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Sequence Variants and Their Nature in an Unselected Cohort of Patients With High-Risk Myeloid Disorders

Dissertation

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For a fluently reading, the generic masculinum was used in this paper. Female and other gender identities are explicitly included. The generic masculinum should be understood independently of its gender.

1. Hypothesis and Study Aims

In myeloid disorders, certain gene mutations are associated with disease status per se or even a certain disease. The number of mutations correlates with progression of the underlying disease. Next to gene mutations, sequence variants of unknown significance and sequence variants that are not pathogenic have been described.

The true nature of sequence variants (not pathogenic, unknown significance, pathogenic mutations) sometimes remains obscure.

Information on sequence variants in addition to clinical appearance, bone-marrow morphology, immunophenotype, and cytogenetic aspects is helpful in diagnostic, and also therapeutic approaches.

In this work, an overview about sequence variants found through next generation sequencing (NGS) panel diagnostic is supplied using a large cohort of high-risk myeloid disorders. The occurrence of specific sequence variants in certain myeloid disorders in this cohort is compared with publicly accessible data. For this, the minimal variant level data and the level of evidence is obtained. Thus, the nature of a sequence variant might be inferred through association with the disease in which it is frequently found.

2. Introduction

Determination of sequence variants is widely used in cancer diagnostics. Difficulties arise in the interpretation of the manifold variant findings as there is neither a universal tool to calculate nor any consent about at which frequency particular sequence variants are expected to have a significant impact on the pathogenesis or prognosis of a neoplasm. During the last years, efforts were undertaken to establish classifications for variants detected through NGS into pathogenic or benign categories. Approaches for classification differ from in silico models to research procedure recommendations in well-established databases and studies for evidence on the nature of these variants.

In the current work, evidence on sequence variants resulting from NGS in a cohort of high-risk myeloid disorders is presented. Data is analyzed by applying structured research on databases (*COSMIC, TCGA, My Cancer Genome, Ensembl*, *Variant Effect Predictor (VEP), OMIM, ClinVar, dbSNP*), predictive calculation algorithms (*FATHMM-MKL, VEP, PolyPhen 2, SIFT*) and professional national health guidelines, based on the *minimal variant level data* (*MVLD)* and the four tier evidence level system proposed as *Joint Consensus Recommendation* by the *Association for Molecular Pathology* (AMP), the *American Society of Clinical Oncology*, and the *College of American Pathologists* on the nature and evidence of sequence variants. In addition, a comparison with the published frequency and impact of sequence variants in the general population and in myeloid disorder cohorts is performed.

2.1 Myeloid Neoplasia

Myeloid neoplasia is a cluster of clonal diseases of haematopoietic stem and progenitor cells and divided into several diseases: myelodysplastic syndrome (MDS), myeloproliferative neoplasia (MPN), MDS/MPN overlap syndrome, mastocytosis and acute myeloid leukemia (AML). Diagnosis is reached through integration of clinical appearance of the patient, certain peripheral blood laboratory results and bone marrow analysis, frequently including morphology, immunophenotypic, cytogenetic and molecular makeup of the diseased cells and tissues.

2.1.1 MPN

In *MPN*, typical gene mutations are considered clonal markers for certain subgroups and as such, together with bone marrow morphology, are part of the diagnostic criteria in the WHO classification of 2016 (Swerdlow et al. 2017) which is the basis for most clinical and country-specific guidelines. MPN includes chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), hypereosinophilic syndrome (HES).

2.1.2 MDS

Dysplastic blood and bone marrow cells with haematopoietic insufficiency and an increased risk of developing AML are characteristic for *MDS*. Therapy management is not easy as patients are usually elderly, display comorbidities. Chemotherapy is very toxic but might not be curative. With the development of NGS and its diagnostic use in haemato-oncologic diseases, the *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues* (Greenberg et al. 2012) was revised and includes now also genetic changes in the *MDS* classification. Diagnosis is based on the cytomorphologic determination of the proportion of dysplastic and monocytic cells and ringed sideroblasts as well as the proportion of peripheral and medullar blast cells. Additionally, karyotyping of cytogenetic abnormalities, such as del(5q), is used as diagnostic criteria. The significance of genes that are typically mutated in MDS patients (like *SF3B1, TET2, SRSF2, ASXL1, DNMT3A, RUNX1, U2AF1, TP53, EZH2*) remains unclear as they can also be found in healthy individuals. Thus, these mutations do not form part of the criteria for the diagnosis of an MDS but they may be useful to calculate prognosis (Swerdlow et al. 2017).

2.1.3 MDS/MPN overlap syndrome

MDS/MPN overlap syndrome can be diagnosed if characteristics of MDS and MPN are found. It describes a stage in the disease evolution of myeloid neoplasia and can develop into MDS, MPN or AML in course of time (Swerdlow et al. 2017).

There are five different types, atypical CML (aCML), chronic myelomonocytic leukemica (CMML), juvenile myelomonocytic leukemia (JMML), MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) and MDS/MPN-Unclassifiable (MDS/MPN-U). In the presented work, CMML was regarded separately (Swerdlow et al. 2017).

Diagnostic criteria of *CMML* are monocytes in the peripheral blood (>1000/µl) with bone marrow dysplasia of myeloid cell lines, exclusion of reactive monocytosis, CML, PMF, PV or ET, proportion of blasts in the peripheral blood and bone marrow of less than 20%, absence of *PDGFRA, PDGFRB, FGFR1, PCM1-JAK2* rearrangements, or *BCR-ABL1*. In addition, the cytogenetic and molecular biological findings are important to determine the clonal character. CMML can be MDS-like (leukocytes <13.000/µl) or MPN-like (leukocytes ≥13.000/µl) (Swerdlow et al. 2017). The CMML-Specific Prognostic Scoring System (CPSS) (Greenberg et al. 2012) and CPSS-molecular (Greenberg et al. 1997) are used to predict the patient specific prognosis. Certain mutations in *ASXL1* and *NPM1* are associated with an aggressive course of disease.

2.1.4 AML

AML is distinguished from other myeloid neoplasms by ≥20% blast cells in the bone marrow or peripheral blood, and/ or the presence of AML defining genetic aberrations such as the balanced chromosomal translocation t(8;21)(q22;q22.1), resulting in the fusion product *RUNX1-RUNX1T1*, the inversion inv(16)/ t(16;16)(p13.1;1q22), corresponding to the *CBFB-MYH11* fusion product, or t(15;17)(q22;q12) (fusion product *PML-RARA)*. AML is divided into subgroups depending on the underlying cause of disease (recurrent genetic aberrations, myelodysplasia-related changes, therapy-related neoplasm, Down-syndrome-related, blastic plasmacytoid dendritic neoplasm, other not specified, ambiguous lineage, myeloid sarcoma) (Swerdlow et al. 2017). AML originating most probably from MDS or MPN are classified as secondary AML (sAML) and therefore as MDS or MPN. AML is defined by the amount of blast cells in the bone marrow or peripheral blood equal to or surpassing 20%, the amount and severity of affected cell lines, and genetic aberrations such as translocations and molecular-genetic changes (as *NPM1* or biallelic *CEBPA* mutations) (Swerdlow et al. 2017).

2.2 Diagnostic of Haematologic Diseases

For the diagnosis of myeloid neoplasia, peripheral blood and bone morrow samples are needed. Usually, differential diagnoses have to be excluded (see 1.3), therefore a differential blood count is taken. Acute myeloid leukemia is diagnosed if blasts in bone marrow or peripheral blood are ≥20%. Also, the bone marrow sample is used for cytology and cytogenetical examination, histology, and immunophenotyping. Mutation analysis through NGS can be performed on peripheral- and bone marrow samples. In a clinical examination, the size of spleen and liver are measured.

2.3 Differential Diagnosis

The reasons for aplasia can be of different cause, so differential diagnostics should be considered during the diagnostic process. This was the reason for outtakes in our cohort as well. Other neoplasia, like lymphoma or bone marrow infiltration of a solid tumor, can lead to aplasia. Apart from neoplasia, it can be seen in aplastic anemia (see 1.3.1), hypersplenism, or be caused by faulty synthesis because of lack of substrate or toxic substrates (e.g. chemotherapy, alcohol, lead), or can occur reactive (sepsis, chronic infection, autoimmune).

2.3.1 Aplastic Anemia

Aplastic Anemias (AA), excluding inherited bone marrow failure syndromes, belong to group of pathogenetically heterogenous bone marrow insufficiencies with pancytopenia through hypo- or aplastic bone marrow without abnormal cells or fibrosis. Three subgroups (non-severe, severe, and very severe AA (SAA)) exist depending on the number of neutrophilic granulocytes, thrombocytes, and reticulocytes. Exclusion of differential diagnoses and etiology is used as diagnostic criteria (Killick et al. 2016).

2.4 Prognosis

To assess prognosis, parameters like age, comorbidities but also disease-specific parameters like blast cell count, cytogenetic variants, and clinical parameters, such as need for transfusions, cytopenia, and LDH are taken into account.

There are different prognostic scoring systems for different types of myeloid neoplasia. They are usually used to calculate a pretreatment risk score to decide together with other parameters on therapeutical options.

For AML, the European LeukemiaNet developed a classification to assess risk groups using molecular cytogenetic findings at primary diagnosis to classify patients into favorable, intermediate and adverse risk groups.

In MDS, the International Prognostic Scoring System (IPSS) and the Revised International Prognostic Scoring System (IPSS-R) are used for prognosis. Parameters for IPSS are the relative number of medullary blasts, karyotype and cytopenia. For IPSS-R, instead of cytopenia, separate values are distributed for absolute count of hemoglobin, platelets and neutrophils. The later developed IPSS-R is not only used for pretreatment but can further be continuously used in the course of disease to assess the risk of developing an AML and to estimate the expected median OS.

In MPN, prognostic factors are disease specific. In ET, thromboembolic complications or severe bleedings together with age >60 years and platelet count of >1.500.000/µL are used in pretreatment diagnosis to decide on therapy options. In PV, the prognosis depends mostly on the thromboembolic risk, which itself depends on age ≥60 years and thrombosis in the past, or cardiovascular risk factors and leukocytosis. For CML,

different scores (Sokal, EURO, EUTOS, ELTS) use parameters such as age, size of spleen, platelets, number of blasts in the peripheral blood and counts of basophils and eosinophils to distribute the pretreatment relative risk into high, intermediate or low. In PMF, there are several scores: the IPSS, the Dynamic International Prognostic Scoring System (DIPSS) for risk calculation at any time in course of disease and the DIPSS-plus using in addition to the IPSS-parameters unfavorable biological parameters (e.g. chromosomal aberrations) to identify the survival probability. The MIPSS-70 and MIPSS-70plus (Mutation-Enhanced International Prognostic Scoring System) combine clinical and cytogenetic or molecular genetic parameters.

To identify high, intermediate and low risk patients in CMML, the CPSS, which uses the leukocytes >13.000/µl and medullary blasts ≥10%, a regular need for transfusions and cromosomal aberrations, and the CPSS-molecular, which additionally uses molecular markers and classifies already from >5% medullary blasts, both defining four risk groups are relied upon.

2.5 Therapy

Therapeutic options vary depending on the respective myeloid disorder. In all cases, they depend on the fitness of the patient, on the risk category of the disease, and on the stage of disease. The diseases evolute over time and often reach the stage of AML. Before this stage, for some diseases in low risk situations,

'watch and wait' is the strategy used, as for low risk MDS. In progression to sAML (secondary AML, developed from a myeloid disease), stem cell transplantation is the only curative option. This is also the situation when we mostly sampled and screened our patients, as they then were presented to a department of stem cell transplantation at a university hospital.

In general, for a first line therapy a combination or single chemotherapy is applied together with immunosuppressive medication, like dexamethason or prednisolon, antihistminices antivirals and antibioitics and antiemetics.

Specific therapeutic options are rare but new options are increasingly available as individual therapy is a research focus in cancer nowadays. Some mutations can be specifically targeted like in CML, where the gene fusion BCR-ABL leads to an overactivation of a tyrosine kinase and causes an uncontrolled growth of leukocytes. This can be specifically hit by specific tyrosine kinase inhibitors such as imatinib.

Another example for specific therapeutic options are *IDH*-inhibitors in AML, ivosidenib (*IDH1*-inhibitor) and enasidenib (*IDH2*-inhibitor). They are approved by the Food and Drug Administration (FDA) to be applied in AML relapse if a mutation in these genes is present.

3. Material and Methods

3.1 Patients

In this study, we analysed material from patients presenting to the Universitätsklinikum Hamburg-Eppendorf (UKE) at the Department of Stem Cell Transplantation with a suspected myeloid neoplasia who underwent sampling and NGS panel diagnostic before stem cell transplantation. 627 patients sampled between 2004 and 2019 were included. All of them had given their written consent to use their data for scientific research purposes. An ethical vote was not necessary as data were anonymized. Inclusion criteria were sampling pre-allogeneic stem cell transplantation (SCT) and information about previous and further diagnostic and treatment of the patient.

Few patients had to be excluded from the final analysis due to lack of complete NGS data or sampling time after stem cell transplantation date.

All relevant and necessary data for the diagnosis criteria were obtained either at the UKE or externally and compiled. The final diagnosis was used as documented by the Department of Stem Cell Transplantation of the UKE.

Patients were classified according to the documented diagnosed disease at time of sampling and then grouped into six major diseases *AML, MPN, MDS, MDS/MPN overlap, CMML, SAA* and *other diseases.* Secondary AML or myelofibrosis developing out of MDS, *MPN, MDS, MDS/MPN overlap* was classified according to the originating disease.

Of the 627 patients, 267 were female and 360 male. The median age was 59 years, with a range of 2 to 80 years.

3.2 Concept of VUS

For VUS, the definition by Richards et al. (2015), is used, replacing the terms *mutation* and *polymorphism* by *variant*. Sequence variants can either be oncogenic mutations or *variants of unclear (*or *unknown) significance* (VUS)*.* Since the increasing establishment of NGS as diagnostic tool, several platforms and classification systems for VUS are used but neither has agreement on one uniform rating system been reached nor has a systematical evidence about the pathogenicity of VUS been established. VUS can be found among patient cohorts and commonly in the general population or in healthy control cohorts.

