Single-cell protein profiling in early therapy response evaluation of acute myeloid leukemia by mass cytometry

Benedicte Sjo Tislevoll

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

During my PhD, 2018-2022, I have been affiliated with the Signalling-Targeted therapy group led by Prof. Bjørn Tore Gjertsen, at the department of clinical science, University of Bergen (UiB). This group is part of the Centre for Cancer Biomarkers (CCBIO) at UiB. Prof. Bjørn Tore Gjertsen has been my main supervisor and Dr. Monica Hellesøy, my co-supervisor.

My affiliation with the group of Prof. Gjertsen started in 2013 when I entered the medical research program at UiB. As a medical research student, I started the collection of samples from AML patients used in this thesis in 2014. The collection of patient material led to a collaboration with Dr. Yngvar Fløisand at Rikshospitalet, Oslo, and the group of Prof. Jorrit Enserink at the Norwegian Radium Hospital. They were responsible for collecting samples from patients treated in Oslo. The Enserink group also contributed with the DSRT data and data analysis. The mass cytometry analysis used in paper I was analyzed in 2016 and in 2019 for paper III, with technical assistance from the Flow Cytometry Core Facility at the University of Bergen. Mass cytometry data analysis and statistics were done in collaboration and with statistical expertise from Nello Blaser and Dimitrios Kleftogiannis at Computational Biology Unit (CBU). NGS was performed at the department of medical genetics at Haukeland university hospital by Randi Hovland, and at the department of Clinical Genomics in Uppsala, Sweden by Baliakas Panagiotis. The Genomics Core Facility (GCF) at the University of Bergen, which is a part of the NorSeq consortium, provided services on RNA sequencing, Aashish Srivastava contributed to the data analysis. Proteomics was performed at the proteomics core facility, PROBE, at the University of Bergen, with technical support from Even Birkeland.

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Being part of the Gjertsen lab has been an exciting journey both scientific and social. I would like to thank past and present members of the group for helping me and for the inspiring environment you have provided me. I am grateful for the contributions of all my collaborators and co-authors, in particular Yngvar Fløisand, Jorrit Enserink, Lars Herfindal, Dimitrios Kleftogiannis, and Vibeke Andresen. I would also like to thank the past and present members of the Reikvam lab for the good conversations during our lunch breaks. I am grateful to the University of Bergen for funding my PhD-fellowship.

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Bergen, September 2022

Benedicte Sjo Tislevoll

Abbreviations

AKT	AKT Serine/Threonine Kinase
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
ASXL1	Additional Sex Combs Like 1, Transcriptional regulator
BCL-2	B-cell Lymphoma 2
BCR-ABL	Breakpoint Cluster Region -Abelson Murine Leukemia Viral Oncogene Homolog 1
CBF	Core binding factor
CyTOF	Cytometry by Time of Flight
CD	Cluster of Differentiation
CML	Chronic Myeloid Leukemia
DNA	Deoxyribonucleic acid
ELN	European Leukemia Net
ERK1/2	Extracellular signal-regulated kinase 1/2
FLT3-ITD	Fms-Like tyrosine Kinase 3 – internal tandem duplication
HMA	Hypomethylating agent
IDH 1/2	Isocitrate Dehydrogenase ½
KRAS	Kirsten Rat Sarcoma Viral Proto-Oncogene
LSC	Leukemic Stem Cell
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia-1
MDM2	Mouse double minute 2 homolog
MFC	Multiparameter flow cytometry
MLL	Mixed Lineage Leukemia
MRD	Minimal residual disease

NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NGS	Next generation sequencing
NPM1	Nucleophosmin 1
NRAS	Neuroblastoma RAS viral oncogene homolog
PI3K	Phosphatidylinositol 3 kinase
PCR	Polymerase Chain Reaction
PML-RARA	Promyelocytic Leukemia -Retinoic Acid Receptor Alpha
PTM	Post-translational modification
P38	p38 MAP kinase
RAS	RAS proto-oncogene
RNA	Ribonucleic acid
RUNX1	Runt-related transcription factor 1
STAT	Signal transducer and activator of transcription
t-SNE	t-Distributed stochastic neighbour embedding
TP53	Tumor protein P53
WHO	World health organization

Abstract in Norwegian

Akutt myelogen leukemi er ein aggressiv og heterogen form for blodkreft med om lag 150 nye tilfelle årleg i Noreg. Med ei total overlevingsrate på 25% har den dårlig prognose, særlig blant eldre. Behandlinga består av eit intensivt kjemoterapi regime, eventuelt supplert med stamcelletransplantasjon. Sjølv om den første responsen på behandlinga er god, er det svært vanleg at pasientar får tilbakefall ettersom behandlinga kan gi opphav til leukemi celler som er resistente mot kjemoterapi. Behandlingsresponsen vert i dag målt ved antall leukemiceller i beinmarg etter behandling (tumor load) eller ved å måle minimal restsjukdom (MRD) ved hjelp av væskestraumscytometri. Denne måten å måle behandlingsrespons på kan ta veker eller månadar etter at pasienten har starta behandling. Det fører til at ein mistar verdifull tid, som kunne vore spart dersom man hadde hatt metodar for å måle respons på behandling tidlegare i forløpet. Tidlegare studiar har vist at når leukemiceller vert eksponert for kjemoterapi, får man ein rask respons i cellas intracellulære nettverk, som kan målast i løpet av minutt. Denne responsen kan også målast i pasienten ved å ta ein blodprøve etter behandlingsstart. I denne avhandlinga presenterer vi den første *in vivo* enkelt-celle, funksjonelle «stress testen» av kjemoterapi respons i AML. Umiddelbar evaluering av intracellulær signalering etter start av behandlinga i maligne og ikkje-maligne celler kan avsløre funksjonelle eigenskapar av resistente AML celler som kan nyttast til å utvikle målretta kreftbehandling. Dette vil kunne brukast til å skilje pasientar som respondera på behandling frå dei som ikkje gjer det, og dermed gi viktig informasjon som kan bli brukt til å endre behandlinga på eit tidleg tidspunkt. Vi har her brukt massecytometri for å kunne profilere dei intracellulære nettverka i enkelt celler. Massecytometri er ein kombinasjon av to teknikkar; massespektrometri og veskestraumscytometri. Med denne metoden kan vi måle opp til 40 antistoff per enkelt celle og dermed oppnå ei samtidig undersøking av signalerings nettverk og immunfenotypen til AML celler og friske celler. Med automatiserte grupperings algoritmar og maskin læring har vi demonstrert korleis endringa i intracellulær signalering i dei leukemiske cellene ved 24 timar etter start av behandling kan predikere pasientens 5 års overleving. I vår pasient kohort viser denne metoden seg overlegen dei

eksisterande genestiske risiko klassifieringane i AML. Dynamikken i den intracellulære signaleringa viser sitt potensiale ved å umiddelbart kunne skilje mellom dei pasientane som har fordel av behandlinga og dei som ikkje har det. Funksjonell presisjons onkologi er derfor tilgjengelig ved tidleg, longitudinell måling på enkeltcelle nivå. Ved å ta i betraktning heterogeniteten som er til stades i AML, kan den funksjonelle presisjonsmedisinen forbetre overleving og til slutt bli eit nyttig verktøy i klinisk kreftbehandling.

Abstract in English

Acute myeloid leukemia is a heterogeneous hematological malignancy with overall 5-year survival approximately 25%. Although high rates of initial chemotherapy response, patients often relapse due to the selection and development of chemotherapy-resistant AML cells. The response to therapy is currently measured by tumor load or through flow cytometry- or genomics-based estimation of measurable residual disease. This tumor-load based response-evaluation takes weeks to months, thereby losing important time. However, an immediate response to chemotherapy in intracellular signalling networks is detected within minutes *in vivo*. In this thesis, we present the first *in vivo* single-cell functional "stress test" of chemotherapy response in AML. Immediate evaluation of intracellular signaling after the start of treatment in malignant and non-malignant cellular subsets could reveal the functional properties of resistant AML cancer clones, and how they are therapeutically targeted. This might be used to determine responders from non-responders and thereby provide valuable information for an early change of treatment. We have applied mass cytometry for single-cell profiling of intracellular networks. Mass cytometry combines the platforms of mass spectrometry with conventional flow cytometry. This allows the detection of over 40 antibodies per single cell and can permit simultaneous analysis of signaling networks and immunophenotypes of AML cells and healthy cells. With automated clustering algorithms and machine learning, we demonstrate how intracellular signalling dynamics of the leukemic cells 24 hours after the start of chemotherapy can unravel the overall five-year survival. In our cohort, this method proved superior to genetic risk stratification and without correlation to mutations in the most frequently mutated genes of signal transduction in AML. Intracellular signalling dynamics demonstrate its potency in a close to real-time method to discriminate responders from non-responders. Functional precision oncology is therefore available by early longitudinal monitoring at the single cell level, taking into consideration the heterogeneity of a tumour and suggesting how functional precision medicine can improve outcomes and eventually become a standard tool in clinical oncology.

List of publications

Paper I

Benedicte Sjo Tislevoll, Monica Hellesøy, Oda Helen Eck Fagerholt, Stein-Erik Gullaksen, Aashish Srivastava, Even Birkeland, Dimitrios Kleftogiannis, Pilar Ayuda-Durán, Laure Piechaczyk, Dagim Shiferaw Tadele, Jørn Skavland, Baliakas Panagiotis, Randi Hovland, Vibeke Andresen, Nima Aghaeepour, Sonia Gavasso, Tor Henrik Tvedt, Kimmo Porkka, Inge Jonassen, Yngvar Fløisand, Jorrit Enserink, Nello Blaser and Bjørn Tore Gjertsen. **Early response evaluation by single cell signaling profiling in acute myeloid leukemia.** (Submitted, 2022) Preprint available at research square. DOI: https://doi.org/10.21203/rs.3.rs-1335315/v1.

Paper II

Dimitrios Kleftogiannis, Benedicte Sjo Tislevoll, Monica Hellesøy, Stein-Erik Gullaksen, Nisha van der Meer, Emmanuel Griessinger, Inga K. F. Motzfeldt, Oda Fagerholt, Andrea Lenartova, Yngvar Fløisand, Jan Jacob Schuringa, Bjørn Tore Gjertsen and Inge Jonassen. **Identifying predictors of survival in leukemia using single-cell mass cytometry and machine learning.** (Submitted, 2022) Preprint available at bioRxiv. DOI: https://doi.org/10.1101/2022.08.13.503587.

