Aus der Medizinischen Klinik mit Schwerpunkt Hämatologie, Onkologie
und Tumorimmunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Prognostic Implications of Cellular Senescence in Acute Myeloid Leukemia

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 25. November 2022

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List of Abbreviations and Acronyms

7+3	Acronym for a specific AML chemotherapy regimen (containing 7 days cytarabine, 3 days anthracycline)
ADC	Antibody-drug conjugate
ARF	Alternative reading frame
AML	Acute myeloid leukemia
AML1–ETO	Acute myeloid leukemia-1 transcription factor and the eight-twenty-one corepressor
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
AraC	Cytarabine
BCL2	B-cell lymphoma 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B
C12FDG	5-dodecanoylaminofluorescein di-β-D-galactopyranoside
CD	Cluster of differentiation
CDK	Cyclin-dependent-kinases
CDKN2A	Cyclin-dependent kinase inhibitor 2A
ChIP-seq	Chromatin immunoprecipitation in combination with DNA-sequencing
CML	Chronic myeloid leukemia
CR	Complete remission
CXCR2	C-X-C motif chemokine receptor 2
DIC	Disseminated intravascular coagulation
DFS	Disease-free survival
DKFZ	Deutsches Krebsforschungszentrum
DMEM	Dulbecco's Modified Eagle's Medium
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EdU	5-Ethynyl-2'-deoxyuridine

e.g.	*exempli gratia*, for example
EMA	European Medicine Agency
ELN	European Leukemia Net
ESR1	Estrogen receptor alpha
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FLT3	FMS-like tyrosine kinase 3
FLT3-L	FMS-like tyrosine kinase 3 ligand
fSA-β-gal	Immunofluorescence-based SA-β-gal
G-CSF	Granulocyte colony-stimulating factor
GEO	Gene Expression Omnibus
GO	Gene ontology
GSEA	Gene set enrichment analysis
H3K9me3	Histone H3 protein trimethylation at the 9th lysine residue
HER2	Human epidermal growth factor receptor 2
HP1	Heterochromatin protein 1
hTERT	Human telomerase reverse transcriptase
i.e.	*id est,* that is
IMDM	Iscove's Modified Dulbecco's Medium
ITD	Internal tandem duplication
IL-3	Interleukin 3
IL-6	Interleukin-6
JAK	Janus kinase
KDM2b/JHDM1b	Lysine Demethylase 2B
Ki67	Antigen KI-67
LDH	Lactate dehydrogenase
LMU	Ludwig-Maximilians-Universität München
MACS	Magnetic activated cell separation
MDS	Myelodysplastic syndrome
MNC	Mononuclear cell

MPN	Myeloproliferative neoplasms
MSigDB	Molecular signature database
MYC	MYC proto-oncogene
NCCN	National Comprehensive Cancer Network
NES	Normalized enrichment score
NF-κB	Nuclear factor of κB
NPM1	Nucleophosmin 1
NGS	Next generation sequencing
NRAS	N-ras proto-oncogene
OS	Overall survival
$P16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A (also denoted as CDKN2A)
PDGFR-β	Platelet-derived growth factor receptor β
PKC	Protein kinase C
PML/RARA	Promyelocytic leukemia gene/ Retinoic acid receptor-alpha
PTEN	Phosphatase and tensin homolog
RAS	Ras proto-oncogene
RB	Retinoblastoma protein
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
SA-β-gal	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
SEM	Standard error of the mean
shRNA	Short harpin RNA
siRNA	Small interfering RNA
SRSF2	Serine and arginine-rich splicing factor 2
STAT	Signal transducer and activator of transcription proteins
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor β
TIS	Therapy-induced senescence
TKD	Tyrosine kinase domain

TNF-α	Tumor necrosis factor-α
TP53	Tumor protein P53
TPE-OLD	Telomere position-effect on long distance
TPO	Thrombopoietin
TU	Technische Universität München
VEGF	Vascular endothelial growth factor
VIPER	Virtual inference of protein activity by enriched regulon analysis
WHO	World Health Organization

1. Summary

Acute myeloid leukemia (AML) is a heterogeneous disease for which biologically grounded predictors of outcome remain a clinical need. In addition to tumor cell death, antineoplastic drugs can mediate a long-lasting growth arrest of vital, metabolically active tumor cells termed therapy-induced senescence, but structured investigations into its prognostic and predictive power are lacking. Besides its occurrence in response to chemotherapeutic treatment, cellular senescence can be evoked by replicative stress or activation of oncogenes and serves as an initial barrier to cancer development. Yet, long-term effects of senescent cancer cells on tumor growth are unclear as they are known to mediate inflammation via a senescence-associated secretory phenotype (SASP) and are subjected to epigenetic remodeling, thereby acquiring cancer stemness characteristics.

In an *ex vivo* analysis of AML blast samples from patients at diagnosis, I aimed to characterize basal as well as treatment-evoked senescence and determine its role as a prognostic and predictive biomarker.

I established assays to detect and therapeutically induce senescence in a primary AML culture setting. Senescence was assessed by senescence-associated β-galactosidase (SA-β-gal) activity and other senescence markers. Gene expression analyses validated my experimental characterization of AML samples as “senescent”, as evidenced by upregulation of senescence-associated gene expression signatures.

For prognostic analysis, clinical outcomes and molecular genetics of AML sample donors were retrieved. I found the intra-individual changes of senescence levels in response to the standard anti-leukemic agent daunorubicin to be positively correlated with better disease-free- and overall patient survival. In line with this, a more favorable molecular risk group, normal karyotype, and *NPM1* as well as *DNMT3A*^{R882} mutations were associated with higher therapy-induced senescence levels. Other therapeutic AML agents, namely hydroxyurea, decitabine and gemtuzumab ozogamicin were also shown to induce senescence. Finally, in a consecutive *ex vivo* treatment with daunorubicin (to induce senescence), followed by the “senolytic” (i.e., selectively cytotoxic to senescent cells) BCL2 inhibitors venetoclax and navitoclax, both growth and viability of AML blasts were additionally reduced compared to single-agent treatments only in senescence-capable samples.

To the best of my knowledge, this is the first study providing direct evidence that cellular senescence, induced *ex vivo* in patient-derived AML blasts by chemotherapeutic drugs, could serve as a predictive biomarker of long-term response to standard therapy. I believe that therapy-induced senescence might explain, at least in part, the underlying biology of current paraclinical risk indicators, and, as an outlook, might serve as a guidance for future personalized treatment of AML.

1. Zusammenfassung

Die akute myeloische Leukämie (AML) ist eine heterogene Erkrankung, für die die Entwicklung neuer pathophysiologisch fundierter prädiktiver Biomarker von großer klinischer Notwendigkeit ist. Zusätzlich zu apoptotischem Zelltod von Tumorzellen können antineoplastische Medikamente zu einem dauerhaften Zellzyklusarrest viabler, metabolisch aktiver Tumorzellen führen, welches Phänomen als Therapie-induzierte Seneszenz in verschiedenen Tumorentitäten charakterisiert wurde. Die prognostische und prädiktive Relevanz Therapie-induzierter Seneszenz für den Verlauf von Tumorerkrankungen ist derzeit unklar. Außer einer Induktion durch Chemotherapeutika kann Seneszenz u.a. durch replikativen Stress oder Onkogen-Expression hervorgerufen werden und dient dadurch als initiale zelluläre Barriere gegen maligne Entartung. Die langfristige Bedeutung von im Organismus persistierenden seneszenten Tumorzellen bleibt jedoch unklar, da diese durch ihren Seneszenz-assoziierten sekretorischen Phänotyp (SASP) auch proinflammatorisch wirken und durch epigenetische Veränderungen Krebsstammzelleigenschaften aufweisen können.

In *ex vivo*-Untersuchungen an aus Patient:innenproben zum Zeitpunkt der Diagnosestellung gewonnenen AML-Blasten konnte ich zunächst „basale“ und Therapie-bedingte Seneszenz in der AML charakterisieren um daraufhin Seneszenz als prädiktiven Biomarker zu analysieren.

Nach Etablierung von Primärkulturbedingungen für die zytostatische Behandlung (und somit mögliche Seneszenzinduktion) aufgereinigter AML-Blasten konnte ich mit zytochemischen und Fluoreszenz-basierten Assays die Zunahme der Seneszenz-assoziierten-β-Galaktosidase (SA-β-gal)-Aktivität und anderer Seneszenzmarker nachweisen. Durch RNA-Sequenzierung konnte meine experimentelle Klassifikation individueller AML-Proben als „Seneszenz-fähig“ anhand Seneszenz-assoziierter Genexpressionssignaturen bestätigt und weiter charakterisiert werden. Zur Analyse der prädiktiven Bedeutung Therapie-bedingter Seneszenz wurden die einzelnen AML Proben weiter molekulargenetisch untersucht und experimentelle Ergebnisse mit dem jeweiligen klinischen Verlauf individueller Patient:innen korreliert.

Ich konnte zeigen, dass die intraindividuelle Induzierbarkeit von Seneszenz durch *ex vivo*-Behandlung mit dem AML-Standardchemotherapeutikum Daunorubicin

positiv mit einem verbesserten erkrankungsfreien Überleben und Gesamtüberleben korrelierte. Zudem waren eine günstigere molekulare Risikogruppe, ein normaler Karyotyp sowie *NPM1*- und *DNMT3A*^{R882}-Mutationen mit höheren Leveln Therapie-induzierter Seneszenz assoziiert. Durch die Behandlung mit anderen AML-Therapeutika wie Hydroxyurea, Decitabin oder Gemtuzumab-Ozogamicin konnte ebenfalls Seneszenz ausgelöst werden. Schließlich konnten ich durch eine konsekutive *ex vivo*-Behandlung mit zunächst Daunorubicin (zur Seneszenzinduktion) und darauffolgend mit den „senolytisch“ wirkenden (d.h. selektiv zytotoxisch gegenüber seneszenten Zellen) BCL2-Inhibitoren Venetoclax und Navitoclax sowohl Zellzahl als auch Viabilität seneszenzfähiger AML-Proben im Vergleich zu einer Therapie mit den Einzelsubstanzen oder zu AML-Proben, welche nicht seneszenzfähig waren, zusätzlich reduzieren.

Nach meinem Kenntnisstand konnte im Rahmen dieses Promotionsprojektes erstmals nachgewiesen werden, dass durch *ex vivo*-Chemotherapie in aus Patient:innen gewonnenen AML-Blasten induzierte zelluläre Seneszenz als prädiktiver Biomarker für das langzeitige Therapieansprechen auf die Standard-Induktionstherapie dienen kann. Möglicherweise erklärt Therapie-induzierte Seneszenz Teilaspekte der etablierten paraklinischen Risikofaktoren zugrundliegenden Tumorbiologie und kann perspektivisch als Marker für personalisierte Behandlungskonzepte in der AML verwendet werden.

2. Introduction

In the introduction to this dissertation, I will briefly characterize acute myeloid leukemia (AML), particularly regarding its current therapeutic standards and prognosis. Subsequently, I will describe cellular senescence with an emphasis on therapy-induced senescence and its prognostic and therapy response predictive value as well as possible implications for future anti-cancer therapy, which will then lead me to a description of the specific research objective of this dissertation.

2.1 Acute Myeloid Leukemia

AML is a hematological neoplasm characterized by the accumulation of proliferative, clonal, poorly differentiated cells, denoted as blasts, which derive from the myeloid hematopoietic lineage. AML manifests clinically through symptoms that are caused by blast infiltration of the blood, bone marrow and other tissues. Without a rapid diagnosis and introduction of chemotherapy, AML takes a fatal course in which the consequential bone marrow failure as well as coagulopathy with disseminated intravascular coagulation (DIC) and primary hyperfibrinolysis lead to severe infections and major bleedings as the most common causes of death.[1]

With an incidence of 3.2 (for female) and 3.6 (for male) newly diagnosed patients per 100,000 people in Germany, AML accounts for approximately 21% of all forms of leukemia and 28% of all myeloid neoplasms (besides myelodysplastic syndrome, MDS, and myeloproliferative neoplasms, MPN). It occurs in an age-related increasing incidence, with a median age at diagnosis of 72 years (and only 2% of cases being diagnoses below the age of fifteen).[2]

Known risk factors for developing an AML are a former exposure to deoxyribonucleic acid (DNA) damaging agents (chemotherapy, radiation, cigarette smoking, benzene),[3, 4] and predisposing hematological diseases, above all MDS and some rare syndromic disorders (Down syndrome, Fanconi anemia, Bloom syndrome, Ataxia telangiectasia, Diamond-Blackfan anemia, Schwachman-Diamond syndrome, Kostmann syndrome).[5] Yet, in most AML patients, a clear cause of disease cannot be identified.

Despite intensive therapy regimes, including aggressive chemotherapy and allogeneic hematopoietic stem cell transplantation, prognosis of adult AML patients still

has to be considered unsatisfactory, with an overall 5-year survival rate of approximately 24%, ranging from 60% in 15- to 34-year-old patients to 5% above 75 years.[6] With the exception of acute promyelocytic leukemia (APL), a distinct subtype of AML carrying a pathognomonic and effectively druggable chromosomal translocation (t(15;17)(q24;q21), denoted as *PML/RARA*), prognosis also does not remarkably differ between cytomorphological subtypes as defined in the French-American-British (FAB) classification, ranging from 20% to 30% of 5-year survival.[6]

Research over the past two decades identified several pathogenetic and prognostically relevant genetic and chromosomal aberrations, which led to a shift away from the importance of the cytomorphological FAB classification to a molecular genetically and cytogenetically based subtyping, prominently implemented with the revision of the WHO (World Health Organization) classification of AML in 2002.[7, 8] As a consequence, in contrast to other malignancies, molecular analyses at diagnosis and relapse of AML have been part of the standard of care for several years already. This has led to the revelation of a heterogeneous landscape of molecular and cytogenetic AML classes.[9, 10]

In a large-scale next generation sequencing (NGS) study of 200 AML samples within The Cancer Genome Atlas (TCGA) program, a potential driver mutation in at least one of the known pathogenetically relevant genes was identified in almost every sample. In addition, a complex interaction of genetic events with cooperation and mutual exclusivity was uncovered which, at least in part, reflects separate paths of clonal evolution in AML.[9] Taking into account that AML genomes have on an average “only” 13 mutations, fewer than most adult cancers, and the mutual exclusivity of certain recurrent genetic lesions, it makes sense to stratify AML by underlying driver mutations, as this could result, in contrast to other cancers with higher mutational count, in a reasonable number of groups with differential prognostic implications and, more importantly, for which group-specific therapies could be established. The molecular heterogeneity of AML has been additionally complicated by insights into the dynamic nature of subclonal evolution under chemotherapeutic treatment in the course of disease.[11] Novel subtype-specific targeted therapeutics in combination with chemotherapy are currently under investigation, or have already been established as standard of care, with the fms-like tyrosine kinase 3 (FLT3)-inhibitor midostaurin being the first one to be approved by the U.S. Food and Drug

Administration (FDA) and European Medicine Agency (EMA) for addition to standard therapy in *FLT3*-mutated AML.[12]

In contrast to the morphological FAB classification, these molecularly and cytogenetically heterogeneous subgroups show a wide range of responses to standard therapy and patient prognosis. The 10-year survival rate in cytogenetically defined subgroups ranges from 4% in the case of AML with an inv(3)/t(3;3) aberration to 65% for AML with a t(8;21) translocation. Similarly, in molecular genetically defined subgroups of cytogenetically normal AML, 10-year survival extends from 10% in *FLT3-ITD* mutant/ *NPM1* wild type AML to 52% in *FLT3-ITD* wild type/*NPM1* mutant AML patients.[13] The broadly accepted European LeukemiaNet (ELN) risk stratification incorporates both cytogenetic and molecular genetic information, and supports the initial therapy decision in an increasingly diverse therapy algorithm. Besides genetic risk stratification and individual expected tolerability of intensive chemotherapy regimens, the presence of the CD33 surface antigen and whether AML occurrence is associated with myelodysplasia-related changes or prior therapies (i.e., chemotherapy, radiation, immunosuppression) are further determinants that are incorporated into treatment decisions.[10]

Table 1. 2017 ELN Risk Stratification by Genetics	
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT3-ITD or with FLT3-ITD^{low} Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD^{high} Wild-type NPM1 without FLT3-ITD or with FLT3-ITD^{low} (without adverse-risk genetic lesions) Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214 t(v;11q23.3); KMT2A rearranged t(9;22)(q34.1;q11.2); BCR-ABL 1 inv(3)(q21.3q26.2) or t(3;3)(q21.3;q25.2); GATA2, MECOM (EVI1) -5 or del(5q); -7; -7/abn(17p) Complex karyotype, monosomal karyotype Wild-type NPM1 and FLT3-ITD^{high} Mutated RUNX1 Mutated ASXL1 Mutated TP53

Table 1. 2017 ELN Risk Stratification by Genetics adapted from Döhner et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel (Blood, 2017).[10]

In general, prognostic and predictive factors can be divided into patient-specific-, e.g. age as most prominent, and disease-specific factors. Of the latter, chromosomal and

molecular genetic aberrations, as discussed above, constitute the strongest risk factors, while laboratory parameters (white blood cell count, lactate dehydrogenase [LDH], β2-microglobulin), leukemic stem cell frequency, bone marrow microenvironment, gene expression levels, epigenetic changes and micro-ribonucleic acids (micro-RNAs) are, in contrast to the factors discussed before, not really part of clinical decision making, but have shown promise in this regard as well. Despite a wide range of prognostic factors which are associated with a certain prognosis, few predictive markers exist which, in contrast to prognostic markers, can predict treatment outcome to a certain therapy.[14]

Except for APL – which, considering its special position amongst AML subgroups (as a result of the existence of a specific and highly effective therapy), will generally not be considered in this study - the highly heterogeneous clinical course of AML is reflected in a diverse therapy algorithm. In cases of initial hyperleukocytosis (i.e., a white blood cell count of >100 x 10^9 leukocytes/L), an upfront cytoreductive therapy with hydroxyurea, low-dose cytarabine or leukocytapheresis might be necessary to prevent tumor lysis syndrome before starting the first intensive chemotherapy course, denoted as induction therapy. To give a simplified description of the current AML treatment paradigm, at first diagnosis, patients eligible for intensive chemotherapy receive an induction therapy scheme called “7+3”, composed of 7 days of cytarabine and 3 days of an anthracycline (e.g., daunorubicin, idarubicin or mitoxantrone), with or without midostaurin. Given that patients achieve a “blast-free” state of their bone marrow, induction treatment is followed by consolidation therapy according to individual ELN risk classification, with further cycles of chemotherapy with cytarabine, high-dose chemotherapy and/or allogeneic hematopoietic stem cell transplantation. Despite these intensive and partially targeted therapeutic regimens, long-term outcome for many patients is still poor, which is illustrated by the 5-year overall survival rate of 24% in the whole patient population, caused by a 40% proportion of the adverse ELN risk group in newly diagnosed AML.[6, 15-17]

Therapeutic options for patients who are not eligible for intensive chemotherapy are low-dose cytarabine, hypomethylating agents (5-azacitidine, decitabine) or participation in a clinical study which evaluates one of the many novel therapeutics (protein kinase inhibitors, epigenetic modulators, other chemotherapeutic agents, mitochondrial inhibitors, agents targeting oncogenic proteins, (bispecific monoclonal) antibodies, immunotherapies, therapeutics targeting the AML environment, chimeric

antigen receptor T cells). Similar approaches might also be part of a salvage therapy, which denotes the treatment of patients with primary refractory- or relapsed disease. Notably, three new AML-targeting drugs have received FDA and EMA approval in recent years: gemtuzumab ozogamicin, an anti-CD33 antibody-drug conjugate, CPX-351, a dual-drug liposomal encapsulation of cytarabine and daunorubicin,[10] and venetoclax, a BCL2 (B-cell lymphoma 2) inhibitor, which targets a protein that acts as a negative regulator of apoptosis. In particular, venetoclax is increasingly integrated in the therapy of relapsed or older AML patients who are not eligible for intensive chemotherapy.[18]

In consideration of the increasing variety of available therapeutic options and the heterogeneity of clinical courses, definition of more integrative predictors of treatment outcome, possibly biologically grounded, constitutes an urgent clinical need for choosing the best individual therapy approach. In contrast to the elaborate molecular characterization of the disease, the biological properties of AML blasts at diagnosis, and especially in response to cytotoxic therapies, are still less well understood, and this is of pivotal importance to this thesis project. In particular, cellular senescence as a terminal cell-cycle exit program in response to oncogenic transformation, and as a potential drug-inducible biological state switch that complements treatment-induced apoptosis, has remained largely unaddressed in this regard.[19-21]

2.2 Chemotherapeutic Agents in AML

Conventional chemotherapeutic agents, such as daunorubicin and cytarabine, the two major drugs established as the standard backbone of AML induction therapy, cause cytotoxicity preferentially on actively proliferating cells, typically through interfering with DNA or RNA metabolism in a wider sense. Combinations of two drugs can lower required doses of the single agents and therefore toxicity, as well as prevent development of drug resistance, and they may have synergistic therapeutic activity beyond merely additive effects.[22]

The main effector principle of daunorubicin is inhibition of topoisomerase II and thereby interference with DNA replication, which leads to DNA double-strand breaks. Furthermore, it has several additional effects with varying (or yet to be determined) degrees of impact on therapeutic activity: inhibition of other DNA/RNA polymerases, formation of free radicals, interference with the cell membrane as well as inhibition of

metallothionine synthesis, mitochondrial oxidative phosphorylation, chelate formation and histone eviction.[23, 24]

Cytarabine is a pyrimidine analogue which inhibits DNA synthesis by misincorporation into DNA and inhibition of DNA polymerase.[25] The chemotherapeutic induction protocol “7+3” (discussed above), comprising a combination of both agents, has been already established as a standard of care in AML for over four decades.[26]

For both daunorubicin and cytarabine, as for most conventional chemotherapeutic agents, the main cell biological effector programs are triggered via DNA damage response signaling, leading to different types of cell death (mainly apoptosis, autophagic cell death and necrosis) and in addition possibly cellular senescence.[23, 27, 28]

2.3 Cellular Senescence

The key feature of malignant cells is an uncontrolled growth overcoming several cellular failsafe mechanisms against malignant transformation, i.e., temporary growth arrest, apoptotic cell death or an irreversible cell-cycle arrest named cellular senescence. In addition, other “Hallmarks of Cancer”, prominently described by Hanahan and Weinberg, include an altered extracellular interaction with the environment, as well as acquired abilities to sustain angiogenesis, to escape the immune system, to invade adjacent healthy tissues, and to form distant metastasis.[28, 29]

Remarkably, some intracellular failsafe mechanisms of malignant cells, which are disabled during tumorigenesis, can be re-provoked by exposition to external stressors like chemotherapeutic drugs or therapeutic irradiation, which have both been used as tumor controlling strategies since the early days of oncology.