The nature of variants can be described best by using the mentioned *MVLD* (Ritter et al. 2016) and *Joint Consensus Recommendation* (Li et al. 2017). The *MVLD* consists of different levels of interpretation and data gathering (F*igure 1*). As there are multiple nomenclatures for different characteristics of variants, the first level, *Allele Descriptive*, serves for collection and easier research on different databases in other levels. The second level, *Allele Interpretive*, establishes a link to a functional and clinical effect of the variant, considering somatic classification, DNA and protein position, variant type and

consequence and PMIDs (Pub-Med-Database ID). In a third level, *Somatic Interpretive*, the focus lies on *Cancer Type (using ontology database), Biomarker Class* (its significance for diagnosis, prognosis and prediction)*, Therapeutic Context,* in the form of drugs *and its therapeutical Effects*, *Level of Evidence (tier I-IV from the Joint Consensus Recommendation),* and *Sub-Level of Evidence (type of used evidence)* (Ritter et al. 2016).

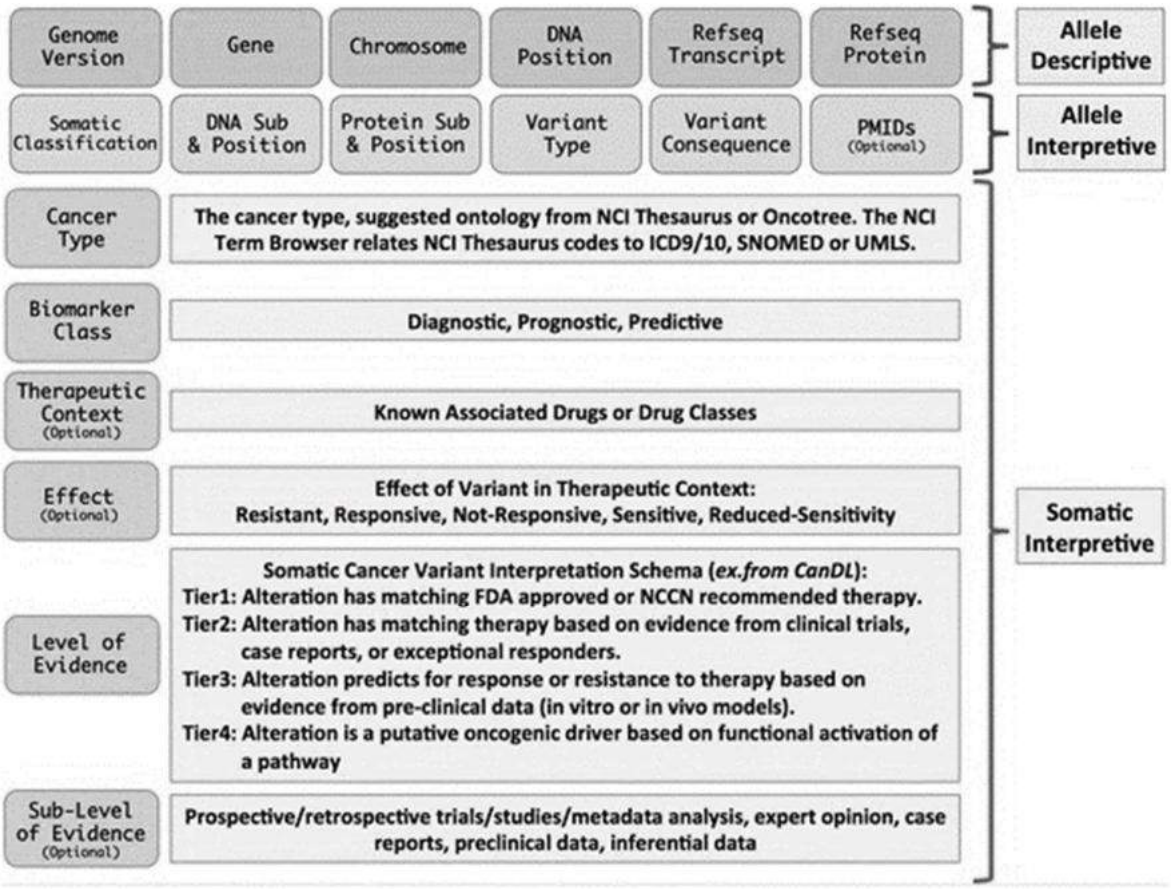

Figure 1. Minimal variant level data for variant classification, *ICD* International Classification of Diseases, *NCNN* National Comprehensive Cancer Network, *NCI* National Cancer Institute, *PMID* PubMed ID, *Sub* substitution, *SNOMED* Systematized Nomenclature of Medicine, *UMLS* Unified Medical Language System, according to Ritter DI et al. 2016.

The focus of this study is the nature of variants in high-risk myeloid disorders in a clinical context, so evidence levels as proposed by the *Joint Consensus Recommendation* were used. Different levels of evidence (*level A*: FDA approved therapies and PG, *level B*: well-founded studies with expert consensus, *level C*: specific approved/investigational therapies or different smaller published studies with less consensus, *level D*: preclinical trials or non-consented case reports) are grouped in the first two tiers, level A and B evidence into *Tier I: Variants of Strong Clinical Significance*, and level C and D evidence into *Tier II: Variants of Potential Clinical Significance*. *Tier III: Variants of Unknown Clinical Significance* contains not yet significantly mentioned variants in subpopulations or

databases or without any founded published evidence. The last part, *Tier IV: Benign or Likely Benign Variants*, includes significantly frequent variants found in general or subpopulation databases without any association to cancer (Li et al. 2017).

3.3 NGS

We applied NGS (PGM (Personal Genome Machine ™), Ion Torrent/ Fisher Lifetechnologies) to an unselected cohort of 627 patients who had been referred to be consulted regarding the indication to potentially undergo allogeneic stem cell transplantation to treat a high-risk myeloid disorder.

Sample collection took place between 2004 and 2019 in course of standard and follow up diagnostics.

Sample material was obtained from bone marrow or peripheral blood, of which DNA was isolated for use in the NGS protocols with different panels.

The NGS-protocol consisted of 4 steps: library preparation, OT2-Hi-Q view, OT2-enrichment (ES) and sequencing at *PGM™*. For the library preparation, the *Ion AmpliSeq™Library Kit 2.0* (Ion Torrent™, ThermoFisher Lifetechnologies) was used as instructed by manufacturer. First, the primer, according to the tested panels, was prepared on a PCR-stripe for the thermocycler. To ligate the adapters to the amplicons, barcode was added and subsequently purified utilizing magnetic *AMPure XP Beads*. After quantifying the library through dilution and the *Ecoli DH108 Calibration Library*, the PCR was prepared with *Ion Library Taqman* on a PCR-plate in the *Light Cycler 480 II.* To start the OT2-Hi-Q view, *Ion PGM™ Hi-Q™ View OT2 Kit (Ion Torrent™)* was filtered through *Ion OneToch™Reaction Filter* and then the *PGM IonOT™ HiQ OT2-Kit 200*-programme of the *IonOT™-Software* was started. In the OT2-enrichment, Magnetic *MyOne Dynabeads* were used with an 8-well strip for washing and binding the sample-DNA. The fourth step, sequencing, was performed as recommended by the manufacturer with the *Ion PGM™ Hi-Q™View Sequencing Kit* for preparation and loading of the *Ion 318™Chip.*Then the sequencing program was started on the *Ion Torrent PGM.*

We used three specific panels (AML, MDS, PMF) for testing, which we applied to the samples in the NGS protocol. The choice of panel depended on the preclinical suspected diagnosis upon anamnesis, clinical examination and blood sampling.

MDS- and PMF-panels were designed by our department through the *IonTorrent platform,* including commonly mutated genes associated with these haemato-oncologic syndromes (MDS, PMF). The AML panel is a standard panel kit from *IonTorrent*. The composition of panels with the target genes can be seen in *tables 1-3*. Each analysis was performed once, for validation, we looked on the sequencing depth.

Table 1. AML panel targets.

Request ID	Gene Symbol	Chromosome	Chromosome start	Chromosome end	Amplicons	Covered bases	Target bases
AML	NRAS	chr1	115256401	115256619	2	219	219
AML	NRAS	chr2	115258651	115258801	1	151	151
AML	DNMT3A	chr2	25457128	25457309	2	182	182
AML	DNMT3A	chr2	25458556	25458714	2	159	159
AML	DNMT3A	chr2	25459785	25459894	1	110	110
AML	DNMT3A	chr2	25461979	25462104	2	126	126
AML	DNMT3A	chr2	25463151	25463339	2	189	189
AML	DNMT3A	chr2	25463489	25463619	1	131	131
AML	DNMT3A	chr2	25464411	25464596	2	186	186
AML	DNMT3A	chr2	25466747	25466871	2	125	125
AML	DNMT3A	chr2	25467004	25467227	3	224	224
AML	DNMT3A	chr2	25467389	25467541	3	153	153
AML	DNMT3A	chr2	25468102	25468221	2	120	120
AML	DNMT3A	chr2	25468869	25468953	2	85	85
AML	DNMT3A	chr2	25469009	25469198	2	190	190
AML	DNMT3A	chr2	25469469	25469665	2	197	197
AML	DNMT3A	chr2	25469900	25470047	2	148	148
AML	DNMT3A	chr2	25470440	25470638	2	199	199
AML	DNMT3A	chr2	25470886	25471141	3	256	256
AML	DNMT3A	chr2	25497790	25497976	2	187	187
AML	DNMT3A	chr2	25498349	25498432	1	84	84
AML	DNMT3A	chr2	25505290	25505600	3	311	311
AML	DNMT3A	chr2	25522988	25523132	1	145	145
AML	DNMT3A	chr2	25536762	25536873	2	112	112
AML	IDH1	chr2	209113073	209113404	3	332	332
AML	GATA2	chr3	128199842	128200181	4	340	340
AML	GATA2	chr3	128200642	128200807	3	166	166
AML	GATA2	chr3	128202683	128202868	2	186	186
AML	GATA2	chr3	128204550	128205231	7	682	682
AML	GATA2	chr3	128205626	128205894	4	269	269
AML	KIT	chr4	55589730	55589884	1	155	155
AML	KIT	chr4	55593364	55593510	3	147	147
AML	KIT	chr4	55593562	55593728	3	167	167
AML	KIT	chr4	55599216	55599378	2	163	163
AML	TET2	chr4	106155080	106158528	26	3449	3449
AML	TET2	chr4	106162476	106162606	2	131	131
AML	TET2	chr4	106163971	106164104	2	134	134
AML	TET2	chr4	106164707	106164955	4	249	249
AML	TET2	chr4	106180756	106180946	3	191	191
AML	TET2	chr4	106182896	106183025	2	130	130
AML	TET2	chr4	106190747	106190924	2	178	178
AML	TET2	chr4	106193701	106194095	4	395	395

AML	TET2	chr4	106196185	106197696	14	1512	1512
AML	NPM1	chr5	170837511	170837589	1	79	79
AML	BRAF	chr7	140453131	140453141	1	11	11
AML	JAK2	chr9	5073678	5073805	1	128	128
AML	WT1	chr11	32413498	32413630	2	133	133
AML	WT1	chr11	32417783	32417973	2	191	191
AML	CBL	chr11	119148856	119149027	2	172	172
AML	CBL	chr11	119149200	119149443	3	244	244
AML	KRAS	chr12	25380148	25380366	2	219	219
AML	KRAS	chr12	25398188	25398338	2	151	151
AML	PTPN11	chr12	112888102	112888336	3	235	235
AML	PTPN11	chr12	112926808	112926999	2	192	192
AML	FLT3	chr13	28589830	28589838	1	9	9
AML	FLT3	chr13	28592604	28592657	1	54	54
AML	FLT3	chr13	28602340	28602342	1	3	3
AML	IDH2	chr15	90631799	90631999	2	201	201
AML	TP53	chr17	7572907	7573028	2	122	122
AML	TP53	chr17	7573907	7574053	2	147	147
AML	TP53	chr17	7576517	7576604	2	88	88
AML	TP53	chr17	7576605	7576677	1	73	73
AML	TP53	chr17	7576833	7576946	2	114	114
AML	TP53	chr17	7576999	7577175	3	177	177
AML	TP53	chr17	7577479	7577628	2	150	150
AML	TP53	chr17	7578137	7578309	3	173	173
AML	TP53	chr17	7578351	7578574	4	224	224
AML	TP53	chr17	7579282	7579610	4	314	329
AML	TP53	chr17	7579680	7579741	1	44	62
AML	TP53	chr17	7579819	7579932	1	96	114
AML	ASXL1	chr20	31022215	31025161	28	2947	2947
AML	RUNX1	chr21	36171578	36171779	3	202	202
AML	RUNX1	chr21	36206687	36206918	3	232	232
AML	RUNX1	chr21	36231751	36231895	3	145	145
AML	RUNX1	chr21	36252834	36253030	2	197	197
AML	RUNX1	chr21	36259120	36259413	3	294	294
AML	RUNX1	chr21	36265202	36265280	1	79	79
AML	CEBPA	chr19	33792224	33793340	9	1117	1117

Table 2. MDS panel targets.

Type	Name	Chromosome	Start	End
GENE_CDS	ZRSR2			
REGION	U2AF1	chr21	44524389	44524539
GENE_CDS	TET2			
GENE_CDS	ASXL1			
REGION	RUNX1/1	chr21	36421396	36421596

REGION	RUNX1/2	chr21	36421046	36421296
REGION	RUNX1/3	chr21	36265146	36265296
REGION	RUNX1/4	chr21	36259046	36259396
REGION	RUNX1/5	chr21	36252796	36253046
REGION	RUNX1/6	chr21	36231696	36231896
REGION	RUNX1/7	chr21	36206896	36206946
REGION	RUNX1/8	chr21	36171546	36171796
GENE_CDS	EZH2			
GENE_CDS	TP53			
GENE_CDS	KRAS			
GENE_CDS	NRAS			
REGION	CBL/8	chr11	119148855	119149025
REGION	CBL/9	chr11	119155655	119155835
REGION	ETV6	chr12	12043837	12043987
REGION	U2AF1	chr21	44514739	44514939
GENE_CDS	IDH1			
GENE_CDS	IDH2			
GENE_CDS	DNMT3A			
GENE_CDS	SF3B1			

Table 3. PMF panel targets.

