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A study of inflammatory cytokine gene polymorphisms in B-cell diseases

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A STUDY OF INFLAMMATORY CYTOKINE GENE POLYMORPHISMS IN B-CELL DISEASES

BY
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PREFACE

This Ph.D. thesis is submitted for evaluation to the Faculty of Medicine at Aalborg University with the purpose of attaining the degree of Doctor of Philosophy. The Ph.D. study was initiated during my training period to become a specialist in Clinical Immunology.

I owe great thanks to my supervisors for support, inspiration and guidance through all aspects of the process. I also thank my employer Kim Varming, for support and valuable input during the project time. The important work of laboratory technicians Birgitte Busk Yssing, Hanne Søndergaard, Lene Funder Hjortshøj, Line Jørgensen and Mariann Møller were essential for completing the laboratory analysis which this thesis is based on.

My deepest and special thanks go to my colleges John Bæch and Rudi Steffensen for essential support, not only as academically mentors but also as good friends.

I would like to thank all my co-authors on the papers on which this thesis is based for their contribution and their valuable expertise. I also thank all my colleagues at the Department of Clinical Immunology not only for outstanding technical and laboratory training and assistance but also for your spirit and the always positive atmosphere – I enjoy every day that I go to work because of you.

CV

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SUMMARY

This thesis consists of four studies examining inherited variants in cytokine genes and their pathogenetic impact in rheumatoid arthritis (RA), multiple myeloma (MM) and B-cell non-Hodgkin's lymphoma (B-NHL).

In the first study, We established a method to investigate functional variants in the *CHI3L1* gene, encoding the pro-inflammatory YKL-40 protein. We genotyped eight single nucleotide polymorphisms (SNPs) in a cohort of 308 RA patients and a 605 healthy controls (HC) and subsequently measured serum levels of YKL-40. We found strong association between serum YKL-40 and the *CHI3L1* -131 C/G (rs4950928) SNP in RA (p=2.4e-08) and HC (p<2.2e-16). We did however not detect association between genotypes and risk of RA. In the second study, We expanded the search for pathogenetic SNPs. The impact of thirteen SNPs was investigated in a cohort of 348 MM patients and 355 HC. We discovered an association between the *TNFA* -238A (rs361525) allele and decreased risk of MM (OR=0.51 (0.29-0.86)). Our findings also suggested a prognostic effect of *TNFA* and *IL10* genotypes, whereas we did not find evidence for an effect of the *CHI3L1* (rs4950928) SNP. Cytokines interact in complex networks, and we aimed to establish a model for studying gene-gene interactions. Our power to study such interactions were limited due to the size of our cohorts, however we suggest a prognostic effect of interactions between *IL6* and *IL10* and *TNFA* and *IL4*. To include a functional approach, we determined the expression of such interacting genes in normal and malignant B-cells. The expression pattern suggests that these genes play a role in different stages of the pathogenetic process, and we were able to use *CHI3L1*, *IL6* and *IL10* expression to predict prognosis, according to the TC classification, in a retrospective dataset of 414 MM patients. In the third study we included 50 SNPs in our screening. Selection of SNPs reflected the findings from the two first studies and recent genome wide association studies (GWAS). Genotyping was performed on a custom designed SNP array, and we developed a method for DNA extraction from the formalin fixed bone marrow samples we had available. 355 B-NHL patients and 307 HC were included. We confirmed associations from recent GWAS and proposed novel associations. Amongst notable findings, the *CHI3L1* (rs4950928) was related to outcome (HR$_{CG}$ = 2.04 (1.17-3.54)) in follicular lymphoma. Interaction was observed between IL-4 and IL-10 related genes and subsequently explored on gene expression level using normal B-cell subpopulations and lymphoma cells. The expression pattern of *IL10*, *IL10RB* and *IL4R* was indicative of an effect in the stepwise oncogenesis believed to precede B-cell lymphomas. In light of these complex findings and the vast amount of studies published in parallel with our studies being carried out, we decided to thoroughly review the literature and described studies of genes associated with lymphoma risk

and prognosis as reported in the fourth study. We used this as a platform for a discussion of the need for new candidate gene studies, whole genome studies and suggested that studies should be consortium driven to ensure statistical power in order to investigate gene-gene interactions. We also suggest, that the functional aspect of such proposed pathogenetic genes are studied. We have taken the first steps towards designing model systems for the investigation of in vivo as well as in vitro function of these proposed pathogenetic genes and we believe that this combined approach in the near future will bring us a more detailed knowledge of the complex biology in B-cell diseases eventually leading to a more rational prognostic classification, improved treatment strategies and the possibility of real personalized medicine.

RESUME (DANSK)

Afhandlingen består af fire studier som undersøger udvalgte nedarvede varianter i gener for inflammatoriske cytokiner og deres patogenetiske betydning ved rheumatoid artritis (RA), myelomatose (MM) og B-celle non-Hodgkin's lymfom (B-NHL).

I det første studie etablerede vi metoden til genotypning af single nucleotide polymorphisms, (SNP's) og tog derefter udgangspunkt i *CHI3L1* genet, der koder for det pro-inflammatoriske protein YKL-40. Vi genotypede 8 SNPs i *CHI3L1* genet i en kohorte af 308 RA patienter og 605 raske kontroller. I undersøgelsen målte vi samtidig serumniveauer af YKL-40 og fandt disse stærkt associeret til *CHI3L1* -131C/G (rs4950928) i RA (p=2.4e-08) og i kontroller (p<2.2e-16). Vi kunne ikke påvise en sammenhæng mellem genotyper og risiko for RA. I det andet studie udvalgte vi i alt tretten funktionelle SNP's. Vi genotypede 348 MM patienter og 355 kontroller og fandt en association mellem *TNFA* -238A (rs361525) og nedsat sygdomsrisiko (OR=0.51 (0.29-0.86)). Vi kunne desuden sandsynliggøre en sammenhæng mellem *TNFA* og *IL10* genotyper og sygdommens prognose. Inflammatoriske cytokiner interagerer i netværk, og vi etablerede derfor en model til at studere gen-gen interaktioner. Med de begrænsninger vi havde i form af et relativ lille patient materiale, kunne vi vise en mulig sammenhæng mellem prognose og interaktion mellem *IL6* og *IL10* samt *TNFA* og *IL4* genotyper. Vi undersøgte derefter ekspression af de pågældende gener i normale B-celler samt i MM celler og fandt et ekspressionsmønster som antyder en betydning af disse gener ved udvikling af den maligne celleklon. Vi inkluderede yderligere et retrospektivt datasæt, og kunne vise at *CHI3L1*, *IL6* og *IL10* ekspression er prognostisk ud fra TC klassifikationen. I det tredje studie genotypede vi 355 B-NHL patienter og 307 kontroller for 50 SNPs. Vi etablerede først en metode til at oprense DNA fra formalinfikseret væv og designede derefter en custom SNP array til genotypning. De udvalgte SNPs afspejlede resultater fra de 2 første studier, og vi inkluderede yderligere SNPs ud fra genome wide association studies (GWAS). Vi bekræftede tidligere påviste sammenhænge mellem såvel sygdomsrisiko og overlevelse og antydede en ny sammenhæng mellem *CHI3L1* (rs4950928) og follikulært lymfom (HR_{CG} = 2.04 (1.17-3.54)). Gen-gen interaktions analyse viste en mulig kombineret effekt af gener af betydning for IL-4 og IL-10 og disse interaktioner blev efterfølgende undersøgt på gen ekspressions niveau med fund af forskellig ekspression af *IL10*, *IL10RB* og *IL4R* mellem diskrete B-celle subpopulationer og lymfomceller. Dette fund antyder en effekt af disse gener i den trinvise onkongenese, man formoder sker i udvikling af disse kræftceller. Baseret på det komplekse billede vi observerede i vores studier og den litteratur, der sideløbende er publiceret, foretog vi en litteraturgennemgang og beskrev i det fjerde studie den

foreliggende litteratur vedrørende gener associeret til sygdomsrisiko og overlevelse. Vi diskuterede behovet for nye consortium drevne studier med den nødvendige statiske power til såvel kandidatgen, helgenom og gen-gen interaktioner. Vi argumenterede for at genetisk screening for nedarvede patogenetiske varianter medfører, at disse efterfølgende undersøges funktionelt i såvel in vivo som in vitro modelsystemer. Målet er at erhverve ny biologisk viden om B-celle sygdommene, og vi arbejder allerede nu med modeller for såvel in vitro og in vivo funktionelle aspekter af disse genetiske variationer. Perspektivet er at denne viden kan anvendes til at designe ny prognostiske systemer samt til at skrædderisy behandlingen for den enkelte patient og dermed opnå egentlig "personalized medicine".

PUBLICATIONS

This Ph.D. thesis is based on four manuscripts aimed for publishing in international peer-reviewed journals. Paper I and IV are published. Paper III is accepted for publications. Paper II is under review.

PAPER I

Promoter polymorphisms in the chitinase 3-like 1 gene influence the serum concentration of YKL-40 in Danish patients with rheumatoid arthritis and in healthy subjects. Nielsen KR, Steffensen R, Boegsted M, Baech J, Lundbye-Christensen S, Hetland ML, Krintel SB, Johnsen HE, Nyegaard M, Johansen JS. *Arthritis Res Ther. 2011 Jun 29;13*

PAPER II

Interactions between inherited inflammatory response genes are associated with multiple myeloma disease risk and survival. Nielsen KR, Rodrigo-Domingo M, Steffensen R, Baech J, Bergkvist KS, Haunstrup TM, Oosterhof L, Schmitz A, Bødker JS, Johansen P, Dybkær K, Bøgsted M, Vogel U, Johnsen HE, Vangsted A. *Under review in Cancer Genetics*

PAPER III

Inherited inflammatory response genes are associated with B-cell non hodgkins lymphoma risk and survival. Nielsen KR, Steffensen R, Bendtsen MD, Rodrigo-Domingo M, Baech J, Haunstrup TM, Bergkvist KS, Schmitz A, Bødker JS, Johansen P, Dybkær K, Bøgsted M, Johnsen HE. *Accepted for publication in PlosOne*

PAPER IV

Inherited variation in immune response genes in follicular lymphoma and diffuse large B-cell lymphoma. Nielsen KR, Steffensen R, Haunstrup TM, Bødker JS, Dybkær K, Baech J, Bøgsted M, Johnsen HE. *Leuk Lymphoma. 2015 Jul 7:1-10*

HYPOTHESIS AND AIMS

HYPOTHESIS

Inherited genetic variation in cytokine genes are of pathogenetic impact in B-cell diseases and could affect disease risk and outcome.

This hypothesis was tested through the following aims. Results and conclusions are reported in Paper I-IV.

AIMS

- Establish a method for genotyping of functional variants in cytokine genes.

- Determine the frequency of functional cytokine genes in patients with B-cell diseases and a healthy control group.

- Determine the association between functional cytokine gene and outcome in B-cell diseases.

- Determine the interaction between cytokine genes in relation to disease risk and outcome.

- Determine the gene expression patterns of pathogenetic genes in normal and diseased B-cells.