Apoptosis, as one of the first failsafe mechanisms to be uncovered, is a physiological, non-inflammatory cell death program and a major cellular effector cascade of several cellular stresses, e.g. chemotherapeutic drugs. Different apoptotic pathways have been studied in depth and are paralleled by specific resistance mechanisms as well as varying magnitudes of induction by oncological drugs. More recently, selective induction of apoptosis in tumors by pharmacological BCL2 inhibitors has gained large interest from a therapeutic perspective.[30, 31] For decades, apoptotic cell death, as measured in short-time cytotoxicity assays, has served as a surrogate marker and potential indicator of therapeutic response to evaluate new chemotherapeutic drugs for subsequent treatment of oncological patients.[32] As a concomitant response to cytotoxic

treatment, or as a complementing cellular response if the apoptotic cellular effector program of malignant cells is blocked, tumor cells may enter senescence as another failsafe mechanism.[33]

The phenomena of cellular senescence as a terminal cell cycle arrest was first described in 1961 by Hayflick and Moorhead.[34] When cultivating human diploid cell strains, these researchers observed that after an approximal number of 40 to 60 cell divisions, cells entered a phase of cessation of cell division while remaining viable and metabolically active, which they hypothesized as reflecting cellular ageing and denoted as “replicative senescence”. The specific number of possible cell divisions until the onset of replicative senescence differs from cell type to cell type and species to species, and was later named the “Hayflick limit”. Progressive telomere shortening with every division has been proposed as the cellular correlate of the Hayflick limit, and, in line with that, expression of the enzyme telomerase can circumvent “replicative senescence” and sustain cellular growth.[35] In contrast to the specifically defined terminal arrest of senescent cells, the term “quiescence” is used to describe a cell state of temporary and often reversible growth arrest that is more broadly used to describe diverse, multifaceted phenomena with less clearly defined biological features.[36]

A similar phenotype to the initially described replicative senescence was later uncovered in the context of cancer with “oncogene-induced senescence” as a mechanism of tumor suppression which restricts the progression of benign neoplasms in response to activated oncogenes.[37] Furthermore, as a stress response of cancer cells to cytotoxic treatment, e.g., irradiation and chemotherapy, “therapy-induced senescence” operates as an anti-tumor mechanism.[38-40] Importantly, this state of senescence is generally not reversible by metabolic, apoptotic or oncogenic stress.[33, 37, 38, 41] Studies in precancerous tissues first detected oncogene-induced senescence as a response to cancer-causing mutations, with the observation that nevi harboring *BRAF* mutations show a strong expression of senescence markers.[42] Similar observations were made in early-stage prostate abnormalities harboring loss of the *PTEN* tumor-suppressor gene[43] and in lymphoma development driven by oncogenic *RAS* activation.[41] In the case of therapy-induced senescence, DNA-damaging chemotherapeutic drugs (as well as radiotherapy) might exploit the same molecular pathways that are activated in oncogene-induced senescence.[33] The evidence of senescence occurring as a response to chemotherapy *in*

patient as well has already been provided in histological preparations of breast cancer resections after neoadjuvant chemotherapy.[40]

Terminally arrested senescent cells typically halt in the G1 phase of the cell cycle, predominantly mediated by two intracellular signaling cascades: besides direct activation of TP53, also denoted as “the guardian of the genome”, which plays a key role in the apoptotic effector program as well, the TP53-interacting retinoblastoma protein (RB)/$p16^{INK4A}$ tumor suppressor pathway constitutes a classical intracellular senescence-mediator.[28] Described in a simplified manner (at first the latter pathway), in the case of cell cycle progression, cyclin-dependent kinases (CDK) phosphorylate and thereby repress the ability of RB protein family members to inhibit E2F family (also called RB-associated proteins) transcription factor activity, which enables cell proliferation. When inhibitors of CDK, such as the tumor suppressors $p21^{Cip1}$ or $p16^{INK4A}$ (the latter encoded by the *cyclin-dependent kinase inhibitor 2A (CDKN2A)* gene), accumulate in the cell, RB proteins are constantly activated with a resulting inhibition of E2F-triggered cell cycle progression. In the other important senescence-inducing pathway, the alternative reading frame (ARF) protein splicing variant of *CDKN2A*, which is structurally unrelated to $p16^{INK4A}$, mediates senescence by TP53 activation. Whereas the senescence-inducing RB/$p16^{INK4A}$ pathway is commonly activated by oncogenic RAS activation, the otherwise pro-proliferative transcription factor MYC can also trigger the ARF/TP53 pathway, thus resulting in apoptosis and/or senescence. In other words, for MYC-driven tumor formation, the net effect of driving proliferation must outweigh its induction of the ARF/TP53 pathway. The complex mechanisms which determine whether TP53 activation leads to an induction of senescence or apoptosis are still poorly understood.[28, 44] Despite a common dependence of senescence induction on one of the described pathways, senescence-reminiscent phenotypes were also observed in TP53-/$p16^{INK4A}$-/$p21^{Cip1}$-deficient scenarios by activation of alternative pathways (e.g. telomere dysfunction), and are in these cases more frequently manifest in the G2 cell cycle phase.[39]

Besides replicative, oncogenic and therapeutic modes of senescence induction, further known factors that can cause a senescent arrest are oxidative stress, potentially by a mechanistically linked mitochondrial dysfunction, and several microenvironmental factors, such as different cytokines. In particular, transforming growth factor β (TGF-β) is a well characterized mediator of senescence.[44, 45] Moreover, excessive mitogenic

signaling can exert opposing impacts on tumors, i.e., resulting in either cancer propagation or growth delay. Exemplarily, tumor necrosis factor-α (TNF-α) mediated activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an important oncogene in many cancer entities such as diffuse large B-cell lymphoma, can lead to hyperproliferation and chemotherapy resistance, but it may also participate in a senescence reinforcing cytokine response.[46]

Importantly, senescent cells are not terminally differentiated and arrested cells, such as neurons or cardiomyocytes, but have undergone characteristic, major metabolic and epigenetic changes, which are also reflected by distinct gene expression patterns. As a consequence, persistent senescent cancer cells have significant long-term implications for tumor biology. In contrast to apoptotic cells, these senescent cancer cells remain in the tumor microenvironment after exposure to chemotherapeutic agents, where they exert a profound influence on the growth and immunogenicity of tumors by secretion of soluble growth factors, cytokines, and extracellular proteases, which has been collectively described as the senescence-associated secretory phenotype (SASP).[45, 47, 48] The predominantly pro-inflammatory and extracellular matrix-active SASP has also been linked to promotion of degenerative non-malignant pathologies, such as neurodegenerative diseases or artherosclerosis.[49-51] Specific SASP components, such as TGF-β, interleukin-6 (IL-6) or C-X-C motif chemokine receptor 2 (CXCR2)-binding chemokines, can also induce senescence, a phenomenon referred to as paracrine senescence.[52] The overall effect and specific composition of SASP, including concentrations of individual cytokines, can differ notably between the various types of senescence and between tissue types, and, in particular, between non-malignant and malignant cells.[53] As a result, on one hand, the SASP contributes to tumor suppression by enforcing the senescent arrest and recruiting defined immune cell populations to the cancer site. On the other hand, the SASP can also act as a tumor promoter by causing paracrine inflammatory signals and by provoking immunosuppressive mechanisms. In a compilation of experimental studies addressing the complexity of SASP interactions, Rao and Jackson[54] concluded that one underlying mechanism of this ambivalence is the ratio of the NF-κB (tumor suppressive, immune system activating) and the janus kinase (JAK)/ signal transducer and activator of transcription proteins (STAT) pathway (tumor promoting, immunosuppressive).

Moreover, the discovery of extensive epigenetic remodeling of senescent cells, including reprogramming into *de novo* cancer stemness, explicitly in AML blasts as well, and occasional escape from cell cycle arrest, have prompted the recent view that tumor cell senescence may account for detrimental effects in the course of malignant diseases. As a prominent property of stem-cell renewal, particularly in the hematopoietic compartment as well as the gastrointestinal tract, primarily the Wnt/ β-catenin pathway was shown to be upregulated in malignant cells undergoing therapy-induced senescence.[55-58]

Between the two extremes of an initial beneficial establishment of a failsafe mechanism (resulting in prevention of tumor progression) and possible negative long-term effects of proinflammatory SASP and stemness reprogramming, the impact of oncogene-induced senescence in already manifest malignancies and, even more, of therapy-induced senescence on treatment response and long-term prognosis in cancer remain to further be elucidated. As a start, Professor Schmitt's research group previously exploited a selection of surrogate markers of oncogene-induced senescence in diagnostic, formalin-fixed paraffin-embedded colorectal tumor specimens that had been obtained in the metastasized (i.e., stage IV) disease condition. In these samples, a high content of senescent cancer cells was associated with superior outcome to 5-fluorouracil-based chemotherapy in a retrospective analysis.[59] With regards to therapy-induced senescence, *in vivo* evidence from murine lymphoma models suggests that it is an important positive prognostic factor and contributes to long-term survival, as mice with lymphomas that were unable to enter senescence had a worse survival.[33] Further studies into the influence of both oncogene- and therapy-induced senescence on the clinical course of patients with solid and hematological malignancies are urgently needed.

The fact that senescent cells are terminally arrested makes them difficult to target by conventional chemotherapy, which primarily interferes with the cell cycle progression of rapidly dividing (tumor) cells. Of high importance to this work are recent studies, primarily from the field of aging and gerontology, that have identified mechanisms and drugs, denoted as senolytics, which can selectively eradicate quiescent and senescent cells. One of the revealed modes of senolysis is an inhibition of the anti-apoptotic proteins BCL2, BCL2L1 (BCL-X) and BCL2L2 (BCL-W) by the drug navitoclax and also by the related substance venetoclax (which is known to solely inhibit BCL2, but not other BCL2

family proteins). Other agents which were found to harbor a senolytic potential in pharmacological screenings were the tyrosine kinase inhibitors dasatinib and quercetin (the latter is also reported to work as an antioxidant and estrogen receptor activator).[60-62] In a murine model system, the elimination of senescent cells by senolytic agents was sufficient to increase physical function as well as lifespan, and to prevent or at least reduce the occurrence of atherosclerosis and neurodegenerative diseases.[49-51] Another scientific report connecting the field of gerontology to hematology provided evidence that quiescent human leukemia stem cells could be selectively targeted through BCL2 inhibition.[63]

Interestingly, almost simultaneously to the discovery of its role in aging and senolysis, the BCL2 inhibitor venetoclax first demonstrated impressive clinical activity in AML patients with relapsed/refractory disease or who were unfit for intensive chemotherapy regimens.[64-67] Consequently, venetoclax has been approved for these indications (in combination with decitabine/ azacytidine or low-dose cytarabine) by the FDA in the United States. The assumed, but not well characterized, main biological mode of venetoclax action in AML is direct stimulation of the mitochondrial apoptotic pathway, a mechanism that was initially established for treatment of chronic lymphocytic leukemia. The senolytic potential of venetoclax has not been thoroughly investigated in the context of its successful clinical application in AML.[31]

2.4 Markers of Cellular Senescence

To characterize cells as “senescent” and, in particular, to discriminate them from quiescent cells (the latter displaying a transient, mitogen-responsive cell arrest), features described above are utilized, such as high metabolic activity and epigenetic changes.[68] The long-established and still accepted “gold standard” marker of senescence is detection of senescence-associated β-galactosidase activity (SA-β-gal), which reflects the altered metabolic state and is not detectable in quiescent or terminally differentiated cells.[69] Further diagnostic features of senescence include morphological changes with an enlarged cell size and multinucleation, epigenetic alteration reflected by chromatin changes (e.g. detection of senescence-associated heterochromatin foci (SAHF), focal histone H3 lysine 9 trimethylation (H3K9me3), focal heterochromatin protein 1 (HP1) recruitment) and short or dysfunctional telomeres. The interaction of H3K9me3 and its co-factor HP1 mediates a persisting and stable epigenetic silencing of E2F-responsive

genes in senescent cells.[70] In addition, an increased detection of protein levels of key regulators of senescence such as $p16^{INK4a}$/ARF, $p21^{CIP1}$ and p53 is established to reflect senescence.

In multi-parameter senescence assays, the absence of markers of proliferation and DNA synthesis (e.g., Ki67, 5-ethynyl-2′-deoxyuridine (EdU) incorporation) is also widely used to confirm the senescent cell cycle arrest.[71, 72]

Currently, there is no single, universally applicable senescence-defining marker. The sole application of the “gold standard” marker SA-β-gal is impaired by its overlap with autophagy, due to the increased lysosomal compartment in both cell states, and as it is not possible to analyze SA-β-gal in formalin-fixed paraffin-embedded tissues, and as a consequence, other, less direct markers need to be used. Therefore, a commonly used strategy to define senescent cells *in vitro* and *in vivo* is the combination of several senescence-associated markers. Whenever technically feasible, detection panels of senescence parameters contain SA-β-gal, which has also been used to validate most other markers of senescence. Of note, the positivity of some of these parameters is not mandatory for classification as “senescent” in every setting (as they depend on the pathway of induction and cell type), but, if detected, they deliver strong evidence of senecence.[68]

2.5 Cellular Senescence in AML

Analogously to the findings of oncogene-induced senescence serving as an important barrier to tumorigenesis, Wang et el.[73] demonstrated that bone marrow mononuclear cells and CD34- (a cell surface marker of hematopoietic stem cells) positive cells from MDS patients show an increased expression of senescence markers (i.e. $p16^{INK4a}$, SA-β-gal) compared to healthy blood donors as well as patients with newly diagnosed AML. These observations were further broadened by Xiao et al.[74] who demonstrated an increased induction of p21-dependent senescence in low-risk compared to high-risk MDS blasts. This phenomenon could be even further enforced by additional DNA damage and by reactive oxygen species (ROS). These results also reflect the biological concept that therapy-induced senescence reutilizes cellular mechanisms by which oncogenic activation induces senescence. In a further descriptive observation, the tumor suppressor and senescence marker $p16^{INK4a}$, which normally increases in healthy aging tissues as an evidence of progressive senescence, was detected at stronger downregulated levels

in AML blasts from patients of higher age. This seemingly paradoxical finding might serve as another hint for loss of oncogene-induced senescence as a barrier in AML leukemogenesis.[75]

There are several molecular features of specific subtypes of AML with recurrent molecular aberrations which can be linked to the intrinsic capability to execute senescence. The frequent chromosomal translocation t(8;21) (with the consequent generation of a fusion protein of acute myeloid leukemia-1 transcription factor and the eight-twenty-one corepressor (AML1–ETO)) has been described as disabling senescence by reducing ARF expression through direct transcriptional targeting.[76] As discussed, specific histone and gene promoter methylation patterns are also key characteristics of the senescence state. For instance, the histone H3 lysine 36 dimethyl-specific demethylase KDM2b/JHDM1b is highly expressed in various human leukemias and shown to be sufficient to transform hematopoietic progenitors to leukemic blasts by suppressing the induction of cellular senescence.[77]

A study by Ablain et al.[78] was the first one to describe therapy-induced senescence in the context of AML, but exclusively for the very specific sub-entity of APL (please refer to section 2.1 for clinical details on APL). The PML tumor suppressor, a cell cycle regulator and key contributor to pathogenesis of APL, normally induces cellular senescence via TGF-β signaling. In the case of PML function loss, the inducibility of senescence as a failsafe mechanism to counteract emerging mitogenic or oncogenic stress is significantly impaired.[79] The loss of function of PML in APL is caused by formation of the fusion protein PML-RARA via chromosomal translocation. By targeted and effectively frequent curative treatment with retinoic acid and/ or arsenic trioxide, PML function can be restored through degradation of the PML-RARA fusion protein, and, consequently, restoring the capability to enter senescence.[78, 80] Of note, these studies have described therapy-induced senescence exclusively in the specific AML sub-entity of APL. Nevertheless, aberrant TGF-β signaling with subsequent deregulation of PML has commonly been detected in leukemogenesis across AML subgroups and might exploit the same mechanisms of senescence repression to benefit blast propagation.[81] Likewise, aberrant TNF-α signaling, as another key mediator of senescence, has also been reported to enable the induction of senescence specifically in human leukemia cells.[82]

Previously, Professor Schmitt's research group unveiled profound metabolic changes in several cancer entities in the context of therapy-induced senescence and

illustrated metabolic targeting as a potential future treatment perspective in apoptosis-resistant but senescence-competent malignancies. In particular, *ex vivo* senescence induction in AML by chemotherapeutics and basic characterization of its key features were already demonstrated exemplarily in this work in a limited number of primary AML blast samples.[83] These initial results have prompted the research project presented in my thesis, with the major goal to more systematically assess therapy-induced senescence in AML.

2.6 Objectives and Research Question

As the central research question of this dissertation, I wanted to investigate the role of senescence, primarily of therapy-induced senescence, in a representative cohort of AML patients as a potential novel predictive biomarker of therapy response. Specifically, I wanted to assess the capacity of individual AML patient samples to enter senescence upon exposure to standard chemotherapeutic drugs (as employed in AML induction therapy) and to correlate these findings with individual patient's clinical courses, laboratory parameters and molecular AML blast specifications.

Given the fact that it is not yet possible to continuously track AML blasts *in patient*, I chose an *ex vivo* approach for chemotherapeutic treatment to subsequently determine individual senescence susceptibility, and to also monitor features of “spontaneous” (oncogenic, mitogenic stress-induced) senescence of the primary AML samples.

To enable this in-depth assessment of senescence capability in primary AML blasts, it became necessary, as an upfront step, to establish an experimental setting applicable across all AML sub-entities, including a primary culture system, a method of blast purification and a consistent drug treatment approach. I then aimed at a further characterization of cellular senescence in AML using various immunohistochemical- and fluorescence markers as well as gene expression profiling. The obtained insights were also validated in the context of novel AML therapeutics.

Finally, another key objective of my work was to utilize the knowledge generated with regards to senescence capability of individual AML samples in order to evaluate possibilities to specifically target senescent AML cells. In this context, I focused on the potential of the recently approved anti-AML compound venetoclax to act in a senolytic fashion.

3. Methods

3.1 Patient Samples and Tumor Material

The studied bone marrow and peripheral blood samples from patients with (highly suspected) AML were obtained as part of routine clinical workup for the initial diagnosis of AML. Blast samples were collected before the introduction of any systemic leukemia-specific therapy and after obtaining patient's informed consent with a subsequent enrolment in this translational study. Anonymized research use of samples was specifically approved by the local Ethics Commission of the Charité – Universitätsmedizin Berlin (Ethical Committee reference number EA4/061/11). Samples from 31 AML patients were collected at the date of initial diagnosis in the period from 03/2012 to 04/2015 at the Charité – Universitätsmedizin Berlin, Campus Virchow-Klinikum. Samples from patients with retrospectively final diagnoses other than AML were excluded.

A validation cohort of 37 AML blast samples was compiled from newly diagnosed AML patients treated at both university medical centers in Munich, i.e., the Medical Clinic and Polyclinic III of the Ludwig-Maximilians-Universität München (LMU) and the III. Medical Clinic of Klinikum rechts der Isar of the Technische Universität München (TU) from 09/2012 to 05/2018. Sampling at both sites was also approved by local ethics committees (Ethical Committee reference number LMU: 204-11; TU: 538/16S). In order to compose a molecularly more homogenous cohort (and to account for covariate factors in follow-up analyses), exclusively patients with a normal karyotype were selected as part of this sample set, referred to as the "Munich cohort" in this thesis.

Clinical data sets were compiled from respective patient files and complemented by the Gießener Tumordokumentationssystem (German documentation system of patients with malignant disease) in a retrospective and anonymous manner, including gender, age, common laboratory parameters, cytogenetics, molecular genetics, immunophenotype, disease-free survival and overall survival. Disease-free survival was defined as the time from complete remission or complete remission with incomplete hematologic recovery according to the ELN response criteria[10] until diagnosis of relapse or death. If no complete remission was achieved, values for disease-free survival were set to 0 months. Overall survival reflects the time between diagnosis and death.

Mononuclear cells (MNCs) were isolated under sterile conditions by Ficoll density-gradient centrifugation, followed by red blood cell lysis (ultrapure water supplemented

with 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA), frozen with controlled temperature decrease (Mr. Frosty™) in a freshly prepared freezing solution (89% fetal bovine serum [FBS], 10% dimethyl sulfoxide, 100 IE/ml penicillin/streptomycin) and stored in liquid nitrogen before experimental use.

Table 2. Clinical and Molecular Features of Both Cohorts in Comparison to an AML Reference Population Reflected by the AML TCGA Research Network Cohort

Characteristic	Value of patients in TCGA Research Network[9]	Value of studied patient cohort ("Berlin cohort")	Value of studied validation cohort ("Munich cohort")
Age at study entry – years	55.0±16.1	62.7±16.2	57.2±12.9
Male sex – no. (%)	108 (54)	18 (58)	14 (38)
Bone marrow blasts at diagnosis – %	69.3±19.1	73.3±26.5	61.8±22.4
Normal cytogenetics – no./total no. analyzed (%)	92/195 (47)	15/28 (54)	37/37 (100)
White-cell count at diagnosis – per mm^3	36,300±48,500	47,600±49,900	62,390±97,300
Immunophenotype – no./total no. analyzed (%)			
CD33 positive	160/198 (81)	26/30 (87)	35/37 (95)
CD34 positive	123/199 (62)	14/30 (47)	19/37 (54)
CD117 positive	174/185 (94)	19/24 (79)	not assessed
Mutation – no./total no. analyzed (%)			
NPM1	54/200 (27)	12/31 (39)	20/35 (57)
FLT3-ITD/TKD	56/200 (28)	10/31 (32)	16/37 (43)
DNMT3A	51/200 (26)	8/24 (33)	12/25 (38)
Median follow-up time – months	52.6	50.8	63.5

Table 2. Clinical and Molecular Features of Both Experimental Cohorts in Comparison to an AML Reference Population (Reflected by the AML TCGA Research Network Cohort):[9] Amongst the listed key characteristics (± standard deviation) of AML patients, age was found to be the only significantly different variable ($p = 0.01$) between the studied test cohort ("Berlin cohort") and the AML patient set analyzed as part of TCGA (a well characterized reference cohort). In the "Berlin cohort" patients were significantly older. In the comparison between the "Berlin cohort" and the validation cohort ("Munich cohort"), only the intended difference in composition of karyotype was significant ($p < 0.001$).

3.2 Cell Culture and Treatment

The myeloid leukemic cell line K562 (chronic myeloid leukemia [CML] in myeloid blast crisis) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ [Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH], Leibniz-Institut, Braunschweig, Germany) in December 2013. BJ_{hTERT} cells (human diploid foreskin fibroblasts immortalized by overexpression of hTERT [human telomerase reverse transcriptase]), which were used as feeders in the established primary AML cell co-culture system, and the cytokine-supplying cell line 5637 (human bladder carcinoma) were obtained from local partner laboratories as well as other cell lines that were initially evaluated for the AML co-culture system and are described in the following section.

All cell lines were cultured in RPMI medium supplemented with 10% FBS and 100 IU/mL penicillin/streptomycin under standard conditions (37°C, 95% humidity, 21% O_2, 5% CO_2) and split when subconfluent. For passaging, adherent cells were detached by trypsin-EDTA treatment for 2-3 minutes, counted and reseeded afterwards. All cell lines were regularly reconstituted from low passage stocks and mycoplasma tests were performed regularly without detection of contamination.

We tested several agents and cell lines to optimize four different components of a primary AML cell culture system: feeder cells, culture media, a cytokine-producing cell line and commercially available individual cytokines to supplement cell culture media. Potential components were chosen based on a literature search focusing on approaches to culture primary human myeloid leukemia cells. As beneficial feeder layers, the following cell lines had been described and were thus tested for their potential impact on growth and viability of primary AML blasts: 3T3 (mouse embryonic fibroblasts),[83] BJ_{hTERT},[84] HS-5 (human bone marrow stroma cells),[85] 5637[86, 87] and WEHI-3 (mouse peripheral blood leukemia cell line).[88] Additionally, primary human bone marrow mesenchymal stromal cells (Pt244),[89] which were also obtained from a local partner laboratory, were incorporated into this panel. Before their utilization as feeder cells, all cell lines were irradiated when subconfluent with optimized arrest-inducing doses of γ-irradiation (to optimize their function as a feeder layer without later overgrowing the primary human AML cells), seeded at a density of 1,200 cells/cm^2 and used as feeders within 5 days. Feeder quality was evaluated by microscopic assessment of cell attachment, morphology, cellular viability and growth arrest (i.e., lack of confluence increase over time).

HS-5 as well as 5637 and WEHI-3 cells displayed an insufficient feeder quality after irradiation and were therefore tested as a cytokine supplier for the inexpensive supplementation of cell culture media. Conditioned media was collected after 48h incubation time, filtered, and added to the carrier media at a concentration of 20%. The carrier medium in which feeders, cytokines and conditioned media were tested consisted of 45% DMEM (Dulbecco's Modified Eagle's Medium)/ 45% IMDM (Iscove's Modified Dulbecco's Medium)/ 10 % FBS culture media, 100 IU/ml penicillin/ streptomycin, 200 mM L-glutamine and 14.3M β-mercaptoethanol. Based on results of my literature research, the following individual human cytokines were tested in several combinations together with the different feeder layers discussed above: SCF (stem cell factor), IL-3 (interleukin 3), IL-6, TPO (thrombopoietin), FLT3 ligand,[84] and G-CSF (granulocyte colony-stimulating factor).[87, 90] In an analysis of variance (ANOVA), a total of 184 different setups comprising 19 individual primary AML samples were tested regarding promotion of AML growth and viability. The definite components of the primary AML culture system were then determined based on their ability to positively impact primary AML blast growth and viability. For feeder layers and conditioned media, only the top candidate was chosen, whereas all cytokines with positive influence on AML growth and viability were included together (Figure 1).

