Therapeutic and prognostic implications of metabolism in acute myeloid leukemia

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

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Supervisors of this work have been Kimberley Joanne Hatfield (MSc, PhD) as main supervisor, Øystein Bruserud (MD, PhD, Prof.) and Håkon Reikvam (MD, PhD, Ass. Prof.).

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Ida

Table of Contents

Abbreviations

2DG	2-deoxy-D-glucose
6AN	6-aminonicotinamide
Akt	Protein kinase B
ALL	Acute lymphoblastic leukemia
allo-HSCT	Allogenic hematopoietic stem cell transplantation
AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinase
Ang-1	Angiopoietin 1
APL	Acute promyelocytic leukemia
AraC	Cytarabin/1-b-arabinofuranosylcytosine
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BPTES	Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide 3
CAR	Chimeric antigen receptor
CBF	Core binding factor
CCL-1	C-type lectin-like receptor 1
CD	Cluster of differentiation
cGVHD	chronic graft versus host disease
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CPT1a	Carnitine palmitoyl transferase 1
CQ	Chloroquine
CR	Complete remission
CXCL12	C-X-C motif chemokine 12 ligand
DFS	Disease free survival
DNA	Deoxyribonucleic acid
ELN	European leukemia Net
ETCI	Electron transport chain complex I
FAB	French-American-British
FAO	Fatty acid oxidation
FLT3	Fms-related tyrosine kinase 3
G6P	Glucose-6-phosphate
GDH	Glutamate dehydrogenase
GLS	Glutaminase

GVL	Graft-versus-leukemia
HDAC	Histone deacetylases
HK	Hexokinase
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
IL	Interleukin
IL1RAP	IL 1 receptor accessory protein
INF	Interferon
ITD	Internal tandem duplication
LSC	Leukemic stem cell
MCT	Monocarboxylate transporters
MDS	Myelodysplastic syndromes
MRD	Minimal residual disease
MSC	Mesenchymal stromal cell
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NPM1	Nucleophosmin
OS	Overall survival
OXPHOS	Oxidative phosphorylation
PET	Positron emission tomography
PFK	Phosphofructokinase
PI3K	Phosphoinositide 3-kinases
PK	Pyruvate kinase
PML	Promyelocytic leukemia
PPP	Pentose phosphate pathway
RIC	Reduced-intensity conditioning
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor 1
SL-IC	SCID leukemia-initiating cell
t-AML	Therapy-related AML
TCA	Tricarboxylic acid
TKD	Tyrosine kinase domain
t-MN	Therapy-related myeloid neoplasms
VCAM-1	Vascular cell adhesion molecule-1
VEGFR2	Vascular endothelial growth factor receptor 2
VLA-4	Vary late antigen-4

WBC	White blood cell
WHO	World Health Organization
αKG	α-ketoglutarat

Genes

ABL	ABL proto-oncogene 1, non-receptor tyrosine kinase
ASXL1	Additional sex combs like-1
BCR	BCR activator of Rho GEF and GTPase
CBF	Core binding factor
CEBPA	CCAAT/enhancer-binding protein alpha
DEK	DEK proto-oncogene
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
EVI1	Ecotropic viral integration site 1
EZH2	Enhancer of zeste homolog 2
FLT3	Fms-related tyrosine kinase 3
GATA2	GATA binding protein 2
GLUT1	Glucose transporter
IDH	Isocitrate dehydrogenase
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
KMT2A	Lysine Methyltransferase 2A
MECOM	MDS1 and EVI1 complex locus
MLLT3	MLLT3 super elongation complex subunit
MYH11	Myosin heavy chain 11
NPM1	Nucleophosmin
NUP214	Nucleoporin 214
PML/RARα	Retinic acid receptor alpha
RBM15	RNA binding motif protein 15
RUNX1	Runt-related transcription factor 1
SMC1A	Structural maintenance of chromosome 1A
SMC3	Structural maintenance of chromosome protein 3
SRSF2	Serine and Arginine Rich Splicing Factor 2
TET2	Tet methylcytosine dioxygenase 2
TP53	Tumor protein p53
U2AF1	U2 small nuclear RNA auxiliary factor 1
WT1	Wilms tumor suppressor gene 1

Summary

Acute myeloid leukemia (AML) is a highly aggressive form of blood cancer, characterized by an interruption in the differentiation process and by rapid proliferation of immature myeloid leukemia cells leading to bone marrow failure. On a molecular level, multiple genetic and epigenetic alterations are detected in hematopoietic progenitors that may contribute to disease progression, which results in the considerable inter- and intraindividual heterogeneity of AML clones. This heterogeneity also contributes to high plasticity in metabolic reprogramming, allowing cancer cells to maintain proliferation and cell growth under the high demands of expanding growth. Leukemia cells may employ different metabolic routes based on the molecular alterations of the disease, and this may in turn imply that certain AML patient subsets have common metabolic characteristics. These AML subsets may either have a more extensive adaptive capacity, or be more sensitive to therapies targeting metabolism. Thus, it may be possible to stratify patients according to metabolic phenotypes/capacities, which in turn may serve as a common therapeutic target.

In the four papers presented in this thesis, we investigated AML cell metabolism, either as a possible target for treatment (**Papers I** and **II**), or to examine if metabolic alterations in AML patients might have prognostic value (**Papers III** and **IV**). In the first two papers, we studied the *in vitro* antileukemic effects of eight different metabolic drugs (metformin, 2DG, 6AN, BPTES, ST1326, lonidamine, AZD3965 and chloroquine (CQ)), alone and in combination with chemotherapy (AraC), targeting different metabolic pathways and proteins involved in the regulation of cancer cell metabolism. In addition, we studied the metabolomic profiles of AML patients, (i) before and after treatment with disease-stabilizing therapy including all-trans retinoic acid (ATRA) and valproic acid, and (ii) in cases of chronic graft versus host disease (cGVHD) after allogenic hematopoietic stem cell transplantation (allo-HSCT). We aimed to see if alterations in systemic metabolite profiles can be used as a prognostic tool to help determine which patients might benefit from cGVHD treatment.

The results presented in this thesis confirm the heterogeneity of AML patients as regards susceptibility to metabolic targeting agents. A subset of patients with generally high sensitivity to multiple metabolic drugs was identified using an unsupervised hierarchical clustering analysis based on the antiproliferative effect of metabolic inhibitors. We saw that the clustering of this subset was largely driven by the patient cells´ sensitivity to metabolic drugs effecting the glycolytic pathway, supporting the idea that glycolysis is the main metabolic pathway for many types of cancer. We saw that the majority of our AML patient cells were sensitive to chloroquine, a known inhibitor of late-stage autophagy, also used to treat malaria. CQ proved

to have antileukemic effects, and interestingly there was also a smaller subset of patient cells that showed high *in vitro* susceptibility to CQ, but on whom standard chemotherapy (AraC) had little or no effect. We did not however identify any significant molecular similarities among the patients with similar response to metabolic inhibitors, nor did we find correlations to any of the known prognostic markers (i.e. secondary versus *de novo*, AML cell differentiation (FAB classification, CD34 expression), karyotype, *FLT3*-ITD or *NPM1* mutations).

Our studies also showed that systemic metabolomic profiling can be used to examine effects of treatment with ATRA and valproic acid. We observed alterations in the amino acid and lipid profiles when studying ATRA and valproic acid as monotherapy, and changes were most apparent after valproic acid treatment. Even though, patients with rapidly progressive disease showed extensively altered lipid metabolism, pretreatment metabolite profiles could not be used to distinguish responders from non-responders to this AML-stabilizing treatment. Metabolic profiles were also altered in patients with cGVHD after allo-HSCT compared to patients without signs of cGVHD. We were able to identify a subset of 11-metabolites not effected by immunosuppressive treatment, that might have both diagnostic and prognostic value for cGVHD.

To conclude, AML patients are heterogeneous with regard to susceptibility to metabolic inhibitors, and further investigation has to be conducted to identify common therapeutic targets. Patients are heterogeneous also with regard to their metabolic profiles, making it challenging to analyze and identify significant changes of single metabolites. However, our studies show that changes in metabolomic profiles may influence both epigenetic regulation and bone marrow microenvironment. These changes may in turn modulate disease progression as well as contribute to therapy resistance, thus giving metabolomic profiling a potential prognostic value.

List of publications

Article I

Ida Sofie Grønningsæter, Håkon Reikvam, Elise Aasebø, Sushma Bartaula-Brevik, Øystein Bruserud and Kimberley Joanne Hatfield. Targeting cellular metabolism in acute myeloid leukemia and the role of patient heterogeneity. *Manuscript.*

Article II

Ida Sofie Grønningsæter, Håkon Reikvam, Karen Marie Hagen, Sushma Bartaula-Brevik, Øystein Bruserud and Kimberley Hatfield. Effects of the autophagy-inhibiting agent chloroquine in acute myeloid leukemia. *Manuscript.*

Article III

Ida Sofie Grønningsæter, Hanne Kristin Fredly, Bjørn Tore Gjertsen, Kimberley Joanne Hatfield and Øystein Bruserud. Systemic Metabolomic Profiling of Acute Myeloid Leukemia Patients before and During Disease-Stabilizing Treatment Based on All-Trans Retinoic Acid, Valproic Acid, and Low-Dose Chemotherapy. *Cells. 2019. 8 (10). 2073-4409.*

Article IV

Håkon Reikvam, Ida Sofie Grønningsæter, Knut Anders Mosevoll, Roald Lindås, Kimberley Joanne Hatfield and Øystein Bruserud. Patients with treatment-requiring chronic graft versus host disease after allogeneic stem cell transplantation have altered metabolic profiles due to the disease and immunosuppressive therapy: potential implication for biomarkers. *Frontiers in Immunology. 2018, 8 (2). 1664-3224.*

1 Introduction

1.1 General background

Acute myeloid leukemia (AML) is an aggressive form of blood cancer [1]. It is characterized by the abnormal proliferation and differentiation of clonal bone marrow myeloblasts, resulting in impaired normal hematopoiesis and thereby bone marrow failure. Even though AML is the most common acute leukemia in adults, its incidence is of only 2.5-3 per 100.000 [2]. The median age at the time of first diagnosis is 65-70 years, meaning there is a majority of elderly patients. Elderly patients tend to have poorer prognostics due to adverse cytogenetics, resulting in refractory disease. These patients should not receive the most intensive and potentially curative treatment due to an unacceptable risk of severe toxicity and treatment-related mortality [3]. AML is highly heterogeneous with regard to both cytogenetic abnormalities and differences in molecular genetics [3]. New genomic sequencing techniques have revealed a spectrum of frequent mutations and distinct mutational patterns [4, 5]. Based on molecular genetics, new targeted therapies have been suggested. However, the therapeutic approaches and the prognoses differ between patients subgroups [6]. More recent studies also suggest that metabolic reprogramming is important for chemosensitivity [7]; metabolic targeting is therefore emerging as a possible therapeutic strategy in the treatment of AML [8].

Metabolic reprogramming plays a key role in cancer cells´ ability to escape the mechanisms of homeostatic control, and contributes to the aggressiveness of the disease. This also applies to AML. High metabolic plasticity helps cancer cells acquire and utilize necessary nutrients from an often nutrient-poor environment. This allows for continued fast proliferation, expansive growth and survival even under stressful conditions, as the cells rapidly outcompete normal hematopoietic cells [9]. As we learn more about AML cells´ ability to alter their metabolism, it is understood that the high heterogeneity of the disease also applies to their metabolic reprogramming. As a result of both direct and indirect oncogenic mutations, different subtypes prefer different nutrients, and utilize different metabolic pathways, making metabolic targeting challenging [9]. However, our knowledge to date of metabolic reprogramming is scarce. More studies are needed to see if researching AML cell metabolism may contribute to improve classification, prognosis and treatment of the disease.

1.2 Diagnosis, subclassification and prognostic evaluation

The only curative treatment for AML as of today comes with severe toxicity and high treatment-related mortality [3]. Refractory disease and disease relapse are common. Finding the patients who will not only tolerate, but also benefit from this treatment is therefore crucial. As the molecular heterogeneity of AML is becoming increasingly apparent, new diagnostic procedures, as well as new subclassifications of the disease are emerging. The prognostic importance of this molecular heterogeneity is now generally accepted [10]. However, localizing new subsets that might benefit from new targeting approaches is proving to be challenging.

1.2.1 Diagnosis and subclassification of human AML

Acute promyelocytic leukemia (APL) is a separate subset of AML; the other AML subsets can be referred to as non-APL variants. All the studies in this thesis include only patients with non-APL variants, and the term AML throughout the thesis refers to the non-APL variants of AML. According to the World Health Organization (WHO) classification, AML is generally characterized by the presence of at least 20% leukemic myeloblasts among nucleated bone marrow cells. There are some exceptions in the groups labelled "AML with recurrent genetic abnormalities" and "acute erythroid leukemia" as explained in detail in the WHO classification system [11]. A small subset of acute leukemia patients cannot be classified as myeloid or lymphoblastic; specific diagnostic criteria are then used for these patient groups with either mixed phenotype or unclassified acute leukemia [12]. Cytogenetic and molecular genetic analyses are mainly used for the prognostic evaluation of the patients [2].

The previous French-American-British (FAB) classification was established several decades ago and was based merely on morphology and cytochemistry. It categorized the leukemic cells into subtypes M0 to M7 based on the degree of lineage differentiation and required at least 30% blast cells as the basis for an AML diagnosis [13]. However, the FAB classification does not reflect the genetic and clinical diversity of the disease that is important for the prognostic evaluation of patients. The first WHO classification for tumors of the hematopoietic and lymphoid tissues was published in 2001 [14], and the last update was published in 2016 [6]. The WHO classification divides AML into: AML with recurrent cytogenetic translocations, AML with myelodysplasia-related changes, therapy-related (secondary) AML, AML not otherwise categorized, Myeloid sarcoma, Myeloid proliferations associated with Down syndrome, Blastic plasmacytoid dendritic cell neoplasm and AML of ambiguous linage (Table 1).

Table 1. *A summary of the World Health Organization (WHO) 2016 classification of acute myeloid leukemia and related neoplasms [11].*

Acute myeloid leukemia (AML) and related precursor neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1);*RUNX1-RUNX1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARα*
AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A*
AML with t(6;9)(p23;q34.1);*DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM (EVI1)*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.1);*RBM15-MKL1*
AML with *BCR-ABL1*
AML with mutated *NPM1*
AML with biallelic mutations of *CEBPA*
AML with mutated *RUNX1*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified (NOS)
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomocytic leukemia
Acute monoblastic and monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed-phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2);*BCR-ABL1*
MPAL with t(v;11q23.3); *KMT2A* rearranged
MPAL, B/myeloid, NOS
MPAL, T/myeloid, NOS
Acute leukemias of ambiguous lineage, NOS

Genetic abbreviations are listed in the list of genetic abbreviations in the front of the thesis.

1.2.2 The prognostic impact of cytogenetic abnormalities

Cytogenetic abnormalities are detected in approximately 55% of all AML patients [15]. Several of these abnormalities are strong and independent predictors for prognosis, and karyotyping is therefore mandatory for the WHO classification. Eight translocations and inversions are included in the WHO category "AML with recurrent genetic abnormalities". Based on cytogenetic alterations, patients can be stratified into three classes with prognostic value, (i) favorable t(8;21)(q22;q22.1) [*RUNX1-RUNX1T1*], inv(16)(p13.1q22) or t(16;16)(p13.1;q22) [*CBFB-MYH11*] and t(15;17)(q24;q21) [*PML-RARα*], (ii) intermediate t(9;11)(p22.3;q23.3) [*MLLT3-KMT2A*], and (iii) unfavorable/adverse t(6;9)(p23;q34.1) [*DEK-NUP214*],

inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) [*GATA2,MECOM(EVI1)*] and t(1;22)(p13.3;q13.1) [*RBM15-MKL1*][11] (Table 1).

Core binding factor (CBF) is a group of heterodimeric transcription factors essential for normal hematopoiesis, and is composed of two subunits: a deoxyribonucleic acid (DNA) binding *CBF*-alpha chain (runt-related transcription factor 1 (*RUNX1)-RUNX2-RUNX3*), and a non-DNA binding *CBF*-beta chain (*CBFβ*). Genetic rearrangements involving components of the *CBF* complex are present in about 5-13% of cases of AML, usually in younger patients, and are associated with a favorable prognosis [11]. Patients with t(8;21)(q22;q22.1) AML have the fusion transcript *RUNX1-RUNXT1* which likely results in transcriptional repression of normal *RUNX1*, through aberrant recruitment of nuclear transcriptional co-repressor complexes [11]. Inv(16)(p13.1q22) or t(16;16)(p13.1;q22) both result in the fusion of *CBFβ* and *MYH11* that codes for smooth muscle myosin heavy chain [11]. Patients with *CBF* leukemias benefit from consolidation chemotherapy with high dose cytarabin (AraC). Monitoring of the fusion transcripts can also detect minimal residual disease (MRD) [15]. Patients with favorable cytogenetics also include PML with the translocation t(15;17) resulting in the oncogene *PML-RARα*. This variant of AML requires a specific treatment different from the other AML variants and has a long-term leukemia-free survival of more than 90% [16]. Patients with t(9;11)(p21.3;q23.3) are categorized as intermediate cytogenetics. The lysine methyltransferase 2A (*KMT2A*) gene (also known as *MLL*) [6] is a histone methyltransferase that assembles protein complexes that regulate gene transcription through chromatin remodeling. In translocations involving 11q23, the *KMT2A* results in a fusion gene, most common with 9p22 giving the fusion transcript *MLLT3-KMT2A*. *KMT2A* (11q23.3) has an overall poor prognosis, but is modified by the fusion partner 9p21.3, giving t(9;11)(p21.3;q23.3) a better prognostic classification than the other 11q23 fusion genes [15].

The abnormalities t(6;9)(p23;q34.1), inv(3)(q21.3q26.2) and t(1;22)(p13.3;q13.1) are all associated with an unfavorable prognosis. The translocation t(6;9)(p23;q34.1) with the fusion gene *DEK/NUP214* is associated with basophilia and multilineage dysplasia. This abnormality is uncommon in AML and more than 70% of the cases are accompanied by fms related tyrosine kinase 3 (*FLT3*) mutations [15, 17]. The inv(3)(q21.3q26.2) causes the distal GATA binding protein 2 (*GATA2)* enhancer to reposition itself, causing activation of ecotropic viral integration site 1 *(EVI1)* instead of *GATA2*[18]. The *EVI1* gene encodes a transcript factor that is essential for self-renewal of HSC and it increases proliferation, prevents differentiation and is involved in epigenetic regulation of transcription. The AML cells may or may not carry signs of

differentiation [17]. Finally, t(1;22)(p13.3;q13.1) forms the *RBM15-MKL1* fusion gene. This abnormality results in acute megakaryoblastic leukemia (AMKL) [19]. It occurs in less than 1% of all AML cases, is uncommon in patients without Down syndrome and a majority of these patients are less than one year of age [11].

1.2.3 The prognostic impact of molecular genetics

Based on high-throughput sequencing techniques a large number of mutations with documented or potential prognostic impact have been identified. These include *FLT3*, nucleophosmin (*NPM1*), CCAAT/enhancer-binding protein alpha (*CEBPA*), *RUNX1*, additional sex combs like-1 (*ASXL1*), tumor protein 53 (TP53), *KMT2A*, isocitrate dehydrogenase (*IDH*), KIT proto-oncogene receptor tyrosine kinase (*KIT*), Wilms tumor suppressor gene1(*WT1*), DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*), Tet methylcytosine dioxygenase 2(*TET2*) and serine and arginine rich splicing factor 2 (*SRSF2*) (Table 3) [17]. The prognostic impact of *FLT3*, *NPM1, CEBPA, RUNX1*, *ASXL1* and *TP53* mutations is generally accepted (Table 2), and is especially important for the prognostic evaluation of the 40-45% of patients that have normal cytogenetics [4].

NPM1 and *CEBPA* mutated AML were incorporated as separate entities in the WHO 2016 classification (Table 1), while *FLT3* was not regarded as a separate entity mainly because of its association with other entities, but its prognostic significance was emphasized [20]. Furthermore, the WHO classification added, as a provisional category, *BCR-ABL1* mutated AML to identify *de novo* AML that benefits from treatment with tyrosine kinase inhibitors and AML with mutated *RUNX1* (Table 1) [6]. The number of mutations in the AML genome is generally lower than for many other human malignancies [21, 22], but it should be emphasized that AML is usually a heterogeneous disease where individual patients have multiple somatically acquired driving mutations and coexisting competing clones [23].

The Cancer Genome Atlas Research Network analyzed 200 adult patients with *de novo* AML by either whole-genome (n=50) or whole-exome (n=150) sequencing, ribonucleic acid (RNA) and microRNA expression and DNA methylation status. They observed an average of 13 mutations per patient; 23 genes were recurrently mutated including *NPM1, FLT3, CEBPA, DNMT3A, IDH1, IDH2,* enhancer of zeste homolog 2 (*EZH2),* U2 small nuclear RNA auxiliary factor 1 (*U2AF1),* structural maintenance of chromosomes 1A *(SMC1A)* and structural maintenance of chromosomes protein 3 (*SMC3)*[21]. Nearly all samples had at least one potential driver mutation, and these mutations could be classified as either transcription-factor fusions (18%), *NPM1* (27%), tumor-suppressor genes (16%), DNA-methylation-related genes

(44%), signaling genes (59%), chromatin-modifying genes (30%), myeloid transcription-factor genes (22%), cohesion-complex genes (13%), and spliceosome-complex genes (14%) [21]. Furthermore, more than half of the patients had at least one subclone in addition to the founding clone, and their observations also suggested that mutations in epigenetic regulators occur early in preleukemic progenitor cells, before leukemogenic events [23]. This last observation is also supported by other studies [24, 25].

A more recent study included 1540 AML patients from three prospective clinical trials. They used karyotyping and targeting resequencing of 111 myeloid cancer genes and identified 5234 driver mutations across 76 genes or genomic regions [22]. At least one driver mutation was identified in 96%, and two or more in 86% of the patients. The genetic patterns in this cohort identified three additional genomic subsets, i.e. AML with mutations in genes encoding chromatin and/or RNA-splicing regulators, AML with *TP53* mutations and chromosomal aneuploidies; and AML with *IDH2* mutations. Patients with chromatin-spliceosome and *TP53*-aneuploidy had poor outcome. The study also showed that co-occurring driver mutations have a significant prognostic value. This was especially pronounced in AML with *NPM1*-mutations, where co-mutations identified groups with favorable or adverse prognosis [22].

Table 2. *The genetic risk stratification of AML based on the 2017 European Leukemia Net (ELN) recommendations [4].*

AML genetic risk stratification based on the 2017 ELN recommendations

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD low
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD ^{high}
	Wild-type NPM1 without *FLT3*-ITD or with *FLT3*-ITD ^{low} (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD ^{high}
	Mutated *RUNX1, ASXL1* and Mutated *TP53*

Genetic abbreviations are listed in the list of genetic abbreviations in the front of the thesis.

1.2.4 Recurrent gene mutations with prognostic value for AML

Based on the 2017 ELN recommendations, the diagnostic workup in AML should include screening for *FLT3*, *NPM1*, *CEBPA*, *RUNX1*, *ASXL1* and *TP53* mutations [4]. These mutations are described in detail; additional mutations are listed in Table 3.

AML with FLT3 mutation

The *FLT3* gene encodes a tyrosine kinase receptor expressed by normal progenitors as well as AML blasts [11]. *FLT3* mutations are detected in 20-40% of patients and are most common in AML with normal karyotype and AML with t(6;9)(p23;q34.1). The most common mutations are (75-80%) internal tandem duplications (ITD) between exons 14 and 15 in the juxtamembrane domain, but missense mutations in exon 20 in the second tyrosine kinase domain (TKD) are also detected. *FLT3* mutations are associated with high blast counts, increased relapse risk and poor overall survival (OS) [26].

AML with NPM1 mutation

NPM1 mutations are one of the most common recurrent genetic lesion in AML, and occur in about one third of AML patients. They are relatively specific for AML [11]. *NPM1* mutations usually involve exon 12 and exceptionally occur in exon 11, and about 40 variations of them have been identified. Mutation A accounts for 70-80% of *NPM1* mutations in adults [27]. *NPM1* mutations are associated with normal karyotype. However, 5-15% of patients with *NPM1* mutations show chromosomal aberrations [27]. Secondary mutations are common in AML: approximately 50% of patients with *DNMT3A* mutations and 40% of patients with *FLT3* mutations also have *NPM1* abnormalities [28] (Figure 3). *NPM1* without *FLT3* mutations have a favorable prognosis. In combination with *FLT3-ITD* the prognosis is worse although better than for patients with *FLT3-ITD* alone [29].

AML with CEBPA mutation

CEBPA encodes a transcription factor involved in the control of proliferation and differentiation of myeloid progenitors. Although mutations can occur throughout the gene sequence, they are mainly out-of-frame insertions or deletions in the N-terminal and in-frame insertions or deletions in the C-terminal [17]. *CEBPA* mutations occur in 6-15% of AML patients and 22-33% of them have *FLT3-ITD* [11]. For patients with the normal karyotype AML the mutations are associated with a favorable prognosis, but whether or not *FLT3-ITD* has an impact in this context is controversial [11].

Mutated RUNX1

RUNX1 is involved in translocations such as (t8;21)(p22;q22) and t(3;21)(q26.2;q22) (*see section 2.2*), but it can also be involved in recurrent intergenic mutations (10% of all AML patients) that lead to a preleukemic AML-predisposing state [30]. *RUNX1* mutations are also associated with radiation exposure [31], inherited disorders such as Fanconi anemia [32], and a rare autosomal dominant familial platelet disorder predisposing to AML [33]. *RUNX1* mutations alone are associated with a favorable prognosis, but the combination of *RUNX1* and cohesion complex mutations (*RUNX1-ASXL1, RUNX1-SRSF2)* is associated with an adverse prognosis[23].

Mutated ASXL1

The *ASXL1* gene encodes a chromatin binding protein of the polycomb group that seems important for epigenetic regulation of gene expression [34, 35]. It acts as a coactivator for the retinoic acid receptor, is involved in histone demethylation and may therefore have a role in leukemogenesis [36]. *ASXL1* mutations occur in approximately 5-18% of all AML patients and are more common in secondary AML. Sequential studies have reported a change of mutation status during disease evolution, suggesting high mutation instability [36]. These mutations are associated with adverse prognosis [36], but may not be an independent prognostic factor [36].

Mutated TP53

TP53 is a tumor suppressor gene [37]. In contrast to many other malignancies, *TP53* mutations are uncommon in AML [38], and they are associated with complex karyotypes involving chromosomes 3, 5 and 17 [37-39]. *TP53* mutations are associated with poor outcome. The mechanisms behind this prognostic impact seem to involve gain of functions with altered intracellular signaling as well as regulation of proliferation/cell cycle progression and apoptosis [40].

Table 3. *Other mutations that have, or may have clinical/prognostic significance according to the WHO 2016 classifications and the ELN 2017 recommendations* (adapted from [23, 41]) [3, 11, 21, 23, 42, 43].

Gene	Characteristics	Prognostic significance
IDH1	Occurs in 6-10% of patients with AML. Associated with early leukemogenesis. Associated with *NPM1* mutation. Associated with clonal hematopoiesis in healthy elderly patients.	The mutation in itself is currently not prognostic, but IDH1 inhibitors are in clinical development. Associated with possible poor outcome in combination with NPM1.
IDH2	Associated with *NPM1* mutation.	The mutation in itself is currently not prognostic, but IDH2 inhibitors are in clinical development, and are associated with favorable prognostics.
DNMT3A	Frequency increases with age (< 60 5-10%, > 60 20-30%). Gene involved in epigenetic regulation. Associated with early leukemogenesis. Associated with clonal hematopoiesis in healthy elderly patients. Associated with *FLT3-ITD, NPM1, IDH* mutations.	Associated with recurrent disease and poor prognosis.
KIT	Occurs in less than 5% of patients. Highest prevalence in patients with core-binding factor (25-35%). Signaling gene, *KIT*, located at 4q11-12 is a member of type III tyrosine kinase family. Most common in exon 8 and 17.	In AML with CBF, in particular t(8;21)(p22;q22), *KIT* is associated with poor prognosis. *KIT* alone is not sufficient to assign patients to a different risk category. There are inhibitors against KIT in clinical development.
KMT2A	Occurs in approximately 5% of AML patients. Gene involved in epigenetic regulation and essential for chromatin-spliceosome class of AML. Associated with RUNX1 mutations, trisomy 11 and CN-AML.	The mutation in itself is currently not prognostic, but KMT2A inhibitors are in clinical development.
TET2	Frequency increases with age (<60: 7-25%, >60: years 20-30%). Gene involved in epigenetic regulation. Early event hematopoiesis. Associated with *IDH1* and *IDH2* mutations. Associated with clonal hematopoiesis in healthy elderly patients	Mutation may lead to recurrent disease and poor prognosis. In the ELN 2010 recommendation, it is classified as a favorable subgroup.
NRAS	Occurs in approximately 15% of all AML patients. Most common with normal karyotype, AML with inv(16)/t(16;16), and AML with inv(3)/t(3;3).	Mutant RAS may be predictive for sensitivity to AraC.
SRSF2, SF3B1, ZRSR2, U2AF1	RNA splicing factors. Tightly correlated cluster of mutated genes. Prevalence uncertain. Associated with early hematopoiesis in healthy elderly people. Associated with antecedent hematological disorder and multilineage dysplasia.	Few studies on prognostic value in AML, but associated with poor outcome.
STAG2, RAD21, SMC1A, SMC3	Cohesion complex genes. Prevalence uncertain. Associated with *NPM1* mutation and t(8;21)	Clinical significance uncertain.

Abbreviations: ELN: European Leukemia Net, ITD: internal tandem duplication, CBF: core binding factor. *Genetic abbreviations are listed in the list of genetic abbreviations in the front of the thesis*.

1.2.5 The prognostic impact of response to the first induction cycle

The response to the first induction cycle is an independent prognostic factor for younger patients receiving intensive chemotherapy. The study by Wheatley *et al*. included 1711 patients below

55 years of age. The relapse risk for patients with complete remission based on morphological evaluation was of 46%. For patients with partial remission the risk increased to 48% and for patients with resistant disease, it reached 69% [44]. There is also a correlation between karyotype and response to first induction therapy [44]; this has also been reported by others [45]. Evaluation of MRD is now emerging as a new prognostic parameter especially for patients classified as having intermediate risk according to conventional criteria. For this reason patients can now be classified as complete remission (CR) with or without MRD (CR_{MRD-}), and this seems to be an independent prognostic factor at least for subsets of AML patients [4].

1.2.6 The prognostic impact of leukemization

High white blood cell (WBC) count at the time of diagnosis is associated with an adverse prognosis at least for certain subsets of AML patients [46, 47]. However, its prognostic impact seems to be relatively weak compared to cytogenetics and response to first induction cycle [44]. Some studies suggest that the use of WBC count as a continuous variable has a stronger prognostic impact than a cutoff of 100 x 10^9/l [48]. WBC counts exceeding 100 x 10^9/L have been associated with increased induction mortality in adult AML; this is mainly due to fatal hemorrhages [48]. Furthermore, *FLT3-ITD* has been associated with a high WBC count [49], and WBC counts exceeding 100 x 10^9/L seem to be associated with an inferior prognosis in patients with *FLT3-ITD* [47]. Alternatively, the bone marrow blast count may also be used for prognostication; patients with 20-29% bone marrow blasts at the time of diagnosis seem to have a better prognosis than patients with >30% blast [50].

1.2.7 Other prognostic parameters

Increasing age seems to be an independent marker of adverse prognosis. This is not only due to poorer performance status, but rather due to an increased frequency of genetic abnormalities associated with chemoresistance [4], and secondary AML (secondary to myelodysplastic syndrome (MDS) [51], chronic myeloid leukemia (CML), myeloproliferative neoplasm [52], or prior cytotoxic therapy [4]). Therapy-related myeloid neoplasms (t-MN) are defined as a one of the main subsets in the WHO 2016 classification, and include treatment-related AML (t-AML), t-MDS and t-MDS/myeloproliferative neoplasm [11]. t-MN have a particularly poor prognosis [11]. The prognosis depends on karyotype; balanced translocations have a generally better outcome except for (i) t(15;17)(q22;12) and (ii) inv(16)(p13.1q22) or t(16;16)(p13.1;q22), and (iii) abnormalities involving chromosome 5 and/or 7 as well as (iv) complex karyotype [11]. Finally, *TP53* mutations are relatively frequent in t-MN (approximately 33%) and contribute to the adverse prognosis [53]. The leukemia stem cells

(LSC) profile may also have prognostic value. LSCs govern the ability of self-renewal and multipotency as seen in normal stem cells (*see section 3.1* "*The hierarchical organization of normal hematopoiesis and the leukemic hematopoiesis in AML"*), and they also show similarities in their global gene expression profiles [54, 55]. A previous study suggests that detection of a global stem cell-like gene expression profile of the primary AML cell population is associated with an adverse prognostic impact in patients with normal karyotype [55]. Finally, the detection of clonal heterogeneity by karyotyping of pretreatment primary AML cells is an independent marker of adverse prognosis [56].

1.3 Epigenetics

The word *"epigenetic"* means *"in addition to changes in genetic sequence",* and is used to describe heritable processes that alter gene expression without changing the DNA sequence [57]. Global epigenetic abnormalities are common in human malignancies and are important both for leukemogenesis and chemosensitivity [5]. The best known epigenetic mechanism is DNA methylation. DNA methylation is the covalent binding of a methyl group to cytosine in CpG regions in the DNA; these regions are concentrated in so called "CpG islands"[5]. AML subsets have distinct DNA methylation profiles that may correlate with specific gene mutations, e.g. *IDH* and *NPM1* mutations [21, 58, 59], and methylation signatures seem to have an independent prognostic impact [59].

Other important epigenetic mechanisms are histone modifications, i.e. posttranslational alterations that are crucial for chromatin structure and gene expression. Modifications can either activate or repress gene expression and include acetylation, methylation, phosphorylation, ubiquitination or sumoylation. Histone modifications are regulated by various enzymes including histone acetyltransferases, histone deacetylases (HDACs), histone methyltransferases and histone demethylases [5]. Acetylation leads to opening of chromatin and promotes gene transcription. Histone deacetylation on the other hand, promotes chromatin condensation and represses gene transcription. HDACs are often overexpressed in cancer cells and are regarded as therapeutic targets [5, 60].

1.4 The leukemic cell population

1.4.1 The hierarchical organization of normal hematopoiesis and the leukemic hematopoiesis in AML

Both normal and leukemic hematopoiesis have a hierarchical organization, and the cells then proceed through an organized developmental hierarchy that is initiated by the hematopoietic stem cells (HSCs) [61]. The HSCs give rise to the different hematopoietic lineages, the first

step being cellular development into committed progenitors and the last step being development into at least nine fully differentiated main effector cells (Figure 1) [62]. Most effector cells have a high turnover and a short life span, whereas the HSCs are characterized and defined by multipotency and long-term self-renewal [61].

In the hematopoietic hierarchy model, the immediate progeny after the HSCs are also multipotent cells and they are therefore referred to as multipotent progenitors. These cells have a limited capacity for self-renewal and the HSCs are therefore the only hematopoietic cells to possess both these properties. There are several models for the differentiation of HSCs into the various lineages, but it is generally accepted that multipotent progenitor cells differentiate into either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP), which determines their subsequent commitment to either a lymphatic or a myeloid pathway [63]. The CLPs give rise to the B-lymphocytes, T-lymphocytes, and natural killer cells, while the CMPs give rise to granulocyte/monocyte progenitors and megakaryocyte/erythrocyte progenitors [62].

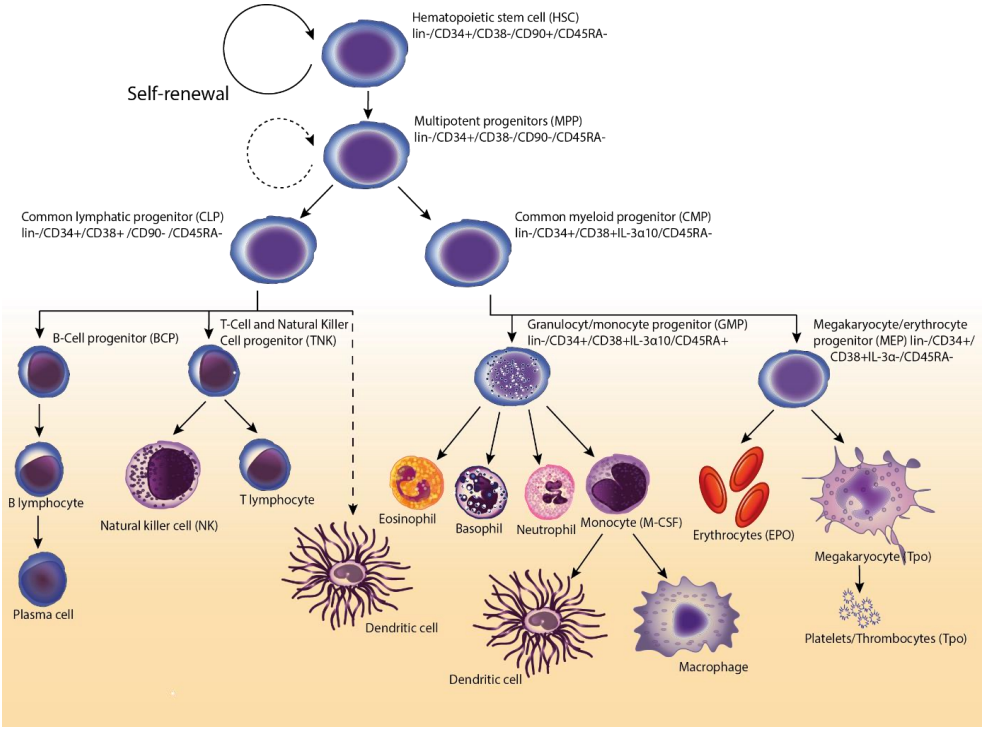

Figure 1. The hierarchical organization of normal hematopoiesis. Human hematopoiesis has a structural hierarchic organization, initiated by the multipotent, self-renewing hematopoietic stem cell (HSC). Downstream from the HSC are the multipotent progenitors, which in turn give rise to the lineage-committed common progenitors (CLP and CMP). The CLP and CMP will ultimately generate fully differentiated blood cells, including thrombocytes, erythrocytes, monocytes, granulocytes, dendritic cells, and the lymphocytes. The darkening of the background color illustrates the increased lineage-commitment in the hierarchic organization. *Abbreviations are listed in the list of abbreviations in the front of the thesis.*

Identifying stem and progenitor cells is challenging and depends on various tools and strategies, including cellular, molecular and functional assays, to distinguish stem cells from other cell population. To make it even more challenging, there are no clear molecular definitions of stem cells. Cluster of differentiation (CD) molecules are cell surface markers frequently used for identifying cell types/subsets. Multipotent cells do not express lineage-specific differentiation markers and are referred to as Lin^-.

The AML cell population is also hierarchically organized, initiated by the LSCs [64]. The heterogeneity within the hierarchical organization is reflected in morphological signs of AML cell differentiation, in the expression of molecular stem cell markers and in various cell surface or intracellular differentiation markers [65]. Genetic changes responsible for causing AML usually occur in cells that have self-renewal potential, i.e. the HSCs or the multipotent progenitors [66], and cells that continue to divide are most likely to accumulate chromosomal abnormalities or molecular mutations. However, the AML cell population may also show an additional heterogeneity that is not caused by the hierarchical organization alone, e.g. some patients show a clonal heterogeneity with cells that differ in their AML-associated genetic abnormalities [56, 67]. Clonal heterogeneity can also be reflected by differences in the activation of intracellular signaling pathways [68]. Both genetic and functional (i.e. constitutive intracellular signaling) evidence of clonal heterogeneity seem to be associated with an adverse prognosis [56, 68].

Only a small subset of cancer cells is capable of extensive growth [69-71], and the first to provide direct evidence of an LSC was Lapidot *et al*. [72] and Bonnet *et al*. [73] in the mid ´90s. They showed that when transplanting primary AML cells into respectively SCID or NOD/SCID mice, only a small subset of the cells could initiate disease development *in vivo* [72, 73]. This population called SCID leukemia-initiating cells (SL-IC), possessed a high self-renewal capacity and was able to differentiate; the cells were thus considered to be LSCs. The SL-ICs were usually (but not always) $CD34^+/CD38^-$ cells regardless of the phenotype of the majority of the blasts within the AML cell population [55, 73]. Finally, the expression of certain molecular markers seems to differ between HSCs and LSCs. This includes Thy^{-1} (CD90) and c-kit (CD117) that are expressed on the surface of HSCs but not on LSCs [74-77], whereas interleukin (IL) -3 receptor α chain (CD123) seems to be expressed mainly by the LSCs together with CD13, CD96, CLEC12A [76, 78], CD25, CD47, IL 1 receptor accessory protein (IL1RAP) and C-type lectin-like receptor 1 (CCL-1) [76, 79-81]. However, the AML stem cells differ between patients and have been found in all four cell subsets defined by the two markers CD34

and CD38 [55], and it is therefore not currently possible to identify a specific AML stem cell phenotype that is similar for all patients.

1.4.2 The stem cell niche

Normal hematopoiesis supplies the human body with more than 100 billion mature blood cells every day [82]. These cells are derived from a small population of HSCs and in adults they are mainly found in a quiescent state in the bone marrow stem cell niches [83]. These niches were first described four decades ago [84], and they function as a specialized microenvironment that consists of various specialized cells that support hematopoiesis and regulate and balance the characteristics of the stem cells, i.e. quiescence (G_0 state), self-renewal and differentiation [85]. The quiescent state ensures their life-long self-renewal [85].

During embryogenesis the HSCs travel between different niches, starting in the yolk sac where immature precursors give rise to erythrocytes [86], and definitive HSCs are later found in the aorta-gonad-mesonephric region and the placenta [87, 88]. After development of vasculature, the HSCs migrate to the fetal liver [86] before they colonize the bone marrow [86]. This last step represents an active recruitment mediated by the release of stromal cell-derived factor 1 (SDF-1) also known as C-X-C motif chemokine 12 ligand (CXCL12) by the bone marrow stromal cells. This chemokine then binds to the chemokine receptor CXCR4 expressed by the stem cells [89]. The CXCL12/CXCR4 interaction is also essential for the maintenance and quiescence of HSCs [90, 91]. In addition, CXC12 up-regulates the stem cell expression of Vascular Cell Adhesion Molecule-1 (VCAM-1) and Very Late Antigen (VLA-4) that are important for homing and retention of HSC in the niches [92].

Stromal cells comprise most of the non-hematopoietic cells in the bone marrow and include cells of endothelial, mesenchymal (adipocytes and osteoblasts) and neural origin. These cells form the stem cell niches and thereby support the homeostatic balance of the stem cells between self-renewal, differentiation and quiescent [93]. However, these cells also maintain the bone marrow framework through their contribution to local vascularization, bone remodeling and metabolic regulation of the hematopoietic cells [93]. Previous research suggests that most HSCs are located in endosteal niches [94], in association with chimeric antigen receptor (CAR) cells or close to sinusoidal blood vessels in perisinusoidal niches [95, 96] (Figure 2).

The bone marrow has a high density of vessels comprised of a single layer of bone marrow endothelial cells surrounded by perivascular cells and non-circulating hematopoietic cells. The endothelial cells can be divided into subtypes based on their phenotype, expression of adhesion molecules and production of hematopoietic growth factors [93]. The bone marrow vessels form

a microvascular tree that starts with arterial vessels surrounded by sympathic nerves, smooth muscle cells and matrix components. These vessels have low permeability. They branch into thinner arterioles that further branch into capillary vessels that are located at the interface between bone marrow and the bone surface where they are important for bone remodeling. The thin-walled venous vessels form sinusoids that are highly permeable and serve as a main site for cell trafficking [97]. The sinusoidal epithelial cells produce high levels of stem cell factor, CXCL12, and E-selectin; they express vascular endothelial growth factor receptor 2 (VEGFR2) and are thereby important for bone marrow regeneration after bone marrow damage [98].

The mesenchymal compartments that surround the endothelial cells are also important for the regulation of hematopoiesis, and these cells also have a hierarchical organization with multipotent mesenchymal stromal cells (MSCs) that can give rise to osteoblasts, adipocytes, chondrocytes and reticular stromal cells [93, 99]. MSCs express several regulators of hematopoiesis, including CXCL12, angiopoietin 1 (Ang-1) and stem cell factor. The CXCR12 abundant reticular cells (CARs) are a subpopulation of mesenchymal progenitor cells with adipogenic and osteogenic potential *in vitro* [100]; they are a main source of CXCL12 and also express several adhesion molecules, e.g. VCAM-1 [93].

The osteoblasts have a role in HSC regulation through their expression of the glycoprotein osteopontin that is important for adhesion, migration and proliferation of HSCs [101]. Osteoblasts also express other adhesion molecules including annexin II, n-cadherin, CD44, CD164, VCAM1 and ICAM1 [96], as well as several soluble regulators of hematopoiesis including G-CSF, GM-CSF, MCSF, IL-1, IL-6, IL-7, CXCL12 [94, 96], thrombopoietin [102], Ang-1 [103], Notch ligands [104] and Wnt ligands [105].

The number of BM adipocytes increases with age and correlates inversely with hematopoietic activity. Naveiras *et al*. showed that mice with impaired adipogenesis had increased hematopoietic activity in the bone marrow at sites that would normally be rich in adipocytes. They also observed that the pharmacological inhibition of adipogenesis after bone marrow transplantation increased the hematopoietic progenitors in the recovering marrow [106]. These observations suggest that adipocytes play an inhibitory role in HCS maintenance.

The stromal compartment of the bone marrow also includes neural cells, e.g. glial fibrillary acidic protein, non-myelinating Schwann cells and adrenergic neurons. The sympathic nervous system regulates the expression of CXC12 through circadian noradrenaline secretion [107]. Adrenergic signaling seems to be important for the circadian regulation of hematopoiesis [108]. The glial cells, especially non-myelinating Schwann cells, are also involved in the regulation

of hematopoiesis [109]. Loss of Schwann cells is associated with decreased TGF-β levels and loss of HSCs. Finally, an extracellular matrix, which is a three-dimensional extracellular macromolecule complex, is essential for stem cell niche homeostasis, maintenance and regeneration [110].

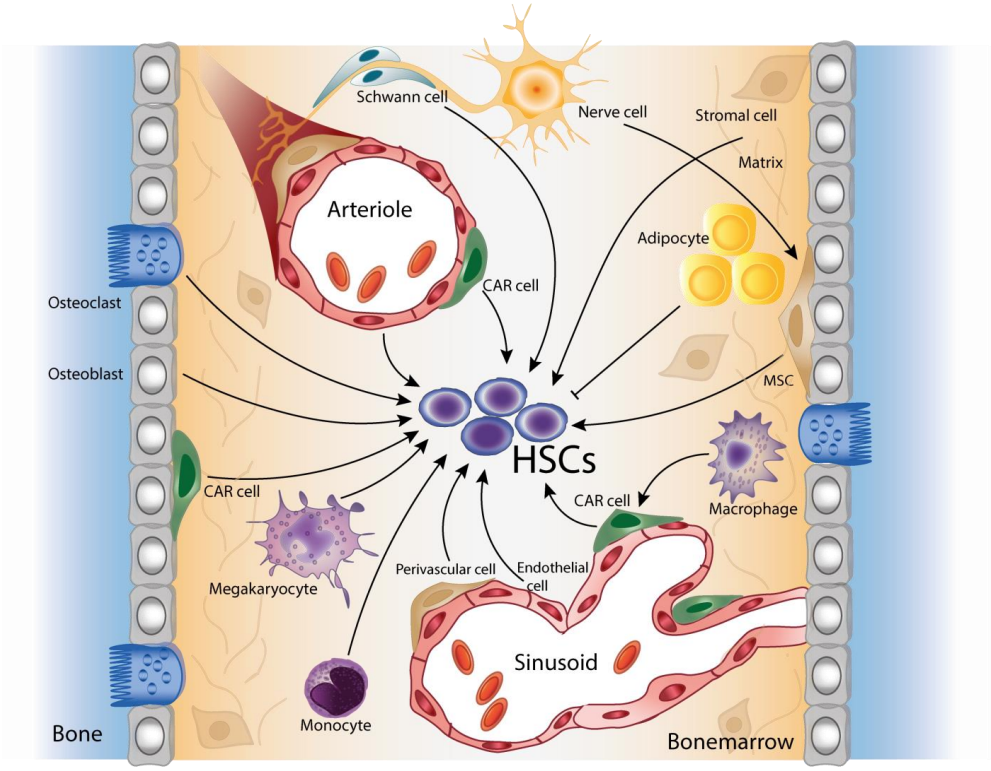

Figure 2. The stem cell niche. Hematopoietic stem cells (HSCs) are found in specialized well vasculated niches, where distinct cells contribute to the maintenance, migration and proliferation of HSCs. A complex group of stromal cells, including endothelial cells, mesenchymal stem cells (MSCs), osteoblasts, osteoclasts and CAR cells are implicated in HSCs maintenance. In addition, sympathic neurons indirectly regulate HSCs through MSCs, and direct regulation from Schwann cells. Finally, hematopoietic cells such as macrophages, megakaryocyte and monocyte also influence the HSCs migration and proliferation. *Abbreviations are listed in the list of abbreviations in the front of the thesis.*

1.4.3 The cytokine network

Cytokines are small proteins that mainly mediate signaling between cells by binding to specific cell surface receptors. They are important for the regulation of cell viability, proliferation, differentiation and migration. Cytokines are thereby important regulators of many processes, including inflammation, angiogenesis and normal as well as leukemic hematopoiesis [111]. Cytokines can act on the cells that release them (autocrine), on other cells close by (paracrine) or on distant cells (endocrine). One cytokine may act on different types of cells (pleiotropy) or different cell types may secrete the same cytokine. Cytokines may also be redundant in their

activity. Thus, effects of cytokine networks can be complex. Cytokines have shown to be important for the communication between AML cells and their neighboring leukemia-supporting stromal cells [111]. Receptor ligation initiates downstream signaling through intracellular pathways. Abnormalities of cytokine and growth factor signaling pathways are common in AML, and these patients also have abnormal systemic cytokine levels that normalize in CR, e.g. increased CXCL8 levels that at least partly reflect the leukemia cell burden [112]. The cytokines that commonly show constitutive release by primary AML cells can be divided into interleukins (IL), chemokines (CCL or CXCL), immune-regulators, hematopoietic growth factors and others (more detailed in Table 4.) [113]. Chemokines are a subgroup of cytokines with low molecular weight that are known to induce chemotaxis, though some also possess other functions. The chemokine system has been investigated in detail in human AML [114], and these studies have shown that patients can be divided into specific subsets based on their chemokine release subset [114-116]. However, primary AML cells release a wide range of soluble mediators [116, 117], and the overall cytokine profile is even associated with prognosis [118]. Finally, the cytokines are not the only soluble mediators that are important, soluble cytokine receptors and soluble adhesion molecules may also contribute to the communication between leukemic and stromal cells [119, 120].

Table 4. *General and simplified classification of cytokines. Cytokines can be divided into six cytokine families based on their biological effects. Table modified from [113].*

Cytokine family	Subgroup	Main physiologic effect
Interleukins	IL-3/IL-7, IL-1/IL-6, IL-2/IL-4/IL-5/IL-12/IL-13 FLT3-ligand	Stimulate hematopoiesis Pluripotent, pro-inflammatory Regulate T and B cell interaction
Chemokines	CXCL1/4/5/6/8/9/10/ 11/12/13 CCL1/2/3/4/5/7/13/17/20 /22/23/24/26/28	Regulate migration of granulocytes, lymphocytes and monocytes Promote angiogenesis and inflammation Enable medullar homing Differentiation induction (CCL17 and CCL22)
Interferons	Type I: INFα/ β/ώ Type II: INFγ	Anti-viral immunity Anti-proliferative effect Anti-neoplastic activity Response to intracellular pathogens
Tumor necrosis factors	TNFα TNFβ	Pro-inflammatory and pyrogenic Activates non-specific immunity Adhesive molecule expression on endothelial surface May induce apoptosis Overproduction causes SIRS Similar effects to TNFα, but produced by T- and B lymphocytes
Colony stimulating factors	G-CSF/GM-CSF/M-CSF Erythropoietin Thrombopoietin	Mobilization of HSC from BM to PB Stimulate proliferation and maturation of myeloid precursors
TGF-β		Stimulates growth of fibroblasts and extracellular matrix production MMP inhibition

Abbreviations are listed in the list of abbreviations in the front of the thesis.

1.5 Treatment

1.5.1 Potentially curative treatment

The therapeutic strategy for patients with AML has not changed significantly during the last 30 years [3], and the only curable treatment is intensive chemotherapy possibly including autologous or allogenic stem-cell transplantation (allo-HSCT) [4].

Induction therapy

The initial intensive induction chemotherapy will often be a continuous infusion of AraC for seven days combined with three days of anthracycline treatment; this is referred to as the "7+3 regime" [4]. The goal of this therapy is to achieve disease control with normalization of peripheral blood cell counts and no morphological signs of leukemia in the bone marrow; this is often referred to as CR. This is achieved for 60-80% of adults below 60 years of age, whereas for elderly patients above 60 years of age the CR rate is of only 40-60% [4]. The reasons for this reduced rate include increased frequency of both high-risk genetic abnormalities and secondary AML as well as co-morbidities (*see section 2.7)* [3, 4].

Consolidation therapy

Patients in CR after induction therapy continue with consolidation therapy. This treatment aims to reduce and eradicate MRD. Standard post-remission treatment strategies include conventional intensive chemotherapy and possibly autologous or allo-HSCT [3]. The decision whether to continue with conventional chemotherapy or HSCT is based on the prognostic evaluation of the patients, i.e. the risk of relapse without intensified treatment versus the risk of severe morbidity or mortality if the treatment is intensified with autologous and especially allo-HSCT [4]. For patients with low-risk AML, conventional chemotherapy will often be preferred, possibly combined with autologous HSCT. Allo-HSCT is considered for younger patients (below 70-75 years of age) without severe comorbidity and intermediate or high-risk disease.

Allo-HSCT is the most effective antileukemic treatment due to the intensive pre-transplant conditioning treatment (with high-dose chemotherapy and/or possibly radiation therapy) and due to the post-transplant immune-mediated graft-versus-leukemia (GVL) reactivity [121]. Due to the increased use of mismatch or unrelated donors as well as cord blood sources, a suitable donor can be found for most patients [4]. Because of this, as well as non-myeloablative or reduced-intensity conditioning (RIC) regimens, more patients can nowadays be allotransplanted. However, because many patients are elderly or unfit, only a minority of AML patients undergo transplantation [122].

If CR is not achieved after the first induction cycle, then a second induction cycle is tried. If CR is still not achieved after a second attempt, the patients are considered to have refractory disease. A second induction is also tried in younger and fit relapse patients. There is no standard salvage regime for primary refractory or relapse AML [4]. However, allo-HSCT is the only curable alternative for both relapsed and refractory disease [123]. To achieve this there are three possible strategies: (i) additional intensive induction cycles to try to achieve CR before allo-HSCT, (ii) intensive chemotherapy cycle to reduce the leukemia burden immediately followed by allo-HSCT [4], or (iii) allo-HSCT without prior CR, though this has significantly lower OS [124].

1.5.2 Complications to AML therapy

The early 4-weeks induction treatment mortality rate is usually of 5-10% of AML patients; this risk depends on age and comorbidity and the most common causes of death are organ failure/toxicity, infections and hemorrhages. The mortality during conventional intensive chemotherapy is also relatively low, and again the most common causes of death are infections and hemorrhages.

The mortality of allo-HSCT is usually higher and depends on several factors, but age and comorbidity are important also in these patients [125]. Hemorrhages are frequent during the early post-transplant period, but during intensive conditioning therapy and allo-HSCT shortly afterwards, the risks of severe infections and occlusive disease are the predominant complications [126]. Infections are also common during the early post-transplant cytopenia (bacterial and fungal infections) and also later after hematopoietic reconstitution (especially viral reactivation). In the long term, immune-mediated complications with organ damage turn out to be the main challenge (i.e. acute and chronic graft versus host disease, GVHD) [127]. These immune-mediated complications are the major long term causes of non-relapse mortality [128] and affect a wide range of organs. Acute GVHD mainly affects the skin, liver and gastrointestinal tract whereas chronic GVHD (cGVHD) may affect many additional organs and the pulmonary form of cGVHD is especially severe [129]. The pathogenesis of GVHD involves several subsets of immunocompetent cells, including donor $CD4^+$ and $CD8^+$ T cells causing immune-mediated tissue damage, but B cells as well as other antigen-presenting cells are also important [130]. Thus, the pathophysiology - especially of cGVHD is not fully understood, but the main contributions seems to be (i) central T cell tolerance failure, (ii) B cell abnormalities, and (iii) formation of prefibrotic and fibrotic lesions [131] as described in detail in a recent review [131].

1.5.3 AML stabilizing treatment

Many elderly and unfit patients will only receive disease-stabilizing treatment due to an unacceptable risk of severe toxicity and/or treatment-related mortality if intensive therapy is tried. This treatment can be low-dose AraC or demethylation agents; low-dose AraC induces CR in 15-25% of patients, but the median survival is of only 5-6 months [132]. Some of the alternative therapies will be briefly described below.

Demethylating agents

DNA methylation can result in gene silencing, which in turn can lead to inactivation of tumor suppressor genes. Demethylating agents induce DNA hypomethylation and thereby re-expression of silenced genes, e.g. tumor suppressor genes [133]. The two drugs 5-azacitidine and 5-aza-2´-deoxycitidine are currently approved for treatment of AML. Both drugs inhibit methyltransferase and thereby have direct cytotoxic or proapoptotic effects. In a randomized trial, decitabine showed a slightly increased survival (7.7 months versus 5.0 months) compared to other treatments (usually low-dose AraC) without any major differences in toxicity [134]. Another randomized trial showed that azacitidine increased median survival (10.4 months

versus 6.5 months) compared with conventional treatment [135]. Both these drugs are recommended by the ELN guidelines [4].

Valproic acid plus all-trans retinoic acid

Valproic acid is an anti-epileptic drug; it is generally well tolerated but has a teratogenic potential and distinct anti-neoplastic properties [136]. Valproic acid is a short-chain fatty acid that functions as a histone deacetylase (HDAC) inhibitor [137]. To form chromatin, DNA is wrapped around histones in the cell nucleus. Acetylation is one of the main histone modificators that lead to opening of the chromatin, promoting gene transcription. HDACs will therefore promote chromatin condensation and repress gene transcription, and are shown to be overexpressed in malignant tissue. HDAC inhibition results in re-expression of gene transcription and has antiproliferative and proapoptotic effects also in AML cells [138-141]. When used as monotherapy in AML, valproic acid has low response rates (3-5%) [142, 143], but the combination of valproic acid and all-trans retinoic acid (ATRA) has a response rate of approximately 30% [133, 144].

ATRA is a vitamin A metabolite used in the treatment of APL where the absence of *ATRA* induces chromatin condensation and transcriptional repression [16]. ATRA binds to the RARα, which leads to decomposition of the *PML/RARα* gene complex in APL and thereby results in growth suppression and induction of myeloid differentiation [145]. Antileukemic effects of ATRA have also been reported in non-APL variants of AML [146-148], and ATRA seems to increase the ability of HDAC-inhibitors to induce differentiation and inhibit proliferation [149, 150]. Even though the combination of ATRA and valproic acid has clinically relevant antileukemic activity, complete remission is very uncommon despite the fact that 30-40% of the patients respond with increased peripheral blood cell counts, lasting for up to 1-2 years in exceptional patients [133]. Thus, the combination of ATRA and valproic acid possibly together with low-toxicity chemotherapy has an acceptable toxicity and a stabilizing potential for a subset of AML patients.

Low-dose cytarabine

This therapeutic strategy has been used for several decades for elderly and unfit AML patients and various doses have been tried [146]. The previous MRC study showed that this treatment has a limited effect especially for patients with low-risk disease characteristics, whereas survival was not improved for patients with high-risk disease [146]. A recent study also

suggested that low-dose cytarabine may be combined with the hedgehog pathway inhibitor glasdegib [151].

Low-dose melphalan

Low dose melphalan as a strategy for elderly and unfit patients has been investigated in patients with AML or MDS. The studies are few, relatively small and for some of only a minority of patients had AML [152-156]. This therapeutic strategy can be considered, but the documentation should be regarded as weak.

1.5.4 Other therapeutic targets in AML

The biological characterization of primary AML cells has identified several possible therapeutic targets [3], and a number of ongoing clinical trials are looking into new areas, including targeting of tyrosine kinases such as FLT3, AXL and KIT inhibitors [157-159], epigenetic regulation (DNA methylation or histone acetylation), as well as promising second-generation hypomethylation agents [160]. BET bromodomain inhibition is also being studied [161], as well as various immunological approaches based on checkpoint inhibition or antibody therapy including both monoclonal antibodies and bispecific antibodies targeting CD33 and CD3 (NCT03971799). Furthermore, strategies to target metabolic regulating enzymes (e.g. mutant metabolic IDH1 and IDH2 enzymes) [162] are being vastly investigated, and FDA has now approved the use of IDH inhibitors for treatment of AML [163].

Finally, an exciting new strategy to target AML is BCL-2 inhibition [164]. BCL-2 is an anti-apoptotic molecule that is found to be overexpressed in LSCs [165]. Moreover LSCs are shown to be uniquely dependent on oxidative phosphorylation (OXPHOS), and BCL-2 inhibition targets OXPHOS, selectively eradicating LSCs [166]. Venetoclax, a BCL-2 inhibitor was recently approved by the FDA in combination with azacitidine or decitabine or low-dose cytarabine for treatment of newly diagnosed AML patients ≥ 75 years, or patients unable to receive intensive chemotherapy due to comorbidities. Recent studies of venetoclax in combination with azacitidine or low-dose cytarabine has given very promising results [167-169], but again the treatment seems most effective for patients with favorable disease characteristics whereas the effect is less impressive for patients with high-risk disease [170].

1.6 Metabolism

Table 5. *Metabolic definitions and characteristics used in this thesis.*

	Definition	Characteristics
Metabolism	The chemical transformations within the cells that allow the organism to perform energy-consuming operations	Metabolism is commonly divided into catabolism (which breaks down organic substances that produce energy, intermediates and NADPH) and anabolism, (which uses energy, intermediates and NADPH to construct cellular components)
Metabolites	All small molecules (<1-1.5 kDa) within an organism.	Metabolites function as intermediates and products of metabolism. Examples include carbohydrates, amino acids, lipids, nucleotides, vitamins and so on.
Metabolon	The total composition of the metabolites in an organism is the metabolon	The composition is determined by the transcription and translation of the genome, molecular protein – protein interaction and enzymatic activity, as well as external factors.
Metabolic profiles	The entire metabolite composition of an organism	The metabolic profile can give us information on the organism´s function, or response to genetic, epigenetic or environmental changes
Metabolomics	The systematic study of the metabolon is metabolomics	During the last decade metabolomics have been increasingly used to identify potential biomarkers in diseases, as well as to improve our understanding of the metabolon and the associated pathways

Abbreviations are listed in the list of abbreviations in the front of the thesis.

1.6.1 Cancer cell metabolism

Since the discovery of cancer cells´ altered metabolism, cancer cell metabolism has emerged as a target for cancer therapy. While normal cells, in the presence of oxygen, mainly prefer OXPHOS, cancer cells are more dependent on alternative metabolic pathways, mainly glycolysis. The conversion of glucose into lactate even in the presence of oxygen is termed "aerobic glycolysis", and was first postulated by Otto Warburg and colleagues in the 1920s, hence the term the Warburg effect [7, 171]. Why cancer cells prefer other, less energy efficient metabolic pathways to OXPHOS is still not fully understood. However, cancer cells are dependent on ATP production for survival only in certain conditions. Under normal circumstances, the cytosolic level of nicotinamide adenine dinucleotide phosphate (NADPH) is the limiting factor for fast proliferation. NADPH has two main functions in mammalian cells: (i) it provides redox to counteract oxidative stress, which is crucial for cancer cells to be able to survive under conditions with high metabolic stress, (ii) it acts as a co-enzyme for anabolic enzymes vital for generating new building blocks to sustain growth and proliferation [172]. Cancer cells´ ability to alter their energetics in order to meet the metabolic demands of rapid proliferation and survive stressful conditions is crucial for cancer development, and is today recognized as one of the hallmarks of cancer [8]. The main metabolic pathways used by cancer

cells to gain energy and NADPH besides OXPHOS and glycolysis, are glutaminolysis, fatty acid β-oxidation and the pentose phosphate pathway (Figure 3).

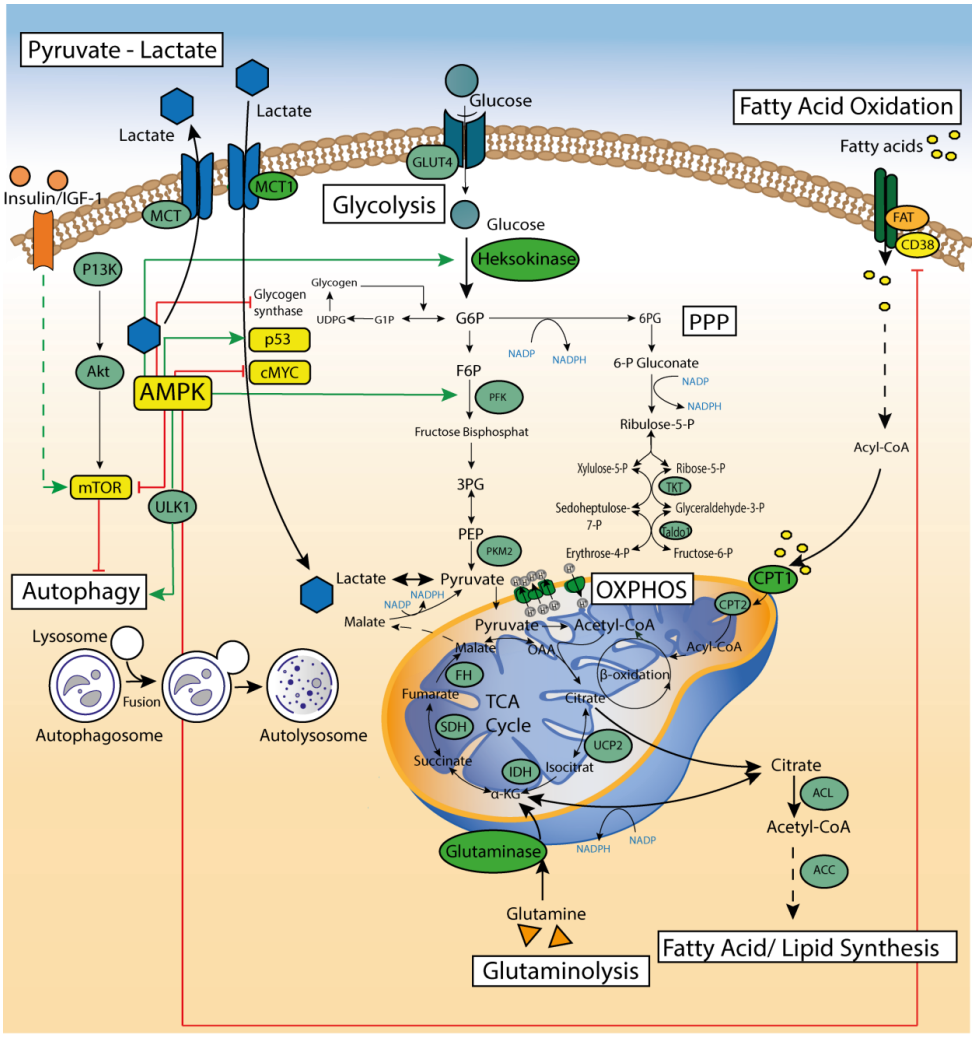

Figure 3. Metabolic pathways in cancer cells. The main metabolic pathways in cancer cells besides from OXPHOS are glycolysis, glutaminolysis, fatty acid β-oxidation, lactate transport and the pentose phosphate pathway. In addition, the figure illustrates the AMPK pathway, and autophagy. *Abbreviations are listed in the list of abbreviations in the front of the thesis.*

1.6.2 Metabolic pathways

Glycolysis

The majority of cancer cells prefer aerobic glycolysis as their main metabolic pathway. This is compounded by the fact that cancer cells take up an enormous amount of glucose compared

with cells from surrounding tissue [7]. Glycolysis is the first step in the catabolism of glucose, where glucose molecules are converted into pyruvate in the cytosol to be further processed to generate ATP. This is a far less energy efficient metabolic pathway compared to OXPHOS, where one glucose molecule can generate up to 36 ATP, while glycolysis only generates 2 ATP [8, 173]. However, glycolysis also produces more intermediates, as well as NADPHs, and building blocks for amino acids and nucleosides that are required in large quantities when synthesizing new cells [9]. Glycolysis is also a faster pathway than OXPHOS, so even though the energetic yield is much lower for each glucose molecule, one can produce ATP at a higher quantity by glycolysis given sufficient excess of glucose [173, 174].

So what causes tumor cells to initiate the high consumption of glucose? Normal cells´ glucose uptake is strictly regulated by growth factor signaling, as well as interactions with the extracellular matrix [175, 176]. In contrast, cancer cells are shown to be significantly less dependent on external requirements, and more dependent on genetic alterations in internal signaling pathways [8, 177]. Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling is the main regulator for glucose uptake. PI3K/Akt signaling causes upregulation of glycolysis by promoting increased expression of glucose transporter *GLUT1* mRNA [178] and translocation of GLUT1 from endomembranes to cell surface. In addition it potentiates the activity of hexokinases (HKs), the rate limiting enzymes of the glycolysis, and phosphofructokinase (PFK), the enzyme that catalyzes the key irreversible step of glycolysis [179]. Studies have shown that Akt-hyperactivation promotes aerobic activation without affecting the rate of OXPHOS, thus promoting aerobic activation through the Warburg effect [180]. The PI3K-Akt pathway is upregulated in a subset of AML cells, potentially contributing to metabolic reprogramming [181]. The PI3K-Akt signaling is not the only signaling pathway that plays a role in glucose metabolism. There are multiple other signaling nods facilitating uptake of glucose that are upregulated in cancer cells - notably, RAS, another oncogenic signaling protein, that has also been shown to upregulate *GLUT1* mRNA expression, increasing intracellular glucose consumption [182]. Cancer cells´ increased glycolytic activities have been shown in various cancers, and have also been linked to treatment resistance in AML [183, 184]. Growing evidence suggests that oncogenes and tumor suppressors may play a role in promoting the switch to glycolysis. This dependency on glycolysis may be seen upon as a weakness that can be exploited therapeutically. A potential target for inhibiting the glycolytic pathway is HKs. HKs are, as mentioned, the rate limiting enzyme for glycolysis, converting glucose to Glucose-6-phosphate (G6P). HKs influence the magnitude of glucose within the cell by facilitating the

entry of glucose. They also determine the direction of flux by converting glucose to G6P, which has a feedback inhibition on HK. There are four major HK isoforms in mammals (KK1-4), all encoded by separate genes. HK1 and HK2 are the two high affinity HKs. HK1 is expressed in most mammalian tissues, while HK2 is expressed in a high level only in a limited number of cells in adult tissues [185]. However, high levels of HK2 are observed in cancers cells [186, 187], meaning that HK2 has a selective overexpression in cancer. This is manifested by the use of positron emission tomography (PET), where the glucose analog [^{18}F] fluoro-2-deoxyglucose is phosphorylated by hexokinase to form fluoro-2-deoxyglucose-phosphate, which can be detected by PET [187]. In addition, *HK2* conditional knockout mice have shown that HK2 is required for tumor initiation and maintenance in a study using mouse models of lung and breast cancer [187]. Drug induced inhibition of HK2 with HK2 inhibitors such as 2-deoxy-D-glucose (2DG) and Lonidamine has also been known to decrease proliferation and/or increase apoptosis in both leukemic cell lines and primary cells [188-190].