ABBREVIATIONS

ABC-DLBCL	Activated B-cell diffuse large B-cell lymphoma.
ACR	American college of rheumatology
APRIL	A proliferation-inducing ligand.
BCR	B-cell receptor.
BLyS/BAFF	B-cell activating factor.
BM	Bone marrow.
B-NHL	B-cell non- Hodgkin's lymphoma.
CB	Centroblast.
CC	Centrocyte.
CHI3L1	Chitinase-3-like protein 1
Chr	Chromosome.
CNV	Copy number varation
CSR	Class-switch recombination.
DLBCL	Diffuse large B-cell lymphoma.
FDC	Follicular dendritic cell.
FL	Follicular lymphoma.
GC	Germinal center.
GCB-DLBCL	Germinal center B-cell diffuse large B-cell lymphoma.
GWAS	Genome wide association study.
HC	Healthy control.
HDT/ASCT	High dose therapy with autologous stem cell transplantation.
HGF	Hepatocyte growth factor.
IFN	Interferon.
Ig	Immunoglobulin.
IgH	Immunoglobulin heavy chain.
IgL	Immunoglobulin light chain.
IL	Interleukin.
LD	Linkage disequilibrium
LN	Lymph node.
LT	Lymphotoxin
LTi	Lymphoid tissue inducer cell.
MIF	Macrophage inhibitory factor.
MGUS	Monoclonal gammopathy of undetermined significance.
MM	Multiple myeloma.
M□	Macrophage.
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OS	Overall survival
PC	Plasma cell.
RA	Rheumatoid arthritis.

SHM	Somatic hyper mutation.
SNP	Single nucleotide polymorphism.
TC	Translocation and cyclin D classification.
TGF	Transforming growth factor.
TLR	Toll-like receptor.
TNF	Tumor necrosis factor.
YKL-40	Chitinase-3-like protein 1

INTRODUCTION

GENERAL INTRODUCTION

Inherited genetic variation in cytokine genes affects gene function in B-cells and the lymph node microenvironment[1–6]. These functional variants could be part of the sustained inflammation observed in chronic inflammatory disorders as rheumatoid arthrtitis (RA) and be involved in the pathogenesis in cancers by promoting malignant transformation and the proliferation and evolution of malignant clones as illustrated in **Figure 1** (Paper IV, Figure 2)
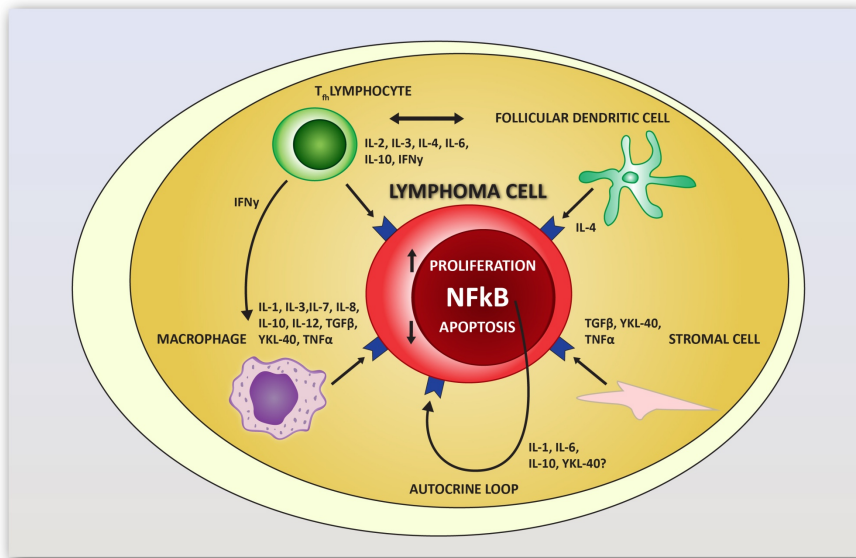
Figure 1. The effect of cytokine signaling in the proliferation of malignant B-cell clones. Sustained activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) enhances autocrine signaling by the lymphoma cells. Interleukin (IL)-6 is expressed by antigen presenting cells in the LN – and by B-cells, whereas IL1B, IL-2, IL-3, and tumor necrosis factor alpha (TNF-α) and YKL-40 are produced by immune cells in the LN. The combined effect of paracrine stimulation and autocrine loops stimulates to uncontrolled proliferation[1–6].

Cytokines play an important role in the proliferation and differentiation of normal B-cells[7,8]. The lymph node (LN) microenvironment include lymphoid tissue inducer

cells (LTi), T follicular helper cells (Tfhs), follicular dendritic cells (FDCs), macrophages (MΦ), stromal cells, and T-cells[9,10]. These cells secrete various pro- and anti-inflammatory cytokines that orchestrate B-cell development[11]. Dysregulation of B-cells is a major pathogenetic factor in RA[12]. RA affects 1% of the population and is known to be associated with more than 2 fold increase risk of developing B-cell non Hodgkin's lymphoma (B-NHL), possibly due to the chronic B-cell activation and stimulation by inflammatory mediators[13]. Hematological malignancies account for 10% of all malignant diseases; 95% of these are of B-cell origin, with B-NHL and multiple myeloma (MM) accounting for more than 50% of the cases[14,15]. Gene expression analyses of malignant B-cells and the tumor microenvironment have classified both B-NHL and MM in discrete groups in relation to prognosis; these findings argue that a pro-inflammatory milieu have a negative effect on disease risk and outcome[16,17]. Suggestive evidence points to a combined effect of these mediators interacting in inflammatory networks[3,18]. These genetic differences could even act as primary genetic hits or alter the cell's response to anti-inflammatory drugs or chemotherapy[19–22]. For these reasons, inherited functional genetic variation is thought to be of pathogenetic impact in B-cell diseases. Investigation of such pathogenetic variations using methods as hypothesis driven candidate gene studies and genome wide association studies (GWAS) combined with gene-gene interaction analyses may prove useful as a screening approach, with the goal of identifying candidate genes for subsequent functional analyses.

THE B-LYMPHOCYTE

B-cells are the central mediators of adaptive humoral immunity, however, the function of B-cells have been recognized only since the early 1970s[23,24]. B-cells are capable of differentiating into long lasting plasma cells (PCs) responsible for the generation of high affinity antigen-specific antibodies (immunoglobulins (Igs))[25] involved in innate immune responses through toll like receptor (TLR) activation. They play an important role in antigen presentation and immune regulation[26,27]. Mature B-cells are defined by the expression of membrane-bound immunoglobulin molecules (the B-cell receptor (BCR)). The variability in the BCR repertoire is the result of gene recombination at the variable (V), joining (J) and diversity (D) gene segments (V(D)J recombination). The process ultimately results in novel amino acid sequences in the antigen binding site of the Igs and in an Ig composed of two immunoglobulin heavy (IgH) chains and two immunoglobulin light (IgL) chains (either κ or λ)[28]. The development and differentiation of B-cells is dependent on the microenvironment in the bone marrow (BM) and LN[6]. In mice, early B-cell development depends crucially on interleukin (IL)-7; however, the cytokine profile required for shaping the human B-cell compartment remains elusive[25]. B-cell development is initiated by D_H to J_H rearrangement at the IgH locus on chromosome (Chr) 14[29]. Surviving pre-B-cells rearrange the V_L to J_L genes at the κ locus at Chr 2 and, if unsuccessful, the λ locus at Chr 22. The mature naïve transitional B-cells are critically dependent on tumor necrosis factor (TNF) family cytokines, B-lymphocyte stimulator (BLys/BAFF) and to some extend A Proliferation Inducing Ligand (APRIL) for survival signals[8]. The B-cell now enters the secondary lymphoid organs[30]. When encountering an antigen, T-cells stimulate the mature naïve B-cells to proliferation; these either form short lived IgM producing PCs or initiate the germinal center (GC) reaction. GC consists of B-cells (termed centrocytes (CC) and centroblasts (CB)), FDCs and Tfhs. The CBs undergo DNA editing in terms of somatic hyper mutation (SHM) and class-switch recombination (CSR)[31]. The GC reaction is dependent on a number of growth factors and cytokines; the exact functions of these are however currently not fully understood. The process of CSR is dependent on both cell mediated signaling and cytokine signaling from Tfh and FDCs producing IL-2, IL-4, IL-10, IL-12, IL-13, IL-17, IL-21, interferon gamma (IFNγ), transforming growth factor beta (TGBβ) and TNFα responsible for survival and proliferation signals and for shaping the antibody repertoire[32] – see **Figure 2** (Paper IV, Figure 1).[33]
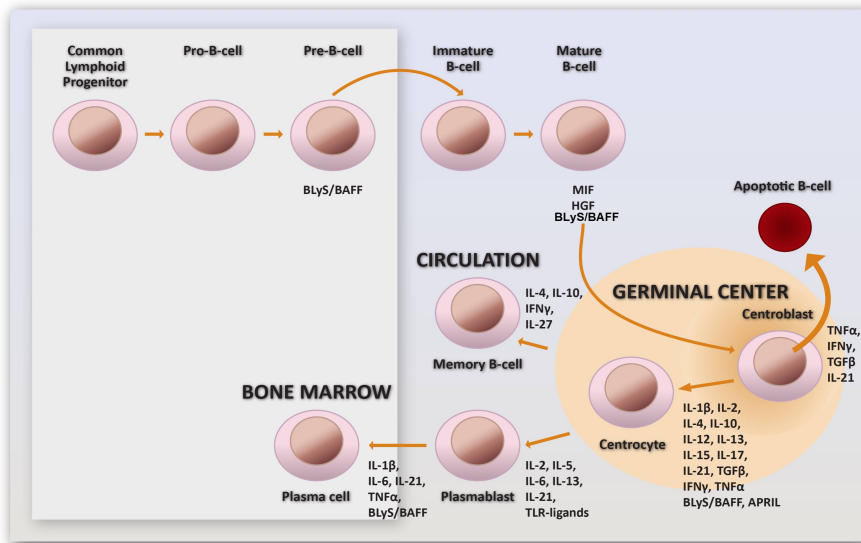
Figure 2. Cytokines shape the normal B-cell differentiation

The somatically mutated, isotype switched B-cells then complete the transition into antibody secreting PCs or memory B-cells. A number of cytokine stimuli, most notable IL-4 and Il-10, promote memory B-cell differentiation[34,35]. Persisting antigenic stimulation, cytokines and TLR signaling may drive memory B-cells into PC differentiation[36,37]. PC differentiation is enhanced by cytokines and IL-2, IL-5, IL-6, IL-13 and most prominently IL-21, whereas TNF family cytokines and IL-6 is important for long term PC survival[38].

THE B-CELL DISEASES

RHEUMATOID ARTHRITIS

RA is a chronic systemic autoimmune disorder that affects approximately 1% of the population. The characteristics of the disease are destructive polyarthritis and signs of systemic inflammation[39]. RA was for a long time considered a T cell driven disease, but clearly B cells play an important role in the RA pathogenesis and RA is associated with a more than two fold increase in the risk of developing B-NHL[13,40,41]. The synovial cellular infiltrates found in RA resembles a GC structure releasing autoantibodies and cytokines, and the B cells support autoreactive T cells by more than 1000-fold efficiency compared to other professional antigen presenting cells[42]. Autoantibodies form immune complexes that contribute to increased inflammation as well as activation of the complement system and are considered a predictor for increased joint destruction[43]. Twin-studies have estimated that genetic factors accounts for about 60% of the disease risk, however, less than half of these can be attributed to already identified genetic loci. The strongest genetic association to RA is with genes within the HLA locus and the tyrosine phosphatase non receptor type 22 (*PTPN22*)[44]. Susceptibility genes associated with RA such as *PTPN22* have been shown to affect BCR signaling pathways[12], and a recent study has found the *BACH2* transcription factor loci to be highly associated with RA risk[45]. There even seems to be an association between post treatment B-cell phenotypes in the synovial infiltrate and clinical treatment efficiency[12,45]. Cytokines are the major factor in the pathogenesis of RA, illustrated by the effect of TNF inhibiting drugs in the RA treatment. Among potential risk loci are the chitinase 3-like 1 (*CHI3L1*) gene encoding the YKL-40 protein. YKL-40 contains HLA-DR4 binding motifs, and has been suggested to function as an autoantigen in RA[46]. Variation in genes encoding signal transducer and activator of transcription (*STAT4*), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), Fc gamma receptor III-a (*FCGR3A*), interleukin-6 signal transducer (*IL6ST*), interleukin-2 receptor alpha (*IL2RA*), interleukin-2 receptor beta (*IL2RB*), *IL21, IL23* and cluster of differentiation 40 (*CD40*) are associated with RA. Recent GWAS data reported TNF receptor-associated factor 1 (*TRAF1*) the third most strongly RA-associated locus. The TRAF1 protein links TNFα to downstream signaling networks. Even though recent GWAS studies have identified novel risk genes, future studies are necessary to elucidate the remaining unknown genetic factors associated with RA.[44,47] The effect of gene-gene interaction have also only been analyzed in a limited number of studies and needs further investigation[48,49].