Feeder systems

	-	3T3	BJ	HS-5	Pt244	5637
Growth	-0,15	-0,049	0,37	-0,11	-0,24	*
Viability	0,55	0,55	0,21	0,35	0,44	*
Sum	0,40	0,50	0,58	0,24	0,21	*

Color scale

Regression coefficient
Negative
0-0.05
0.05-0.1
>0.1

Conditioned media

	-	5637	HS-5	WEHI
Growth	-0,23	-0,05	0,04	-0,11
Viability	0,05	0,21	0,02	-0,27
Sum	-0,18	0,17	0,06	-0,39

Cytokines

	IL-3	IL-6	FTL3-L	TPO	G-CSF	SCF
Growth	0,26	0,09	-0,08	0,09	-0,35	0,05
Viability	0,02	-0,09	0,09	0,03	-0,07	0,13
Sum	0,28	0,00	0,00	0,12	-0,41	0,17

Figure 1. ANOVA of Possible Components of a Primary AML Co-Culture System: In a 5-day co-culture assay with different primary AML samples of the "Berlin cohort", the ability of cell lines to act as feeder layer and providers of cytokine-conditioned media as well as single cytokines to positively impact growth (defined as cell number at day 5 relative to day 0) and viability (defined as cell viability at day 5 relative to day 0) were assessed in different combinations and biological replicates (i.e., testing different primary AML samples after blast purification by magnetic-activated cell separation). The following components were tested: No addition of feeder system or conditioned media ("-"), the cell lines 3T3, BJ_{hTERT} ("BJ"), 5637, HS-5, Pt244, WEHI-3 and the cytokines SCF, IL-3, IL-6, TPO, FLT3-L (FLT3 ligand), and G-CSF. In preceding experiments (not displayed), optimal irradiation conditions for each individual feeder layer were tested. 5637 did not show sufficient feeder quality, due to a significant rate of cell death with cell detachment at the lowest irradiation dose mediating cell arrest (therefore marked with "*"). In an analysis of variance (ANOVA) of the 184 different setups in total, the regression coefficients of the different components are displayed with color coding regarding their impact. "Sum" reflects the addition of regression values of growth and viability. Chosen components for the finally established primary AML blast culture system were chosen by their "Sum" value and have thick borders. In the case of feeder systems and sources of conditioned media, only the cell line with the highest "Sum" value was chosen. For cytokines, all components with positive values were included.

As a result, the established primary human AML co-culture system consisted of (20 Gy) irradiated BJ_{hTERT} feeder cells in a culture medium containing 35% DMEM/ 35% IMDM/ 20% 5637 cell line-conditioned media and 10% FBS supplemented with 100 lU/ml penicillin/ streptomycin, 200 mM L-Glutamine, 14.3 M β-Mercaptoethanol, 100 ng/ml SCF, 20 ng/ml IL-6, 10 ng/ml IL-3, 10 ng/ml TPO and 10 ng/ml FLT3 ligand.

AML blast purification was conducted after a 3-day pre-culture period via positive immunobead separation with the MACS (magnetic activated cell separation) system

choosing the leukemic surface antigens CD34 or CD33 depending on the individual expression profile (favoring CD34 over CD33 if both markers were present) according to the manufacturer's protocol with adjusted immunobead concentrations: Briefly, CD34 positive cells were incubated in 300 µl MACS buffer (PBS [phosphate-buffered saline] complemented with 0.5% bovine serum albumin, 2mM Na_2-EDTA), 100 µl F_cR Blocking Reagent™ and 100 µl CD34 MicroBeads™ for 30 minutes at 4°C, whereas CD33 positive cells were incubated with 40 µl MACS buffer, 20µl F_CR Blocking Reagent™ and 40µl CD33 MicroBeads™ for 15 minutes at 4°C, followed by one washing step, and subsequently rinsed through MACS LC Columns™, which were placed in the magnetic field. After removing the columns from the magnetic field, magnetically labeled AML cells were flushed out. After another washing step, cells were added to the primary AML culture system. In only one of 59 AML samples in total analyzed here were both CD33 and CD34 not present as a surface marker, as well as other myeloid differentiation antigens (CD133, CD117). In this case (sample no. 93248), experimental use of the undifferentiated AML cells was continued without blast purification due to the high reported blast percentage (77%) in the corresponding bone marrow aspirate.

Separation efficacy was confirmed by immunophenotyping with flow cytometry. AML cells were washed in PBS, incubated with directly fluorescence-conjugated antibodies against CD33 (CD33-APC [allophycocyanin] antibody, 1:20) and CD34 (CD34-APC antibody, 1:20) for 30 minutes at 2-8°C and washed again. Immunofluorescence intensity was then detected on a Becton Dickinson FACSCalibur flow cytometer. Flow cytometric forward- and side-scatter characteristics were recorded in order to gate viable cells. FlowJo software was used to analyze immunofluorescence intensities and quantify marker-positive cells.

As described in the introduction, therapy-induced senescence might occur concomitantly with apoptosis, or predominate when apoptosis is blocked.[33] Yet, at least in an *in vitro* setting, the final cellular fate will be dependent on the magnitude of stress that is externally applied. In a simplified explanation of observed dose dependencies, higher toxicities would more likely be expected to result in apoptosis, while lower doses of a chemotherapeutic drug might trigger therapy-induced senescence instead.[91] Hence, I chose two different doses each of daunorubicin and cytarabine, within ranges as defined by other researchers for *in vitro* treatments,[92, 93] to expose primary AML blasts to a variable magnitude of stress. For *in vitro/ ex vivo* drug treatment, daunorubicin at

concentrations of 10 and 50 ng/ml and cytarabine at 25 and 100 ng/ml were added once to K562 cells on the day of seeding at a density of $1x10^5$ viable cells/ml, or to primary AML cells at a density of $1x10^6$ viable cells/ml after blast purification and seeding on fresh feeder layers. In both settings, cell cultures were subjected to a regular medium exchange every 3 days without additional drug administration, and, if necessary, cell density was adjusted to the intended density at treatment start (in order to prevent cell culture saturation). In case of hydroxyurea (30 µM), decitabine (1 µM), gemtuzumab ozogamicin (1 µg/ml), midostaurin (1 µM), navitoclax (1 µM) or venetoclax (1 µM) treatment, drug administration was repeated after every regular cell culture media exchange. This difference in dosing is also reflected by the established treatment schedules in patients, i.e., an intermittent treatment in therapy cycles with daunorubicin and cytarabine as compared to continuous (oral) intake of the denoted inhibitors.

Cultured cells were harvested for senescence assessment 5 days after treatment start. This time point was described as the median of *in vitro* senescence induction in a meta-analysis of experimental conditions to detect therapy-induced senescence.[91] After preliminary validation experiments, the treatment duration of 5 days was also chosen in accordance with the experimental approach followed by Dörr et al. in their precedential exemplarily demonstration of therapy-induced senescence in primary AML samples.[83] Another advantage of this endpoint was avoidance of a second, potentially stressful culture medium exchange (required after a total 6 days of culture).

3.3 Analysis of Growth Parameters, Viability and Cellular Senescence

Cell viability and cell density were assessed by the Guava ViaCount reagent using the Guava easyCyte flow cytometer (8 HT and 12 HT) and the Guava CytoSoft software package. After staining of cells with the Guava ViaCount reagent, viable and dead cells were separated using the viability (PM1) vs. nucleated cells (PM2) plot. In some experiments, such as early evaluations of potential components of the primary AML co-culture system (please refer to section 3.2 for details on components of the co-culture system), cells were also manually counted in a Neubauer improved counting chamber after dead cell exclusion with trypan blue.

Cytohistochemical staining of SA-β-gal activity was detected in cytospin preparations of suspension cell cultures in untreated vs. treated matched pairs of individual samples as described by Dimri and colleagues.[69] Cells were washed in

PBS_{MgCl2} (PBS supplemented with 1 mM $MgCl_2$), fixed in PBS_{MgCl2} containing 2% paraformaldehyde and 0.25% glutaraldehyde, and incubated in PBS_{MgCl2} containing 2.5% 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal) and 5.0% KCN at pH 6.0 and 37°C for 3-12 hours depending on the cell type and reactivity of individually matched untreated controls. Mechanistically, the added X-Gal substrate is quantitatively cleaved, depending on intracellular β-galactosidase activity, to form a cytoplasmic blue stain of 5,5'-dibromo-4,4'-dichloro-indigo. Representative photomicrographs were taken using a CKX41 microscope equipped with a XC30 camera.

For quantitative detection of immunofluorescence-based SA-β-gal (fSA-β-gal) reactivity, a protocol was adapted from Debacq-Chainiaux and colleagues.[94] Briefly, cells were incubated in their respective culture medium with 150 µM chloroquine at 37°C for 1 hour to induce lysosomal alkalinization, followed by a 30 minute exposure to the fluorogenic β-galactosidase substrate 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG). Subsequently, cells were washed, stained with a viability dye (Ghost Dye™ Violet 450, 1:1,000) in PBS for 15 minutes at 4-8°C, washed again and fixed in freshly prepared 2% paraformaldehyde for 10 minutes. For additional staining with the proliferation marker EdU, cells were fixed with BD Cytofix™ fixation buffer and stained with the Click-iT™ Plus EdU Cell Proliferation Kit for Imaging Alexa Fluor™ 647 dye according to manufacturer's recommendation. For flow cytometry detection of Ki67 (directly fluorescence-conjugated antibody, dilution 1:20), p16 (directly fluorescence-conjugated antibody, dilution 1:50) and H3K9me3 (primary unconjugated antibody, dilution 1:500) cells were fixed with BD Cytofix™ fixation buffer, permeabilized with Click-iT™ saponin-based permeabilization and wash reagent, and incubated at 4-8°C for 16 hours, and in the case of H3K9me3, this was followed by staining with a corresponding secondary fluorescence-conjugated anti-rabbit IgG antibody (1:2,000) at room temperature for 1 hour. For combined immunofluorescence staining of CD34 and fSA-β-gal intensities, CD34 detection was conducted as described in the previous section. Exemplary immunofluorescent stainings for all markers are depicted in Figures 2-7.

Immunofluorescence image-based flow cytometry was conducted on an Amnis ImageStreamX Mark II Imaging Flow Cytometer. Analysis of senescence was restricted to viable cells, which were selected by viability dye exclusion of the Ghost Dye™ Violet 450, in all displayed immunofluorescent stainings. Detected immunofluorescence intensities were normalized by cell size to minimize interferences by autofluorescence

alterations due to changes in cell size between conditions for individual samples, particularly as an increased cell size is a main feature of senescent cells (Figures 2 and 3). Cell size was determined by a 2-dimensional area assessment with the IDEAS software (implemented software package on the Amnis ImageStreamX Mark II Imaging Flow Cytometer). Cell size normalization denotes the ratio of recorded fSA-β-gal intensities divided by the number of detected pixels within the defined area of each individual cell. The treatment-induced SA-β-gal ratio and treatment-induced EdU-ratio were then calculated as the ratio of immunofluorescence-reactive viable cells under treatment divided by immunofluorescence-reactive viable untreated cells at the same time point (after cell size normalization) and reflect the capacity to execute therapy-induced senescence, particularly in case of EdU proliferation capacity, of individual AML samples.

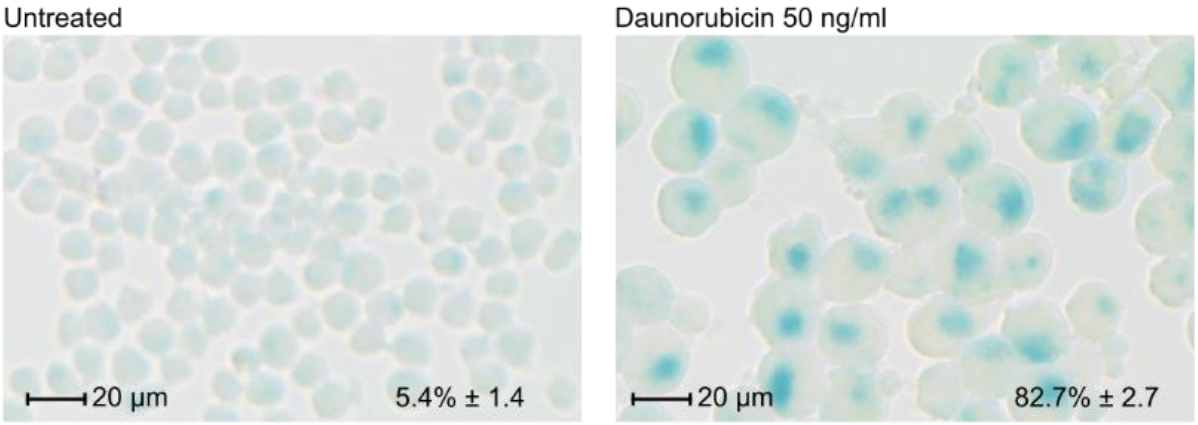
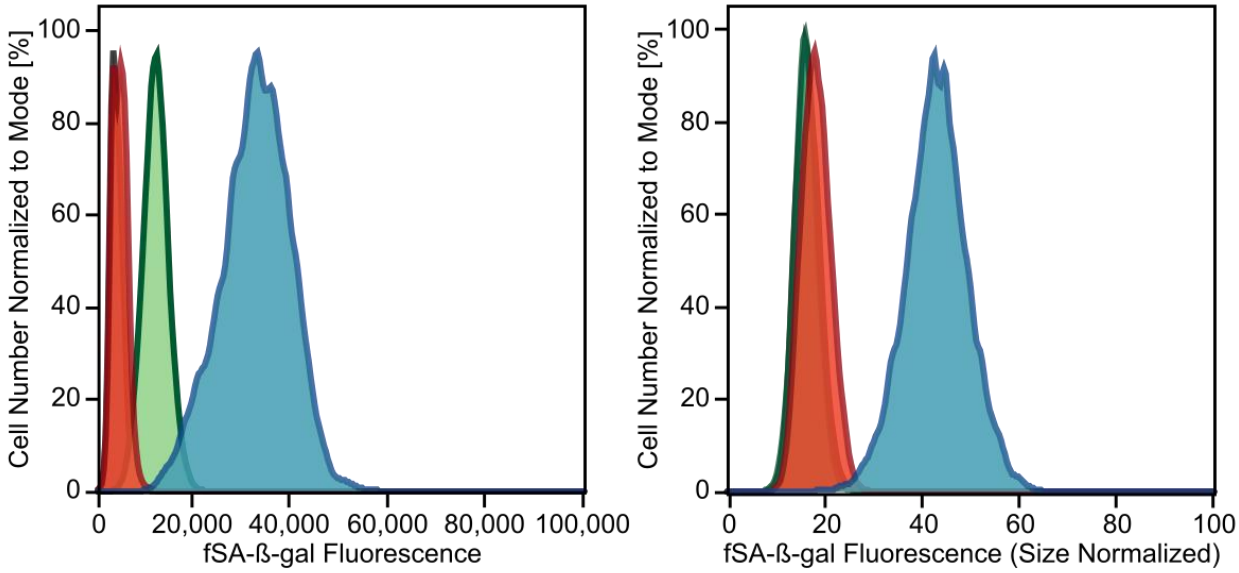


Figure 2. Cytohistochemical SA-β-gal Stainings of the Blastoid Myeloid Leukemia Cell Line K562: This figure depicts representative photomicrographs of cytohistochemical SA-β-gal reactivity in K562 cytospin preparations which were processed according to the reference protocol by Dimri and colleagues.[69] The K562 leukemia cell line was treated *in vitro* for 5 days with daunorubicin (50 ng/ml) and compared to an untreated control. Numbers (right bottom corner) reflect mean percentages of SA-β-gal-reactive (blue colored) cells ± standard deviations derived from 3 independent experiments. A legend for size measurement is displayed in the left bottom corner. Beside a strong gain in cytoplasmic SA-β-gal reactivity in the daunorubicin-treated leukemic cells, an accompanying significant gain in cell size is visible as another feature of senescence.



	DNR Treatment	fSA-ß-gal Staining	Mean Intensitiy fSA-ß-gal	Mean Intensitiy fSA-ß-gal (Size Normalized)
	Untreated	Unstained	4,867	16.6
	Untreated	Stained	5,463	18.2
	Treated	Unstained	12,768	16.3
	Treated	Stained	33,128	43.0

Figure 3. Normalization of Fluorescent SA-β-gal Intensity to Cell Size: K562 cells were treated (as in Figure 2) with daunorubicin (DNR, 50 ng/ml) *in vitro* for 5 days to strongly induce senescence. A main feature of senescent cells is an increase in size as displayed in Figure 2. Histogram depictions are presented of detected fluorescent SA-β-gal (fSA-β-gal, x-axis) intensities before (left) and after (right) correction of raw signals for individual cell sizes, i.e., fluorescence intensities per pixel. Cell size was determined using a two-dimensional area assessment tool provided as part of the IDEAS software. For easier comparison, cell numbers of indicated fluorescence intensities were divided by the mode of their distributions (y-axis). Note that higher raw fluorescence levels of unstained DNR-treated (i.e., larger) cells (green, left plot) become virtually indistinguishable from untreated controls after normalization for cell size (green vs. grey, right plot). Without correction for cell size (left side), daunorubicin-treated (senescent) cells already show a strong shift in the measured fSA-β-gal intensity without actual staining for fSA-β-gal reactivity, most likely through a distinct increase of autofluorescence by cell enlargement. After correction for cell size with calculated intensities per pixel (right side), a shift in detected fSA-β-gal intensities is only observed in the DNR-treated (senescent) cell sample after specific staining for fSA-β-gal.

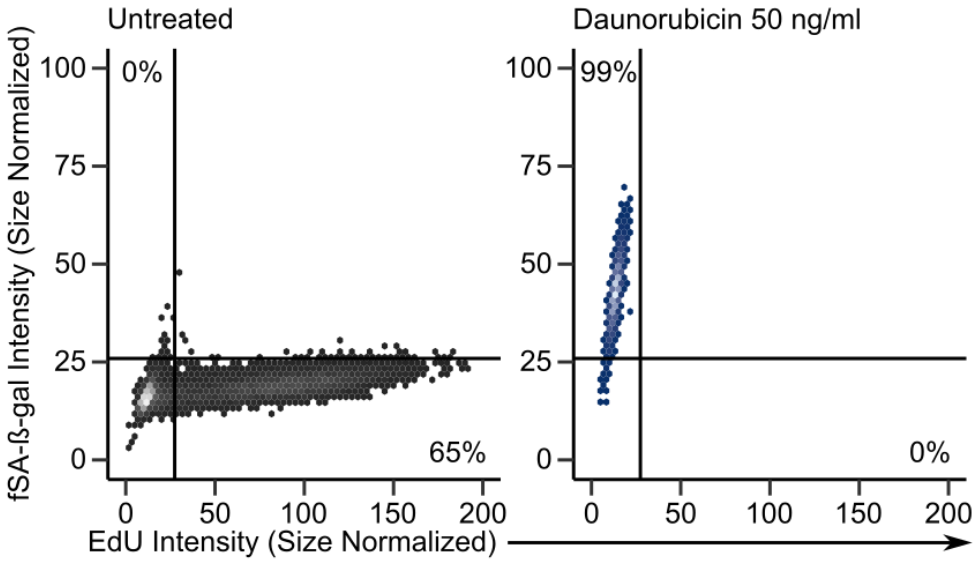


Figure 4. Performance of the Newly Established Fluorescence-Based Assay for the Quantitative Detection of Therapy-Induced Senescence: Presented above are combined flow cytometric density plots of fluorescent SA-β-gal intensities (fSA-β-gal, y-axis), together with fluorescence intensities from detection of the proliferation marker EdU (x-axis) in the leukemic K562 cell line after *in vitro* daunorubicin treatment and cell size normalization (as described in Figures 2 and 3). Percentages reflect fractions of proliferating (lower right field) and senescent (upper left field) cells of the entire displayed cell population. Only viable cells were analyzed after viability dye exclusion. The fluorescence-based triple staining of a viability (Ghost Dye™ Violet 450), senescence (fSA-β-gal) and proliferation (EdU) marker in the same sample as illustrated here was subsequently used for the quantitative assessment of senescence in the primary AML samples.

3.4 Mutational Profiling Analyses

Total DNA extraction of primary human AML blast samples was achieved with the Quick-DNA™ Universal Kit according to the manufacturer's protocol. Concentration and purity of DNA was validated by photometric measurement of light extinction. Maximum absorption of the purine and pyrimidine bases of DNA is observed at 260 nm. Aromatic amino acid groups absorb at 280 nm, so that an E_{260}/E_{280} ratio of 1.8 - 2 is an indicator of a sufficient DNA purity without protein or phenol contamination.

Library preparation for targeted next-generation sequencing was performed using the TruSight Myeloid Sequencing Panel according to the manufacturer's protocol. The resulting sequencing ready libraries were normalized, pooled (10 samples per pool) and sequenced on a MiSeq System with a 2x221 cycle paired-end sequencing using the V3 flow cell and corresponding reagents. On-board analysis was performed using the MiSeq

Reporter Software, and the resulting variant-calling file was annotated and filtered with Variant Studio using certain filter criteria (i.e., passed quality filter, read depth > 100, and variant allele frequency > 4%) for the following myeloid genes-of-interest panel adjusted from the genomic landscape in AML as described by the Cancer Genome Atlas project:[9] *ASXL1, BRAF, CALR, CEBPA, CSF3R, DNMT3A, FLT3, IDH1, IDH2, JAK2, JAK3, KIT, KRAS, MPL, NPM1, NRAS, RUNX1, SETBP1, SF3B1, SRSF2, STAT3, TET2 and TP53*. Known single-nucleotide polymorphisms were excluded from further analysis.

3.5 Transcriptional Profiling Analysis

Total RNA was prepared from untreated and 5-day daunorubicin-treated primary human AML blasts using the RNAeasy™ Plus Mini Kit according to the manufacturer's instructions. RNA concentration and purity were validated with the RNA 6000 Pico Kit using the Agilent Bioanalyzer 2100. Samples were submitted to the High Throughput Sequencing Unit of the Deutsches Krebsforschungszentrum (DKFZ, Heidelberg, Germany) for RNA sequencing. Libraries were prepared with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing according to the manufacturer's protocol and sequenced on a HiSeq 2000 (V4: 50 bp single read).

Gene set enrichment analysis (GSEA) on the resulting obtained transcriptome data of my AML sample cohort as well as on publicly available transcriptome datasets generated from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) was performed with the GSEA v4.0.3 software (Broad Institute of MIT [Massachusetts Institute of Technology] and Harvard, http://www.broad.mit.edu/gsea).

Additionally, most differentially expressed genes in untreated vs. treated and senescence-incapable vs. senescence-capable AML samples were checked for their inclusion in senescence-associated gene signatures after an upfront definition of relevant gene sets from the > 10,000 gene sets of the molecular signature database (MSigDB).[95] Senescence-related gene sets were selected from 5 categories of interest: direct association with senescence, growth arrest, signaling pathways associated with senescence mediation, inflammation, and immune response. At first, signatures were searched for in the well-defined and expert-curated hallmark collection (which reflects 50 major, non-redundant biological states and processes).[96] Hallmark signatures covered aspects of growth arrest, senescence mediation and inflammation, but neither directly senescence-associated nor cellular or humoral immune response signatures are part of

the Hallmark signature collection. The following directly senescence-associated signatures are commonly cited and were thus further investigated: a general senescence signature by Fridman et al.,[97] as well as signatures representing replicative senescence[98] and oncogene-, in particular RAS-, induced senescence.[99] As a SASP reference gene set, I included a well-compiled review of relevant genes by Lasry and Ben-Neriah[53] (which assembled three different experimental generations of SASP signatures).[100-102] As immune response signatures, I selected gene sets from the gene ontology (GO) database, which provides detailed annotations of gene sets of biological processes, cellular components and molecular functions.[103, 104] To reflect a certain range within several immune response pathways, representative signatures for innate, adaptive, humoral, T-cell-regulated, and toll-like receptor pathway-regulated immune responses were chosen. In summary, a total of 23 different gene signatures which illustrate various typical molecular features of cellular senescence and its possible implications were further employed in this analysis.

Transcription factor activities were assessed by virtual inference of protein activity by enriched regulon analysis (VIPER)[105] with the VIPER R-System Package (http://califano.c2b2.columbia.edu/viper), with a confidence score based on the level of evidence of transcription factor-target interactions.[106] In this approach, aberrant protein activities of transcription factors are predicted by gene expression of their targets (denoted as their transcription factor regulon) to identify dysregulated oncoproteins. As genetic alterations are only partially predictive, this analysis also evaluates the functional relevance of detected genetic alterations. Data sets to link transcription factors and target genes are derived from manually curated resources, chromatin immunoprecipitation in combination with DNA-sequencing (ChIP-seq), *in silico* prediction and reverse engineering of gene expression data sets. In my analysis of transcription factors out of a panel of >1,500 candidates tested, only transcription factor activities with the highest level of evidence (category A), which are required to contain at least one manually curated resource, were further used.

3.7 Statistical Analysis

Experimental data were evaluated for statistical significance using SPSS (version 24), R (version 3.5.3) and GraphPad Prism (version 6).