Paper III

Tislevoll BS, Oda Helen Eck Fagerholt, Monica Hellesøy, Stein-Erik Gullaksen and Bjørn Tore Gjertsen. **Early modulation of BCL-2 family proteins after start of chemotherapy predicts AML survival and therapy response**. (Manuscript)

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1. Introduction

1.1 Cancer as a signaling disease

Cancer is defined as a group of diseases that have the ability of unregulated cell growth and invasion of cells from the site of origin to other sites of the body. Although cancers of different origins have distinct features, Hanahan and Weinberg defined ten conceptual hallmarks of cancer in 2011 with an updated edition in 2022 to better understand the biology of cancer. These were the following: the ability to sustain proliferative signaling, evade growth suppressors, activate invasion and metastasis, enable replicative immortality, induce angiogenesis, resist cell death, the ability to deregulate cellular energetics, avoid immune destruction, genome instability, and mutation and tumor-promoting inflammation.(1, 2, 3)

The development of cancer (carcinogenesis) is a multi-step process where mutations and chromosomal aberrations accumulate in cells over time.(4) This explains the increased risk of cancers with age and the increasing prevalence of cancer as the human lifespan has increased over the centuries. When these alterations in the DNA affect genes involved in apoptosis, cell growth or differentiation, the result is unregulated cell growth. Cancer has traditionally been referred to as a "genetic disease at the cellular level" as mutations are passed down to daughter cells after mitosis. Analogous to the concept of Darwinian evolution, the mutations of cancer cells confer changes in phenotype that give selective advantages in adapting to the environment. Mutations that recurrently are observed among cancer patients have been extensively studied as they are potential therapeutic targets. The crown example that researchers have been striving towards ever since is the successful history of targeted therapy in chronic myeloid leukemia (CML). In 2001 the treatment of CML was revolutionized by the introduction of imatinib, which inhibits the BCR-ABL tyrosine kinase.(5, 6) This led to a 10-year survival rate of 83.3% for patients with CML.(7) Similar results have been obtained in the highly aggressive acute promyelocytic leukemia if treated early with retinoic acid, and hairy cell leukemia treated with cladribine.(8, 9) However, the same success has not been achieved in other cancer types, despite an extensive effort in identifying

potential targets. The lack of success might be that the evolutionary advantages of these recurrent mutations in carcinogenesis are not necessarily reflected by their frequency. Evidence suggests that the frequency of recurrent mutations depends not only on the fitness advantage of the mutation but also on the mutagenic processes which are enriched in the tissue types of a tumor.(10) Our knowledge of these tissue-specific mutational processes is limited, and the complexity increases when several mutational processes are present at once. One mutation might affect several downstream signaling pathways, and as our knowledge increases, we realize that there is substantial crosstalk between these pathways.

The ten hallmarks of cancer mainly describe functional aspects of cancer cells caused in aberrant signaling.(11) Mutations in genes of intracellular signal transduction and cell surface receptor proteins are frequently present in cancers, and protein kinases are the most common domain encoded by cancer genes.(12) Most molecularly targeted therapeutics have been developed to target such signaling molecules. Recent exceptions are the BCL-2 family inhibitors of protein-protein interaction (e.g navitoclax, venetoclax). Therefore, profiling signaling pathways is crucial for a better understanding of the function of tumor cells and the response to therapy.

Phosphorylation is one of the most common ways a eucaryotic cell regulates these signaling pathways.(13) Over two-thirds of the 21.000 proteins encoded by the human genome can be phosphorylated. Evidence suggests that more than 90% of proteins are subject to this post-translational modification (PTM). As for the other PTMs, acetylation occurs in 85% of proteins,(14) while methylation and ubiquitination is less frequent. Protein phosphorylation involves an enzyme-catalyzed attachment of a phosphate group to one of the amino acid side chains on the protein, which alter the activity/properties of the protein. Phosphorylation can tell us something about the activity of a protein. Therefore, most of the signaling proteins investigated in this thesis are phosphorylated proteins. This thesis investigates the early signaling response to standard induction therapy for the heterogeneous, aggressive hematological malignancy, acute myeloid leukemia (AML)

1.2 AML

Acute myeloid leukemia (AML) originates in the myeloid lineage of blood cells. It involves a differentiation block and excessive proliferation of immature blast cells that suppresses the normal function of the bone marrow. It is a heterogeneous disease at the molecular, phenotypic, and clinical levels and has poor overall survival.(15-17) It is the most common type of acute leukemia in adults and has an incidence of 3,7 per 100.000 year in Europe. The median age is 70 years, and the incidence above this age is higher.(18) Due to low therapy efficiency and high relapse rate, it is the most frequent cause of leukemia-related death.(19, 20)

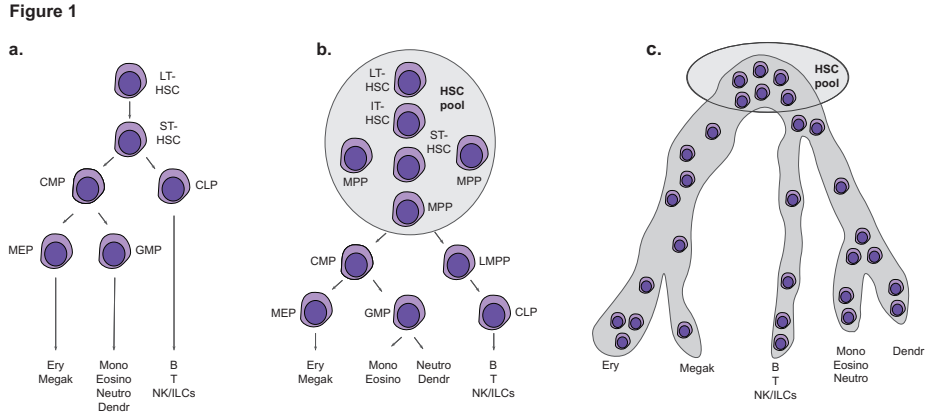

Figure 1 The evolving model of hematopoiesis. a, in the early 2000 HSCs were represented as a homogenous cell population, which separated into the common lymphoid (CLP) and common myeloid (CMP) cell populations. b, From 2005-2015 this visualization acknowledged the heterogeneity within the pool of HSCs, which can have different capabilities of self-renewal and differentiation. The lymphoid and myeloid lines were more tightly connected down in the hierarchy, than first presumed, via the lymphoid-primed multipotential progenitor (LMPP). The GMP population was shown to be more heterogeneous. c, Since 2016, single-cell transcriptomics has unraveled the fluidity of the hierarchy of hematopoiesis. The distinction between different stages of differentiation is more complex than first presumed, and cell identity is not discrete but there is a continuum of differentiation. This figure is reconstructed from Laurenti et al. Nature 2018. LT-HSC, Long term-hematopoietic stem cells, ST, short-term, GMP, Granulocyte-monocyte progenitors; MEP, megakaryocyte-erythrocyte progenitors; MPP, multi potent progenitors. Ery, erytrocytes; Megak, Megakaryocytes; Mono, monocytes; Neutro, neutrophils; Dendr, dendritic cells; B, B-cells; T, T-cells; ILCs innate lymphoid cells; NK, natural killer cells.

1.2.1 Molecular landscape of AML

A generally accepted understanding of leukemogenesis is the "2-hit model", where one class of mutations confers proliferative and survival advantages (class I) and the second serves impairment of differentiation (class II).(21) FLT3-ITD (25%), N-and K-RAS (15-25%) mutations are examples of class I mutations, PML/RARa (9-12%), AML1/ETO (15-22%), and MLL (3-4%) fusions are examples of class II mutations.(22) Mutations within one class are commonly mutually exclusive; for example, a coexistence of FLT3-ITD and NRAS mutations is rarely seen within the same patient. The most frequent mutation in AML, NPM1 (30%), is frequently present in the same cells as FLT3-ITD.(22)

Initiating mutations of leukemogenesis might persist in the patient for years before an AML diagnosis. In myeloproliferative disease, these mutations are estimated to be created in utero or in the first years of living while the disease emerges 30-60 years later, in some cases transforming to AML.(23) Sometimes, this is detected in peripheral blood as an expanded clone of cells, termed clonal hematopoiesis.(24) Mutations like ASXL1, DNMT3A, and TET2 are considered early events, while FLT3-ITD, RUNX1, and NRAS mutations tend to appear later in leukemogenesis.

In 2013 whole-genome and exome genetic profiling of 200 AML cases by The Cancer Genome Atlas AML substudy was conducted.(25) Only 23 genes were commonly mutated among the 200 AML cases, whilst 237 genes had a pattern of co-occurrence and mutual exclusivity. These mutated genes were functionally divided into nine categories: tumor suppressor genes, DNA methylation-related genes, transcription factor fusions, the NPM1 gene, signaling genes, myeloid transcription factor genes, chromatin-modifying genes, cohesion complex genes, and spliceosome complex genes. Interestingly, mutations of genes affecting receptor tyrosine kinase (RTK)/RAS signaling proteins were found in 55% of the samples.

In this thesis, we have investigated several key signaling pathways for AML at the protein level, including the MAPK-, PI3K/Akt-, STAT3/STAT5- signaling pathways, the transcription factor p53, the BCL-2 protein family and cell-cycle regulation. These

proteins have been shown to contribute to leukemic transformation, to be involved in the survival and proliferation of immature progenitors, myeloid differentiation, and resistance of AML cells to chemotherapy.(26-29) I will only describe the pathways most central to our results in detail.

1.2.2 Leukemic stem cells in AML

Stem cells are defined as cells that can differentiate into mature cells of a particular tissue and have the ability to self-renew. Through the ability of self-renewal, there is a striking similarity to cancer cells, where the same signaling pathways regulate self-renewal.(30, 31) The cancer stem cell theory claim that some cancer cells have the property of indefinite potential for self-renewal and thereby sustain cancer.(26) AML has served as a model for investigations of this theory, and the theory was experimentally supported by Dominique Bonnet, John Dick, and co-workers in 1997.(32) Bonnet and Dick demonstrated the hierarchical organization of AML, with the Leukemic stem cell (LSC) at the apex of the leukemic hierarchy, as the cell susceptible to leukemia transformation. The definition of an LSC is functional and described as the capability of initiating AML in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID-mice). High expression of gene signatures associated with LSCs is associated with adverse outcomes in AML, which underscores their clinical importance in AML.(33) These cells are thought to be the cause of resistance to chemotherapy as they can enter a reversible dormancy/quiescence state, thereby re-initiating the disease after chemotherapy. Evidence of the healthy human HSC as the cell of origin for leukemia was supported by the seminal studies of Jaiswal and Genovese in 2014. (34, 35) These studies showed that mutations of DNMT3A and other recurrently mutated genes in AML were present in healthy elderly individuals with clonal hematopoiesis and that these cells were generated from a single mutated HSC.

