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Genotype – phenotype relationships in response to external stressors in clonal hematopoiesis and hematologic malignancies

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Abbreviations (Abkürzungen)

ABC	activated B cell-like
AML	acute myeloid leukemia
ANCA	anti-neutrophil cytoplasmic antibody
ARCH	age-related clonal hematopoiesis
ASXL1	additional sex combs-like 1
BCL2	B cell lymphoma 2
BCL6	B cell lymphoma 6
BCL10	B cell lymphoma/leukemia 10
BCR	B cell receptor
BCR-ABL	breakpoint cluster region - Abelson murine leukemia viral oncogene homolog 1
BTK	Bruton's tyrosine kinase
CALR	calreticulin
CARD11	caspase recruitment domain-containing protein 11
CAR-T	chimeric antigen receptor T cell
CBL	Casitas B lineage lymphoma
CCND1	Cyclin D 1
CCUS	clonal cytopenia of undermined significance
CD10	cluster of differentiation 10
CD79A	cluster of differentiation 79A
CD79B	cluster of differentiation 79B
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
cGvHD	chronic graft versus host disease
CHEK2	checkpoint kinase 2
CLL	chronic lymphocytic leukemia
cIAP1	cellular inhibitor of apoptosis 1
cIAP2	cellular inhibitor of apoptosis 2
CHIP	clonal hematopoiesis of indeterminate potential
COO	cell of origin
COPD	chronic obstructive pulmonary disease
DCL	donor cell leukemia
DLBCL	diffuse large B cell lymphoma
DNA	deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
ET	essential thrombocytosis
EZH2	enhancer of zeste homolog 2
FOXP1	forkhead box protein 1
GCB	germinal center B cell-like
GEP	gene expression profiling
GvHD	graft versus host disease
GvL	graft versus leukemia
HSC	hematopoietic stem cell
ICUS	idiopathic cytopenia of undetermined significance
IDH1	isocitrate dehydrogenase 1
IDH2	isocitrate dehydrogenase 2
IGH	immunoglobulin heavy chain
IGHV	immunoglobulin heavy chain variable region
IκB	inhibitor of kappa B
IKK	IκB kinase
IKZF1	Ikaros family zink finger protein 1
IL-1β	interleukin-1 beta
IL-6	interleukin 6

IL-13	interleukin 13
IRAK	interleukin-1 receptor associated kinase
ITAM	immunoreceptor tyrosine-based activation motif
JAK	janus kinase
JAK2	janus kinase 2
MALT	mucosa associated lymphoid tissue
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAP3K14	mitogen-activated protein kinase kinase kinase 14
MCL	mantle cell lymphoma
MDS	myelodysplastic syndrome
MF	myelofibrosis
MPL	myeloproliferative leukemia protein
MPN	myeloproliferative syndrome
mTOR	mechanistic target of rapamycin
MUM1	multiple myeloma 1
MYD88	myeloid differentiation primary response 88
NET	neutrophil extracellular trap
NF1	neurofibromin 1
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NHL	non-Hodgkin lymphoma
NIK	NFκB-inducing kinase
NLRP3	NLR family pyrin domain containing 3
NOS	not otherwise specified
NOTCH2	neurogenic locus notch homolog protein 2
NRAS	neuroblastoma RAS viral oncogene homolog
ORR	overall response rate
PCNSL	primary central nervous system lymphoma
PI3K	phosphoinositide 3-kinase
PKCβ	protein kinase C beta
PLCγ	phospholipase C gamma
PMF	primary myelofibrosis
PPM1D	protein phosphatase 1D
PV	polycythemia vera
PTL	primary testicular lymphoma
RIC	reduced intensity conditioning
RNA	ribonucleic acid
SF3B1	splicing factor 3B subunit 1
SOX11	SRY-related HMG-box 11
SRSF2	serine and arginine rich splicing factor 2
STAT	signal transducer and activator of transcription
STN	sotrastaurin
SYK	spleen tyrosine kinase
TET2	Tet methylcytosine dioxygenase 2
TLR	toll-like receptor
TMN	therapy-related myeloid neoplasm
TNFα	tumor necrosis factor alpha
TP53	tumor protein p53
TRAF2	TNF receptor associated factor 2
TRAF3	TNF receptor associated factor 3
TRAF6	TNF receptor associated factor 6
U2AF1	U2 small nuclear RNA auxiliary factor 1
VAF	variant allele frequency
WHO	World Health Organization

1. Introduction (Einleitung)

Cells interact with their cellular and non-cellular environment. Growth, expansion, survival, and death of a cell depend on a bouquet of cell-extrinsic factors that may be organism-intrinsic (microenvironment) or organism-extrinsic (all environmental influences, *e.g.* viruses, microorganisms, nutrition, toxic agents, medication, radiation). Of note, human cells acquire coding mutations starting at the beginning of life(1, 2), but some resulting phenotypic features will only have selective advantage, increase fitness, or become clinically "visible" under the influence of certain cell-extrinsic stressors. Analysis of these complex genotype – phenotype relations in response to various stressors and in various hematologic settings spanning both premalignant (clonal hematopoiesis) and malignant conditions (diffuse large B cell lymphoma (DLBCL), mantle cell lymphoma (MCL), primary and secondary myelofibrosis (MF), secondary acute myeloid leukemia (AML)) is the overarching topic of this assembly of research papers.

1.1 B cell lymphomas

B cell lymphomas are a heterogeneous group of neoplasms arising from various maturation stages of physiological B cells and respective precursors. Lymphoid neoplasms are classified according to the World Health Organization (WHO) classification. The classification is updated in regular intervals to ensure incorporation of latest clinical and scientific achievements (3). DLBCL and MCL were investigated for this work and described in detail below.

1.1.1 DLBCL, not otherwise specified (NOS)

DLBCL not otherwise specified (NOS) are a heterogeneous group of aggressive lymphomas and disease courses are fatal without treatment. They are the most frequent B cell neoplasms in humans with an incidence of 7/100.000(3, 4). About 50% of patients can be cured with rituximab-based immunochemotherapy(5).

Gene expression profiling (GEP), *i.e.* phenotypic, analyses have revealed distinct subgroups based on the cell of origin (COO) of the neoplasm. "Germinal center B cell-like" (GCB) DLBCL express genes also known to be expressed in normal germinal center B cells. In most cases, they are associated with good response to R-CHOP chemotherapy (rituximab, cyclophosphamide, hydroxydaunorubicine, vincristine, prednisone/prednisolone) and favorable prognosis compared to "activated B cell like"

(ABC) DLBCL. GCB DLBCL often have translocated *BCL2* and carry mutations in *EZH2*(3). ABC DLBCL express genes associated with physiologic activated B cells or plasmablasts. They are typically characterized by chronic nuclear factor kappa B (NFκB) signaling (outlined in detail in 1.1.3), poorer response to rituximab-based immunochemotherapy and unfavorable prognosis(5-7). Hence, great efforts are made to improve dismal outcome of ABC DLBCL, for example, to more specifically target dysregulated NFκB signaling(8-10), which is also outlined in section 1.1.5. A third group of DLBCL does not match criteria for either category(7). Since comprehensive gene expression analyses are difficult to be broadly implemented in routine diagnostics for various reasons, efforts have been made to simplify sub-classification. The "Hans classifier" based on immunohistochemical analysis of CD10, BCL6, MUM1, FOXP1, Cyclin D2, and BCL2 distinguishes "GCB" and "non-GCB" DLBCL(11). Though classification according to the Hans classifier is not completely identical with classification according to GEP, it has turned out to be a useful tool in routine diagnostics(3).

Other phenotypic features of relevance include the expression of MYC and BCL2. Genotypic sub-classification probably yields great potential, but is not yet part of the current WHO classification(3) (also see section 1.1.4.1).

1.1.2 MCL

MCL is a B cell neoplasm mostly characterized by the translocation t(11;14) (q13;q32), which juxtaposes *CCND1* to the *IGH* gene and results in overexpression of Cyclin D1 (12) and can be considered a disease defining event. Patients with MCL are mostly of older age, predominantly male and usually present in advanced stage of disease. SOX11 expression status correlates with clinical course, with absence of SOX11 representing a more indolent disease(3, 13, 14). Depending on clinical features, age, and fitness of the patient, MCL are treated with immunochemotherapy, high dose chemotherapy followed by autologous stem cell transplantation(15) or chemo-free regimens including rituximab, lenalidomide, ibrutinib, and temsirolimus in the relapsed/refractory situation(16-19).

1.1.3 NFκB pathway

Constitutive activation of the NFκB signaling pathway is a key pathogenic event in various B cell malignancies. In general, we distinguish a canonical (or classical) and non-canonical (or alternative) NFκB pathway.

NFκB comprises a family of transcription factors: p50 and its precursor p105, p52 and its precursor p100, p65 (=RelA), RelB, and c-Rel. In B cells, NFκB transcription factors play an important physiological role in the control of genes associated with proliferation, differentiation, survival, apoptosis, and immune and stress response. Usually, they are kept inactive in the cytoplasm by the inhibitor of kappa B (IκB)(20, 21). Upstream activation of NFκB signaling leads to phosphorylation of the α-subunit of IκB (IκBα) by the IκB kinase (IKK)(22), leading to release of the NFκB subunits and subsequent translocation to the nucleus. p65, p50, and c-Rel nuclear homo- or heterodimers are typically activated by signaling through the canonical NFκB pathway. Activation and assembly of p100 (and its degraded form p52) and RelB is seen when the non-canonical pathway is activated(20, 21).