Amino acid metabolism in cancer cells

Metabolic reprogramming goes far beyond cancer cells´ increased glucose assumption and the Warburg effect. Other metabolic pathways are required for the production of nutrients, and three major classes of macromolecules (proteins, lipids and nucleic acids) are essential for cell growth and proliferation [191]. Amino acids play a dual role as they are used as both building blocks for the protein synthesis and as metabolites for biosynthetic reactions [192]. Studies have shown that cancer cells (including AML) have altered amino acid metabolism compared to healthy cells [192-194]. This is supported by our own findings in **Paper III** [195]. Amino acid, particularly glutamine, also provides a carbone source, which allows malignant cells to rely on high glycolytic rates [191]. Through glutaminolysis, the amino acid glutamine is converted to lactate, providing reduced NADPH that is needed in the lipid synthesis, and a carbon source for the TCA cycle intermediates to maintain mitochondrial function under aerobic glycolysis [196, 197]. Glutamine also contributes to reduced nitrogen that is used in the *de novo* synthesis of nitrogen-containing compounds such as purine, pyrimidine, G6P and non-essential amino acids [9]. Proliferating tumor cells have a high demand for glutamine. This was first described by the American physiologist Harry Eagle in the 1950s [198], and has later been confirmed in various studies [9]. The main signaling pathways that regulate glutaminolysis are still not fully understood, but key oncogenes such as *c-myc* and *RAS*, which are upregulated in AML, have been demonstrated to stimulate glutamine metabolism [199, 200].

The main glutaminolytic enzymes are the glutamine-utilizing enzymes glutaminase (GLS), the gatekeeper enzyme which catalyzes the hydrolysis of glutamine to glutamate in mitochondria, and glutamate dehydrogenase (GDH) which produces α-ketoglutarat (αKG) from glutamate to fuel the TCA cycle. In mammalian cells, the GLS family has two isoforms, known as GLS1 and GLS2. GLS2 is a known p53 target gene [201]. Several studies have shown that drugs targeting these enzymes, such as bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide 3 (BPTES), a selective GLS1 inhibitor, exert an antitumor effect in different types of tumors [202-206], including AML [207]. As certain cancer cells have been shown to be addicted to glutamine, drugs targeting glutaminolysis are emerging as a potential novel therapeutic strategy to "starve" the cancer cells of energy for growth, and trigger apoptosis [207].

Fatty acid synthesis and oxidation

In addition to glucose and glutamine, fatty acids are an important energy source for proliferating cells. *De novo* synthesis of fatty acids is required for synthesis of membranes, and therefore crucial for cell growth and proliferation [208]. Fatty acid synthesis is an anabolic process that converts acetyl CoA to malonyl CoA through acetyl CoA carboxylase. Fatty acids are catabolized through fatty acids oxidation (FAO), also known as β-oxidation [208]. FAO generates one molecule of acetyl CoA in each oxidation cycle, and two in the last cycle (Figure 3), which in turn can be used to produce cytosolic NADPH. The production of FAO-derived cytosolic NADPH has been reported to be crucial to counteract oxidative stress in human glioblastoma cells, where inhibition of FAO has led to a decrease in NADPH levels and an increase in reactive oxygen species (ROS) -dependent cell death [209]. Furthermore, a high-throughput screening study of primary AML cells, found that Avocatin B, a lipid derived from avocado, reduced human primary AML cells´ viability by inhibiting FAO, decreasing NADPH, resulting in ROS-dependent leukemia cell death [210]. Human AML cell lines are also reported to be dependent on FAO for survival. Sanudio *et al*. showed that etoxomir, a carnitine palmitoyl transferase 1 (CPT1a) inhibitor, decreased leukemia progenitor cells in approximately 50% of primary AML samples [211]. Targeting FAO by inhibiting CPT1a, one of the enzymes that transports fatty acids into the mitochondria, has shown promising results. CPT1 is considered the rate limiting enzyme in FAO, and inhibition of CPT1a prevents production of ATP from fatty acids [208]. Etoxomir induces apoptosis in several studies with AML [211], but has exhibited toxic effects (hepatotoxicity) in clinical trials [212]. Other studies using the CPT1 inhibitor ST1326 to inhibit FAO have reported impaired cancer cell survival and decreased

proliferation in AML cell lines and primary AML cells [213], as well as *in vivo* Burkitt's lymphoma cells [214].

Pyruvate to lactate, the preferred fate for pyruvate in cancer cells

Pyruvate is a central metabolite in a number of metabolic processes. In the last step of the glycolytic breakdown of glucose, pyruvate is synthesized by pyruvate kinase (PK). From here, pyruvate can convert to lactate in a reversible process by lactate dehydrogenase, which facilitates the rapid recycling of NAD^+ necessary to maintain a high glycolytic rate. Alternatively, it can enter the oxidative route, where pyruvate is imported into the mitochondrial matrix where it can fuel the TCA cycle. Pyruvate may also be converted into alanine or malate through alanine aminotransferase or malic enzyme [215]. In healthy cells most of the pyruvate converts to Acetyl-CoA to fuel the TCA cycle. In tumor cells however, pyruvate converting to lactate is the preferred reaction, due to the upregulation of lactate dehydrogenase in tumor cells [216]. This benefits the cancer cells as it facilitates regeneration of NADPH. The serum concentration of lactate is directly related to tumor differentiation, treatment resistance and metastasis. It can also be a diagnostic indicator of disease progression [8]. Interestingly, some tumors are found to contain two subpopulations of cancer cells that differ in their energy generating pathways. One subpopulation consists of glucose-dependent (the Warburg effect) cells that secrete lactate, whereas cells from the second population preferentially import and utilize the lactate produced by their neighbors as the main energy source, employing part of the citrate cycle to do so. These two subpopulations function symbiotically [217]. This symbiotic relationship is an efficient way for cancer cells to maintain a sufficient metabolism under nutrient-poor conditions. Furthermore, converting pyruvate to lactate reduces ROS, thereby promoting tumor survival [216].Transportation of lactate occurs primarily through the monocarboxylate transporters (MCT) 1-4, which function as an important modulator for this metabolic symbiosis [217]. MCTs have also emerged as promising therapeutic targets for cancer treatment, since inhibiting MCT1 leads to accumulation of intracellular lactate, which disables cell growth and eventually leads to cell death [218]. AZD3965 is a selective MCT1 inhibitor that has shown to decrease proliferation in human gastric and small cell lung cancer tumor xenografts *in vivo* [219, 220], and is now in a clinical trial for adult solid tumors (clinical trial information: NCT01791595).

Pentose phosphate pathway

Pentose phosphate pathway (PPP) is a parallel metabolic pathway to glycolysis, and a major pathway for glucose catabolism. The PPP consists of two distinct phases: one oxidative branch

and one non-oxidative branch. In the oxidative branch, G6P is converted into ribulose-5-phosphate which generates NADPH [216], NADPH is further used for both intracellular ROS detoxification and catabolic metabolism. The non-oxidative branch synthesizes pentose, which is required for the synthesis of ribonucleotides [221]. Therefore, the PPP is frequently referred to as one of the main antioxidant cellular defense systems, and important for the production of nucleotides [222]. Furthermore, studies have shown that the PPP has distinct carcinogenic impact, and that PPP is important for cancer cell proliferation, apoptosis, invasion, drug resistance and metastasis [222]. In addition, Parkhitko *et al.* have discovered that some cancer cells have distinct autophagy-dependent PPP alterations, revealing that PPP may be an autophagy dependent compensatory metabolic mechanism [223].

The history of drugs inhibiting PPP started as early as 1926, when the anti-malaria drug, plasmochin, led to the first drug-induced hemolytic anemia, affecting G6PD, which is one of the key enzymes of PPP [224]. G6PD has later shown to have strong anti-tumor effects. The inhibition of G6PD decreases proliferation in both *in vitro* in cell lines and *in vivo* xenograft mice models [225]. Inhibition of 6-phosphogluconate dehydrogenase (6PGH), the third enzyme in the PPP, can decrease proliferation and tumor-growth in xenograft mice models [226]. 6-aminonicotinamide (6AN) is a combined G6PD and 6PGH inhibitor that has shown promising anti-cancer results. In a study done on a PML cell line, 6AN suppressed growth, migration and invasion of the cancer cells [227].

AMPK and inhibition of OXPHOS induce a metabolic shift to catabolic state

Metformin is a well-known drug used to treat diabetes mellitus type II. However, after several epidemiological studies linking metformin to a reduced risk of developing various cancer types [228-230], metformin's potential as an anti-cancer drug has received increased attention. The two major effects of metformin are (i) activation of AMPK, the key energy sensor in the cells that acts as a metabolic switch which alters the cells from an anabolic to a catabolic state in response to stress [231, 232], and (ii) decrease of electron transport chain complex I (ETCI) activity [233-235]. Targeting ETCI will induce ROS, leading to a metabolic shift to a catabolic state. This means that metformin decreases energy-consuming processes such as protein synthesis, and increases energy-producing processes like glycolysis for ATP and lactate production, PPP for purine biosynthesis, fatty acid metabolism, as well as anaplerosis and mitochondrial gene expression [233]. Notably, leukemic cells with high basal AKT phosphorylation are more resistant towards metformin-induced apoptosis; the same applies to leukemic cells that relay mainly on glycolysis [232]. Consequently, glucose starvation,

treatment with glycolytic inhibitors such as 2DG, or AKT inhibitors will increase these cells´ sensitivity to metformin [232]. Some studies have shown that LSCs, which generally have low ROS, are more dependent on OXPHOS than other leukemic cells, and that these cells cannot compensate OXPHOS by increasing glycolysis [232]. This might render LSCs more vulnerable towards metformin.

1.6.3 AML cell metabolism

Metabolic alterations play a central role in AML cells´ leukemogenesis and disease progression. As previously mentioned, there is a large heterogeneity among the different AML cells, this also applies to their metabolic preferences. Studies have shown that different leukemic cells seem to differ in their dependency on glycolysis or oxidative phosphorylation. A study of AML cells derived from a small amount of patients (n=23) reported that highly glycolytic primary AML cells were more resistant to apoptosis induced by antileukemic therapy *in vitro*, indicating resistance to induction chemotherapy similar to that observed in solid tumors [183]. However, the same study also found that a higher level of glycolytic metabolism in leukemic cells at the time of diagnosis was associated with a significantly improved duration of CR and OS after induction chemotherapy. These results, though seemingly contradictory, suggest that glycolytic metabolism plays a significant role in leukemic survival and chemoresistance. Metabolic alterations linked to AML cells´ disease progression have been found in several of the major metabolic pathways, but there is a clear need for more knowledge regarding AML cells´ metabolic reprogramming. Optimally, characterization of metabolic pathways in leukemia cells may identify proteins/pathways correlated with drug responsiveness or disease aggressiveness as shown by Jain *et al*. for the role of glycine in breast cancer using metabolic profiling [236]. However, due to the heterogeneity of the disease, identifying common therapeutic targets has been challenging. The pharmaceutical development and testing of metabolic inhibitors are at an early stage, and even though a lot of research has been done on AML cells both *in vitro* and *in vivo*, there are to our knowledge just a few clinical trials with metabolic targeting drugs on AML patients (Table 8).

1.6.4 The systemic metabolic profiles in patients with AML

Metabolomic profiling of patients with AML has revealed a panel of altered metabolites with potential risk stratification [237]. A large metabolomic study compared the pretreatment serum samples of 400 AML patients to 446 health controls. This showed alterations in 45 metabolic pathways, demonstrating increase in the pyruvate production and downregulation of free fatty acids, indicating increased FAO. In addition, the study revealed a distinct glucose metabolism

in the AML patients, with significant alterations in six out of 10 identified serum metabolites in this pathway having prognostic value. The result also showed that enhanced glycolysis contributed to increased resistance to chemotherapy, and inhibition of glycolysis decreased proliferation, and increased sensitivity towards chemotherapeutic cytotoxicity [237]. Metabolic profiling has also shown prognostic value for predicting the outcome of GVHD after allo-HSCT. In a large retrospective study, serum asymmetric dimethylarginine (ADMA) levels of 938 patients with GVHD after receiving allo-HSCT were investigated. This showed that high levels of pre-transplant serum ADMA were associated with a higher risk of non-relapse mortality. ADMA is an endogenous metabolite that inhibits nitric oxide synthase, and is closely related to L-arginine [238].

Table 6. *Brief description of drugs targeting metabolism used in this PhD project*

Metabolic drug	Metabolic pathway targeted	Main target	Characteristics	AML
2DG [188, 239, 240]	*Glycolysis* – the first step of catabolism of glucose. Preferred metabolic pathway for most cancer cells.	Hexokinase – the first enzyme in glycolysis and the rate controlling step.	Glucose analogue. 2DG is the most widely tested agent targeting glucose metabolism. Clinical trials: 2DG as monotherapy has so far been disappointing due to severe side-effects when given in high concentrations. Lower doses of 2DG in combination with radiotherapy in patients with glioblastoma have so far showed promising results.	*In vitro* and *in vivo* studies confirm that 2DG induces cell death in AML. Cells with FTL3-ITD and mutated c-KIT seem more sensitive to 2DG. 2DG decreases MCL-1 protein expression *in vivo*. 2DG may sensitize resistant cells to other treatment. To our knowledge there are no clinical trials with 2DG and AML.
Lonidamine [189]	*Glycolysis* *OXPHOS*	Hexokinase II inhibitor. Inhibits lactate efflux. Increases mitochondrial permeability	Originally used to treat African sleeping sickness, and has been through several clinical trials against different types of solid tumors. It has shown to have a multisite effect in tumor cells. It targets, in addition to HKI I, the mitochondrial pyruvate carrier (MCT), the electron transport chain and mitochondrial permeability. Overall it sensitizes cancer cells to chemotherapy, hyperthermia and radiotherapy.	Lonidamine has a generally low toxicity when used as monotherapy, but used in combination with other antileukemic agents it induces apoptosis *in vitro*. Stimulates ROS. Anti-oxidants in AML cell lines dampen Lonidamine toxicity. There are to our knowledge no ongoing clinical trials with lonidamine and AML.
AZD3965 [219, 220]	*Glycolysis* *Pyruvate/lactate metabolism* – an alternative energy source for cancer cells, drives tumorigenesis.	Monocarboxylate Transporter 1(MCT1). MCT1 is important for the uptake of lactate.	A second generation selective MCT1 inhibitor. AZD3965 increases intracellular lactate and reduces lactate uptake. Induces metabolic reprogramming and promotes tumor inflammation and angiogenesis. AZD is currently in a Phase I clinical trial for diffuse large B-cell lymphoma and adult solid cancers (NCT01791595).	To our knowledge no studies have been published with regard to AZD3965´s effect against AML.
BPTES [241]	*Glutaminolysis* - important energy pathway for many cancer cells.	Inhibits glutaminase, the enzyme which converts glutamine to glutamate, which is further oxidized to α-ketoglutarat.	Glutaminase is upregulated by Myc, which is highly expressed in many cancers. Poor aqueous solubility. Glutamine is an important source of energy for cancer cells, especially for IDH mutated cells (in leukemia and glioblastoma).	BPTES slows growth of primary AML cells. AML cells with IDH1 mutation are more sensitive to BPTES inhibition. There are to our knowledge no clinical trials with BPTES and AML.

Metformin [242-244]	*AMPK signaling pathway* – *mTOR* *OXPHOS*	Activates AMPK pathway Inhibits mTOR	A well-known type II diabetes drug. Activates LKB1/AMPK causing indirect inhibition of mTOR. Inhibits fatty acid metabolism, which in turn increases glycolysis.	Metformin increases proliferation and induces apoptosis in AML cell lines, while not effecting normal CD34+ HSCs. Also induces apoptosis in AMKL, inhibits mTOR, and triggers autophagic response that proceeds to apoptosis. Metformin affects protein synthesis, and decreases c-myc and Bcl-xL levels. Synergistic effect with other antileukemic agents. To our knowledge there are no clinical trials with metformin and AML.
6-Amino-nicotinamide (6-AN) [223, 227]	*Pentos phosphate pathway* – a parallel metabolic pathway to glycolysis. PPP directs glucose to its oxidative branch, producing NADPH.	Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). G6PD is the first and rate limiting enzyme of PPP.	A nicotinamide analogue. Competitive inhibitor of PPP. Also mentioned in studies as a potential diabetogenic agent. 6AN has also been suggested as a key autophagy-dependent compensatory metabolic mechanism.	6AN suppresses growth, migration and invasion of AML cells *in vitro*. It decreases glucose oxidation and increases fatty acid oxidation. In combination with autophagy inhibition it suppressed proliferation and prompted the activation of NF-κB and CASP1 in AML cells *in vivo*. To our knowledge there are no clinical trials with 6AN and AML.
Chloroquine [245, 246]	*Autophagic pathway* – cancer cells may digest their own cell-organelles to provide energy (ATP) to maintain metabolism and unlimited proliferation under stressful and hypoxic conditions.	Inhibits autophagy by disrupting acidification of the lysosomes that fuse with the autophagosomes.	Originally made to prevent and treat malaria. Potent blocker of autophagy, and can sensitize cancer cells to radiotherapy, chemotherapy and potentially other anti-cancer drugs. Used in clinical trials.	Increases cell death. Sensitizes AML cells to chemotherapy. AML cells have been shown to be more sensitive to hydrochloroquine than normal bone marrow.

Abbreviations are listed in the list of abbreviations in the front of the thesis.

1.7 Autophagy

1.7.1 Definition and regulation

The word autophagy is derived from the Greek words *auto*, meaning "self" and *phagein*, "to eat", and can be seen as the body's own “cleansing process”. This catabolic process starts with the formation of a phagophore from a membrane, which engulfs intracellular waste and subsequently forms double membraned vesicles (Figure 4). These vesicles, called autophagosomes, are transported to lysosomes where their contents are digested by lysosomal acid proteases and “recycled”. Products from this degradation process, such as amino acids, can then be re-used in metabolic pathways or for building macromolecules. This way autophagy provides an endogenous source of nutrients, which may be exploited as a survival strategy during stressful conditions [223, 247].

In mammalian cells autophagy is controlled by several protein complexes composed of autophagy-related genes (*ATG*). To date, about 40 ATG proteins have been identified [248], and these can be classified into three main functional groups according to the stage of autophagy they control (Table 4) [249]: (i) initiation-step proteins, (ii) phagophore nucleation proteins and (iii) proteins involved in autophagosome completion. The main regulator of induction of autophagy is the mammalian target of rapamycin complex (mTORC) 1[250]. Under conditions with sufficient nutrients, mTORC1 inhibits autophagy, whereas nutrient deprivations lead to inhibition of mTORC1, activating autophagy [251]. Through mTORC1 inhibition, different extracellular stress factors activate proteins such as unc-51 like autophagy activating kinase 1(ULK1)/ATG1, PI3K, mATG13, FIP200 and ATG101 [249, 250]. The key players involved in phagophore nucleation are BECLIN-1/ATG6, ATG14, VPS34, VPS15, UVRAG and BIF-1. Finally, the vesicle completion consists of two conjugation systems: the ATG12-ATG5-ATG16 system and the ATG8 family. These two systems are essential for the elongation and the closure of the autophagosomes [249] (Figure 4). Autophagy can be presented as both a non-selective and a selective process [250]. An example of selective autophagy is mitophagy, which is selective degradation of damaged mitochondria [252]. To be able to distinguish cargoes ready for degradation from their functional counterparts, selective autophagy depends on autophagy receptors and adaptor proteins like p62, neighbor of BRCA1 gene 1 protein (NBR1) and autophagy-linked FYVE protein (ALFY) [253, 254]. The autophagy receptors that recognize the cargo have specific ligand-binding domains (e.g. an ubiquitin-binding domain) that interact with the ATG8/LC3 family proteins (Figure 4). Non-selective

autophagy seems to share the same core machinery as selective autophagy; however, the autophagy receptors and adaptors do not seem to be required for non-selective autophagy [253].

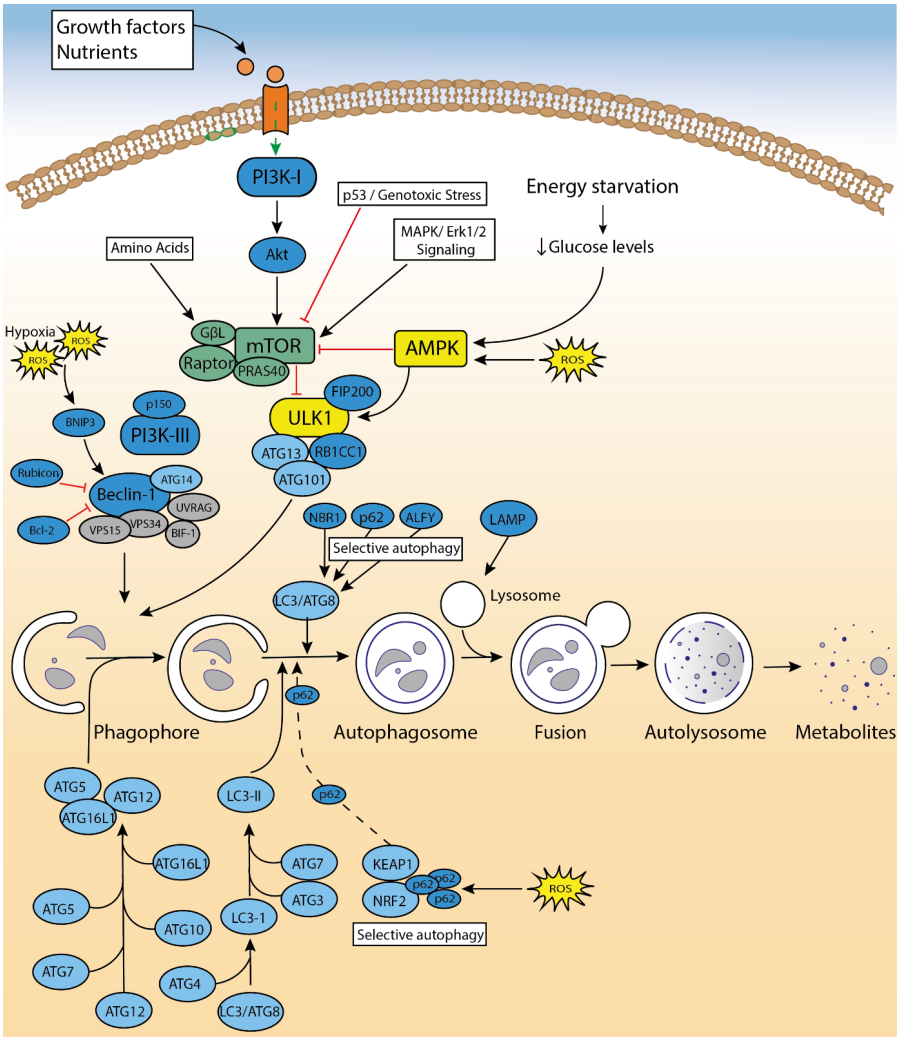

Figure 4. The main machinery and regulation of the autophagic network. Autophagy is controlled by several protein complexes composed of autophagy-related (*ATG*) genes. The machinery can be divided into three main steps, (i) autophagy induction mTORC1 ULK1/ATG1, PI3K, mATG13, FIP200 and ATG101, (ii) phagophore nucleation; BECLIN-1/ATG6, ATG14, VPS34, VPS15, UVRAG and BIF-1, and (iii) vesicle completion; the two conjugation systems ATG12-ATG5 and the ATG8 family. Selective autophagy depends on autophagy receptors and adaptor proteins such as p62, NBR1 and ALFY. The autophagy receptors that recognize the cargo have specific ligand-binding domains (e.g. an ubiquitin-binding domain). They also interact with the ATG8/LC3 family proteins, like p62, which can attach to either ubiquitin or kelch-like ECH-associated protein 1 (KEAP1) on the autophagosomes, leading to their selective degradation. *Abbreviations are listed in the list of abbreviations in the front of the thesis.*

1.7.2 The role of autophagy in cancer cells

In healthy tissue, the autophagic process can be tumor suppressive by eliminating damaged organelles, proteins or oncogenic substrates [255]. Autophagy may also play a role in cancer development, as cancer cells may use autophagy to maintain their metabolism and unlimited proliferation under stressful and hypoxic conditions, by digesting their own cell-organelles to provide energy (ATP). Indeed, autophagy is often reported to be upregulated in cancer cells, promoting cancer cell survival and drug resistance [255]. The effect of autophagy on cancer development and growth seems to correlate with the autophagic level, which in turn is affected by intracellular and extracellular stressors, such as ROS accumulation [250]. Autophagy in healthy cells is an important tumor suppressive mechanism, which contributes to the degradation of damaged organelles and proteins that may have oncogenic potential. This property has also been exploited in AML treatment. Interestingly, in a study on APL, autophagy facilitated therapy-induced degradation of the *PML-RARα* oncogene, thereby facilitating leukemia treatment [256]. Watson *et al*. also found that loss of autophagy in an MLL-ENL model of AML led to increased proliferation in vitro, a glycolytic shift and more aggressive leukemias *in vivo* [257]. On the other hand, AML cells can use autophagy as a prosurvival response to treatment, providing substrates for metabolism under stressful conditions, making them more treatment resistant [255]. Autophagy is upregulated in human AML cell lines exposed to hypoxia, enabling them to overcome an initial growth restriction [245]. Pharmacological inhibition of hypoxic AML cells has also been shown to increase cell death, and to enhance sensitivity to chemotherapy [245]. Folkerts *et al.* showed that AML CD34+ cells were more sensitive to the autophagic inhibitor hydrochloroquine (HCQ) than normal bone marrow CD34+ cells. The fraction of AML CD34+ cells with low ROS levels had higher basal autophagy, with reduced survival after HCQ treatment compared to the CD34+ fraction with high ROS, indicating that autophagy inhibition affects the immature sub-fraction of AML [246]. Recent studies examined the autophagic interactions between the cancer cells and the surrounding stromal cancer cells [258, 259], postulating that cancer cells induce oxidative stress, increasing ROS production which activates transcriptional factors such as HIF-1α and NF-κB. This induces autophagy and mitophagy in the microenvironment surrounding the cancer cells, making the surrounding stromal cells secrete high energy nutrients such as ketones and lactate that the cancer cells can then feed on. This enables them to maintain proliferation under nutrient scarce conditions [258]. This hypothesis, also called the reverse Warburg effect, states that cancer cells are fed by nutrients from increased autophagy of adjacent stromal cells, while mainly relying on OXPHOS, not glycolysis, for energy production [259].

2 Main hypothesis and aim

AML is a heterogeneous disease, as regards to morphology, growth characteristics, cytogenetics and epigenetics. In addition, recent data suggests that leukemia cells have abnormal cellular metabolism and that the cells may employ different metabolic routes to control their growth and survival.

Our main hypothesis is that systemic regulation of metabolism and cellular metabolism play important roles in the development of AML. We propose that AML cells derived from certain AML patient subsets have common metabolic characteristics. These AML subsets may either (i) have a more extensive adaptive capacity (in which case this metabolic adaptation may render cells more resistant to drug induced cytotoxicity), or (ii) be more sensitive toward metabolic-targeting drugs. Thus, it may be possible to stratify patients according to metabolic phenotypes/capacities, which may then serve as a common therapeutic or prognostic target.

Based on our hypothesis, the primary objective of this Ph.D. project is to investigate systemic metabolic profiles of AML patients, and characterize the metabolic phenotype of AML cell populations, in particular how they respond to inhibitors of metabolic pathways. We aim to study the heterogeneity of AML cell metabolism to reveal potential new therapeutic targets, and also contribute to increased knowledge about how leukemia cells achieve treatment resistance.

3 Summary of papers

Paper I: Targeting cellular metabolism in acute myeloid leukemia and the role of patient heterogeneity

Authors: Ida Sofie Grønningsæter, Håkon Reikvam, Elise Aasebø, Sushma Bartaula-Brevik, Tor Henrik Tvedt, Øystein Bruserud and Kimberley Joanne Hatfield.

We examined the antileukemic effect of metabolic drugs targeting (i) glycolysis (lonidamine and 2DG), (ii) pentose phosphate pathway (6AN), (iii) the glutaminolysis pathway (BPTES), (iv) the AMPK pathway (metformin), (v) fatty acid oxidation (ST1326) and (vi) the pyruvate pathway (AZD3965). Primary AML cells taken from 81 unselected patients were treated with drugs either alone or in combination with standard chemotherapy (AraC). Cell proliferation was determined using the [^{3}H]-thymidine incorporation assay, and viability/apoptosis/necrosis frequencies were analyzed by flow cytometry. Metformin, 2DG, 6AN, BPTES and ST1326 result in significant antiproliferative and/or pro-apoptotic effects on patients` leukemia cells, while lonidamine and AZD1326 generally did not show the same cytotoxicity. As expected due to the heterogeneity of AML patients, we observed a wide variation among patient samples regarding sensitivity to the different metabolic inhibitors, although no correlations were found between sensitivity and characteristics of AML patients, i.e. FAB classification, cytogenetics or gene mutations of *FLT3* and *NPM1* were found. Additive inhibitory effects on AML cell growth were seen when the inhibitors metformin, 2DG, 6AN and ST1326 were used in combination with AraC. Moreover, we identified a subset of 29 patients with generally strong antiproliferative effects after drug treatment. Gene expression and proteomic studies revealed different metabolic and transcriptional regulations in this subset. Furthermore, antiproliferative effects of 2DG on AML cells were still observed after coculture of AML cells with growth-inducing mesenchymal stem cells (MSCs). Our findings suggest that inhibitors of metabolic pathways represent a novel targeted approach for treatment of AML that should be investigated further.

Paper II: Effects of the autophagy-inhibiting agent chloroquine in acute myeloid leukemia

Authors: Ida Sofie Grønningsæter, Håkon Reikvam, Karen Marie Hagen, Sushma Bartaula-Brevik, Tor Henrik Tvedt, Øystein Bruserud and Kimberley Hatfield

We evaluated the effects of CQ, an inhibitor of autophagy, on cell viability and proliferation in 81 AML patient samples. Cell viability after 48-hour drug treatment was measured using flow cytometry, and cell proliferation was analyzed after 7-day drug treatment using the [3H]-thymidine incorporation assay. A subgroup of AML patients was more sensitive to treatment

with CQ, showing a greater antiproliferative effect and decreased viability after drug treatment. We also identified a small subset of patients that were sensitive to CQ, but on which AraC had little or no antiproliferative effect. We then compared gene expression profiles based on the strong/low antiproliferative effects of CQ, and identified 99 genes upregulated in the patient subset with high susceptibility to CQ. Among these genes were several linked to AML leukemogenesis. In addition, we examined at the constitutive mediator release after CQ treatment. A subset of 18 patients showed generally increased mediator levels after CQ treatment, and this subset showed increased upregulation of genes, of which several are associated with metabolic processes.

Furthermore, we investigated the expression of ROS, autophagic flux, cells cycle phases, and viability, apoptosis and necrosis in HL60 and MOLM-13 cell lines treated with CQ alone or in combination with the metabolic drugs metformin and 2DG. We found that CQ leads to accumulation of lysosomes, thus inhibiting autophagy in both cell lines, though different responses were seen for the different cell lines. Both metformin and 2DG seemed to decrease autophagy when used in combination with CQ. CQ also decreased viability and increased apoptosis in both cell lines, though no effects were seen on ROS levels or cell cycle distribution after CQ treatment. To conclude, CQ has antileukemic effects on AML and should be explored further for its antileukemic activity, alone or in combination with other drugs.

Paper III: Systemic metabolomic profiling of acute myeloid leukemia patients before and during disease-stabilizing treatment based on all-trans retinoic acid, valproic acid, and low-dose chemotherapy

Authors: Ida Sofie Grønningsæter, Hanne Kristin Fredly, Bjørn Tore Gjertsen, Kimberley Joanne Hatfield and Øystein Bruserud

This study is based on results from two previously published clinical studies conducted at Haukeland University Hospital [144, 260]. Older and unfit AML patients were included to investigate the combination of the drugs ATRA, valproic acid and low-dose therapy with hydroxyurea, 5-mercaptopurine, and/or AraC, as disease-stabilizing treatment. Before and during treatment, serum samples were collected from all patients included in the clinical studies. In the current study, we compared the metabolic profiles of 44 patients stratified as either responders (n=18) or non-responders (n=26, six of them showing rapidly progressive disease) to treatment. Furthermore, we investigated the metabolomic changes during ATRA and valproic acid monotherapy, which were compared to the corresponding untreated controls (n=20). Metabolomic analysis was performed by Metabolon®, and the global metabolite

profiling measured a total of 886 biochemicals. Analysis revealed that ATRA and valproic acid induced changes in fatty acid and lipid metabolism. In addition, the lipid metabolism of patients with rapidly progressive disease was extensively altered. As monotherapy, only valproic acid caused significant alterations; twenty-three metabolites were significantly altered by seven-day valproic acid treatment ($p < 0.05$, $q < 0.05$). The majority of altered metabolites belonged to lipid (especially fatty acid) and amino acid pathways, including several carnitines. These metabolomic effects, and especially the effects on lipid metabolism, may be important for the antileukemic and epigenetic effects of this treatment.

Paper IV: Patients with treatment-requiring chronic graft versus host disease after allogeneic stem cell transplantation have altered metabolic profiles due to the disease and immunosuppressive therapy: potential implication for biomarkers

Authors: Håkon Reikvam, Ida Sofie Grønningsæter, Knut Anders Mosevoll, Roald Lindås, Kimberley Joanne Hatfield and Øystein Bruserud.

cGVHD is the most common and severe long-term complication of allo-HSCT. New biomarkers are needed both for the diagnosis and for the evaluation of treatment in allo-HSCT patients with cGVHD. We analyzed the serum metabolomic profiles of patients with and without cGVHD one year after transplantation, revealing potential associations between the serum metabolomic profiles and the development of cGVHD. Furthermore, we explored the effects of immunosuppressive therapies on the metabolomic serum profiles of 51 hematology patients who underwent allo-HSCT from 2006-2014 at Haukeland University Hospital. Of these patients, 31 had signs of cGVHD, while 20 had no sign of cGVHD one-year post-transplant, and all but two patients with cGVHD received immunosuppressive treatment (i.e. cyclosporine, steroids). Metabolomics analysis was performed by Metabolon®, and a total of 755 metabolites were identified in all samples. A random forest analysis of serum profiles revealed 30 top-ranked metabolites to be highly discriminative for cGVHD, whereof 18 were related to fatty acid metabolism, most of them belonging to the annotation's sphingolipids, plasminogens, lysolipids and phospholipids. We also compared the systemic metabolomic profiles of patients receiving or not receiving immunosuppressive treatment and found differences in amino acid and lipid metabolites between these groups. The overall effect on metabolomic alterations is most likely due to both the cGVHD, and immunosuppressive treatment. However, we did identify a subset of 11 metabolites that seem to mainly reflect the diagnosis and severity of cGVHD, reflecting a unique metabolomic signature of cGVHD.

4 Material and methodological considerations

4.1 Patient selection

All the studies in this thesis are based on samples from patients treated for hematological diseases at Haukeland University Hospital during a 20–year period. This is the primary/referral hospital for patients in the largest part of the western region of Norway, which has a population of approximately 580 000 inhabitants, accounting for around 11% of the country´s population [261]. In **Papers I, II** and **III** we analyzed samples from patients with AML. The AML cancer incidence and rate in western Norway is similar to that of the rest of the country. Thus our material is considered to reflect a population based on an unselected patient group of Norwegian AML cancer. Furthermore, previous studies have shown that our patient cohorts are representative of AML in general with regard to cytogenetic analysis and the most important genetic abnormalities related to prognostics and chemosensitivity, i.e. *FLT3* mutations [262]. In **Papers I** and **II**, we investigated primary AML cells taken from 81 unselected patients. AML blasts were taken from peripheral blood, and all patients had high, relative or absolute levels of circulating AML cells in peripheral blood. We obtained highly enriched AML cells by performing gradient separation alone, thereby avoiding the induction of functional alterations in the leukemic cells – a potential problem during cell isolation that has been discussed in detail previously [263, 264]. Peripheral blood cells have also proved to be representative of the corresponding bone marrow cells, and although quantitative differences may be seen, the major characteristics of the leukemic cells are maintained [181, 265]. In **Paper III,** we analyzed serum samples from AML patients, which were collected during the course of two clinical studies conducted at Haukeland University Hospital between 2004 and 2007 [266], and 2008 and 2012 [144]. Both clinical studies were based on a combined treatment of ATRA and valproic acid in addition to low-toxicity conventional therapy with hydroxyurea, 5-mercaptopurin or low-dose AraC. This is considered a low-toxicity or disease stabilizing treatment and was offered to patients that were found unfit for intensive treatment during these time periods. Our present study included an unselected subset of samples which were taken from 44 patients included in these two studies. 18 patients were classified as responders and 26 did not respond to this treatment. As there is no general agreement with regard to the response criteria for low-toxicity or disease stabilizing treatment [144], our patients were evaluated on the basis of both the conventional criteria for patients receiving intensive AML chemotherapy [267] and the MDS criteria [268]. In **Paper IV** we analyzed serum samples from 51 patients with hematological diseases who were allotransplanted between 2006-2014 at Haukeland University Hospital, all with HLA-matched family donors. Ninety-five patients were

transplanted at our institution during this period; 25 of them died from treatment-related causes, 6 patients relapsed, and 13 were lost to follow-up. The decision to perform an allo-HSCT was taken by the Norwegian Advisory Board for Stem Cell Transplantation and based on national guidelines.

Taken together, all our studies are considered as population-based and include an unselected and consecutive group of well-characterized patients that reflect the general AML and allo-HSCT patient population. Written informed consent was obtained from all patients included in this thesis.

4.2 AML patient cell preparation and cryopreservation

The methods for inclusion of patients [262], preparation, cryopreservation and thawing are highly standardized [269, 270], and the same methods are used for all samples in our biobank. Density gradient centrifugation was used to isolate AML cells, resulting in a highly enriched AML cell population >90%. This method offers a minimal risk of inducing functional alterations, and just a small degree of contamination that mainly consists of small lymphocytes that release cytokines only after activation [271]. In our experience, normal peripheral blood mononuclear cells, cultured in medium alone without specific activation, do not show detectable proliferation or detectable cytokine release. Thus, though we cannot rule it out, they are unlikely to have any major impact on our results.

The use of cryopreserved patient derived cells instead of freshly prepared cells gives the opportunity to analyze and compare a larger subset of patients simultaneously. This does not only make it possible to analyze different patient characteristics in parallel, it also reduces the risk of variation between analyses, which is unavoidable when multiple analyses are done over time. In addition, using cryopreserved samples makes it possible to reproduce the results. The disadvantage of using DMSO in cryopreservation includes a decline of viability of the AML cell population through the freezing and thawing processes. DMSO can have other effects on cells, e.g. affecting cell differentiation and chromosome stability [272]. Still, cryopreserved cells are the preferred cells for analysis as long as there are no known effects on attributes to be studied.

4.3 The relevance of investigating the overall AML cell population

Chemoresistant relapse is still a main cause of death in AML [4], and relapse is thought to be derived from remaining LSCs [67]. However, the results from several previous studies suggest that biological characteristics of the total AML cell population are important markers for *in vivo*

chemosensitivity and thereby probably reflect important common biological characteristics for the majority of the hierarchically organized AML cell population. Firstly, the response to the first chemotherapy cycle with induction of complete hematological remission after only one chemotherapy cycle is an important prognostic parameter. CR can be regarded as a characteristic of the total AML cell population [4], because the clinical experience has shown that additional cycles are needed to eradicate remaining AML (stem) cells [4, 273, 274]. Secondly, several biological characteristics of the overall AML cell population are associated with prognosis, including global as well as selected gene expression profiles [55, 275], epigenetic regulation [276], single molecular markers detected at the mRNA or protein level [277, 278] and functional characteristics (e.g. constitutive mediator release) [116, 279]. Finally, the use of bone marrow examination 17 days after induction treatment (i.e. morphological evaluation of blast percentage) to guide the later chemotherapy should also be regarded as evaluation of the overall AML population [4]. The same may be true for the evaluation of MRD based on the membrane molecule profile detected at the time of diagnosis [280]. Thus, studies of the total AML cell population seem to reflect biological characteristics that are relevant for *in vivo* chemosensitivity.

4.4 Cell lines

Two human leukemia cell lines were used in this thesis, HL60 and MOLM-13. These cell lines were chosen for their different genotypic properties representing two different AML populations. HL60 is p53-null, representing FAB M2 [281], and MOLM-13 is a M5 cell line expressing FLT3-internal tandem duplication (ITD) [282]. They are both well-established cell lines and widely used for experiments within the field of AML. The cell line models were used in **Paper II**, and cell lines were chosen to avoid the influence of spontaneous stress induced apoptosis that occurs during *in vitro* culture of primary human AML cells [283]. However, it is important to remember that immortalized cell lines are not “normal” anymore; many cell lines have been passaged for decades which makes the cells prone to genotypic and phenotypic changes, thus altering cell characteristics; in addition fast growing subclones with biological advantages may arise and can effect results during experimental studies [284].

To ensure the authentication of our cell lines, an authentication test using short tandem repeat (STR) profiling was performed on the two cell lines used in this thesis. Both were an exact match for HL60 and MOLM13 profiles. Furthermore, cell lines were kept in culture for only 3-4 weeks before a new batch of cells (same passage) were thawed and used in studies, thus reducing the chance of introducing changes and/or contamination cells. To sum up, cell lines

are helpful to use for standardization and confirmation of experiments where a large number of cells are needed. Cell lines have less variability than primary cells and are easier to culture, and more viable. Ideally several cell lines of the disease should be examined in experimental studies, and patient cells should be used for further validation of results to confirm biological relevance and reveal potential variability between patient subsets.

4.5 Drug selection

The doses used in this thesis were chosen based on reported *in vitro* studies, as well as on *in vivo* serum levels and tolerable concentrations for drugs that have been tested in the clinic (Table 7). AraC (1-b-arabinofuranosylcytosine), CQ, metformin, 2DG, and lonidamine have all been used *in vivo*, and their clinical tolerance has been well documented (Table 8). 6AN, BPTES, ST1326 and AZD3965 have not yet been established in the clinic and our doses were chosen based on *in vitro* and *in vivo* preclinical studies (Table 7). There is, however, one study from 1961 investigating the clinical toxicity and tumor-inhibitory effects of 6AN on 30 patients with advanced solid tumors [285]. For AZD3965 there is an ongoing trial where AZD3965 is given to patients with B cell lymphoma and Burkitt's lymphoma (NCT01791595); this is to our knowledge the first time this type of drug is given to patients [286]. BPTES has been shown to have poor aqueous solubility and an unfavorable pharmacokinetic profile that has limited its clinical development. As far as we know there are no clinical trials with BPTES, but preclinical studies are investigating BPTES delivered in nanoparticles to improve drug solubility and efficacy [287]. A clinical trial with another glutaminase inhibitor (CB-839) for treatment of advanced or metastatic renal cell carcinoma (NCT03428217) is currently taken place.

Inhibitors of CPT-1, are not new to the field either: etomoxir, an irreversible inhibitor of CPT-1 has already been through phase II clinical development for treatment of type II diabetes and heart failure, but due to adverse side effects, the phase II trial with etomoxir was ended [212]. Based on our information, the clinical development of etomoxir as an anti-carcinogenic agent has also been terminated due to the severe hepatotoxicity associated with the treatment [288]. For this reason, we chose to study the less characterized CPT-1 inhibitor ST1326. An *in vitro* study of CLL cells showed no cytotoxic effect of ST1326 on normal CD34+ cells, thus ST1326 might be a good alternative to etoxomir for further treatment based on CPT-1 inhibition [289]. However, in this study we found the solubility of the drug to be challenging, which explains why we did not pursue the use of this drug in all our experimental assays. A summary of the references used to choose drugs and doses can be found in Table 7.

Table 7. *Overview of the main references used to choose the drug doses used in this thesis.*

Drug [Reference]	Title of article	Doses used	Main findings in article
Metformin			
Sabins *et al.* [290]	Synergistic cell death in FLT3-ITD positive acute myeloid leukemia by combined treatment with metformin and 6-benzylthioinosine	10 mM	Metformin, in combination with 6-BT, showed an increased cytotoxicity in AML cell lines. This was associated with inhibition of FLT3-ITD activated STAT5 and reduced c-Myc and GLUT-1 expression, resulting in reduced glycolysis and increased cell death.
Wang *et al.* [243]	Metformin synergistically sensitizes FLT3-ITD-positive acute myeloid leukemia to sorafenib by promoting mTOR-mediated apoptosis and autophagy	0.2 - 16 mM	Synergistic effect between metformin and sorafenib in MV4-11 cell line and FLT3-ITD-positive cells. Metformin alone decreased the viability and proliferation of AML cells in a dose-dependent manner, exerting an antileukemic effect through activation of AMPK.
Xie *et al.* [291]	Metformin induces growth inhibition and cell cycle arrest by upregulating MicroRNA34a renal cancer cells	0.2, 1 and 5 mM	Metformin inhibits cell proliferation in a dose dependent manner. Growth is inhibited through G0/G1 phase arrest and delayed entry into S-phase, partially by inhibiting miRNA34a.
Jagannathan *et al.* [292]	Pharmacologic screens reveal metformin that suppresses GRP78-dependent autophagy to enhance the anti-myeloma effect of bortezomib	0.5 mM	Metformin suppresses GRP78, a critical UPR effector, impairing autophagosome formation and enhancing apoptosis. This enhances the effect of bortezomib on myeloma patients.
Saito *et al.* [293]	Chemical genomics identifies the unfolded protein response as a target for selective cancer cell killing during glucose deprivation	1, 3, and 10 mM	Metformin disrupts UPR transcription, inhibiting autophagy. This leads to massive cell death during glucose deprivation.
Sahra *et al.* [294]	Targeting cancer cell metabolism: The combination of metformin and 2-Deoxyglucose induces p53-dependent apoptosis in prostate cancer cells	1 - 5 mM metformin 1mM 2DG	Metformin, in combination with 2DG, induces p53-dependent apoptosis. The combination of metformin and 2DG induces G2-M cell cycle arrest. Metformin inhibits 2DG induced autophagy.
2DG			
Raez *et al.* [295].	A phase I dose-escalation trial of 2-deoxy-D-glucose alone or combined with docetaxel in patients with advanced solid tumors	63 mg/kg/day	2-DG has a clinical tolerable dose of 63 mg/kg/day. This gave a mean maximum plasma concentration of 116 µg/ml (0.7 mM). Higher doses led to adverse side effects such as hyperglycemia, QTc prolongation and gastrointestinal bleeding.
Chen *et al.* [296]	A distinct glucose metabolism signature of acute myeloid leukemia with prognostic value	0 – 50 mM	2DG suppresses cell proliferation and potentiates the cytotoxicity of AraC.
6AN			
Parkhitko *et al.* [297]	Autophagy-dependent metabolic reprogramming sensitizes TSC2-deficient cells to the antimetabolite 6-aminonicotinamide	0.1 mmol/L	Supplementation with 0.1 mmol/L of NADPH was sufficient to prevent accumulation of cleaved caspase-1 under chloroquine/6-AN treatment for 96 hours (Fig. 6C), consistent with the rescue of cell proliferation by NADPH
Chen *et al.* [227]	Inhibition of pentose phosphate pathway suppresses acute myelogenous leukemia	5 mM	PPP inhibited by 6AN at 5 mM, treated for 72 hours, caused increased glucose oxidation, inhibited the migration ability of AML and the invasiveness of AML. It decreased glutathione level significantly and inhibited AML growth.
BPTES			
Lee *et al.* [298]	Dual targeting of glutaminase and thymidylate synthase elicits death synergistically in NSCLC	*In vitro:* 10 µM *In vivo:* 10 mg/kg/200 µL	Glutaminase 1 inhibition induced cell cycle arrest with ATP depletion by glutamate reduction. Synergistic effect of BPTES and thymidylate synthase inhibitor on cell death through cell cycle arrest and anti-tumor effect in xenograft model of NSCLC .
Seltzer *et al.* [299]	Inhibition of glutaminase preferentially slows growth of glioma cells with mutant IDH1	10 µM	BPTES preferentially slows cell growth in D54 cells with mutant IDH1. BPTES also inhibits glutaminase activity, lowers glutamate and α-KG levels, and increases glycolytic intermediates
Xiang *et al.* [300]	Targeted inhibition of tumor-specific glutaminase diminishes cell-autonomous tumorigenesis	10 µM	BPTES also inhibits growth of MYC-dependent cells by blocking DNA replication, leading to cell death and fragmentation.
Elgogary *et al.* [287]	Combination therapy with BPTES nanoparticles and metformin targets the metabolic heterogeneity of pancreatic cancer	10uM	Metformin and BPTES in combination significantly reduced the pancreatic tumor more than metformin alone.
Emadi *et al.* [241]	Inhibition of glutaminase selectively suppresses the growth of primary acute myeloid leukemia cells with IDH mutations	20 and 40 uM	BPTES inhibits glutaminase, suppresses proliferation of IDH positive AML cells, compared to AML cells with wt IDH.

ST1326			
Ricciardi *et al.* [213]	Targeting the leukemia cell metabolism by the CPT1a inhibition: functional preclinical effects in leukemias	1 to 50 µM	ST1326, CPT1 inhibitor, highly selective for the CPT1a isoform. ST1326 has been tested in phase 2 studies to treat diabetes, and has so far demonstrated a strong safety profile.
Lonidamine			
Robustelli della Cuna *et al.* [301]	Toxicity and clinical tolerance of lonidamine	300-450 mg	Lonidamine has been found to have no conventional side effects when daily doses range from 300-450 mg, and, given in combination with radiotherapy, results in no additional toxicity. In doses ranging between 300-900 mg. reported side effects are myalgia, testicular pain, asthenia, ototoxicity, nausea, vomiting, gastric pain, drowsiness, hyperesthesia, and photophobia.
AZD3965			
Halford *et al.* [286]	A first-in-human first-in-class (FIC) trial of the monocarboxylate transporter 1 (MCT1) inhibitor AZD3965 in patients with advanced solid tumors	20 mg	20 mg orally per day is well tolerated. The doses found to be safe in Part I are given to patients with diffuse large cell lymphoma and Burkitt´s lymphoma in the ongoing clinical trial Part II. NCT01791595
Polanski *et al.* [219]	Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer	8 nmol/L	8 mmol/L AZD3965 caused a significant reduction in cell lines. There were large variations in response, two of four cell lines were only sensitive under hypoxia, and two were relativly resistant.
Bola *et al.* [220]	Inhibition of monocarboxylate transporter-1 (MCT1) by AZD3965 enhances radiosensitivity by reducing lactate transport	10 nM – 1 µM	Inhibition of MCT1 results in an increase in glycolysis in human tumor cell lines and xenografts, Combining MCT1 inhibition with radiotherapy provided a significantly greater therapeutic effect than the use of either modality alone.
Chloroquine			
WHO [302, 303]	WHO. Guidelines for treatment of malaria. Third Edition	25 mg base/kg, over three days.	Total dose of 25 mg base/kg bw given over three days is efficient and well tolerated. Lower total doses are not recommended. This report found a median **AUC** ranging from 8.2-140 µg/mL/h (25 – 440 µM) over three days.
Augustijns *et al.* [304]	Chloroquine levels in blood during chronic treatment of patients with rheumatoid arthritis	250 mg/day	Mean serum levels of CQ for 29 patients treated for rheumatological illnesses were 815 (±743) ng/ml, equivalent to 2.55 uM CQ.
Ette *et al.* [305]	Pharmacokinetics of chloroquine: Saliva and plasma levels relationship	500 mg CQ (diphosphate)	Mean plasma levels of CQ in five heathy adults given 500 mg CQ dephosphat were 4 mg/ml equivalent to 12.54 uM CQ.
Marmor *et al.* [306]	Recommendations on screening for chloroquine and hydroxychloroquine retinopathy (2016 Revision)	Recommended daily doses < 5.0 mg/kg	CQ and hydroxychloroquine (HCQ) are widely used for the treatment of autoimmune inflammatory and dermatologic conditions. Due to retinal toxicity from CQ and HCQ, recommended daily doses are less than < 5.0 mg/kg for long time use.
Orfali *et al.* [307]	Induction of autophagy is a key component of all-trans-retinoic acid-induced differentiation in leukemia cells and a potential target for pharmacologic modulation.	10 uM with 1 uM ATRA for four days	Enhancing autophagy promotes ATRA-induced granulocytic differentiation of an ATRA-resistant derivative of the non-APL AML HL60 cell line (HL60-Diff-R). This supports the strategies to stimulate autophagy as a novel approach to promote differentiation in AML.
Egger *et al.* [308]	Inhibition of autophagy with chloroquine is effective in melanoma	0–100 uM (0, 20, 40, 60, 80 and 100 uM)	Induced cytotoxicity in human melanoma cell lines under both normoxia and hypoxia. Chloroquine combined with echinomycin achieved synergistic cytotoxicity under hypoxic conditions in multiple melanoma cell lines (BRAF wild-type and mutant).
Zheng *et al.* [309]	Inhibiting autophagy with chloroquine enhances the anti-tumor effect of high-LET carbon ions via ER stress-related apoptosis	50 mg/kg/day	CQ blocks autophagy through the IRE1/JNK/Beclin-1 axis, and enhances apoptosis. Using CQ to inhibit autophagy might be a promising therapeutic strategy to enhance radiotherapy via aggravating ER stress-related apoptosis.
AraC			
Hubeek *et al.* [310]	Cytosine arabinoside. Cancer drug discovery and development: Deoxynucleoside analogs in cancer therapy	100-200 mg/m2 (0.1-0.5 uM).	Conventional dose varies from 100 to 200 mg/m2. The steady-state plasma concentration generally ranges between 0.1-0.5 uM. High dose AraC is administered at 1-3 g/m2, resulting in a peak concentration in the range of 100 µM which falls rapidly with a t ½ of 7-20 min. AraC has also been given at very low doses (20 mg/m2/d), giving plasma levels as low as 100 nM.

Abbreviations are listed in the list of abbreviations in the front of the thesis.

4.6 Measurement of cell proliferation and viability/apoptosis

In **Papers I** and **II** we evaluated the AML cell proliferation after seven days of *in vitro* culture using a [^{3}H]-thymidine assay as described in detail [311]. The cells were cultured in the presence of metabolic drugs or in medium alone (or added vehicle control) for six days before [^{3}H]-thymidine isotope was added, and the following day [^{3}H]-thymidine incorporation was estimated. During the first six days an increased spontaneous or stress induced apoptosis can be observed [283]. Thus, this proliferation assay reflects the characteristics of a minor subset of cells that are able to survive and still proliferate after seven days of *in vitro* culture. This subpopulation seems to reflect the enrichment of more primitive colony-forming progenitor-like cells [312], allowing for the analysis of less-differentiated AML cells. It is suggested that progenitor cells are one of the major causes of disease relapse, which is the main cause of death in AML. [^{3}H]-thymidine assays therefore provide a beneficial way of evaluating the effect of metabolic drugs on the progenitor population.

In the same articles, we determined by flow cytometry the amount of cell viability, apoptosis and late apoptosis/necrosis in cells exposed to drug treatment [283]. Previous studies indicate that *in vitro* cultured AML cells undergo spontaneous *in vitro* apoptosis, and the percentage of viable cells decreases during the first days of the culture until it reaches a low level [283]. We estimated the cell viability after 48 hours (24 and 48 hours for cell lines in **Paper II**). This assay may thus reflect the characteristics of the major subsets of mature cells in the hierarchically organized AML cell population. There is an array of preventive measures to avoid spontaneous apoptosis in cells. In our proliferation assay and our viability/apoptosis assay we cultured cells in medium enriched with growth factors. However, there will always be some level of spontaneous apoptosis when cells are removed from their microenvironment and subjected to *in vitro* treatment.

4.7 Measurement of cytokine release

There are various methods for analyzing mediators such as cytokines. In this thesis we have used Luminex® assays, purchased from R&D Systems™ (Minnesota, MN, US), which involve fluorescent bead-based multiplex technology. This capture/detection sandwich type immunoassay allows the measurement of up to 100 different analyses in a single 50 µL sample [313]. Because multiplex assays only require a small sample volume and are time-saving, they have become an attractive method to investigate immune responses [313]. However, analytical differences have been reported between different manufacturers, product lots and storage time [314], but kits purchased from R&D Systems™ have been described as some of the most

accurate kits for the measurement of cytokine concentrations in supernatants, with good recovery ranges and reproducibility for most cytokines [313]. As with most lab techniques, Luminex® is highly operator-dependent, and this will always be a possible source of error. Intra-plate variability may occur, which can be reduced by running more than one assay plate, and a standardized inter-assay control is preferable. All samples analysed with Luminex® assays in this thesis were cell culture supernatant samples. This is considered to be more robust than serum or plasma samples since it contains lower levels of interfering substances (i.e. proteins, metabolites, hetrophilic antibodies) [315]. All samples had similar storage time and the Luminex® kits had the same lot number. In Luminex® assays, the same dilution of a sample is used when analyzing all mediators in a sample. For some cytokines, there can be a considerable number of cytokines whose levels are below or above the detection range. Optimally, samples below or exceeding the lowest or highest point of the standard range should be re-analyzed at a different dilution, but due to the cost of Luminex® assays, this was not done. In order to perform statistical analysis, values below (or above) detection range were set lower than the detection limit (or higher in a few cases). This should not affect the results, as rank sum testing was used to evaluate cytokine data. It could however impact the logistic regression and hierarchical clustering, and has to be taken into consideration when using these types of analyses. Furthermore, the mediator concentrations measured in supernatants can be affected by AML cell autocrine and paracrine loops, as well as the degradation by proteases, but the levels in supernatants are still relevant as they reflects the levels that are available for communication between cells in the cell culture.

4.8 Liquid Chromatography-Tandem Mass Spectrometry methods

In this thesis, mass spectrometry methods have been used to perform metabolomics in **Papers III** and **IV** and proteomics in **Paper I**. The mass spectrometer measures the intensity of ionized biochemicals (in this thesis metabolites or peptides,) differing in their mass to charge ratio (*m/z)*. The relative abundance of the individual *m/z* detected in the mass spectrometer generates a mass spectrum. To separate the biochemicals, a high-performance liquid chromatograph (HPLC) is directly coupled to the mass spectrometer. The untargeted metabolomic profile analyses in this thesis were performed in collaboration with Metabolon, Inc. (Durham, NC, US), and the preparation and analysis of serum samples are described in more detail in **Paper III**. Metabolon® uses ultra-high-performance liquid chromatography/tandem accurate mass spectrometry (UHPLC/MS/MS) to analyze samples. All systems are operated in a rigorously controlled environment to remove as much noise from the process as possible. Compounds

were identified by comparison to Metabolon®´s library entries of purified standards or recurrent unknown entities. Metabolon, Inc. maintains a library based on authenticated standards that contains retention time/index (RI), mass to *m/z* and chromatographic data (including MS/MS spectral data on all molecules present in the library).

As for mediators, human plasma and serum are widely used matrices for investigating metabolites. Here too, different collecting procedures will influence the concentration since both the collecting procedures and the coagulation cascade influence concentration of proteins and metabolites in these matrices [316]. Serum has been obtained from coagulated blood. Blood cells, fibrin and coagulation factors are then separated from serum by centrifugation. During the centrifugation process, platelets may release metabolites, and there is therefore a higher metabolite concentration in serum than in plasma where heparin or EDTA is added before the removal of blood cells [316]. Several studies have examined differences between plasma and serum for metabolomics [317-319]. They report good reproducibility rates for both plasma and serum, while revealing a clear difference between the metabolic profiles in the two matrices [316, 319]. As expected, the metabolite concentration is generally higher in serum samples, suggesting that serum samples are more sensitive for detection of biomarkers [316]. In addition, plasma was shown to be more affected by incubation time than serum [319]. Serum samples have therefore been recommended as the sample of choice in metabolomic studies [319].

4.9 Statistical methods and terminology

For our studies, two types of statistical analysis have been used; significance tests, and classification analyses. In **Papers I** and **II**, all significance tests were done using SPSS® (IBM Statistical Package for the Social Sciences v. 23.0; IBM SPSS statistics Inc., Chicago, IL, USA) and GraphPad® (Graph Pad Software, San Diego, CA, USA) software. The data obtained from experimental studies of AML patient cells could not be considered as having a normal distribution, and therefore non-parametric tests (Mann-Whitney *U,* Wilcoxon signed rank test and Kruskal-Wallis H-test with Dunn´s Post Hoc test) were used for statistical comparisons. In **Papers III** and **IV,** standard significance tests were performed in ArrayStudio (https://arraystudios.com/), R (http://cran.r-project.org/) and the data analysis software program JMP® (JMP®, Statistical Discovery™ from ©SAS Institute Inc. Lane Cove, NSW 2066, Australia) by Metabolon, Inc. (Durham, NC, USA) on log transformed data. Welch´s two sample *t*-test was used to identify metabolites that differed significantly between groups. The paired *t*-test was used for comparison of paired samples, and ANOVA contrasts were used to analyze pharmacological effects for responders and non-responders separately. Since statistics

provides the basis for our research, as for most medical research, it is imperative that the right test is used. We have based our choice of methods on the type of research question being asked and the type of data to be analyzed (e.g. is the data normally distributed, do multiple comparisons have to be taken into account, are the groups being tested paired or unpaired, etc.).

In this thesis, the statistical significance of data was set to p-values < 0.05. The lower the p-value, the more evidence we have that the null hypothesis (that two populations are equal) is not true. A statistical significance set to 0.05, means that 5 % of the time we incorrectly conclude that the populations were different, when they were in fact the same. The p-value of 0.05 is the false positive rate when there is only one test. However, for a large number of tests, as in **Papers III** and **IV**, we need to account for false positives. There are different methods to correct for multiple testing. The oldest methods are family-wise error rate adjustments (Bonferroni, Tukey etc.), but these tend to be extremely conservative for a very large number of tests. For large scale analysis involving microarrays, proteomics and metabolomics, using the q-value to calculate the False Discovery Rate (FDR), is therefore more common, in contrast to the family-wise error rate adjustments which allow for zero false discoveries. FDR allows for a small number of false discoveries; this needs to be taken into account when evaluating the results. Optionally, one can use z-statistics which focus on fold change. This makes it possible to correct false positives while capturing the biochemicals with the fold change that could be most biologically interesting. The American Statistical Association (ASA) stated that "No single index should substitute for scientific reasoning"; thus in addition to statistical analysis, other validation methods should preferably be performed and findings should be reviewed critically [320].

Classification analyses that have been used in this thesis are first and foremost hierarchical clustering analyses (**Papers I, II** and **IV**). Hierarchical clustering is an unsupervised method for clustering data and can show large scale differences. With these analyses we aim to build a hierarchy of clusters where objects (in this case AML patients) that have similar features cluster together, and more dissimilar objects are dispersed further apart. This makes it possible to identify subgroups within the patient cohort. However, hierarchical clustering analysis should be interpreted with care. Hierarchical clustering mainly shows large scale differences and should not be used for specific analysis at patient level. There are many types of hierarchical clustering and many distance metrics that can be used. In our papers, we have used the program J-express 2012 software for all our clustering analysis (MolMine AS, Bergen, Norway).

In **Papers III** and **IV**, random forest analysis was used to provide an unbiased method to predict which variables (in this case metabolites) make the largest contribution to the classification. To determine the "variable importance", a random forest analysis uses a supervised classification technique based on an ensemble of decision trees [321]. To do this, Mean Decrease Accuracy (MDA) was used as the metric. MDA is determined by randomly permuting a variable, running the observed values through the trees, and then reassessing the prediction accuracy. This allows a random forest analysis to provide an "impotency" rank ordering of the variables. When the full forest is grown, the class predictions are compared to the true classes, generating the "out-of-bag error rate" as a measure of prediction accuracy. Thus, the prediction accuracy is an unbiased estimation of how well one can predict sample class in a new data set. A random forest analysis has several advantages: it makes no parametric assumption, variable selection is not needed, it does not overfit, it is invariant to transformation and it is fairly easy to interpret.

Metabolite pathway enrichment analysis was used for biological interpretation of metabolite data at a system level using the MetaboLync analysis tool (MetaboLync® Portal, supplied by Metabolon, Inc.). This analysis is based on the same concept as gene set enrichment analysis (GSEA) [322]. A metabolite pathway enrichment analysis tests whether the metabolites involved in a predefined pathway occur towards the top or the bottom of a ranked query compound list. Instead of focusing on single metabolites in a sample group, it helps to discover metabolic pathways or networks that are altered in the experiment, and it is designed to handle many-to-many relationships that may occur between the compounds and the metabolite annotations [323]. The advantage with this type of analysis is that it helps overcome analytical challenges that are typical for single–metabolite analysis with large scale data, such as a low number of significant alterations after correcting for multiple hypothesis testing, because the relevant biological differences are modest relative to the noise inherent to the technology. Interpretation is dependent on the researcher's area of expertise which may miss important effects on pathways, especially since a small increase in a set of metabolites within the same metabolic pathway may have a much higher biological effect than a 20-fold increase of one single metabolite [322].

5 Discussion

5.1 Targeted therapy

Over the past decades, tremendous progress in understanding the molecular mechanisms of cancer has been made, and specific genes and pathways crucial for cancer progression have been identified, laying the foundation for target-specific treatment. Some major discoveries have been done for a subset of cancer patients, exemplified by the differentiation agent ATRA for treatment of PML patients, imatinib (also known as Glivec®) for CML and ALL patients (targeting the *BCR-ABL* fusion gene), and gefitinib in epidermal growth factor receptor mutated cancers. However, the more we reveal about the underlying mechanisms of cancer development, the more complex and heterogenic the disease is proving to be. Even within a specific cancer type such as AML, the malignant population is not only heterogenic, it is in constant change, both because of genetic instability and epigenetic alterations. In addition, the malignant cells can change their metabolic pathway preference based on their microenvironment, which may be in constant change (e.g. altered nutrient availability, pH, oxygen levels). It is therefore difficult to target a single specific gene or pathway, and combination treatment or multiple target agents may be required to combat cancer.

5.2 Targeting metabolic reprogramming in AML

One of the most prominent abilities of cancer cells is the way they can alter their metabolic preferences, making them more resilient. A promising target for new cancer treatment has therefore been to target metabolic alterations, making cancer cells more vulnerable. Cancer cells generally have higher metabolic plasticity which allows the cells to use different sources of nutrients, including glucose, glutamine and fatty acid, to fuel the TCA cycle. This makes them able to survive under stressful metabolic conditions, outcompeting normal hematopoietic cells [324]. This highly diverse and flexible metabolism contributes to both the aggressiveness of the disease and the high percentage of refractory disease. It has also made metabolic targeting challenging since blocking one metabolic pathway does not necessary kill cancer cells. It can simply make the cells shift to utilize another pathway for cell growth and survival [191]. Simultaneously targeting more than one metabolic pathway may therefore be an approach to eliminate the cancer cells´ ability to survive under stressful conditions and render them vulnerable to chemotherapy. In **Paper I** we used a hierarchical cluster analysis to identify a subset of patients with high sensitivity to several metabolic inhibitors. The thought behind this was to identify a subset that might benefit from a treatment combining multiple metabolic targets. This analysis identified a subset of 29 patients, sensitive to several metabolic inhibitors.

We were not able to identify common molecular or biological similarities for the patient subsets that had either high or low sensitivity to metabolic drugs. Nor did the known prognostic markers for chemotherapeutic sensibility (i.e. secondary AML, karyotype, *FLT3*-ITD, *NPM1* mutations, CD34 expression) show any significant correlations with metabolic vulnerability. Thus, the susceptibility to the antiproliferative effects of metabolic inhibitors seems to depend at least partly on other cellular/molecular mechanisms than the susceptibility to conventional chemotherapy.

In 2009, recurrent mutations in two key metabolic enzymes were identified, namely the cytosolic and mitochondrial isocitrate dehydrogenases (*IDH1* and *IDH2*, respectively). *IDH* mutations are found in 15-20% of all AML patients [325]. The mutation in itself is currently not prognostic, but as IDH inhibitors are in clinical development, the mutation is associated with a favorable outcome [4]. The IDH enzymes are required to provide NADPH for lipid biogenesis, protect against oxidative damage, and facilitate α-KG-dependent dioxygenases. As IDH is a key enzyme in the TCA cycle, mutated *IDH* leads to the production of the oncometabolite 2-hydroxyglutarat (2-HG). 2-HG promotes DNA and histone hypermethylation, which in turn blocks cellular differentiation, promoting leukemic transformation and disease initiation. Mutant IDH inhibitors (enasidenib and ivosidenib) have shown promising results and have recently obtained FDA approval for *IDH* mutated AML patients. Interestingly, *IDH* mutated AML cells have shown to be especially sensitive to inhibition with the glutaminase inhibitor BPTES [241, 326]. There are however, to our knowledge no clinical trials with BPTES in *IDH* mutated AML patients (Table 7). In our patient cohort (**Paper I**), there were only five patients (out of 81) with *IDH* mutations. This was not enough to make any statistically significant assumptions, but all five patients were significantly inhibited by BPTES in this study (Wilcoxon Signed Rank-test), compared to their respective untreated controls. Glutaminase inhibitors are showing promise: CB-389 has been FDA-approved for renal cell carcinoma and is in clinical trials for several other malignancies (including hematological cancers, though not for AML, see Table 7) [327].

FLT3 inhibitors have failed to achieve long-term remission as monotherapy [328], even though experimental studies suggest that they can impair glutamine metabolism and thereby have proapoptotic activity [329]. A combination of glutamine and FLT3 inhibitors has therefore been suggested as a possible therapeutic strategy, and CB-389 combined with AC220 induces increased apoptosis and cell death in *FLT3* mutated AML cells [330]. However, we did not find any significant association between the effects of BPTES and *FLT3* mutations (**Paper I).**

FLT3 mutations lead to abnormal activation of the PI3K-Akt-mTOR pathway [331]. Metabolic reprogramming is mainly driven by the nutrient-sensors AMPK, mTOR and PI3K/Akt; dysregulation of these sensors is seen in more than 60 % of AML patients and is associated with cancer cell survival and proliferation [332-334]. These observations suggest there is a crosstalk between various metabolic pathways, the regulators of these pathways and driver mutations (including *IDH, FLT3, p53* and *NPM1*) in AML. AML cells with different mutations may therefore depend on the same metabolic pathways.