Type	Name	Chromosome	Start	End
GENE_CDS	TET2			
REGION	JAK2 V617F	chr9	5073684	5073804
REGION	JAK2 Exon 12	chr9	5069904	5070074
REGION	MPL	chr1	43814924	43815054
REGION	LNK	chr12	111855901	111856701
REGION	CBL/8	chr11	119148855	119149025
REGION	CBL/9	chr11	119155655	119155835
REGION	IDH1	chr2	209113044	209113407
REGION	IDH2	chr15	90631759	90631959
GENE_CDS	IKAROS			
REGION	ASXL1	chr20	31022235	31025196
REGION	EZH2/10	chr7	148514892	148515192
REGION	EZH2/18	chr7	148506342	148506492
REGION	EZH2/20	chr7	148504692	148504842
REGION	SRSF2	chr17	74732794	74733494
REGION	SF3B1/14	chr2	198267222	198267572
REGION	SF3B1/15	chr2	198266622	198266872
REGION	DNMT3A/15	chr2	25466960	25467210
REGION	DNMT3A/16	chr2	25466710	25466860
REGION	DNMT3A/17	chr2	25464360	25464610
REGION	DNMT3A/18	chr2	25463460	25463610
REGION	DNMT3A/19	chr2	25463110	25463360
REGION	DNMT3A/20	chr2	25461910	25462110

REGION	DNMT3A/21	chr2	25459710	25459910
REGION	DNMT3A/22	chr2	25458510	25458710
REGION	DNMT3A/23	chr2	25457060	25457310
REGION	RUNX1/1	chr21	36421396	36421596
REGION	RUNX1/2	chr21	36421046	36421296
REGION	RUNX1/3	chr21	36265146	36265296
REGION	RUNX1/4	chr21	36259046	36259396
REGION	RUNX1/5	chr21	36252796	36253046
REGION	RUNX1/6	chr21	36231696	36231896
REGION	RUNX1/7	chr21	36206896	36206946
REGION	RUNX1/8	chr21	36171546	36171796
REGION	FLT3/14	chr13	28608130	28608380
REGION	FLT3/15	chr13	28607930	28608130
REGION	NRAS/12-13	chr1	115258666	115258816
REGION	NRAS/61	chr1	115258616	115258816
REGION	KRAS/12-13	chr12	25398205	25398355
REGION	KRAS/61	chr12	25380205	25380305

3.4 Statistical Analysis

Statistical analysis was performed via *IBM SPSS Statistics 25* operating *chi-squared test (χ^2 test)*, *Fisher's exact test*, *median*, with a significance level if $p < .05$. For the analysis we included the following data: testing of the gene in more than 25% of our cohort and if ≥ 5% of included patients were affected by the found sequence variant. Respectively, excluded from analysis were tested genes with low measurement rates.

In the collected data we calculated and looked for frequencies of somatic mutations and VUS in association with haematologic diseases, like *AML, MPN, MDS, MDS/MPN overlap, CMML, SAA* and *other diseases.*

3.5 Database Research

Database research was performed on variants found with a frequency of ≥ 5% in tested samples. Since there is no consensus yet about how to analyze variants, we used the *MVLD* scheme (Ritter et al. 2016) and integrated the four-tier evidence level classification and recommended databases of the *Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists* (Li et al. 2017). As we focused on diseases association of sequence variants in the spectrum of myeloid disorders of found sequence variants, detailed research was not performed on the functional aspects of the sequence variants (e.g. pathway involvement) and predictive calculating models. Also, we did not consider specific actual and possible future treatment options (e.g. *FDA*).

Research included *PubMed*, online free public available somatic (*COSMIC, TCGA, My Cancer Genome, Ensembl VEP, OMIM*), germline (*ClinVar*) and population databases

(*ESP, dbSNP*), including predictive software scores (*FATHMM-MKL, VEP, PolyPhen 2, SIFT*), professional (national health) guidelines on the pathology and significance of detected sequence variants, known mutations and pathogenic variants on screened genes in association with haemato-oncologic malignancies and in general population cohorts. A table proposed by Ritter et al. (2016) for *MVLD*, was modified and used to structure, gather, and compare data (*table 4*).

Table 4: Table used for structured research of MVLD, adjusted (Ritter et al. 2016).

Sequence variant	e.g. *IDH1*V178I
Genome Version	GRCh38
Chromosome	
DNA Position (HGVS)	4:…
rs ID	rs…
RefSeq Transcript	NM_...
RefSeq Protein	NP_...
ENST	ENST…
COSM	COSM…
OMIM	OMIM…
DNA Sub & Position	c.…
Protein Sub & Position	p.…
Variant Type	SNV
Variant Consequence	e.g. missense
Somatic Classification	
Pathogenicity evidence	
COSMIC	
OMIM	
ClinVar	
Ensembl	
ESP	
dbSNP	
TCGA	
MyCancer Genome	
Therapeutic Context	Specific medication
PMIDs	
study details	
Level of Evidence Classification	I-IV
Cancer Type	
Sub-level of evidence	
pro/ret trials/studies	
case reports	
preclinical data	

The cutoff for the systematical research in the above-mentioned manner were gene variants in ≥5% of tested genes, which is a common selection criteria (McClure et al. 2018).

4. Results

4.1 Sequence Variants in Myeloid Neoplasia

Patients suffered MPN (198), MDS (173), AML (172), CMML (31), MDS/MPN overlap (17), SAA (10), and other, non-malignant haematologic diseases (19), (*table 5*).

Table 5: Overview about study characteristics and included patients.

	Characteristics	**Number abs.**	**% of total**
Patients	male	360	57.4
	female	267	42.6
Age	Median	59	
	Min	2	
	Max	80	
Disease	AML	172	27.4
	MPN	198	31.1
	MDS	173	27.6
	CMML	31	4.9
	SAA	10	1.6
	MDS/MPN overlap syndrome	17	2.7
	other	19	3.0
	Not classified	7	1.1

More than 2,000 sequence variants were called, about one third occurred frequently (≥5%). Amongst 32 genes analyzed in 3 amplicon panels, the *TET2* sequence was most frequently altered (71.0% of sampled patients; *TET2*I1762V (41.5% of sampled patients, VUS, FATHMM score .14), *TET2*P363L (21.0%, .70), *TET2*L1721W (18.8%, .78), *TET2*H1778R (5.3%, VUS)). This was trailed by *ASXL1* (23.6%; *ASXL1*E1102D (2.4%) .95), *CEBPA* (22.5%; *CEBPA*P198S (59.5%) VUS), *DNMT3A* (21.2%; *DNMT3A* R882H (3.4%) .98), *JAK2* (16.7%; *JAK2*V617F (16.7%) .94), *TP53* (13.7%; *TP53*P72R (3.7%) .36), *IDH1* (11.0%; *IDH1*V178I (4.2%) .99), *RUNX1* (10.2%; *RUNX1*L56S (1.5%) .90), *GATA2* (9.3%; *GATA2*A164T (21.1%) .91), *EZH2* (8.6%; *EZH2*D185H (7.6%) 1.0), *SF3B1* (7.2%, *SF3B1*K141K (4.1%), VUS), *NRAS* (7.0%; *NRAS*G12D (2.4%) .91), *IDH2* (6.7%; *IDH2*R140Q (4.5%) .98), *KRAS* (5.9%; *KRAS*R161R (2.2%) VUS), *CALR* (5.1%, *CALR*L367fs* (5.2%), VUS), and *KIT* (4.5%; *KIT*M541L (12.0%) .74).

Patients displayed a median of 3 sequence variants (AML, MPN and patients with other, non-malignant haematologic diseases each 3; MDS, CMML and SAA each 4; MDS/MPN overlap patients 5).

Sequence variants found most frequently in AML were *CEBPA*P198S (14.2% of all sequence variants in patients with AML, $p<10^{-3}$, chi² test), *TET2*I1762V (14.0%, p=.013),

*GATA2*A164T (5.4%, $p<10^{-3}$), and *KIT*M541L (2.4%, $p<10^{-3}$). *JAK2*V617F (13.4%, $p<10^{-3}$) was primarily found in patients with MPN, and *EZH2*D185H (2.7%, $p<10^{-3}$) in patients with MDS. *IDH2*R140Q (2.3%, p=.912) and *ASXL1*E1102D (2.3%, p=.111) were most frequently found in MDS/MPN overlap syndrome. *TET2*H1778R (3.0%, p=.466) was seen equally frequent in CMML and in other, non-malignant haematologic diseases, where also *IDH1*V178I (4.5%, p=.027) and *TP53*P72R (3.0%, $p<10^{-3}$) occurred most frequently, and *DNMT3A*R882H (3.0%, p=.011) in CMML. Sequence variants characterizing our SAA patient cohort were *TET2*L1721W (7.5%, p=.101), *SF3B1*K141K, *SF3B1*V1219V, and *TET2*G355D (2,5% each, $p<10^{-3,}$ $p<10^{-3}$ and p=.758). *TET2*P363L (12.0%, p=.671) and *TET2*V128M (6.1%, p=.507) were frequent in other, non-malignant haematologic diseases.

The circos-plot (*figure 2*) shows results of our data. The lables around the plot indicate the diseases, which also determine the colors of the ribbons, protruding lefthand, and the genes, righthand. There are two perspectives to view this plot: Absolute numbers of sequence variants in each analyzed gene are connected via colored ribbons to the diagnosed disease in which they appeared. Or, diseases are connected to genes, showing sequence variants in this disease. This plot gives an overview of frequent mutated genes and the specific mutated genes in the analyzed diseases. *TET2* is the most frequently mutated gene, appearing in approximately equal amount in AML, MDS, MPN. Sequence variants in *CEBPA* appear mostly in AML, and in MPN, there are approximately equal amounts of mutations in *ASXL1* and *JAK2*.

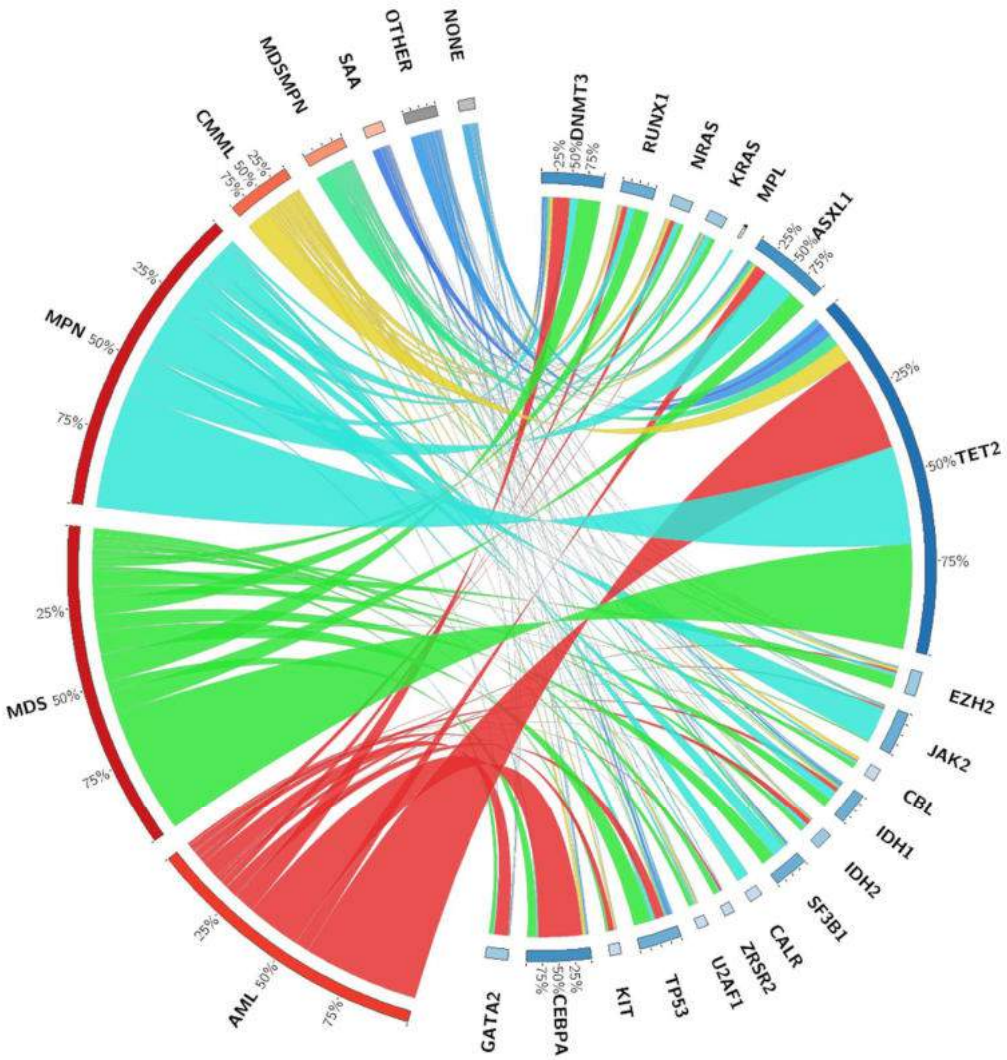

Figure 2. Circos plot, showing diseases (AML, MDS, MPN, CMML, MDSMPN, SAA, other, none) and their mutated genes (*DNMT3, RUNX1, NRAS, KRAS, MPL, ASXL1, TET2, EZH2, JAK2, CBL, IDH1, IDH2, SF3B1, CALR, ZRSR2, U2Af1, TP53, KIT, CEBPA, GATA2*) in absolute numbers, indicated by ribbons. Stroke scale at 0, 25%, 50%, 75% and 100% of each disease and mutated gene. Results of our data analysis.

Among the classified *other diseases* were anemia (4 patients), unclear cytopenia (5), platelet associated disorder (4), other malignant, but non-haematologic diseases (3) and non-malignant haematologic diseases (3).

4.2 Database Research Results

4.2.1 MVLD

Difficulties during categorization arose because of discordant evidence. Determination of the variant strongly depends on data and studies submitted to the database. As at meantime there is no universal database where all data about variants is gathered, determination of pathogenic or benign or anything in between remains fluctuant,

constantly depending on latest data and quality of studies considered in the database or for the scores.

An example of the table designed for the assembly of data of the database research can be seen in *table 3.*

In our data, discordance occurred between pathogenic *FATHMM*- and benign *PolyPhen2*-scores (*KIT*M541L, *IDH1*V178I), between pathogenic predictive scores and databases describing the variant as benign (*RUNX1*L56S, *EZH2*D185H), between neutral FATHMM-score, but benign studies and possibly damaging *PolyPhen2*-score (*TP53*P72R).

Lacking information led to classification difficulties, e.g. pathogenic *FATHMM*- or *PolyPhen2*-score and less information on databases about clinical significance (*TET2*L1721W, *TET2*P336L, *TET2*H1178R), and benign or no scores, but results from *The AACR GENIE* study (2017) showing association to cancer (*CEBPA*P198S, *GATA2*A174T).