In the healthy B cell, antigen binding to the B cell receptor (BCR) leads to activation of the canonical NFκB pathway by phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR subunits CD79A and CD79B by a SRC family kinase. This leads to further activation of a series of downstream kinases involving SYK, PI3K, BTK, PLCγ, and PKCβ. PKCβ then phosphorylates the scaffold protein CARD11, initiating the formation of a multiprotein complex consisting of CARD11, MALT1, BCL10, and the ubiquitin ligase TRAF6, finally leading to activation of IKK and release of NFκB transcription factors by IκB. Alternatively, the canonical NFκB pathway can be activated through toll like receptors (TLR). The TLR adapter molecule MYD88 then interacts with TRAF6 supported by IRAKs, finally leading to IKK activation and nuclear translocation of NFκB transcription factors (5). A20 is a negative regulator of NFκB activation(23).

Signaling through the non-canonical pathway depends on stabilization of NFκB inducing kinase (NIK = mitogen-activated protein kinase 14/MAP3K14) and subsequent IKKα phosphorylation, finally leading to the partial degradation of p100 to p52 and nuclear translocation of the heterodimer p52/RelB. In the resting cell, NIK is destabilized by a protein complex consisting of cIAP1/2 (=BIRC2/3), TRAF2 and TRAF3. Upon activation through tumor necrosis factor (TNF) family receptors, the cIAP1/2 and TRAF2/3 proteins assemble at the receptor, are subject to ubiquitination and proteasomal degradation, and hence are no longer capable of NIK destabilization(20).

1.1.4 Mutations in B cell malignancies

Due to the central role of NFκB signaling in B cell biology, oncogenic mutations in genes encoding proteins essential for NFκB pathways are a frequently seen in B cell malignancies. In the following, oncogenic mutations in NFκB pathway members in DLBCL and MCL are presented.

1.1.4.1 NFκB mutations in DLBCL

In DLBCL, mutations of NFκB pathway components are frequent. Addiction to chronic NFκB signaling is a key characteristic of mostly ABC DLBCL and respective mutations are therefore predominantly found in this subtype(5). Oncogenic mutations in NFκB pathway members were first discovered using either targeted sequencing of NFκB pathway genes in preselected ABC DLBCL patients samples(24) or RNA interference screens in DLBCL cell lines with subsequent sequencing of genes that had turned out to be vital(25-28). Using this approach, it could be shown that approximately 10% of ABC DLBCL harbor activating mutations in the coiled-coil domain of *CARD11*, resulting in permanent activation of all downstream NFκB pathway activity(26). Likewise, mutations in the ITAMs of either *CD79A* or *CD79B* can be found in approximately 20% of ABC DLBCL, leading to chronic activation of BCR signaling including the NFκB pathway(27). About 30% of all ABC DLBCL harbor the activating *MYD88*L265P mutation and one third of these lymphomas has a concurrent *CD79A* or *CD79B* mutation(28). Interestingly, these mutations seem to cooperate by multiprotein supercomplex formation, explaining the high above average sensitivity of *MYD88/CD79A* or *MYD88/CD79B* double mutant lymphomas to ibrutinib(29). Inactivating mutations in *A20*, a gene encoding a negative regulator of NFκB, can be found in approximately 30% of ABC DLBCL(24).

Mutations in *CD79B*, *MYD88* as well as *CD79B/MYD88* double mutants have been more frequently detected in lymphomas of immune-privileged sites, such as the central nervous system and testes. Various studies report high percentages of mutations in these genes in DLBCL occurring at these sites(30-33).

As outlined above, for two decades, DLBCL were mostly classified according to their gene-expression phenotype, using either array-based classifiers or reduced-scale classifiers such as the Hans classifier(3). However, next generation sequencing techniques have led to more comprehensive genotypic characterization of DLBCL, leading to new genetic – yet preclinical – classifiers of DLBCL. In an

approach by Schmitz and colleagues, 47% of all 574 investigated DLBCL samples could be classified into one of four distinct genetic subgroups. Of note, *MYD88/CD79A* or *MYD88/CD79B* double mutants account for a distinct category termed MCD accounting for 8% of all DLBCL, with 96% of MCD cases being ABC DLBCL by gene expression analysis and 23% of ABC DLBCL can be classified as MCD. Other mutations associated with NFκB signaling were particularly frequent in the BN2 group (defined by *BCL6* fusions and *NOTCH2* mutations) that accounted for 15% of all DLBCL. Interestingly, only 41% of BN2 cases were classified as ABC DLBCL by gene expression data, whereas 40% accounted for "unclassified" cases, indicating that mutations in NFκB pathway components are not exclusively of pathogenic relevance in ABC DLBCL(34). In another study, Chapuy and colleagues reported comprehensive genetic analysis of 304 DLBCL cases and subsequent classification into five different genetic clusters. Comparable to the study by Schmitz and colleagues, mutations of NFκB pathway components were enriched in defined clusters and predominantly found in cluster 1 and cluster 5, which were also enriched for ABC DLBCL(35).

1.1.4.2 NFκB pathway activation in MCL

Chronic signaling through the BCR and activation of the canonical NFκB pathway also plays an important role in the biology of MCL(36, 37). Approximately two thirds of MCL cases are sensitive to BTK inhibition, making ibrutinib a powerful agent in the therapy of MCL(19, 38). Though comprehensive sequencing studies have revealed mutations in BCR/canonical NFκB pathway members such as *A20*(39) or *CARD11*(40, 41), these are not as frequent as in ABC DLBCL and do, for most parts, not explain the addiction of MCL to chronic NFκB signaling(42). Therefore, stimulation of the BCR by (auto)antigens has been discussed as pathogenic mechanism for at least a subset of MCL(37, 43).

Signaling through the non-canonical NFκB pathway is essential for another subset of MCL and identification and description of this pathogenically relevant mechanism including oncogenic mutations is part of this work and will be presented in chapter 2.1.1.

1.1.5 BCR/NFκB pathway inhibition

As signaling through the NFκB pathway is essential for a wide range of B cell lymphomas, selective pharmacologic targeting of pathway components has become a major therapeutic strategy. Inhibition of kinases in close proximity to the BCR – first of all inhibition of BTK – have revolutionized lymphoma

treatment, as they lead to impressive and often long-lasting clinical responses in relevant subsets of patients with B cell lymphoma addicted to chronic NFκB signaling(19, 42, 44, 45). In addition, a series of selective inhibitors used in (pre)clinical studies inhibiting, for example, SYK(46), PKCβ, and IKK, have contributed to deepen our understanding of BCR/NFκB pathway (patho)biology.

One of the clinically most relevant agents is the BTK inhibitor ibrutinib. Ibrutinib covalently binds to BTK and interrupts signaling through the NFκB pathway(47). Clinical trials have documented impressive responses in various B cell lymphomas and is for example standard care in first line treatment of chronic lymphocytic leukemia (CLL)(48, 49). With regard to relapsed or refractory ABC DLBCL, an overall response rate (ORR) of 37% and a complete response rate of 10% was reported. In contrast, GCB DLBCL only have an ORR of 5%(44). In relapsed or refractory MCL, the ORR is described as 68-78% and complete response rate as 21-33%, respectively(19, 45). By now, the more selective, second generation BTK inhibitors acalabrutinib, tirabrutinib, and zanubrutinib have gained or are in process of gaining agency approval(50, 51).

The kinase PKCβ is located downstream of BTK and its activity is essential for activation of the canonical NFκB pathway. Inhibition of PKCβ by the PKCβ inhibitor enzastaurin has shown therapeutic benefit in some patients with relapsed or refractory DLBCL(52) and MCL(53), but maintenance therapy with enzastaurin in high-risk DLBCL did not improve disease free survival in a randomized phase III trial(54). In addition, in preclinical studies, the PKCβ inhibitor sotrastaurin (STN) has shown activity in ABC DLBCL cell lines with mutant *CD79A* or *CD79B*, but not in those with mutant *CARD11*, again highlighting the addiction of ABC DLBCL to chronic NFκB signaling. As CARD11 is located downstream of PKCβ, PKCβ inhibition remains without consequence in the presence of mutant *CARD11*(55). As described in detail in part 2.1.1 of this work and in a later study by Rauert-Wunderlich and colleagues, sotrastaurin is also effective in a subset of MCL(56, 57).

As outlined above, both canonical and non-canonical NFκB pathways lead to IKK activation and subsequent translocation of NFκB subunits to the nucleus. Hence, IKK inhibition is toxic to lymphomas addicted to either pathway activation. As clinical safety profiles of IKK inhibitors of these agents seem

to be rather disadvantageous, implementation of IKK inhibitors in the clinical setting remains problematic(58). However, they are valuable tools in preclinical research(25, 55, 56).

1.2 Clonal hematopoiesis

Clonal hematopoiesis is a condition – not disease – defined by the presence of acquired, cancer-associated mutations in the blood of predominantly elderly individuals without hematologic malignancy. It first gained broad attention when in 2014 three large studies almost in parallel reported this phenomenon in huge population studies comprising more than 32,000 individuals(59-61). Mutations appear in an age-dependent manner with a strong increase in mutational frequency in the elderly population. We now know that a substantial part of all individuals older than 60 years harbor clonal hematopoiesis(59, 60, 62). When reporting on clonal hematopoiesis, different definitions and terminologies have been established, complicating reports on frequencies and prevalence. While "clonal hematopoiesis" describes the condition in general, "clonal hematopoiesis of indeterminate potential" or "CHIP" is used only if the respective clone has a variant allele frequency (VAF) of at least 2%(63). It is estimated that about 10-20% of individuals of 60 years and older harbor CHIP(64). In contrast, "age related clonal hematopoiesis" or "ARCH" is used without VAF cut-off(65) and depending on sequencing depth, mutations with a very low VAF can be detected in almost all individuals(66). Though a VAF cut-off of 2% as used for CHIP could be to a certain extent perceived as arbitrary, it mirrors considerations regarding biological relevance and technical limitations of reliable detection of clones at the time this definition was established. Discussion about the meaningfulness of VAF cut-offs is still controversial and from a technical point of view the detection of much smaller clones is now possible with acceptable effort(62, 65). However, the classical definition of CHIP is widely used and published and all publications on clonal hematopoiesis that are part of this work (2.2.1 and 2.2.2) use the definition of CHIP.