Normal distribution was assessed by Shapiro-Wilk test and, if present, data were further evaluated for variance homogeneity by Levene's test. Data following normal distribution and variance homogeneity were analyzed by unpaired two-sample *t*-test, and when lacking homogeneity with Welch's correction. Data not following normal distribution were analyzed by nonparametric Mann-Whitney U test. The Kruskal-Wallis test by ranks was applied in the case of more than two groups to be evaluated. *P* values <0.05 were considered statistically significant. Statistical analysis of Kaplan-Meier survival plots was based on the log-rank (Mantel-Cox) test.[107] Median follow-up time is exactly defined as median time to censoring.[108] For GSEA, the nonparametric Kolmogorov–Smirnov test was applied. Normalized enrichment scores (NES) reflect a statistically significant enrichment with false discovery rate (FDR) values (calculated by the Benjamini-Hochberg procedure) <0.05. Due to the rather small number of RNA-seq analyzed AML samples, a stricter FDR value of <0.05 was applied compared to the otherwise commonly used FDR <0.25 in larger sample cohorts.

Bar plot diagrams in this work reflect mean values with error bars showing standard deviation (SD), or, where indicated, standard error of the mean (SEM). Whisker plot boxes indicate first and third quartiles with median, upper and lower bars minimum and maximum values. Outliers were defined as points beyond a 1.5-fold interquartile range. Censored values in Kaplan-Meier plots mark the last point of an observation.

No data were excluded, which means that all probes or samples that met proper experimental conditions were included in the analyses presented in this thesis.

3.8 Materials

All common chemicals, tubes, pipettes, dishes, other plastic disposables (which are not explicitly listed) and indicated materials were purchased from: Abcam (Cambridge, UK), Agilent (Santa Clara, CA, USA), BD Bioscience (Heidelberg, Germany), Bio-Rad (Hercules, CA, USA), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Eppendorf (Wesseling-Berzdorf, Germany), Illumina (San Diego, CA, USA), Luminex (Austin, TX, USA), Merck Millipore (Billerica, MA, USA), Miltenyi Biotec (Bergisch Gladbach, Germany), Qiagen (Hilden, Germany), Sigma-Aldrich (St. Louis, MO, USA), Stemcell Technologies (Vancouver, Canada), Thermo Scientific (Waltham, MA, USA), Tonbo Biosciences (San Diego, CA, USA), VWR (Radnor, PN, USA), Zymo Research (Freiburg, Germany).

Chemicals

5-Bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal)	Carl Roth
Ammonium chloride (NH_4Cl)	Sigma-Aldrich
β-Mercaptoethanol	Bio-Rad Laboratories
Bovine serum albumin (BSA)	New England Biolabs
Chloroquine	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Carl Roth
Disodium phosphate (Na_2HPO_4)	Carl Roth
DNAse I	Stemcell Technologies
Ethanol	Carl Roth
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal bovine serum (FBS)	Thermo Fisher Scientific
Ficoll (Biocoll Separating Solution)	Merck Millipore
Formaldehyde	Merck Millipore
Glutaraldehyde	Carl Roth
Isopropanol	Carl Roth
L-Glutamine	Sigma-Aldrich
Magnesium chloride ($MgCl_2$)	Carl Roth
Monopotassium phosphate (KH_2PO_4)	Sigma-Aldrich
Moviol 4-88	Sigma-Aldrich
Paraformaldehyde (PFA)	Carl Roth
Penicillin/ streptomycin	Merck Millipore
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich
Potassium chloride (KCl)	Sigma-Aldrich
Potassium cyanide (KCN)	Sigma-Aldrich
Potassium ferricyanide ($K_3Fe(CN)_6$)	Sigma-Aldrich
Potassium ferrocyanide ($K_4FE(CN)_6$ x 3 H_2O)	Sigma-Aldrich
Sodium chloride (NaCl)	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Trypsin-EDTA	Sigma-Aldrich
Ultrapure water (Milli-Q^{TM})	Merck Millipore

Buffer and solutions

KCN concentrate

- 25 ml PBS containing
  - 820 mg $K_3Fe(CN)_6$
  - 1050 mg $K_4FE(CN)_6$ x 3 H_2O

MACS buffer

- PBS supplemented with
  - 0.5% bovine serum albumin
  - 2mM Na2-EDTA

Phosphate-buffered saline (PBS)

- Ultrapure water supplemented with
  - 137mM NaCl
  - 27mM KCl
  - 10mM Na_2HPO_4
  - 1.8mM KH_2PO_4

Red blood cell lysis buffer

- Ultrapure water supplemented with
  - 155 mM NH4Cl
  - 10 mM KHCO3
  - 0.1 mM EDTA

Senescence associated β-galactosidase fixation solution

- PBS containing
  - 1mM $MgCl_2$ at pH 6
  - 2% Paraformaldehyde
  - 0.25% Glutaraldehyde

Senescence associated β-galactosidase staining solution

- PBS containing
  - 1mM $MgCl_2$ at pH 6
  - 2.5% 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal)
  - 5.0% KCN concentrate

Antibodies

Anti-Ki-67-APC, human and mouse (clone: REA183), 130-100-332	Miltenyi
Anti-rabbit IgG antibody, A-11008	Thermo Fisher Scientific
CD33-APC, human (clone: AC104.3E3), 130-098-864	Miltenyi
CD33 MicroBeads, human, 130-045-501	Miltenyi
CD34-APC, human (clone: AC136), 130-098-864	Miltenyi
CD34 MicroBead Kit, human, 130-046-702	Miltenyi
FcR Blocking Reagent, human, 130-059-901	Miltenyi
FITC Mouse Anti-Human p16 Set	BD Biosciences
Ghost Dye™ Violet 450, 13-0863-T500	Tonbo Biosciences
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, A-11008	Thermo Fisher Scientific
Guava ViaCount reagent, 4000-0040	Luminex
H3K9me3 primary antibody, ab8898	Abcam

Commercial kits

BD Cytofix™ fixation buffer	BD Biosciences
Click-iT™ Plus EdU Alexa Fluor 647 Kit for Imaging Alexa Fluor™ 647 dye	Thermo Fisher Scientific
Click-iT™ saponin-based permeabilization and wash reagent	Thermo Fisher Scientific
ImaGene Green C12FDG lacZ Gene Expression Kit, Molecular Probes, I2904	Thermo Fisher Scientific
MACS LS cell separation columns	Miltenyi
RNA 6000 Pico Kit	Agilent
RNAeasy™ Plus Mini Kit	Qiagen
SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing TruSight Myeloid Sequencing Panel	Illumina
Quick-DNA™ Universal Kit	Zymo Research

Cell culture media solutions and additives

Dulbecco's Modified Eagle's Medium (DMEM)	Thermo Fisher Scientific
Human Flt3-Ligand, research grade	Miltenyi Biotec
Human IL-3, research grade	Miltenyi Biotec

Iscove's Modified Dulbecco's Medium (IMDM)	Thermo Fisher Scientific
Recombinant Human G-CSF	PeproTech
Recombinant Human IL-6	PeproTech
Recombinant Human SCF	PeproTech
Recombinant Human TPO	PeproTech
RPMI 1640 medium (RPMI)	Thermo Fisher Scientific

Cell line culture medium

90% RPMI, 10% FBS, 100 I/mL penicillin/ streptomycin

Freezing solution

89% FBS, 10% dimethyl sulfoxide, 100 IE/ml penicillin/streptomycin

Primary human AML medium

35% DMEM, 35% IMDM, 20% 5637 cell line-conditioned media, 10% FBS, 100 l/ml penicillin/ streptomycin, 200 mM L-Glutamin, 14.3 M β-Mercaptoethanol, 100 ng/ml SCF, 20 ng/ml IL-6, 10 ng/ml IL-3, 10 ng/ml TPO, 10 ng/ml FLT3 ligand.

Drugs

Cytarabine	Sigma-Aldrich
Daunorubicin	Sigma-Aldrich
Decitabine	Selleck Chemicals
Gemtuzumab ozogamicin	Pfizer
Hydroxyurea	Sigma-Aldrich
Midostaurin hydrate	Sigma-Aldrich
Navitoclax	Selleck Chemicals
Venetoclax	Selleck Chemicals

Instruments

Amnis ImageStreamX Mark II Imaging Flow Cytometer	Luminex
Bioanalyzer 2100	Agilent Technologies
BioPhotometer	Eppendorf
CKX41 microscope with a XC30 camera	Olympus
FACSCalibur	Becton Dickinson
GSR D1 irradiation unit	Gammaservice

Guava easyCyte flow cytometer 8 HT	Merck Millipore
Guava easyCyte flow cytometer 12 HT	Merck Millipore
HiSeq 2000	Illumina
MiSeq System	Illumina
Nanodrop 2000	Thermo Fisher
Neubauer improved counting chamber	Carl Roth

Software

FlowJo Software, version 10	Flow Jo
GraphPad Prism, version 6	GraphPad Software Inc.
GSEA software, version 4.0.3	Broad Institute of MIT (Massachusetts Institute of Technology) and Harvard
Guava CytoSoft, version 3.1	Merck Millipore
IDEAS software, version 6.2	Amnis
InkScape, version 0.92	InkScape Community
MiSeq Reporter Software, version 2.6.2.3	Illumina
R, version 3.5.3 (R packages used downloaded from https://www.bioconductor.org)	The R Foundation for Statistical Computing
R Studio, version 1.2.5019	R Studio
SPSS, version 25	IBM
Variant Studio, version 3.0	Illumina

4. Results

4.1 Primary Human AML Blasts Display Features of Therapy-Induced Senescence

Previous work by Dr. Jan Dörr and co-workers[83] in our laboratory has already demonstrated that primary AML blasts may enter senescence in response to anthracycline-based chemotherapy *ex vivo* via staining for the senescence "gold standard marker" of SA-β-gal (according to the protocol by Dimri and colleagues[69]). Moreover, Dörr et al. found an altered metabolism in therapeutically-induced senescent cells to be a potential mechanism for pharmacologically targeting them.

As a first prerequisite to systematically assess and characterize the role of senescence in the context of AML treatment, AML patient samples from bone marrow punctures and peripheral blood were collected at the Charité – Universitätsmedizin Berlin (for further description see Table 2 in the Methods Section). In addition, a robust primary co-culture system to enable *ex vivo* treatment and senescence detection in AML blast samples was established after systematic evaluation of several potential components described in the scientific literature as positively impacting *ex vivo* growth and viability of primary AML blasts (see paragraph 3.2 in the Methods Section). As a third technical requirement, performance of the well-established cytochemical protocol to quantify SA-β-gal reactivity[69] and the alternative fluorescence-based assessment[94] were quantitatively compared in the blastoid leukemia cell line K562 after senescence induction via daunorubicin treatment. Results of both assays were similar with 83% SA-β-gal positive blasts in the cytohistochemical protocol (Figure 2) and 99% in the fluorescent protocol (Figure 4) under identical experimental conditions. Importantly, differences in detected senescence levels can potentially be explained by the preceding viability dye exclusion of dead cells in the fluorescent, but not cytochemical variant of the protocol. Finally, the fluorescent variant was chosen as it enables a more standardized and objective senescence assessment and allows detection of senescence in a small number of AML blast populations. Fluorescent senescence assessment was conducted with an image-based flow cytometric immunofluorescent measurement on the Amnis ImageStreamX Mark II Imaging Flow Cytometer. Initially observed, prominent effects on detected fluorescence levels by differences in autofluorescence due to a significant increase in cells size in senescent cells could successfully be compensated for by cell size normalization, thereby defining reliable measuring conditions (Figure 3).

In the established primary culture setting, I treated primary AML blast samples from the obtained sample cohort *ex vivo* for 5 days with the drugs daunorubicin and cytarabine that are commonly used for induction therapy in AML. As an initial step to confirm the occurrence of therapy-induced senescence in primary AML blasts, to further validate established detection methods and to additionally characterize senescence in these cells, several senescence-related biomarkers were analyzed in comparison between untreated and treated AML blasts of individual patients. Thereby, initial observations of therapy-induced senescence in AML by Dörr et al.[83] could be confirmed with my newly established methods in the sample cohort and further deepened by not only showing morphological changes of blasts upon treatment with an increase in size and granulation, and cytohistochemical and fluorescence-based evidence for upregulation of SA-β-gal, but also detection of other senescence markers such as an elevated expression of the cell-cycle inhibitor $p16^{INK4a}$ and the E2F target gene/S-phase-repressive chromatin mark H3K9me3. Also, the virtual absence of the proliferation markers Ki67 and EdU incorporation could confirm the cell cycle arrest and ceased DNA replication (by loss of EdU incorporation) of senescent cells and potentially serve as a future approach for senescence assessments (Figure 5).

For the envisaged systematic assessment of senescence in my primary AML sample test cohort (“Berlin cohort”), I decided to choose an image-based quantification of a three-color immunofluorescence staining. This assay was comprised of a viability dye (Ghost Dye™ Violet 450, generating a negative signal in viable cells), the fluorescent variant of SA-β-gal detection (as the gold standard and most universal readout of senescence), and a proliferation marker (in order to confirm cell cycle arrest by negativity). Since EdU signals were found to be more stable and robust as part of this co-staining compared to the “classical” proliferation-associated marker Ki67, I decided to proceed with EdU in my newly established detection method. Both markers, fluorescent SA-β-gal and EdU, which were subsequently quantitatively compared in untreated vs. treated individual AML samples, were subjected to cell size normalization as described in Methods Section 3.3.

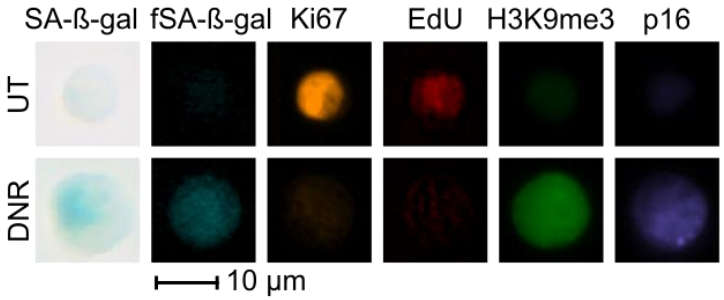


Figure 5. *Ex Vivo* Detection of Therapy-Induced Senescence: Shown above is cytohistochemical and image-based immunofluorescence-based detection of the indicated marker images in a representative example of primary AML blasts after 5 days of *ex vivo* treatment with daunorubicin (DNR, 50 ng/ml) compared to the untreated (UT) control. Both samples were subjected to conventional (i.e., cytohistochemical) SA-β-gal and fluorescence-based SA-β-gal (fSA-β-gal) detection as well as immunofluorescence analysis of further senescence markers and proliferation indicators (Ki67, EdU, H3K9me3 and $p16^{INK4a}$ [p16]). Prior to analysis, signals were gated for viable populations by viability dye exclusion (Ghost Dye™ Violet 450). Notably, besides an upregulation of senescence markers (SA-β-gal, H3K9me3, $p16^{INK4a}$) and an absence of markers of proliferation (Ki67, EdU), a distinct cell size increase is detected in the DNR-treated AML blast samples. The visible granular pattern in the $p16^{INK4a}$-stained treated AML samples might potentially reflect $p16^{INK4a}$-associated senescence-associated heterochromatic foci (SAHFs), another prominent characteristic of cellular senescence.[109]

After confirmation of the detectability of therapy-induced senescence in AML in my *ex vivo* setting and determination of experimental parameters for further systematic analyses, I next aimed at uncovering if senescence truly occurs in chemotherapeutically treated leukemic AML patients (i.e., *in patient)*. Leukemic blast samples from AML patients undergoing induction treatment with the standard chemotherapeutic regimen “7+3” were collected from peripheral blood at day 0 and day 5 (i.e., after 4 days of cytarabine and 2 days of daunorubicin application) and were stained for CD34 (in order to mark AML blasts) and fSA-β-gal. In comparison to untreated AML blasts at day 0 and at day 5, a sizeable proportion of CD34-positive cells displayed the fSA-β-gal positive senescent phenotype in response to therapy (Figure 6). This result demonstrates that induction of senescence by antineoplastic chemotherapy is not an artificial *in vitro* phenomenon but has a biological *in vivo* correlate.

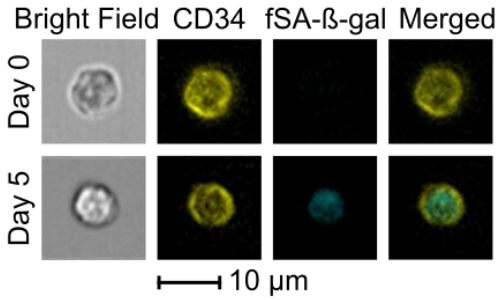


Figure 6. *In Vivo* Detection of Therapy-Induced Senescence: Depicted above are representative pictures of image-based immunofluorescence analyses of leukemic blasts from peripheral blood of one representative, newly diagnosed AML patient before (Day 0) and at day 5 of "7+3" induction treatment (Day 5). AML blasts were detected by CD34 after viability dye exclusion (Ghost Dye™ Violet 450) of dead cells and co-stained for the senescence marker fSA-β-gal. Bright field microscopy images of the cells in the image-based flow cytometry are included for completeness. Merged pictures demonstrate detection of both fluorescent markers (i.e., CD34- and fSA-β-gal) in the same individual blasts.

Despite the technical possibility of senescence detection *in vivo*, I reasoned that a systematic assessment of different AML subtypes would be more reliable and reproducible in an *ex vivo* setting, given the ambiguity of leukemic blast kinetics in the peripheral blood during the first days of induction therapy (with potential blast clearance and compartment shifts between peripheral blood, bone marrow, and spleen). For instance, peripheral blood blast clearance in AML is an independent prognostic factor,[110] and hyperleukocytosis at diagnosis, as another probably interfering variable, correlates with certain AML characteristics, especially FLT3 mutational status.[111] In contrast to apoptotic cell death of tumor cells, which is typically induced within 24-48 hours by chemotherapeutic drugs, the full onset of cellular senescence, with its characteristic expression of SA-β-gal, both *in vivo* and *in vitro*, requires at least 3-7 days after initial treatment exposure.[91] However, after 5 days of induction treatment, only 10 percent of AML patients still have detectable peripheral blasts.[112] The combination of these well-known clinical characteristics leaves only a very narrow, if not unfeasible window for *in vivo* detection of therapy-induced senescence. A technically possible multi-compartment analysis (including bone marrow and spleen histology) at day 5 would probably enable a sufficiently sensitive AML blast detection, but is, purely for explorative research, not justifiable for ethical reasons. Another caveat of an invasive *in vivo* assessment could be the common occurrence of infections due to the immunocompromised status of AML

patients. Of note, viral infections especially are known to mediate senescence and complexly interact with senescent cells, and therefore emerging infections potentially interfere with the quantification of the AML drugs' capacity to induce senescence.[113, 114]

In conclusion, to investigate the prognostic impact of therapy-induced senescence in my AML patient cohort, the immunofluorescent triple staining of the senescence marker fSA-β-gal, the proliferation marker EdU and the viability marker Ghost Dye™ Violet 450 was systematically conducted after the described *ex vivo*-exposure to daunorubicin and cytarabine in the newly established cell culture setting. Chemotherapy was applied after an initial 3-day adaptive pre-culturing phase of the primary patient material and subsequent $CD34^+$-, or, if not expressed, $CD33^+$-based magnetic positive selection of blasts.

In this assay, I found that, in response to either chemotherapeutic agent, individual AML blast samples exhibited varying degrees of shifts from EdU-positive to EdU-negative states and partly gained fSA-β-gal reactivity, in line with the onset of senescence (Figure 7). Whilst some of the assessed AML samples did not display significant changes in EdU- and fSA-β-gal intensities after *ex vivo* treatment, others demonstrated only shifts from an EdU-positive to an EdU-negative condition without changes of fSA-β-gal reactivity. Finally, all samples of my cohort could be assigned to one of the following three biological scenarios (with varying intensities) according to their response to chemotherapeutic treatment *ex vivo*:

1. EdU-negative and fSA-β-gal-positive (senescence-capable)
2. EdU-negative and fSA-β-gal-negative (cell cycle arrested, senescence-incapable)
3. EdU-positive and fSA-β-gal-negative (proliferating, treatment-unresponsive)

Encouragingly, and in line with biological expectations, a condition of high fSA-β-gal reactivity and concomitantly preserved EdU-positivity was not observed in response to treatment, which argues in favor of the utility of this senescence/proliferation marker combination to reliably detect senescent cells.

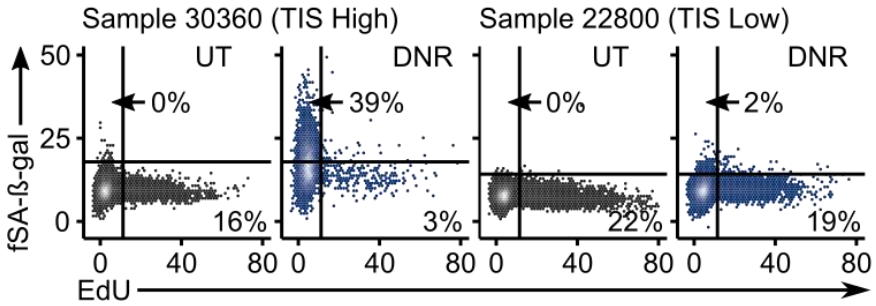


Figure 7. Differential Capability of Therapy-Induced Senescence (TIS): Proliferation (EdU, x axis) vs. senescence (fSA-β-gal, y axis) density plots by image-based flow cytometry of untreated (UT) vs. 5 days daunorubicin (DNR) *ex vivo* treated viable AML blasts are exemplified for one senescence-capable AML sample (sample no. 30360, "TIS High", left) and one TIS-incapable sample (sample no. 22800, "TIS Low", right). In contrast to sample 30360, sample 22800 does not demonstrate significant population shifts in either fSA-β-gal or EdU intensity. Percentages indicate respective cell fractions in proliferation (lower right) or senescent growth arrest (upper left). Classification into "TIS High" and "TIS Low" by the treated vs. untreated ratios of fSA-β-gal intensities are defined in the following section and Figure 8.

At first glance, the apparent parameter to compare senescence capacities of individual AML blast samples would be the fraction of SA-β-gal reactive cells after a defined chemotherapeutic treatment. Yet, due to profound differences in baseline fSA-β-gal intensities between individual untreated samples, an identical, static fSA-β-gal intensity to define the threshold for therapy-induced senescence for all samples would over- and underestimate individual senescence capacities. On the other hand, individual gating strategies to distinguish fluorescence-positive and -negative populations in untreated vs. treated sample groups would complicate a quantitative comparison between different samples and potentially introduce a notable observer bias. Therefore, I reasoned that the ratio of fSA-β-gal intensities between the treated and untreated condition of each AML sample would serve as the most robust and reliable output parameter to compare individual AML senescence capacities in an unbiased way without the need for sample-specific gating strategies.

When applied to my "Berlin" test cohort of 31 newly diagnosed AML patients, isolated primary AML blast samples presented with varying fSA-β-gal intensity shifts in response to *ex vivo* daunorubicin treatment (as already exemplarily depicted in Figure 7). Individual senescence capacities were subsequently classified into three groups (therapy-induced senescence capacity high vs. intermediate vs. low). Corresponding

cutoff values for daunorubicin-induced fSA-β-gal ratios were generated in order to result in three groups of equal sizes (Figure 8). Similarly, intra-individual changes in EdU intensities between untreated and treated conditions were analyzed.

Importantly, exposure of the same AML blast samples to cytarabine *ex vivo* largely reproduced the individual extents of fSA-β-gal signal induction by daunorubicin (Figure 8). This finding suggests therapy-induced senescence to act as a general cellular response to cytotoxic stress, with the extent of senescence inducibility as an AML sample-intrinsic characteristic.

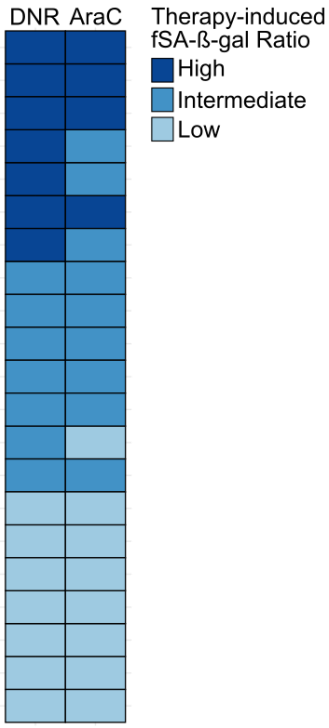


Figure 8. Correlation of Daunorubicin (DNR)- with Cytarabine (AraC)-Induced Senescence in Individual AML Samples: An ordinal heatmap comparison is shown with individual levels of fSA-β-gal intensity ratios (treated-to-untreated) of 21 AML patient samples (one sample per row) in response to exposure to DNR 50 ng/ml and AraC 100 ng/ml *ex vivo*. Samples were ranked by their DNR-induced fSA-β-gal ratios (on the left) from highest (at the top) to the lowest value (bottom). The color code reflects classification into three equal subgroups based on individual DNR-inducible fSA-β-gal ratios as “Low” (treatment-induced fSA-β-gal ratio <1.14), “Intermediate” (1.14-1.38) and “High” (>1.38). The matching AraC-responses of each sample are shown in the right column and were color-coded using the same cutoff values to show consistency of senescence capacities between both treatment groups. Only 21 of the total 31 AML samples of the “Berlin” test cohort could be analyzed with comparative treatments of both agents due to limited availability of biological material. For samples with insufficient primary material, experiments for the analysis of DNR-evoked senescence were prioritized as anthracycline-induced senescence has been more commonly described.