5.3 Signaling mechanisms, glycolytic metabolism and autophagy

The nutrient sensors AMPK, mTOR and P13K/Akt are important for metabolic regulation [332-334]. Both mTOR1 and AKT support glycolysis in AML cells, and starvation or glucose deprivation leads to AMPK activation that indirectly inhibits mTOR and induces apoptosis in AML, as suggested by our observations described in **Paper I.** AMPK activation will also lead to direct and indirect (through mTOR inhibition) activation of ULK1, the major autophagy initiation protein [249, 250]. Furthermore, there seems to be a close connection between PPP and autophagy and certain cancer cells show distinct autophagy-dependent PPP modulations [223]. In **Paper II** we studied antiproliferative and proapoptotic effects on AML cells (i.e. patient cells, the AML cell lines HL60 and MOLM-13) of the autophagy inhibitor CQ alone or in combination with AraC or the metabolic drugs metformin and 2DG. Metformin is an AMPK activating drug, but it also seems to inhibit autophagy through suppression of UPR [292]. This too was suggested by our own results in **Paper II.** Similarly, 2DG showed an additional inhibitory effect when combined with CQ both in the present and previous studies [294]. CQ as monotherapy showed a generally lower autophagic flux for HL60 than for MOLM 13; this is consistent with previous studies showing that HL60 cells display a glycolytic profile that is associated with Akt/mTOR1 activation and low autophagy flux [246, 335]. Furthermore, previous studies have also shown that autophagy limits glycolysis in AML and this is associated with reduced autophagy flux [246, 335]. This may explain the weak effect of CQ monotherapy on the viability of HL60 cells (which mainly depend on glycolysis) but the additive effect when combined with metformin **(Paper II).** Taken together these observations suggest that combined treatment with different autophagy inhibitors should be considered.

5.4 Cancer cell culture media

The availability of different nutrients influences the plasticity of the AML cells [336]. Commercial media have been designed to supply cancer cells with nutrients essential for continuous proliferations [337] and do not therefore recreate physiological conditions. They

often lack metabolites normally present in human fluids, while others metabolites important for growth and proliferation (e.g. glucose, glutamine, pyruvate) are usually present in very high concentrations [338]. The culture medium may influence cell metabolism and thereby susceptibility to metabolic targeting [339]. Such effects may contribute to the generally lower susceptibility to CQ, metformin and 2DG for AML cell lines (**Paper II)** compared to patient cells (**Papers I** and **II)**. All our studies of patient cells were performed with serum-free StemSpan SFEM^{TM} medium supplemented with growth factors, whereas cell lines were cultured in RPMI-1640 supplemented with 10 % inactivated fetal calf serum. RPMI-1640 contains both glucose and amino acids (e.g. glutamine, arginine, proline) in addition to the supplemented serum that contains high levels of growth factors. StemSpan SFEM^{TM} does not contain serum. Thus, *in vitro* AML studies require the use of carefully characterized experimental models that are described in detail in the scientific reports.

5.5 Toxicity of metabolic drugs and their potential as combination therapy

Combination therapies have provided some of the most effective results with regard to AML treatment. The most common chemotherapy regime to treat AML today is a combination of three days with an anthracycline, and seven days with standard-dose AraC, hence the name "3+7" (*see section 1.4.1, Potentially curative treatment).* Combination therapy is also used as a consolidation therapy, as in **Paper III** of this thesis, with ATRA and valproic acid [144, 266]. As the role of epigenetic alterations in cancer development has become more apparent, clinical trials with a combination of epigenetic modifiers are becoming more frequent. Both ATRA and valproic acid have been used in various combination regimes for AML treatment. HDAC inhibitors in particular have emerged as important agents in clinical trials, as HDAC dysregulation is associated with many forms of cancer. Valproic acid, which is a well-known epileptic drug, has generally low toxicity and is well tolerated. However, results for valproic acid as monotherapy in the clinic have been somewhat disappointing. Nonetheless, used in combination therapy it has shown promising results, and has therefore been included in several clinical trials also for AML [133].

To combine two or more different agents against metabolic signaling pathways allows for the simultaneous targeting of multiple pathways, which can minimize treatment resistance since the cancer cells have to adapt to different toxic effects simultaneously. In addition, the resistance of AML cells towards treatment often depends on certain dysregulated cellular signaling pathways, so targeting these dysregulations might sensitize the cancer cells to other agents. This might make cells that were initially refractory to standard treatment receptive to

this treatment, or it may result in the need of much lower doses of chemotherapy or radiation to have clinical effects. Lower doses of treatment will reduce unwanted toxicity, which in turn means that more patients could tolerate the treatment.

However, combination treatments also have disadvantages. The combination of multiple agents can have an additive effect on unwanted side effects in patients. When using multiple agents, it could be difficult to assess which agent is poorly tolerated, and adjusting doses could be challenging. The best doses and order of the drug would have to be carefully selected, to optimize the use and minimize the side effects. Especially if the drugs have similar side effect profiles, toxic substances may accumulate. Furthermore, the metabolizing capacity of drugs differs from one patient to another. The pharmacokinetics of a particular drug can be influenced by genetic variations or alterations in drug-metabolizing enzymes. Patient reactions can also be influenced by simultaneous treatment with other drugs, especially drugs that modulate metabolism. Therefore, drug interactions and side effect profiles have to be thoroughly investigated when creating new combination treatment regimes. Nevertheless, simultaneously targeting one or more pathways might be exactly what it takes to outmaneuver the cancer cells´ plastic metabolism. The challenge is to find the right balance.

Side effects of the metabolic drugs described in this thesis have been reported after use in clinical studies, and are summarized in Table 7. The most common ones are gastrointestinal side effects (including diarrhea, anorexia and nausea), fatigue, and dizziness. More severe side effects such as electrocardiographic changes (2DG and CQ) auditory changes due to toxic effect on the eighth cranial nerve (6AN) and retinopathies (CQ) have also been reported. However, severe hematological and gastrointestinal toxicity seems to be relatively uncommon, suggesting that combination of metabolic targeting with conventional antileukemic treatment should be further investigated.

Table 8. *An overview of metabolism inhibitors in this thesis; clinical trials pertaining to cancer and reported side effects (2019.11.01).*

Clinical trial registered on *ClinicalTrials.gov*	**AML/other leukemias**	**Doses**	**Side effects**
Metformin: 189 clinical trials with metformin in cancer treatment (registered per October 2019).	Five clinical trials with leukemias; three with ALL, one with CML and one with AML (NCT01849276).	Recommended therapeutic doses are 500-2500 mg; 500 mg daily gives a plasma concentration of approximately 5 μM whereas 1500 mg daily gives a plasma concentration of approximately 18 μM. The drug accumulates in the liver, liver concentrations can reach 50-100μM [340].	Lactic acidosis with metformin in therapeutic doses is rare. Metformin side effects include gastrointestinal toxicity (diarrhea, anorexia and nausea) in about 20–30% of patients, resulting in discontinuation of metformin treatment in about 5% of cases. Other side effects are abdominal distension/bloating, acidosis (metabolic or otherwise), dizziness, fatigue, heartburn/dyspepsia, nausea, rash, taste alteration, ulcer (GI), and vomiting [341, 342].
2DG: We found three completed or for other reasons ended clinical trials on *ClinicalTrials.gov*, in addition we found one clinical trial from 1995 not listed in *ClinicalTrials.gov* [343]. NCT00096707 completed, NCT00633087 ended 2014, NCT00247203 withdrawn.	We found no clinical trials with AML or other leukemias.	The dose 63 mg/kg/day gave a mean maximum plasma concentration of 116 μg/ml (0.7 mM) [295]. Other investigated doses are 200 mg/kg body weight in combination with whole brain irradiation (5 Gy) [343] and 30 mg/kg/day for two weeks (days 1-14) of a 21 day cycle.	High doses can lead to adverse side effects such as hyperglycemia, QTc prolongation and gastrointestinal bleeding, and less severe fatigue, sweating, dizziness and nausea (mimicking hypoglycemic symptoms) [295].
6AN: We found no clinical trials involving 6AN in *ClinicalTrials.gov*. We found one study from 1961 investigating the clinical toxicity and tumor-inhibitory effects of 6-AN in 30 patients not listed in *ClinicalTrials.gov* [285].	We found no clinical trials with AML or other leukemias.	Doses were administrated from 200 to 250 mg/day	Frequent toxicities are nausea, headache, tinnitus; less frequent are vomiting and inflammatory lesions involving the mucous membranes of conjunctiva, nose, and oropharynx. Severe toxicity of in the eighth cranial nerve occurred in almost all patients receiving >0.6 mg/kg/day for more than 10 days (initial tinnitus, later high frequency deafness, finally total hearing loss). No hematological toxicity.
BPTES: There are to our knowledge no clinical trials with AML patients or other cancer patients with BPTES. Another GLS1 inhibitor CB-389 has been used in several clinical trials including hematological tumors (NCT02071888) and leukemias (NCT02071927). CB-389 has so far been reported to be well tolerated. The most severe adverse side effect reported is reversible asymptomatic elevation in transaminase [344].			
ST1326: There are to our knowledge no clinical trials with AML patients or other cancer patients with ST1326. Etoxomir, another CPT-1 inhibitor, was through phase I and II clinical trials, but the trials were terminated due to hepatotoxicity associated with the treatment.			
Lonidamine: There were two studies on the safety and efficacy on Benign Prostatic Hyperplasia (NCT00435448, NCT00237536); none on cancer *(ClinicalTrials.gov)*. We found one phase II study on glioblastoma multiform, and one study on toxicity and clinical tolerance not listed in *ClinicalTrials.gov* [345] [301].	We found no clinical trials with AML or other leukemias.	Doses were administered from 300-450 mg/day.	Lonidamine has been found to have no conventional side effects when daily doses range from 300-450 mg, and given in combination with radiotherapy results in no additional toxicity [301]. There were no therapy-related deaths. Side effects such as asthenia, muscular pain and testicular pain were reported. There was no hematological toxicity [345].
AZD3965: One ongoing clinical trial *(ClinicalTrials.gov)*. Ongoing clinical trial in patients with advanced solid tumors, diffuse large B cell lymphoma or Burkitt´s lymphoma. NCT01791595 [286].	We found no clinical trials with AML or other leukemias.	5, 10, 20 and 30 mg were given orally once or twice a day for 28 days.	A dose of 20 mg daily was well tolerated. Nausea and fatigue were the most frequent side effects. A single DLT of cardiac troponin increase was observed at 20 mg. Asymptomatic, reversible retinal ERG changes were first observed at 20 mg; this is the DLT. One patient with tumor-associated undiagnosed lactic acidosis experienced DLT with exacerbation, following a single dose of 10 mg [286].
Chloroquine: Eighteen clinical trials with chloroquine in treatment of cancer in *ClinicalTrials.org*. Eight that were ended; NCT01575782, NCT00969306, NCT01446016, NCT01777477, NCT01023477, NCT01438177, NCT01727531, NCT00224978	We found no clinical trials with AML or other leukemias.	Daily doses have been 100 - 600 mg/day; the WHO guidelines for treatment of malaria recommend 25 mg base/kg, over three days [302, 303]. Mean plasma levels in five healthy adults given 500 mg CQ dephosphat were 4 mg/ml equivalent to 12.54 uM CQ [305].	Side effects are minimal at low doses, while many more toxic effects occur at higher doses, such as visual disturbances, gastrointestinal symptoms, electrocardiographic changes, headache, and pruritus. For long time use of CQ (normally used for rheumatic disease), recommended daily dose is < 5.0 mg/kg due to risk of retinopathy [306].

Abbreviations are listed in the list of abbreviations in the front of the thesis.

5.6 Metabolic inhibition is not specific to AML cells

The ability to preferentially kill cancer cells without increasing toxic effect to normal cells, so-called targeted therapy, is the most important aim when trying to find new cancer therapy. To achieve this, we need to understand the biological differences between normal cells and cancer cells. In this Ph.D. project we have mainly focused on leukemic cells` response to metabolic treatment (**Papers I** and **II**), and systemic metabolic alterations in AML patients reacting to disease-stabilizing treatment, and treatment-requiring GVHD after allo-HSCT (**Papers II** and **IV**).

Toxic effects on normal cells have not been a main focus of this study. However, it is important to test and compare effects on normal cells too. It is nonetheless difficult to determine which cell-type can be used as an appropriate control. We chose to use human MSCs and UCB cells as healthy controls (**Papers I** and **II**), but they were derived from few subjects (only five UCB donors and one MSC donor). In **Paper I**, the metabolic inhibitors had similar antiproliferative effects on the UCB and the AML cells. However, metformin had an overall stronger effect on the proliferation of AML cells compared to UCB cells. In the viability assay, the UCB cells were generally less sensitive towards all metabolic drugs (except AZD3965). In our coculture assay with MSCs, 2DG had an antiproliferative effect for the majority of AML patients, even in the presence of MSCs, and the AML cells were significantly more sensitive to 2DG than the MSC cells. In **Paper II,** CQ had a similar inhibitory effect on the proliferation of UBC and AML cells, and in addition, the MSCs were less inhibited in MSC-AML cocultures, compared to the AML cells. As all MSCs originated from the same donor, it is difficult to conclude on the overall MSCs sensitivity towards CQ compared to AML cells in our assays. However, this shows that the AML cells do not protect the MSCs from CQ-induced toxicity.

In our studies we have primarily investigated the effect of metabolic inhibitors on AML cells, and the healthy control cells were merely included to give a brief guidance as to how these drugs might affect the surrounding environment. As we used UCB and not isolated CD34+ stem cells, there are no guarantees that the proliferating cells do not contain T-cells. However, T-cells would normally require a specific antigen signal, as well as a co-stimulatory protein to be fully activated and proliferate [346], neither of which are found in the serum-free StemSpan SFEM^{TM} medium used in these assays. The growth factors G-CSF, SCF and FLT3-L, which were added to the medium, should not affect T-cell proliferation. However, certain drugs, such as streptokinase, can initiate antigen-stimulated T-cell activation, without antigen present. Response peaks after 6-7 days of culture [347]. Therefore, a possible T-cell activation cannot

be ruled out, although it is unlikely, as all of our drugs showed a dose-dependent inhibition of proliferation. Thus, the proliferating cells in our UCB controls are most likely CD34+ cells. It would of course have been optimal to use isolated bone marrow CD34+ cells as controls in our assays. However, these commercially available cells are expensive, and since LSCs were not the main focus in these studies, isolating CD34+ cells was not prioritized.

5.7 T-cell metabolism

In **Paper IV** we looked at the metabolic alterations of allo-HSCT recipients, identifying metabolic characteristics of patients with treatment requiring cGVHD. Allo-HSCT is commonly used to treat severe hematological malignancies, including AML. Allo-HSCT relies on the ability of engrafted immune systems, mainly the T-cells, to remove residual leukemic cells, known as the GVL effect [348, 349]. However, the greater the GVL effect, the greater the risk of developing GVHD. We examined the metabolic profiles of allo-HSCT patients one-year post-transplantation, to see if the metabolic profiles could be used to diagnose and determine the severity of cGVHD requiring systemic immunosuppression, and to study the effect of immunosuppression on the metabolic profiles of cGVHD patients. As patients were examined at a defined time point, the metabolic profiles of the patients were likely to be influenced both by the pathology and the duration of cGVHD, and differences in the immunosuppressive treatment. The pathology of cGVHD is still not fully understood, but it is generally accepted that it involves inflammatory mechanisms related to T-cells [349]. Emerging evidence has revealed that metabolic alterations impact the activation and differentiation of T-cells. Whereas naïve, regulatory and memory T-cells rely mainly on OXPHOS for energy production, the metabolism of activated T-cells is in many ways similar to cancer cells [350]. Like tumor cells, activated T cells mainly rely on aerobic glycolysis instead of OXPHOS [350]. Activated T cells can undergo metabolic reprogramming, upregulating other metabolic pathways like glutaminolysis and FAO, if they need to secure different energy sources for cell growth, proliferation, differentiation and cytokine production [351]. Furthermore, recent studies support the hypothesis that metabolic pathways are important for shaping the T-cell responses and are recognized as key players in T-cell activation and differentiation. They play an important role in modulating the T-helper cell lineages, and do not only function as passive support and as nutrient sources [352]. This supports our findings in **Paper IV**, that systemic metabolic profiles may be used as a prognostic indicator and help identify patients with treatment-requiring cGVHD. However, this metabolic reprogramming is strongly influenced by signals from the cells´ microenvironment and intracellular energy status, where, among other factors, AMPK,

mTORC1 and HIF are important signaling pathways. This is supported by studies showing that immunosuppressive molecules, such as rapamycin which inhibits mTORC1, also have an impact on T-cell metabolism [351]. 51 patients were included in our study (**Paper IV**), 31 with cGVHD. All except two patients with active cGVHD received immunosuppressive treatment (i.e. cyclosporine, steroids). One could therefore speculate about the possibility that the metabolic alterations we detected in these patients are, at least in part, related to drug-induced modifications of T-cell metabolism.

5.8 The antileukemic effect of valproic acid; epigenetics and the emerging role of lipid metabolism in AML

In **Paper III** the aim of the study was to compare the pretreatment metabolomic profiles found in non-responders and responders to AML-stabilizing treatment with ATRA and valproic acid (to see if responders/non-responders could be identified). We aimed to characterize how these profiles altered during the early period of this treatment (two-day or seven-day monotherapy with either ATRA or valproic acid, respectively). Valproic acid is an epigenetic targeting drug that functions as an HDAC inhibitor (*see section 1.1.8 Epigenetics*). We found a highly significant change in the lipid metabolite profiles after treatment with valproic acid; especially levels of fatty acid metabolites were altered**.** In coherence with our findings in **Paper III**, lipid dysregulation has been established as an important phenotype in cancer cells, and reprogrammed FAO has been reported for various cancers, also for AML [353, 354]. In recent years, the literature has increasingly focused on altered lipid metabolism both intracellular in AML cells, and signaling to adipocytes in the cells´ microenvironment (mainly the bone marrow) [353-358]. In addition, there seem to be several links between epigenetic regulation and fatty acid metabolism in AML [359].

Firstly, epigenetic regulation is important for general fatty acid metabolism. Acetyl-CoA is the precursor of fatty acid synthesis and is produced in the mitochondria from fatty acid oxidation. Acetyl-CoA is converted to citrate in the TCA cycle, and transported into the cytoplasm by the citrate transporter, where it can be used to regenerate Acetyl-CoA that can be used in the fatty acid synthesis. This process is catalyzed by the two Acetyl-CoA-carboxylase (ACC) isoforms ACC1 and ACC2, and the fatty acid synthase (FAS) complex [359]. This is reflected in our findings in **Paper III**, where we, in addition to an increase in fatty acid metabolites, also see an increase in TCA- metabolites between non-responders with rapidly progressive AML and non-responders with less aggressive disease. This suggests an increase in the transition of fatty acids to the energy metabolism in patients with rapidly progressive disease. Expression of ACC2 is

also a key driver of fatty acid β-oxidation in AML cells [360]. Repression of this gene by histone deacetylation allows for simultaneous β-oxidation and fatty acid synthesis to take place [360]. Secondly, changes in the expression of genes encoding fatty acid synthesis enzymes have been seen in response to dietary changes. A carbohydrate-rich diet increases ACC1 and FAS, promoting fatty acid synthesis. On the other hand, fasting has been shown to decrease fatty acid synthesis and inhibit ACC1 and FAS expression. Obesity has been shown to favor AML growth [361]. The mechanisms behind this include the expression of both fatty acid binding protein 4 (FABP4) and DNA methyltransferase 1. High-fat diet-induced obese mice, with increased FABP4, have a much higher leukemia burden. This diet accelerates AML cell growth [362]. These findings are supported by a recent study showing that genetic disruption of FABP4 in AML cells or mice blocked cell proliferation *in vitro* and induced leukemia regression *in vivo* [362]. Finally, valproic acid has been shown to alter lipid metabolism and fatty acid oxidation [363, 364]. Our results in **Paper III** thereby support the hypothesis that valproic acid alters epigenetic regulation in AML both through direct effects on histone acetylation and indirectly through modulation of fatty acid metabolism.

5.9 Metabolic preferences of LSCs may influence the treatment strategy

While conventional chemotherapy eliminates most of the proliferating bulk of AML cells, some of the disease-initiating LSC population is spared, leading to disease progression and relapse [66, 365-367]. This may indicate that LSCs display some different molecular or metabolic advantages, making them more resistant to conventional therapy and increasing risk of relapse. One could therefore argue that also targeting LSCs is likely to result in better outcomes for AML patients. Recently, striking characteristics of LSCs have been discovered: firstly, LSCs depend mainly on OXPHOS fueled by amino acids to drive the TCA cycle. Secondly, LSCs have low ROS levels, while mature blasts generally exhibit higher ROS levels. Thirdly, LSCs have been shown to have an overexpression of the anti-apoptotic molecule BCL-2 [165]. Not only does targeting BCL-2 increase apoptosis, it also targets LSCs selectively, partly by mechanisms impairing OXPHOS [165, 166, 368]. An ongoing multicenter clinical trial (NCT02203773) investigating Venetoclax, a BCL-2 inhibitor, in combination with the hypomethylating agents azacitidine or decitabine is showing promising preliminary results, demonstrating CR/CRi rates reported from 61-85% [166]. Furthermore, metabolomic analyses of circulating blasts from the patients before and after venetoclax/azacitidine treatment, revealed alterations of metabolites in the TCA cycle, reduction of the central antioxidant glutathione and reduction of amino acids in the LSCs, which were not found for the majority

of blast cells after treatment [166]. Further studies have shown that metabolic plasticity is important for resistance of venetoclax [368]. To conclude, these studies suggest that combination treatment targeting cancer cell metabolism has potential in AML, and gives rise to an exciting new therapeutic regimen.

6 Concluding remarks

All four articles included in this thesis support the evidence that the heterogeneity of AML also applies to AML cell metabolism. The plasticity of AML cells enables them to adapt to environmental conditions, and optimize the consumption of what is available in terms of nutrients (i.e. glucose, amino acid and fatty acids). This allows the AML cells to maintain fast proliferation and cell growth, thus rapidly outgrowing normal hematopoietic cells. However, the results from **Papers I** and **II** demonstrate that this heterogeneity also renders sub-populations more sensitive to metabolic targeted therapy. These subpopulations do not seem to have any associations to the well-established risk factors for chemoresistance after conventional intensive chemotherapy (i.e. secondary AML, karyotype, *FLT3*-ITD, *NPM1* mutations, CD34 expression) or long-term AML-free survival. Thus, the mechanisms behind metabolic vulnerability seem to depend, at least partly, on other cellular and molecular mechanisms than conventional chemotherapy. The literature suggests that metabolic rewiring is highly dependent on both cellular and environmental contexts. This, including the heterogeneity among the AML cells, as well as the limitations of experimental models, has made it difficult to find specific metabolic targets. Future preclinical and clinical studies have to be performed in order to identify subsets with common characteristics suitable as metabolic targets, or combinations of targets, better suited to harm AML cells, while simultaneously sparing healthy cells.

Our results show that different subsets are more sensitive to the inhibition of different metabolic pathways, and rely not only on glycolysis and OXPHOS, but also on amino acid and lipid metabolism. Lipid metabolism is emerging as a potential therapeutic target, given the alterations found in AML cells, as is supported by our own findings in **Paper III**. Even so, targeting lipid metabolism has proven to be challenging, and successful results from clinical trials are still lacking. Our studies presented in **Papers III** and **IV**, suggest nonetheless that the alterations seen in AML cells´ lipid and amino acid metabolism might have prognostic value, predicting both disease progression and treatment responses. In this context, it is tempting to suggest patient metabolic profiles as a potential prognostic tool for AML treatment and diagnosis in future.

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I

Article

TARGETING CELLULAR METABOLISM IN ACUTE MYELOID LEUKEMIA AND THE ROLE OF PATIENT HETEROGENEITY

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Abstract:

Acute myeloid leukemia (AML) is an aggressive blood cancer resulting in accumulation of dysfunctional white blood cells in the bone marrow. Changes in cell metabolism are features of many cancers, including AML, and this may be exploited as a therapeutic target. In this study, we investigated the *in vitro* antileukemic effects of drugs inhibiting different metabolic pathways. Drug-efficacy on AML cells from 81 patients was evaluated using proliferation and viability assays; we also compared global gene expression and proteomic profiles for various patient subsets. The drugs metformin, 2DG, 6AN, BPTES and ST1326 had strong antiproliferative and proapoptotic effects for most patients, whereas lonidamine and AZD3965 had an effect only for a minority. Antiproliferative effects on AML cells were additive when combined with the chemotherapeutic agent AraC. Drug-effects showed no associations with clinical or biological characteristics of patients. Using hierarchical clustering, we identified a subset of 29 patients with a strong antiproliferative effect towards metabolic drugs. Gene expression and proteomic studies suggested that this subset was characterized by altered metabolic and transcriptional regulation. Antiproliferative effects of drugs were maintained in the presence of mesenchymal stem cells (MSCs), and the metabolic inhibitors also had anti-proliferative effects on MSCs and umbilical cord blood cells. Therapeutic targeting of cell metabolism may have potential in AML, but the optimal strategy will probably differ between patients.

Keywords: acute myeloid leukemia, glycolysis, metabolism, oxidative phosphorylation

1. Introduction

Acute myeloid leukemia (AML) is the most common leukemia in adults. It is an aggressive hematologic malignancy characterized by accumulation and enhanced cell proliferation of immature myeloid precursors in the bone marrow, leading to marrow failure [1]. AML is a heterogeneous disease with regard to phenotypic, molecular and clinical features, with variations in differentiation, karyotype, molecular genetics and epigenetics of the leukemic cells. There is also heterogeneity with respect to the susceptibility of chemotherapeutic treatment [2]. Current treatment is based on high-intensity induction chemotherapy; however, not all patients are eligible for this potentially curative treatment, and a large amount of AML patients relapse, which is associated with impaired survival. The 5-year overall survival rate after intensive therapy is below 50% and even lower for patients older than 70 years [3], and new treatment options for AML are therefore warranted.

Changes in cell metabolism have been described as hallmarks of cancer and metabolic targeting is considered as a possible therapeutic strategy in various cancers, including AML [4]. Cancer cells are often more dependent on glycolysis and have increased energy requirements in comparison to normal cells that mainly employ oxidative phosphorylation (OXPHOS) to gain energy in the presence of oxygen [5, 6]. The ability of malignant cells to alter their energy metabolism to meet their increasing bioenergetic demands is crucial for cancer development [7]. Complex metabolic capacities may contribute to the aggressiveness of cancer, including AML, allowing cells to survive under higher metabolic demands. However, AML patients are heterogeneous with regard to metabolic regulation [8-11], and in this study we wanted to further investigate if patients are heterogeneous also with regard to the antileukemic effects of metabolic targeting.

Several metabolic pathways can be utilized by cancer cells to gain energy and building blocks for growth and survival, including OXPHOS, glycolysis, glutaminolysis, fatty acid β-oxidation, the pentose phosphate pathway (PPP) and the lactate synthesis pathway [12]. Hematological malignancies have been shown to rely on different metabolic routes depending on their genetic abnormalities [13-18], an observation suggesting that AML patients can be subclassified based on their metabolic phenotype and may vary in their susceptibility to metabolic targeting. To further study patient heterogeneity, we investigated the *in vitro* effects of seven drugs that affect various metabolic pathways (see Table 1) including metformin, 2-deoxy-D-Glucose (2DG), lonidamine, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide (BPTES), 6-aminonicotinamide (6AN), AZD3965 and ST1326 on leukemic cells derived from a large group of consecutive patients.

Materials and Methods

2.1 Primary human cells.

Primary human AML cells. This study was approved by the Regional Ethics Committee (REK III 060.02, (100602), REK Vest 2013-634 (190313), REK Vest 2015/1410 (190615), 2017/305 (070417) and samples were collected after written informed consent. The main characteristics of the 81 consecutive patients are presented in Table 1; acute promyelocytic leukemia (APL) patients were excluded. Peripheral blood mononuclear cells were isolated from patients with at least 80% AML cells among blood leukocytes and generally >15 x 10^6/L blood leukocytes. Enriched AML cell populations could then be prepared by density gradient separation alone (density of 1.077, Ficoll-Hypaque; Nycomed, Oslo, Norway) and included >90% AML cells; contaminating cells consisted mainly of small lymphocytes [19-21]. Primary AML cells were cryopreserved in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, USA) with 10% dimethyl sulfoxide and 20% heat-inactivated fetal bovine serum (FBS) and stored in liquid nitrogen until thawed and used in experiments.

Table 1. Biological and clinical features of the 81 AML patients in this study.

CLINICAL CHARACTERISTICS					
Gender			*Age*		
Female	30		Median (yrs)		67.5
Male	51		Range (yrs)		17-87
Previous hematological disease			*Survival*		
AML relapse	5		Yes		15
MDS	11		No		24
CMML	2		nd		42
Polycythemia Vera	1				
Li-Fraumeni syndrome	1				
AML CELL DIFFERENTIATION					
FAB classification			*CD34 expression*		
AML-M0/M1/M2	38		Negative (<20%)		22
AML-M4/M5	36		Positive (>20%)		52
nd	7		nd		7
GENETIC ABNORMALITIES					
Cytogenetics		*FLT3*		*NPM-1*	
Favorable	10	wt	45	wt	45
Intermediate	48	ITD	22	INS	28
Adverse	14	TKD	2	nd	8
nd	9	ITD/TKD	3		
		nd	9		

Abbreviations: CMML, Chronic myelomonocytic leukemia; FAB, French-American-British; INS, insertion; ITD, internal tandem duplication MDS, Myelodysplastic syndrome; nd, not determined; TKD, tyrosine kinase domain; wt, wild type; yrs, years.

Primary umbilical cord blood (UCB) cells. Cells were obtained from five donors after written informed consent, REK Vest 2015/1759 (051115), 2017/305 (070417). Mononuclear cells were enriched in a similar manner to AML cell populations, by using density gradient separation, and stored in liquid nitrogen until used in experiments.

2.2 Reagents

Culture medium used in all experiments, except the coculture studies, was serum-free Stem Span SFEM^{TM} medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with granulocyte-colony stimulation factor (G-CSF), stem cell factor (SCF) and fms-related tyrosine kinase 3 ligand (FLT3L). All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA) and used at a final concentration of 20 ng/ml. The seven metabolic inhibitors tested on AML cells are described in more detail in Table 2. Metformin hydrochloride (PHR1084), 2-Deoxy-D-Glucose (abbreviated 2DG; 154-17-6), lonidamine (L4900) and BPTES (SML0601) were all obtained from Sigma-Aldrich (St. Louis, MO, USA), while 6AN (100-093-15) and AZD3965 (199-12) were purchased from Cayman Chemical (Ann Arbor, MI, USA), and ST1326 (870853; also known as Teglicar) from Avanti Polar Lipids (Alabaster, AL, USA). AraC (Cytosine Arabinoside) was provided by Pfizer (New York, NY, USA). The same lot numbers of drugs were used in all experiments. Stock solutions were prepared according to the distributors' datasheets. Metformin and 2DG were passed through a 0.22 µm filter after being dissolved, and all drugs were aliquoted and stored at -20°C. Aliquots were thawed only once and diluted with their respective solvents (DMSO or PBS).

Table 2. The seven metabolic inhibitors tested on AML cells and their main targets.

Metabolic inhibitor	Molecular target – *metabolic pathway*	Biological effects
Targets cell surface		
AZD3965	MCT1 – *Glycolysis (Lactate/Pyruvate)*	AZD3965 is a second generation selective MCT1 inhibitor. It inhibits both lactate influx and efflux into cells obstructing the metabolic interplay between cell populations within tumors. In addition, it increases glycolysis and upregulates glycolytic enzymes [1, 2].
Cytoplasmic targets		
Metformin	*OXPHOS and AMPK signaling pathway*	Metformin inhibits mTOR indirectly through effects on LKB1/AMPK. It inhibits OXPHOS and fatty acid metabolism, which in turn increases glycolysis [3-5].
2DG	Hexokinase – *Glycolysis.*	2DG is a glucose analogue that inhibits hexokinase, the first and rate-limiting enzyme in glycolysis [6, 7].
Lonidamine	Hexokinase II – *Glycolysis.* MCT, *OXPHOS*	Lonidamine has a multisite effect. It targets hexokinase II, the mitochondrial pyruvate carrier, mitochondrial permeability, MCT and the electron transport chain [8, 9].
6AN	G6PD and 6PGD - *PPP*	6AN is a nicotinamide analogue and a competitive inhibitor of PPP, a parallel metabolic pathway to glycolysis. PPP directs glucose to its oxidative branch leading to production of NADPH, but it is also a key autophagy-dependent compensatory metabolic pathway [10, 11].
AZD3965	MCT1 - *Glycolysis (Lactate/Pyruvate)*	See above.
Mitochondrial targets		
BPTES	Glutaminase - *Glutaminolysis*	It inhibits glutaminase, the enzyme that converts glutamine to glutamate which is further oxidized to α-ketoglutarate. Glutamine is an important source of energy for many cancer cells. This is especially seen in *IDH* mutated cells. Glutaminase is upregulated by the *Myc* oncogene [12-14].
ST1326	CPT1 – *Fatty acid metabolism*	FAO is an important source of fuel for cancer cells proliferation during metabolic stress. CPT1 catalyzes the rate-limiting step of FAO. ST1326 is a selective CTP1 inhibitor [15].

2.3 Analysis of cell proliferation, viability and constitutive cytokine release AML cell proliferation.

Proliferation was analyzed using a [^{3}H]-thymidine incorporation assay [22]. AML cells were seeded (5 x 10^4 cells/well, 200 µL medium/well) in flat-bottomed 96-well microtiter plates (Nuncleon™; Nunc, Roskilde, Denmark). Cell cultures were added drugs at various concentrations, medium controls and solvent/vehicle controls. After 6 days of incubation at 37ºC in a humidified atmosphere of 5% CO_2, [^{3}H]-thymidine (37 kBq in 20 µL saline per well; TRA 310; Amersham International, Amersham, UK) was added and cultures incubated for additional 22 hours before nuclear incorporation was determined by liquid scintillation counting. For each treated culture, proliferation (uptake of thymidine) was calculated by subtracting values obtained for untreated controls (medium alone) from drug-treated cultures, and detectable proliferation was defined as at least 1000 counts per minute (cpm). The median of triplicate cultures was used in all calculations.

Cocultures of MSCs and AML cells. Normal human mesenchymal stem cells (MSC) (Lonza, Cambrex BioScience, Walkersville, MD, USA) derived from a healthy donor (MSC24539) were expanded in complete mesenchymal stem cell growth medium (MSCGM™; Lonza) with 10% heat-inactivated FBS and 4 mM L-glutamine. The MSCs were trypsinized and used in cocultures in passage four. AML-MSC cocultures were prepared in MSC medium, and the cells were then separated by a semipermeable membrane (0.4 µm pore size) using transwell plates (Costar 3401 plates; Costar, Cambridge, MA, USA), allowing only cytokine-mediated crosstalk with no direct MSC-AML cell contact. Cell proliferation of both the AML cells and MSCs were also analysed using the [^{3}H]-thymidine incorporation assay after three days of coculture as described in detail previously[23].

Viability. The percentages of viable, apoptotic and necrotic AML cells were determined by flow cytometry using the Apoptest™–FITC kit according to the manufacturer`s protocol (NeXins Research, Kattendijke, the Netherlands) [24]. Briefly, primary cells were cultured for 48 hours in Stem Span SFEM™ medium supplemented with cytokines (1 x 10^6 cells/ml in 1 ml/well) in 24-well culture plates with or without drugs. Cells were double-stained with AnnexinV fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the distributors staining protocol. Acquisition of data was done using a FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA, USA), with a minimum of 10,000 events collected for each sample. The flow cytometry data was analyzed using FlowJo v10.3 software (Tree Star, Inc., Ashland, OR, USA).

Soluble mediator release. AML cells (1x 10^6/ml, 1 ml/well) were cultured for 48 hours in Stem Span SFEM™ medium supplemented with G-CSF, SCF and FLT3L (1 x 10^6 cells/ml and 1 ml per well) in 24-well culture plates (Nucleon™; Nunc, Roskilde, Denmark). Culture supernatants were harvested and stored at -80° until analyzed using human magnetic Luminex multiplex assays (LXSAHM-17, R&D Systems; Minnesota, MN, US). Supernatants from cocultures of MSCs and AML cells were also harvested and analysed with the Luminex multiplex assays. Nineteen analytes were measured: (i) the chemokines CXCL1, CXCL10, CXCL5, CXCL8/IL-8, CCL2, CCL3, CCL4, CCL5, (ii) the interleukins IL-1β, IL-1RA, IL-6, (iii) the matrix metalloproteinases MMP-1, MMP-2, MMP-9, (iv) tumor necrosis factor-α (TNF-alpha), (v) the growth factors GM-CSF and HGF, and (vi) the proteinase inhibitors Cystatin C and Serpin E1/PAI-1.

2.4 Mutational profiling, global gene expression profiling and proteomic analysis

Mutational profiling.

The TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA, USA) was used to assess 54 genes frequently mutated in myeloid leukemias as described previously [25], and data from this analysis was available for 35 of our AML patients.

Global gene expression profiling. Analysis of global mRNA profiles were performed on 21 patients using the Illumina iScan Reader based on fluorescence detection of biotin-labeled cRNA as described previously [25]. For each sample, 300 ng of total RNA was reversely transcribed, amplified and biotin-16-UTP-labeled (Illumina TotalPrep RNA Amplification Kit; Applied Biosystems/Ambion, USA). The quantity and quality of the biotin-labeled cRNA was assessed using the NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Biotin-labeled cRNA (750 ng) was hybridized to the HumanHT-12 V4 Expression BeadChip that targets 47,231 probes mainly derived from genes in the NCBI RefSeq database (Release 38). Data from the array scanning were investigated in GenomeStudio and J-Express 2012 [26]. All arrays within each experiment were quantile normalized before being compiled into an expression profile data matrix. The PANTHER version 14.1 was used for functional classification of genes [27, 28].

Proteomic analyses. Proteomics preparation and analysis was performed on 14 patients, as described previously [29, 30]. Briefly, filter-aided sample preparation (FASP) procedure [29] was used to prepare samples, which were further analyzed on the Q Exactive HF Orbitrap mass spectrometer (MS) (Thermo Fisher Scientific; Waltham, MA, USA) coupled to an Ultimate 3000 Rapid Separator liquid chromatography (LC) system (Thermo Fisher Scientific). The LC-

MS raw files were searched against a Swiss-Prot Homo sapiens FASTA file (downloaded 28.08.18) and the label-free quantitation (LFQ) count was set to 1 in MaxQuant version 1.6.1.0 [31].

2.8 Statistical analyses

Mann-Whitney *U*-test, Kruskal-Wallis *H*-test with Dunn´s test for multiple comparisons, Wilcoxon signed rank test, Welch's *t*-test and Kendall's test were used for statistical analyses. Differences were regarded as statistically significant when $p < 0.05$. SPSS (IBM Statistical Package for the Social Sciences v. 23.0; IBM SPSS statistics Inc., Chicago, IL, USA) and GraphPad prism v. 5.02 (Graph Pad Software, San Diego, CA, USA). J-Express 2012 was used to perform all cluster-analysis; all cluster-analysis were made using complete linkage. The statistical analyses used for proteomics analysis have been described elsewhere [30]. Significantly different proteins were analyzed in the STRING database (version 11.0) [32] using experiments and databases as interaction sources at a confidence cut-off score of 0.7, and visualized in Cytoscape (version 3.3.0) [33]. Since data was collected over multiple days, a data normalization step was performed to correct inter-day and plate variation. Essentially, this was done by registering the medians to equal one (1.00) and normalizing each data proportionally, termed "block correction".

3. Results

3.1. Drug screening study; effects on proliferation of AML and UCB cells after exposure to metabolic drugs

A proliferation assay ([^{3}H]-thymidine incorporation assay) was used for the initial testing of several metabolic drug concentrations on AML cells, i.e. we evaluated the ability of AML cells to survive after seven days of culture and still be able to proliferate after drug treatment. Six concentrations of each drug were initially tested (Figure S1). Metformin (1.25-40 mM), 2-DG (0.625-20 mM) and 6AN (50-500 μM) showed strong antiproliferative effects for all 17 patients investigated. The other four drugs had divergent effects, and especially lonidamine (25-300 μM) and AZD3965 (6.25-200 nM) showed no or minor antiproliferative effects. Based on these initial results, the following drug concentrations were chosen for further experiments: metformin 1.25 and 2.5 mM, 2DG 0.3 and 0.6 mM, 6AN 50 and 100 μM and BPTES 10 and 20 μM. Lonidamine and AZD3965 were further tested at their highest screening concentration of 300 μM and 200 nM, respectively, which were the maximum achievable concentrations in DMSO without having a toxic effect on AML cells. ST1326 was also further tested at its highest

screening concentration of 50 μM; higher concentrations were not tested due to issues with poor solubility. Ara-C (0.0125-0.3 μM) had significant antiproliferative effects at all concentrations tested, with responses differing among patients (data not shown). Both DMSO and PBS alone showed no effect on AML cell proliferation or viability at the concentrations used in this study (data not shown).

The effects of metabolic inhibitors on the proliferation of human UCB-derived mononuclear cells were also tested in our [^{3}H]-thymidine incorporation assay. Five samples where tested while four UCB samples showed detectable proliferation (> 1000 cpm) in untreated control cultures. Treatment with metabolic inhibitors had similar antiproliferative effects on UCB cells as seen on primary AML cells (Figure S1), however, metformin 5 mM inhibited proliferation of AML cells stronger than UCB cells at the same doses (p = 0.030, Mann-Whitney U-test).

3.2. Several metabolic inhibitors have antiproliferative effects on primary AML cells, with no association with secondary AML, AML cell differentiation, karyotype, FLT3-ITD, NPM1 mutation or patient survival

We investigated the effects of drugs on cytokine-dependent AML cell proliferation for all 81 patients. Detectable proliferation, defined as [^{3}H]-thymidine incorporation >1000 cpm, was observed in the control cultures for 69 of the 81 patients (85% of patients). For these patients with detectable proliferation, drug treatment resulted in a highly significant antiproliferative effect compared to untreated controls ($p \leq 0.0001$, Kruskal Wallis test, Dunn´s post-hoc test), for all drugs except lonidamine and AZD3965. The latter two drugs did not show any significant inhibitory effects on proliferation, though proliferation was decreased after drug treatment for certain patients (Figure 1). When comparing the effect of drugs on proliferation for individual patients, we observed that the antiproliferative effect varied considerably between patients. For certain patients, cell proliferation was inhibited completely by drug treatment while other patients had no effect with the same drug concentration. The strongest overall antiproliferative effect was seen after treatment with 2DG (Figure 1). Furthermore, we defined the antiproliferative effect as >20 % inhibition compared to untreated controls and investigated whether these antiproliferative effects showed any associations with AML cell differentiation (FAB classification, CD34 expression), cytogenetics, *FLT3 o*r *NPM1* mutations or occurrence of previous disease (versus *de novo* AML). A few associations of borderline significance were detected ($p < 0.05$, Table S1). Due to the large number of comparisons, and small sample numbers, we conclude that the antiproliferative effects show no significant associations in these analyses.

Our patient cohort included 40 younger patients who completed intensive induction and consolidation therapy; 15 of them remained in remission for a median of 105 months (range 39-164) whereas the others died from resistant/relapsed AML. The antiproliferative effects observed with the metabolic inhibitors did not differ between patients when comparing long-term survivors and patients dying from resistant leukemia (Table S1).

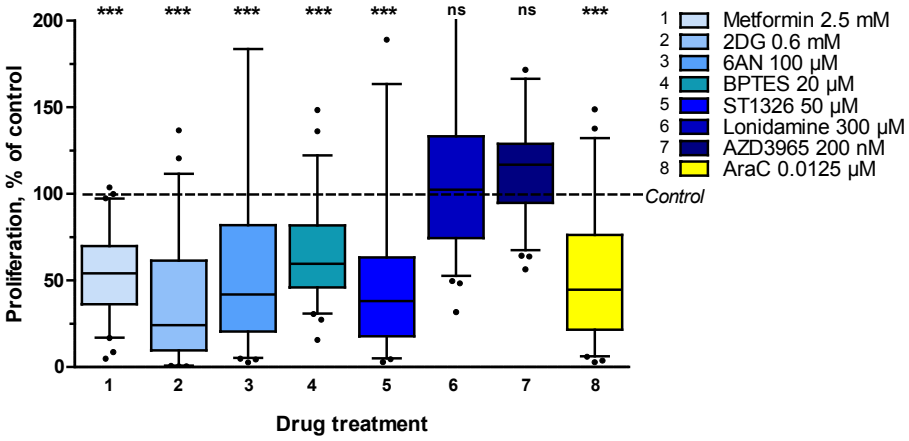

Figure 1. Effects of treatment with metabolic inhibitors or AraC on AML cell proliferation. AML cells were cultured alone or treated with metabolic inhibitors or AraC for 7 days in suspension cultures, before proliferation was measured using the [^{3}H]-thymidine incorporation assay. Detectable proliferation defined as >1000 cpm was found for 69 out of the 81 control cultures; except ST1326 which included 54 patients with detectable proliferation among 62 patients tested. The figure illustrates the results only for patients with detectable proliferation. The median proliferation (% of untreated controls) for all patients are presented (25/75 percentiles, 5/95 percentile whiskers). The drug-free (untreated) controls were set to 100% (stippled line). Statistically significant effects are shown (ns, not significant, * p-value < 0.05, *** p-value < 0.0001). The Kruskal Wallis, with Dunn´s post-hoc test was used for statistical analyses.

3.3. Metabolic inhibitors have additive antiproliferative effects when combined with AraC

The antiproliferative effects of metabolic inhibitors were also tested alone or in combination with AraC 0.0125 µM (81 patients examined, 69 showing detectable proliferation). The following concentrations of drugs were then used, metformin 1.25 mM and 2.5 mM, 2DG 0.3 and 0.6 mM, 6AN 50 and 100 µM, BPTES 10 and 20 µM, ST1326 50µM, lonidamine 300 µM and AZD3965 200 nM. Significant additive effects (i.e. combined effects, being greater than their separate effects) were found after treatment with metformin 2.5 mM, 2DG 0.6 mM, 6AN 50 and 100 µM and ST1326 50 µM in combination with AraC compared to AraC alone (Figure 2; *p*-value < 0.05, Kruskal Wallis test, Dunn`s post-hoc test).

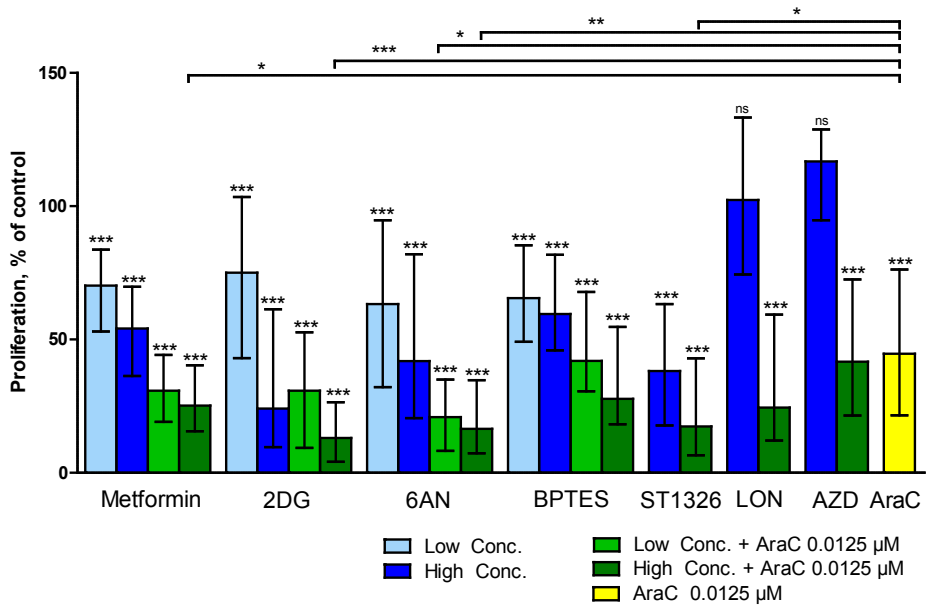

Figure 2. The combined treatment of metabolic inhibitors with AraC on AML cell proliferation, using the [^{3}H]-thymidine incorporation assay. The metabolic inhibitors were tested at the following concentrations: metformin 1.25 and 2.5 mM, 2-DG 0.3 and 0.6 mM, 6AN 50 and 100 µM, BPTES 10 and 20 µM, ST1326 50 µM, lonidamine 300 µM and AZD3965 200 nM alone and in combination with AraC 0.0125 µM. All 69 patients with detectable proliferation in untreated control cultures were included in this analysis, except ST1326 which included 54 patients with detectable proliferation among 62 patients tested. The results are presented as the median percentage of proliferation in cell cultures treated with metabolic inhibitors or in combination with AraC compared to the corresponding control cultures (column), ±SD (whiskers). The Kruskal-Wallis test with Dunn`s multiple comparison test was used for statistical comparisons between monotherapy and combination therapy compared to untreated controls (significance shown above each column), and between combination therapy with metabolic drugs and AraC, and AraC alone (illustrated above brackets at the top of the figure). ns = not significant, * p-value < 0.05 and, ** p-value 0.001 and ***, p-value < 0.0001; LON, Lonidamine; AZD, AZD3965.

3.4. The antiproliferative effect of metabolic inhibitors differs between patients and a subset of patients show increased susceptibility to several inhibitors

One of the most prominent alterations in cancer cell metabolism is the increased consumption of glucose. We therefore did an unsupervised hierarchical cluster analysis based on the effects of the metabolic inhibitors that can affect glucose metabolism, which include metformin, 2DG and 6AN (lonidamine was not included due to lack of significant effects on cell proliferation, Figure 1). This analysis was based on the percent inhibition of AML cell proliferation after drug treatment, i.e. the proliferation in treated cultures relative to the corresponding untreated control cultures. This clustering identified a subset of 22 patients with overall high sensitivity to these drugs (Figure 3A). These 22 patients did not differ significantly from the other patients with

regard to cause of AML (secondary versus *de novo*), AML cell differentiation (FAB classification, CD34 expression), karyotype, *FLT3*-ITD, *NPM1* mutations, age, gender or survival of the younger patients receiving intensive therapy.

We thereafter did an unsupervised hierarchical cluster analysis based on the five drugs with significant antiproliferative effects when analyzing the overall results (shown in Figure 1) which included metformin, 2DG, 6AN, BPTES and ST1326 (Figure 3B). We now identified a subset of 29 patients (the 29 upper patients) characterized by a strong antiproliferative effect by all or most of the five inhibitors. These 29 patients did not differ significantly from the other patients with regard to cause of AML (secondary versus *de novo*), AML cell differentiation (FAB classification, CD34 expression), karyotype, *FLT3*-ITD, *NPM1* mutations or survival of the younger patients receiving intensive therapy (Table S2). Furthermore, the 29 upper patients in Figure 3B did not differ from the other patients with regard to degree of cytokine-dependent proliferation in control cultures.

We also performed a hierarchical cluster analysis based on the effects of cell proliferation after drug treatment relative to the corresponding untreated control cultures when all metabolic inhibitors and drug concentrations were included (Figure S2). Based on this analysis, the patients could be divided into three subsets (Figure S2).

The two hierarchical cluster analyses that were based on the antiproliferative effects of (i) drugs that can affect glucose metabolism (metformin, 2DG and 6AN) (Figure 3A) and (ii) the five metabolic drugs that have overall significant effects on cell proliferation (metformin, 2DG, 6AN, BPTES and ST1326) (Figure 3B), did not differ much with regard to patient distribution in the different subclusters. This shows that the results obtained with the three drugs metformin, 2DG and 6AN, in particular 2DG, weighed heavily in the clustering analyses.

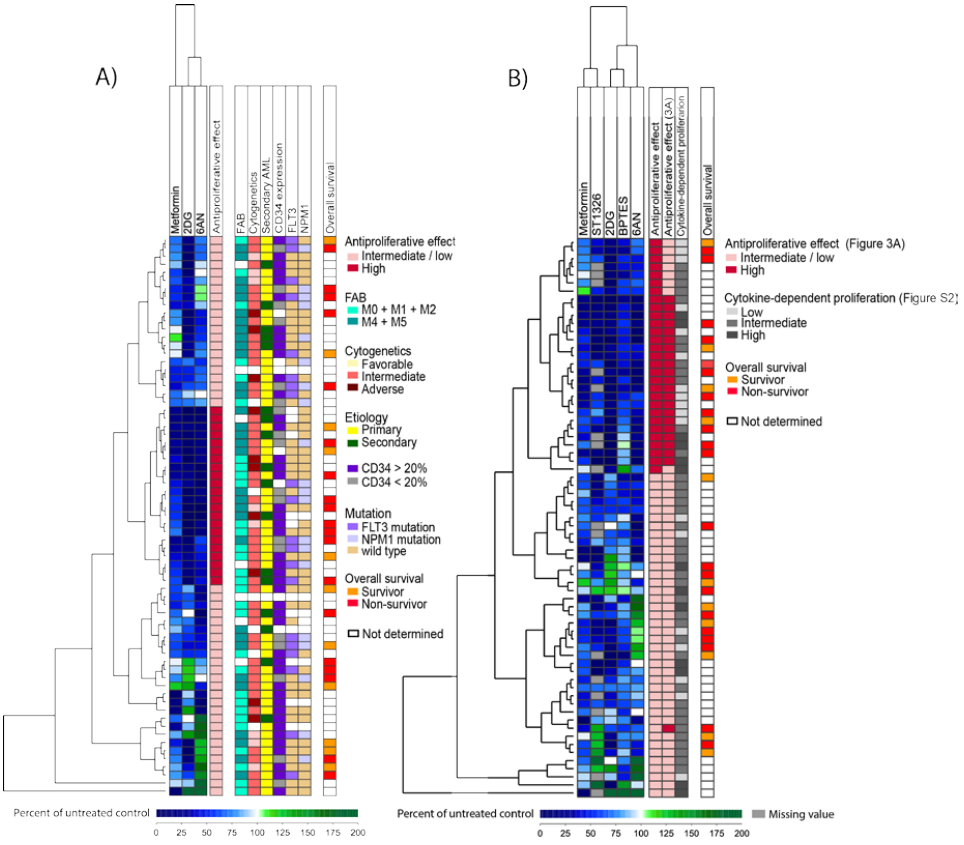

Figure 3. Unsupervised hierarchical cluster analysis based on the effects of metabolic inhibitors on proliferation of primary human AML cells. Primary AML cells were grown in suspension cultures in medium supplemented with exogenous growth factors, and treated with metformin 2.5 mM, 2DG 0.6 mM, 6AN 100 µM, BPTES 20 µM, ST1326 50 µM or added medium (untreated controls) for 7 days before cell proliferation was analysed using the [^{3}H]-thymidine incorporation assay. The percent inhibition (i.e. proliferation in treated cultures relative to untreated controls) was estimated for each patient/drug combination **A) Clustering of patient samples based on effects of metabolic inhibitors that can influence glucose metabolism.** The cluster analysis was based on the effect of metformin, 2DG and 6AN on AML cell proliferation, and identified a subset of 22 patients with overall higher sensitivity towards these drugs (illustrated in dark red in the first column to the right of the cluster). The next columns to the right indicate biological and clinical characteristics for each individual patient (FAB, cytogenetics, secondary versus *de novo*, CD34 expression, *FLT3*-ITD and *NPM1* mutations). **B) Clustering based on significant antiproliferative effects of metabolic inhibitors on AML cells.** The cluster is based on the results for all five inhibitors that showed significant antiproliferative effects on AML cells when analyzing the overa ll results (see Figure 1), and two main patient subsets were then identified. The upper cluster included 29 patients, shown as dark red in the first column to the right of the cluster (with stronger antiproliferative effects of drugs), and the lower cluster included 40 patients. Columns are also shown for patient subsets identified from clustering based upon inhibitors influencing glucose metabolism (from Figure 3A), and clustering based upon cytokine dependent proliferation after treatment with all metabolic inhibitors (data from Figure S2) and overall survival.

3.5. A proteomic comparison of patient samples showing either generally strong or weak antiproliferative effects after treatment with metabolic inhibitors

We did a proteomic analysis of AML cells derived from six patients with a generally strong antiproliferative effect after treatment with five metabolic inhibitors (Figure 3B, six patients in the upper cluster of 29 patients), versus eight patients that showed either no or little effect of treatment (Figure 3B, lower cluster). Forty-nine proteins showed a significant difference between the two groups (Welch's t-test, $p < 0.05$) and thirty-one of these also had a significant fold change ($p < 0.05$) (Table S3 and S4). Among these, the largest group of proteins belonged to the classification Metabolism (19 proteins, including 4 mitochondrial proteins) and/or were involved in transcriptional regulation (9 proteins). We also did a functional network analysis (STRING database v. 11.0) based on the 49 proteins that showed a significant difference only based on the Welch's *t*-test (Table S5). We then identified 17 proteins that interacted with other significant proteins, where the majority of proteins were involved in the formation of a network of 12 proteins that are important for regulation of autophagy/metabolism and/or transcription (Figure S6). Although this proteomic data and analyses should be interpreted with great care because few patients were included, they support that antiproliferative effects of metabolic inhibitors is influenced especially by the metabolic context (i.e. different expression of proteins involved in cellular metabolism) of the AML cells and in addition also by their transcriptional regulation.

3.6. A comparison of global gene expression profiles for AML samples that differ in their general susceptibility toward metabolic inhibitors

Our proteomic analysis suggested that differences in the susceptibility to the metabolic inhibitors are associated with differences in metabolism and transcriptional regulation. We therefore compared the global gene expression profiles for 21 consecutive patients, eight patients with strong antiproliferative effects after drug treatment (Figure 3B; upper subcluster of 29 patients) and 13 with intermediate/low antiproliferative effects (Figure 3B; lower subcluster of 40 patients). We performed a feature subset selection (FSS) analysis and by selecting genes with an R-score > ±3.5, we identified 265 differentially expressed genes, 133 upregulated in the intermediate/low antiproliferative subset and 132 upregulated in the high antiproliferative subset. We then performed a hierarchical cluster analysis that clearly separated the two patient subsets (Figure S3). We thereafter performed gene ontology (GO) mapping using the PANTHER analytic tool based on the 265 upregulated genes. Among the categories Protein class and Biological process, we found several ontologies to be overrepresented in the

two patient subsets. Genes belonging to transcription factors and involved in metabolic processes seem to be upregulated in the intermediate/low antiproliferative subset (cluster BI, Figure S3), while genes belonging to different enzyme systems (hydrolase, oxido reductase and transferase) and nucleic acid binding as well as cellular processes were upregulated in the subset with strong antiproliferative effects after drug treatment (cluster AII, Figure S3).

In addition, an extended molecular genetic analysis was available for 35 unselected patients (Figure S4). Most mutations had expected low frequencies [34], and for most mutations, there were too few patients to do any significant comparisons. For those mutations detected in more than five patients (i.e. *FLT3*, *NPM1*, *TET2* and *DNMT3A*) we found no significant associations with the five metabolic inhibitors that showed significant overall effects on AML cell proliferation (i.e. percent inhibition after drug treatment compared with controls), nor did we see any significant associations with mutational class or the number of mutations per patient.

3.8. Metabolic inhibitors decrease AML cell viability through proapoptotic effects

For 78 of the 81 patients, AML cell viability was analyzed after 48 hours of culture with or without treatment with metabolic inhibitors. The highest concentrations of drugs, which were also tested in the proliferation assay, were tested in the apoptosis flow cytometric assay, i.e. metformin 2.5 mM, 2DG 0.6 mM, 6AN 100 μM, BPTES 20 μM, ST1326 50 μM, lonidamine 300 μM and AZD3965 200 nM (Figure 4 A and B). The gating strategy to define viable, early apoptotic and late apoptotic/necrotic cells is illustrated for a representative sample in Figure S5. Of the 78 patients analyzed, six of the patients showed less than 5% viable cells in medium (untreated) controls and were excluded from the statistical analyses. The leukemic cell viability after 48 hours of culture showed a wide variation between patients, the median viability for untreated controls was 58% (range 14.6-89.1%), and the median viability ranged from 39.4% after treatment with 2DG to 58.6% after treatment with AZD3965. All drugs except AZD3965 decreased AML cell viability compared to untreated controls for a significant majority of patients (Wilcoxon signed rank test, p-value < 0.05) (Figure 4A). The percentage of apoptotic cells was generally relatively low after 48 hours of drug treatment; still, apoptosis was significantly increased by all drugs compared to untreated controls except for lonidamine and AZD3965 (Wilcoxon signed rank test, p-value < 0.05) (Figure 4B).

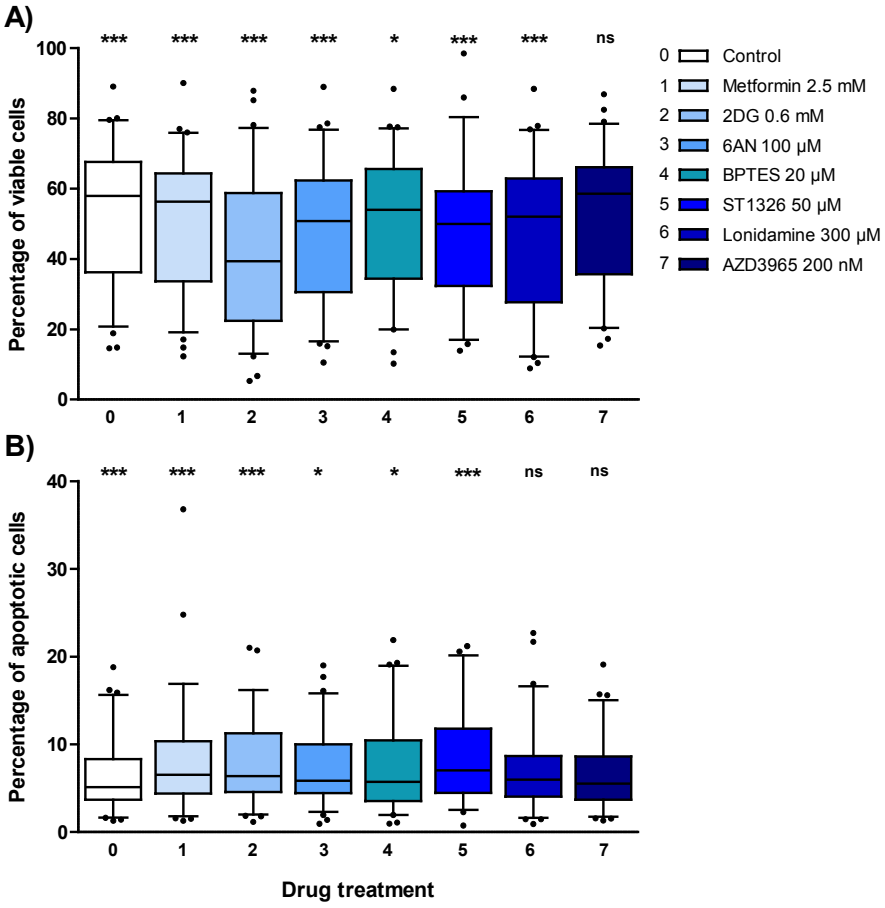

Figure 4. Effects of metabolic drugs on AML cell viability and apoptosis. AML cells derived from 78 consecutive patients were cultured for 2 days in suspension cultures in medium supplemented with cytokines with or without metabolic inhibitors, before the percentage of viable and apoptotic cells were determined by flow cytometry using the AnnexinV/PI apoptosis assay. 72 patients had more than 5 % viable cells in untreated controls and were used in the statistical analysis. The percentage of **A)** viable and **B)** early apoptotic cells (median percentage, 25/75 percentiles, 5/95 percentile whiskers) is shown for all 72 patients. Significant effects of drug treatment compared to untreated controls are shown. (Wilcoxon signed rank test, * p-value < 0.05, *** p-value < 0.0001; ns, not significant).

Effects of metabolic inhibitors on the viability of UCB-derived mononuclear cells were also analyzed by flow cytometry after 48 hours of treatment with metformin 2.5 mM, 2DG 0.6 mM, ST1326 50 µM, BPTES 20 µM, AZD3965 200 nM and lonidamine 300 µM. The UCB cells from five donors were generally less sensitive towards all the metabolic drugs, with at least 90% viability after treatment compared to drug-free controls (Figure S7).

3.9. Identification of patient subsets based on AML cell viability after treatment with metabolic inhibitors

To further study the patient heterogeneity, we did a hierarchical cluster analysis based on AML cell viability after treatment with or without drugs normalized to the corresponding median level (Figure 5A). We could then identify three patient subsets; an upper patient subset with a low cell viability (i.e. less than the median) both for untreated and treated cultures, patients in the lower cluster showed higher viability (i.e. higher than the median) after treatment with most drugs, while the middle cluster showed variable effects. Thus, the variation between patients with regard to spontaneous *in vitro* apoptosis is largely maintained in the presence of drugs targeting metabolic pathways.

We next did an unsupervised hierarchical cluster analysis based on the relative effect of the metabolic inhibitors on viability, i.e. percent cell viability in cultures treated with metabolic drugs, compared to control cultures (cells in medium alone) and the clustering analysis then identified two clusters (Figure 5B). The upper 24 patients in this analysis formed a separate cluster characterized by decreased viability (more apoptotic cells) after treatment with metabolic inhibitors and also a relatively strong spontaneous *in vitro* apoptosis in control cultures. When comparing the viability after drug treatment for the two patient clusters, less viable cells were seen after treatment with 2DG ($p = 0.0005$) and 6AN ($p = 0.015$) for the upper cluster of 24 patients (Table S6). Furthermore, the cluster with the strongest proapoptotic effect after drug treatment did not differ with respect to cause of AML (secondary versus *de novo*), AML cell differentiation, karyotype, *FLT3*-ITD or *NPM1* mutations. Finally, we could not detect any correlations between the antiproliferative and proapoptotic effects of individual inhibitors, except for 2DG and lonidamine that showed a highly significant but weak correlation (Figure S6).

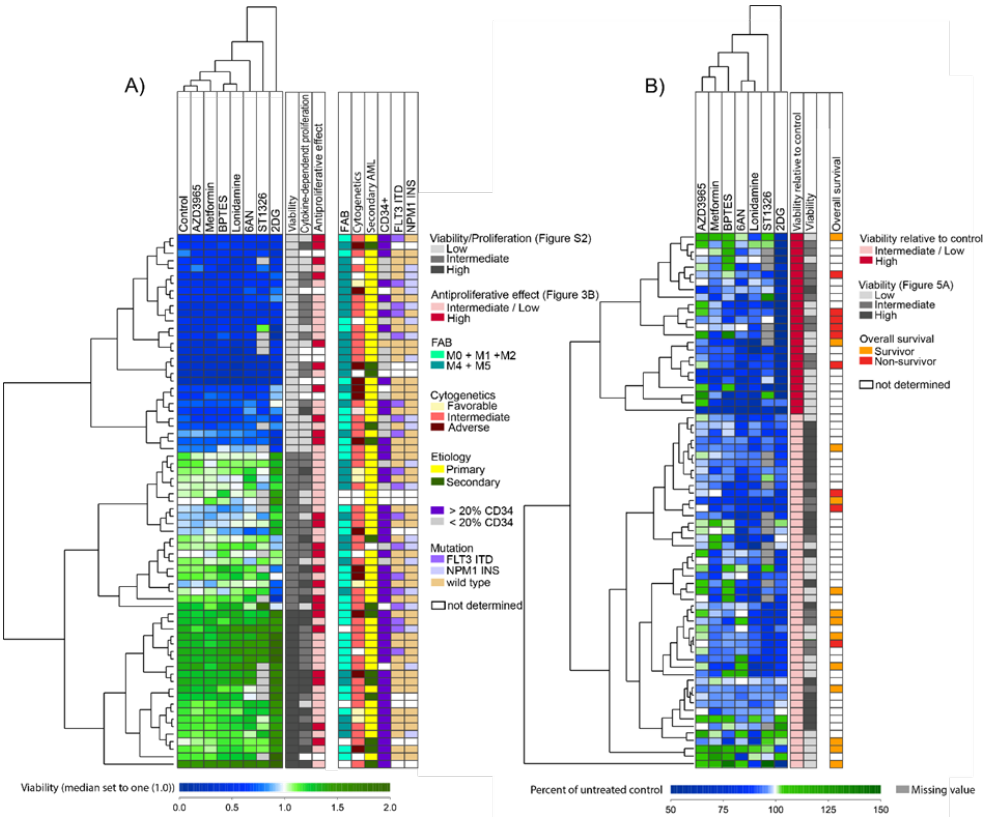

Figure 5. Unsupervised hierarchical cluster analysis based on the effects of metabolic inhibitors on AML cell viability. Primary AML cells were grown in suspension cultures in medium supplemented with exogenous growth factors. The cells were treated with metformin 2.5 mM, 2DG 0.6 mM, 6AN 100 µM, BPTES 20 µM, ST1326 50 µM, lonidamine 300 µM and AZD3965 200 nM or untreated controls (medium alone) before effects on cell viability were determined using the AnnexinV/PI flow cytometric assay. **A) Cluster analysis based on the viability of primary AML cells after treatment with metabolic inhibitors**. The analysis is based on the percentage of viable cells in cultures after 48 hours with or without drug treatment. The data obtained from the 72 patient samples were normalized to the corresponding median level (median set as 1.0). Patients could then be divided into three main subsets; one upper subset showing low viability, one subset with intermediate viability, and one subset with generally high viability after drug treatment. These subsets are shown in the column to the right of the figure, also with a column showing the degree of AML cytokine-dependent cell proliferation (based on Figure S2) and the effect of metabolic inhibitors on cell proliferation (based on Figure 3B), as well as biological and clinical characteristics for the individual patients (i.e. FAB-classification, karyotype, etiology, CD34 expression, *FLT3*-ITD and *NPM1* mutations). **B) Cluster analysis based on the relative viability of AML cells after treatment with metabolic inhibitors for 48 hours.** The analysis is based on the relative effect of metabolic inhibitors on AML cell viability after 48 hours treatment, i.e. the cell viability in cultures treated with drugs relative to the viability in control cultures (untreated cultures, set to 100%). The 72 patients could be divided into two main subsets; one upper patient subset showing reduced viability (shown in dark red) and one with intermediate/low effects on viability (light red) after drug treatment. The subsets are indicated in the column to the right of the figure, along with a column showing drug effects on AML cell viability normalized to median levels (based on Figure 5A), and the classification of younger patients receiving intensive chemotherapy as survivors or nonsurvivors (i.e. dying from resistant/relapsed AML).

3.10. Effects of metabolic inhibitors on the constitutive release of soluble mediators by primary AML cells

We analyzed the concentration of 19 soluble mediators in culture supernatants collected after 48 hours in *vitro* culture of AML cells derived from 72 patients. The release profiles varied considerably between patients, however based on these profiles, patients could be clustered into three main subsets using hierarchical cluster analysis (Figure S8). Still, no significant associations were found between the release of mediators and the effect of drugs on proliferation (Figure 3B) or viability (Figure 5B). Furthermore, the metabolic inhibitors had divergent effects on the release of soluble mediators (Figure 6A) that were relatively small compared with the large variations between individual patients that were maintained in the presence of inhibitors (data shown for metformin, 2DG and 6AN, Figure 6B).

