society 2005-07.

5.2.9 Bronchoalveolar lavage (BAL) analysis for infectious agents, inflammatory cytokines and impact on aetiology and prognosis in patients with antibiotic resistant pulmonary infiltration following chemotherapy for haematological malignancies. 1999-2009
Coordinator: OJ Bergmann OJ, R Dessau, G Lisby and HE Johnsen. Private Grants.

5.2.10 Chemotherapy prediction (CHEPRE) in malignant lymphoma by genomic signature for drug sensitivity – from clinical guidance to pathogenetic understanding
Coordinator: K Dybkaer and HE Johnsen
Granted by the Danish Research Council 2008-2010

5.3 Clinical protocol status **(Project description=protocols are available)**

Participation in multicenter treatment protocols, activated or in processing:

5.3.1 "Biobank Haematology Aalborg-Aarhus"
Principal Investigators: HE Johnsen & P Hokland
February 2007 – 2009 (follow up and ongoing)

5.3.2 Chemosensitivity index based on DNA chip analysis in all newly diagnosed patients with malignant haematological diseases (local protocol CHEPRE).
Principal Investigator: A Bukh and HE Johnsen
2008 - 2009

5.3.3 A prospective randomized trial of second line therapy in lymphoma patients comparing outcome of conventional with individual DNA chip based experimental chemotherapy (CHEPRE).
Principal Investigators: A Bukh and HE Johnsen
Protocol in preparation – to start 2009-2010

5.3.4 3rd Nordic Mantle Cell Lymphoma Phase II Protocol. High-dose therapy with autologous stem cell support in first line treatment of mantle cell lymphoma – 90y-Ibritumomab Tiuxetan in combination with BEAM or BEAC to improve outcome for patients not in CR after induction treatment.
Principal Investigators: G Thorsell, I Christiansen
2007 – 2009 (follow-up)

5.3.5 AIHA. Rituximab added to Prednisolone in patients with warm antibody mediated autoimmune haemolytic anaemia.
Principal Investigator: I Helleberg
June 2005 - ongoing

5.3.6 AML 15. A randomised controlled Phase III trial for patients with AML/APL.
Principal Investigators: HE Johnsen and NN
2006 – 2009 (follow-up)

5.3.7 AML 16. A Trial for Older Patients with Acute Myeloid Leukaemia and High Risk Myelodysplastic Syndrome.
Principal Investigators: HE Johnsen and NN
2009 -

5.3.8 BDR Waldenström trial. Phase II Study of Combination Bortezomib, Dexamethasone, and Rituximab in previously untreated Patients with Waldenstrom's Macroglobulinemia: A multicenter Trial of the European Myeloma Network.
Principal Investigators: HE Johnsen and I Petruskevicius
2009 -

5.3.9 Bortezomib-HD-melphalan in relapse. NMSG #16/07. Phase II study of bortezomib-dexametasone and high-dose melphalan in patients relapsing after high-dose melphalan with autologous stem cell support.
Principal Investigator: H Gregersen
2007 – 2009 (follow-up)

5.3.10 CLL-8/ML17102 Protocol of the GCLLSG. A Phase III trial of combined immuno- chemotherapy with Fludarabine (F), Cyclophosphamide (C) and Rituximab (FC-R) versus chemotherapy with Fludarabine and Cyclophosphamide (FC) alone in patients with previously untreated chronic lymphocytic leukaemia.
Principal Investigators: I Christiansen
2005 - 2006 (follow-up)

5.3.11 FLUDARABINE NMSG #13/03. Fludarabine added to Induction Treatment in Untreated Multiple Myeloma Patients: A randomised, placebo controlled, double blind Phase II Trial.
Principal Investigator: HE Johnsen & H Gregersen
2005 – 2006 (Closed and published)

5.3.12 HOVON 68. A randomized phase III study in previously untreated patients with biological high-risk CLL: Fludarabine + cyclophosphamide (FC) versus FC + low-dose alemtuzumab
Principal Investigators: I Christiansen, E Andersen, A Kristensen
January 2007 -

5.3.13 HOVON 84 NHL. Randomised phase III study on the effect of early intensification of rituximab in combination with 2-weekly CHOP chemotherapy followed by rituximab maintenance in elderly patients (66-80 years) with diffuse large B-cell lymphoma.
Principal Investigator: A Bukh, G Thorsell, T El-Galaly
2009 -

5.3.14 IMPACT. A Retrospective and Prospective Observational Study reviewing Supportive Care Management of NHL Patients treated with CHOP-14 or 21 (with or without Rituximab).
Principal Investigators: HE Johnsen & J Madsen
2007 – 2008 (follow-up)

5.3.15 ITP. A randomised Phase III Study of the Efficacy of High-dose Dexametasone versus High-dose Dexametasone combined with Rituximab in patients with untreated Idiopathic Thrombocytopenic Purpura (ITP).
Principal Investigator: I Helleberg
May 2005 -