CHIP mutations can be predominantly found in several functional groups of genes, comprising epigenetic modifiers, genes associated with the spliceosome, signal transduction, and DNA repair. Epigenetic modifiers regulate transcription by modification of DNA and histones. They constitute the largest group of mutated genes with *DNA-methyltransferase 3A* (*DNMT3A*), *Ten-Eleven Translocation-2* (*TET2*), and *Additional sex combs-like 1* (*ASXL1*) accounting for most mutations in this group.

DNMT3A is the gene that is by far most frequently mutated in CHIP(59-61). It encodes a *de novo* DNA methyltransferase that modifies unmethylated DNA(67). TET2 catalyzes the conversion of 5-methyl-C (5mC) of DNA to 5-hydroxyl-methyl-C (5hmC), a process regulating DNA methylation patterns(68). In pre-clinical models, mutations in *DNMT3A* and *TET2* lead to increased self-renewal capacities, reduced differentiation (*DNTM3A* and *TET2*), and immortalization (*DNMT3A*) of hematopoietic stem cells (HSCs), but rarely cause full malignant disease(68-72). In contrast, *ASXL1* mutations cause hypocellularity and myelodysplasia(73). Other pathways associated with CHIP involve the spliceosome (e.g. *SF3B1, SRSF2, U2AF1*), DNA repair (e.g. *TP53, PPM1D*), and signal transduction (e.g. *MYD88, JAK2*)(59, 60, 74). As outlined in detail below, these mutations are also commonly found in hematological malignancies like AML, myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), and lymphomas(75-78)

Though clonal hematopoiesis itself is not a disease, it is associated with an increased risk for various adverse medical conditions, as increased risk of hematologic cancer and cardiovascular disease, finally leading to a significantly decreased overall survival(59, 60).

1.2.1 Clonal hematopoiesis as premalignant condition

Clonal hematopoiesis is a premalignant state, as individuals harboring this condition have a substantially increased risk to develop a full hematologic malignancy. Though most individuals with clonal hematopoiesis will never be diagnosed with hematologic disease, hazard ratio for the development of blood or lymphoid cancer is clearly above 10 (11.1 in (60) or 12.9 in (59)). Pathogenically, this is explained by the stepwise acquisition of additional genetic aberrations that increase clonal fitness and lead to malignant transformation(63, 74). Of note, various external stressors can promote this disadvantageous evolution as further explained in section 1.4.2.1.

Newer studies have refined the characteristics of clonal hematopoiesis that promote leukemic progression to AML, underlining the heterogeneous nature of the conditions subsumed under this term. They report mutations in genes associated with the spliceosome (e.g. *U2AF1*, *SRSF2*, *SF3B1*), *IDH1, IDH2,* and *TP53* to be associated with higher risk of leukemic transformation(79-81). With regard to the most frequently mutated genes *DNMT3A* and *TET2*, Abelson and colleagues could not find an increased risk of AML(79). In contrast, the studies performed by Desai and colleagues could find an increased

risk of leukemic progression for *DNMT3A* and *TET2* mutations(80), while Young and colleagues identified an increased risk for *DNMT3A* R882H/C mutations only(81). In addition, clone size and number of mutations were uniformly reported to increase the risk of transformation(79-81).

With regard to unexplained cytopenias that do not fulfill the diagnostic criteria of MDS, discovery of CHIP led to the newly established term "clonal cytopenia of undetermined significance" (CCUS), which comprises a subgroup of "idiopathic cytopenia of undetermined significance" (ICUS) patients who have co-occurring CHIP. In this definition, CCUS is a condition intermediate between CHIP and MDS(63).

1.2.2 Clonal hematopoiesis and its interrelatedness with inflammatory conditions

Individuals with CHIP are at much higher risk to suffer from cardiovascular disease(60, 82) and other inflammatory processes such as chronic obstructive pulmonary disease (COPD)(83, 84) and ANCA associated vasculitis(85).

While it was first assumed that the presence of CHIP simply might be indicative of a (pre-)aged phenotype with increased risk for age-associated conditions including cardiovascular diseases, we now consider CHIP to be part of a complex interplay between clonal mutations, inflammation and aging ("inflammaging")(86-88).

In vitro functional research has proven a direct pathogenic link between CHIP and atherosclerosis for mutations in the *TET2* gene. Fuster and colleagues were the first to show that atherosclerosis-prone, low-density lipoprotein receptor–deficient (Ldlr–/–) mice with partial Tet2 deficiency had an accelerated course of atherosclerosis compared to control mice. Of note, VAFs of the mutant *Tet2* were comparable to mean VAFs observed in humans. Pathogenically, the increased size of atherosclerotic plaques could be explained by altered monocyte/macrophage activity by showing increased NLRP3 inflammasome–mediated interleukin-1β (IL-1β) secretion and a consecutive enhanced chronic inflammatory condition(89). Similar functional studies were reported by Jaiswal and colleagues with concordant results(82). In line with these results, in an exploratory analysis, patients with mutant *TET2* showed a superior response to therapy with the monoclonal antibody against IL-1β canakinumab following myocardial infarction and increased levels of C-reactive protein(90, 91).

With regard to the most frequently mutated gene *DNMT3A*, *in vivo* analyses in mice with CRISPR-mediated *Dnmt3a* inactivation have shown increased evasion of the myocardium by macrophages and increased expression of inflammation markers following angiotensin II induced cardiac dysfunction, probably leading to impaired resolution of inflammation(92). This observation could potentially explain the higher re-hospitalization rate and poorer outcome of patients with *DNMT3A* mutations and chronic heart failures(93). In summary, however, functional connections between *DNMT3A* and cardiovascular disease are yet less clear than for mutant *TET2*. For *JAK2* mutations, elevated risk of cardiovascular disease(82) and thrombotic events is described, probably due to increased early lesion formation and neutrophil extracellular traps (NET)(87, 94). Likewise, in a murine model, atherosclerotic plaque formation and inflammation were enhanced by the presence of *Jak2*V617F mutation(95).

With regard to other frequently mutated genes such as *ASXL1* and *SF3B1* very little is known about the impact on innate immune cells/inflammation and causal connection with cardiovascular disease(87). Therefore, in summary, description of the interrelatedness of CHIP, aging and inflammatory conditions like atherosclerosis is still in the beginning.

1.3 Myelofibrosis

Myelofibrosis (MF) is a rare disease with an incidence of about 1.5/100,000 persons/year. It affects mostly elderly individuals who typically present with hepatosplenomegaly, anemia, early satiety and hypercatabolic symptoms including fatigue, fever, night sweats, bone pain, and weight loss(96). MF belongs to the classical BCR-ABL1-negative chronic myeloproliferative neoplasm (MPN). This group of hematologic disorders also includes essential thrombocythemia (ET) and polycythemia vera (PV)(97). MF can either appear *de novo* (primary MF [PMF]) or following ET or PV (post-ET or post-PV MF)(98). Pathogenically, it is a clonal proliferation of pluripotent hematopoietic stem cells. The abnormal cell population releases cytokines and growth factors in the bone marrow that lead to marrow fibrosis and stroma changes, alterations that finally lead to colonization of extramedullary organs such as the spleen and liver by hematopoietic cells. Leukemic transformation occurs in about 20% of MF patients and is a detrimental factor for the high variability observed in the clinical course of MF patients(96).

1.3.1 Mutational landscape of myelofibrosis

MPN are characterized by constitutive activation of the JAK-STAT (janus kinase-signal transducer and activator of transcription) pathway caused by somatic mutations. Approximately 50-60% of PMF and ET patients carry the activating V617F mutation in *janus kinase 2* (*JAK2)*. In PV, even 95% of patients harbor the *JAK2* V617F mutation(99-101). Frequent copy-number neutral loss of heterozygosity of a fragment of the short arm of chromosome 9 (9pLOH) leads to homozygosity of *JAK2* mutations (101, 102). JAK2 is a non-receptor tyrosine kinase and *JAK2* V617F mutations leads to constitutive activation of the associated the pathway involving STATs, ultimately resulting in enhanced cell proliferation(103). Mutations in calreticulin (*CALR*) and the thrombopoietin receptor (= myeloproliferative leukemia protein/*MPL*) have also been identified to be pathogenically relevant in *JAK2* wildtype MPN. These mutations likewise lead to constitutive activation of JAK-STAT signaling(77, 104-106) and are usually mutually exclusive(107). *CALR* mutations can be found in approximately 25-30% of PMF(77, 104, 107).

Though activation of the JAK-STAT pathway is considered to be the key pathogenic event in BCR-ABL negative MPN(107), *JAK2* mutations can sometimes be found in the healthy elderly population with clonal hematopoiesis(59, 60) (see section 1.2), indicating that additional pathogenic events are necessary for disease initiation and progression of myelofibrosis. Comprehensive, next generation sequencing studies have revealed the complex and diverse mutational landscapes underlying MF. Most mutated genes are not specific for MF, as recurrent mutations can be found in other hematologic neoplasms and clonal hematopoiesis. Epigenetic modifiers, particularly *TET2, ASXL1*, *DNMT3A, IDH1, IDH2* and *EZH2* and genes encoding components of the spliceosome machinery like *SRSF2*, *SF3B1* and *U2AF1* are the functional groups most frequently mutated(77, 107-109). These mutations often seem to shape the course of the disease in terms of progression and/or transformation to secondary AML. Progression to secondary AML is an extremely disadvantageous event in MF and is associated with the presence of mutations in various genes, as for example *ASXL1, EZH2, SRSF2, IDH1/2, CBL, NRAS, NF1, TP53*, and *IKZF1* (107, 108, 110).

