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DISSERTATION

Donor engrafted clonal hematopoiesis of indeterminate potential in allogeneic hematopoietic stem cell transplantation

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Summary

Abstract German

Einführung: Klonale Hämatopoese von unbestimmtem Potential (clonal hematopoiesis of indeterminate potential, CHIP) beschreibt das Vorkommen von Blutkrebs-assoziierten, somatischen Mutationen in mindestens 4% der kernhaltigen peripheren Blutzellen, ohne dabei weitere Kriterien für hämatologische Neoplasien zu erfüllen. Das Hauptprojekt untersuchte CHIP im Kontext von allogenen hämatopoetischen Stammzelltransplantationen (HSZT). Aufgrund der stetig zunehmenden Zahl älterer Patient*innen, die sich einer allogenen HSZT unterziehen, nimmt auch der Anteil älterer Spender*innen zu, da Verwandte häufig geeignete Spender*innen darstellen. Diese Entwicklung wird begleitet von einer höheren Prävalenz von CHIP unter den Spender*innen, da die Prävalenz altersabhängig zunimmt. Da CHIP mit Gesundheitsrisiken wie kardiovaskulären Erkrankungen oder hämatologischen Neoplasien assoziiert ist, wurden die Auswirkungen von transplantierter Spender-CHIP auf den klinischen Verlauf von Empfänger*innen einer HSZT systematisch untersucht. Ein Nebenprojekt befasste sich mit CHIP im Kontext solider Tumoren unter Einfluss von (Radio-)Chemotherapie sowie mit der Ursprungszelle und Verteilungsmustern von CHIP-Mutationen während der hämatopoetischen Differenzierung in nicht-onkologischen Patient*innen. Ein weiteres Nebenprojekt untersuchte die Rolle von CHIP im Setting von antineutrophilen zytoplasmatischen Antikörpern (ANCA)-assoziierten Vaskulitiden.

Methoden: DNA-Proben aus peripherem Blut wurden mittels paired-end-Sequenzierung auf CHIP getestet. Die Sequenzierdaten wurden mittels einer angepassten bioinformatorischen Pipeline ausgewertet und anschließend statistisch auf klinische Assoziationen untersucht.

Ergebnisse: 80/500 Stammzellspendern wurden positiv auf CHIP getestet (16%). Die Transplantation von Spender-CHIP war in den Empfänger*innen assoziiert mit einer höheren kumulativen Inzidenz für chronische Graft-versus-Host-Reaktionen sowie mit einer niedrigeren kumulativen Inzidenz für Rezidive/Progress. Zudem bestand ein erhöhtes Risiko für die Entwicklung von Spenderzellleukämien. Die therapiebedingte Mortalität und das Gesamtüberleben wurden jedoch durch den CHIP-Status nicht beeinflusst. In Patient*innen mit soliden Tumoren war CHIP mit einer häufigeren Reduktion der Chemotherapiedosis verbunden. Unter Ausschluss von einzig im Gen *DNMT3A* mutierten Patient*innen, war CHIP mit einem höheren Bedarf an Erythrozytentransfusionen assoziiert. Als Ursprungszelle wurden Lin$^-$CD34$^+$CD38$^-$ hämatopoetische Stammzellen identifiziert und es zeigte sich eine Expansion der CHIP-Klone zugunsten der myeloischen Zellreihe sowie natürlicher Killerzellen. Für AAV-Patient*innen wurde erstmalig eine unerwartet hohe CHIP-Prävalenz von 30.4% (34/112) nachgewiesen. Zudem ging CHIP mit einer geringeren renalen und neuronalen Beteiligung einher.

Schlussfolgerung: Unserer Ergebnisse weisen auf neue klinische Assoziationen von CHIP hin und erweitern Kenntnisse über die Rolle von CHIP im Rahmen fehlgesteuerter Immunantworten

bei chronisch entzündlichen Erkrankungen. Die Übertragung einer Spender-CHIP scheint das Gesamtüberleben nach HSZT nicht zu beeinflussen und wirkt somit sicher.

Abstract English

Introduction: Clonal hematopoiesis of indeterminate (CHIP) describes the presence of hematologic cancer-associated somatic mutations in at least 4% of peripheral nucleated blood cells in the absence of further criteria for hematological malignancies. The main project investigated CHIP in the setting of allogeneic hematopoietic stem cell transplantation (HSCT). Due to continuously growing numbers of older patients undergoing allogeneic HSCT, the proportion of older donors has been increasing, too, since siblings are often eligible donors. This is accompanied by a higher CHIP prevalence among donors as the prevalence increases in an age dependent manner. Since CHIP is known to be associated with adverse conditions such as cardiovascular diseases or hematologic cancers, our study investigated the effects of transplanted donor-CHIP on the clinical outcome of hematopoietic stem cell transplant recipients systematically. A side project addressed CHIP in the context of (radio-)chemotherapy in solid tumor patients and additionally studied the cell of origin and distribution patterns of CHIP-mutations among the hematopoietic differentiation tree in non-cancer patients. A second side project focused on CHIP in the setting of anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis.

Methods: DNA samples from peripheral blood were tested for CHIP using paired-end sequencing. Sequencing data were analyzed using a customized bioinformatic pipeline and statistically tested for clinical associations.

Results: 80/500 stem dell donors were tested positive for CHIP (16%). Transplantation of donor-CHIP to the recipient was associated with a higher cumulative incidence of chronic graft-versus-host disease and a lower cumulative incidence of relapse/progression. Also, an elevated risk for the development of a donor cell leukemia was observed. However, non-relapse mortality and overall survival were not affected by CHIP-status. In the setting of solid tumor patients CHIP was linked to a more frequent chemotherapy dose reduction. After exclusion of solely *DNMT3A*-mutated patients CHIP was also associated with a higher need for red blood cell transfusions. Lin^- $CD34^+CD38^-$ hematopoietic stem cells were identified as cells of origin and expansion of CHIP-clones showed a bias towards the myeloid compartment and natural killer cells. In AAV patients an unexpectedly high CHIP prevalence of 30.4% (34/112) was observed. Also, the renal and nervous system were less frequently affected in $CHIP^+$ individuals.

Conclusion: Our results provide evidence for new clinical associations of CHIP and extend reported findings linking CHIP with aberrant immune response in inflammatory diseases. Transplantation of donor-CHIP does not affect overall survival after HSCT and therefore appears safe.

1. Introduction

1.1. Clonal hematopoiesis of indeterminate potential

Clonal hematopoiesis (CH) describes the acquisition of hematologic cancer-associated somatic mutations, which can be detected in the peripheral blood and lead to disproportionate clonal expansion of mutated hematopoietic cells. In 2014, three large-scale retrospective studies using next generation sequencing (NGS) methods revealed that such mutations occur in the peripheral blood of approximately 10% of non-cancer patients older than 60 years with an overall prevalence rising in an age dependent manner (1-3). Most frequently affected are the genes *DNMT3A, TET2* and *ASXL1* (1, 2) that encode for epigenetic regulators of transcription and are essential for cellular differentiation. In 2015, the term clonal hematopoiesis of indeterminate potential (CHIP) was introduced by Steensma et al. as a working definition to describe a premalignant state for hematological neoplasms with a low progression rate to overt malignancy of 0.5 – 1% per year similar to monoclonal gammopathy of undetermined significance and multiple myeloma (4). CHIP was defined by the sole presence of such a respective somatic mutation above an arbitrary threshold of 2% variant allele frequency (VAF) in the peripheral blood and the absence of other criteria for hematological malignancies (4). Using highly sensitive error corrected sequencing (ECS) approaches, CH at very low levels was shown to be almost ubiquitous in middle-aged healthy adults, however the relevance of such small clones remains unclear (5).