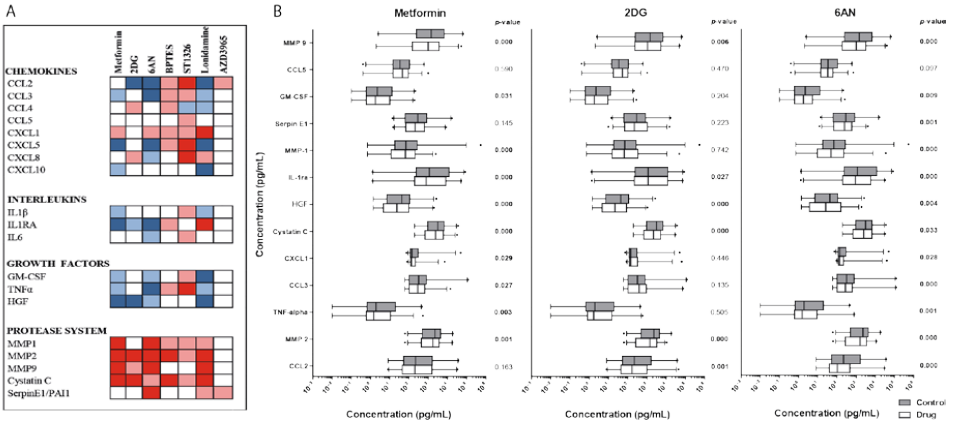

Figure 6. The effects of metabolic inhibitors on the constitutive release of soluble mediators by primary human AML cells. Primary AML cells from 72 patients were cultured for 48 hours with and without drug treatment before the concentrations of 19 soluble mediators were determined in supernatants using multiplex analysis. Concentration of drugs were: metformin 2.5 mM, 2DG 0.6 mM, 6AN 100 µM, BPTES 20 µM, ST1326 50 µM, lonidamine 300 µM and AZD3965 200 nM. **A) Overall effects of metabolic inhibitors on the release of soluble mediators by AML cells.** Mediator levels measured in drug-treated cultures were compared to concentrations in untreated control cultures. Increased levels after drug treatment are shown in red, while decreased levels are shown in blue. Significant changes are indicated by the colors ($p < 0.05$ light color, $p < 0.001$ dark color, Wilcoxon signed rank test). **B) Changes in the constitutive release of soluble mediators by metformin, 2DG and 6AN.** Mediator concentrations measured in cultures after 48-hour drug treatment (white boxes) and untreated control cultures (grey boxes) are presented, with the median levels, 25/75 percentiles (boxes), 5/95 percentiles (whiskers) and outliers. The mediator concentrations are given on the X-axis as log-values.

3.11. 2DG has an antiproliferative effect on AML cells even in the presence of AML-supporting MSCs

2DG was the metabolic inhibitor with the strongest antiproliferative or proapoptotic effect when analyzing the overall results (Figure 1 and Figure 4). Using the [3H]-thymidine incorporation assay, we therefore investigated whether 2DG (0.6 mM) also inhibited AML cell proliferation in the presence of normal MSCs when the different cell types were separated by a semipermeable membrane with no direct contact. Primary AML cells derived from 18 unselected patients were tested, while only 14 patients showed detectable AML cell proliferation in the medium controls. Proliferation decreased after treatment with 2DG for the majority of patients even in the presence of MSCs (Figure 7A). Furthermore, MSC proliferation was also decreased after 2DG treatment of cocultures (Figure 7A), though 2DG had a stronger antiproliferative effect on AML cells than on MSCs (Mann-Whitney *U-test*, $p = 0.0016$). The constitutive release of soluble mediators was also measured in cocultures with and without treatment with 2DG. Only minor changes in the concentrations of soluble mediators were found in drug-treated cocultures compared to untreated cocultures , and overall the mediator release profile seemed to remain similar in the absence and presence of the glycolytic inhibitor 2DG (Figure 7B).

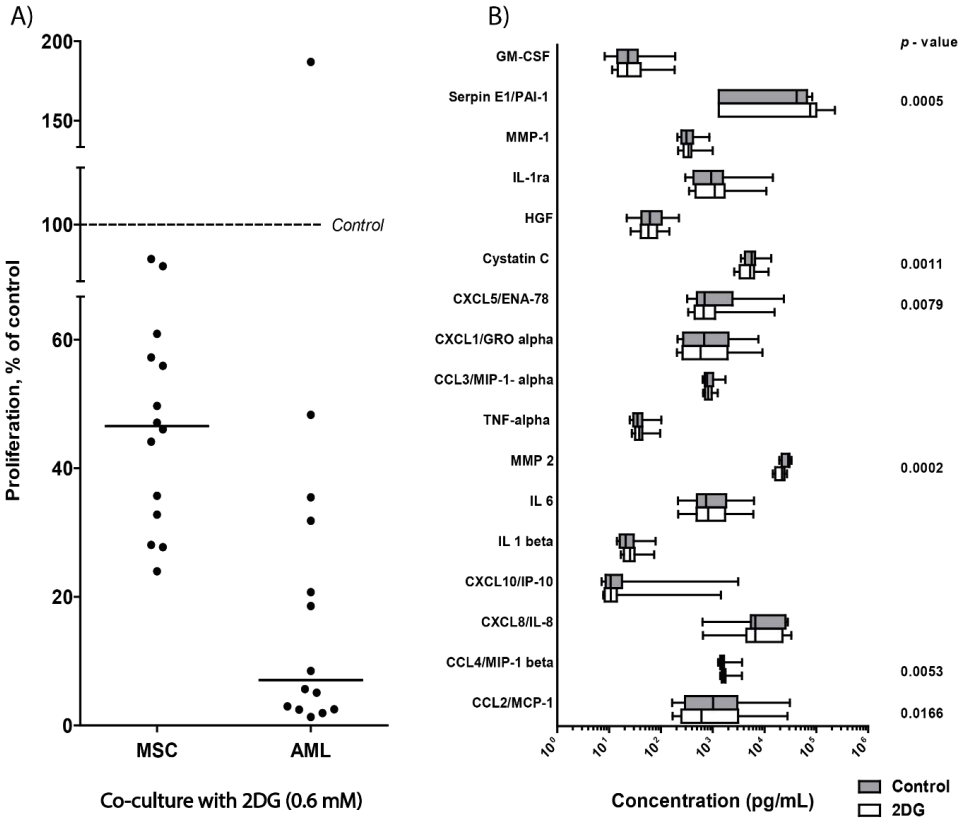

Figure 7. Cell proliferation and mediator release after *in vitro* coculture of primary human AML cells and normal MSCs after treatment with the glycolytic inhibitor 2DG. Primary AML cells derived from 14 patients were cultured together with normal MSCs in transwell cocultures for 3 days, before proliferation was assayed using the [^{3}H]-thymidine incorporation assay, and cytokine release was determined after 48 hours in supernatants using multiplex analysis. **A) Effect of 2DG on cell proliferation in MSC-AML cocultures.** Proliferation of MSCs and AML cells in 2DG-treated cocultures relative to the drug-free control cocultures (shown as stippled line at 100%) was calculated, and an antiproliferative effect by 2DG was then observed both for the MSCs (derived from one donor) and the AML cells (derived from 14 patients) during coculture. The median proliferation of the MSCs and the AML patient cells after treatment is indicated in the figure (–). **B) The constitutive soluble mediator release in cocultures with and without 48-hour treatment with 2DG.** The results are presented as the median levels, 25/75 percentiles (boxes), 5/95 percentiles (whiskers) and outliers. The mediator concentrations (pg/mL) are given on the X-axis as log-values. Grey boxes illustrate mediator release measured in untreated cocultures, and white boxes are after treatment with 2DG.

4. Discussion

Metabolic pathways are dysregulated in different types of cancer, including AML, to meet the energetic and biosynthetic demands of cancer cells. A recent study described a prognostic scoring system for cytogenetically normal AML based on the systemic levels of a limited

number of metabolites, suggesting that a glucose signature had prognostic value [35]. Furthermore, glycolytic metabolism at diagnosis was associated with favorable clinical outcome in another study [36]. It seems that AML patients are heterogeneous with regard to metabolic regulation and its prognostic impact, and targeting of metabolism may be a promising therapeutic strategy in AML [4]. In this study, we have investigated the direct *in vitro* effects of metabolic inhibitors targeting main metabolic pathways on the survival and proliferation of primary human AML cells, and our results show that patients are heterogeneous with regard to direct effects of metabolic inhibitors.

We examined primary AML cells from patients with relative and/or absolute high levels of circulating AML cells, resulting in highly enriched AML cell populations (>90%) after density gradient separation, and our results are thus maybe only representative for this patient subset [37, 38]. [39]. However, our patients are representative for AML in general with regard to karyotypic abnormalities and molecular genetics [20, 25, 30]. Our methods for cryopreservation and thawing are highly standardized, but the cells have decreased viability after thawing, and will undergo spontaneous or stress-induced *in vitro* apoptosis during further culture [24]. However, most patient samples will have a viability of approximately 60-70% after thawing and may show reduced expression of certain cell surface molecules [40]. Our model to analyze effects of metabolic inhibitors on AML cell viability is thus based on a combined effect of stress-induced and inhibitor-induced apoptosis. Patient samples with low stress-induced apoptosis (i.e. high viability after thawing in control cultures) also had a weak proapoptotic effect induced by metabolic inhibitors. Finally, there was no significant association between the proapoptotic and antiproliferative effects, though the assays likely evaluate effects of metabolic inhibitors on different AML cell populations.

We used the [^{3}H]-thymidine incorporation assay for our dose-response studies. [^{3}H]-thymidine is added after six days and nuclear incorporation is assayed one day later, i.e. we investigated the AML cell subset that is able to survive and still proliferate after six/seven days of culture. We selected the concentrations to be used based on (i) dose-response curves, (ii) no effect of DMSO concentrations ($\leq 0.2\%$) used for dissolving drugs; (iii) previous experimental studies and (iv) clinical tolerated doses for those drugs that have so far been used in the clinic (Table 1). Metformin, 2DG, 6AN, BPTES and ST1326 all showed significant antiproliferative and proapoptotic effects, while lonidamine and AZD3965 only showed effects for a minority of patients (Figure 1 and Figure 4).

Patients with cytogenetically normal AML have been reported to have a distinct glucose metabolism [11], and the glycolytic profile seems important for therapy response, as well as

having an impact on the clinical prognosis [36]. Glycolytic activity has been reported to be involved in the progression of several cancer types, and has also been shown to contribute to chemoresistance in patients with AML [41, 42]. Metformin, 2DG and 6AN are drugs that have effects on glucose metabolism. 2DG and 6AN directly inhibit glucose metabolism through inhibition of glycolysis and PPP, while the main effect of metformin on glucose metabolism is through its activation of AMPK, (which switches the cells from an anabolic to a catabolic state, inhibiting gluconeogenesis and hepatic glucose output) and through inhibition of OXPHOS. In addition, metformin decreases OXPHOS in cancer cells, which can be compensated for by increasing their glycolysis [43]. Some studies have shown that leukemic stem cells (LSCs) are more dependent on OXPHOS than more mature leukemic cells, and that these cells cannot use the glycolytic pathway to compensate for reduced OXPHOS [44]. It has recently been demonstrated that Venetoclax, a Bcl2 inhibitor, selectively targets LSCs by inhibiting OXPHOS [45]. Thus, LSCs might also be more vulnerable towards metformin. However, metformin has also been shown to have a direct inhibitory effect on hexokinase II, thereby inhibiting glycolysis [46], and another study showed that the proapoptotic effect of metformin is dependent on the glucose levels [47]. Thus, it seems that the antileukemic effects of metformin as well as 2DG and 6AN is affected by both extracellular and intracellular glucose levels, and will be most efficient on glucose dependent (glycolysis-driven) leukemia cells [47, 48]. We therefore did an unsupervised hierarchical cluster analysis based on the effect of metformin, 2DG and 6AN (Figure 3A) (we left out lonidamine since we saw no significant effect of this drug in our patient cohort). This identified a subset of 22 patients with strong antiproliferative effects of all three drugs. However, we found no association between this subpopulation and any of the well-established risk factors for chemoresistance after conventional intensive chemotherapy (i.e. secondary AML, karyotype, *FLT3*-ITD, *NPM1* mutations, CD34 expression) or long-term AML-free survival.

The high metabolic plasticity of AML cells may allow fluctuation between the sources of nutrients needed to fuel the TCA cycle. This highly diverse and flexible metabolism of AML cells, not only contributes to the aggressiveness of the disease and the high percentage of refractory disease, but it can also make metabolic targeting challenging since blocking one metabolic pathway can simply make the cells shift to use another pathway instead of inhibiting their ability to proliferate. Simultaneously targeting multiple metabolic pathways may therefore be an approach to remove their ability to survive under stressful conditions and render them vulnerable to therapy. Venetoclax with azacitidine is currently enrolled in an ongoing multicenter clinical trial (NCT02203773), the preliminary results have been very promising

with a CR reported between 61-85% [49]. However, LSCs from relapsed AML patients does not seem to have the same dependence on amino acids, as LSCs in these patients seems to be able to compensate through increased fatty acid metabolism [50]. Thus, adding the fatty acid inhibitor sulfo-N-succinimidyl oleate (SSO) seemed to resensitize the cells to venetoclax/azacitidine treatment [50]. These findings highlight the potential in targeting multiple metabolic vulnerabilities, as well as highlights the importance of understanding metabolic properties of AML cells. To further investigate this, we did a hierarchical cluster analyses, identifying a patient subset with high susceptibility to five metabolic inhibitors with overall significant effects (Figure 3B, upper 29 patients). This effect was independent of the proliferative capacity of the AML cells, the spontaneous or stress-induced *in vitro* apoptosis and the capacity of constitutive cytokine release by the AML cells and showed no association with any of the well-established risk factors for chemoresistance after conventional intensive chemotherapy or AML-free survival. Thus, the susceptibility to the antiproliferative effects of metabolic inhibitors at least partly depend on other cellular/molecular mechanisms than the susceptibility to conventional chemotherapy; this is supported by our findings of enhanced antiproliferative effects of metabolic inhibitors when tested in combination with AraC. Synergistic effects of drugs that target glycolytic activity in combination with chemotherapeutics have been considered as a potential therapeutic strategy in AML [51], and our data supports the further exploration of this treatment strategy.

The two patient subsets identified in our hierarchical cluster analysis of antiproliferative effects after treatment with metabolic inhibitors (Figure 3B) did not differ with regard to established biological and clinical characteristics associated with survival after intensive chemotherapy. We therefore tried to identify other AML cell characteristics associated with the antiproliferative effects of metabolic inhibitors. First, our proteomic comparisons suggest that the two patient subsets differ in metabolic regulation, and also in transcriptional regulation and possibly intracellular trafficking. We would emphasize that these proteomic results should be interpreted with great care because few patients were available for these studies. Second, our comparison of global gene expression profiles showed that the two patient subsets could be identified based on mRNA profiling, and these observations support the proteomic data and suggest that susceptibility to metabolic inhibitors is associated with differences in metabolic and transcriptional regulation.

The metabolic inhibitors decreased primary AML cell viability and had proapoptotic effects, and we could identify a subset of patients with increased susceptibility to the inhibitors (Figure 5B, upper 24 patients). This antiproliferative effect is probably not due to the effect on the

constitutive release of growth factors by the AML cells, because the effects of the inhibitors on the constitutive release were divergent and relatively small compared with the persisting wide variation between individual patients.

MSCs have a cytokine-mediated supporting effect on AML cell growth and survival during *in vitro* culture [23], and MSCs also localize to the bone marrow in stem cell niches [52]. 2DG had a very strong antiproliferative effect for a majority of AML patients, and its antiproliferative effect could be maintained even in the presence of the AML-supporting MSCs. In addition, 2DG also inhibited proliferation of cocultured normal MSCs, which was most likely mediated by a direct effect on the MSCs and not an indirect effect mediated by altered cytokine release by AML cells as 2DG had relatively weak effects on the constitutive cytokine release and the wide variation between patients persisted in the presence of 2DG (Figure 7). The metabolic inhibitors also had an antiproliferative effect on UCB cells. However, only one donor of MSCs and four UCB donors were tested, so the effects of metabolic inhibitors on normal cells should be investigated further, possibly also using CD34+ stem cells. Still, this shows that the antiproliferative effects of metabolic inhibitors are not specific to AML cells. AraC is one of the most effective drugs used in the treatment of AML, but has serious side effects. As with AraC, the maximal tolerated dose of a drug is not necessarily the most effective therapeutic dose. Furthermore, it may be more effective to combine chemotherapeutics with other drug candidates such as metabolic inhibitors, to combat AML.

5. Conclusions

Our present studies show that *in vitro* metabolic targeting has anti-leukemic effects for many patients; however, patients are heterogeneous with regard to susceptibility to such targeting. Metabolic targeting, in particular targeting of the glycolytic pathway and OXPHOS should be further investigated as a treatment strategy for AML. A future focus should include further characterization of patient heterogeneity and finding optimal concentrations and combinations of metabolic inhibitors, and/or metabolic inhibitors combined with chemotherapeutics. In our current study we did not characterize effect of metabolic drugs on LSCs, something that should be considered for future studies, to determine whether this therapeutic strategy can be effective in defined AML patient subsets.

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SUPPLEMENTARY INFORMATION

TARGETING CELLULAR METABOLISM IN ACUTE MYELOID LEUKEMIA AND THE ROLE OF PATIENT HETEROGENEITY

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Table S1. Associations between the antiproliferative effects of metabolic inhibitors and clinical/biological characteristics of the AML patients. The comparisons (secondary vs. *de novo*, FAB; M0-M2 vs. M4/M5, cytogenetics; favorable, intermediate and adverse, CD34; positive vs. negative, *FLT3*; mutation *(ITD)* vs. wt, *NPM1* mutation *(INS)* vs. wt, and survivors vs. nonsurvivors) are based on the percent inhibition of the proliferative responses for each mediator and patient (i.e. 69 patients showing detectable proliferation in control cultures; 54 patients for ST1326). Differences were regarded as statistically significant when $p < 0.05$; shown in bold. The Mann-Whitney *U-test* and the Kruskal-Wallis test were used for the statistical analyses, and the *p*-values are not corrected for the number of comparisons.

Metabolic inhibitors and concentrations	Secondary versus *de novo*	FAB	Cytogenetics	CD34+	*FLT3 ITD*	*NPM1 INS*	Survivors versus nonsurvivors
Metformin 2.5 mM	0.549	0.071	0.190	0.114	0.903	0.385	0.736
Metformin 1.25 mM	0.303	0.205	0.303	0.073	0.942	0.198	0.911
2DG 0.6 mM	0.242	0.065	0.074	0.207	0.261	0.472	0.881
2DG 0.3 mM	0.578	0.508	**0.032**	0.438	0.680	0.403	0.600
6AN 100 µM	0.075	0.910	0.098	0.605	0.512	0854	0.295
6AN 50 µM	**0.010**	0.422	**0.045**	0.879	0.104	0.815	0.575
BPTES 20 µM	0.956	0.367	0.889	0.117	0.633	0.462	0.501
BPTES 10 µM	0.989	0.662	0.971	0.124	0.599	0.341	0.575
ST1326 50 µM	0.790	0.590	0.899	0.431	0.015	0.103	0.910
Lonidamine 300 µM	0.606	0.833	0.389	0.148	0.512	0.155	1
AZD3965 200 nM	0.824	0.135	0.421	0.162	0.544	0.483	0.765

Table S2. A comparison of the two patient subsets identified in the unsupervised hierarchical cluster analysis (Figure 3B) based on the antiproliferative effects of five metabolic inhibitors. The percent inhibition by each metabolic drug was estimated for each of the 69 patients showing detectable proliferation in the [^{3}H]-thymidine incorporation assay (i.e. 7 days of suspension cultures). Hierarchical clustering was done based on the percent inhibition by metabolic drugs, and we then identified two clusters (Figure 3B), where the upper cluster included 29 patients that showed a strong antiproliferative effect towards drugs. We compared the effect of metabolic inhibitors for these two clusters (Figure 2). The table shows (from left to right); the inhibitor tested, the median relative proliferation (proliferation in drug-treated cultures relative to drug-free controls) and the *p-value* (Mann-Whitney *U-test*). Differences were regarded as statistically significant when $p \leq 0.05$ (no corrections were made for the number of comparisons, significant *p-values* marked in bold).

Inhibitor	Cluster (i.e. anti-proliferative effect)	Number of patients in each group	Median proliferation, drug-treated cultures relative to the corresponding drug-free controls	*p*-value
Metformin	Weak effect	40	63.96	
	Strong effect	29	48.57	**0.024**
2DG	Weak effect	40	61.64	
	Strong effect	29	12.25	**0.000**
6AN	Weak effect	40	78.89	
	Strong effect	29	26.47	**0.000**
BPTES	Weak effect	40	70.90	
	Strong effect	29	47.18	**0.003**
ST1326	Weak effect	31	52.35	
	Strong effect	22	27.71	0.097

Table S3. Proteomic comparison of primary AML cells derived from patients where AML cell proliferation is either strongly inhibited by metabolic inhibitors (6 patients) or AML cells show no or minimal antiproliferative effects by metabolic inhibitors (8 patients) (Figure 3B). The analysis identified 31 differentially expressed proteins, and the table shows a functional classification of the individual proteins (for a detailed description see Table S5). Some of the proteins are included in more than one functional class.

Functional classification	INCREASED in subset susceptible to metabolic inhibitors (18 proteins)	DECREASED in subset susceptible to metabolic inhibitors (13 proteins)
Metabolism (n=15)		
Protein/proteinases/molecular degradation/amino acid	PRTN3, CFD, THEM6, HM13,	UBXN7, QRICH1, DDAH2, ISOC1
Lipid	SGPL1, AGPS	
Energy	FDXR	DDAH2, ISOC1, ACO1
Detoxification	SQRDL	
Mitochondria (n=4)	SQRDL, FDXR	DDAH2, ACO1
Transcription, RNA (n=9)	RNASE3, RNASE 2, SRPK1, ASCC3	RFX1;RFX2, QRICH1, SMNDC1, GRWD1, ACO1
DNA damage (n=2)	ASCC3	GRWD1
Cell cycle regulation, cytoskeleton (n=3)		NAE1, MAPRE2, MPP1
Intracellular signaling (n=8)	BST1, IL1RAP	PIP4K2A, MPP1
G protein coupled receptor	SGPL1, CD97	RAPIGAP2
Kinase/phosphatase	SRPK1	
Intracellular transport, Golgi, endoplasmatic reticulum, microtubule (n=3)	TMEM214, HM13,	MAPRE2
Cell membrane, adhesion, migration (n=6)	BST1, HLA-B, CD97, IL1RAP, CPNE2,	MPP1
Apoptosis (n=1)	TMEM214	
Cancer association (n=7)	BST1, CD97	RFX1, MAPRE2, GRWD1, MPP1, ISOC1

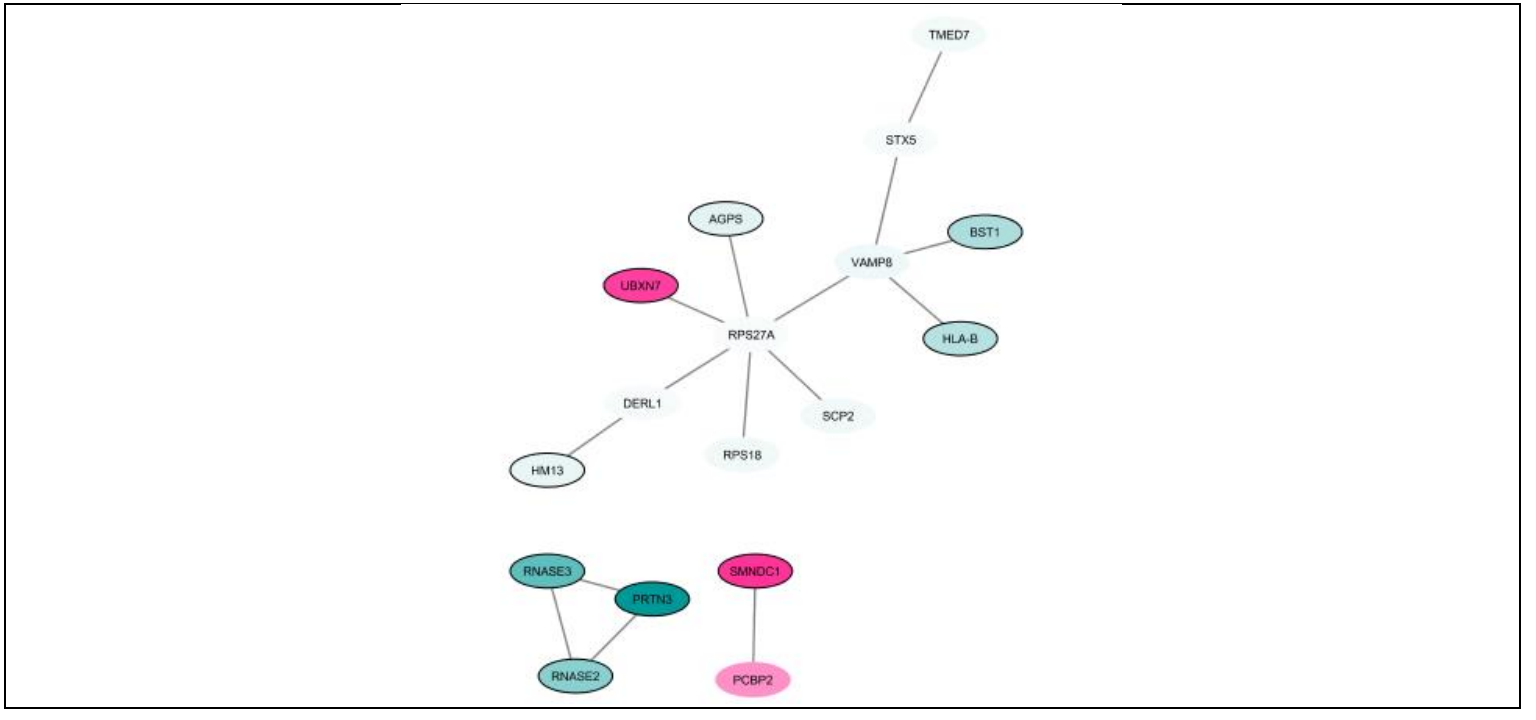
Table S4. Description of the 31 differentially expressed proteins found after performing a proteomic comparison between a subset of patient samples showing a strong antiproliferative effect of metabolic inhibitors (n=6) versus patient samples that show no or low effect of metabolic inhibitors (n=8) (Figure 3B). Table is based on information from https://www.ncbi.nlm.nih.gov/gene/.

Gene names	Protein name	Key words
INCREASED (18 proteins)		
PRTN3	*Myeloblastin/proteinase 3.* Restricted expression to bone marrow. This is an AML-associated antigen.	Proteinase
RNASE3	*Eosinophil cationic protein/ ribonuclease A family member 3.* The protein belongs to the pancreatic ribonuclease family, a subset of the ribonuclease A superfamily. Its expression is regulated by GATA transcription factors. Restricted expression to bone marrow.	RNAse
CFD	*Complement factor D.* This gene encodes a member of the S1, or chymotrypsin, family of serine peptidases. This protease catalyzes the cleavage of factor B, the rate-limiting step of the alternative pathway of complement activation. This protein also functions as an adipokine, a cell signaling protein secreted by adipocytes, which regulates insulin secretion.	Serine proteinase Insulin secretion
RNASE2	*Non-secretory ribonuclease/ ribonuclease A family member 2.* The protein is a non-secretory ribonuclease that belongs to the pancreatic ribonuclease family, a subset of the ribonuclease A superfamily. Restricted expression to bone marrow.	RNAse
BST1	*ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2/ bone marrow stromal cell antigen 1.* This is a glycosylphosphatidylinositol-anchored molecule with high expression in normal bone marrow. CD157/Bst1 is a dual-function receptor and β-NAD^+-metabolizing ectoenzyme of the ADP-ribosyl cyclase family. CD157 interacts with extracellular matrix components and regulates leukocyte diapedesis via integrin-mediated signaling. CD157 also regulates cell migration and is a marker of adverse prognosis in certain cancers.	Metabolism Cell adhesion Signaling Cancer
HLA-B	*HLA class I histocompatibility antigen, B alpha chain.* Belongs to the HLA class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). Class I molecules play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. They are expressed in nearly all cells.	Cell surface HLA
SGPL1	*Sphingosine-1-phosphate lyase 1.* Low, but detectable expression in normal bone marrow. *SGPL1* is the intracellular enzyme responsible for the irreversible final breakdown of the lipid molecule S1P, which is cleaved to ethanolamine phosphate and *trans*-2-hexadecenal. Through G-protein-coupled receptor activation, it has been proven that S1P has important regulatory functions in normal physiology. The molecule is localized in the endoplasmatic reticulum.	Lipid metabolism G-protein coupled receptor
SQRDL	*Sulfide Quinone oxidoreductase, mitochondrial.* The protein may function in mitochondria to catalyze the conversion of sulfide to per-sulfides, thereby decreasing toxic concentrations of sulfide.	Mitochondria Sulfide detoxification
CD97	*CD97 antigen/adhesion G protein-coupled receptor E5.* This protein is a member of the EGF-TM7 subfamily of adhesion G protein-coupled receptors, which mediate cell-cell interactions. These proteins are cleaved by self-catalytic proteolysis into a large extracellular subunit and seven-span transmembrane subunit, which associate at the cell surface as a receptor complex. The encoded protein may play a role in cell adhesion as well as leukocyte recruitment, activation and migration, and contains multiple extracellular EGF-like repeats which mediate binding to chondroitin sulfate and the cell surface complement regulatory protein CD55. Expression of this gene may play a role in the progression of several types of cancer.	Cell surface GPCR Cell adhesion Cell migration Carcinogenesis

IL1RAP	*Interleukin-1 receptor accessory protein.* This protein is a component of the interleukin 1 receptor complex. Alternative splicing of this gene results in membrane-bound and soluble isoforms differing in their C-terminus. The ratio of soluble to membrane-bound forms increases during acute-phase induction or stress.	Signaling
FDXR	*NADPH adrenodoxin oxidoreductase, mitochondrial/ferredoxin reductase.* This mitochondrial flavoprotein initiates electron transport for cytochromes P450 receiving electrons from NADPH.	Mitochondria Metabolism
AGPS	*Alkyldihydroxyacetonephosphate synthase, peroxisomal.* This protein is a member of the FAD-binding oxidoreductase/transferase type 4 family and catalyzes the second step of ether lipid biosynthesis in which acyl-dihydroxyacetonephosphate (DHAP) is converted to alkyl-DHAP by the addition of a long chain alcohol and the removal of a long-chain acid anion. The protein is localized to the inner aspect of the peroxisomal membrane and requires FAD as a cofactor.	Metabolism Lipid
TMEM214	*Transmembrane protein 214.* Endoplasmic reticulum (ER) stress caused by excessive aggregation of misfolded proteins induces apoptosis. TMEM214 is suggested to be a critical mediator of ER stress-induced apoptosis. Overexpression of TMEM214 induced apoptosis, whereas knockdown of TMEM214 inhibited ER stress-induced apoptosis. TMEM214 can localize on the outer membrane of the ER where it is constitutively associated with procaspase 4, which is also critical for ER stress-induced apoptosis.	Apoptosis Endoplasmatic reticulum
SRPK1	*SRSF protein kinase 1.* This serine/arginine protein kinase specific for the SR (serine/arginine-rich domain) family of splicing factors. The protein localizes to the nucleus and the cytoplasm. It is thought to play a role in regulation of both constitutive and alternative splicing by regulating intracellular localization of splicing factors.	RNA splicing Kinase
THEM6	*Protein THEM6/thioesterase superfamily member 6.* THEM6 is a protein-coding gene.	Esterase
CPNE2	*Copine-2.* Calcium-dependent membrane-binding proteins may regulate molecular events at the interface of the cell membrane and cytoplasm. This gene encodes a calcium-dependent protein containing two N-terminal type II C2 domains and an integrin A domain-like sequence in the C-terminus.	Cell membrane Calcium
HM13	*Minor histocompatibility antigen H13.* The protein localizes to the endoplasmic reticulum, catalyzes intramembrane proteolysis. Its activity is required to generate signal sequence-derived HLA-E epitopes that are recognized by the immune system.	Endoplasmatic reticulum Proteolysis
ASCC3	*Activating signal cointegrator 1 complex subunit 3.* This protein belongs to a family of helicases that are involved in the ATP-dependent unwinding of nucleic acid duplexes. The encoded protein is the largest subunit of the activating signal cointegrator 1 complex that is involved in DNA repair and resistance to alkylation damage.	Helicase
DECREASED (13 proteins)		
PIP4K2A	*Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha.* Phosphatidylinositol-5,4-bisphosphate, the precursor to second messengers of the phosphoinositide signal transduction pathways. The protein encoded by this gene is one of a family of enzymes capable of catalyzing the phosphorylation of phosphatidylinositol-5-phosphate This gene is a member of the phosphatidylinositol-5-phosphate 4-kinase family.	Phosphoinositide Signaling
RFX1;RFX2	*MHC class II regulatory factor RFX1; DNA-binding protein RFX2.* This gene encodes a member of the regulatory factor X (RFX) family of transcription factors. RFX1 contains an N-terminal activation domain and a C-terminal repression domain, and may activate or repress target gene expression depending on cellular context. This transcription factor has been shown to regulate a wide variety of genes involved in cancer. RFX2 is a transcriptional activator that can bind DNA as a monomer or as a heterodimer with other RFX family members.	Transcription factor

NAE1	*NEDD8-activating enzyme E1 regulatory subunit.* The protein binds to the beta-amyloid precursor protein, a cell surface protein with signal-transducing properties. It can form a heterodimer with UBE1C and bind and activate NEDD8, an ubiquitin-like protein. This protein is required for cell cycle progression through the S/M checkpoint.	Cell cycle S/M checkpoint
MAPRE2	*Microtubule-associated protein RP/EB family member 2.* The protein shares significant homology to the adenomatous polyposis coli (APC) protein-binding EB1 gene family. It is a microtubule-associated protein that is necessary for spindle symmetry during mitosis. It is thought to play a role in control of proliferation.	Microtubule Mitosis
QRICH1	*Glutamine-rich protein 1.* The molecule has a caspase activating domain, it is predicted to localize to the nucleus and may be involved in transcriptional regulation.	Protease Transcription
UBXN7	*UBX domain-containing protein 7.* UBXN7 over-expression converts the scaffold protein CUL2 to its neddylated form and causes the accumulation of non-ubiquitylated HIF1α.	Transcription
SMNDC1	*Survival of motor neuron-related-splicing factor 30.* This is a nuclear protein that has been identified as a constituent of the spliceosome complex.	RNA Splicing
GRWD1	*Glutamate-rich WD repeat-containing protein 1.* This protein may play a critical role in ribosome biogenesis and may also have a role in histone methylation through interactions with CUL4-DDB1 ubiquitin E3 ligase. GRWD1 negatively regulates p53 via the RPL11-MDM2 pathway and promotes tumorigenesis.	Ribosome Histone Transcription p53
RAP1GAP2	*Rap1 GTPase-activating protein 2.* This gene encodes a GTPase-activating protein that activates the small guanine-nucleotide-binding protein Rap1. The protein interacts with synaptotagmin-like protein 1 and Rab27.	GTP-ase
MPP1	*55 kDa erythrocyte membrane protein/ membrane palmitoylated protein 1.* This gene encodes the prototype of the membrane-associated guanylate kinase (MAGUK) family proteins. MAGUKs interact with the cytoskeleton and regulate cell proliferation, signaling pathways, and intercellular junctions. The encoded protein is an extensively palmitoylated membrane phosphoprotein containing a PDZ domain, a Src homology 3 (SH3) motif, and a guanylate kinase domain. This gene product interacts with various cytoskeletal proteins and cell junctional proteins. It may be important for tumor suppressor activity.	Guanylate kinase Cytoskeleton Cell junctions
DDAH2	*N(G),N(G)-dimethylarginine dimethylaminohydrolase 2.* This dimethylarginine dimethylaminohydrolase functions in nitric oxide generation by regulating the cellular concentrations of methylarginines, which in turn inhibit nitric oxide synthase activity. The protein may be localized to the mitochondria.	Mitochondria Nitric oxide
ISOC1	*Isochorismatase domain-containing protein 1.* This protein has putative isochorismatase activity and is a regulator of proliferation and survival in pancreatic cancer cells.	Hydralase Puryvate Carcinogenesis
ACO1	*Cytoplasmic aconitate hydratase.* The protein is a multi-functional, cytosolic protein that functions as an essential enzyme in the TCA cycle and interacts with mRNA to control the levels of iron inside cells. When cellular iron levels are high, this protein binds to a 4Fe-4S cluster and functions as an aconitase. Aconitases are iron-sulfur proteins that function to catalyze the conversion of citrate to isocitrate. When cellular iron levels are low, the protein binds to iron-responsive elements (IREs), which are stem-loop structures found in the 5' UTR of ferritin mRNA, and in the 3' UTR of transferrin receptor mRNA. When the protein binds to IRE, it results in repression of translation of ferritin mRNA, and inhibition of degradation of the otherwise rapidly degraded transferrin receptor mRNA. The protein is involved in the metabolic shift seen during development of acute lymphoblastic leukemia.	Mitochondria Metabolism Transcription Leukemia

Table S5. Functional network analysis based on a proteomic comparison of a subset of patients showing a strong antiproliferative effect towards metabolic inhibitors versus patients that show no or low antiproliferative effect towards metabolic inhibitors *in vitro* (Figure 3B). Based on 49 proteins with significant difference, 17 proteins interacted with other proteins, forming protein networks; and a description of the network-associated proteins are shown below. A black circle around the node indicates a significant fold change, while a statistically significant difference is indicated as * in the table. Description of proteins is based on information from https://www.ncbi.nlm.nih.gov/gene/.



Gene names	Protein names	Key words
INCREASED (14 proteins)		
*PRTN3**	*Myeloblastin/proteinase 3.* Restricted expression to bone marrow. This is an AML-associated antigen.	Proteinase
*RNASE3**	*Eosinophil cationic protein/ ribonuclease A family member 3.* The protein belongs to the pancreatic ribonuclease family, a subset of the ribonuclease A superfamily. Its expression is regulated by GATA transcription factors. Restricted expression to bone marrow.	RNAse
*RNASE2**	*Non-secretory ribonuclease/ ribonuclease A family member 2.* The protein is a non-secretory ribonuclease that belongs to the pancreatic ribonuclease family, a subset of the ribonuclease A superfamily. Restricted expression to bone marrow.	RNAse
*BST1**	*ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2/ bone marrow stromal cell antigen 1.* This is a glycosylphosphatidylinositol-anchored molecule with high expression in normal bone marrow. CD157/Bst1 is a dual-function receptor and β-NAD^+-metabolizing ectoenzyme of the ADP-ribosyl cyclase family. CD157 interacts with extracellular matrix components and regulates leukocyte diapedesis via integrin-mediated signaling. CD157 also regulates cell migration and is a marker of adverse prognosis in certain cancers.	Metabolism Cell adhesion Signaling Cancer
*HLA-B**	*HLA class I histocompatibility antigen, B alpha chain.*	Cell surface HLA
*AGPS**	*Alkyldihydroxyacetonephosphate synthase, peroxisomal.* This protein is a member of the FAD-binding oxidoreductase/transferase type 4 family and catalyzes the second step of ether lipid biosynthesis in which acyl-dihydroxyacetonephosphate (DHAP) is converted to alkyl-DHAP by the addition of a long chain alcohol and the removal of a long-chain acid anion. The protein is localized to the inner aspect of the peroxisomal membrane and requires FAD as a cofactor.	Metabolism Lipid
*HM13**	*Minor histocompatibility antigen H13.* The protein localizes to the endoplasmic reticulum, catalyzes intramembrane proteolysis. Its activity is required to generate signal sequence-derived HLA-E epitopes that are recognized by the immune system. The encoded protein is an integral membrane protein with sequence motifs characteristic of the presenilin-type aspartic proteases.	Endoplasmatic reticulum Proteolysis
TMED7	*Transmembrane p24 trafficking protein 7.* This protein inhibits MyD88-independent toll-like receptor 4 signaling. TMED7 overexpression inhibits the ability of TRAM, an adaptor utilized by toll-like receptor (TLR) 4 to activate the interferon regulatory factor 3-signalling pathway, whereas TMED7 knockdown enhances production of the CCL5 chemokine following TLR4 ligation. Upon lipopolysaccharide stimulation, TMED7 co-localizes with TRAM and TLR4 in late endosomes where it encounters the negative regulator of TRAM, TAG. TMED7 is essential for TAG-mediated disruption of the TRAM/TRIF complex and the degradation of TLR4.	TLR4 CCL5 Myd88
STX5	*Syntaxin 5.* This gene encodes a member of the syntaxin or t-SNARE (target-SNAP receptor) family. These proteins are found on cell membranes and serve as the targets for v-SNAREs (vesicle-SNAP receptors), permitting specific synaptic vesicle docking and fusion. The encoded protein regulates endoplasmic reticulum to Golgi transport and plays a critical role in autophagy.	Endoplasmatic reticulum Golgi Autophagy
VAMP8	*Vesicle associated membrane protein 8.* This gene encodes an integral membrane protein that belongs to the synaptobrevin/vesicle-associated membrane protein subfamily of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). The encoded protein is involved in the fusion of synaptic vesicles with the presynaptic membrane.	Endoplasmic reticulum Golgi Autophagy
RPS27A	*Ribosomal protein S27a.* Ubiquitin, a highly conserved protein that has a major role in targeting cellular proteins for degradation by the 26S proteosome, is synthesized as a precursor protein consisting of either polyubiquitin chains or a single ubiquitin fused to an	Ribosome Proteasome

	unrelated protein. This gene encodes a fusion protein consisting of ubiquitin at the N-terminus and ribosomal protein S27a at the C-terminus. Ribosomal protein S27a is a component of the 40S subunit of the ribosome and belongs to the S27AE family of ribosomal proteins. It contains C4-type zinc finger domains and is located in the cytoplasm.	Ubiquitin
SCP 2	SCP2 protein is thought to be an intracellular non-specific lipid transfer protein. Alternative splicing of this gene produces multiple transcript variants, some encoding different isoforms.	Lipid metabolism
RPS18	*Ribosomal protein S18.* Ribosomes, the organelles that catalyze protein synthesis, consist of a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. This gene encodes a ribosomal protein that is a component of the 40S subunit. The protein belongs to the S13P family of ribosomal proteins. It is located in the cytoplasm. As is typical for genes encoding ribosomal proteins, there are multiple processed pseudogenes of this gene dispersed through the genome.	Ribosome Proteasome Ubiquitin
DRL1	*DRL1 protein. T*he DRL1 gene may encode a homolog of the Elongator-associated protein KTI12 of yeast, and is possibly a chromatin-associated protein.	Chromatin
DECREASED (3 proteins)		
*SMNDC1**	*Survival of motor neuron-related-splicing factor 30.* This is a nuclear protein that has been identified as a constituent of the spliceosome complex.	RNA Splicing
*UBXN7**	*UBX domain-containing protein 7.* Its over-expression converts the scaffold protein CUL2 to its neddylated form and causes the accumulation of non-ubiquitylated HIF1α.	Transcription
PCBP2	*Poly(rC) binding protein 2*. The protein appears to be multifunctional. Along with PCBP-1 and hnRNPK, it is one of the major cellular poly(rC)-binding proteins. It contains three K-homologous (KH) domains which may be involved in RNA binding. Together with PCBP-1, this protein also functions as a translational coactivator It has also been implicated in translational control of the 15-lipoxygenase mRNA.	RNA binding Translation Metabolism

Table S6. Identification of two patient subsets based on AML cell viability after treatment with metabolic inhibitors; a comparison of the two subsets identified in the unsupervised hierarchical cluster analysis (Figure 5B). The cell viability percentage was estimated for each of the 72 patients after treatment with each metabolic drug (48-hour treatment). Hierarchical clustering was done based on the percent reduction of viability, and then two clusters were identified; with the lower cluster of 48 patients showing more viable cells (a weaker proapoptotic effect) after drug treatment while the upper cluster including 24 patients had a stronger reduction of viability after drug treatment (strong apoptotic effect). We compared the median viability found after drug treatment for these two patient clusters separately (Figure 5B). The table shows (from left to right) the inhibitor tested, the median relative viability (viability in drug-treated cultures relative to the drug-free controls) and the *p-value* (Mann-Whitney *U-test*). Differences were regarded as statistically significant when $p \leq 0.05$ (no correction for the number of comparisons, significant *p-values* marked in bold).

Median viability (%), for drug-treated cultures relative to corresponding drug-free controls			
Inhibitor	**Upper cluster Strong proapoptotic effect (24 patients)**	**Lower cluster Weak proapoptotic effect (48 patients)**	***p*-value**
Metformin	95.85	92.55	0.076
2DG	90.28	57.86	**0.000**
6AN	93.88	85.13	**0.013**
BPTES	96.52	94.55	0.202
ST1326	94.50	91.92	0.761
Lonidamine	90.55	89.28	0.515
AZD3965	99.57	99.68	0.662

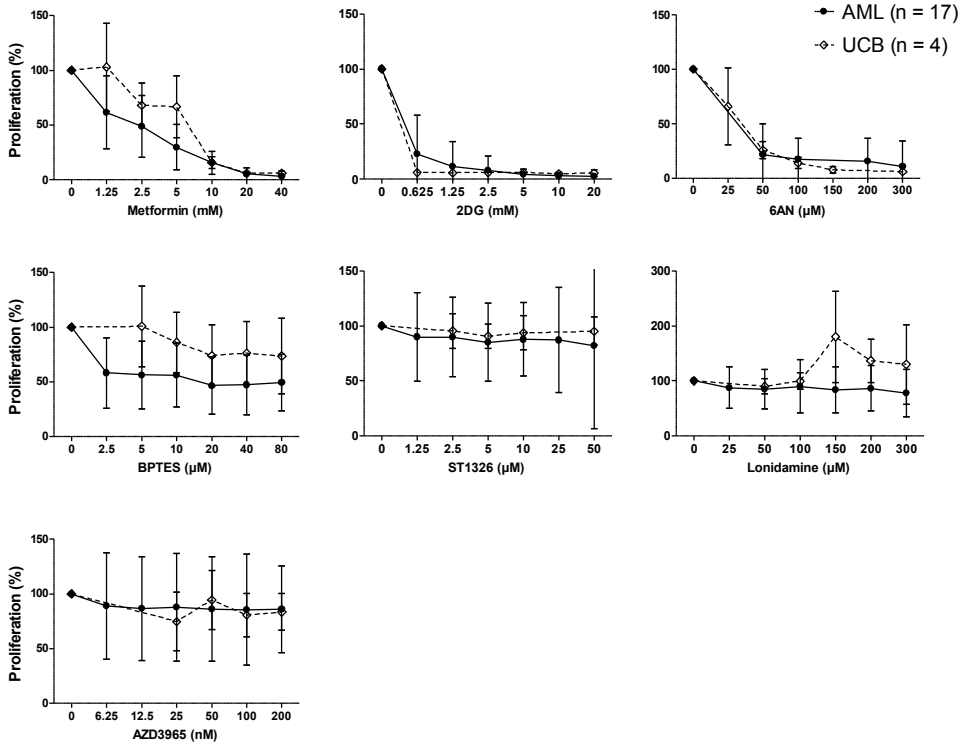


Figure S1. Initial screening of seven metabolic inhibitors; effects on proliferation of primary human AML cells and umbilical cord blood (UCB) mononuclear cells. The [^{3}H]-thymidine incorporation assay was used to assess the effect of metabolic inhibitors on AML cell proliferation after 7 days of treatment. Primary AML cells derived from 17 patients (solid line, filled circles) and umbilical cord blood (UCB) cells derived from four donors (stippled line, open squares) were tested. The drug concentrations are shown on the x-axis, while the y-axis shows percent proliferation compared to drug free controls (set as 100%). The drug-response of AML patient samples and UCB samples are presented as the mean proliferation ± standard deviation.

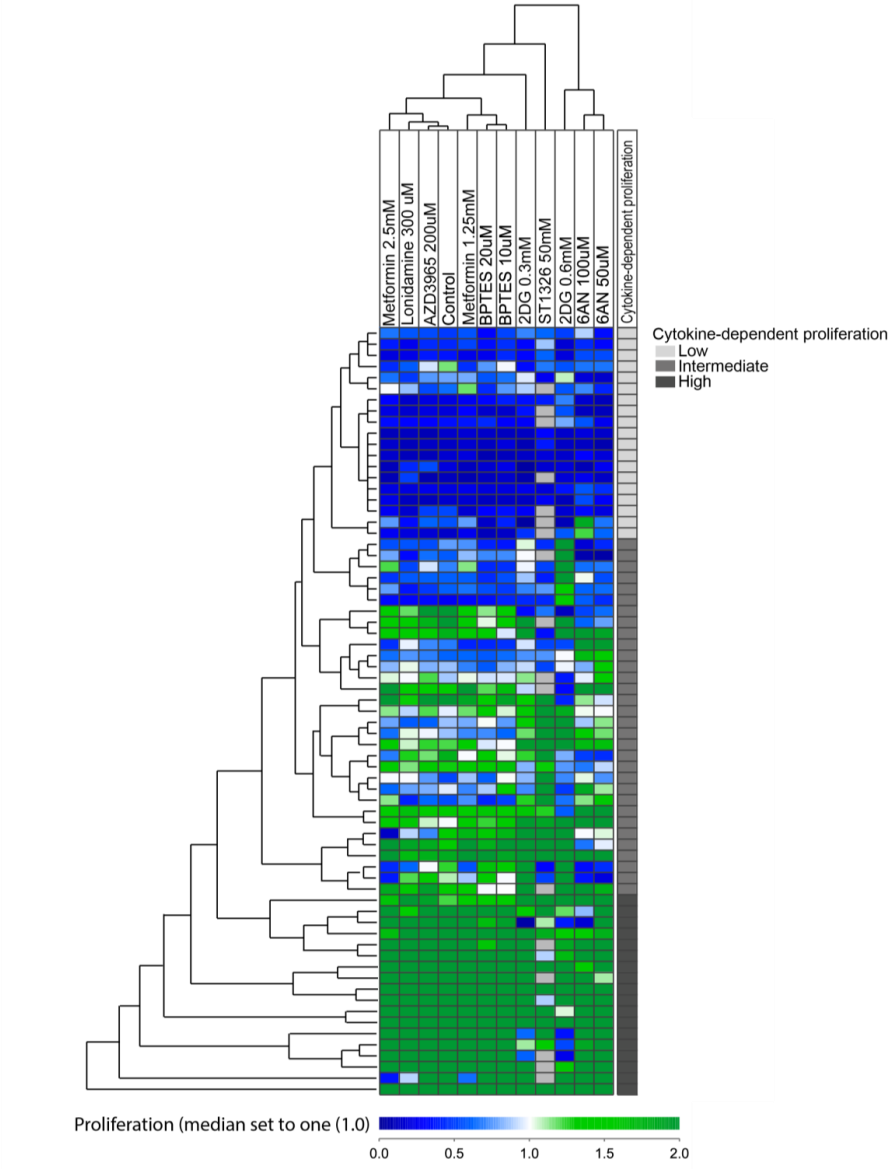


Figure S2. Hierarchical cluster analysis based on AML cell proliferation in cultures with and without treatment of metabolic inhibitors. Proliferation was tested using the [^{3}H]-thymidine incorporation assay, and the unsupervised hierarchical cluster analysis was based on effects of all metabolic inhibitors and drug concentrations. The proliferative responses for each inhibitor were normalized to the corresponding median before analysis. The metabolic inhibitors and the concentrations tested are shown at the top of the figure. We identified three main patient subsets: an upper subset generally showing low proliferation in all cultures compared to other patients (light grey), a lower subset showing generally high cytokine-dependent proliferation (dark grey color), and a large intermediate group with divergent effects (middle grey).

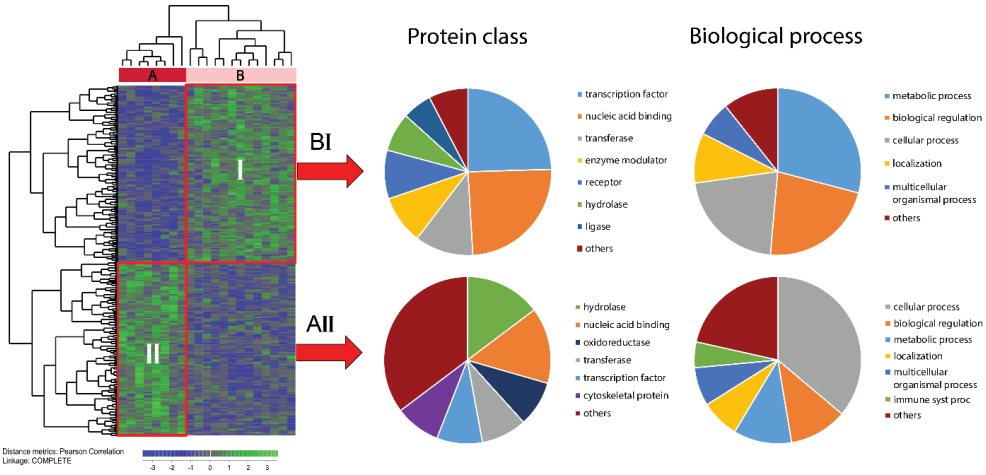


Figure S3. A comparison of global gene expression profiles for AML samples showing a strong antiproliferative effect towards metabolic inhibitors (based upon Figure 3B, upper cluster) versus no or minimal antiproliferative effect (based upon Figure 3B, lower cluster). We compared the global gene expression profiles that were available for 21 unselected AML patients; eight of these patients had strong antiproliferative effects by metabolic inhibitors (based on cluster Figure 3B), while 13 patients had weaker antiproliferative or no effects (based on cluster Figure 3B). A feature subset selection (FSS) analysis based on R-score > ± 3.5 was performed and identified 265 differentially expressed (upregulated) genes. We further performed a hierarchical cluster analysis that separated both the genes (I and II) and the patients (A and B) into two separate subclusters, subcluster BI (weak antiproliferative effects after treatment with metabolic inhibitors) and subcluster AII (strong antiproliferative effects after treatment with metabolic inhbitors). Based on the identified upregulated genes, we performed gene ontology (GO) mapping based on the PANTHER database. By use of the categories Protein class and Biological process, we found several ontologies to be overrepresented in the two patient subclusters (shown in chart figures).

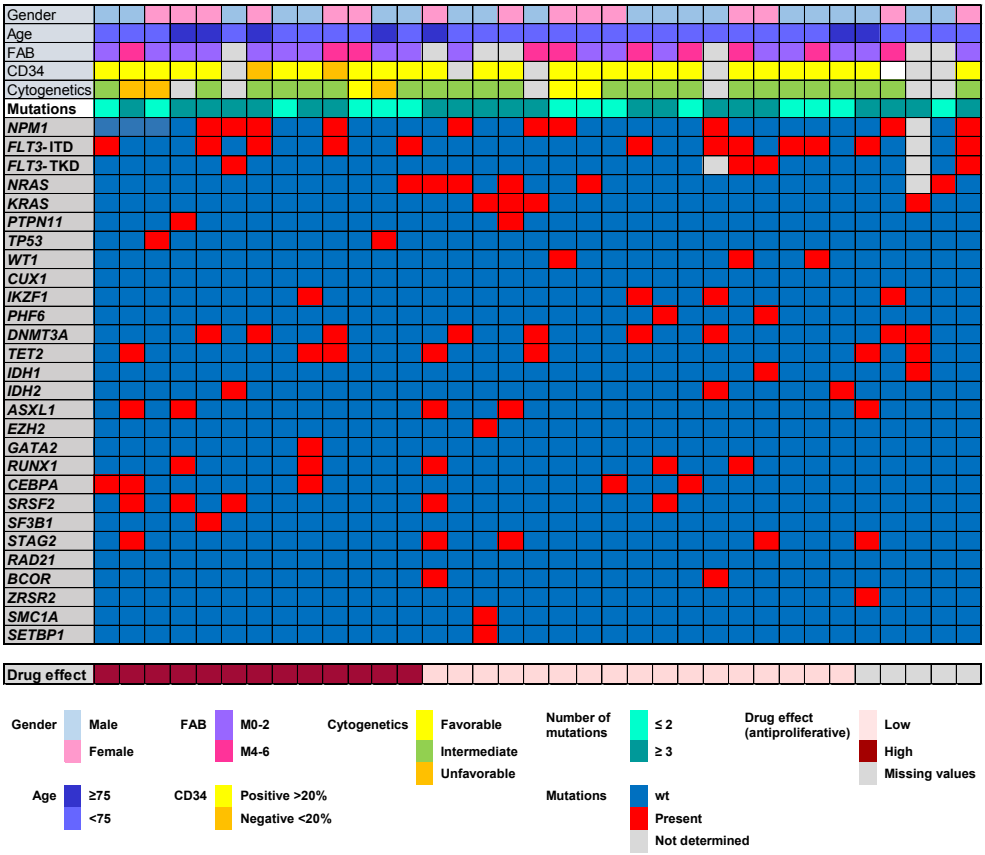


Figure S4. AML associated molecular genetics for 35 unselected patients, a summary of the overall results.

The mutations are listed to the left in the figure, each column represent one patient and the presence of mutations is shown in red. Molecular genetics were analyzed for 35 patients, and as shown in the bottom row (drug effect), these patients included 13 patients with a generally strong antiproliferative effect toward the metabolic inhibitors (based on Figure 3B, upper cluster of 29 patients), while 17 patients showed a weaker antiproliferative effect (see Figure 3B) and the five last patients did not have detectable cytokine-dependent proliferation (> 1000 cpm). Patient gender, age, FAB classification, CD34 expression and cytogenetics for each patient is shown at the top. A considerable heterogeneity can be seen between patients/patient groups with regard to the molecular genetics.

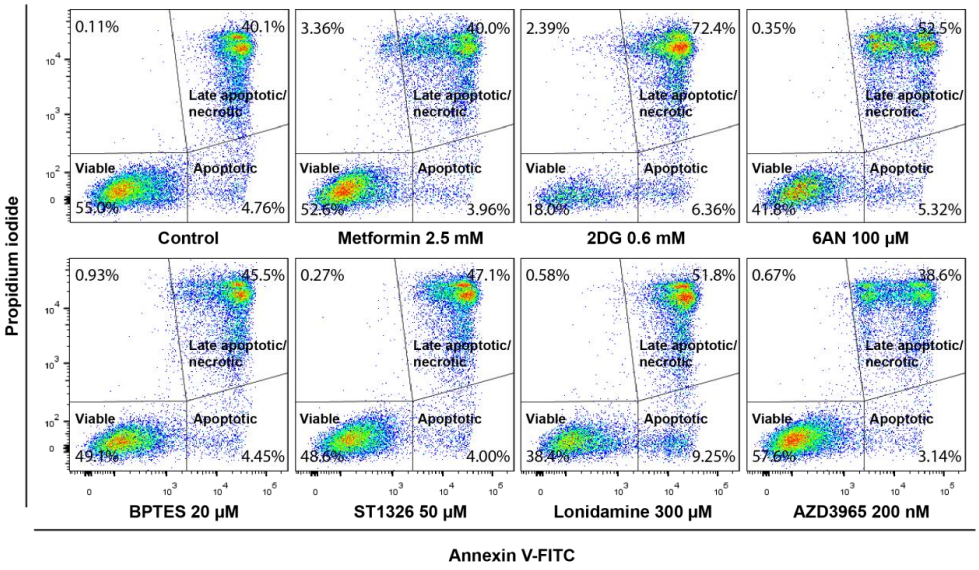


Figure S5. Flow cytometric analysis of AML cell viability; fraction of viable, apoptotic and late apoptotic/necrotic AML cells in control and metabolic inhibitor-treated cultures. These plots show the gating strategy used to define the percentage of viable, apoptotic and late apoptotic/necrotic cells, illustrated for one representative patient sample. The cells were incubated with medium alone (upper left panel, control), or treated with metabolic drugs for 48 hours. Cell viability, necrosis and apoptosis were measured by flow cytometry using the ApoptestTM–FITC kit and 10,000 events collected and analysed per sample.

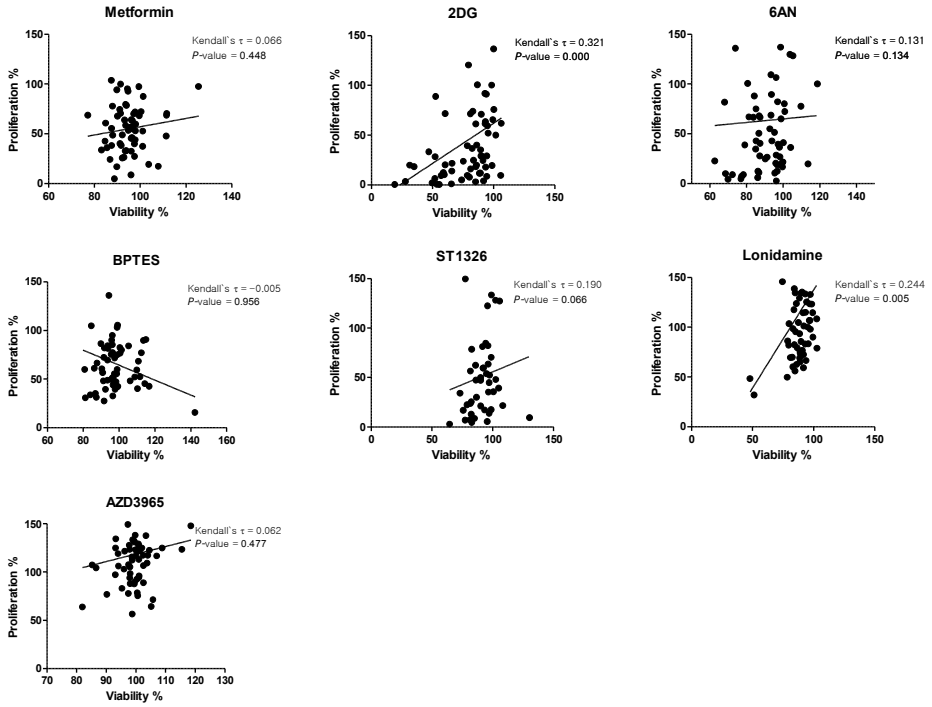


Figure S6. Antiproliferative and proapoptotic effects of metabolic inhibitors on primary human AML cells. We investigated whether the proapoptotic and antiproliferative effects of the various inhibitors showed significant correlations. The correlation plots show the percent alteration of cytokine-dependent proliferation (Y-axis) versus the percent alteration of AML cell viability (X-axis) for each drug treatment. Thus, both proliferation and viability are presented as percent (%) alteration after treatment with drugs compared with drug-free control cultures. Kendall`s τ test was used for correlation analyses. The strength of the associations is presented as Kendall`s τ (-1 = 100% negative association, 1 = 100 % positive correlation), and the significance of correlation is presented as *p*-values (significant *p*-value < 0.05). Only 2DG and lonidamine showed a significant, but weak, correlation between proliferation and viability (Kendall`s τ 0.321 and 0.244, and *p*-value 0.0004 and 0.005, respectively).

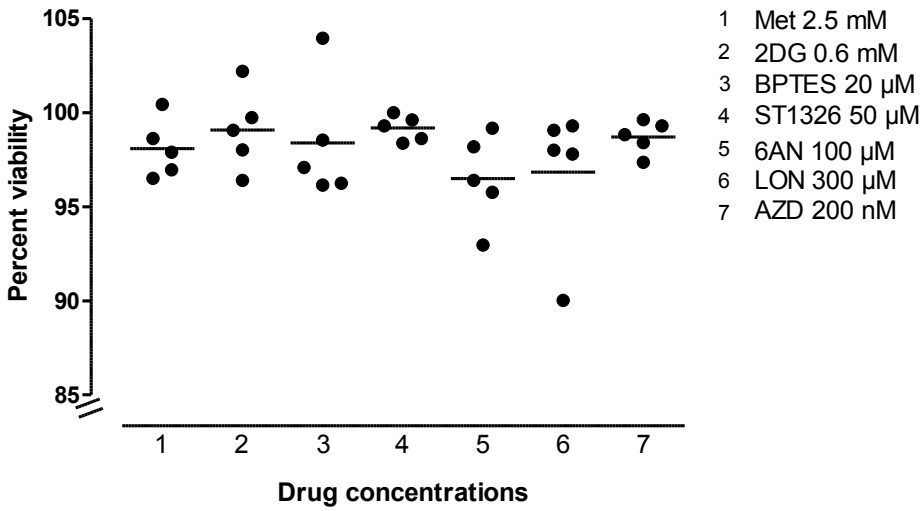


Figure S7. Viability of UCB cells after 48-hours treatment with metabolic inhibitors compared to untreated controls. UCB mononuclear cells were cultured for 48 hours in suspension cultures in StemSpan medium supplemented with cytokines with or without metabolic inhibitors. The fraction of viable cells was then determined using the flow cytometric AnnexinV/PI assay. The percentage of viable cells for each UCB donor sample (after treatment relative to untreated controls) is shown for each drug tested. 10,000 events of each sample were collected and analyzed. The solid line indicated mean values.

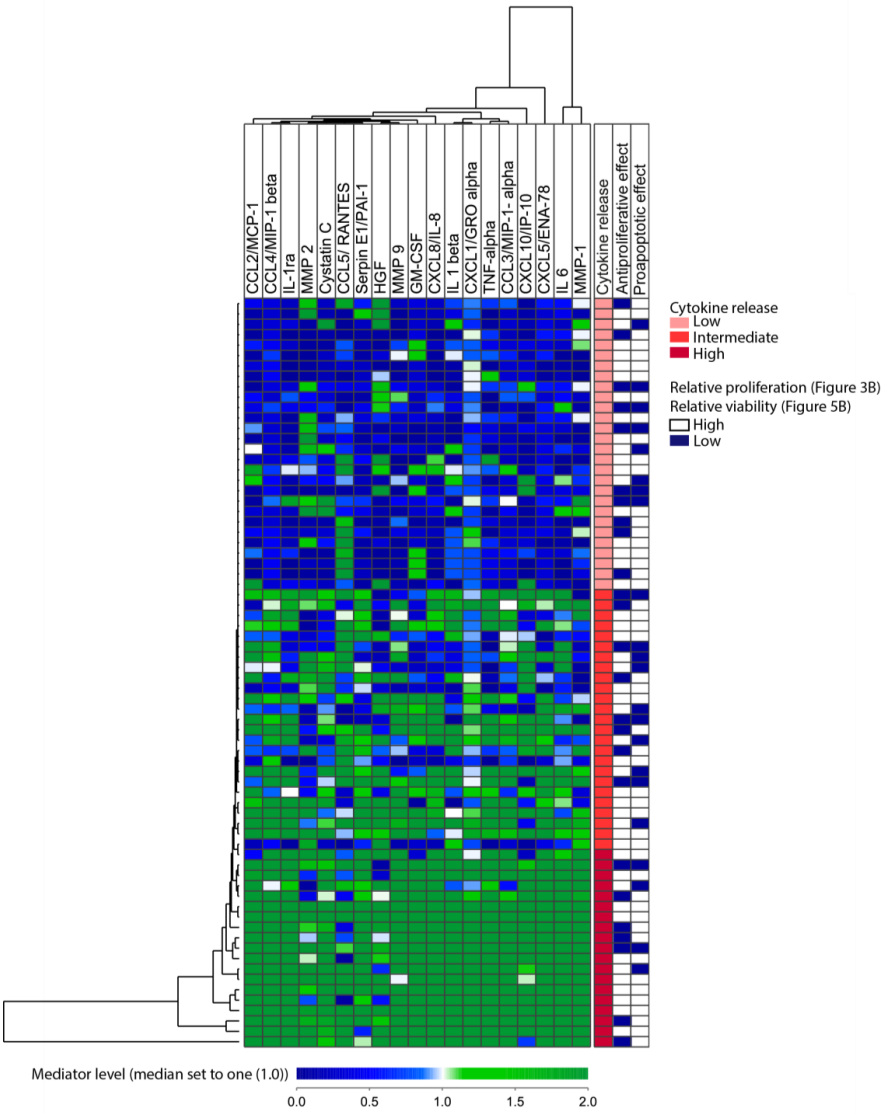


Figure S8. Unsupervised hierarchical cluster analysis of the constitutive release of soluble mediators by primary AML cells derived from 72 unselected patients. The constitutive mediator release during 48 hours of *in vitro* culture was determined for 19 soluble mediators. The concentrations were normalized to the corresponding median before analysis. Low release of mediators is shown in blue, while green indicates higher release. Three patient subsets could be identified based on this analysis, indicated by the red column to the right of the cluster: an upper subset showing generally low release of cytokines, a lower subset with a high release of cytokines and a relatively large group of patients showing intermediate/divergent release. Antiproliferative and proapoptotic effects after treatment with metabolic inhibitors showed no association with the capacity of constitutive mediator release (see right part of the figure; based on cluster analyses presented in Figure 3B and 5B).

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II

EFFECTS OF THE AUTOPHAGY-INHIBITING AGENT CHLOROQUINE IN ACUTE MYELOID LEUKEMIA

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Abstract

Autophagy is a highly conserved cellular degradation process that prevents cell damage and promotes cell survival, and autophagy inhibition is considered as a therapeutic strategy in cancer. Chloroquine (CQ) is a well-known antimalarial agent that inhibits late-stage autophagy. We evaluated the effects of CQ on cell viability and proliferation of AML cells derived from 81 AML patients. Our results show that CQ decreased AML cell viability and proliferation for the majority of patients. Furthermore, a subgroup of AML patients showed a greater susceptibility to CQ, and using hierarchical cluster analysis, we identified 99 genes upregulated in this patient subgroup, including several genes related to leukemogenesis. Combination of CQ with low dose AraC had an additive inhibitory effect on AML cell proliferation. Furthermore, a small subset of patients had little or no effect of AraC, but still showed strong antiproliferative effect of CQ. Using two leukemia cell lines, HL60 and MOLM-13, we further investigated the expression of reactive oxygen species (ROS), autophagic flux, apoptosis and cell cycle distribution after treatment with CQ, also in combination with the metabolic drugs metformin and 2DG. We observed a decrease in cell viability and a slight proapoptotic effect after treatment with CQ in both cell lines. None of the drugs had any effect on ROS levels. CQ did not alter the cell cycle distribution, however, metformin resulted in accumulation of HL60 cells in G1 phase, which was further increased when combined with CQ treatment. Our results show that CQ inhibits autophagy in both AML cell lines, though responses varied, and both metformin and 2DG seem to further inhibit autophagy when used in combination with CQ. In summary, CQ has anti-leukemic activity and should further be investigated as a therapeutic drug against AML in combination with other cytotoxic or metabolic drugs.

1. INTRODUCTION

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. It is an aggressive malignancy characterized by accumulation of immature leukemic cells in the bone marrow [1]. Current treatment of AML relies largely on intensive chemotherapy, potentially followed by allogeneic hematopoietic stem cell transplantation (allo-HSCT), and this is the only curative treatment of the disease. However, the median age at diagnosis is 65-70 years, and patients >70 years have a dismal outcome with less than 5% long-term AML-free survival, as many elderly patients cannot tolerate this intensive therapy [2, 3]. The long-term AML-free survival is only 35-40 % even for younger patients who can tolerate the most intensive conventional chemotherapy [1]. Although advances in treatment have significantly improved outcome in younger patients, there is still a strong need for new effective agents, or efficient combinations of agents, with a less toxic effect, to treat AML, also including patients unable to tolerate intensive treatment [4].

Targeting of autophagy is a therapeutic strategy being considered for treatment of cancer [5], including AML [6, 7]. Autophagy is a cellular recycle mechanism where cells digest their own cytoplasmic components; damaged proteins and organelles are thereby eliminated, and cellular homeostasis is maintained. The autophagic process can be tumor suppressive through removal of damaged organelles or oncogenic substrates [8, 9], but it can also contribute to carcinogenesis by providing energy and maintaining metabolism for cancer cells under stressful conditions [10], and sustain growth and survival when cancer cells are challenged by cytotoxic therapies [11]. Given its essential role in cell growth and survival, autophagy is therefore investigated as a target for therapeutic intervention.