The immunophenotypic characterization of LSCs is not discrete. In the first studies, LSCs were thought to be exclusively $CD34^{+}$,$CD38^{-}$.(32, 36)However, in subsequent

animal studies, some cells previously thought to be nontumorigenic could also initiate disease, including the $CD34^-$ cell compartment in AML patients with NPM1 mutations.(37, 38) Several surface markers have been linked to poor prognosis in AML, including CD123 and CD56.(39) The disconnection of functional leukemia-initiating cells and their immunophenotype has been recognized, and therefore signaling profiling might be a better substitute for LSC identity.(40, 41)

1.2.3 MAPK/ERK signaling pathway in AML

The MAPK/ERK signaling pathway regulates apoptosis, differentiation, and cell proliferation in most eukaryote cells. It is deeply involved in regulating cell fate in the myeloid lineage.(42) ERK1/2 is a serine/threonine kinases that can be activated through various cytokines and growth factors. Activation of ERK1/2 is mediated through phosphorylation of MEK 1/2 and mainly mediates antiapoptotic and mitogenic signals. (43) ERK1/2 is a crucial regulator of multiple signaling pathways and has over 200 identified substrates.(44, 45) These include nuclear targets such as the transcription factors c-Fos, Elk1, and c-Jun. ERK1/2 signaling induces primary response genes (immediate early, delayed early, and immediate late genes), which are genes that do not require de novo protein synthesis. The mRNA expression of immediate-early genes is highest within 30-60 post-stimulation. The delayed early genes peaks within 2 hours post-stimulation. Immediate-late genes have a response time of 3.5 hours and half-lives exceeding 2 hours. FOSL-1/FRA-1 is one example of an immediate-late gene. (46) c-Fos, c-Jun, and FOSL-1 are components of the AP-1 transcription complex. AP-1 proteins consist of members of the Jun and Fos families, Jun dimerization partners, and the activating transcription factors (ATF) subfamilies. AP-1 is a nuclear target of ERK1/2 and plays a central role in cell proliferation and survival. Martinez et al. identified AP-1 as a pioneer transcription factor in senescence that imprints a reversible transcriptional program of senescent cells.(47) This might represent a mechanism for cancer cells to survive chemotherapy. Interestingly, a senescence-like state was recently revealed in AML cells resilient to induction therapy.(48)

The ERK1/2 pathway is hyperactivated in many tumor types, this can be due to activating mutations in the BRAF, NRAS, or KRAS genes, or the pathway is affected by mutations in other genes such as KIT, FLT3, and PDGFR. Approximately 15-25% of patients with AML have RAS mutations.(25) However, RAS mutations in AML do not have an impact on the clinical outcome.(49) In contrast to solid tumors, there may be a lack of correlation between NRAS mutations and constitutive activation of the MAPK kinase pathway in AML.(50) This might explain the lack of success in clinical trials with MEK inhibitors in RAS mutated AML.(51,52)

ERK phosphorylation is elevated in AML samples compared to CD34+ cells from healthy individuals.(53) Activation of the ERK1/2 signaling protects cancer cells specifically against apoptosis induced by chemotherapeutic cytotoxic drugs and drives the selection of resistant clones during induction therapy for AML in vitro.(54) Elevated ERK1/2 activation has also been observed in AML patients who have developed resistance to FLT3-targeted inhibitors.(55)

1.2.4 TP53 in AML

The tumor suppressor p53 is the primary regulator of various signaling and cell-fate decision pathways.(56-59) In healthy cells, p53 is inhibited by continuous ubiquitylation by MDM2(HDM2) until it is required. When cells are exposed to environmental stress, genetic damage, abnormal metabolic activity or aging, p53 is activated and orchestrates the expression of hundreds of genes to initiate the cell's response, such as DNA repair or apoptosis. Therefore, p53 is called the "guardian of the genome" because it protects against cancer.

p53 is regulated on the RNA level, on the protein level and by post-translational modifications (PTMs). On the RNA level, p53 is regulated by regulatory RNAs, long non-coding RNAs, micro RNAs, and circular RNAs. At the protein level, p53 is mainly regulated by the MDM2 protein. MDM2 is an E3 ubiquitin ligase that inhibits p53 by directly binding to the transactivation domain of p53 or by ubiquitination and

proteasomal degradation. The MDM2 gene is a transcriptional target of p53, and an upregulation of p53 will result in increased levels of MDM2 in a negative feedback loop.(60) During cell stress, the MDM2-p53 interaction needs to be inhibited to stabilize and activate p53. This is mainly performed by the kinases ATM, ATR, Chk1/2 and DNA-PK which phosphorylate serine residues on p53 and p53 acetylation by CBP/p300.(61) Furthermore, MDM2 can be inhibited by ARF($p14^{ARF}$) or the ARF binding partner ARF-BP1. In addition, p53 is heavily modified by post-translational modifications (PTM) such as phosphorylation, methylation, and acetylation, which affect the expression of p53 target genes. Acetylation of p53 is, for example, an essential modification after DNA damage and regulates p53s transcriptional activity.(62)

Apoptosis or cell cycle arrest are the main consequences of p53 activation by DNA damage. This is orchestrated by p53 target proteins. $P21^{WAF1/CIP1}$, encoded by the CDKN1A gene, is required for p53-mediated cell cycle arrest at the G/1 and G2/M checkpoints. Both the intrinsic and extrinsic apoptosis pathways are induced by p53. The TNF/Fas/FasL pathway controls the extrinsic apoptosis signaling pathway and leads to caspase-dependent apoptosis. In response to DNA damage, p53 increases the cell surface level of Fas and Fas ligand (FasL).(63) The BCL-2 protein family controls the intrinsic apoptosis pathway where cytochrome c is released from the mitochondria. P53 can directly bind and activate several of the pro-apoptotic members of the BCL2 family, including BBC3(PUMA), NOXA, and BAX.

Approximately 50% of all cancers have mutations in p53, while in AML, p53 mutations are only present in about 8% of de novo AML patients.(25) P53 mutations are more common in treatment-associated AML (t-AML) and AML with complex karyotypes (approximately 30 and 70%, respectively). It is also more frequent in clonal hematopoiesis and MDS. The association to t-AML is not mainly explained by induction of TP53 mutations by chemotherapy but might rather be due to the resistance of rare age-related TP53 mutated founding clones that expand after chemotherapy.(64) This demonstrates that TP53 mutations are early events in leukemogenesis. AML patients with TP53 mutations are classified as a distinct subgroup of AML with dismal prognosis and resistance to chemotherapy.(22, 65)

Although TP53 mutations are not frequent in AML, there is a lack of normal p53 activity in AML that affects the biology and treatment response of the disease. This is called non-mutational wtp53 inactivation.(66) TP53 mutations are mutually exclusive with mutations in NPM1, MDM2, FLT3, ARF, RUNX1, t(15;17)(PML-RARA), t(8;21) an inv (16). This suggests that the p53 dysfunction of these subgroups of AML is different than for those with TP53 mutations. Non-mutational wtp53 inactivation might occur at the protein level through low ARF expression or MDM2 overexpression, frequently observed in AML (40-50%).(67) In NPM1 mutated AML; cytoplasmatic mutated NPM1 forms a complex with ARF and wtNPM1 leading to cytoplasmic mis-localization and attenuation of ARF induced p53 stabilization, which induces MDM2 mediated degradation of p53.(68) In FLT3-ITD mutated AML several mechanisms lead to wtp53 dysfunction: SIRT1 deacetylation of p53, activation of PI3K/AKT which promotes MDM2 mediated p53 degradations, and the STAT and MAPK signaling pathways with BCL-2 accumulation which again opposes p53 activation. Aberrant PI3K/AKT and MAPK/ERK signaling are also frequently present in most AML cases and give wild type p53 dysfunction by MDM2 activation and through the Wnt/beta-catenin and NK-kB signaling.(69, 70)

1.2.5 BCL-2 in AML

The BCL-2 protein family has an important role in the survival and death of AML blasts and is a key mediator of the mitochondrial apoptotic pathway.(71, 72) Members of the BCL-2 family share one to four of the characteristic BCL-2 homology (BH) domains. They are traditionally categorized into three groups: the anti-apoptotic members (BCL-2, BCL-XL, and MCL-1), the pro-apoptotic pore formers which form the mitochondrial outer membrane permeabilization (MOMP) (BAX and BAK), and the pro-apoptotic BH3 only proteins (BAD, BID, BIM, NOXA, and PUMA).(73, 74) The cell's fate is decided by the interaction between the different members of the BCL-2 family and their affinity to one another. When the outcome is apoptosis, the BAX and BAK proteins form pores in the mitochondrial outer membrane, with leads to the release of pro-

apoptotic proteins such as cytochrome c, and thereby the activation of the caspase cascade.(75) The MOMP is a switch-like event determined by the interaction of the BCL-2 family members. The phenomenon of mitochondrial priming measures how close a cell is to the apoptotic threshold. A primed cell will more easily undergo apoptosis than an unprimed cell in response to apoptotic stimuli. This can be measured in a functional assay as the response of isolated mitochondria to pro-apoptotic peptides from the BH3 domains of the BH3-only proteins. The ability of these peptides to induce cytochrome C release from the mitochondria is then measured, called BH3-profiling. BH3 profiling can be done in tumor cells and has been shown to correlate to chemosensitivity in multiple cancers.(76, 77) BH3 profiling in AML might be used to determine response to induction therapy, relapse, and the need for allogeneic bone marrow transplantation.(28)

1.3 Classification of AML

Leukemia has pioneered the genetic classification of cancer, starting with the identification of the Philadelphia chromosome in 1960, leading to the identification of the pathognomonic BCR::ABL1 chimeric kinase and tyrosine kinase inhibitor therapy of chronic myeloid leukemia.(78) The genetic classification of AML has, like for other cancers, shifted from a morphological classification toward a genetic-based classification. There are two well-known and widely used classification systems for AML: the French-American-British (FAB) classification and the World Health Organization (WHO) classification for myeloid neoplasms and acute leukemias. The FAB classification describes the cytomorphology of AML with seven distinct classes. The WHO classification was revised in 2016 and 2022 with increasing use of molecular genetics, cytogenetics, germline variant associated, cytomorphology, and etiology-related subgroups. AML with recurrent genetic abnormalities is a defined category embedded in the WHO 2016 edition and constitutes 11 subgroups of AML. The same year Papaemmanuil *et al.* proposed a purely genetic classification of AML based on targeted sequencing of 111 myeloid cancer genes from 1540 AML patients treated with

intensive therapy.(22) In addition to the known disease classes of the WHO classification, three classes were defined: AML with TP53 mutations and/or chromosomal aneuploidies, AML with mutations in chromatin and RNA splicing regulators, and AML with $IDH2^{R172}$ mutations, respectively. The ELN risk classification of AML was now purely based on genetics from 2017 and was recently revised in 2022.(79)

1.3.1 Risk classification of AML

The assessment of somatic mutations and chromosomal alterations is currently the cornerstone of AML risk assessment and therapeutic decision-making. The guidelines for diagnosis and management of acute myeloid leukemia by the European LeukemiaNet (ELN) are widely used and were first published in 2010.(80) The prognostic factors were then divided into patient-related and AML-related factors. Increasing age and comorbidities were the most important patient-related factors. AML-related factors were the cytogenetic and molecular alterations in the leukemic cells at diagnosis, previous cytotoxic therapies, prior MDS, and white blood count (WBC). Patients were divided into four risk groups: Favorable, Intermediate I and II, and adverse risk group based on cytogenetic and molecular characteristics. In 2017, these guidelines were revised, and the genomic landscape of AML was given a more central place in the risk assessment of the disease. The 2017 ELN risk classification by genetics was based on the WHO 2016 classification and the proposed genetic classification by Papaemmanuli *et al.* and divided patients into: favorable, intermediate, and adverse risk groups. Since 2017, our knowledge has increased, and the ELN risk classification was revised in July 2022 with a hierarchical risk classification that may be a novel framework for future updates, that include new targeted therapies, e.g., against IDH1/2 mutations and against TP53 mutations.

FMS-like tyrosine kinase 3 (FLT3) mutation is the second most common mutation in AML and occurs in approximately 30% of patients. Internal tandem duplication (ITD) is the most common type of FLT3 mutation (approximately 25% of AML patients). In

the 2017 ELN classification, a high allelic ratio of FLT3-ITD was considered a poor prognostic factor. However, in the 2022 revision, the allelic ratio of FLT3-ITD is abolished. This is due to methodological challenges in standardizing the assay for measuring the allelic ratio between sites, the clinical success of FLT3 inhibitors like midostaurin in FLT3-ITD mutated AML, and the increasing use of MRD in treatment decisions. Therefore, in the 2022 edition, all FLT3-ITD mutated AML are classified as intermediate risk, irrespective of allelic ratio or the presence of NPM1 mutations.

