The PI3K-Akt-mTOR intracellular signaling pathway in human acute myeloid leukemia

Ina Nepstad

Avhandling for graden philosophiae doctor (ph.d.)
Universitetet i Bergen
2019

UNIVERSITETET I BERGEN

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Disputasdato: 08.03.2019

År: 2019

Tittel: The PI3K-Akt-mTOR intracellular signaling pathway in human acute myeloid leukemia

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Trykk: Skipnes Kommunikasjon / Universitetet i Bergen

SCIENTIFIC ENVIRONMENT

During my PhD, all laboratory work was performed at the Leukemia Research Group, Department of Clinical Science at the University of Bergen, headed by Prof. Øystein Bruserud.

From February to July 2013, I worked at The Cochin Institute, University Paris Descartes, in collaboration with Prof. Patrick Mayeux and Prof. Didier Bouscary.

My PhD fellowship was funded by grants from the University of Bergen, Helse-Vest and the Norwegian Cancer Society, and I was enrolled as a PhD student at the Department of Clinical Science, University of Bergen.

ACKNOWLEDGEMENT

My deepest gratitude to my supervisor, Øystein Bruserud – Thank you for giving me the opportunity to work in your research group and for introducing me to the field of hematology. I am very grateful for your excellent guidance and support. I have been amazed so many times by how you can turn every small result into something to useful. You have been a great motivator throughout the whole project, and your enormous work capacity and great knowledge is truly impressive.

My extended gratitude also goes to my two fabulous supervisors, Håkon Reikvam and Kimberley Joanne Hatfield – You have contributed to all aspects of this PhD project, and I am very grateful for all your support and guidance. I appreciate our numerous discussions and your comments to the articles and thesis, and I admire your knowledge and enthusiasm. A special thanks to you, Håkon, for convincing me to take the PhD fellowship in the first place.

The work in the publications included in this thesis would not have been possible without all of my co-authors; Elise Aasebø, Kristin Paulsen Rye, Marie Hagen, Sushma Bartaula-Brevik, Tor Henrik Anderson Tvedt, Maria Hernandez-Valladares, Jørn Skavland, Frode Selheim, Frode Berven, Annette Katharina Brenner, and Bjørn Tore Gjertsen – Thank you all for your valued contributions to my PhD project.

Moreover, I would like to thank everyone who has been part of the Bruserud lab during my years as a PhD candidate; Annette Katharina Brenner, Astrid Olsnes Kittang, Elise Aasebø, Elisabeth Ersvær, Guro Kristin Melve, Ida Marie Rundgren, Ida-Sofie Grønningsæter, Jenny Kristine Rosmer Ihle, Marie Hagen, Knut Anders Mosevoll, Kristin Paulsen Rye, Kristoffer Sand, Maria Hernandez-Valladares, Sushma Bartaula-Brevik, and Tor Henrik Anderson Tvedt, and of course Håkon and Kimberley – I am thankful for all the valuable discussions, for the technical and scientific support, and for your friendship. I have appreciated the many laughs during lunchtime and of course the wonderful annual “gruppetur”. A special thanks to Kristin and Marie – I am grateful for all your help and expertise in the lab.

I would like to acknowledge all of my past and present colleagues in the Gjertsen lab – The workday would not be the same without any of you (and our shared love for chocolate, cake and science).

I also appreciated the opportunity to work in the lab with Jerome Tamburini and Pierre Sujobert at Institute Cochin in Paris, France – Thank you for including me in your remarkable research group with Nathalie Jacque, Marie-Anne Hospital, Adrien Grenier, Laury Poulain, Alexa Green, Lise Willems and Patrick Mayeux.

To my parents, Lise and Tore – Thank you for all your loving support and encouragement through this process, and for always being there for me. A special thanks to my dear sister, Kaia – My best friend and personal on-call "administrative support" during my PhD. I have also much appreciated the encouragement of my family in-law, Jarl, Anette, Anne-Grete, Terje, Kjell-Olav and Åse. Thank you for always being supportive. I would also like to express my gratitude for the support from all my friends.

My deepest appreciation goes to my dear partner, Tore – Thank you for your unconditional support and encouragement, and for just being you. Last, but not least; our children Ida and Nora – Thank you for your love and for keeping my mind focused on the important things in life.

Bergen, October 2018
Ina

ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous malignancy where disruption of normal intracellular signaling occurs due to mutations or abnormal external signaling. Many pathways have emerged as potential targets for pharmacological intervention, including the PI3K-Akt-mTOR pathway. Aberrantly upregulated PI3K-AKT-mTOR signaling characterizes many types of cancers and may represent an adverse prognostic parameter; this is possibly also true for AML since several observations suggest that this pathway is important in leukemogenesis. Targeting of this pathway with specific inhibitors could therefore result in suppression of leukemic cell growth. Our hypothesis was that AML patients differ with regard to the impact of PI3K-Akt-mTOR signaling in leukemogenesis and chemosensitivity, and that patients thereby differ with regard to their susceptibility to various PI3K-Akt-mTOR inhibitors.

The aim of this thesis was to further characterize the patient heterogeneity in human AML through studies of (i) the constitutive expression of selected mediators in the PI3K-Akt-mTOR pathway; (ii) how the signaling pathway is modulated by the agonist insulin and by various pathway inhibitors; and (iii) through molecular studies to try to elucidate possible mechanisms behind the heterogeneity of PI3K-Akt-mTOR signaling in AML.

Our studies confirmed that constitutive activation of the PI3K-Akt-mTOR pathway differed between patients and two main subsets with high and low pathway activation were identified based on PI3K-Akt-mTOR activation profiles. Our overall results described in this thesis showed that AML patients are heterogeneous with regard to constitutive PI3K-Akt-mTOR activation, and the same was observed when studying the effect of agonistic insulin and the effects of various pathway inhibitors in the presence of insulin. Insulin increased the phosphorylation of mediators in the PI3K-Akt-mTOR pathway, and this effect was especially seen for mediators upstream of mTOR.

Our studies showed that patients differed with regard to the energy, amino acid, and arachidonic acid metabolism in their leukemic cells, and the metabolic profile seemed to differ between primary AML cells that were susceptible or resistant to the

antiproliferative effect of PI3K-Akt-mTOR pathway inhibitors. Susceptible and resistant cells to pathway inhibitors then seemed to differ especially in arachidonic acid, proline and/or energy metabolism. However, our studies also suggest that differences in redox homeostasis may be important.

In the final work, we demonstrated that clonal heterogeneity could be reflected in the activation status of mediators in the PI3K-Akt-mTOR pathway. We found that this heterogeneity was associated with adverse prognosis, i.e. the survival after intensive antileukemic treatment was decreased for patients showing evidence for clonal heterogeneity when analyzing the pathway activation profile. Patients with and without evidence for clonal heterogeneity differed in their global gene expression profiles, especially with regard to expression of genes encoding proteins that are important for G protein coupled receptor signaling.

To conclude, the possible importance of the differences found between AML patients should be considered when designing and/or analyzing the results in future clinical studies of PI3K-Akt-mTOR inhibitors, and possibly also when considering combination of pathway inhibitors with other targeting therapies or conventional chemotherapy. Biological heterogeneity reflected in the intracellular signaling status should also be further investigated as a potential prognostic biomarker in human AML both in patients receiving intensive and possibly curative treatment, and in elderly/unfit patients receiving AML-stabilizing treatment.

LIST OF PUBLICATIONS

Article I

Two acute myeloid leukemia patient subsets are identified based on the constitutive PI3K-Akt-mTOR signaling of their leukemic cells; a functional, proteomic and transcriptomic comparison

Authors: Ina Nepstad, Kimberley Joanne Hatfield, Elise Aasebø, Maria Hernandez-Valladares, Annette K. Brenner, Sushma Bartaula-Brevik, Frode Berven, Frode Selheim, Jørn Skavland, Bjørn Tore Gjertsen, Håkon Reikvam, and Øystein Bruserud

Article II

Insulin-initiated activation of the PI3K-Akt-mTOR pathway in acute myeloid leukemia cells; a study of patient heterogeneity and pathway inhibitors

Authors: Ina Nepstad, Kimberley Joanne Hatfield, Elise Aasebø, Maria Hernandez-Valladares, Karen Marie Hagen, Kristin Paulsen Rye, Frode Berven, Frode Selheim, Håkon Reikvam and Øystein Bruserud

Article III

Resistance to the Antiproliferative *In Vitro* Effect of PI3K-Akt-mTOR Inhibition in Primary Human Acute Myeloid Leukemia Cells Is Associated with Altered Cell Metabolism

Authors: Ina Nepstad, Håkon Reikvam, Annette K. Brenner, Øystein Bruserud, Kimberley J. Hatfield

Article IV

Clonal heterogeneity reflected by PI3K-Akt-mTOR signaling in human acute myeloid leukemia cells and its association with adverse prognosis

Authors: Ina Nepstad, Kimberley Joanne Hatfield, Tor Henrik Anderson Tvedt, Håkon Reikvam and Øystein Bruserud

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LIST OF ABBREVIATIONS

4EBP1	eIF4E binding protein
AGC kinase	Group of kinases named after protein kinase family A, G, and C
AGM	Aorta-gonad-mesonephros
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinase
ANGPT1	Angiopoietin 1
APL	Acute promyelocytic leukemia
ARG	Arginine
AS16O	Akt substrate of 160 kDa
ASN	Asparagine
ASP	Asparat
BAD	Bcl-2-associated agonist of cell death
BCL-2	B-cell lymphoma 2
CAR	CXCL12-abundant reticular
CCL-1	C type lectin-like molecule
CEBPA	CCAAT/enhancer-binding protein alpha
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CREB	cAMP response element binding protein
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C receptor
Cyc D1	Cyclin D1
Deptor	DEP domain-containing mTOR- interacting protein
EBMT	European Group for Blood and Marrow Transplantation
eIF4E	Eukaryotic initiation factor-4E
EPO	Erythropoietin
ERK	Ras- extracellular signal-regulated kinase
ERα	Estrogen receptor alpha
FKBP38	FK506-binding protein 38
FLT3	Fms-like tyrosine kinase 3
FLT3/ITD	FLT3/internal tandem duplications
FLT3/TKD	FLT3/tyrosine kinase domain
FOXO	Forkhead box O
GAB2	GRB2 - associated binding protein 2
GAP	GTPase-activating protein
GLN	Glutamine
GLS	Glutaminase
GLU	Glutamate
GLUT	Glucose transporter
GMP	Granulocyte-macrophage progenitors
GPCRs	G protein-coupled receptors

GRB2	Growth factor receptor bound protein 2
GS	Glutamine synthase
GSA	Glu 1-semialdehyde
GSK3	Glycogen synthase kinase 3
HIF	Hypoxia-inducible factor
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem/progenitor cell
IC50	The half maximal inhibitory concentration
IDH	Isocitrate dehydrogenase
IGF	Insulin growth factor
IGF-1R	IGF-1 receptor
IL	Interleukin
IL1RAP	IL1 Receptor Accessory Protein
IRS1	Insulin substrate receptor 1
LCO	Initial leukemia cell-of-origin
LIC	Leukemia initiating cells
LSC	Leukemic stem cell
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MEK	MAPK ERK kinase
MEP	Megakaryocyte-erythroid progenitors
mLST8	Mammalian lethal with SEC13 protein 8
MPL	Myeloproliferative leukemia protein
MPPs	Multipotent progenitors
MRD	Minimal residual disease
mRNAs	Messenger RNAs
MSC	Mesenchymal stem cells
mSIN	Mammalian stress-activated protein kinase-interacting protein 1
mTOR	Mechanistic target of rapamycin
mTORC 1/2	mTOR complex
NAD+	Nicotinamide adenine dinucleotide
NK cells	Natural killer cells
NPM1	Nucleophosmin 1
OPN	Osteopontin
ORN	Ornithine
OXA	Oxaloacetate
P5C	Pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
P5CS	P5C synthase
PDK1	3’phosphoinositide-dependent kinase 1
PF4	Platelet factor 4
PFK2	Phosphofructokinase-2
PH	Pleckstrin-homology
PI3K	Phosphoinositide 3-kinase

PIKK	PI3K-related kinase
PIP2	Phosphatidylinositol-4,5 bisphosphate
PIP3	Phosphatidylinositol-3,4,5 trisphosphates
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMT	Photomultiplier tubes
POX	Proline dehydrogenase/oxidase
PRAS40	Proline-rich Akt substrate of 40 kDa
PRO	Proline
PRODH	Proline dehydrogenase
PRX1	Paired related homeobox 1
PTEN	Phosphate and homologue protein deleted on chromosome 10
PYCR1/2	Pyrroline-5-carboxylate reductase 1 and 2
PYCRL	Pyrroline-5-carboxylate reductase L
RHEB	RAS homologue enriched in brain
ROS	Reactive oxygen species.
RSK	p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinase
S6	Ribosomal protein
S6K	Ribosomal protein S6 kinase
S6K1	S6 kinase beta 1
SCF	Stem Cell Factor
SDF1	The stromal cell-derived factor 1
Ser	Serine
SH2	Src Homology 2
SHIP1/SHIP2	SH2 domain-containing inositol 5-phosphatases
SIN1	Mammalian stress-activated protein kinase interacting protein 1
SKG3	Serum/glucocorticoid regulated kinase family, member 3
SREBP 1C	Sterol regulatory element-binding transcription factor 1C
SYK	Spleen tyrosine kinase
TCA cycle	Tricarboxylic acid/ Krebs cycle
TGF-β1	Transforming growth factor beta-1
Thr	Threonine
TPO	Thrombopoietin
TSC1/2	Tuberous sclerosis complex 1 and 2
WHO	World Health Organization
YB1	Y-box-binding protein 1

1 INTRODUCTION

1.1 Acute myeloid leukemia

1.1.1 Definition and classification

Acute myeloid leukemia (AML) is an aggressive malignancy characterized by the proliferation of immature myeloid leukemia cells [1, 2]. In most cases, this disease involves a bone marrow malignancy, but malignant cells may also be detected in peripheral blood or as extra medullary infiltration. In exceptional patients, soft tissue infiltrates can be the first and/or only manifestation of the disease [1, 3, 4]. The acute promyelocytic leukemia (APL) variant of AML is characterized by specific genetic abnormalities in the leukemic cells and severe coagulopathy, and treatment differs from that of other types of AML [5-7]. In this thesis, we use the term AML to refer to non-APL variants of the disease.

For a significant majority of patients, non-APL variants of AML primarily affect the bone marrow, and for a large percentage of these patients, at least 20% of the nucleated bone marrow cells are immature or undifferentiated leukemia blasts [2]. However, for patients with inv(16), t(16;16) and t(8;21) abnormalities, the minimum threshold of 20% of bone marrow blasts is not required. The erythroleukemic variant of AML also has other distinct diagnostic criteria [2]. A detailed description of the various subsets of AML is given in the original publication [2]. Additional strategies for subclassification, such as micro-RNA, epigenetic, transcriptomic, and proteomic characterization, have been suggested but are not a part of our present, routine handling of these patients, [8-12].

Table 1. A summary of the World Health Organization (WHO) 2016 classification of AML [2, 13].

Categories
Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified (NOS)
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis (syn.: acute myelofibrosis; acute myelosclerosis)
Myeloid sarcoma (syn.: extramedullary myeloid tumor; granulocytic sarcoma; chloroma)
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (syn.: transient myeloproliferative disorder)
Myeloid leukemia associated with Down syndrome
Comments:
More details can be found in Döhner et al. [1, 13].

1.1.2 Treatment of AML

Intensive chemotherapy is the only treatment that can cure AML, and autologous or allogeneic stem cell transplantation can be a part of this intensive treatment [3]. Treatment is initiated with an induction cycle that often consists of treatment with an anthracycline together with a nucleoside analogue such as cytarabine. This treatment is

referred to as standard 7 + 3 induction therapy and combines cytarabine for seven days with an anthracycline for three days. The goal of this first induction cycle is to achieve complete remission, i.e. a normal number of bone marrow blasts based on a morphological evaluation and at the same time a normalization of peripheral blood cell counts.

If complete remission is not reached after the first cycle, a second induction cycle is attempted. If remission still is not achieved after this cycle, the patient is considered to have resistant AML. For patients achieving complete remission, a second phase of treatment, often referred to as consolidation therapy, is begun. The intention of consolidation therapy is to eradicate any residual disease and thereby avoid a later AML relapse; the intensity of this treatment is therefore based on a prognostic evaluation of the individual patient with regard to the risk of treatment-related complications versus the risk of AML relapse (see below).

Intensive chemotherapy is administered either as intensive conventional chemotherapy alone, or in combination with autologous or allogeneic stem cell transplantation. Conventional chemotherapy with autotransplantation has a relatively low risk of treatment-related mortality. Allotransplantation has a stronger antileukemic effect, resulting in a lower risk of later AML relapse than chemotherapy alone or with autotransplantation; however, this alternative is associated with a higher rate of treatment-related mortality

Patients classified as chemoresistant can be treated with (i) additional intensive induction cycles to try to achieve complete remission; (ii) allogeneic stem cell transplantation either directly after the second induction cycle or when remission is achieved after a later induction cycle; or (iii) supportive or less intensive AML stabilizing treatment [3]. A small percentage of primary chemoresistant patients will become long-term survivors if treated with allotransplantion without previous complete remission.

For patients who cannot tolerate intensive treatment (e.g. elderly patients over 70-80 years of age and patients with severe comorbidity) supportive treatment with antibiotics

and transfusions should be offered, and a less intensive AML-stabilizing treatment should be considered. The most commonly used antileukemic treatments for these patients are demethylating agents (azacitidine, decitabine) and low-dose cytarabine [3, 14-17].

1.1.3 Prognostic evaluation of patients with AML

AML is a very aggressive disease and survival based on supportive treatment alone (including low-toxicity chemotherapy to control peripheral blood leukocytosis), without antileukemic treatment, is short and depends on the patient's age [18]. The average survival for patients between 65 and 75 years of age, given only supportive treatment, is approximately three months. Whereas, for patients over 85 years of age, it is only one month. However, there are exceptional patients who survive as long as two years, given supportive treatment alone [19].

The most commonly used disease-stabilizing treatments in AML are low-toxicity cytarabine and demethylating agents. The effect of these agents is not dependent on induction of complete remission. However, the median overall survival is less than 12 months for both strategies, and low-dose cytarabine does not prolong survival for patients with adverse prognoses [17, 20-22].

Several strategies for prognostic classification (i.e. the probability of long-term, leukemia-free survival) of AML patients treated with intensive chemotherapy have been used. These are usually based on AML-associated cytogenetic abnormalities; additional mutations detected by molecular analyses (especially fms-like tyrosine kinase 3 (*FLT3*), nucleophosmin 1 (*NPM1*) and CCAAT/enhancer-binding protein alpha (C*EBPA*) abnormalities); the response to the first induction chemotherapy cycle (whether a complete hematological remission is reached); and, for certain subsets of patients, the peripheral blood AML blast counts [23]. Cornelissen et al. used these four parameters for the classification of patients receiving intensive chemotherapy/autologous stem cell transplantation and further subdivided patients into four risk categories: (i) favorable, with 35-40% relapse risk; (ii) intermediate, with 55-60% relapse risk; (iii) Poor, with 70-80% risk; and (iv) very poor, with >90% relapse risk. The prognostic impact of the

peripheral blood AML blast count has been questioned, but a recent retrospective study suggested that the blast count has an independent impact on prognosis after allogeneic stem cell transplantation [24]. However, the prognostic impact of a marker may be treatment-dependent and therefore may change in relation to new therapies.

Risk stratification can also be based on genetic abnormalities alone [1]; the stratification given by the European Leukemia Net is summarized in Table 2. This classification system defines the *FLT3* allelic ratio as low when it is <0.5 and high when it is ≥0.5 (i.e. *FLT3*-ITD versus *FLT3*-wt).

Table 2. The ELN 2017 risk stratification based on genetics alone [1].

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*
	Mutated *ASXL1*
	Mutated *TP53*

Comments:
The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse–risk gene mutations. Complex karyotype mean three or more unrelated chromosome abnormalities in the absence of 1 of the WHO–designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3). Monosomal karyotype is defined by the presence of 1 single monosomy (excluding loss of X or Y) in association with at least 1 additional monosomy or structural chromosome abnormality (excluding core–binding factor AML). Mutated *RUNX1* or *ASXL1* should not be used as an adverse prognostic marker if they co–occur with favorable–risk AML subtypes. *TP53* mutations are significantly associated with AML with complex and monosomal karyotype. More details can be found in Döhner et al. [1]

These prognostic stratifications are primarily used along with comorbidity scores and the European Group for Blood and Marrow Transplantation (EBMT) score when deciding whether an allogeneic stem cell transplantation should be recommended for younger patients [23].

1.2 Leukemic cell populations in human AML

1.2.1 Hierarchical organization of AML cell populations

The normal presentation of hematopoietic organization is one of highly regulated cellular hierarchy. Hematopoietic stem cells (HSCs) reside in the bone marrow, and mature cells are continuously generated from progenitors, giving rise to the different types of blood cells including erythrocytes, platelets, monocytes, granulocytes, natural killer cells (NK-cells), and T-cell and B-cell lineages [25] (Figure 1). The HSCs have a unique ability to both self-renew and differentiate, which enables life-long blood cell production due to the replacement of a stem cell with daughter cells during mitosis [25].

Normal HSC are defined as linage negative (Lin-) CD34+CD38-CD90+CD45RA- cells and generate multipotent progenitors (MPPs) with lymphomyeloid potential, defined as Lin-CD34+CD38-CD90-CD45RA- cells [26]. Downstream progenitor cells, such as common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) all have the same Lin-CD34+CD38-CD90-CD45RA- phenotypes. They can be further distinguished based on their differential expressions of the IL3 receptor subunit α (IL3Rα+/CD123), the thrombopoietin receptor CD110/myeloproliferative leukemia (MPL) protein, and CD45RA [27, 28].

AML was one of the first diseases for which the existence of a population of cancer stem cells was suggested [29]. Consequently, human AML LSCs represent one the most well characterized populations of cancer stem cells [30]. However, these cells are defined by functional assays (i.e. long-term culture or xenograft models), and these functionally defined stem cells can be detected in different cell subsets (i.e. they differ in their expression of cell surface markers) [31, 32].

Reduced differentiation in AML results in the excess production of undifferentiated/immature leukemic blasts that show limited proliferative capacity and acquired critical genetic or epigenetic alterations that disrupt important growth-regulatory pathways [33]. AML is expressed as abnormal development in one of the major blood lineages; however, the blasts from patients are heterogeneous with respect to the lineage antigens they express [33].

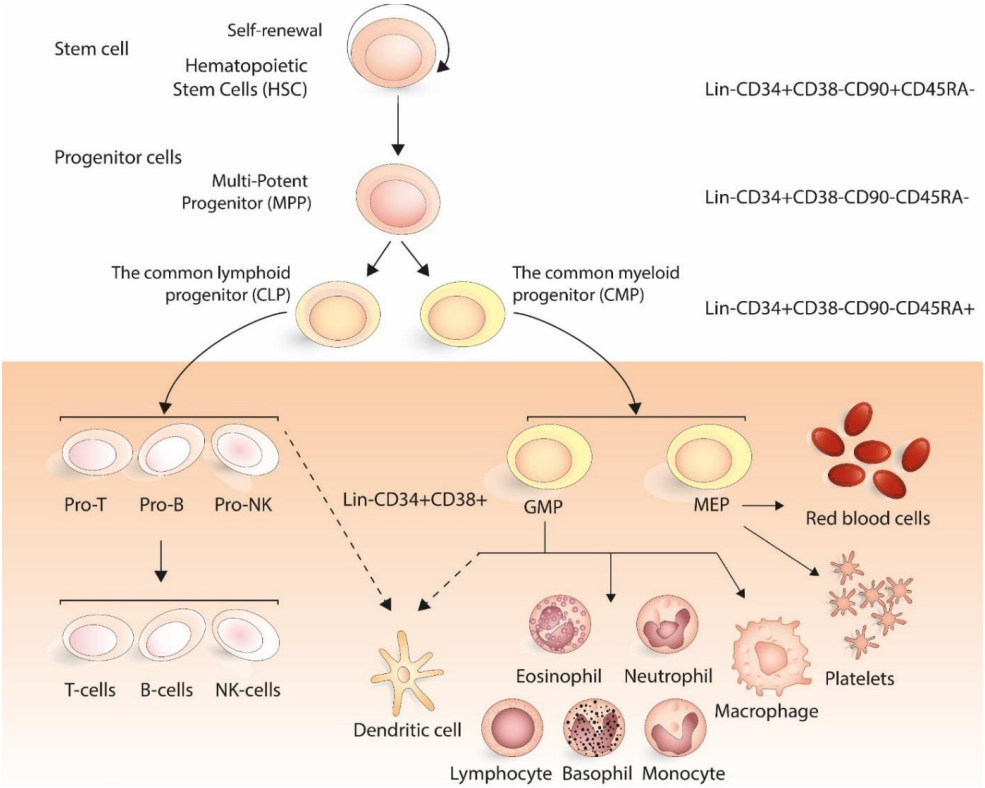

Figure 1. Normal human hematopoiesis. Normal human hematopoiesis is structured as a cellular hierarchy initiated and maintained by self-renewing HSC. Self-renewing HSCs are located at the top of the hierarchy, generating multipotent progenitors, which subsequently generate lineage-committed progenitors that ultimately give rise to terminally differentiated blood cells. These include erythrocytes, platelets, granulocytes, macrophages, dendritic cells and the lymphocytes T, B and NK cells. The lineage-committed progenitors and the mature cells are shown in the colored box. The abbreviations shown in the figure can be found in the list of abbreviations.

Thus, a balance between cell proliferation, differentiation, and survival controls the normal hematopoiesis, whereas the leukemic hematopoiesis in AML shows disturbances

in all three fundamental cell characteristics. The leukemic cells have an increased proliferative capacity but, at the same time, a limited capacity for differentiation and an increased ability to survive (i.e. resistance to apoptosis). This leads to disease progression with an increasing accumulation of immature leukemic myeloblasts [34].

The hierarchical organization within the leukemia clone is also reflected in its cellular heterogeneity (e.g. with respect to morphology, cell surface markers, functional characteristics) and is similar to that of normal hematopoiesis [35]. However, further definition of the phenotype of LSCs has revealed several differences between LSCs and normal HSCs. Most leukemic cells in AML express CD34, which is the normal surface marker for hematopoietic stem/progenitor cell (HSPC) [36, 37]. Within the total AML cell population, LSCs are always a minority, and patients can have AML stem cells with CD34+38-, CD34+CD38+ and/or CD34- phenotypes [38, 39]. However, for approximately 25% of AML patients, more than 90% of the total AML cell population is CD34- [38, 40, 41], and this phenotype can be associated with the *NPM1* mutation [39, 40]. Finally, the Lin-CD34+ fraction of AML patients can be divided into two subpopulations based on AML stem cells. These two populations are similar to normal lymphoid-primed MPPs (CD38-CD90-CD45RA+), and GMP-like cell populations (CD38+CD123+CD45RA+) [41]. In most patients, both populations coexist and possess LSC potential [41].

The phenotypic characterization of AML stem cells can be further distinguished based on the presence of antigens. These include CD25 [42], CD123 [43], CD47 (integrin-associated signal transducer) [44], interleukin (IL)1 receptor accessory protein (IL1RAP) [45], and C-type lectin-like receptors (CCL-1) [46]. Furthermore, markers such as CD13, CD96 (adhesion molecule), and CLEC12A [46, 47] are expressed on AML stem cells but not on normal HSC, while the expression of others, such as CD90 and CD117 (c-Kit), is found on HSCs but not on AML stem cells [45, 48, 49].

1.2.2 The pre-leukemic versus the leukemic stem cells

Hematopoietic cells that have acquired genetic and/or epigenetic modifications can give rise to leukemic cells and may eventually form the self-renewing LSC, or leukemia

initiating cells (LIC), that sustain the disease (Figure 2). The first mutation in HSPC originates in the leukemia cell-of-origin (LCO). These pre-leukemic stem cells preserve the ability to differentiate, but need additional mutations to be fully transformed [50]. In the LCO and subsequent pre-LSC stage, the cells gradually obtain more abnormalities, finally generating fully transformed LSC/LIC. The different aberrations in the hematopoietic pathway may include genes that control DNA methylation (*DNMT3A, IDH2, TET2*); the cohesion expression complex (*RAD21, MAU2, SMC1A*); chromatin modification (*SAP130, MIER3, ASXL1, MTA2, KAISO, CTCF*); the fusion protein inv(16); and *NPM1* mutations [34, 50-52]. Due to their involvement in global chromatin changes, these genes have formerly been denoted as landscaping genes [34]. LSC/LIC are self-renewing with genetic and epigenetic alterations that block differentiation and results in the accumulation of dysfunctional leukemic blasts.