Chloroquine (CQ) is a clinically available drug, that is shown to inhibit the last stage of autophagy. The drug was initially discovered and used to treat malaria, for which it has obtained approval from the Food and Drug Administration (FDA). These last decades, CQ has further been widely tested for its anti-cancer activity against a variety of cancer types [12]. The precise anti-cancer mechanism of CQ remains unclear, however, scientific reports suggest that the autophagy-specific mechanism of action of CQ is at least partially responsible, though other molecular mechanisms independent of autophagy may also contribute [13]. CQ inhibits autophagy by raising lysosomal pH that leads to inhibition of the fusion of autophagosomes with lysosomes, disrupting the lysosomal protein degradation [14]. CQ and its derivate hydroxychloroquine (HCQ) are currently the only FDA-approved drugs that also are autophagy-inhibitors, and they are therefore the main agents that have been tested in the clinic

to treat cancer through inhibition of autophagy. There are multiple ongoing clinical trials with these drugs alone or in combination with other anticancer agents [5, 15]. In addition, CQ has been shown to have an effect on chemosensitization of cancer cells, independent of its autophagy-dependent anticancer effects [16, 17].

In this study, we aimed to evaluate the anti-leukemic effects of CQ, in particular effects on proliferation, survival and soluble mediator release of primary AML cells derived from a large group of consecutive AML patients. We also tested the anti-leukemic effects of CQ in combination with the chemotherapeutic agent Ara-C, and investigated whether we could identify a subset of patients that might benefit from treatment with CQ. Finally, we examined the effects of combining CQ with the glycolytic inhibitor 2-Deoxy-D-Glucose (2-DG) and the anti-diabetic drug metformin on AML cells.

2. MATERIAL AND METHODS

2.1 Preparation of primary AML cells

The study was conducted after approval by the Regional Ethics Committee (REK) III 060.02, (100602), REK Vest 2013-634 (190313), REK Vest 2015/1410 (190615) and samples collected after written informed consent from all patients. The characteristics of AML patients are shown in Table 1. Patients with the FAB M3 subtype of AML were excluded. Peripheral blood mononuclear cells were isolated from patients with at least 80% AML cells among circulating leukocytes. Leukemia cell preparation was performed using density gradient separation (density 1.077 g/ml; Lymphoprep, Serumwerk Bernburg AG for Alere Technologies AS, Oslo, Norway), resulting in cell populations with >90% leukemia cells for all patients, where contaminating cells were mainly small lymphocytes [18-20]. The isolated AML blasts were immediately cryopreserved in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, US) with 10% dimethylsulfoxide and 20% inactivated fetal calf serum (Biowest, Riverside, MO, US). Cells were stored in liquid nitrogen until the cryopreserved cells were thawed, counted and used directly in the experimental studies.

2.2 Normal primary cells

Umbilical cord blood (UCB) cells were obtained from five donors after written informed consent (REK Vest 2015/1759 (051115), 2017/305 (070417)). Mononuclear cells were enriched in a similar manner to AML cell populations, by using density gradient separation, and stored in liquid nitrogen until used in experiments.

Cryopreserved human primary mesenchymal stem cells (MSCs) the bone marrow of a healthy donor (MSC24539, 24-year old female Caucasian) were purchased from Lonza in passage two (Cambrex BioScience, Walkersville, MD, US) and were expanded in complete MSC growth medium (MSCGM™; Lonza) with 10% inactivated fetal bovine serum (Biowest, Riverside, MO, US) and 4 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, US).

2.3 AML cell lines

The human AML cell lines, HL60 and MOLM-13, were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, US), supplemented with Streptomycin-Penicillin (50 µg/mL), 2mM L-glutamine (Sigma-Aldrich, St. Louis, MO, US) and 10% heat-inactivated fetal calf serum (Biowest, Riverside, MO, US), which was used in all experiments with cell lines. Both cell lines were mycoplasma negative (tested using MycoAlert™ detection kit, Lonza) and short-tandem repeat (STR) profiling was used to confirm the identity of cells (American Type Culture Collection (ATCC) cell line authentication service, Virginia, US; 100% exact match of HL60 to ATCC, and 100% exact match of MOLM-13 to Deutsche Sammlung von Mikroorganismen und Zellkulturen, (DSMZ)). HL60 cell line is a myeloblastic AML M2 subtype and has a biallelic deletion of the *TP53* gene [21], and carries amplified *MYC*. MOLM-13 is a monocytic M5 subtype leukemia cell line expressing an internal tandem duplication (ITD) of *FLT3*, both a mutated and wild-type allele, with no activation of FLT3 protein [22] and also carries the MLL-AF9 fusion protein [23].

2.4 AML and UCB cell culture medium

Serum-free Stem Span SFEM™ medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with exogenous granulocyte-colony stimulating factor (G-CSF), stem cell factor (SCF) and fms-related tyrosine kinase 3 ligand (FLT3-L) was used in all cell culture experiments with patient AML cells except coculture studies (described below), and also in experiments with UCBs. All three cytokines were purchased from Peprotech (Rocky Hill, NJ, US) and used at a final concentration of 20 ng/mL.

2.5 Reagents

Stock solutions of CQ (C6628), metformin hydrochloride (PHR1084) and 2-Deoxy-D-Glucose (154-17-6) were purchased from Sigma-Aldrich (St. Louis, MO, US), while cytarabine (Ara-C) was obtained from Pfizer (NY, US). All drugs were prepared according to datasheets provided from the distributors. Stock solutions were sterile-filtered (0.22 µm) and stored in small aliquots at -20°C until used. Aliquots were thawed only once and diluted with their

respective solvents to obtain the desired final concentrations. The concentration of solvents alone did not affect cell proliferation nor viability.

2.6 *In vitro* culture studies of primary cells

Suspension cultures of AML cells alone. Cells were seeded in triplicates (1 x 10^6 /ml, 200 µl medium/well) in flat-bottomed 96-well microtiter plates (Nucleon™; Nunc, Roskilde, Denmark), and cultures were then incubated with or without drugs in StemSpan medium supplemented with exogenous cytokines. AML cell proliferation was investigated using a [^{3}H]-thymidine incorporation assay [24]. After 6 days of incubation at 37ºC in a humidified atmosphere of 5% CO_2, 20 µL of 37 kBq [^{3}H]-thymidine (TRA 310, Amersham, UK) in saline was added per well, and nuclear incorporation was determined 22 hours later. For each drug concentration, the effect on proliferation was calculated by comparing cell proliferation (cpm values) as the percentage of untreated cultures. The median of triplicate cultures was used for all calculations, and detectable incorporation was defined as >1000 counts per minute (cpm).

Cocultures of AML cells and primary mesenchymal stem cells (MSCs). The MSCs were trypsinated and used in cocultures in passage four. Cocultures were prepared as described previously [25], by adding MSCs to the lower chamber and AML cells to the upper chamber of transwell plates (Costar 3401; 0.4 µm pore size, Costar, Cambridge, MA, US) in complete MSC medium, thus allowing no direct MSC-AML cell contact. Cocultures were incubated for 2 days with or without 5 µM CQ before 280 kBq/well [3H]-thymidine was added, and proliferation of MSCs and AML cells after 3-day coculture was determined as described in detail previously [26]. For cocultures, the median value of three samples derived from the same transwell culture was used in all calculations.

Analysis of cytokine levels in culture and coculture supernatants. AML cells were cultured for 48 hours in cytokine-supplemented Stem Span SFEM™ medium (1 x 10^6 /ml, 1 ml per well) in 24-well culture plates (Nucleon™; Nunc) with or without 5 µM CQ. Supernatants were collected and stored at -80°C. In addition, cell culture supernatants were harvested from MSC-AML cocultures (prepared in MSC medium) with or without 5 µM CQ for two days. Subsequently, 19 mediators were analyzed by Luminex® bead-based multiplex assays strictly according to the distributors protocol (LXSAHM-17, R&D Systems; Minnesota, MN, US).

2.6 RNA preparation, labeling and microarray hybridisation

All microarray experiments were performed using the Illumina iScan Reader, which is based upon fluorescence detection of biotin-labeled cRNA. 300 ng of total RNA from each sample was reversely transcribed, amplified and Biotin-16-UTP–labeled, using the Illumina®

TotalPrep™ RNA amplification kit (Applied Biosystems/Ambion, CA, US). Amount and quality of the biotin-labeled cRNA was controlled by both NanoDrop® spectrophotometer (NanoDrop Technologies, Inc. Delaware, US) and Agilent 2100 Bioanalyzer (Agilent Technonlogies, Inc. CA, US). 750 ng of biotin-labeled cRNA was hybridized to the HumanHT-12 V4 Expression BeadChip according to manufacturer's instructions. The HumanHT-12 V4 BeadChip targets 47,231 probes derived primarily from genes in the NCBI RefSeq database (Release 38). The data from the scanning of arrays on Illumina iScan Reader was investigated in GenomeStudio (Illumina Inc. CA, US) and J-Express 2012 (MolMine AS, Bergen, Norway) for quality control measures [27]. Before being compiled into an expression profile data matrix, all arrays within each experiment were quantile normalized to be comparable. We used the analysis of variance (ANOVA), and by setting an F-score >1.0 and a fold change (FC) value >1.0, we identified genes differently expressed between the two patient populations. The genes encoding proteins with a known function were classified using the PANTHER (protein annotation through evolutionary relationship) classification system (version 14.0) [28].

2.7 Flow cytometric analyses of cell viability and cell cycle progression

Cell viability analysis. The percentage of viable, apoptotic and necrotic primary AML cells were determined by flow cytometry using the Apoptest™–FITC kit (NeXins Research, Kattendijke, the Netherlands) as previously described [29]. AML cell lines, HL60 and MOLM-13, were analysed using the Pacific Blue™ AnnexinV apoptosis detection kit with propidium iodide (PI) (BioLegend®, San Diego, CA, US). Cell lines were analysed in accordance with the manufacturer's instructions. Briefly, cells were seeded in medium at 10^6 cells/ml in flat-bottomed 24-well plates (Nucleon™, Nunc, Roskilde, Denmark), and different concentrations of CQ were added cultures, or CQ in combination with either metformin or 2DG at different concentrations. Cells cultured in medium alone were used as controls. After 24 and 48 hours of incubation (37ºC, humidified atmosphere of 5% CO_2), cells were analysed using a BD FACSVerse 8-color flow cytometer (BD Biosciences; Franklin Lakes, NJ, US). Doublets were excluded by gating forward scatter (FSC) height and FSC-area, and side scatter (SSC)-height and SSC-area. 10,000 events were collected for each sample.

Cell cycle analysis. HL60 and MOLM-13 cells were seeded in medium at 10^6 cells/ml in flat-bottomed 24-well plates and treated with drugs for 24 and 48 hours before cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) and then fixated in 70% ethanol. The cells were thereafter stored at -20ºC until analysed by flow cytometry. On the day of analysis, the cells were washed twice with PBS, and re-suspended in PBS containing 100 µg/mL RNaseA

(Sigma Aldrich) and 50 µg/ PI. The cells were incubated for 30 minutes at 37ºC in the dark before being analysed using a BD FACSVerse 8-color flow cytometer (BD Biosciences), and 10,000 events were collected for each sample using low acquisition flow rate.

2.8 Flow cytometric analysis of reactive oxygen species (ROS)

HL60 and MOLM-13 cells were seeded into 24-wells plates (Nucleon™; Nunc) and incubated for 6 hours in the presence or absence of drugs; this incubation time was based on pilot studies performed to optimize the assay. The CellROX® Green flow cytometry assay kit (Invitrogen by Thermo Fisher Scientific, Eugene, OR, US) was used to measure levels of ROS. The CellROX® assay kit is specifically formulated for flow cytometry, where the cell-permeable CellROX® Green reagent stains the cell nucleus and mitochondria. It is essentially non-florescent while in reduced state, but exhibits a strong fluorogenic signaling upon oxidation, providing a reliable measure of ROS generation in live cells. Three controls were included in all experiments; (i) cells incubated in medium alone, (ii) cells cultured with tert-Butyl hydroperoxide (TBHP) (final concentration 300 µM) as a positive control, and (iii) cells cultured with N-acetylcystein (NAC) (final concentration 10 mM) as a negative control. The assay was performed strictly in accordance with the manufacturer's instruction, and analysed using a BD FACSVerse 8-color flow cytometer (BD Biosciences). For each sample, 10,000 events were collected.

2.9 Flow cytometric assay for detection of autophagy

HL60 and MOLM-13 cell lines were seeded at 10^6 cells/ml in 24-well plates (Nucleon™; Nunc) for 18 hours with or without drugs before the level of autophagy was measured using the CytoID® autophagy detection kit (ENZO Life Sciences, Farmingdale, NY, US). The assay was performed strictly in accordance with the manufacturer's instructions. After incubation, cells were harvested and washed in PBS before being resuspended in 250 µL of diluted CytoID® stain solution and incubated for 30 minutes at room temperature in the dark. The cells were thereafter pelleted, washed with assay buffer, then resuspended in fresh assay buffer and finally analyzed by flow cytometry. Rapamycin 0.5 µM was included as a positive control (autophagy inducer) in all experiments, CQ 60 µM was added as an inhibitor of autophagy. Vesicles produced during autophagy become brightly fluorescent after staining with CytoID, and the dye allows for minimal staining of lysosomes while exhibiting bright fluorescence upon incorporation into autophagic compartments.

2.10 Statistical analysis

Mann-Whitney *U,* Wilcoxon signed rank test and Kruskal-Wallis H-test with Dunn´s post hoc test, and Fisher´s exact test were used for statistical comparisons of AML patient cells, and paired *t*-test and ANOVA with Dunnett´s or Tukey post hoc test for cell lines. ANOVA, using F-score >1.0 and a fold change (FC) value >1.0, was used to identify genes, in our gene expression analysis. Analysis and graphical presentations were done using IBM Statistical Package for the Social Sciences® (SPSS®) v.23.0 (IBM SPSS statistics Inc., Chicago, IL, US) and GraphPad® prism version software v.5.02 (Graph Pad Software, Inc., San Diego, CA, US). The flow cytometry data was analyzed using FlowJo™ v.10.3 software (Tree Star, Inc., Ashland, OR, US). Differences were regarded as significant when $p < 0.05$.

3 RESULTS

3.1 Initial *in vitro* screening study of CQ on the proliferation of AML primary cells and UCB mononuclear cells

Initial studies to evaluate the effects of various concentrations of the drug CQ (2.5 μM - 100 μM) were conducted using the [^{3}H]-thymidine assay for AML cells derived from 17 patients and UCB cells derived from 5 donors, though only 4 UCB donors had detectable proliferation in untreated controls. CQ had dose-dependent antiproliferative effects both for primary AML cells and UCB cells, although the sensitivity towards drugs varied considerably between AML patients at the lowest concentrations of 2.5 μM and 5 μM (Supplementary Figure 1). None of the AML patient cells showed any proliferation after treatment with 50 μM CQ or higher concentrations (Supplementary Figure 1).

3.2 AML cell proliferation is inhibited by CQ alone and in combination with AraC

We expanded our initial study and investigated the effects of CQ alone (2.5 and 5 μM) on AML cell proliferation for 81 patients, using the [^{3}H]-thymidine incorporation assay. The two lowest concentrations (2.5 and 5 μM) were chosen for further assays, to characterize patient heterogeneity and also to be able to test CQ in combination with Ara-C. Detectable cell proliferation (>1000 cpm) was observed in drug free controls (cells cultured in medium alone) for 69 patients, and further statistical analysis was therefore based on the results for these patients. A highly significant antiproliferative effect of CQ was observed with both concentrations of CQ compared to untreated control cultures (p-value < 0.0001, Mann Whitney *U*-test; Figure 1). As expected, AraC (0.0125 μM) also had a significant inhibitory effect on AML cell proliferation compared to untreated controls (Figure 1). However, the effect of AraC

was not significantly different from any of the CQ concentrations tested alone when comparing overall effects on all patients (Kruskal Wallis test, Dunn´s post hoc test; Figure 1). Furthermore, we combined CQ (2.5 and 5 μM) with AraC (0.0125 μM), and both concentrations of CQ in combination with AraC 0.0125 μM showed an additive effect compared to CQ or AraC alone (*p-value* < 0.05, Kruskal Wallis test, Dunn´s post hoc test; Figure 1).

3.3 CQ inhibits AML cell proliferation in cocultures with MSCs

We further investigated the antiproliferative effect of CQ on AML cells when AML cells were cocultured in the presence of normal MSCs. Cocultures allowed no direct cell contact between AML cells and MSCs, and the [3H]-thymidine incorporation assay was used to measure proliferation of cells in cocultures after incubation with or without 5 μM CQ for three days. Of the 18 AML patients tested, 14 showed detectable AML cell proliferation in untreated controls (cpm >1000). MSCs increased AML cell proliferation for the majority of patients (data not shown). Still, AML cell proliferation was decreased after treatment with 5 μM CQ in cocultures for the majority of AML patients (*p*-value = 0.017, Wilcoxon signed rank test) (Supplementary Figure 2). CQ also inhibited MSC proliferation and the antiproliferative effect was stronger for the MSCs (derived from one donor) than for the AML cells (Supplementary Figure 2).

3.4 An antiproliferative effect of CQ is detected for most patients and even for patients insensitive to cytarabine

We performed an unsupervised hierarchical cluster analysis where we compared the AML cell proliferation (i.e. normalized to the median cpm value for each group) after cells had been cultured in medium alone, in the presence of CQ or after combined treatment with CQ and AraC for 7 days. In this analysis we only included the 69 patients demonstrating detectable proliferation (>1000 cpm) in untreated control cultures. We could then detect three patient subsets: a small subset with generally low proliferation in untreated cultures as well as in drug-treated cultures (upper blue subcluster, light gray column, Figure 2A), a larger subset with generally strong proliferation in both treated and untreated cultures (bottom green subcluster, shown as a dark gray column, Figure 2A), and an intermediate subset with diverse proliferation in both untreated and drug-treated cultures (middle subcluster, shown as a gray column, Figure 2A).

Next, we performed an unsupervised hierarchical clustering analysis based on the relative proliferation of the drug-treated cell cultures, i.e. the proliferation in drug-treated cultures (CQ or AraC) relative to the proliferation in the control cultures prepared in medium alone after seven days of culture (Figure 2B). Patients showed a varied response to drug treatment. Effects

on cell proliferation after treatment with both CQ (5 μM) and AraC (0.0125 μM) alone ranged from 0 - 99% inhibition, with a median inhibitory value of 83.4% and 57.9% compared to control cultures, respectively. Sixty-two of the 69 patients treated with CQ 5 μM, and 58 of the 69 patients treated with CQ 2.5 μM demonstrated an antiproliferative effect (> 20% inhibition compared to untreated controls), whereas the last nine patients had no or minor effects of both drugs (Figure 2B, lower patient sub-cluster). However, we also identified a subset of patients that were sensitive towards CQ (strong antiproliferative effect), although no or low inhibitory effects on cell proliferation were seen after treatment with AraC (Figure 2B, the upper 16 patients).

The antiproliferative effect of CQ showed no significant associations with age, cause of AML (*de novo* versus secondary), morphological signs of differentiation (FAB classification), expression of the stem cell marker CD34, karyotype, or *FLT3*-ITD or *NPM1* mutations (data not shown).

3.5 The AML antiproliferative effect of CQ is associated with a distinct gene expression profile

Based on the antiproliferative effects of 2.5 μM CQ we created a heat map, which sorted the patients according to the antiproliferative effects of CQ on AML cell proliferation compared to control cultures. The heat map indicates a strong antiproliferative effect to the right (high sensitivity to CQ) and patients with the lowest antiproliferative effect of CQ to the left (low sensitivity to CQ) (Figure 3, upper part). Next, we divided the patient cohort into four quartiles, and compared gene expression profiling (GEP) data from the lowest and highest quartiles; i.e. patient with strong antiproliferative effects and patients with low or no antiproliferative effects of CQ (Figure 3, upper part). GEP data was available for six patients with high sensitivity to CQ, and 11 patients with low sensitivity to CQ treatment (marked in boxes). Based on ANOVA (F-score >1.0 and FC value >1.0), we identified 99 genes upregulated among patients with high sensitivity (Supplementary Table 1). Among the 99 identified genes, several have previously been linked to AML leukemogenesis, and the full list with references to AML involvement is presented in Supplementary Table 1. Furthermore, we did an unsupervised hierarchical cluster analysis that illustrates the upregulated genes in the group of patients with the highest or lowest sensitivity to CQ. Finally, we used the PANTHER classification system to further classify the upregulated genes, and we then selected the category molecular function, which included catalytic activity and binding as the largest subterms within this category (Figure 3, lower part).

We further identified single genes belonging to these subterms (Figure 3, lower part and Supplementary Table 1).

3.6 Treatment with CQ significantly decreased primary AML cell viability and increased apoptosis and necrosis

Primary cells derived from 78 of the 81 consecutive AML patients were cultured with or without CQ 5 μM for 48 hours before the percentages of viable, early apoptotic and late apoptotic/necrotic cells were determined by flow cytometry. Six patients showed less than 5% viable cells in untreated controls and were excluded from the statistical analyses. There was a wide variation between patients with regard to the percentage of viable ($AnnexinV^-PI^-$) cells in drug-free control cultures, with only a slight decrease in overall viability after treatment with CQ (median 55.7%, range 5.9-89.1%) and the CQ-treated cultures (median 52.0%, range 4.8-88.9%) (Figure 4A). The percentage of early apoptotic cells ($AnnexinV^+PI^-$) was generally low for both treated and untreated cultures, with a slight increase in apoptosis after CQ-treatment (Figure 4A). When comparing samples pairwise, there was a small but statistically significant decrease in the percentage of viable cells after CQ treatment (p-value < 0.0001, Wilcoxon signed rank test) and an increase om the percentage of early apoptotic cells (p-value = 0.0008, Wilcoxon signed rank test). The effect of 5 μm CQ on AML cell viability was relatively weak, certain exceptional patients showed a small increase in the percentage of viable AML cells, and patients with a high viability in untreated control cultures generally showed the highest viability also in the presence of the drug (Figure 4B, p-value = 0.0001, Wilcoxon signed rank test). Finally, neither the AML cell viability in control cultures nor the effect of CQ on AML cell viability (i.e. increased versus decreased viability compared to median) showed significant associations with age, cause of AML (*de novo* versus secondary), morphological signs of differentiation (FAB classification), expression of CD34, karyotype, *FLT3*-ITD or *NPM1* mutations (data not shown).

3.7 CQ alters the constitutive release of only a few soluble mediators by primary human AML cells

The effect of CQ on the constitutive soluble mediator release by primary AML cells after 48 hours of culture was investigated for the same 72 patients tested in the viability assay. No significant changes were found between mediator levels when comparing overall results, but when comparing pairwise samples (untreated versus CQ-treated), a significant effect of CQ on the release of mediators was observed for four mediators, MMP9, MMP2, cystatin-C and CCL2 (Supplementary Figure 3; p-value < 0.05, Wilcoxon signed rank test).

Furthermore, we performed a hierarchical cluster analysis that identified a subset of 18 patients where treatment with CQ generally increased the mediator levels, whereas CQ had divergent effects for the other patients with unaltered or decreased levels after CQ treatment for most mediators and patients (Figure 5). The three main subsets identified in the cluster analysis showed no significant differences with regard to age, cause of AML (*de novo* versus secondary), morphological signs of differentiation (FAB classification), expression of CD34, karyotype, *FLT3*-ITD or *NPM1* mutations (data not shown).

3.8 High CQ-mediated soluble mediator release shows a gene expression profile associated with genes involved in metabolic processes

Gene expression profiling (GEP) data was available for a total of 33 patients that were examined for their release of soluble mediators after CQ treatment. Nine of these 33 patients belong to the previously identified group of patients with high CQ-mediated mediator release, while the remaining 24 belong to the group of patients with intermediate or low cytokine release (Figure 5). Using ANOVA (F-score >1.0 and FC value >1.0) we identified 74 genes upregulated among patients with high CQ-mediated mediator release (Supplementary Table 2). We investigated if these genes were able to separate the two patient groups by performing a hierarchical cluster analysis. Only two of the nine patients with high CQ-mediated mediator release clustered outside the identified subcluster including patients with high CQ-mediated mediator release (p-value = 0.001, Fisher´s exact test) (Supplementary Figure 4). Among the 74 genes that were upregulated among patients with high CQ-mediated release of mediators were several genes encoding proteins found to be involved in AML pathogenesis. All genes are presented in Supplementary Table 2. To investigate a broader biological context of the upregulated genes, we used the PANTHER classification system to further classify these genes. The four most frequent subterms were metabolic process, cellular process, localization and response to stimulus when using the main category biological process. We further identified single genes belonging to these subterms, which included genes known for involvement in AML leukemogenesis, noteworthy, *SNX2*, *FLT3*, *PFKP* and *CCL23* (Supplementary Figure 4, Supplementary table 2).

3.9 Effects of CQ alone or in combination with 2DG or metformin on cell viability, apoptosis and cell cycle of AML cell lines

We investigated the effects of CQ (5-40 μM) on the viability of HL60 and MOLM-13 cell lines using flow cytometry. Treatment with CQ alone for 24 or 48 hours did not significantly alter the cell viability of HL60 nor MOLM-13, even at the highest concentration of 40 μM

compared to untreated controls (Figure 6; data shown for 48 hours' treatment). Both cell lines were also treated with CQ in combination with two other metabolic inhibitors (2DG and metformin). 2DG alone (0.6-4.8 mM) had only minor effects on cell viability except for the highest concentration, where 4.8 mM 2DG decreased cell viability for both HL60 and for MOLM-13 (changes were not significant compared to untreated controls, Figure 6, upper part of figure), and CQ did not have any additional effects on cell viability when combined with 2DG (Figure 6). In contrast, treatment with metformin alone caused a significant and dose-dependent decrease in viability of HL60 cells, with an additive effect in combination with CQ (Figure 6, lower part of figure); though metformin showed a less effect on the viability of MOLM-13 cells, which was not additionally decreased by combination with CQ (Figure 6). The ability of CQ, 2DG and metformin to induce apoptosis in cell lines was also examined. The drugs, in particular CQ seemed to induce apoptosis in MOLM13 cells with higher concentrations, and metformin increased apoptosis in HL60 and MOLM13 cells, though none of these differences were statistical significant. (Supplementary Figure 5).

We also examined cell cycle phase distribution following treatment of cell lines with CQ alone or when CQ was combined with 2DG or metformin. Both cell lines had rapid cell growth; the doubling time for HL60 and MOLM-13 was approximately 24 hours and 48 hours, respectively, during our culture conditions. For HL60 cells, cell cycle phase distribution did not differ after treatment with any drug, alone or in combination, after 24 hours' treatment; however, after 48 hours' drug-treatment, a significant accumulation of cells in S phase was seen after treatment of HL60 cells with metformin alone and also when metformin was combined with CQ (Figure 7C, one-way ANOVA with Dunnett`s multiple comparison test, $p < 0.05$). The G2/M phase of HL60 cells was also significantly decreased after treatment with metformin, 2-DG, and the combination of CQ with metformin, and also CQ combined with 2-DG (one-way ANOVA with Dunnett`s Multiple Comparison Test, $p < 0.05$). For MOLM-13 cells, the cell cycle phase distribution was not altered after treatment with drugs for 24 or 48 hours (Figure 7E and 7F). A slight increase in the number of cells in fraction sub G1 was seen both after 24 and 48 hours of treatment with CQ (data not shown), and representative figures of the distribution of cell cycle phases are shown in Supplementary Figure 6.

3.10 Effects of CQ on levels of reactive oxygen species and autophagy

We investigated the effect of CQ (5 μM, 10 μM, 20 μM), metformin (2.5 mM, 5 mM, 10 mM) and 2DG (0.6 mM, 2.4 mM, 4.8 mM) on the generation of ROS in both cell lines during 6 hours of *in vitro* culture. This time interval was based on pilot experiments investigating

different time intervals (data not shown), however, none of the drugs had any significant effects on cellular levels of ROS (Supplementary Figure 7).

We used a flowcytometric-based method to detect autophagy in cells. CQ is a well-known inhibitor of late-stage autophagy [30-32], and treatment with CQ resulted in a significant accumulation of lysosomal vesicles in a dose-dependent manner for both HL60 and MOLM-13 (p-values < 0.05), although MOLM-13 seems to have a higher autophagic flux than HL60 (one-way ANOVA, with Dunnett´s multiple comparison test) (Figure 8A). Both metformin and 2DG caused a further dose-dependent inhibition of autophagy in combination with CQ, though changes were not statistically significant (Figure 8B and C).

4. DISCUSSION

Today, the main treatment for AML consists primarily of intensive chemotherapy followed by consolidation therapy or allo-HSCT. However, a large portion of adults with AML, mostly older adults, is not given the standard intensive chemotherapy due to treatment-related toxicity and comorbidities [4]. There is a strong need for new effective agents, or efficient combinations of agents, to treat AML including those patients unable to tolerate intensive treatment. Repurposing of antimalarials as inhibitors of autophagy to treat cancer is gaining immense interest, in particular in combination with other therapies [33]. This may also become a potential therapy in AML [34], and we therefore investigated the potential of chloroquine as an anti-leukemic drug.

CQ has been reported to arrest the last step of autophagy i.e. fusion of autophagosomes with lysosomes, and lysosomal protein degradation (Supplementary Figure 8), thus it inhibits autophagy mainly by targeting the lysosomes [35]. As far as we know, this is the first study to investigate effects of CQ on a relative large group of consecutive AML patients. First, we tested the effect of CQ over a wide concentration-range of the drug on leukemia cells derived from 17 AML patients and mononuclear UCB cells from four individuals. CQ had an inhibitory effect on proliferation for all patients, and based on these experiments we selected the concentrations used in further experiments with primary AML cells. The inhibitory effect of CQ on cell proliferation was not specific for AML cells; the drug had a similar effect for all umbilical cord cell donors. Although CQ is FDA-approved and considered well-tolerated as an antimalarial drug, higher doses of CQ can be toxic and hematological effects have been reported, including leukopenia and agranulocytosis [36]; and future studies of CQ in AML therapy should investigate the toxicity of this treatment carefully. We further evaluated CQ on cells derived

from 81 AML patients, and found that CQ inhibited cell proliferation for the majority of AML patients, though with a wide variation between patients.

To assess the efficacy and clinical relevancy of CQ against blasts, we used concentrations within the clinical recommendations for patients treated with CQ over longer periods (250-500 mg CQ, with reported plasma levels of 2.5 – 12.5 uM), as is common for inflammatory and dermatologic conditions [37, 38]. In addition, we tested higher concentrations that are closer to what is seen in patients' plasma during treatment of malaria (ranging from an area under the curve (AUC) of 8.2-140 μg/mL, equivalent to 25-440 μM over three days) [39]. In this study, we also examined the anti-leukemic effects of CQ combined with the metabolic drugs metformin and 2DG. With regard to metformin, serum levels obtained at clinical doses are highly dependent on kidney function [40]. When metformin accumulates in the plasma due to kidney failure, toxic plasma concentrations over > 5 mg/L can be seen, and is found when metformin is implicated as cause of lactic acidosis [41]. This may be challenging when trying to transfer results from *in vitro* studies, which commonly use higher doses (including our current study), to *in vivo* studies [42-46]. For 2DG, the concentrations used in our present study correspond to plasma concentrations that are found tolerable in clinical studies [47]. The same applies for cytarabine, were the concentrations tested on AML blasts in our present experiments correspond to the serum concentrations reached during low-dose subcutaneous cytarabine therapy [48]. Thus, the drug concentrations used in our experimental studies are likely within clinical relevant concentrations, though several factors can influence drug concentrations tolerated in humans [49].

For our proliferation studies, we used a seven-day [^{3}H]-thymidine incorporation assay, i.e. the cells were cultured for six days before [^{3}H]-thymidine was added for additional 24 hours and then cell proliferation was assessed. During the first six days of *in vitro* culture of blasts, an increasing spontaneous or stress-induced apoptosis is generally observed [29]. Thus, our [^{3}H]-thymidine incorporation assay reflects the characteristics of the minor subset of cells that are able to survive and still proliferate after seven days of *in vitro* culture. Our results showed that CQ had a significant and dose-dependent effect on AML cell proliferation for a large majority of patients, though the inhibitory effect varied between patients and was absent for a small minority. In addition, CQ had an additive antiproliferative effect on leukemia cells when tested together with cytarabine. These results are supported by the literature, were several studies have shown that both leukemic stem cells (LSCs) and leukemic blasts can utilize autophagy to counteract chemotherapeutic-induced stress, and that blocking autophagy can enhance sensitivity to standard chemotherapy, thus killing cancer cells [34]. As expected,

cytarabine also had an antiproliferative effect for a majority of patients, however, the antiproliferative effect of CQ could be detected also for a few patients who showed no effect of low-dose cytarabine. Furthermore, CQ decreased AML cell viability partly through induction of apoptosis, but this effect showed no significant association with the antiproliferative effect of the drug.

In the present study we included a consecutive group of AML patients with relatively high percentage of AML cells among circulating leukocytes, thus there is a selection of patients only based on the degree of leukemization, and our results may therefore be representative only for this subset of patients with peripheral blood leukemization. Differences may also be present between samples taken from bone marrow and peripheral blood, however, a previous study reported that no significant difference in autophagy flux was shown between AML cells derived from bone marrow compared to peripheral blood [50]. However, it is plausible that the effect of CQ, as a lysosomal inhibitor, may depend on the autophagic activity of AML cells. A study by Folkerts *et al*. [50] showed that the basal levels of autophagy vary among leukemic cell lines and also in human CD34+ leukemia cells, with a large variability between AML patients. In this study, higher levels of genes related to autophagy were found in AML patients classified as poor risk (complex karyotype) and they also had a higher autophagic flux; though no differences in autophagy levels were seen in AML samples harboring prognostic mutations. In our study, we did not observe any associations between the effect of CQ and mutational status of AMLs, nor were effects of CQ associated with karyotype or changes of gene involved in autophagy when comparing gene expression profiles of patients with strong antiproliferative effects towards CQ with low or none antiproliferative effects of CQ; however some reports have shown anti-leukemic effects of CQ which seem to be independent of its autophagy-inhibitory mechanisms [13, 35].

We investigated the effect of CQ on the constitutive extracellular release of soluble mediators by AML cells, and found significantly decreased levels only for MMP-2, MMP-9, Cystatin C and increased levels of CCL2 when comparing samples pairwise with and without CQ treatment. CQ has been reported in other studies to affect the matrix metalloproteinase network [51] and may also induce expression of chemokines through NF-kappaB activation [52]. However, the mediator levels showed no significant differences between treated and untreated cultures when comparing overall results; this may possibly be explained by the finding of a subset of patients with generally increased release of soluble mediators after treatment with CQ, while another subset showed decreased or no alteration of soluble mediator release after CQ treatment. To investigate the effect of CQ on the release of soluble mediators further, we

examined the global gene expression profiles for 33 of the patients included in this study, nine of which showed a generally higher mediator release after CQ treatment, while the others had decreased or no changes in mediator levels. Among the identified genes upregulated in the subset with increased levels of mediator were several genes known for involvement in AML leukemogenesis, *SNX2*, *FLT3*, *PFKP* and *CCL23*. Furthermore, CQ also altered the release of soluble mediators after coculture of AML cells with mesenchymal stromal cells (MSCs). We have previously shown that MSCs can support the growth and survival of AML cells through a cytokine-mediated crosstalk between AML cells and MSCs [25], and effects of CQ on the release of soluble mediators by the leukemia-supporting nonleukemic stromal cells may possibly further influence the direct anti-leukemic effects of CQ on the leukemic cells [29].

Other metabolic inhibitors such as the AMPK inhibitor metformin and the inhibitor of glycolysis 2DG have been shown to have anti-leukemic effects that are increased by combination therapy [42, 43, 53-56], and in addition a recent clinical trial with metformin and CQ was described for glioblastoma, implying a potential role for IDH1/2 mutations with regard to sensitivity towards CQ [57], and for this reason we also investigated the anti-leukemic effect of combining these two metabolic inhibitors with CQ. These experiments were performed using two AML cell lines, which then avoids the influence of spontaneous or stress-induced apoptosis that occurs during *in vitro* culture of primary human AML cells [29]. CQ increased the accumulation of lysosomal vesicles in both MOLM-13 and HL60 in a dose-dependent manner. Furthermore, the autophagic activity decreased after combination treatment with 2DG and metformin. One of the most important activators of autophagy is glucose deprivation that leads to activation of the unfolded protein response (UPR) and thereby autophagy. Metformin inhibits mTOR indirectly through inhibition of AMPK; an effect that one would expect to increase autophagy. However, our results for both cell lines showed that metformin decreased autophagy (Figure 8B and 8C). Decreased autophagy together with increased apoptosis after exposure to metformin, has also been described in myeloma cells through suppressed induction of the critical UPR effector glucose-regulated protein 87 (GRP78) [46]. Another experimental study confirmed that metformin could be an autophagy inhibitor through this mechanism [45]. Furthermore, 2DG inhibits glucose metabolism through inhibition of hexokinase, the first and rate limiting step of glycolysis, and thereby induces autophagy [58]. In contrast, our present results showed that 2DG decreased autophagy in the presence of CQ. Furthermore, a previous study of prostate cancer cells described a synergistic anti-autophagy effect of 2DG when combined with metformin [53]. Thus, the effect of 2DG on autophagy seems to depend on the biological context.

In conclusion, our study shows that CQ has anti-leukemic activity, and recent findings that other mechanisms besides CQ`s autophagy-inhibitory effect can explain the anti-cancer activities of CQ necessitate a further investigation of this drug as an anti-cancer agent. Therapy utilizing CQ in combination therapy should be evaluated further as a strategy for the treatment of AML.

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LEGENDS TO FIGURES

Figure 1.

Effect of chloroquine, alone or in combination with AraC, on cytokine-dependent AML cell proliferation. AML cells from 81 consecutive patients were cultured for seven days in medium alone (control cultures), or treated with CQ (2.5 μM and 5 μM), AraC (0.0125 μM) or CQ in combination with AraC. The [^{3}H]–thymidine assay was used to measure cell proliferation. Detectable proliferation defined as >1000 cpm in untreated control cultures, was observed for 69 patients. Proliferation is shown as the median levels (with 25-75% percentiles) for patients, and proliferation in treated cultures is shown as percent proliferation of the drug-free controls (set to 100%). Significant effects were calculated using Mann-Whitney U-test for comparison to untreated controls (shown as asterisks directly above bars), and Kruskal Wallis with Dunn´s post hoc test for comparison between the different groups (asterisks above brackets) (ns = not significant, * p-value = 0.05, *** p-value = 0.0001).

Figure 2.

An unsupervised hierarchical cluster analysis based on the effect of CQ and AraC on AML cell proliferation. AML cells from 81 consecutive patients were cultured for seven days with CQ (2.5 μM and 5 μM), AraC (0.0125 μM), CQ in combination with AraC or medium alone (control). Proliferation was investigated using [^{3}H]-thymidine incorporation assay. Detectable proliferation was defined as >1000 cpm, and data is presented for the 69 patients with detectable proliferation. **A)** The cluster illustrates the cytokine-dependent AML cell proliferation for the untreated controls and drug-treated cultures (CQ or a combination of CQ and AraC) after results were normalized to the corresponding median for each group. The cluster could be divided into three main subsets based on the degree of proliferation as illustrated in the first column to the right: (i) low proliferation (light gray), (ii) intermediate proliferation (gray), and (iii) high proliferation (dark gray). The figure also shows the distribution of biological and clinical characteristics for each individual patient (columns on the right part of figure). **B)** The cluster shows the relative AML cell proliferation (i.e. percent proliferation compared to untreated controls) for the 69 AML patients after treatment with CQ and AraC. As shown in the cluster, the majority of patients had a strong inhibitory effect of CQ, AraC or both drugs (two top

subclusters, shown as blue and dark blue in the column to the right) a strong inhibitory effect of CQ, AraC or both. A small subcluster of nine patients (bottom subcluster, shown as light blue in the column to the right) had little or no effect of these treatments. Shown in different columns to the right of the figure are different patient subsets based on clustering of cytokine-dependent proliferation (based on Figure 2A), proapoptotic effects, CQ-mediated cytokine release (based on Figure 5), and survivors.

Figure 3.

Profiling of gene expression data based on antiproliferative effects of CQ on primary AML cells. For 69 AML patients, the proliferation of AML cells after treatment with 2.5 μM CQ was compared to the AML cell proliferation of untreated controls. Proliferation was analyzed using the [^{3}H]-thymidine incorporation assay, and we used the log2 ratio of the proliferation values to create a heat map (illustrated in upper part of figure). Based on this heat map, the patient cohort was further divided into quartiles, where the quartile at the far left side of the heat map (shown in red) included patients that had the most decreased proliferation after CQ treatment (high sensitivity to CQ), while the quartile to the far right (shown in blue) had unaltered or high proliferation after CQ treatment (low sensitivity to CQ). Gene expression data for six patients found in the quartile with high sensitivity to CQ was then compared with 11 patients found in the quartile with low sensitivity to CQ. Based on ANOVA (F-score >1.0 and FC value >1.0), we identified 99 genes upregulated among patients that were highly sensitive to CQ (a strong antiproliferative effect), illustrated by the unsupervised hierarchical cluster analysis shown in the middle part of the figure. The genes encoding proteins with a known function were classified using the PANTHER database, by using the term molecular function we identified the subcategories Catalytic activity and Binding as the two most prominent terms among the upregulated genes. Furthermore, we identified the three largest subterms belonging to these subcategories, and the figure show the genes upregulated in each subterm (see colored circles at bottom of figure). All 99 identified genes are listed in Supplementary Table 1.

Figure 4.

Effect of 5 µM CQ on primary AML cell viability, apoptosis and necrosis after 48 hours of culture. Primary cells from 78 consecutive AML patients were cultured with or without 5 µM CQ for 48 hours before viability, early apoptosis and late apoptosis/necrosis were determined by flow cytometry using the AnnexinV/Propidium iodide (PI) assay. **A)** The figure shows the overall results for the 72 patients with more than 5% viable cells in drug-free controls. The percentage of viable ($AnnexinV^{-}$ PI^{-}), early apoptotic ($AnnexinV^{+}$ PI^{-}) and end stage apoptotic and necrotic cells ($AnnexinV^{+}$ PI^{+}) were determined in patient samples after treatment with 5 µM CQ (white boxes) and after culture in medium alone (gray boxes) for 48 hours. Data is presented as median levels, 25/75 percentiles and 5/95 percentile whiskers, • = outliers. The overall effect of CQ on viability/apoptosis/necrosis was not significant (Mann-Whitney U-test), but when analyzing samples pairwise, treatment with 5 µM CQ significantly decreased viability (p-value < 0.0001), and increased apoptosis and late apoptosis/necrosis (p-values = 0.0008 and < 0.0001, respectively; Wilcoxon signed rank test). **B)** The percentage of viable primary AML cells cultured in medium alone compared to treatment with 5 µM CQ for 48 hours. A wide range of cell viability is seen among patients, with a significant decrease in viability after treatment with 5 µM CQ (p-value = 0.0001, Wilcoxon signed rank test).

Figure 5.

Unsupervised hierarchical cluster analysis based on alterations in the release of soluble mediators by primary AML cells derived from 72 unselected patients after treatment with 5 µM CQ for 48 hours. Primary AML cells were cultured with 5 µM CQ or medium alone for 48 hours, before the levels of soluble mediators in harvested supernatants were determined using luminex analysis. A hierarchical cluster analysis was performed based upon the alteration of soluble mediators compared to untreated controls (control set to 100%). The cluster analysis identified three patient subsets: (i) a subset with increased release of soluble mediators after treatment with CQ (upper subset, shown as dark green in first column to the right), (ii) one intermediate subset, with divergent release of soluble mediators (middle subset, shown as turquoise in column to the right), and (iii) a subset of patients with decreased release of soluble mediators after treatment with CQ (light turquoise). There is one outlier at

the bottom of the cluster with very high cytokine release of all soluble mediators after treatment with CQ (marked in dark green).

Figure 6.

Effect of CQ combined with the metabolic drugs 2DG and metformin on the viability of AML cell lines HL60 and MOLM-13. The cells were treated with CQ (5 μM, 10 μM, 20 μM and 40 μM), metformin (2.5 mM, 5 mM, 10 mM and 20 mM), and 2DG (0.6 mM, 1.2 mM, 2.4 mM and 4.8 mM), alone and in combination for 48 hours. Cell lines incubated in medium alone were used as drug-free controls. Viability was measured using the flow cytometric AnnexinV/PI assay and 10,000 events were collected per sample. CQ and 2DG did not significantly alter the viability of cell lines alone or as drug combinations when compared to control cultures, however HL60 cells were sensitive to treatment with metformin (p-value = 0.015), especially when used in combination with CQ (p-value = 0.002, compared to control cultures). The results are shown as mean values (±SD) of three repeated experiments. (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.0001; one-way ANOVA, with Tukey's multiple comparison posttest).

Figure 7.

Effect of CQ combined with the metabolic drugs 2DG and metformin on cell cycle distribution of HL60 and MOLM-13 cells lines after 24 and 48 hours' culture. Cell lines were cultured for 24 and 48 hours in the presence or absence of drugs and cell cycle analysis was performed using flow cytometry. Drug concentrations tested were: CQ 40 μM, metformin 20 mM, 2DG 4.8 mM, and CQ 40 μM in combination with either metformin 20 mM or 2DG 4.8 mM. **A) and D)** The histogram plots shows the gating used to determine the distribution of cells in sub-G1, G0/G1, S and G2/M phase in HL60 and MOLM-13 cell lines, respectively, **B)** and **C)** show the cell cycle distribution in HL60 cells while **E) and F)** show the cell cycle distribution in MOLM-13 cells for each drug and drug combination tested after 24 and 48 hours culture. CQ alone did not alter the cell cycle distribution. Only HL60 cells showed a significant increase in S-phase when treated with metformin alone for 48 hours, which was also seen when cells were treated with metformin in combination with CQ. In addition, a significant decrease of

G2/M phase in HL60 cells was seen after treatment with metformin alone and in combination with CQ. A significant alteration of the G1 phase and G2/M phase was also seen for HL60 cells treated with CQ in combination with 2DG. The results are shown as mean values (±SD) of three repeated experiments. (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.0001 (one-way ANOVA, with Dunnett´s multiple comparison posttest).

Figure 8.

Differences in autophagic activity after treatment of HL60 and MOLM-13 cell lines with CQ alone or in combination with metabolic drugs. The CytoID® autophagy detection kit for flow cytometry was used to detect changes in autophagic activity of HL60 and MOLM-13 cells after treatment with CQ alone and in combination with metformin and 2DG for 18 hours. **A)** Cell lines were treated with different concentrations of CQ (2.5-60 μM) (shown on the X-axis) and autophagy measured as fluorescence (percent of untreated controls set as 100% on the Y-axis). CQ resulted in a significant accumulation of lysosomal vesicles in a dose-dependent manner for MOLM-13 cells, and MOLM-13 seems to have a higher autophagic flux than HL60 (* = p-value < 0.05, *** = p-value < 0.0001, one-way ANOVA, with Dunnett´s multiple comparison post test). Results are shown as mean values (±SD) of three repeated experiments. **B)** HL60 and MOLM-13 cells were treated with 30 μM CQ in combination with metformin at the following concentrations: (1) 2.5 mM, (2) 5 mM, (3) 10 mM and (4) 20 mM, or 2DG at the following concentrations: (1) 0.3 mM, (2) 0.6 mM, (3) 1.2 mM and (4) 2.4 mM. Autophagic activity decreased in a dose dependent manner after treatment with both metformin and 2DG for both cell lines, but there were no significant differences (p-value < 0.05) between untreated controls and cells treated with CQ in combination with metformin or 2DG (pairwise t-test). CQ 60 μM in combination with the mTOR inhibitor Rapamycin 500 nM was used as positive control in all experiments (upper stippled line), and cells cultured in medium alone were used as controls. Results are shown as mean values (±SD) of three repeated experiments.

Table 1. Biological and clinical features of the 81 AML patients included in this study.

CLINICAL CHARACTERISTICS					
Gender			*Age*		
Male	51		Median (yrs)		67.5
Female	30		Range (yrs)		17-87
Survival (in younger patients n = 39)			*Secondary AML (n = 20)*		
Yes	15		AML relapse		5
No	24		MDS		11
nd	42		CMML		2
			Polycythemia Vera		1
			Li-Fraumeni syndrome		1
DIFFERENTIATION					
FAB classification			*CD34 expression* (> 20%)		
AML-M0/M2	38		Negative		22
AML-M4/M5	36		Positive		52
nd	7		nd		7
GENETIC ABNORMALITIES					
Cytogenetics		*FLT3*		*NPM-1*	
Favorable	10	wt	45	wt	45
Intermediate	48	ITD	22	INS	28
Adverse	14	TKD	2	nd	8
nd	9	ITD/TKD	3		
		nd	9		

Abbreviations: CMML, Chronic myelomonocytic leukemia; FAB, French-American-British; INS, insertion; ITD, internal tandem duplication MDS, Myelodysplastic syndrome; nd, not determined; TKD, tyrosine kinase domain; wt, wild type; yrs, years.

Figure 1.

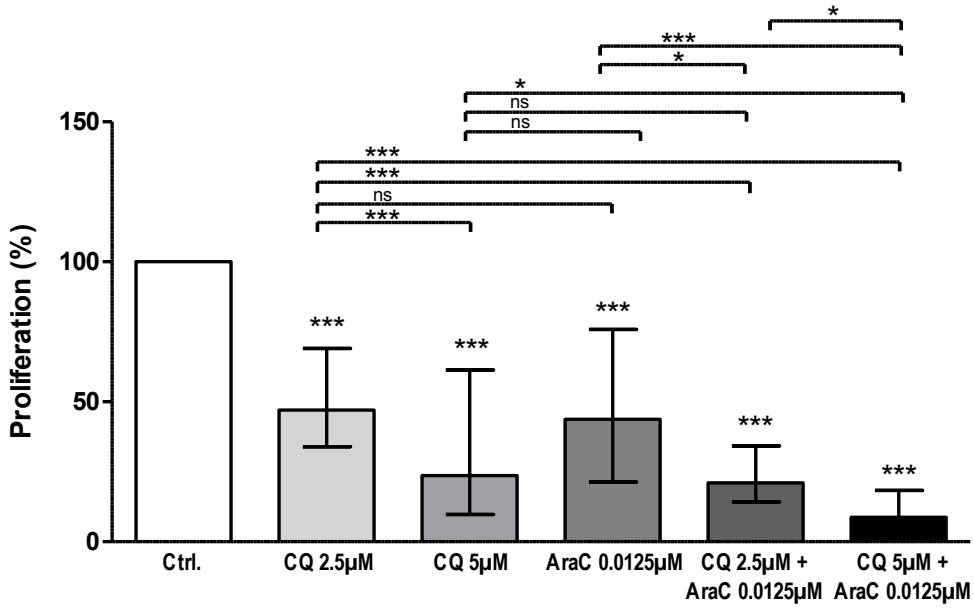

Proliferation (%)
150
100
50
0
Ctrl.
CQ 2.5µM
CQ 5µM
AraC 0.0125µM
CQ 2.5µM + AraC 0.0125µM
CQ 5µM + AraC 0.0125µM
***
ns
*

Figure 2.

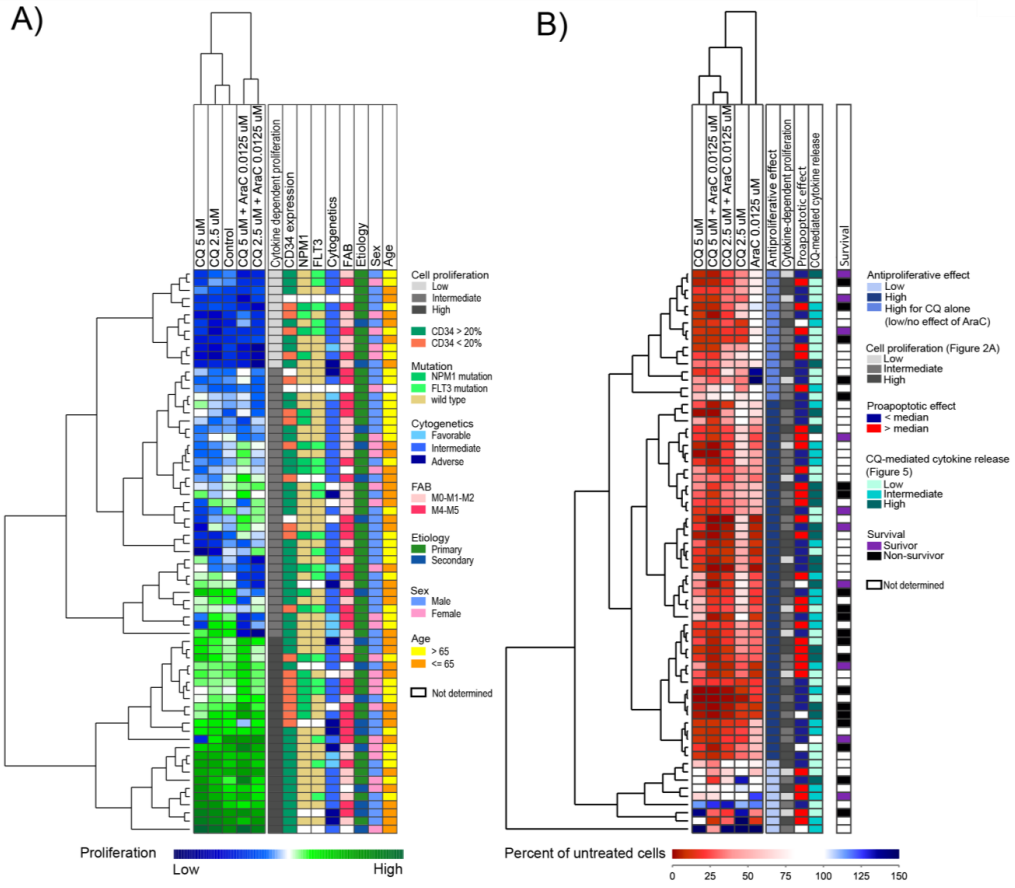

A)
CQ 5 uM
CQ 2.5 uM
Control
CQ 5 uM + AraC 0.0125 uM
CQ 2.5 uM + AraC 0.0125 uM
Cytokine dependent proliferation
CD34 expression
NPM1
FLT3
Cytogenetics
FAB
Etiology
Sex
Age
Cell proliferation
Low
Intermediate
High
CD34 > 20%
CD34 < 20%
Mutation
NPM1 mutation
FLT3 mutation
wild type
Cytogenetics
Favorable
Intermediate
Adverse
FAB
M0-M1-M2
M4-M5
Etiology
Primary
Secondary
Sex
Male
Female
Age
> 65
<= 65
Not determined
Proliferation
Low
High
B)
CQ 5 uM
CQ 5 uM + AraC 0.0125 uM
CQ 2.5 uM + AraC 0.0125 uM
CQ 2.5 uM
AraC 0.0125 uM
Antiproliferative effect
Cytokine-dependent proliferation
Proapoptotic effect
CQ-mediated cytokine release
Survival
Antiproliferative effect
Low
High
High for CQ alone
(low/no effect of AraC)
Cell proliferation (Figure 2A)
Low
Intermediate
High
Proapoptotic effect
< median
> median
CQ-mediated cytokine release
(Figure 5)
Low
Intermediate
High
Survival
Surivor
Non-survivor
Not determined
Percent of untreated cells
0
25
50
75
100
125
150

Figure 3.

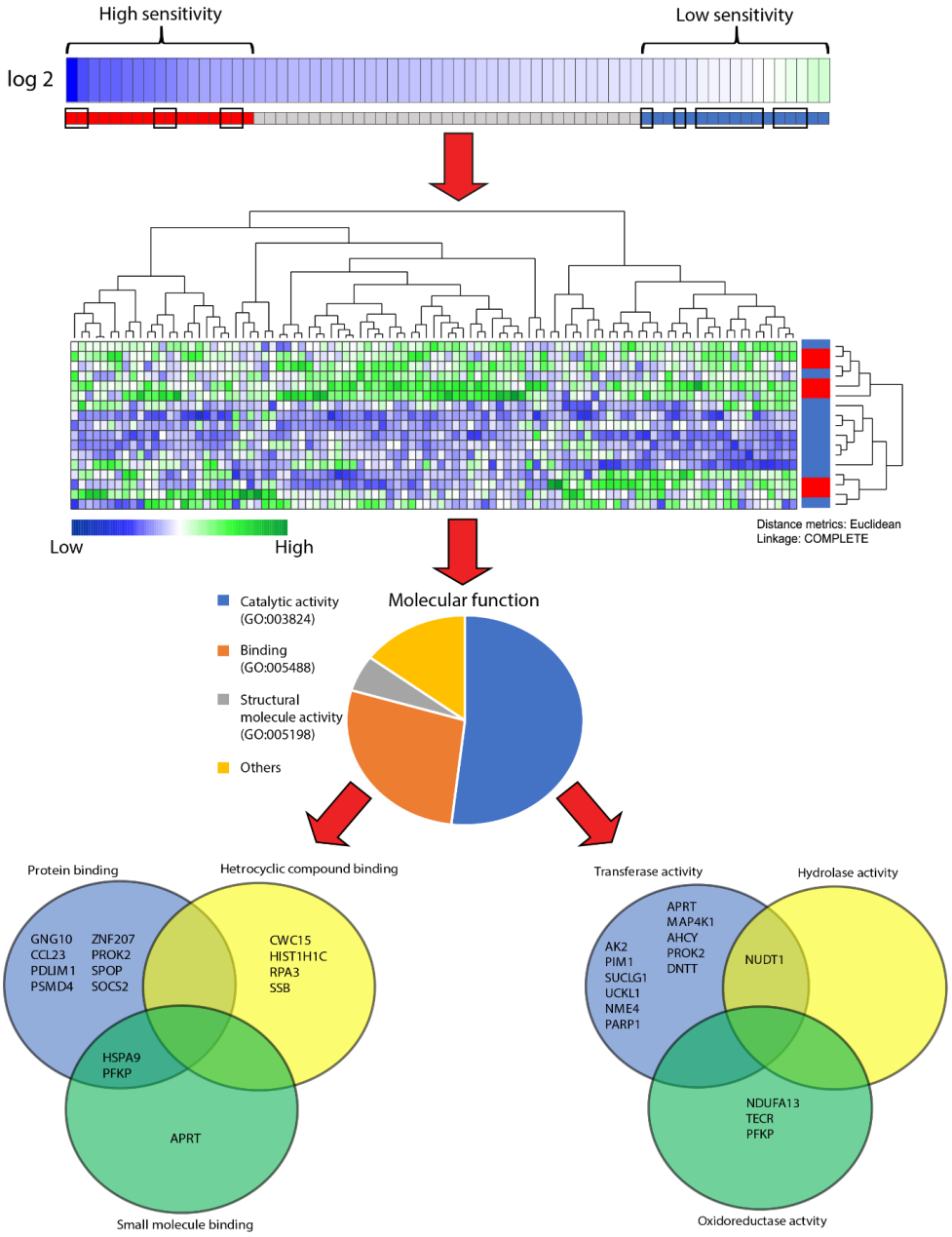

High sensitivity
Low sensitivity
log 2
Low
High
Distance metrics: Euclidean
Linkage: COMPLETE
Molecular function
Catalytic activity (GO:003824)
Binding (GO:005488)
Structural molecule activity (GO:005198)
Others
Protein binding
GNG10 ZNF207
CCL23 PROK2
PDLIM1 SPOP
PSMD4 SOCS2
Hetrocyclic compound binding
CWC15
HIST1H1C
RPA3
SSB
HSPA9
PFKP
APRT
Small molecule binding
Transferase activity
APRT
MAP4K1
AHCY
PROK2
DNTT
AK2
PIM1
SUCLG1
UCKL1
NME4
PARP1
Hydrolase activity
NUDT1
NDUFA13
TECR
PFKP
Oxidoreductase actvity

Figure 4.

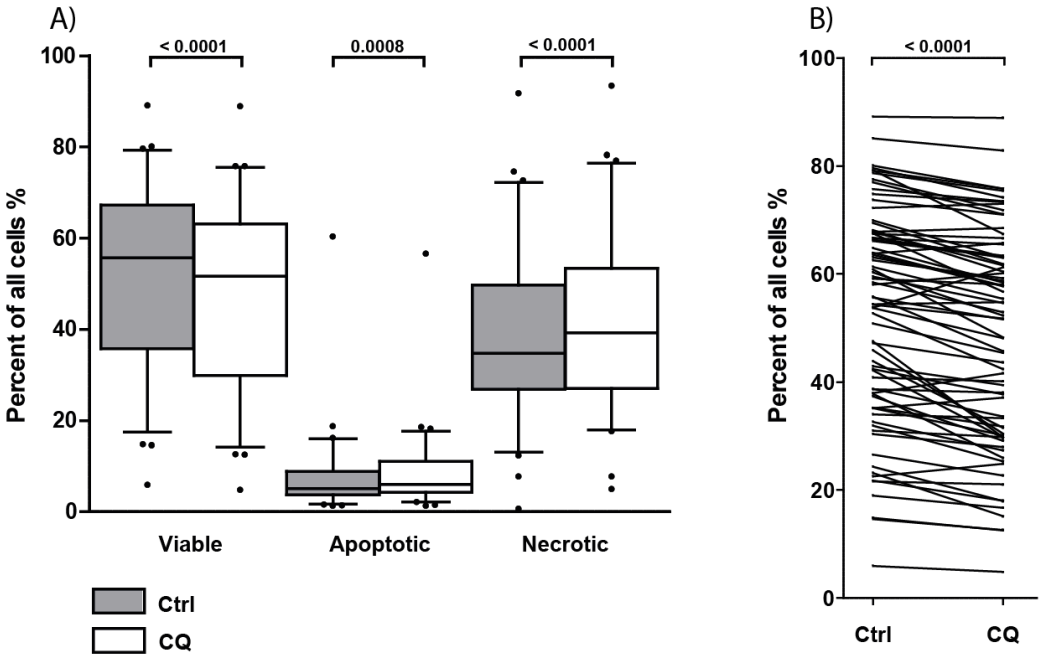

A)
< 0.0001
0.0008
< 0.0001
100
80
60
40
20
0
Percent of all cells %
Viable
Apoptotic
Necrotic
Ctrl
CQ
B)
< 0.0001
100
80
60
40
20
0
Percent of all cells %
Ctrl
CQ

Figure 5.

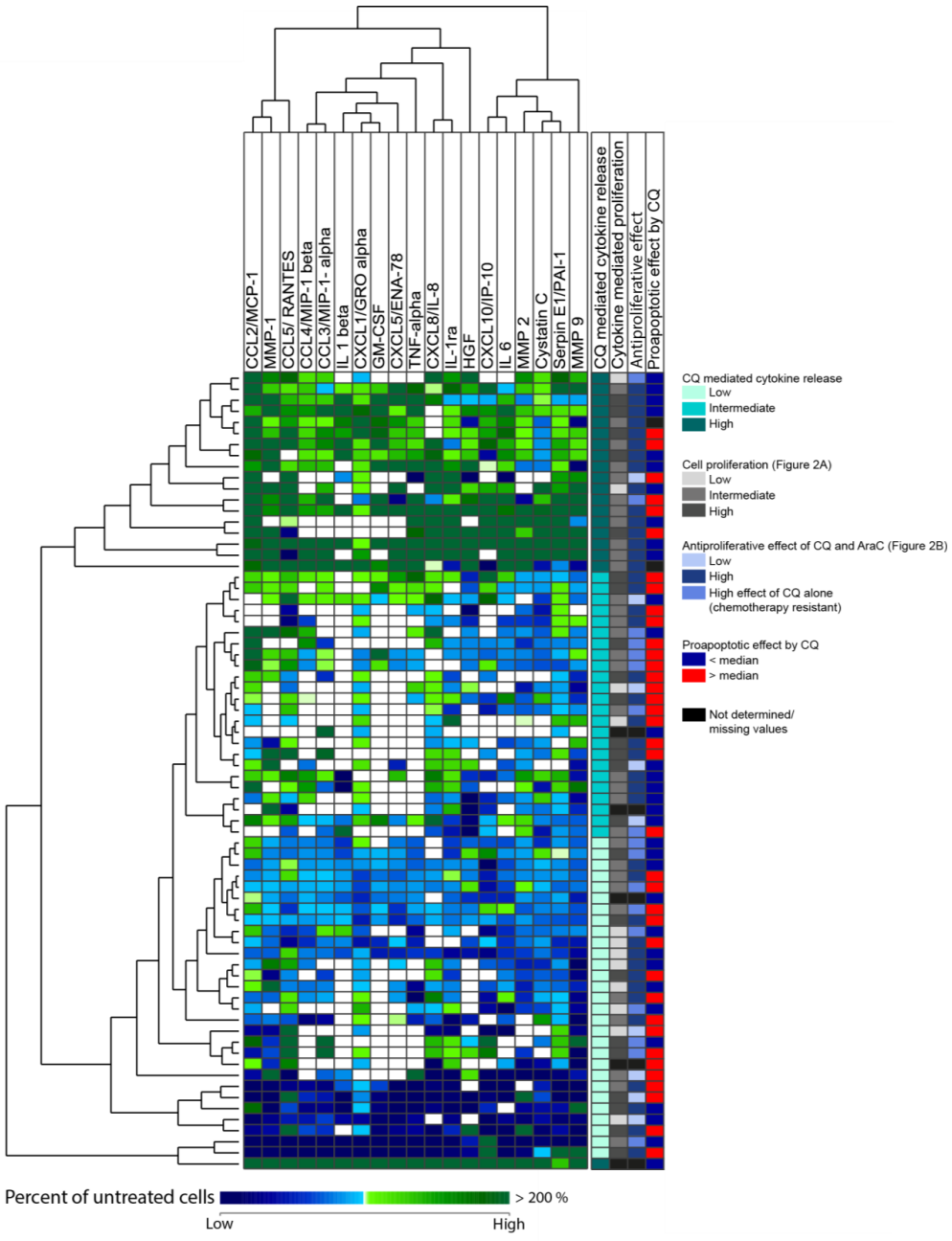

CCL2/MCP-1
MMP-1
CCL5/ RANTES
CCL4/MIP-1 beta
CCL3/MIP-1- alpha
IL 1 beta
CXCL1/GRO alpha
GM-CSF
CXCL5/ENA-78
TNF-alpha
CXCL8/IL-8
IL-1ra
HGF
CXCL10/IP-10
IL 6
MMP 2
Cystatin C
Serpin E1/PAI-1
MMP 9
CQ mediated cytokine release
Cytokine mediated proliferation
Antiproliferative effect
Proapoptotic effect by CQ
CQ mediated cytokine release
Low
Intermediate
High
Cell proliferation (Figure 2A)
Low
Intermediate
High
Antiproliferative effect of CQ and AraC (Figure 2B)
Low
High
High effect of CQ alone
(chemotherapy resistant)
Proapoptotic effect by CQ
< median
> median
Not determined/
missing values
Percent of untreated cells
> 200 %
Low
High

Figure 6.

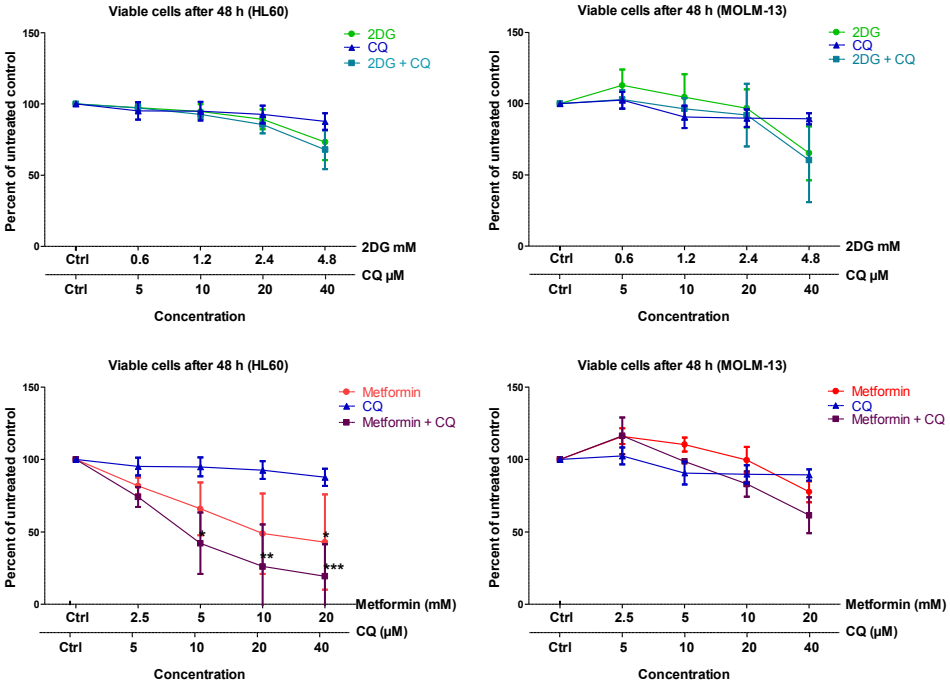

Viable cells after 48 h (HL60)
Percent of untreated control
150
100
50
0
2DG
CQ
2DG + CQ
Ctrl
0.6
1.2
2.4
4.8
2DG mM
Ctrl
5
10
20
40
CQ µM
Concentration
Viable cells after 48 h (MOLM-13)
Percent of untreated control
150
100
50
0
2DG
CQ
2DG + CQ
Ctrl
0.6
1.2
2.4
4.8
2DG mM
Ctrl
5
10
20
40
CQ µM
Concentration
Viable cells after 48 h (HL60)
Percent of untreated control
150
100
50
0
Metformin
CQ
Metformin + CQ
*
**
***
Ctrl
2.5
5
10
20
Metformin (mM)
Ctrl
5
10
20
40
CQ (µM)
Concentration
Viable cells after 48 h (MOLM-13)
Percent of untreated control
150
100
50
0
Metformin
CQ
Metformin + CQ
Ctrl
2.5
5
10
20
Metformin (mM)
Ctrl
5
10
20
40
CQ (µM)
Concentration

Figure 7.