Nucleophosmin 1 (NPM1) mutations are the most common genetic abnormality in AML, counting about 30% of all cases, and it often co-occurs with FLT3. In the ELN 2017 risk classification, NPM1 mutations were associated with a favorable prognosis when FLT3-ITD was negative or had a low allelic ratio. It was assumed that this was true, regardless of the presence of other cytogenetic abnormalities. However, a meta-analysis by Angenendt et al. in 2019 showed that when adverse risk cytogenetics is present, patients with NPM1 mutation share the same poor prognosis as patients with wild-type NPM1.(81) Therefore, in the 2022 ELN risk classification, patients with NPM1 mutations and adverse risk cytogenetics are classified as adverse risk. NPM1 mutated AML without FLT3-ITD is still considered favorable.

Another adjustment in the 2022 ELN risk classification is that AML with myelodysplasia-related gene mutations is classified as adverse risk. These genes include the ASXL1, RUNX1, BCOR, EZH2, SRSF2, SF3B1, U2AF1, STAG2, and ZRSR2. In the previous classification, only myelodysplasia-related cytogenetic abnormalities were considered adverse risk.

An essential change in the 2022 guidelines is the change in the blast threshold defining AML. All the recurring genetic abnormalities that represent the different subtypes of AML, except AML with BCR-ABL1 mutations (which still require > 20%), can establish an AML diagnosis if there are more than 10% blasts in the bone marrow (BM) or peripheral blood (PB). The previous requirement for an AML diagnosis was more than 20% blasts in BM. AML patients with TP53 mutations have been classified as their own entity in the 2022 edition.

The 2022 guideline revision illustrates the importance of co-occurring mutations in AML, and how they affect one another. The complexity of the disease is increasing as new knowledge is gained, and we still have much to learn. Treatment response is very heterogeneous between different AML risk groups. AML patients with core binding factor AML (t(8;21), inv(16), or t(16;16) have a survival of 60-70% with chemotherapy alone, while patients with complex karyotype have survival of approximately 10%.

The 60-70% survival of favorable AML is not a decent number and advocates a need for a better risk classification system.

1.4 Treatment of AML fit for intensive chemotherapy

Newly diagnosed AML patients fit for intensive treatment are initially treated with a combination of an anthracycline, dauorubicine (60-90mg/m^2/d) or idarubicine (10-12 mg/m^2/d) for 3 days, and cytarabine (Ara-C) (200mg/ m^2/d) for 7 days.(16) This is referred to as the "7+3" treatment. The induction therapy is usually given in two cycles, then the patient receives consolidation therapy with cytarabine or hematopoietic stem cell transplantation (HSCT), dependent on the ELN 2022 risk classification. Importantly, MRD if available can be used to downgrade good risk disease for allogeneic HSCT. MRD in intermediate risk disease is used by HOVON/SAKK to select autologous versus allogeneic HSCT.

Targeted therapy as an addition to "7+3" is available for patients with certain mutations patients with FLT3 kinase mutations and will likely in the future be available for patients with IDH1/2 mutations. For patients with FLT3 mutations, per oral midostaurin is given from day 8 to 21.

There are several strategies for p53 targeted therapy in AML. These therapeutic strategies aim to either abolish mutated TP53 leukemic cells, rescue the p53 mutational activation or target the mechanisms of wtp53 inactivation present in AML. Several MDM2 inhibitors are currently in clinical trials with AML. However, they have

challenges with on-target effects of normal cells, especially hematological toxicity, as MDM2 has a crucial role in hematopoiesis.(82)

Hypomethylating agents (HMAs), such as azacitidine and decitabine, are cytosine analogs with the capability to incorporate into DNA, trap DNA methyltransferases and target them for destruction. DNA synthesis in the absence of DNA methyltransferases results in hypomethylation and reactivation of silenced genes. In this way, these agents affect epigenetic changes, which have a crucial role in AML development.(83) These agents are frequently used for older patients who are not fit for intensive chemotherapy but have also been shown to be advantageous in AML patients with TP53 mutations.(84)

Venetoclax is a selective small molecule inhibitor of BCL-2 and can induce apoptosis in BCL-2 dependent AML cells. (85) It has a modest effect in AML patients treated with venetoclax in monotherapy. (86) However, combination treatment of venetoclax and azacitidine in elderly patients not fit for intensive induction therapy showed an increase in overall survival from 9.6 to 14.7 months.(87) Azacitidine is thought to down-regulate MCL-1 and BCL-XL and induce the expression of pro-apoptotic proteins NOXA and PUMA.(88) This combination therapy is now the front-line therapy for elderly unfit-AML patients.

Despite intensive research and multiple clinical trials for new therapies in AML, no other induction regimen has demonstrated more efficiency than “7+3”. Complete remission (CR) is achieved in 60-85% of patients <60 years of age.(89) However, the treatment is highly toxic, and most patients who initially achieve CR will eventually relapse.

1.4.1 Anthracyclines

Anthracyclines are a class of cytotoxic antibiotics extracted from Streptomyces bacterium, including daunorubicin (DNR), idarubicine (IDA), epirubicin, and doxorubicin. They are used to treat a variety of cancers (leukemia, lymphoma, bladder

cancer, prostate cancer, and breast cancer) due to their effective capability to induce apoptosis in cancer cells. Anthracyclines are ranked among the most effective cancer drugs in current clinical use. DNR and IDA are central in AML treatment, and most patients in this study have received induction therapy including one of them. The main side effect of anthracyclines and the biggest challenge in their usefulness is cardiotoxicity. Other side effects include severe or febrile neutropenia, vomiting, and due to damage to cellular DNA; treatment is also associated with secondary cancers.

Anthracycline's cytotoxic activity combines multiple mechanisms, such as intercalation into the DNA, stabilization of the topoisomerase II enzyme, and induction of oxidative stress. However, studies have indicated that intercalation into DNA might not be required for the cytotoxicity of anthracyclines.(90) In addition to the direct effect on DNA, anthracyclines induce apoptosis through modifying intracellular signaling pathways.(91) One of the central pathways of anthracycline signaling is the sphingomyelin-ceramide pathway.(91) Sphingomyelin is a type of sphingolipid, that consist of phosphocholine and ceramide. Sphingomyelin is found in cell membranes, especially in the myelin surrounding nerve cell axons. During the early 1990s, evidence suggested that DNR induced ceramide generation, which is capable of inducing apoptosis in leukemic cells.(92) Ceramide induces a variety of signaling proteins, such as Raf-1, ERK, and stress-activated protein/c-Jun N-terminal kinase (SAPK/JNK), which leads to activation of the AP-1 transcription complex. However, in 1996, the ceramide metabolite, sphingosine-1-phosphate (S1P), was discovered.(93) S1P can inhibit ceramide-mediated apoptosis, stimulate the ERK pathway and contract the ceramide-induced action of SAPK/JNK. This proposed the concept of the "sphingolipid rheostat" as a major determinant of cell fate.(94) Sphingosine kinase 1 (SPHK1) is responsible for the conversion of sphingosine to S1P. It has been shown to have increased expression in AML and in AML cells resistant to daunorubicin.(95, 96) A recent study by Lewis *et al*. showed that inhibition of SPHK1 in AML depleted the pro-survival MCL-1 protein and synergized with the BCL-2 inhibitor navitoclax. It was also able to overcome resistance to BCL-2 inhibitors in AML blasts.(97) Overexpression of BCL-2 and BCL-XL has been shown to protect AML cells against anthracycline-

induced apoptosis and are involved in cancer cell survival and chemoresistance.(98) MCL-1 is considered one of the main resistance mechanisms to BCL-2 inhibitors in AML. Current MCL-1 inhibitors may give cardiotoxicity that limit the use in AML patients. (99)

In the early 2000s, the BAX/BCL-2 ratio at diagnosis was thought to predict the outcome in AML treated with induction therapy. Cytotoxicity by anthracyclines is also associated with increased caspase activity. Increased caspase activity has been observed in circulating mononuclear cells from AML patients 15 hours after the start of anthracycline treatment.(100)

1.4.2 Cytarabine (Ara-C)

Cytarabine (cytosine arabinoside,Ara-C) combines a cytosine base with an arabinose sugar. After cellular uptake, it is converted to the therapeutically active triphosphate metabolite, Ara-CTP, which competes with cytidine to incorporate itself into DNA and thereby inhibit DNA synthesis in proliferating cells.(101) Cytarabine has been the major drug for AML treatment the recent decades and is also the primary choice of consolidation therapy after induction therapy.(102) In 2017 Farge et al. showed that the cytarabine-resistant leukemic cells were not enriched in the quiescent LSCs but were characterized by a high oxidative phosphorylation status. This study demonstrated that essential mitochondrial function contributed to cytarabine resistance and can be used as a therapeutic target to increase cytarabine sensitivity in AML.(103) Another study showed that the cellular enzyme SAMHD1, a dNTPase, confers resistance to cytarabine in AML. SAMHD1 reduces cytarabine toxicity by hydrolyzing Ara-CTP, which dramatically reduces Ara-CTP in leukemic cells. The authors also showed that the patient's response to cytarabine was inversely correlated with SAMHD1 expression.(104) Both of these studies present potential biomarkers for the stratification of patients who might respond to cytarabine-based therapy regimes.

1.5 Response evaluation in AML

The first assessment of therapy response in AML is by morphological evaluation of the bone marrow on day 14 to 17 after the start of induction therapy and is mandatory before the second cycle. The patient is in complete remission if there are less than 5% blasts in the bone marrow and peripheral blood values are normalizing. If there are more than 6% blasts in the bone marrow, the patient is not in complete remission (non CR).

Measurable residual disease (MRD) is an important biomarker for monitoring therapy response and if detected, also a strong prognostic factor for relapse and short survival. It can be measured through multiparameter flow cytometry (MFC) or by qPCR (molecular MRD) after the first or second cycle of chemotherapy. Bone marrow is preferred for MRD, although peripheral blood appears as a robust alternative for selected markers (q/dPCR of NPM1, CBF-AML) and is currently included in the ELN 2022 algorithm for MRD.(79, 105) MFC-MRD is the most used method for MRD detection. Leukemia-associated immunophenotype (LAIP) or an immunophenotype different from normal (DfN) is defined in the diagnostic sample for each specific patient and used to track diagnostic and emerging clones. The core MRD markers are CD34, CD117, CD45, CD33, CD13, CD56, CD7, and HLA-DR; often, CD38 is also included. (106) For molecular MRD, there are two methods: PCR and NGS. The pertinence of PCR-MRD is limited to those patients with one or more targetable cytogenetic abnormalities like mutated NPM1, PML-RARA, CBFB-MYH11, and RUNX1-RUNX1T1.(107)

MRD and morphological assessment of the bone marrow is the current state of the art in response evaluation. However, earlier response evaluation could provide superior by allowing changing treatment early in suboptimal responders. Several studies have investigated early blast clearance after the start of induction therapy and found that the elimination of peripheral blasts by day 5 or 6 was a good prognostic predictor. (108, 109) Another study found that clinical response to induction therapy could be detected after two days by PET-CT.(110)

2. Aims of the thesis

In this thesis, we investigated changes in intracellular signaling in single cells by mass cytometry to:

1) Examine if single cell signaling profiling hours after the start of induction therapy in AML may distinguish responders from non-responders, and predict therapy response, relapse, and survival in AML.
2) Examine if single cell profiling may predict therapy response, relapse, and survival in AML.
3) Increase the understanding of AML biology and reveal potential new targets for therapy by profiling intracellular signaling networks with single-cell resolution.