1.3.2 Current treatments of myelofibrosis

Currently, allogeneic stem cell transplantation is the only curative treatment available for MF(111). As two large phase III trials (COMFORT-I and COMFORT-II) highlighted, the oral JAK inhibitor

ruxolitinib can effectively control constitutional symptoms and splenomegaly. Interestingly, response to treatment with JAK inhibitors is effective in MF patients with and without a *JAK2*V617F mutation. (112, 113). However, ruxolitinib does not appear to reduce the rate of leukemic transformation(113, 114). In addition, ruxolitinib-therapy rarely leads to molecular remissions in MF. Changes in *JAK2* allele burden are usually minor and do not mirror the clinical benefit(115, 116).

1.4 Genotype – phenotype relationships in response to cell-extrinsic stressors

While genotype is defined as the nucleic acid or polypeptide sequence, phenotype is understood as the trait of a cell or organism. Phenotypic descriptions can range from molecular to organism scale. Mapping of genotype – phenotype relationships has multiple implications. In hemato-oncology, it is of great use to characterize the fitness – or clinical aggressiveness – of individual mutations or to predict response to pharmacological measures(117). In this line, genotype – phenotype correlations are also of high interest when studying the influence of cell-extrinsic stressors.

Though the differentiation of cell-extrinsic stressors into organism-intrinsic and organism-extrinsic factors has its limitations due to border-crossing effects (*e.g.* microenvironmental changes induced by chemotherapy or infection), the following explanations make use of this categorization for reasons of clarity.

1.4.1 Organism-intrinsic factors: the microenvironment

(Tumor) microenvironment is the non-malignant surrounding of (pre)malignant cells, including stromal and immune cells, extracellular matrix and cytokines. In the last years we have come to understand that cancer cells cannot be observed in an isolated fashion, but that the surrounding plays a vital role for the fate of a malignant cell(118).

1.4.1.1 Lymphoma microenvironment

Though lymphoma are the malignant counterparts of normal lymphocytes and hence are often found in structures related to the immune system such as lymph nodes, spleen and bone marrow, they can occur at virtually all sites of the human body(3). With regard to DLBCL, occurrence at various specific localizations – predominantly extranodal sites – is associated with characteristic genetic lesions(34),

pinpointing to selection and survival benefits of malignant cells with certain unique phenotypic features in a certain microenvironment. While certain microenvironments such as the gastrointestinal system provide a multitude of allo-antigens by ingested food and microorganisms(119), barrier-protected, immune-privileged tissues, provide a relatively stimulus-poor microenvironment with additional protection against pathogens and inflammatory processes(31, 120). In this work, relation of primary anatomical occurrence of DLBCL and association with certain genetic alterations was investigated (section 2.1.2).

1.4.1.2 CHIP and the microenvironment

It is estimated that protein coding mutations in HSC occur with a rate of 1 in 10 years per HSC(121, 122). Most of these mutations are considered to be neutral or disadvantageous to the HSC and its progeny and hence do not play a major role in the constitution of the blood cells. However, in some cases, mutations confer selective advantage to the cells thus leading to measurable clonal expansion and over-representation of a certain clone within the pool of blood cells(123-125). While cell-intrinsic mechanisms of selective advantage like increased self-renewal and proliferation have been well-described (described in detail in section 1.2), cell-extrinsic factors leading to selective, over-proportional growth and evolution also need to be considered. The bone marrow niche – physiological home of the HSC – changes with age and under environmental influences with regard to composition of the cellular and extracellular components as well as to the cytokine milieu. Specifically, aging is accompanied by chronic, low-grade inflammation, measurable, for example, by increased levels of TNF-α, IL-6, IL-1β, and C-reactive protein (122, 126-128). Interestingly, the presence of clonal hematopoiesis was shown to be associated with increased levels of IL-6(129) and TNF-α(130). In addition, various preclinical mouse studies have shown a preferential expansion *Tet2* mutant cells in the presence of inflammatory cytokines(131, 132) and of *Dnmt3a* mutant HSCs in the aged bone marrow(133). In summary, these observations pinpoint to the fact that certain phenotypic features of the mutated cells gain selective advantage and favor clonal expansion in the aging and/or inflamed bone marrow.

1.4.2 Therapeutic interventions as extrinsic stressors

1.4.2.1 Chemotherapy and radiation therapy

Chemotherapy and/or radiation therapy are key components of almost all anti-cancer therapies. However, their mode of action is not specific to cancer cells, as almost all rapidly dividing cells – malignant or benign – are swept away. Therefore, application of chemotherapy (and radiation, if bone marrow is within the radiation field) and subsequent blood cell reconstitution imposes strong replicative stress on the hematopoietic cells(134). This fact raises the question whether subtle differences in the HCS clones, *e.g.* clonal hematopoiesis, gain relevance by the bottleneck effect caused by the therapeutic measures. Possible scenarios include a differential sensitivity to the therapeutic agent, hence leading to different degrees of extinction or, in case of equal sensitivity, competitive advantage and over-proportional expansion of a clone during reconstitution(135). Before our study was published (section 2.2.1), three papers had described an association of therapy related myeloid neoplasms with the presence of clonal hematopoiesis before initiation of primary chemotherapy(136-138). *TP53* was identified as a gene conferring particular vulnerability for TMN to the affected patients(136, 137). Regarding the potential association with other complications associated with (radio-)chemotherapy, as for example necessity of dose reduction, transfusions, and neutropenic complications, no systematic analyses were published to the best of our knowledge.

1.4.2.2 Targeted therapy

While chemo- or radiotherapy are rather unspecific in their mode of action, the opposite is true (and already implied in the terminology) for targeted therapies. Effectiveness of targeted therapies first requires a powerful target that is key to oncogenic signaling or other oncogenic mechanisms, often compared with Achilles' heel. Once the target is identified and attacked, malignant cells are usually deprived of their basis of survival. However, even with the same diagnosis, usually not all patients respond to targeted therapies, indicating that for a subset, the stressor "targeted therapy" is not effective due to primary resistance. Primary resistance is often difficult to predict with current routine diagnostics – though efforts to broadly implement precision medicine make this a realistic vision – and identification of underlying mechanisms is often arduous(139, 140). Concerning MCL, ibrutinib is a powerful therapeutic component, but about one third of MCL do not respond to ibrutinib indicating primary

resistance(19). Our study introduced in section 2.1.1 untangles the underlying genotypic differences and suggests alternative targets.

Secondary resistance is frequent and implies that the malignant cells lose sensitivity of a previously effective treatment. In this scenario, clonal evolution and dynamics come into the play. Secondary resistance implies that malignant diseases are not static, but change due to selective pressure of a therapeutic agent. There are at least four scenarios for secondary resistance: Resistance of the target by mutations, re-activation of the oncogenic pathway by mutations in downstream pathway-components, bypass of the oncogenic pathway, and transformation of the underlying disease(141). Clonal dynamics and tumor evolution under the JAK inhibitor ruxolitinib were studied in section 2.2.3.

1.4.2.3 Hematopoietic stem cell transplantation

Autologous and allogeneic HSC transplantation constitute a very exceptional therapeutic setting, as hematopoiesis is completely (myeloablative) or nearly completely (reduced-intensity conditioning/RIC) eradicated by conditioning therapy and rebuilt by the own (autologous) or foreign (allogeneic) HSCs transplanted after completion of conditioning therapy. In both settings, HSCs are confronted with a bone marrow microenvironment altered by the preceding conditioning therapy, in the case of allogeneic transplantation even in a foreign setting. In addition, patients after HSC transplantation are highly vulnerable for infections and many other adverse events, and receive plenty of supportive medication(142). All these circumstances can potentially influence hematopoietic reconstitution and the role of clonal hematopoiesis in this setting was almost unexplored. Before our study (presented in 2.2.2) it had been shown for autologous transplantations that the presence of clonal hematopoiesis in the transplanted HSCs, was associated with an increased risk of TMN, with mutations in *TP53* and *PPM1D* being of particular relevance(74). In addition, unexplained cytopenias after allogeneic transplantation were shown to be often due to donor clonal hematopoiesis(143). Case reports hinted at an increased risk of donor cell leukemia (DCL) derived from clonal mutations in the transplanted HSCs(144). Of note, by the nature of allogeneic transplantation, it is difficult to draw clear boundaries with regard to the rather artificial categorization of “organism-intrinsic” and “organism-extrinsic” stressors, particularly with regard to foreign microenvironmental influences. However, leaving semantic discussions behind, the investigation of the role of donor clonal hematopoiesis in the context of allogeneic transplantation

is of high clinical relevance. As the age of graft recipients steadily increases due to improvements in the peri-transplantational setting(145), the number of elderly related donors rises in parallel(146), leaving us with a growing pool of donors with high risk of clonal hematopoiesis. With regard to important outcome parameters like overall survival, relapse risks, severe complications like acute and chronic graft versus host disease (GvHD), and infections, no data was available before our study.