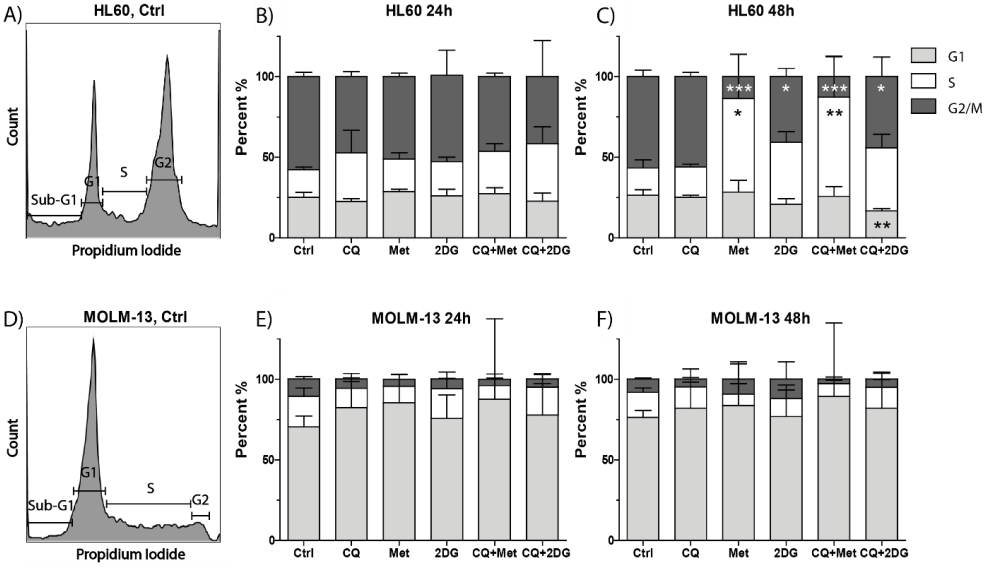
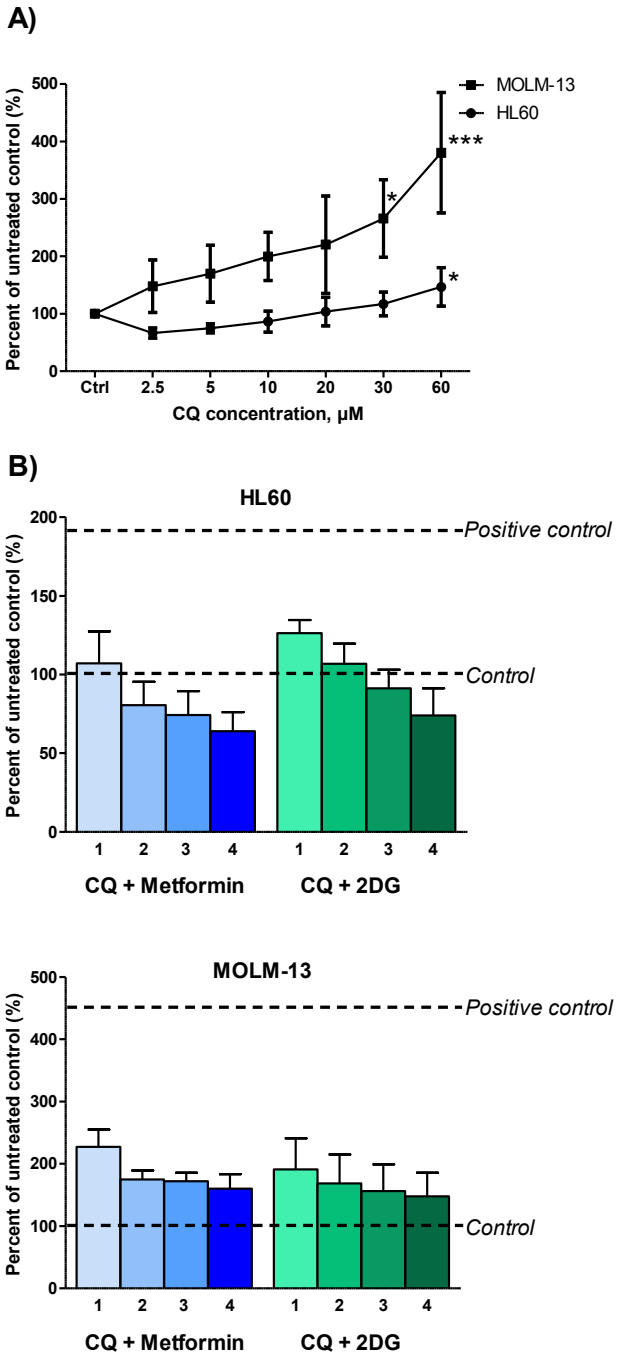

A)
HL60, Ctrl
Count
G1
S
G2
Sub-G1
Propidium Iodide
B)
HL60 24h
Percent %
100
50
0
Ctrl
CQ
Met
2DG
CQ+Met
CQ+2DG
C)
HL60 48h
Percent %
***
*
*
***
**
*
**
G1
S
G2/M
D)
MOLM-13, Ctrl
Count
G1
S
G2
Sub-G1
Propidium Iodide
E)
MOLM-13 24h
Percent %
F)
MOLM-13 48h
Percent %

Figure 8.


A)
MOLM-13
HL60
Percent of untreated control (%)
500
400
300
200
100
0
***
*
*
Ctrl
2.5
5
10
20
30
60
CQ concentration, μM
B)
HL60
Positive control
Control
Percent of untreated control (%)
200
150
100
50
0
1
2
3
4
1
2
3
4
CQ + Metformin
CQ + 2DG
MOLM-13
Positive control
Control
Percent of untreated control (%)
500
400
300
200
100
0
1
2
3
4
1
2
3
4
CQ + Metformin
CQ + 2DG

SUPPLEMENTARY INFORMATION

EFFECTS OF THE AUTOPHAGY-INHIBITING AGENT CHLOROQUINE IN ACUTE MYELOID LEUKEMIA

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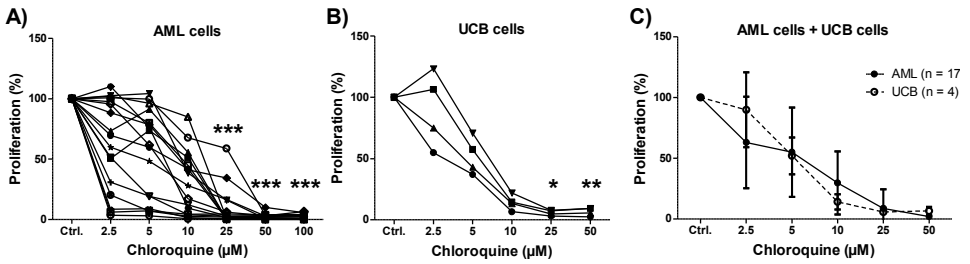
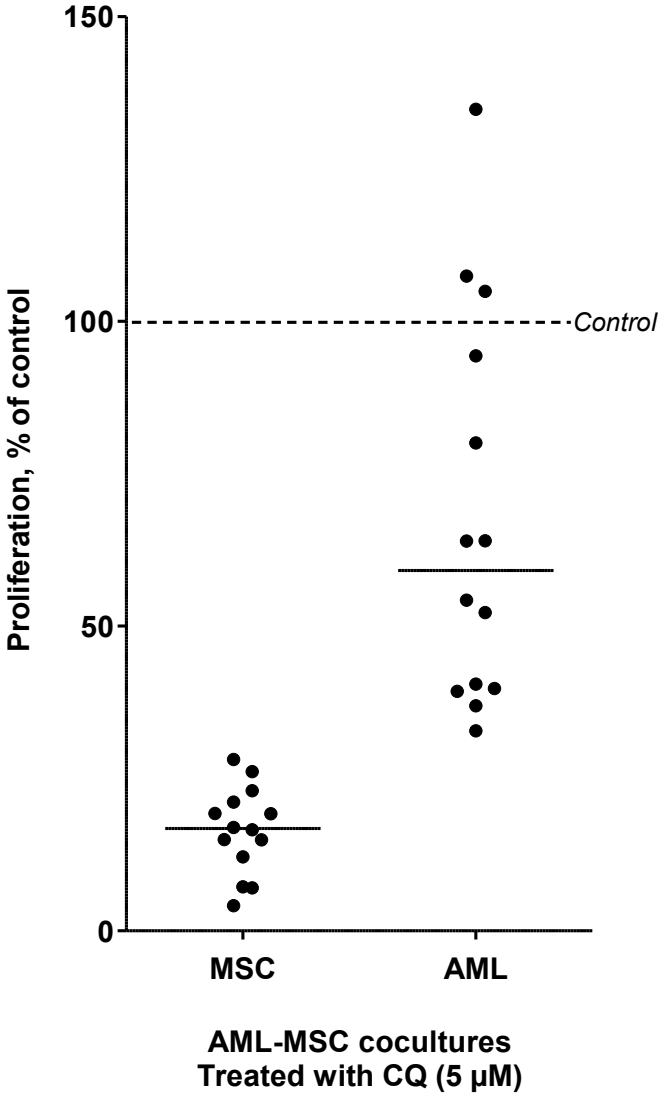
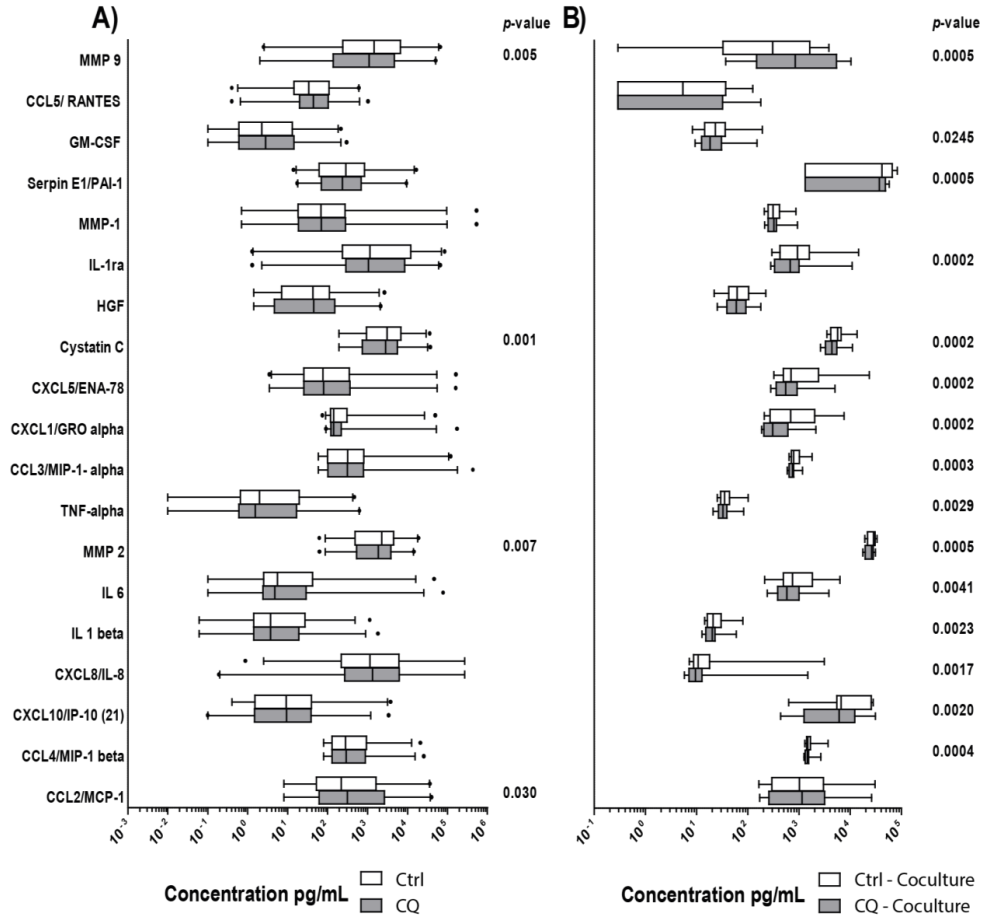


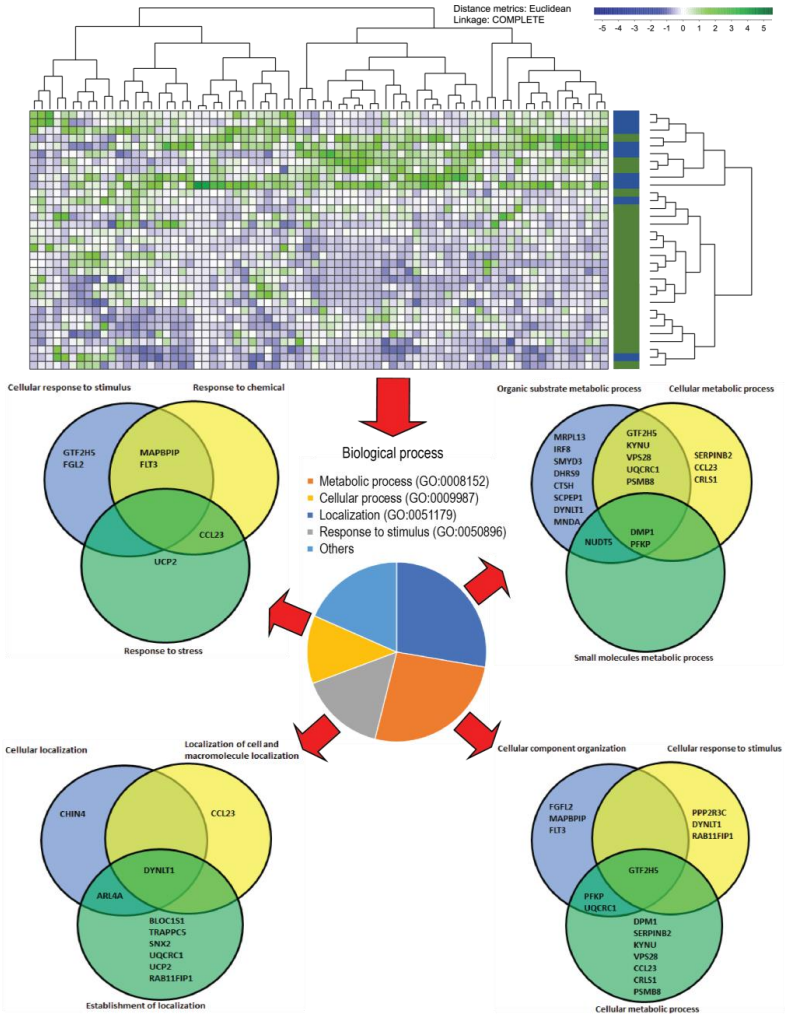
Supplementary Figure 1. Initial *in vitro* drug screening study of chloroquine (CQ) on patient-derived AML cells and UCB mononuclear cells. Cells were treated with CQ for six days before [^{3}H]-thymidine was added to cultures for an additional 22 hours of incubation, before proliferation (nuclear incorporation) was determined by liquid scintillation counting. **A)** Leukemic cells derived from 17 AML patients were treated with CQ at six different concentrations (2.5 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM), and **B)** UCB mononuclear cells from four donors were treated with CQ at five different concentrations (2.5 µM, 5 µM, 10 µM, 25 µM and 50 µM). Detectable incorporation was defined as > 1000 counts per minute (cpm). Results are shown as the percent proliferation of CQ-treated cultures compared to the respective untreated control cultures (set to 100%). At lower concentrations (2.5 µM and 5 µM) there was a varied sensitivity towards CQ, but at higher concentrations (10 µM - 100 µM) all samples showed decreased proliferation compared to untreated controls, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.0001, Kruskal-Wallis, Dunn´s post-hoc test. **C)** The figure shows the overall mean cell proliferation with SD, for all 17 AML patients and four UCB donors. There were no significant differences between the anti-proliferative effects of CQ on AML cells compared to UCB cells.



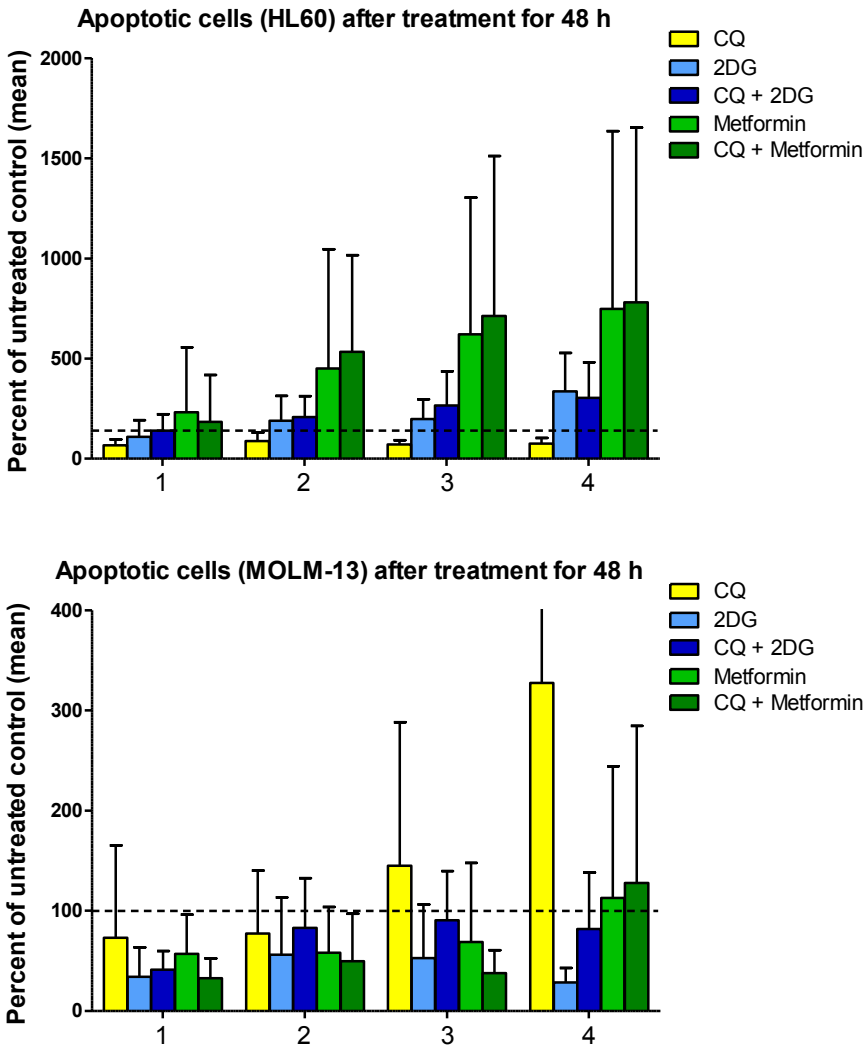
Supplementary Figure 2. The effect of CQ on cell proliferation of primary AML cells and normal MSCs grown in coculture. Cocultures of AML cells derived from 14 patients and normal MSCs derived from one donor were treated with 5 μM CQ for three days. Proliferation was measured using the [^{3}H]-thymidine incorporation assay, and percent proliferation was then determined for both AML cells and MSCs in cocultures, with and without CQ-treatment. The results are shown as percent proliferation of CQ-treated cocultures compared to untreated cocultures. CQ inhibited proliferation of both AML cells and MSCs in cocultures.



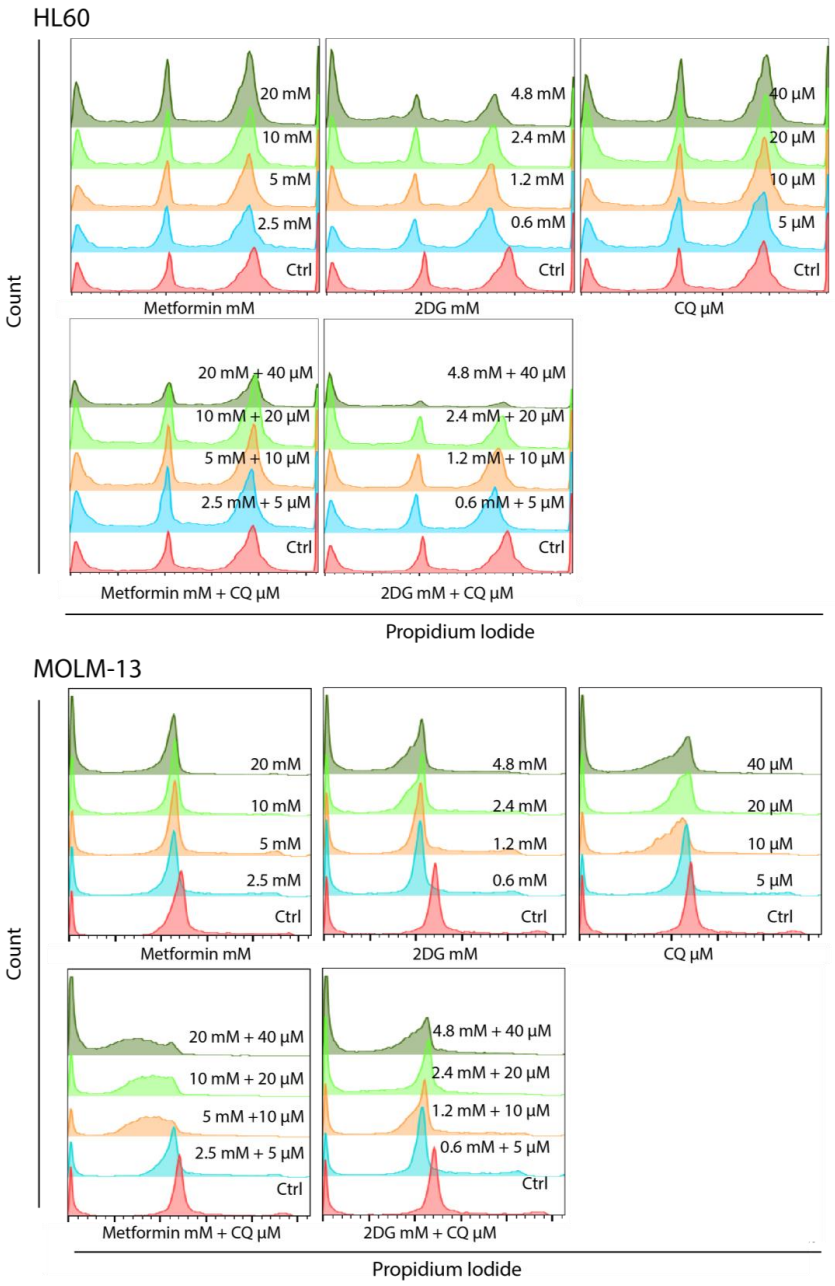
Supplementary Figure 3. Effect of chloroquine treatment on the constitutive release of soluble mediators in AML cell cultures and in AML-MSC cocultures. A) Primary AML cells from 72 patients were incubated for 48 hours with CQ 5 µM or medium alone before supernatants were harvested and the concentrations of the soluble mediators were determined. The results are presented as the median levels, 25/75 percentiles (box), 5/95 percentiles (whiskers) and outliers, for CQ treated cultures (CQ) and untreated cultures (Ctrl). Significant alterations are shown in the figure (Wilcoxon signed rank test). **B)** Primary AML cells derived from 14 patients were cocultured with normal MSCs derived from one donor. The cells were cultured together in transwell cocultures, i.e. the two cell types have no direct contact. After 48 hours' incubation with CQ 5 µM or in medium alone, supernatants were harvested and the concentrations of the soluble mediators were determined. The results are presented as the median levels, 25/75 percentiles (box), 5/95 percentiles (whiskers) and outliers, for CQ treated cocultures (CQ - Coculture) and untreated cocultures (Ctrl - Coculture). Significant alterations (p-value <0.05) are shown in the figure (Wilcoxon signed rank test).



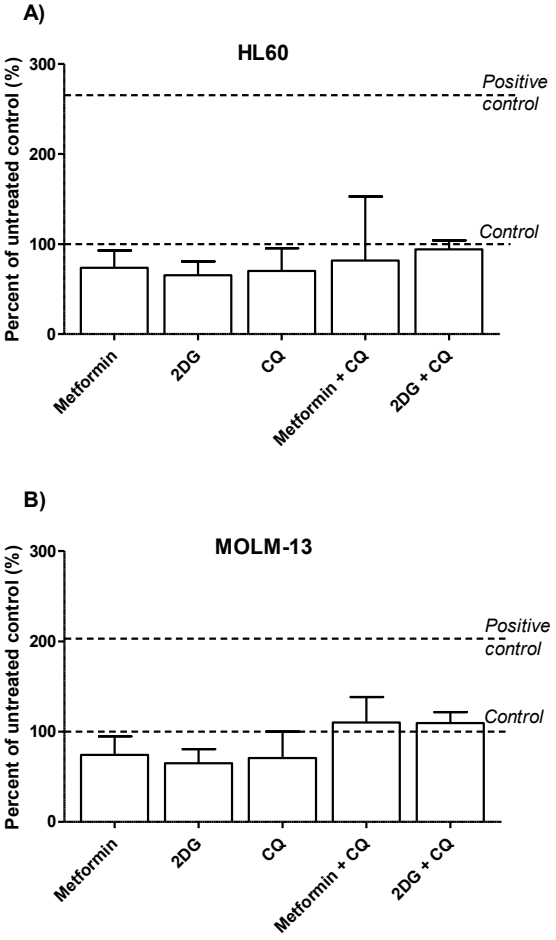
Supplementary Figure 4. Gene expression profiles associated with levels of soluble mediators released by AML cells after CQ treatment. Total gene expression profiling was available for 33 AML patients included in the study, and we compared the 24 patients with low/intermediate cytokine release after CQ-treatment (Figure 4, the two lower subclusters) with the nine patients showing high cytokine release (Figure 4, top subcluster). Using ANOVA, and by setting an F-score >1.0 and a fold change (FC) value >1.0, we identified 74 genes differently expressed between the two patient populations. These genes were used to perform an unsupervised hierarchical cluster analysis (Euclidean distance matrix, with complete linkage), which could discriminate the two patient's subsets (Upper part). Patients with high release of soluble mediators after CQ-treatment are shown in blue, while patients with low/intermediate release are shown in green. The genes encoding proteins with a known function were classified using the PANTHER database. By using the term biological process, we identified metabolic process, cellular process, localization and response to stimulus as the four most frequent subgroups. Finally, we searched for GO-terms in these four subgroups and the singles genes for each of these subgroups are shown in the colored circles (lower part).



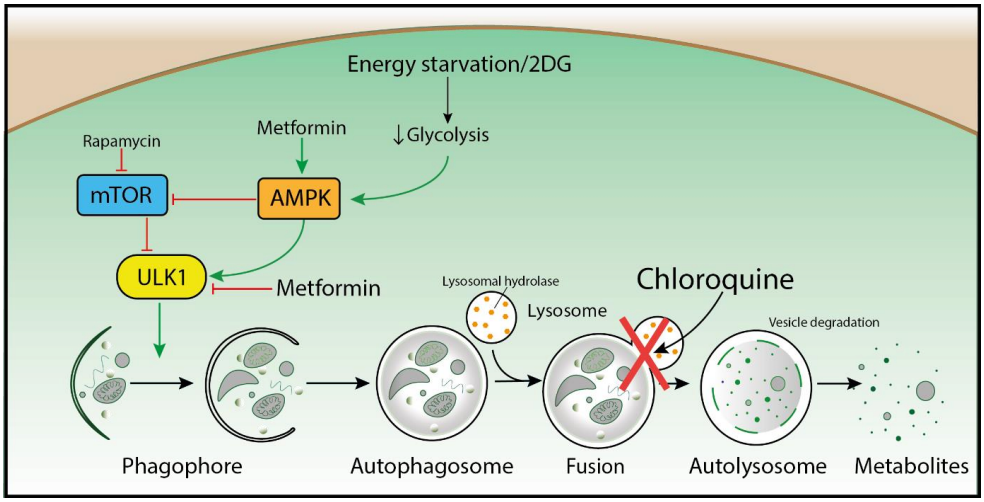
Supplementary Figure 5. Apoptosis in HL60 and MOLM-13 cell lines after treatment with increasing concentrations of drugs. Apoptosis was measured by flow cytometry after treatment of cell lines for 48 hours with drugs alone (CQ, 2DG or metformin) or drugs in combination (CQ combined with either 2DG or metformin). Increasing concentrations (2x) of drugs were tested: **(1)** CQ 5 µM, metformin 2.5 mM and 2DG 0.6 mM, **(2)** CQ 10 µM, metformin 5 mM and 2DG 1.2 mM, **(3)** CQ 20 µM, metformin 10 mM and 2DG 2.4 mM and **(4)** CQ 40 µM, metformin 20 mM and 2DG 4.8 mM. Cells incubated in medium alone were used as drug-free controls (illustrated with stippled line at 100%). The flow cytometric Annexin V/PI assay was used to measure apoptotic cells. No significant differences were found using statistical analyses (one-way ANOVA, with Dunnett´s multiple comparison posttest), but as illustrated in the figure, there was a dose dependent increase in apoptosis after treatment of HL60 cells with all drugs except CQ, though CQ induced apoptosis in MOLM-13 cells. Data is shown as mean values (percent of untreated controls) ±SD, of three repeated experiments.



Supplementary Figure 6. Cell cycle distribution of cell lines with and without drug treatment. Representative results of three repeated experiments, showing the distribution of cells in sub-G1, G1, S and G2/M phase in HL60 and MOLM-13 cells treated with CQ (5 µM, 10 µM, 20 µM and 40 µM), metformin (2.5 mM, 5 mM, 10 mM and 20 mM), and 2DG (0.6 mM, 1.2 mM, 2.4 mM and 4.8 mM), alone and in combination for 48 hours. Cell cycle distribution was analyzed using flow cytometry after staining DNA with propidium iodide (PI).



Supplementary Figure 7. No significant alteration in ROS production in cell lines after treatment with CQ, metformin or 2DG, alone or in combination. Levels of ROS were measured in HL60 and MOLM-13 cells using the CellROX flow cytometric assay. **A)** HL60 and **B)** MOLM-13 cells were incubated with CQ 5 μM, metformin 2.5 mM and 2DG 0.6 mM alone and in combination for 6 hours before measuring ROS levels. A slight non-significant decrease in ROS production was seen after treatment with the metabolic drugs alone for both HL60 and MOLM-13 cells; however, combination of drugs did not alter ROS production. Cells treated with TBHP 300 μM were used as a positive control (indicated with a stippled line at the top) which greatly induced ROS generation in both cell lines. The data are presented as percent of untreated controls (indicated with the lower stippled line), each column represents mean of three repeated assays, with SD.



Supplementary Figure 8. Schematic illustration of the main steps of autophagy, including the target points for chloroquine, metformin and 2DG. Cytosolic material is sequestered by an expanding membrane sac, the phagophore. The phagophore expands into a double membrane vesicle, an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, forming an autolysosome, this leads to degradation of the material inside the autophagosomes, as well as vesicle degradation. *Chloroquine* inhibits autophagy by raising the lysosomal pH, prohibiting the fusions of autophagosomes with lysosomes and disrupting lysosomal protein degradation. *Metformin* is reported to have conflicted roles in autophagy, it activates AMPK, indirectly inhibiting mTOR and activating ULK1. At the same time, it has been shown to directly inhibit ULK1. *2DG* triggers glucose starvation, activating autophagy through AMPK. However, in contrast, in combination with other autophagy inhibitors, 2DG seems to have an additive effect on inhibition of autophagy.

Supplementary Table 1. The complete list of 99 significantly increased genes (F-score >1.0 and FC value >1.0) in patient samples with high sensitivity towards CQ. List is shown in alphabetical order.

GENE ID	FULL NAME	POTENTIAL ROLE IN AML	PUBMED ID
ADA	Adenosine deaminase	High levels of adenosine deaminase are associated with unfavourable outcome in AML	7049266
AHCY	Adenosylhomocysteinase		
AK2	Adenylate kinase 2		
APRT	Adenine phosphoribosyltransferase		
ARMET/ MANF	Mesencephalic astrocyte derived neurotrophic factor		
ATP5G1	ATP synthase membrane subunit c locus 1		
C11orf1	Chromosome 11 open reading frame 1		
C5orf15	Chromosome 5 open reading frame 15		
CCDC56/ COA3	Cytochrome c oxidase assembly factor 3		
CCL23	C-C motif chemokine ligand 23	Chemokine involved in leukemogenesis	17339182
CNST	Consortin, connexin sorting protei	Connexins are expressed in AML	25529637
COPB1	COPI coat complex subunit beta 1		
CTSC	Cathepsin C		
CWC15	CWC15 spliceosome associated protein homolog		
DCTN3	Dynactin subunit 3		
DKC1	Dyskerin pseudouridine synthase 1		
DNTT	DNA nucleotidylexotransferase		
DUSP23	Dual specificity phosphatase 23		
EBNA1BP2	EBNA1 binding protein 2		
EBPL	EBP like		
FMC1	Formation of mitochondrial complex V assembly factor 1 homolog		
GAPT	GRB2 binding adaptor protein, transmembrane		
GLO1	Glyoxalase I	Decrease of glyoxalase I activity is associated with differentiation in AML	3166382
GNG10	G protein subunit gamma 10		
GNL2	G protein nucleolar 2		
GPR56	G protein-coupled receptor 56	Possible involvment in AML leukemogenesis	27063597
GSTM1	Glutathione S-transferase mu 1	Polymorphisms in GSTM1 increase the risk of developing AML	24854448
GSTM2	Glutathione S-transferase mu 2		
GUCY1A3	Guanylate cyclase 1 soluble subunit alpha 1	Associated with a leukemic stem cell gene signature in AML	21177505
HIST1H1C	H1.2 linker histone, cluster member		
HSH2D	Hematopoietic SH2 domain containing		
HSPA9	Heat shock protein family A (Hsp70) member 9	HSP70 inhibitors have antileukemic activity	23586877
HSPD1P5	Heat shock protein family D (Hsp60) member 1 pseudogene 5		
IGLL3	Immunoglobulin lambda like polypeptide 3, pseudogene		
ITGAE	Integrin subunit alpha E		
ITGB1BP1	Integrin subunit beta 1 binding protein 1		
LAT2	Linker for activation of T cells family member 2	Possibly involved in the t(8;21)((q22;q22)) translocation in acute leukemia	21488857
LPIN1	Lipin 1		
LYRM1	LYR motif containing 1		
LYSMD2	LysM domain containing 2		

MAGED1	MAGE family member D1	An AML-associated antigen	25092142
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	High expression of MAP4K1 has been associated to poor prognosis in AML	31522654
MIR155HG	MIR155 host gene	miR-155 has potentail negative prognostic impact in AML	23650424
MRPL21	Mitochondrial ribosomal protein L21		
MRPL24	Mitochondrial ribosomal protein L24		
MRPL36	Mitochondrial ribosomal protein L36		
MRPL45	Mitochondrial ribosomal protein L45		
MRPL54	Mitochondrial ribosomal protein L54		
NDUFA13	NADH:ubiquinone oxidoreductase subunit A13		
NDUFA8	NADH:ubiquinone oxidoreductase subunit A8		
NDUFB6	NADH:ubiquinone oxidoreductase subunit B6		
NENF	Neudesin neurotrophic factor		
NME4	NME/NM23 nucleoside diphosphate kinase 4		
NT5C3	5'-nucleotidase, cytosolic IIIA	NT5C3 polymorphism associated with response to chemotherapy in AML	25000516
NUDT1	Nudix hydrolase 1		
NUDT5	Nudix hydrolase 5		
PAICS	Phosphoribosylaminoimidazole carboxylase and Phosphoribosylaminoimidazole-succinocarboxamide synthase		
PARP1	Poly(ADP-ribose) polymerase 1	High PARP-1 expression is a predictor for poor survival in AML patients	30472087
PBX3	PBX homeobox 3	Found to interact in MLL-rearranged AML	26747896
PDLIM1	PDZ and LIM domain 1		
PFKP	Phosphofructokinase, platelet		
PHB2	Prohibitin 2		
PIM1	Pim-1 proto-oncogene, serine/threonine kinase	Expressed in AML patients and possible prognostic marker	28851457
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	Increased expression in AML, and a potential pharmacological target	29848341
POLR3GL	RNA polymerase III subunit G like		
PRMT1	Protein arginine methyltransferase 1	Promotes survival and growth of FLT3-ITD^{+} AML cells	3121718
PROK2	Prokineticin 2		
PSMD4	Proteasome 26S subunit, non-ATPase 4		
RAB37	RAB37, member RAS oncogene family		
RPA3	Replication protein A3		
RPL12P6	Ribosomal protein L12 pseudogene 6		
RPS26P2	Ribosomal protein S26 pseudogene 2		
RPS26P53	RPS26P53 ribosomal protein S26 pseudogene 53		
RPS26P55	Ribosomal protein S26 pseudogene 55		
SELS			
SLC2A5	Solute carrier family 2 member 5	Enhanced fructose utilization in AML cells , and a potential therapeutic target	27746145
SLC39A3	Solute carrier family 39 member 3		
SLC9A3R1	SLC9A3 regulator 1		
SMC4	Structural maintenance of chromosomes 4	Involved in leukemogenesis related to chromosomes condensing	29043883
SOCS2	Suppressor of cytokine signaling 2	High expression of SOCS2 associated with negative outcome in AML	24559289
SPOP	Speckle type BTB/POZ protein		

SSB	Small RNA binding exonuclease protection factor		
SUCLG1	Succinate-CoA ligase GDP/ADP-forming subunit alpha	Associated with possible prognostic gene profile in AML	29138577
TBCB	Tubulin folding cofactor B		
TECR	Trans-2,3-enoyl-CoA reductase		
TMED9	Transmembrane p24 trafficking protein 9		
TOMM22	Translocase of outer mitochondrial membrane 22		
TSEN34	tRNA splicing endonuclease subunit 34		
UCKL1	Uridine-cytidine kinase 1 like 1		
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1		
WDR61	WD repeat domain 61		
ZMYM6	Zinc finger MYM-type containing 6		
ZNF207	Zinc finger protein 207		

Supplementary Table 2. The complete list of 74 significantly increased genes (F-score >1.0 and FC value >1.0) in patient samples with upregulated release of soluble mediators after CQ treatment. List is shown in alphabetical order.

GENE ID	FULL NAME	POTENTIAL ROLE IN AML	PUBMED ID
ADAP1/ CENTA1	Arf GAP with dual PH domains 1		
ANXA2P1	Annexin A2 pseudogene 1		
ANXA5	Annexin A5		
ARL4A	ADP ribosylation factor like GTPase 4A		
BLOC1S1	Biogenesis of lysosomal organelles complex 1 subunit 1		
BLOC1S1	Biogenesis of lysosomal organelles complex 1 subunit 1		
C5orf15	Chromosome 5 open reading frame 15		
CCL23	C-C motif chemokine ligand 23	Chemokine involved in leukemogenesis	
CDKN1A	Cyclin dependent kinase inhibitor 1A		
CKS1B	CDC28 protein kinase regulatory subunit 1B		
CNIH4	Cornichon family AMPA receptor auxiliary protein 4		
COMMD8	COMM domain containing 8		
CRLS1	Cardiolipin synthase 1		
CTSH	Cathepsin H		
DHRS9	Dehydrogenase/reductase 9		
DPM1	Dolichyl-phosphate mannosyltransferase subunit 1		
DYNLT1	Dynein light chain Tctex-type 1	Possible involved in the t(7;11)(p15;p15) translocation in acute leukemia	12446457
EAF2	ELL associated factor 2	Possible involved in the (11;19)(q23;p13.1) translocation in acute leukemia	12446457
EIF5A	Eukaryotic translation initiation factor 5A-2	Involved in chemoresistance in AML	30745844
EVI2A	Ecotropic viral integration site 2A		
FABP5	Fatty acid binding protein 5		
FAM45A	Family with sequence similarity 45		
FGL2	Fibrinogen like 2		
FLT3	Fms related tyrosine kinase 3	Both FLT3 mutation and FLT3 overexpression have prognostic impact in AML	
FUT4	Fucosyltransferase 4		
GLB1	Galactosidase beta 1		
GRN	Granulin precursor		
GTF2H5	General transcription factor IIH subunit 5		
HEBP2	Heme binding protein 2		
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1		
HLA-DRB5	Major histocompatibility complex, class II, DR beta 5		
HMGB1P37	High mobility group box 1 pseudogene 37		
IAH1	Isoamyl acetate-hydrolyzing esterase		
IGFBP7	Insulin like growth factor binding protein 7	Induces differentiation and loss of survival in LSCs	30540936
IRF8	Interferon regulatory factor 8	Possible tumor-suppressor role for IRF8 in AML	30266821
ITGAE	Integrin subunit alpha E		
KYNU	Kynureninase	Found downregulated in IDH mutated AML cases	21647152

LSM3P4	U6 small nuclear RNA and mRNA degradation associated pseudogene 4		
LY86	Lymphocyte antigen 86	Identified in gene signature for LSCs, associated with chemoresistance	20371479
LY96	Lymphocyte antigen 96		
MAPBPIP	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 2		
MCUB/ CCDC109B	Mitochondrial calcium uniporter dominant negative subunit beta		
MMADHC	Metabolism of cobalamin associated D		
MNDA	Myeloid cell nuclear differentiation antigen	Correlated with myeloid and monocytic differentiation in acute leukemia	2259228
MRPL13	Mitochondrial ribosomal protein L13		
MRPL36	Mitochondrial 54S ribosomal protein YmL36		
MS4A7	Membrane spanning 4-domains A7		
NDUFA13	NADH ubiquinone oxidoreductase subunit A13		
NDUFB9	NADH: ubiquinone oxidoreductase subunit B9		
NUDT5	Nudix hydrolase 5		
PECAM1	Platelet and endothelial cell adhesion molecule 1	Bone marrow retention of AML cells depends on PECAM1 expression	17875702
PFKP	Phosphofructokinase, platelet		
PGAM1	Phosphoglycerate mutase 1		
PPP2R3C	Protein phosphatase 2 regulatory subunit B"gamma		
PQLC3	Solute carrier family 66 member 3		
PSMB8	Proteasome 20S subunit beta 8	Immunoproteasome subunits critical for malignant cells to escape immune recognition	23770850
RAB11 FIP1	RAB11 family interacting protein 1		
RPL21P14	Ribosomal protein L21 pseudogene 14		
SCPEP1	Serine carboxypeptidase 1		
SERPINB2	Serpin family B member 2	Important for stem cells in response to various existing chemicals	29925837
SLC35A1	Solute carrier family 35 member A1		
SLC7A7	Solute carrier family 7 member 7		
SMYD3	SET and MYND domain containing 3		
SNHG8	Small nucleolar RNA host gene 8		
SNX2	Sorting nexin 2	Found to interact in MLL-rearranged AML	11438682
TRAPPC5	Trafficking protein particle complex 5		
UCP2	Uncoupling protein 2		
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1		
UTP6	UTP6 small subunit processome component		
VCAN	Versican	Possible prognostic biomarker in AML	29861382
VPS28	VPS28 subunit of ESCRT-I		
WDR61	WD repeat domain 61		
ZFR	Zinc finger RNA binding protein		

III

cells

MDPI

Article

Systemic Metabolomic Profiling of Acute Myeloid Leukemia Patients before and During Disease-Stabilizing Treatment Based on All-Trans Retinoic Acid, Valproic Acid, and Low-Dose Chemotherapy

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Abstract: Acute myeloid leukemia (AML) is an aggressive malignancy, and many elderly/unfit patients cannot receive intensive and potentially curative therapy. These patients receive low-toxicity disease-stabilizing treatment. The combination of all-trans retinoic acid (ATRA) and the histone deacetylase inhibitor valproic acid can stabilize the disease for a subset of such patients. We performed untargeted serum metabolomic profiling for 44 AML patients receiving treatment based on ATRA and valproic acid combined with low-dose cytotoxic drugs (cytarabine, hydroxyurea, 6-mercaptopurin) which identified 886 metabolites. When comparing pretreatment samples from responders and non-responders, metabolites mainly belonging to amino acid and lipid (i.e., fatty acid) pathways were altered. Furthermore, patients with rapidly progressive disease showed an extensively altered lipid metabolism. Both ATRA and valproic acid monotherapy also altered the amino acid and lipid metabolite profiles; however, these changes were only highly significant for valproic acid treatment. Twenty-three metabolites were significantly altered by seven-day valproic acid treatment ($p < 0.05$, $q < 0.05$), where the majority of altered metabolites belonged to lipid (especially fatty acid metabolism) and amino acid pathways, including several carnitines. These metabolomic effects, and especially the effects on lipid metabolism, may be important for the antileukemic and epigenetic effects of this treatment.

Keywords: acute myeloid leukemia; all-trans retinoic acid; lipids; metabolomics; valproic acid

1. Introduction

Acute myeloid leukemia (AML) is an aggressive malignancy [1], and the only possible cure is intensive therapy possibly including stem cell transplantation [2]. The median age at first diagnosis is 65–70 years [3,4], and the elderly patients above 70–75 years of age have an increased frequency of high-risk disease [4] where remission is unlikely [5,6]. In addition, these patients are often not eligible to receive intensive treatment [7]. Furthermore, intensive chemotherapy is not always recommended for patients with refractory or relapsed disease either [8]. Such patients may instead receive low-toxicity AML-stabilizing treatment, e.g., a histone deacetylase (HDAC) inhibitor combined with all-trans retinoic acid (ATRA) and low-dose cytotoxic drugs [9].

HDAC inhibitors increase acetylation of various proteins, including histones [10], and this last effect increases gene transcription [11], especially in AML cells where HDACs often are overexpressed [12]. Valproic acid is a short-chain fatty acid that functions as an HDAC inhibitor [13] and has antiproliferative and proapoptotic effects in AML cells [14–18]. The toxicity of this drug is low, and its antileukemic activity alone or in combination with ATRA has been documented in several clinical studies [19,20]. ATRA is a derivative of vitamin A that binds to retinoid-responsive nuclear receptors [12,21]; it has antileukemic activity [22–24] and seems to increase the antileukemic effects of HDAC inhibitors [25,26]. Thus, the combination of ATRA and valproic acid seems to be a low-toxicity AML-stabilizing treatment; 30–40% of the patients respond to this therapy and responses may last up to one to two years [19,20]. This treatment can also be used in elderly and unfit patients [19,27]. Finally, AML cell metabolism is important for chemosensitivity in AML [28,29], and both ATRA and valproic acid can modulate cellular and/or systemic metabolism [30–37].

Even though the antileukemic in vivo effects of both ATRA and valproic acid are limited, both drugs should still be regarded as candidates for the treatment of non-acute promyelocytic leukemia (non-APL) variants of AML, especially in combination with other antileukemic agents in selected subsets of patients. First, although a large clinical study could not find a general improvement of overall survival when ATRA was added to intensive chemotherapy, increased survival was observed for subsets of patients, especially patients with a favorable prognosis (i.e., patients with genetic abnormalities affecting epigenetic regulation) [38]. A preliminary report from the randomized DECIDER study suggests that ATRA improves survival of AML patients receiving hypomethylating agents [39], and recent experimental studies also suggest that ATRA induces degradation of the mutated NPM1 protein [40], sensitizes AML cells to FLT3 inhibitors [41], and is effective in AML cells carrying the metabolism-modulating IDH mutations [42]. Second, clinical studies suggest that valproic acid improves survival in certain AML subsets (i.e., patients with NPM1 mutations) [43], and recent experimental studies suggest that it has synergistic effects when combined with autophagy-inhibiting chloroquine [44] or inhibitors of DNA methyltransferase [45]. HDAC-induced epigenetic reprogramming may also increase the susceptibility to ATRA [46]. For future studies, predictive molecular networks may become important to identify responders to valproic acid [47].

Metabolomics is emerging as a powerful approach to study cancer metabolism, and a metabolomics study showed that systemic metabolic markers (i.e., an altered glucose metabolism signature) was associated with prognosis in AML [28]. Such metabolomic signatures probably reflect both the characteristics of the AML cells/disease and the influence of a severe disease on systemic metabolic regulation. The overall systemic metabolomic profiles may also be important for leukemic growth and chemosensitivity. AML cells show metabolic plasticity; however, leukemia growth and chemosensitivity also seem to depend on the environmental context, possibly including the systemic supply of nutrients and especially amino acid and fatty acid metabolism [48,49]. The systemic metabolomic effects of ATRA and valproic acid may thus further contribute to the effects of these agents on AML cells. In this context, we have performed systemic metabolomic profiling of AML patients treated with the combination of ATRA, valproic acid, and low doses of conventional cytotoxic drugs, and we also examined the effects of both ATRA and valproic acid monotherapy on systemic metabolite profiles of AML patients. In our present study, we have focused on metabolite pathways rather than single metabolites. The aims of our study were to compare the pretreatment metabolomic profiles found in responders and non-responders to the AML-stabilizing treatment, and to characterize how these profiles are altered during the early period of this antileukemic treatment (two-day or seven-day monotherapy with either ATRA or valproic acid, respectively).

2. Materials and Methods

2.1. Patients

The study was approved by the Regional Ethics Committee (REK 2017/305 070417, REK Vest 2015/1410 190615, 215.03 120504, 231.06 150307) and included 44 elderly or unfit patients (25 men and 19 women; median age 76.4 years with range 61–86 years) with high-risk leukemia, i.e., AML relapse, secondary AML, complex karyotype and/or TP53 mutations (Table S1). Patients with APL were excluded. The patients represent an unselected subset of the consecutive patients included in two clinical studies [19,27]. Eighteen of the patients were classified as responders to the treatment according to the myelodysplastic syndrome (MDS) criteria [50]; the others 26 patients did not respond to the treatment and six of them showed a rapidly progressive disease (patients 39–44).

2.2. The Antileukemic Treatment Based on ATRA, Valproic Acid, and Low-Dose Cytotoxic Drugs

All serum samples were collected from patients included in two previously published clinical studies conducted at Haukeland University Hospital in Bergen, based on combined treatment with oral ATRA (22.5 mg/m^2 twice daily for 14 days every 12th week), continuous valproic acid therapy at maximal tolerated doses, and low-dose therapy with hydroxyurea, 5-mercaptopurine, and/or cytarabine [19,27]. Samples were taken prior to treatment, and also after receiving ATRA alone (after two days of treatment) or valproic acid alone (after seven days of treatment), as described in the following sections (see Figure S1).

Patients included in the first study [27] received oral ATRA as described above. On day three of the first cycle the patients received valproic acid as an initial intravenous infusion with a loading dose (5 mg/kg) followed by continuous infusion (28 mg/kg/24 h), and theophylline with a loading dose (5 mg/kg) followed by continuous infusion (0.65 mg/kg/hour). After five days (day 8), the patients received oral valproic acid (serum levels between 300–600 μmol/L) and oral theophylline adjusted to reach the therapeutic level of 50–100 μM. Patients with (i) peripheral blood blasts exceeding 50×10^9/L at diagnosis or (ii) later increasing blast counts received cytotoxic drugs to control leukocytosis.

Patients included in the second study [19] received valproic acid monotherapy for the first seven days with loading dose and subsequent continuous infusion for 24 h before oral valproic acid treatment (serum levels 300–600 μmol/L). On days 8–22, the patients received ATRA, and on days 15–24, they received subcutaneous cytarabine (10 mg/m^2 once daily). ATRA and cytarabine treatment were repeated at 12-week intervals. Patients with later increasing peripheral blood blasts (>50×10^9/L) received hydroxyurea or 5-mercaptopurine instead of cytarabine.

There is no general agreement with regard to which response criteria should be used for patients receiving low-toxicity AML stabilizing treatment [20], and our patients were therefore evaluated both with regard to the conventional AML criteria for remission induction [51] and the MDS response criteria [52]. Two responders in the second study achieved complete hematological remission; the other responders fulfilled the criteria for hematological improvement according to the MDS criteria and lasting for at least eight weeks before progression.

2.3. Preparation and Analysis of Serum Samples

Venous peripheral blood was collected onto sterile serum-clot activator tubes with gel separator (Greiner Bio-One GmbH, Kremsmünster, Austria or BD Vacutainer SST, Becton-Dickenson; Franklin Lakes, NJ, USA). Samples were left upright for 30–120 min at room temperature before being centrifuged (1300× *g*, 10 min). The serum supernatants were immediately allocated in plastic cryotubes (Nunc^{TM,} Roskilde, Denmark) and stored at –80 °C. The patients had no dietary restrictions, and all samples were collected from fasting patients, between 7:30 and 8:30 in the morning before breakfast.

The untargeted metabolomic profile analyses were performed in collaboration with Metabolon Inc (Durham, NC, USA) as described previously [53]. Samples were prepared using the automated MicroLab STAR®® system (Hamilton Company, Bonaduz, Switzerland) and extracted using

Metabolon's standard solvent extraction method [53]. Four recovery standards were added prior to the first extraction step, DL-2-fluorophenylglycine, tridecanoic acid, d6-cholesterol and 4-chlorophenylalanine [53]. To remove proteins, dissociate small molecules bound to proteins or trapped in the protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for two minutes (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into two fractions for analysis by two separate reverse phase (RP)/ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS, Waters ACQUITY, Milford, MA, USA) methods with positive ion mode electrospray ionization (ESI, Thermo Scientific, Waltham, MA, USA) RP/UPLC-MS/MS with negative ion mode ESI, and hydrophilic interaction liquid chromatography (HILIC)/UPLC-MS with negative ion mode ESI. A last sample was stored for backup. Samples were placed briefly on a Zymark TurboVap®® (McKinley Scientific, Sparta, NJ, USA) to remove the organic solvent; they were thereafter stored overnight under nitrogen before further preparation. Extracted water samples served as process blanks and solvents used during the extraction process served as solvent blanks. A pooled matrix sample generated by taking a small volume of each experimental sample, and also a large pool of human plasma extensively characterized and prepared by Metabolon, served as technical replicates throughout the data set. Furthermore, a combination of quality control standards that were carefully chosen not to interfere with the analyzes, were spiked into every analyzed sample to allow monitoring of instrument performance and chromatographic alignment as described previously [53].

Instrument variability was determined for each sample by calculating the median relative standard deviation (RSD) prior to injection into the mass spectrometers. The overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples (serum samples). The experimental samples were randomly spaced across the platform and the control samples were evenly spread.

Metabolon Inc maintains a library based on authentical standards that contains the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS spectral data); the metabolites were identified by comparison to this library. Peaks were quantified using area-under the-curve.

2.4. Statistical Analyses

Welch's two sample t-test was used to identify metabolites that differed significantly between different groups, the paired t-test was used comparison of paired samples, and ANOVA contrasts used to analyze pharmacological effects for responders and non-responders separately. Random Forest analysis was used to provide an unbiased estimate of how well individuals can be classified into each group based on the metabolomic data. Metabolite pathway enrichment analysis was used for biological interpretation of metabolite data at a system level using the MetaboLync analysis tool (MetaboLync®® Portal). Differences were regarded as statistically significant when p-values were < 0.05. Statistical analyses were performed with the programs ArrayStudio, R (http://cran.r-project.org/) and the data analyze software program JMP (JMP®®, Statistical Discovery™ from ©SAS Institute Inc., Lane Cove, NSW, Australia).

3. Results

3.1. The Pretherapy Serum Metabolomic Profiles Could Not Predict Responsiveness to Antileukemic Treatment Based on ATRA, Valproic Acid, and Low-Dose Chemotherapy

The serum metabolomic profiles were analyzed in pretreatment serum samples derived from 44 patients (18 responders and 26 non-responders). Untargeted metabolomic profiling identified 886 metabolites, of which approximately 45 significant changes would be expected to occur by chance alone when using $p < 0.05$ as significance level; however, only 36 metabolites showed a significant difference ($p < 0.05$, Welch two-sample t-test) when comparing pretreatment levels of responders and non-responders, and all of these metabolites had high q-values ($q > 0.4$). These results suggest

that some of the statistically significant changes represent false discoveries (Figure S2). First, 10 of the 36 significantly altered metabolites are classified as peptides (gamma-glutamyltyrosine), amino acids (tyrosine, methionine, histidine, lysine, threonine, homoarginine), or amino acid metabolites (indolepropionate, 2-methylbutyrylcarnitine, 5-hydroxylysinepropionyl) (Figure S2). Second, 11 of the 36 significantly altered metabolites were classified as lipid metabolites (Figure S2). Thirdly, there were also significantly altered levels of five metabolites classified as xenobiotics between responders and non-responders (i.e., metabolites not naturally produced by the organism) and two of these were vitamin A metabolites. Finally, only two metabolites from the subclass carbohydrate/energy metabolism (mannose, citrate) differed significantly when comparing responders and non-responders, and both showed only borderline significance ($0.04 > p > 0.05$).

As stated above, the significantly altered metabolites between responders and non-responders showed high q-values in our analysis (Figure S2). Although both fatty acid and amino acid metabolism seem important for the growth and chemosensitivity in human AML [48], and a distinct pretherapy systemic glucose metabolism signature is associated with prognosis (i.e., survival) of younger patients receiving intensive and potentially curative AML therapy [28], we could not identify any highly significant differences in pretreatment systemic metabolite profiles between responder and non-responder patients receiving low-intensity AML-stabilizing treatment. Finally, a random forest analysis based on the overall metabolite profiles showed a predictive accuracy of only 48% for responders versus nonresponders (data not shown).

We also did a metabolite pathway enrichment analysis based on the 36 metabolites with $p < 0.05$ (Figure S3). This analysis showed enriched pathways associated with amino acid and lipid metabolism, although vitamin A metabolism also had a high ranking (two metabolites differed between groups). Taken together, these analyses suggest that, in contrast to patients receiving intensive and potentially curative antileukemic treatment, there is no (or only a minor) prognostic impact of the pretherapy metabolomic profiles in our study for patients receiving leukemia-stabilizing treatment. Although vitamin A metabolism was enriched in the pathway analysis this should be interpreted with great care because only two of the five identified metabolites had borderline significance between responder and non-responder groups (see Figure S2).

3.2. Pretherapy Differences in Fatty Acid Metabolism Are Found between Non-Responders with Rapidly Progressive AML Compared with Other Non-Responders with Less Aggressive Disease

Six non-responders had rapidly progressive disease with increasing peripheral blood blast counts and survival ≤12 days (Table S1, patients 39–44). We compared the metabolomic profile of these six patients with the 20 other non-responders because, in clinical practice, it will be important to identify such patients and start early with alternative therapy. This early cutoff was chosen because a clinically relevant response is usually seen after 2–3 weeks of treatment with ATRA/valproic acid [19,27]. Eighty of the 886 metabolites differed significantly between these two groups (Figure 1, Table S2). Most of the metabolites had a relatively high q-value, but when considering the relatively high number of significantly altered metabolites (80 out of 886 metabolites) and the overall metabolomic profiles/pathways (instead of individual metabolites), the most striking difference between these two groups was the high number of lipid metabolites (45 out of 80 metabolites) of which patients with rapidly progressive disease showed increased levels for 42 out of these 45 lipid metabolites (a more detailed classification of metabolites is presented in Table S2). Thus, the six non-responders with rapidly progressive disease seem to differ from the 20 other non-responders especially with regard to lipid and fatty acid metabolites. Finally, a random forest analysis showed a predictive accuracy of 77% (Figure 2), suggesting that it might be possible to use metabolite profiles to segregate these groups; however, the group size of aggressive non-responders is very small, and the results therefore need to be confirmed in a larger study. The 30 top-ranked metabolites from the random forest analysis included 26 metabolites with a p-value < 0.05 (six amino acid metabolites, 16 lipid metabolites, and four other metabolites; see Figures 1 and 2).

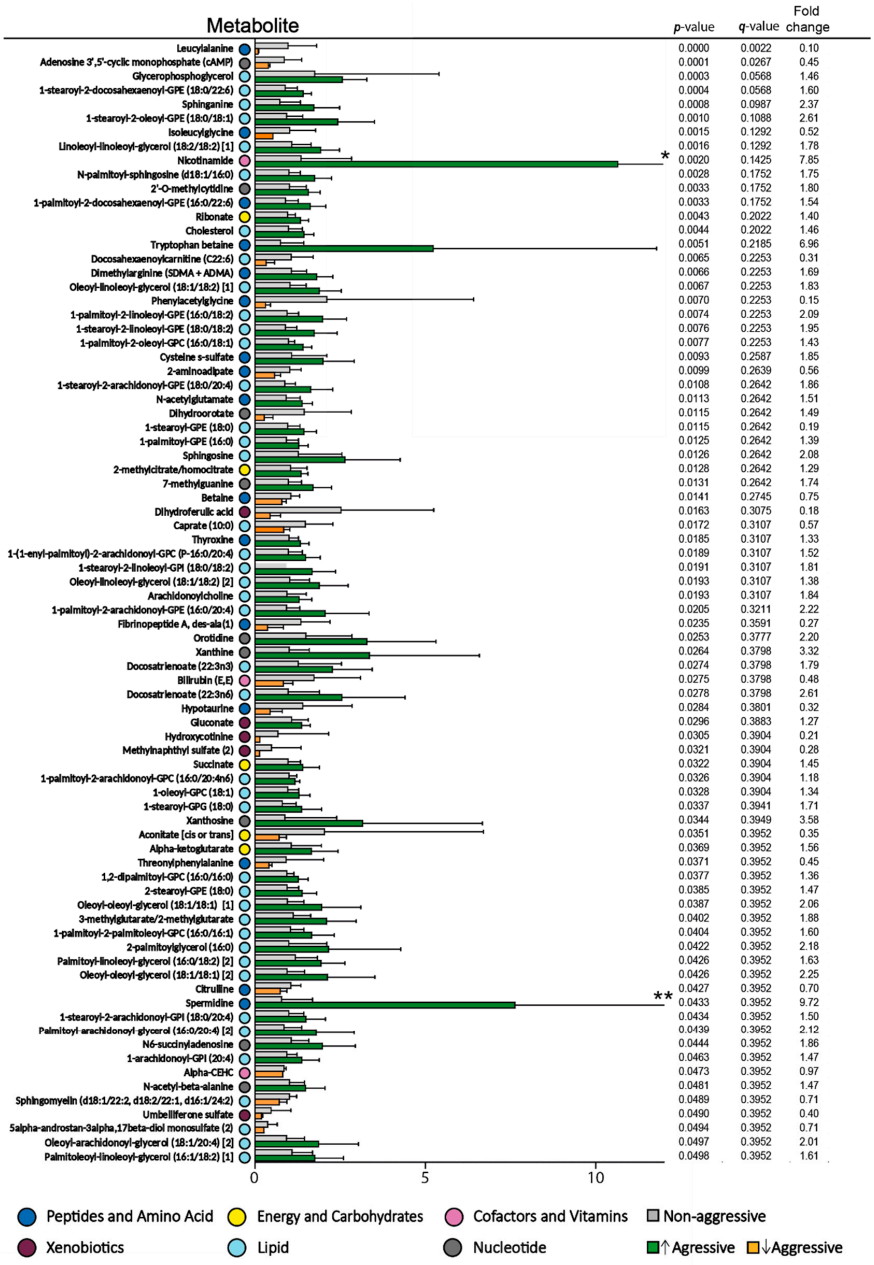

Figure 1. Significantly altered metabolites when comparing the pretreatment levels of non-responders with rapidly progressive disease versus non-responders with less aggressive disease to antileukemic treatment based on all-trans retinoic acid (ATRA) and valproic acid. The *p*-values, *q*-values and mean fold change values for each metabolite are listed to the right in the figure (ranked by *p*-value), and a

fold change >1 indicates that the levels were increased in non-responder patients with very aggressive disease compared to non-responder patients with less aggressive disease. Levels of serum metabolites in non-responders with less aggressive disease are shown in grey, increased levels found in non-responders with aggressive disease are shown in green, while decreased levels in non-responders with aggressive disease are shown in yellow. Color codes for classification of metabolites are explained at the bottom of the figure. Error bars show Standard deviation (SD). * Nicotidamine SD 6.761, ** Spermidine SD 9.051.

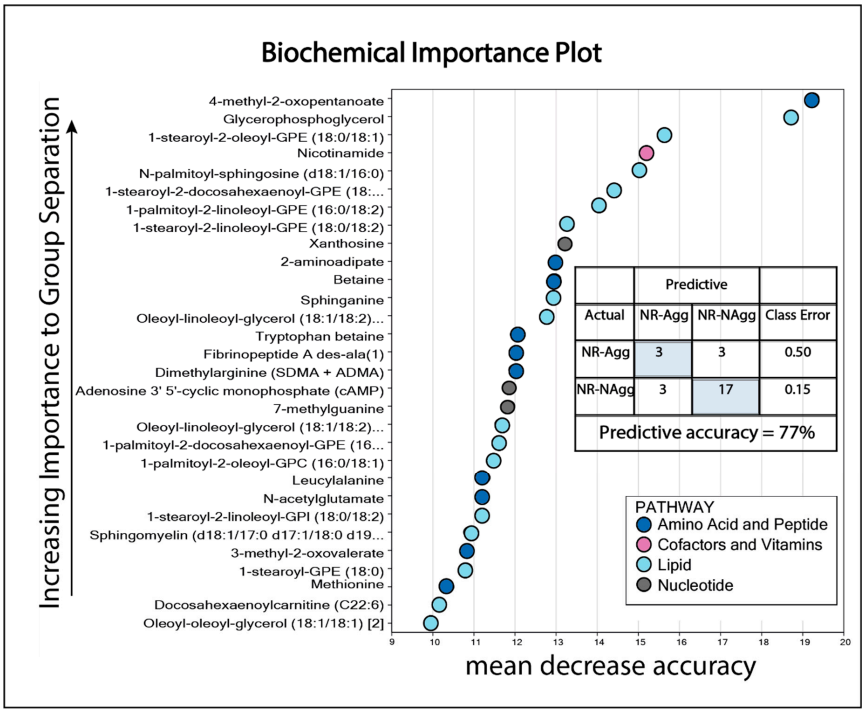

Figure 2. Random forest analysis based on pretreatment serum metabolites when comparing non-responders with rapidly progressive disease versus non-responders with less aggressive disease to antileukemic treatment based on ATRA and valproic acid. The figure shows the 30 top-ranked metabolites from this analysis, which can segregate the two patient groups with a predictive accuracy of 77%. Amino acid and lipid metabolites constituted the majority of the top-ranked metabolites. Color codes for classification of metabolites are shown to the lower right. This analysis included 26 metabolites with a p-value < 0.05 (see Figure 1; six amino acid metabolites, 16 lipid metabolites, and four other metabolites). Abbreviations; NR-Agg, non-responders aggressive disease (i.e., rapidly progressive); NR-NAgg, non-responders non-aggressive disease.

We did a pathway enrichment analysis (Figure 3) based on metabolites with p-value below 0.05, and mostly fatty acid pathways were enriched between non-responders with rapidly progressive AML and non-responders with less aggressive disease. The metabolites important for the tricarboxylic acid cycle (five metabolites, see Figure 1) all reflect transition of fatty acid metabolites to the energy metabolism/mitochondria. Thus, our results suggest that rapidly progressive AML is associated with alterations especially in lipid (i.e., fatty acid) metabolism.

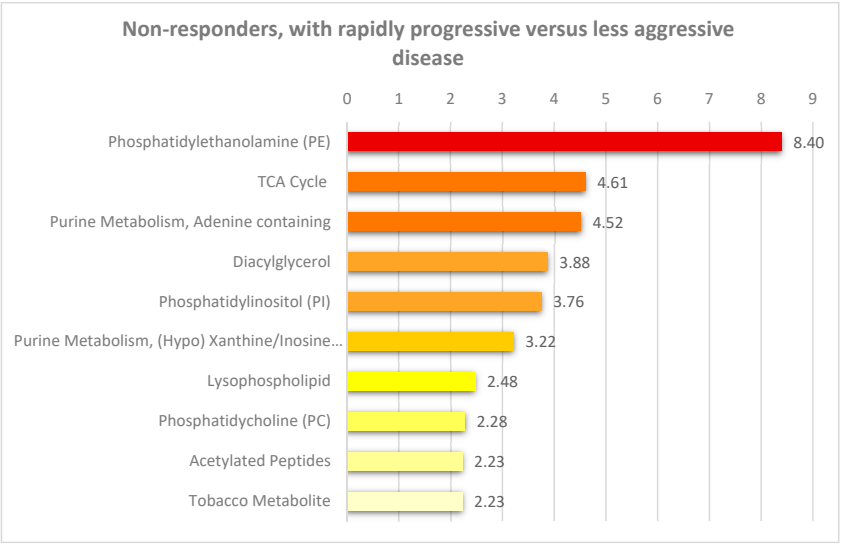

Figure 3. Pathway enrichment analysis based on altered metabolites between non-responder patients with rapidly progressive disease (i.e., aggressive disease) versus non-responders with less aggressive disease. This analysis was based on significantly altered metabolites $p < 0.05$ (see Figure 1). Only pathways with an enrichment value greater than two and at least two metabolites within each pathway are shown in the figure.

3.3. Effects of ATRA Monotherapy on Serum Metabolite Profiles

We compared the serum metabolite profiles before and after two days of ATRA monotherapy for ten patients from the first clinical study (Table S1, the first five responders and five non-responders) [27]. A total of 54 metabolites were then significantly altered after ATRA monotherapy (paired *t*-test, $p < 0.05$, Figure 4), though all metabolites had high *q*-values ($q > 0.1$, Figure 4). However, of the 54 altered metabolites, a large majority of 50 metabolites were increased after ATRA treatment.

A large fraction of these 54 metabolites were amino acid metabolites (24/54), including isoleucine/leucine and tryptophan together with several of their metabolites as well as histidine, lysine, methionine/cysteine/taurine arginine, lysine, and valine metabolites (Figure 4). Nineteen lipid metabolites were also altered by ATRA; especially sphingolipid/sphingomyelin but also plasmalogen metabolites were increased. The largest subgroup of altered lipid metabolites was sphingolipids that are largely synthesized from serine and palmitoyl-CoA and are important as cell membrane components and regulators of cell proliferation and survival [54–56]. Although several sphingomyelins were increased, their second messenger ceramide was not altered. Finally, several one-carbon/methylated metabolites were increased after ATRA therapy together with the methylation reaction product S-adenosylhomocysteine.

We performed a pathway enrichment analysis (Figure S4) based on the significantly altered metabolites observed after ATRA treatment. This analysis also showed that especially lipid metabolites but also amino acid metabolites before and during ATRA therapy. In addition, we finally also investigated the effects of ATRA monotherapy for the five responders and the five non-responders separately. A similar profile was then observed where significantly altered metabolites ($p < 0.05$, but $q > 0.05$) consisted mainly of amino acid and lipid metabolites in both groups (data not shown).

To conclude, our present metabolomic results suggest that ATRA mainly increases levels of metabolites involved in amino acid and lipid metabolism, but these observations must be interpreted with great care because the effects were relatively weak and showed high *q*-values. However, previous

animal studies have also shown that ATRA can alter the systemic levels of amino acid and fatty acid metabolites [37]; these results are thus consistent with our present observations in AML patients.

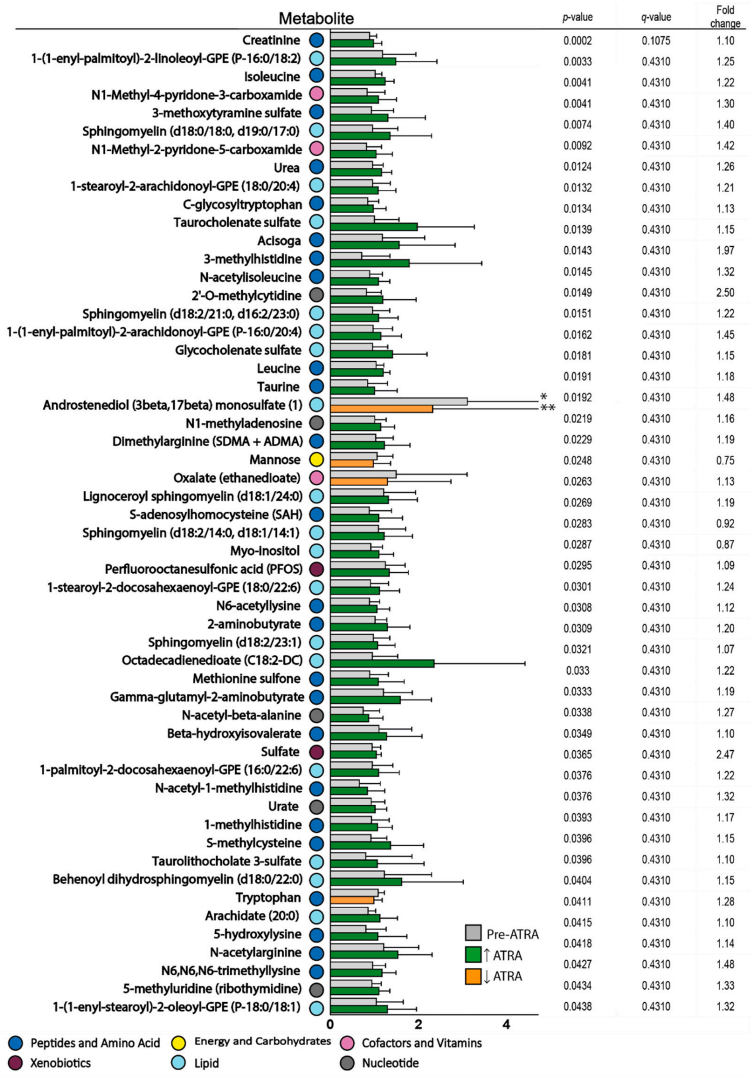

Figure 4. The effect of ATRA monotherapy on the serum metabolomic profiles of patients after two days of treatment. Fifty-four metabolites were significantly altered after ATRA treatment ($p < 0.05$). The *p*-values, *q*-values, and mean fold change values for each metabolite are listed to the right in the figure (ranked by *p*-value), and a fold change >1 indicates that the levels were increased in responders compared with non-responders. Pretherapy levels of serum metabolites for the ten patients are presented in grey, increased levels during ATRA treatment are presented in green and decreased levels presented in yellow. Color codes for classification of metabolites are explained at the bottom of the figure. Error bars show Standard deviation (SD). * Androstenediol (3 beta, 17 beta) pre-ATRA SD 3.862, ** Androstenediol (3 beta,17 beta) post-ATRA SD 2.542.