Differences between LSCs and the majority of the AML cell population make them more difficult to eliminate with standard chemotherapy, a property that may give rise to later chemoresistant AML relapse. Consistent with LSCs showing high therapy resistance and being a source of relapse in AML [53], the presence of a stem cell expression signature in the main AML cell population as well as high LSC frequencies correlate with poorer survival [54]. Identification of the presence of therapy-resistant cells at the time of diagnosis indicates two major patterns of relapse in AML [53]. Relapse can either originate from infrequent LSCs with HSPC phenotypes or from the development of larger subclones of committed leukemia cells with immune phenotypes that contain strong stemness transcriptional signatures [53]. With regard to genetic abnormalities, relapses are thought to be derived from (i) the original predominating AML clone; (ii) a minor subclone also existing at the time of first diagnosis; or (iii) development from LSCs [51].

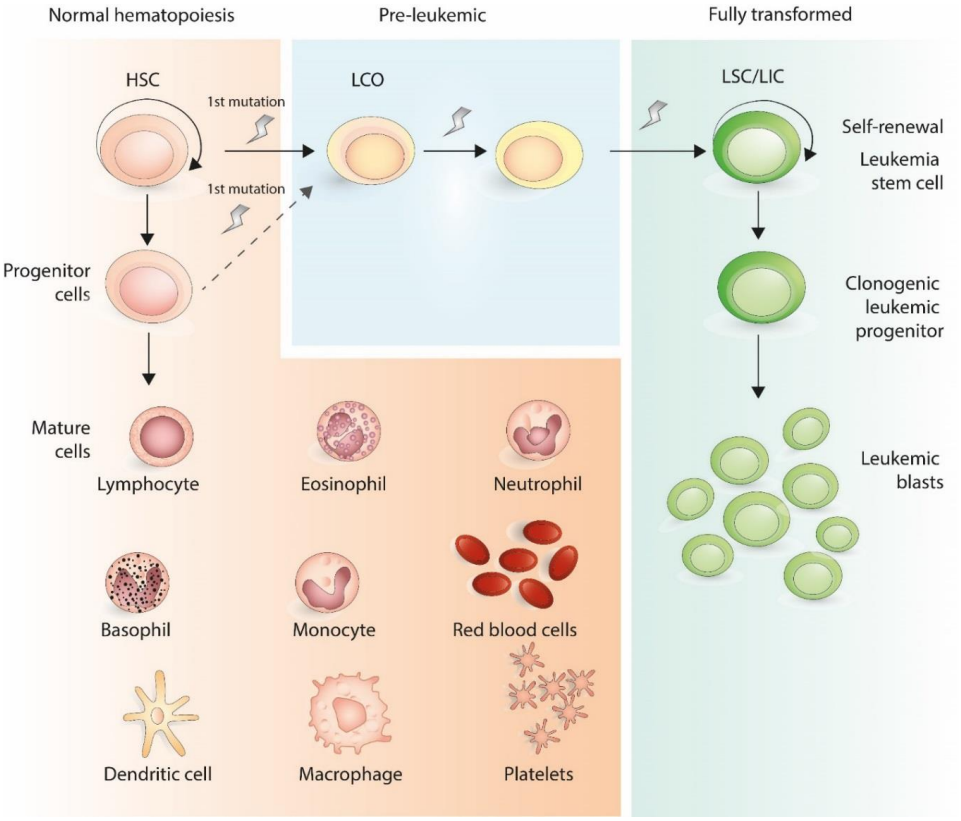

Figure 2. Overview of the transformation of LSC. Leukemia cells develops from HSCs with genetic and/or epigenetic modifications and are transformed to self-renewing LSC/LIC. The transforming events (e.g. oncogenic mutations) can occur in either long-term HSC or in progenitor cells. Mutations in HSC may give rise to a pre-leukemic state associated with an increase in HSCs and genetic instability. A second genetic event (here indicated by lightning bolts) is necessary for the development of the full leukemic phenotype. Mutations occurring within progenitor cells may counter the property of self-renewal, giving rise to the full leukemic phenotype. The abbreviations shown in the figure can be found in the list of abbreviations.

1.2.3 Stem cell niche

A stem cell niche is a spatial structure that houses and maintains stem cells by facilitating self-renewal in the absence of differentiation. Niches are specialized local cellular and molecular microenvironments that control and preserve stem cells, and regulate the balance between HSC self-renewal and differentiation [55]. Hematopoiesis provides a model for understanding mammalian stem cells and their niches (Figure 3). HSC niches

are located in the aorta-gonad-mesonephros (AGM) region, fetal liver, placenta, and spleen during embryonic development, and the bone marrow serves as the primary post-natal reservoir for HSC maintenance and hematopoiesis [55].

The production of blood cells happens continuously inside a complex tissue framework established by highly systematized stromal cell networks of mesenchymal, neural, and vascular origin inside the bone marrow cavities. Together, stromal cells have two main functions: first as primary contributors to bone remodeling and metabolism, and second as main controllers of the various stages of production and development of blood cells [56, 57].

Stromal cells include almost all non-hematopoietic cells in the bone marrow microenvironment, including endothelial cells, osteoblasts, macrophages, adipocytes, fibroblasts, HSCs and HSC progeny cells. These cells support the maturation of precursor blood cells and produce the signals that drive the commitment, differentiation, and maturation of hematopoietic cells. Within the bone marrow environment, the stromal cell types are located in unique combinations and distinct anatomical areas in order to generate suitable niches for the hematopoietic system [58].

The stromal cell-derived factor 1 (SDF1), also known as C-X-C motif chemokine 12 ligand (CXCL12), is one of the growth factors that contributes to bone marrow microenvironments and local cytokine networks. It is an essential chemokine for maintenance of the quiescent HSC pool through CXCL12-CXC receptor 4 (CXCR4) signaling in adult bone marrow [59, 60]. Maintenance and self-renewal of HSCs in the niches are performed by various cells including perivascular, nestin-positive, immature mesenchymal stem cells (MSCs) and endothelial cells together with macrophages/monocytes, sympathetic nerves, non-myelinated Schwann cells and osteoclasts [61]. Osteoblasts affect HSC self-renewal directly through the secretion of CXCL12 and other factors [62-64]. The CXCL12-abundant reticular (CAR) cells, leptin receptor-positive stromal cells, and the nestin-GFP-positive stromal cells are all cell populations that expresses high levels of CXCL12 [56]. CAR cells are defined as mesenchymal progenitors with adipogenic and osteogenic potential *in vitro* [56] and are

the main source of CXCL12 in the bone marrow. In addition, CAR cells can prominently express other adhesion molecules, such as VCAM-1 [57]. Adipocytes are stromal components of the bone marrow that control the function of HSCs in a negative manner, by reducing the number of HSCs [84]. Moreover, the transcriptional coactivator paired-related homeobox 1 (PRX1) and the hypoxia-inducible factors (HIF) -1α and HIF-2α are identified as determinants of HSC maintenance [65, 66].

Due to the heterogeneity of AML, the relative involvement of the various non-leukemic cells in leukemogenesis can differ among patients [34]. However, osteoblasts [67-69], endothelial cells [70-72], MSCs [73-75] and monocytes [76, 77] appears to function as important AML supporting cells in a majority of patients. Multiple soluble mediators released by the primary AML cells and the non-leukemic stromal cells can support or influence leukemogenesis. Among these are cytokines, soluble adhesion molecules, and various proteases [49, 69, 73, 78-80]. Several of these soluble mediators are important for autocrine or paracrine AML cell stimulation, and they can be constitutively released by both leukemic and non-leukemic cells. The bidirectional crosstalk between leukemic and non-leukemic cells can further increase local production levels of many cytokines [81-83].

In the presence of MSCs, an increased long-term *in vitro* proliferation of primary human AML cells that is dependent on both direct cell-to-cell contact and cytokine-mediated crosstalk has been described [75], with integrins and cadherin/catenin proposed to be important mediators [84]. The expression of integrin mRNA varies between AML patient cells, and for a subgroup of patients, this is associated with chemokine-induced growth enhancement [79]. Thus, despite the patient heterogeneity and possible differences between individual patients, normal leukemia-supporting crosstalk involves both distant mechanisms (e.g. soluble mediator release) and direct cell-to-cell contact.

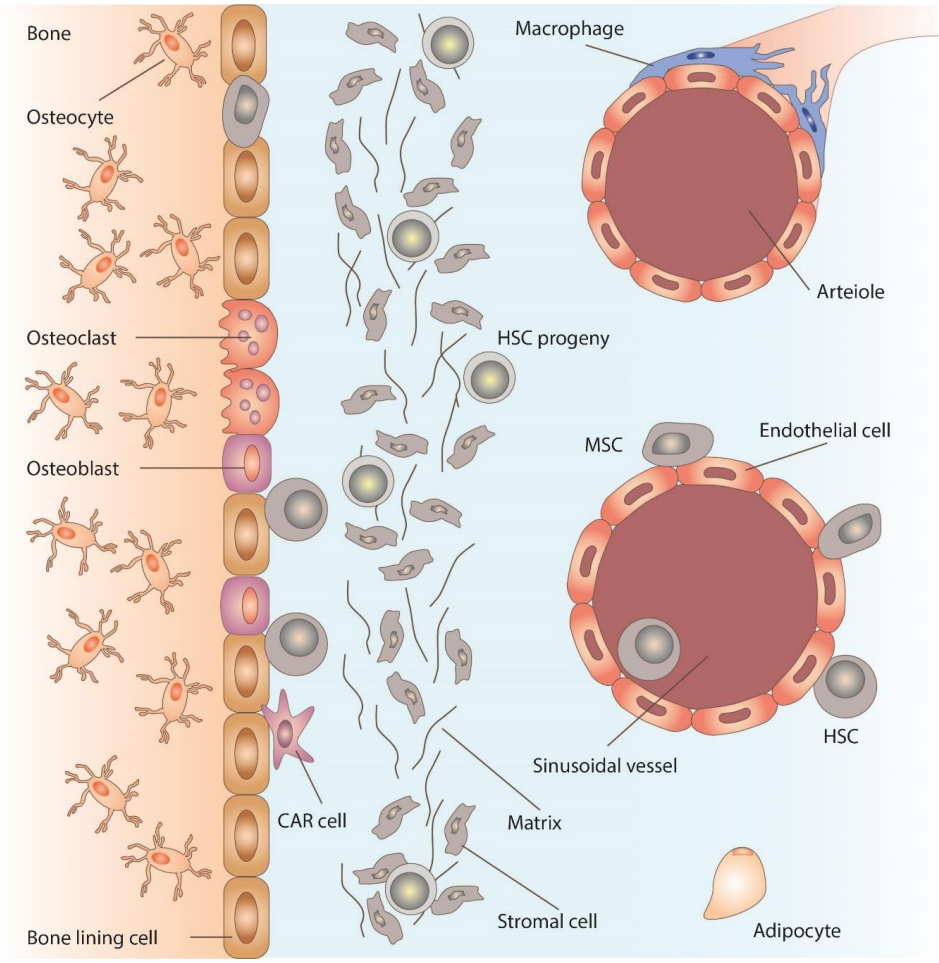

Figure 3. Bone marrow stem cell niches. A bone marrow niche is made up of a heterogeneous mixture of cell types, including MSCs, osteoblasts, osteoclasts, and endothelial cells. The combination of bone lining cells, a hypoxic environment, as well as proximity of MSCs and sinusoidal vessels provide a unique environment for HSCs and MSCs. HSC can be associated with CXCL12-abundant reticular CAR cells close to the endosteal surface and in the periphery of sinusoids and perivascular nestin-expressing cells. Blood vessels in bone marrow are often located near the bone. Osteoblast cells express factors that participate in HSC retention; osteoclasts control the osteoblastic cell function by inducing bone remodeling, while the macrophages regulate osteoblastic cell activity and the retention of HSCs. These cells are all located at the endosteal surface. In the bone marrow stroma, HSCs are associated with CAR cells, which express factors that promote HSC retention, whereas adipocytes negatively regulate HSCs, causing a reduction in HSCs. The abbreviations shown in the figure can be found in the list of abbreviations.

The marrow vasculature is composed of a dense network of various types of vessels such as arterioles and sinusoids, showing differences with regard to surface markers and function. They consist of a single layer of endothelial cells, surrounded by perivascular MSCs and different non-circulating hematopoietic cells [85]. However, arterioles and sinusoids are structurally different components of bone marrow. Arterioles are surrounded by sympathetic nerves, layers of smooth muscle cells, and matrix components, and together they form an important niche that preserves HSC quiescence in the bone marrow [86]. Arterioles are associated with quiescent HSCs, suggesting that the vessel itself may be a critical gatekeeper of stem cell quiescence in the bone marrow [86]. Bone marrow MSCs provide an environment for regulation of HSC proliferation and quiescence. Bone marrow MSCs are located close to the arteriole and sinusoid vessels and are in direct contact with the non-luminal side of the endothelial cells [87]. MSCs are important both for the extravascular microenvironment of HSCs and for cell trafficking to and from this microenvironment. In addition, MSCs can be located in the extravascular space found between the vessels and close to the osteoblasts in the endosteum, where they release extracellular matrix molecules [85]. Quiescent HSCs are known to associate with arterioles that are preferentially found in endosteal bone marrow.

Different signals are provided locally by the bone marrow niche cells [88, 89], including secreted factors such as stem cell factor (SCF), transforming growth factor beta-1 (TGF-β1), platelet factor 4 (PF4 or CXCL4), angiopoietin 1 (ANGPT1), and thrombopoietin (TPO), and they are important contributors to HSC quiescence [90]. Various selectins and extracellular matrix proteins constitute some of the essential components for the regulation of HSC homing and anchoring, while cell-bound molecules like Notch ligands or locally secreted cytokines like IL-7 or erythropoietin (EPO) act as important regulators of HSC proliferation and differentiation [90].

Osteopontin (OPN) is a glycoprotein found in the bone marrow microenvironment, where it is produced by osteoblasts and MSCs [91]. OPNs contributes to bone marrow adhesion as well as to the migration of HSCs [92, 93], and it can function as a negative

regulator of HSCs through inhibition of cell division [93]. Hematopoietic cells can be found next to the endosteal bone surface, which is primarily lined with osteoblasts (Figure 3). This anatomic organization suggests a mutual communication between the osteoblasts and HSCs as well as a possible role for osteoblasts in the regulation of HSCs [94]. Most studies have shown that OPN levels are increased at both the mRNA and blood protein levels in AML patient cells [95], and high levels of OPN have been associated with poor prognoses of AML patients [96]. However, our group found that high levels of OPN released in AML supernatants could be associated with favorable prognoses [97]. In addition, in the bone marrow environment, the leukemic cells are physiologically exposed to low oxygen levels (20-21%), and studies applying low oxygen pressure showed increased levels of *in vitro* cytokine expression, including OPN, in primary AML cells [98].

1.2.4 AML cell metabolism and its possible clinical importance

One of the hallmarks of cancer cells, as proposed by Hanahan and Weinberg in 2000, is their altered metabolic state [33]. The interest in clarifying how metabolism is altered in cancer cells, based on observations that components of signal transduction pathways frequently regulate nutrient metabolism, has increased in the past decade. Central for this research is the notion that metabolic pathways are reprogrammed in cancer cells to divert nutrients towards anabolic processes for heightened growth and proliferation. Normally, access to and subsequent utilization of nutrients is highly regulated by physiological cellular signaling mechanisms, providing an important barrier to transformation. Cancer cells are able to meet their bioenergetic needs by altering their metabolism to promote growth, survival, proliferation, and long-term maintenance. In normal cells, mitochondria are the main components in the generation and regulation of cellular bioenergetics and are responsible for the main production of ATP by oxidative phosphorylation. Cancer cells do not follow the normal pathway for energy production, and this altered metabolism found in cancer cells includes a switch from mitochondrial oxidative phosphorylation to provide an increased glucose uptake and fermentation of glucose to lactate; this is observed even in the presence of fully functioning mitochondria and is referred to as the Warburg effect. Metabolism in cancer cells can

be reprogrammed from a homeostatic state, with high nutrient catabolism or storage, to an anabolic state where nutrients are transformed into biomass [99].

Lately, it has been shown that the switch to aerobic glycolysis provides tumor cells with a proliferative advantage, prompting an investigation into the mechanisms by which this process is triggered and regulated in order to determine the best means of exploiting this pathway for therapeutic gain [100]. Consequently, it is becoming more evident that given the importance of promotion of nutrient uptake and utilization in cell building processes, cancer cells repeatedly select for mutations that augment signal transduction through pathways that unite upon a common set of metabolic processes.

The phosphoinositide 3-kinase (PI3K) -Akt -mechanistic target of rapamycin (mTOR) pathway in normal cells responds to extracellular stimuli, such as insulin and growth factors, by regulation of a wide range of cellular functions. Cell proliferation requires energy and the synthesis of cellular molecules, and the PI3K-Akt-mTOR pathway contributes to the regulation of cellular metabolism by upregulation of glucose transporters/transport, lipid metabolism, and protein synthesis [101, 102].

AML cells can adapt to meet the increased energy or substrate demand during stress conditions in the bone marrow microenvironment. While normal HSCs apply glycolysis mainly as a source for energy homeostasis, recent studies show that AML cells, including LICs, are dependent on oxidative phosphorylation for survival [103]. In newly diagnosed patients, variable proportions of both high and low oxidative phosphorylation are found in AML cells, whereas in post-chemotherapy patients, there is an excess of high oxidative phosphorylation cells. This indicates that mitochondrial oxidative phosphorylation may be associated with AML chemoresistance. High oxidative phosphorylation signatures and metabolism are identified as hallmarks of chemoresistance *in vivo* [103]. Malignant cells often have a reprogrammed and upregulated metabolism with glucose consumption for energy generation, use of glutamine for the refilling of the intermediates of the tricarboxylic acid (TCA) cycle (also called citric acid cycle or Krebs cycle), and fatty acid synthesis for the building of cellular membranes [104], and this has also been shown for AML cells [8, 105, 106].

The PI3K-Akt-mTOR pathway is upregulated in AML cells, potentially contributing to metabolic reprogramming, e.g. a study reported that preserved hematopoietic cells altered by a constitutively active mutant of Akt show aerobic glycolysis, displaying increased rates of glycolysis without effects on the rate of oxidative phosphorylation. As a regulator of glucose metabolism, Akt causes upregulation of the glycolysis phenotype [107]. There is no correlation between this increased glycolysis and oxygen consumption rates, suggesting that Akt hyperactivation promotes aerobic activation through the Warburg effect [107]. In addition, Akt interacts with PDK1, influencing the entrance of pyruvate into the mitochondrial metabolism [108].

In addition to its effect on glycolysis, Akt may also affect oxidative phosphorylation: Akt could promote an indirect oxidative phosphorylation through elevated levels of substrates essential to activity of the TCA cycle and oxidative phosphorylation, such as pyruvate, ADP, and NADH.

Regulation of cellular metabolism by mTOR is more complex, as it involves activation of Akt by mTORC1 and upregulation of HIF1, for the promotion of glycolysis by converting pyruvate to ATP molecules and lactate [109]. During normal oxygen concentrations, HIF1 is deregulated, but can accumulate with increased signaling of mTORC1 and the downstream mediators, 4E-BP and eIF4E [110]. Furthermore, by promoting translation of particular mitochondria-related mRNAs, mTORC1 can regulate mitochondrial function and oxidative metabolism [99]. mTORC1 also responds to intracellular and environmental stresses, such as low ATP levels, hypoxia, or DNA damage, and participates in regulation of cell growth and metabolism by inducing a shift in glucose metabolism from oxidative phosphorylation to glycolysis [99]. Activation of mTORC1 is reliant on growth factors and amino acids, such as glutamine, leucine, and arginine; this facilitates feedback mechanisms for increased uptake of nutrients to act as fuel for anabolic reactions. However, the activity of mTORC1 is reduced upon starvation [99], and during conditions of high-energy stress, the metabolic regulator AMPK is activated. AMPK represses mTORC1 activity indirectly through phosphorylation and activation of TSC2, and directly through the phosphorylation of Raptor [99].

Interestingly, besides promoting the expression of the enzymes of lipid synthesis and the pentose phosphate pathway, mTORC1 has also been found to increase expression of GLUT1 and other enzymes of glycolysis [110]. Activated mTORC1 participates in regulation of autophagy, mRNA translation, lipid synthesis, glycolysis and the pentose phosphate pathway. Poulain and colleagues demonstrated that constitutive activation of mTORC1 signaling makes AML cells dependent on glucose metabolism [111], implementing mTORC1 as one of many contributors to the glycolytic switch contained in most cancer cells.

1.3 Intracellular signaling in leukemogenesis - The importance of PI3K-Akt-mTOR signaling in AML

The cells in multicellular organisms are continuously exposed to and thereby must respond to a wide range of extracellular signals, such as growth factors, cytokines, and nutrients, which impact cell fate, including proliferation, growth, survival, differentiation, motility, and metabolism. To effectively process these signals, cells have established intricate signaling networks that allow them transduce extracellular signals into cellular decisions [112]. Receptor ligation initiates signal transmission from the cell surface through a series of molecular events often including protein phosphorylation catalyzed by protein kinases, and this ultimately results in a cellular response. There are numerous receptors and pathways involved in intracellular signal transduction, and there is extensive cross-talk and cross-activation between different signaling pathways [113]. Disturbed signaling caused by mutations in pathway components are important factors in leukemogenesis [114-116].

1.3.1 The PI3K-Akt-mTOR pathway

The PI3K-Akt-mTOR pathway has been extensively studied in normal and malignant cells [113]. The signaling cascade is activated by a wide variety of extracellular stimuli, including receptor tyrosine kinases, various integrins, B and T cell receptors, and G-protein-coupled receptors (GPCRs). Family members of PI3K are Serine (Ser)/Threonine (Thr) kinase heterodimers, which can be divided into three different

classes based on their structural characteristics and substrate specificity [117]. Class I enzymes are separated into class IA and class IB enzymes, both of which are activated by cell surface receptors. Class IA enzymes can be activated by receptor tyrosine kinases (RTKs), GPCRs, and various oncogenes such as the small G protein Ras, whereas class 1B enzymes are activated solely by GPCRs.

Class 1A PI3K enzymes include a catalytic (p110) and a regulatory subunit (p85 or p101) [118, 119]. In response to extracellular stimuli, recruitment scaffolding proteins, such as the growth factor receptor-bound protein 2 (GRB2) -associated binding protein 2 (GAB2) or insulin receptor substrates (IRS) 1/2, bind to the regulatory p85 subunit of PI3K. Sequentially, the catalytic subunits of PI3K are activated, and phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) generates the second messenger phosphatidylinositol 3,4,5- trisphosphates (PIP3) [120]. This facilitates the recruitment of proteins that contain pleckstrin-homology (PH) domains, including the Ser/Thr kinase Akt (also known as protein kinase B or PKB) and its upstream activator 3-phosphoinositide-dependent kinase-1 (PDK1)

Akt can function as a proto-oncogene, and there are three structurally active forms of Akt in mammalian cells termed Akt1, Akt2, and Akt3 or PKB α, β, γ, respectively [121]. All three isoforms comprise an N-terminal PH domain, a T-loop region of the catalytic domain containing a Thr308 phosphorylation site, and a C-terminal regulatory tail with a Serine-473 Ser473 phosphorylation site [121, 122]. Whereas Akt is cytosolic in unstimulated cells, an activation mediated by PI3K requires translocation of Akt to the membrane, where PIP3 serves as an anchor [123]. At the plasma membrane, PDK1 phosphorylates Akt at Thr308, leading to its partial activation. A subsequent phosphorylation at Ser473 is required for full enzymatic activation. This phosphorylation is achieved by the mTOR complex 2 (mTORC2) as well as by members of the PI3K-related kinase (PIKK) family [121, 122]. Phosphorylation of homologous residues in Akt2 and Akt3 occurs in the same way. This activation leads to the relocation of Akt to the cytosol or the nucleus, and it has been postulated that Akt then can phosphorylate more than 9000 downstream substrates [124], thereby regulating important cellular processes such as cell metabolism, proliferation, transcription, and

survival. The mTOR complex 1 (mTORC1) is an important downstream target of Akt. This is summarized in Figure 4.

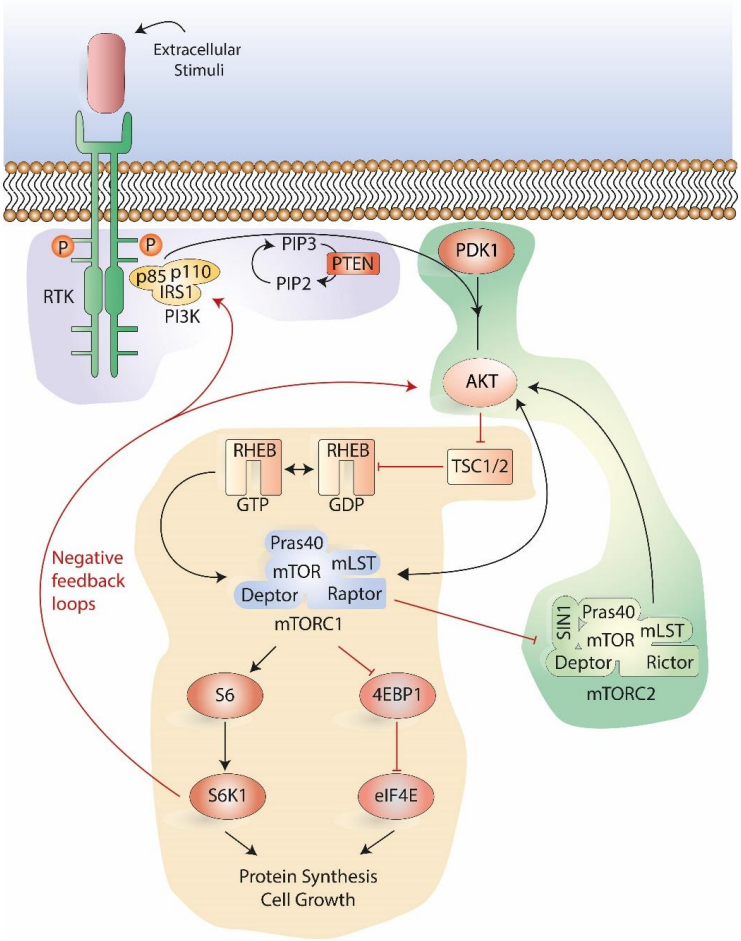

Figure 4. Overview of the PI3K-Akt-mTOR signaling pathway. Following ligation of cell surface receptors (e.g. growth factor receptors) phosphorylated RTK recruits scaffolding proteins, which bind to the regulatory p85 subunit of PI3K. A subsequent activation of the catalytic subunits of PI3K generates PIP3. PDK1 and Akt proteins are then recruited to the plasma membrane, inducing the phosphorylation of Akt on Thr308 by PDK1. This is followed by activation of Akt on Ser473 by the mTORC2 complex; this second phosphorylation is necessary for complete activation. Akt controls the activation of mTORC1 by constraining the GTPase activity of the TSC1/TSC2 complex towards the Ras-related GTP-binding protein RHEB that associates to mTORC1 and phosphorylates mTOR. mTORC1 induces cap-dependent mRNA translation by phosphorylating 4EBP1, leading to the formation of eIF4F and the inhibition of autophagy. The abbreviations shown in the figure can be found in the list of abbreviations.

The Ser/Thr protein kinase mTOR was first identified in the budding yeast Saccharomyces cerevisiae during a trial for resistance to the immunosuppressant drug rapamycin [125]. It belongs to the PIKK family and has a COOH-terminal catalytic domain with sequence homology to PI3Ks. It is a large, multi-domain protein with protein kinase activity, adding phosphate groups to Ser or Thr residues in a wide range of substrates, many of which are involved in anabolic pathways [126]. Insulin and insulin growth factors (IGFs) [127], nutrients (such as amino acids) [128], various forms of stress (e.g., hypoxia and DNA damage), and the accessibility of ATP are the main regulators of mTOR.

The activity of mTOR is accomplished by the two separate multi-protein complexes, mTORC1 and mTORC2. The two complexes differ in their protein components, substrate specificity, and regulation, and they have dissimilar responses to rapamycin and its derivatives (rapalogs). The mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (Deptor) and the proline-rich Akt substrate of 40 kDa (PRAS40) [129]. Both Deptor and PRAS40 function as inhibitors. The mTORC2 is composed of mTOR, mLST8, Deptor, the rapamycin insensitive companion of mTOR (Rictor), the mammalian stress-activated protein kinase-interacting protein 1 (mSIN1), and Protor [127, 130].

Raptor is responsible for the phosphorylation of downstream substrates, and this action is blocked by rapamycin. As mTORC2 contains Rictor rather than Raptor, it phosphorylates a different set of substrates, although the regulation of mTORC2 activity remains poorly understood. The mTOR complexes have different upstream mechanisms of activation as well as different downstream substrates. The most common substrates of TORC1 are the ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor-4E (eIF4E) -binding proteins (4EBP1), while the main substrates of TORC2 are Akt and related kinases [131]. The S6K protein plays a central yet moderately defined role in cellular and organismal physiology. There are two identified isoforms of S6K, termed p70 and p85, produced by differential splicing from a common gene. Both isoforms are implicated in regulation of cell growth, but the p70S6K isoform has been given the most

attention, and the function of the p85S6K remains poorly characterized. Downstream targets of mTORC1 play critical roles in the regulation of translation. The translation initiation complex eIF4F is a heterotrimeric protein complex composed of eIF4E, eIF4A, and eIF4G [131]. The eIF4E binds to the messenger RNA (mRNA) 5′-cap structure to promote the initiation of translation. In the unphosphorylated state, 4EBPs binds to eIF4E, hindering its association with the complex, and blocking it from binding to mRNA. However, in response to stimuli such as growth factors, mitogens, and amino acids, mTORC1 phosphorylates 4EBPs, causing it to lose its inhibitory effect. This allows the formation of the eIF4F complex and the subsequent initiation of translation [131]. In addition, PDK1 and mTORC1 phosphorylates the S6 kinase, which in turn phosphorylates numerous substrates that are involved in translation.