4.2.2 Level of Evidence

Among most frequent found variants (20), three could be classified in tier I of the *Joint Consensus Recommendation* (*JAK2*V617F, *IDH2*R140Q, *NRAS*G12D), one in II C (*DNMT3A*R882H), five in II D (*KIT*M541L, *CALR*L367fs, *TET2*I1762V, *TP53*P72R, *GATA2*A164T), and four within a category in between II and III (*IDH1*V178I, *RUNX1*L56S, *TET2*L1721W, *EZH2*D185H). None was classified in tier IV.

Table 6. Database research, example of data collection table for *IDH2*R140Q.

Gene		***IDH2*R140Q**
Genome Version		GRCh38
Chromosome		15q26.1
DNA Position (HGVS)		15:90088702C>T
	rs ID	rs121913502
RefSeq Transcript		NM_002168.3
RefSeq Protein		NP_002159.2
	ENST	ENST00000540499/ENST00000559482/ENST00000330062
	COSM	COSM41590
	OMIM	1.476.500.001
DNA Sub & Position		c.419G>A
Protein Sub & Position		p.R140Q
Variant Type		SNV
Variant Consequence		single base substituion
Somatic Classification		
Pathogenicity	**COSMIC**	pathogenic FATHMM 0.98, >760 samples (mostly haematopoietic/lymphoid), 70 studies (ext.)
Evidence	**OMIM**	samples with D2HGA2, study 1.+ 2.

	ClinVar	pathogenic in D2HGA2/AML, likely pathogenic MDS/MM/squamous cell carcinoma head and neck/neoplasm large intestine, study 2.-14.
	Ensembl	likely pathogenic/pathogenic, study 3., 5.5.-11., 13.-19.
	ESP	n
	dbSNP	n
	TCGA	VEP: moderate, SIFT deleterious low confidence, score=0, PolyPhen=1 probably damaging
	My Cancer Genome	in 0.31% of AACR GENIE cases (AML(8%)/MDS(3.3%)/NSCLC/OMF/melanoma/CMML (5.3)), >120cases with leukemia listed
Therapeutic Context		Enasidenib (approved for AML-therapy by FDA), Venetoclax, AGI-6780
PMIDs		1. 20847235, 2. 24049096, 3. 26619011, 4. 25326635, 5. 25157968, 6. 24606448, 7. 23949315, 8. 23815907, 9. 23558173, 10.22397365, 11. 22160010, 12. 21889589, 13. 20946881, 14. 20171147, 15. 25741868, 16. 20847235, 17. 22417203, 18. 24049096, 19. 30514800
Level of Evidence Classification (Tier I-IV, level A-D)		I

4.3 NGS in Professional Guidelines

4.3.1 MDS Guidelines

The occurrence of *TET2*-variants and -mutations is age- and subclone-dependent, so that it serves as additional diagnostic factor in the Nordic MDS Study Group (NMDS)-guideline (Kittang et al. 2017). The ESMO- guideline (Mesa et al. 2016) notes *TET2* mutation frequency 15-25%, *ASXL1* 10-20%, *DNMT3A* 10%. Additional genetic testing is used for diagnostics in questionable cases, and only in few centers (Mesa et al. 2016). Mutations in *ASXL1* and *DNMT3A* confer a bad prognosis (Yu et al. 2020).

4.3.2 MPN Guidelines

The NCNN-guideline on MPN (Mesa et al. 2016) is set up using the WHO diagnostic criteria from 2016. According to these criteria, genetic testing depends on type of MPN. For PMF and ET, *JAK2, CALR*, and MPL should be included, for PV *JAK2*V617F and *JAK2* exon 12. MF-patients with *CALR* mutation have a higher OS compared to *JAK2*V617F or *MPL*W515. Patients with *TET2*- or *TP53* mutations also show a worse overall prognosis.

In case of questionable hematopoietic stem cell transplantation, so called *high-molecular risk mutations* on *ASXL1, EZH2, TET2, IDH1, IDH2, SRSF2*, and *TP53* should be tested, as they go along with shorter overall- and leukemia-free survival. The amount of high-molecular risk mutations seems to be of importance for the OS, the more mutations the shorter the OS, and especially the occurrence of *ASXL1* mutations. Testing for mutations is used to estimate the prognosis of MF.

A Korean MPN guideline mentions the use of *JAK2*V617F as major criterion for PMF and ET. Molecular testing is done stepwise if MPN is suspected, first *JAK2*V617F, if negative *JAK2* exon 12, then *CALR* and *MPL* (Choi et al. 2015).

4.3.3 AML Guidelines

The NCNN-guideline from 2019 (Tallmann et al. 2019) states the importance of several genes for prognosis and special treatment of patient groups, so that determination of mutations and variants is recommended for *KIT*, *FLT3*, *NPM1*, *CEBPA*, *IDH1*/*IDH2*, *RUNX1*, *ASXL1*, and *TP53.* Marrow sampling should be taken before treatment and quickly in view for rapid decision of additional therapeutically options. *FLT3* mutation status should be determined first, as it is associated with intermediate risk and midostaurin as specific therapeutic option from beginning on of therapy. Patients with *FLT3-ITD, NPM1* and *DNMT3A* seem to profit from a higher daunorubicin dose. Ivosidenib and enasidenib are explored as potential therapeutic options for *IDH-1* and *IDH-2* mutated *AML* patients.

RUNX1, *ASXL1*, and *TP53* are associated with poor risk. The European guideline is from 2013 and was excluded from this work due to massive diagnostic and therapy development since then.

4.3.4 CML Guidelines

The ESMO CML guideline includes only genetic testing by RT-PCR for BCR-ABL and additional mutational testing in case of TKI-therapy failure (Fey and Buske 2013).

4.4 NGS in Patient Cohort Publications

To find out about the nature of the frequently found sequence variants and mutated genes in our cohort, research was performed on studies with comparable cohorts, similar diseases and sequence variant findings. Not for all frequently found sequence variants studies could be found (seetable 7 for our data).

More information on the detail of the single sequence variants can be found in the appendix to this work.

Table 7: Overview of sequence variants in our study cohort.

Gene	**Sequence variants** [gene altered in % of sampled patients; variant (variant altered in % of sampled patients, FATHMM score .14)]
TET2	-71.0% of sampled patients; -*TET2*I1762V (41.5% of sampled patients, VUS, .14), *TET2*P363L (21.0%, .70), *TET2*L1721W (18.8%, .78), *TET2*H1778R (5.3%, VUS)
ASXL1	23.6%; *ASXL1*E1102D (2.4%, .95)
CEBPA	22.5%; *CEBPA*P198S (59.5%, VUS)
DNMT3A	21.2%; *DNMT3A* R882H (3.4%, .98)
JAK2	16.7%; *JAK2*V617F (16.7%, .94)

TP53	13.7%; *TP53*P72R (3.7%, .36)
IDH1	11.0%; *IDH1*V178I (4.2%, .99)
RUNX1	10.2%; *RUNX1*L56S (1.5%, .90)
GATA2	9.3%; *GATA2*A164T (21.1%, .91)
EZH2	8.6%; *EZH2*D185H (7.6%, 1.0)
SF3B1	7.2%, *SF3B1*K141K (4.1%, VUS)
NRAS	7.0%; *NRAS*G12D (2.4%) .91)
IDH2	6.7%; *IDH2*R140Q (4.5%, .98)
KRAS	6.7%; *IDH2*R140Q (4.5%, .98)
CALR	6.7%; *IDH2*R140Q (4.5%, .98)
KIT	4.5%; *KIT*M541L (12.0%, .74)

4.5 Sequence Variants in the General Population and Healthy Control Groups

4.5.1 Variants in Healthy Population Cohort Studies in General

To determine the nature of VUS, data about VUS frequencies among healthy persons is important. Some studies measure existence and frequencies of malignancy associated sequence variants listing all sequence variants in detail.

Auton et al. (2015) report over 88 million variants (84.7 million SNPs, 3.6millio indels, 60,000 structural variants) in genomes of 2,504 people from 26 populations. More than 99.9% of the found variants are SNPs but structural variants affect a greater part of the genome (bases). Also, there were 2,100 to 2,500 common structural variants. Africans had most variant sites assembled. Most of the variants are rare, but paradox, most of the variants in a single genome occur commonly. In a smaller cohort of 681 healthy individuals, Bodian et al. (2014) found genomic variations in 158 genes (average 68 variants per individual, total 2,688 variants). 75% of the variants occurred just in one to two individuals, 43% were novel variants. 326 were mentioned in the *Human Gene Mutation Database* (HGMD) to be possibly disease associated, whereas 2,297 were VUS. A very large cohort of 31,717 cancer patients was compared to 26,136 cancer free individuals using data of genome-wide association studies. Mosaic chromosomal abnormalities were found in 0.89% (517) of cases, variants' frequency rising with age (0.23% <50y., 1.91% 75-79y*),* supporting the Age-related clonal haemopoiesis (ARCH) concept (Jacobs et al. 2012). This effect is also the key issue of a study by McKerrell et al. (2015), looking for mutations in 15 genetic hotspots in 4,219 individuals via ultra-deep sequencing, revealing increasing clonal haemopoiesis with age (0.8% <60 years, 19.5% ≥

90 years). Spliceosome mutations (like *SF3B1*, *SRSF2*) were found solely in older individuals (>70 years).

4.5.2 Sequence Variants in the general Population and Healthy Control Groups in Detail

To prove accuracy of circulating exosome DNA, Yang et al. (2017) compared results of *KRAS*G12D and *TP53*R273H mutations in patients with pancreatic pathologies and healthy individuals (n=114). *KRAS*G12D was present in 39.6% of pancreatic patients and in 2.6% of healthy individuals, *TP53*R273H in 4.2% of pancreatic cases and none in healthy individuals. Unfortunately, no follow-up data is available to see what happened to the positive tested healthy individuals.

In the European Prospective Investigation into Cancer and Nutrition study, Gormally et al. (2006) looked at *KRAS2* (present in 1.2%, 13/1098) and *TP53* (3.6%, 20/550) mutations in cfDNA in lung, bladder, or upper aerodigestive tract cancers or leukemia compared to matched (follow-up, age, sex, area of recruitment, smoking status) healthy individuals. In the follow-up (up to 18.3 months), 15 developed cancer. Six of the 13 mutations in *KRAS2* were present in the patients' group (5 bladder cancers), and 7 in the healthy control group (one developed skin cancer). Ten of 20 mutations in *TP53* were in the patients' group (7 bladder cancers), and ten in the control group (one developed pancreatic cancer).

Evidence on *CEBPA* mutations in healthy individuals is given in a study (Fuchs et al. 2010) comparing patients with peripheral artery disease (61 mutated of 264 tested), ischaemic heart disease (17/45) and hyperlipidemia (10/24) compared to healthy individuals (38/98). 16 variations in *CEBPA* were found with no significant difference in patient control group, except for 580_585 dup, increased in ischaemic heart disease.

Using NGS with a very sensitive targeted error-corrected sequencing (0.0003 variant allele fraction), Young et al. (2016) demonstrated *DNMT3A* and *TET2* mutations in 95% of healthy individuals (121,701 females from the longitudinal Nurses' Health Study). This high frequency in healthy population and long stability of mutations typically associated with clonal haematopoiesis stands in contrast to other studies. Frequent mutations in epigenetic regulators can increase self-renewal capacity, compensate, and preserve long-term blood production, without increasing proliferation.

The dynamic of current mutations of haematologic cancers in healthy individuals (n=17,182) was observed by Jaiswal et al (2014) (rarely <40years; 9.5% (219 of 2300 persons) aged 70-79; 11.7% (37 of 317) aged 80-89; 18.4% (19 of 103) aged 90-108). Most of the mutations were found in *DNMT3A* (403 variants), *TET2* (72), *ASXL1* (62), *TP53* (33), *JAK2* (31), associated with increased risk of haematologic cancer, mortality, incident coronary heart disease and ischaemic stroke. Follow-up data of a part of the

cohort (3,342 individuals, 96 months) showed somatic mutations in 134 cases, 16 developed haematologic cancers.

In a study applying a similar method as we did, but testing exhaled breath condensate (EBC) on mutation hotspot regions (22 genes) of healthy individuals (n=20), 35 somatic mutations were found in 15 cases (75%), (*TP53* (in 8 subjects), *KRAS* (3, one with G12V), *SMAD4* (3), *NRAS* (2)) and 106 not yet reported novel mutations in *DDR2, SMAD4, MET, ERBB4, ALK, EGFR, FGFR3, PIK3CA, PTEN, AKT1, ERBB2, KRAS, STK11, NRAS, FGFR1, CTNNB1, FBXW7, BRAF, FGFR2, MAP2K1* (Youssef et al. 2017).

4.6 Evolution of SNPs

In a study by Kong et al. (2016), evolution of SNPs is described: the average de novo mutation rate of 1.2×10^{-8} per nucleotide per generation (increasing by 2 nucleotides / year) in 78 Icelandic parent offspring trios, supposing a correlation between the diversity in SNP mutation rate with the fathers's age at conception. This is a small cohort study in a specific population with a questionable significance but it gives an idea of motion in SNP evolution.

A closer look at the *JAK2*V617 mutation was taken by Nielsen et al. in several studies: evaluation of data from the Copenhagen City Heart Study with 10,507 individuals showed *JAK2*V617 mutation prevalence of 0.2% (18). During follow-up (up to 17.6 years), all individuals who had been tested positively died, 14 developed cancer (7 haematologic cancer, 5 MPN) and 4 did not develop any cancer (Nielsen et al. 2011). In a second study, Nielsen et al. (2014) tested 49,488 individuals of the Copenhagen General Population Study and found *JAK2*V617 in 0.1% (63). A retesting was done 4 to 9 years later with 48 of the 63 positive tested individuals (8 had not developed a disease, 20 ET, 13 PV, 7 PMF). These results again underline the pathogenicity for MPN of the *JAK2*V617 mutation.

The ***KIT*M541L** polymorphism was found in a cohort of 64 unrelated subjects with a frequency of 0.09 and in a two-generation family positive for this mutation, unrelated to any disease (Nagata et al. 1995).

4.7 Co-Mutations

Spectra of concurrent sequence variants are observed in myeloid disorders, at points specifying the disease subtype but also therapy options and prognosis.

Our data (*figure 3*) show a high incidence of co-mutations between *TET2* and *CEBPA* (113 sequence variants, *TET2* and *ASXL1* (103), *TET2* and *DNMT3A* (93), and *TET2* with *JAK2*V617F (78). As *TET2* is frequently mutated in our cohort and in myeloid disorders, *TET2* is involved in all of the most frequent co-mutations. The high incidence of co-mutations in our study can be explained by the characteristics of the patients we included, most of them presenting with a high-risk myeloid disease and more than one mutation.