3.4. Valproic Acid Monotherapy Alters the Systemic Amino Acid and Lipid Metabolite Profiles in Both Responders and Non-Responders to the Antileukemic Treatment

We compared the systemic metabolomic profiles before and after seven days of valproic acid monotherapy for ten patients included in the second clinical study, including the first five responders and non-responders (Table S1) [19]. A total of 109 metabolites were significantly altered in patients treated with valproic acid compared to pretreatment levels; 55 metabolites were increased, and 54 were decreased ($p < 0.05$; see Figure S5). Among these, 36 metabolites had both a p-value < 0.05 and a q-value < 0.1 (Figure 5), and 23 of these metabolites also had a q-value below 0.05. Four of these 36 metabolites were valproic acid metabolites, whereas the rest included 13 amino acid metabolites and 12 lipid metabolites. Only one of these metabolites reflected energy/carbohydrate metabolism.

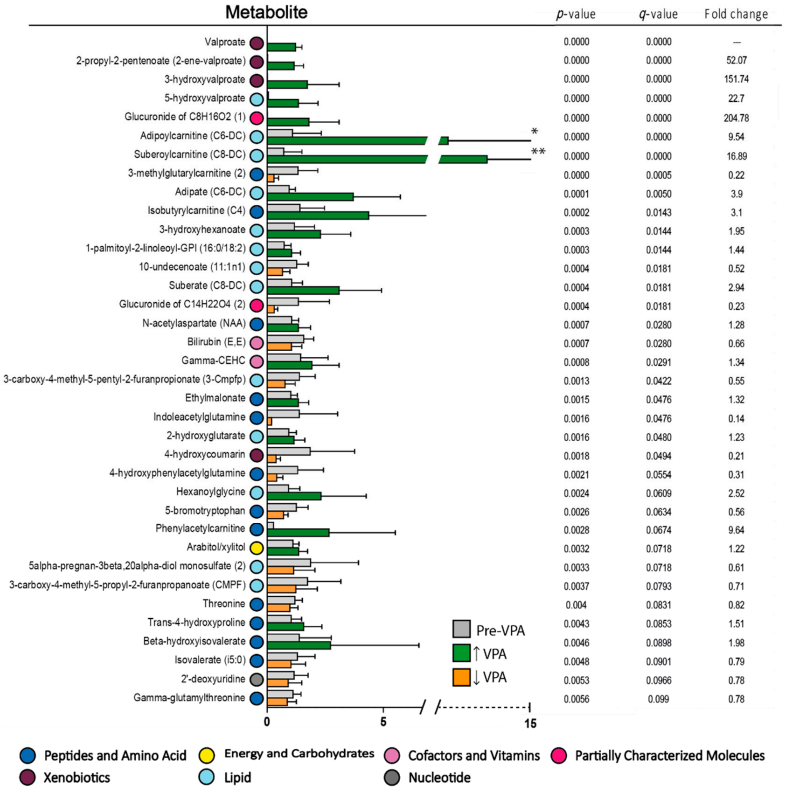

Figure 5. Identification and classification of serum metabolites that differed significantly when comparing samples taken prior to treatment and after seven days of valproic acid (VPA) therapy. Thirty-six metabolites differed significantly between untreated and VPA-treated samples ($p < 0.05$, Welch's two sample t-test), with q-value < 0.1 (the upper 23 metabolites with $q < 0.05$). The p-values, q-values, and mean fold change for each metabolite are listed to the right in the figure (ranked by p and q-value), and a fold change >1 indicates that the levels were increased after valproic acid therapy. Metabolite levels found in pretreatment samples are shown in grey, while increased levels during treatment are shown in green (22/36 increased) and decreased levels during treatment are shown in orange (14/36 decreased). Color codes for classification of metabolites are explained at the bottom of the figure. Error bars show Standard deviation (SD). *Adipoylcranitine (C6-DC) SD 6.928 **Suberoylcarnitine (C8-DC) SD 8.50.

A random forest analysis was performed after excluding the four valproic acid metabolites, and then 14 lipid and seven amino acid metabolites were among the 30 top-ranked metabolites of this analysis, with a predictive accuracy of 90% (Figure 6). Twenty-two of these 30 metabolites were significantly altered by the valproic acid treatment ($p < 0.05$, see Figure S5), including six of the highly significant carnitine metabolites (Figure 6). In addition, another random forest analysis was performed including all identified metabolites (not excluding valproic acid metabolites) and the results showed that the four top-ranked metabolites were all valproic acid metabolites as expected (Figure S6; predictive accuracy of 100%). Hence, responsiveness to the combination of ATRA and valproic acid is probably not determined by a difference in valproic acid metabolism.

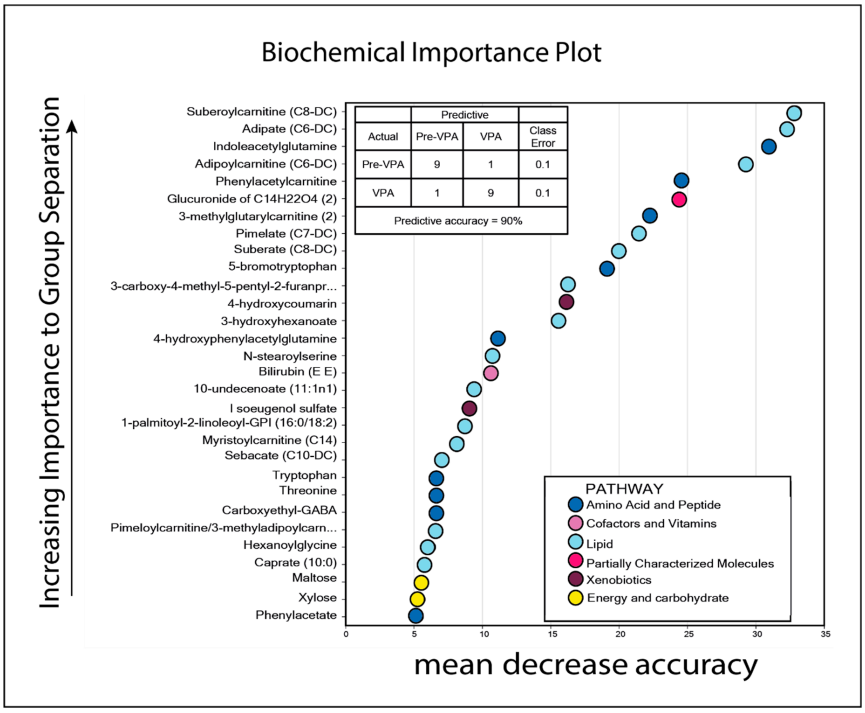

Figure 6. The effect of 7-day valproic acid (VPA) monotherapy on the serum metabolomic profiles for ten patients (five responders and five non-responders). The random forest analysis was based on the identification of 886 metabolites in pretherapy samples and samples collected after seven days of valproic acid monotherapy. The analysis showed a predictive accuracy of 90% (see the insert table) after exclusion of the four valproic acid metabolites. The top-30 most important metabolites for separation of the two groups are shown in ranking order. Color codes indicate the classification of individual metabolites at the lower right part of the figure.

A pathway enrichment analysis for differential metabolites after valproic acid treatment revealed enrichment of pathways relating especially to lipid metabolism as well as amino acid metabolism (Figure 7). As discussed above, several lipid and fatty acid metabolites were altered after valproic acid therapy, including increased levels of several fatty acid-carnitines (Figure 5, Figure S5). This is consistent with impaired β-oxidation, and the increased levels of five out of nine significantly altered dicarboxyl fatty acids suggests that ω-oxidation is used as an alternative mechanism in the liver to compensate for impaired β oxidation [57]. Furthermore, a total of 33 amino acid metabolites were significantly altered by valproic acid, in particular metabolites reflecting tryptophan (six metabolites)

and valine/leucine/isoleucine metabolism (eight metabolites). Seven amino acid metabolites were also included among the top-ranked metabolites in the random forest analysis (Figure 6). In our analysis, the 109 metabolites with significant p-values < 0.05 (but high q-value) had a similar overall profile as that found for metabolites with $p < 0.05$ and $q < 0.05$ (Figure 5), mainly consisting of metabolites belonging to amino acid (see Figure S7) and lipid pathways (see Figure S8), whereas metabolites reflecting the carbohydrate and energy metabolism were scarce.

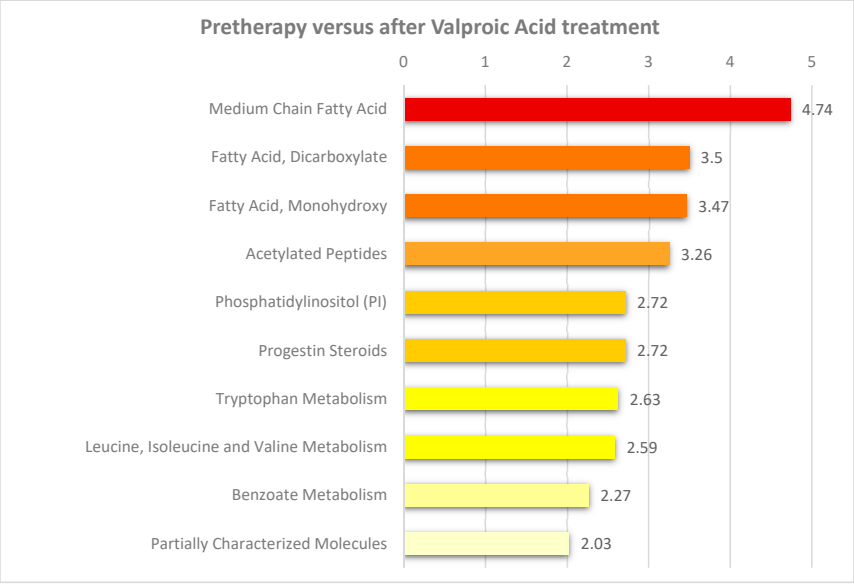

Figure 7. Pathway enrichment analysis based on metabolites altered after seven days of valproic acid treatment compared to pretherapy levels. This analysis was based on significant altered metabolites $p < 0.05$ (see Figure S5), and only pathways with an enrichment value greater than two and at least two metabolites within each pathway are shown. The most significant pathway is shown in red and less significant pathways in light yellow.

We finally analyzed and compared the effects of valproic acid monotherapy for the five patients classified as responders and five non-responders separately (Table 1). Then, 78 metabolites were significantly altered after valproic acid treatment for the responders, including divergent effects for 17 amino acid and 32 lipid metabolites, while 105 metabolites were significantly altered after valproic acid treatment for the non-responders, including divergent effects for 21 amino acid and 49 lipid metabolites (Table 1, Tables S3 and S4). Thus, a similar pattern was observed for both responders and non-responders as described above; valproic acid mainly alters amino acid and lipid metabolism. The metabolites showing both p- and q-value < 0.05 are listed in Table 1. These metabolites included the valproic acid metabolites and also several carnitines, which also reflect differences in lipid and amino acid metabolism.

Table 1. An overview of significantly altered serum metabolites ($p < 0.05$ and $q < 0.05$) after seven-day valproic acid therapy.

Biochemical Name	Classification	During VPA Therapy/Pretherapy		
		p-Value	*q*-Value	Fold Change
RESPONDERS				
*Valproate	Drug concentration	0.0000	0.0000	
*2-propyl-2-pentenoate (2-ene-valproate)	Valproic acid metabolite	0.0000	0.0000	55.81
*5-hydroxyvalproate	Valproic acid metabolite	0.0000	0.0000	13.83
*3-hydroxyvalproate	Valproic acid metabolite	0.0000	0.0000	25.77
Glucuronide of C8H16O2 (1)	Partially characterized	0.0000	0.0000	28.34
*Suberoylcarnitine (C8-DC)	Fatty acid metabolism, acyl carnitine	0.0000	0.0000	30.82
Phenylacetylcarnitine	Acetylated peptide	0.0000	0.0000	17.30
*Adipoylcarnitine (C6-DC)	Fatty acid metabolism	0.0000	0.0000	17.06
*3-methylglutarylcarnitine (2)	Leucine/isoleucin/valine metabolism	0.0000	0.0000	0.18
*Adipate (C6-DC)	Fatty acid, dicarboxylate	0.0000	0.0027	3.41
10-undecenoate (11:1n1)	Medium chain fatty acid	0.0001	0.0043	0.49
*Isobutyrylca'rnitine (C4)	Leucine/isoleucin/valine metabolism	0.0001	0.0093	3.06
Glucuronide of C14H22O4 (2)	Partially characterized	0.0003	0.0182	0.22
4-hydroxycinnamate sulfate	Tyrosine metabolism	0.0004	0.0259	3.00
Isoeugenol sulfate	Food component, plant	0.0005	0.0264	0.04
NON-RESPONDERS				
*Valproate	Drug concentration	0.0000	0.0000	
*2-propyl-2-pentenoate (2-ene-valproate)	Valproic acic metabolite	0.0000	0.0000	48.32
*5-hydroxyvalproate	Valproic acic metabolite	0.0000	0.0000	31.58
*3-hydroxyvalproate	Valproic acic metabolite	0.0000	0.0000	93.38
*Glucuronide of C8H16O2 (1)	Partially characterized	0.0000	0.0000	16.16
*Suberoylcarnitine (C8-DC)	Fatty acid, dicarboxylate	0.0000	0.0000	12.96
*Adipoylcarnitine (C6-DC)	Fatty acid metabolism	0.0000	0.0000	7.34
*Adipate (C6-DC)	Fatty acid, dicarboxylate	0.0000	0.0001	4.33
*3-methylglutarylcarnitine (2)	Leucine/isoleucin/valine metabolism	0.0000	0.0001	0.25
Suberate (C8-DC)	Fatty acid, dicarboxylate	0.0000	0.0022	3.43
3-carboxy-4-methyl-5-pentyl-2-Furanpropionate (3-Cmpfp)	Fatty acid metabolism, dicarboxylate	0.0001	0.0054	0.44
Hexanoylglycine	Fatty acid metabolism, acyl glycine	0.0001	0.0077	3.03
3-hydroxyhexanoate	Fatty acid metabolism, monohydroxy	0.0001	0.0100	2.03
Androstenediol (3beta,17beta) disulfate (1)	Androgenic steroid	0.0003	0.0197	1.45
*Isobutyrylcarnitine (C4)	Leucine/isoleucin/valine metabolism	0.0004	0.0208	3.15
Gamma-CEHC	Cofactors/vitamins	0.0004	0.0234	1.56
Isoursodeoxycholate	Secondary bile acid metabolism	0.0005	0.0278	0.06
Indoleacetylglutamine	Tryptophane metabolism	0.0006	0.0298	0.09
4-hydroxyphenylacetylglutamine	Acetylated peptide	0.0008	0.0352	0.28
1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)	Phosphatidylinositole	0.0008	0.0352	1.51
5-bromotryptophan	Tryptophane metabolism	0.0010	0.0387	0.49
4-allylphenol sulfate	Food component, plant	0.0010	0.0387	0.29
N-acetyltyrosine	Tyrosine metabolism	0.0011	0.0413	2.42
Trans-4-hydroxyproline	Urea cycle. Proline metabolism	0.0011	0.0411	1.73
Galactonate	Carbohydrate metabolism	0.0012	0.0430	0.38
3-aminoisobutyrate	Pyrimidine metabolism, thymine	0.0013	0.0433	1.86

The metabolites are ranked according to their *p*- and *q*-values, and the metabolites that were altered in responders and non-responders to the antileukemic AML-stabilizing therapy are listed separately. Overlapping metabolites between these two groups are marked with *. VPA, valproic acid.

4. Discussion

Epigenetic targeting based on combination therapy with ATRA and valproic acid can stabilize the disease for a subset of AML patients. Furthermore, both drugs can also modulate the cellular and/or systemic metabolism [32–37]. Such pharmacological effects may influence the metabolic regulation of the leukemia cells through altered levels of nutrients in their microenvironment [48,49], and these effects may even contribute to the epigenetic effects of the drugs [58,59]. We have therefore performed metabolomic profiling of serum samples collected from AML patients before receiving disease-stabilizing treatment to compare responders and non-responders to treatment, and we also

compared systemic metabolite profiles before and after drug monotherapy. Our study suggests that differences in pretreatment amino acid and lipid metabolite profiles are associated with disease aggressiveness, and both ATRA and valproic acid cause further modulation of the systemic metabolic signature (i.e., amino acid and lipid metabolism). This is different from patients receiving intensive and potentially curative chemotherapy where a glucose metabolism signature has been shown to be associated with chemoresistance and survival after chemotherapy [28].

Untargeted metabolomics generates large amounts of data that can be complex and challenging to analyze. A p-value of 0.05 is the false positive rate when one test is performed, but for a large number of tests on the data one has to account for false positives. There are different methods to correct for multiple testing. One strategy is to use the family-wise error rate adjustment (e.g., Bonferroni correction), but this is regarded as very conservative when one has a large number of tests; the use of false discovery rates (i.e., the q-values) is therefore more common although it allows for a small number of false discoveries [60]. A relatively high q-value (e.g., $q > 0.10$) indicates diminished confidence but it does not necessarily rule out the biological significance of a result. In our opinion other evidence should also be considered, for example (i) significance in another dimension of the study, (ii) inclusion in a common pathway together with highly significant compounds, and (iii) residing in a similar metabolic family with other statistically significant metabolites. Throughout the present study we have therefore for each biological comparison listed all metabolites with the corresponding p-value < 0.05, we have presented p-values together with the corresponding q-values and fold change values for all these metabolites, and when we discuss the importance of single metabolites we focus on the q-values (i.e., the studies of the valproic acid effects). We also performed pathway enrichment analysis based on significantly altered metabolites ($p < 0.05$) which revealed enriched pathways instead of focusing on single metabolites. In our opinion such an identification of a metabolic pathway can be justified because it is based on several metabolites and not a single metabolite. However, we would like to emphasize that all background information (p- and q-values and fold change values) are provided in the text or in figures/tables for all metabolites included in our analyses, so the level of confidence in the results can be further evaluated by the reader.

Our pretreatment profiles were probably determined by the metabolic characteristics of a large leukemia cell burden together with the effects of this burden on systemic metabolic regulation. The lipid and amino acid profiles were further modulated by metabolic effects of ATRA but especially of valproic acid. The drugs have direct (i.e., epigenetic) effects on the AML cells, and metabolic modulation may then represent an additional indirect effect on the leukemia cells by modulation of metabolite/nutrient levels in their bone marrow microenvironment. Thus, altered metabolomic profiles may contribute for the prognostic evaluation for such patients, but they may also influence AML cell chemosensitivity and/or contribute to the antileukemic effects of the treatment.

The clinically relevant responses to this AML-stabilizing treatment are usually seen after 14–20 days of treatment [19,27]. Thus, in this study, the effects of ATRA and valproic acid on metabolomic profiles were evaluated early before improvement of peripheral blood cell counts could be expected. Despite the late clinical responsiveness, alterations in metabolomic signatures were found during the first days of treatment and these effects differed between patients whom about two weeks later became responders or non-responders to treatment.

There is no general agreement on how responses to AML-stabilizing treatment should be classified. We used the generally accepted definition of complete hematological remission for AML patients [51] and the MDS criteria for stable disease that require improvement/stabilization of normal peripheral blood cell counts for at least two months [61]. However, the median survival for elderly patients not receiving AML-directed therapy is only two-three months [7,62,63] and we therefore regarded such disease stabilization as unexpected and classified this as a response.

Amino acid profiles in healthy individuals and cancer patients have been compared in several previous studies [64]. The plasma amino acid profiles for patients with non-small-cell lung cancer or colorectal cancer is considerably different from healthy controls, and altered amino acid profiles

have been detected also in early stage breast cancer [64]. Our present study found altered amino acid profiles in AML after both ATRA and valproic acid treatment, and we observed a considerable heterogeneity in pretherapy metabolite levels among AML patients when comparing (i) responders versus non-responders and (ii) non-responders with rapidly progressive disease versus other non-responders. The mechanisms behind altered amino acid profiles in cancer patients may be related to the risk of cancer [65] or reflect nutritional or microbiotic alterations, progression of the disease, cachexia or weight loss [66–68]. In our opinion, it is most likely that the mechanism behind the differences in amino acid metabolism between various AML patient groups is also multifactorial.

To the best of our knowledge, the present study is the first to investigate metabolomic profiles in patients receiving AML-stabilizing treatment. We investigated elderly and unfit patients that were included in two previous clinical studies; in both clinical studies the patients received the same doses of ATRA, guidelines for adjustment of valproic acid treatment were similar, and we used low-dose (i.e., low-toxicity) cytarabine/mercaptopurin/hydroxyurea. Our results showed that responders and non-responders showed relatively small differences in pretherapy metabolic profiles, but non-responders with rapidly progressive disease seemed to have more extensive differences compared with the other non-responders. Fatty acid oxidation may be important for AML growth [69], and increased levels of lipids/fatty acids in non-responders could then be a strategy to provide energy substrates for the growing leukemia cells. In addition, the long chain fatty acid oleoyl ethanolamid is suggested to contribute to drug resistance in other patients with hematological malignancies [70]. The increased oleoyl ethanolamid levels may also reflect cachexia that is associated with poor prognosis in other malignancies [68]. Our responders and non-responders differed in their pretherapy levels of the three endocannabinoids oleoyl ethanolamide, linoleoyl ethanolamide (both increased in non-responders) and *N*-palmitoylserine (decreased in non-responders). Oleoyl ethanolamide activates the nuclear peroxisome proliferator-activated receptor alpha (PPAR-alpha) that induces lipolysis and fat utilization [71]. Consistent with increased lipolysis and energy utilization, non-responders also showed significantly altered serum levels of four free fatty acids and one fatty acid metabolite, suggesting release of fatty acids. Oleoyl ethanolamide may therefore represent a link between endocannabinoid and fatty acid metabolism in different patient groups. In addition, responders and non-responders also differed in xenobiotic metabolites including gut-bacteria derived metabolites. Taken together, these observations suggest that differences in nutritional status or gut microbiome contribute to the metabolomic heterogeneity of elderly patients with newly diagnosed AML.

Our analyses showed that the non-responders are not only heterogeneous with regard to survival but also with regard to pretherapy metabolomic profiles. Patients with rapidly progressive disease differed considerably from other non-responders especially in lipid metabolism but also amino acid metabolism. AML is a very aggressive disease and if the first treatment fails the patients may become unfit for further treatment. Our present observations have to be interpreted with great care because few patients with very aggressive disease were studied and further studies are needed. Still, they suggest that pretreatment metabolomic profiling may be helpful to select patients that should not receive treatment based on ATRA plus valproic acid, especially patients that have rapidly progressive disease because it takes 15–20 days before clinical responses can be detected.

Neither our comparisons of pretherapy metabolomic profiles of responders and non-responders nor our studies of metabolite profiles during monotherapy detected any major differences in carbohydrate or energy metabolism (glycolysis, citric acid cycle), even though such differences have a prognostic impact for AML patients receiving intensive therapy [28]. However, clinically relevant responses to our disease-stabilizing therapy can be seen even for patients with high-risk AML (e.g., high-risk karyotype, relapse; Table S1). Thus, the conventional high-risk criteria, and also glucose metabolism, seem less important for patients receiving treatment based on ATRA and valproic acid. Instead, differences in or altered lipid and amino acid metabolism seem to be important for patients receiving this disease-stabilizing treatment, and effects on systemic levels of metabolites induced by valproic acid generally seem to be stronger than the ATRA-induced effects. Previous studies have also described an

effect of ATRA on one-carbon metabolism [72], and similar effects were also seen in our AML patients although they seemed to be relatively weak.

We also examined the effects of ATRA and valproic acid on metabolomic profiles for ten patients, i.e., five responders and five non-responders analyzed together, after relatively short duration of treatment (two and seven days, respectively); whereas clinically relevant responses are usually detected after two-three weeks [19,27]. Thus, the observed alteration of metabolite profiles should be regarded as early pharmacological effects that are common for responders and non-responders.

The most striking effects by the two drugs in our studies were altered lipid and amino acid metabolism. ATRA increased serum levels of sphingolipids and sphingomyelins in non-responders; a hypothesis is therefore that ATRA contributes to chemoresistance in the non-responders through a growth-enhancing and antiapoptotic effect of these metabolites as described for other malignancies [54–56]. Several sphingomyelins were altered by ATRA, but their second messenger ceramide derived from hydrolysis of sphingomyelin was not altered. Altered ceramide synthesis has been observed in other malignancies [73], and a subset of AML patients may even have mutations in the sphingomyelin/ceramide pathway [74]. Taken together, these observations suggest that ATRA also has complex effects on this pathway. Moreover, the levels of several plasmalogens were also altered by ATRA. These glycerophospholipid derivatives are thought to be protective against reactive oxygen species [75], their levels are high in inflammatory cells [76], and their levels increase during differentiation-induction in the HL60 human AML cell line [77]. Thus, increased plasmalogens may reflect ATRA-induced effects on AML cells that contribute to chemoresistance at least for certain patients.

Furthermore, several methylated metabolites were increased after ATRA treatment, including 3-methylhistidine, *N*6,*N*6,*N*6-trimethylhistidine, S-methycysteine, 2′-*O*-methylcytidine, *N*1-methyl-4-pyridone-3-carboxamide, as well as the methylation reaction product S-adenosylhomocysteine. ATRA has been shown to increase both glycine *N*-methyltransferase that regulates the methyl group supply for S-adenosylmethionine-dependent transmethylation reactions [77] as well as the activation of histone methyltransferase SUV39H2 that is important for epigenetic regulation of gene transcription [78]. ATRA-induced alteration of DNA methylation has also been described in the HL60 AML cell line [79]. Thus, these observations suggest that ATRA influences the general methylation potential in AML cells.

Lipid and amino acid metabolism were both altered during valproic acid therapy, and especially altered levels of fatty acid metabolites were observed. There seem to be several links between epigenetic regulation and fatty acid metabolism in AML [58], including expression of fatty acid binding protein 4 (FABP4) in AML cells that is important both for fatty acid uptake and epigenetic regulation [80]. Epigenetic regulation is also important for the expression of acetyl-CoA carboxylase 2, a key driver of fatty acid β-oxidation in AML cells [81] and a regulator of the general lipid metabolism [82]. Repression of this gene by histone deacetylation allows for simultaneous β-oxidation and fatty acid synthesis to take place [81]. Finally, valproic acid has been shown to alter lipid metabolism and fatty acid oxidation [33,35]. Our overall results thereby support the hypothesis that valproic acid alters epigenetic regulation both through direct effects on histone acetylation and indirectly through modulation of fatty acid metabolism.

We compared the effects of ATRA and valproic acid for five responders and five non-responders; these results have to be interpreted with great care due to the low number of samples. However, non-responders showed more extensive effects on amino acid and lipid metabolism after two days of ATRA monotherapy but especially after seven days of valproic acid monotherapy. In our opinion, these more extensive effects in non-responders may partly reflect pharmacological effects, but may also be influenced by disease progression [30–36].

5. Conclusions

Both ATRA and valproic acid have been used in the treatment of AML, and this is the first study to investigate systemic metabolomic profiles in AML patients receiving leukemia-stabilizing treatment based on ATRA plus valproic acid. Our study shows that especially amino acid and lipid metabolism

varies between patients and during treatment. The effect of valproic acid on the regulation of lipid and amino acid metabolism is particularly strong and may contribute to the antileukemic and/or epigenetic effects of this drug, whereas the effects of ATRA and the differences between patient subsets are weaker. Metabolites related to carbohydrate or energy metabolism showed only minor variations in our study. Systemic metabolomics should be further investigated to identify biomarkers for pretreatment evaluation of susceptibility to AML stabilizing treatment, though larger studies are needed. Finally, changes in metabolomic profiles may influence the bone marrow microenvironment and thereby modulate AML cell metabolism as well as epigenetic regulation and contribute to therapy resistance.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/8/10/1229/s1, Figure S1: Timeline of treatment schedule for patients included in two clinical studies, Figure S2: Identification and classification of metabolites in pretherapy serum samples that differed significantly between responders and non-responders to the antileukemic treatment of ATRA plus valproic acid, Figure S3: Comparison of pretreatment metabolomics profiles for responders and non-responders to antileukemic treatment based on ATRA and valproic acid; a metabolite pathway enrichment analysis, Figure S4: Comparison of metabolomics profiles before and during ATRA treatment for responders and non-responders to antileukemic treatment based on ATRA and valproic acid; a metabolite pathway enrichment analysis, Figure S5: The effect of seven-day valproic acid (VPA) monotherapy on the serum metabolomic profiles of AML patients, Figure S6: The effect of valproic acid monotherapy for seven days on the serum metabolomics profiles for 10 patients (five responders and five non-responders; patients included in the study described in PMID 23915396, valproic acid metabolites included in the study), Figure S7: The effect of seven days of valproic acid monotherapy on serum metabolomic profiles; significantly altered amino acid and peptide metabolites when comparing samples derived from 10 patients (five responders and five non-responders; all patients included in the study by Fredly et al. PMID 23915396), Figure S8: The effect of seven days of valproic acid monotherapy on serum metabolomic profiles; significantly altered lipid metabolites when comparing samples derived from 10 patients (five responders and five non-responders; all patients included in the study by Fredly et al. PMID 23915396), Table S1: Clinical and biological characteristics of the included patients, Table S2: Significantly altered serum metabolites between subsets of non-responders to antileukemic treatment based on ATRA and valproic acid; a comparison of non-responders with very aggressive (i.e., rapidly progressive) and less aggressive disease, Table S3: Significantly altered metabolites after seven days of valproic acid monotherapy; a comparison of pretreatment samples versus samples collected during treatment for patients classified as responders to antileukemic therapy based on ATRA and valproic acid, Table S4: Significantly altered metabolites after seven days of valproic acid therapy; a comparison of pretreatment samples versus samples collected during treatment for patients classified as non-responders to antileukemic treatment based on ATRA and valproic acid.

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SUPPLEMENTARY INFORMATION

Systemic Metabolomic Profiling of Acute Myeloid Leukemia Patients before and During Disease-Stabilizing Treatment Based on All-Trans Retinoic Acid, Valproic Acid, and Low-Dose Chemotherapy

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Table S1. Clinical and biological characteristics of the included patients[1]. Responders to treatment are listed at the upper part of the table; the non-responders are listed at the lower part.

ID[1]	Gender	Age	Previous disease	FAB	Membrane molecule expression[2]					Karyotype	FLT3	NPM1	Additional mutations	WBC counts (x10^8)	Survival (days)[3]
					CD13	CD14	CD15	CD33	CD34						
RESPONDERS															
1*	M	73	MDS	M1	+	-	-	+	+	multiple	ITD, TKD	wt	PHF6, RUNX1	<0.5	392
2*	F	61	1st relapse	M1	+	-	+	+	+	multiple	wt	wt	NRS, SF3B1	55.8	644
3*	M	62	2nd relapse	M2	+	-	+	+	+	-7	wt	wt	nt	4.9	350
4*	M	80	*de novo*	M1	+	-	-	+	+	multiple	wt	wt	nt	8	58
5*	M	78	MDS	M1	+	-	-	+	+	nt	nt	nt	nt	142	69
6*	F	68	1st relapse	M1	+	-	+	+	+	normal	wt	wt	TET2, ASXL1, BCOR	15.6	105
7*	F	81	MDS	M1	+	-	-	+	+	normal	wt	wt	TET2, ASXL1, GATA2, CEBPA, SRSF2	6	147
8*	M	86	*de novo*	M4	+	+	+	+	-	nt	nt	nt	nt	18.7	59
9	M	81	Polycytemia-vera	M2	-	-	-	-	+	-7	wt	wt	ASXL1, SRSF2, RAD21	22.3	610
10	M	77	MDS	M1	+	-	-	+	+	normal	wt	wt	NRAS, RUNX1, CEBPA, SRSF1, STAG2	2.1	419
11	F	85	MDS	M1	-	-	-	-	+	multiple	nt	nt	nt	3.9	171
12	F	83	MDS	M2	+	-	+	-	+	normal	nt	nt	nt	0.3	196
13	M	66	MDS	M2	-	-	+	-	-	14q	nt	nt	nt	2,6	239
14	M	83	MDS	M0	-	-	-	-	+	normal	wt	wt	nt	1.1	102
15	M	73	*de novo*	M1						nt	wt	Ins	TKD, IDH2, SRSF1	12.1	383
16	M	74	*de novo*	M0	+	-	-	+	+	multiple	wt	wt	TP53	18.7	151
17	F	72	MDS	M2	+	-	-	+	-	t(1;5), t(2;3)	ITD	wt	KMT3, RUNX1	42.6	132
18	F	77	MDS	M2	+	-	-	+	+	normal	wt	wt	NRAS, TET2, ASXL1, RUNX1, SRSF1, STAG2, BCDR	142	132
NON-RESPONDERS															
19*	F	81	*de novo*	M1	+	-	-	+	+	multiple	nt	nt	nt	1.7	192
20*	M	74	*de novo*	M0	+	-	-	-	+	multiple	wt	wt	IDH2	13.3	112
21*	F	70	MDS	M1	+	-	-	+	+	multiple	wt	wt	TP53	3.6	142
22*	M	67	1st relapse	Nt	nt	nt	nt	nt	nt	normal	TKD	wt	nt	15.6	73

23*	M	71	1st relapse	M2	+	-	-	+	+	normal	TKD	Ins	TET2, GATA2, STAG2	1.5	49
24	F	82	*de novo*	M5	+	-	+	+	+	normal	ITD, TKD	wt	WT1, DNMT3	142	37
25	F	79	*de novo*	M2	+	-	+	+	+	multiple	wt	wt	TP53, BCORL1	1.7	42
26	M	73	MDS	M1	+	-	-	+	+	multiple	wt	wt	TP53, CUX1	11.1	33
27	M	58	Relapse	M1	+	-	-	+	+	-7	wt	wt	nt	0.9	65
28	F	65	Relapse	M6	+	-	-	+	+	multiple	wt	wt	TP53, IKZF1, RUNX1	1.3	65
29	M	77	Polycytemia vera		-	-	-	-	+	nt	nt	nt	nt	8.3	41
30	F	86	*de novo*							del5q	wt	wt	GATA2	249	17
31	F	65	Relapse	M6	+	-	-	+	+	multiple	wt	wt	nt	1.3	65
32	F	77	*de novo*	M1	-	-	-	-	-	normal	ITD	Ins	DNMT3A	68.5	32
33	M	62	MDS	M0	-	-	-	-	+	multiple	nt	nt	nt	8.2	57
34	M	68	MDS	M0	-	-	-	-	+	normal	wt	wt	TET2, ASXL1, BEBPA, SRSF2, STAG2	1.5	24
35	F	83	*de novo*		+	-	-	+	-	normal	ITD	Ins	nt	105	38
36*	M	78	MDS	nt	+	-	-	+	+	nt	ITD	wt	ASXL1, STAG2, ZRSR2	5.6	55
37*	M	62	3rd relapse	M1	+	nt	-	+	+	t(4;20)	wt	wt	DNMT3, IDH2	1.5	78
38*	M	68	Myelofibrosis	M1	+	-	-	+	+	Normal	wt	wt	KRAS	34.3	56
39*	F	80	*de novo*	M2	-	-	-	+	-	normal	ITD	Ins	PHF6, TET2, BCDFIL1, CSF3R	217	5
40*	F	70	Chemotherapy	M4	+	-	+	+	-	normal	wt	Ins	NRAS, DNMT3A, IDH1	73.7	7
41*	M	60	2nd relapse	M4	+	-	+	+	+	normal	ITD	wt	WT1	66	6
42	M	71	Chemotherapy	M4						nt	wt	Ins	KRAS, DNMT3A, TET2	104	2
43	M	48	Relapse	M4					-	normal	ITD, TKD	Ins	DNMT3A, IDH1	30.4	8
44	F	60	Relapse	M4	+	-	+	+	-	normal	ITD	Ins	DNMT3A, TET2	16.7	12

Abbreviations: MDS, myelodysplastic syndromes; Ins, insertion; ITD, internal tandem duplications; nt, not tested; wt, wild type.

1) Patient IDs marked with (*) indicate that these patients were included in the study by Ryningen *et al.* (PMID 19007987) (n=19) [1], while unmarked patient IDs were included in the study by Fredly *et al.* (PMID 23915396) [2].

2) Expression of a marker was defined as at least 20% positive cells compared with the corresponding negative control.

3) Survival is presented as the survival from start of treatment.

Table S2. Significantly altered serum metabolites between subsets of non-responders to antileukemic treatment based on ATRA and valproic acid; a comparison of non-responders with very aggressive (i.e. rapidly progressive) and less aggressive disease. The arrows (↑↓) indicate whether the metabolite levels were increased or decreased in patients with very aggressive disease compared to patients with less aggressive disease.

Main class / Subclassification		Metabolite	*p*-value	*q*-value	Fold change, very aggressive versus less aggressive
Amino Acid					
Glycine, Serine and Threonine Metabolism	↓	Betaine	0.0141	0.2745	0.75
Glutamate Metabolism	↑	N-acetylglutamate	0.0113	0.2642	1.51
Lysine Metabolism	↓	2-aminoadipate	0.0099	0.2639	0.56
Tyrosine Metabolism	↑	Thyroxine	0.0185	0.3107	1.33
Tryptophan Metabolism	↑	Tryptophan betaine	0.0051	0.2185	6.96
Methionine, Cysteine, SAM and Taurine Metabolism	↑	Cysteine s-sulfate	0.0093	0.2587	1.85
	↓	Hypotaurine	0.0284	0.3801	0.32
Urea cycle; Arginine and Proline Metabolism	↓	Citrulline	0.0427	0.3952	0.70
	↑	Dimethylarginine (SDMA + ADMA)	0.0066	0.2253	1.69
Polyamine Metabolism	↑	Spermidine	0.0433	0.3952	9.72
Peptide					
Dipeptide	↓	Isoleucylglycine	0.0015	0.1292	0.52
	↓	Leucylalanine	0.0000	0.0022	0.10
	↓	Threonylphenylalanine	0.0371	0.3952	0.45
Fibrinogen Cleavage Peptide	↓	Fibrinopeptide A, des-ala(1)	0.0235	0.3591	0.27
Acetylated Peptides	↓	Phenylacetylglycine	0.0070	0.2253	0.15
Carbohydrate					
Pentose Metabolism	↑	Ribonate	0.0043	0.2022	1.40
Energy					
TCA Cycle	↓	Aconitate [cis or trans]	0.0351	0.3952	0.35
	↑	Alpha-ketoglutarate	0.0369	0.3952	1.56
	↑	Succinate	0.0322	0.3904	1.45
	↑	2-methylcitrate/homocitrate	0.0128	0.2642	1.29
Lipid					
Medium Chain Fatty Acid	↓	Caprate (10:0)	0.0172	0.3107	0.57
Polyunsaturated Fatty Acid (n3 and n6)	↑	Docosatrienoate (22:3n3)	0.0274	0.3798	1.79
	↑	Docosatrienoate (22:3n6)	0.0278	0.3798	2.61
Fatty Acid, Dicarboxylate	↑	3-methylglutarate/2-methylglutarate	0.0402	0.3952	1.88

Fatty Acid Metabolism(Acyl Carnitine)	↓	Docosahexaenoylcarnitine (C22:6)	0.0065	0.2253	0.31
Fatty Acid Metabolism (Acyl Choline)	↑	Arachidonoylcholine	0.0193	0.3107	1.38
Phosphatidylcholine (PC)	↑	1,2-dipalmitoyl-GPC (16:0/16:0)	0.0377	0.3952	1.36
	↑	1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)	0.0404	0.3952	1.60
	↑	1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	0.0077	0.2253	1.43
	↑	1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6)	0.0326	0.3904	1.18
Phosphatidylethanolamine (PE)	↑	1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	0.0074	0.2253	2.09
	↑	1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	0.0205	0.3211	2.22
	↑	1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6)	0.0033	0.1752	1.80
	↑	1-stearoyl-2-oleoyl-GPE (18:0/18:1)	0.0010	0.1088	2.61
	↑	1-stearoyl-2-linoleoyl-GPE (18:0/18:2)	0.0076	0.2253	1.95
	↑	1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	0.0108	0.2642	1.86
	↑	1-stearoyl-2-docosahexaenoyl-GPE (18:0/22:6)	0.0004	0.0568	1.60
Phosphatidylinositol (PI)	↑	1-stearoyl-2-linoleoyl-GPI (18:0/18:2)	0.0191	0.3107	1.81
	↑	1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)	0.0434	0.3952	1.50
Lysophospholipid	↑	1-oleoyl-GPC (18:1)	0.0328	0.3904	1.34
	↑	1-palmitoyl-GPE (16:0)	0.0125	0.2642	1.39
	↑	1-stearoyl-GPE (18:0)	0.0115	0.2642	1.49
	↑	2-stearoyl-GPE (18:0)	0.0385	0.3952	1.47
	↑	1-stearoyl-GPG (18:0)	0.0337	0.3941	1.71
	↑	1-arachidonoyl-GPI (20:4)	0.0463	0.3952	1.47
Plasmalogen	↑	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P16:0/20:4)	0.0189	0.3107	1.52
Glycerolipid Metabolism	↑	Glycerophosphoglycerol	0.0003	0.0568	1.46
Monoacylglycerol	↑	2-palmitoylglycerol (16:0)	0.0422	0.3952	2.18
Diacylglycerol	↑	Palmitoyl-linoleoyl-glycerol (16:0/18:2) [2]	0.0426	0.3952	1.63
	↑	Palmitoleoyl-linoleoyl-glycerol (16:1/18:2) [1]	0.0498	0.3952	1.61
	↑	Palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]	0.0439	0.3952	2.12
	↑	Oleoyl-oleoyl-glycerol (18:1/18:1) [1]	0.0387	0.3952	2.06
	↑	Oleoyl-oleoyl-glycerol (18:1/18:1) [2]	0.0426	0.3952	2.25
	↑	Oleoyl-linoleoyl-glycerol (18:1/18:2) [1]	0.0067	0.2253	1.83
	↑	Oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	0.0193	0.3107	1.84
	↑	Linoleoyl-linoleoyl-glycerol (18:2/18:2) [1]	0.0016	0.1292	1.78
	↑	Oleoyl-arachidonoyl-glycerol (18:1/20:4) [2]	0.0497	0.3952	2.01
Sphingolipid Metabolism	↑	Sphinganine	0.0008	0.0987	2.37

	↑	Sphingosine	0.0126	0.2642	2.08
	↓	Sphingomyelin (d18:1/22:2, d18:2/22:1, d16:1/24:2)	0.0489	0.3952	0.71
Ceramides	↑	N-palmitoyl-sphingosine (d18:1/16:0)	0.0028	0.1752	1.75
Sterol	↑	Cholesterol	0.0044	0.2022	1.46
Androgenic Steroids	↓	5alpha-androstan-3alpha,17beta-diol Monosulfate	0.0494	0.3952	0.71
Nucleotide					
Purine Metabolism, (Hypo)Xanthine/Inosine containing	↑	Xanthine	0.0264	0.3798	3.32
	↑	Xanthosine	0.0344	0.3949	3.58
Purine Metabolism, Adenine containing	↓	Adenosine 3',5'-cyclic monophosphate (cAMP)	0.0001	0.0267	0.45
	↑	N6-succinyladenosine	0.0444	0.3952	1.86
Purine Metabolism, Guanine containing	↑	7-methylguanine	0.0131	0.2642	1.74
Pyrimidine Metabolism, Orotate containing	↓	Dihydroorotate	0.0115	0.2642	0.19
	↑	Orotidine	0.0253	0.3777	2.20
Pyrimidine Metabolism, Uracil containing	↑	N-acetyl-beta-alanine	0.0481	0.3952	1.47
Pyrimidine Metabolism, Cytidine containing	↑	2'-O-methylcytidine	0.0033	0.1752	1.54
Cofactors and Vitamins					
Nicotinate and Nicotinamide Metabolism	↑	Nicotinamide	0.0020	0.1425	7.85
Tocopherol Metabolism	↓	Alpha-CEHC	0.0473	0.3952	0.97
Hemoglobin and Porphyrin Metabolism	↓	Bilirubin (E,E)	0.0275	0.3798	0.48
Xenobiotics					
Tobacco Metabolite	↓	Hydroxycotinine	0.0305	0.3904	0.21
Food Component/Plant	↑	Gluconate	0.0296	0.3883	1.27
	↓	Dihydroferulic acid	0.0163	0.3075	0.18
	↓	Umbelliferone sulfate	0.0490	0.3952	0.40
Chemical	↓	Methylnaphthyl sulfate	0.0321	0.3904	0.28

Table S3. Significantly altered metabolites after seven days of valproic acid monotherapy; a comparison of pretreatment samples versus samples collected during treatment for patients classified as responders to antileukemic therapy. The comparison is based on the results for 5 responders from the study by Fredly *et al.* (PMID 23915396) [2]. Seventy-eight metabolites were significantly altered after valproic acid therapy; 48 were significantly increased and 30 decreased. The arrows (↑↓) indicate whether the metabolite was increased or decreased by the treatment. Metabolites with $q<0.05$ are marked with yellow.

Main class / Subclassifications		Metabolite
Amino Acid (n=17)		
Glutamate Metabolism	↑	Glutamate
Lysine Metabolism	↓	N6,N6,N6-trimethyllysine
	↑	2-aminoadipate
Phenylalanine Metabolism	↑	Phenylacetate
Tyrosine Metabolism	↑	4-hydroxycinnamate sulfate
	↑	Catechol glucuronide
Tryptophan Metabolism	↓	Tryptophan
	↓	Kynurenine
	↓	Indolelactate
	↓	Indoleacetylglutamine
	↓	5-bromotryptophan
Leucine, Isoleucine and Valine Metabolism	↑	Isovalerylcarnitine (C5)
	↑	Beta-hydroxyisovalerate
	↓	3-methylglutarylcarnitine (2)
	↑	Isobutyrylcarnitine (C4)
Methionine, Cysteine, SAM and Taurine Metabolism	↑	Cysteine sulfinic acid
Urea cycle; Arginine and Proline Metabolism	↑	Homocitrulline
Peptide (n=3)		
Dipeptide	↑	Glycylvaline
Acetylated Peptides	↑	Phenylacetylcarnitine
	↓	4-hydroxyphenylacetylglutamine
Carbohydrate (n=3)		
Disaccharides and Oligosaccharides	↑	Lactose
Fructose, Mannose and Galactose Metabolism	↓	Mannose
Aminosugar Metabolism	↓	N-acetylneuraminate
Lipid (n=32)		
Medium Chain Fatty Acid	↓	10-undecenoate (11:1n1)
	↓	5-dodecenoate (12:1n7)
Polyunsaturated Fatty Acid (n3 and n6)	↓	Adrenate (22:4n6)
Fatty Acid, Dicarboxylate	↑	Adipate (C6-DC)
	↑	Suberate (C8-DC)
	↑	Sebacate (C10-DC)
	↓	3-carboxy-4-methyl-5-pentyl-2-furanpropionate (3-Cmpfp)
Fatty Acid, Amino	↓	2-aminooctanoate
Fatty Acid Metabolism (also BCAA Metabolism)	↑	Propionylcarnitine (C3)
Fatty Acid Metabolism(Acyl Glycine)	↑	Hexanoylglycine
Fatty Acid Metabolism(Acyl Carnitine)	↑	3-hydroxybutyrylcarnitine (2)
	↑	Suberoylcarnitine (C8-DC)
	↑	Adipoylcarnitine (C6-DC)
Fatty Acid, Monohydroxy	↓	2-hydroxynervonate
	↑	3-hydroxyhexanoate

	↓	3-hydroxydecanoate
	↓	3-hydroxyoleate
	↓	3-hydroxylaurate
	↑	5-hydroxyhexanoate
	↑	5-hydroxyvalproate
Phosphatidylethanolamine (PE)	↑	1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)
	↑	1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)
Phosphatidylinositol (PI)	↑	1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)
Diacylglycerol	↑	Diacylglycerol (14:0/18:1, 16:0/16:1) [1]
	↑	Diacylglycerol (14:0/18:1, 16:0/16:1) [2]
	↑	Palmitoyl-palmitoyl-glycerol (16:0/16:0) [2]
	↑	Oleoyl-oleoyl-glycerol (18:1/18:1) [2]
	↓	Linoleoyl-linolenoyl-glycerol (18:2/18:3) [1]
Sterol	↑	Beta-sitosterol
Androgenic Steroids	↓	Dehydroisoandrosterone sulfate (DHEA-S)
	↓	Androsterone glucuronide
Primary Bile Acid Metabolism	↑	Tauro-beta-muricholate
Nucleotide (n=2)		
Pyrimidine Metabolism, Uracil containing	↓	2'-deoxyuridine
Pyrimidine Metabolism, Thymine containing	↑	3-aminoisobutyrate
Cofactors and Vitamins (n=3)		
Ascorbate and Aldarate Metabolism	↓	Oxalate (ethanedioate)
Tocopherol Metabolism	↑	Gamma-CEHC
Hemoglobin and Porphyrin Metabolism	↓	Bilirubin (E,E)
Xenobiotics (n=16)		
Benzoate Metabolism	↑	3-(3-hydroxyphenyl)propionate sulfate
	↑	3-(3-hydroxyphenyl)propionate
Food Component/Plant	↑	Cinnamoylglycine
	↑	Dihydroferulic acid
	↑	Ferulic acid 4-sulfate
	↑	Glycyrrhetinate
	↓	Naringenin 7-glucuronide
	↓	Isoeugenol sulfate
Drug - Cardiovascular	↓	4-hydroxycoumarin
	↓	Candesartan
Drug - Neurological	↑	3-hydroxyvalproate
	↑	2-propyl-2-pentenoate (2-ene-valproate)
Chemical	↑	3-acetylphenol sulfate
	↑	HEPES
	↑	1,2,3-benzenetriol sulfate (2)
	↑	2-methoxyresorcinol sulfate
Partially Characterized Molecules (n=2)		
Partially Characterized Molecules	↑	Glucuronide of C8H16O2 (1)
	↓	Glucuronide of C14H22O4 (2)

Table S4. Significantly altered metabolites after seven days of valproic acid therapy; a comparison of pretreatment samples versus samples collected during treatment for patients classified as non-responders to antileukemic treatment. The comparison is based on the results for 5 non-responders from the study by Fredly *et al.* (23915396). A total of 105 metabolites were significantly altered after valproic acid therapy; 52 were significantly increased and 53 were decreased. The arrows (↑↓) indicate whether the metabolite was increased or decreased by the treatment. Metabolites with $q<0.05$ are marked with yellow.

Main class / Subclassifications		Metabolite
Amino Acid (n=21)		
Glycine, Serine and Threonine Metabolism	↑	Dimethylglycine
Alanine and Aspartate Metabolism	↑	N-acetylalanine
Glutamate Metabolism	↑	Carboxyethyl-GABA
	↓	S-1-pyrroline-5-carboxylate
Histidine Metabolism	↑	N-acetylhistidine
Lysine Metabolism	↑	6-oxopiperidine-2-carboxylate
Phenylalanine Metabolism	↑	N-acetylphenylalanine
Tyrosine Metabolism	↑	N-acetyltyrosine
Tryptophan Metabolism	↓	Tryptophan
	↑	N-acetyltryptophan
	↑	Tryptophan betaine
	↓	Indoleacetate
	↓	Indoleacetylglutamine
	↓	5-bromotryptophan
Leucine, Isoleucine and Valine Metabolism	↑	Beta-hydroxyisovalerate
	↓	3-methylglutarylcarnitine (2)
	↑	Ethylmalonate
	↑	Isobutyrylcarnitine (C4)
	↑	2,3-dihydroxy-2-methylbutyrate
Methionine, Cysteine, SAM and Taurine Metabolism	↑	N-acetylmethionine
Urea cycle; Arginine and Proline Metabolism	↑	Trans-4-hydroxyproline
Peptide (n=2)		
Acetylated Peptides	↓	4-hydroxyphenylacetylglutamine
	↓	Phenylacetylglycine
Carbohydrate (n=4)		
Pentose Metabolism	↑	Ribitol
	↑	Xylose
	↑	Arabitol/xylitol
Fructose, Mannose and Galactose Metabolism	↓	Galactonate
Energy (n=1)		
TCA Cycle	↓	Succinylcarnitine (C4-DC)
Lipid (n=49)		
Medium Chain Fatty Acid	↓	Caprate (10:0)
	↓	10-undecenoate (11:1n1)
Fatty Acid, Dicarboxylate	↑	Adipate (C6-DC)
	↑	Pimelate (C7-DC)
	↑	Suberate (C8-DC)
	↓	Dodecanedioate (C12-DC)

	↓	Tetradecanedioate (C14-DC)
	↓	Hexadecenedioate (C16:1-DC)
	↓	Octadecanedioate (C18-DC)
	↓	Octadecenedioate (C18:1-DC)
	↓	Eicosanodioate (C20-DC)
	↓	3-carboxy-4-methyl-5-pentyl-2-furanpropionate (3-Cmpfp)
Fatty Acid Metabolism(Acyl Glycine)	↑	Hexanoylglycine
Fatty Acid Metabolism(Acyl Carnitine)	↑	Hexanoylcarnitine (C6)
	↑	5-dodecenoylcarnitine (C12:1)
	↑	Cis-4-decenoylcarnitine (C10:1)
	↑	Laurylcarnitine (C12)
	↑	Myristoylcarnitine (C14)
	↑	Myristoleoylcarnitine (C14:1)
	↑	Suberoylcarnitine (C8-DC)
	↑	Adipoylcarnitine (C6-DC)
	↑	Pimeloylcarnitine/3-methyladipoylcarnitine (C7-DC)
Fatty Acid, Monohydroxy	↓	2-hydroxyoctanoate
	↓	2-hydroxydecanoate
	↑	3-hydroxyhexanoate
	↑	3-hydroxysebacate
	↑	5-hydroxyvalproate
Endocannabinoid	↓	N-stearoylserine
Phosphatidylcholine (PC)	↓	1-stearoyl-2-docosahexaenoyl-GPC (18:0/22:6)
Phosphatidylinositol (PI)	↑	1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)
	↑	1-stearoyl-2-linoleoyl-GPI (18:0/18:2)
Lysophospholipid	↓	1-palmitoyl-GPC (16:0)
	↓	1-palmitoleoyl-GPC (16:1)
	↓	1-stearoyl-GPC (18:0)
Diacylglycerol	↓	Diacylglycerol (14:0/18:1, 16:0/16:1) [1]
	↓	Diacylglycerol (14:0/18:1, 16:0/16:1) [2]
	↓	Palmitoyl-oleoyl-glycerol (16:0/18:1) [2]
	↓	Palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]
Sphingolipid Metabolism	↓	Sphinganine-1-phosphate
Progestin Steroids	↓	5alpha-pregnan-3beta,20alpha-diol monosulfate (2)
	↓	Pregnanediol-3-glucuronide
Corticosteroids	↑	Cortisone
Androgenic Steroids	↑	Androstenediol (3beta,17beta) disulfate (1)
	↑	Androstenediol (3beta,17beta) disulfate (2)
	↑	Andro steroid monosulfate C19H28O6S (1)
Primary Bile Acid Metabolism	↓	Glycochenodeoxycholate glucuronide (1)
Secondary Bile Acid Metabolism	↓	Isoursodeoxycholate
	↓	7-ketolithocholate
	↓	3b-hydroxy-5-cholenoic acid
Nucleotide (n=6)		
Purine Metabolism, (Hypo)Xanthine/Inosine containing	↑	N1-methylinosine
Purine Metabolism, Adenine containing	↑	N6-carbamoylthreonyladenosine

Purine Metabolism, Guanine containing	↑	7-methylguanine
Pyrimidine Metabolism, Uracil containing	↑	3-ureidopropionate
Pyrimidine Metabolism, Cytidine containing	↑	Cytidine
Pyrimidine Metabolism, Thymine containing	↑	3-aminoisobutyrate
Cofactors and Vitamins (n=4)		
Nicotinate and Nicotinamide Metabolism	↑	N1-Methyl-2-pyridone-5-carboxamide
Pantothenate and CoA Metabolism	↓	Pantothenate
Tocopherol Metabolism	↑	Gamma-CEHC
Hemoglobin and Porphyrin Metabolism	↓	Bilirubin (E,E)
Xenobiotics (n=15)		
Benzoate Metabolism	↓	4-hydroxyhippurate
	↓	Catechol sulfate
	↓	Guaiacol sulfate
	↓	4-ethylphenylsulfate
Xanthine Metabolism	↑	3-methylxanthine
Food Component/Plant	↓	Ferulylglycine (1)
	↓	Ferulylglycine (2)
	↓	Acesulfame
	↓	Thymol sulfate
	↓	4-allylphenol sulfate
Drug - Analgesics, Anesthetics	↓	Lidocaine
Drug - Cardiovascular	↓	4-hydroxycoumarin
Drug - Neurological	↑	3-hydroxyvalproate
	↑	2-propyl-2-pentenoate (2-ene-valproate)
Chemical	↑	O-sulfo-L-tyrosine
Partially Characterized Molecules (n=3)		
Partially Characterized Molecules	↑	Glucuronide of C8H16O2 (1)
	↓	Glucuronide of C10H18O2 (7)
	↓	Glucuronide of C14H22O4 (2)

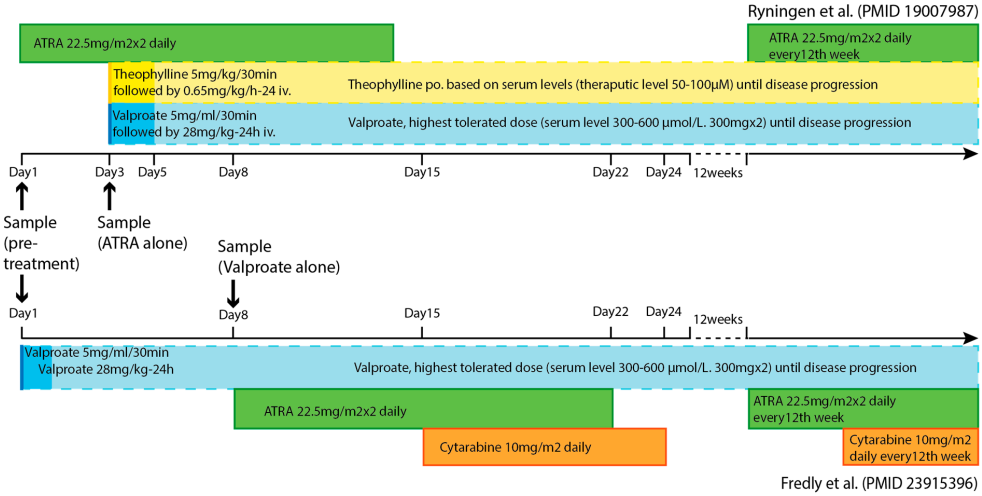


Figure S1. Timeline of treatment schedule for patients included in two clinical studies. In the first study shown at the top (Ryningen *et al.*), patients were give ATRA alone for two days before theophylline and valproic acid were given on day 3. Samples were collected pretherapy (day 1) and after 2-days of ATRA treatment. In the second study shown at the bottom (Fredly *et al.*), patients received valproic acid alone for 7 days before ATRA and then subsequently cytarabine were given. Samples were collected pretherapy (day 1) and after 7 days of valproic acid therapy.

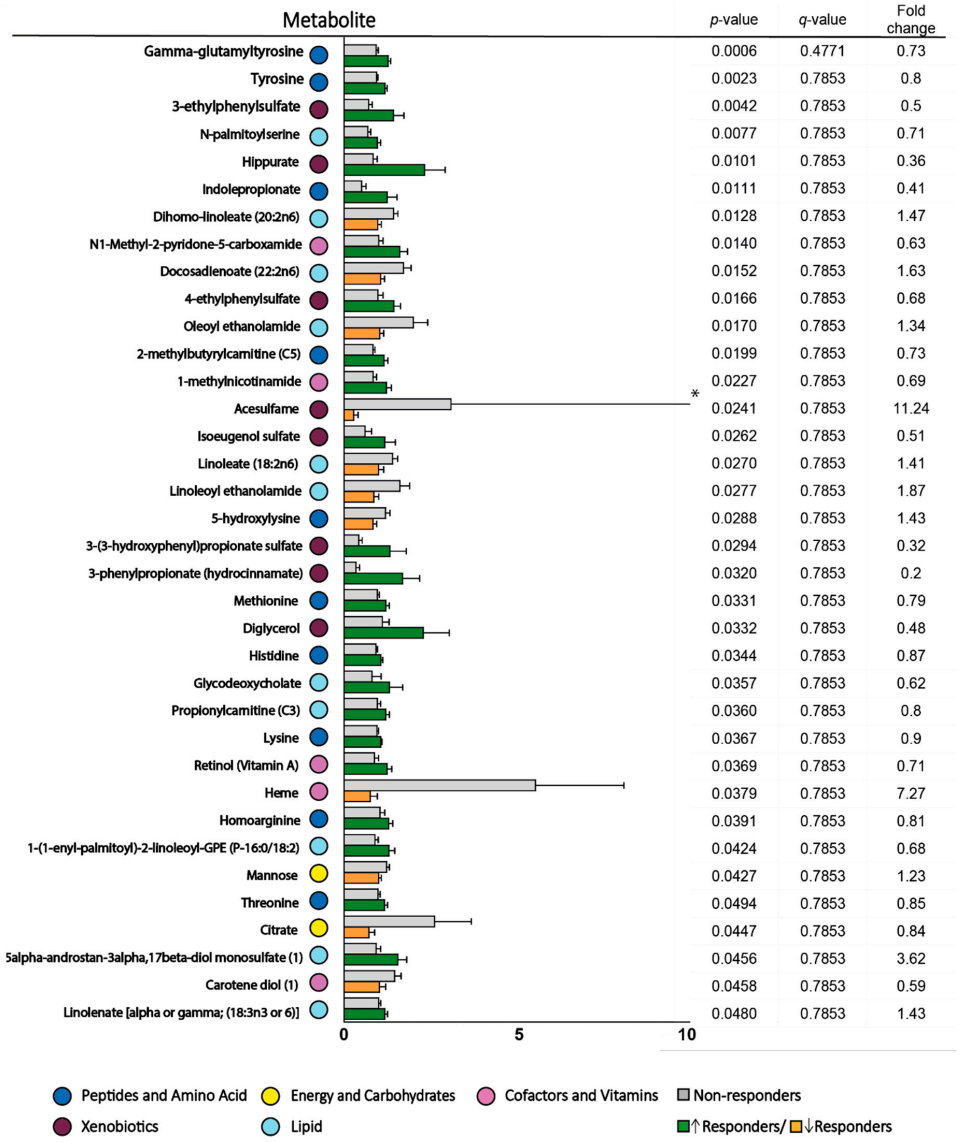


Figure S2. Identification and classification of metabolites in pretherapy serum samples that differed significantly between responders and non-responders to the antileukemic treatment of ATRA plus valproic acid. Thirty-six metabolites differed significantly between responders and non-responders (p<0.05, Welch´s two sample t-test). The p-values, q-values and mean fold change values for each metabolite are listed to the right in the figure (ranked by p-value), and a fold change >1 indicates that the levels were increased in responders compared with non-responders. Metabolite levels for non-responders are shown in grey, while increased levels in responders are shown in green (25/36 increased), and decreased levels in responders are shown in orange (11/36 decreased). Color codes for classification of metabolites are explained at the bottom of the figure. Error bars show Standard deviation (SD). * Acesulfame SD 8.963.

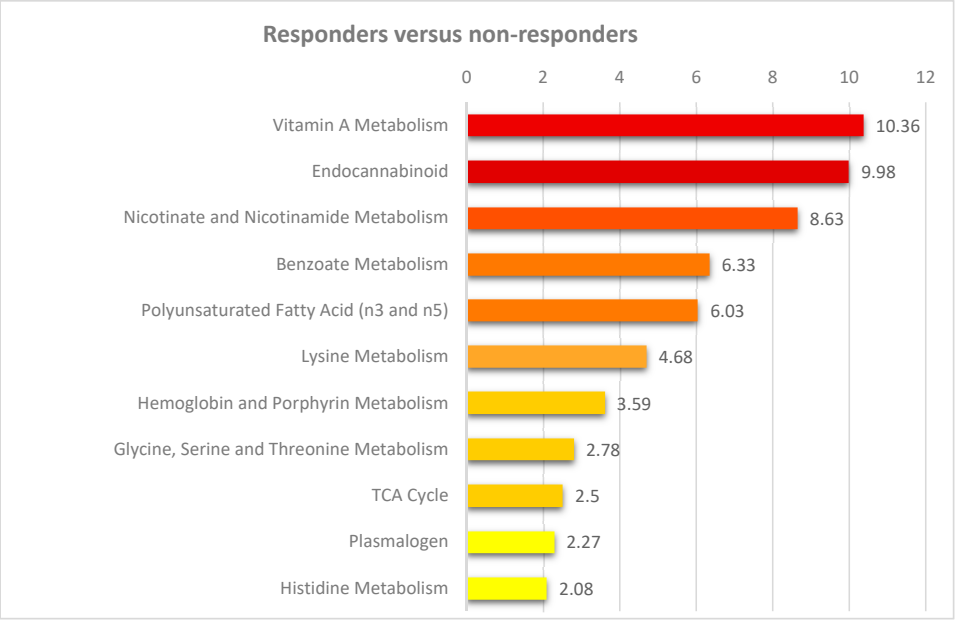


Figure S3. Pathway enrichment analysis based on pretreatment levels of metabolites that differed between responders and non-responders to antileukemic treatment based on ATRA and valproic acid. The analysis is used for visualization and biological interpretation of the metabolomics data and was based on significantly altered metabolites ($p<0.05$). Only signaling pathways with an enrichment value greater than two and at least two metabolites within each pathway are shown. The most significant pathway is shown in red and less significant pathways in light yellow.