The FK506-binding protein 38 (FKBP38, also known as FKBP8) is a unique member of the FKBP-family and acts as an upstream regulator of the PI3K-Akt-mTOR pathway [132]. FKBP38 was identified as an endogenous inhibitor of mTORC1 that binds to the FKBP-C domain of mTOR. This binding interferes with mTOR function in a manner comparable to the FKBP12-rapamycin complex [132]. Under conditions rich in growth factors and nutrients, the FKBP-C domain might interact with Ras homologue enriched in brain (RHEB) -GTP, releasing mTOR from FKBP38 and activating downstream mTOR signaling [132, 133].

A negative feedback loop can be formed in the PI3K-Akt-mTOR pathway by activation of mTORC1. Phosphorylated S6K will sequentially phosphorylate IRS proteins, triggering their proteasomal degradation and inhibiting insulin/IGF-1-mediated PI3K activation (Figure 4).

The PI3K-Akt-mTOR pathway is negatively controlled by the phosphate and homologue protein deleted on chromosome 10 (PTEN) protein and Src Homology 2 (SH2) domain-containing inositol 5-phosphatases (SHIP1 and SHIP2) that directly antagonize PI3K by dephosphorylating PIP3 back to PIP2, thus averting activation of Akt [117]. *PTEN* is the third most commonly mutated gene in human cancers, underlining its functional significance [134]. The activity of mTOR is also negatively controlled through the tuberous sclerosis complex (TSC: comprising TSC1 and TSC2).

Upon activation, Akt will phosphorylate and inhibit heterodimeric TSC, which then acts as a GTPase-activating protein (GAP) for small GTPase RHEB [135]. This activation leads to hydrolysis of bound GTP with subsequent RHEB inhibition. The inactivation of TSC2 maintains RHEB in its GTP-bound state, thereby supporting augmented activation of mTOR [135].

The mTORC1 can also be regulated by cellular stress and energy status through TSC, in addition to growth factors. Moreover, a low energy status can activate AMP-activated protein kinase (AMPK), which in turn phosphorylates Raptor and TSC2, leading to the inhibition of mTORC1 [135, 136]. Amino acids regulate mTORC1 in a TSC-independent pathway. Thus, multiple stimuli modulate mTORC1 to control cell growth and autophagy.

1.3.2 PI3K-Akt-mTOR signaling in AML

Dysregulation of the PI3K-Akt-mTOR signaling pathway subsequent to oncogene activating mutations, oncogene amplification, upstream activation of RTKs, or inactivation of tumor suppressor genes has been demonstrated in many human malignancies, including AML. The PI3K-Akt-mTOR pathway plays an important role in the regulation of proliferation, differentiation, and survival of hematopoietic cells. Constitutive activation of this pathway has been observed in more than 60% of AML patients and is associated with decreased overall survival [137-139]. Mutations in membrane bound-proteins, such as RTKs or GTPases, are major causes of dysregulated PI3K-Akt-mTOR signaling and are observed in 55% of AML cases [11, 134].

Signaling initiated through *FLT3* is one of the most important causes of the dysregulation of PI3K-Akt-mTOR signaling in AML, and mutations in this gene lead to abnormal activation of the pathway [140]. Mutations in *FLT3* are among the most frequent mutations seen in AML. These mutations can be classified as internal tandem duplications (*FLT3*/ITD) and mutations in the tyrosine kinase domain (*FLT3*/TKD), with the former being more common in AML [141]. Because *FLT3*/ITDs are located in or near the juxtamembrane domain of the RTK, they affect multiple processes within

the activation loop of the TDK, while other mutations are effectively isolated, resulting in the substitution of single amino acids in the loop[141].

The three classes of PI3Ks (Class I-III) have different structure, cellular distribution, mechanism of action, and preference of substrates [142]. Class I PI3Ks are most frequently linked to carcinogenesis [119]. Expression of the regulatory p85α subunit of PI3K Class IA was examined in a previous study, which included 40 AML patients; expression of the regulatory subunit was then detected in nearly all samples, and 21 cases exhibited increased PI3K activation [143]. Furthermore, PI3K expression correlated with proliferation capacity of AML blasts [143]. The catalytic subunits termed p110α, p110β, p110γ and p110δ are responsible for activation of Akt, and p110δ is the only form that constantly shows elevated expression in human AML [119, 144].

Constitutive phosphorylation activates Akt in human AML cells, and the constitutive activation of the PI3K-Akt-mTOR pathway is necessary for the survival of AML cells [138, 145]. Furthermore, the constitutive activation of Akt is facilitated by phosphorylation at Thr308 by PDK1 and at Ser473 by mTORC2. Constitutive activation at both Thr308 and Ser473 has been detected in a majority of AML patients [138, 146]. Akt phosphorylation at these two sites has been associated with decreased overall survival in several studies [138, 147] [113], though independently, phosphorylation at Ser473 may constitute a favorable prognostic factor [146]. Finally, aberrant AML cell activation of mTORC1, causing the phosphorylation of downstream targets such as p70S6K, S6RP, and 4EBP1, has also been detected for a large majority of patients [148], but this activation of mTORC1 may not depend on PI3K/Akt activity alone in human AML [149].

The IGF-1/IGF-1 receptor (IGF-1R) signaling pathway is important in the development of many malignancies [150]. IGF-1/IGF-1R-initiated signaling is facilitated by PI3K activation both in myeloid leukemia cell lines and in primary AML cells [146, 151]. The constitutive activation of Akt in AML is supported by autocrine IGF-1/IGF-1R signaling and inhibition of IGF-1R results in decreased activation of Akt for most patients with such autocrine signaling [150].

Even though *PTEN* is the third most frequently mutated gene in human cancer, it is rarely mutated in AML [11, 134]. However, abnormal PTEN have been detected in a small subset of AML patients with adverse prognoses; this abnormality leads to a lack of inhibitory pathway control and thereby increased Akt signaling [152, 153]. Activation of the PI3K-Akt-mTOR pathway due to mutations of its components is observed in nearly 30% of all human cancers [154] but is rarely seen in human AML [147, 155].

1.3.3 SYK as a regulator of PI3K-Akt-mTOR signaling

Spleen tyrosine kinase (SYK) is a cytoplasmic protein-tyrosine kinase known to facilitate interaction between immune cell receptors and the intracellular signaling pathways important for the regulation of cellular responses to extracellular antigens as well as the antigen-immunoglobulin complexes central to initiation of inflammatory responses [156]. SYK is widely expressed in hematopoietic cells and is involved in proliferation, differentiation, and phagocytosis. The importance of SYK in hematological malignancies has been demonstrated in lymphoma, leukemia, and myelodysplastic syndrome (MDS) [156, 157]. Targeting SYK diminishes viability and stimulates differentiation in AML. However, knowledge related to downstream signaling effectors for SYK in AML is scarce. There has been much interest in mTOR as a target in AML, and mTOR is activated in most primary AML blasts [158]. Furthermore, the inhibition of mTOR in AML has been linked to both anti-proliferative and pro-differentiating effects [148, 149, 158]. Consequently, studies suggest that inhibition and constitutive activation of SYK leads to corresponding activation and inhibition of mTOR signaling, and simultaneous inhibition of SYK and the PI3K pathway is postulated to promote differentiation and inhibit viability in AML cells [159].

SYK is an important mediator of FLT3 in AML, and patients often display mutations in the *FLT3* gene resulting in constitutively active RTK [160]. In an active and phosphorylated state, FLT3 associates with SYK through its C-terminal SH2 domain, and this association increases the activity of FLT3 through phosphorylation. This cooperative activation of FLT3 and SYK results in an increase in the expression of c-Myc and the target genes of c-Myc [161]. SYK is a regulator of mTOR and MAPK

signaling in AML, and inhibition of PI3K-Akt-mTOR pathway activity enhances the effects of SYK inhibition on AML cell differentiation and viability [160, 161].

Chemical inhibition of SYK has been tested in multiple cells lines and results in a dose-dependent inhibition of mTOR [159]. Furthermore, in some AML cell lines and primary cells, both p70S6K and 4EBP1/eIF4E signaling pathways are influenced by manipulations in SYK activity, and combined chemical inhibition of SYK and eIF4E has shown a synergistic effect on AML viability in multiple AML cell lines [159]. This is supported by inhibition of eIF4E through SYK knockdown, indicating that there are enhanced inhibitory effects of SYK on cell viability. Inhibition of eIF4E enhanced AML differentiation when combined with SYK inhibition using either chemical or SYK-directed short hairpin RNA [159].

1.3.4 The importance of PI3K-Akt-mTOR for the bone marrow stem cell niche

Through interaction between leukemic cells and bone marrow-derived MSC, a pleiotropic range of proliferative and anti-apoptotic signaling pathways can be activated, including the PI3K-Akt-mTOR signaling pathway.

This pathway is central to stress responses, such as the adaptation of MSCs to the hypoxic microenvironment in the bone marrow, induction of autophagy, and regulation of MSC metabolism. MSCs possess functional alterations depending on the age of the patient, and changes in pathway activation can influence the aging process and age-dependent variations of bone marrow MSCs. Additionally, the PI3k-Akt-mTOR pathway is associated with MSC differentiation, and AML-supporting osteoblasts and adipocytes are among the major differentiated mesenchymal cells in bone marrow. Finally, the PI3K-Akt-mTOR pathway is central to the balance between osteogenic and adipogenic differentiation and supports the regulation of communication between MSCs and their adjacent cells.

Studies have confirmed the upregulation of numerous survival signaling pathways, including PI3K-Akt-mTOR, in primary AML cells co-cultured with stromal MSC [162], indicating the importance of this pathway for the regulation of several biological characteristics of MSCs. An mTOR kinase inhibitor, PP242, successfully prompted

apoptosis in primary samples cultured with or without stroma [163]. PP242 functions by weakening the activities of both mTOR complexes, which results in their inhibition by phosphorylated Akt, S6K, and 4EBP1. Simultaneously, chemokine receptor CXCR4 expression in primary leukemic cells, as well as stromal cells cultured alone or co-cultured with leukemic cells, was suppressed following treatment with PP242. These results were confirmed in mouse models, where PP242 inhibited mTOR signaling in leukemic cells with a greater anti-leukemia effect than rapamycin. Combined, studies with inhibitors targeting the PI3K-Akt-mTOR pathway show that disrupting mTOR-Akt signaling with selective kinase inhibitors could successfully target leukemic cells within the bone marrow microenvironment [163-165]. Additionally, the PI3K-Akt-mTOR signaling pathway has been found to play a central role in stromal cell-mediated resistance to FLT3 inhibition. Following combined treatment with selective PI3K inhibitor GDC-0941 and protein kinase inhibitor Sorafenib, a reversion of the protective effects of bone marrow stromal cells was found on FLT3-mutant AML cells in hypoxia. An association between the effect of this combined treatment and the downregulation of Pim-1 and Mcl-1 expression was noted [166]. Hence, PI3K-Akt-mTOR signaling is central for aging, adaptation, communication, differentiation, and proliferation in bone marrow MSCs and the inhibition of this pathway could affect and alter both MSC functional characteristics and AML cell microenvironments, including those of the stem cell niches.

1.3.5 PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway cross talk

Together, the Ras- extracellular signal-regulated kinase (ERK) and PI3K-Akt-mTOR signaling pathways are the main mechanisms for regulating cell survival, differentiation, proliferation, metabolism, and motility in response to extracellular stimuli in the cell, and the pathways crosstalk to effect positive and negative regulation of each other [167, 168]. Many mechanisms and methods of crosstalk between the two pathways have been identified, including cross-inhibition, cross-activation, negative feedback loops, and pathway convergence on substrates (Figure 5 and figure 6).

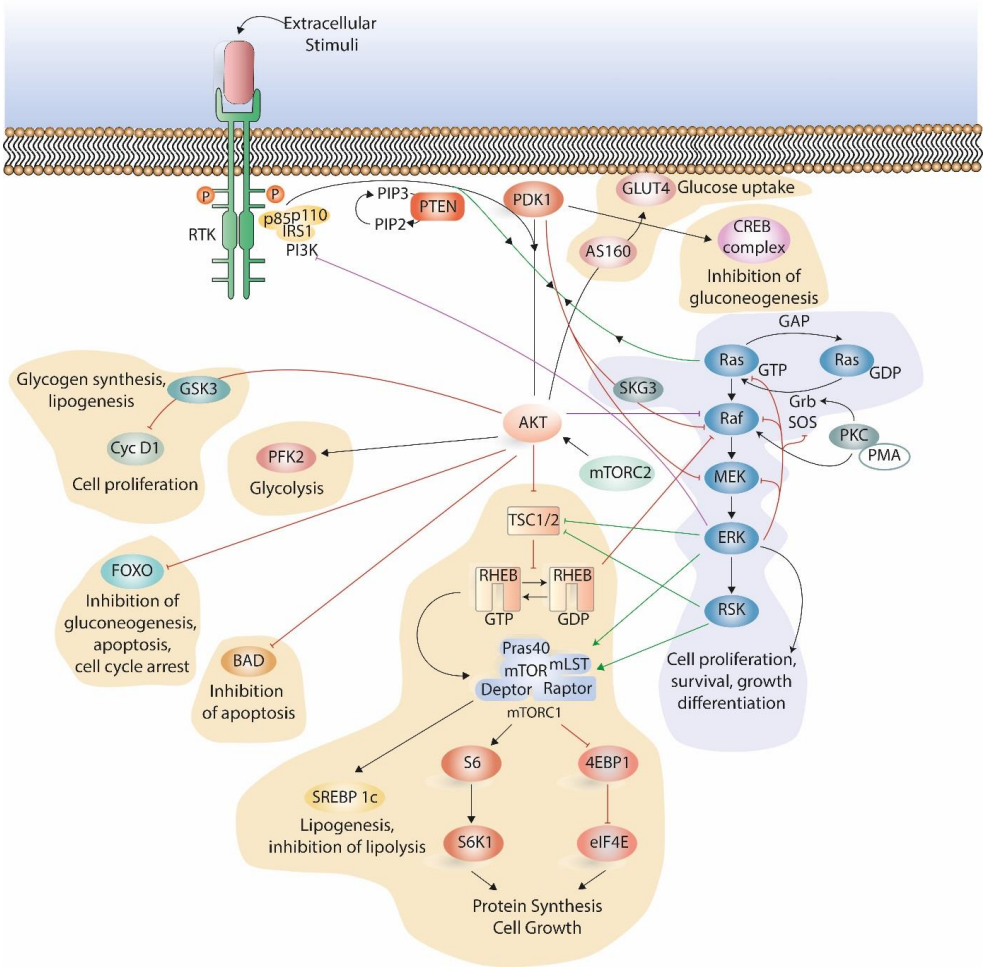

Figure 5. Main pathway components of Ras-MAPK and PI3K-Akt-mTOR and pathway crosstalk. Ras-MAPK and PI3K-Akt-mTOR pathways respond to extracellular and intracellular signals to control cell survival, proliferation, motility, and metabolism, and they are both activated by the binding of a growth factor to an RTK. In the Ras-MAPK pathway, this binding produces binding sites for the Src homology and collagen (SHC) and GRB2 adaptor molecules that recruit SOS to the membrane. SOS catalyzes the exchange of Ras GDP to Ras GTP, followed by the recruitment and activation of Raf. PKC is directly bound and activated by PMA through its mimicking of the natural ligand of PKC. The mechanism by which PKC activates ERK remains unclear. Following a Ras activation of MEK, ERK is activated through an activation loop phosphorylation. The pathway is further regulated by a negative feedback loop from ERK. Pathway crosstalk is regulated through cross-inhibition (purple) and cross-activation (green) between the components of the Ras-MAPK and PI3K-mTOR pathways. Each pathway has a mechanism to negatively feed onto the other: ERK phosphorylation of GAB connected to PI3K and Akt

phosphorylation of Raf. Components of the Ras-ERK pathway also exhibit positive regulation of the PI3K-Akt-mTORC1 pathway; TSC2 and mTORC1 are key integration sites that have several inputs from both the Ras-ERK and PI3K signaling. Positive regulation of the pathways are shown as arrows, and negative regulation of the pathways are represented as blunt-ended lines. PI3K-Akt (shaded in pink) and Ras-ERK (shaded in blue) signaling network exhibit examples of crosstalk between intracellular networks. PI3K and ERK signaling meet on mTORC1, which is the master controller of protein translation. PTEN acts as a strong negative regulator of both pathways. The abbreviations shown in the figure can be found in the list of abbreviations.

While some of the kinase, such as Raf, MEK, and mTORC1, that are involved in these pathways possess narrow substrate specificity, others such as ERK, RSK, Akt, and S6K, phosphorylate several members of the main signaling pathways in addition to several effector proteins. Consequently, a significant part of pathway integration occurs through these latter kinases.

Mitogen-activated protein kinase (MAPK) is a Ser/Thr protein kinase that is broadly present in eukaryotic cells, and Ras-Raf-MEK-ERK is an important signaling pathway in MAPKs, with ERK as the key effector of the Ras oncoprotein [168]. Cell surface receptors activates the signal transduction and the production of the appropriate biological response. By conducting signals through the receptor, the Ras-Raf-MEK-ERK pathway activates transcription factors and regulates gene expression [169]. The pathway is activated by growth factors, polypeptide hormones, neurotransmitters, chemokines, and phorbol esters (e.g. phorbol myristate acetate (PMA)), which signal through their related RTKs and GPCRs, or by the direct activation of protein kinase C (PKC). Ras recruits and activates the Ser/Thr protein kinase Raf, which promotes the MEK1/2 (MAPK/ERK kinase) dual-specificity protein kinase and the activation of ERK1/2. Activated ERK1/2 phosphorylates several downstream targets and controls transcription factors.

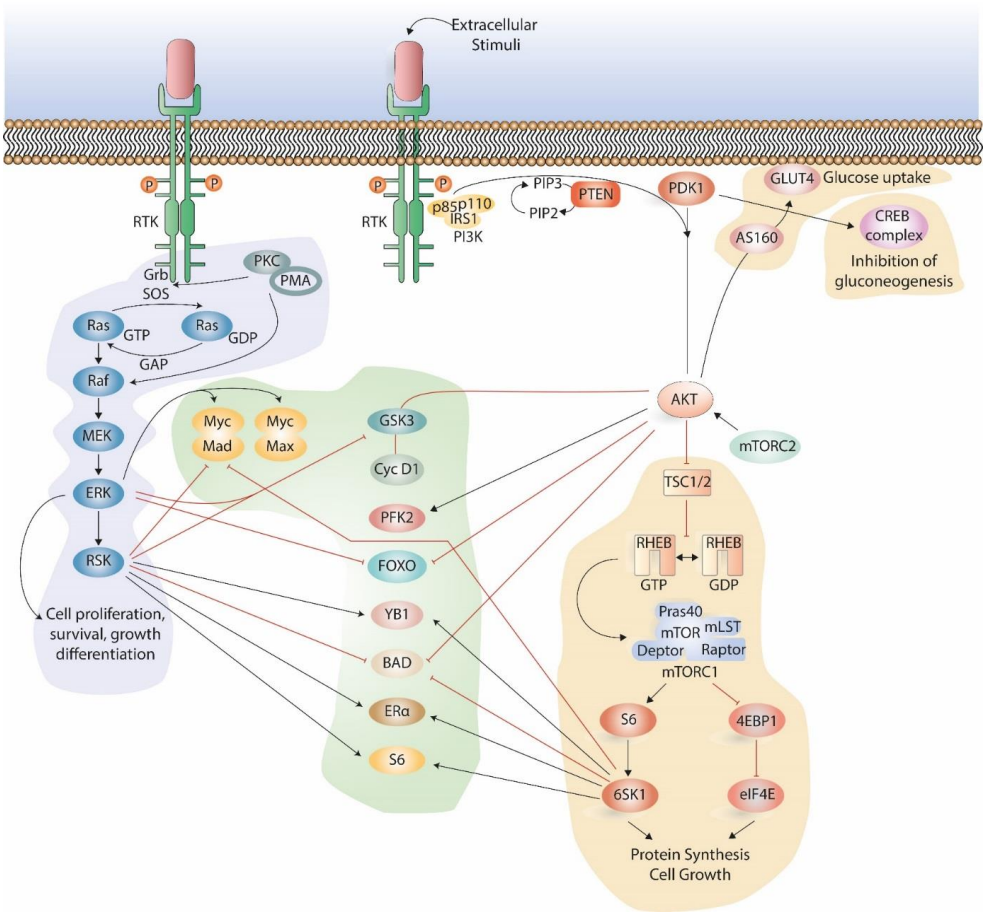

Figure 6. Pathway Convergence. ERK and AGC kinases often regulate the same substrates to produce the same phenotypic effects. FOXO and GSK3 are examples of Ras-Raf-MEK-ERK and PI3K-Akt-mTOR convergence at different residues on the same substrate through ERK and AGC kinase inputs. The Myc-Mad1 and Myc-Max dimers are examples of two pathways converging on different members of the same complex. BAD and S6 are examples of AGC kinases regulating different motifs within the same substrate. YB1 and ERα are examples of AGC kinase promiscuity, in which several AGC kinases phosphorylate the same residue. The abbreviations shown in the figure can be found in the list of abbreviations.

The Ras-Raf-MEK-ERK pathway cross-activates PI3K-Akt-mTOR signaling by regulating three main substrates: PI3K, TSC2, and mTORC1. Ras-GTP can directly bind and allosterically activate PI3K [170-172]. Furthermore, strong activation of the Ras-Raf-MEK-ERK pathway can activate mTORC1 indirectly through ERK and RSK signaling to the TSC complex. Constitutively active Ras mutants, phorbol esters, and EGF can phosphorylate TSC2 through ERK and RSK [167, 173]. The ERK and RSK sites functions to promote mTORC1 activity and tumorigenesis, but they are different from those phosphorylated by Akt [167] in that they induce a PI3K-independent mTORC1 phosphorylation of 4EBP1 [167, 174, 175]. In an active state, ERK and AGC kinases can act on the same substrate, often at the same time, to promote cell survival, proliferation, metabolism, and motility. Examples include the forkhead box O (FOXO) and c-Myc transcription factors, BAD (B-cell lymphoma (BCL) 2-associated agonist of cell death), and glycogen synthase kinase 3 (GSK3).

Briefly, FOXOs are regulators of the expression of cell cycle components and apoptotic proteins that oversee cell survival and proliferation. c-Myc is an obligate heterodimer that, along with its activation partner Max, is responsible for the positive regulation of growth and survival transcriptional mechanisms. The related transcription factor Mad1 competes with Max to bind to c-Myc, and the Myc/Mad heterodimer functions as a repressor of gene transcription of growth and survival genes [176]. BAD is a pro-apoptotic BH3-only protein in the BCL-2 family. This protein positively regulates cell apoptosis by forming heterodimers with BCL-xL and BCL-2, reversing their death-repressor activity. Different growth factors (e.g. Wnt, insulin) are known to deactivate GSK3 α/β Ser/Thr kinases. This deactivation releases the inhibitory effect of GSK3 of pro-survival, proliferation, and motility proteins, such as adhesion proteins and the β-catenin transcription factor that drives the expression of cyclin D and MYC.

1.3.6 PI3K-Akt-mTOR signaling pathway inhibition in AML

It is commonly accepted that a disparity in the regulation of protein kinases may be one of the primary causes of genetic-based human diseases, and a major part of new drug development over the last decade has been dedicated to the development of protein

kinase inhibitors. The PI3K-Akt-mTOR pathway has emerged as a possible therapeutic target in human malignancies, and several pharmacological inhibitors have been developed, including isoform-selective or pan-class I PI3K inhibitors, Akt inhibitors, rapamycin, and rapalogs as well as dual PI3K-mTOR inhibitors. The rapalogs everolimus and temsirolimus represent allosteric inhibitors directed towards mTORC1 and are now in clinical use. Early studies indicated that PI3K inhibition has proapoptotic and antiproliferative effects in primary human AML cells through the dephosphorylation of Akt and BAD [145].

The efficiency of several selective inhibitors towards the PI3K-Akt-mTOR pathway has been studied in AML cell lines and primary cells, and results have shown that Akt can be reactivated through negative feedback mechanisms subsequent to rapamycin-mediated mTOR inhibition. Inhibitors targeting the catalytic domain of mTOR have also been developed [177], and pre-clinical studies of rapamycin combined with rapalogs targeted at mTOR showed inhibition of clonogenic AML cell proliferation without inhibition of normal $CD34^+$ cells [148]. The antileukemic effects were further enhanced by combination with conventional cytotoxic drugs [158]. However, the relevant antileukemic activity of rapamycin and rapalogs has not been demonstrated in clinical trials [178-181].

Both PI3K and mTOR are members of the PIKK superfamily and share structural domains, and some inhibitors therefore act on both kinases. An important consequence of inhibiting mTORC1/S6K by rapalogs has been the increased phosphorylation of Akt [177, 182]; this has been observed in both experimental and clinical studies. Dual inhibition of PI3K and mTOR blocks both the upstream and downstream pathways of Akt, consequently circumventing activation of Akt subsequent to the reduced mTORC1-S6K-IRS1 mediated negative feedback loop [183]. Possible reasons for the failure to demonstrate clinically relevant antileukemic effects by pathway inhibitors include limited activity in human patients [184], intrinsic molecular defects [185], and dose-limiting toxic effects [186]. Akt is important for a wide range of cellular functions and interacts with an immense number of substrates [122]. Only a few Akt inhibitors have been developed, and they have not produced convincing effects in AML [187, 188].

Results from selected representative and relatively large clinical studies of PI3K-Akt-mTOR inhibitors were recently reviewed by Herscbein-Liesveld and colleagues [189] and Table 3 represents the most relevant studies. These clinical studies justify the conclusions that (i) pathway inhibitors used alone have only modest antileukemic effects; (ii) pathway inhibition in combination with intensive chemotherapy have acceptable toxicity; (iii) the doses used in clinical studies can alter the activation of pathway mediators; and (iv) the most important toxicities revealed by clinical studies are hematological and gastrointestinal toxicity. However, while there is some evidence it may be more effective in certain subsets of patients, clinical experience with mTOR inhibition is extremely limited and has not produced convincing result.

Table 3. Important and representative clinical studies of PI3K-Akt-mTOR inhibitors in human AML (including more than 20 AML patients). The table presents important studies of PI3K-Akt-mTOR inhibitors used alone or in combination with other targeted therapies or conventional cytotoxic drugs. For a more detailed presentation of additional studies and a more extensive discussion please see the recent review by Herschbein and Liesveld [189].

STUDY	PATIENTS AND TREATMENT	SUMMARY OF RESULTS	TOXICITY
Rizzieri [180]	A total of 55 patients, including 23 patients with AML and three with other myeloid malignancies. Most patients were heavily pretreated. Deforolimis 12.5 mg intravenous infusion for 5 days every 2 weeks.	No complete or partial responses. Stabilization of the disease for a minority of patients: one AML patient showed normalization of neutrophil counts and three patients' diseases were stabilized for 2-6 weeks. Decreased levels of phosphorylated 4E-BP1 in 9 of 11 leukemia/MDS patients after therapy.	Common treatment-related adverse events, which were generally mild and reversible, were mouth sores, fatigue, nausea, and thrombocytopenia.
Perl [178]	Twenty-nine patients with refractory or relapsed AML. Sirolimus in a 12 mg loading dose on day 1 followed by 4 mg/d on days 2 to 7, in parallel with MEC chemotherapy.	Complete or partial remissions in 6 (22%) of the 27 subjects who completed chemotherapy, including 3 (25%) of the 12 subjects treated at the maximal tolerated dose. Measured rapamycin trough levels within therapeutic range for solid organ transplantation. However, direct measurement of the mTOR target p70 S6 kinase phosphorylation in marrow blasts from these subjects only showed definite target inhibition in one of five samples.	Dose-limiting toxicities were irreversible marrow aplasia and multi organ failure. The maximum tolerated dose of sirolimus; 12 mg loading dose on day 1 followed by 4 mg/d on days 2 to 7, concurrent with MEC chemotherapy. Median time to neutrophil recovery was 32 days.
Park et al [190]	Phase Ib trial including 28 patients below 65 years of age in first relapse (median duration of remission 20 months; median bone marrow blasts 20%). Everolimus in increasing doses from 10 to 70 mg, administrated orally on days 1 and 7 in combination with conventional 3 + 7 daunorubicin + cytarabine induction therapy.	Complete remission in 68% percent of patients. Subsequent intensification with allogeneic stem cell transplantation in 29% of patients. Strong plasma inhibition of P-p70S6K was observed after RAD001 administration, still detectable at d7 (d7) at the 70 mg dosage. Complete remission rates in patients with RAD001 areas under or above the curve median were 53% versus 85%.	Maximal tolerated dose was not reached. Treatment was well tolerated with <10% toxicity, mainly involving the gastrointestinal tract and lungs.
Amadori et al [191]	Fifty-three evaluable patients with primary refractory of first relapse AML (median age 69 years). Clofarabine 20 mg/m^2 on days 1-5 and temsirolimus 25 mg on days 1, 8 and 15. Patients achieving complete remission with or without (incomplete complete remission) full hematological recovery could receive monthly temsirolimus maintenance therapy.	Overall remission rate of 21%: complete remission in 8% and incomplete complete remission in 13%. Median disease-free survival of 3.5 months. Median overall survival of 4 months (9.1 months for responders). Laboratory data from 25 patients demonstrated that a >50% *in vivo* inhibition of S6 ribosomal protein phosphorylation was highly correlated with response rate (75% with inhibition versus 0% without inhibition; P=0·0001).	The most common non-hematological severe adverse events included infection (48%), febrile neutropenia (34%) and transaminitis (11%). Thirty-day mortality was 13%.