3. Methodological considerations

3.1 Biobanking and preservation of biological material

In this thesis, we used peripheral blood and bone marrow samples from leukemia patients and healthy donors. Patients were consecutively included in this study between 2014 and 2016. They were treated at Haukeland University Hospital, Bergen and Rikshospitalet, Oslo. These patients received continuous infusions with Daunorubicine and Cytarabine. Therefore, samples collected after the patient started chemotherapy were drawn from a peripheral vein for the 4-hour sample and not from the implanted central venous line (Hickman catheter). This was to avoid getting a too high concentration of chemotherapy in the sample. The 24-hour sample was usually drawn from the catheter before the second daily dose of chemotherapy was administrated. The cohort size is unfortunately small. This is mainly because the patient material we present in this study is unique. Samples are collected shortly after start of treatment and processed using fix/lysis right after sample collection to preserve the phosphorylation status of intracellular signaling proteins. This requires immediate on-site sample processing by qualified personnel in appropriately equipped labs, which limits sample collection significantly. Additionally, AML is a relatively rare diagnosis, with approximately 150 new cases diagnosed per year in Norway. Thus, to our knowledge, no other comparable datasets of AML patient material exist to date. Cryopreserved material will likely not allow a similar analysis of signaling at baseline and 24 h after start of chemotherapy.

Samples that were going to be analyzed by mass cytometry were fixated as soon as possible (usually within 20 min after sampling), and red blood cells were lysed using a Lyse/Fix buffer from BD Phosphoflow. This was to minimize potential ex vivo alterations in the intracellular signaling networks and to preserve the in vivo signaling.

For samples to be analyzed with RNAseq, proteomics, and targeted DNA sequencing (TruSight myeloid panel), mononuclear cells were prepared and sampled in a BC Vacutainer CPT^{TM} with sodium heparin. The isolation of mononuclear cells was done by density gradient separation (Lymphoprep), where the CPT vial was centrifuged for

20 min. Then the white blood cell layer was washed in saline. DNA was stored as a dry pellet of cells, RNA was dissolved in TRIzol™ Reagent (Thermo Fisher), and protein was precipitated in 7% trichloroacetic acid (TCA) and stored at -80°C until analysis.

For samples drawn after the start of chemotherapy, the samples are unavoidably incubated for some time in chemotherapy, which is present in the blood sample, while the sample is processed. Although the sample processing takes less than 2 hours, changes in the signaling networks and apoptosis might be induced. These changes are minimal for the lyse/fixed cells due to a quick fixation (within 30 min). However, for the samples processed by Lymphoprep, the whole process might take 1,5 -2 hours. It should be noted that this is a significant source of potential error/technical variation/artifacts in this study. Although the sample processing with Lymphoprep is longer, the initial high chemotherapy concentration present in the blood sample is reduced by washing the cells after centrifugation for 30-40 min.

Studies have shown that apoptotic cells are immediately removed from circulation in vivo and that apoptosis cannot be detected in vivo after the start of chemotherapy for leukemia. (111, 112) Another study has shown induction of BAX but not p53 in lymphocytes after the start of chemotherapy in vivo. (113) The authors explained this to be due to a difference between cycling/activated and resting lymphocytes. P53 could only be detected in vitro in cell lines and stimulated lymphocytes but not during in vivo chemotherapy. A former study in our group showed that p53 was induced in Molm-13 cells after gamma-irradiation with 25Gy in vitro, measured by mass cytometry.(114) However, in this thesis (manuscript III), there was no induction of p53 (DO-7) after the start of induction therapy measured in vivo by mass cytometry. This contrasts with our former studies by Anensen et al., which showed significant induction of p53 after the start of induction therapy in AML.(115) In the study by Anensen et al, cell samples were lysed and p53 was detected by gel electrophoresis and immunoblotting. These samples were purified by Lymphoprep, and the p53 detection might therefore be influenced by cell incubation in chemotherapy during sample preparation. A more plausible explanation is the analysis of p53 in cell lysate versus intact but fixated cells. If stabilized p53 acts as a transcription factor it will form protein complexes that is

dissolved in the lysate and detected as an increase in protein by immunoblot. The fixed cells preserve protein complexes which may hide p53 protein epitopes, and the fixation and preparation by itself may influence antibody binding. This need to be tested in additional experiments.

3.2 Mass cytometry

Mass cytometry or Cytometry by Time of Flight (CyTOF) is the main technology used in this thesis (Figure 2). Mass cytometry is a technology that combines the principles of conventional flow cytometry with time-of-flight mass spectrometry to allow analyses of multiple parameters simultaneously in single cells.(116, 117) Applying stable heavy metal isotopes instead of fluorophores for antibody detection dramatically increases the number of antibodies that can be applied. Compared to conventional flow cytometry, where spectral overlap limits the number of antibodies that can be analyzed per cell, mass cytometry enables single cell characterization and can measure over 50 parameters per single cell. (118, 119) This includes antibody-based measurement of DNA, and proteins, nucleotide amplification-based detection of RNA, measures of the cell cycle, live/dead cell staining, and sample multiplexing by unspecific metal tags. Mass cytometry is an ideal tool for analyzing dynamic signaling responses, as sample multiplexing permits direct comparison of serial samples from the same patient.

Flow cytometry is the standard technique in the clinic and has the benefit of years of experience and optimization of antibody panels and gating strategies. The transfer of experience from the flow cytometry field to mass cytometry is challenging as these two technologies have several differences. For example, is the granularity of a cell and cell size central to the analysis and gating of cells by flow cytometry. This information is not available by mass cytometry. The detection of low abundance antigens might be better in flow cytometry and the acquisition rate for flow cytometry is also much higher (thousands of events/s) than for mass cytometry (approximately 500 events/s). Another disadvantage of mass cytometry is the atomization/destruction of the cells before analysis. This prevents the ability to sort viable cells after analysis.

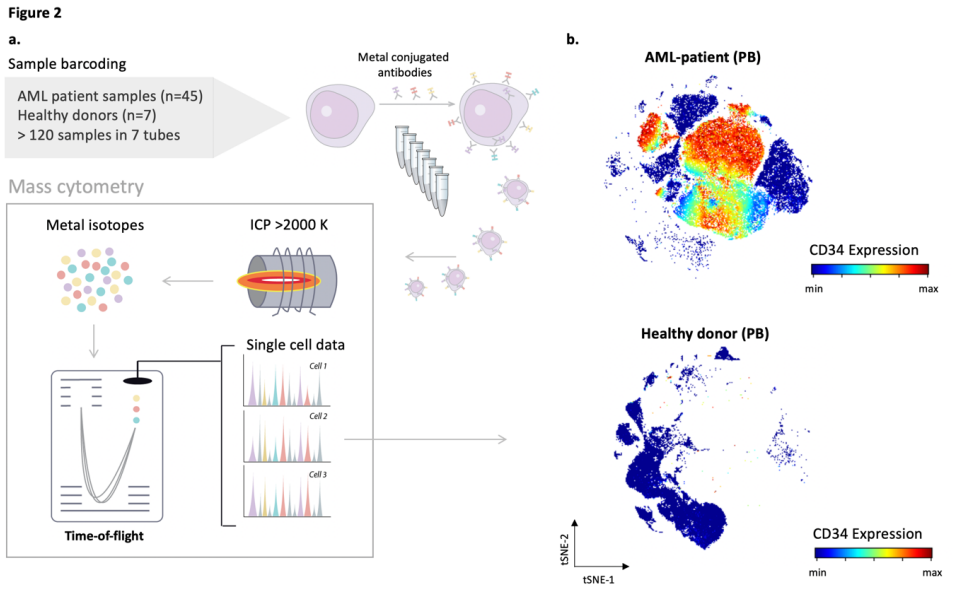

Figure 2: Mass cytometry and data analysis. a, 140 samples from 45 patients and 7 healthy donors were barcoded into 7 barcodepools. Cells were stained with metal conjugated antibodies, detecting both intracellular and surface markers. The single cell suspension is pushed through a nebulizer which generates a spray of single cell droplets. Each cell is atomized and ionized by an argon plasma which holds a temperature of 5000-10.000 K (ICP: Inductively coupled plasma). The resulting ion cloud is then passed through a quadrupole, removing irrelevant elements, and keeping the metal isotopes. The cloud of metal isotopes is then measured by time of flight and the atomic composition of each cloud is then analyzed. These data are integrated over time, and recorded as dual counts for each channel, and exported as .fcs files. b, The data can be visualized with dimensionality reduction tools such as t-SNE. This t-SNE map shows a typical AML patient in our dataset with lots of CD34+ leukemic blast cells. A peripheral blood sample from a healthy donor is shown below, and almost no CD34+ cells are detected.

3.2.1 Cell fixation and staining

In this thesis, we have used the lyse/fix buffer from BD that simultaneously fixes white blood cells and lyses red blood cells. This buffer contains (7.15% methanol, 20.35% formaldehyde, and 15.65 diethylene glycol. The lyse/fix solution might give rise to irrelevant antibody binding sites and block the binding of some fixation-sensitive antibodies. This might happen by cross binding of proteins which can lead to hiding of epitopes from antibody detection. Another source of artifacts is that nucleated red blood

cells might not be lysed by the lyse/fix solution and this might vary between patients. Granulocytes are removed when cells are prepared with lymfoprepp, but they are not removed by the lyse/fix. These cells are known to have more unspecific antibody binding. However, granulocyte-specific antibodies are used in the panel to recognize these cells. Other methods such as ProtStab should be considered in the future.(120) The cells are stained with intracellular antibodies after cell permeabilization with methanol. This can also disrupt the binding of the already bound surface marker antibodies. The best staining quality is obtained with live cell staining. This is also the standard in diagnostic flow cytometry. However, live cell staining might alter the intracellular signaling pathways when antibodies detecting surface markers bind to receptors on the cell surface.

3.2.2 Antibody panel design for mass cytometry and signal spillover

Although the spillover between different antibodies by mass cytometry is minimal compared to flow cytometry, there are three sources of signal spillover in mass cytometry. These can come from isotopic impurities in the metal isotopes, signal overlap from highly abundant metal isotopes into adjacent channels (+/- 1 dalton) and isotope oxidation (+16 dalton). (121) Therefore, the antibody panel must be designed and titrated carefully. This is especially important when measuring intracellular signaling, which often has lower signal intensities than surface antibodies. The intracellular antibodies should therefore be conjugated to the metal tags with the highest intensity (60-70Da), and the more robust surface markers should be placed on the less intense metal tags. (122) To limit the possibility of spillover between antibody channels, there has been developed a compensation pipeline with bead-based compensation that allows for compensation of channels after acquisition.(123) In this thesis, our mass cytometry acquisitions were performed before the publication of this pipeline. Therefore, we used compensation beads created in a later experiment. The beads were conjugated to the same metal isotopes but different antibodies. We assume that the difference in metal abundance between the two experiments are negligible,

and that the potential difference in metal abundance between the two experiments will be outweighed by the advantage of compensating the data.