2. Own original work (Eigene Arbeiten)

Scientific questions

The following, scientific questions connect the individual pieces of research presented in this collection of papers:

1) Which are the genotypic and phenotypic/functional differences in lymphoma sharing the same diagnosis (MCL) but showing differential sensitivity to targeted therapy? (section 2.1.1)
2) In various specific hematologic circumstances (DLBCL, CHIP), are certain genotypes/phenotypes selected or gain advantage in a specific microenvironment (specific anatomical location, altered bone marrow after allogeneic transplantation) and what are the clinical consequences? (sections 2.1.2 and 2.2.2)
3) In various specific hematologic conditions (CHIP, myelofibrosis), are certain genotypes/phenotypes selected or gain advantage under therapeutic pressure (*i.e.* (radio-)chemotherapy, allogeneic transplantation, targeted therapy) and what are the clinical consequences? (sections 2.2.1 – 2.2.3)
4) How do genotypes evolve under selective pressure on their phenotype? (sections 2.2.1 -2.2.3)

2.1 Genotype – phenotype relationships in B cell lymphoma

2.1.1 Genotypic differences in MCL cause differential sensitivity to inhibition of BCR/NFκB signaling

Rahal R*, **Frick M***, Romero R, Korn JM, Kridel R, Chan FC, Meissner B, Bhang HE, Ruddy D, Kauffmann A, Farsidjani A, Derti A, Rakiec D, Naylor T, Pfister E, Kovats S, Kim S, Dietze K, Dörken B, Steidl C, Tzankov A, Hummel M, Monahan J, Morrissey MP, Fritsch C, Sellers WR, Cooke VG, Gascoyne RD, Lenz G, Stegmeier F.
Pharmacological and genomic profiling identifies NF-κB-targeted treatment strategies for mantle cell lymphoma. Nature Medicine. Jan 2014, 20(1):87-92. DOI: 10.1038/nm.3435 ***equal contribution**

My research on genotypic-phenotypic relationships started with a study investigating primary drug resistance in MCL with respect to targeted therapies attacking BCR/NFκB signaling. From clinical observations, it was reported that about two thirds of MCL patients respond extraordinary well to BCR/NFκB inhibition with the BTK inhibitor ibrutinib while about one third of patients had no clinical benefit(19). The molecular background for these diverging responses was unknown. Our data from pharmacological profiling using 119 cell lines from hematological malignancies and 16 compounds were in line with these reports, as only a subset of MCL cell lines was sensitive to BCR/ NFκB inhibition with the PKC inhibitor sotrastaurin and the BTK inhibitor ibrutinib. Further functional studies confirmed two distinct phenotypes of MCL: One that was sensitive to BCR/NFκB inhibition and one that was not. Surprisingly, both MCL types were sensitive to IKKβ/NFκB inhibition, indicating addiction to NFκB signaling across all MCL cell lines. Transcriptome sequencing revealed mutations in components of the non-canonical NFκB pathway in insensitive cell lines, and extensive functional analyses confirmed addiction of ibrutinib/STN insensitive cell lines to non-canonical NFκB signaling. Targeted sequencing of 165 MCL patients confirmed mutations in *BIRC3*, *TRAF2*, and *MAP3K14* (all components of the non-canonical NFκB pathway) in 15% of patients.

Therefore, our study provides an in-depth genetic and functional dissection of MCL with different phenotypic responses (highly sensitive versus insensitive) with respect to inhibition of BCR/NFκB signaling. To our knowledge, it was the first publication describing and providing functional analyses regarding activation of the non-canonical NFκB pathway by oncogenic mutations as an alternative pathogenic mechanism in MCL.

https://doi.org/10.1038/nm.3435

2.1.2 Anatomical location of DLBCL is associated with distinct genetic features

Frick M, Bettstetter M, Bertz S, Schwarz-Furlan S, Hartmann A, Richter T, Lenze D, Hummel M, Dreyling M, Lenz G, Gaumann A.
Mutational frequencies of CD79B and MYD88 vary greatly between primary testicular DLBCL and gastrointestinal DLBCL. Leukemia & Lymphoma. May 2018, 59(5):1260-1263
DOI: 10.1080/10428194.2017.1370546

In our next study, we investigated distinct genetic features associated with chronic canonical NFκB pathway activation with regard to anatomical presentation of DLBCL. In total, 335 DLBCL samples were screened for mutations in *CD79A*, *CD79B*, and *MYD88* using Sanger sequencing or real time PCR high resolution melting analysis (HRMA). Our study cohort included 208 nodal DLBCL and 95 extranodal DLBCL, of which 15 were primary testicular lymphoma (PTL) and 38 had gastrointestinal manifestation. Interestingly, mutational frequencies in CD79A, *CD79B*, and *MYD88* greatly varied in the subgroup of extranodal DLBCL. While mutations in the respective genes were frequent in PTL (*CD79B*:10/14 evaluable, *MYD88*:11/14 evaluable), they occurred clearly below average in the subgroup of DLBCL with gastrointestinal manifestation (*CD79B*: 2/37 evaluable, *MYD88*:1/35 evaluable). In the subgroup of PTL, lymphoma with mutations in both *CD79B* and *MYD88* were particularly frequent (9/13 evaluable for both genes), pinpointing to an addiction to canonical NFκB pathway activation, which is even enhanced by known synergistic effects of these mutations(29). With regard to DLBCL, subclassification into GCB/ABC DLBCL (GEP) or GCB/non-GCB DLBCL (Hans-classifier), PTL were mostly of ABC/non-GCB origin, which is in line with reports linking mutations in *CD79A*, *CD79B*, and *MYD88* with ABC DLBCL phenotype(27, 28).

Our findings support the idea that specific genetic lesions and associated phenotypes gain selective advantage in a specific microenvironment. While testes are considered an immune-privileged site, the opposite is true for the gastrointestinal system, where a multitude of antigens from food and microorganisms is present.

In view of possible clinical implementations, our study could be of help to identify DLBCL subpopulations suitable for further investigation of pharmacological BCR/NFκB pathway inhibition based on their anatomical presentation.

https://doi.org/10.1080/10428194.2017.1370546

2.2 Clinical impact, clonal dynamics and evolution in response to extrinsic stressors in CHIP and myelofibrosis

With few exceptions, hemato-oncologic treatment strategies can be classified as organism-extrinsic stressors. Therapeutic measures used in cancer patients undoubtedly impose heavy external stress on hematopoietic stem cells. In order to investigate if and how these measures interfere with the clonal dynamics, evolution and selection processes of CHIP and myelofibrosis, and to objectify if these processes have an impact on important standard clinical outcome parameters, the following three studies were performed. Of note, all three studies include serial measurements of patient samples to observe long-term clonal dynamics and determine the impact of various stressors on a time dimension scale.

2.2.1 Role of CHIP in the context of radio-chemotherapy

Arends CM, Galan-Sousa J, Hoyer K, Chan W, Jäger M, Yoshida K, Seemann R, Noerenberg D, Waldhueter N, Fleischer-Notter H, Christen F, Schmitt CA, Dörken B, Pelzer U, Sinn M, Zemojtel T, Ogawa S, Märdian S, Schreiber A, Kunitz A, Krüger U, Bullinger L, Mylonas E, **Frick M***, Damm F*. *Hematopoietic lineage distribution and evolutionary dynamics of clonal hematopoiesis*. Leukemia. Sep 2018, 32(9):1908-1919 DOI: 10.1038/s41375-018-0047-7 ***equal contribution**

As CHIP is a frequent condition in the elderly population, additional information on potential effects of CHIP in the setting of anti-cancer treatments is urgently needed. This is particularly true, as cancer incidence likewise increases with age(147). To our knowledge, this study was the first to investigate the immediate effects of CHIP and (radio-)chemotherapy on elderly patients.

In our study, 72 patients with non-hematological cancer who were 55 years of age or older were screened for the presence of CHIP prior to (radio-)chemotherapy initiation using a targeted gene panel covering genes frequently mutated in clonal hematopoiesis and hematologic malignancy (TruSight Myeloid Panel and Customized TruSight Myeloid Panel , Illumina, San Diego, USA). Twenty-two patients harbored a total of 33 mutations, with *DNMT3A* (n = 13) and *TET2* (n = 5) being the most frequently mutated genes. Mutations were verified using an amplicon based targeted deep sequencing approach or digital droplet PCR. Follow-up samples were obtained after several cycles of (radio-)chemotherapy and 32 of the 33 mutations could be tracked by targeted deep sequencing over treatment courses. We could observe three different patterns of clonal dynamics: While 12 of 13 *DNMT3A* mutations remained stable (defined as

< 50% change in baseline VAF), suggesting minor clinical relevance, we could observe clonal expansion in six cases including a *PPM1D* mutation. In contrast, seven clones had a decreasing VAFs, including all three cases with mutated *SF3B1*.

Correlation with clinical data indicated that patients with antecedent CHIP required chemotherapy dose reductions more frequently. Taking solely *DNMT3A* mutated cases aside due to postulated minor clinical relevance, patients with CHIP mutations other than *DNMT3A* were significantly more likely to present with lower hemoglobin levels after six cycles of chemotherapy ($P = .017$) and hence to be in the need of red blood cell transfusions ($P = .025$). Though more homogeneous, larger, and prospective studies are needed, these findings indicate that depending on the mutated gene, CHIP does have an impact on the immediate clinical outcome, as clonal mutations seem to be capable of influencing the way HSCs react to the external stressor "radio-chemotherapy".