	DNMT3A	FLT3	RUNX1	N-RAS	K-RAS	MPL	ASXL1	TET2	EZH2	IKAROS	JAK2-V617F	CBL	IDH1	IDH2	SF3B1	SRSF2	CALR	ZRSR2	U2AF1	ETV6	TP53	WT1	KIT	NPM 1	CEBPA	GATA2	PTPN11
DNMT3A		1	13	9	8	0	28	**93**	18	3	12	8	29	11	20	1	6	9	8	4	24	1	3	7	28	16	1
FLT3	1		0	1	1	0	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	1	0
RUNX1	13	0		13	5	0	29	**51**	7	2	11	9	6	6	8	4	4	1	2	0	12	1	3	0	15	9	3
N-RAS	9	1	13		6	0	13	32	6	1	4	4	7	5	7	1	0	3	4	1	5	0	2	0	8	6	3
K-RAS	8	1	5	6		0	13	28	8	0	4	2	7	4	12	3	5	4	2	1	14	1	1	0	5	1	1
MPL	0	0	0	0	0		3	8	0	0	0	0	0	0	1	2	1	0	0	0	0	0	0	0	0	0	0
ASXL1	28	2	29	13	13	3		**103**	17	6	34	6	18	15	13	8	9	1	5	0	22	2	5	0	19	16	2
TET2	**93**	5	**51**	32	28	8	**103**		40	9	**78**	19	**49**	28	27	12	20	17	17	2	**57**	4	22	7	**113**	**45**	7
EZH2	18	0	7	6	8	0	17	40		1	4	1	8	5	9	1	3	5	1	1	10	0	0	0	1	2	0
IKAROS	3	0	2	1	0	0	6	9	1		7	0	3	1	2	0	0	0	0	1	0	0	0	0	0	0	0
JAK2-V617F	12	0	11	4	4	0	34	**78**	4	7		3	9	6	8	9	0	1	1	1	4	0	1	0	5	1	0
CBL	8	0	9	4	2	0	6	19	1	0	3		3	3	3	2	2	3	4	0	3	0	0	0	5	1	0
IDH1	29	0	6	7	7	0	18	**49**	8	3	9	3		2	10	2	4	3	4	2	13	0	1	1	6	3	0
IDH2	11	0	6	5	4	0	15	28	5	1	6	3	2		3	3	2	6	3	0	3	0	0	2	10	2	2
SF3B1	20	0	8	7	12	1	13	27	9	2	8	3	10	3		2	5	5	5	4	19	1	0	0	0	2	0
SRSF2	1	0	4	1	3	2	8	12	1	0	9	2	2	3	2		0	0	0	0	1	0	0	0	0	0	0
CALR	6	0	4	0	5	1	9	20	3	0	0	2	4	2	5	0		2	0	1	5	0	0	0	0	0	0
ZRSR2	9	0	1	3	4	0	1	17	5	0	1	3	3	6	5	0	2		5	1	7	0	0	0	0	0	0
U2AF1	8	0	2	4	2	0	5	17	1	0	1	4	4	3	5	0	0	5		1	2	0	0	0	0	0	0
ETV6	4	0	0	1	1	0	0	2	1	1	1	0	2	0	4	0	1	1	1		1	0	0	0	0	0	0
TP53	24	0	12	5	14	0	22	**57**	10	0	4	3	13	3	19	1	5	7	2	1		1	4	1	13	5	1
WT1	1	1	1	0	1	0	2	4	0	0	0	0	0	0	1	0	0	0	0	0	1		1	0	3	3	1
KIT	3	1	3	2	1	0	5	22	0	0	1	0	1	0	0	0	0	0	0	0	4	1		3	18	7	1
NPM 1	7	1	0	0	0	0	0	7	0	0	0	0	1	2	0	0	0	0	0	0	1	0	3		5	3	1
CEBPA	28	2	15	8	5	0	19	**113**	1	0	5	5	6	10	0	0	0	0	0	0	13	3	18	5		37	7
GATA2	16	1	9	6	1	0	16	**45**	2	0	1	1	3	2	2	0	0	0	0	0	5	3	7	3	37		4
PTPN11	1	0	3	3	1	0	2	7	0	0	0	0	0	2	0	0	0	0	0	0	1	1	1	1	7	4	

Figure 3. Co-occurrence of sequence variants, grey box between 50-100 co-occurrences, dark grey >100 co-occurrences, absolute numbers, results of our data analysis.

Most studies with co-mutations report cases with AML, as during the course of the disease, like evolution from MDS to AML, and with age more mutations develop. Also, treatment, relapse, or stem cell transplantation can lead to changes in mutation patterns.

In de novo-AML, co-mutations occur more frequently in the intermediate cytogenic risk group, with average 2.7 mutations, and are associated with shorter OS and higher cumulative incidence of relapse (Wakita et al. 2016). In the study, three or more mutations were classified as *complex molecular genetic abnormalities* (CMGA) having a negative impact on prognosis. CMGA patients often had mutations in *NPM1*, *DNMT3A*, *FLT3*-ITD, *TET2* and *IDH1/2*. *CEBPA* single mutations occurred more frequently in patients with CMGAs, but *CEBPA* double mutations more frequently in patients without CMGAs (Wakita et al. 2016). Higher incidence of co-mutations at the time of diagnosis is associated with relapse after MR, but no additional co-mutations seem to occur at relapse compared to time of diagnosis (Höllein et al. 2019). A case study shows co-occurrence of variants in *DNMT3A*, *FLT3*, *KRAS*, *NPM1*, *PTPN11*, and *TET2* in an AML patient with an aggressive disease progress (Lee et al. 2019).

AML patients often have *NPM1* mutations with *FLT3*-ITD and mutations in methylation regulation genes (*DNMT3A*, *TET2*, and *IDH1/2). NPM1*-mutated AML patients with mutations in *TET2* and *IDH1/2* are rare but have a longer relapse-free and a better chance for OS than patients with CD34 and or HLA-DR positivity (Mason et al. 2018). Co-occurrence of *NPM1* mutations in AML patients with mutations in DNA methylation genes is also stated by Patel et al. 2017. Additionally, *NPM1* mutations are seen together with mutations in RNA splicing genes (*SRSF2*, *SF3B1*), cohesion complex genes, and cell signaling pathways genes (*FLT3*, *NRAS*, and *PTPN11*). Apparently, *NPM1* mutations to

not co-occur with *IDH2*R172. Concluding, *NPM1* mutations seem to happen late in the course of AML (Patel et al. 2017). A third study provides evidence for co-mutation of *FLT3*-TKD and *NPM1* in AML patients, associated with a more favorable response and survival rate compared to *NPM1* mono mutation (Boddu et al. 2017).

Yu et al. (2020) looked for differences in molecular patterns between MDS and AML patients. Mutations on *CEBPA*, *FLT3-ITD*, *DNMT3A*, *NPM1* and *IDH1/2* have a higher incidence in AML patients, and *TET2* and *U2AF1* occur less often than in MDS patients (Yu et al. 2020).

5. Discussion

This study examines the presence of VUS and their possible influence on diagnosis, therapy, and prognosis in a cohort of patients with high-risk myeloid disorders from a university department of stem cell transplantation.

The first aim of the study was to assemble evidence on the occurrence and frequency of sequence variants from a real life patient cohort.

Secondly, it should be shown that the nature of sequence variants can be more clearly determined with this additional information.

Thirdly, the effect of results from NGS in treatment of patients with myeloid disorders should be lighted.

The first aim could be reached for some VUS significantly, as these results show:

In AML most frequently altered were *CEBPA*P198S (14.2% of all sequence variants in patients with AML, $p<10^{-3}$, chi² test), *TET2*I1762V (14.0%, p=.013), *GATA2*A164T (5.4%, $p<10^{-3}$), and *KIT*M541L (2.4%, $p<10^{-3}$), in MPN it was *JAK2*V617F (13.4%, $p<10^{-3}$) and in MDS *EZH2*D185H (2.7%, $p<10^{-3}$). In MDS/MPN overlap syndrome *IDH2*R140Q (2.3%, p=.912) and *ASXL1*E1102D (2.3%, p=.111) could be detected. In CMML it was *TET2*H1778R (3.0%, p=.466), which was also seen in equal frequency in other, non-malignant haematologic diseases, together with *IDH1*V178I (4.5%, p=.027) and *TP53*P72R (3.0%, $p<10^{-3}$). In CMML, *DNMT3A*R882H (3.0%, p=.011) occurred also in a significant frequency. In the SAA patient cohort, there were *TET2*L1721W (7.5%, p=.101) and *SF3B1*K141K, *SF3B1*V1219V and *TET2*G355D (2,5% each, $p<10^{-3,}$ $p<10^{-3}$ and p=.758) found in remarkable frequency.

For the second aim, the minimal variant level and the level of evidence (Ritter et al. 2016) were obtained through data base research and compared to published and publicly accessible data.

Difficulties arose using the proposed data research. If looking for evidence of a specific VUS, the published data often is incomplete and rarely related just to myeloid disorders.

The greater the number of collected data, the more diverse subjective evaluations about the significance and impact of VUS one gets (see table 3). So, awareness of different

qualities of databases or scores is an important part of the interpretation of the nature of VUS. Estimating the level of evidence of underlying comparing data, the tendency and definitiveness of own assumptions can be revealed. It is important to deeply study underlying data, possibly sort out, depending on significance and relevance for the own data.

As we wanted to give a broader overview on the process of interpretation of VUS, we did not restrict the data research on specific publications on myeloid disorders, which should be done in future research for a more specific interpretation. Yet this might carry the problem of reduced available knowledge on especially the rarer VUS.

The nature of sequence variants we found in our cohort often remains unclear and data base research did just partly help to reach a clearer interpretation.

To approach the third aim, we studied existing national disease specific guidelines (Kittang et al. 2017, Mesa et al. 2016, Choi et al. 2015, Tallman et al. 2019, Fey et al. 2013, Hochhaus et al. 2018).

The use of NGS in diagnosis of myeloid disorders serves as an addition to cytomorphology and histopathology. In MDS, *TET2*-variants and -mutations are used additionally in the NMDS-guideline (Kittang et al. 2017). The presence of *ASXL1* and *DNMT3A* confer a bad prognosis. In MPN, NGS can be used to define disease subtypes (Mesa et al. 2016). In PMF and ET, variants in *JAK2, CALR*, and *MPL* can be found, in *PV JAK2*V617F and *JAK2* exon 12 (Mesa et al. 2016). In MF, the presence of *CALR* mutation indicates a higher OS, and *TET2*- or *TP53* mutations lead to a worse prognosis (Mesa et al. 2016). To decide on transplantation, as additional point, high-molecular risk mutations (in *ASXL1, EZH2, TET2, IDH1, IDH2, SRSF2*, and *TP53)* are assessed as they are associated with shorter overall- and leukemia-free survival (Mesa et al. 2016). The *JAK2*-inhibitor ruxolitinib is used for treatment in patients with corresponding mutation (Eghtedar et al. 2012). The Korean guideline uses *JAK2V617F* as major criterion for PMF and ET (Choi et al. 2015). In AML, the NCCN-guideline recommends looking for variants and mutations, as FLT3 mutations are associated with intermediate risk and can be treated with midostaurin, patients with *IDH1* and *IDH2* mutations might in future be treated with specific inhibitors, e.g. ivosidenib and enasidenib. Mutations in *RUNX1*, *ASXL1*, and *TP53* show a poor risk. In CML, genetic testing by RT-PCR for *BCR-ABL* is used, additional genetic testing does not play an important role in first line therapy (Tallman et al. 2019).

There were unexpected findings, sequence variants we found quite often in our cohort of high-risk myeloid disorders which could not be proven by database research to be known in association with haematologic malignancies. *CEBPA*P198S and *KRAS*R161R could not be found in any study or database. For *IDH1*V178I, no association to haemato-oncologic malignancies could be found.

*SF3B1*K141K is listed as SNP, which means that it is classified as not having a malignant effect, but the evidence is not linked in any database. The role of these sequence variants in myeloid disorders and in our cohort remains unclear. It can even be discussed if this might be a bias through the NGS panel diagnostic used.

There were some limitations in our study. For a complete picture, matched material, tumor and germline of the same patient, should be analyzed to separate germline from somatic origin of disease. As NGS is used more and more, the obtained data on VUS is growing constantly. Together with continuous optimization of data processing tools and data exchanging platforms, interpretations of VUS are changing quickly. Research for the presented thesis has been completed in 2019. Meanwhile, the interpretation of some sequence variants might have changed and also specific therapeutic options might have been further developed.

Data about pre-treatment was not obtained, this might be a confounding factor, because treatment can induce genetic variation. As the patient cohort consists of high-risk myeloid disorders, it is very likely that treatment was given to most patients before presenting for sampling. Samples analyzed in this study were taken before stem cell transplantation. Considering that usually induction chemotherapy is performed before stem cell transplantation and sampling time was sometimes within less than one month before stem cell transplantation was done, this aspect should not be neglected. This might as well be a reason for the amount of variants found in the AML cohort.

Some subgroups, like SAA (10 patients) and MDS/MPN overlap syndrome (17), were rather small. For more precise data on subgroup comparisons, greater populations are needed.

Also, this study can give just a snapshot on sequence variants in myeloid disorders. Knowing that the genetic pattern changes in the course of disease and treatment, this should be analyzed in future studies, together with patient survival data.

To sum up, this study thoroughly examines the association and role of sequence variants in high-risk myeloid disorders. For some sequence variants, a significant correlation to a myeloid disease can be established. The role of some other sequence variants in myeloid disorders remains unclear. It could be shown in this work that some of the data obtained through NGS is already used in diagnostic, treatment and prognosis of myeloid disorders, which is for example reflected in disease specific guidelines. Still, for a lot of sequence variants, evidence on their role is missing, as most of them are rare. This gap might be filled in course of time as with the increasing use in well-established, comparable NGS-protocols and patient cohorts more data is produced which can then be analyzed more systematically, reaching higher significance. So this task should be continued to gather data in regular diagnostic. Even more it seems to be important to make the obtained data

accessible on publicly available databases, so that huger analysis can be performed in the near future to develop uniform valid and applicable tools to better and faster assess for example the individual risk of a sequence variants profile of a patient.

6. Conclusion

VUS on frequently mutated genes in haematologic malignancies occur with high abundancy in this high-risk cohort of patients and differ in frequency between various myeloid disorders.