All antibody-based assays have the undesirable issue of non-specific antibody binding. Therefore, there is a need for positive and negative controls in antibody-based studies. Ideally, the panel should be titrated on the same material you will analyze in your mass cytometry study. In this thesis we titrated our antibody panel on lyse/fix peripheral blood from 5 healthy donors, 9 of the patients included in this study and bone marrow from 5 healthy donors. In addition, all antibodies have been validated by the supplier. Several of the antibodies included in this study have been validated in Gullaksen et al. Cytometry part A, 2019 and by Fagerholt et al Cancers 2020. The pERK-1/2 (T202/Y204) antibody was validated by western blot on several of the patients in this study, with Molm13 treated with GM-CSF and MEK inhibitor- UO126 as positive and negative control (data not shown). Ideally, we should have used stimulated cells as positive controls for the titration of the intracellular antibodies in our mass cytometry panel. An overview of the antibody panels used in Paper I and Paper II is shown in Figure 3

Figure 3

Antibody panel paper I

Surface (n=21)	
Pr141Di	CD66b
Nd143Di	CD117 (c-Kit)
Nd144Di	CD38
Nd145Di	CD4
Nd146Di	CD64
Sm147Di	CD20
Nd148Di	CD16
Eu151Di	CD123 (IL-3R)
Gd155Di	CD56 (NCAM)
Gd160Di	CD14
Dy163Di	CD33
Er168Di	CD34
Dy162Di	CD8
Er170Di	CD3
Yb176Di	CD7
Yb174Di	HLA-DR
Y89Di	CD45
Bi209Di	CD11b
Tb159Di	CD90 (Thy-1)
Tm169Di	CD25 (IL-2R)
Yb171Di	AXL

Intracellular (n=15)	
Nd142Di	Cleaved Caspase 3 (D3E9)
Gd156Di	p-p38 [T180/Y182] (D3F9)
Sm152Di	pAkt [S473] (D9E)
Sm149Di	p4E-BP1 [T37/T46] (236B4)
Nd150Di	pSTAT5 [Y694] (47)
Eu153Di	pSTAT1 [Y701] (4a)
Sm154Di	pRB [S807/S811] (J112-906)
Gd158Di	pSTAT3 [Y750] (4/P-STAT3)
Dy161Di	pAxl [Y779] (Y779)
Dy164Di	CyclinB1 (GNS-1)
Ho165Di	pCREB [S133] (87G3)
Er166Di	pNFkB p65 [S529] (K10895.12.50)
Er167Di	pERK 1/2 [T202/Y204] (D13.14.4E)
Yb172Di	pS6 [S235/S236] (N7-548)
Lu175Di	pHistone3 [S28] (HTA28)

Antibody panel paper III

Surface (n=19)	
Pr141Di	CD66b
Nd143Di	CD117 (c-Kit)
Nd144Di	CD38
Nd145Di	CD4
Nd146Di	CD64
Sm147Di	CD20
Nd148Di	CD16
Eu151Di	CD123 (IL-3R)
Gd155Di	CD56 (NCAM)
Gd160Di	CD14
Dy163Di	CD33
Er166Di	CD34
Er168Di	CD8
Er170Di	CD3
Yb176Di	CD7
Yb174Di	HLA-DR
Bi209Di	CD11b
Y89Di	CD45
Yb173Di	CD300e

Intracellular (n=17)	
Ce142Di	Cleaved caspase 3 (D3E9)
Gd156Di	p-p38 [T180/Y182] (D3F9)
Sm152Di	pAkt [S473] (D9E)
Sm149Di	pATR [S428] (EPR2184)
Nd150Di	MCL-1 (Y37)
Eu153Di	P53 (DO-7)
Sm154Di	BAX (2D2)
Gd158Di	Mdm2 (SMP14)
Tb159Di	p21 (12D1)
Dy161Di	pBad [S112] (40A9)
Dy162Di	Ki67 (B56)
Dy164Di	P53 delta133
Ho165Di	Bcl-2
Er167Di	Acetyl-P53 [Lys382] (REA529)
Tm169Di	pBcl-2 [S70] (EPR21162)
Yb171Di	PUMA (EP512Y)
Lu175Di	Mutated TP53 (Y5)

Figure 3 Side-by-side comparison of the two antibody panels used in this thesis. The first row in the table indicates the metal to which the antibody was conjugated to. For the intracellular antibodies, the phosphorylation site is shown in brackets and the clone is shown in parenthesis. The surface marker panels were almost identical, however, CD90, CD25, and AXL were present in the antibody panel used in paper I. In paper III these three

antibodies were removed, and CD300e was included. The intracellular panels were different, except for cleaved caspase 3, p-p38, and pAkt which were present in both panels.

3.2.3 Variation of antibody-derived signal over time and batch effect

The sensitivity of a mass cytometer varies over time. There is temporal variation between different days and even during the same run, with a drop in sensitivity over time. Therefore, the data is routinely normalized after acquisition. This is achieved by normalization beads, which contain a blend of four different metal salts. These beads are mixed with the single-cell suspension and used for normalization. This type of normalization can correct for fluctuations in machine sensitivity, and the data is normalized to EQ bead standard.(124) However, the normalization is only based on four metals. The other metals in the antibody panel are normalized based on linear estimates from the variation of these four metals. In addition, this type of normalization does not account for the other factors that can create variability between different measurements such as variability in reagent lots, technical variability in sample preparation, and the ratio of antibody concentration to cell number per measurement. These variabilities are often referred to as batch effects. Sample multiplexing, where different samples are barcoded with Palladium, allows up to 20 samples to be stained in the same tube. However, larger experiments might still require multiple batches or barcode pools. The variability between different batches is often referred to as a batch effect.(125)

To minimize the batch effect in this thesis, all samples from each patient were placed in the same barcode pool. We also included one identical reference sample in each of the seven barcode pools. This reference sample consisted of pooled peripheral blood samples from seven healthy donors. The mix of several healthy donors was done to get a representative reference sample and to get enough material for all barcodes.

In paper, I the seven different barcode pools were standardized according to this reference sample in barcode pool 7. We used the CytoNorm approach without clustering in 101 quantiles. (126) However, after the experiments in paper I were completed, we

realized that healthy donors might not be the ideal reference sample due to the absence of specific surface markers present in the patient samples, such as CD34+, CD117 and CD90. Furthermore, the healthy samples were expected to have lower intracellular signaling levels in healthy donors. Thus, signals that are not present in the reference sample could not be normalized. An ideal reference sample should therefore reflect the material analyzed in the study and ideally express all the antibodies analyzed. Therefore, to perform a better normalization in manuscript III, we added a cisplatin-stained AML patient sample to the barcodes. This sample was used for normalization by the CytoNorm approach in manuscript III. Both mass cytometry measurements were done before the CytoNorm approach was published in 2020. Therefore, we had only included one reference sample in the paper I and two different samples in manuscript III (one healthy donor and one AML patient). A barcode pool should ideally contain two identical reference samples, to control the effect of the normalization in the second reference sample.

3.2.4 Mass cytometry data pre-processing

After normalization of the acquired data by the standard of the beads, cells were gated by DNA-Ir191 versus event length and DNA-Ir191 vs DNA-Ir192 to exclude cell doublets. This method was applied in Paper I. For Manuscript III we also used a Gaussian gating where cells were gated on the Gaussian parameters (Center, Width, Ofset and Residual versus Time).(127) The Gaussian parameters are measurements that give information of the quality of the total ion current pulse. Unwanted non-Gaussian pulses are then removed from the data, making it easier to gate the final event length and DNA gates. This removal and data cleanup of unwanted events should be done carefully, as important data might be lost during these steps. The DNA staining in mass cytometry is done by an iridium-based intercalator and the DNA staining is likely different between cell types and for different stages of the cell cycle. Therefore certain cell types, especially blast cells, might have more DNA staining than other cells.(128) This initial gating of our data might be a considerable source of artifact in mass

cytometry data, as cell doublets might be included in the data analysis if the singlet gating is not strict enough. These cell doublet populations might be misinterpreted as blast cells as they have aberrant surface marker expression.

3.3 Automated data analysis and machine learning

Mass cytometry produces high dimensional data, and an essential task in the data analysis pipeline is to identify different cell subsets within the dataset. The traditional way of analyzing flow cytometry data relies on the bi-axial gating of cell populations. This method is challenging in a mass cytometry dataset with increased dimensionality. However, bi-axial gating is an important validation of cell clusters identified by clustering algorithms. In paper I we used manual gating to validate our results. A clustering algorithm might artificially divide cells into many subsets if too many surface markers are used for clustering. For instance, if the researcher would like to investigate all the CD8+ T-cells, without sub-stratifying with e.g. HLA-DR, a manual concatenation of clusters would have to be done. Or this might be done more easily by manual gating.

3.3.1 Dimensionality reduction and clustering algorithms

There are two sets of common tools for identifying cell-subsets in mass cytometry data, namely the dimensionality reduction algorithms like PCA, t-SNE, and UMAP and the clustering algorithms like SPADE, FlowSOM, Phenograph and x-shift. The dimensionality reduction algorithms are methods for visualizing these high-dimensional data by giving each datapoint (cell) location on a two- or three-dimensional map, making the data easier to interpret.

Clustering algorithms distribute cells into distinct clusters that can be considered entities. The advantage of using clustering algorithms is that they are data-driven, reproducible, and avoid subject bias from manual gating. Therefore, they can be used

to identify both canonical and new cell populations, which is especially useful in exploratory datasets and cancer with aberrantly expressed surface markers. However, there is still some requirement for user input in the clustering algorithms, such as determining the number of clusters or neighbors to be considered.

Clustering algorithms rely on manual annotation of the identified cell populations. When many samples are analyzed, this can be time-consuming and limits the reproducibility of identifying cell populations across different samples. Furthermore, there is a loss of single-cell resolution when the average of a signal across a cell cluster is used in the data analysis.

In paper I, we applied the clustering algorithm FlowSOM. FlowSOM is a clustering algorithm that uses a self-organizing map (SOM) for cell clustering. The pipeline consists of four steps: reading fcs-files with scaling of the data, building, and training a self-organizing map, building a minimal spanning tree for visualization, and finally, metacustering.(129) We chose this analysis because it was one of the fastest clustering algorithms at the time, and allowed us to cluster cells without downsampling. The application of FlowSOM requires the selection of grid size, which represents the number of nodes the cells should be plotted to, and the number of desired meta clusters.

The challenge with manual gating and all clustering algorithms is that cell identity is not distinct but lies in a continuum of mixed phenotypes or is in transition between cell states. The appliance of clustering algorithms always needs manual expertise for interpretation. And often, a concatenation of similar cell clusters is needed or preferred, depending on the research question. An advantage when not applying too many meta clusters is that there is less probability of different clusters being a result of batch effect. The choice of clustering algorithm will always be a compromise between time and biological representability.