The fact that hematologic cancer-associated mutations occur in the normal elderly population has raised enormous interest and concerns over the last years. Various studies have linked CHIP with health risks such as hematologic cancers, cardiovascular diseases, and an elevated all-cause mortality (1, 2, 6). A direct causal link between *TET2*-deficient clonal hematopoiesis and the development of cardiovascular disease in terms of accelerated atherosclerosis was demonstrated in a murine model (7). CH was also shown to be common in unexplained cytopenias, which are therefore referred to as clonal cytopenias of unknown significance (8). So far, CHIP has hardly been studied in the setting of allogenic stem cell transplantation (HSCT).

1.2. Allogeneic hematopoietic stem cell transplantation and clonal hematopoiesis of indeterminate potential

Allogeneic HSCT often represents the only curative treatment approach for various hematologic malignancies (9). It consists of radio-/chemotherapy as a conditioning treatment to eradicate or reduce malignant cells and to clear up space in the marrow and subsequent transplantation of HSCs from a healthy, matching donor to repopulate the marrow. Conventional myeloablative conditioning is associated with high morbidity and mortality, especially in elderly patients, making it suitable only for younger patients in good physical condition until recently. With the discovery and increasing understanding of the graft versus leukemia (GVL) effect, reduced intensity conditioning regimens were introduced (10). These milder regimens made elderly patients eligible for stem cell therapy, too (11). Ever since, the number of allogeneic transplant recipients aged 60 years and

older has been continuously increasing, accounting for approximately 40% of all HSCTs in the US in 2018 (12). Since relatives, particularly HLA-matched siblings, represent an important source of stem cell donors, the age of related donors is increasing simultaneously (13). As the frequency of CHIP is increasing in an age dependent manner, a growing number of donors is suspected to harbor CHIP, which is associated with health risks as described above. This prompted the question of the effects and risks of donor derived CHIP, a condition in which stem cells carrying hematologic cancer-associated mutations are transplanted from the donor to the recipient. As CHIP has been directly linked to alterations in immune function in terms of inflammatory cytokine expression, macrophage function and innate immune trafficking in native hematopoiesis in a murine model (7), donor engrafted CHIP might also affect transplantation outcomes such as occurrence of graft versus host disease, infections or relapse. There has been one retrospective study showing that CHIP was enriched in recipients that developed unexplained cytopenias after allogeneic stem cell transplantation (n = 6) (14) suggesting possible causal associations between CHIP and impaired graft function. Further, several case reports of donor cell leukemia (DCL) have been reported (15, 16). DCL derives from donor engrafted CHIP and is a rare complication affecting an estimated 0.1% of transplanted patients (17, 18). Finally, in the setting of autologous HSCT, CHIP has been linked to an increased non-relapse mortality (NRM) and an increased risk for therapy-related myeloid neoplasms (19, 20).

1.3. Aims

The aim of the main project (21) was to investigate the effect of donor derived CHIP on the clinical outcome of recipients of allogeneic HSCs and by this means evaluate if individuals with CHIP (referred to as CHIP^{+}) were suited for hematopoietic stem cell donation. The fact that CHIP has been linked to diverse adverse clinical outcomes, especially in the setting of autologous stem cell transplantation (19, 20), lead to the apprehension that donor engrafted CHIP might as well affect the clinical course of allogeneically transplanted recipients. Additionally, we were interested in clonal dynamics of transplanted CHIP-clones under the replicative stress and altered bone marrow microenvironment caused by the conditioning regimens in HSCT (22-24). So far, there has been little investigation of CHIP in the setting of allogeneic stem cell transplantation.

A side project focused on evolutionary dynamics of CHIP-clones in follow-up samples of solid cancer patients over the course of chemotherapy treatment (25). We assumed that these clones might show a selective advantage under cytotoxic stressors and or even malignant transformation, as CHIP has been linked to an increased risk for therapy-related myeloid neoplasms in solid cancer patients undergoing chemotherapy (26, 27). Additionally, this project aimed at identifying the cell of origin and lineage distribution patterns of CHIP in cell fractions during hematopoietic differentiation.

A third project addressed the role of CHIP in the setting of ANCA-associated autoimmune vasculitis (AAV) (28). Aberrant expression of target antigens in myeloid cells plays a crucial role in the

pathogenesis of AAV and was demonstrated to be linked to disrupted gene silencing due to epigenetic dysregulation (29). Since CHIP has been associated with chronic inflammation and affects mainly genes encoding for epigenetic regulators, we assumed it might also influence the expression of target antigens and the clinical course of AAV patients.

2. Samples and methods

The following will focus on detailed explanation of the main methods used in the project studying the role of donor CHIP in the setting of allogeneic stem cell transplantation (21). Screening, validation, and follow-up of CHIP-clones were performed analogously for all three projects. Additional information regarding used methods is listed in the original publications (21, 25, 28). All three projects were conducted in accordance with the Declaration of Helsinki and ethical approval was obtained from the local ethics committees for each project.

2.1. Samples

To study CHIP in the setting of allogeneic stem cell transplantation, we obtained blood and/or bone marrow samples from 500 elderly healthy HSC donors collected at the time of stem-cell donation as well as corresponding relevant clinical and demographic data from both donor and recipient from 10 transplantation centers in Germany and France. The samples had been collected between 1993 and 2017 and derived from donors related to the recipients, collected at a minimum donor age of 55 years. The vast majority of samples were DNA samples extracted from peripheral blood, except for 26 cases extracted from bone marrow. 52 samples were frozen cells, of which DNA was extracted using the QIAGEN DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. DNA concentration of all samples were quantified by Quantus Fluorometer (Promega, Fitchburg, Wisconsin, USA). Diagnoses leading to transplantation in our cohort were acute myeloid leukemia (AML) (n=249; 49.8%), followed by lymphoma (n=62; 12.4%), myeloproliferative neoplasms (MPN) (n=55; 11%), myelodysplastic syndromes (MDS) (N=53; 10.6%), multiple myeloma (n=41, 8.2%), acute lymphatic leukemia (n=25; 5%), MDS/MPN overlap (n=3; 0.6%) and non-hematological cancer associated diagnosis (n = 12, 2.4%). To study clonal dynamics of donor CHIP, we obtained follow-up samples from 22 recipients who had received a CHIP containing graft with an average of five follow-up samples per patient representing a time period of 0,17 to 10,79 years after HSCT.

2.2. Mutation screening

Screening of the 500 DNA samples was performed by next generation paired-end targeted deep sequencing (TDS). First, genomic libraries were constructed using two customized versions of the Trusight Myeloid Sequencing Panel (Illumina, San Diego, California, USA), targeting genes related to myeloid malignancies and which we adapted to the state of research over the course of the study. The first version targeting 60 genes was used for 345 samples, whereas the second version aiming at 66 genes was applied to 155 samples. Details regarding target regions are listed

in the supplement of the paper (21). Library preparation was performed based on the manufacturer's protocol. Steps of library preparation included hybridization of up- and downstream oligos to 10µl of 5ng/µl DNA sample, extension und ligation of bound oligos, introduction of different combinations of indices for multiplexing as well as adapters for cluster generation on the flow cell, and final PCR amplification of the targeted regions. Successful amplification was tested by verifying the expected product size using gel electrophoresis. Normalization of the libraries was performed using magnetic beads (AMPure XP, Beckman Coulter, Brea, California, USA). This method is based on the idea that a given volume of beads can only bind a consistent quantity of DNA molecules, which is then retained by a magnetic field, guarantying a balanced representation of all libraries. Unbound molecules were washed away subsequently, and bound molecules eluted off the beads. Normalized libraries were then pooled, diluted, and loaded on the sequencing cartridge.