To further characterize transcriptional changes occurring in AML as a response to chemotherapeutic treatment with particular focus on therapeutically-induced senescence, I next compared gene expression profiles obtained by RNA sequencing (RNA-seq) from twelve matched pairs of daunorubicin-treated vs. untreated AML blast samples (please refer to Methods Section 3.5 for depiction of the 23 selected senescence-associated gene signatures).

Senescence-Capable AML Samples: Treated vs. Untreated

A first exploratory analysis compared six therapy-induced senescent AML blast samples (defined by an individual daunorubicin-induced fSA-β-gal ratio above the median of all 12 RNA-seq samples which is likewise the median of the whole “Berlin cohort”) against their untreated controls. Within the 100 most differentially regulated genes, I found, as part of the selected gene signatures, directly senescence-associated genes, other cell-cycle regulators, SASP factors, regulators of the Wnt/ β-catenin signaling pathway and several components of immune responses, thus further underscoring the senescent nature of these drug-exposed AML blast samples (Table 3).

Table 3. Top 100 Differentially Expressed Genes in Daunorubicin-Senescent Compared to Untreated AML blasts

Upregulated differentially expressed genes			
Gene	Log2 fold change	*p*-value	Senescence-associated gene set (Source)
CLCA2	6.36	2.26E-10	p53 pathway (Hallmark)
GJA5	4.98	9.17E-10	
GFRA1	4.96	1.94E-06	
TPBGL	4.91	1.06E-07	
PSG9	4.64	5.48E-10	
SERTAD4	4.61	5.94E-06	
SFRP1	4.51	2.26E-08	
KRTAP3-1	4.49	2.54E-06	
AJAP1	4.44	7.85E-11	
MARK1	4.40	6.45E-08	
KRT19	4.36	1.68E-07	
PCLO	4.31	1.36E-03	
ANGPTL2	4.24	6.20E-09	

IFI44L	4.22	4.96E-06	IFNα response (Hallmark), IFNγ response (Hallmark)
PSG8	4.22	9.52E-06	
PI3	4.20	2.13E-10	Humoral immune response (GO), RAS-induced senescence (Mason et al.)
LHX9	4.17	3.17E-08	
PSG11	4.11	7.87E-08	
CNTN3	4.11	9.69E-13	
KRT34	4.09	1.25E-11	
SH3GL2	4.03	1.69E-05	Protein secretion (Hallmark)
GUCY1A2	3.95	3.48E-07	
SIM1	3.95	2.16E-11	
GABBR2	3.93	2.39E-06	
IFIT1	3.92	8.60E-08	Innate immune response (GO), IFNγ response (Hallmark)
PCDH7	3.92	7.21E-07	Inflammatory response (Hallmark)
ADGRL2	3.92	6.90E-05	
HOXC11	3.83	1.07E-09	
CCDC85A	3.77	1.87E-06	
PRSS12	3.75	6.73E-08	
SOX9	3.69	2.31E-05	
PAX3	3.67	7.51E-04	
CADPS2	3.64	3.04E-05	
TRNP1	3.62	1.69E-12	
RGMB	3.62	1.12E-11	
RP3-509I19.1	3.57	3.15E-03	
PTN	3.52	3.44E-07	Il-6/ JAK/ STAT3 signaling (Hallmark)
SPATA18	3.49	3.68E-06	
DAZL	3.46	1.24E-04	
MAP6	3.44	4.90E-03	Il-2/ STAT5 signaling (Hallmark)
CPZ	3.43	7.03E-08	
SNX7	3.43	3.97E-06	
AMOTL2	3.41	1.39E-07	
WLS	3.40	3.03E-04	Il-2/ STAT5 signaling (Hallmark)
FBLN1	3.40	2.02E-13	
MOXD1	3.39	5.30E-13	
PDGFRL	3.38	4.27E-03	
SLIT2	3.36	1.14E-12	

CDKN1C	3.36	1.90E-03	Senescence (Fridman et al.), Il-2/ STAT5 signaling (Hallmark), TGFβ signaling (Hallmark)
GPR37	3.34	1.52E-05	
IFI6	3.32	6.07E-06	Innate immune response (GO)
SHC3	3.31	2.02E-05	
HTRA1	3.31	9.06E-09	
XKR3	3.30	1.19E-03	
SSTR1	3.29	2.60E-07	
FLNC	3.28	9.95E-09	
ACKR4	3.27	1.92E-13	
DKK1	3.25	2.11E-09	Wnt/ β-catenin signaling (Hallmark)
GDF15	3.24	1.87E-10	SASP (Lasry et al.), RAS-induced senescence (Mason et al.)
SLC16A2	3.23	1.73E-09	Replicative senescence (Zhang et al.)
CORO2B	3.23	5.76E-07	RAS-induced senescence (Mason et al.)
FGF7	3.22	1.08E-09	RAS-induced senescence (Mason et al.), SASP (Lasry et al.)
TJP1	3.21	1.71E-05	TGFβ signaling (Hallmark)
THY1	3.20	4.59E-10	
SH2D5	3.19	3.24E-03	
NTN4	3.16	7.95E-09	
SYNM	3.16	2.42E-04	
PSG5	3.16	4.02E-09	Replicative senescence (Zhang et al.)
RBM24	3.16	4.38E-07	
GLI2	3.15	2.92E-04	
ROBO2	3.15	2.04E-04	
MMP8	3.13	8.28E-04	
CXCL12	3.12	5.46E-03	
GFRA2	3.11	4.29E-07	
PSG4	3.10	6.40E-05	
Downregulated differentially expressed genes			
ZDHHC19	-3.08	1.50E-03	
CDK1	-3.10	2.61E-07	E2F targets (Hallmark)
NRXN2	-3.11	2.87E-03	
CCNB1	-3.12	4.95E-14	
FAM72D	-3.13	8.93E-07	
NUF2	-3.14	1.16E-08	

HIST1H4C	-3.15	1.84E-09	
SKA3	-3.17	5.45E-08	
PBK	-3.18	1.50E-08	
KIF2C	-3.21	1.91E-06	E2F targets (Hallmark)
CDC20	-3.22	2.17E-08	E2F targets (Hallmark), Mμc targets 1 (Hallmark)
KIF4A	-3.27	3.58E-07	E2F targets (Hallmark)
ASPM	-3.27	5.11E-06	
HIST1H1B	-3.35	1.94E-08	
DLGAP5	-3.38	5.88E-07	E2F targets (Hallmark)
E2F8	-3.63	1.17E-11	E2F targets (Hallmark)
ANLN	-3.74	1.17E-11	
NOSTRIN	-3.86	4.08E-06	
KIF20A	-4.12	4.17E-12	
TSLP	-4.29	2.24E-03	
DEPDC1	-4.34	2.18E-08	E2F targets (Hallmark)
CEACAM6	-4.65	4.41E-06	
CCR8	-5.00	7.70E-05	
EPX	-5.55	1.59E-05	
MPO	-6.88	9.51E-14	

Table 3. Top 100 Differentially Expressed Genes in Daunorubicin-Senescent Compared to Untreated AML Blasts: This table shows the 100 transcripts with highest absolute significant values of fold mRNA expression changes obtained in six matched pairs of daunorubicin-treated vs. untreated AML blast samples that had been characterized as capable of therapy-induced senescence (i.e., those exhibiting a daunorubicin-induced fSA-β-gal ratio above the median of all "Berlin cohort" samples). Genes are ranked by values of log2 fold change from highest (top) to lowest (bottom). Senescence-associated gene sets (of the selected 23 representative gene sets discussed in the Methods Section) which contain the listed genes are indicated in the right column with the respective sources of gene set in brackets: Hallmark collection,[96] gene ontology (GO),[103, 104] or specific literature reference.[53, 97-99]

As a next step, the six matched pairs of senescence-capable AML blasts were further analyzed regarding their prominent and central transcription factor activities. When assessed by VIPER (please refer to section 3.5 for details on this method),[105] transcription factors in the daunorubicin-treated condition with the highest confidence score based on their transcription factor-target interactions[106] comprised important key players of senescence, such as active TP53 as well as suppressed E2F family members and

MYC.[28, 44] Likewise, other senescence-associated moieties occurred in the strongest transcription factor alterations after daunorubicin treatment, which included upregulation of FOXO1 (triggering senescence via its target p21[44, 115]), and downregulation of FOXM1, a transcription factor that drives G2/M gene expression,[116] as well as increased activities of the tumor suppressor SMAD4, mediator of TGF-β signaling,[117] and, importantly, of STAT1, a SASP regulator (via JAK/STAT and NF-kB signaling[54]) and component of senescence and cancer stemness gene signatures.[97, 99, 118] For other transcription factors that were found altered in response to daunorubicin treatment, such as GATA3 (NF-kB target gene[119] and part of cancer stemness signatures[118]) and the estrogen receptor 1 (ESR1, a regulator of the JAK/ STAT pathway),[120] preliminary evidence also suggests potential implications in senescence, but with a yet to be determined role. In collaboration with members of Professor Schmitt's group, activation and dysregulation of FOS and JUN, both part of the transcription factor complex activator protein 1 (AP-1) and also strongly altered in my transcription factor analysis, have recently been shown to play an important role in the epigenetic and transcriptional initiation and maintenance of the senescence state switch. Nevertheless, reports addressing the complex (dys)regulation of AP-1 superfamily members remain in part contradictory regarding senescence propagation and suppression (Figure 9).[121, 122]

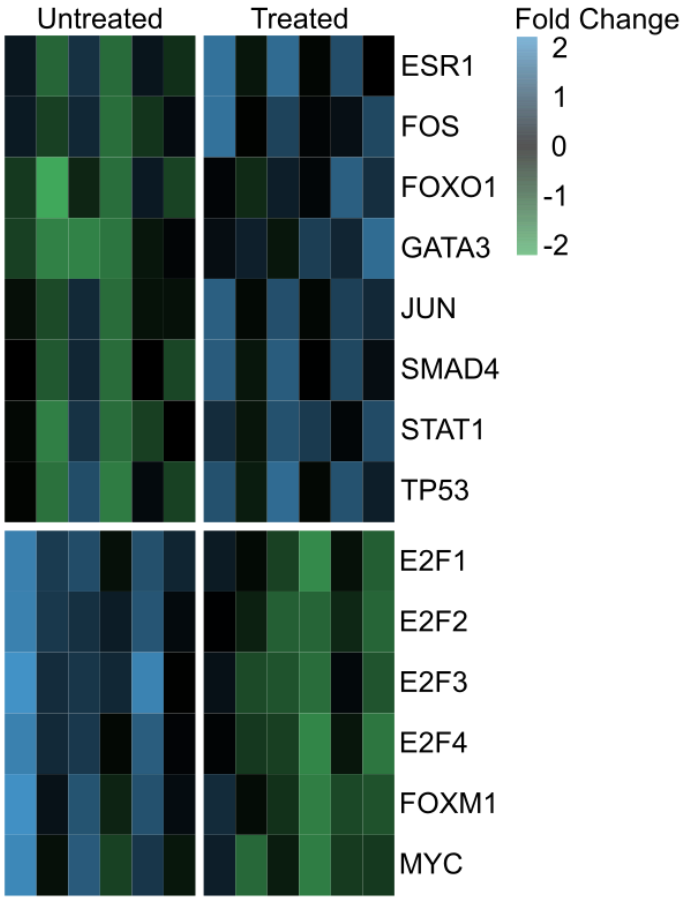


Figure 9. Transcription Factor Activity Modulation in Therapy-Induced Senescence: Modulation of transcription factor activities in senescence-capable AML blast samples is displayed in a heat map format. RNA sequencing analysis (as in Figure 9, right side) reflects 6 matched pairs of daunorubicin-treated vs. untreated AML blast samples (presented in identical order for untreated and daunorubicin-exposed conditions) which had been classified as capable of executing therapy-induced senescence by daunorubicin-induced fSA-β-gal ratios above median. Transcription factor activities were assessed through virtual inference of protein activity by enriched regulon analysis (VIPER) and displayed color-coded by their log2 fold change of transcript expression ("Fold Change").[105] Only results with a top confidence score (based on the level of evidence of transcription factor-target interactions, category A)[106] are displayed here.

Whole AML Cohort: Treated vs. Untreated

Importantly, the following analysis included AML samples which had displayed the entire spectrum of therapy-induced senescence capability (i.e., from a profound to a small or no shift of fSA-β-gal intensity upon daunorubicin exposure *ex vivo*). The resulting GSEA for the depicted gene signatures indicated transcriptional induction of typical molecular senescence features upon AML sample treatment in the *ex vivo* drug assay. These molecular features included, beside the upregulation of directly associated senescence

signatures, enrichment of the apoptosis and the TP53 pathway, suppression of largely S-phase entry-conferring E2F targets and MYC targets, transcriptional induction of senescence-mediating pathways (TGF-β, NF-κB, Wnt/β-catenin) as well as inflammation (SASP, INFα and INFγ response, JAK/STAT signaling). In addition, upregulation of innate, adaptive and humoral immune responses, but suppression of T-cellular immune mechanisms, was noted (Figure 10, left side).

Treated AML Samples: Senescence-Capable vs. Senescence-Incapable

When chemotherapy-treated AML samples were classified by their extent of fSA-β-gal signal shift (i.e., separated into groups above vs. below median), expression of senescence-associated transcripts in the same analysis was, again, found even more skewed towards the particularly strong fSA-β-gal reactive group (Figure 10, right side).

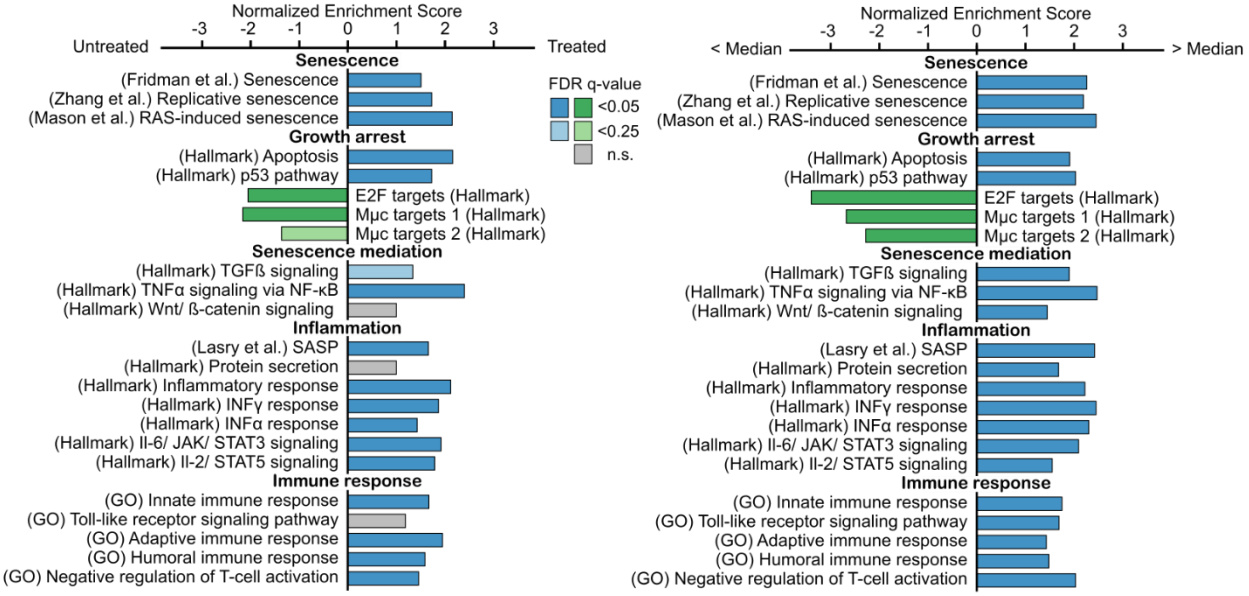


Figure 10. Transcriptional Profiles Confirm Therapy-Induced Senescence in AML: Comparison of gene set enrichment analyses (GSEA) profiles for senescence-associated signatures in transcriptomes of twelve matched pairs of daunorubicin-treated ("Treated") vs. untreated ("Untreated") AML blast samples. On the left side, an analysis of all 12 matched pair samples is depicted comparing all treated vs. untreated samples independent of individual senescence capability. On the right side, only data derived from *ex vivo* daunorubicin-treated samples are shown and classified as senescence capable or incapable by fSA-β-gal ratios above ("> Median", 6 samples) or below median ("< Median", 6 samples). The 23 interrogated signatures included directly senescence-defining gene sets,[53, 97-99] as well as senescence-related (growth arrest, senescence mediation, inflammation) gene signatures from the Molecular Signatures Databases (Hallmark)[96] and immune response gene signatures from the Gene Ontology Resource (GO),[103, 104] reflecting further cellular states and aspects of senescence as discussed in the main text. Displayed GSEA results were color-coded by significance levels (FDR q-values). All normalized enrichment scores in the right panel are significant with an FDR q-value <0.05.

In summary, the newly established fSA-β-gal assay was validated to detect cellular senescence in AML blasts by association with several individual established parameters of senescence and proliferation as well as in transcriptome analyses. It consistently identified senescent primary AML blasts after *ex vivo* daunorubicin and cytarabine treatment, and served as a differentiating marker to classify samples by varying extents of senescence inducibility. In addition, AML blasts evaluated as therapy-induced senescent presented a wide spectrum of molecular features that are typically associated with cellular senescence, including growth arrest (TP53 activation, E2F inhibition),

immune attraction, a proinflammatory SASP, cancer stemness and dysregulation of immune response.

4.2 Basal Senescence Does Not Provide Prognostic Value in AML

As discussed in the introduction of this thesis, Haugstetter et al.[59] reported spontaneously occurring (i.e., oncogene-, replicative or stress-induced) senescence, also termed “basal senescence”, as a positive predictor of outcome to 5-fluorouracil/leucovorin-based first-line cancer therapy in metastasized colorectal cancer. I tested the prognostic power of basal senescence in two different approaches. Firstly, I conducted a gene expression analysis in a publicly available AML reference cohort as provided by TCGA Research Network.[9] More precisely, I analyzed transcriptomic data of 173 AMLs regarding corresponding patient outcome based on expression of the three previously described senescence signatures, i.e., a compiled (universal) cellular senescence gene set (Fridman et al.),[97] a replicative senescence signature (Zhang et al.)[98] and an oncogene (RAS)-induced senescence signature (Mason et al.).[99] In the analysis of disease-free and overall survival of this cohort after stratification into high and low expression of the indicated basal senescence gene signatures, significant differences regarding the clinical course of AML patients could not be detected (Figure 11).

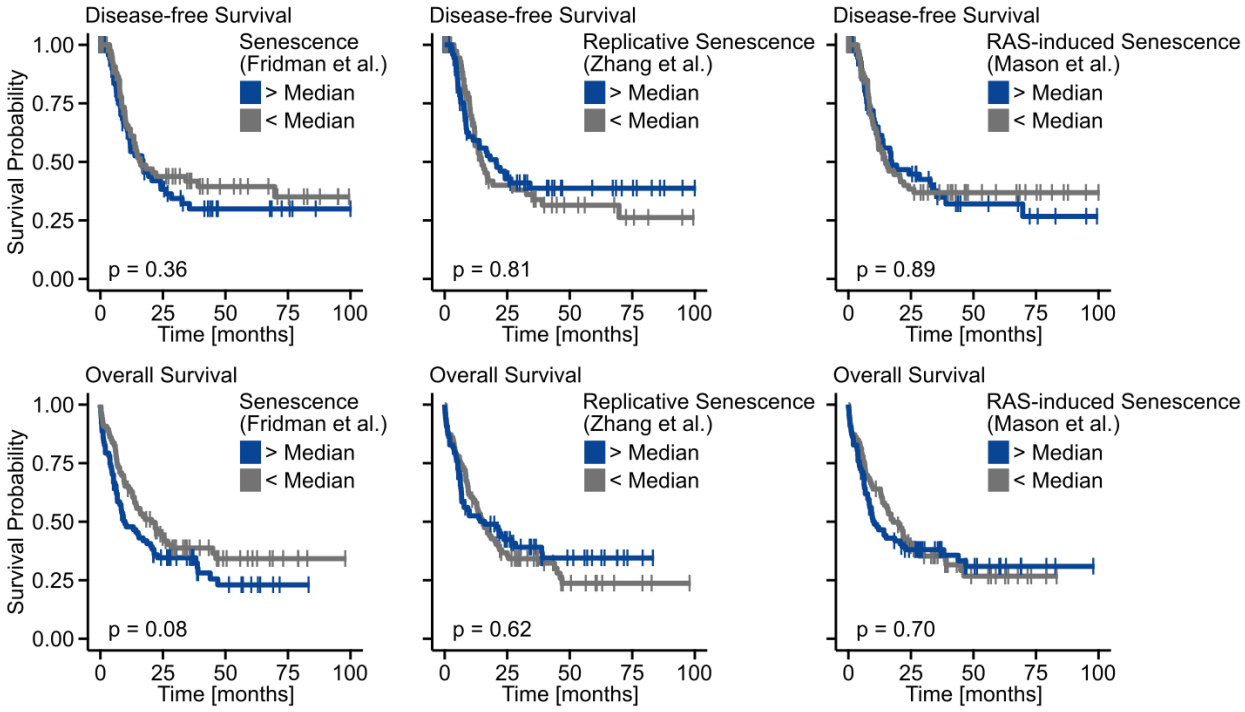


Figure 11. Prediction of Patient Outcome Based on Senescence-Defining Gene Signatures in The Cancer Genome Atlas Research Network AML Cohort:[9] Kaplan-Meier analysis of disease-free survival (top) and overall survival (bottom) of 173 patients from The Cancer Genome Atlas Research AML cohort stratified by expression of the indicated senescence-defining gene signatures (original references in brackets)[97-99] in AML blasts at initial diagnosis (i.e., before the start of induction therapy). All patients were stratified into two groups based on log2 mRNA expression values above and below median.

In a second approach to assess the long-term impact of basal senescence levels in AML, I analyzed fSA-β-gal intensities of untreated AML patients' samples in my "Berlin cohort". In line with the gene expression analysis in the (independent) TCGA cohort, basal senescence did not show any predictive or prognostic value with regards to disease-free or overall survival (Figure 12A). Accordingly, neither sample stratification by ELN risk group nor by karyotype status resulted in significantly different basal fSA-β-gal intensities of untreated AML blast sample groups (Figure 12B).

In conclusion, unlike previously reported for metastasized colorectal cancer,[59] assessment of spontaneous senescence might not be a risk predictor or indicator for therapy outcome in the case of AML. Haugstetter et al. assumed a correlation of basal senescence (as a senescence susceptibility) with therapy-induced senescence as the reason for the reported predictive value of basal senescence in colorectal cancer. In the

analysis of my AML cohort with available data on basal and therapy-induced senescence (i.e., fSA-β-gal intensities of untreated and daunorubicin-induced fSA-β-gal ratios of treated AML blast samples), I did not find any correlation between these two parameters (R^2 = 0.02, p = 0.44) and therefore any indication of therapy-induced senescence predictability by assessment of baseline senescence in untreated AML samples.