3.3.2 LASSO Cox regression

For survival analyses in paper I, we applied a Cox Lasso regression model with automatic feature selection and nested leave-one-out cross-validation to determine the regularization parameter. LASSO is applied to the cox regression model for variable selection and shrinkage of coefficients.(130) This makes the results from the Cox regression easier to interpret and selects only the best predictive marker for survival. Feature input in the model included, for each MC, the measured dual count (arcsinh transformed, 90th percentile) of the intracellular markers: cCaspase3, CyclinB1, p-4E-BP1(T37/T46), p-AKT(S473), p-AXL(Y779), p-CREB(S133), p-ERK1/2(T202/Y204), p-Histone3(S28), p-NF-kB p65(S529), p-p38(T180/Y182), p-Rb(S807/S811), p-S6RP(S235/S236), p-STAT1(Y701), p-STAT3(Y750) and p-STAT5(Y684). Additionally, we included the change in the level of each feature from baseline to 24h (delta 24h), size of the 10 MCs, age, and sex as features in the model. Survival of the individual patients was followed until five years post-induction chemotherapy. For each selected feature we performed time-to-event analyses in a regularized Lasso Cox proportional hazards model. We split the resulting features that were significantly associated with survival at the median and plotted the survival curves for patients with high and low values of each feature. Since we used leave-one-out cross-validation and only had 32 patients, we could use all possible 1-patient subsets as test samples, all 1-patient subsets as validation samples and all 30-patient subsets as training data. CyTOF data are multidimensional and complex. Thus, the downstream analysis requires effective computational methods that can handle such data. In this case, we used lasso regression, which is commonly used in the mass cytometry literature.

3.3.3 Lineage discriminant analysis (LDA) and K-nearest neighbor

In paper II we used a semi-automated gating strategy applying FlowDensity on the peripheral blood and bone marrow samples of the healthy donors in our cohort. We then trained two supervised single cell classifiers, namely the lineage discriminant analysis (LDA) and the K-nearest neighbor (KNN) algorithms, based on the cell types identified

in the healthy donors. These two algorithms were then compared in their performance of identifying healthy cell populations. The K-nearest neighbor algorithm combines the classification of the K nearest points to determine the classification of a point. It is a supervised algorithm as you classify each point based on the known classification of other points.

3.3.4 DREMI: Conditional density resampled estimate of mutual information.

Signaling biology is complex; the methods we typically use to understand these networks, eg graph diagrams, fail to capture the complexity of signaling networks. DREMI (Conditional-Density Resampled Estimate of Mutual Information) is a data-driven approach that can quantify signaling interactions in molecular circuits. Given a relationship between two proteins, X and Y, DREMI considers the abundance or activity of protein Y as a stochastic function of the abundance or activity of protein X. (131) This algorithm is based on the statistical methods: conditional probability and density estimation. An important strength of this approach compared to previous approaches is that DREMI computes mutual information on the estimated conditional probability of Y/X rather than the joint probability of X and Y. Joint probability is like a traditional scatter- or density plot and describes the density of cell states. Whereas conditional probability describes how the state of Y varies with different states of X and thereby reveals signaling behavior along its full dynamic range. Conditional-Density Rescaled Visualization (DREVI) is used to visualize and characterize the estimated conditional density function as a rescaled heatmap. It visualizes the stochastic function of how X influences Y. DREMI is like mutual information (MI) a shape-agnostic measure that scores how predictive X if of Y. Still, it is not symmetric and might therefore inform about the directionality and strength of the relationship between X and Y. DREMI start with the conditional density estimate from DREVI. Then resamples the data evenly through the range of conditional probability density, and MI is computed on the resampled data. The entire X range is covered, and each slice of the X axis contains the same number of cells. The challenge of the DREMI/DREVI method is

understanding the results and how to interpret them. Laboratory validation of the results is also challenging, and the choice of an appropriate validation method is not obvious.

3.3.5 VoPo

For paper III we applied the VoPo algorithm for data analysis. Vopo, or Vox populi which means “voice of the people”, is based on the principle that heterogeneity in estimations can be used for a more precise prediction. This concept was published by Francis Galton in Nature in 1907. In this study, he applied statistics to a guessing contest. Villagers were invited to guess the weight of an ox after it was slaughtered. 800 people participated in this contest, and to his surprise, he found that the median of the 800 guesses was exactly the weight of the ox. Vopo is a bioinformatics pipeline for the prediction and visualization of single-cell data. (132) First each sample is clustered into 1000 clusters, then VoPo does a repeated metaclustering on all samples, in paper III we used the default which is 50 metaclustering solutions. We used only the surface markers for this metaclustering. VoPo uses the k-means clustering method for all clustering steps. The k-means method is an unsupervised clustering algorithm that places each point into a cluster such that the points in each cluster are near each other. Unsupervised Laplacian score-based feature selection is applied to these 50 metaclusters. In paper III we used the change in intracellular markers from pre-treatment to 4 or 24 hours as our features. The result is a collection of features that is measured across the independent metaclustering solutions. The results from each single cell can then be visualized in a UMAP. Cells are then colored by their degree of association with the change in intracellular marker between the two timepoints (pre-treatment to 4 hours or pre-treatment to 24 hours) and their likelihood of belonging to a particular metacluster. The different metacluster solutions are not visualized in these plots as this would be too difficult to visualize or interpret. VoPo uses single cell dual counts for metaclustering. And then calculate the median intensity difference in signal between two timepoints for each of the 50 metaclusters. The significance is calculated by a grouped Wilcoxon Rank Sum test.

3.4 Proteomics

The proteome is fundamentally different from the genome. The genome is static and well-defined for an organism, while the proteome changes continually in response to external stimuli and internal events. The proteome defines the cell's functional properties and determines its phenotype. In this thesis, we applied labeled mass-spectrometry-based proteomics to validate changes in the proteome during chemotherapy. The patient samples were mixed with a super-SILAC mix composed of five heterogenous AML cell lines labeled with Arg6 and Lys8 isotopes. The super-SILAC mix serves as an internal standard for the MS analysis.(133) In MS-based proteomics, trypsin is used to cut the proteins into smaller peptides identified by the machine. The principles of mass spectrometry are similar to mass cytometry, but instead of a time-of-flight measurement of the ions, they are measured by an Orbitrap. The orbitrap analyzes the ions as a function of their rotational frequencies and converts it to mass spectra. The peptides and subsequent protein identifications is then performed through database searches by computational algorithms. In this thesis, we used MaxQuant and the Andromeda search engine. A major challenge in proteomics is the proteome coverage of biological samples. There might be extensive overlap between peptides which makes protein identification difficult. The SILAC method has improved the proteome coverage to some extent, but many proteins are still not identified. Some heavily modified proteins, such as p53, might be difficult to detect by proteomics. Our proteomics data did not detect several of our proteins of interest, such as p53 and several of the BCL-2 family members. Another important question is the detection/identification of proteins in cancer. Mutations and cytogenetics might make the proteins unrecognizable to the search engine.

In this thesis, we have used proteomics for the validation of our mass cytometry results. Although both methods investigate proteins the challenge of comparing these methods is that proteomics is bulk analysis while mass cytometry analysis is single-cell resolution. Ideally, since we in our mass cytometry analysis mostly look at

phosphorylated proteins, the proteomics should also be phospho-specific. This was initially the plan, but due to limited amounts of protein extracted from these samples, we did not have enough material for phospho-proteomics.

3.5 RNA sequencing

The transcriptome is all the messenger RNA (mRNA) transcripts produced in a particular cell. In contrast to the genome and like the proteome, the transcriptome is changing to environmental conditions. Initially, sanger sequencing and microarray methods were used for sequencing the transcriptome. These approaches have limitations such as low throughput, high costs, and for microarray, the reliance on existing knowledge about the genome sequence. RNA-sequencing (RNA-seq) is a high-throughput method that uses deep-sequencing technologies. RNA is converted to a library of RNA fragments or cDNA with adaptors attached to both or one end. Each molecule is then sequenced in a high throughput manner. The resulting reads are aligned to a reference genome or transcript. (134) One of the challenges with RNA-seq is that large RNA molecules must be fragmented into smaller pieces, making their identification difficult. Short reads can be identical to one another, making it difficult to determine whether these are abundant RNA or PCR artifacts. Another important issue is the sequencing coverage and depth, which has cost implications. In addition, RNA is very unstable, and therefore variations in sample preprocessing time and other technical variations are important sources of artifacts in these data.

3.6 Drug sensitivity and resistance testing

In this thesis, drug sensitivity and resistance (DSRT) testing was performed on fresh bone marrow or blood from 23 of the patients included in the study. These samples were screened for sensitivity to 349 drugs (anti-cancer Selleck Chemical library) at five different concentrations. Cells were incubated for 72 hours, and cell viability was measured by the ATP-based cell viability assay CellTiter-Glo. The result from each

patient was then normalized to the median of the positive (cells treated with BzCl) and negative controls (Cells treated with DMSO) and presented as a selective drug sensitivity score (sDSS).

Ex vivo DSRT has successfully been used to identify new drug targets and functional drug response patterns for AML.(135) With molecular profiling of the patient, the information from the DSRT assays can be used for clinical treatment decisions.(136) However, the clinical applicability of these high throughput screens in a clinical routine setting has to be investigated further. DSRT assays rely on cell viability or proliferation measurements in an *ex vivo* setting. Functional drug response might be different *in vivo*, with the influence of the tumor microenvironment. The readout of the Cell-Titer Glo assay used in this thesis is an average measurement of the remaining cells in the well. It does not account for different responses across a heterogenous blast population present in the leukemia patient. The leukemic blast cells might have different responses to the drugs based on functional or genetic heterogeneity between different blast cells. Ideally, the assay should be combined with a flow or mass cytometry analysis to reveal which cells were sensitive or resistant to the chemotherapy. Additionally, there might be variations between patients on how long the cells will survive in vitro. Some presumably healthy cell populations might not survive in vitro but can have effects on drug response in vivo. The results from these assays might also be affected by growth factors present in the incubation media.(137) The results from this assay should therefore be interpreted with caution and might not be transferable to the in vivo setting.

4. Summary of papers

Paper I

Early response evaluation by single cell signaling profiling in acute myeloid leukemia

A fundamental hallmark of cancer cells is their ability to sustain proliferative signaling and cell survival, reflected in a cellular chemotherapy response that is poorly understood. We questioned whether chemotherapy modulated phospho-signaling at 4 and 24 h *in vivo* could provide information about long-term survival in acute myeloid leukemia (AML), and if the signaling response to therapy was more informative than analysis at time of diagnosis. Peripheral blood was collected from 32 younger AML patients (age 16-74 years), before, 4- and 24 hours after start of induction chemotherapy. Samples were analyzed by 36-dimensional mass cytometry to assess alterations in immunophenotypes and intracellular signaling using unsupervised and supervised machine learning approaches. Results were validated by RNA sequencing and mass spectrometry proteomics (Super SILAC). Targeted sequencing was used to characterize patient samples for recurrent AML mutations. Drug sensitivity and resistance testing *ex vivo* was compared to activation of relevant signal transduction pathways and mutational profile. 5-year patient survival was accurately predicted in the leukemic cell population at 24 hours after therapy onset by phospho-proteins p-ERK1/2 (T202/Y204) and p-p38 (T180/Y182). RNA sequencing showed induction of MAPK target gene expression and the AP-1 transcription complex in patients with high p-ERK1/2. Super-SILAC proteomics confirmed an increase in the abundance of p38 prime target MAPKAPK2(MK2) 24 hours after start of induction therapy. *Ex vivo* drug sensitivity testing demonstrated high sensitivity to MEK inhibitors in the patient cells with high p-ERK1/2 measured at diagnosis or 24 hours after start of chemotherapy. Early single cell signaling response to chemotherapy provided precise prognostic information independent of stratification by genetics. We propose that early functional measurement of chemotherapy-potentiated MAPK pathway signaling could identify non-responders to intensive chemotherapy allowing precise treatment adjustment.