Samples were paired-end sequenced either on a HiSeq (n = 62, Rapid Duo cBot v2 Sample Loading Kit), MiSeq (n = 286, Reagent Kit v2 300 cycles) or NextSeq system (n = 152, 500/550 Mid Output Kit v2 300 Cycles) (all kits by Illumina, San Diego, California, USA) (30). To this end, the generated fragments were bound to the flow-cell surface and clusters were generated via bridge amplification, which function as signal enhancers during sequencing. Bridge amplification was repeated numerous times for millions of clusters simultaneously resulting in up to a thousand of copies locally clustered around each initially bound fragment. Sequencing by synthesis was performed for all clusters in parallel using fluorescently labeled nucleotides (deoxynucleoside triphosphates, dNTPs). Every sequencing cycle, only one dNTP molecule is incorporated in each nucleoid acid chain at a time as dNTPs need to be enzymatically cleaved first prior to incorporation of the next molecule. The emitted fluorescent signals of all clusters are then imaged to identify the bases at the end of each cycle.

For bioinformatic analysis, we used a customized computational pipeline. First, sequences were demultiplexed and FastQ files were generated for each sample. Similar sequences were locally clustered and forward and reverse strands paired and aligned to the GRCh37 reference genome with additional Epstein-Barr virus and decoy sequences (hs37d5) using Burrows-Wheeler algorithm (BWA-MEM version 0.7.15-r1140) (31). Local realignment around small insertions and deletions (indels) to correct mapping errors often generated by genome aligners was performed using the GATK IndelRealigner (3.6-0-g89b7209) (32). For variant calling we used the software SAMtools (version 1.3.1) (33) and Varscan (version 2.4.2) (34) and set the filters to a minimum VAF of 2% and a minimum of 10 variant supporting reads. Variant annotation was performed based on the dbSNP database (b146/GRCh37p13) and the COSMIC database to identify reported polymorphisms and confirmed somatic mutations, respectively using the Jannovar software (version 0.16) (35) and the ensembl transcript set. Further filtering of variants was performed following an R-based algorithm (R version 3.3.1): Variants were excluded if annotated as intronic

or synonymous variants, if the VAF was between 45% and 55% or > 95%, if being listed as polymorphisms in the dbSNP database and if never confirmed as somatic mutations in the COSMIC database. Following hotspot variants were rescued *DNMT3A* R882C/H, *GNB1* K57E, *JAK2* V617F, *SF3B1* K666N and K700E, *SFRS2* P95L, *U2AF1* S34F and Q157P/R. All variants passing these filters were manually checked using the Integrative Genome Viewer (IGV, Broad Institute, version 2.3), a visualization tool for genomic datasets, and selected for subsequent validation.

2.3. Mutation validation

Validation of candidate variants was performed either by TDS for the vast majority of cases, digital droplet PCR (ddPCR) for hotspot variants or Sanger sequencing. For TDS, fragments of up to 200 base pairs of length covering the respective regions of interest were amplified from genomic DNA via standard PCR. First, primers were designed using the *Primer3web* program version 4.1.0 and produced by Eurofins (Eurofins Scientific, Luxembourg). After quality controls by gel electrophoresis, PCR fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), quantified by Quantus (Promega, Fitchburg, Wisconsin, US), and subsequently pooled in equimolar amounts for library construction. PCR products were pooled together to form one library, if they did not cover the same DNA sequence. Libraries were then indexed using the NEBNext Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, Massachusetts, USA). Next, the indexed libraries were quantified on a Bioanalyzer (Agilent, Santa Clara, California, USA), diluted and combined to form a final pool. The fragments were paired-end sequenced on a MiSeq system using the MiSeq Reagent Kit v2 300 cycles by Illumina as described above. Bioinformatic analysis was conducted in analogy to screening, except for variant calling which was performed with freeBayes (version 1.0.2) (36). Read counts and VAFs of known mutated positions were extracted and manually checked in IGV. TDS was also performed for quantification of mutational burden in serial follow-up samples from 22 recipients transplanted with CHIP as well as for sorted cell fractions of one donor, who carried a mutation that failed to engraft in the recipient.

DdPCR is based on the use of two types of fluorophore labelled oligonucleotides which are specific for each variant and complimentary to either the mutant or the wildtype sequence, resulting in two different fluorescent signals if activated. For each sample approximately 20000 nanoliter-sized water-oil emulsion droplets were generated by a QX200 Droplet Generator with the target and background DNA being randomly distributed into the droplets. After PCR-amplification and labelling within the droplets, the fluorescent signal of each droplet was then measured individually by a QX200 Droplet. Counts of wildtype and mutant droplets were used to calculate the VAF of the respective mutation using the QuantaSoft software (V.1.7.4) (generator, reader and software by Bio-Rad, Hercules, California, USA).

Sanger sequencing was performed by Eurofins (Eurofins GATC, Cologne, Germany). Regular deoxynucleotide triphosphates and fluorescently labelled dideoxynucleotide triphosphates

(ddNTPs) were used during PCR amplification. Due to the lack of a hydroxyl group, incorporation of ddNTPs lead to the termination of the DNA strands resulting in fragments of all lengths, each terminated by a fluorescently labelled ddNTP. Fragments were sorted by size using gel electrophoresis and the fluorescent dye of each band was activated by UV radiation. The emitted light was measured by a fluorometer and translated into the corresponding nucleotides.

2.4. Statistical analysis

We used the Statistical Package for the Social Sciences (SPSS) for Windows version 23.0 (IBM Corporation, Armonk, NY) and the statistical program R version 3.3.1 with packages “survival,” “cmprsk,” and “forestplot.” The overall survival (OS, time from transplantation to death of any cause) probabilities were estimated with the Kaplan Meier method and differences in survival time between groups were tested for significance using the log rank test. Follow-up time was calculated using the reverse Kaplan-Meyer method. Sensitivity analyses were performed for non-relapse mortality (NRM, time from transplantation to death without relapse or progression) and cumulative incidence of relapse and progression (CIR/P, time from transplantation to relapse or progression of the primary hematologic cancer).

The cumulative incidence in the presence of competing risks was estimated and compared between groups using Gray´s test and reported with 95% CIs using the method of Fine and Gray for outcome parameters *leucocyte and thrombocyte engraftment (*increase in thrombocyte counts > 20000/µl or no need for thrombocyte infusion for at least five days and increase of leucocyte counts >1000/µl or for neutrophils >500/µl, respectively*), acute graft-versus-host disease (aGVHD), chronic graft-versus-host disease (cGVHD), cytomegalovirus (CMV) reactivation, NRM, and CIR/P*, with competing risks being either *death, relapse/death or non-relapse mortality.* For multivariate analysis we used a Fine and Gray competing risks regression model to estimate the cumulative incidence with adjustments for selected baseline parameters with an unadjusted univariate P value ≤0.1. The cumulative incidence of cGVHD was adjusted for donor age, antithymocyte globulin (ATG) application and CHIP/*DNMT3A* mutations status, whereas CIR/P was adjusted for disease type, conditioning regimen intensity, ATG application and CHIP/*DNMT3A* mutation status. Further, exploratory analysis was performed using either two-sided Mann-Whitney tests for numerical variables or two-sided X^2 and Fisher´s exact tests for categorical variables to identify pairwise associations with the level of significance set to p<0.05 without corrections for multiple testing.