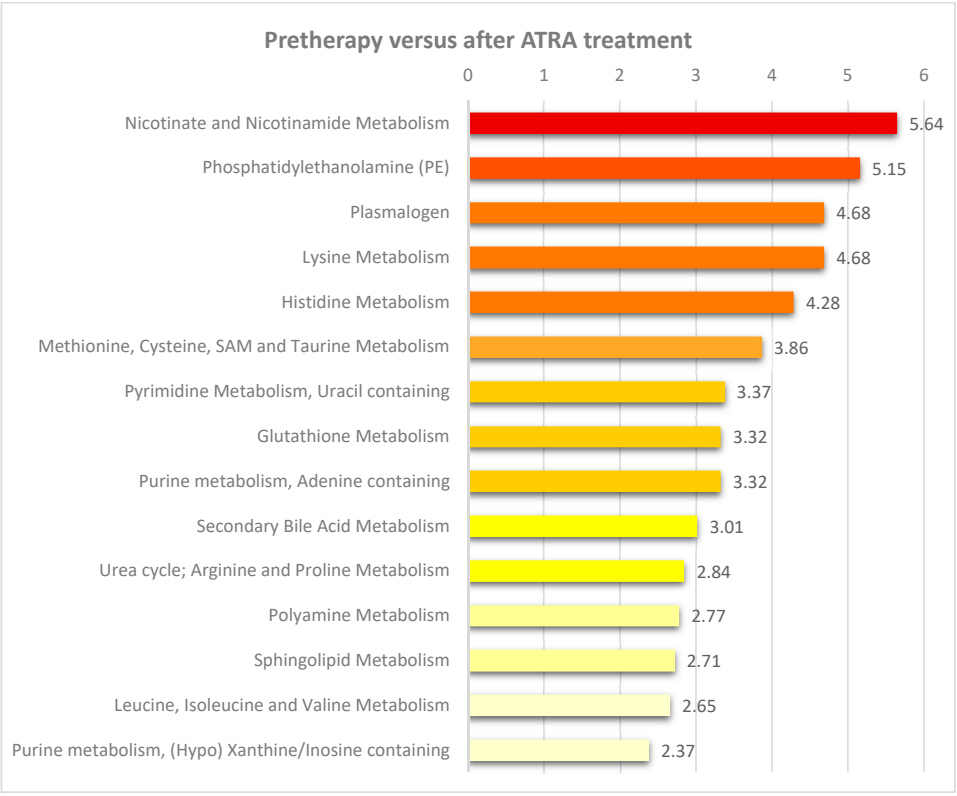
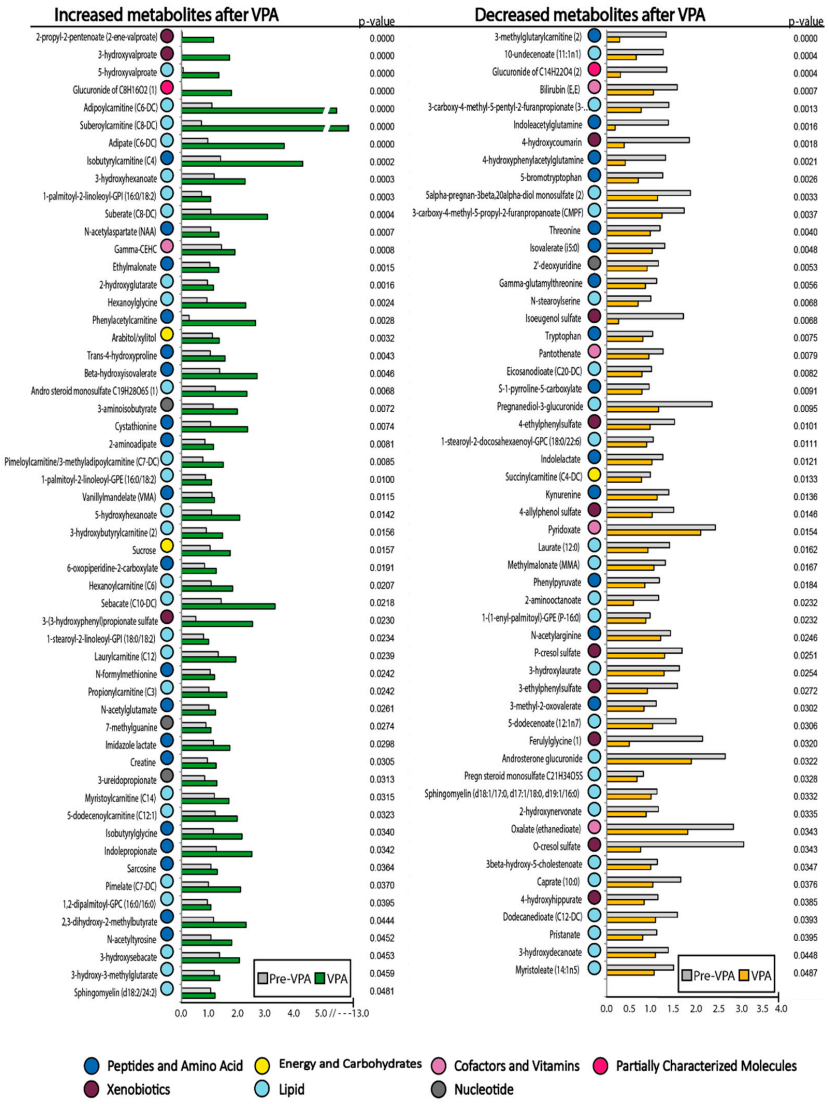
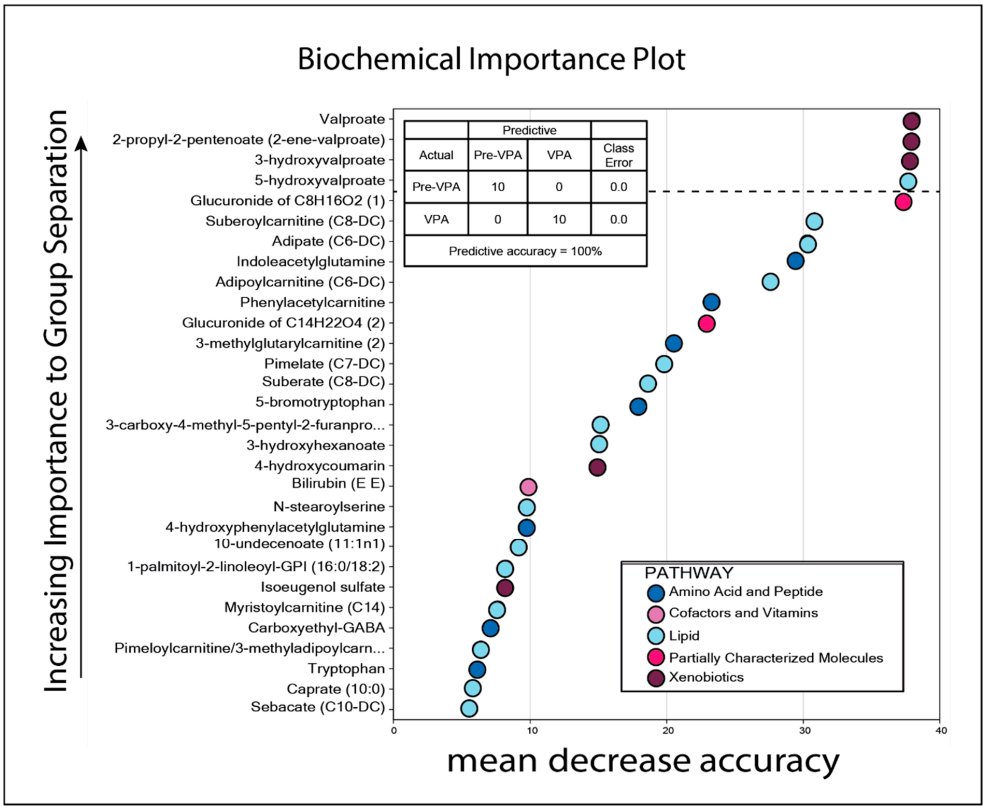


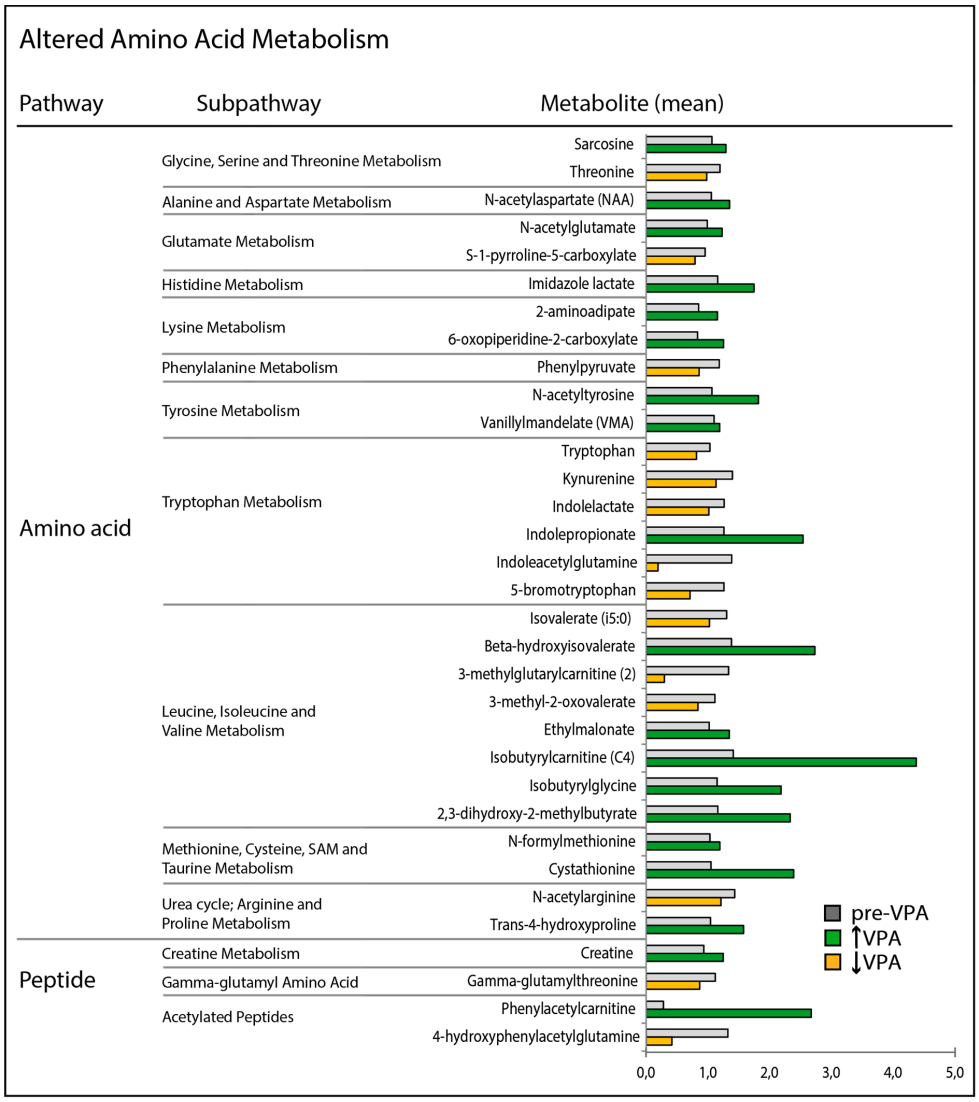
Figure S4. Pathway enrichment analysis based on metabolite levels that differed in patients pretherapy compared to during ATRA treatment for responders and non-responders to antileukemic treatment. The analysis was based on significantly altered metabolites ($p<0.05$), and only signaling pathways with an enrichment value greater than two and at least two metabolites within each pathway are shown in the figure. The most significant pathway is shown in red and less significant pathways in light yellow.



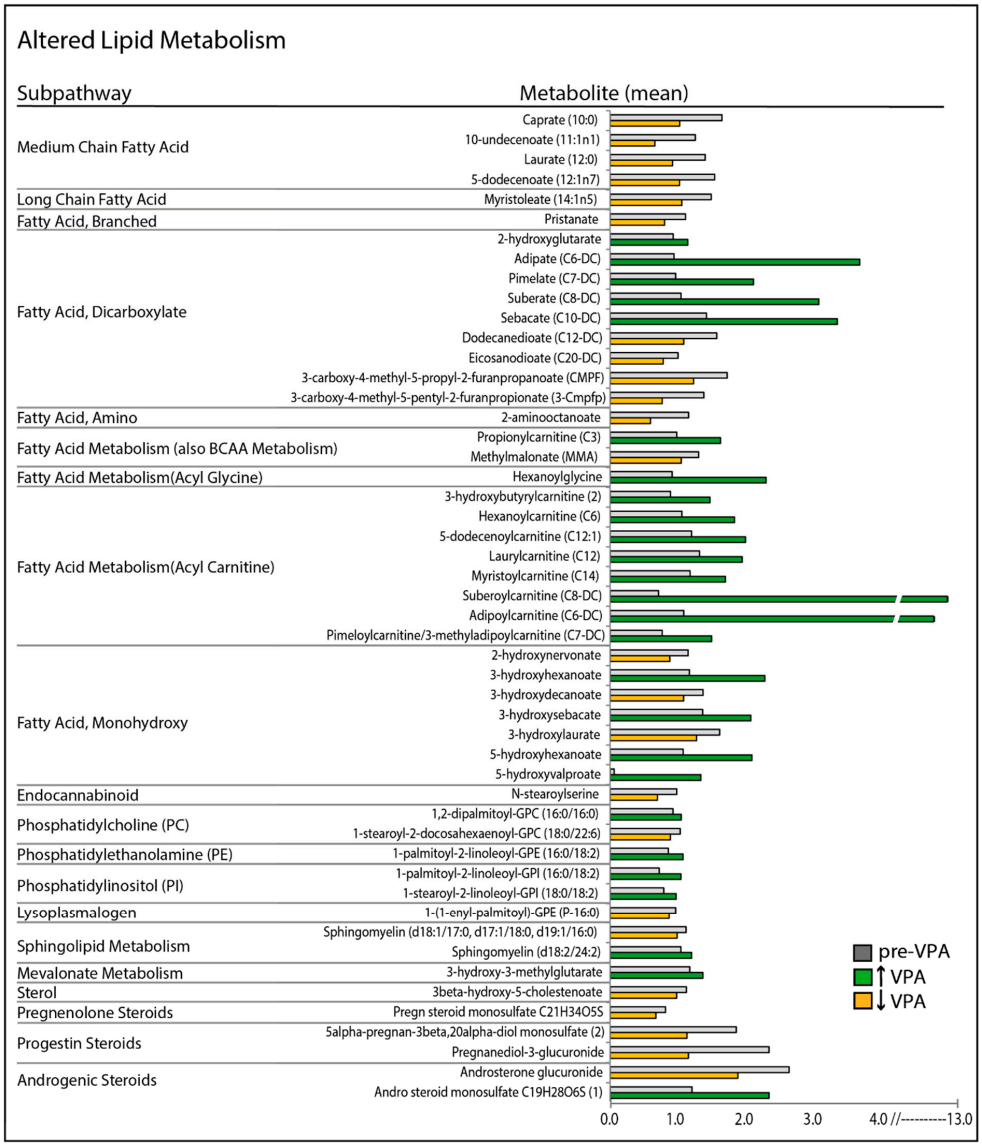
Supplementary Figure 5. The effect of 7-day valproic acid (VPA) monotherapy on the serum metabolomic profiles of AML patients. A total of 109 metabolites were significantly altered after VPA treatment ($p < 0.05$). The *p*-values for each individual metabolite are listed to the right, ranked according to *p*-values. Pretherapy systemic levels of metabolites are presented in light grey, increased levels during VPA treatment are presented in green and decreased levels in yellow. Color codes for classification of individual metabolites are explained at the bottom of the figure.



Supplementary Figure 6. The effect of valproic acid monotherapy for seven days on the serum metabolomics profiles for 10 patients (5 responders and 5 non-responders; patients included in the study described in PMID 23915396, valproic acid metabolites included in the study). The random forest analysis was based on the identification of all 886 metabolites in pretherapy samples and samples collected after seven days of treatment. The analysis showed a predictive accuracy of 100% (insert table). The importance plot shows the metabolites listed after their importance for separation of the two sets of samples. The figure shows the top-30 ranked metabolites. Color codes for classification of metabolites are shown to the lower right.



Supplementary Figure 7. The effect of 7-day valproic acid monotherapy on serum metabolomic profiles; significantly altered amino acid and peptide metabolites when comparing samples derived from 10 patients (5 responders and 5 non-responders; all patients included in the study by Fredly et al. PMID 23915396). The pretreatment samples are presented in grey; metabolites increased during valproic acid therapy are presented in green and decreased levels after valproic acid therapy are presented in yellow.



Supplementary Figure 8. The effect of 7-day valproic acid monotherapy on serum metabolomic profiles; significantly altered lipid metabolites when comparing samples derived from 10 patients (5 responders and 5 non-responders; all patients included in the study by Fredly et al. PMID 23915396). The pretreatment samples are presented in grey; metabolites increased during valproic acid therapy are presented in green and decreased levels after valproic acid treatment are presented in yellow.

IV

frontiers in Immunology

ORIGINAL RESEARCH
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Patients with Treatment-Requiring Chronic Graft versus Host Disease after Allogeneic Stem Cell Transplantation Have Altered Metabolic Profiles due to the Disease and Immunosuppressive Therapy: Potential Implication for Biomarkers

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Chronic graft versus host disease (cGVHD) is a common long-term complication after allogeneic hematopoietic stem cell transplantation. The objective of our study was to compare the metabolic profiles for allotransplant recipients and thereby identify metabolic characteristics of patients with treatment-requiring cGVHD. The study included 51 consecutive patients (29 men and 22 women; median age: 44 years, range: 15–66 years) transplanted with peripheral blood stem cells derived from human leukocyte antigen-matched family donors. All serum samples investigated by global metabolomic profiling were collected approximately 1 year posttransplant (median 358 days). Thirty-one of the 51 patients (61%) had cGVHD 1 year posttransplant. The affected organs were (number of patients) liver/bile duct (23), eyes (15), gastrointestinal tract (14), skin (13), mouth (10), lungs (3), and urogenital tract (1). We compared the metabolic profile for patients with and without cGVHD, and a Random Forrest Classification Analysis then resulted in 75% accuracy in differentiating the two groups. The 30 top-ranked metabolites from this comparison included increased levels of bile acids, several metabolites from the cytokine-responsive kynurenine pathway for tryptophan degradation, pro-inflammatory lipid metabolites, phenylalanine and tyrosine metabolites derived from the gut microbial flora, and metabolites reflecting increased oxidative stress. However, nine of these 30 top-ranked metabolites were probably altered due to cyclosporine or steroid treatment, and we therefore did a hierarchical clustering analysis including all 51 patients but only based on the other 21 cGVHD-specific metabolites. This analysis identified three patient subsets: one cluster included mainly patients without cGVHD and had generally low metabolite levels; another cluster included mainly patients with cGVHD (most patients with at least three affected organs) and high metabolite levels, and the last intermediate group including cGVHD patients

with limited organ involvement. We conclude that allotransplant recipients with cGVHD have an altered metabolic profile caused both by the disease and its immunosuppressive treatment.

Keywords: metabolomics, chronic graft versus host disease, stem cell transplantation, biochemical profiling, biomarkers

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is used in the treatment of severe bone marrow failure and aggressive hematological malignancies, including acute leukemia (1, 2). The treatment approach depends on the ability of the engrafting immune system to remove residual leukemia cells *via* a graft-versus-leukemia effect (1). Allo-HSCT is then a potentially curative treatment, although at the same time the treatment is associated with a relatively high risk of morbidity and mortality due to severe transplant-related complications (3). Chronic graft versus host disease (cGVHD) is then the most common cause of late non-relapse mortality (4–6). Guidelines for the diagnosis and treatment of this complication have recently been published (7). However, the complex immunopathology of cGVHD is still poorly understood (8), and preclinical models have weakness and limitations in the study of the disease (9). An increasing interest for biomarkers, to confirm diagnosis and prognosis in cGVHD, has evolved the last decade (10–13), although still no biomarkers are established in routine clinical practice (10, 13). Among the risk factors for cGVHD are older patient age, previous acute GVHD (aGVHD), reduced intensity conditioning, female donor to male recipient, peripheral blood stem cell (PBSC) grafts and human leukocyte antigen (HLA) mismatched donors (14–19).

Graft versus host disease can be considered an exaggerated manifestation of normal inflammatory mechanisms in which donor lymphocytes encounter foreign antigens in a pro-inflammatory milieu, and this inflammation involves several donor immunocompetent cell subsets (8, 9, 20–22). Metabolic regulation is important for immunoregulation, and we have previously demonstrated that pretransplant cytokine profiles as well as the pretransplant metabolic status of allotransplant recipients is associated with a risk of later aGVHD (23–25).

Our present study was initiated to compare patients with and without cGVHD 1 year posttransplant and thereby identify possible associations between the serum metabolic profile, the diagnosis and severity (i.e., organ involvement) of cGVHD requiring systemic immunosuppression, and the effects of this immunosuppressive (i.e., cyclosporine, steroids) on the metabolic profiles in cGVHD patients.

Abbreviations: aGVHD, acute graft versus host disease; allo-HSCT, allogeneic hematopoietic stem cell transplantation; cGVHD, chronic graft versus host disease; GC, gas chromatography; G-CSF, granulocyte-colony stimulating factor; GVL, graft-versus-leukemia; HLA, human leukocyte antigen; LC, liquid chromatography; MS, mass spectrometry; PBSC, peripheral blood stem cell; PCA, principal component analysis; RIC, reduced intensity conditioning; TNF-α, tumor necrosis factor-alpha.

MATERIALS AND METHODS

Patients' Characteristics

The study was approved by the local Ethics Committee (Regional Ethics Committee III, University of Bergen, Norway; REK), and the samples were collected after obtaining written informed consent from the patients. The study included 51 consecutive allotransplant recipients (29 men and 22 women; median age: 44 years with range: 15–66 years) with HLA-matched family donors; these patients were transplanted during the period March 2006–December 2014. Ninety-five patients were transplanted in our institution during this period; 25 of them died from treatment-related causes, 6 patients relapsed, and 13 were lost to follow-up. The decision to perform an allo-HSCT was taken by the Norwegian Advisory Board for Stem Cell Transplantation and based on national guidelines. Thus, our study is population-based and includes an unselected and consecutive group of well-characterized patients with family donors. All samples were collected approximately 1 year post-transplant (median 358 days). The patient characteristics are given in **Table 1** and **Figure 1**. Patients were transplanted with granulocyte colony-stimulating factor mobilized PBSC. Most patients received GVHD prophylaxis with cyclosporine A plus methotrexate ($n = 50$), only one patient received cyclosporine A alone.

Detailed information about individual patients is given in **Figure 1**, including previous aGVHD, the presence of treatment-requiring cGVHD and when this was diagnosed, the type of cGVHD. All these patients were able to travel with public communication to come to the hospital for controls and blood sampling. All except two patients had active cGVHD requiring continued immunosuppression, but only four patients (patients 37, 38, 42, and 43) had platelet counts observed below 100×10^9/L at the time of sampling (this was also true for the time of diagnosis).

Diagnosis of cGVHD

Chronic graft versus host disease was diagnosed according to generally accepted criteria based on careful clinical evaluation and additional biopsies for histological confirmation (7, 26).

Preparation of Serum Samples

All venous blood samples were collected into sterile plastic tubes (BD Vacutainer® SST™ Serum Separation Tubes; Becton-Dickenson, Franklin Lakes, NJ, USA) and allowed to coagulate for 120 min at room temperature (18°C) before centrifugation ($300 \times g$ for 10 min) and serum collection. All samples were immediately frozen and stored at −70°C until analyzed.

TABLE 1 | Demographical, clinical, and laboratory data for the 51 patients included in the study.

Patient characteristics		Observation	+cGVHD	−cGVHD
Demographic data and disease history				
Gender (numbers)	Male/female	29/22	19/12	10/10
Age (years, median and range)		44 (15–66)	44 (18–62)	43 (15–66)
Height (cm, median and range)		172 (149–193)	169 (158–190)	172 (149–193)
Weight (kg, median and range)		69 (42–133)	72 (47–133)	66 (42–98)
BMI (kg/m^2, median and range)		23.4 (16.9–39.7)	23.7 (17.9–39.7)	22.2 (16.9–28.5)
Diagnosis (numbers)	AML/MDS	31	22	9
	ALL	13	8	5
	CLL	2	0	2
	MF	4	1	3
	AA	1	0	1
Conditioning regimen (numbers)	BU + CY	39	25	14
	ATG + CY	1	0	1
	TBI + CY	1	1	0
	BEAM	1	1	0
	TBI + ETO	1	0	1
	FLU + BU	5	3	2
	FLU + CY	2	0	2
	FLU + BU + ATG	1	1	0
cGVHD organ involvement	Liver (23 patients), eyes (15), gastrointestinal tract (14), skin (13), mouth (10), lungs (3) and urogenital tract (1).			

Unless otherwise stated the values are presented as median with range given in parenthesis. Height and weight were registered at the start of conditioning therapy. BMI, body mass index; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MF, myelofibrosis; AA, aplastic anemia; BU, busulfan; CY, cyclophosphamide; ATG, anti-thymoglobulin; TBI, total body irradiation; ETO, etoposide; FLU, fludarabine; cGVHD, chronic graft versus host disease.

Analysis of Metabolite Serum Levels

Metabolomic analysis was done in collaboration with Metabolon® (27). Briefly, samples were prepared using the automated MicroLab STAR® system (Hamilton Company, Bonaduz, Switzerland). A recovery standard was added prior to the first step in the extraction process for quality control. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min followed by centrifugation. The resulting extract was divided into four fractions: one for analysis by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)/MS with positive ion mode electrospray ionization, the second for analysis by UPLC-MS/MS with negative ion mode electrospray ionization, the third for analysis by gas chromatography-mass spectrometry, and the last sample was reserved as a backup. Samples were placed briefly on a Zymark TurboVap® (McKinley Scientific, Sparta, NJ, USA) to remove the organic solvent. The samples for liquid chromatography (LC) were stored overnight under nitrogen before preparation for analysis. For GC, each sample was dried under vacuum overnight before preparation for analysis. A total of 755 metabolites of known identities (named biochemicals) were analyzed in all samples (Table S1 in Supplementary Material).

Bioinformatical and Statistical Analyses

Bioinformatical analyses were performed using the J-Express (MolMine AS, Bergen, Norway) (28). For hierarchical clustering, all values were median variance standardized and log(2) transformed. The complete linkage was used as the linkage method, and for distance measured the Pearson correlation was used. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA). The Mann–Whitney *U*-test was used to identify biochemicals that differed significantly between groups. The Chi-Square test was used for analysis of categorized data. Unless otherwise stated p-values < 0.05 were regarded as statistically significant.

RESULTS

Allotransplant Recipients Are Heterogeneous with regard to Their Serum Metabolic Profile When Tested 1 Year Posttransplant

31 of the 51 patients included in the study (61%) had signs of cGVHD 1 year posttransplant; 29 of the 31 cGVHD patients required systemic immunosuppressive treatment either as prolonged or increased treatment with cyclosporine A (27 patients). Eleven of these 27 cyclosporine-treated patients (22% of the whole cohort) received combination treatment also including systemic steroid therapy, and two additional patients received mycophenolate mofetil monotherapy. The two last patients were diagnosed with cGVHD of the skin and received only topical steroid treatment. Thus, the large majority of the 31 cGVHD patients (29/31) received systemic treatment either as prolonged

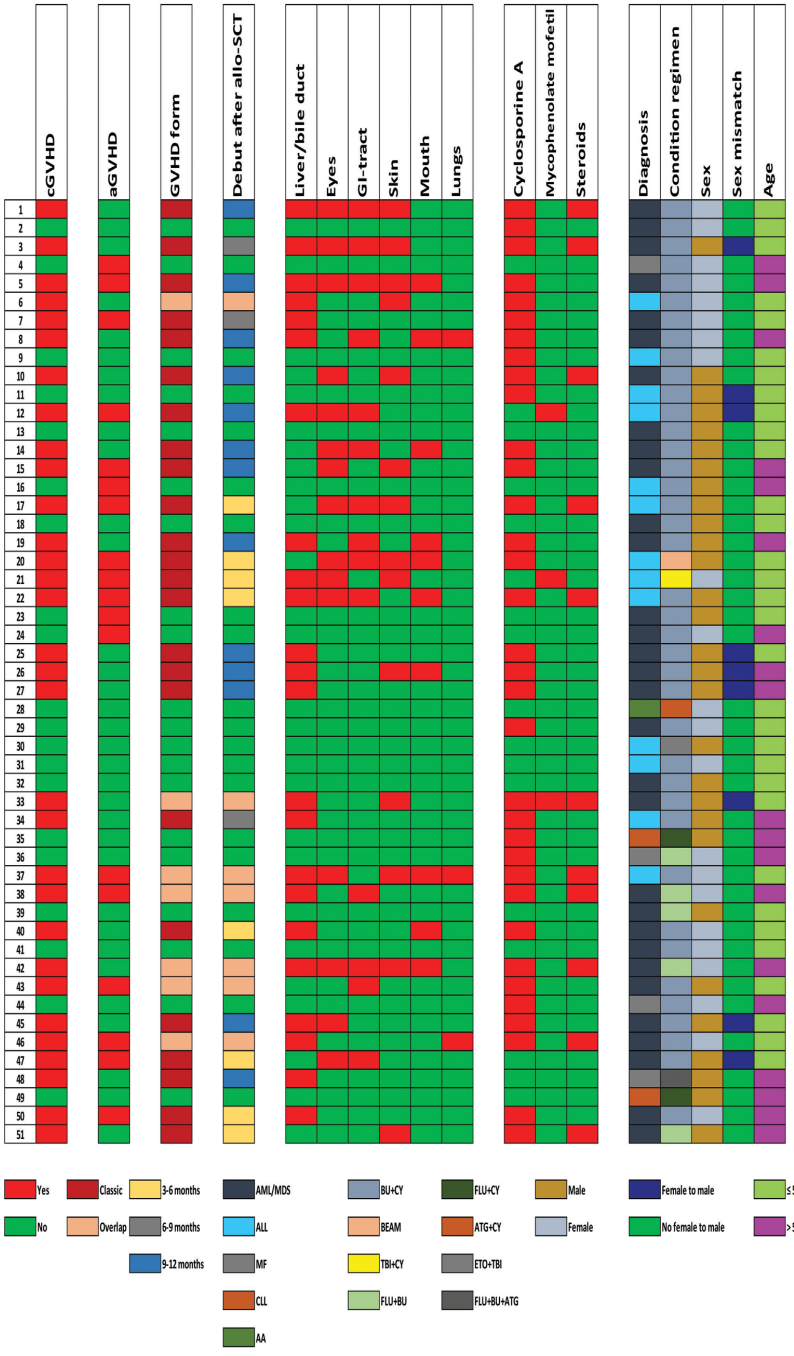

FIGURE 1 | Continued

FIGURE 1 | Clinical data of the 51 patients included in the study. The figure presents the clinical and demographical characteristics of the patients, including (from left to right) the presence of chronic graft versus host disease (cGVHD) and acute GVHD, the form of GVHD and the time when developing treatment-requiring cGVHD, organ involvement, ongoing immunosuppressive treatment at the time of sampling, and the clinical characteristics of the patients (hematological diagnosis, conditioning treatment, gender, female to male transplantation, and age). Abbreviations: AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MF, myelofibrosis; AA, aplastic anemia; BU, busulfan; CY, cyclophosphamide; ATG, antithymoglobulin; TBI, total body irradiation; ETO, etoposide; FLU, fludarabine.

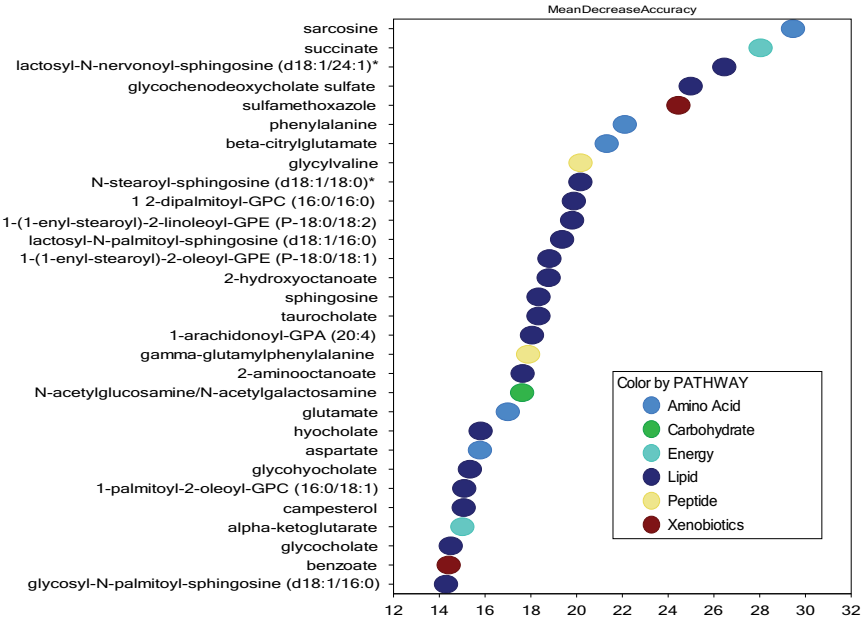

FIGURE 2 | Random forest analysis of the systemic metabolite profiles including all 51 patients; a comparison of patients with and without chronic graft versus host disease (cGVHD) and identification of the 30 top-ranked metabolites showing increased levels in patients with posttransplant cGVHD. Random forest analysis could distinguish between the metabolic signatures of patients with and without acute GVHD with a predictive accuracy of 75.0%. The figure presents the 30 top-ranked metabolites and their classification (indicated in the figure, lower right) based on their importance for the identification of the two patient subsets.

or increased cyclosporine A therapy, or they received additional immunosuppression with oral prednisolone (daily doses 2.5–40 mg) to maintain disease control. The most commonly affected organ was the liver/bile duct (23 patients; for additional details on organ involvement see **Figure 1** and **Table 1**).

Seven of the 20 patients without signs of cGVHD received prolonged cGVHD prophylaxis at 1 year posttransplant due to either previous severe aGVHD and/or the presence of other risk factors for the development of cGVHD. Thus, a total of 34 patients (67%) received cyclosporine A 1 year posttransplant.

We first used principal component analysis (PCA) and hierarchical clustering to analyze the overall metabolic profiles of the patients. However, these analyses could not distinguish between the 31 patients with and the 20 patients without cGVHD (data not shown); this is probably due to the metabolic heterogeneity for both patient subsets.

Patients with and without cGVHD Differ in Fatty Acid and Bile Acid Metabolism

In contrast to PCA and unsupervised hierarchical clustering, random forest analysis is an unbiased and supervised classification technique based on an ensemble of a large number of decision trees. In addition to producing a metric of predictive accuracy (**Figure 2**), this analysis also gives a list of the metabolites ranked according to their importance for the classification scheme, i.e., their degree of difference between the two compared groups. Random forest analysis of serum metabolic profiles differentiated patients with and without cGVHD with a predictive accuracy of 75%. Eighteen of the 30 top-ranked metabolites from this comparison reflected differences in lipid/fatty acid/bile acid metabolism (**Figure 2**), most of them belonging to the annotations sphingolipids, plasmalogens/lysoplasmalogens, lysolipids, and phospholipids. We therefore compared the levels

of all metabolites from these classes for patients with and without cGVHD. These subclasses include a total of 122 metabolites (Table S1 Supplementary Material), and 46 of them were significantly increased in patients with cGVHD.

Four of the 30 top-ranked metabolites (i.e., the secondary bile acid hyocholate and the primary bile acids glycochenodeoxycholate sulfate, taurocholate, and glycocholate) reflect differences in bile acid metabolism and were increased in patients with cGVHD (**Figure 2**). The subclass primary bile acids include 10 metabolites, and eight of them were significantly increased in patients with cGVHD. We therefore performed a hierarchical cluster analysis based on the serum levels of the 10 primary bile acid metabolites (**Figure 3**). This analysis identified a minor subset of 14 patients with generally low levels of these metabolites and including 12 of the 20 patients without cGVHD; only two patients with cGVHD were included and both patients required systemic immunosuppression with only cyclosporine A alone. The remaining patients (a major subset of 36 patients together with one outlier) showed relatively high levels of most primary bile acid metabolites and thus included 28 of the 31 patients with cGVHD, i.e., all patients requiring systemic steroids were included among these 37 patients. The difference in frequency of patients with cGVHD between these two groups was statistically significant (Chi-square test, $p < 0.0001$). Finally, neither the cGVHD patients receiving cyclosporine alone, receiving additional systemic steroids nor having cGVHD with liver involvement clustered together in this analysis.

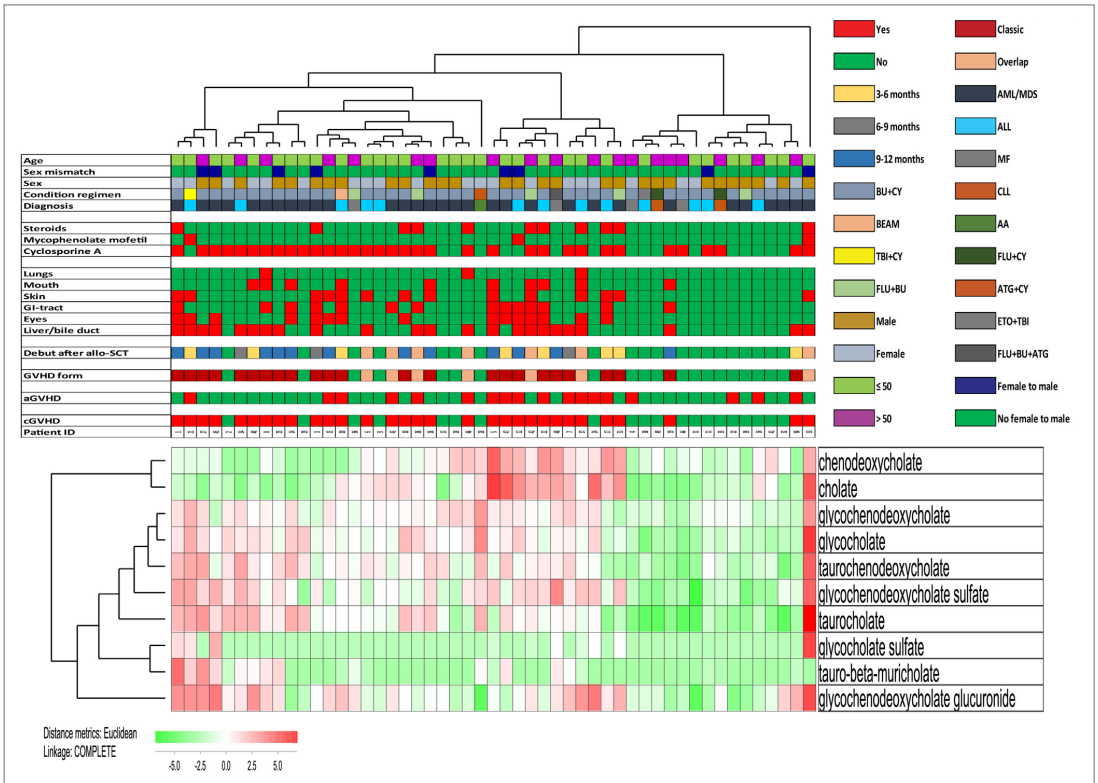

FIGURE 3 | Hierarchical clustering analysis including all 51 patients and based only on 10 primary bile acid metabolites, identification of patient subsets with high frequency of chronic graft versus host disease (cGVHD) patients. We performed a hierarchical clustering analysis (Euclidian Correlation, complete linkage) based on the bile acid metabolites indicated to the right in the figure. The heat map and the corresponding dendrograms are shown in the figure. As indicated to the lower left in the figure red indicates high metabolite levels and green low levels. The clinical characteristics of each individual patient are presented in the upper part of the figure; for the lower horizontal bars the presence of a factor is indicated by red and the absence by green, whereas the color codes for the upper horizontal bars are explained in the figure. We identified two main clusters; the left included a major part of patients with cGVHD, whereas the right cluster included mainly patients without cGVHD. 28 of the 31 cGVHD patients clustered in the group with high bile acid metabolite levels, two patients clustered in the group with low levels, and the last cGVHD patient was an outlier. The frequency of cGVHD patients differed significantly between the two main clusters (Chi-square test, $p < 0.0001$).

A Comparison of Patients with and without cGVHD by Metabolic Pathway Analysis—Increased Levels of Metabolic Markers of Inflammation, Protein Degradation and Oxidative Stress in cGVHD

We next performed a metabolic pathway analysis to compare patients with and without cGVHD (**Figure 4**). The nine highest ranked metabolic classes included (1) amino fatty acid metabolism (a small class only including two metabolites in our analysis); (2) sphingolipid, plasmalogen, and lysoplasmalogen metabolites; (3) sterol and primary bile acid metabolites; (4) amino acid metabolites (alanine and aspartate, glycine, serine, and threonine); and (5) amino sugar metabolism. Thus, this alternative analysis, which is based on the overall results and not only the highest ranked metabolites, shows that fatty acid/triglyceride/bile acid metabolism differs between patients with and without cGVHD not only when comparing the highest ranked metabolites but also when comparing the overall results.

The presence of cGVHD was associated with a metabolic signature consistent with ongoing inflammation and significantly increased levels of (1) the three lysolipid metabolites 1-linoleoyl-GPC (18:2), 1-oleoyl-GPC (18:1), 1-palmitoleoyl-GPC (16:1), (2) the eicosanoid 12-HETE; and (3) the sphingolipid sphingosine (**Figure 2**) (29, 30). These signs of inflammation could still be detected despite the systemic immunosuppressive treatment for the large majority of the cGVHD patients. Furthermore, patients with cGVHD showed a significant increase in phennylacetat, 3-(4-hydroxyphenyl) lactate, phenylalanine, and tyramine *o*-sulfate compared with patients without cGVHD; a possible explanation for these differences is altered gastrointestinal function (31), probably caused by gastrointestinal disturbances and altered microbial flora (**Figure 5**). Furthermore, increased levels of several markers for proteolysis and accelerated protein catabolism were also detected in the cGVHD patients (32), including *N*-acetylserine, *N*-acetylaspartate, *N*-acetylasparagine, *N*-acetylglutamate, and 1-methylhistidine (**Figure 5**).

Increased oxidative stress seems to be important in the pathophysiology of GVHD (33). The significantly increased levels in cGVHD patients of gamma-glutamyl amino acids (e.g., gamma-glutamylglutamate, gamma-glutamyltryptophan, gamma-glutamylphenylalanine, and gamma-glutamylthreonine) are consistent with an oxidative stress phenotype and increased activity of the gamma-glutamyl cycle that is important for recycling and regeneration of the antioxidant glutathione (34). Similarly, a significant increase in other oxidative stress markers, including alpha-tocopherol, cysteine sulfonic acid, and methionine sulfoxide (35), was also observed in cGVHD patients (**Figure 5**). Taken together, these observations suggest altered protein metabolism with disturbed redox homeostasis in cGVHD patients, and we therefore performed a hierarchical clustering analyses based on the 10 metabolites included in the term "oxidative stress" (Table S1 in Supplementary Material) (**Figure 6**). Two main clusters were then identified, and the frequency of cGVHD patients was significantly higher for the subset showing generally high levels of these metabolites ($p = 0.0010$, Chi-square test).

Steroid Treatment of cGVHD Is Associated with Increased Levels of Phospholipids, Lysolipids, Plasmalogen, Monoacylglycerol, and Diacylglycerol Metabolites

We first compared the metabolic profiles for the 11 patients receiving systemic steroid therapy versus all the other 40 patients. The 30 top-ranked metabolites are presented in **Figure 7**. 26 of the 30 top-ranked metabolites altered by systemic steroids were classified as lipid metabolites, and 20 of these lipid metabolites

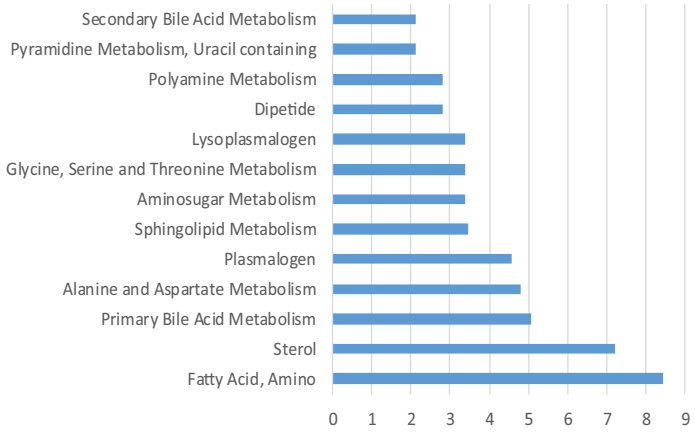

FIGURE 4 | Metabolic pathway analysis based on the analysis of all 51 patients; a comparison of patients with and without chronic graft versus host disease (cGVHD). The figure presents the metabolic pathways with an enrichment score > 2 when comparing patients with and patients without cGVHD.

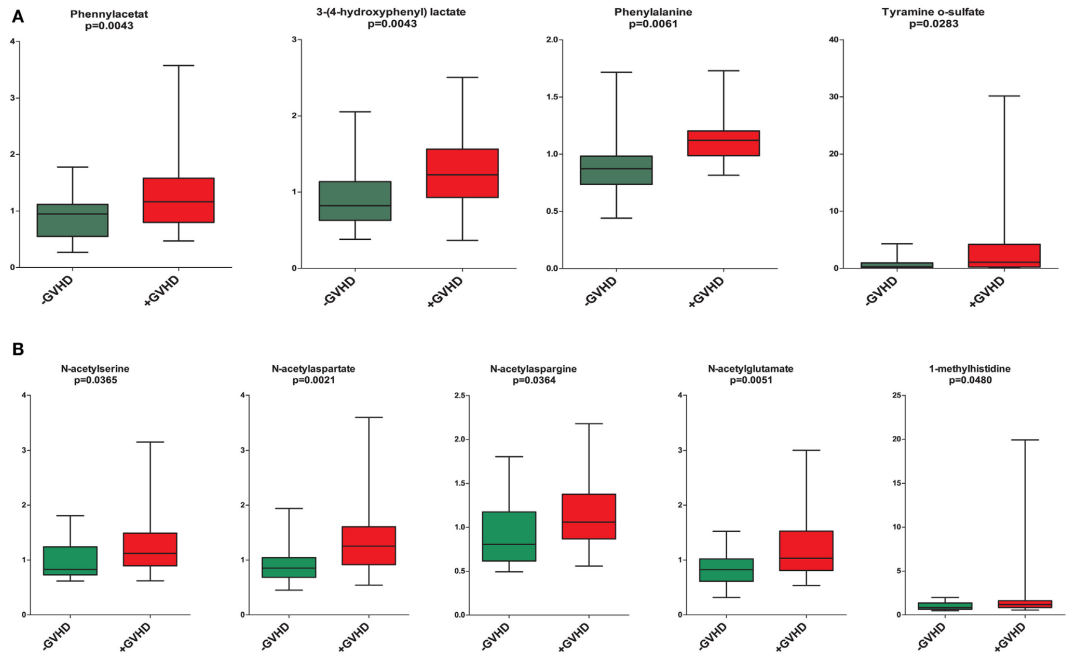

FIGURE 5 | Altered phenylalanine/tyrosine metabolism **(A)** and altered proteolysis **(B)**; a comparison of single metabolites including all patients with and without chronic graft versus host disease (cGVHD). [**(A)**, upper part] The serum levels of the four metabolites phennylacetat, 3-(4-hydroxyphenyl) lactate, phenylalanine, and tyramine *o*-sulfate were significantly increased in cGVHD patients compared to patients without cGVHD. [**(B)**, lower part] The serum levels of the proteolysis markers *N*-acetylserine, *N*-acetylaspartate, *N*-acetylasparagine, *N*-acetylglutamate, and 1-methylhistidine were significantly increased in cGVHD patients (marked with red) compared with patients without cGVHD (marked with green). All results are presented as the median, the 25%/75% percentiles and the variation range; results for cGVHD patients are presented as red boxes whereas patients without cGVHD are marked with green. The metabolites and the corresponding *p*-values (Mann–Whitney *U*-test) are shown at the top of each individual figure.

belonged to the subclasses phospholipid (5 metabolites), lysolipid (7), plasmalogen (4), monoacylglycerol (3), and diacylglycerol (1). Thus, a major effect of systemic steroid treatment seems to be altered triglyceride/fatty acid metabolism.

We then did an alternative analysis and compared the metabolic profiles for the nine cGVHD patients receiving systemic steroids together with cyclosporine versus the cGVHD patients only treated with cyclosporine, i.e., comparing two patient subset with similar cGVHD/cyclosporine status but differing with regard to steroid treatment (see **Figure 1** for identification of the patients in these two subsets). The top-ranked metabolites from this comparison also included a large number of lipid metabolites (20 metabolites), and most of these metabolites were classified as phospholipid (three metabolites), lysolipid (8), plasmalogen (2), monoacylglycerol (2), and diacylglycerol (1). Thus, this comparison also suggests that a major effect of systemic steroid treatment of patients with cGVHD is an altered fatty acid/triglyceride metabolism.

Finally, the steroid-associated pattern presented in **Figure 7** was also reflected in our overall comparison of patients with and without cGVHD (**Figure 2**), although the phospholipid (2 metabolites), lysolipid (1), plasmalogen (2), monoacylglycerol (none), and diacylglycerol (none) metabolites only constituted a minor subset (5 metabolites) among the 30 top-ranked metabolites from this cGVHD comparison. These metabolites may reflect the systemic steroid treatment, but several of them are also increased during inflammation (29, 30), and alternatively reflect the more severe manifestation of cGVHD for patients requiring systemic steroids.

Cyclosporine Has Diverse Effects on the Systemic Metabolic Profile of Allotransplant Recipients and These Effects Are Similar for Patients with and without cGVHD

We first compared the profile of all patients receiving cyclosporine (34 patients, including nine patients receiving combination treatment with systemic steroids) versus all the other allotransplant recipients (17 patients); the 30 top-ranked metabolites are shown in **Figure 8**. It can be seen that cyclosporine treatment had diverse effects and was associated with altered levels of many different

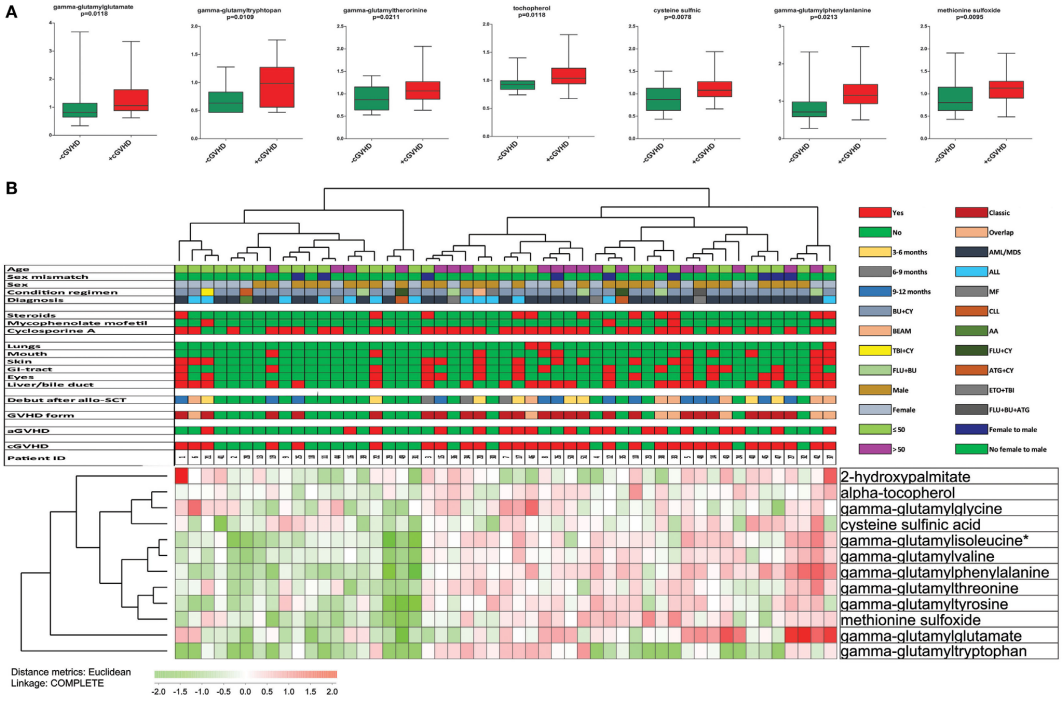

FIGURE 6 | Altered serum levels of oxidative stress markers in patients with chronic graft versus host disease (cGVHD); a comparison of patients with and without cGVHD and including all 51 patients. [**(A)**, upper part] We observed increased levels of seven metabolites classified as oxidative stress markers in cGVHD patients (red boxes) compared with patients without cGVHD (green boxes). All results are presented as the median, the 25%/75% percentiles and the variation range. The metabolites and the corresponding *p*-values (Mann–Whitney *U*-test) are shown at the top of each individual figure. [**(B)**, lower part] We performed a hierarchical clustering analysis based on the 10 metabolites belonging to the group oxidative stress. The clinical characteristics of each individual patient are presented in the upper part of the figure; for the lower horizontal bars the presence of a factor is indicated by red and the absence by green, whereas the color codes for the upper horizontal bars are explained in the figure. Two main clusters could then be identified; one with generally high metabolite levels and another with generally low levels. The cluster characterized by generally high levels had a significantly higher frequency of patients with cGVHD (25 out of 32 cGVHD patients; $p = 0.0010$, Chi-square test).

metabolites/metabolite subsets. Firstly, eight of these 30 metabolites belonged to the subset amino acid metabolites, and none of them overlapped with the top-ranked metabolites for those patients receiving systemic steroids. However, 12 lipid metabolites were also included among the top-ranked metabolites; 5 of these lipid metabolites belonged to the subclasses phospholipid/lysolipid/plasmalogen and may therefore reflect alterations in the 11 cGVHD patients receiving systemic steroid, although it should be emphasized that only 3 of the 12 lipid metabolites overlapped with the 30 top-ranked metabolites identified in the comparison of patients with and without steroid treatment (**Figure 7**).

We then compared the metabolic profiles for a subset of patients without cGVHD and receiving no immunosuppressive treatment with the subset of seven patients characterized by without cGVHD but still receiving prophylactic cyclosporine treatment 1 year posttransplant. These subsets can be identified from **Figure 1**; in this subset analysis we thus could compare two groups of patients with a similar cGVHD status (i.e., no cGVHD) but differing with regard to cyclosporine treatment. Even though the ranking of individual metabolites differed, one should emphasize that 27 of the 30 top-ranked metabolites from this comparison overlapped with the 30 top-ranked metabolites identified in the previous comparison of all patients receiving cyclosporine versus all the other patients (**Figure 8**).

Finally we compared (1) the 30 top-ranked metabolites previously identified by the comparison of all patients with versus all without cGVHD (**Figure 2**) versus the (2) 30 top-ranked metabolites identified when we compared all patients with and without cyclosporine treatment (**Figure 8**). There was only a minor overlap including 5 heterogeneous metabolites between the 30 top-ranked metabolites identified in each of these 2 analyses, and the 5 metabolites included *N*-acetylneuraminate (amino sugar metabolism), 2-aminooctanate (fatty acid, amino), 2-hydroxyoctanoate (fatty acid, monohydroxy), lactosyl-*N*-nervonoylsphingosine (d18:1/24:1) (sphingolipid), and sulfamethoxazole (drug). Thus, even though cyclosporine seems to have distinct

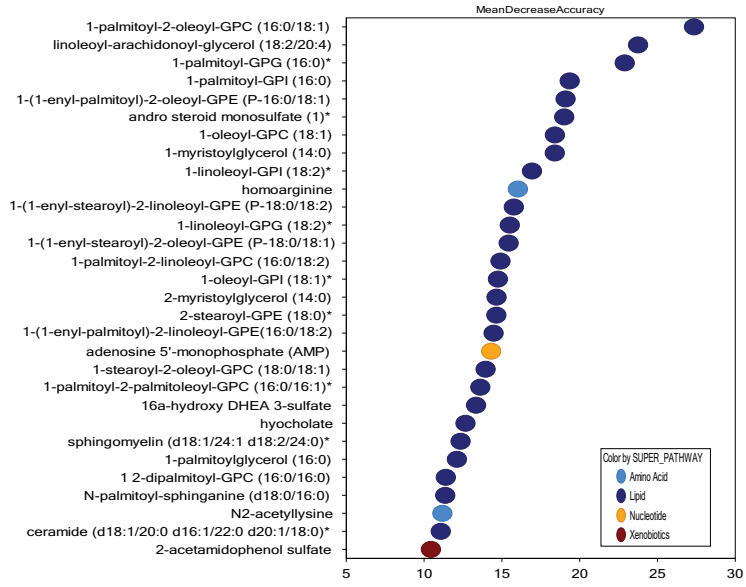

FIGURE 7 | Random forest analysis based on the overall metabolomics profile for all 51 patients included in the study; a comparison of patients receiving and not receiving treatment with systemic steroids. Random forest analysis could distinguish between these two patient subsets with a predictive accuracy of 73.0%. The figure presents the 30 top-ranked metabolites and their classification (indicated in the figure, lower right) based on their importance for the identification of the two patient subsets.

effects on the systemic metabolic profile in allotransplant recipients, the effects of cGVHD by itself seem to be stronger than the cyclosporine effects.

Clustering Analysis Based Only on Metabolites Identified as cGVHD-Associated in Random Forest Analyses Identifies Three Patient Subsets with Different Frequencies of cGVHD

We compared the 30 top-ranked metabolites for the random forest analysis of all patients with and without cGVHD (**Figure 2**) and the 30 top-ranked metabolites from the analysis of all patients with and without cyclosporine treatment (**Figure 8**), and we then found 4 overlapping metabolites [lactosyl-*N*-nervonoyl-sphingosine (d18:1/24:1), sulfamethoxazole, 2-hydroxyoctanoate, and 2-aminooctanoate]. Similarly, when comparing the top-ranked metabolites for the cGVHD analysis (**Figure 2**) with the 30 top-ranked metabolites from the comparison of all patients with and without additional steroid treatment (**Figure 7**), we identified the five overlapping metabolites 1,2-dipalmitoyl-GPC (16:0/16:0), 1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2), 1-(1-enyl-stearoyl)-2-oleoyl-GPE (P-18:0/18:1), hyocholate, and 1-palmitoyl-2-oleoyl-GPC (16:0/18:1). Thus, the 30 top-ranked metabolites from the comparison of all patients with and without cGVHD included 21 metabolites that only were associated with cGVHD but not with cyclosporine or steroid therapy.

We then did a clustering analysis of all 51 patients based on the 21 non-overlapping metabolites from the cGVHD random forest analysis (**Figure 9**). First, the middle cluster showed a low frequency of patients with cGVHD (3 out of 16), and this is significantly different from the other 35 patients (28/35; Chi-Square test, $p < 0.0001$). Second, patients with cGVHD were mainly included in the two other clusters (11 out of 13 in the left and 17 out of 22 in the right cluster). The frequencies of patients with cGVHD did not differ significantly between these two clusters, but the frequency of patients with extensive cGVHD affecting at least three organs was significantly higher in the right cluster (10 out of 22 patients) than in the left cluster (2 out of 13 patients; Chi-Square test, $p = 0.027$). Third, the frequencies of patients receiving cyclosporine treatment and additional steroid treatment did not differ between the right and left clusters, i.e., the two clusters including the majority of cGVHD patients. Thus, differences in immunosuppressive therapy cannot explain the localization of severely affected cGVHD patients mainly into one cluster. Finally, the two cGVHD patients without immunosuppressive therapy also clustered within the intermediate (left) cluster together with several other cGVHD patients.

Seven exceptional patients without cGVHD clustered together with the majority of cGVHD (**Figure 9**, left and right patient

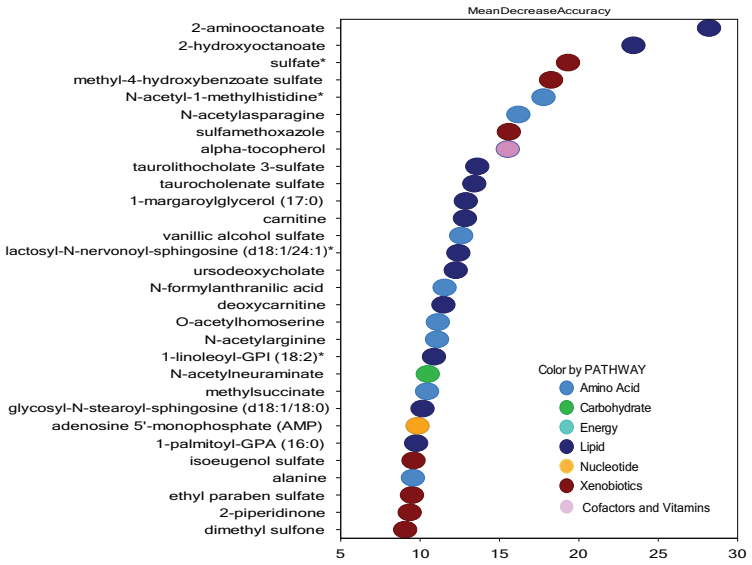

FIGURE 8 | Random forest analysis based on the overall metabolomics profile for all 51 patients included in the study, a comparison of patients receiving and not receiving treatment with cyclosporine. Random forest analysis could distinguish between these two patient subsets with a predictive accuracy of 60%. The figure presents the 30 top-ranked metabolites and their classification (indicated in the figure, lower right) based on their importance for the identification of the two patient subsets.

clusters). Five of these exceptional patients (patients 2, 4, 24, 32, and 41) had a minor increase in liver enzymes at the time of blood sampling without other signs of cGVHD, but additional diagnostic procedures were not indicated. Thus, they were classified as not having treatment-requiring GVHD in our study; the two last exceptional patients had no signs of cGVHD.

The three patient clusters were separated mainly due to the variation of 11 metabolites that clustered together in the lower metabolite cluster and included 5 amino acid metabolites (beta-citrylglutamate, alpha-ketoglutarate, glutamate, aspartate, and glycylvaline) and 4 bile acid metabolites (glycochenodeoxycholate, glycocholate, glycohyocholate, and taurocholate) together with the 2 additional lipid metabolites sphingosine and 1-arachidonoyl-GPA (20:4).

DISCUSSION

Graft versus host disease is characterized by immune dysregulation/deficiency, organ damage, and decreased survival (8, 9). Alloreactive T-cells have been implicated in its pathogenesis, but the precise role of specific T-cell subsets, autoantigens, alloantigens, and B-cells as well as the contribution from immunoregulatory soluble mediators is not known (8, 9, 20–22). Thus, GVHD reflects an exaggerated response of inflammatory mechanisms that involve donor T cells as well as multiple innate and adaptive cells and various mediators. Moreover, the involvement of inflammatory and profibrotic cytokines, such as transforming growth factor beta or platelet-derived growth factor receptors, are also important for GVHD-targeted organ injury (8, 9, 36).

We decided to investigate the patients 1 year posttransplant. This time point was selected because the impact of pretransplant and early posttransplant factors on the metabolic profile was then expected to be low, the early hematological and immunological defects in reconstitution would be less important, a substantial number of patients would have developed cGVHD but the impact on the metabolic profiles from more severe organ failures was expected to be limited.

The present metabolomic profiling study was conducted to identify serum metabolic changes associated with cGVHD. To the best of our knowledge, this is the first study to investigate the metabolic profile of patients with cGVHD. Our results have to be interpreted with care because we investigated a relatively small group of patients, but the patients are relatively homogenous because all patients received grafts from matched family donors, most of them received the same GVHD prophylaxis and a limited number of conditioning treatments were used. Our study should also be regarded as population-based because our patient cohort represents all allotransplanted patients with a family donor from a defined geographic area and during a defined time period. Additional studies are therefore needed to investigate whether our results are representative also for other subsets of allotransplant recipients (e.g., other donors).

We first compared all patients with and all patients without cGVHD, and this comparison suggests that cGVHD patients

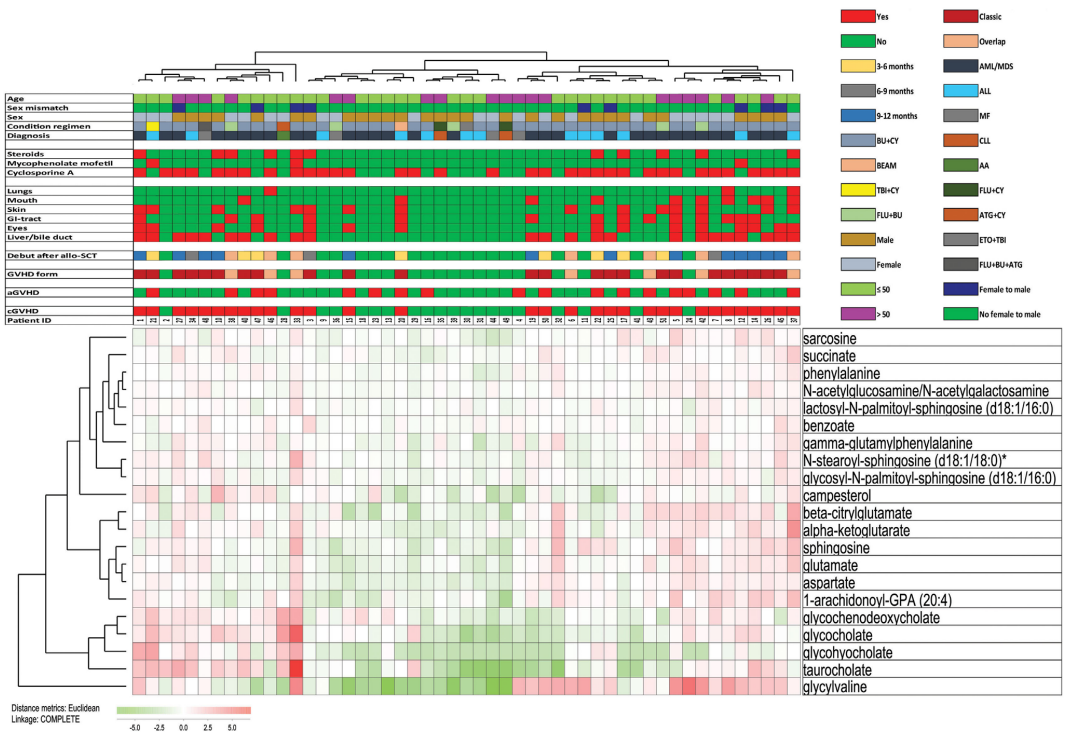

FIGURE 9 | Hierarchical clustering analysis including all 51 patients and based on 21 selected metabolites. The analysis was based on the 30 top-ranked metabolites identified by the random forest analysis of the overall metabolic profile of all 51 patients and comparing patients with and without chronic graft versus host disease (cGVHD) (see **Figure 2**), but 9 of these 30 metabolites were excluded from this analysis because they overlapped with the 30 top-ranked metabolites identified by the comparison of patients with/without cyclosporine treatment (**Figure 8**) or with/without systemic steroid treatment (**Figure 7**). Based on the 21 remaining metabolites we performed a hierarchical clustering analysis (Euclidian Correlation, complete linkage). The heat map and corresponding dendrograms are shown in the figure together with the clinical characteristics of individual patients (see horizontal bars in the upper part). The metabolites are listed to the right in the figure; red color means high metabolite levels and green color low levels as indicated to the lower left in the figure. The clinical characteristics of each individual patient are presented in the upper part of the figure; for the lower horizontal bars the presence of a factor is indicated by red and the absence by green, whereas the color codes for the upper horizontal bars are explained in the figure.

have a unique metabolic signature (**Figure 2**). We examined the patients at a defined time point and the metabolic profile of our patients may therefore be influenced both by differences in immunosuppressive treatment, different duration of cGVHD and thereby also differences in cumulative effects by the ongoing pathological process. Despite this heterogeneity our random forest analysis could distinguish between patients with and without cGVHD with a predictive accuracy of 75%. The identification and validation of biomarkers in cGVHD remain very challenging (10–12), but our study suggests that metabolic markers may become useful in these patients.

As stated above, the altered metabolic profile in our cGVHD patients can be caused either by the disease itself or its treatment, i.e., cyclosporine and/or systemic steroids. Several observations suggest that cyclosporine can affect systemic metabolic profiles. First, we analyzed all the 51 patients in our patient cohort and compared all patients with and all patients without cyclosporine treatment (**Figure 8**); the 30 top-ranked metabolites from this comparison showed a minimal overlap (only four metabolites) with the 30 top-ranked metabolites from the comparison of all patients with versus all patients without cGVHD (**Figure 2**). Second, to further identify metabolic effects probably caused by cyclosporine treatment we compared our patient subset without cGVHD and still receiving cyclosporine with another subset also being without cGVHD but not receiving cyclosporine, i.e., these two subsets had similar cGVHD status and differed only with regard to cyclosporine treatment. The 30 top-ranked metabolites from these two comparisons of patients with and without cyclosporine treatment showed a large degree of overlap (27 metabolites). Thus, both these comparisons suggest that cyclosporine has diverse effects on systemic metabolic profiles, and our present observations are consistent with

previous observations in kidney transplant recipients (37). We conclude that cyclosporine treatment can alter systemic metabolic profiles in allotransplant recipients, but our observed differences between patients with and without cGVHD cannot be explained by cyclosporine alone because the 30 top-ranked cGVHD-associated metabolites (**Figure 2**) included only four of the cyclosporine-associated metabolites (**Figure 8**).

We used the same strategy as for cyclosporine when we investigated the contribution from steroid treatment. First, we examined the whole patient cohort and compared all patients with and all patients without steroid treatment. When comparing the results from this analysis (**Figure 7**) with the results from the with/without cGVHD comparison (**Figure 2**), we identified five overlapping metabolites, i.e., lipid metabolites associated both with cGVHD and systemic steroid therapy. Taken together these observations suggest that the increased levels of these overlapping metabolites in cGVHD patients are mainly due to the steroid treatment rather than the cGVHD. Our present results are consistent with previous studies of steroid-treated myasthenia gravis patients showing that steroids alter triglyceride/fatty acid metabolism (38). However, one cannot exclude the possibility that even these steroid-associated effects may be at least partly due to the more severe and thereby steroid-requiring cGVHD of these patients. This last possibility is actually supported by previous observations suggesting that increased levels of these lipid metabolites are also associated with inflammation (29, 30).

The altered levels of bile acid as well as tyrosine and phenylalanine metabolites in cGVHD patients may reflect at least partly an altered microbiome composition. Previous studies have demonstrated the complex and multidirectional interactions between inflammation, microbiota, and immune reconstitution in allotransplant recipients (39–43). Allo-HSCT can alter the intestinal flora and this may then be more pronounced in individuals with cGVHD (39–41). The human gut microbiome is involved in vital biological functions, such as maintenance of immune homeostasis, modulation of intestinal function and metabolic regulation; disturbances of the intestinal microbiota can thereby be associated with development and progression of inflammation, including GVHD (44). The microbial intestinal flora is responsible for the generation of several metabolites derived from amino acids, bile acids, heme, and dietary sources; several of these metabolites are absorbed and can bind specific receptors on host cells. The metabolism of aromatic amino acids, phenylalanine, and tyrosine is partly due to enzymes encoded within the microbiome (44). Thus, a change in microbiome-derived metabolites might be due to a shift in the flora with translocation of pro-inflammatory metabolites or bacterial components into the systemic circulation and thereby further acceleration of GVHD through the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha and IL-1 (45, 46). Thus, our present observation supports the hypothesis that effects of altered microbiota on the systemic metabolic profile contribute to the biological and clinical impact of microbiota in allotransplant recipients (39–43).

Chronic graft versus host disease was associated with increased levels of primary bile acid metabolites. Bile acids are derived from cholesterol in the liver and released into the small intestine to facilitate dietary lipid absorption. Differences in serum bile acid levels may be caused by altered synthesis, release, or reabsorption. Bile acid malabsorption has previously been reported in GVHD (47), but an altered gut microbiome is an alternative explanation as discussed earlier. The increased levels of bile metabolites may then induce hepatic cell dysfunction and induction of pro-inflammatory mediators (48).

Serum levels of multiple markers of inflammation and oxidative stress were increased in our cGVHD patients (36), possibly reflecting an increased risk of inflammatory complications after allo-HSCT. Uremic toxicity, metabolic acidosis, and pro-inflammatory soluble mediators may activate protein degradation in cGVHD (25, 32, 49), and cGVHD may thereby be associated with altered metabolic and endocrine functions of several organs (8, 31, 36). An altered balance between protein synthesis and catabolism may then be the final result.

The altered lipid profiles in cGVHD may be due to differences in membrane lipid turnover. Immunocompetent cells switch from resting to activated state after stimulation, and this requires increased energy metabolism to fuel cell proliferation and acquire effector functions (50, 51). Disruption of lipid synthesis can reduce GVHD in murine models (52), indicating an important role of lipid metabolism in the pathogenesis of GVHD. Our findings are in concordance with these observations.

Based on our overall results we did a final clustering analysis of our entire patient cohort to distinguish between cGVHD-associated and treatment-associated metabolic effects in our patient cohort. Because we analyzed a relatively small number of patients, this analysis was based on the 30 top-ranked metabolites from the initial with/without cGVHD analysis (**Figure 2**). We then excluded from these 30 metabolites the 9 overlapping metabolites identified by the comparisons of patients with/without cyclosporine (**Figure 8**, four metabolites) and patients with/without steroid (**Figure 7**, five metabolites). Thus, this analysis was based on 21 cGVHD-associated metabolites and included all patients in our cohort. We did not leave out from the analysis those metabolites that may be associated with an altered gastrointestinal microbiome because we regard the microbiome as a part of the overall clinical status of allotransplant recipients. The results from this last clustering analysis (**Figure 9**) showed that the patients were distributed in three main clusters; one cluster included mainly patients without cGVHD, a second cluster included mainly patients with cGVHD, but the disease involved only one or two organs for the large majority of these patients; and a third cluster including a majority of patients with cGVHD and many of them having involvement of at least three organs. This identification of three patient subsets could not be explained by pharmacological differences, and it was mainly caused by differences in the systemic levels of five amino acid and six lipid metabolites (most of them being bile acids) that clustered together in **Figure 9** (lower metabolite cluster). Finally, our two cGVHD patients not receiving immunosuppressive therapy clustered within the intermediate (left) cluster together with several other cGVHD patients; this observation further supports our view that this clustering analysis reflects differences in cGVHD-induced metabolic alterations.

To the best of our knowledge, this is the first study of systemic metabolic profiles in allotransplant recipients. We describe altered

metabolic profiles for patients with treatment-requiring cGVHD, and the overall profile includes effect due to both cGVHD itself and the immunosuppressive treatment. However, our study identified a subset of 11 metabolites that seem to reflect both the diagnosis and the severity of cGVHD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of local Ethics Committee (Regional Ethics Committee III, University of Bergen, Norway; REK). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regional Ethics Committee III, University of Bergen, Norway; REK.

AUTHOR CONTRIBUTIONS

HR carried out the analyses, made the data for presentation, and wrote the manuscript. I-SG, KM, and RL collected the clinical data. KH participated in the study and helped to draft the manuscript. ØB planned and organized the study, collected the data and patient informed consent, coordinated the work, and wrote the manuscript. All the authors have approved the version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01979/full#supplementary-material.

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