Paper II

Identifying predictors of survival in patients with leukemia using single-cell mass cytometry and machine learning

In this paper, we used the same dataset as in paper I. The aim was to use other methods to identify predictors of chemoresistance and lack of treatment response. Here we applied an automatic cell population annotation and combined this with analysis of the interactions between the signaling proteins. To identify the cells' cellular phenotypes, we used a semi-automated gating strategy by FlowDensity on the peripheral blood and bone marrow samples of the healthy donors in our cohort. We then trained a supervised single cell classifier (K-nearest neighbor- KNN) based on the cell types identified in the healthy donors. Furthermore, we applied this automated gating strategy to the 45 leukemia samples. Cellular abundance was compared between the leukemia samples and the healthy donors and between patient groups with specific mutations and structural re-arrangements. Patients with FLT3-ITD had a lower abundance of CD4+ and CD8+ T helper and cytotoxic cells. In a cox proportional hazard modeling, patients with higher T helper cell abundance at diagnosis showed better survival. We used Conditional Density Resampled Estimate of Mutual Information (DREMI) to investigate the signaling profiles of the identified cell populations. For each cell type, a DREMI score was calculated for each pairwise combination of the 14 intracellular markers. The DREMI scores were then ranked using a minimum redundancy maximum relevance (mRMR) criterion. LASSO was applied to train models and make predictions. The DREMI scores of the MPP cells achieved the best results in predicting short-term survivors from long-term survivors. Patients with higher pCREB-pSTAT5 and pP38-pSTAT5 DREMI scores had better survival. Then we clustered the DREMI scores of the MPP cells to see if this could identify different subgroups of survival. Kaplan-Meier analysis showed the worst prognosis for cluster C2, which had a low DREMI score. One possible explanation for this might be that a low DREMI score reflects an increased heterogeneity within the MPP cell population in patients in cluster C2. Others have

shown that increased DREMI scores are higher within homogenous cell populations. The DREMI scores of pCREB-pSTAT5 and pp38-pSTAT5 were finally added to the existing risk classification of the AML patients in our cohort and improved the risk classification of the patients.

Paper III

Early modulation of BCL-2 family proteins after start of chemotherapy may predict AML survival and therapy response

In paper III we used the same patient material as in paper I and paper II. This material was originally barcoded for paper I, frozen down and then analyzed by an antibody panel focusing on the BCL-2 protein family and p53 for paper III. The BCL-2 protein family is central in leukemic cell resistance to chemotherapy. The in vivo changes in BCL-2 proteins and p53 during induction therapy are incompletely understood. Due to the importance of these proteins in cell fate, the investigation of these proteins in vivo might elucidate how leukemic cells respond to chemotherapy and reflect clinical response. In this paper we applied an antibody panel of 17 intracellular markers (cCaspase3, CyclinB1, p-4E-BP1(T37/T46), p-AKT(S473), p-AXL(Y779), p-CREB(S133), p-ERK1/2(T202/Y204), p-Histone3(S28), p-NF-kB p65(S529), p-p38(T180/Y182), p-Rb(S807/S811), p-S6RP(S235/S236), p-STAT1(Y701), p-STAT3(Y750) and p-STAT5(Y684)) and 21 surface markers. The surface marker panel was very similar to the surface marker panel applied in paper I, with a few exceptions as shown in Figure 3. Mass cytometry data was analyzed by the bioinformatics pipeline VoPo, which applies repeated metaclustering. The anti-apoptotic protein MCL-1 showed a decrease at 4 hours in the CD34+ cell population for all patients who were alive 5 years after start of induction therapy. Similar changes were observed for BCL-2. Proteomics analysis confirmed a decrease of BCL-2 in patients alive at 5 years as observed in our mass cytometry data. RNA sequencing data showed an induction of p53 associated genes as reported before. We conclude that early *in vivo* detection of

MCL-1 and BCL-2 in the leukemic cell compartment by mass cytometry might reflect an early chemotherapy response.

5. Discussion

Profiling of signaling networks in cancer cells might reveal the behavior of cancer cells. By exposing the cancer cells to potentiating inputs such as cytokines and growth factors, cancer network profiles that correlate to disease outcome and genetics can be revealed. (138, 139) The investigations of potentiated signaling networks in cancer cells has mainly been carried out in vitro. In this thesis, we hypothesized if the chemotherapy itself could be used as a potentiator for these signaling networks. A peripheral blood sample after start of chemotherapy could thereby represent an *in vivo* stress test of the cancer cells to reveal the function of the signaling networks. This could tell us something about the cancer cells' response to therapy, the mechanism of resistance, and the functionality of these signaling networks *in vivo*. Like an exercise stress test of the heart to reveal heart disease, we do a stress test on the cancer cells to reveal weaknesses and new therapeutic targets in the signaling networks of the cancer cells.

Previous studies performed in our group have shown that genes and proteins related to chemotherapy resistance, p53, and cell survival are altered only hours after the start of induction therapy in AML.(115, 140) Mass cytometry could bring us to the next level by elucidating therapy response at the single cell level, in different cell subsets, both healthy and leukemic cells.

The immunophenotype of AML cells is elusive but most often characterized by aberrant expression of surface markers. There is extensive heterogeneity in immunophenotypes between patients and within the same patient. Whilst flow cytometry relies on multiple panels of antibodies for MRD detection, mass cytometry can measure over 40 antibodies per single cell. This gives a new level of resolution, which can reveal novel cell types. Mass cytometry has been used previously to detect changes in immunophenotypes after the start of induction therapy in AML.(141) Levine *et al.* used mass cytometry to investigate signaling responses to cytokines and growth factors in AML ex vivo. They found that there was a disconnection between the immunophenotype and signaling in AML cells. In healthy cells, there was a tight link between surface and signaling phenotype in primitive and mature cell types. This was

not present in the leukemic samples, where the signaling profile resembled the phenotype of primitive cells but was not associated with expression of the primitive marker CD34.(41) The authors, therefore, claimed that the signaling phenotype might be a better surrogate for the functional state of the cell. The results presented in paper I, II and III support this hypothesis.

Few studies have used mass cytometry for early response evaluation so far. In the clinical trial ENEST1st for chronic phase CML, monotherapy with nilotinib induced a signaling response 7 days after the start of therapy that could identify optimal responders. This was confirmed by quantitative PCR of BCL-ABL1 at 18 months.(142) Behbehani *et al.* investigated samples from AML patients receiving hydroxyurea (HU) by mass cytometry. In vitro treatment of AML cells with HU induces a complete S-phase arrest and cell cycle exit. But this was not observed in the samples from patients undergoing treatment. This discrepancy indicates that we have much to learn from in vivo functional profiling of signaling networks and might give us a better understanding of why some patients respond or don't respond to therapy.

6. Concluding remarks

The primary aim of this thesis was to investigate if early signaling changes in response to chemotherapy in AML could tell apart responders from non-responders. In paper I, we successfully found that pERK1/2 at 24 hours in a specific myeloid cell cluster was able to patient 5-year outcomes of AML patients undergoing induction therapy. Here we used a standard approach for mass cytometry data analysis with a clustering algorithm and LASSO Cox regression analysis. The mass cytometry results were validated through manual gating of the data. We found pERK1/2 targets upregulated after therapy by proteomics and RNA sequencing. The DSRT data showed an increased sensitivity towards azacytidine and MEK inhibitors for patients with high pERK1/2. In paper II, we evaluated if the interactions between the different signaling molecules could, in the same dataset as paper I, give further information on patient outcomes. In this study, we found that the mutual information between the signaling proteins could give prognostic information at the time of diagnosis. In paper III, the same patients as in paper I were analyzed with a new antibody panel focused on the transcription factor p53 and the BCL-2 protein family. These proteins decide the fate of the cells, and might further elucidate the effect of the signaling pathways investigated in paper I. In paper III we found that cells with expression of anti-apoptotic proteins are reduced within 4 hours after start of induction therapy in patients who are responding to therapy.

The same patient material, consisting of consecutive samples from 45 leukemia patients, was used in all three papers. The cohort size is small but deeply characterized in these three studies, with large amounts of data. The patient material we present in this study is unique. Samples are collected shortly after the start of treatment and processed using fix/lysis right after sample collection to preserve the phosphorylation status of intracellular signaling proteins. This requires immediate on-site sample processing by qualified personnel in appropriately equipped labs, which limits sample collection significantly. To our knowledge, no other datasets like this exist. Through these three papers, we have successfully increased the understanding of AML cell's functional response to chemotherapy. We have used machine learning to generate hypotheses from a complex mass cytometry dataset. We used it to dissect the data by

linking single cells to predictive markers. We aimed to present this as a framework that can be used in future larger studies, not only for AML but possibly other cancer forms.

7. Future perspectives

Most studies of cancer cell signaling have been performed by removing the cells from their tissue context and investigating signaling *in vitro*. This introduces perturbation in the signaling state and response, and essential information might be lost. One of the strengths of these studies is that we performed a quick fixation of the cells after sampling to preserve the *in vivo* signaling state as close as possible to *in situ*. In an ideal world, we would not have to remove the cells from the patient's body to investigate. This might be achievable through future imaging technologies, e.g., PET-CT with suitable probes.

A common question to our concept of investigating signaling responses after the patient has started treatment is: "would it not be better to know which treatment to give in advance". This is the goal we are aiming at, but we believe precision medicine based on genomics still may fail or be suboptimal through a multitude of unknown mechanisms of the cancer cell and the host. Therefore, early investigation of how cancer cells respond to therapy *in vivo* may be a necessary key to improving cancer therapy.

In the era of personalized medicine, powerful multi-omics tools continue to revolutionize the characterization and treatment of cancer. In AML research, our understanding of the disease has dramatically improved since the first whole-genome sequencing of an AML patient in 2008. With the vast amount of information from multi-omics data, there is a need for a close collaboration between molecular scientists, physicians, and bioinformaticians to apply this knowledge to clinical practice. The countless amounts of data will dramatically increase in the future as scientists unravel the information from epigenetics and the non-coding genome and the application of new technologies like single cell proteomics emerge.(143)Another interesting technology is CITE-seq. This method performs RNA sequencing along with surface antibody staining on the single-cell level. In this thesis, we found our results with single-cell mass cytometry. However, our results were validated by bulk proteomics and RNA sequencing. Technologies like single cell proteomic and single cell RNAseq or CITE-seq would be the techniques that could genuinely validate our results. This

necessitates the development of better antibodies for the detection of specific mutations like TP53 and BCR-ABL. Then we could be more confident that we are accurately investigating the malignant cells.

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