3. Results

3.1. Clonal hematopoiesis of indeterminate potential in the setting of allogeneic hematopoietic stem cell transplantation

The following is based on the results from the original publication (21).

3.1.1. Donor sequencing results and baseline characteristics

For screening a mean coverage of 2,033 reads per amplicon per sample was achieved. Screening of 500 donor DNA samples and subsequent validation of candidate variants identified a total of 92 mutations in 80 out of 500 donors. This corresponds to a CHIP prevalence of 16% at a median donor age of 64.5 years at the time of sampling. The prevalence increased by donor age from 10.3% (60 to 64 years) to 20.3% (65 to 69 years), 22.5% (70 to 74 years), and 28.6% (75 to 79 years). However, there was no statistically significant association between CHIP frequency and age, likely due to the fact that donors 60-69 years of age accounted for 82% of our cohort and thus were overrepresented. Variants were unevenly distributed among mutated genes with the most frequently affected ones being *DNMT3A* (44/92 = 47.8%), followed by *TET2* (11/92 = 12.0%) and *ASXL1* (7/92 = 7.6%). Prevalences within the cohort for *DNMT3A*, *TET2* and *ASXL1* were 8% (40/500), 2.2% (11/500) and 1.4% (7/500), respectively (Fig 1). 70 donors showed one single mutation, in nine donors two mutations and in one case four mutations were detected. Among multiply mutated donors the most frequent combinations were *DNMT3A/DNMT3A* (n = 4) and *DNMT3A/ASXL1* (n = 3). The median screening VAF was 5.9% (range 2% to 43%). In 20 patients, 25 mutations were detected at a VAF of at least 10%. Out of 61 SNVs 40 were transitions (65.6%) with the most frequent alteration being cytosine to thymine substitutions (49.2%), which is considered a signature characteristic of aging (37), matching our older donor cohort. There were no significant differences in baseline characteristics of donors (age, sex, hemoglobin levels, thrombocyte numbers, leukocyte numbers, red cell distribution width) depending on the donor CHIP status. In particular, the number of harvested HSCs was not affected by the CHIP status. The same was true for baseline and clinical characteristics of corresponding recipients (age, sex, remission status at transplantation, ECOG score, conditioning regimen, anti-thymocyte globulin application, transplanted CD34+ cells, stem cell source, HLA mismatch) except for a higher CHIP frequency in donors related to recipients with myeloid malignancies compared to lymphoid malignancies leading to transplantation (19.2% vs. 6.3%; P ≤ .001).

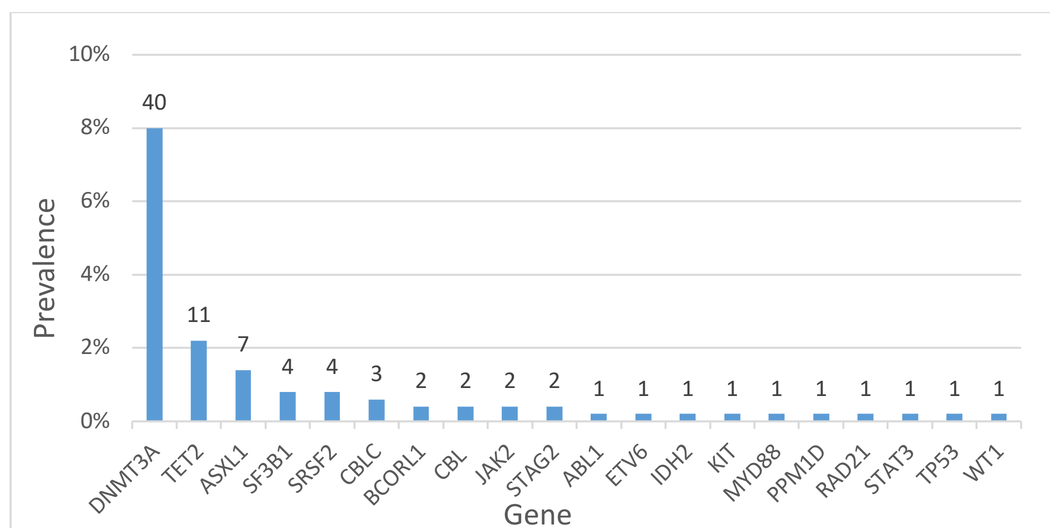

Fig 1. Prevalence of mutated CHIP genes in 500 healthy, elderly stem cell donors (21).

3.1.2. Associations between donor clonal hematopoiesis of indeterminate potential and transplantation outcomes

Endpoints included time to engraftment, cytomegalovirus (CMV) reactivation, occurrence of aGVHD and cGVHD, donor cell leukemia and relapse/progression after stem cell transplantation. We were unable to test for associations between CHIP and unexplained cytopenias due to a lack of uniform data. CHIP status did not affect the occurrence of aGVHD, CMV reactivation or thrombocyte engraftment time. In univariate analysis donor CHIP led to a faster leukocyte engraftment (cumulative incidence of engraftment after 15 days, 64.1% v 51.4%; P = .023). Donor engrafted CHIP was also associated with a higher 5-year cumulative incidence of cGVHD (52.9% v 35.7%; P = .008) and was identified as an independent risk factor for cGVHD development in multivariate analysis when adjusted for ATG application and donor age (hazard ratio, 1.73; 95% CI, 1.21 to 2.49; P = .003). Gene specific testing proofed *DNMT3A* mutations to be the predominant CHIP factor for cGVHD development in univariate (58.5% v 36.6%; P =.006) and multivariate analysis (HR, 1.99; 95% CI, 1.28 to 3.12; P = .002).

Donor engrafted CHIP as well as *DNMT3A* mutations were also associated with a lower CIR/P (P = .027, P = .029, respectively) and were confirmed as independent risk factors in multivariate analysis (Donor CHIP: HR, 0.633; 95% CI, 0.41 to 0.98; P = .042; *DNMT3A*: HR, 0.494; 95% CI, 0.26 to 0.93; P = .029). In separate competing risk analysis based on remission status, this was true only for patients transplanted in non-complete remission (n-CR) (n = 301; CHIP: HR, 0.52; 95% CI, 0.27 to 0.88; P = .019; *DNMT3A* mutation: HR, 0.36; 95% CI, 0.15 to 0.85; P = .015), which was confirmed in multivariate analysis (CHIP: HR, 0.46; 95% CI, 0.25 to 0.88; P = .019; *DNMT3A*: HR, 0.29; 95% CI, 0.12 to 0.74; P = .01). NRM was not affected in any setting. As expected, donor engrafted CHIP was significantly linked to an increased risk for DCL. There were two cases of DCL in 82 recipients transplanted with donor CHIP compared to no case within the group transplanted without donor CHIP (P = .026, Fisher´s exact test). One case derived from an unrelated donor that had been disqualified from our study. Since DCL represents a severe complication, we included also the eight cases of unrelated donors in our DCL analysis, of which two were tested positive for CHIP.

Median follow-up time for patients alive was 3.3 years (range 0.1 to 20.6 years), median OS was 2 years, and the 5-year OS rate was 37.6%. Neither follow-up times nor OS were affected by donor CHIP or donor *DNMT3A* status. Patients diagnosed with MDS and AML who underwent transplantation in n-CR (n = 152) showed a survival benefit when transplanted with donor CHIP (HR, 0.49; 95% CI, 0.28 to 0.86; P = .011).