2 AIMS OF THE THESIS

The PI3K-Akt-mTOR signaling network shows constitutive activation in human AML. This pathway is important for regulation of cell metabolism, cell cycle progression, gene transcription and translation, cell differentiation and apoptosis. However, AML is heterogenic disease and previous experimental studies suggest that the antileukemic effects of pathway inhibitors differ among patients.

The main aim of this thesis was to map patient heterogeneity based on cell signaling. This included mapping constitutive PI3K-Akt-mTOR signaling within a group of AML patients. The scientific and technological objectives were as follows:

- Study constitutive PI3K-Akt-mTOR signaling with and without activation/stimulation
- Evaluate the effects of pathway inhibitors among subgroups of patients
- Study the metabolic regulation.

3 SUMMARY OF THE RESULTS

3.1 Article I

Two acute myeloid leukemia patient subsets are identified based on the constitutive PI3K-Akt-mTOR signaling of their leukemic cells; a functional, proteomic, and transcriptomic comparison

Authors: Ina Nepstad, Kimberley Joanne Hatfield, Elise Aasebø, Maria Hernandez-Valladares, Annette K. Brenner, Sushma Bartaula-Brevik, Frode Berven, Frode Selheim, Jørn Skavland, Bjørn Tore Gjertsen, Håkon Reikvam, and Øystein Bruserud

Background. The aim of this article was to study the constitutive PI3K-Akt-mTOR activation of primary AML cells for a large group of unselected patients.

Methods. The expression and phosphorylation of 18 mediators in the PI3K-Akt-mTOR main track for AML cells derived from 77 patients was examined by flow cytometry.

We compared global gene expression profiles, proteomic and transcriptomic profiles, and the difference in susceptibility to antileukemic agents among patients.

Results. Patients were separated into one subset showing high constitutive activation and one subset showing low constitutive activation based on their constitutive pathway activation profiles. The high activation subset was characterized by reduced frequency of cells showing monocytic differentiation, increased frequency of adverse karyotypes, decreased constitutive cytokine release, and increased expression of certain integrins.

Conclusion. The two subsets differed in their expression of genes encoding regulators of protein phosphorylation, whereas phosphoproteomic analyses showed differences related to transcriptional regulation. These variations are a part of complex phenotypic differences.

3.2 Article II

Insulin-initiated activation of the PI3K-Akt-mTOR pathway in acute myeloid leukemia cells; a study of patient heterogeneity and pathway inhibitors

Authors: Ina Nepstad, Kimberley Joanne Hatfield, Elise Aasebø, Maria Hernandez-Valladares, Karen Marie Hagen, Kristin Paulsen Rye, Frode Berven, Frode Selheim, Håkon Reikvam and Øystein Bruserud

Background. Constitutive signaling through the PI3K-Akt-mTOR pathway is present in AML cells, and this pathway is considered a possible therapeutic target. Insulin can be a growth factor for AML cells, and we characterized the effect of insulin on PI3K-Akt-mTOR activation.

Methods. We investigated the phosphorylation level of 10 proteins involved in PI3K-Akt-mTOR signaling by flow cytometry for AML cells derived from 76 unselected patients. Patient subsets were compared by global gene expression, and proteomic and phosphoproteomic profiling.

Results. Patients were classified in two main subsets based on the constitutive activation of their AML cells; the overall results indicated insulin significantly increased the phosphorylation of all investigated mediators.

Strong insulin responders were characterized by a specific gene expression profile as well as proteomic and as phosphoproteomic differences with regard to regulators of transcription, RNA metabolism, and protein modification. Even though the overall effects of pathway inhibitors differed between patients, PI3K and Akt inhibition was characterized by a generally strong inhibition of AktpT308 and 4EBP1pT36pT45 whereas mTOR inhibition caused a strong inhibition of mTORpS2448 and S6pS244.

Conclusion. Insulin modulates PI3K-Akt-mTOR signaling in primary human AML cells, but the insulin effect differs between patient subsets, which can be identified through their mRNA or proteomic profiles. The effects of pathway inhibitors on activation differs between patients and depends on the molecular target of the inhibitor.

3.3 Article III

Resistance to the antiproliferative *in vitro* effect of PI3K-Akt-mTOR Inhibition in primary human acute myeloid leukemia cells is associated with altered cell metabolism

Authors: Ina Nepstad, Håkon Reikvam, Annette K. Brenner, Øystein Bruserud, Kimberley J. Hatfield

Background. The PI3K-Akt-mTOR pathway plays a central role in the regulation of proliferation, differentiation, and survival of hematopoietic cells, and constitutive signaling through this pathway has been observed in AML cells. This pathway is a possible therapeutic target in human AML and we therefore investigated possible associations between cellular metabolism and sensitivity to PI3K-Akt-mTOR pathway inhibitors, in relation to heterogeneity of the disease.

Methods. A non-targeted metabolite profiling was performed to compare the metabolome variances of primary human AML cells derived from patients susceptible or resistant to the *in vitro* antiproliferative effects of inhibitors to the pathway. Additionally, using flow cytometry, we investigated the phosphorylation profile of 18 proteins involved in PI3K-Akt-mTOR signaling, together with the effect of the cyclooxygenase inhibitor indomethacin on their phosphorylation status.

Results. Inhibitors of the PI3K-Akt-mTOR pathway have antiproliferative effects on leukemia cells for only a subset of patients. We compared the metabolite profiles of AML cells defined as either susceptible to or resistant to *in vitro* treatment with pathway inhibitors and found 627 metabolites could be detected. Non-responders showed increased levels of metabolites reflecting energy metabolism (citric acid, isocitric acid), amino acid metabolism (proline, aspartic acid, glutamine, taurine), and arachidonic acid metabolism (4,7,10,13-eicosateraenoic acid, 4,7,10,13,16-doocosapentaenoic acid). Decision tree analysis showed that the two patient groups could be identified based on the levels of cysteinyl-cysteine and threonic acid. Furthermore, the cyclooxygenase inhibitor indomethacin altered the phosphorylation of mTOR and its downstream mediators.

Conclusion. Differences were found in leukemia cells that are susceptible or resistant to PI3K-Akt-mTOR inhibitors in energy, amino acid, and arachidonic acid metabolism. Results shows that modulation of arachidonic acid metabolism alters the activation of mTOR and its downstream mediators.

3.4 Article IV

Clonal heterogeneity reflected by PI3K-Akt-mTOR signaling in human acute myeloid leukemia cells and its association with adverse prognosis

Authors: Ina Nepstad, Kimberley Joanne Hatfield, Tor Henrik Anderson Tvedt, Håkon Reikvam and Øystein Bruserud

Background. Clonal heterogeneity is seen for a subset of AML patients, and detection of separate subclones based on karyotyping is associated with adverse prognoses. However, the use of another methodological strategy is required for a more general evaluation of the prognostic impact of clonal heterogeneity in human AML. Constitutive activation of the PI3K-Akt-mTOR pathway is present in AML cells, and this pathway is considered a possible therapeutic target in human AML. However, the degree of pathway activation varies between patients. In this study, we suggest that this pathway reflects biologically important clonal heterogeneity.

Methods and Results. We investigated constitutive PI3K-Akt-mTOR pathway activation in primary human AML cells derived from 114 patients, together with 18 mediators central to the signaling in this pathway. The cohort included patients with normal karyotype or single karyotype abnormalities, with an expected heterogeneity regarding molecular genetic abnormalities. Clonal heterogeneity reflected as pathway mediator activation was detected for 39 patients, and the study shows that primary AML cells derived from patients with and without dual PI3K-Akt-mTOR cell copulations have differences in their global gene expression profiles. Finally, detection of AML subclones based on PI3K-Akt-mTOR signaling was associated with adverse prognosis for patients receiving intensive antileukemic treatment.

Conclusion. Clonal heterogeneity, as shown in the activation status of selected mediators in the PI3K-Akt-mTOR pathway, was related to differences in gene expression profiles and had an independent prognostic impact. This biological heterogeneity reflected in the intracellular signaling status should be further investigated as a potential prognostic biomarker in human AML.

4 METHODOLOGICAL CONSIDERATIONS

The following section describes and elucidates the main methods and techniques used during the thesis.

4.1 Selection of patients

By definition, AML involves leukemic blast counts of 20% or more in the bone marrow or peripheral blood [13]. In addition, AML can be defined by the presence of an extra medullary tissue infiltrate or documented t(8;21), inv(16), or t(15;17) in the appropriate clinical setting, regardless of blast percentage [192]. In our studies, AML blasts derived from the peripheral blood of patients with high peripheral blood blast count was used. As will be discussed in detail below, our results may be relevant for AML in general despite this selection, *but they should be interpreted carefully and may only be representative for the selected patient subset.*

We investigated primary human AML cells derived from patients with high, relative, or absolute levels of circulating AML cells in the peripheral blood. This methodological approach was used to allow preparation of highly enriched AML cells by density gradient separation alone, thereby avoiding the induction of functional alterations in the leukemic cells - a potential problem that has been discussed in detail previously [32, 193]. Furthermore, our previous studies showed that patient cohorts are reasonably representative for AML in general with regard to cytogenetic analysis and *FLT3* abnormalities, i.e. the most important genetic abnormalities related to prognostication and *in vivo* chemosensitivity [81]. Peripheral blood cells were also shown to be representative with regard to the corresponding bone marrow AML cells; even though quantitative differences may be seen, the major characteristics of the leukemic cells are maintained [194, 195]. Finally, the peripheral blood cell count is a weak prognostic parameter in human AML [196], and patients selected based on their peripheral blood extravasation are likely to be relevant for AML in general with regard to clinically relevant chemosensitivity.

Even though remaining immature leukemic and pre-leukemic stem cells are thought to be responsible for AML relapse, several previous reports suggest that functional characteristics of the total AML cell population are important markers for chemosensitivity and thereby reflect fundamental common characteristics between the more immature minority of AML cells and the majority of the hierarchically organized AML cell population:

- Genetic abnormalities are common for the cells within the hierarchically organized AML cell population genetics [1].
- Chemoresistant relapse is the most frequent cause of death in AML; treatment-related mortality is usually low for younger patients receiving intensive chemotherapy alone, while non-relapse mortality is higher for patients treated with allogeneic stem cell transplantation [23, 197]. The prognostic impact of global mRNA profiles as well as selected mRNA profiles (e.g. leukemic stem cell profiles) [31, 198], epigenetic regulation [199], single molecular markers detected at the mRNA [200] or protein level [34, 201], and constitutive cytokine release profiles

[202] has been demonstrated through analysis of the total AML cell population. These observations illustrate that the common biological characteristics of the overall AML cell populations are correlated with the chemosensitivity of the disease and the risk of chemoresistant relapse. In our opinion, studies using the total leukemia cell population should therefore be regarded as relevant.

- *In vivo* evaluation through observation of bone marrow blast counts 17 days after the start of induction therapy, as well as the use of minimal residual disease (MRD) estimation by flow cytometry during consolidation therapy, is also based on evaluation of common AML cell characteristics [202].

Taken together, these observations suggest that the biological characteristics of the total AML cell population reflect clinically relevant chemosensitivity in primary human AML cells.

4.2 Cell preparation and cryopreservation

Density gradient centrifugation was used for the isolation of AML cells for all patients, and highly enriched blast populations were prepared [32]. Most contaminating cells were small lymphocytes. In our opinion, this small degree of contamination was unlikely to have any major impact on our results; and in our experience, normal peripheral blood mononuclear cells cultured in medium alone without any specific activation signal do not show detectable proliferation or detectable cytokine release.

Our methods for cryopreservation were highly standardized, and the same methodology was used for all samples in our biobank [203]. Even though more recent studies of mobilized stem cell grafts in human peripheral blood suggests that cells can be cryopreserved by using 5% dimethyl sulphoxide [204, 205], all our samples were preserved with 10% dimethyl sulphoxide and a standard concentration of inactivated fetal calf serum.

The advantage of using cryopreserved cells instead of freshly prepared cells is the reduction in variations between analysis of different patients in different experiments that results from the ability to reproduce results and examine different patients in

parallel. The disadvantage is the reduction in the viability of the AML cell population though the freezing and thawing processes.

4.3 Sample storage time

We did not evaluate the possible influence of storage time on the protein levels and phosphorylation levels of the mediators in the PI3K-Akt-mTOR pathway. For this reason, we compared the relative storage times for samples with high and low pathway activation as identified in our study of constitutive pathway activation (Figure 1, article I). Patients with low constitutive PI3K-Akt-mTOR activation had an average storage time of 6.5 years whereas the patients with high activation had an average storage time of 6.0 years; this difference was not statistically significant. Thus, the storage time of cryopreserved samples is not considered to have any major impact on our analysis of constitutive pathway activation.

4.4 Assay for the detection of apoptosis and the proliferation assays

We used a standardized flow cytometric assay for the detection of apoptosis and the estimation of viable cells. 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 [206]. We estimated the cell viability after one or two days, and our studies of cell viability thus reflect the characteristics of the major subset of more mature cells in the hierarchically organized AML cell population.

For article I, II, and the pilot study of article III, we evaluated AML cell proliferation after seven days of *in vitro* culture using a ^{3}H-thymidine assay. The isotope was added after six more days of culture, and ^{3}H-thymidine incorporation was estimated on the following day. Thus, this proliferation analysis reflects the characteristics of a minor subset of cells that survive for at least six days and still are able to proliferate. The different time points for evaluation of apoptosis/viability and proliferation may thus reflect the characteristics of different AML cell subsets.

4.5 Flow cytometry as a tool for the investigation of PI3K-Akt-mTOR signaling in AML

We used flow cytometry, a laser-based analysis technique, for the study, characterization, and detection of the cells. A flow cytometer performs simultaneous and rapid quantitative measurements of numerous physical and chemical characteristics. The basic principle behind the method is the passage of particles in single file in front of a laser, which detects, counts, and sorts them. The cells are fluorescently labelled using antibodies and are excited by the laser though the emission of light at different wavelengths. The fluorescence can then be measured to determine the type and quantity of cells present in the given sample.

Prior to measurement, particles in suspension are hydrodynamically focused to maintain proper alignment and separation of the cells within the fluid stream. The stream is run through one or more lasers, resulting in the detection of florescent and scattered light by photomultiplier tubes (PMT) (Figure 7). The signal is then amplified and converted to a voltage pulse and finally transformed to a digital value. While the run of each sample includes measurements of a large number of cells, the strength of the method is that each cell is measured by itself, allowing for the identification and grouping of individual cells. The technique uses fluorescent reagents, usually monoclonal antibodies, for the identification and stratification of cells.

Fluorochromes are excited at a specific wavelength of light provided by the laser and emitted at a lower specific wavelength. Emitted light of a specific wavelength is distinguished by the use of suitable optical filters prior to the detection of the emitted light. This increases the number of parameters that can be detected.

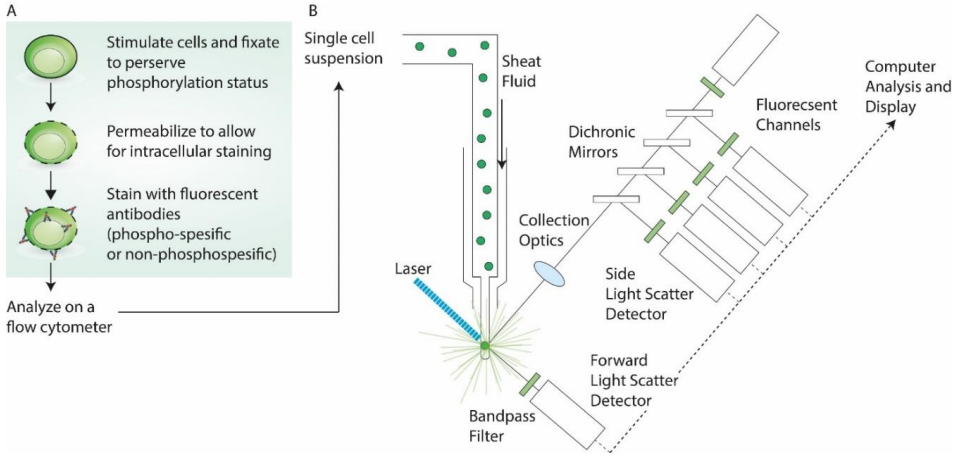

Figure 7. Schematic of the basic steps of flow cytometry. A) A heterogeneous cell sample can be stimulated to prompt specific signaling cascades and phosphorylation of the proteins of interest. The cells are fixed, permeabilized, and stained with fluorophore-conjugated for identification of cell type and mediator of interest. The cells are then analyzed on a flow cytometer. B) A cell suspension is hydrodynamically focused to intersect with a laser. The signals from the laser are collected by a forward light scatter detector, a side light scatter detector, as well as various detectors for fluorescence emissions. Finally, the signals are amplified and transformed into digital data for further analysis.

4.6 Intracellular flow cytometry

The main method used in these studies is flow cytometric detection of total protein and protein phosphorylation, and all experiments (article I-IV) were performed on a FACS Verse flow cytometer. Flow cytometry offers several benefits for studies investigating cell signaling based on its ability to assemble data at the individual cell level. Detection of intracellular signaling responses mediated through protein phosphorylation for defined cell subsets within a complex cell population can be accomplished through the combined use of fluorescent subset-identifying antibodies (e.g. detection of specific cell surface molecules) and antibodies specific to protein phosphorylation sites.

The processes that occurs within a cell are important for many aspects of cell biology and medicine. The understanding of the function of normal cell metabolism, cell response to environmental factors, and eventually, cell aging and cell death, can provide insight into how diseases develop and functions. More importantly, knowledge of how

and when these normal processes malfunction is essential for the understanding of disease and the development of more effective treatments and cures. The ideal method of analysis would be to study and obtain information from living cells, without the need for labels. Instead, cell fixation is often used to allow a snapshot of a cell state to be measured. To detect specific phosphorylated proteins using flow cytometry, cells have to be fixed to preserve the phosphorylated state of the signaling proteins, and then the cell membrane must be permeabilized to allow antibodies to enter the cells and bind to their target epitopes.

Aldehydes, including paraformaldehyde, function as cross-linking agents that react with proteins and nucleic acids within the cell, and are widely used in fixation. They provide good preservation of the cellular structure and their use does not appear to result in significant structural changes to the proteins.

Following paraformaldehyde, methanol was used for permeabilization in all our studies (article I-IV); and cells were then stained with phosphospecific antibodies. Organic solvents, such as methanol, acetone, and ethanol preserve both cell morphology and nucleic acid content [207, 208]. Moreover, these fixatives have been shown to be more effective for the preservation of nucleic acid content in cells than aldehyde-based fixatives [208]. Methanol combined with low non-specific staining has the ability to increase the reactivity of antibodies to certain nuclear antigens [209], and cold methanol is often used as a permeabilization agent when flow cytometry is used to detect phosphorylated proteins and transcription factors. However, one of the potential problems with methanol is the denaturation of surface receptors [207, 208]. We tested our surface markers for studies I-IV before and after treatment with methanol and detected the same markers at both times.

Protein phosphorylation is temporary and is regulated by protein phosphatases. Most methods for detection of protein phosphorylation require prompt inactivation of phosphatases immediately following stimulation. All cell samples were therefore swiftly fixed to sustain phosphorylation. In order to avoid unspecific antibodies binding to cells, the antibodies must be titrated. The degree to which this is a problem may differ between different antibodies that are specific for the same antigenic epitope. We used the same

well-characterized antibodies throughout all of our studies to avoid the problem of variation in nonspecific binding between different experiments. During titration testing, we also found that the most effective concentration of antibodies for a staining mixture was essentially independent from the number of cells being stained but strongly dependent on stain volume. Hence, the same stain volume was used in all experiments.

The normal level of cell fluorescence, referred to as autofluorescence, may present an issue for data analysis of flow cytometry. Mammalian cells contain many compounds that are excited by the 488 nm laser that is commonly used in flow cytometry. Hence, the signal to noise ratio is reduced resulting in a decreased sensitivity and more false-positive cells. Spillover refers to the overlap among the emission spectra of certain commonly used fluorochromes, and spillover from one channel to another may mask low expressers. This can be corrected using unstained controls, and fluorophores that emit above 600 nm will have less autofluorescence interference. Fluorophore brightness is a relative indication of the intensity of a fluorophore, measured above the background (dim=1 to bright=5). The use of a bright fluorophore will diminish the effect of autofluorescence. In addition, we stained cells separately with each antibody as a compensation control.

Cell signaling studies often investigate cellular responses to treatment with stimulatory or inhibitory molecules, making unstimulated or untreated cells the best controls for evaluation of background staining. In the past, isotype controls were widely used to correct for unspecific binding of antibodies. However, an unstimulated cell control considers the background characteristics of each antibody as well as the basal phosphoprotein expression levels within the cells of interest, which is different from an immunoglobulin isotype control. For this reason, we included three unstimulated cell controls for all flow cytometric assays in articles I-IV. For stimulation studies, we also included unstained controls with each stimulus or inhibitor, as well as stimulus plus inhibitor controls.

4.7 Selection of antibodies

All antibodies used during the course of the thesis were titrated to provide the best staining for phenotypic and phosphoantigens. For articles I-IV, antibodies to phosphoantigens were titrated using unstimulated samples, and for articles II-IV, we did an additional titration with stimulated samples to determine the concentration necessary to maximize fold change. Final concentrations were selected based on the point at which small changes in antibody concentrations caused little to no fold change. Additionally, during titration studies, we tested the effect of media conditions. We found that storage time of RPMI-1640 media affected the activation of some substrates. Hence, we always used fresh media for the studies to minimize unwanted effects, thereby minimizing irregularities in the results.

We chose the fluorophores for this antibody panel based on the wavelength of available lasers and filters on the flow cytometer. The expression density of an antigen is also important in the selection of fluorophores. Some cellular antigens have a high expression density and can be combined with a variety of fluorophores with both high and low brightness, whereas others have a low expression density and should be combined with a bright fluorophore. We tested different fluorophores to determine which was best suited to differentiate between positive and negative populations. Selections were also based on the ability of the fluorophore to minimize spillover into central and sensitive channels to diminish the need for compensation and subsequent introduction of error.

The selection of antibodies used for the identification of the correct cells was based on general knowledge of AML surface markers. Antibodies used in the analysis of intracellular cell signaling pathways were chosen to give the best coverage of the main track of the PI3K-Akt-mTOR signaling pathway. However, PI3K signaling is extremely complex, and there is a wide variety of available antibodies. For practical reasons, it was not possible to include all mediators that at some level influence or crosstalk with the main track PI3K-Akt-mTOR in this network. Our intention was to describe patient heterogeneity with regard to the activation status of the PI3K-Akt-mTOR pathway and to elucidate whether patient heterogeneity in this axis may be an explanation for why pathway inhibitors (i.e. mainly mTOR inhibitors) do not show clinically relevant

antileukemic activity in many patients. Due to the complexity of this signaling network, it will always be possible to criticize a selection and to suggest inclusion of additional mediators. However, for our purpose, we feel that the main substrates are included.

4.8 Bioinformatic approaches

Initial visualization and analysis of flow cytometry data was achieved with FlowJo (Tree Star) (articles I-IV). Identification of immune cell populations was based on light scatter properties or relative expression of CD markers. In articles I-IV, the mean fluorescence intensity (MFI) was recorded in channels used to measure phosphospecific antibodies.

Unsupervised hierarchical clustering was used for identification of subgroups within patients in articles I-IV (not included in article IV). With hierarchical clustering analysis we aim to build a hierarchy of clusters where objects (in this thesis; AML patients) that are more similar cluster closer together, and more dissimilar objects are dispersed farther apart. Dissimilarities between clusters of objects can be defined in several ways: maximum dissimilarity (complete linkage), minimum dissimilarity (single linkage), or average dissimilarity (average linkage). Clustering categorizes big datasets into minor groups of similar or comparable entities, presenting data in a manageable format for further elucidation. However, hierarchical clustering analyses should be interpreted with care. The results for clustering analyses in articles I-III are dependent on the specific patients and mediators/effectors used in the study. The main differences that appear by changing the mediators and/or patients used for the cluster analysis, is that the order of patients in the cluster may be altered, while the trend with two signaling subsets is still present. One should therefor use at this type of analysis to generate an overview of the dataset and arrange objects into relatively homogeneous groups based on multivariate observations rather than for specific analysis at the patient level. For all articles, hierarchical clustering analysis was performed with the J-Express 2012 software (MolMine AS, Bergen, Norway).

5 GENERAL DISCUSSION

AML is an aggressive malignancy characterized by a proliferation of immature myeloid leukemia cells [1, 2]. The main aim of this thesis was to describe the heterogeneity of AML patients by studying PI3K-Akt-mTOR signaling. We analyzed the same flow cytometric parameters in all four articles (I-IV) included in this thesis, and these mediators represent the main steps in the PI3K-Akt-mTOR pathway. The panel was selected to provide an overview of the signaling/phosphorylation status of this pathway, and by studying the same panel in all four articles, we were able to make an overview of the relationship between the pathway and AML patient heterogeneity, and thus evaluate if such signaling profiles could be correlated to clinical diagnosis or treatment.

Due to the complexity of PI3K signaling, which is studied in all four articles, several key mediators should be included in this kind of study. For practical reasons, it was not possible to include all mediators that, at some level, influence or crosstalk with the main track PI3K-Akt-mTOR in this network. We selected a panel of 18 mediators (see article I-IV), and we do not argue that this selection is correct but rather that it is relevant; the identification of PTEN as total and as phosphorylated/inactive proteins (S380) is of fundamental importance because of its role in PI3K signaling regulation. However, as PTEN mutations have been shown to occur in less than 1% of AML patients, this mediator is less important in AML than in many other malignancies. As PDK1 is located between PI3K and Akt in the main track of the pathway, the activity of PTEN is reflected in the immediate downstream phosphorylation of PDK1 [210].

In addition, GSK3 could constitute an important mediator, as analysis of GSK3 reveals whether PI3K signaling affects cell metabolism. In our opinion, for article I, this complex acts more like branch from the main track than an integral part of the main track.

In retrospect, it would have been beneficial to include this or other mediators in our studies on insulin and metabolism. However, the effect of many of these mediators is partly reflected in our analysis through mediators related to Akt and mTOR [211].

5.1 Patients included in the various studies

The various studies included patients with high peripheral blood cell counts or high percentages of leukemic blasts among circulating leukocytes. This was the only criteria for selection, and the patient populations consist of consecutive patients fulfilling this criterion, as discussed above. In a previous study, we compared patients with increased peripheral blood blasts with other AML patients without this degree of extravasation over a defined period of time [81]. This comparison showed negligible differences in the frequency of *FLT3* mutations, with regard to cytogenetic abnormalities. Thus, the patients do not differ with regard to the most important biological characteristics associated with risk of relapse and clinical chemosensitivity. However, the degree of extravasation is also associated with an adverse prognosis [196], though this impact is much weaker. A final important prognostic parameter for chemosensitivity is whether complete hematological remission is achieved after the first induction cycle [196]. This parameter was not considered in our present studies because we included consecutive patients with a high median age, and as many of these patients did not receive intensive chemotherapy, this parameter is not available for these patients. Thus, in the present study we generally investigated associations with biological characteristics for which data were available for most or all patients: cytogenetics, *FLT3* and *NPM1* mutation, and differentiation.

An extended mutational analysis was only available for a subset of patients; some of these mutations are now regarded as established prognostic parameters and should be included in routine clinical practice [1]. As a result, the prognostic classification of some of our patients might have changed had these data been available for all patients. However, as most of these mutations occur infrequently, additional mutational analyses might change the prognostic classification for only a relatively small number of patients.

Because we included consecutive patients admitted to our hospital with diagnoses of AML, patients with both first diagnoses and relapsed leukemia are included in our studies. The median age at the time of first diagnosis of AML is 65-70 years, and this was the median age for the patients included in the present studies [1]. The study

population included a relatively low percentage of patients with favorable cytogenetic abnormalities, compared to percentages typically seen in clinical studies of AML, because these abnormalities occur more frequently in younger AML patients; many clinical studies include intensive leukemic therapy, which requires the inclusion of patients below 60 or 65 years of age. Thus, our patient populations are representative for AML in general, but they should be interpreted carefully as they may not be representative for patients without extravasation or for subsets of younger patients.