Apart from analysis of clonal dynamics in the context of (radio-)chemotherapy, our study also provided valuable insights into clonal expansion patterns within the hematopoietic differentiation tree using targeted sequencing of flow-sorted hematopoietic subfractions. Major findings are the predominant expansion of CHIP mutations into the myelo-monocytic compartment, with underrepresentation in B and particularly T cells, but not NK cells, and, in cases with more than one mutation, expansion patterns indicating both monoclonality and oligoclonality.

https://doi.org/10.1038/s41375-018-0047-7

2.2.2 Role of donor CHIP in the context of allogeneic hematopoietic stem cell transplantation

Frick M*, Chan W*, Arends CM, Hablesreiter R, Halik A, Heuser M, Michonneau D, Blau O, Hoyer K, Christen F, Galan-Sousa J, Noerenberg D, Wais V, Stadler M, Yoshida K, Schetelig J, Schuler E, Thol F, Clappier E, Christopeit M, Ayuk F, Bornhäuser M, Blau IW, Ogawa S, Zemojtel T, Gerbitz A, Wagner EM, Spriewald BM, Schrezenmeier H, Kuchenbauer F, Kobbe G, Wiesneth M, Koldehoff M, Socié G, Kroeger N, Bullinger L, Thiede C, Damm F.
Role of donor clonal hematopoiesis in allogeneic hematopoietic stem cell transplantation. Journal of Clinical Oncology. 2019 Feb 10;37(5):375-385. DOI: 10.1200/JCO.2018.79.2184 ***equal contribution**

In a second paper, we explored the impact of donor CHIP in the setting of allogeneic HSC transplantation from a related older donor. While HSCs are not directly exposed to high doses of chemotherapy in the setting of allogeneic transplantation, they have to engraft and proliferate in a foreign microenvironment altered by conditioning regimen and frequent infections(142). These circumstances likely impose heavy stress on the transplanted HSCs and the role of donor CHIP in this setting was almost unexplored before our study. One study related donor CHIP to unexplained cytopenias after transplantation(143) and case report data linked donor CHIP and donor cell leukemia(144). However, systematic and comprehensive analyses regarding various essential standard transplantation outcome parameters and clonal dynamics were lacking.

We therefore designed a retrospective study including 500 older (55 years or older) related donors from nine transplantation centers in Germany and France. Donors were screened for CHIP in pre-transplant blood samples using customized versions of the Trusight Myeloid Panel (Illumina, San Diego, USA), and donor CHIP status was correlated with standard outcome parameters. Whenever available, mutations were tracked in the recipient post transplantation.

Ninety-two clonal mutations were detected in 80 donors with a median VAF of 5.9%. While overall survival was not affected with regard to donor CHIP status (HR: 0.88; 95% CI, 0.65 to 1.321; $P = .434$), cumulative incidence of chronic GvHD was significantly increased (HR: 1.73; 95% CI, 1.21 to 2.49; $P = .003$). On the contrary, cumulative incidence of relapse or progression (CIRP) was significantly lower in the presence of donor CHIP (HR, 0.62; 95% CI, 0.40 to 0.97; $P = .027$). These findings remain significant after adjustment for confounders and are indicative of an increased inflammatory activity of

or evoked by donor CHIP in the setting of allogeneic HSC transplantation. Of note, non-relapse mortality was not affected by the presence of donor CHIP.

Tracking of clonal mutations proved engraftment of 24 of 25 clones and showed a disproportionate expansion in half of them, suggesting proliferative advantages and in this biologically exceptional setting. In addition, two cases of donor cell leukemia arising from a donor CHIP clone were comprehensively characterized with regard to clonal evolution and malignant transformation, including whole exome sequencing in one case. No cases of donor cell leukemia were detected in recipients transplanted from donors without CHIP.

In summary, our study was the first comprehensive analysis of the effects of donor CHIP in the setting of allogeneic transplantation. Our results indicate that CHIP clones tend to respond differently than unmutated HSCs to the multitude of stressors associated with allogeneic transplantation, as deduced from the significant differences in the cumulative incidences of cGvHD and CIRP. Future clinical trials and functional analyses are urgently warranted to further refine our knowledge on the role of donor CHIP in the setting of allogeneic HSC transplantation.

https://doi.org/10.1200/JCO.2018.79.2184

2.2.3 Clonal evolution of myelofibrosis under ruxolitinib therapy

Mylonas E*, Yoshida K*, **Frick M***, Hoyer K, Christen F, Kaeda J, Obenaus M, Noerenberg D, Hennch C, Chan W, Ochi Y, Shiraishi Y, Shiozawa Y, Zenz T, Oakes CC, Sawitzki B, Schwarz M, Bullinger L, le Coutre P, Rose-Zerilli M, Ogawa S, Damm F.
Single-cell analysis based dissection of clonality in myelofibrosis. Nature Communications. 2020 Jan 11(1):73 DOI: 10.1038/s41467-019-13892-x ***equal contribution**

Not only CHIP clones come under pressured by therapeutic measures implemented in hemato-oncology, pre-existing hematologic malignancies can likewise evolve under external stressors. As we know from various contexts, targeted therapies can function as bottleneck and promote the selection of resistant clones(148). When evolving, some diseases become more aggressive. This is the case with myelofibrosis, where disease evolution can lead to transformation into secondary AML(96).

To explore the clonal dynamics and evolution of myelofibrosis treated with the JAK inhibitor ruxolitinib, we obtained serial peripheral blood samples from 15 patients with primary or secondary myelofibrosis who all received ruxolitinib treatment. The mean follow-up time was 3.9 years. Three patients suffered from disease progression: two had transformation into secondary AML and one had evidence of disease acceleration.

Whole exome sequencing was performed for 42 time points, allowing the description of new and vanishing mutations and clonal dynamics. Targeted deep sequencing with a mean coverage of 15,250 reads/amplicon was then implemented to more precisely determine VAFs even at low mutational burden. Data obtained from the exomes and targeted sequencing were then used for calculations of copy number aberrations and mutation clusters. While genomic complexity increased over time, ruxolitinib did not seem to specifically increase evolutionary pressure, as *JAK2* and *CALR* mutational burdens remained at relatively high levels in most patients. In line with these molecular observations, clinical disease courses were stable in these patients. However, in those patients with disease progression under ruxolitinib treatment, we observed greater genetic heterogeneity and mutations in genes associated with Ras-Raf-MEK-ERK signaling. Of note, aggressive mutations could be detected at low VAFs months before transformation was clinically diagnosed. In addition, one patient acquired a *JAK2* R867Q mutation associated with resistance to ruxolitinib(149).

Single cell sequencing data provided additional insights into clonal architecture of myelofibrosis. Using phylogenetic reconstruction, we could show that loss of heterozygosity and parallel evolution are frequent features of clonal diversification in myelofibrosis.

https://doi.org/10.1038/s41467-019-13892-x

3. Discussion (Diskussion)

3.1 Genotype – phenotype relationships in MCL and DLBCL

3.1.1 Genotypic – phenotypic differences in MCL – clinical implications with regard to targeted therapy

Our study presented in section 2.1.1 provides a comprehensive genetic and functional analysis of MCL with primary resistance to BCR/NFκB inhibition by STN/ibrutinib. Of note, these phenotypic differences with regard to activation of either the canonical or non-canonical NFκB pathway had not become clinically apparent before implementation of specific compounds, underlining the fact that certain traits only gain visibility under certain stressors. MCL cases with mutations in *BIRC3*(150) and *TRAF2*(151) had been described in two sequencing studies published in 2013, and several later sequencing studies performed in MCL confirmed our results, as they likewise report recurrent mutations in *BIRC3* and *TRAF2*(41, 152-154).

Two years after our study was published in 2014, the WHO revised its classification of lymphoid neoplasms, now differentiating two subtypes of MCL based on phenotypic differences. The conventional or classical MCL (cMCL) has unmutated/minimally mutated IGHV and mostly expresses SOX11, and the leukemic, non-nodal MCL (nnMCL) with mutated IGHV which usually does not express SOX11 and has a rather indolent clinical course(3). A recent study published by Nadeu and colleagues investigated genetic and epigenetic characteristic of both subtypes using a combination of various high-throughput techniques. Of 82 MCL investigated, 61 (=74%) were cMCL and 21 (=26%) were nnMCL. Mutations in *BIRC3* were more frequent in cMCL (16/61 = 26.2%) than in nnMCL (2/21 = 9.5%)(41), but statistical significance was not reached ($p = 0.11$, Chi-Square, calculated by the author). Therefore, additional studies are required to evaluate how canonical and non-canonical NFκB pathway activation is distributed within the new, phenotype-based sub-classification of MCL by the WHO.

Identification of activation of the alternative NFκB pathway by oncogenic mutations as mechanism of primary resistance to BTK inhibition in MCL bares great therapeutic potential, as it both offers a possibility to predict response by pre-therapeutic mutation testing and indicates potential novel targets. However, it is important to note that activation of the alternative NFκB pathway by oncogenic mutations constitutes an important but by far not the only mechanism of resistance to BTK inhibition. Other

mechanisms of primary and/or acquired resistance include mutations in BTK or downstream components of the canonical NFκB pathway, as well as mutations in the PI3K/mTOR pathway, *TP53*, *CCDN1*, or epigenetic modifiers(155-157). In addition, there are further mechanisms of drug resistance beyond genetic evolution and selection of resistant clones. For example, interaction of MCL cells with the tumor microenvironment leading to adaptive reprogramming of the kinome and bypass of the canonical NFκB signaling by activation of PI3K-AKT-mTOR and integrin-1β signaling has been described as another mechanism of ibrutinib resistance(158).

There are various strategies to overcome ibrutinib resistance in MCL. Selective pharmacologic targeting of alternative NFκB signaling by a NIK inhibitor or targeting of the converging part of both NFκB pathways by an IKKβ inhibitor has shown relevant activity in our preclinical *in vitro* and *in vivo* models. However, implementation of NIK or IKKβ inhibitors in the clinical setting yet face substantial problems due to challenging drug design and toxicity(159). Therefore, current concepts favor, for example, the use of BCL2 inhibitors such as venetoclax(160, 161), the CDK4/6 inhibitor palbociclib(162), and the anti-CD19 directed chimeric antigen receptor T (CAR-T) cell product brexucabtagene autoleucel(163).