3.1.3. Clonal dynamics in recipient follow-up samples

Clonal dynamics of donor engrafted CHIP-clones were studied by TDS of follow-up samples of 22 recipients with an average of five time points per patient. Recipient samples prior to transplan-

tation were available for 20 patients, in which the respective donor mutations could not be detected. This proves that the mutations originated from the donors. A mean coverage of 51278 reads/amplicon was achieved. Clonal dynamics were classified into three categories: No engraftment, linear expansion, and disproportionate expansion, with disproportionate expansion being defined as a constant doubling of the allelic ratio over time. Sequencing results were matched with chimerism data to ensure a most precise analysis. Engraftment was observed in 21 of 22 cases and the mutations persisted throughout follow-up. Linear expansion was observed in 10 cases, of which one half developed a relapse during the course of follow-up. 11 patients showed disproportionate expansion, of which in contrast only one relapsed. In all cases of relapse, donor chimerism and VAF of the respective mutation decreased simultaneously. Engraftment failure was observed in one recipient (041R) allografted with cells containing a *SF3B1* K700E mutation. TDS of sorted cells of the respective donor (041D) for the *SF3B1* K700E mutation demonstrated its stem-cell origin. Engraftment failure could possibly be explained by clonal disadvantage and negative selection.

3.1.4. Clonal evolution in two cases of donor cell leukemia

In both cases of DCL we proved clonal evolution by detecting additional, newly acquired mutations in follow-up samples at full donor chimerisms. In one case, the recipient initially showed a single *CBLC* mutation after transplantation, which rapidly increased from 8% to 37% and remained stable until the end of the follow up. Panel sequencing of the DCL sample collected at day 550 after transplantation identified an additional *TP53* R175H (VAF, 25.9%) and *BCORL1* A971G (x-chromosomal adjusted VAF, 32.2%) mutation. For the second case of DCL whole exome sequencing of the DCL sample was performed. Beside of the initial donor engrafted *ASXL1* and *DNMT3A* mutations, nine additional mutations were acquired within four years, including a *RUNX1* splice site alteration, leading to the full-blown donor derived malignancy.

3.2. Evolutionary dynamics and lineage distribution of clonal hematopoiesis of indeterminate potential

The following is based on the results from the original publication (25). TDS of 72 solid cancer patients and 365 non-cancer patients showed no differences between the cohorts regarding CHIP prevalence (solid cancer cohort: 22/72 (30.6%); non-cancer cohort: 99/365 (27.1%)), affected genes, number of mutations per individual and their VAFs. Therefore, mutation analysis was described for both cohorts combined (n = 437). 168 variants were validated in 121 patients (27.7%). 87 patients showed one mutation, 23 patients two, nine patients three and two patients four mutations. Again, the most frequently affected genes were *DNMT3A* (59/437 = 13.5%), *TET2* (34/437 = 7.8%) and *ASXL1* (11/437 = 2.5%). CHIP frequency increased in an age-dependent manner from 16.8% to 29.3%, 35.2% and 66.7% among individuals 55-69, 70-79, 80-89 and 90-99 years of age, respectively ($P < .001$).

To gain insights into clonal dynamics of CHIP under evolutionary pressure, TDS was performed for 32 mutations in 121 follow-up samples from 22 solid cancer patients undergoing (radio-)chemotherapy treatment. By applying a cutoff of ±50% VAF change, clonal dynamics were split into three categories: 19 mutations remained stable, six showed an increase and seven a decrease of the VAF. Of 13 *DNMT3A* mutations, only one showed relevant changes in VAF, whereas the remaining 19 variants not affecting *DNMT3A* showed changes in 13 cases (7.7% vs. 68.4%; P < .001). CHIP status did not affect common chemotherapy-associated complications such as transfusion necessity, neutropenic fever, need for G-CSF application or therapy delay, except for a more frequent chemotherapy dose reduction in CHIP$^+$ patients (P = .035 after adjustment for radiation and sex). However, after exclusion of single *DNMT3A*-mutated cases (n = 7), a positive CHIP status correlated with significantly lower hemoglobin levels after six cycles of chemotherapy (P = .017) and an elevated overall red cell transfusion necessity (P = .025 after adjustment for radiation, treatment with alkylating agents, and topoisomerase inhibitors) with a mean of 1.2 transfused erythrocyte concentrates in CHIP$^-$ cases vs. 3.9 in CHIP$^+$ patients (P = .004).
To identify lineage distribution patterns and the cell of origin in CHIP, TDS of sorted cell fractions for 91 mutations of 63 non-cancer patients was performed, revealing a strong distribution bias toward the myeloid compartment and NK-cells. The five most frequently mutated genes (*DNMT3A, TET2, ASXL1, SF3B1*, and *TP53*) showed similar repartition patterns, however we observed a higher median VAF of *DNMT3A* mutations in T-cells compared to mutations in other genes (P = .001). By determining the mutational burden in sorted cells from bone marrow of nine patients, Lin$^-$CD34$^+$CD38$^-$ HSCs could be identified as cells of origin in all cases. By calculating expansion ratios (VAF(granulocytes) / VAF(HSCs) or VAF(monocytes) / VAF(HSCs)) three categories of clonal dynamics pattern could be observed: There were three cases of modest expansion (ER <2), three cases of intermediate expansion (ER ≈ 3) and three cases of high expansion patterns (ER 6.5 – 88) with expansion occurring mainly during early differentiation from HCSs to progenitor cells. Regarding clinical aspects, an elevated frequency of peripheral artery disease was observed in CHIP$^+$ individuals (P = 0.43 after adjustment for age) in the non-cancer cohort (n=365). Also, the presence of multiple mutations (n = 24) was significantly associated with peripheral artery disease and diabetes (P = .001 and P = .034 after adjustment for age).

3.3. Clonal hematopoiesis of indeterminate potential in the setting of anti-neutrophil cytoplasmic antibodies-associated autoimmune vasculitis

The following is based on the results from the original publication (28). 46 mutations were identified in 34 out of 112 AAV patients (30.4%). TDS of a constructed sex- and age-matched control cohort using the same sequencing technology confirmed this unexpectedly high prevalence (30.4% vs 17.9%, P = .042), thus showing a new association between CHIP and AAV. 25 patients harbored one single mutation, eight patients showed two and one patient five mutations. Again, the most frequently affected genes were *DNMT3A* (18/112 = 16.0%), *TET2* (7/112 = 6.3%) and

ASXL1 (4/112 = 3.6%). TDS was performed to determine mutational burden in follow-samples of 19 CHIP$^+$ patients with a median follow-up time of 2.3 years and two to four time points per patient. In twelve cases the mutations remained stable, five patients showed increasing and two decreasing VAFs over the course of follow-up.

Statistical analysis showed differences of disease manifestations in granulomatosis with polyangiitis patients (n=76) depending on CHIP-status: In CHIP$^+$ patients the renal and nervous system were less frequently affected than in CHIP$^-$ patients (68.2% vs. 88.5%, P = .049 and 0% vs. 19.2%, P = .028, respectively). Further associations (e.g. necessity of immunosuppressive treatment prior to sampling, comorbidities, relapse risk) could not be observed. Quantitative PCR showed reduced *RUNX3* mRNA levels in CHIP$^+$ AAV patients compared to healthy controls (0.28 vs. 0.79, P = .007) which would lead to the assumption of higher expression of the autoantigens PR3 and MPO in CHIP$^+$ patients according to previous research results (29). However, PB mRNA levels of MPO and PR3 were increased in CHIP$^-$ AAV patients compared to CHIP$^-$ healthy controls (MPO: 1.94 vs. 0.86, P = .026; PR3: 2.02 vs. 0.58, P = .057) and was less marked in CHIP$^+$ AAV patients. In line with lower MPO and PR3 levels, ANCA-induced *in vitro* neutrophil activation was reduced in CHIP$^+$ AAV patients compared to CHIP$^-$ patients (anti-MPO: 6.29 vs. 13.01 Stimulation index (SI), P = .057; anti-PR3: 7.72 vs. 13.00 SI, P = .026), whereas no difference in membrane expression index or percentage of positive cells was observed.