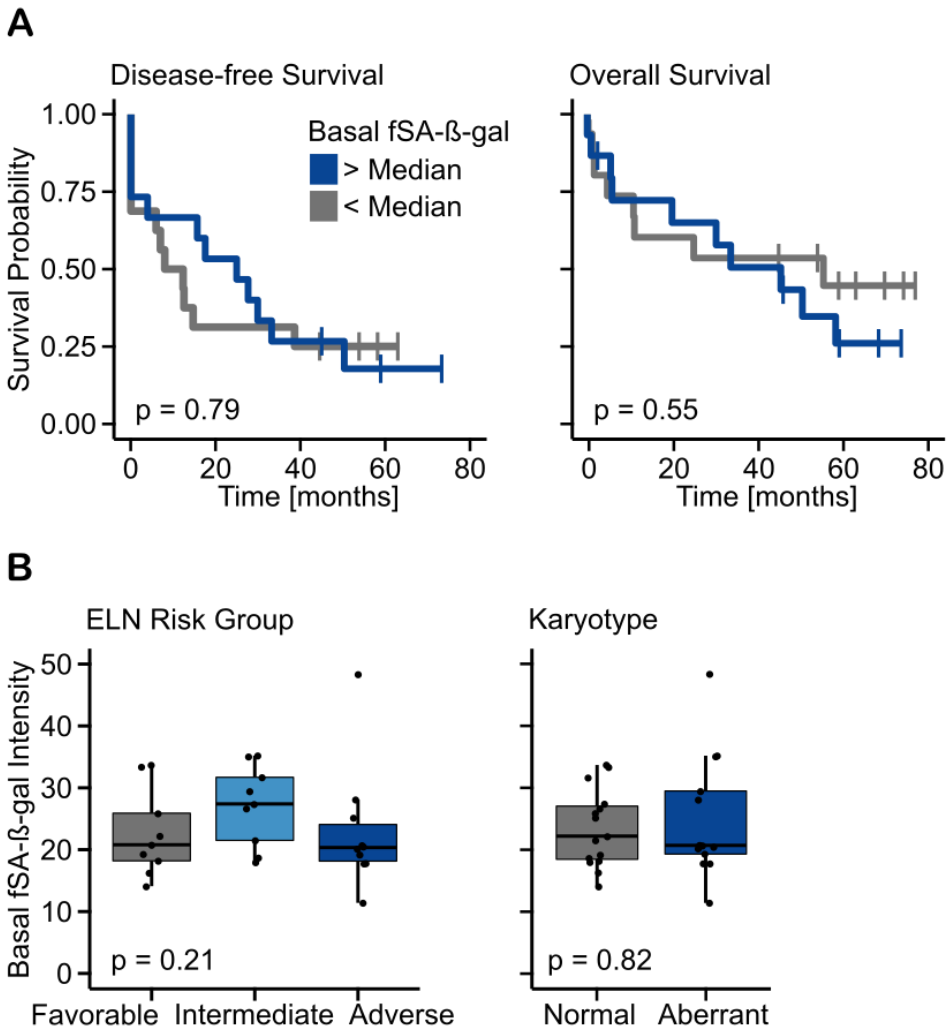


Figure 12. Impact of Basal Blast Senescence on Outcome and Established Risk Prognosticators in AML Patients (Berlin Cohort): Panel A shows Kaplan-Meier analysis of patient outcome (disease-free survival, left, and overall survival, right) in AML patients from the "Berlin Cohort" stratified by individual basal AML blast senescence in untreated samples (detected as fSA-β-gal intensity) into above and below cohort median. fSA-β-gal intensities were assessed in a purified blast population after magnetic bead separation and 5 days of cultivation in the established *ex vivo* assay. Panel B depicts box plot and scatter plot (of single values) overlays of individual basal fSA-β-gal activities (samples as in panel A) grouped by genetic risk groups as defined by the European Leukemia Net (ELN, left) or presence of karyotypic alterations (right). Notably, the depicted untreated fSA-β-gal intensities also served as reference values for the treated vs. untreated ratios of fSA-β-gal intensities to assess therapy-induced senescence.

4.3 Therapy-Induced Senescence Capacity is a Long-Term Prognosticator in AML

To probe the potential of therapy-induced fSA-β-gal intensity changes to serve as a predictor of treatment outcome in AML patients, I performed the established *ex vivo* daunorubicin treatment assay for all 31 primary AML blast samples comprising the "Berlin cohort". Subsequently, I linked individual senescence capacities, reflected by individual untreated vs. treated fSA-β-gal intensity changes, to clinical outcome of the respective patients, who had in most cases received the "7+3" daunorubicin and cytarabine-based induction regimen. In contrast to my findings for basal senescence, higher daunorubicin-induced senescence capacities (as stratified by the cohort median) were associated with a significantly longer disease-free survival (p = 0.04), and, even more pronouncedly, a significantly longer overall survival (p = 0.02, Figure 13A) of AML patients in this cohort.

To confirm this observation, I conducted an analysis employing the same *ex vivo* daunorubicin treatment assay in an independent validation cohort ("Munich cohort") of 37 newly diagnosed AML patients compiled by both university medical centers in Munich (i.e., the Ludwig-Maximilians-Universität München [LMU] and the Klinikum rechts der Isar of the Technische Universität München [TU]). Furthermore, to unravel the role of therapy-induced senescence in a molecularly more homogeneous subgroup, this validation cohort was generated from AML patients with a normal karyotype only (for details of both cohorts and comparison to a reference cohort, please see Table 2). Complementing my results obtained in the initially investigated "Berlin cohort", I found that in the "Munich cohort" as well, AML patients with a corresponding daunorubicin-induced fSA-β-gal ratio above sample cohort median exhibited a significantly longer disease-free survival (p = 0.03) and, again, more pronounced overall survival (p = 0.02, Figure 13B).

Notably, median levels of therapy-induced fSA-β-gal intensities differed between both cohorts, potentially due to differences in pre-processing protocols (times from bone marrow aspiration to isolation of mononuclear cells, freezing protocols, and components of freezing solutions). Since storage of "Munich cohort" AML blast samples had not primarily been optimized for *ex vivo* cultivation, a pooled analysis of both patient cohorts was ruled out *a priori*.

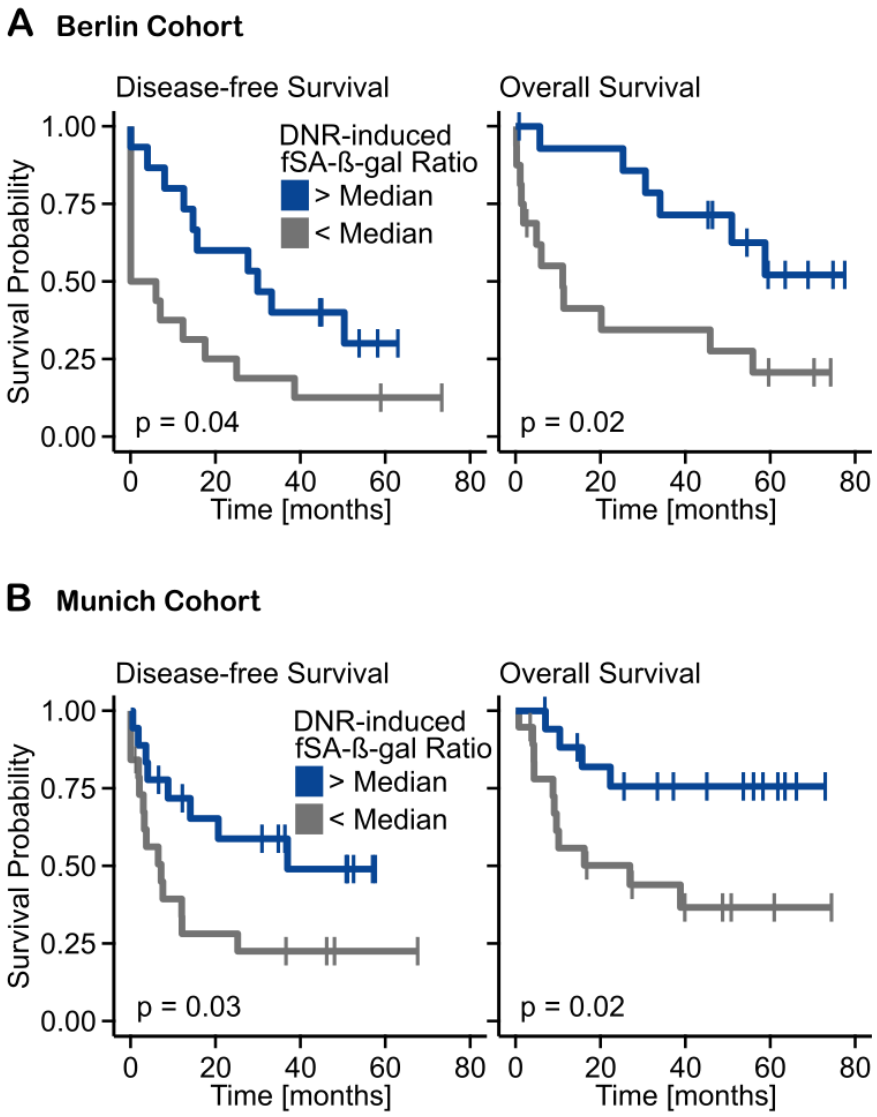


Figure 13. *Ex vivo* Therapy-Induced Senescence Capacity as a Stratifier for Clinical Outcome of AML Patients in the "Berlin Cohort" and in the "Munich Cohort": Panel A shows outcome in AML patients (*n* = 31, "Berlin cohort") as disease-free survival (defined as time from complete remission to relapse or death, left) and overall survival (right) stratified into above and below median by their daunorubicin (50 ng/ml, DNR)-induced fSA-β-gal ratios *ex vivo*. Panel B shows results of the same stratification obtained in 37 karyotype-normal AML patients (representing the "Munich cohort"). Please note the generally superior outcome of patients in the "Munich cohort", which is expected in a cytogenetically normal AML subgroup as, despite existence of favorable karyotypic alterations, presence of an aberrant AML karyotype is an independent negative prognostic factor.[10]

The strongest surrogate marker of clinical patient outcome and basis for initial therapy decisions is the ELN genetic risk stratification, which integrates and categorizes molecular and cytogenetic risk prognosticators. In a further analysis of the "Berlin cohort"

(i.e., in order to confirm a predictive value of *ex vivo* determination of therapy-induced senescence by fSA-β-gal), I tested whether individual fSA-β-gal ratios also differed between ELN genetic risk groups (see Table 1 for description).[10] Indeed, AML samples within the favorable risk group presented with the strongest fSA-β-gal inducibility, whereas those in the adverse group showed lowest fSA-β-gal ratios (Figure 14, left side). Another strong predictor of better therapy outcome is a normal karyotype, which I also found to correlate with higher daunorubicin inducible senescence capacity (Figure 14, right side), irrespective of the above reported power that the *ex vivo* fSA-β-gal assay retains within this group (reflected by the "Munich cohort", Figure 13B). Notably, AML samples carrying one of the two favorable cytogenetic aberrations (RUNX1-RUNX1T1, CBFB-MYH11) were found at the top end of fSA-β-gal ratios in the aberrant karyotype group.

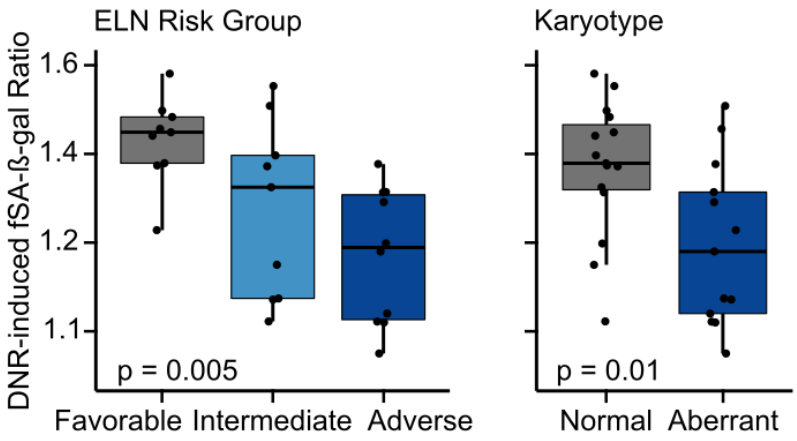


Figure 14. Association of Daunorubicin (DNR)-Induced Senescence Capability with Established AML Risk Prognosticators ("Berlin Cohort"): This panel unveils an association between senescence capability and other AML risk prognosticators (analyzed in the "Berlin cohort"). European Leukemia Net (ELN) genetic risk stratification (*n* = 30, left) and karyotype status (*n* = 28, right) were correlated to individual *ex vivo* DNR-induced fSA-β-gal ratios in primary AML blast samples (assessed as in Figure 13). Individual AML sample fSA-β-gal ratios are displayed as an overlay scatter plot. In 3 of the total 31 "Berlin Cohort" patients, a karyotype could not be determined by initial routine diagnostics due to technical reasons. Yet, an ELN genetic risk group alignment was still possible for 2 of the 3 samples by the presence of adverse mutational aberrations. Notably, AML blast samples with more favorable cytogenetic aberrations (*n* = 3), RUNX1-RUNX1T1 (t(8;21)(q22;q22.1)) and CBFB-MYH11 (inv(16)(p13.1q22) or t(16;16)(p13.1;q22)), showed fSA-β-gal ratios distinctly above the median of the aberrant karyotype group (right panel).

In essence, I was able to show that the *ex vivo* fSA-β-gal-based determination of the individual leukemic blast therapy-induced senescence capacity has profound predictive potential for AML patients undergoing a daunorubicin- and cytarabine-based induction therapy. These results obtained in my “Berlin (test) cohort” were also validated in an external sample group (“Munich cohort”). Moreover, the association between senescence capacity (determined in response to AML chemotherapeutic drugs *ex vivo*) and ELN genetic risk group as well as AML karyotype indicates that therapy-induced senescence capability might actually underlie clinical risk groups and cytogenetic features as a decisive drug-inducible biological effector principle.

4.4 AML Genetics Impact Therapy-Induced Senescence Capacity

Beside karyotypic aberrations, mutations in *NPM1, FLT3, DNMT3A* and other gene loci as well as common gene fusions such as *MLL* and *RUNX1* reflect AML-associated molecular lesions that, alone or in combination with other recurrent defects, mark distinct AML biologies.[10, 11, 15] Some of these mutations have prognostic implications, like more favorable *NPM1* mutations or the prognostically adverse *FLT3-ITD*, *RUNX1, ASXL1* and *TP53* mutated status.[10] AML-driving genetic aberrations of *FLT3*, *IDH1* and *IDH2* can even be directly targeted by small molecules.[12, 123, 124] The prognostic implications of some other recurrent mutations (like the common *DNMT3A* alteration) remain to be determined.

Therefore, I asked whether recurrent AML genetic defects impinge on the ability to execute senescence in response to conventional AML chemotherapeutic agents detected in the “Berlin cohort”. Importantly, in the analysis of this heterogeneous cohort of limited size, the genetic background of AML blast samples had been taken into account: Most samples harbored more than three recurrent mutations and half of the samples displayed an aberrant karyotype in addition. Consequently, the presented analysis does not reflect a comparison of individual genetically altered vs. genetically intact wild type AML blast samples. Therefore, it was not surprising to me that most of the 28 recurrent lesions assessed in total did not exhibit differences in fSA-β-gal inducibility between the respective wild type and mutated sample groups. Yet, despite extensive confounding genetic aberrations, *NPM1*- and *DNMT3A*^{R882}-mutant blasts robustly displayed a significantly higher fSA-β-gal inducibility upon daunorubicin exposure *ex vivo* than unmutated primary samples, while serine and arginine-rich splicing factor 2 (*SRSF2*)-

mutant blasts presented with a trend towards reduced fSA-β-gal inducibility. Likewise, N-ras proto-oncogene (*NRAS*)-mutant samples showed a trend towards lower fSA-β-gal treatment inducibility compared to wild type samples, though the mutational frequency was very limited (*n* = 3), and its association should be validated in a larger sample cohort (Figure 15, analyses for other recurrent genetic events are shown in Table 4). In the case of *DNMT3A*, the missense mutation in codon R882 constitutes by far the most common aberration and a pathology-driving event. Therefore, this variant had to be distinguished from variants of uncertain significance (and single nucleotide polymorphisms as already stated in the Methods Section), which were also present in my dataset. To specify the pathogenic *DNMT3A*^{R882} variant and to distinguish it from other *DNMT3A* lesions, it is explicitly indicated in the following sections.

Unlike the relatively intuitive positive correlation between a stronger fSA-β-gal readout and prognostically favorable *NPM1*-mutational status,[125] the underlying biological implications of superior senescence inducibility with rather less favorable DNA methyltransferase *DNMT3A*^{R882} mutations or clinically less obvious splicing factor *SRSF2* mutations[126-128] still need to be functionally elucidated in greater detail, also in the context of other accompanying genetic lesions.

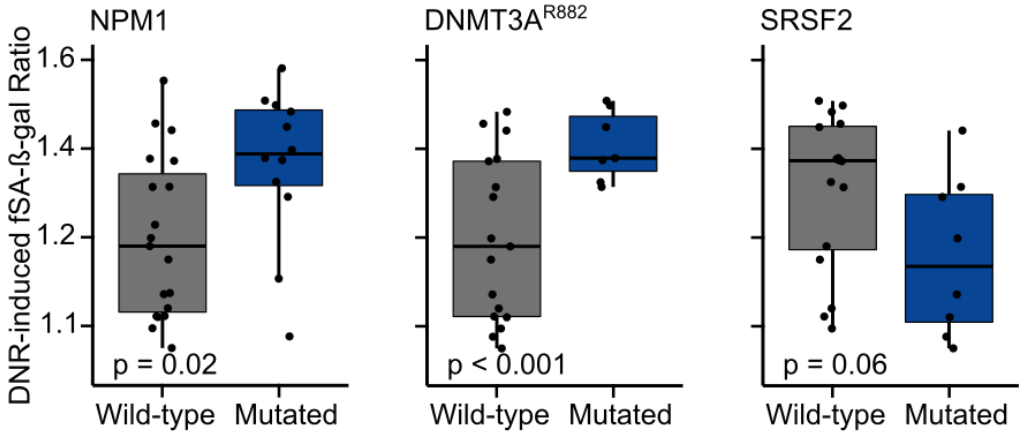


Figure 15. Association of Therapy-Induced Senescence Capability with Recurrent AML Mutations in the "Berlin Cohort": This boxplot panel depicts *ex vivo* daunorubicin (DNR)-induced fSA-β-gal ratios in individual AML samples of the "Berlin cohort" (as in Figure 14) with or without the indicated recurrent mutations. Individual values are displayed as an overlay scatter plot.

Table 4. Association of Therapy-Induced Senescence Capability with Recurrent AML Mutations (Berlin Cohort)

Genetic lesion	Mutation – no./total no. (%)	Mean fSA-β-gal ratio in wild type samples (±SD)	Mean fSA-β-gal ratio in mutated samples (±SD)	*p*-values
ASXL	9/24 (38%)	1.25 (±0.21)	1.27 (±0.16)	0.80
CSF3R	3/24 (13%)	1.27 (±0.18)	1.15 (±0.26)	0.32
DNMT3A^{R882}	7/24 (29%)	1.20 (±0.19)	1.41 (±0.08)	**<0.001**
FLT3-ITD	6/30 (20%)	1.26 (±0.19)	1.33 (±0.19)	0.42
FLT3-TKD	4/30 (13%)	1.26 (±0.19)	1.34 (±0.17)	0.44
IDH1/2	4/24 (17%)	1.25 (±0.18)	1.30 (±0.24)	0.63
NPM1	12/31 (39%)	1.20 (±0.18)	1.36 (±0.17)	**0.02**
NRAS	3/24 (13%)	1.28 (±0.19)	1.09 (±0.10)	0.09
RUNX	4/24 (17%)	1.25 (±0.18)	1.30 (±0.25)	0.63
SRSF2	8/24 (33%)	1.31 (±0.18)	1.16 (±0.18)	0.06
TET2	10/24 (42%)	1.25 (±0.19)	1.27 (±0.20)	0.87

Table 4. Association of Therapy-Induced Senescence Capability with Recurrent AML Mutations ("Berlin Cohort"): Tabular depiction of a stratification of daunorubicin-induced fSA-β-gal ratios in primary AML blast samples of the "Berlin cohort" according to presence of recurrent genetic lesions as in Figure 15. Only aberrations with a detection in samples of $n \geq 3$ patients are displayed.

4.5 Targeted AML Agents Induce Cellular Senescence

I next investigated the role of modern targeted AML therapeutics, which have recently emerged in the standard therapy of AML, both as an addition to induction therapy as well as a component of salvage regimens or therapeutic options of low intensity for elderly or frail patients. I wondered whether these new agents, which, in contrast to the conventional chemotherapeutics cytarabine and daunorubicin, directly (e.g. signaling inhibitors) or indirectly (i.e., antibody-drug conjugates) utilize specific molecular principles, may, at least in part, exert their anti-neoplastic effects via induction of cellular senescence. Hence, I tested *ex vivo* fSA-β-gal inducibility in response to the anti-CD33 antibody-drug conjugate gemtuzumab ozogamicin, the FLT3 inhibitor midostaurin, the DNA-hypomethylating agent decitabine, and the BCL2 inhibitors venetoclax or navitoclax (results for venetoclax and navitoclax are discussed in section 4.6). To complete the list

of commonly used AML drugs, I also investigated the unspecific S-phase blocker hydroxyurea in this regard.

Indeed, hydroxyurea, gemtuzumab ozogamicin and decitabine were found to induce senescence in my fSA-β-gal *ex vivo* assay, albeit to varying extents and, in the case of gemtuzumab ozogamicin, only when stratified for presence or absence of the presumed cellular target principle, i.e., by CD33 surface expression. In respect of midostaurin, some individual AML blast samples showed an increased fSA-β-gal intensity upon treatment, yet the overall effect of midostaurin remained insignificant for the whole tested sample cohort and independent of *FLT3* mutational status (Figure 16). Regarding the latter aspect, it is known that midostaurin also inhibits other tyrosine kinases than FLT3 and thus exerts effects in *FLT3* wild type AML patients as well.[129]

In conclusion, with the exception of midostaurin, senescence induction seems to be a general effector principle of susceptible AML blasts in response to a variety of commonly applied leukemia-targeting agents.

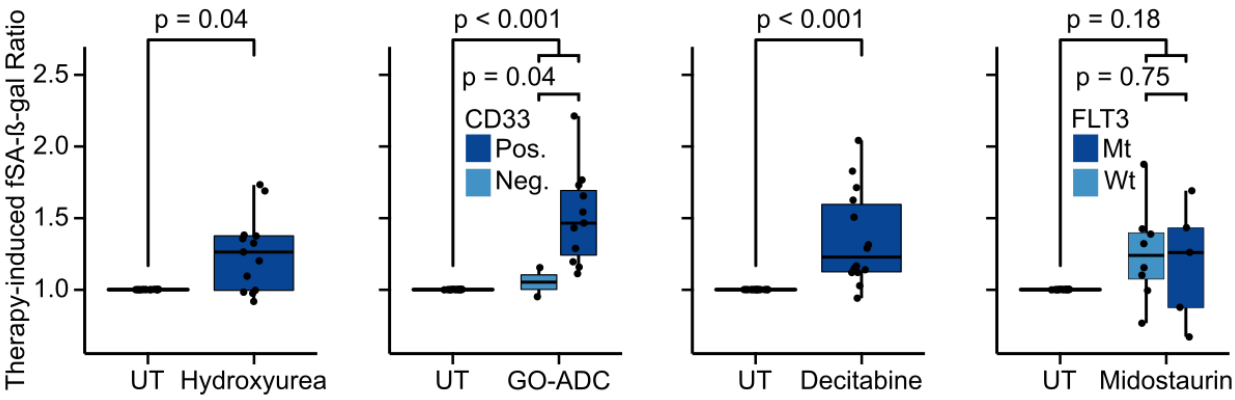


Figure 16. Senescence Induction by Various Anti-Leukemic Agents: Presentation of the pro-senescent potential (as fSA-β-gal ratios) in primary AML blasts from the "Berlin cohort" (*n* = 14) 5 days after *ex vivo* exposure to the anti-leukemic compounds hydroxurea (30µM, left), the anti-CD33 antibody-drug conjugate gemtuzumab ozogamicin (1 µg/ml, GO-ADC, second from left), the demethylating agent decitabine (1µM, third from left) and the FLT3 inhibitor midostaurin (1µM, right) compared to the respective untreated conditions (UT) and stratified by their molecular target expression status (CD33, *FLT3*), where indicated. Unlike daunorubicin and cytarabine, all drugs were readministered after medium exchange and reseeding on day 3 of the *ex vivo* treatment assay.

4.6 BCL2 Family Inhibitors Exhibit Senolytic Potential in AML

Remarkably, in contrast to daunorubicin, cytarabine and the other novel AML therapeutics discussed above, *ex vivo* exposure of otherwise untreated AML blasts to the BCL2 family inhibitor venetoclax significantly reduced the treated-to-untreated fSA-β-gal ratio, i.e., it lowered senescent cell fractions in comparison to basal frequencies (Figure 17).

Importantly, BCL2 family inhibitors have gained enormous interest lately because of their high potential in AML care and recently, the BCL2 inhibitor venetoclax, initially registered for treating chronic lymphocytic leukemia (CLL), was, in combination with azacytidine or low-dose cytarabine, also approved for AML.[130, 131] Notwithstanding its remarkable therapeutic activity, the mode of venetoclax action in AML patients is mechanistically less understood.[18, 64, 65, 130, 132] In the context of other malignancies and age-related diseases in general, BCL2 family inhibitors were shown to mediate secondary cell death in senescent cells, i.e., to promote their selective “senolytic” removal[60, 61] and to thereby alleviate potentially detrimental long-term implications of senescent cells in cancer and chronic conditions.[49, 56, 133]

Consistently with the results obtained with venetoclax, the somewhat broader BCL2-, BCL2L1 (BCL-X_L)- and BCL2L2 (BCL-w)-co-inhibitor navitoclax, which was demonstrated to have an even higher senolytic potential, and is under current clinical investigation in hematological as well as solid malignancies,[134] also caused a reduction of fSA-β-gal intensities after *ex vivo* single-agent treatment in comparison to patient-matched untreated AML blasts (Figure 17).