In summary, our work contributes to the further understanding of the pathogenesis of phenotypically different MCL and provides valuable information on potential therapeutic targets. Therefore, with regard to future implications of our study, the identified and functionally characterized oncogenic mutations should be part of diagnostic targeted sequencing panels for MCL to more precisely characterize genotypic properties and prospectively evaluate and/or predict response to therapy.

3.1.2 Highly different mutation patterns in DLBCL – considerations on anatomical lymphoma presentation and associated microenvironmental influences as intrinsic stressors

Various research papers, including section 2.1.2 of this work have described over-average occurrence of characteristic mutations in DLBCL with regard to anatomical manifestation. Specifically, lymphoma of immune-privileged sites such as PCNSL and PTL show a much higher rate o.f mutations in the TLR-adaptor molecule *MYD88* and the BCR subunit *CD79B*(30-33). In line with these reports, the cohort investigated in section 2.1.2 comprised 15 PTL, with 11/14 (79%) of evaluable samples having mutations in *MYD88*, 10/14 (71%) of evaluable samples having mutations in *CD79B*, and 9/14 (64%) of evaluable samples having both(85). In addition, the high frequency of mutations in *MYD88* and/or

CD79B is in line with the reported over-representation of ABC phenotypes in PTL(164), also matching the MCD DLBCL subtype that was described later by Schmitz and colleagues(34) or C5 subtype later described by Chapuy and colleagues(35).

Looking for a possible explanation, we have to understand what defines the immune-privilege of the testes. Post-meiotic spermatids have a haploid chromosome set and express possible autoantigens. As inflammatory processes could be of harm to the developing sperm, baring the danger of infertility, Sertoli cells form the blood-testes barrier (or seminiferous epithelium) that provides protection against microorganisms and immune cells(165). To fight microorganisms without invasion of immune cells, Sertoli cells can release various defense molecules (e.g. defensins and interferons)(166). A possible explanation for the remarkably homogeneous landscape of oncogenic lesions in PTL – and lymphoma of other immune-privileged sites such as brain and eye – could therefore be suspected in the stimulus-poor microenvironment of this immune-privileged site. Only those B cells that obtain pro-survival and pro-proliferative NFκB pathway signals through acquired BCR-independent NFκB signaling generated by mutations in *MYD88* and/or *CD79B* can survive and proliferate(31). Other authors hypothesize that due to high mutational load and ongoing somatic hypermutation, PTL are highly immunogenic but can escape immune surveillance most likely in immune-privileged sites(120, 167).

On the contrary, mutations in *MYD88* and *CD79B* occurred clearly below-average in lymphoma of the gastrointestinal system (2/37 and 1/35 evaluable cases) in our study. This is in line with data reported by Kraan and colleagues(31). Two groundbreaking next generation sequencing studies by Schmitz and colleagues(34) and Chapuy and colleagues(35) did not introduce a separate subgroup of DLBCL of gastrointestinal origin, prohibiting comparison of our data with these valuable datasets. By its nature, the gastrointestinal system is rich in allo-antigens both from ingested materials and microorganisms(119), building a microenvironment fundamentally different from the immune-privileged sites discussed above. Extranodal lymphoma frequently arise from the gastrointestinal system, with DLBCL being the most frequent NHL entity(119, 168, 169). Occurrence of at least a subgroup of gastrointestinal DLBCL is associated with clinically relevant inflammatory processes such as chronic infection with *Helicobacter pylori*(168, 170), though associations with further pathogens are not as well documented as for example for mucosa associated tissue lymphoma (MALT). However, with

the emergence of high-throughput microbiome research in the last decade, we yield promising new techniques for the systematic description of possible further associations. Though functional proof is lacking, we can hypothesize that the abundance of antigens present in the gastrointestinal system can lead to chronic stimulation of B cells and activation of BCR signaling, which then promotes and/or sustains proliferations of malignant clones, bypassing the necessity of oncogenic mutations activating associated pathways.

In conclusion, different anatomical localizations representing highly divergent microenvironments promote the selection of different genetic subtypes of DLBCL, most probably reflecting different biological needs of the malignant cells in terms BCR stimulation in an antigen-poor or antigen-rich surrounding, respectively. In terms of clinical relevance, different genotypes at different anatomical locations can be predictive for the response to specific targeted therapies, as for example, ibrutinib, which is a knowledge particularly relevant as long as targeted sequencing of oncogenic genes is not everywhere part of routine diagnostics.

3.2 Genotype – phenotype relations in Clonal hematopoiesis/CHIP

3.2.1 Radio-Chemo

To our knowledge our study introduced in 2.2.1 was one of the first to investigate the impact of (radio-)chemotherapy on the clonal evolution of CHIP. Considering the fact that about 10-15% of individuals older than 60 years have CHIP, it is easy to estimate that also a substantial part of elderly cancer patients in need of (radio-)chemotherapy is affected by this condition, highlighting the clinical relevance of this investigation.

Our study allows a differentiated view on the urgent question if CHIP clones exhibit specific patterns of expansion or extinction in response to the bottleneck effect evoked by myelotoxic therapies. Of note, clones harboring mutations in the most frequently mutated gene *DNMT3A* showed a stable clone size over the course of therapy in 12 of 13 cases, indicating minor clinical relevance of *DNMT3A* mutations in this setting. This notion is also supported by the fact that the presence of CHIP in general was not associated with complications or adverse events with the exception of an increased need of chemotherapy dose reductions. However, when excluding mutations in *DNMT3A* due to postulated minor clinical significance in this specific setting, we suspect premature exhaustion of at least the

erythroid cells, mirrored in significantly lower hemoglobin levels ($P = .017$) and an increased transfusion necessity ($P = 0.25$, after adjustment for confounders) after six cycles of therapy.

Other studies primarily focused on the risk of developing therapy associated myeloid neoplasms (TMN), while effects of clonal hematopoiesis on treatment related toxicities or adverse events were not systematically investigated. These studies uniformly report an in increase in clone size of *PPM1D* and *TP53* mutations(138, 171, 172) in the context of classic chemotherapy. This was most impressively shown in a very recent study by Bolton and colleagues, in which 10.138 individuals with cancer were screened for CHIP in pre- or post-therapeutic samples. While *ASXL1* mutations were significantly associated with smoking, mutations in *PPM1D*, *TP53*, and *CHEK2* were enriched by previous cytotoxic therapy. Of note, this was not the case for mutations in the epigenetic modifiers *DNMT3A* and *TET2*, or the splicing regulators *SF3B1*, *SRSF2*, and *U2AF1(138)*. Among the six expanding clones in our study, we find a *PPM1D* mutation, while another *PPM1D* mutation and a *TP53* mutation remained stable according to our definition (< 50% increase in VAF).

Our prospective study is limited by the heterogeneous chemotherapeutic regimes applied in a relatively small number of cases and a follow-up time that is too short to evaluate risk of TMN. However, together with data generated by other studies, characteristic profiles of individual CHIP mutations in response to specific stressors become apparent. While mutations in *DNMT3A* and *TET2* are rather associated with increased HSC self-renewal in conditions involving inflammatory processes (also see section 1.2.2), mutations in DNA repair genes such as *TP53* and *PPM1D* lead to increased survival, consecutive clonal expansion and clonal evolution in the context of cytotoxic stress. Together, these factors culminate in a dramatically increased risk of therapy associated myeloid neoplasm (TMN)(74, 136, 137, 173, 174). But while the presence of a *TP53* or *PPM1D* mutation is a clear intrinsic risk factor for future TMN, the type of cytotoxic agent applied also plays an important role. Though additional studies are needed to precisely determine the risk of TMN for individual mutation – drug combinations, we already know that platinum and topoisomerase II inhibitors are associated with a high risk of TMN in the presence of *TP53* and *PPM1D* mutations(138, 173). The same is true for therapeutic radiation(138).

In summary, our study was one of the first to untangle the role of CHIP in the context of (radio-)chemotherapy. As shown by us and other studies, a differentiated view on the risks of CHIP in this setting is required that both considers the mutated gene (or even type of mutation) and the type of stressor (specific cytotoxic reagent, dosage, *etc.*) administered. To more precisely evaluate this complex interplay, joint efforts and the integration of respective analyses in prospective clinical trials are needed. The same is of course true for the effect of clonal mutations in the presence of targeted therapies (and other novel agents) as a type of external stressor with different principle of action (further discussed in section 3.3 for paper 2.2.3). Further knowledge about the interplay of clonal mutations and specific therapies will enable physicians to weigh up the short-term (*e.g.* necessity of dose reduction, transfusions) and long-term (*e.g.* TMN) risks for each patient, and hence design a "hand-tailored" regimen best fitting the individual prerequisites and needs of the patient.

3.2.2 Microenvironmental stressors

In our study presented in 2.2.2 investigating the role of donor CHIP in the setting of allogeneic transplantation, CHIP clones are likewise exposed to heavy stressors. Like all transplanted HSCs, they have to newly engraft in a foreign bone marrow that has previously been subjected to highly toxic conditioning therapy, repopulate this empty niche and reconstitute the complete hematopoietic system, and in parallel deal with additional stressors caused by treatment related complications like organ failures or viral, bacterial, fungal or parasite infections leading to inflammation. As outlined in 1.2.2, inflammatory processes, aging, and clonal hematopoiesis are closely linked, but cause and consequence relationships yet need to be untangled.