4. Discussion

We showed that approximately one sixth of donors at a median age of 64.5 years harbored CHIP mutations. The mutated clones engrafted in the recipients in the vast majority of cases (21/22 recipients) and persisted in the recipient for the time of follow-up. We demonstrated that the clones could expand in size and undergo clonal evolution as observed in two cases of DCL. Our CHIP prevalence and mutation spectrum was in line with previous results studying non-cancer populations using similar sequencing technologies (38). As mentioned earlier, ECS has revealed that somatic, hematologic cancer-associated mutations are almost ubiquitous in healthy, middle-aged adults (5), however, the clinical relevance of CH at such low levels remains unclear. A recently published study using an ECS approach demonstrated that CH was present in 44% (11/25) of considerably younger, unrelated donors (median age, 26 years), at low VAFs (median VAF, 0.247%) (39). The authors showed that CH-clones could engraft, persist and expand in the recipients regardless of the clone size, stating that also rare clones possess the potential to become biologically and clinically relevant, once having expanded in size beyond a certain threshold. Additionally, they detected new mutations in post-HSCT samples, of which some were de-novo mutations and others represented donor derived clones that expanded under cytotoxic stressors. These rare preexisting clones, initially too small to be detected by ECS, could only be discovered by ddPCR in pre-HSCT donor samples. As we worked with a threshold of 2% VAF and did not panel sequence recipient follow-up samples for new mutations, it is likely that we missed potential

clinically relevant clones. Assumingly, clonal expansion of clones at such low VAFs, however, would require a long time of positive selection until a clinically relevant clone size was reached. Due to a small sample number and a short follow-up of one year, the authors were unable to test for clinical associations (39). Large studies using ECS with long follow-ups are warranted in the future to investigate the relevance of rare clones and to revise the threshold for CHIP based on clear clinical associations.

Our finding of a higher CHIP frequency in donors related to patients diagnosed with myeloid compared to lymphoid malignancies could indicate familial aggregation of hematologic cancer-associated mutations, as CHIP and myeloid malignancies share many mutations. Familial aggregation has also been reported for *TET2* in healthy individuals (38) and for the *JAK2* V617F mutation which is linked to *JAK2* 46/1 haplotype (40). This prompted the question if an inherited predisposition to acquire CHIP exists. However, two recently published twin studies addressing this issue showed no evidence for a genetic predisposition to develop CHIP as no higher concordance for CH could be observed within monozygotic twin pairs compared to dizygotic twin pairs, neither overall nor in gene specific subanalyses for *DNMT3A* or *TET2* (41, 42). Hence, one possible explanation for familial aggregation of CH could be shared environmental factors rather than inherited factors.

Allogeneic HSCT from a $CHIP^+$ donor appears safe in the setting of related, elderly donors as CHIP status did not influence OS. Also, specific gene mutations, mutational burden or number of mutations did not affect OS. In subanalyses, we even noticed a survival benefit in the subset of patients diagnosed with MDS and AML if transplanted with donor CHIP in n-CR. However, we observed an increased risk for DCL, which represents a rare but severe complication.

As in the second side project multipotent $Lin^-CD34^+CD38^-$ HSC have been identified as cells of origin of somatic mutations (25), pleiotropic effects on both mature myeloid and lymphoid lineages are conceivable. Regarding lineage distribution, however, a strong bias towards the myeloid compartment and NK-cells was observed. For *DNMT3A* mutations the bias was less pronounced as the mutational burden in T-cells was significantly higher compared to mutations in other genes. This finding could presumably indicate that mutations in this gene occur early in less committed hematopoietic stem cells and is consistent with our assumption that a higher incidence of cGVHD might be mediated by immunological effects of *DNMT3A* mutations in T-cells.

For the first time, we describe a higher frequency of cGVHD in recipients transplanted with donor engrafted CHIP. Interestingly, gene specific testing revealed that this association was predominantly driven by mutations in *DNMT3A. DNMT3A* functions among others as an epigenetic regulator of Th1 and Th2 cell differentiation and has been reported to limit the expression of proinflammatory cytokines like IL-13 in Th2-cells (43, 44). Dysregulated T cell differentiation and a proinflammatory cytokine profile are essential elements of the pathophysiology of cGVHD (45). IL-13, in particular, plays an important role in the development of fibrosis as shown in several experimental models (46-49). Additionally, mutations in the second most commonly affected

gene, *TET2*, have been reported to contribute to chronic inflammation in the pathogenesis of atherosclerosis. Here, *TET2*-deficient macrophages lead to an increased NLRP3 inflammasome-mediated interleukin-1b secretion, resulting in accelerated atherosclerosis in a mouse model (7). At the same time, the NLRP3 inflammasome is also known to play a crucial role in the pathogenesis of GVHD by controlling pro-IL 1ß cleavage (50). Therefore, it seems conceivable that mutations in *DNMT3A* and *TET2* might foster the development of cGVHD.

The higher frequency of cGVHD was accompanied by a reduced CIR/P for CHIP^{+} and *DNMT3*-mutated patients if transplanted in n-CR. This effect could be directly linked to the higher incidence of cGVHD in the group of recipients with donor engrafted CHIP. Weiden et al. described an beneficial anti-leukemic effect of GVHD, referred to as GVL effect, that resulted in a lower incidence of relapse in patients that received allogeneic HSCT (51). Therefore, a reduced CIR/P might be a direct consequence of a higher frequency of cGVHD, which in this case is directed against the cells of the hematopoietic system of the host including remaining malignant cells. Additionally, CHIP-clones might have a competitive advantage compared to residual malignant cells in the post transplantation setting. This is backed by our observations in longitudinal tracking of mutational burden in recipient follow-up samples as the occurrence of relapse was less frequent in patients with disproportionate expansion of CHIP-clones compared to linear expansion (1/11 vs 5/10, respectively). This advantage is likely driven by mutations in the most commonly affected genes *DNMT3A* and *TET2* as inactivation of these genes was demonstrated to enhance self-renewal of HSCs (52, 53). Notably, the authors also observed perturbed subsequent differentiation. However, the differentiation blockage was proven to be not completely penetrant. Although not in proportion to the strong expansion of the stem cell compartment, both *DNMT3A* and *TET2*-deficient HSCs showed increased contribution to peripheral blood production compared to controls. Therefore, they contribute to homeostasis and host defense, too, and presumably foster GVHD and GVL effects, resulting in a lower CIR/P. Additionally, the increased self-renewal potential might also explain the faster leukocyte engraftment time in recipients transplanted with donor CHIP.