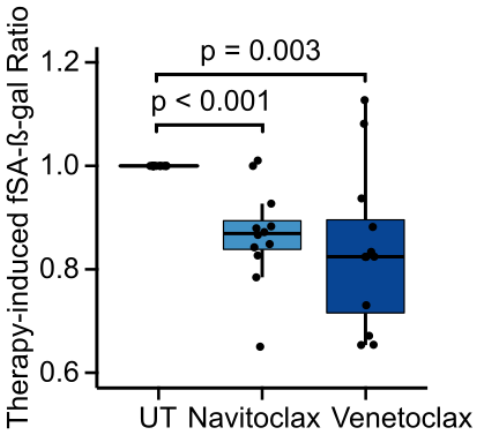


Figure 17. Changes of Senescence Levels by BCL2 Inhibitors: As in Figure 16, this panel presents fSA-β-gal ratios as the relative basal extent of senescent cells (normalized to untreated blasts of the same individual patients, UT, left) after a 5-day *ex vivo* exposure to the BCL2 family inhibitors navitoclax (1µM, middle) or venetoclax (1µM, right) in AML samples of the "Berlin cohort" (*n* = 12). The indicated inhibitors were readministered on day 3 after medium exchange.

Finally, to further investigate this senolytic potential of both BCL2 family inhibitors in the context of AML therapy, I conducted a sequential treatment with daunorubicin followed by venetoclax or navitoclax in a selection of daunorubicin-induced senescence-capable and senescence-incapable AML specimens. This approach enabled me to assess the anti-leukemic efficacy of the combination and its dependence on senescence capability of the individual AML blast samples. Indeed, in this "second-hit" approach, both BCL2 inhibitors effectively reduced viability and cell number in daunorubicin-pretreated blasts. Importantly, these effects were especially pronounced in blast populations with a higher therapy-induced senescence capacity and therefore at least in part senescence-dependent, as illustrated by the only minor effects in AML samples with low senescence capacity (Figure 18).

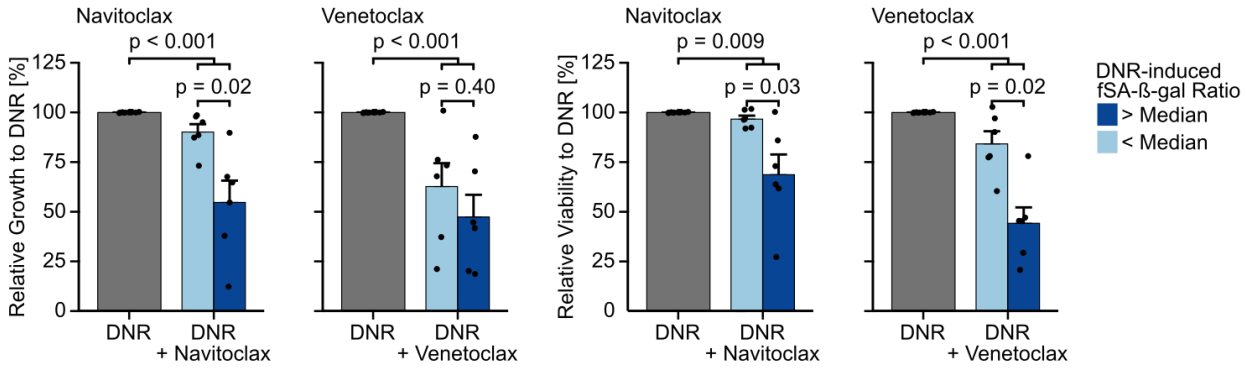


Figure 18. Changes of Growth and Viability by Sequential Senolytic Treatment of AML Blasts After Chemotherapy: These bar plots display relative growth (1st and 2nd plot from left) and viability (3rd and 4th from left) of AML blasts after 5 days daunorubicin (DNR) treatment without or with subsequent exposure to the indicated BCL2 inhibitors (navitoclax, venetoclax, both at 1µM) for additional 72 hours. Results of sequential treatments are substratified into data from senescence-capable and incapable AML blast samples (according to the cohort median of *ex vivo* daunorubicin-induced fSA-β-gal ratios as a marker of therapy-induced senescence capacity). DNR treatment (depicted as the relative control) was solely administered on day 1.

In essence, my data show that the ability to execute therapy-induced senescence is a rather universal response principle to a variety of AML-relevant drugs and may contribute to the long-term effects of conventional chemotherapeutic agents as well as modern targeted therapeutics. With the special potential of BCL2 family member inhibitors to also clear remaining viable blasts via senolysis, a potentially very important but underrecognized mode of action of this substance class in AML care has been additionally demonstrated.

5. Discussion

5.1 Strengths and Limitations of the Newly Established Senescence Assay

Early treatment response assessment in AML focusses on the morphological elimination of leukemic blasts from the bone marrow, and more sensitive DNA-based quantification of minimal residual leukemia burden subsequently.[135] While reduction in tumor load undoubtedly is key to disease control not only in AML, the contribution of alternative, non-apoptotic stress responses in those leukemic blasts that survive the initial exposure to chemotherapy, at least for a while, remains largely understudied. Indeed, this limitation does not only apply to leukemia, but even more to solid malignancies, as repeated tumor biopsies for a dynamic disease assessment are even less accessible and therefore less commonly conducted. Studying cellular senescence as one of these therapy-induced stress responses in patient specimens, although of pivotal importance, has been difficult due to additional, technical constraints, i.e., the lack of a simple, singular senescence-defining marker that is applicable to fresh and fixed tissue material as well as the potentially short timeframe for the detection of a sufficient number of tumor cells to characterize as senescent in re-biopsies. The important question of whether the successful competition of a given tumor cell for senescence over apoptosis as the drug-inducible cell fate might impair long-term outcome beneficially or adversely remains an issue of intense research.[44, 56, 58]

The scope of this dissertation was to investigate this ambiguity of cellular senescence, primarily of therapy-induced senescence in response to leukemia-relevant agents, in AML. In a broader context, the objective of my work was to explore the role of therapy-induced senescence in cancer in a clinically oriented manner and to contribute to the hitherto sparse body of experimental senescence analyses in primary human cancer tissues. On the one hand, while oncogene-induced senescence serves as an initial failsafe mechanism against tumor development,[33, 59] therapy-induced senescence has been demonstrated as a cellular correlate of chemotherapy responsiveness[91] and its SASP possesses tumor-controlling properties in certain scenarios. On the downside, long-term persistence of senescent cells might lead to tumor progression or relapse through an immunosuppressive and tumor-propagating SASP[54] as well as possible epigenetic reprogramming of senescent cells into cancer stemness.[56]

AML appeared to be a suitable tumor entity for an investigation of this general research question as, in contrast to many other (and especially to solid) malignancies, *ex vivo* cultivation of primary tumor cells had already been demonstrated as feasible, even as part of larger scale experiments.[90] Generally, only a few scientific studies have focused on analyses of cellular senescence in patient samples, with some of these reports mostly histopathologically examining replicative, stress-, or oncogene-induced senescent cells in tumor resections.[42, 43, 59] In the context of a breast cancer treatment, it was even possible to demonstrate therapy-induced senescence in response to neoadjuvant chemotherapy.[40] Preclinical *in vivo* data addressing all-trans retinoic acid-induced senescence in APL support a disease-controlling role of therapy-induced senescence in this unique AML subtype.[78, 136]

Prior to my more systematic investigation into the predictive role of senescence in AML, Dörr et al.[83] had already exemplarily demonstrated therapy-induced senescence in an *ex vivo* assessment of primary AML blasts by conventional SA-β-gal staining. Employing my newly established immunofluorescence-based senescence characterization, I was able to replicate, and strengthen these results by demonstration of further senescence features, including upregulation of the cell-cycle inhibitor $p16^{INK4A}$ and of the chromatin methylation mark H3K9me3 as well as downregulation of the proliferation markers Ki67 and EdU incorporation.

My initial assessment of senescence was conducted by conventional SA-β-gal since this assay still represents the gold standard for detection of cellular senescence and is considered independent of particular pathways of senescence induction. Yet, the broad utilization of SA-β-gal is not unproblematic: There are drastic individual differences in β-galactosidase expression levels of the normal/non-senescent condition, depending on tissue and cell type, and, more importantly for my assay, its enzymatic colorigenic substrate degradation is distinctly time dependent. In practice, the assessment of therapy-induced senescence by classical cytohistochemical staining is thus commonly time point controlled against the matched non-senescent (e.g. untreated) control to determine the optimal staining duration (i.e., until the untreated control begins to slightly show the characteristic blue staining, average of 3-12 hours), at which both samples are then quantitatively analyzed. Although the variation of the immunofluorescent staining is smaller, the choice of an approach enabling an inter-sample comparison of therapy-

induced senescence capacities proved difficult. I reasoned that treated vs. untreated ratios of sample-matched fSA-β-gal marker reactivities might represent the best comparative output parameter as it truly reflects intra-individual changes upon treatment and allows an unbiased comparison of all samples without subjective flow cytometry gating strategies.

As part of a 3-marker-fluorescence-based assessment, I chose to combine SA-β-gal with a viability parameter (to exclude dead cells) and EdU as a universal marker for proliferation, and, conversely, for cell cycle arrest if downregulated, to detect senescence. Importantly, except if caused by unspecific fluorescence signals, a double positive staining for EdU and fSA-β-gal is biologically not possible. I decided against a double staining for two senescence markers as it was not clear to me how robustly these less universal (senescence induction pathway-dependent) markers would occur in AML. In summary, there are of course multiple other potentially valid staining and analyzing strategies (e.g., the mentioned combination of two positive senescence staining markers, the comparison of percentage values instead of untreated-to-treated ratios, or the development of a senescent surrogate score with multiple markers as suggested by Haugstetter and colleagues[59]), but, for the reasons discussed above, I decided for my unique analytical strategy. To my knowledge, in particular, the treated vs. untreated ratio approach has not yet been conducted in immunofluorescent fSA-β-gal staining assays and bears the potential for a broad application in senescence cancer research to assess senescence capacities.

Given the complex and unpredictable compartment dynamics that AML blasts may follow during initial treatment in peripheral blood, and considering aleukemic courses, I also decided against an *in vivo/ in patient* approach to assess treatment-induced senescence. The consequently established image flow cytometry-based *ex vivo*-assay was eventually able to faithfully detect viable senescent blasts in response to 5-day incubation with the standard AML induction therapy agents daunorubicin and cytarabine, and allowed a quantitative assessment of senescence capability in primary AML samples.

For the benefit of enabling a systematic investigation of therapy-induced senescence across primary AML samples, my artificial *ex vivo* assay could not reflect the microenvironment of the bone marrow niche as a key player in the pathogenesis of AML, therapy resistance of the disease and the varied pharmacokinetics of AML drugs (e.g.,

via induction of the cytochrome P450 system in bone marrow stromal cells).[137, 138] It has even been demonstrated that AML blasts can induce $p16^{INK4A}$-driven senescence in the bone marrow environment, thereby creating a feedback loop to promote AML blast survival via effects of the SASP.[139] Furthermore, a potential impact of inter-blast interactions via SASP on the resulting overall senescence levels *in vivo* was probably also affected by the constant leveling of cytokines in my *ex vivo* culture system. Repeated bone marrow punctures would theoretically allow detection of senescent bone marrow AML blasts, but are prohibited for ethical reasons and would also not allow further investigations of other (e.g. experimental) drugs or combination therapies.

The *in vivo* monitoring of therapy-induced AML blast senescence in peripheral blood was conducted to confirm that therapy-induced senescence is not just an artificial laboratory culture phenomenon, but, due to the described blast clearance and compartment shifts, the *ex vivo* approach was eventually favored. In addition, peripheral blasts detected in *in vivo* assays do not necessarily reflect AML biology in the bone marrow niche, especially the quantity of senescent cells. (Please note that my primary *ex vivo* leukemic blast cultures were predominantly derived from diagnostic bone marrow punctures.)

In my view, the further development of my *ex vivo* assay into two directions offers the opportunity to circumvent the described limitations: Firstly, a more advanced primary AML *ex vivo* culture system with co-cultivation of individual patient's bone marrow mesenchymal stromal and immune cells could further elucidate AML blast – bone marrow interactions. Secondly, flow cytometric minimal residual disease monitoring with the integration of senescence parameters during AML induction therapy would possibly enable reasonable *in patient* monitoring of senescence.

5.2 Implications of Therapy-Induced Senescence as a Predictor of Outcome

My analyses based on primary patient material from two independent patient cohorts demonstrated the ability of AML blasts to execute senescence as a relatively universal response principle to a variety of AML-relevant drugs. Moreover, using my newly established *ex vivo* assay, I found that senescence capacity in response to daunorubicin is able to predict superior long-term outcome in AML as a biomarker with respect to both disease-free and overall survival. The ability to launch a senescence response was significantly correlated with established prognostic factors known to correlate with a better

clinical course of patients, such as particularly a more favorable risk group in the ELN genetic risk stratification, a normal AML karyotype or *NPM1* mutational status. I speculate that therapy-induced senescence might in part explain the underlying biology of these current paraclinical risk indicators. In general, my findings strongly argue for an at least initially beneficial effect of senescence induction in AML, as the ability of leukemic blasts to enter this failsafe mechanism constitutes a barrier to tumor progression and enables responsiveness to senolytic treatment approaches.

A brief remark regarding the view of therapy-induced senescence as a predictive (and not solely prognostic) marker: Only a few biomarkers and risk factors are, by formal definition, predictive in patients with AML, i.e., can tell *a priori* if a specific therapy modality has a preferential prognostic impact compared to other treatment options.[14] Following this interpretation, therapy-induced senescence could be regarded as a predictive marker for a sequential senolytic treatment approach in AML, as the detected senescence capability would enable the successful application of senolytic agents after induction therapy, in particular venetoclax or navitoclax. By strict definition, the value of therapy-induced senescence capacity in both AML cohorts regarding disease-free and overall survival is not a predictive marker of a certain therapy response to different treatment modalities (and this was not the subject of my investigation). Yet, the investigated patient cohorts were predominantly treated with a “7+3” induction therapy, and disease-free and overall survival remain the most relevant and generally applied measures of long-term treatment efficacy. Moreover, therapy-induced senescence is, by definition, an anti-cancer treatment-evoked phenomenon, even if in my investigation it was merely analyzed *ex vivo*. Hence, I suggest to further validate and characterize it as a predictive marker of AML induction therapy and possible treatment guidance for senolytic treatment sequences, but also to be aware of its general prognostic impact in AML.

Beyond the mere detection of senescence capability and characterization of its predictive/ prognostic value, I was also able to validate a whole range of senescent features in daunorubicin-treated AML blasts by gene expression analyses. These not only included upregulation of senescence markers but also different features of SASP and its pro-inflammatory microenvironment, aberrant TGF-β- and Wnt-signaling (reflecting possibly paracrine mediation of senescence, also denoted as secondary senescence), as well as modulation of immune responses. This in-depth characterization of senescent AML blasts

is a prerequisite to the incorporation of my findings into the discussion about diverse implications of therapeutically-induced senescent cancer cells and their potential impact on clinical courses of patients beyond the investigated immediate treatment response.

Even before the described barrier of therapy-induced senescence against tumor progression, senescence is a well characterized tumor-suppressive mechanism during imminent oncogenic transformation in pre-neoplastic lesions. Concomitant senescence is suspected to account, via its associated SASP, for chronic inflammation as an underlying pathogenic principle of various age-related diseases.[44] In cancer, therapy-induced senescence as a lasting proliferative arrest certainly complements apoptosis as a terminal cell-cycle exit program, and paracrine spreading of senescence to neighboring cancer cells as well as enhanced clearance of senescent cells by host immunity might further explain its tumor-controlling role.[58] My work was further able to demonstrate the role of therapy-induced senescence as an initially tumor-controlling mechanism when comparing AML blasts capable of senescence to their counterparts that had overcome the barrier of senescence. Conversely, pro-inflammatory SASP, reprogramming of senescent tumor cells into latent cancer stem cells, and the option of an occasional cell-cycle re-entry of those cells collectively raise concerns as to how desirable senescence might be as a persistent tumor cell state.[44, 56, 78] The impact of remaining senescent cells with an altered metabolic status and features of cancer stemness promoting inflammation and tumor growth in the bone marrow niche is more difficult to conclude from my work. However, I was able to validate that daunorubicin-induced senescent AML blasts indeed displayed features of a SASP, possibly induced by an upregulation of the rather tumor propagating and immunosuppressive JAK/STAT-pathway, as well as transcriptional induction of the Wnt-/β-catenin-pathway, which has been, explicitly in primary AML blasts, described to serve as a surrogate marker of cancer stemness and possible senescence escape.[56] In general, senescence-centered clinical long-term observations are still sparse in the scientific literature, but preclinical tumor models, cross-species translation of findings made therein to patient cohorts, and indirect analyses in patient materials at diagnosis suggest a primarily beneficial role of senescence as a drug effector principle *in vivo*.[33, 69, 140] Yet, in particular, the difficulty to directly or indirectly assess therapy-induced senescence at diagnosis due to the need for a therapeutic trigger to reveal the senescence phenotype instead makes these investigations difficult to conduct. Importantly, my thesis, as well as most presented studies, compares therapy-induced

senescence capability to its loss, but not long-term persistence of senescent cells to their eradication, which is likely to have different prognostic implications for the course of a malignant disease. I believe that further functional analysis in preclinical tumor models and implementation of innovative approaches in minimal/measurable residual disease monitoring with the integration of biological characterizations of detected AML blasts would provide deeper insights into possible long-term implications of senescent AML blasts remaining in the bone marrow niche.[141] In particular, multiparameter flow cytometry, already established in AML diagnostics, could combine the monitoring of minimal residual disease and biological state changes (such as senescence) of the detected AML blasts in the future.

From my point of view, analyses of other malignancies are likely to eventually uncover therapy-induced senescence as a rather uniform and prognostically relevant drug effector principle across cancer entities. My study also strongly underscores the need for a more dynamic view on cancer state switches during the course of disease and therapy.

In my analysis of the publicly available TCGA AML cohort as well as in my own AML test cohort, I did not find any prognostic relevance of a basal assessment of spontaneous, possibly oncogene and/or replicative stress-induced senescence. Furthermore, there was no evidence for a correlation between spontaneous senescence and susceptibility to treatment-induced senescence in my AML cohort, unlike previously suggested for metastasized colorectal cancer.[59] Thus, basal and typically low fractions of senescent cells in untreated blast populations were found not to indicate superior or inferior patient outcome. It was only the treatment-inducible change in biology that unveiled novel prognostic information.

However, whether spontaneous or oncogene-induced senescence might play a role in leukemogenesis, particularly in the secondary development of AML out of MDS, was not addressed within this project. Observational studies of senescence markers (SA-β-gal, $p16^{INK4a}$, p21) in MDS and AML patients as well as in healthy donors suggest a similar senescence-associated barrier as operating against melanogenesis, but functional studies of its role in leukemogenesis and MDS are still pending.[42, 73-75] Additionally, the contribution of bone marrow mesenchymal stromal cells to the pathogenesis of MDS is well-acknowledged. Interestingly, these cells have recently been

characterized with a prominent senescent phenotype, explicitly in MDS, and might thus contribute to paracrine (secondary) induction of senescence in MDS blasts as well. Albeit, similarly, MDS blasts could also cause the senescent phenotype of bone marrow mesenchymal stromal cells.[142] I think that further investigations of senescence in MDS, and explicitly in a MDS to AML comparison, could lead to therapeutical implications for MDS treatment (which are discussed in the “senolysis” section 5.5).

5.3 Interpretation of Varying Senescence Capacities in AML Genetic Subgroups

Despite the association of two particular karyotypic aberrations (t(8;21), RUNX1-RUNX1T1; inv(16), CBFB-MYH11) with a more favorable prognosis, cytogenetic abnormalities in general, and especially a complex karyotype, are important prognostic parameters in AML which indicate adverse prognosis and poor therapy response. In the “Berlin cohort”, I showed a strong correlation of senescence capacity with normal karyotype status and therefore genomic integrity.[10] In the karyotypically normal “Munich cohort”, I observed that therapy-induced senescence is also a predictive marker independent of the profound impact of karyotype aberrations.

The strong interaction of genomic integrity, reflected by a normal karyotype status, and senescence capability most likely not only corresponds to the reciprocal correlation of two strong prognosticators, but also has a biological rational. As early as in the first description of (replicative) senescence by Hayflick and Moorhead, telomere shortening was assumed as the primary effector principle for senescence induction and, therefore, telomerase activity as an important senescence bypass mechanism.[35] In the pathogenesis of karyotypically altered AML, intense telomere loss by shortening of telomeric DNA or distinct telomere-capping proteins leads to a strong activation of telomerase and, consequently, genetic instability, which in turn results in chromosomal abnormalities. Notably, the shortest telomere lengths as well as strongest expression of telomerase reverse transcriptase (hTERT) were found in AML patient samples with multiple karyotypic aberrations. Concomitantly, telomerase activity can prevent AML blasts from replicative senescence, thereby enabling their clonal selection and, ultimately, disease progression.[143, 144] Despite the strong evidence of this mechanism of action and further detailed validation in other entities, i.e., development of malignant lesions from human mammary epithelial cells,[145] direct co-staining assays of SA-β-gal and hTERT in (*ex vivo*) treated AML blasts are needed to further validate these findings.

In my analysis of recurrent genetic aberrations in respect of their association with AML blast senescence capacity, I identified three lesions with significant differences between mutated and wild type samples regarding their ability to execute senescence upon chemotherapeutic treatment. First, AML blast samples carrying an *NPM1* mutation presented with a significantly higher therapy-induced senescence capacity than their wild type counterparts. Mutated *NPM1* status on its own is a strong prognostic factor in AML indicating a considerably superior long-time prognosis after induction therapy. Therefore, detection of *NPM1* mutation in the absence of adverse prognostic factors can guide treatment towards less intense consolidation therapies (i.e., without allogeneic hematopoietic stem cell transplantation). Accordingly, my observation that therapy-induced senescence positively correlates with mutated *NPM1* status in AML could also be explained by the mere interaction of two favorable risk factors. Yet, as discussed for karyotypic aberrations already, there is again scientific evidence for a biological link to senescence. Cheng and colleagues[146] have already demonstrated that a pathogenic *NPM1* mutation can specifically cause oncogene-induced senescence in classical transformation assays (conducted in primary mouse embryonic fibroblasts), although by a yet unresolved pathway, as it seems to also block $p19^{ARF}$ induction (the murine equivalent to human $p14^{ARF}$), which is generally regarded as a pro-senescenct tumor suppressor. This mechanism of oncogene-induced senescence could potentially be reinforced by cytotoxic chemotherapy.

DNMT3A is a de novo DNA methyltransferase and regulator of hematopoietic stem cells and its mutated status is strongly associated with age and clonal hematopoiesis. As with *NPM1*, mutated *DNMT3A* status was found to display a higher therapy-induced senescence capacity in comparison to *DNMT3A* wild type AML blasts. The prognostic implications of similarly common *DNMT3A* mutations on therapy response and/or prognosis in AML are not as clear as for *NPM1*. A mutation in *DNMT3A*, in AML predominantly a missense mutation in codon R882, results in lower DNMT3A protein levels, reduction of DNMT3A methyltransferase activity and, consequently, in a genome-wide hypomethylation.[147] There are no studies which directly connect *DNMT3A* aberrations to senescence in myeloid neoplasms, but a scientific analysis in colorectal cancer suggested that low levels of DNMT3A, as occurring with a mutated variant, increase the extent of anthracycline (i.e., doxorubicin)-induced senescence via upregulation of $p21^{CIP}$.[148]

In contrast to *NPM1* and *DNMT3A*, mutated *SRSF2* status was associated with a trend towards reduced therapy-induced senescence capacity in my study. *SRSF2* is a member of the serine and arginine protein family which is involved in constitutive and alternative mRNA splicing. Mutations in *SRSF2* are associated with an adverse clinical outcome[11] and known to drive oncogenesis by influencing widespread modifications in multiple RNA processing and splicing proteins.[127] Several scientific reports highlight the (dys)regulation of senescence by the differential activity and functional status of splicing factors, especially from the SRSF family, which leads to an alternative splicing of DNA repair-, telomere maintenance- and chromatin components, and thus genomic instability.[149, 150] Possibly, the most common missense variant (both in the general AML population and in my "Berlin cohort") *SRSF2*^{P95H} also negatively affects genomic integrity by reducing levels of SRSF2, and thereby undermines senescence capacity, which could have led to the observed trend for lower levels of therapy-induced senescence in *SRSF2* mutant samples. Yet, this assumption remains purely speculative at present and certainly needs to be further investigated, especially since a SRSF2-caused oncogenic transformation (which would be expected to lead to reduced genomic integrity) could potentially also trigger oncogene-induced senescence.