5.2 Strategies for therapeutic targeting of the PI3K-Akt-mTOR pathway

In article I, the focus of our study was to characterize the activation profiles, which included the mediators of the main steps of the PI3K-Akt-mTOR signaling network, for a large group of AML patients. By investigating a large group of patients, we could describe patient heterogeneity. Results show that alterations in constitutive PI3K-Akt-mTOR signaling are part of more complex genetic, as well as phenotypic, differences in AML cells, including constitutive cytokine release and proteomic and transcriptomic differences. In accordance with our clustering analysis, patients could be classified into two main subsets based on the described activation profile of the main track in this pathway/network. As this was our primary observation, we continued our study as a comparison of these two main subsets. Our initial intention was not to create a yes/no classification system, but as the analysis of our data showed that patients could be classified in this way, we regarded the comparison of these two subsets as important for the characterization of patient heterogeneity. In addition, the same findings were observed when examining other patient groups with the same antibody panel (article II-IV). Simultaneously, the subsets also differed with regard to cell differentiation, but the association between differentiation and protein phosphorylation was relatively weak. In article I, the high phosphorylation group included an increased frequency of patients with adverse cytogenetic abnormalities, while frequencies of *FLT3* or *NPM1* mutations were not significantly different from those of the low phosphorylation group. However, in articles II-IV, only a minor increase in frequency of patients with adverse cytogenetic

abnormalities was found in the subsets with high phosphorylation. Studies have shown that isocitrate dehydrogenase (IDH) mutations can upregulate Akt-mTOR expression [212, 213]. However, only a minority of the patients included in our four articles show detectable levels of *IDH* mutations, and therefore this factor cannot explain the overall activation/expression pattern.

5.3 Constitutive versus insulin-dependent pathway activation

Insulin regulates cellular proliferation, survival, and metabolism by binding to its receptor, with PI3K-Akt-mTOR as one of many downstream pathways. PI3K-Akt-mTOR is closely linked to insulin signaling through its insulin substrate receptor (IRS1), which can phosphorylate PI3K directly, thereby regulating the PI3K-Akt-mTOR pathway according to insulin levels. In contrast, constitutive activation of the pathway has been shown to inhibit the activity of IRS1 through a negative feedback loop caused by the activation of mTOR and p70S6K, resulting in phosphorylation of IRS1 [183].

PI3K, Akt and mTOR are important regulators of the PI3K-Akt-mTOR pathway and are able to sense both external and internal signals to control cellular growth and survival. For the adjustment of both uptake of glucose into muscle and fat, and gluconeogenesis in the liver, insulin serves as a main controller of blood glucose levels. Previous studies show that, for some subsets of patients, insulin has an enhancing effect of growth for primary human AML cells [214], and article III shows that the cellular metabolism differs between patients that are susceptible and unsusceptible to the antiproliferative effect of pathway inhibitors. In article IV, we characterized the heterogeneity of human AML based on the effects of insulin, both alone and combined with various pathway inhibitors (PI3K, Akt and mTOR inhibition), and the effect of PI3K-Akt-mTOR pathway activation in primary human AML cells. Results from articles II, III, and IV show that the heterogeneity found among AML patients is preserved after the activating effect of stimulation by insulin on PI3K-Akt-mTOR signaling in their leukemic cells as well as the effects of various inhibitors on the pathway activation. However, the general activation of the pathway increased significantly following insulin stimulation, and inhibition of the pathway did not reduce signaling to the levels seen in unstimulated

samples. The purpose of this study was not to investigate the inhibitors, but rather to study the heterogeneity of the patients in relation to the pathway. However, inhibitors that target mTOR will block the activity of mTORC2/Akt and hence further impair insulin insensitivity. Therefore, further targeting of mTOR complex could provide valuable information.

5.4 Proline, energy, and arachidonic acid metabolism in malign cancers

Previous experimental studies have proposed a large variation in the antileukemic effects of pathway inhibitors among patients. The aim of study III was to further characterize this patient heterogeneity in relation to the constitutive activation/signaling of the PI3K-Akt-mTOR pathway and study the effect of pathway inhibition and metabolic regulation. The observations described in article III suggest that primary AML cells derived from patients with high/low activation are heterogeneous with regard to their metabolic profile, and differences in the glutamine/proline/arachidonic acid metabolism are part of this heterogeneity.

Proline and glutamine are nonessential amino acids that have a close metabolic association, but dissimilar physiologic purposes (See Figure 8) [215, 216]. Glutamine is a central signaling molecule, and like many other amino acids, it can activate mTOR [217]. In addition, it is the main source of endogenous proline, and during normal metabolic regulation, proline is incorporated into collagen [215, 216]. As collagen is the most abundant protein in the body, this stored pool of proline serves as an important source of amino acids and energy in conditions of metabolic stress.

Arachidonic acid is a polyunsaturated fatty acid that is central to the processes of cell metabolism, especially the synthesis of prostanoids and leukotrienes, and the metabolism of arachidonic acid is important for the survival and proliferation of hematopoietic cells [218-220]. Our observations, described in article III, suggest that this may be true also for leukemic hematopoiesis. However, many questions remain with regard to our understanding of PI3K-Akt-mTOR signaling in malignant cells and its role in the modulation of cancer metabolism, including energy metabolism. Additional

studies are needed to further characterize AML cell metabolism, the metabolic heterogeneity of AML patients, and the possibility of metabolic targeting as a part of future AML therapies [221].

Amino acid metabolism is important for the energy metabolism of cells [215, 216]. However, previous studies show that oncogenic stimulation or activation of the PI3K-Akt-mTOR signaling network stimulates post-translational regulation of glucose transporter movement to the plasma membrane; this represents an alternative mechanism for modulation of energy metabolism [222, 223]. This effect can be further strengthened through transcriptional upregulation of glucose transporter genes [224]. The combined effect of such mechanisms may provide strong stimulation of glucose uptake by cancer cells, thereby providing the amount of substrate necessary for aerobic glycolysis.

Although the mechanisms that regulate metabolism through PI3K-Akt-mTOR signaling need to be studied in greater depth, our analysis suggest that the metabolic differences observed in cancer cells do not occur as isolated effects but as part of more complex biological phenotypes that are established by oncogenic signaling. The uptake and consumption of nutrition represent coordinated events that support the survival, growth, and proliferation of the malignant cells. The constitutive activation of PI3K-Akt-mTOR signaling may be caused by or may contribute to the metabolic signature of the AML cells.

The proteomic studies in article II suggest that a subset of patients has a leukemic cell phenotype characterized by a general PI3K-Akt-mTOR activation/phosphorylation in response to insulin and a difference in several proteins that regulate cell metabolism; at the same time, these cells are characterized by low constitutive PI3K-Akt-mTOR activation and increased susceptibility to the antiproliferative effects of pathway inhibitors. Article III also describes different metabolomic profiles for AML cells that are susceptible to the antiproliferative effects of pathway inhibitors, and these two patient subsets especially differ with regard to glutamine/proline metabolism.

The interrelatedness of glutamine-glutamate-proline is well recognized [215, 216]. The free pool of proline is limited, but protein-bound proline can be released extracellularly, and collagen can then function as a proline reservoir [216]. Proline can be synthesized from glutamine/glutamate and from ornithine by ornithine aminotransferase [216]. Furthermore, the conversion of pyrroline-5-carboxylate (P5C) by P5C reductase to proline alters the redox balance in the cells and releases nicotinamide adenine dinucleotide (NAD+).

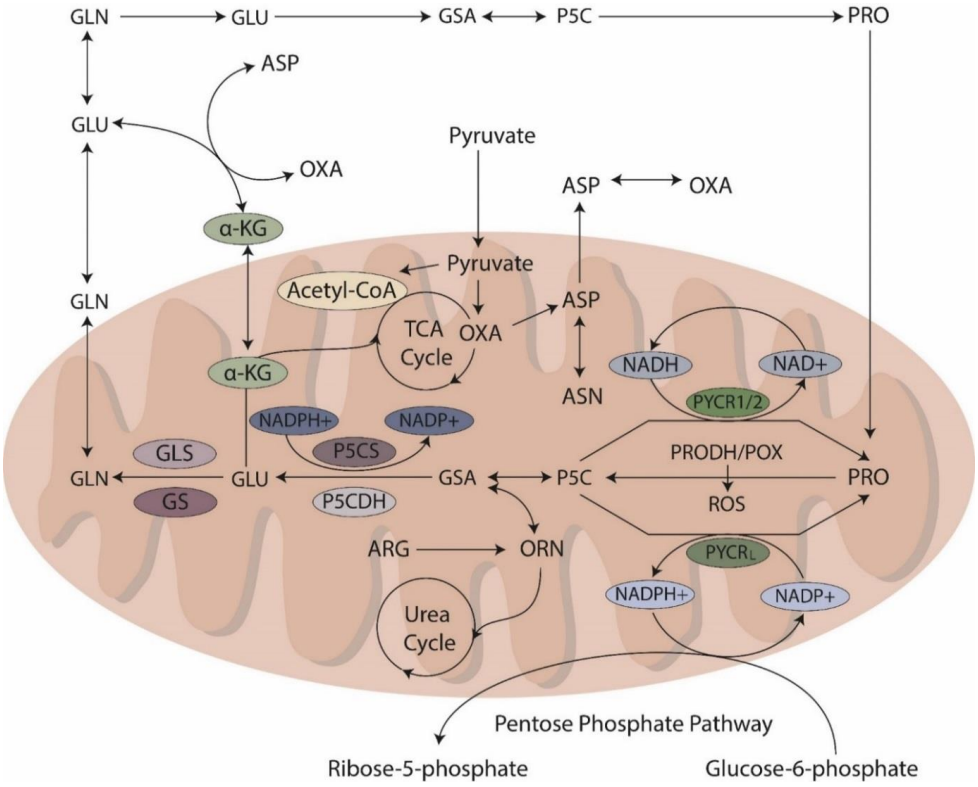

Figure 8. Proposed schematic of the proline metabolic pathway. Proline metabolism, or the proline cycle, is closely linked to the TCA cycle, the urea cycle, and the pentose phosphate pathway, and acts as a redox transport that transfers redox potential generated by the pentose phosphate pathway into the mitochondria for the production of ROS. The oxidation of proline to glutamate occurs in the mitochondria in two enzymatic steps, with the use of P5C which is the common intermediate for both biosynthesis and catabolism of proline. P5C is synthesized in mitochondria through the catabolism of proline by the enzyme PRODH. The abbreviations shown in the figure can be found in the list of abbreviations. Some of the pathways in this presentation are simplified, and a single arrow may represent multiple steps.

Recent studies have described the poor cell permeability of aspartate, and cancer cells may lack asparaginase, making them able to convert asparagine to aspartate [225]. Aspartate can function as an endogenous metabolic limitation for malignant cell proliferation; thus, cancers that express asparaginase may grow faster [225]. Aspartate has also been shown to support cellular redox homeostasis in addition to enhancing growth [226]. However, the importance of cytosolic aspartate in carcinogenesis may depend on the metabolic context because its antiapoptotic effect is most evident in low-glutamine environments [226]. Though glutamine is a non-essential amino acid, malignant cells may consume glutamine in excess of other amino acids and thereby become dependent on extracellular glutamine for survival; malignant cells may also be dependent on serine [221]. Proline, glutamine, aspartate, and serine are functionally linked to one another, and they may serve as regulators of cellular proliferation and survival. Contribution to the cellular redox homeostasis is a characteristic they hold in common [221]. Furthermore, aspartate is linked to proline metabolism through the urea cycle and ornithine metabolism [221].

It is not known whether the molecular mechanisms outlined above are operative in primary human AML cells or whether they are responsible for the resistance/susceptibility of primary AML cells to the antiproliferative effects of PI3K-Akt-mTOR inhibitors. However, maintenance of NAD+ is important for protein deacetylation, ADP ribosylation, calcium signaling, generation of oxidized molecules necessary for biomass oxidation, support of anabolic reactions, and regulation of proliferation [227-231]. Thus, our hypothesis is that differences in proline/glutamine metabolism are important factors for chemosensitivity in AML through different mechanisms including their contribution in energy metabolism (i.e. the TCA cycle); their direct effects on redox homeostasis; and their indirect effects on the redox homeostasis, amino acid metabolism (aspartate, glutamine, serine) and ornithine metabolism in the urea cycle.

5.5 Clonal heterogeneity in human AML – a prognostic parameter?

Clonal heterogeneity has been detected in several malignancies, and it is regarded as a driving mechanism of disease development and progression because a high degree of genetic variability is associated with an increased risk for development of subclones with a proliferative advantage, leading to clonal expansion [232, 233]. Clonal heterogeneity is also associated with disease that is more aggressive; this association is shown at the molecular level and is true for the pre-leukemic myelodysplastic syndrome [234]. Acute lymphoblastic leukemia (ALL) is also a hematological bone marrow disease characterized by infiltration of morphologically immature and undifferentiated hematopoietic cells; these characteristics are similar to those of AML.

In pediatric ALL, the original clone often gives rise to several subclones, and some of these subclones lead to further subclonal diversification [235]. A recent publication investigating karyotypic clonal heterogeneity suggests that such heterogeneity is associated with adverse prognoses [236]. The authors concluded that the detection of subclones by karyotyping adds prognostic impact, especially in the cytogenetic adverse risk group. They could not detect any prognostic impact in patients with intermediate or core binding–factor AML, which, for this last group, is in line with previous observations that additional cytogenetic abnormalities do not have any additional negative prognostic impact [237]. The present study is too small to allow analysis of patient subsets, but only a minority of our patients had an adverse karyotype, and our results suggest that detection of clonal heterogeneity has a prognostic impact for other patient subsets, not only those with adverse cytogenetic abnormalities.

5.6 Chemosensitivity in patients with different pathway activation

The experience from AML patients treated with BCL-2 inhibitors suggest that high BCL-2 expression is associated with high susceptibility to BCL-2 inhibitors; this observation suggests that AML cells with high BCL-2 levels are highly dependent on this mediator and thereby have an increased susceptibility to and low expression of the half maximal inhibitory concentration (IC50) [238, 239]. However, there are exceptional patients, suggesting that effective antileukemic effects can be detected at relatively low

BCL-2 levels; this may indicate that for these patients, the low levels allow effective inhibition at low drug concentrations. Taken together, these observations suggest that effective antileukemic activity at low concentrations is due to either high dependency on the targeted mediator or low expression levels that allow therapeutic effects at low drug concentrations.

The antiproliferative *in vitro* effect of PI3K-Akt-mTOR inhibitors differs between patients; our *in vitro* studies suggest that low constitutive activity is associated with an increased antiproliferative effect, but several exceptional patients were seen (article II and III). Thus, both mechanisms for significant antileukemic effects may exist for these pathway inhibitors as they do for the BCL-2 inhibitors described previously. Low constitutive activation may facilitate pathway activation below a critical level, but exceptional patients clearly illustrate this is not the only factor that determines therapeutic effect.

6 CONCLUDING REMARKS

In all four articles, we were able to show that AML patients display different constitutive activation profiles for the PI3K-Akt-mTOR pathway. These dissimilarities are part of more complex phenotypic variations, including transcriptional regulation, cross signaling, and cell communication. We examined large groups of patients in each study and were able to generate an overview of the heterogeneity of AML patients based on the PI3K-Akt-mTOR signaling pathway. By stimulation of leukemic cells with insulin, we showed that AML patients are heterogeneous with regard to the effect of insulin on PI3K-Akt-mTOR pathway activation, as well as the effects of various inhibitors on pathway activation. In the metabolomic studies, we showed AML patient heterogeneity relative to PI3K-Akt-mTOR signaling by examining susceptibility or resistance to PI3K-Akt-mTOR inhibitors and showing that leukemic cells differ in energy, amino acid, and arachidonic acid metabolism. The results also showed that modulation of arachidonic acid metabolism alters the activation of mTOR and its downstream mediators. Finally, we studied the clonal heterogeneity in leukemic cells from AML

patients revealed by the activation status of selected components in the PI3K-Akt-mTOR pathway and found that it was associated with different gene expression profiles and an independent prognostic impact.

7 FUTURE PERSPECTIVES

Our present studies support previous observations suggesting that the PI3K-Akt-mTOR pathway is important for disease development and chemosensitivity in AML. They also show that patients are heterogeneous with regard to pathway activation profiles. Though the results from the initial clinical studies using PI3K/mTOR inhibitors are regarded as disappointing by many clinicians [189], one should remember that results in relapsed/refractory patients may not be representative of effects in patients treated at the time of first diagnosis. Thus, our results suggest that biological identification of patient subsets should be evaluated in future clinical studies using this therapeutic strategy. Combined treatment, e.g. the combination of different pathway inhibitors (i.e. dual pathway inhibition) and the combination of pathway inhibitors with other targeted therapies or conventional chemotherapy should also be considered.

Although the contributions of nearly all components of this pathway can now be described in mechanistic detail, there are issues that need to be addressed further, such as the signaling pathways may cooperate with other pathways. Thus, further analysis of the interrelations of this pathway with other signaling mediators, pathways, and/or genetic factors is needed.

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III

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Resistance to the Antiproliferative In Vitro Effect of PI3K-Akt-mTOR Inhibition in Primary Human Acute Myeloid Leukemia Cells Is Associated with Altered Cell Metabolism

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Received: 6 December 2017; Accepted: 23 January 2018; Published: 27 January 2018

Abstract: Constitutive signaling through the phosphatidylinositol-3-kinase-Akt-mechanistic target of rapamycin (PI3K-Akt-mTOR) pathway is present in acute myeloid leukemia (AML) cells. However, AML is a heterogeneous disease, and we therefore investigated possible associations between cellular metabolism and sensitivity to PI3K-Akt-mTOR pathway inhibitors. We performed non-targeted metabolite profiling to compare the metabolome differences of primary human AML cells derived from patients susceptible or resistant to the in vitro antiproliferative effects of mTOR and PI3K inhibitors. In addition, the phosphorylation status of 18 proteins involved in PI3K-Akt-mTOR signaling and the effect of the cyclooxygenase inhibitor indomethacin on their phosphorylation status was investigated by flow cytometry. Strong antiproliferative effects by inhibitors were observed only for a subset of patients. We compared the metabolite profiles for responders and non-responders towards PI3K-mTOR inhibitors, and 627 metabolites could be detected. Of these metabolites, 128 were annotated and 15 of the annotated metabolites differed significantly between responders and non-responders, including metabolites involved in energy, amino acid, and lipid metabolism. To conclude, leukemia cells that are susceptible or resistant to PI3K-Akt-mTOR inhibitors differ in energy, amino acid, and arachidonic acid metabolism, and modulation of arachidonic acid metabolism alters the activation of mTOR and its downstream mediators.

Keywords: acute myeloid leukemia; metabolism; mTOR; PI3K; phosphorylation

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by proliferating myeloblasts in the bone marrow [1,2]. Abnormal constitutive signaling through intracellular pathways is often observed in AML, including the phosphatidylinositol-3-kinase-Akt-mechanistic/mammalian target of rapamycin (PI3K-Akt-mTOR) pathway that seems to be important both in normal and leukemic hematopoiesis [3–5]. This pathway is important for regulation of proliferation, apoptosis, differentiation, and metabolism [6,7].

Constitutive signaling through the PI3K-Akt-mTOR pathway is found in 50–80% of AML patients and correlates with poor prognosis [4,8]. This abnormal signaling may be initiated by various mechanisms, e.g., oncogenes or mutated receptor tyrosine kinases, cell adhesion molecules, G-protein-coupled receptors, or other cytokine or hormonal receptors.

When signaling is initiated in response to extracellular stimuli, scaffolding proteins are recruited and bind to the regulatory subunit of PI3K. Sequentially, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which facilitates recruitment and binding of proteins containing pleckstrin–homology domains, including Akt and its upstream activator 3'phosphoinositide-dependent kinase 1 (PDK1) [9]. PDK1 phosphorylates Akt at T308, leading to its partial activation. However, a subsequent phosphorylation at S473 is required for full enzymatic activation of Akt [9,10], and this can be achieved by mTOR complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK) [10,11]. The mTOR kinase is part of two complexes, mTORC1 and mTORC2 with different biochemical structures and substrate specificity. The interactions between Akt and mTORC1/2 are complex. Akt phosphorylates the inhibitor of mTORC1 and proline-rich Akt-substrate-40 (PRAS40), preventing the suppression of mTORC1 signaling. Additionally, an Akt-driven inactivation of tuberous sclerosis complex (TSC) 1/2, leads to activation of mTORC1 through Ras homolog enriched in brain (RHEB). mTORC1 is an important regulator of cellular metabolism and protein synthesis through phosphorylation and activation of both the S6 ribosomal protein kinase (S6PK) and the repressor of messenger RNA (mRNA) translation initiation factor 4E-binding protein 1 (4EBP1) [6].

The PI3K-Akt-mTOR signaling pathway is one of the most frequently dysregulated pathways in human malignancies, including AML [12,13]. Though PI3K-Akt-mTOR is rarely mutated in AML, these patients harbor several mutations that may activate the pathway [12,14] and, hence, contribute to chemoresistance [4,15]. Based on extensive experimental studies, this pathway has been regarded as a possible therapeutic target in human AML [3,16]. However, despite this evidence, the initial clinical studies suggest that the tested mTOR inhibitors have only a modest antileukemic effect [16]. However, it should be emphasized that previous experimental studies also suggest that therapeutic targeting of the PI3K-Akt-mTOR pathway will be effective only for a subset of patients [17], and pathway inhibition may be more effective when using combined treatment strategies [18].

As discussed in a recent review, previous studies of resistance towards PI3K-Akt-mTOR inhibitors have focused on the possible hyperactivation of upstream mediators through feedback loops (e.g., PI3K and Akt) and compensatory activation of other pathways [16]. However, this cannot be the only explanation because chemoresistance is also seen for inhibitors upstream to Akt [17]. Our present study is to the best of our knowledge the first to suggest that metabolic alterations are a part of the therapy-resistant AML cell phenotype, and the metabolic differences seem to involve pathways that are involved in cellular energy and amino acid metabolism.

2. Results

2.1. Selection of Patients for the Metabolomics Comparison of Primary Human Acute Myeloid Leukemia (AML) Cells

We investigated the antiproliferative effect of four PI3K-Akt-mTOR inhibitors on primary human AML cell proliferation in the presence of exogenous cytokines [17]. The antiproliferative effects of the inhibitors differed considerably between the 56 patient cell samples studied, and for a subset of patients, even increased proliferation was seen in the presence of pathway inhibitors. In contrast, these previous studies showed that pathway inhibitors had only minor effects on the spontaneous stress-induced in vitro apoptosis that occurs during culture of primary human AML cells [19], and only minor differences could then be detected between different patients [17]. Patient subsets could thus be identified based solely on differences in our proliferation assay, i.e., these cells survived for 6 days in the presence of the pathway inhibitors and could still proliferate and incorporate ^{3}H-thymidine during the period from day 6 to day 7 of the in vitro culture. This means that our subclassification was based on the pharmacological effects on a cell subset within the hierarchically organized AML cell population that were able to both survive and still be able to proliferate.

We also investigated the effects of pathway inhibitors on the in vitro proliferation of primary human AML cells for a second and larger cohort including 76 additional consecutive patients; in these

experiments, we only examined the effects of rapamycin and GDC-0941. The overall results are presented in Figure 1. The studies of this second cohort confirmed that the antiproliferative effects of PI3K-Akt-mTOR pathway inhibition varied among individual patients, and a variation of the effect between the two drugs was observed. We also investigated the susceptibility to stress-induced or spontaneous in vitro apoptosis for these 76 patients, but we could not observe any correlation between this susceptibility to apoptosis and the antiproliferative effects of the two pathway inhibitors. Taken together, our results from the two patient cohorts showed that neither the general regulation of apoptosis, as reflected in the degree of spontaneous in vitro apoptosis, nor the viability of the AML cell population after in vitro exposure to pathway inhibitors showed any significant association with the variation in antiproliferative effects of pathway inhibitors that was detected in our proliferation assay.

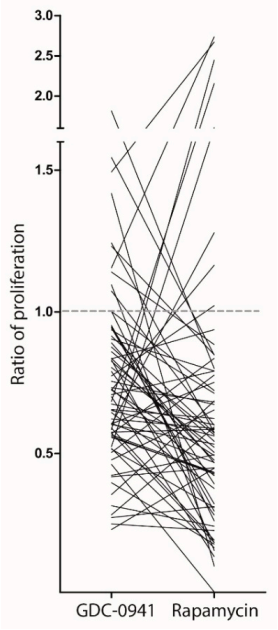

Figure 1. The effect of phosphatidylinositol-3-kinase-mechanistic target of rapamycin (PI3K-mTOR) inhibitors on cytokine-dependent in vitro acute myeloid leukemia (AML) cell proliferation. Leukemic cell proliferation was assayed as ^{3}H-thymidine incorporation after six days of culture. We compared the proliferation of primary human AML cells cultured in the presence of the PI3K-inhibitor GDC-0941 and the mTOR-inhibitor rapamycin. The results are presented as the ratio of proliferation, i.e., nuclear incorporation of ^{3}H-thymidine in drug-exposed cells relative to the incorporation in corresponding drug-free control cultures. The patient cohort included 76 patients, but detectable proliferation was only seen for the 68 AML patients whose results are presented in the figure. Each line represents the results for one patient. The dashed line indicates a ratio of 1.0, i.e., no change in proliferation.

The data presented in Figure 1 clearly illustrate that pathway inhibitors can increase AML cell proliferation for a subset of patients, whereas for other patients, a strong inhibition corresponding to more than 50% inhibition could be detected for different mediators. For further analysis of the possible association between metabolic characteristics and the antiproliferative effects of pathway inhibitors on primary human AML cells, we compared two contrasting groups of selected patients based on the studies of the two patient cohorts. We then selected 15 patient samples with significantly decreased proliferation after inhibition with both rapamycin and GDC-0941; these samples are referred to as responders to the treatment. The other group included 15 patient samples showing no significant

alteration of proliferation (corresponding to <10% inhibition) or even growth enhancement in the presence of pathway inhibitors. These are referred to as non-responders to treatment.

2.2. Patient Samples with Different Drug Sensitivity towards PI3K-mTOR Inhibitors Also Differ in Energy, Amino Acid and Arachidonic Acid Metabolism

Previous studies suggest that metabolic regulation of chronic myeloid leukemia cells is important for their susceptibility towards targeted therapy with kinase inhibitors [20]. We compared the metabolic profiles of the two contrasting patient groups that were sensitive and insensitive to PI3K-Akt-mTOR inhibition in vitro. As described above, these groups were selected based on their susceptibility to the antiproliferative effect of PI3K and mTOR inhibitors [17]. The metabolic analysis of the AML cells detected a total of 627 metabolites, and 128 of these metabolites were annotated. A principal component analysis was performed to illustrate the variance between the different sample groups. The responders and non-responders showed a great overlap in the plot, though four of the non-responders clustered separately from the rest (Figure 2). Thus, responders and non-responders could not be separated by an analysis of the overall metabolic profile, and non-responder patients seem to be heterogeneous with regard to their global metabolite profiles even though they show a similar resistance to PI3K-Akt-mTOR pathway inhibition.

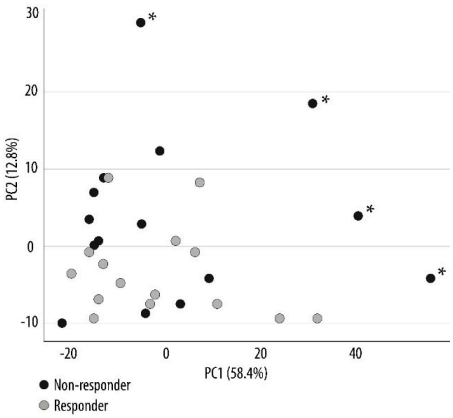

Figure 2. Principal component analysis (PCA) comparing the metabolic profiles of responders and non-responders to PI3K-mTOR inhibitors. The analysis was performed to generate an overview of the metabolic variance among the entire set of samples. Prior to this analysis, primary AML cells from 30 patients were separated into two contrasting groups based on their susceptibility to the in vitro antiproliferative effect of pathway inhibitors. The metabolic profiles for the primary AML cells derived from patients being susceptible (15 responders; grey circle) or resistant (15 non-responders; black circle) to PI3K-mTOR inhibitors were compared. The PCA depicts 71.2% (58.4 + 12.8% as indicated at the *X* and *Y* axis) of all variances in the data set. A separation of four non-responders (indicated by the asterisks *) from the rest of the sample was seen. Each circle represents the results for one patient.

Of the 627 detected metabolites, 23 metabolites differed significantly between the two contrasting groups of responders and non-responders, and among these, 15 were annotated (Table 1). These significantly altered metabolites are involved in energy (citric acid, isocitric acid, glutamine), amino acid (proline, glutamine, taurine), and lipid metabolism (two phosphatidylinositols (PI), the arachidonic acid metabolites 4,7,10,13-eicosatetraenoic acid, and 4,7,10,13,16-docosapentaenoic acid).