5.3.16 Kepivance. Amgen Protocol No. 20050219 entitled "A Double-Blind, Randomised, Placebo-controlled Study of Two Different Schedules of Palifermin (Pre- and Post Chemotherapy and Pre-Chemotherapy only) for Reduction in Severity of Oral Mucositis in Subjects with Multiple Myeloma (MM) Receiving High Dose Melphalan followed by Autologous Peripheral Blood Stem Cell Transplantation (PBSCT)"
Principal Investigators: I Christiansen & HE Johnsen
2007 – 2008 (follow-up)

5.3.17 MDS/AML Lenalidomide NMDSG07A. A multicenter phase II study of the efficacy and safety of lenalidomide in high-risk myeloid disease (high-risk MDS and AML) with karyotype including del(5q) or monosomy 5.
Principal Investigator: HE Johnsen
2008 -

5.3.18 PRIMA - "Primary Rituximab and Maintenance". A multicentre, phase III, open-label, randomized study in patients with advanced follicular lymphoma evaluating the benefit of maintenance therapy with Rituximab (MabThera®) after induction of response with chemotherapy plus Rituximab in comparison with no maintenance therapy
Principal Investigator: A Bukh
2005 - 2007 (follow-up)

5.3.19 Revlimide/Lenalidomide. A Phase III, Multicentre, Randomised, Double-Blind, Placebo-Controlled, 3-Arm Parallel Group Study to Determine the Efficacy and Safety of Lenalidomide (Revlimid®) in Combination with Melphalan and Prednisone versus Placebo Plus Melphalan and Prednisone in Subjects with Newly Diagnosed Multiple Myeloma who are 65 Years of Age or older.
Principal Investigators: H Gregersen & HE Johnsen
2007 – 2008 (follow-up)

5.3.20 Thalidomid vs. Velcade in patients with melphalan refractory multiple myeloma. NMSG #17/07.
Principal Investigator: H Gregersen
2008 -

5.3.21 VELCADE NMSG#15/05. Bortezomib consolidation in patients with myeloma following treatment with high-dose melphalan and autologous stem cell support. A randomised NMSG trial.
Principal Investigators: H Gregersen, I Christiansen, I Petruskevicius
2006 – 2009 (follow-up)

5.4 PhD programme ongoing (Project description=protocols are available)

5.4.1 "Identification, quantitation of blood circulating mesenchymal progenitors following bone fracture and regeneration" 2004-7
PhD student H Gottlieb; Supervisor HE Johnsen & J Kastrup

5.4.2 "MicroRNA targets in Diffuse Large B-Cell Lymphoma: evaluation by an experimental approach including gene expression profiling following knockdown by RNAi" 2006-9
PhD student: Anders Petersen; Supervisor K Dybkaer

5.4.3 "Identification and characterization of microRNA profiles in Diffuse Large B-cell lymphomas of nodular and extra nodular manifestations" 2006-9
PhD student: Charlotte Mandrup Petersen; Supervisor K Dybkaer

5.4.4 "Inflammatory cytokine polymorphism in malignant B cell diseases." 2006-10
PhD student KR Nielsen; Supervisor HE Johnsen & J Baech/R Steffensen and K Overvad

5.4.5 "Stem cell derived transcription factors in normal and malignant lymphopoeises with focus on germinal centre B-cells" 2008-2010
PhD student: Cand Scient Humanbiolog Malene Krag Pedersen; Supervisor K Dybkaer

5.4.6 "The role of miRNA and AID in B-cell malignancies" 2009-2011
PhD student: Maria Bach Laursen, M.Sc. Molecular Biology; Supervisor K Dybkaer

5.4.7 "The role of PAX5, BCL6, and PRDM1 isoforms in B cell differentiation and malignancies" 2009-2011
PhD student: Maria Bro Kloster MSc Medical biotechnology; Supervisor K Dybkaer

5.4.8 "Studies of low number sorted B cells by array technology - In search of stem cell associated genes in B cell malignancies"
PhD student: Kim Steve Bergkvist MSc.Medical biotechnology; Supervisor HE Johnsen Linda Pilgaard

5.4.9 "Resistance to melphalan in B cell malignancies studied by protein array technology" PhD student: Torben L Andersson Cand Scient; Supervisor HE Johnsen and Kirsten Fogd

5.4.10 "Impact of immune phenotyping by multiparametric flow cytometry on diagnosis, prognosis and therapy outcome" 2010-12
PhD student: NN; Supervisor HE Johnsen and Alexander Schmitz

5.5 Scholarships ongoing (Project description=protocols are available)

5.5.1 "Identification of useful housekeeping miRNAs for real time Q-RT,PCR" 2009 (ongoing)
Scholar: Stud Med K Quistgaard; Supervisor KD Sorensen