MULTIPLE MYELOMA

MM is a clonal B-cell neoplasm that affects terminally differentiated B-cells. The disease is characterized by BM infiltration of malignant plasma cells, monoclonal protein in serum and/or urine and the presence of lytic bone lesions. A monoclonal gammopathy of undetermined significance (MGUS) or smouldering multiple myeloma precedes MM in most cases. Here, precursor conditions are characterized by varying levels of monoclonal protein in the blood and/or urine and the presence of clonal plasma cells in the BM[50]. Patient's ≤ 70 years of age are usually treated aggressively with high-dose treatment with autologous stem cell support (HDT/ASCT). Despite intensive treatment strategies the disease is still regarded incurable with a median survival time of 5 years after diagnosis. However, significant variations in treatment outcomes are seen, even in patients with equal prognostic markers[51]. Since family histories of either MM or other lymphoid malignancies are known risk factors, and racial and ethnic variations in disease risk are observed, an inherited genetic component affecting the risk of MM development is suggested. This has been studied intensively, primarily based on knowledge from cytokines known to stimulate the growth of PCs. Findings are overall contradictory and most studies until now have been small in sample sizes[52,53]. The interleukin-1 beta (*IL1B)* gene seems to be rather consistently associated with MM[54], whereas findings with respect to the *TNFA* and *IL10* genes have been more contradictory[55–59]. The *IL6* gene has only been limitedly studied[59–62], and the *IL4* and *CHI3L1* genes have not yet been investigated in relation to MM. To further complicate the picture, recent GWAS studies have not been in consistence with earlier findings, and confirmatory and novel screening studies are still needed[63]. Other possible risk factors for MM are immunodeficiency and autoimmune diseases, however, data on these risk factors remain contradictory[64]. Malignant plasma cells arise from a myeloma propagating cell with the capacity to self renew and proliferate. An initiating event is thought to be translocations leading to cyklin-D deregulation, plasma cell proliferation and hyperdiploidia[65]. However, inherited genetic variation might precede these translocations[66] . Secondary hits drive disease progression, and MM cells express genes that are target of nuclear factor kappa-light-chain-enhancer of activated B cell activation (NF-κB) which explains for the dependence on BM microenvironment. As a consequence of accumulating genetic alterations, constitutive activation of these pathways take over and, in the case of plasma cell leukemia, make these cells less dependent on growth factors. MM is often a mix of 3-6 clones with different mutational status; in average, every clone has 35 mutations. Gene expression profiles have shown that expression of NF-κB-related genes are associated with a discrete prognostic group emphasising the importance of these pathways[67]. Growing evidence suggests, that the interaction between normal host cells, genetically altered premalignant cells and inflammatory mediators in the microenvironment are indispensable for the proliferation of the malignantly transformed clone. The BM microenvironment plays an important role in

differentiation, drug resistance, proliferation and survival of the malignant cells. Genes encoding key inflammatory mediators have been associated with disease risk and outcome[52,53]. Growth factors such as IL-1β, IL-4, IL-6, IL-10, YKL-40, BLys/BAFF, APRIL, TGFβ and insulin-like growth factor-1 (IGF-1) are regulated through paracrine as well as autocrine pathways. In vitro proliferation of MM cells are supported by primarily IL-6. Osteoclasts and osteoblasts produce IL-6 and YKL-40. IL-1β produced from MM cells further upregulates IL-6 production and NF-κB activation[68–70]. TNFα and lymphotoxin alpha (LTα) are both potent inducers of IL-6 production and are shown to stimulate growth of malignant plasma cells[71].

B-CELL NON HODGKIN'S LYMPHOMA

B-NHL is a malignant disease accounting for 80-85% of all malignant lymphomas in the western world and roughly 5% of all cancers[14,72] with a slightly increasing incidence[73]. B-NHL is caused by malignant transformation of mature B lymphocytes; the two most common subtypes are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). These subtypes account for 35% and 20% of all cases, respectively[74]. The etiology of B-NHL remains unknown; however, among known risk factors, immune suppression is the best described and 8% of patients with B-NHL display autoimmune manifestations suggesting immune dysregulation[75–77]. Family- and twin studies do not support strong penetration by a single gene. Increased lymphoma risk is probably caused by inheriting a deleterious genotype, which include a number of genetic variations distributed over a number of risk alleles that combine to form a high-risk phenotype[78–81]. A large number of studies have investigated these inherited genetic risk factors. However, lack of consistence between studies has been a major problem for interpretation of these findings. Moreover, recent GWAS studies are pointing in different directions and only show limited consistence with earlier candidate gene studies. Gene-gene interactions have only been studied for a limited number of genes and functional approaches are lacking. As reported in Paper IV, *TNFA*, *IL10* and *HLA* loci have been relatively constitutively associated with disease risk[82–95] and *TNFA* genotypes with outcome[96–99],however, most findings still need further confirmation.

DIFFUSE LARGE B-CELL LYMPHOMA

DLBCL is characterized by autonomous growth of B-cells that harbor a number of different genetic alterations; typically 30-100 genetic lesions pr case but displaying great variability between individual patients. In general, around 50% of patients with DLBCL are cured by conventional chemotherapy and a minority of relapsed patients is cured by HDT/ASCT[100]. DLBCL occur in two major prognostic subtypes; germinal center B-cell–like (GCB)-DLBCL and activated B-cell–like (ABC)-

DLBCL[101–103]. The two subtypes present different genetic profiles based on "cell of origin" classifications and oxidative phosphorylation genes[104,105].

The mutational landscape of DLBCL is identified primarily by SNPs and copy number variation (CNV) with few translocations being present. Compared to most other malignancies, tumor protein p53 (*TP53*) mutations are rare. However, lesions in histone/chromatin modification enzymes and acetyl transferases are found in more than 40% of cases leading to increased B-cell lymphoma 6 (BCL-6) activity and decreased p53 activity[106]. Around 50% of DLBCL cases harbor translocations placing the anti-apoptotic B-cell lymphoma 2 gene (*BCL2*) (t14;18) or pro proliferative *Cyklin-D* (t11;14) gene under control of the IgH promoter. Recently, gene expression profiles of the tumor microenvironment were used to stratify patients in relation to outcome in DLBCL; these studies showed that a monocyte/macrophage and matrix protein signature (Stromal-I) was related to a favorable outcome whereas a an microenvironment characterized by angiogenesis and endothelial cells were associated with a dismal outcomes[11,107–109]. Cytokines seems to play a significant role in DLBCL. Recent studies reported pre-treatment markers, here among interleukin-1 receptor antagonist (IL-1RA), IL-6, IL-8, IL-10, IFN-γ, IFN-gamma-inducible protein 10 (IP-10), vascular endothelial growth factor (VEGF), soluble interleukin-2 receptor (sIL-2R), monokine induced by gamma interferon (MIG), and IL-12, hepatocyte growth factor (HGF), and macrophage inflammatory protein 1 alpha (MIP-1α), associated with DLBCL.[110–112] High IL-10 is associated with decreased overall survival (OS) possibly through an JAK2 /STAT3 pathway leading to proliferation[113]. Serum levels of TNFα, IL-6 , VEGF, basic fibroblast growth factor (bFGF), YKL-40, IL-8, IP-10 and sIL-2R have similarly been linked to outcome[114–118]. Malignant B-cells have also been shown to express cytokine genes, however, for the DLBCL subgroup this topic remains largely unknown[119].

FOLLICULAR LYMPHOMA

FL is mostly a low grade lymphoma with an indolent clinical course and a median survival of 10 years[14]. The disease is often disseminated at time of diagnosis and is at that stage incurable with chemotherapy. A fraction of FL will transform into a DLBCL like lymphoma with a dismal prognosis. The vast majority of FL cases exhibit the (14;18) translocation, which up-regulates BCL-2 expression. Translocation of the *BCL2* locus to the *Igκ* locus t(2;18) or the *Igλ* locus t(18;22) may also occur. The BCL-2 upregulation allows for escape from apoptosis. B-cells harboring the t(14;18) translocations are seen in a small fraction of B-cells in >50% of healthy persons - of which most do not develop lymphoma after follow up[120], pointing to the fact that single translocations are neither necessary nor sufficient for causing overt malignant disease. FL may in fact originate from a pre-B-cell harboring the initiating translocation, acquiring secondary transforming events as

p53 mutations and chromosomal gains/deletions[106]. FL cells are difficult to grow in vitro and this gave a clue to the need of stimuli from the tumor environment, primarily activating NF-κB pathways, providing growth signals to the FL cells[121]. Studying the LN microenvironment in FL revealed two signatures of prognostic impact. The Immune response-I signature rich in T-cells predicted a favorable outcome compared with an LN environment with macrophages and dendritic cells – Immune response-II – which was associated with a poor outcome. This finding argues that a pro-inflammatory milieu has a negative effect and also suggests, that T-cells may play a role in inhibiting tumor progression[122–124]. Cytokines acts as proliferation and growth signals for FL cells. Increased levels of tumor necrosis factor receptor 2 (sTNFR2) and vascular endothelial growth factor receptor type 2 (sVEGR2) can forego clinical FL by more than 8 years[125]. Serum level of IL-10 and TNFα[112,125], IL-2R, IL-1RA, HGF, MIG and MIP-1a is associated with FL at time of diagnosis[110] whereas serum levels of IL-2R, IL-1RA and chemokine (C-X-C motif) ligand 9 (CXCL9)[126],   TGF-β and VEGF[127] have all been associated with disease outcome.