In our cohort of 627 patients with high-risk myeloid disorders, we found more than 2,000 sequence variants, with a median of 3 sequence variants per patient. Patients suffered from MPN (198), MDS (173), AML (172), CMML (31), MDS/MPN overlap (17), SAA (10), and non-malignant haematologic diseases (19). We could confirm existing data about frequent mutated genes in this patient group. Frequently mutated genes were *TET2* (71.0% of sampled patients; *TET2*I1762V (41.5% of sampled patients, VUS, FATHMM score .14), *TET2*P363L (21.0%, .70), *TET2*L1721W (18.8%, .78), *TET2*H1778R (5.3%, VUS)). This was trailed by *ASXL1* (23.6%; *ASXL1*E1102D (2.4%) .95), *CEBPA* (22.5%; *CEBPA*P198S (59.5%) VUS), *DNMT3A* (21.2%; *DNMT3A*R882H (3.4%) .98), *JAK2* (16.7%; *JAK2*V617F (16.7%) .94), *TP53* (13.7%; *TP53*P72R (3.7%) .36), *IDH1* (11.0%; *IDH1*V178I (4.2%) .99), *RUNX1* (10.2%; *RUNX1*L56S (1.5%) .90), *GATA2* (9.3%; *GATA2*A164T (21.1%) .91), *EZH2* (8.6%; *EZH2*D185H (7.6%) 1.0), *SF3B1* (7.2%, *SF3B1*K141K (4.1%), VUS), *NRAS* (7.0%; *NRAS*G12D (2.4%) .91), *IDH2* (6.7%; *IDH2*R140Q (4.5%) .98), *KRAS* (5.9%; *KRAS*R161R (2.2%) VUS), *CALR* (5.1%, *CALR*L367fs* (5.2%), VUS), and *KIT* (4.5%; *KIT*M541L (12.0%) .74). Our data revealed differences between existing data in associated variants and diseases in some cases: *CALR*L367fs* more frequently in MPN, existing studies more in MF, *GATA2*A164T and *SF3B1*K141K were not pre-described pathogenic, *IDH1*V178I not mentioned in studies with myeloid disorders, no research data was found for *DNMT3A*K456fs* and *KRAS*R161R. Level of evidence for database research was mostly II/ III. Difficulties arise with discordant, incomplete published data. As most sequence variants are very rare, there are studies with evidence of sequence variants in healthy general population but mostly, details about the found sequence variants are not published and follow-up data is lacking.

Applying structured research on various databases and using predictive calculation models together with professional guidelines and data from publications on patient cohort, healthy control groups, and general population cohorts, can help to gather profound evidence. This can serve as a support to determine the nature and role of VUS and gives an overview about existing evidence for variants, which can be used as template for future research, but should be updated regularly. This approach might be very time-consuming

in daily routine, but necessary to develop uniform classification systems and approaches for future findings in diagnostic NGS, as for risk stratification of patients, of single mutations or mutation profiles, or subgroup analysis for specific therapeutically options. Constantly ameliorating sequencing tools and data processing methods could help to simplify and shorten this process. As for now, the nature and role of VUS often remains uncertain.
We will consistently have to reconsider our guidelines and question their finiteness.

VUS häufig mutierter Gene bei malignen hämatologischen Erkrankungen konnten in diesem Hochrisiko-Patientenkollektiv häufig nachgewiesen werden. Die Häufigkeit ist hierbei je nach Krankheitsentität unterschiedlich. In unserer Kohorte von 627 Patienten mit Hochrisiko-hämatologischen Erkrankungen, detektierten wir mehr als 2.000 Sequenzevarianten, im Median 3 Sequenzvarianten. Die Patienten waren an MPN (198), MDS (173), AML (172), CMML (31), MDS/MPN overlap (17), SAA (10) und anderen, nicht-malignen hämatologischen Erkrankungen (19) erkrankt. Wir konnten die bereits bestehenden Daten über häufig mutierte Gene in malignen-hämatologischen Erkrankungen in dieser Patientenkohorte bestätigen. Häufig mutierte Gene waren *TET2* (71.0% der untersuchten Patienten; *TET2*I1762V (41.5% der untersuchten Patienten, VUS, FATHMM score .14), *TET2*P363L (21.0%, .70), *TET2*L1721W (18.8%, .78), *TET2*H1778R (5.3%, VUS)). Dies wurde nachgewiesen durch *ASXL1* (23.6%; *ASXL1*E1102D (2.4%) .95), *CEBPA* (22.5%; *CEBPA*P198S (59.5%) VUS), *DNMT3A* (21.2%; *DNMT3A*R882H (3.4%) .98), *JAK2* (16.7%; *JAK2*V617F (16.7%) .94), *TP53* (13.7%; *TP53*P72R (3.7%) .36), *IDH1* (11.0%; *IDH1*V178I (4.2%) .99), *RUNX1* (10.2%; *RUNX1*L56S (1.5%) .90), *GATA2* (9.3%; *GATA2*A164T (21.1%) .91), *EZH2* (8.6%; *EZH2*D185H (7.6%) 1.0), *SF3B1* (7.2%, *SF3B1*K141K (4.1%), VUS), *NRAS* (7.0%; *NRAS*G12D (2.4%) .91), *IDH2* (6.7%; *IDH2*R140Q (4.5%) .98), *KRAS* (5.9%; *KRAS*R161R (2.2%) VUS), *CALR* (5.1%, *CALR*L367fs* (5.2%), VUS), und *KIT* (4.5%; *KIT*M541L (12.0%) .74). Unsere Daten zeigten Unterschiede im Vergleich zu bereits existierenden Daten über genetische Veränderungen in Assoziation mit myeloischen Erkrankungen: *CALR*L367fs* war häufiger in MPN mutiert, vorbeschrieben ist es öfter in MF. *GATA2*A164T und SF3B1K141K sind nicht als pathogen vorbeschrieben, IDH1V178I ist nicht vorbeschrieben in myeloischen Erkrankungen, DNMT3AK456fs* und KRASR161R waren gar nicht vorbeschrieben. Das Evidenzniveau für die Datenbankrecherche beträgt meistens II/II. Diesbezügliche Schwierigkeiten der Einschätzung ergeben sich aufgrund fehlender oder unvollständig publizierter Daten. Die meisten Sequenzvarianten sind sehr selten. Große Populationsstudien, in denen das Vorkommen in gesunden Probanden untersucht wird, publizieren ihre Daten häufig

unvollständig, gerade bezogen auf die seltenen Sequenzvarianten, und es gibt zuallermeist keine Verlaufsdatenerhebung. Strukturierte Recherche anhand verschiedener Datenbanken, prädiktive Kalkulationsmodelle sowie (inter)nationale Leitlinien und Daten von Patientenkohorten, gesunden Kontrollgruppen und Bevölkerungsstudien können helfen, fundierte Evidenz zusammenzustellen über die Auswirkungen und die Rolle von VUS. Dies kann als Grundlage für zukünftige Recherchen dienen, sollte aber regelmäßig aktualisiert warden. Solche Herangehensweise ist sehr zeitaufwändig, aber notwendig, um ein allgemeingültiges Klassifikationssystem für Befunde aus zu diagnostischen Zwecken durchgeführtem NGS zu erstellen, beispielsweise zur Risikostratifizierung und für Therapieoptionen. Dieser Prozess wird durch regelmäßige Aktualisierung und Verbesserung der Datenprozession an Genauigkeit und Schnelligkeit gewinnen. Gegenwärtig bleiben die Rolle und Auswirkung von VUS häufig unklar Es erscheint daher erforderlich und unabdingbar, unsere Leitlinien laufend zu überarbeiten und deren Endgültigkeit zu überdenken.

7. Nonstandard Abbreviations

AACR GENIE	American Association for Cancer Research, Genomic Evidence Neoplasia Information Exchange (project)
aCML	Atypical Chronic Myeloid Leukemia
AML	Acute Myeloid Leukemia
AMP	Association for Molecular Pathology
ARCH	Age-Related Clonal Haemopoiesis
APL	Acute Ppromyelocytic Leukemia
BC	Bronchial Carcinoma
BCBM	Breast Cancer Brain Metastasis
CEL	Chronic Eosinophilic Leukemia
CEL-NOS	Chronic Eosinophilic Leukemia - not otherwise specified
CKB	Clinical Knowledgebase (Jackson laboratory)
ClinVar	Clinical Variants Archive
CLL	Chronic Lymphocytic Leukemia
CMGA	Complex Molecular Genetic Abnormalities
CML	Chronic Myelogenous Leukemia
CMML	Chronic Myelomonocytic Leukemia
CMPN	Chronic Myeloproliferative Neoplasia
CN-AML	Cytogenetically Normal Acute Myeloid Leukemia
CNL	Chronic Neutrophilic Leukemia
COSM	ID of COSMIC Database,
COSMIC	Catalogue of Somatic Mutations In Cancer
CPSS	CMML-Specific Prognostic Scoring System
CRC	Colorectal Cancer
CTC	Common Toxicity Criteria
dbSNP	Single Nucleotide Polymorphism Database
DFS	Disease Free Survival
DIPSS	Dynamic International Prognostic Scoring System
D2-HGA	2-Hydroxyglutaric Aciduria
Ensembl	Genome Browser
Ensembl VEP	Ensembl Variant Effect Predictor
ENST	Ensembl Transcript ID
ESMO	European Society for Medical Oncology
ESP	Exome Sequencing Project
ET	Essential Thrombocythemia
ETP-ALL	Early T- Cell Precursor Acute Lymphoblastic Leukemia

FAB M1-7	French-American-British Classification of AML
FATHMM-MKL	Functional Analysis Through Hidden Markov Models
FDA	Food and Drug Administration
GIST	Gastrointestinal Stroma Tumor
GRCh38	Human Reference Genome
HES	Hypereosinophilic Syndrome
HGVS	Human Genome Variation Society
HLA-DR	Human Leukocyte Antigen – DR isotype
HSC	Haematopoietic Stem Cell
IBM SPSS	International Business Machines Corporation-Statistical Product and Service Solutions
ICD	International Statistical Classification of Diseases and Related Health Conditions
IMF	Idiopathic Myelofibrosis
IPSS	International Prognostic Scoring System
IPSS-R	Revised International Prognostic Scoring System
JMML	Juvenile Myelomonocytic Leukemia
mCRC	Metastatic Colorectal Cancer
MDS	Myelodysplastic Syndrome
MDS/MPN-RS-T	Myelodysplastic/Myeloproliferative Syndrome with Ring Sideroblasts and Thrombocytosis
MDS/MPN-U	Myelodysplastic/Myeloproliferative Syndrome-Unclassifiable
MDS-RS	Myelodysplastic Syndrome with Ringed Sideroblasts
MF	Myelofibrosis
MIPSS	Mutation-Enhanced International Prognostic Scoring System
MPD	Myeloproliferative Disease
MPN	Myeloproliferative Neoplasia
MVLD	Minimal Variant Level Data
My Cancer Genome	Database About Clinical Impact of Molecular Biomarkers
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
NCNN	National Comprehensive Cancer Network
NGS	Next Generation Sequencing
NMDS	Nordic MDS Study Group
NSCLC	Non Small Cell Lung Carcinoma
OMF	Myelofibrosis
OMIM	Online Mendelian Inheritance in Man (database)

OS	Overall survival
PC	Prostate Cancer
PGM	Personal Genome Machine™
PMF	Primary Myelofibrosis
PMID	PubMed-database ID
PolyPhen 2	Polymorphism Phenotyping v2 (score)
pro	Prospective
PSA	Prostate-Specific Antigen
PV	Polycythemia Vera
RefSeq	Reference Sequence (database)
ret	Retrospective
RT-PCR	Reverse Transcription Polymerase Chain Reaction
rsID	Reference SNP cluster ID
SAA	Severe Aplastic Anemia
sAML	Secondary Acute Myeloid Leukemia
SCT	Stem Cell Transplantation
SIFT	Sorting Intolerant From Tolerant (prediction of amino acid substitution affecting protein function)
SNOMED	Systematized Nomenclature of Medicine
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
TCGA	The Cancer Genome Atlas
TKI	Tyrosine Kinase Inhibitor
UMLS	Unified Medical Language System
VEP	Variant Effect Predictor
VUS	Variants of Unclear (or Unknown) Significance
wt	Wild Type (mutation)

8. Appendix

8.1 CALR

In cohorts of MPN patients, *CALR* mutations occur in *JAK2*- and *MPL* non-mutated patients (67% in ET, 88% in PMF (Klampfl et al. 2013), 84% in MPN, 70% in follow-up sub-group (Nangalia et al. 2013). The most frequent sequence variant in our data, *CALR*L367fs*, was not mentioned specifically in either of the studies.

COSMIC, OMIM, ESP and TCGA do not list *CALR*L367fs*. ClinVar classifies it pathogenic, associated with MF and somatic thrombocythemia, but in the underlying studies the variant cannot be found due to incomplete published data. dbSNP is similar to ClinVar. My Cancer Genome has *CALR*L367fs in 0.13% of AACR GENIE cases (25 cases MF, 4 MPN, 2 leukemia, The AACR Project GENIE Consortium 2017).

More than 90% of *CALR* mutations in our cohort are in the MPN-subgroup.

8..2 CEBPA

Mutations of *CEBPA* can be found in different cancers, most frequently in AML (7.7%) and MDS (1.1%) (Gombart et al. 2002). Leroy et al. (2005) found *CEBPA* mutations exclusively in AML patients, in M1, M2 and M4 FAB subtypes. Pabst et al. (2001) point out, that the *CEBPA* mutation is expressed in myelomonocytic cells and therefore leads to a differentiation block in AML. They found frameshift *CEBPA* mutations more frequent in AML-M2 patients (16%) than overall in AML patients (7.3%). These findings are as wll depicted by Snaddon et al. (2003), having 10-15% of AML-patients with *CEBPA* mutation, 40% in the M2 subgroup.

Overall, a *CEBPA* mutation can be seen in about 8 to 16% of AML-patients. We detected a little higher rate with 22.5% in our cohort. The sequence variant we found most frequently, *CEBPA*P198S, does not have any database entry, so it is a SNV (single nucleotide variant) and its nature remains unclear.

8.3 EZH2

Overexpression of *EZH2* correlates with cell proliferation, invasion, adhesion, and metastasis in several cancer types (Shen et al. 2013). In patients with MDS, Nikoloski et al. (2010) found heterozygous acquired somatic mutations in 23% of the patients, in 40% *TET2* mutations were present as well. Ernst et al. (2010) identified *EZH2* mutations in MDS/MPN (12%) and in PMF (13%).