We did a metabolic pathway mapping based on the identified metabolites for further characterization of differences between responders and non-responders based on the 80 top-ranked metabolites. Purine metabolism (including the annotated metabolites glutamine, glutamic acid, glycine,

and hypoxanthine) and Warburg effect (i.e., energy metabolism; including the annotated metabolites NAD, glutamic acid, glutamine citric acid, and isocitric acid) were then the two highest-ranked terms. This metabolic pathway mapping analysis further supports our conclusion that responders and non-responders to PI3K-mTOR inhibitors show metabolic differences.

2.3. Responders and Non-Responders to PI3K-Akt-mTOR Inhibition Could Be Identified Based on Metabolic Differences

No single metabolite could be used to identify responders and non-responders. However, a decision tree analysis was performed showing that samples can be differentiated based on the levels of cysteinyl-cysteine and threonic acid (Table 1). The patient subset sensitive to PI3K/mTOR inhibitors in vitro was then characterized by low levels of both these metabolites (Figure 3). Cysteinyl-cysteine is a dipeptide composed of two cysteine residues and an incomplete breakdown product of protein catabolism, whereas threonic acid is probably derived from glycated proteins or degradation of ascorbic acid (Human Metabolome Database). This additional and alternative analysis of our metabolomic data further illustrates that our contrasting groups of responder and non-responder AML cells show metabolic differences.

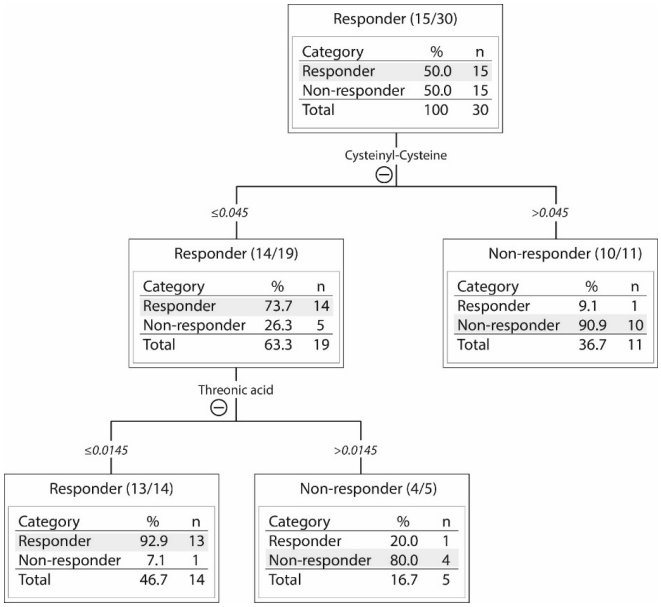

Figure 3. A decision tree analysis of the metabolic differences between 15 responders and 15 non-responders to PI3K-Akt-mTOR inhibition. The levels of two metabolites, cysteinyl-cysteine and threonic acid, allowed for discrimination between responders and non-responders. The 30 patients (see the upper box) were first classified into two subsets based on their cysteinyl-cysteine levels ($\leq$ or >0.045). In the box with high cysteinyl-cysteine (>0.045; right box), there were 10 non-responders and 1 responder. The 19 patients (14 responders and 5 non-responders) with low levels of cysteinyl-cysteine ($\leq$0.045; left box) were further subclassified into two subsets based on the level of threonic acid ($\leq$ or >0.0145). Thirteen of the 14 responders with low levels of cysteinyl-cysteine also showed low levels of threonic acid ($\leq$0.0145; left box). Whereas four of five non-responders among the 19 patients with low levels of cysteinyl-cysteine showed high levels of threonic acid (>0.0145; right box). Approximately ninety percent of patients were then correctly classified as responders or non-responders.

Table 1. A description of annotated metabolites that differed significantly between the two patient groups and were sensitive (responders) or insensitive (non-responders) to the in vitro antiproliferative effect of phosphatidylinositol-3-kinase-Akt-mechanistic/mammalian target of rapamycin (PI3K-Akt-mTOR) inhibition.

Metabolite	*p*-Value	Ratio * Responder versus Non-Responder	Short Description
↓Allose	0.037	−0.875	Sugar metabolism. Possibly involved in cell cycle regulation.
↓Citric acid	0.005	−1.262	Energy metabolism, citric acid cycle.
↓Cysteinyl-cysteine	0.006	−1.471	Dipeptide
↓Glutamine	0.029	−0.737	Non-essential amino acid, important for nucleic acid synthesis. Energy metabolism, conditionally essential during catabolic states.
↓Indoleacrylic acid	0.047	−0.426	Involved in tryptophan metabolism.
↓Isocitric acid	0.029	−0.698	Substrate of the citric acid cycle.
↑Phosphatidyl inositol (18:0/0:0)	0.040	0.765	Lipid metabolism, cell membrane constituents.
↑Phosphatidyl inositol (15:1(9Z)/22:6(4Z,7Z,10Z,13Z16Z19Z))	0.025	0.809	Lipid metabolism, cell membrane constituents.
↓Phosphonic acid (8:0/8:0)	0.009	−1.660	Lipid metabolism
↓Proline	0.046	−0.611	Non-essential amino acid, synthesized from glutamic acid and also other amino acids, energy metabolism.
↓Taurine	0.035	−1.0524	Sulfur amino acid not incorporated into protein; adults can synthesize taurine from cysteine. Stabilizes cell membranes, regulates ion transport.
↓2-amino-4-hydroxy-propiophenone	0.021	−0.744	Lipid metabolism
↓4-phenyl-1,2,3-thiadiazole	0.041	−1.024	Inhibitor of cytochrome P450 enzymes that regulate arachidonic acid metabolism.
↓4,7,10,13-eicosatetraenoic acid	0.021	−0.983	Arachidonic acid metabolite, possibly influencing the leukotriene B4 (LTB4) pathway; expression of the LTB4 receptor (BLT1) may be altered in myeloid leukemia cells.
↓4,7,10,13,16-docosapentaenoic acid	0.042	−0.766	Fatty acid and arachidonic acid metabolism, an intermediate between eicosapentaenoic acid and docosahexaenoic acid, precursor of prostanoids that are only formed from docosapentaenoic acid.

* Responders versus non-responders were compared as the $\log_2$-ratio. The arrows to the left in the table indicate whether the mean metabolite levels were decreased (↓) or increased (↑) in responder cells relative to the non-responder cells. The information in this table is based on PubChem and Human Metabolome databases.

2.4. Modulation of Arachidonic Acid Metabolism Alters PI3K-Akt-mTOR Signaling

In a previous study, we used Western blot to analyze phosphorylation mediators downstream of mTOR in a small group of patients treated with PI3K-mTOR inhibitors [17]. Even though these results have to be interpreted with great care as few patients were studied, the observations suggested that (i) patients differed considerably with respect to the degree of constitutive signaling through the PI3K-Akt-mTOR pathway; and (ii) the heterogeneous antiproliferative effects of PI3K-mTOR inhibitors seen among patients could not be explained by differences in constitutive pathway activation.

Arachidonic acid metabolism seems to be important for survival and proliferation of various cells, including myeloid cells [21,22]. Arachidonic acid can be metabolized by cyclooxygenase, lipoxygenase, or the cytochrome P450 pathways into a number of metabolites, referred to as eicosanoids. These arachidonic acid derived eicosanoids belong to a complex family of lipid signaling mediators that control many important cellular processes, including cell proliferation, apoptosis, and cell metabolism [21,23]. Therefore, we wanted to investigate whether modulation of the balance between the various pathways of arachidonic acid metabolism would influence PI3K-Akt-mTOR signaling in primary human AML cells.

In these experiments, we modulated the balance of arachidonic acid metabolism by incubating the cells with indomethacin (a nonselective cyclooxygenase 1/2 inhibitor), and we investigated the effects of this inhibitor on PI3K-Akt-mTOR signaling in primary AML cells derived from five patients showing constitutive signaling throughout this pathway. These five patients showed a wide variation in constitutive pathway activation; this activation was also observed in previous Western blot analyses of the downstream mTOR mediators P70SK6 and p4E-BP1 (see above) [17]. Thus, a variation in the degree of constitutive pathway activation can be detected by both Western blot and phospho-flow, and, therefore, we selected five patient samples with a constitutive, though wide variation of signaling. The variation between these five patients was, in addition, reproduced/documented in independent analysis of cells derived from separate freezing ampullas.

Using a flow cytometry technique, we explored the effects of indomethacin of the PI3K-Akt-mTOR pathway (10 μg/mL, 15-min incubation) for cells incubated in medium alone (i.e., constitutive signaling) and medium supplemented with insulin 10 μg/mL (Figures 4 and 5). Insulin was studied because PI3K-Akt-mTOR is an important pathway downstream of the insulin receptor [24,25], and in vitro studies have shown that insulin is an important growth factor for primary AML cells for a major subset of patients [26]. When performing an unsupervised hierarchical clustering of the overall results, we observed that the four combinations tested (medium alone ± indomethacin, insulin ± indomethacin) for each individual patient sample generally clustered together; showing that differences in pathway signaling between patients were maintained even in the presence of cyclooxygenase inhibition. An indomethacin-induced decrease of mTOR pS2448, S6 pS235 pS236, and S6 pS244 was seen for all patients in insulin-free and/or insulin-supplemented cultures, and for four of the five patients a decrease was seen for Akt pS473 and S6 pS240 (Figure 4).

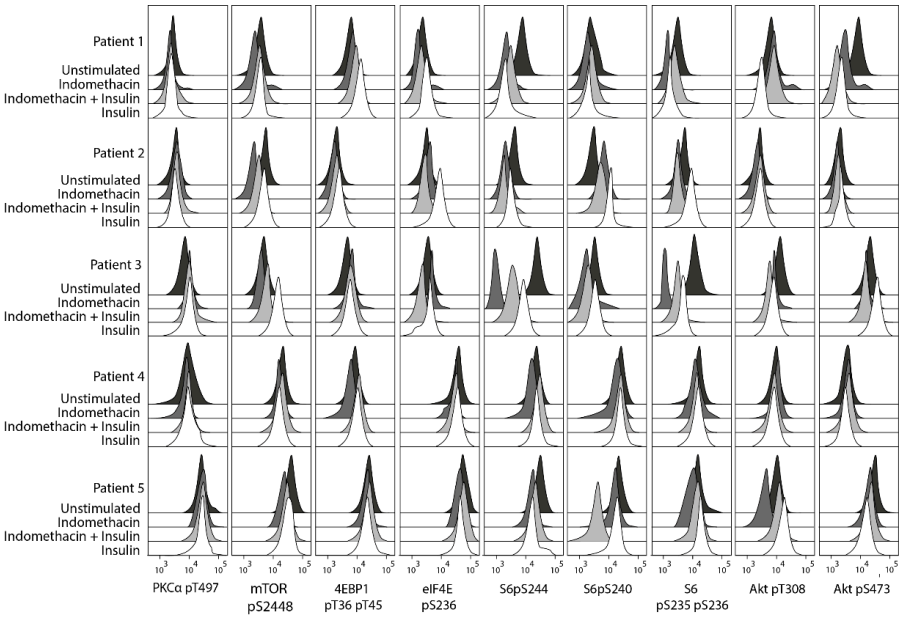

Figure 4. In vitro phospho-signaling analysis of primary AML cells derived from five patients to explore the effects of indomethacin on the PI3K-Akt-mTOR pathway. AML cells were incubated in medium alone, in medium supplemented with 10 μg/mL of either indomethacin or insulin, and in medium supplemented with the combination of insulin and indomethacin. Phosphorylation status of nine mediators were examined. An indomethacin-induced decrease of mTOR pS2448, S6 pS235 pS236, and S6 pS244 was seen for all patients in insulin-free and/or insulin-supplemented cultures, and a decrease of S6 pS240 and Akt pS473 was seen for four of the five patients. The *X*-axis is a log-scale for fluorescence intensity; the *Y*-axis indicates the number of cells.

Based on our current observations, we conclude that modulation of arachidonic acid metabolism by exposure to indomethacin has only minor effects on the phosphorylation of certain mediators in the PI3K-Akt-mTOR pathway; a similar conclusion can be made also for insulin. Only minor effects were observed on the overall pathway activation profile compared with the observed wide variation in constitutive pathway activation between different patients. Accordingly, our results in Figure 5 illustrate that the wide variation between patients in constitutive pathway activation seems to be maintained also after exposure of the leukemic cells to insulin, as well as after drug-induced modulation of arachidonic acid metabolism, i.e., samples from an individual patient cluster together whereas samples from insulin/indomethacin exposed cells do not cluster together.

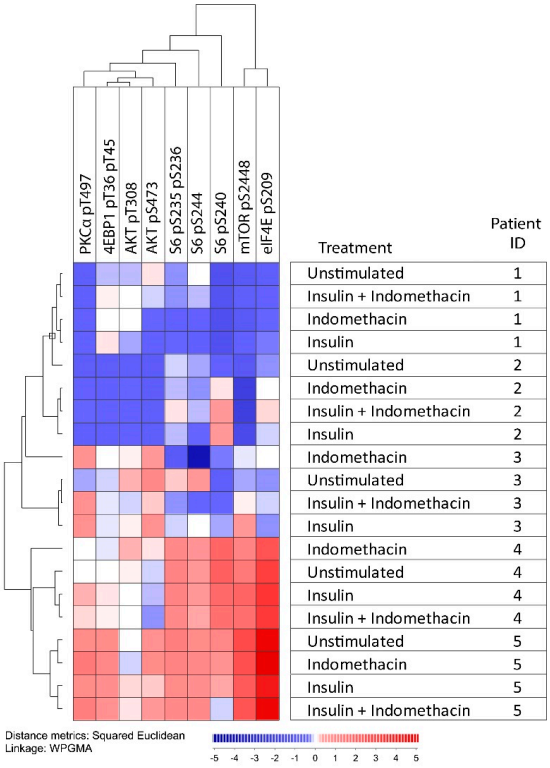

Figure 5. The effect of indomethacin on the activation of PI3K-Akt-mTOR signaling. We investigated the effects of indomethacin on PI3K-Akt-mTOR signaling in primary AML cells derived from five patients. For each sample, we tested AML cells incubated in medium alone, with only indomethacin 10 μg/mL, in medium supplemented with 10 μg/mL insulin, and with the combination of insulin and indomethacin. Phosphorylation status of nine mediators were examined. Red indicates high and blue indicates low phosphorylation/expression of the mediators. All combinations tested for each patient sample also clustered together in the same subclusters for all patients. All values from the flow cytometric analyses were calculated using fold change on the Inverse hyperbolic sine (Arcsinh) scale.

3. Discussion

The PI3K-Akt-mTOR signaling network shows constitutive activation in human AML [12]. However, previous experimental studies [17] suggest that the antileukemic effects of pathway inhibitors differ between patients, and the aim of the present study was to further characterize this patient heterogeneity with regard to constitutive PI3K-Akt-mTOR activation/signaling, pathway inhibition, and metabolic regulation.

For younger patients receiving the most intensive chemotherapy, the overall long-term AML-free survival is only 40–50%. However, the large group of patients above 70–75 years of age are not able to receive this intensive therapy, and are therefore, treated with AML-stabilizing treatment [27]. Many of these elderly patients, as well as younger unfit patients, have an expected survival of only 1–3 months. Thus, new therapeutic strategies are needed both for younger as well as for elderly and unfit patients who can only receive AML-stabilizing treatment; combination therapy including PI3K-Akt-mTOR inhibitors may then be an alternative therapeutic strategy [3,16,18]. However, due to the short survival of many elderly and unfit patients [28,29], they may get only one chance of antileukemic therapy

because of the rapid disease progression if this first treatment fails. For this reason, pretreatment identification of patients with high risk of resistant disease will be important. Our present study suggests that metabolic characterization should be further explored as a possible strategy to identify patients with a high risk of resistance to PI3K-Akt-mTOR inhibition, and such patients should then try an alternative strategy as their initial treatment.

In vitro cultured hierarchically organized AML cell populations show spontaneous apoptosis during the first 4–5 days of culture; for most patients, this is an extensive process [19]. Our previous study showed that PI3K-mTOR inhibitors only have weak influence on this spontaneous or stress-induced in vitro apoptosis, and the variation between patients is limited so that it cannot be used for subset classification [17]. However, a wide variation between patients can be detected when using our ^{3}H-thymidine incorporation assay after six days of in vitro culture, i.e., an analysis of the AML cell minority that has been able to survive the initial six days of in vitro culture and still are able to proliferate. Inhibitors of the PI3K-Akt-mTOR pathway were added at the start of the cultures. Our patient classification reflects a combined effect of pathway inhibitors on both survival and proliferation, i.e., the presence of the drug during the initial six days characterized by spontaneous in vitro apoptosis and the ability of the remaining viable cells to still show cytokine-dependent proliferation in the presence of the pathway inhibitors when ^{3}H-thymidine incorporation is assayed from day six to seven of in vitro culture.

We investigated the effect of insulin on phosphorylation of the PI3K-Akt-mTOR-pathway as this pathway is important for insulin signaling [24,25], and insulin is also an important growth factor for in vitro cultured primary human AML cells for a large subset of patients [26]. Our present studies showed that insulin altered the activation/phosphorylation of several mediators; however, the effects were minor and differed between patients. Also, the wide variation in PI3K-Akt-mTOR pathway activation between patients was maintained in the presence of insulin (i.e., the samples exposed to insulin did not cluster together with each other but rather together with the corresponding insulin-free control (Figures 4 and 5)).

Even though constitutive activation of PI3K-Akt-mTOR signaling in the enriched leukemic cells is seen for most AML patients, resistance to the antiproliferative effect of pathway inhibitors is relatively common. In our present study, we show that resistant patient-derived leukemia cells differ with regard to their metabolomic profile, including the metabolites involved in amino acid and arachidonic acid metabolism. Similar abnormalities are also associated with chemoresistance in other myeloid malignancies [30].

Arachidonic acid metabolism is important for survival and proliferation of hematopoietic cells [21,23,31–33] and several of its metabolites can influence activation/signaling through the PI3K-Akt-mTOR pathway, e.g., pathway-activating prostaglandins and eicosatetraenoic acid derivatives [31–35]. Our comparison of the metabolite profiles of cell samples representing either responders or non-responders to PI3K-Akt-mTOR inhibition supports the hypothesis that arachidonic acid metabolism is important with regard to susceptibility to these inhibitors. This hypothesis was also supported by our observed effects of modulated arachidonic acid metabolism by indomethacin on mediator phosphorylation, and previous studies in both human chronic myeloid leukemia and in animal models of leukemic stem cells indicating that arachidonic acid metabolism is important for both leukemogenesis and chemosensitivity [20,36].

For other cell types, there are functional links between redox balance, purine metabolism, NADH, proline, and glutamine metabolism, and the citric acid cycle [37–41]. Even though few studies of myeloid cells are available, observations in other cell types suggest links between such metabolic steps and PI3K-Akt-mTOR signaling. Firstly, arachidonic acid metabolites can function as regulators of the PI3K-Akt-mTOR pathway [21,23,42,43], and our present results suggest that this may also be true in human AML. Secondly, there are links between PI3K-Akt-mTOR signaling via free oxygen radicals/redox homeostasis to the NAD/NADH/proline/glutamine/glutamate system [44,45]. Thirdly, proline and glutamine are interconvertible, and glutamine is an important substrate for the energy metabolism in many malignant cells; a link between arachidonic acid and

energy metabolism/the citric acid cycle is therefore possible [37,39]. Finally, both arachidonic acid metabolism and PI3K-Akt-mTOR signaling are important for regulation of the peroxisome proliferator activated receptors, a group of transcription factors [21,40]. However, additional studies are needed to clarify the possible contributions of these various steps in human AML.

Previous experimental studies suggest that altered proline metabolism can be important for the development of cancer chemoresistance, and proline oxidase has been suggested as a possible target in cancer treatment [37,39,41,46]. Our present study suggests that proline/glutamine metabolism may also contribute to resistance of PI3K-mTOR inhibitors in human AML. Our observation of increased levels of eicosatetraenoic acid and docosapentaenoic acid indicates that arachidonic acid may be one of these interacting factors. However, an alternative explanation could be that effects on proline/glutamine only reflect differences in energy metabolisms, and differences in arachidonic acid metabolites may reflect the altered energy/lipid metabolism. This is further supported by previous studies showing that arachidonic acid metabolism is important in murine leukemogenesis and for chemoresistance in human chronic myeloid leukemia [20,36]. The PI3K-Akt-mTOR pathway may represent a link between these two systems through the effect of arachidonic acid metabolites on this pathway and the regulatory effect of mTOR on proline oxidase.

The role of arachidonic acid and its metabolites in normal and malignant hematopoiesis has been reviewed previously [21]. Increased expression of lipoxygenase enzymes has been detected in malignant myeloid cells, and products from this pathway of arachidonic acid metabolism often seem to mediate growth-enhancing and antiapoptotic effects. Our present observations of increased levels of eicosanoids in cells that are resistant to PI3K-Akt-mTOR inhibitors suggest that these metabolites may have such a role in human AML. Furthermore, the effect of arachidonic acid itself seems to differ between cell lines, but proapoptotic effects have been described. Finally, a previous study of primary human AML cells showed that even low levels of indomethacin could reduce the AML cell levels of prostaglandin E_2, and in their model PGE_2, could enhance both the spontaneous proliferation as well as Toll like receptor mediated growth enhancement of primary human AML cells [47].

4. Materials and Methods

4.1. AML Patients

The study was approved by the Regional Ethics Committee (REK) (REK III 060.02, 10 June 2002; REK Vest 215.03, 12 March 04; REK III 231.06, 15 March 2007; REK Vest 2013/634, 19 March 2013; REK Vest 2015/1410, 19 June 2015), The Norwegian Data Protection Authority 02/1118-5, 22 October 2002, and The Norwegian Ministry of Health 03/05340 HRA/ASD, 16 February 2004. All AML cell samples were collected after written informed consent.

The clinical and biological characteristics of those 30 patients included in the metabolic studies are summarized in Table 2. All patients had a high number and/or percentage of peripheral blood blasts; leukemic peripheral blood mononuclear cells could, therefore, be isolated by density gradient separation alone (Lymphoprep, Axis-Shield, Oslo, Norway) and generally contained at least 95% leukemic blasts. The contaminating cells were small lymphocytes. These enriched AML cells were stored in liquid nitrogen until used in the experiments [48]. All the 15 responder patients selected for metabolic profiling had a strong inhibition (i.e., >50% inhibition) of cytokine-dependent AML cell proliferation by both PI3K and mTOR inhibitors, whereas PI3K and mTOR inhibition either increased the proliferation or had a weak antiproliferative effect corresponding to <10% inhibition for the 15 non-responders.

Table 2. Important clinical and biological characteristics of responders and non-responders to of phosphatidylinositol-3-kinase- mechanistic/mammalian target of rapamycin (PI3K-mTOR) inhibitors.

ID	Gender	Age	Previous Hematological Malignancy or Chemotherapy	FAB	CD34	Karyotype		*Flt3* Mutation	*NPM-1* Mutation
						Abnormality	Classification		
Responders									
1	F	45	Chemotherapy	M4	Negative	Normal	Normal	wt	ins
2	F	63		M4	Positive	Normal	Normal	ITD	wt
3	M	72		M5	Negative	Normal	Normal	wt	ins
4	M	29	Relapse	M4	Positive	Normal	Normal	ITD	ins
5	F	80		M2	Positive	Complex	Adverse	wt	wt
6	F	36		M4	Positive	Normal	Normal	wt	nt
7	F	75		M1	Positive	nt		ITD	wt
8	M	71	Relapse	M2	Negative	Normal	Normal	G835	
9	M	35		M2	Positive	Normal	Normal	wt	wt
10	M	72	Myelodysplastic syndrome	M1	Positive	Complex	Adverse	wt	
11	F	64	Chemotherapy	M2	Negative	Normal	Normal	ITD	ins
12	F	59	Chemotherapy	M5	Negative	Normal	Normal	ITD	ins
13	M	58		M5	Positive	Normal	Normal	wt	wt
14	F	59	Chemotherapy	M4	Negative	Normal	Normal	ITD	ins
15	F	75		M4	Positive	Normal	Normal	ITD	wt
Non-responders									
16	F	29	Chemotherapy	M5	Positive	Normal	Normal	ITD+Asp835	wt
17	M	24		M2	Positive	Multiple	Adverse	nt	wt
18	F	82		M4	Positive	Normal	Normal	ITD	wt
19	F	77		M1	Negative	nt		nt	ins
20	M	84		M1	Positive	Multiple	Adverse	wt	wt
21	M	53		M0	Positive	13	Intermediate	wt	wt
22	M	65		M5	Negative	Normal	Normal	ITD	ins
23	F	46		M1	Positive	inv(16)	Favorable	wt	wt
24	F	70		M4	Negative	nt		wt	ins
25	M	33	Chemotherapy	M1	Positive	Normal	Normal	wt	wt
26	F	77		M1	Positive	nt		nt	wt
27	M	76		M0	Positive	Normal	Normal	wt	wt
28	M	60		M4	Positive	Normal	Normal	ITD	wt
29	M	36		M5	Positive	+8, +22, inv(16)	Favorable	ITD	wt
30	F	67		M5	Negative	t(9,11), +19	Intermediate	wt	wt

The table shows the gender (M, male; F, female) and age (years) of the individual patients at diagnosis. The FAB classification was used to classify morphological and/or histochemical signs of differentiation. Cytogenetic abnormalities were classified according to the medical research council (MRC) criteria. The detection of Fms like tyrosine kinase 3 (Flt3) (ITD, internal tandem duplications) or nucleophosmin (NPM)-1 insertions (ins) is also indicated in the table. Complex karyotype means at least three abnormalities [1]. FAB: The French-American-British (FAB) classification system; nt: not tested; wt: wild type.

4.2. Drugs

Drugs used in this study included the mTOR inhibitor rapamycin (LC Laboratories, Woburn, MA, USA), the PI3K class I specific inhibitor GDC-0941 (Axon Medchem BV, Groningen, The Netherlands), human insulin (Sigma-Aldrich, St. Louis, MO, USA), and the nonselective cyclooxygenase 1/2 inhibitor indomethacin (Sigma-Aldrich; dissolved in dimethyl sulfoxide (DMSO)). Stock solutions were sterile filtered and stored at −20 °C until used in experiments, thawed only once, and diluted with their respective solvents to obtain the desired final concentrations.

Indomethacin (Sigma-Aldrich) was tested at a final concentration of 10 μg/mL (corresponding to 28 μM). Previous studies in human as well as murine AML cells often used indomethacin concentrations in the range of 10–50 μM (3.6–18 μg/mL) [49–51], and the conventional cyclooxygenase-blocking concentration of indomethacin is considered to be 10–20 μM (for original reference see [50]). However, even indomethacin concentrations as low as 1 μM (0.4 μg/mL) will decrease the in vitro prostaglandin production by primary human acute leukemia cells [47]. Our use of indomethacin 10 μg/mL was based on these previous studies. Finally, in pilot experiments we investigated pharmacological effects after incubation for 7, 10, 15, 30, and 45 min before analyzing the PI3K-Akt-mTOR pathway activation. We decided to incubate cells with the drugs for 15 min because additional effects could not be detected when using longer incubations.

4.3. Analysis of PI3K-Akt-mTOR Activation

Flow cytometry was used to examine the basal expression of 18 mediators in the PI3K-Akt-mTOR pathway/network in the AML cells. Cryopreserved and thawed primary leukemic cells were incubated for 20 min in RPMI-1640 (Sigma-Aldrich) before being directly fixed in 1.5% paraformaldehyde (PFA) and permeabilized with 100% methanol. The cells were subsequently rehydrated by adding 2 mL phosphate-buffered saline (PBS), gently re-suspended, and then centrifuged. The cell pellet was washed twice with 2 mL PBS and resuspended in 150 μL PBS supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich). Washed cells were blocked with immunoglobulin (Octagam; Octapharma, Jessheim, Norway) and 1% BSA, and then split evenly into nineteen new tubes (1×10^5 cells per sample) before staining. All staining panels included the same live/dead discriminator, either FITC or Alexa Fluor® 647 Mouse anti-Cleaved PARP (Asp214); an unstained sample was also included. Three directly conjugated dyes were used: (i) Alexa Fluor® 647 was used for PTEN, PDPK1 pS241, PKCα, PKCα pT497, Akt pS473, 4EBP1 pT36 pT45, elF4E pS209, S6 pS244, and mTOR; (ii) phycoerythrin (PE) for Akt total, Akt pT308, mTOR pS2448, and S6 pS240; and (iii) V450 for S6 pS235 pS236. Antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ, USA), except for anti-mTOR that was purchased from Cell Signal Technology (Danvers, MA, USA). Four of the antibodies were unconjugated (anti Raptor, Tuberin, FKBP38, and RHEB; all from Abcam; Cambridge, UK) and required secondary antibody-conjugated Alexa Fluor® 647 (BD Pharmingen). Together these mediators represent the main steps in the PI3K-Akt-mTOR pathway and they were selected to provide an extended phosphorylation profile of this pathway. Finally, in our pharmacological studies, AML cells were incubated with human insulin 10 μg/mL (Sigma-Aldrich) and/or indomethacin 10 μg/mL (Sigma-Aldrich; dissolved in DMSO); final concentration in the medium 0.5%) or a DMSO control solution for 15 min before flow cytometric analysis as described above. Flow cytometry analysis was acquired on a BD FACS Verse 8-color flow cytometer (BD Biosciences, San Jose, CA, USA) and data analysis performed using FlowJo 10.0.7 software (Tree Star, Inc., Ashland, OR, USA).