GENETIC POLYMORPHISMS AND GENE-GENE INTERACTIONS

SNPs are defined as evolutionarily stable, single nucleotide DNA sequence variations that occur in at least 1% of the general population. The human genome contains more than 10^7 SNPs, which make up 90% of all human genetic variations. Functional SNPs located in the promoter region can affect transcription factor binding which changes the expression of the gene – see **Figure 3** (Paper IV, Figure 3A). If the SNP is located in the coding regions it could result in a change in protein structure and function, or introduce a stop codon, leading to a truncate protein – see **Figure 4** (Paper IV, Figure 3B). SNPs located in non-coding regions can affect gene splicing – see **Figure 5** (Paper IV, Figure 3C). SNPs can also interfere with microRNA binding or long non-coding RNA binding sites and even induce epigenetic modification[128].
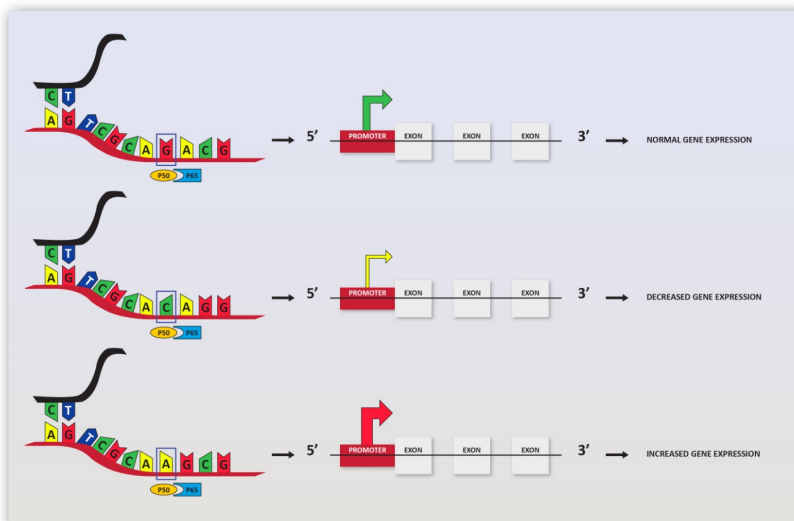
Figure 3. SNP located in promoter region.
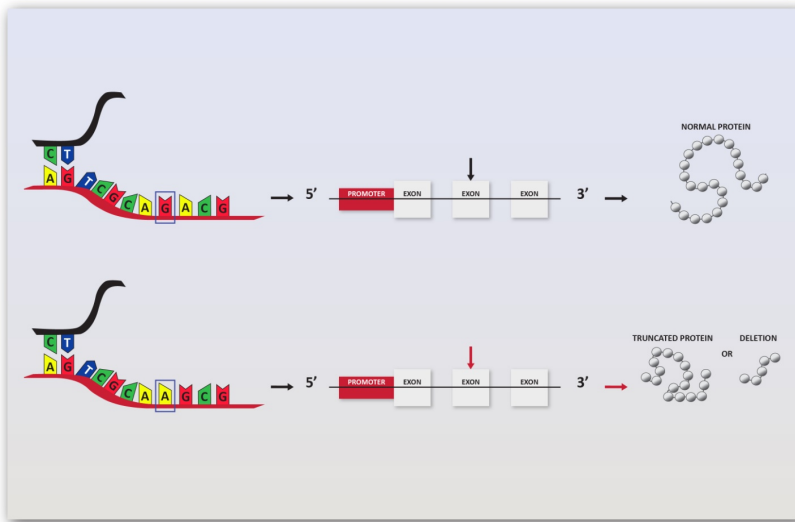
Figure 4. SNP located in coding region of the gene.
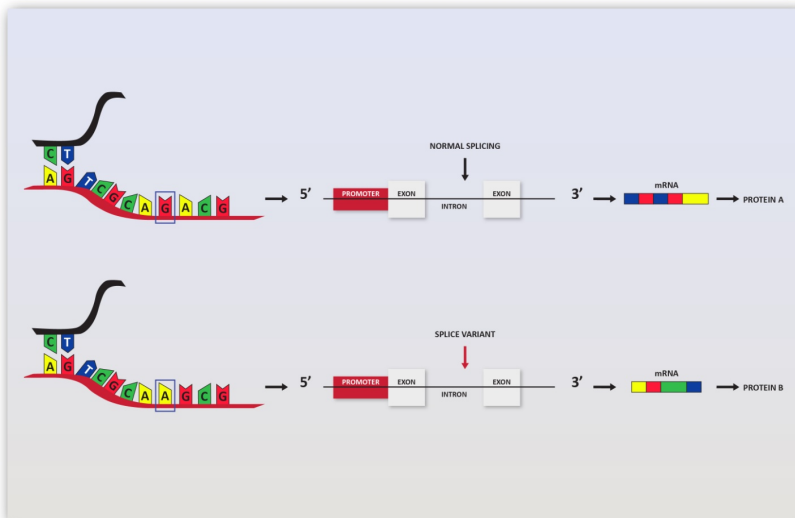
Figure 5. SNP located in the non-coding region of the gene.

In genes that encode inflammatory response proteins, SNPs are estimated to be responsible for up to 75% of protein variation[129]. By their functional effects, these

SNPs can affect the inflammatory response through paracrine and autocrine secretion of inflammatory cytokines. Apart from these indirect effects, SNPs may affect cells directly with a genetic hit that affects the tumorigenesis process[20,21]. Furthermore, recent studies have shown that genetic hits in progenitor cells can lead to epigenetic changes that affect the phenotypes of mature cells[21]. Recently, growing interest has focused on the fact that inflammatory mediators work in complex networks. Thus, studying single SNPs is less informative than previously thought and the genetic of cancer cells and the cytokine network in the tumor microenvironment is viewed with increasingly complexity and simple mechanisms is no longer suspected[130,131]. Cytokine genes may interact to cause an epistatic effect. Epistasis is a non-linear association between genotype combinations and the clinical phenotype. Genetic predisposition to B-cell diseases may be caused by interactions between loci or genes that individually show little or no contribution to risk, further complicating the functional aspect of genetic variation in inflammatory response genes[132,133].

FUNCTIONAL GENETIC VARIATION IN CYTOKINE GENES

Controversy still exists regarding the exact functional effect of SNPs in cytokine genes. Some –but not all- loci associated with risk and prognosis in hematogical malignancies have been investigated with respect to their functional properties using rather different techniques and methods; the topic has recently been reviewed by Smith et al[134]. Amongst the most intensively studied genes are the *IL6* gene and, to a lesser extent, the *IL6R* gene. The genetic variation in the *IL6* gene is restricted to the promoter region and the RefSNP cluster ID number (rs) 1800795 polymorphism has been intensively studied. Luciferase reporter assays have showed a 2.4-3.6 fold increase in expression from the G allele, however a number of in vivo and in vitro studies have not been able to show a simple association between alleles and gene activity. More distal promoter SNPs may be responsible for most of the functional genetic variation in the *IL6* gene; this seems only to affect certain cell types and certain inflammatory condition adding another layer of complexity to these functional genetic variants[128]. The *IL10* locus has also been studied as the promoter region harbors several SNPs. The rs1800896, rs1800871 and rs1800872 are best investigated, showing lower transcriptional activity and lower IL-10 production in stimulated whole blood by the ATA haplotype[135] and proposing the rs1800896 as of most importance[136]. However, a SNP in the distal promoter (rs1800890) has also been suggested functional[137], and although it is well established that the *IL10* gene promoter holds functional variants, further investigation of this gene is warranted. Studies of the functional properties of the *TNFA* locus have also been investigated. In general there is lack of consistence between studies, and it is to be concluded that the regulation of the TNFA locus, which harbors a large number of SNPs in high LD, is very complex and that SNPs may have cooperative effect[138]. The functional variants in the IL-4 loci are still not resolved. The *CHI3L1* gene is also investigated for functional SNPs, and evidence points to variations in the proximal promoter region[139]. Similar findings have been published for variations in other key inflammatory mediators and further studies of these functional polymorphism are awaited[134].

CHITINASE-3-LIKE PROTEIN 1 (YKL-40, CHI3L1)

The YKL-40 protein, encoded by the *CHI3L1* gene, is emerging as a new biomarker of severe disease activity and poor prognosis in patients with cancer and autoimmune diseases[140]. The functional properties of the *CHI3L1* promoter is still under investigation. Several polymorphisms are suggested to be functional including the *CHI3L1* -247G/A (rs10399805) and *CHI3L1* -131C/G (rs4950928) polymorphisms[141–144]. The exact biological function of the YKL-40 protein is still largely elusive. The YKL-40 protein is expressed by various malignant cells and tumor associated macrophages and neutrophils[145]. YKL-40 regulates inflammatory responses via IL-13Rα2 activating pathways and is involved in apoptosis, oxidative injury, pyroptosis, angiogenesis, inflammasome activation and TGFβ mediated anti-apoptotic signaling as shown in malignant melanoma[146–148]. YKL-40 is regulated by IL-1β and IL-6 by activation of STAT3 / NF-κB pathways, leading to *CHI3L1* gene transcription and by this YKL-40 protein up-regulation[149]. To the best of our knowledge, no studies have been made exploring the effect of *CHI3L1* genotypes in B-cell diseases. However, the association to serum levels of the protein have been investigated; our group has recently reported that high circulating levels of YKL-40 is a marker of non-complete remission following salvage therapy before HDT in aggressive NHL[114]. Earlier studies have linked plasma levels of YKL-40 to prognosis in primary nervous system NHL and Hodgkin's disease[150,151]. In MM, high levels of YKL-40 are associated with high risk disease, and a recent study has confirmed this association between serum-YKL-40 and disease risk, increased bone resorption and early radiographic progression[152,153]; in RA, YKL-40 is also associated with disease prognosis[154].

METHODS

STUDY POPULATIONS AND SAMPLES

Four different study populations were examined in this thesis:

Study population 1: A Danish RA cohort (Paper I)

Study population 2: A Danish MM cohort (Paper II)

Study population 3: A Danish B-NHL cohort (Paper III)

Study population 4: A healthy Danish blood donor population (Paper I, II and III)

Study population 1:

RA patients were included from the Department of Rheumatology, Hvidovre Hospital, Hvidovre, Denmark. The patients were diagnosed with RA according to the ACR 1987 criteria[155]. Clinical data from each patient was acquired from the DANBIO registry. DANBIO was started in October 2000 and collects clinical data from all medically treated Danish RA patients. Three-hundred and eight patients were eligible for this study as they fulfilled the diagnostic criteria and had blood samples (serum and whole blood) available in the local bio-bank. Blood samples were drawn on all patients before the start of treatment with biological drugs. All samples were immediately transported to the lab and handled within 3 hours after sampling. Serum and whole blood was subsequent stored at -80°C.

Study population 2

Three hundred forty-eight MM patients were recruited from four participating centres in Denmark. All patients were treated with HDT/ASCT. One hundred eighty-five patients participated in HDT protocols (Nordic Myeloma Study Group (NMSG) no. 5/94, 7/98 and 11/00); the remaining 163 patients were not registered in the NMSG protocols, but followed similar treatment regimes[156]. Patients were included from August 1994 to August 2004 and the following diagnostic criteria where used for inclusion; A: serum monoclonal compartment concentration of immunoglobulin IgG > 30 g/L, IgA > 20 g/L, the presence of an M-protein of IgD or IgE regardless of concentration, or Bence-Jones proteinuria > 1 g/24 hr; B: M-protein in serum or urine at a concentration lower than described under A; C: ≥10% plasma cells in bone marrow aspirate or biopsy-verified plasmacytoma; and D: osteolytic bone lesions. Assignment of diagnosis demanded fulfilling: A + C, A + D,

B + C + D. Staging were performed using the Durie and Salmon criteria[157]. Biochemical data was available for all patients. OS was calculated from the day of diagnosis. For all eligible patients, the following material was available from the bio-bank: peripheral blood mononuclear cells (PMBCs) stored at -180°C (292 patients) and formalin fixed paraffin-embedded (FFPE) bone-marrow samples (56 patients) taken for routine analysis and stored at room temperature at the local department of pathology.

Study population 3.

DLBCL or FL patients diagnosed at Aalborg University Hospital in the period 1999-2005 was recruited for the study. 216 DLBCL and 139 FL cases were identified. For each case, the clinical and biochemistry data was abstracted from the Danish lymphoma database (LYFO). Forty-five patients did not have a complete dataset or were not registered in LYFO at all. For 32 patients, hospital medical records were used to abstract biochemistry and clinical data. Thirteen patients died within a few days after diagnosis, and it was not possible to extract sufficient clinical data from this small group why they were excluded from survival analysis. DLBCL and FL cases were histological classified according to the World Health Organization (WHO) classification[74]. The national Danish death registry was used to extract vital data on all patients. From all patients, tissue for genotyping was available as FFPE BM samples. Included patients did not have dissemination to the BM. All samples were initially taken for routine analyses and stored at room temperature at the local department of pathology.

Study population 4.

The control group consisted of 605 donors from the Aalborg University Hospital blood bank. All donors reported Danish ethnicity and were therefore considered Caucasian. The donors routinely sign a statement declaring that they do not take any medication and are not under investigation for any disorder. All donors were clinically healthy at the time of blood drawing. All samples were handled within 3 hours after sampling. Serum was separated immediately and stored at -80C. DNA was extracted from whole blood within 24 hours and subsequent stored at -80C.

ETHICAL CONSIDERATIONS

Study population 1

DANBIO has been approved by The Danish Data Registry since the year 2000 (j.nr. 2007-58-0014 and j.nr. 2007-58-0006) and since October 2006 as a national quality registry by the National Board of Health (j.nr. 7-201-03-12/1).