In summary, donors harboring CHIP mutations appear suitable for stem cell donation as CHIP did not influence overall survival. Nevertheless, we observed an increased risk for development of cGVHD and DCL. Donor screenings for CHIP could serve as a risk stratification tool in the future to improve patient safety. However, an evidence-based consensus on the definition of CH is needed that considers a VAF threshold and a defined set of relevant genes that are based on clear clinical associations. The relevance of many mutations that are currently tested in CH remains unclear and testing for them appears questionable. To decipher the individual effects of these various genes, gene specific analyses are needed, which require a larger sample number to ensure also rarely affected genes are well represented and can be tested for clinical associations. High risk mutations for the development of DCL need to be identified that require further monitoring. Also, long follow-ups are needed to study long-term effects of CHIP in the setting of

allogeneic HSCT. With 3.3 years the median follow-up of our study was relatively short. One recent study investigated follow-up samples of 22 recipients at a median time point of 9.8 years after HSCT and a median age of 78 years at sampling who had been transplanted from younger donors (≤ 40 years at donation) (54). Despite of a high median age at sampling only one CHIP mutation was reported (1/22 = 4.5%), which was proven not to be donor derived. However, pyrosequencing was used as sequencing method, which is characterized by a detection limit of 5% mutant alleles. The respective recipient developed a peripheral artery disease (PAD), which in contrast occurred only in one of 21 CHIP$^-$ patients of the cohort, potentially extending former findings regarding associations of CHIP with cardiovascular diseases. A second study tested both follow-up samples of 42 long-term survivors of HSCT (median age at sampling = 61 years) and their respective sibling donors (median age at sampling = 57 years) for CH at a median follow-up time point of 16 years using an error corrected sequencing approach (55). No significant difference between CH frequencies in donors (10/42 = 23.8%) and recipients (13/42 = 31.0%) was observed. In four of five cases of donor engrafted CH there was a significant, but mild increase of clone size within the recipients compared to the donors, indicating a growth advantage in the post-transplant setting, to a certain extent. However polyclonal hematopoiesis was maintained even after a long follow-up. One fifth case evolved into MDS in both recipient and donor. Colony forming units (CFUs) showed no difference in telomere length depending on their CHIP status. The findings of these two studies provide evidence that the inflammatory milieu and the enhanced proliferation of HSCs after transplantation do not seem to promote the development of CH and demonstrate compatibility of CH with long-term survival.

In the first side project we studied CHIP in the setting of solid-cancer patients undergoing (radio-) chemotherapy (25). The CHIP-prevalence of 30.6% is comparable to former results in solid cancer patients (25.1%) (56). Using ECS, a recently published study revealed that the CH prevalence and also the number of detected mutations per individual were higher after chemotherapy treatment compared to no such exposure in patients diagnosed with lymphoid malignancies (57). This increase in mutations in the setting of cytotoxic stressors can be attributed to positive selection of priorly preexisting clones at VAF below the detection limit (58). In contrast to CH in the setting of allogeneic HSCT (39), new mutations under influence of chemotherapy were hardly ever de-novo mutations but resulted from positive selection (58). Also, the mutational spectrum seems to change under chemotherapy as DNA repair genes such as *PPM1D, TP53* and *SRCAP* were demonstrated to be enriched, indicating a fitness advantage of the respective clones (20, 56, 57, 59, 60). However, selected genes might differ dependent on chemotherapy doses and regimens (58, 60). Considering that we only tracked mutations that were already present at the initiation of chemotherapy and therefore missed potential newly emerging clones and taking into account that our detection limit was set to a VAF of 2%, it stands to reason that we observed a mutational spectrum comparable to those in chemotherapy-naive cohorts (1, 2, 38). Therefore,

mutations in PPM1D and TP53 were not enriched in our cohort, whereas *SRCAP* was not included in our sequencing panel. Among these DNA repair genes, the leukemogenic potential was predicted to be highest for *TP53* mutations as *TP53* mutations were also common in therapy-related AML and MDS samples, whereas *PPM1D* and *SRCAP* mutations were rare (57). Clonal dynamic patterns of CHIP mutations varied under selective pressure of chemotherapy with clones expanding, diminishing, or remaining stable, most likely dependent on specific mutations and affected genes but also different applied treatment regimens within our cohort. *SF3B1* mutated clones decreased in clone size in 3/3 cases, indicating negative selection. Variants in *DNMT3A* were barely affected by cytotoxic treatment as dynamics remained stable in 12/13 cases. This finding is consistent with results of another study, where the prevalence of *DNMT3A* mutations was similar between patients with and without cytotoxic exposure (57). Regarding clinical associations we investigated common chemotherapy-related complications such as transfusion necessity, neutropenic fever, need for G-CSF application or therapy delay. We did not observe any associations between a positive CHIP status and these outcomes; however, it was linked to an increased necessity for chemotherapy dose reduction. Since *DNMT3A* mutations showed no fitness advantage under chemotherapy, but clone size remained stable in 12/13 cases, we hypothesized that DNMT3A mutations might have little impact on clinical outcomes. Exclusion of single *DNMT3A*-mutated cases (n = 7) from analysis revealed reduced red blood cell counts and an increased need for red blood cell transfusions in the $CHIP^+$ group. This finding might indicate impairment of erythropoiesis in patients mutated in genes other than *DNMT3A*.

New associations between CHIP and AAV patients were identified in the third project since the CHIP prevalence was surprisingly high in this cohort (30.4%) and disease manifestation differed as the renal and nervous system were less frequently affected in $CHIP^+$ individuals. Against expectations, autoantigen mRNA expression was upregulated in $CHIP^-$ but not $CHIP^+$ patients concomitant with a reduced ANCA-induced neutrophil activation in $CHIP^+$ patients. Therefore, mutations in CHIP-defining epigenetic regulators like *DNMT3A* do not seem to affect ANCA antigen expression as assumed. For $CHIP^+$ AAV patients the pathogenesis might be based on a different mechanism. ANCA-induced production of reactive oxygen species (ROS) was demonstrated to lead to downregulation of inflammasome activation by inhibiting the inflammasome-caspase-1-interleukin-1ß cascade (61). In reverse, reduced ANCA-induced ROS production in $CHIP^+$ leukocytes as demonstrated in our leukocyte stimulation assays might augment activation of the inflammasome and by this means foster inflammation and contribute to the pathogenesis of AAV.

Taken together, we provide evidence for new potential clinical associations of CHIP and extend reported findings linking CHIP with aberrant immune response in inflammatory diseases. Nevertheless, these findings should be treated with caution due to heterogeneity of the cohorts regarding underlying diseases and treatment regimens or a limited number of patients. An evidence-based consensus on the definition of CHIP is warranted and individual risks for mutated genes need to be determined. Retro- and prospective multicenter studies of larger and homogenous

cohorts with long follow-ups are needed to confirm and extend these results as well as functional experiments to understand the molecular mechanisms behind it before these findings can be translated into clinical use.

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Statutory declaration (Eidesstattliche Versicherung)

„Ich, Willy Chan, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema „Donor engrafted clonal hematopoiesis of indeterminate potential in allogeneic hematopoietic stem cell transplantation" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst."

Datum, Unterschrift

Declaration of Contribution (Anteilserklärung)

Willy Chan hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Frick M*, **Chan W*,** Arends CM, Hablesreiter R, Halik A, Heuser M, Michonneau D, Blau O, Hoyer K, Christen F, Galan-Sousa J, Noerenberg D, Wais V, Stadler M, Yoshida K, Schetelig J, Schuler E, Thol F, Clappier E, Christopeit M, Ayuk F, Bornhäuser M, Blau IW, Ogawa S, Zemojtel T, Gerbitz A, Wagner EM, Spriewald BM, Schrezenmeier H, Kuchenbauer F, Kobbe G, Wiesneth M, Koldehoff M, Socié G, Kroeger N, Bullinger L, Thiede C, Damm F. Role of Donor Clonal Hematopoiesis in Allogeneic Hematopoietic Stem-Cell Transplantation. Journal of Clinical Oncology. 2019 Feb 10.