4.4. Analysis of Cytokine-Dependent Proliferation in Presence of PI3K-mTOR Inhibitors

As described in detail previously [52,53]; AML cells (5×10^4 cells/well) were cultured in flat-bottomed microtiter plates (150 μL/well) in Stem Span SFEM™ serum-free medium (Stem Cell Technologies; Vancouver, BC, Canada) alone or in medium supplemented with granulocyte-macrophage colony-stimulation factor (GM-CSF), stem cell factor (SCF) and Fms like tyrosine kinase 3 ligand

(Flt3L) [54]. All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA) and used at 20 ng/mL. All drugs were added on the first day of culture, whereas 37 kBq/well of ^{3}H-thymidine (Perkin Elmer; Waltham, MA, USA) was added after 6 days, and nuclear incorporation was assayed after seven days of culture. The mTOR inhibitor rapamycin and the PI3K inhibitor GDC-0941 were added at a final concentration of 100 nM on the first day of culture [55].

4.5. Metabolomic Analysis

The metabolomic analyses and sample preparations were performed by Metabolomic Discoveries GmbH (Potsdam, Germany) [56]. Briefly, non-targeted metabolite profiling of cells included analyses by gas chromatography/mass spectrometry (GC-MS) and Liquid Chromatography Quadrupole-Time of Flight (LC-QTOF)/MS; metabolites could then be analyzed in the range of 50–1700 Da with an accuracy up to 1–2 ppm and a resolution of mass/Δmass = 40,000. Metabolites measured in the LC were annotated according to their accurate mass and subsequent sum formula prediction. Metabolite profiles were explored by the platforms of Metabolomic Discoveries and Small Molecule Pathway Database (BioVariance, Munich, Germany). The lists of the 627 detected and the 128 annotated metabolites are presented in the Supplementary Materials presenting the identity/accurate mass@retention time, the *p*-value, and responder/non-responder ratio.

4.6. Bioinformatical and Statistical Analyses

Bioinformatic analyses were performed using the J-Express 2012 software (MolMine AS, Bergen, Norway). For hierarchical clustering analysis, all values from cytometric analyses were calculated using fold change on the Inverse hyperbolic sine (Arcsinh) scale. The median signal for each phospho-protein was used as reference value for the calculation of basal phosphorylation. Complete linkage and Squared Euclidean correlation were used as linkage method and distance measurement, respectively. Statistical analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) version 23 (Chicago, IL, USA), and p-values < 0.05 were regarded as statistically significant.

5. Conclusions

Despite the metabolic heterogeneity of the non-responders to PI3K-Akt-mTOR inhibitors, there are distinct metabolic differences between responders and non-responders. Our present results are consistent with the hypothesis that differences in arachidonic acid metabolism together with differences in proline and/or energy metabolism are associated with differences in susceptibility to pathway inhibitors. The possible importance of such differences should be considered when planning or analyzing future clinical studies with PI3K-Akt-mTOR inhibitors and when designing combination therapy for various AML patient subsets [57].

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/2/382/s1.

Acknowledgments: The technical assistance of Marie Hagen and Kristin Paulsen Rye is gratefully acknowledged.

Author Contributions: Øystein Bruserud is responsible for the AML biobanks. Ina Nepstad established the flow-cytometric methodology. All flow-cytometric analyses of PI3K-Akt-mTOR activation in primary human AML cells were performed by Ina Nepstad. Annette K. Brenner did the proliferation studies presented in Figure 1. Håkon Reikvam and Annette K. Brenner performed statistical analyses regarding AML cell proliferation. Kimberley J. Hatfield contributed to preparing samples for metabolic studies. Bioinformatic and statistical analyses were performed by Ina Nepstad, Håkon Reikvam, and Øystein Bruserud. Ina Nepstad, Øystein Bruserud, and Kimberley J. Hatfield contributed to writing the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

4EBP1	Translation initiation factor 4E-binding protein 1
AML	Acute myeloid leukemia
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
FAB	The French-American-British () classification system
Flt3	Fms like tyrosine kinase 3
Flt3L	Flt3 ligand
GC-MS	Gas chromatography-mass spectrometry
GM-CSF	Granulocyte-macrophage colony-stimulation factor
ins	Insertions
ITD	Internal tandem duplications
LC-QTOF/MS	Liquid Chromatography Quadrupole-Time of Flight MS
mRNA	Messenger RNA
mTOR	Mechanistic/mammalian target of rapamycin
mTORC	mTOR complex
NPM	Nucleophosmin
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDK1	3′phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PRAS40	Proline-rich Akt-substrate-40
RHEB	Ras homolog enriched in brain
S6PK	S6 ribosomal protein kinase
SCF	Stem cell factor
SPSS	Statistical Package for the Social Sciences
TSC	Tuberous sclerosis complex
eIF4E pS209	eukaryotic translation Initiation Factor 4E
PKCα	Protein kinase C α
PTEN	Phosphatase and tensin homolog

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Article

Clonal Heterogeneity Reflected by PI3K-AKT-mTOR Signaling in Human Acute Myeloid Leukemia Cells and Its Association with Adverse Prognosis

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Received: 24 July 2018; Accepted: 13 September 2018; Published: 14 September

Abstract: Clonal heterogeneity detected by karyotyping is a biomarker associated with adverse prognosis in acute myeloid leukemia (AML). Constitutive activation of the phosphatidylinositol-3-kinase-Akt-mechanistic target of rapamycin (PI3K-Akt-mTOR) pathway is present in AML cells, and this pathway integrates signaling from several upstream receptors/mediators. We suggest that this pathway reflects biologically important clonal heterogeneity. We investigated constitutive PI3K-Akt-mTOR pathway activation in primary human AML cells derived from 114 patients, together with 18 pathway mediators. The cohort included patients with normal karyotype or single karyotype abnormalities and with an expected heterogeneity of molecular genetic abnormalities. Clonal heterogeneity reflected as pathway mediator heterogeneity was detected for 49 patients. Global gene expression profiles of AML cell populations with and without clonal heterogeneity differed with regard to expression of ectopic olfactory receptors (a subset of G-protein coupled receptors) and proteins involved in G-protein coupled receptor signaling. Finally, the presence of clonal heterogeneity was associated with adverse prognosis for patients receiving intensive antileukemic treatment. The clonal heterogeneity as reflected in the activation status of selected mediators in the PI3K-Akt-mTOR pathway was associated with a different gene expression profile and had an independent prognostic impact. Biological heterogeneity reflected in the intracellular signaling status should be further investigated as a prognostic biomarker in human AML.

Keywords: acute myeloid leukemia; PI3K; Akt; mTOR; phosphorylation; clonal heterogeneity

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by proliferating myeloblasts in the bone marrow [1–3]. Abnormal or constitutive signaling through intracellular pathways is often observed in the leukemic cells, including the phosphatidylinositol-3-kinase-Akt-mechanistic target of rapamycin (PI3K-Akt-mTOR) pathway that seems to be important both in normal and leukemic hematopoiesis [4–6]. Such abnormal signaling can be initiated by various mechanisms, e.g., various oncogenes or mutated receptor tyrosine kinases, cell adhesion molecules, G-protein-coupled receptors (GPCR) or other cytokine receptors.

Primary AML cell populations may include various subclones at the time of diagnosis, and relapse can occur due to regrowth of the originally dominating clone, a subclone detectable at the

time of first diagnosis, or a new clone derived either from the original clone or from remaining preleukemic stem cells [7,8]. Detection of remaining leukemic cells with cytogenetic abnormalities at the time of allogeneic stem cell transplantation is associated with an adverse prognostic impact even when residual disease cannot be detected by flow cytometry, and morphological examination confirms that the patient is still in complete hematological remission [8]. One possible explanation for such a discrepancy may be the detection of a remaining minor subclone with a different immunophenotype compared with the dominating clone at the time of diagnosis [7]. The detection of subclones (i.e., clonal heterogeneity) based on karyotyping at the time of initial diagnosis is an independent adverse prognostic factor [9]. However, karyotyping for detection of clonal heterogeneity has several limitations as it is not a sensitive methodological approach and it cannot be used to detect heterogeneity for the majority of patients with normal karyotype or single cytogenetic abnormalities.

Clonal heterogeneity of AML cell populations can also be detected by flow cytometric analysis of constitutively activated intracellular signaling pathways, and this may therefore be an alternative methodological strategy [10] that can be used also for evaluation of the large group of patients with normal karyotype. Such analyses may also reflect a functional heterogeneity caused by driving mutations and thereby reflect characteristics that are important for leukemogenesis and/or chemosensitivity. In our present study, we therefore investigated how clonal heterogeneity is reflected in the activation status of the PI3K-Akt-mTOR pathway. This pathway was chosen because (i) it is usually activated in primary human AML cells; (ii) integrates signaling from a wide range of upstream mediators/receptors; (iii) shows crosstalk with and thereby also reflects the activation status of parallel intracellular pathways; and (iv) targets a wide range of downstream mediators that are important for essential cellular processes e.g., regulation of energy metabolism, gene transcription, protein synthesis, induction of apoptosis, and cellular communication [4,6,11]. In this context, we have therefore investigated samples from a group of 114 unselected patients to clarify whether analysis of constitutive PI3K-Akt-mTOR signaling can be used to detect clonal heterogeneity, whether such heterogeneity is a biomarker associated with any clinical or biological patient characteristics, and whether clonal heterogeneity has an independent prognostic impact.

2. Results

2.1. Clonal AML Cell Heterogeneity Reflected by PI3K-Akt-mTOR Signaling Is Seen for a Subset of Patients

In our flow cytometric analysis, we first identified the viable AML cells; this gating strategy is shown in Figure S1. The viable cell population was thereafter analyzed for expression levels of mediators and their phosphorylation. The viability of primary cells was analyzed both immediately after thawing and after the incubation steps by live dead gating. The viability did not differ significantly when comparing these two time points. The median frequency of dead cells after the incubation steps was 16.3% (range 0–48%). The viability of the AML cells did not differ when comparing AML cell samples with and without dual leukemic cell populations.

We investigated the 18 mediators from the PI3K-Akt-mTOR pathway in leukemic cell samples from 114 unselected AML patients. Dual populations were detected in samples from 49 of patients and these overall results are summarized in Figure 1. The flow cytometric evidence for clonal heterogeneity is presented more in detail in Figure S1, and it can be seen that a minor population was clearly separated from the main AML cell population for all the 49 patients.

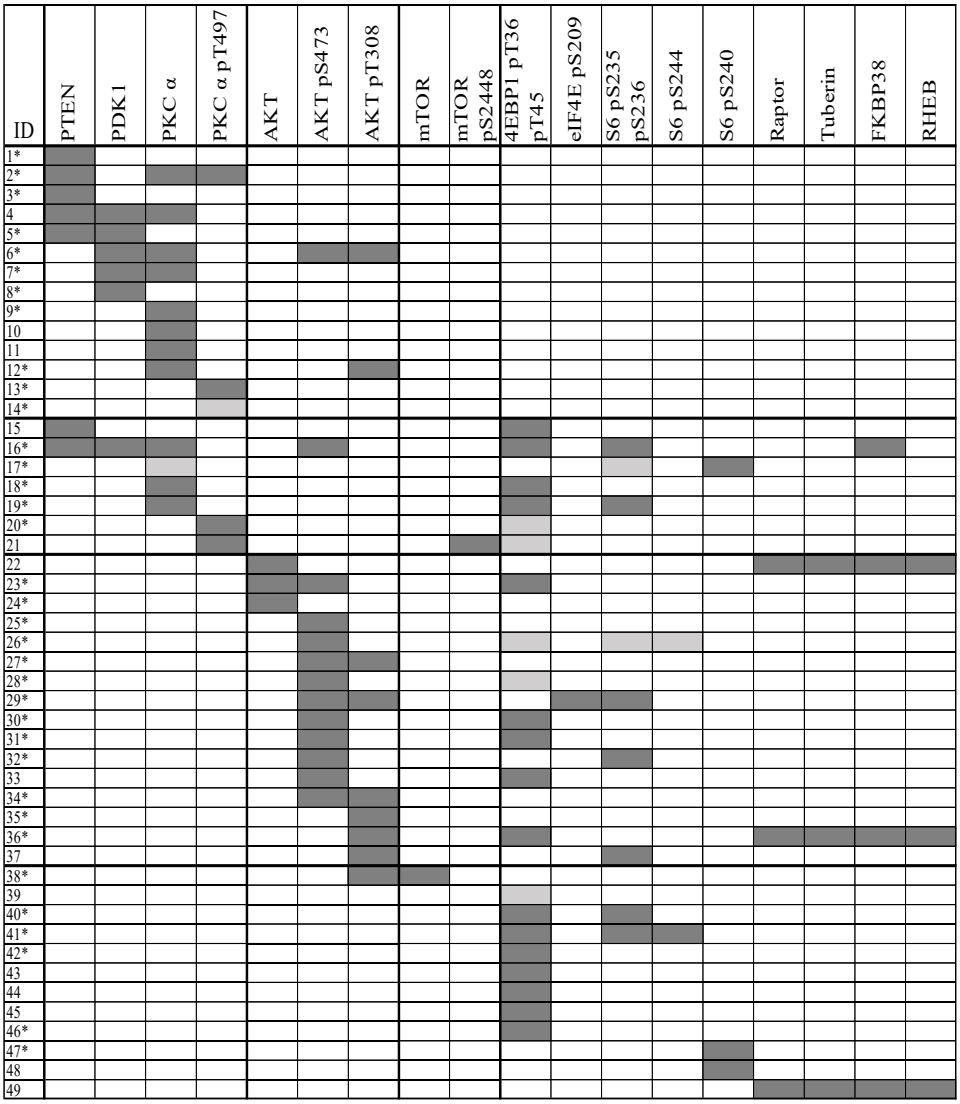

Figure 1. Clonal heterogeneity of primary human acute myeloid leukemia (AML) cell samples; an overview of the 49 patients showing dual populations when investigating activation of the phosphatidylinositol-3-kinase-Akt-mechanistic target of rapamycin (PI3K-Akt-mTOR) pathway. The cells were incubated in medium alone (all patients), with insulin alone and with insulin and a pathway inhibitor (rapamycin, GDC-0941; only an unselected subset of patients). Dark grey means that dual populations were detected in all cultures with and without treatment, and light grey indicates detection only for some cultures. Patient samples analyzed for inhibition by mTOR inhibitor rapamycin and PI3K inhibitor GDC-0941 are indicated by an asterisk (*).

For each of the 49 patients, clonal heterogeneity was detected only for some of the 18 mediators. Nineteen of the patients showed dual population for only one of any of the 18 mediators, 16 patients showed dual populations for two mediators, and dual populations for three mediators was found for seven patients. Thus, for the majority of these patients, dual AML cell populations were detected only by one or a few of the tested mediators, and dual populations for four to seven pathway mediators were observed only for seven patients.

Dual populations for mediators upstream to Akt were detected for 20 patients, whereas dual populations for Akt and mTOR were only detected for four of these 20 patients. Dual populations for mediators downstream to mTOR were detected for 10 patients. Furthermore, clonal heterogeneity reflected in constitutive Akt activation but not reflected by mediators upstream to Akt was found in 17 patients. This heterogeneity reflects differences in activation alone because the total protein levels of Akt did not differ between cell subsets. Finally, it was uncommon for mTOR total protein level or phosphorylation/activation to reflect clonal heterogeneity.

Taken together, the observations described above show that 49 out of 114 patients with clonal heterogeneity differ with regard to how the heterogeneity is reflected in the PI3K-Akt-mTOR expression/phosphorylation profile.

The patient subsets with and without clonal heterogeneity were also compared with regard to their molecular genetics (i.e., analysis of 54 different mutations), morphological as well as molecular signs of differentiation and the overall PI3K-Akt-mTOR activation profile; though the two patient subsets did not show any significant differences with regard to these biological characteristics (see Table S1).

2.2. Primary AML Cells Derived from Patients with and without dual PI3K-Akt-mTOR Cell Populations Differ in Their Global Gene Expression Profiles

We compared the global gene expression profiles for 12 AML samples with and 27 samples without dual AML cell populations. A feature subset selection (FSS) analysis was performed for identification of the most discriminative genes between the two groups, and 1209 genes were then identified (i.e., p-value <0.05, see the complete gene list is included in Table S5 and Table S6). This criterion was chosen because AML is a highly heterogeneous disease with regard to differentiation, karyotype and molecular genetics [1–3]; and this can be seen from the characteristics of our present patient cohort (Tables 1 and 2 below, Table S1). For this reason, a relatively large number of differentially expressed genes will probably be needed to distinguish between patient subsets. The identified genes were used in a hierarchical clustering model (Pearson's correlation distance measure with complete linkage; and this analysis identified two main patient subsets that corresponded to the patients with and without subpopulations. Furthermore, a correlation visualization with distance matrix displays the pairwise correlation between the 39 patients and the classification of patients into two main subsets corresponding to the subsets with and without subclones, respectively (Figure 2).

Table 1. A comparison of overall survival for patients with and without dual populations based on the analysis of PI3K-Akt-mTOR activation. The table presents the results from univariate and adjusted or multivariate analyses (Cox Proportional Hazard Model). Significant p-values in the adjusted analysis are marked in bold.

Covariate	Crude Analysis			Adjusted Analysis		
	***p*-Value**	**HR**	**95%-CI**	***p*-Value**	**HR**	**95%-CI**
Age (per decade)	<0.01	1.64	1.24–2.17	<0.01	1.69	1.22–2.36
Subpopulation versus no subpopulation	0.03	2.15	1,01–4,26	0.04	2.28	1.03–5.04
Adverse cytogenetics	0.519	0.759	0.33–1.75	0.11	0.33	0.11–1.04
NPM1-wt and Flt3-wt	NA	1 (reference)		NA	1 (reference)	
NPM1-mutated and Flt3-wt	0.92	1.62	0.58–4.56	0.693	1.24	0.42–3.75
NPM1-wt and FLT3-mutated	0.21	1.86	0.71–4.92	0.03	3.88	1.18–12.71
NPM1-mutated and FLT3-mutated	0.05	2.33	1.00–5.34	0.48	1.44	0.57–3.61

Abbreviations: CI, confidence interval; HR, hazard ratio. NPM1-wt: Nucleophosmin 1-wild type; Flt3-wt: fms-like tyrosine kinase 3-wild type.

Table 2. The biological and clinical characteristics of the 114 AML patients included in the study.

Patient characteristics			
Age		**Secondary AML**	
Median (years)	67	chemo	5
Range (years)	18–87	CML	2
		CML-RELAPSE	1
Gender		CMML	4
Females	49	*de novo*	81
Males	65	LiFraum, chemo	1
		MDS	8
		MDS, AML relapse	1
		MDS, CHEMO	1
		Myelofibrosis	3
		Polycytemia vera	1
		Relapse	5
Total	114	Relapse, chemo	1
Cell Morphology			
FAB Classification		**CD34 Receptor**	
M0	8	Negative (<20%)	30
M1	28	Positive (>20%)	78
M2	22	n.d.	6
M4	27		
M5	21		
M7	1		
n.d.	7		
Cell Genetics			
Cytogenetics		**Mutations**	
		NPM1 mutations	35
Adverse	20	Mutated	
Favorable	11	Wild-type	62
Intermediate	11	n.d.	17
Normal	60		
n.d.	12	*Flt3* mutations	
		ITD	41
		Wild-type	55
		n.d.	18

The European Leukemia Net classification was used; n.d.: not determined; CML: Chronic myeloid leukemia; CMML:Chronic myelomonocytic leukemia; FAB: French-American-British.

The differentially expressed genes were thereafter used to identify gene ontology terms that were overrepresented among the differentially expressed genes. We then selected Gene Ontology (GO) terms based on the criteria (i) False Discovery Rate (FDR) ad modum Benjamini <0.05 and (ii) *p*-values <0.05. Only two GO-terms were identified in each of the two analyses based on Biological processes or Molecular function, respectively. Based on Molecular function, the two groups G-protein coupled receptor signaling pathway ($p = 0.0000078$; 43 genes included) and Detection of chemical stimulus involved in sensory perception of smell ($p = 0.000015$; 26 genes included) were identified. Based on analysis of Biological processes we identified the two groups G-protein coupled receptor activity ($p = 0.00002$; 36 genes included) and Olfactory receptor activity ($p = 0.000024$; 26 genes included). The complete gene lists for each of these four GO terms are presented in Tables S2 and S3. None of the two patient subsets showed generally higher expression for all differentially expressed genes, but the two subsets differ in their pattern of high/low expressed genes.

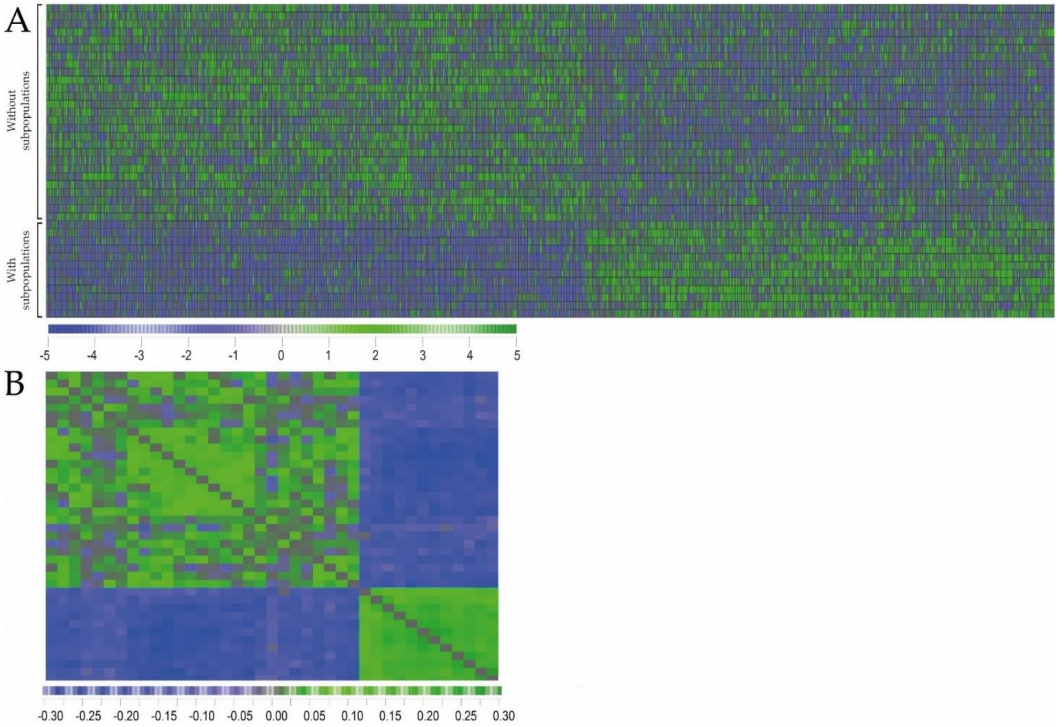

Figure 2. Comparison of global gene expression profiles and gene ontology for primary human AML cells with and without clonal heterogeneity based on the analysis of PI3K-Akt-mTOR activation. Global gene expression profiles (GEP) were available for 39 unselected AML patients included in the study, 12 samples were derived from patients showing dual cell populations and 27 leukemic cell populations did not show dual cell populations. (**A**) A feature subset selection (FSS) was performed to identify the most discriminative genes between the two groups, and 1209 genes were identified (p-value <0.05). These genes were used in a hierarchical clustering model (Pearson's correlation distance measure with complete linkage) demonstrating a highly discriminative expression pattern for the groups with and without subclones. (**B**) A correlation visualization with distance matrix displays the pairwise correlation between the 39 patients. Blue and green colors highlight the negative and positive correlation between samples. The genes found differently expressed were thereafter used to identify gene ontology terms (using the David Database for Gene Ontology) that were overrepresented among the genes differently expressed. A more detailed version is shown in Figure S3.

2.3. Detection of AML Subclones Based upon PI3K-Akt-mTOR Signaling Is Associated with Decreased Patient Survival

In a recent study, karyotyping could detect clonal heterogeneity for approximately 15% of patients, and this heterogeneity was then associated with an adverse prognosis in patients receiving intensive AML therapy [9]. We therefore investigated whether detection of subclones determined by analysis of constitutive PI3K-Akt-mTOR activation had a similar prognostic association. In our present study, we investigated a large group of unselected patients that included several elderly and unfit patients who could not receive intensive treatment [12]. However, 44 of our patients completed their planned intensive induction treatment followed by at least two consolidation cycles, autologous or allogeneic stem cell transplantation after their first diagnosis of AML. We compared the survival of 17 patients with and 27 patients without subpopulations; the overall survival was significantly higher for patients without subpopulations (Figure 3; $p = 0.027$). This association between prognosis and heterogeneity was significant both in crude (Table 1, Cox Proportional Hazard Model, $p = 0.03$) and adjusted analyses ($p = 0.04$). To summarize, the previous study [9] showed that detection of clonal heterogeneity in patients with abnormal karyotype had an adverse prognostic impact. In our present study we used an alternative methodological strategy for detection of subclones independent of the karyotype (i.e., also including patients with normal karyotype), and we could detect an association between clonal heterogeneity and adverse prognosis.

We also did an adjusted analysis of the prognostic impact of age, clonal heterogeneity, cytogenetics, *Flt3*/*NPM1* status (Table 1). After correcting for these adverse factors, clonal heterogeneity still had a significant association with survival.

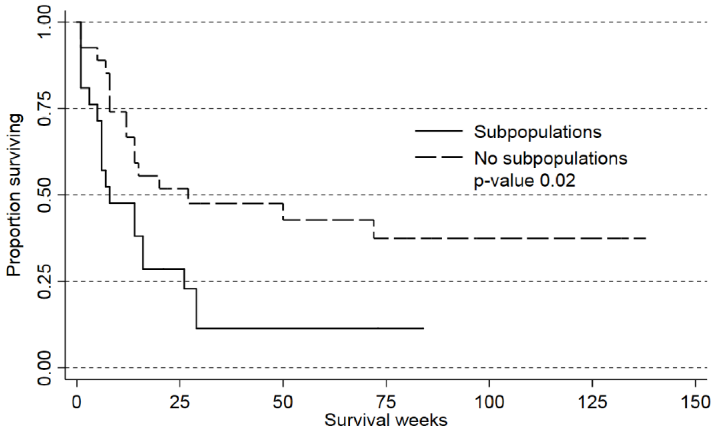

Figure 3. Overall survival of patients with (17 patients) and without (27 patients) clonal heterogeneity when investigating PI3K-Akt-mTOR pathway activation; an analysis of 44 patients who completed their intensive induction treatment followed by at least two consolidation cycles, autologous or allogeneic stem cell transplantation after their first diagnosis of AML.

3. Discussion

The PI3K-Akt-mTOR pathway shows constitutive activation in human AML and is therefore regarded as a possible therapeutic target, but despite this, the results from initial clinical studies suggest that pathway inhibitors have only modest antileukemic activity [13]. Possible explanations for this could be that patients are heterogeneous with regard to their susceptibility [14] due to differences in the crosstalk with other pathway [15], or there is clonal heterogeneity with variation in constitutive pathway activation between leukemic subclones for individual patients [7]. In the present study, we used flow cytometric analysis of PI3K-Akt-mTOR activation to detect clonal

heterogeneity. We investigated a large group of samples derived from unselected AML patients (i.e., the large majority of the patients had normal karyotype or only a single cytogenetic abnormality), and clonal heterogeneity was detected for the majority of these patient samples. However, for each of these patients the clonal heterogeneity was reflected in the basal expression of only one or a few of the 18 investigated pathway mediators, i.e., this heterogeneity was not associated with a difference in activation status throughout the pathway. A possible explanation for this limited pathway heterogeneity could be that the activation status of each mediator not only reflects the downstream signaling from receptor ligation, but also the crosstalk between specific mediators of the PI3K-Akt-mTOR pathway and neighboring intracellular pathways.

Most of our patients were elderly or unfit patients that could not receive intensive antileukemic treatment. Our patients are thus representative with regard to AML cell biology, but they are heterogeneous with regard to antileukemic treatment and the elderly/unfit patients usually received only disease-stabilizing or supportive treatment [12].

Aberrant expression of lymphoid markers is relatively common in AML, and according to the World Health Organization (WHO) classification an uncommon subset of acute leukemia patients also shows a mixed phenotype with both myeloid and lymphoid leukemic cell subpopulation [1]. However, among our heterogeneous AML cell populations neither patients with mixed leukemic phenotype nor aberrant expression of lymphoid markers (CD2, CD3, CD4, CD8, CD19, CD20) were detected. Furthermore, the presence of clonal heterogeneity in the PI3K-Akt-mTOR pathway showed no association with cytogenetic abnormalities, mutational status, morphological or molecular signs of differentiation. Thus, our identification of two patient subsets with and without clonal heterogeneity based on pathway activation seems independent of the conventional subclassification of AML patients.