Study population 2

All tissue samples were collected from myeloma patients, healthy controls and BM donors as described by research protocols N-20100090, and N-20080062MCH, respectively. These protocols were approved by the local scientific ethics committee of the North Denmark Region.

Study population 3

Research protocols were approved by the local scientific ethics committee of the North Denmark Region, approval N-20100059.

Study population 4

Research protocols were approved by the local scientific ethics committee of the North Denmark Region, approval N-20090018.

DNA PURIFICATION

From cryopreserved buffycoat PBMCs, DNA was extracted using a salting-out technique where DNA was precipitated with a saturated NaCl solution as described in details elsewhere[158]. FFPE samples were cut into five 10 μm thick slices. The microtome was cleansed with a biocleaner and 98% ethanol between samples to ensure no cross contamination. Extracting sufficient amount of high quality DNA for SNP array analysis was known to be a challenging task, why three methods were evaluated thoroughly. An automated standard Maxwell® 16 tissue DNA purification method (Promega, Madison, WI, USA) using deparaffinization by heat did not yield sufficient DNA for the planned array analyses in study 3. Instead, a manual xylene/ethanol method was evaluated for sample preparation. After deparaffinization by this method, samples were resuspended in ATL buffer (Qiagen) with 0.1 mg/ml proteinase K at 56°C overnight and DNA was extracted with the Maxwell® 16 tissue DNA purification method (Promega, Madison, WI, USA). The manual xylene/ethanol yielded higher DNA output, however it was labour intensive and for BM samples with low cellular content, did not always prove sufficient DNA yield. Finally a novel automated Maxwell® 16 low elution volume tissue DNA purification kit (Promega,Madison, WI, USA) was evaluated. Using this kit, FFPE tissue was heat-deparrafinized and processed directly using a Maxwell 16 DNA purification method based on a magnetic bead separation technique. The process was automated on a Maxwell® 16 Instrument. This method proved superior for BM samples with low cellular content, and even samples initially discarded were reprocessed with favourable DNA outcome. When whole blood samples were available, DNA was extracted directly from 200μl ethylenediaminetetraacetic acid (EDTA)-stabilized whole blood using Maxwell 16 blood DNA purification kits, according to manufactures protocol. DNA concentration and purity was determined using nanodrop 2000 or nanodrop 8000 UV spectrophotometers (NanoDrop products, Wilmington, DE 19810 USA). DNA concentration was finally adjusted to 25 ng/ul for TaqMan single gene analysis or 50 ng/ul for TaqMan open array analysis.

REAL-TIME PCR ASSAY

Real-time polymerase chain reaction (rt-PCR) allows for the monitoring of the PCR as it occurs, and the reactions are characterized by the point in time during cycling when amplification of a target is first detected. TaqMan based genotyping assay were used in all studies [159–162]. The TaqMan genotyping assays used consists of two specific primers (forward and reverse)- see **Figure 6 -** and two short sequence specific minor groove binder (MGB) hydrolysis probes design to discriminate between the two polymorphic alleles of the genotyped SNPs amplified within a short

PCR product. The specific probes are labeled with two different high-energy fluorescent dyes at the 5´ end and termed a reporter. The 3´end is labeled with a quencher absorbing the fluorescence from the dyes. When the quencher probe is intact and excited by a light source, the reporter dye's emission is suppressed by the quencher dye as a result of the close proximity of the dyes. When the probe is cleaved by the 5' nuclease activity of the enzyme, the distance between the reporter and the quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease. The fluorescent emission can be visualized. With samples of good quality and comparable quantity, the end-point scatter plot can be used for genotype calling. This allows for allelic discrimination between the wildtype homozygous, heterozygous and mutant homozygous individuals as illustrated in **Figure 7**.
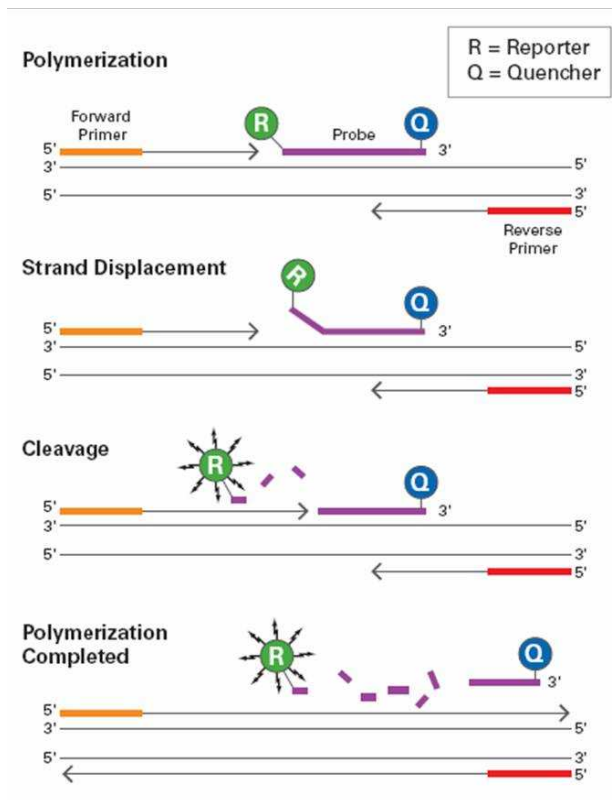
Figure 6. Principles in TaqMan based genotyping assays.
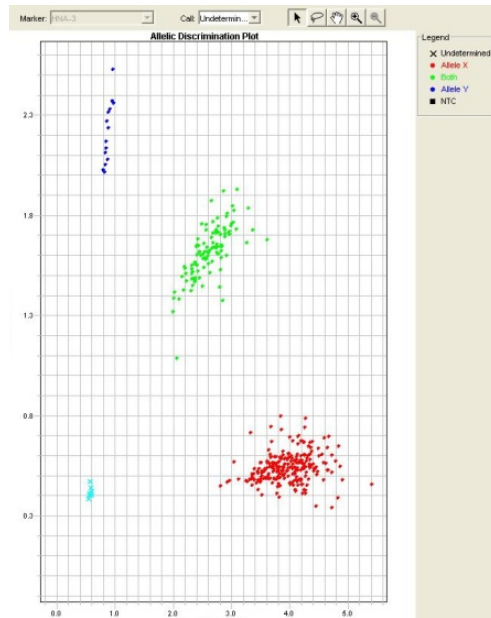
Figure 7. Allelic discrimination plot. wildtype homozygous (red dots), heterozygous (green dots) and mutant homozygous (blue dots) individuals.

TAQMAN SINGLE GENE ASSAY GENOTYPING METHOD (PAPER I AND PAPER II)

SNPs were selected using literature studies and the NCBI SNP database (http://www.ncbi.nlm.nih.gov). In Paper I, a total of eight *CHI3L1* SNPs were genotyped. Four SNPs was located within the promoter region (rs6691378, rs10399931, rs10399805, rs4950928) and four SNPs located within the coding region of the gene (rs7515776, rs1538372, rs2071579, rs880633). In Paper II, thirteen SNPs were selected from the promoter regions of the *TNFA*, *IL4*, *IL6*, *IL10*, and *CHI3L1* genes (rs1800795, rs1800630, rs1799964, rs1799724, rs1800629, rs361525, rs2243248, rs1800797, rs1800890, rs1800896, rs1800871, rs1800872, and rs4950928). Two SNPs (rs1800795 and rs1800630) were genotyped as previously described[163,164]. The remaining SNPs were genotyped using available TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). For all assays, DNA amplification was carried out in a 5 µl volume containing 20 ng DNA, 0.9 µM primers and 0.2 µM probes (final concentrations) and amplified using TaqMan Universal PCR Master. A 384-well plate format was used on a GeneAmp PCR 9700 or a 7900 HT Sequence Detection System. The protocol used were 95°C for 10

minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. To determine genotypes, end-point fluorescence was read on the 7900 HT Sequence Detection Systems using SDS version 2.3 software (Applied Biosystems, Foster City, CA, USA).

TAQMAN OPEN-ARRAY GENOTYPING ASSAY (PAPER III)

A custom SNP array was designed for genotyping. The TaqMan OpenArray genotyping system from Applied Biosystems (Applied Biosystems, Foster City, CA, USA) uses TaqMan chemistry in a predesigned array. The system was initially evaluated and found to provide a high-throughput automated system while allowing for high specificity genotyping comparable to single SNP assays. Seven SNPs was typed using custom-designed assays and 43 SNPs was typed using predesigned TaqMan SNP assays, see Paper III for details. A non-template control (NTC) was introduced within each set of assays and TaqMan OpenArray master mix (ABI, Foster City, CA, USA) was used in this study according to the manufacture's protocol. Samples were loaded into OpenArray plates using the OpenArray NT Autoloader and cycled using GeneAmp 9700 thermal cycler with PCR conditions according to the manufacturer's protocol (ABI, Foster City, CA, USA). The arrays were read using the OpenArray NT Imager and the allele calls and scatter plots were generated with the Biotrove OpenArray SNP Genotyping Analysis Software package version 1.0.3. For all genotyping data the default threshold for the Quality value was set to 0.95. The score is an estimate of how closely a given data point belongs to those underlying models of angle and amplitude distributions for each genotype cluster. The call rate was >80% for all genotypes tested.

AFFYMETRIX GENE EXPRESSION ANALYSIS (PAPER II AND III)

LN tissue, BM samples and DLBCL cells were analyzed as previously described in detail[165–167]. Normal LN tissue and BM samples was obtained from healthy individuals by routine surgical procedures and was immediately disrupted into smaller pieces. Mononuclear cells (MNCs) were enriched using Ficoll-Paque Plus (GE Health Care, Uppsala, Sweden) centrifugation before storage in liquid N2 at -196°C as vital single cell suspensions, frozen in RPMI-1640 medium (GIBCO) supplemented with 1% antibiotics, 15% fetal calf serum and 10% dimethyl sulfoxide (DMSO) (GIBCO) in a temperature gradient controlled proces[165]. Cells were sorted by a FACSAria2 cell sorter directly into 450 µl of lysis/binding buffer (Miltenyi Biotech, Bergisch-Gladbach, Germany), using a single tube 8 color antibody panel for classification of B-cells. Messenger RNA (mRNA) was isolated and amplified using the µMACS mRNA isolation kit (Miltenyi Biotechnologies, Bergisch

Gladbach, DE) and the WT-Ovation Pico RNA Amplification System (NuGEN, San Carlos, CA, USA) according to the manufacturer's instructions. Total RNA was isolated using a modified TRIzol Reagent protocol (Invitrogen, Paisley, UK) and mirVana (Ambion/Invitrogen, Paisley, UK).[165] The samples were prepared for hybridization to Affymetrix GeneChip Human Exon 1.0 ST Arrays following the manufacturer's instructions. CEL-files were generated by Affymetrix Gene Chip Command Console Software (AGCC) and deposited in the NCBI Gene Expression Omnibus (GEO) repository.[165] Retrospective on line available public data on MM plasma cell gene expression analysis was available as mRNA expression profiles in CD138-enriched plasma cells from 414 newly diagnosed patients who went on to receive HDT and tandem stem cell transplants.[168] Gene expression data was analyzed using Partek Genomics Suite version 6.5 (Partec Inc., St. Louis, MO, USA).

BIOCHEMICAL ANALYSIS (PAPER I)

The YKL-40 serum concentration was measured using a commercial two-site sandwich type ELISA (Quidel, Mountain View, CA, USA). For this assay, the detection limit was 10 ng/ml. The intra-assay coefficient of variations (CV) was 5% and the inter-assay CV was < 6%[154].