*EZH2*D185H is mentioned by the Jackson Laboratory to be situated in an interaction region with *DNMT1 / 3A / 3B*. It has been sequenced, in a study with HCC patients (3 homozygous, 36 heterozygous), not showing a significant distribution difference compared to healthy population (Gao et al. 2015). In a study about childhood ALL, Schäfer et al.

(2016) found loss of function *EZH2* mutations in 1.3% (2/152) in common- and ETP-ALL, 17.1% of these were *EZH2*D185H.

8.4 GATA2

Hou et al. (2015) show, that *GATA2* mutations in AML are unstable during disease evolution. *GATA2* mutations were found in AML patients with *CEBPA* mutations, *CEBPA* double-mutation (27.4%), single-mutation (6.7%), and *CEBPA* wt (1%). It is supposed to be a second hit in the leukemogenesis of AML, as it is expressed in haematopoietic progenitor cells (Briegel et al. 1993) and regulates the gene expression in haematopoietic cells (Tsai et al. 1994). Hahn et al. (2011) mention the *GATA2* gene a predisposition for familiar MDS or familial AML. In the study of Spinner et al. (2014) with 57 patients, *GATA2*-mutations were found in MDS patients (84%), 14% developed AML, and 8% CMML.

According to the Jackson Library, *GATA2*A164T is not situated in a known functional domain, but there is one study measuring sequence variants of *GATA2* in correlation with DFS in a cohort of Chinese colorectal cancer patients, where a significant association between DFS and colorectal cancer could be seen, but none between *GATA2*A164T and genotype or other clinical pathologies (Liu et al. 2015). NCBI ClinVar mentions it to be benign/likely benign (2 samples, primary myelodysplasia and lymphedema). Interestingly, we had *GATA2*A164T in 9.3% of patients in our cohort, most frequently (5.4%) in AML patients, indicating a possible pathogenic role. Follow-up sampling would have been interesting here. MVLD research reveals an exclusion from COSMIC, as *GATA2*A164T is a SNP (single nucleotide polymorphism). A triple negative ET case study shows a frequency of 0.6 (Zaidi et al. 2017) A Chinese study demonstrates an association with CRC (Liu et al. 2015). Benign diseases found in combination with *GATA2*A164T are Parkinson's disease (Kurzawski et al. 2010), without significant difference in controls, and coronary artery disease (Horne et al. 2009, Connelly et al. 2006).

8.5 IDH1

Chou et al. (2010) mention *IDH1* mutations in AML patients (5.5%).

Most studies about *IDH1* mutations in AML report *IDH1*R132 mutation: in 8.5% (16/188) of patients (Mardis et al. 2009), in 6.6% somatic heterozygous *IDH1* mutations, 54.8% of these *IDH1*R132 (Schnittger et al. 2010). Losman et al. (2013) assume that the importance of the *IDH1*R132H mutation lies in its role as promotor for growth factor independence and impaired differentiation, whereas Dang et al. (2010) showed that the change of *IDH1*R132 to histidine leads to an accumulation of the onco-metabolite 2HG, elevated also in malignant gliomas. In a meta-study, Yang et al. (2012) point out that IDH1-/2-mutations can be found in more than 20% of AML patients, this was confirmed by the Cancer Genome Atlas Research Network. Marcucci et al. (2010) report about *IDH1*

mutations in CN-AML (49/358 patients), with *IDH1*R132 (46/49), *IDH1*V71 (2/49) and both mutations (1). Tefferi et al. (2010) present a study with 1,473 MPN patients screened for *IDH1/2* mutations, with *IDH1*R132 (18) and *IDH2* mutations (20), occuring significantly more often in the blast-phase of the disease. No separate analysis of IDH1/2 mutations was published. As there are quite diverse rates of *IDH1* mutations in myeloid neoplasms, our frequency (14.3%) lies in the lower range.

No study mentions the role of *IDH1*V178I, which was the sequence variant we found most frequent (4.2%) in *IDH1*, in relation to haemato-oncologic malignancies, although it has a FATHMM score of 0.99, indicating its pathogenicity. *IDH1*V178I mutation was found in thyroid carcinomas, where it made up to 42% of the *IDH1* mutations (Yang et al. 2012).

COSMIC lists *IDH1*V178I with a pathogenic FATHMM score (0.99) with underlying evidence of studies with thyroid carcinomas, where *IDH1*V178I is the most prevalent, and co-occurs with *IDH1*G105G (Hemerly et al. 2010), in a cohort of metastatic cancers (322/10,000, Zehir et al. 2017) and in subependymal giant cell astrocytoma-like astrocytoma of *NF1* patients (Palsgrove et al. 2018). One study shows a sample in the validation cohort (Shankar et al. 2014). The PolyPhen2 score is also pathogenic (0.992). Ensemble lists *IDH1*V178I benign, co-occurring with *IDH1*G105G equally in malignant and benign pediatric lymphoid nodules (Pekova et al. 2019), as tolerated mutation in glioblastoma (Muscat et al. 2017), in association with adipositas in a Hispanic Americans cohort (Gao et al. 2015) and with low frequency in a healthy ancestral cohort (Bodian et al. 2014). Ivosidenib represents a specific FDA approved therapy option for *IDH1* mutated AML. None of the studies from MVLD refers to myeloid disorders.

8.6 KIT

Iurlo et al. (2014) mention a somatic *KIT*M541L mutation in CEL-NOS. Inokuchi et al. (2002) found *KIT*M541L significantly more frequent in their cohort of Japanese CML patients (7.5%) than in healthy individuals, therefore proposing that this mutation is not a polymorphism and clinically relevant. This could not be confirmed by the study of Krüger et al. (2006) with a cohort of Caucasian CML patients (*KIT*M541L in CML 8.3% vs. control group 8.1%). Our data suggest a relation to AML, as it made up 2.4% of all sequence variants in AML.

MVLD research reveals very diverse opinions: *KIT*M541L is listed in COSMIC, is described as somatic, and has a pathogenic FATHMM (0.74) with more than 20 samples, mostly from breast cancer. Unfortunately, not all linked studies in COSMIC publish data with detected *KIT*M541L, so evidence cannot be verified. The published data show associations to primary melanoma (Goswami et al. 2015), high grade metastatic colorectal cancer (Yoshioka et al. 2015) in circulating tumor cells of metastatic breast cancer (De Luca et al. 2016), primary breast cancerand breast cancer brain metastases, in this study

without any difference to matched pairs (Lee et al. 2015), in pancreatic adenocarcinoma, but also in benign samples of breast fibroadenomas (Da Cruz et al. 2017), in validation cohort of Von Hippel-Lindau associated hemangioblastoma (Shankar et al. 2014). The association of *KIT*M541L is mostly not significant, due to low sample size. In ClinVar, *KIT*M541L is of uncertain effect, as samples are from GIST, mast cell disease, CML and Piebald trait. Linked studies show *KIT*M541L as variant in a healthy ancestral cohort (Bodian et al. 2014), as being rather benign in the ClinSeq-data (Johnston et al. 2012) and occurring equally in CML and healthy control group (Krüger et al. 2006). Ensembl has *benign*, *likely benign* and *not provided* as status for *KIT*M541L, as cited studies show samples in healthy individuals (Bodian et al. 2014), equally distributed in patients (CMPN) and controls (Aranaz et al. 2010), in benign disease cohorts (glandular odontogenic cyst) (Siqueira et al. 2017); COPD mouse model (Vishweswaraiah et al. 2018), cemento ossifying fibroma (Pereira et al. 2018), or as germline in the mentioned cases (Kirschner et al. 2015 and Saini et al. 2012) with questionable higher risk for hematologic malignancies. Just one study establishes an association to pathogenicity with aggressive prostate cancer in a cohort of elevated PSA patients (Martinez-Gonzalez et al. 2018) dbSNP shows a similar diverse picture of *KIT*M541L, as it lists mostly the same studies as Ensembl and samples of patients with mastocytosis, partial albinism, GIST, CML, and two not specified. The multikinase inhibitor midostaurin also targets *KIT*-mutations. In systemic mastocytosis that sometimes is treated with midostaurin mutations occur in KITD816.

8.7 TET2

Rasmussen and Helin (2016) state that *TET* is mutated in a lot of different cancers. In haemato-oncologic cancers, *TET2* is involved in leukemic transformation through decreasing DNA methylation in enhancers of haematologic tissue. Thienport et al. (2016) established a relation between hypoxic microenvironment which decreases *TET* hydroxylase activity in tumors and leads to an accumulation of 5-hydroxymethylcytosine (5hmC) resulting in hypermethylation.

Delhommeau et al. (2009) found somatic mutations in early stages of myeloid cancers (15%), with MDS (19%), MPN (12%), sAML (24%) and CML (22%). Kosmider et al. (2009), had 22.9% *TET2* mutated patients in their MDS-cohort and Smith et al. (2010) found functional relevant mutations in MDS- (12%) and CMML- (46%) patients, not further specifying them.

The applicability of NGS on samples of haemato-oncologic patients was the aim of the study of Bernard et al (2014) with CMML patients. They found that 84.6% of the samples were mutated in *TET2*. The frequency of the different variants was not evaluated.

Frequencies of specific sequence variants can be found in a study of Ibrahim and Khalil (2017). The mutation *TET2*L1721W occurred in 2% in a cohort of Sudanese ET-patients

(n=50), concluding that this rather low frequency shows a minor role in the pathogenesis of MPN or resulting from their small cohort. Li et al. (2016) report a cohort of childhood AML which showed *TET2*I1762V (45%) and *TET2*V218M (12%).

Also, a study of Scopim-Ribeiro et al. (2016) had *TET2* mutations in 42% of patients with myeloid neoplasms, and identified 17 variants, among them several SNPs that were also quite frequent in our cohort, like *TET2*V218M, *TET2*P363L, *TET2*H1778R, *TET2*I1762V and *TET2*L1721W. No correlation between *TET2* expression and mutation status to diseases could be established, suggesting that other mechanisms must contribute to the development of myeloid neoplasms. Our cohort seems best comparable to Scopim-Ribeiro's study cohort, regarding frequencies of overall *TET2* mutation (71.0% in our cohort) and found sequence variants, but no correlation between sequence variants and disease could be established, other than in our analysis.

Just one study mentions all the sequence variants *TET2*V218M, *TET2*P363L, *TET2*L1721W, *TET2*I1762V and *TET2*H1778R with frequencies which were found in human gliomas (WHO-Grade II-IV) and healthy brain samples while looking for a correlation with 5hmC, instead of the usually occurring 5mC, which is due to *TET* mutations. For human gliomas, this correlation could not be confirmed. Frequencies of *TET2* variants were as follows, gliomas vs. controls: *TET2*V218M 3% vs. 0%, *TET2*P363L 4% vs. 0%, *TET2*L1721W 25% vs. 15%, *TET2*I1762V 50% vs. 61% and *TET2*H1778R 0% vs. 8% (Kraus et al. 2015).

The role of TET2 variants is rather undecided, with a tendency to a benign (bystander) effect. *TET2*I1762V (FATHMM 0.14 neutral), *TET2*L1721W (FATHMM 0.78 pathogenic) and *TET2*V218M (SNP) are mentioned in COSMIC, and none of them, including *TET2*P363L and *TET2*H1778R in OMIM. Evidence for these variants can be found in a healthy ancestrally cohort study (Bodian et al. 2014), which describes frequencies of 0.2526 (*TET2*I1762V), 0.1065 (*TET2*L1721W), 0.047 (*TET2*P363L), 0.0507 (*TET2*H1778R) and 0.0683 (*TET2*V218M). The PolyPhen2-score ranges from benign with 0.002 (*TET2*V218M) and 0.012 (*TET2*I1762V) to possibly damaging 0.573 (*TET2*P363L) and 0.794 (*TET2*L1721W) to probably damaging with 0.994 (*TET2*H1778R). ClinVar describes samples as *all highly penetrant* for *TET2*I1762V, *TET2*P363L, *TET2*H1778R and *TET2*V218M. *TET2*I1762V is associated with increased OS and EFS in childhood intermediate risk AML in a Chinese cohort (Wang et al.2018). Three studies mention high frequencies of *TET2*I1762V in healthy individuals, in comparison with childhood AML (50-54%, Kutny et al. 2015), CLL (Chronic Lymphocytic Leukemia, 54% in CLL vs. 83%, (Hernández-Sánchez et al. 2014) and MDS (13.3% in MDS vs. 42.7%, Hu et al. 2019). A putative pathogenic effect is demonstrated in an APL study without control group (25/41) (Ottone et al. 2012) and in a triple negative therapy unresponsive ET case study (Zaidi et

al. 2017). In NAFLD, *TET2*I1762V is associated with liver PPARGC1A-methylation and Diabetes mellitus type II (Pirola et al. 2015). *TET2*L1721W, *TET2*P363L, *TET2*H1778R, *TET2*V218M are documented in a study about myeloid neoplasm (Scopim-Ribeiro et al. 2016), where they were excluded as SNPs. Also, *TET2*L1721W and *TET2*P363L are associated to APL (Ottone et al. 2012), in a study without a control group. There are no differences in DNA hypo- and hypermethylation between patients with common *TET2*-SNPs and controls (Kaasinen et al. 2019).

8.8 TP53

In general, TP53 is a tumorsuppressor gene and mutations leading to a TP53 inactivation cause cancer. Somatic mutations may result in family cancer syndromes, like Li Fraumeni. The first suggestion that *TP53*P72R is a polymorphism bases on evience was presented by Ara et al. (1990) via PCR. In 1995 then, Krawczak et al. (1995) supposed that endogenous cellular mechanisms cause somatic *TP53* mutations and could lead to different human cancers. Thomas et al. (1999) mention that *TP53*R72 has a stronger interaction with MDM2 and a different activation of transcription, apoptosis and transformation of primary cells. Litviakov et al. (2010) show an association with high frequency of aberrant cells and chromatid breaks, a step in the evolution of cancer.

Ørsted et al. (2007) looked for the role of the SNP *TP53*R72P, finding among their Danish general population cohort (n>9,000) a significant association with increased longevity, but not with cancer.

One study shows ethnic differences among the observed frequencies of Pro72 (Weston et al. 1992), which might be due to winter temperature, a low average temperature is associated with a high *TP53*R72 frequency (Shi et al. 2009). In their review, Olivier et al. (2010) summarize the diverse assessmentsopinions upon the ethnic difference of Pro72-frequency (north-south gradient) resulting from an adaption to differences in winter temperature.