In our study, we observed engraftment of all but one CHIP clone (*SF3B1* mutation in R041) followed by sequential analyses of pre- and post-transplantation samples. Moreover, more than half of these clones showed a disproportionate expansion in the recipient compared to the donor VAF, indicating increased proliferative activity of the mutated clones compared to the wildtype ones. Of note, among the clones with disproportionate expansion, we find six with a *DNMT3A* mutation, two with a *TET2* mutation, and one with an *SRSF2* mutation. Successful engraftment of clonal hematopoiesis with increased clone size in the recipient were also described in a later study(175).

From murine models we know that inflammatory cytokines promote the expansion *Tet2* mutated clones(131, 132). Likewise, the presence of *TET2* and *DNMT3A* mutations is associated with increased levels of IL-6 and TNFα in humans, respectively(129). Keeping the plethora of possible and frequent complications of allogeneic HSC transplantation in mind, it is not surprising that levels of inflammatory cytokines are elevated in the bone marrow microenvironment after conditioning therapy compared to healthy individuals(176). It is therefore reasonable to hypothesize that the successful engraftment and over-average expansion of *DNTM3A* and *TET2* mutated HSCs is due to their known increased capability of self-renewal which receives an additional boost by the inflamed microenvironment. On the contrary, the pro-inflammatory microenvironment does not regularly seem to induce new clonal mutations in HSCs transplanted from a young donor to elderly recipients, as shown by a recent study by Heumüller and colleagues(177). This allows the conclusion that HSCs with pre-existing mutations – best studied for *DNMT3A* and *TET2* – find a microenvironment particularly favorable with regard to clonal expansion in the context of allogeneic transplantation, but the rate of newly induced mutations does not seem to be increased. Clonal evolution and malignant transformation of pre-existing CHIP clones should however be excluded from this model.

Inflammation not only seems to support the engraftment and expansion of clonal hematopoiesis – clonal hematopoiesis itself promotes the release of pro-inflammatory cytokines and activity of immune cells. This was shown in various studies for mutations in *Dnmt3a* and *Tet2*(82, 129, 178, 179). In specific, in murine models, the presence of *Dnmt3a* and *Tet2* mutant clones led to increased levels of IL-1β, IL-6, IL-13, and TNFα(82, 179). We can therefore postulate a positive feedback loop – or vicious circle – with an inflamed milieu promoting the expansion of various mutations of clonal hematopoiesis, which in return sustain and enhance inflammation(122). This hypothesis is further supported by one of our key findings: The cumulative incidence of chronic graft versus host disease (cGvHD) – a severe inflammatory complication with reactivity of the transplanted immune system against structures of the recipient(142) – is significantly higher in recipients transplanted from a donor with CHIP, in particular with a *DNMT3A* mutation. In return, incidence of relapse or disease progression after transplantation in patients with residual disease is significantly lower. This beneficial fact is most likely due to the well-described graft versus leukemia (GvL) effect that often goes in line with GvHD and can be considered

the positive side of the coin(142). The association of CHIP with GvHD was also described by Oran and colleagues, though their yet not fully published data (last update: 5th January 2021) suggests an increased incidence of acute GvHD rather than chronic GvHD(180).

Though functional analyses are required to dissect the causal relation between CHIP and cGvHD/GvL, the data generated in our study strongly support the idea that the presence of CHIP clones, especially *DNMT3A* and *TET2* mutations, is promoted by and itself promotes inflammatory processes. Yet, with our current knowledge, it is still too early to judge how this ultimately affects patients in general and how a sensible risk stratification could look like. Risks (increased risk of cGvHD) and benefits (reduced risk of relapse/progression) seem to be quite balanced, at least, if the unchanged overall survival observed in our study is taken as outcome parameter of greatest meaningfulness.

At this point, it is important to address the fact that the presence of donor CHIP is associated with an increased risk of donor cell leukemia in our study. Though the two cases observed have no impact on overall survival strata with regard to donor CHIP status, successful engraftment, clonal evolution and malignant transformation of the transplanted clone are of devastating consequence for the affected recipient. Future studies should therefore carefully monitor the occurrence of DCL to identify additional factors that drive malignant transformation in the presence of donor CHIP. Conceivable factors could be mutations in specific genes, as well as extrinsic stressors such as conditioning regimens applied, medication, radiation, infections, and smoking.

3.3 Myelofibrosis – Disease evolution in the context of JAK-targeting therapy

As delineated for the development of DCL from donor HSCs with CHIP, hematologic malignancies are dynamic diseases following Darwinian principles. Disease evolution can therefore be understood as an interplay of mutagenesis and selection processes by intrinsic and extrinsic stressors(181). Therapeutic actions, especially those with a targeted mode of action, hence bare the threat to select for resistant subclones, as for example seen in CML(148), and unintentionally promote unfavorable disease progression.

Myelofibrosis bares the inherent risk to progress to secondary AML with dismal prognosis(96). Factors promoting leukemic transformation by imposing evolutionary pressure on the malignant cells are

therefore of great clinical interest. Though long-term data do not seem to indicate an increased risk for leukemic progression of ruxolitinib therapy compared to best available therapy – in fact, a decreased risk is discussed – current data prohibit final conclusions, as long-term data from the COMFORT-II trial also include cross-overs of the control study population to the treatment arm(182).

Our study provides valuable insights into the clonal architecture, and subclonal evolution and dynamics of 15 patients with primary or secondary myelofibrosis treated with ruxolitinib over a cumulative time of 58.5 years. With a mean follow-up of 3.9 years/patient and samples from 42 time points, our data allow a detailed insight into various disease courses (stable diseases, progression, leukemic transformation) using whole exome sequencing with additional resolution at single cell level.

The study further supports the above described notion that disease progression and leukemic transformation are not the result of extrinsic selection pressure imposed by targeted JAK inhibition, but rather the result of aggressive subclones that are detectable long time before progression and gain predominance over less aggressive clones. As already described, *JAK2* mutational burden usually remains stable or decreases only slightly under ruxolitinib therapy(115), making ruxolitinib an effective, but palliative treatment to relieve MF associated symptoms. In our study, we likewise observe only two molecular remissions, while *JAK2*V617F mutations and *CALR* mutations remain well detectable in the rest of patients despite durable clinical responses.

We observed one subclonal *JAK2* mutation (R867Q) associated with ruxolitinib resistance(149) in a patient who died of sepsis in disease progression. As shown by single cell analysis of CD34+ cells and targeted sequencing of flow-sorted hematopoietic sub-fractions, this mutation particularly dominates the stem cell compartment, while a clone with classical *JAK2*V617F mutation and a mutation in *KRAS* occupies only minor clonal space in the stem cell compartment, but significantly expands into the monocytic compartment. Disease progression in this patient might therefore indeed be due to potential selective advantage of the ruxolitinib resistant clone in the presence of ruxolitinib as external stressor. However, our data provide evidence that there are major, ruxolitinib independent, cell-intrinsic factors that favor disease progression. First, we see increased genomic instability in terms of mutational frequencies and copy number alterations in those patients who progress compared to those with stable

disease. And second, all three patients with disease progression yielded clones harboring mutations in the RAS-RAF-MEK-ERK pathway: two patients in *NRAS* and one patient in *KRAS*. In other myeloid diseases, mutations in these genes are known drivers of progression and indicate poor prognosis(183, 184), hence a similar role in myelofibrosis is probable.

With the data available, it is impossible to rule out a role of ruxolitinib as external stressor in disease progression for any patient. Importantly, with regard to the high-resolution molecular data provided by our study, we have to note that for ethical reasons, data for an untreated control population are not available. However, improved overall survival(182) (with the known limitations of the clinical trials) and our molecular data strongly argue against ruxolitinib as major stressor in genetic evolution and disease progression.

4. Summary (Zusammenfassung)

This series of research papers explores the influence of various stressors on genotype – phenotype relationships in the context of premalignant and malignant hematologic conditions. Mutations translate into phenotypic changes and exposure to organism-intrinsic or organism-extrinsic stressors may reveal differences in clonal fitness with preferential expansion of clones harboring certain traits, as well as primary or secondary drug resistance. Investigation of the effects of pharmacological and other medical interventions on genotype and phenotype is of particular relevance, as this may have profound clinical consequences and therefore is part of the responsibility and due diligence we owe our patients.

Using in-depth genetic and functional analysis of MCL cell lines, mouse models, and patient samples, we comprehensively characterized the genetic background of an important mechanism responsible for primary resistance to the BTK inhibitor ibrutinib in mantle cell lymphoma. Here, activation of the non-canonical NFκB pathway was for the first time described as mechanism of primary resistance to ibrutinib. In another research paper, mutational patterns known to be responsible for chronic active NFκB signaling and potentially predictive of response to targeted BCR/NFκB inhibition were described in DLBCL with respect to anatomical location. Likewise, clonal evolution of primary and secondary myelofibrosis and genetic background of leukemic transformation under the JAK inhibitor ruxolitinib were characterized in long-term serial patient samples using whole exome sequencing, ultra-deep targeted sequencing and single cell analysis. With regard to the premalignant condition of clonal hematopoiesis, we described clinical outcome and genetic evolution under the pressure of myelotoxic therapies and allogeneic hematopoietic stem cell transplantation. Interestingly, mutations in the most frequently mutated gene *DNMT3A* rarely show clonal expansion and seem to be of minor clinical relevance under the pressure of cytotoxic therapies. In contrast, they are associated with immunological effects in the context of allogeneic transplantation, as we observe an increased cumulative incidence of cGvHD and a reduced cumulative incidence of relapse and progression. The later is most probably due to an enhanced GvL reaction when donor CHIP is present in allogeneic HSCs. In addition, disproportionate clonal expansion can be observed in about have of all donor CHIP cases.

Together, these findings indicate mutation and context specific behavior of (pre)malignant hematopoietic cells, underlining the need of individual mutation – stressor and genotype – phenotype investigations to approach the overall goal of individualized precision medicine.

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7. Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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