STATISTICS

Statistical analyses were carried out using the statistical software R R-3.2.0 (http://www.r-project.org/) and STATA version 12.1 (StataCorp, Texas, USA), Helix Tree SNP analysis software package (Golden Helix Software, Bozeman, MT, USA) and SHEsis software (Bio-X Center, Shanghai Jiao Tong University,1954 Huashan Road, Shanghai 200030, China).

HARDY-WEINBERG EQUILIBRIUM

SNPs in the control group were in all studies tested for Hardy-Weinberg equilibrium (HWE) using Helix Tree SNP analysis software package. Deviations from HWE in the control group lead to exploration of genotyping quality, and eventually retesting of samples. Deviation from HWE in the patient cohort was an expected phenomenon. Calculation was made by calculating the expected and observed allele frequencies using the Hardy-Weinberg theorem: $(p + q)^2 = p^2 + 2pq + q^2 = 1$ representing alleles with the frequencies p and q. A chi-square test with 5% significance level was subsequently applied to test deviation from HWE.

LINKAGE DISEQUILIBRIUM

The degree of LD between SNPs known to be located in haplotype blocks was determined using the SHEsis software. Findings were compared to data acquired from the international HapMap project database (http://hapmap.ncbi.nlm.nih.gov/). LD were reported as the disequilibrium coefficient D' explaining the quantitative deviation of the observed frequency of a haplotype from the expected. The correlation coefficient r^2 further include the allele frequencies in the calculation since this can be of importance if rare alleles are included in the calculation[169].

MODELING THE GENOTYPE EFFECT ON SERUM PROTEIN LEVELS

The serum YKL-40 protein concentrations were log transformed resulting in normal distribution in patients and controls. A non-linear and a linear model were explored to adjust for the age-dependent increase in YKL-40. The linear model was

acceptable and included in a multiple linear regression model containing case-control status, age, gender, genotypes and YKL-40 concentration. This analysis further included interactions between explaining variables, and this model could then define independent factors associated with serum concentration. The effect of individual genotypes was explored by including the genotypes one-by-one as dependent variables in a linear age-adjusted model. The most significant SNP was then used as a covariate to determine the influence of the remaining SNPs.

CORRECTION FOR MULTIPLE COMPARISONS

When appropriate, we used correction for multiple comparisons to exclude over-interpretation of results. With an increasing number of comparisons in the analysis, it becomes more likely to observe differences between groups by random chance alone. Since strict correction unfortunately also holds a risk of discarding important findings as random variation, this aspect should be considered before implying correction and should be avoided in explorative studies[170]. In the initial approach of single SNP associations we used uncorrected statistics. However, in gene-gene interaction analyses and gene-expression analyses we decided to apply a strict correction for multiple comparisons using the conservative Bonferroni method. In praxis, the significance level used in single comparison analysis (0.05) was divided by the number of comparisons. As an example if 23 genes were compared we used a significance level of $0.05/23 = 0.0022$.

GENOTYPE MODELS

We analyzed each SNP in three different ways, namely using additive, wild type, and variant models. The three genotypes of each SNP are kept in the additive model. In contrast, the wild type and variant models have two levels per SNP. For example, for TNFA -863C/A, C is the most common allele. Accordingly, the additive model compares CC, CA, and AA, the wild type model compares CC and CA + CC, and the variant model compares AA and CA + CC.

ANALYSIS OF CASE-CONTROL DATA

The effect on individual SNPs and haplotypes was estimated as an odds ratio (OR):

$$OR = \frac{\text{(n) exposed cases / (n) unexposed cases}}{\text{(n) exposed non-cases / (n) unexposed non-cases}} = \frac{\text{(n) exposed cases x (n) unexposed non-cases}}{\text{(n) exposed non-cases x (n) unexposed cases}}$$

The association between disease status and various explaining variables were quantified by odds ratios (OR) using logistic regression models. The regression model was used to estimate the associations between disease status and single genotypes, haplotypes, and the interactions between SNPs and between haplotypes [171]. The significance of individual variables was assessed by likelihood ratio tests that compared the model with and without the variable using the anova.glm function in the stats package.

SURVIVAL ANALYSIS

An important component of survival data analyses is the Kaplan-Meier estimator for generation of Kaplan-Meier survival curves which give a valuable visual impression of the effect of genotypes on survival. Patients were stratified according to genotype, survival time was calculated from time of diagnosis until death or censoring. In case of comparison of survival curves for two or more groups we used the non-parametric log rank test. The Kaplan-Meier curves with log rank test were used to describe differences in OS between genotype groups; however, to include the effect of more variables on survival, the Cox proportional hazard model was applied. Cox proportional hazard model was used to generate hazard ratios for a specified outcome (death) between individuals with different characteristics (genotypes, sex, prognostic score, etc.). The survival model are reported as hazard ratios (HRs) adjusted for significant prognostic factors with 95% CI and p-values [171].

GENE-GENE INTERACTION MODEL

The purpose of performing gene-gene interaction analyses was to identify pairs of genes for which the interaction between them was significantly associated with the

risk of lymphoma diagnosis (performed by logistic regression) or significantly associated with survival among lymphoma patients (performed by Cox regression). We performed the gene-gene interaction analyses by comparing the models with and without the interaction term using a likely hood ratio test. Highly significant interactions were selected using the p-value from the likely hood ratio test as proposed by Cordell,[172] by comparing the saturated model including interaction and additive effects with the purely additive model. We corrected for the number of individual loci (31 loci) investigated, why only interactions with a p-value of < 0.002 were considered significant. As 9 possible combinations of genotypes exist for each pair of genes, we have for the survival part reported hazard ratio estimates for each combination of genotypes with the combination of the most frequent homocygotes as reference.

RESULTS AND DISCUSSIONS

PAPER I

Promoter polymorphisms in the chitinase 3-like 1 gene influence the serum concentration of YKL-40 in Danish patients with rheumatoid arthritis and in healthy subjects

AIM AND DESIGN

This Paper aimed to establish a method for evaluating the functional effect of SNPs in the *CHI3L1* gene in relation to the pro-inflammatory YKL-40 protein and explore if this gene had pathogenetic impact in RA. This was evaluated by genotyping a cohort of RA patients (study population 1) and a cohort of healthy blood donors (study population 4). We investigated 8 SNPs in the CHI3L1 gene and measured serum levels of YKL-40.

RESULTS AND DISCUSSIONS

Investigation of serum YKL-40 levels in both populations revealed no difference with respect to gender, however, serum levels increased with increasing age. After log transformation, serum YKL-40 levels were found to be normally distributed and up till the age of 65, the association between age and serum YKL-40 was linear why we decided to included only persons <= 65 years. When testing each genotype individually the -131C/G (rs4950928) SNP was most strongly associated with YKL-40 levels in patients (p=2,4e-08) and controls (p= 2.2e-16). A dose effect of the genotypes was found, establishing the G allele as low producer and C allele as high producer as shown in **Figure 8** (Paper I, Figure 3)
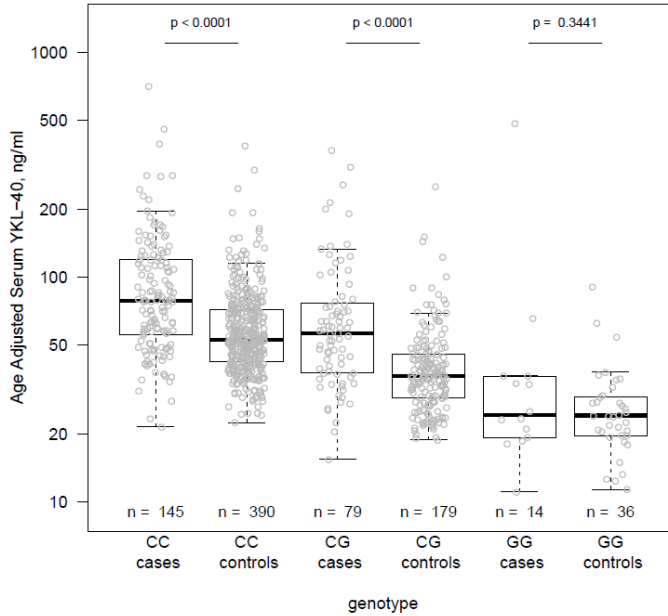
Figure 8. Association between the *CHI3L1* -131C/G (rs rs4950928) SNP and serum concentrations of YKL-40. Box plot for 238 patients with rheumatoid arthritis (RA) ≤ 65 years of age (P < 2.0e-16 and 605 healthy controls (P < 1.1e-8). The x-axis represents genotypes. The y-axis represents serum YKL-40, horizontal bars represents median serum YKL-40 and quartiles for patients with RA and controls.

In this study we did not find any association between genotypes or haplotypes and the risk of developing RA. The -131C/G (rs4950928) allele have earlier been associated with YKL-40 expression by Zhao et al. who studied *CHI3L1* gene expression in a small study of 29 schizophrenic patients and 54 controls, they also found lower activity of transcriptional factor activity associated with this SNP[141]. Ober et al. reported association between several SNPs in the *CHI3L1* gene and serum YKL-40, due to very high LD it was difficult to explore further into the exact functional SNP[173]. Sohn et al reported the -247G/A (rs10399805) SNP associated with serum YKL-40 levels in an Asian population, however allele frequencies and LD was different from Caucasian populations, clearly emphasizing the problem in extrapolation genetic findings between ethnically different populations. Our findings have subsequent been confirmed in a large Danish cohort[142] and an US cohort[174] both suggesting a pronounced functional effect of the -131C/G (rs4950928) allele. We therefore suggest that this SNP must be included in future association studies if the functional aspects of the *CHI3L1* promoter is to be studied in relation to disease risk or outcome.

LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

We studied an ethnically homogenous but relatively small cohort. The sample size results in relatively few persons with the homozygous mutant genotype affecting the strength of the study. Moreover, the *CHI3L1* locus seem to have different allele frequencies and LD between different ethnic groups why our findings may only be valid in a north European Caucasian cohort. We did not include a functional assay to determine if the region close to the -131C/G (rs4950928) SNP is actually the binding site for transcription factors and if transcription could be blocked by interfering with this specific area of the promoter, as suggested by a few investigators[139]. This clearly needs to be studied in more detail before we can conclude on the functional properties of this locus. Another puzzle is the fact that although genotypes and a number of autoimmune and malignant disease seem to be independently associated with serum YKL-40 levels, the direct association between genotypes and disease has been less clear[174,175] and must be explored in future studies.

PAPER II

Interactions between inherited inflammatory response genes are associated with multiple myeloma disease risk and survival.