*Diese Autoren haben in gleichen Teilen zur Arbeit als Erstautoren beigetragen

Der Schwerpunkt meiner Arbeit lag vor allem im experimentellen Teil dieses Projekts.

Beitrag im Einzelnen:

- Verwaltung und Aufbereitung aller Proben (inklusive Lagerung, DNA-Extraktion aus gefrorenen Zellen (n=52), DNA-Quantifizierung)
- Durchführung und Koordination des Mutationsscreenings
    - Erstellung genomischer Bibliotheken und Durchführung der Sequenzieranalysen für den Großteil der Proben (n=450)
    - Bioinformatorische Auswertung für den Großteil der Proben (n=450)
- Validierung von Mutationen und Quantifizierung der Mutationslast in Verlaufsproben
    - Durchführung der targeted sequencing-Analysen (inklusive Primerdesign, Durchführung der PCRs, Pooling) für den Großteil der Proben (n=200)
    - Bioinformatorische Auswertung für den Großteil der Proben (n=200)
- Mitarbeit bei der Erstellung des Manuskripts mit Schwerpunkt auf Sequenzierergebnissen und Methoden
- Mitarbeit bei der Erstellung von Abbildungen und Tabellen
    - Eigenständige Erstellung der Abbildungen Figure 1, A2, A3 und de Tabelle A4
    - Mitarbeit bei der Erstellung der Abbildungen Figure 5 und A11
    - Darüber hinaus führte die von mir durchgeführten Experimente und Datensammlungen in Kombination mit den klinischen Daten zur Erstellung weiterer Abbildungen und Tabellen

Publikation 2: Arends CM*, Galan-Sousa J*, Hoyer K*, **Chan W**, Jäger M, Yoshida K, Seemann R, Noerenberg D, Waldhueter N, Fleischer-Notter H, Christen F, Schmitt CA, Dörken B, Pelzer U, Sinn M, Zemojtel T, Ogawa S, Märdian S, Schreiber A, Kunitz A, Krüger U, Bullinger L, Mylonas E, Frick M, Damm F, Hematopoietic lineage distribution and evolutionary dynamics of clonal hematopoiesis, Leukemia, 2018 Sep.

*Diese Autoren haben in gleichen Teilen zur Arbeit als Erstautoren beigetragen

Beitrag im Einzelnen:

- Mitarbeit bei der Patientenrekrutierung und Probenakquirierung beider Kohorten
- Mitarbeit bei der Verarbeitung der Patientenproben (inklusive Ficoll-Dichtenzentrifugation)
- Mitarbeit bei dem Mutationsscreening in Form von der Erstellung genomischer Bibliotheken für einen Teil der Proben
- Mitarbeit bei der Validierung von Mutationen und Quantifizierung der Mutationslast in Verlaufsproben, beziehungsweise Zellfraktionen für einen Teil der Proben beider Kohorten (Durchführung von PCRs, Durchführung von targeted sequencing-Analysen)

Publikation 3: Arends CM*, Weiss M*, Christen F, Eulenberg-Gustavus C, Rousselle A, Kettritz R, Eckardt K, **Chan W**, Hoyer K, Frick M, Bullinger L, Bieringer M, Schreiber A, Damm F. Clonal Hematopoiesis in Patients with ANCA-associated Vasculitis. Haematologica. 2020 Jun.

*Diese Autoren haben in gleichen Teilen zur Arbeit als Erstautoren beigetragen

Beitrag im Einzelnen:

- Mitarbeit bei der Erstellung genomischer Bibliotheken und Sequenzierung für einen Teil der Proben
- Mitarbeit bei der Erstellung der Kontrollkohorte

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Unterschrift, Datum und Stempel des erstbetreuenden Hochschullehrers

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Unterschrift des Doktoranden

Selected publications

Publication 1

Frick, M., Chan, W., Arends, C.M., Hablesreiter, R., Halik, A., Heuser, M., Michonneau, D., Blau, O., Hoyer, K., Christen, F., Galan-Sousa, J., Noerenberg, D., Wais, V., Stadler, M., Yoshida, K., Schetelig, J., Schuler, E., Thol, F., Clappier, E., Christopeit, M., Ayuk, F., Bornhauser, M., Blau, I.W., Ogawa, S., Zemojtel, T., Gerbitz, A., Wagner, E.M., Spriewald, B.M., Schrezenmeier, H., Kuchenbauer, F., Kobbe, G., Wiesneth, M., Koldehoff, M., Socie, G., Kroeger, N., Bullinger, L., Thiede, C. & Damm, F. Role of Donor Clonal Hematopoiesis in Allogeneic Hematopoietic Stem-Cell Transplantation. J Clin Oncol 37, 375-385 (2019).

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Publication 2

Arends, C.M., Galan-Sousa, J., Hoyer, K., Chan, W., Jager, M., Yoshida, K., Seemann, R., Noerenberg, D., Waldhueter, N., Fleischer-Notter, H., Christen, F., Schmitt, C.A., Dorken, B., Pelzer, U., Sinn, M., Zemojtel, T., Ogawa, S., Mardian, S., Schreiber, A., Kunitz, A., Kruger, U., Bullinger, L., Mylonas, E., Frick, M. & Damm, F. Hematopoietic lineage distribution and evolutionary dynamics of clonal hematopoiesis. Leukemia 32, 1908-1919 (2018).

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Publication 3

Arends, C.M., Weiss, M., Christen, F., Eulenberg-Gustavus, C., Rousselle, A., Kettritz, R., Eckardt, K.U., Chan, W., Hoyer, K., Frick, M., Bullinger, L., Bieringer, M., Schreiber, A. & Damm, F. Clonal hematopoiesis in patients with ANCA-associated vasculitis. Haematologica (2019).

https://doi.org/10.3324/haematol.2019.223305

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of publications

- Arends CM*, Weiss M*, Christen F, Eulenberg-Gustavus C, Rousselle A, Kettritz R, Eckardt K, **Chan W**, Hoyer K, Frick M, Bullinger L, Bieringer M, Schreiber A, Damm F.
  Clonal Hematopoiesis in Patients with ANCA-associated Vasculitis. ***Haematologica.*** 2020 Jun. *equal contribution.

  IF 2019 = 7.116

- Mylonas E, Yoshida K, Frick M, Hoyer K, Christen F, Kaeda J, Obenaus M, Noerenberg D, Hennch C, **Chan W**, Ochi Y, Shiraishi Y, Shiozawa Y, Zenz T, Oakes CC, Sawitzki B, Schwarz M, Bullinger L, le Coutre P, Rose-Zerilli MJJ, Ogawa S, Damm F.
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  IF 2019 = 12.121

- Christen F*, Hoyer K*, Yoshida K*, Hou HA*, Waldhueter N, Heuser M, Hills RK, **Chan W**, Hablesreiter R, Blau O, Ochi Y, Klement P, Chou WC, Blau IW, Tang JL, Zemojtel T, Shiraishi Y, Shiozawa Y, Thol F, Ganser A, Löwenberg B, Linch DC, Bullinger L, Valk PJM, Tien HF, Gale RE, Ogawa S, Damm F.
  Genomic landscape and clonal evolution of acute myeloid leukemia with t(8;21): an international study on 331 patients. ***Blood.*** 2019 Mar 7. *equal contribution.

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  Role of Donor Clonal Hematopoiesis in Allogeneic Hematopoietic Stem-Cell Transplantation. ***J Clin Oncol.*** 2019 Feb 10. *equal contribution.

  IF 2019 = 32.956

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

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Willy Chan

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