5.5.2 "In silico analysis of cell lines – in search of cancer stem cell genes" 2007 (ongoing)
Scholar Stud Med TU Jakobsen (now cand med); Supervisor HE Johnsen

5.5.3 The role of SOX4 in differentiation and malignant B-cell disorders. 2009-10
Scholar Stud Biotechnology Alice Østergaard; Supervisor PhD student Malene Krag Pedersen

5.5.4 "Predictors of melphalan chemosensitivity in multiple myeloma" 2009-10
Scholar: Stud Med Johanne Marie Holst; Supervisor Senior Scientist M Boegsted

5.5.5 "Cell line models for chemoresistance" 2009-10
Scholar: Stud Stat S Steffen Falgreen Larsen; Supervisor Senior Scientist M Boegsted

5.6 Summer Students (Project description=protocols are available)

This summer four medical students have been employed as research assistants to contribute to the ongoing research at the Department of Haematology. Common for all projects are the use of Affymetrix gene expression data as well as B-cell differentiation:

5.6.1 Jakob Virenfeldt (June 23 – September 15) on haematopoietic differentiation and their involvement in lymphomagenesis

5.6.2 Katrine Gade (May 15 – September 30) on characterization of B-cell markers by flow cytometry and gene expression analysis of haematopoietic cell lines

5.6.3 Sofie Hallager (June 23 – December 31) on analyzing and comparing gene expression in embryonic stem cells, B-cell lines, normal B-cells and normal tissue cells

5.6.4 Jonas Svenningsen (June 23 – August 21) on identification of disease specific genes in haematological cancers by in silico analysis with the corresponding cell lines.

6.0 PRACTICAL CONDITIONS

A well-educated staff and necessary technologies are available in the research laboratory the Department of Haematology at Aalborg Hospital Science and Innovation Center AHSIC University of Aarhus, Aalborg, Denmark. This programme is an attempt to establish a regional strategy based on national and international collaborations and integration between laboratory science and clinical lymphoma researchers.

6.1 The actual and future scientific staff

The actual ongoing research programme involves 1 clinical professor, 1 associate professor, 2 senior scientists, 3 post docs, 8 PhD students and 5 research technicians as well as 3 research nurses, 2 secretary assistants and 1 data manager/bioinformatician as well as 1 statistician.

The staff needs to be extended in the proposed programme as listed below with a short description of the working areas:

6.1.1 One Cand Scient, post doc with experience in and responsible for running the multiparametric flow cytometer and sorting.

6.1.2 Two junior fellows per year (cand med) responsible for clinical implementation protocols.

6.1.3 One technician responsible for i) cell culturing, ii) multiparametric flow cytometry and iii) cell sorting.

6.1.4 One research nurses responsible for patient information, monitoring and data sampling.

6.1.5 Running costs will cover purchase of monoclonal antibodies, primers and probes, cDNA and protein chips, and culture flasks, medium including recombinant cytokines.

7.0 ORGANIZATION AND MANAGEMENT

Scientists and clinicians in the field will be involved from the research laboratories associated to the Department of Haematology at Aalborg Hospital. The project will be based on clinical and laboratory protocols. The protocols will be coordinated by the Research Secretariat. An important perspective is to extend both the clinical and laboratory research by collaboration with other departments. The main emphasis of collaboration will primarily take place by an active participation in the Danish Leukemia, Lymphoma and Myeloma Group. Data registration will continue through the "national clinical databases" as well as by associated protocol databases.

The intention with the local research database is to obtain further data needed in more complicated research and to ensure that the quality of treatment is at an international standard. The local statistician will supervise data entry and statistical analysis. The Lymphoma Biobank currently exists on subgroups of patients and collection of biological samples needs to be extended to all lymphoma patients. The department of Haematology at Aalborg Hospital covers region North Jutland. It is, therefore, possible to obtain a population-based consecutive material.

8.0 THE PROPOSED BUDGET, MILESTONES AND TIME PLAN

The budget for the different activities is within the 50% of the sum of the participating partner's budget. The total annual budget estimate is 10 mills DKK for running costs (including salaries), education and training, management and promotion of results.

An annual report and budget is prepared and published 1. September.

9.0 DISSIMINATION OF KNOWLEDGE

Results from the projects will be disseminated through:

9.1 Reviews in scientific journals;

9.2 Publication, disseminating results to a large audience through the journals where articles are published;

9.3 Conferences and workshops, at which the results will be published to a large audience;

9.4 Annual progress reports and public web site www.blodet.dk

10.0 ETHICAL ASPECTS

All clinical studies and laboratory protocols are or will be approved by the ethics committee system including Danish Medicines Agency. All studies will be in accordance with the Helsinki II declaration.

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