Several scientific studies have demonstrated an impact of further recurrent genetic lesions on senescence induction in AML, namely the leukemogenic fusion proteins BCR-ABL, CBFB-MYH11 and RUNX1-ETO[151] as well as *ASXL1* mutations.[152] Yet, their potential effect on drug-induced senescence could not be revealed or validated in my study, possibly due to the multi-genetic and aberrant karyotypic background of my primary AML cohort of limited size and low prevalence of some mutations. Pro-senescent and senescence-repressive mechanisms influence each other in a complex signaling network,[44] so that when wild type and mutated status for one genetic lesion were compared across my AML blast sample cohort, a strong impact of concomitant additional genetic aberrations had to be expected. With an increasing sample number and higher frequency of individual recurrent mutations, the effect of some lesions on therapy-induced senescence will probably become clearer as effects of "genetic background noise" become less confounding. Nevertheless, in clinical reality, every individual AML represents a disease with several genetic lesions which, in addition, interact complexly and determine each other's biological effect. Therefore, my finding of an increased therapy-induced senescence capacity in AML blasts carrying a *NPM1* or *DNMT3A*^{R882}

mutation and a tendency towards a decreased ability to undergo senescence of samples with a *SRSF2* mutated status can be considered even more meaningful.

In conclusion, the predictive value of senescence capacity for individual patient outcome and its correlation with selected established risk stratificators (i.e., ELN genetic risk stratification, normal karyotype status as well as *NPM1* mutational status) characterize therapy-induced senescence as a fundamental biological principle which contributes to favorable clinical outcome in AML. Yet, higher senescence capacity of the clinically rather unfavorable *DNMT3A* mutational status, and a tendency towards a suppressed senescence susceptibility in AML samples of my cohort with *SRSF2* mutated status, underscore the need for mechanistic follow-up investigations in order to fully elucidate the impact of these recurrent genetic lesions on therapy-induced senescence capacity.

5.4 Potential of Targeted AML Agents in Senescence Induction

My findings indicate that, besides cytotoxic chemotherapeutic drugs, in addition targeted non-chemotherapeutic agents and ADCs, which have recently been more commonly employed in AML, may induce senescence. The mechanism behind this finding, i.e., whether it is a universal ability of senescence-capable AML blasts to recapture a stress-responsive cell-intrinsic machinery or a more targeted, drug-specific effect, remains unclear. Although the mere observation that almost all investigated agents held the potential to mediate senescence in AML blasts indicates the former, there are several studies which elucidate drug-specific effects on senescence of the indicated AML therapeutics, which will be discussed in the following section.

Hydroxyurea is a cytoreductive agent which is often used as a pre-phase treatment before AML induction therapy (e.g. in the cases presenting with initial hyperleukocytosis) and was found in my study to be capable of senescence induction. One of the main antineoplastic mechanisms of hydroxyurea is a non-alkylating inhibition of cell cycle progression by inhibiting the enzyme ribonucleotide reductase.[153] Additionally, it was shown to induce erythroid differentiation in leukemic cell lines via stimulation of JUN synthesis.[154] Hydroxyurea-mediated induction of senescence has already been demonstrated and characterized in fibroblasts, including upregulation of H3K9me3 and $p16^{INK4a}$, SA-β-gal staining and formation of senescence-associated heterochromatin foci,[155] as well as in leukemic cell lines, evidenced by upregulation of the senescence

markers $p16^{INK4a}$, $p21^{Waf1}$ and $p27^{Kip1}$.[156] Whether cells are driven into differentiation or senescence by hydroxyurea is complexly regulated by hydroxyurea-mediated induction of FOS and JUN as part of the AP-1 transcription network.[121] In a recent collaboration with two other research groups that further investigated the role of FOS/JUN as part of the AP-1 complex, our laboratory proved in different senescence models that AP-1 constitutes a key component for an epigenetic and transcriptional senescence initiation process.[122] Notably, both FOS and JUN are among the most prominently activated players in my transcription factor analysis of daunorubicin-induced senescent AML blasts (Figure 10).

Gemtuzumab ozogamicin is an antibody-drug conjugate which targets CD33 surface positive AML blasts with the CD33 antibody-linked DNA-damaging calicheamicin derivate ozogamicin. In my *ex vivo* treatment assay, this compound was also able to induce senescence under strict dependence on the expression of its surface target. To my knowledge, my study is the first one to report that gemtuzumab ozogamicin, or the class of calicheamicins in general, can mediate senescence. However, this finding is easily explainable, as ozogamicin-induced DNA double-strand breaks are difficult to repair in cycling cells and senescence can be induced as a cellular chemotherapy effector program, as it was established for other drugs which induce DNA double-strand breaks.[157, 158]

The DNA hypomethylating agent decitabine also mediated significant senescence responses in my *ex vivo* treatment of primary AML blasts. Previous studies in fibroblasts have indicated that the induction of senescence by decitabine strongly reflects features of replicative-induced senescence, as demonstrated by an overlap of gene expression profiles and with telomere shortening as the proposed underlying mechanism of action.[159] Yet, besides the mere description of senescence induction by decitabine in cancer cells, as for AML in my study, or likewise for other entities (CML,[160] lymphoma,[161] cholangiocarcinoma[162]) as well, its precise mechanism of action in malignant cells remains unclear.

In contrast to the other investigated non-chemotherapeutic agents, treatment with the multi-tyrosine kinase inhibitor midostaurin, which is approved for the treatment of *FLT3*-positive AML, was not able to cause significant senescence levels across my AML blast sample cohort in the untreated vs. treatment comparison, albeit some individual AML blast samples did show an increased degree of fSA-β-gal positivity. In line with this,

in my senescence association analysis for recurrent genetic lesions, *FLT3* mutations did not show significant differences in senescence capability between wild type and mutational status.

Mutations of the FLT3 receptor appear in two different manners: as an internal tandem duplication (ITD) and in the tyrosine kinase domain (TKD). *FLT3-ITD* mutational status is an adverse prognostic risk indicator, whose impact on prognosis depends on its mutant-to-wild-type allelic ratio and the accompanying *NPM1* mutational status. The prognostic implications of the *FLT3-TKD* variant are controversial.[10, 163-166] Both types of mutations (i.e., ITD and TKD) constitutively activate the FLT3 kinase activity and thereby act as oncogenic AML drivers.[167] As a conclusion from my study demonstrating therapy-induced senescence as a positive predictor of therapy response, one could have expected an impaired therapy-induced senescence capacity of *FLT3-ITD* mutated compared to *FLT3* wild type AML blast samples. However, this hypothesis was not supported by my data. One of the confounding factors which could have masked a possible impact of *FLT3-ITD* in this regard is the high concomitant occurrence of the senescence prone *NPM1* mutations (in 66% of all samples with *FLT3-ITD* mutations in my cohort). Additionally, the wide range of *FLT3-ITD* allelic ratios with diverging prognostic implications might have further complicated my analysis of *FLT3-ITD* aberrations regarding their effect on senescence capacity. For a valid subgroup analysis without these confounding factors, a significantly larger AML sample cohort would have been required.

Alongside my investigations of the potential of other AML therapeutics to induce senescence, I also investigated midostaurin, which inhibits the autophosphorylation of FLT3, but also other tyrosine kinases such as protein kinase C (PKC), c-kit, platelet-derived growth factor receptor β (PDGFR-β) and vascular endothelial growth factor (VEGF).[168] To the best of my knowledge, there are no scientific studies which report the ability of midostaurin to induce cellular senescence. However, therapy-induced cancer cell senescence has been described in response to other tyrosine kinase inhibitors, such as dasatinib[169, 170, 171] or lapatinib.[172] In summary, although neither *FLT3* mutational status nor FLT3 inhibition by midostaurin significantly altered therapy-induced senescence capacity in my AML sample cohort, due to the presented complex biological properties of the *FLT3-ITD* mutation as well as the attractive option of its pharmacological

inhibition, a possible interaction with senescence certainly deserves further investigations in functional assays.

Likewise, further investigations of other established targeted AML agents, e.g. IDH inhibitors, and uncovering of senescence-inducing mechanisms of targeted AML therapeutics might help to distinguish between general stress-responsive and targeted drug effects. AML therapeutics which greatly induce senescence in AML blasts might be of special interest for sequential senolytic treatments (which are further described in the following section). Concurrently, purely speculative, novel agents which are effective in AML treatment, but without a notable senescence induction in senescence-capable blasts by utilizing other drug-effector principles, might be interesting as they possibly circumvent the undesirable long-term effects of remaining senescent AML blasts.

5.5 Senolysis as a "Second Hit" Treatment Approach in AML

Despite its positive contribution to long-term outcome primarily, therapy-mediated senescence potentially exerts detrimental long-term effects if those senescent AML blasts persist for extended periods of time. These adverse influences on the courses of diseases could not be detected in my correlation of senescence capacity with patient data due to a domination of the beneficial effects of senescence induction. However, negative long-term implications of senescence have been convincingly demonstrated by experimental studies in mouse tumor models[56] and are also reflected by my gene expression analysis of senescence-capable AML blasts, indicating an upregulation of the inflammatory SASP, deregulation of immune responses and acquisition of stemness characteristics (through upregulation of the Wnt/β-catenin pathway). Such considerations of the long-term effects of remaining senescent AML blasts are important from my point of view and require dynamic in-depth analyses in future studies.

To address the probable downsides of persisting treatment-induced senescent AML blasts, I tested the emerging idea of senolytic treatments, which describes the potential of drugs to selectively eliminate senescent cells, in my *ex vivo* therapy setting. So far, primarily the field of gerontology/aging has pursued the concept of senolytic therapies to experimentally treat SASP-mediated chronic inflammatory diseases, e.g., atherosclerosis and neurodegenerative disease, improve physical dysfunction and ultimately extend the healthy lifespan.[49-51] Senescent (cancer) cells are difficult to target selectively as, by their defining biology, they do not proliferate but are cell cycle arrested

instead. This largely precludes the successful application of (cell cycle-interfering) cytotoxic (chemo)therapeutics. To permanently persist in their respective tissues, senescent cells, and especially senescent cancer cells, are notably resistant to apoptosis, which protects them from DNA damage as well as signaling that is triggered by their own pro-inflammatory secretions and reactive metabolites.[173, 174] Instead, the most likely mechanism of physiological elimination of senescent cells *in vivo* is clearance by the immune system, especially the innate immune system.[47]

In small interfering RNA (siRNA) screening studies for mechanistic senolytic principles, in particular, the inhibition of BCL2L1 (BCL-X_L) and other BCL2 family members was effective in causing apoptosis of senescent cells, and, consequently, BCL2 family inhibitors were among the first, most promising senolytic drug candidates.[62, 175] This makes sense from a physiological perspective as senescent cells are protected from an induction of the mitochondrial-mediated apoptosis pathway by a redundant upregulation of antiapoptotic BCL2 family members.[60, 61, 176] Importantly, senolysis via BCL2 inhibition was not observed in all senescent cell types under investigation. Hence, it is of particular significance that the broad BCL2-/ BCL2L1-/ BCL2L2 inhibitor navitoclax was shown to deplete senescent hematopoietic stem cells.[60, 61]

In my work, I clearly demonstrated a potential of BCL2 family inhibitors to operate as senolytics in AML. Of the various anti-leukemic agents that I assessed as mediators of senescence, venetoclax and navitoclax were the only compounds that reduced basal frequencies of senescent cells in previously untreated AML blasts *ex vivo*, an indication of senolysis acting against replicative- or oncogene-induced senescent cells in this setting. More importantly from a therapeutic perspective, both agents also selectively eliminated leukemia cells as a “second hit” if AML blasts had entered senescence in response to the classic AML induction drug daunorubicin as the “first hit”. This observation might help to further elucidate the less intuitive mode of action venetoclax appears to utilize with great promise in AML in combination with azacytidine and low-dose cytarabine,[130] two anti-leukemic drugs which we found to be capable of inducing senescence as well. In essence, my finding of BCL2 inhibitors effectively operating as a senolytic treatment against previously therapy-induced senescent AML blasts implies the option to therapeutically address the previously discussed concerns of a relapse-promoting potential of persistent senescent tumor cells after an initially beneficial pro-senescent chemotherapeutic treatment.

In my gene expression analysis, the estrogen receptor alpha (ESR1) was among the most differentially upregulated transcription factors in daunorubicin-induced senescent AML blasts. ESR1 is druggable by the multi tyrosine kinase inhibitor and antioxidant quercetin, the anti-tumor activity of which has explicitly been characterized as ESR1-dependent.[177] Interestingly, quercetin has also recently been identified as another senolytic agent[61] and might therefore represent another particularly interesting candidate to further investigate sequential senolytic "second hit" treatment strategies in AML.

Certainly, further *in vivo* animal studies are necessary to investigate the efficacy and tolerability of senolytic sequential treatment schedules in AML. A conceivable integration of senolytic concepts into the established AML treatment algorithm could comprise "7+3" induction chemotherapy, followed by cytarabine-based conventional consolidation therapy with prior, simultaneous and/or subsequent short-lasting senolytic treatment cycle(s), e.g. with venetoclax or navitoclax. My established *ex vivo* treatment and senescence detection assay might serve as a predictive biomarker for the identification of individual AML patients who are likely to profit from such senolytic treatment sequences. However, additionally if only a small fraction of AML blasts turns senescent upon induction therapy, i.e., if classified as senescence-incapable specimen by my assay, corresponding patients might profit from such a "second hit" senolytic treatment in the long-term, as even a few remaining senescent cells could be sufficient for the creation of an adverse inflammatory environment in the bone marrow niche and/ or reprogramming into relapse-driving cancer stem cells.

In general, sequential combination treatment schemes in cancer therapy not only offer the possibility to more effectively eliminate malignant cells and reduce the risk of a subsequent relapse, but also to decrease toxicities to the host by reduction of individual drug doses or number of therapy cycles. In respect to AML, this could translate into reduction of chemotherapeutic consolidation cycles and potentially even circumvention of the need for allogenic stem cell transplantation. Due to the impressive clinical activity of venetoclax in elderly or treatment-refractory AML patients, several clinical trials are currently being conducted with the aim to integrate venetoclax into first line "7+3" induction therapy in patients who are eligible for intensive chemotherapy regimens (ClinicalTrials.gov [Internet]. National Library of Medicine [U.S.] Identifier: NCT03629171, NCT02115295, NCT04628026). However, these clinical trials administer venetoclax simultaneously to induction and consolidation treatment cycles. This schedule might not

be ideal in light of my evidence for senescence induction and senolysis as efficacious biological effector principles with consecutive or alternating treatment cycles.

In comparison to the BCL2-inhibitor venetoclax, the broader BCL2-/ BCL2L1-/ BCL2L2-inhibitor navitoclax seemed to have a more pronounced senolytic potential in my *ex vivo* treatment assay. This effect of navitoclax has already been specifically described by Chang and colleagues for hematopoietic stem cells[60] and is also validated from a genetic perspective: Due to the redundant activity of BCL2 and BCL2L1, only short hairpin RNA (shRNA)-mediated simultaneous downregulation of both transcripts was sufficient to selectively clear senescent hematopoietic stem cells. In contrast to venetoclax and despite its assumed stronger therapeutic effect, navitoclax has not yet been approved by the FDA or EMA for any medical indication due to dose-dependent thrombocytopenia that occurred as grade 3-4 adverse event in more than half of the phase 1 dose escalation trial patients.[134] However, in this trial, as well as other ongoing clinical studies, navitoclax was applied as a single agent or as part of a combination therapy regimen for 14 days of repetitive 21-day cycles. In a suggested sequential senolytic “second hit” strategy, less intense navitoclax treatment cycles might be sufficient and less likely to result in dose-limiting thrombocytopenia. Additionally, a more biologically grounded application of navitoclax and venetoclax as senolytic drugs in sequential therapy schemes holds promise of increased treatment efficacy.

Another interesting perspective, which has not been addressed in this work, is the *a priori* application of senolytic drugs in high-risk MDS patients to prevent the detrimental effects of senescent MDS blasts so as to ultimately prevent transformation into AML. This approach clinically translates the concept of senescence as an (oncogene-induced) barrier to tumor propagation (similar to the naevi-to-melanoma transformation as another attractive clinical setting for a potential senolytic therapy).[42] Moreover, it also incorporates the idea of eliminating senescent dysfunctional tissue cells to mitigate age-related illnesses such as atherosclerosis and neurodegenerative diseases by “rejuvenating” the aged tissue stem cell pool.[49-51]

5.6 Outlook

In an initial evaluation of senescence, I confirmed my image-based detection of immunofluorescent SA-β-gal as a robust marker of AML blast senescence (*ex vivo* and *in vivo*) by cross-validation with other senescence- and proliferation-associated

parameters as well as senescence-characteristic gene expression patterns. My newly established method for longitudinal, intra-individual senescence quantification by an enzymatic two-time-point assay, which I conducted *ex vivo* in freshly isolated, untreated vs. daunorubicin-exposed AML blasts, allowed me to directly measure therapy-induced senescence susceptibility in primary individual patient material.

I suggested and validated drug-induced senescence capacity, reflected by the ratio of fSA-β-gal reactivity in treated vs. untreated blasts, as a potential novel predictive biomarker in AML in two separate patient cohorts and proposed its future application for personalized treatment guidance, in particular for sequential cytotoxic - senolytic therapy strategies. Although my assays were carried out using an elaborate cell culture system and a high-end imaging flow cytometer to scientifically study a variety of senescence features in greater depth, they could potentially become available, in a simplified version, in any larger leukemia-specialized routine laboratory with standard flow cytometers.

To date, extensive cytogenetic and molecular genetic tests at initial diagnosis or at disease recurrence and peripheral blood- or bone marrow-derived minimal residual disease analyses during therapy are used to guide treatment decisions in AML. However, none of these approaches is suited to detect markers of senescence or anticipate senescence potentials of individual AMLs. My data argue for the implementation of biological, therapy-evoked conditions as another component of personalized leukemia care. Therefore, I suggest integrating these dynamic biological properties, particularly cellular senescence, more vigorously into molecularly informed future AML care. A first logical step would be the implementation of senescence diagnostics in AML trials testing innovative treatment sequences of conventional or novel drugs that have, in part, already been characterized with regards to their property to mediate AML senescence in my work. Based on the striking activity of BCL2 family inhibitors in my assessment and their emerging senolytic potential, I argue for a deeper investigation of venetoclax and navitoclax in experimental tumor studies as well as the integration of senescence-specific assays into translational programs of clinical studies employing these drugs. A wider application of senescence measurements in larger AML cohorts would also allow the investigation of senescence capacities of more infrequent subgroups, e.g., *IDH 1/2* mutated or core binding factor-altered AML samples, and the respective targeted therapies.

Moreover, I expect my major findings, the prognostic and predictive relevance of therapy-induced senescence, not to be restricted to AML, as studies in other entities have already suggested.[33, 43, 59, 172] I did not detect a similarly predictive role of baseline, potentially oncogene- or replicative stress-induced senescence in AML as has been described for colorectal cancer.[59] Yet, the integration of markers into clinical routine testing with a broad *ex vivo* and *in vivo* assessment of baseline senescence and therapy-induced senescence capabilities at diagnosis, during the course of therapy and in relapsed situations could further unravel the diverse clinical implications of senescence as a double-edged sword. Intriguingly, while my study emphasizes the beneficial property of a drug-responsive senescence effector program in contrast to a reduced or absent senescence capacity, Duy et al.[178] very recently exposed its “dark side” by demonstrating that primary AML cells which enter a senescence-like phenotype in response to chemotherapy can facilitated AML relapse in the long term. These two new studies illustrate the dual nature of AML senescence and demonstrate the high relevance of, and the need for, further investigation of senescence in AML as well as other tumor entities.

6. Appendix

6.1 References

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6.2 Statutory Declaration

"I, Martin Schönlein, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Prognostic Implications of Cellular Senescence in Acute Myeloid Leukemia", independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

[In the case of having conducted your doctoral research project completely or in part within a working group:] Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Curriculum Vitae

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

List of Publications

Peer-Reviewed Publications

Däbritz JHM, Yu Y, Milanovic M, Schönlein M, Rosenfeldt MT, Dörr JR, Kaufmann AM, Dörken B, Schmitt CA. CD20-Targeting Immunotherapy Promotes Cellular Senescence in B-Cell Lymphoma. Mol Cancer Ther. 2016;15(5):1074-81.

Conference Contributions

Schönlein M, Fründt T, von Felden J, Schulze K, Behrends B, Renné T, Adam G, Lohse AW, Wege H. First real-life experience with atezolizumab plus bevacizumab in the treatment of advanced hepatocellular carcinoma. EASL Liver Cancer Summit 2021. (Poster).

von Felden J, Schönlein M, Behrends B, Casar C, Fründt T, Jung C, Krause J, Ittrich H, Haddad M, Renné T, Heumann A, Li J, Fischer L, Lohse AW, Pantel K, Riethdorf S, Wege H, Schulze K. The combination of EpCAM-positive circulating tumor cells and serum AFP/AFP-L3/DCP predicts outcome after curative resection of hepatocellular carcinoma. Journal of Hepatology. 73;S379. EASL Liver Cancer Summit, Prague 2020. (Poster).

Schönlein M, Däbritz JHM, Fan DNY, Ihlow J, Schwarzer R, Vick B, Spiekermann K, Burmeister T, Westermann J, Jeremias I, Schmitt CA. Therapy-Induced Senescence Is a Predictor of Treatment Outcome in Acute Myeloid Leukemia. Blood. 2017;130:1393. The American Society of Hematology Annual Meeting and Exposition, Atlanta 2017. (Poster).

Acknowledgements

Mein Dank gilt zunächst Prof. Clemens Schmitt für die Möglichkeit in seiner Arbeitsgruppe zu promovieren, eine immer offene Tür für Fragen und intensive Diskussionen von Experimenten, einem vermittelten Enthusiasmus für die Wissenschaft und einen immerwährenden Glauben an das Projekt, und auch an meine persönlichen Fähigkeiten.

Meinem wissenschaftlichen Partner und direktem Betreuer, Dr. Henry Däbritz, danke ich für die Einführung in das wissenschaftliche Arbeiten und Erlernen von Experimentaltechniken, einer unendlichen Motivation und Ausdauer bei deren Durchführung, der kritischen Diskussion von Ergebnissen und vor allem für die (über alles Erwartbare hinaus gehende) andauernde Unterstützung.

Darüber hinaus gilt mein Dank vielen weiteren Wissenschaftler:innen und Mitarbeiter:innen der Arbeitsgruppe von Prof. Schmitt für eine engagierte gegenseitige Unterstützung, konstruktive wissenschaftliche Diskussionen und familiäre multikulturelle Atmosphäre. Besonders alle Büronachbar:innen im Raum 2.0203 ermöglichten es mir immer mit Spaß und Enthusiasmus anstehende Experimente anzugehen.

Ich möchte mich auch bei Kooperationspartner:innen aus Berlin, Linz, München und Mailand bedanken, mit deren Unterstützung das Thema in einen größeren Kontext eingeordnet und weiter präzisiert werden konnte, und hoffentlich bald veröffentlich wird.

Vor allem gilt mein allergrößter Dank meinen Eltern, die mit ihrem biochemischen Hintergrund wahrscheinlich hauptverantwortlich für meinen wissenschaftlichen Werdegang und mein onkologisches Interesse sind, mir gemeinsam mit meiner Schwester Lisa bedingungslosen Rückhalt während meiner Promotionszeit boten und mich mit viel Liebe immer darin bestärkt haben meinen Weg zu gehen.

Bei vielen meiner Freund:innen aus Lörrach und Berlin, aber vor allem bei Tim, Marten und Patrick, möchte ich mich für ihr aufmerksames (jahrelanges) begleitendes Interesse und ihre unverzichtbare moralische Unterstützung bedanken. Ich bin mir sicher, dass es wenige größere Seneszenz-Expert:innen ohne naturwissenschaftlichen Hintergrund gibt. Bei der besten Seminargruppe der Welt bedanke ich mich für die unvergessliche Zeit des „nebenbei“ laufenden Medizinstudiums und bei Marina, Maike und (nochmals) Tim für die antreibende Motivation in den finalen Tagen der Fertigstellung dieser Arbeit.