Two meta-analyses could not show a significant effect between *TP53*R72P and leukemia, but an increased risk to develop ALL in a subgroup analysis (Tian et al. 2016) and significantly increased NHLs (Weng et al. 2012. In our cohort, we could see a significant correlation between *TP53* mutation and myeloid malignancies (13.7% of all patients), but the most frequent sequence variant *TP53*R72P made just 3.7% of all *TP53* variants, indicating that diverse effects on the tumor suppressor *TP53* are involved in its silencing.

The database research revealed a *neutral* FATHMM-score (0.36) and a *possible damaging* PolyPhen-score (0.745). dbSNP also lists *TP53*R72P as benign, with association to Li Fraumeni, but also hereditary cancer-predisposing syndrome.

8.9 ASXL1

Gelsi-Boyer et al. (2009) mention *ASXL1* as a tumor suppressor in myeloid malignancies. They find heterozygous somatic mutations in exon 12 in MDS and AML (16%, 5/38), in CMML (43%, 19/44) and in MPN (7.8%, 5/64). A little less frequent are the rates of exon 12 mutations in *ASXL1* found by Chou et al. (2010) in de novo AML (10.8%, 54/501), mutually exclusive with *FLT3*, *NPM1* and *WT1* variations. Schnittger et al. (2011) report *ASXL1*E1102D as rare polymorphism in myeloid malignancies (15/273). Carbuccia et al. (2009) report *ASXL1*E1102D as SNP, whereas the Jackson Laboratory (CKB) notices about *ASXL1*E1102D that it does not lie within any known functional domain of *ASXL1* protein. It has been identified in sequencing studies but no biochemical characterization has been completed to further determine its effect. We could not establish a significant correlation of *ASXL1*E1102D and a myeloid disease but 26.4% of our patient carried *ASXL1* variants.

8.10 DNMT3A

Shlush et al. (2014) proofed proved that *DNMT3* mutations in HSCs are an early event in AML evolution by creating a pool of preleukemic cells. *DNMT3* mutations are quite frequent in de novo AML, ranging from 17% (Stegelmann et al. 2011) over 20.5% (23/112, AML-M5, Yan et al. 2011) and 22.1% (62/281, Yan et al. 2011), with our mutation rate being quite similar with 22.9%, but in the whole cohort, not AML specific.

*DNMT3A*R882H can be found in AML, 9.6% (Ley et al. 2010), in AML-M4, 13.6% (Yan et al. 2011), in MDS 2.7% (4/150, of 13 *DNMT3A* mutations, Walter et al. 2011), in MPN 10%, and MF 15% (Stegelmann et al. 2011), showing higher rates than we found (3.4%), but clearly indicating its pathogenicity. COSMIC provides more than 850 samples, most of them from haematopoietic/ lymphoid tissue in more than 60 publications and a pathogenic FATHMM (0.98). PolyPhen2 (0.651) reveals a possibly damaging effect. This is supported by the occurrence in 6.94% of cases in the TCGA-LAML-Project and 0.26% in AACR GENIE cases (The AACR Project GENIE Consortium 2017). Association to intermediate risk AML (Ley et al. 2010), NK-AML (Ahmad et al. 2014), AML (Li et al. 2012, Qiao et al. 2014, Ley et al. 2010), dominant negative effect of *DNMT3A*R882H over *DNMT3A*wt (Russler-Germain et al. 2014), and its occurrence in 60% of *DNMT3* mutations in myeloid neoplasms (Shih et al. 2012) clearly corroborate its pathogenic potential. Two studies show *DNMT3A*R882H in combination with TBRS (Kosaki et al. 2017, Shen et al. 2017).

*DNMT3A*K456fs* has a COSMIC-ID (COSM4383572) but no studies are linked, so that its effect remains unknown. *DNMT3A*K456fs* occurred in 3.3% of all sequence variants in SAA. MVLD research did not reveal any evidence for *DNMT3A*K456fs*.

8.11 JAK2V617F

The first description of the *JAK2*V617F mutation was provided 2005 by four different groups (Green, Vainchenker, Gilliland, Skoda) in cohorts of MPD patients. In the meta-study of McLornan et al. (2006) a table is published listing the results of several studies with *JAK2*V617F frequencies in different diseases per disease. For PV, the frequency ranges from 65% (Kralovics et al., 2005) to 97% (Baxter et al. 2005), for ET from 23% (Kralovics et al., 2005) to 57% (Baxter et al. 2005) and for PMF from 35% (Levine et al. 2005) to 95% (Jelinek et al. 2005). For MDS and CMML, two are studies mentioned which show *JAK2*V617F frequencies for MDS of 1.5% (Jelinek et al. 2005), 5% (Steensma et al. 2015), 3% (Steensma et al. 2015), and, for CMML specifically, of 13% (Jelinek et al. 2005). A more recent study by McKerrell et al. (2017) shows the evolution of *JAK2*V617F malignancies from clonal haematopoiesis over year at very different rates (0.36% to 6.2% / year), accelerated by co-mutations. As there are quite diverse but rather high mutation rates of *JAK2*V617F in myeloid malignancies, we can confirm the correlation between the *JAK2*V617F and myeloid neoplasia, in our cohort it was 16.7%, and in MPN 13.4%.

Database research revealed hundreds of studies reporting on *JAK2*V617F so that the most recent studies (2016 until now) were chosen. FATHMM (0.94) and PolyPhen 2 (0.996) classify *JAK2*V617F pathogenic. TCGA mentions its occurrence in two cancer studies (hematopoietic and intrahepatic) at a frequency of <1% and in AACR GENIE in 0.56%, of this 4.43% in MDS (The AACR Project GENIE Consortium 2017). Associated cancers are mostly MPN and AML in different frequencies (e.g.: 4/222 AML, 3 post-MDS, 9/116 CMML/aCML (Levine et al. 2005), 65% PV, 53% IMF (Kralovics et al. 2005), 71/73 PV, 29/51 ET, 8/16 IMF (Baxter et al. 2005), 94% in PV, 53% in ET, 67% in PMF, 97.5% in MPN, n=268 (Kim et al. 2016), 97% PV, 57% ET and 50% IMF (Baxter et al., 2005), 7.25% in CMML (Mason et al. 2016), 3/56 CMML (Palomo et al. 2016). A high allele burden seems to be associated with transformation to MF (Passamonti et al. 2010), a higher platelet count (Jekarl et al. 2010), and thrombosis (Kim et al. 2016, Malak et al.2012). MDS is also associated (1.4%) (Ramos et al. 2016). Also, there is diverse evidence for an association of *JAK2*V617F with pregnancy loss, most probably due to increased thrombosis (Mercier et al. 2007, Dahabreh et al. 2009, Campbell et al. 2005). Budd-Chiari-Syndrome is also, most probably as a consequence of increased thrombosis, increasingly observed in association with *JAK2*V617F (Chung et al. 2006, Patel et al. 2006, Sozer et al. 2009, Colaizzo et al. 2008), and one case with *JAK2*V617F in myeloid sarcoma was described (Yoshiki et al. 2011).

8.12 RUNX1

RUNX1 mutations can be frequently found in leukemia, AML-M0, (MDS)-AML and therapy related MDS/AML (Osato et al. 2004). Zimmermann et al. (2015) found RUNX1 mutations

in a cohort of CN-AML (6.4%, 32/538). The frequency of 10.2% that we observed seems to reflect the AML-/MDS-cohorts, and the most frequent variant *RUNX1*L56S makes 0.6% of MPN variants. Mendler et al. (2012) mention in their study inconsistent data for the determination of *RUNX1*L56S as pathogen or benign sequence variant. ClinVar mentions *RUNX1*L56S samples from familial platelet disorder patients and therefore this variance is classified as benign/ likely benign. The FATHMM score is pathogenic (0.90) with sixteen linked samples, fifteen from haematopoietic/ lymphoid tissue, as is the PolyPhen2 score (0.999). Unfortunately, 11/12 linked studies do not cite *RUNX1*L56S, just one study mentions it in 5.7% of 77 MDS-patients.

8.13 NRAS

Hobbs et al. (2016) prove *RAS* as driver initiation and maintenance, missense *RAS* mutations in approximately 25% of human cancers, *NRAS*G12 favored in AML. Matsuda et al. (2007) found NRAS mutations in JMML patients (12.5%, 10/80), most frequently *NRAS*G12D. The effect of the *NRAS*G12D mutation was established by Kong et al. (2016): it leads to hydrolysis and accumulation of Ras-GTP which results in a hyperactivation signal downstream of *RAS*. *KRAS*- and *NRAS*-mutations are described in 15-60% of myeloid disorders. Wang et al. (2013) showed in a mouse model that haematopoietic stem cells (HSCs) which express *NRAS*G12D initiate MPN through moderate hyperproliferation with increased self-renewal and a hyperactivation of ERK1/2 in HSCs. In an earlier mouse-model, Wang et al. (2011) proved that bone marrow-expression of *NRAS*G12D does not support development of MPD phenotypes but with higher rates of T-Acute Lymphoblastic Leukemia. They concluded that different lineages of HSCs need different HSC activity and *NRAS*G12D signals. Primary *NRAS*G12D-mutated mice developed MPD which is explained by microenvironmental factors as transient phenomenon but the evidence is rather rests rather uncertain In our study, 1.6% of the sequence variants in CMML were *NRAS*G12D and the overall mutation rate of *NRAS* was 7.0%. This is lower than in the studies mentioned which could be due to the diverse myeloid diseases in our cohort.

An abundance of studies is linked to *NRAS*G12D but not all publish the corresponding data completely, so that evidence of *NRAS*G12D in diseases is often not retraceable. The FATHMM score (0.91, COSMIC) and ClinVar show a pathogenic effect of *NRAS*G12D with samples deriving in more than 75% from haematopoietic/ lymphoid tissue (COSMIC) and somatic epidermal nevus, JMML, neoplasm of the large intestine, NSCLC, and melanoma in ClinVar. Evidence for pathogenic effect of *NRAS*G12D derives from OncoMap (MacConaill et al. 2014), several TCGA-projects (READ, LAML, COAD, NOS, TGGT, UCEC, SKCM), a lung cancer study (Ohashi et al. 2013), with not smoking associated, MEK-inhibitor responsive lung cancer, CRC studies (Vaughn et al. 2011,

Irahara et al. 2010, De Roock et al. 2010), cutaneous melanoma (van 't Veer et al. 1989), probably caused through exogenic sunlight exposure, AML cohorts (Tyner et al. 2009, Bacher et al. 2006, Vogelstein et al. 1990) and samples from CMML/ MF patients (Janssen et al. 1987). One study compares frequency of *NRAS*G12D in skin (0.66%), tumor (43%) and cDNA (42%) samples, and clearly shows a much higher rate in tumorous tissue (Mardis et al. 2009). One mouse model examines colon epithelial growth and detects increasing resistance to apoptosis (Haigis et al. 2008). Benign effect is shown in KEN samples (Hafner et al. 2012). Therapy model studies (Xu et al. 2012, Adjei et al. 2008) show positive effects of MEK-inhibitors and palmostatin B. Interestingly, one study mentions *NRAS*G12D in connection with the choice of dbSNP taken for research, as in the latest version *NRAS*G12D is not mentioned as oncogenic somatic mutation whereas the authors of the abovementioned study claim oncogenicity (Lao et al. 2019). Interestingly, the PolyPhen score was calculated on two different databases, showing different results: benign (0.372) at ESP and possibly damaging (0.726) at TCGA, which could be due to differences in data used for the scores, ESP possibly from 2011 and TCGA from 2019.

8.14 IDH2

Ashraf et al. (2013) showed in their study with AML patients (n=120) *IDH2*R140Q (14.16%, 17/120) and *IDH2*R172 (4.16%, 5/120). Lin et al. (2018) had *IDH2* mutations (3.2%, 9/281) in their MDS cohort with *IDH2*R140Q (7/9, 78%). A higher transformation to sAML could be noticed for patients with *IDH2*R140Q (66.7% vs. 23.9% *IDH2*-wt) and an increased co-occurrence of *IDH2* mutations with *DNMT3A* or *SRSF2* could be seen. We had *IDH2*R140Q in 2.3% of MDS/MPN overlap syndrome. *IDH2*R140Q is classified pathogenic in COSMIC with FATHMM (0.98). PolyPhen2 is probably damaging and TCGA lists *IDH2*R140Q in 0.31% of AACR GENIE cases (8% in AML, 3.3% in MDS, and in 5.3% in NSCLC/OMF/melanoma/CMML), more than 120 cases with leukemia and *IDH2*R140Q are described in TCGA. OMIM gives evidence for association of *IDH2*R140Q and D-2-HGA II with proof for normal D-2-HGA function, but less catalytic effect in D-2-HGA patients (14/17) probably through *IDH2*R140Q.

8.15 KRAS

KRAS is a well-known proto-oncogene in colon cancer. Evidence on the nature of *KRAS*R161R cannot be found in any database. For KRAS variants we could not see a correlation to a myeloid disease, although found in 5.9% of all patients and *KRAS*R161R in 2.2%, which might be due to its general effect in the evolution of cancer, as we examined a cohort with diverse dysplastic and proliferative malignancies.

8.16 SF3B1

A meta-study on MDS patients (Tang et al. 2019), showed a frequency of *SF3B1* mutations from 7.0 to 62.1% in 15 studies and a strong association with the occurrence of RS, no significant impact on OS, but a significant lower LFS, as it co-occurs with outcome-negative associated mutations on other genes, *TET2* in 28% and *DNMT3A* in 15% in an MDS-RS-cohort, and *SF3B1* mutations being among the first events (Mortera-Blanco et al. 2017). *SF3B1* mutations are proposed to be among the first events in MDS-RS, occurring in lymphomyeloid HSCs and consequently functionally negative influencing lymphoid reactions. Tefferi et al. (2017) found *SF3B1* mutations in 20% in a MDS-cohort, associated with inv(3)(q21q26.2) and del(11q). In a CLL-cohort, *SF3B1* mutations were found in 9.7%, associated with faster disease progression and poor OS (Quesada et al. 2011).

In our cohort, two sequence variants occurred equally frequent, *SF3B1*K141K and *SF3B1*V1219V, mostly in SAA (2.5% each), whereas *SF3B1* sequence variants in general occurred mostly in the MDS subgroup (3.3% of all sequence variants in the MDS subgroup).

Systematic research according to the MVLD scheme did reveal entries in COSMIC for *SF3B1*K141K, with the additional note of SNP, so that *SF3B1*K141K is now excluded from the database. Ensembl lists PolyPhen 2 score unknown and Reference SNP cluster ID (rsID) No studies for evidence are linked in any database, so in conclusion its role in myeloid disorders, in this case, remains unclear.

9. References

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11. Curriculum Vitae

Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

12. Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe. Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

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