AIM AND DESIGN

In this Paper we aimed to explore if genetic variation in cytokine genes *CHI3L1, TNFA, IL4, IL6 and IL10* was of pathogenetic impact in MM. We investigated a cohort of MM patients (study population 2) and a healthy control group (study population 4)

RESULTS AND DISCUSSIONS

The -238A (rs361525) allele was associated with decreased MM risk (OR=0.51 (0.29-0.86)) suggesting that low producer *TNFA* genotypes[176] may protect against the development of MM. Association between MM and the *TNFA* -238A allele was however not reported in two earlier studies including 181 Caucasian MM patients and 210 Asian MM patients[55,177]. The *TNFA* -308G/A polymorphism has been associated with MM in British and Hungarian patients[55,57]. We did not find this association in our Danish study population, supporting the findings by Zheng et al. and the recent meta-analysis by Vangsted et al.[53,61]. We examined the prognostic effect of genotypes and found the *TNFA* -857 and *IL10* -1082 SNPs highly associated with 5 year OS. IL-6, IL-4, IL10 and TNFα are individual B-cell proliferation factors and may work in a network[3]. Gene-gene interaction analysis revealed impact of *IL6*, *IL10, TNFA* and *IL4* genotypes on OS, see **Figure 9** (Paper II, Figure 1C) and **Figure 10.** (Paper II, Figure 1D)
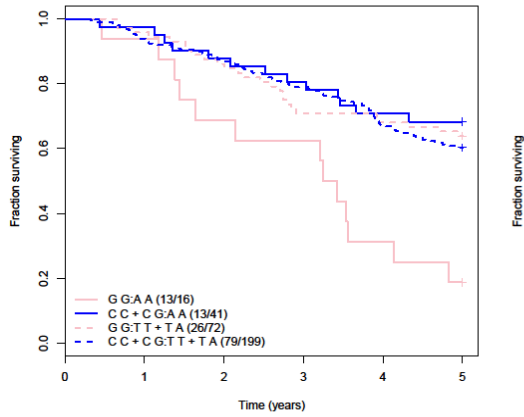
Figure 9. Association between 5-year overall survival and genotypes. Hazard ratios (HR) was adjusted for sex, age, Durie-Salmon stage, creatinin, and β2-microglobulin. *IL6 -174G/C* and *IL10 -3575T/A* genotypes on OS. $HR_{GG*AA}=3.17$ (1.69-5.94), P=0.007.
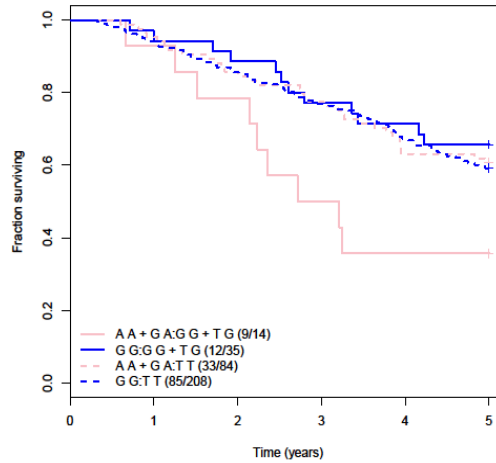
Figure 10. Association between 5-year overall survival and genotypes. Hazard ratios (HR) was adjusted for sex, age, Durie-Salmon stage, creatinin, and β2-microglobulin. *TNFA -308G/A* and *IL4 -1098T/G* genotype. $HR_{AA/AG*GG/GT}=3.47$ (1.64-7.37), *P*=0.03.

The findings of these interaction is very interesting but also difficult to interpret as the functional aspects of these individual SNPs still remain partly elusive[134]. The

existence of premalignant B-cells with a memory phenotype harboring IgH translocation have been described in MM[22]. We found *TNFA*, *IL6* and *CHI3L1* genes up regulated in normal memory B-cells suggesting that cytokine genes in these B-cell subsets may play a role in premalignant transformation. *CHI3L1, IL6* and *IL10* gene expression in MM cells was also significantly associated with prognosis when assigned to the TC classification system (**Figure 11),** indicating that these inherited genetic variants may play a role in response to treatment. Interaction between *IL6* and *IL10* was also seen at expression level supporting our findings from the SNP studies.
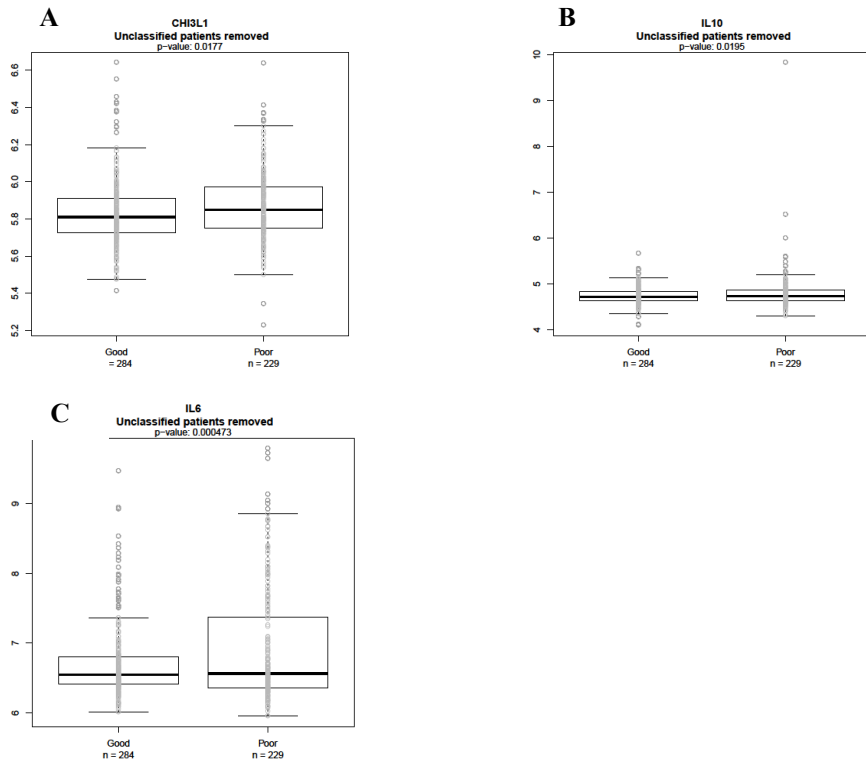
Figure 11. Gene expression in enriched MM cells from patients grouped in poor and good prognosis based on TC classes. **(A)** *CHI3L1* gene expression, **(B)** *IL10* gene expression and **(C)** *IL6* gene expression.

The functional *CHI3L1* SNP (Paper I) was not associated with risk of MM and no association with outcome was seen in univariate analysis. We did observe highly

significant interaction between the *CHI3L1* genotypes and *TNFA* and *IL10*. Due to low number of patients in the rare allele group we could, unfortunately, not obtain precise risk estimates. We believe the *CHI3L1* gene needs further investigation in MM.

LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

Our results add another layer to the complexity of the roles of cytokines in MM by suggesting that gene-gene interactions must be included when analyzing the effect of cytokine genes. This approach needs to be considered in design of such studies to ensure statistical power for these investigations. Our study cohorts were uniform with regards to ethnicity, and the genotyping method used gives a high call-rate and very high allele specificity. However, our sample size was too small with regards to in depth study of rare alleles and gene-gene interactions. Inconsistence between studies on risk and prognosis genes is a major challenge[52,53]. We observed small sample size in some of these earlier studies, and ethnic differences make direct comparisons difficult. With regard to sample size and the "curse of dimensionality" using regression based interaction analysis, we did explore other statistical methods as multifactor dimensionality reduction (MDR)[178] for evaluation of gene-gene interactions, however, these methods are still under development. This emphasizes the important role for biostatisticians in genetic research. Future studies should address these problems and must include functional analyses of the respective genetic loci, as we discovered that the functional knowledge of these genes is rather sparse.

PAPER III

Inherited inflammatory response genes are associated with B-cell non-Hodgkin's lymphoma risk and survival.

AIM AND DESIGN

We aimed to investigate if genetic variation in 50 highly selected and interacting cytokine genes was of pathogenetic impact in B-NHL. We investigated a cohort of B-NHL patients (study population 3) and a healthy control group (study population 4).

RESULTS AND DISCUSSIONS

The case-control study revealed two major findings; an effect of genes encoding inflammatory mediators known to act as B-cell proliferation and differentiation factors such as IL1-β, IL-10 and TLR9 and an effect of genes located in the HLA-II region (*TAP2*) confirming the importance of genes in this region for lymphoma risk[179]. A novel finding was that the *TAP2* SNP was also associated with DLBCL prognosis and this high LD region needs further studies before the exact gene/genes related to this increased risk are identified and the functional mechanisms established[89,91,180]. We have recently reported serum levels of the YKL-40 protein associated with outcome in NHL[114]. In the present study we observed outcome associated with a functional *CHI3L1* genotype in FL as shown in **Figure 12** (Paper III, Figure 1A) and although this further strengthens a suggested effect of this inflammatory protein in B-lymphomas, this needs further investigation.
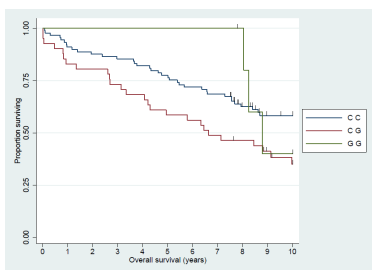
Figure 12. Association between FL 10 year overall survival and CHI3L1 (rs4950928). HRCG = 2.04 (1.17-3.54).

An interaction between *IL4* and *IL10* loci were of prognostic significance. The isolated effect of *IL10* and *IL4* polymorphisms is somewhat contradictory throughout the literature (see Paper IV), however, these inflammatory mediators play a major role in the GC reaction. B-cell proliferation is enhanced by IL-10 in the presence of IL-4 and both cytokines are involved in the CSR procees[33,181,182]. We found that carriers of the *IL10* (rs1800890) AA in combination with the *IL4RA* (rs1805010) AA genotype had a significantly improved OS (HR=0.11 (0.20-0.50)). The rs1805010 polymorphism is a 'gain-of-function' mutation, which results in substitution of isoleucine for valine (I75V) in the extracellular domain of IL-4Rα subunit. The G variant results in abnormal activity of the STAT6 transcription factor even without IL-4 stimulation[183]. IL-10 has earlier been proposed as an important proliferation factor for lymphoma cells and gene expression of the *IL10* gene has been shown in FL and IL10RA in Waldenström's macroglobulinemia.[184] Results of gene expression analyses are shown in **Figure 13**.
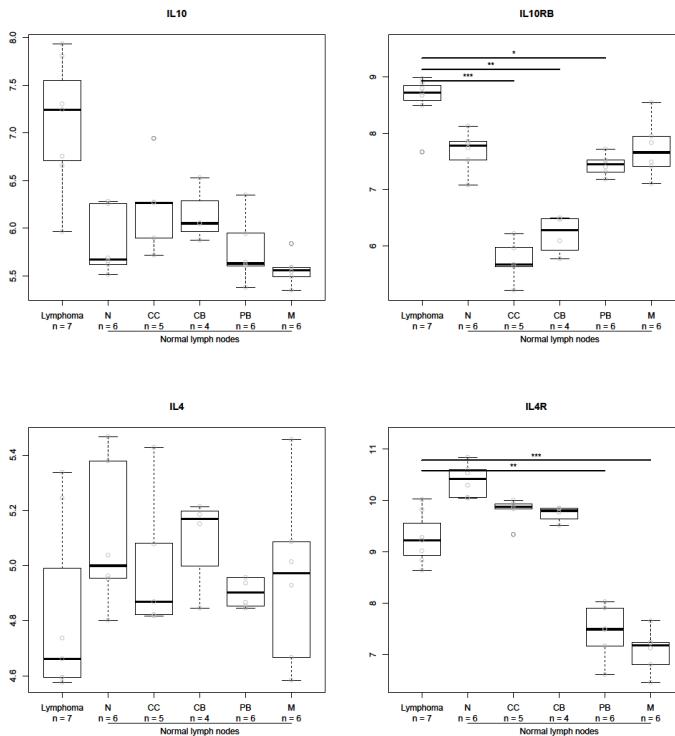
Figure 13. Gene expression analysis of the *IL10, IL10RB, IL4 and IL4RA* genes. The (*) represents significant difference in expression after Bonferroni correction. Gene expression is presented on a log2 scale. Lymphoma: DLBCL B-cells, N: Naïve B-cells, CC: Centrocytes, CB: Centroblasts, PB: Plasmablasts, M: Memory B-cells.

We found an up-regulation of the *IL10* and the *IL10RB* gene in DLBCL cells when compared to normal GC B-cell subpopulations. This supports a role for these cytokines in the pathogenesis of GC derived lymphomas, as supported by earlier findings in chronic lymphocytic leukemia (CLL)[185]. These findings add functional support to the reported protective effect of the low producer *IL10* (rs1800872) AA genotype as well as the protective effect of the *IL10RB* (rs1058867) G allele[82,186]. The decreased expression of the *IL4* gene in DLBCL cells and the lower expression of the *IL4R* gene in post GC B-cell subpopulations suggest an effect of these genes at different stages of the oncogenetic process in B-cell malignancies[103], and that such genes might be important in the multi step process of malignant transformation even if they are not found to be expressed in malignant tissue[21]. Although these data need confirmation in future studies, these two cytokines seem to interact in relation to both risk and outcome. The functional properties of these pathways obviously need further investigation; as an example, the functional properties of *IL4* gene and the distal *IL10* promoter still remain largely unknown as does the effect of these interactions on protein level[134].

LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

Our results were not always consistent with findings in other B-NHL cohorts. This can be a result of small sample sizes as well as ethnical differences between study populations. Our patient cohort was ethnically homogenous with a long follow up period. However, our sample size was under powered for gene-gene interaction analyses making exploration of cytokine networks difficult. Expanding analyses beyond two-way interaction would require a larger patient cohort. Non-regression based statistical methods could also be considered. Another limitation is the lack of functional knowledge for a number of the genes analysed. The age distribution in the control group and patient group was not identical, and the sample material differed between groups. We found no association between age and genotypes within the groups, and we included a cohort with no reported BM involvement; however, these differences between groups still could have introduced bias to our results. Since DLBCL has been shown to consist of different subtypes with regard to gene expression profiles, this also needs to be considered in future genetic studies.

PAPER IV

Inherited variation in immune response genes in follicular lymphoma and diffuse large B-cell lymphoma.

AIM AND DESIGN

We aimed to review the literature to investigate the current knowledge with respect to SNPs in immune regulatory genes in the pathogenesis of B-NHL with focus on the use of the candidate gene approach, the GWAS approach and the pathogenetic effect of gene-gene interactions.

RESULTS AND DISCUSSIONS

After a MESH driven literature search strategy, abstracts were evaluated and 89 Papers were finally included for review. Of these Papers, the majority was related to disease risk (63 Papers) whereas 26 Papers explored genes in relation to outcome.

In brief, we discovered that polymorphisms in the *TNFA* and *IL10* loci were relatively consistently associated with DLBCL risk. Variations in the HLA class I loci at chromosome 6p31-32 were associated with FL risk. In addition variations in the TNFA locus were associated with DLBCL outcome. Although clear differences seem to exist for genetic susceptibility between FL and DLBCL, genes in the *HLA-TNFA-LTA* region on chromosome 6 seem to be a common risk factor in both lymphoma subtypes. GWAS studies will in the future detect many novel genes associated with lymphoma. However, this approach must be combined with carefully designed candidate gene studies, exploring functional properties of investigated genotypes and providing sufficient coverage of the investigated genes. Ethnic differences, effects of rare variants and gene-gene interactions must be included when planning future studies.

LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

Studies were generally heterogeneous in terms of size, ethnic groups, included SNPs, genotyping methods and statistical methods making direct comparison difficult. Functional studies of the investigated genotypes only rarely exist; however,

it would have strengthened our review if functional properties were discussed in depth. We could also have missed studies in our search strategy, and in general, a publication bias could be present. Since minor changes in the expression of genes and the effect of gene-gene interactions can have dramatic effects, these factors need to be addressed in further studies.; it was not possible to thoroughly

review these aspect of genotype/phenotype associations in the present review.

CONCLUSIONS

The research presented in this thesis can be summarized in the following conclusions:

In our first study we established genotyping methods for exploring the functional properties of the *CHI3L1* gene promoter. Furthermore, we discovered a functional SNP strongly associated to serum levels of the proinflammatory YKL-40 protein, a known marker for RA and MM disease activity[140,153,154] and as we recently published, a marker of remission status in NHL[114]. We do acknowledge that our cohorts were small, however, after our studies were published, other groups have explored this functional SNP and confirmed our findings[142,174]. The combination of in vivo functional studies as ours and in vitro functional studies of *CHI3L1* gene transcription clearly points to the -131C/G rs4950928 as a functional SNP. The direct association between this SNP and autoimmune or malignant disease seem less obvious[144,173,175,187]; we did not find association between the *CHI3L1* gene and RA, which was later reproduced[175]. The evidence could suggest, that in inflammatory diseases as RA, the high YKL-40 level is a consequence of the inflammatory process itself more than a causal factor, whereas in malignant diseases *CHI3L1* genotypes may be part of a disease risk genotype[187]. In hematological malignancies the effect of *CHI3L1* genotypes is only scarcely investigated, however, our studies propose the needs for investigation in diseases where YKL-40 expression seems to be of prognostic impact[114,150–152].

In the second study, we evaluated the pathogenetic effect of proposed functional cytokine SNPs in MM. We found low producer *TNFA* genotype associated with decreased disease risk, in accordance with some, but not all earlier findings[55–57,61]. Discrepancy between studies of different sample size, patient characteristics, ethnicity, and methods is well known[134,188,189], and we argue for confirmatory studies sufficiently powered and of multicenter design. External validity is a general challenge in genetic studies as they are often only reproducible in the exact same ethnic groups. Cytokines are expected to be part of a network and not just acting as single independent factors[3,190] why we established a method for studying interactions between pathogenetic genes. Interaction on gene level has been suggested by some authors[178,191–195], and we did find disease outcome related to gene interaction. We were, however, not able to study high order interactions as our sample size was too small to acquire sufficient statistical power. We hypothesized that these inflammatory response genes were of pathogentic impact. We therefore included gene expression analyses of these selected genes in normal B-cell subpopulations and malignant PCs. We were able to display different expression patterns confirming a possible effect of these genes. To further support these findings we

included a retrospective MM cohort of 414 patients and found that expression of the *IL6, IL10* and *CHI3L1* genes were related to prognostic TC classes. We argue that these inflammatory response genes interact and that the effect of them goes beyond pure paracrine stimulatory effects.This has not been investigated earlier and clearly needs confirmation as our study is a small scale explorative study.

In the third study we established a method for using routine FFPE BM samples in SNP array analyses. Extracting sufficient high quality DNA from stored samples is known to be difficult why we evaluated three different methods before deciding on a novel automated method. We then tested 50 highly selected SNPs and investigated single genes, haplotypes and gene-gene interactions. We discovered several novel associations, for example the *CHI3L1* (rs4950928) associated with disease outcome in FL. Moreover, we confirmed earlier findings with regards to SNPs in the *TNFA* and *HLA* region. Interaction analyses pointed to the importance of IL-10 and IL-4 related genes in DLBCL. We also found these genes differently expressed between different B-cell compartments and DLBCL cells. This supports a role for these genes in the step-wise oncogenetic process and suggests that functional aspects should be investigated in the B-cell hierarchy using a "cell of origin" approach. We did not always find results consistent with earlier findings and therefore decided to review the literature of inherited genetic variation in relation to FL and DLBCL. We found some consistence for genes in key inflammatory mediators as *TNFA*, *IL10* and *HLA*, however, we conclude that both novel GWAS studies and carefully designed candidate genes studies are needed to explore new and confirm suspected risk alleles. Moreover, the functional aspects of many SNPs still remain elusive needing further studies. Gene-gene interactions are important factors in future studies and biostatistical expertise are required in the study designs.

PERSPECTIVES

In RA, MM, DLBCL and FL inherited variations in inflammatory response genes seem to have pathogenetic impact[14,44,196]. We added new knowledge to this assumption by studying effects of a novel functional *CHI3L1* variant, and we investigated the expression of these proposed pathogenetic genes in different B-cell subpopulations. We propose an effect of inherited variants in inflammatory tumor/host reaction as well as in direct inherited genetic hits in the process of malignant transformation. Interactions between genes could explain the difficulty in predicting a phenotype from single genotypes, as the system is extremely complex. Combinations of corticosteroids and chemotherapeutic agents have for decades remained the backbone of treatment in MM, NHL and even RA. However in recent years, novel diagnostic approaches using high resolution genetic analysis instead of classic caryotyping together with a broad range of newer treatments as immunomodulatory drugs, cytokine inhibitors, monoclonal antibodies and proteasome inhibitors has been introduced[14,39,197]. Such development can only be achieved by knowing more about the biology in B-cell diseases; genetic analyses could be part of the solution towards true personalized medicine. We have already learnt more about pathogenetic genes from GWAS studies than would have been possible by hypothesis driven studies alone. The combination of biological driven research and the hypothesis free whole genome studies promise new knowledge in the coming years. Future options, including next generation sequencing, miRNA analyses and epigenetic analyses may prove a way to achieve more insight in the biology of B-cell diseases. The findings of cytokine genes being expressed in both diseased tissue and normal microenvironment argues for a more broad effect of these genes by changing signaling mechanisms and providing genetic hits leading a normal B-cell towards a pathogentic pathway. These same genes may also affect the microenvironment and provide a niche for the pathogenetic cells[196]. Genetic studies are typically difficult to interpret due to inconsistence; small study populations are included, techniques differ and the ethnicity of study populations differ - challenges we have faced in our own studies. Novel genotyping approaches, statistical methods and consortium driven analyses as the Myeloma Genetics International Consortium (MAGIC) [198], the International Lymphoma Epidemiology Consortium (InterLymph)[82] and the Wellcome Trust Case Control Consortium[47] now promise the insight we lack from earlier smaller studies. When designing novel studies it is important to have enough resolution in the techniques used to study rare variants. Gene-gene interaction analyses are required to account for the fact that cytokines act in networks[3,189]. We then need to address the functional properties of these genetic variants through in vitro and in vivo model systems. This may clarify associations between proposed pathogenetic variants and gene-expression, protein expression, splicing and epigenetic modifications[134,199–201]. These models must include cells believed to represent normal counterparts of the pathogenetic cells and different

pathogenetic cell lines in combination with in vivo studies of protein expression between individuals with variant genotypes[19,54,166,202,203]. The ultimate goal of our studies is true personalized therapy, first allowing for correct prognostic evaluation and then applying the right drug in the right dose for the right patient. We have taken the first step towards this direction in DLBCL[204] and have planed future studies both as participants in consortium based genetic studies, in vivo studies of cytokine expression in healthy individuals and persons with B-cell diseases and in vitro studies of gene- and protein expression in various cell lines and normal tissue. We believe that these approaches will bring us a more detailed knowledge of the complex biology of B-cell diseases and through this a more rational prognostic classification and treatment strategy.

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APPENDIX

PAPER I

Promoter polymorphisms in the chitinase 3-like 1 gene influence the serum concentration of YKL-40 in Danish patients with rheumatoid arthritis and in healthy subjects. Nielsen KR, Steffensen R, Boegsted M, Baech J, Lundbye-Christensen S, Hetland ML, Krintel SB, Johnsen HE, Nyegaard M, Johansen JS. *Arthritis Res Ther. 2011 Jun 29;13(3):R109. doi: 10.1186/ar3391.*

PAPER II

Interactions between inherited inflammatory response genes are associated with multiple myeloma disease risk and survival. Nielsen KR, Rodrigo-Domingo M, Steffensen R, Baech J, Bergkvist KS, Haunstrup TM, Oosterhof L, Schmitz A, Bødker JS, Johansen P, Dybkær K, Bøgsted M, Vogel U, Johnsen HE, Vangsted A. *Under review in Cancer Genetics*

PAPER III

Inherited inflammatory response genes are associated with B-cell non hodgkins lymphoma risk and survival. Nielsen KR, Steffensen R, Bendtsen MD, Rodrigo-Domingo M, Baech J, Haunstrup TM, Bergkvist KS, Schmitz A, Bødker JS, Johansen P, Dybkær K, Bøgsted M, Johnsen HE. *Accepted for publication in PlosOne*

PAPER IV

Inherited variation in immune response genes in follicular lymphoma and diffuse large B-cell lymphoma. Nielsen KR, Steffensen R, Haunstrup TM, Bødker JS, Dybkær K, Baech J, Bøgsted M, Johnsen HE. *Leuk Lymphoma. 2015 Jul 7:1-10. DOI:10.3109/10428194.2015.1058936*