Clonal heterogeneity can be detected by various methodological strategies [7,9,10], including karyotyping which identified 15% of patients with clonal heterogeneity [9]. We observed clonal heterogeneity for 49 out of 114 patients (42%) using our flow cytometric approach and this higher frequency is most likely due to an additional molecular heterogeneity not reflected by karyotyping.

We used flow cytometry to evaluate constitutive PI3K-Akt-mTOR activation and the criteria for detection of two AML cell subpopulations were (i) two distinct and clearly separated cell subsets for one or more of the 18 mediators; and (ii) the smallest subpopulation being at least 20% of the total viable cell population. Considering the limited number of metaphases analyzed by karyotyping, the study by Bochtler et al. [9] suggests that the clonal heterogeneity has to reach a certain (i.e., detectable) level to have a prognostic impact. By using 20% as our cutoff it was possible to identify distinct cell populations, and this cutoff has also been used to define positivity for differentiation markers by flow cytometry [16].

PI3K-Akt-mTOR is a part of a complex signaling network involving several single mediators and showing crosstalk with other pathways [13]. We selected 18 flow cytometric parameters that reflect the status of the main track of the pathway, including absolute levels and phosphorylation status of important upstream mediators, the key mediators Akt and mTOR and mediators downstream to mTOR (Table S1). It can be argued that for example phosphorylated PTEN should also be included, even though this mediator may be less important in AML than in many other malignancies at least with regard to PTEN mutations; PTEN seems to be mutated in less than 1% of AML patients. However, PDK1 is located between PI3K and Akt in the main pathway track, and the activity of PTEN will be reflected in the immediately downstream PDK1 phosphorylation [4].

The viability of the cryopreserved cells after thawing was determined for all patients and despite a variation between patients, all samples showed more than 50% viable cells. One would expect less than 100% viability for most patients when testing cryopreserved cells [17] and the viability did not differ between patients with and without detectable subclones. Thus, the detection of subclones is not associated with altered susceptibility of the AML cells to stress-induced or spontaneous in vitro apoptosis.

The AML cell population from a single patient may consist of various subclones [7,9] that can be detected by single cell analyses (i.e., flow cytometry) of constitutive pathway activation [10]. We

never observed more than two subsets (dual populations) in a patient sample, independently of which mediator was analyzed. However, our methodological approach does not allow an accurate estimation of the total number of subpopulations when dual populations were detected for two or more pathway mediators because the same two subpopulations may be detected when analyzing various pathway mediators, or different subpopulations identified by different mediators. For this reason, we could classify our patients as either showing or not showing clonal heterogeneity, but we could not estimate the number of subclones in this assay.

We compared the global gene expression profiles for AML cell samples with and without clonal heterogeneity; gene expression data were then available only for an unselected subset of our patients. We first investigated whether gene expression profiling could be used to identify patients with and without detectable clonal heterogeneity in the flow cytometric analysis. AML is a very heterogeneous disease [12], and as would be expected the patient heterogeneity is also illustrated by the clinical and biological characteristics (Tables 1 and 2, Table S2) of the patients included in our present study. We performed a clustering analysis (Figure 2) based on the differentially expressed genes and identified two patient subsets corresponding to the patient samples with and without dual subpopulations. Thus, despite the extensive heterogeneity of the AML disease, the patient subsets that are showing dual populations can be identified by analysis of gene expression profiles.

To further investigate the biological differences between patients with and without clonal heterogeneity, we performed a GO term analysis, and we then identified the terms with FDR <0.05 and statistical significance with p <0.05. This analysis was based on a correction for multiple hypothesis testing and ontologies including few genes were left out. We identified only two GO-terms both when the analysis was based on Biological processes (G-protein receptor signaling, Detection of stimulus smell) and on Molecular function (G-protein receptor activity, Olfactory receptor activity).

Our present studies showed that patients with and without clonal heterogeneity differed with regard to the expression of genes encoding olfactory receptor components and proteins involved in downstream signaling from G-protein coupled receptors (GPCRs) signaling. The olfactory receptors are a subset of the GPCRs [18–20]. Olfactory receptors are expressed in many tissues and by many different normal cells outside the olfactory system. This includes several normal leukocytes (e.g., monocytes, neutrophil granulocytes, erythrocytes, T and B cells, NK cells) [21] as well as adipose tissue, heart, skeletal muscles, kidney, prostate, gastrointestinal tract, liver, lung, several endocrine organs, ovary and testes [22]. It can also be expressed by various malignant cells [23–28], including human AML cells [29,30]. Thus, both normal and malignant myeloid cells are among the cells that show ectopic expression of olfactory receptors, but to the best of our knowledge, our present study is the first to describe an association between olfactory receptor expression and prognosis in a hematological malignancy. Furthermore, within these tissues certain olfactory receptors seem to have a more limited expression, whereas other receptors have a more widespread expression [22]. Limited data are available for the functional roles of ectopic olfactory receptors, but the overall data suggest that they can be involved in modulation and regulation of important cellular processes like cell survival/apoptosis induction, cell-cell recognition, proliferation and migration [22,28]. For cancer cells, these receptors seem to influence migration and development of metastases from solid tumors [31–34]. Our present study is one of the first to describe the broad olfactory receptor expression by primary human AML cells, and to the best of our knowledge, it is the first to suggest an association between chemosensitivity/survival in a hematological malignancy.

Ectopically expressed olfactory receptors influence signaling through several signal transduction pathways, including the PI3K-Akt-mTOR pathway as well as NFκB, MEK-ERK1/2 and p42/44, and they seem to regulate calcium metabolism [22]. Several of these pathways are also important in AML [35]. The bone marrow ligands of these receptors are not known, but one possibility is binding of various metabolites [36]. Several metabolites and metabolic intermediates are ligands for olfactory receptors, including lactate, short- and medium-chain fatty acids, ketones and steroids [22,28,37]. Other metabolic intermediates share structural similarities with known ligands [22,28]. Our hypothesis is that the ectopic olfactory receptors function as metabolic sensors and the

metabolic/metabolite profile of the bone marrow microenvironment thereby becomes important for leukemogenesis and/or AML cell chemosensitivity. The local metabolic profile will probably also be influenced by the systemic metabolic profile, and this may explain why differences in the systemic (i.e., serum) metabolite profile has a prognostic impact in human AML [38]. Finally, these receptors can also be expressed by various stem cells [39], but their expression at the protein level has not been characterized, and it is not known whether they are expressed by leukemic or normal hematopoietic stem cells either. However, the observation that ectopic olfactory receptors are expressed at all stages of erythroid cell development [28,40] suggests that they have a more widespread expression at least in normal hematopoietic cells.

Clonal heterogeneity detected by cytogenetic analysis has an adverse prognostic impact [9]. We used a methodological approach that enabled us to investigate all patients with respect to clonal heterogeneity, including the large group of patients with normal karyotype and the same single abnormality in all investigated AML cells. Our present analysis also showed an association between clonal heterogeneity and adverse prognosis, and clonal heterogeneity was an independent prognostic parameter in our present study of a patient cohort mainly including patients with normal karyotype or favorable genetic abnormalities and only a small subset of patients having a complex karyotype.

Karyotyping is not suitable for rapid detection of clonal heterogeneity in routine clinical practice and this methodological strategy cannot be used for the majority of patients, e.g., patients with single abnormalities or normal karyotype, whereas our present strategy based on flow cytometry can detect clonal heterogeneity within a few hours. Our present study identifies detection of heterogeneity in PI3K-Akt-mTOR activation as a possible biomarker with prognostic impact in human AML. However, the activation status of this pathway may not only be used as a biomarker; the pathway is involved in several important cellular functions and therefore it may be a possible therapeutic target in human AML.

4. Materials and Methods

4.1. AML Patients

The study was approved by the Regional Ethics Committee (REK) (REK III 060.02, 10 June 2002; REK Vest 215.03, 12 March 2004; REK III 231.06, 15 March 2007; REK Vest 2013/634, 19 March 2013; REK Vest 2015/1410, 19 June 2015), The Norwegian Data Protection Authority 02/1118-5, 22 October 2002, and The Norwegian Ministry of Health 03/05340 HRA/ASD, 16 February 2004. All AML cell samples were collected after written informed consent. The clinical and biological characteristics of the 114 unselected patients included in the study are summarized in Table 2 (49 females and 65 males; median age 67 years with range 18–87 years). All patients had a high number and/or percentage of peripheral blood blasts; leukemic peripheral blood mononuclear cells could therefore be isolated by density gradient separation alone (Lymphoprep, Axis-Shield, Oslo, Norway) and generally contained at least 95% leukemic blasts. The contaminating cells were small lymphocytes. These enriched AML cells were stored in liquid nitrogen until used in the experiments [41].

4.2. Flow-Cytometric Analysis of PI3K-Akt-mTOR Activation

Pathway-associated clonal heterogeneity was defined as flow-cytometric detection of at least one extra cell population including at least 20% of the leukemic cells and being detection by the analysis of at least one of the PI3K-Akt-mTOR mediators.

Flow cytometry was used to examine the constitutive expression of 18 mediators in the PI3K-Akt-mTOR pathway/network in the AML cells. Cryopreserved and thawed primary leukemic cells were incubated for 20 minutes in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) before being directly fixed in 1.5% paraformaldehyde (PFA) and permeabilized with 100% ice-cold methanol. The cells were thereafter rehydrated by adding 2 ml phosphate-buffered saline (PBS), gently re-suspended and then centrifuged. The cell pellet was washed twice with 2 mL PBS and resuspended in 150 μL PBS supplemented with 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA). Washed cells were blocked with immunoglobulin (Octagam; Octapharma, Jessheim, Norway) and

1% BSA, and thereafter split evenly into nineteen new tubes (1×10^5 cells per sample) before staining. All staining panels included the same live/dead discriminator, either FITC or Alexa Fluor® 647 Mouse anti-Cleaved PARP (Asp214); a blank sample was also included. Three directly conjugated dyes were used (Table S4): (i) Alexa Fluor® 647 was used for PTEN, PDPK1 (pS241), PKCα, PKCα (pT497), Akt (pS473), 4EBP1 (pT36/pT45), elF4E (pS209), S6 (pS244), and mTOR; (ii) phycoerythrin (PE) for Akt total, Akt (pT308), mTOR (pS2448), and S6 (pS240); and (iii) V450 for S6 (pS235/pS236). Antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ, USA) except for anti-mTOR that was purchased from Cell Signal Technology (Danvers, MA, USA). Four of the antibodies were unconjugated (anti Raptor, Tuberin, FKBP38, and RHEB; all from Abcam; Cambridge, UK) and required secondary antibody-conjugated Alexa Fluor® 647 (Franklin Lakes, NJ, USA). Together all these mediators represent main steps in the PI3K-Akt-mTOR pathway and they were selected to provide an extended phosphorylation profile of this pathway.

Viable leukemic cells were identified and analyzed in flow cytometric analysis based on live/dead staining, doublet discrimination, CD45 staining and forward/side scatter. Dead cells, doublets and the minor contaminating lymphocyte populations were excluded from further analyses. Our flow cytometric analyses were thus based on the analysis of viable leukemic cells, and the gating strategy used to identify the viable cell population is shown in Figure S2.

AML cells were also incubated with human insulin 10 μg/mL (Sigma-Aldrich, St. Louis, MO, USA); the non-specific mTOR inhibitor rapamycin (LC Laboratories, Woburn, MA, USA) and the PI3K class I specific inhibitor GDC-0941 (Axon Medchem BV, Groningen, Netherlands) were added at a final concentration of 100 nM. Cells were incubated with these agents for 15 minutes before fixation/staining and flow cytometric analysis as described above.

4.3. Analysis of Global Gene Expression Profiles and Mutation Analyses

Our methods for RNA preparation, labelling and microarray hybridization have been described in detail previously [42]. All microarray experiments were performed using the Illumina iScan Reader, which is based upon fluorescence detection of biotin-labelled cRNA that was hybridized to the HumanHT-12 V4 Expression BeadChip according to the manufacturer's instructions. The HumanHT-12 V4 BeadChip targets 47 231 probes that are mainly derived from genes in the NCBI RefSeq database (Release 38). Preprocessing, normalization and annotations of the microarray data has also been described in detail in this previous publication; the data from the array scanning were investigated in GenomeStudio and J-Express 2012 for quality control measures.

Submicroscopic mutation profiling of 54 genes frequently mutated in myeloid leukemias was done using the Illuminas TruSight Myeloid Gene Panel and sequenced using the MiSeq system and reagent kit v3 (all from Illumina, San Diego, CA, USA) as described in detail previously [42]. The methods for fragment analysis of *Flt3* exon 14–15 and *NPM1* exon 12 and analysis of *CEBPA* mutations have also been described previously [42].

4.4. Data Collection, Bioinformatical and Statistical Analyses

Flow cytometry analysis was acquired on a BD FACSVerse 8-color flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA) and data analysis performed using FlowJo 10.0.7 software (Tree Star, Inc., Ashland, OR, USA). The J-Express software (J-Express 2012, MolMine AS, Bergen, Norway) was used for bioinformatical analyses. For unsupervised hierarchical clustering analysis, all values were calculated using fold change on the Inverse hyperbolic sine (Arcsinh) scale and with median values for each target group as control. Complete linkage (euclidean distance) and Pearson correlation were used as linkage method and distance measurement, respectively. Statistical analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) version 23 (IBM Corporation, Armonk, NY, USA). The Mann-Whitney *U*-test was used to compare different groups; the Chi square/Fischer's exact test for analysis of categorized data and the Kendall's tau-b correlation test for correlation analyses. The Cox Proportional Hazard Model was used for analysis of survival data. p-values <0.05 were regarded as statistically significant. The gene ontology enrichment analysis of differentially expressed genes was performed by using online bioinformatics tools of DAVID

Bioinformatics Resources 6.8, Laboratory of Human Retrovirology and Immunoinformatics (LHRI) [43,44].

5. Conclusions

To conclude, clonal heterogeneity in human AML cell samples is reflected in the activation of mediator in the PI3K-Akt-mTOR pathway, and this heterogeneity had an independent prognostic impact in our patient cohort. Our present study suggests that that clonal heterogeneity as reflected in intracellular signaling pathways should be further investigated as a possible adverse prognostic biomarker in future clinical studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/10/9/332/s1. Figure S1: Detection of clonal heterogeneity for 49 AML patients; Figure S2. Cell preparation and gating strategy; Figure S3. A correlation visualization with distance matrix; Table S1. A comparison of patients with and without dual AML cell populations; studies of molecular genetics, differentiation and pathway activation profiles; Table S2. The GO-term showing significant differences when comparing AML cell populations for patients with and without clonal heterogeneity; the comparison being based on analysis of Molecular function; Table S3. The GO-term showing significant differences when comparing AML cell populations for patients with and without clonal heterogeneity; Table S4. Monoclonal antibodies used in the flow-cytometric studies; Table S5. The complete gene list from patients with dual AML cell populations; Table S6. The complete gene list from patients without dual AML cell populations.

Author Contributions: Conceptualization, Ø.B; Methodology, I.N.; Software, I.N.; Validation, I.N.; Formal Analysis, I.N., H.R., T.H.A.T., and Ø.B.; Investigation, I.N. and K.J.H.; Resources, Ø.B.; Data Curation, I.N., H.R., T.H.A.T., and Ø.B.; Writing-Original Draft Preparation, I.N. and Ø.B.; Writing-Review & Editing, I.N., H.R., T.H.A.T., K.J.H. and Ø.B.; Visualization, I.N., Ø.B. K.J.H. and HR.; Supervision, Ø.B.; Project Administration, Ø.B.; Funding Acquisition, Ø.B.

Funding: This research was funded by the University of Bergen, Helse-Vest [grant 911788 and 912051] and the Norwegian Cancer Society [grants 100933, 17813, 62370, 62371, 145008, 15752 and 145007].

Acknowledgments: The technical assistance of Karen Marie Hagen and Kristin Paulsen Rye is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials: Clonal Heterogeneity Reflected by PI3K-AKT-mTOR Signaling in Human Acute Myeloid Leukemia Cells and its Association with Adverse Prognosis

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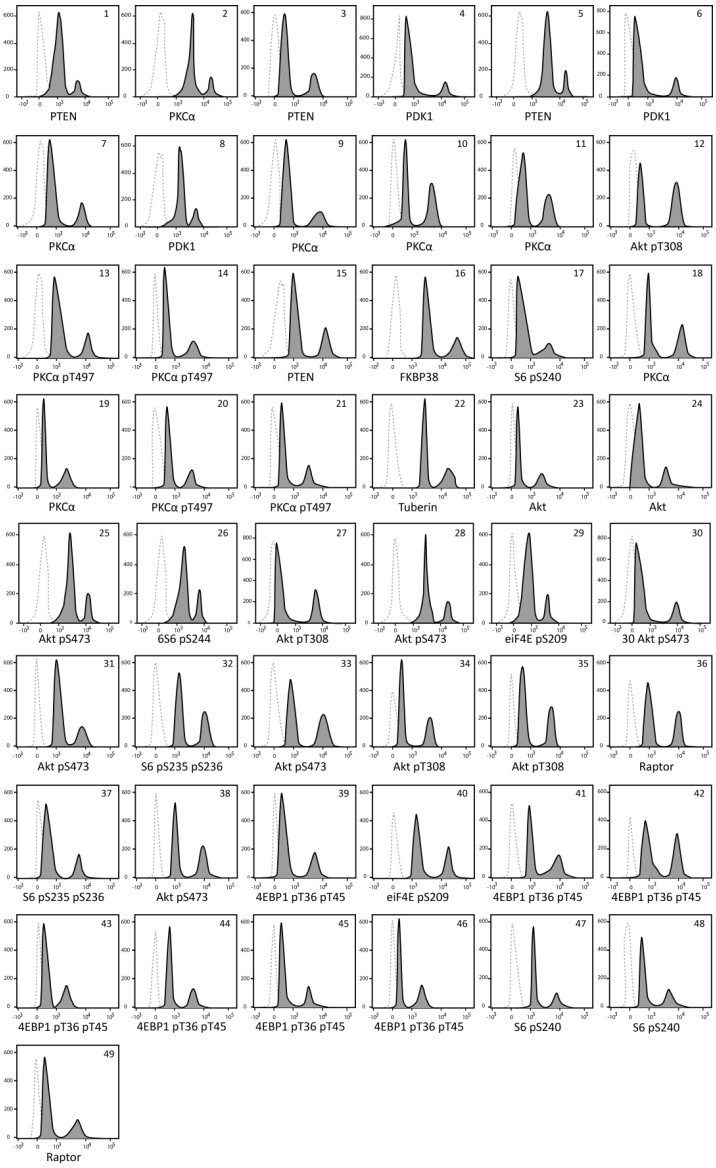

Figure S1. Detection of clonal heterogeneity for 49 acute myeloid leukemia (AML) patients; the results from representative flow cytometric analyses of phosphatidylinositol-3-kinase-Akt-mechanistic target of rapamycin (PI3K-Akt-mTOR) activation. For each patient clonal heterogeneity was detected by analysis

of at least one mediator in the PI3K-Akt-mTOR pathway. Patient ID is shown in the upper right corner of each histogram. The figure documents the detection of dual populations for all patients, showing the results from one representative flow cytometric analysis for each of these 49 patients. The Y-axis represents the amount of cells, and the X-axis represents the fluorescence intensity. The stippled line shows the negative/unstained controls.

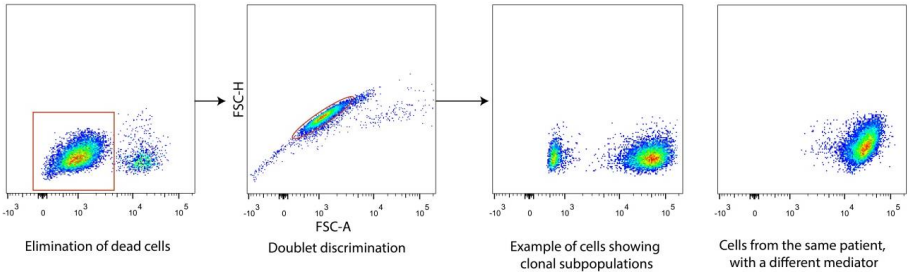

Figure S2. Cell preparation and gating strategy. Flow cytometry was used for examination of the constitutive expression of the mediators in the PI3K-Akt-mTOR pathway/network in primary AML cells. Cryopreserved cells were thawed and washed before suspension cultures were prepared as described in Materials and methods. Briefly, cryopreserved and thawed primary leukemic cells were incubated for 20 minutes in RPMI-1640 (Sigma-Aldrich) before being directly fixed in 1.5% paraformaldehyde (PFA) and permeabilized with 100% ice-cold methanol. The cells were thereafter rehydrated by adding 2 mL phosphate buffered saline (PBS), gently re-suspended and then centrifuged. The cell pellet was washed twice with 2 mL PBS and resuspended in 150 µL PBS supplemented with 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, Missouri). Washed cells were blocked with immunoglobulin (Octagam; Octapharma, Jessheim, Norway) and 1% bovine serum albumin (BSA), and thereafter split evenly into nineteen new tubes (1 x 10^5 cells per sample) before staining. The first step in the gating was to distinguishing populations of cells based on their forward and side scatter properties. Doublet discrimination was performed based on both forward and side scatter, and elimination of dead cells was performed based on the live/dead discriminator, either FITC or Alexa Fluor® 647 Mouse anti-Cleaved PARP (Asp214). The identified viable cells were further analyzed based on each of the mediators/antibodies used in the study. The colors refer to the density of the cells relative to one another, where blue and green correspond to areas of lower cell density, yellow is mid-range, and red and orange are areas of high cell density.

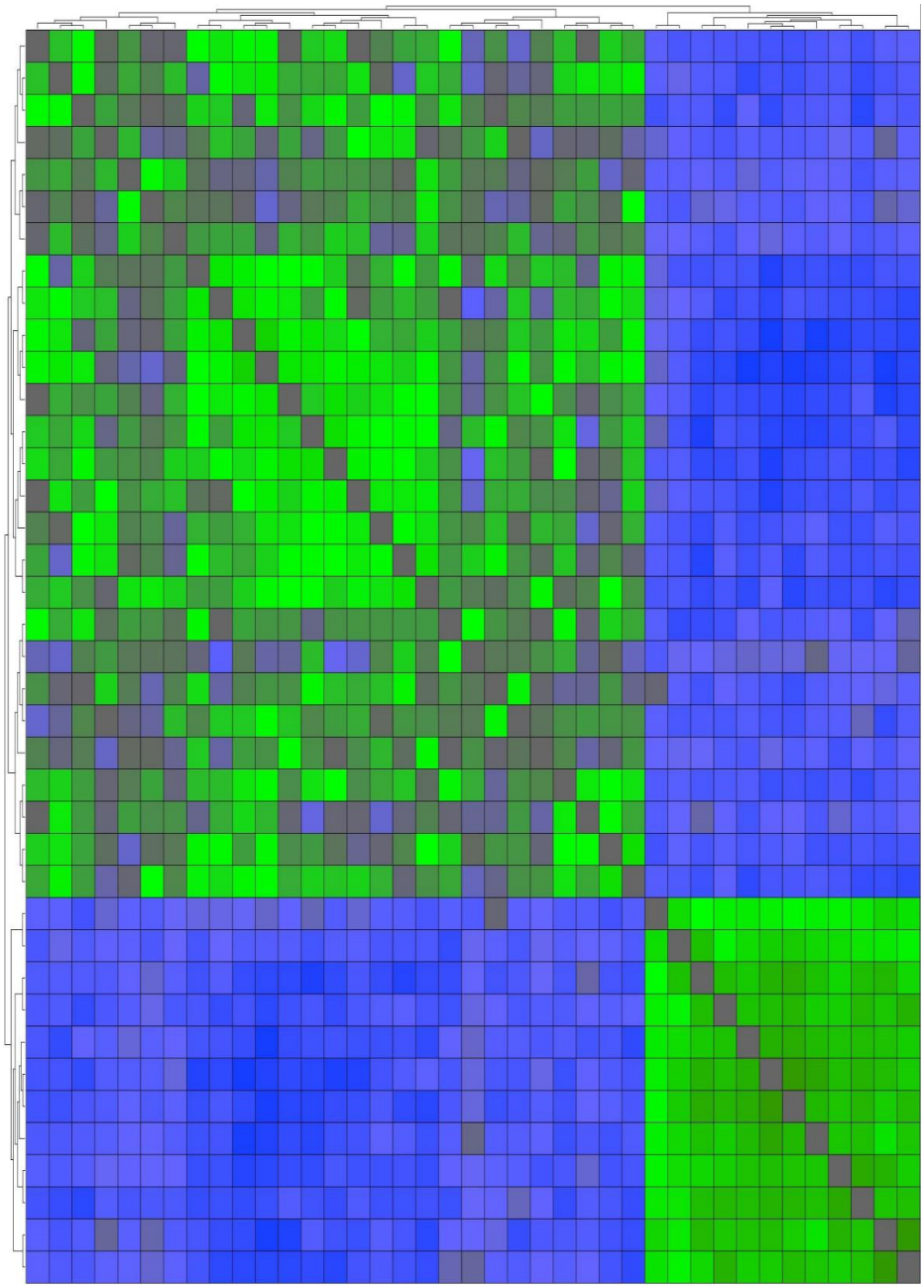

Figure S3. A correlation visualization with distance matrix displays the pairwise correlation between the 39 patients. Blue and green colors highlight the negative and positive correlation between samples. The genes found differently expressed were thereafter used to identify gene ontology terms (using the David Database for Gene Ontology) that were overrepresented among the genes differently expressed。

Table S1. A comparison of patients with and without dual AML cell populations; studies of molecular genetics, differentiation and pathway activation profiles. The two subsets do not differ with regard to frequencies of mutations, expression of differentiation Markers or level of PI3K-Akt-mTOR Activation.

Additional Analysis of AML-Associated Mutations		
Classification of Mutations and Mutated Genes[1]	**Dual Population**	**Single Population**
NPM1	28 %	32 %
Activated signaling Flt3-ITD, Flt3-TKD, NRAS, KRAS, JAK2, PTPN11	67 %	64 %
Tumor Suppressor TP53, WT1, CUX1, IKZF1, PHF6	33 %	24%
DNA methylation DNMT3A, TET2, IDH1, IDH2	67 %	48 %
Chromatin modifiers ASXL1, EZH2, GATA2	22 %	24 %
Myeloid transcription factors RUNX1, CEBPA	28 %	24 %
Spliceosome SRSF2, SF3B1, STAG2, RAD21, BCOR, BCORL1, CSF3R, ZRSR2	39 %	24 %
Cohesin SMC1A	0	4 %
Others NOTCH1, SETBP1	0	4%
Molecular and Morphological Signs of Differentiation		
Expression of differentiation markers. According to the WHO classification a minor subset of acute leukemia patients show expression of both myeloid and lymphoid markers by their leukemic cells, either as expression of both myeloid and lymphoid markers by the same cells or as separate myeloid and lymphoid subpopulations [2]. However, patients with and without dual populations in the PI3K-Akt-mTOR pathway activation analysis did not differ with regard to the expression of T cell (CD2, CD3, CD4, CD8) of B cell markers (CD19, CD20), and we could not detect any differences between the two patient subsets with regard to myeloid marker (DCD11b, CD11c, CD13, CD14, CD15, CD33), stem cell markers (CD34, CD117) or HLA-DR expression. **Morphological signs of differentiation.** The FAB classification did not differ between the two subsets.		
Activation Profile of The PI3K-AKT-mTor Pathway		
The constitutive activation of mediators within the PI3K-Akt-mTOR pathway varies between patients [3]. Hence, we performed a clustering analysis to identify patients with similar pathway activation profiles, but the profiles did not differ between patients with and without detectable clonal heterogeneity.		

[1] The percentages of various mutations for patients without and with dual acute myeloid leukemia (AML) cell populations are presented. A total of 54 AML-associated mutations were investigated. The table lists only those mutations that were detected in the 43 unselected patients that were investigated, i.e. 18 patients with dual AML cell populations and 25 patients without clonal heterogeneity based on pathway activation analysis. [2] Referred in [1] [3] Referred in [2].

Table S2. The GO-term showing significant differences when comparing AML cell populations for patients with and without clonal heterogeneity; the comparison being based on analysis of Molecular function. The genes included in the analysis were identified by the comparison of 12 AML patient samples with and 27 samples without evidence for clonal heterogeneity when analyzing the PI3K-Akt-mTOR pathway. Two GO-terms were then identified based; G-protein coupled receptor activity (GO:0004930; *p*-value 0.00002; 36 genes included) and Olfactory receptor activity (GO:0004984; *p*-value 0.000024; 26 genes included). Several genes were included in both these terms; genes that did not overlap between these two terms are shaded in grey. Increased or decreased gene expression for patients with detectable subpopulations when analyzing constitutive PI3K-Akt-mTOR inhibition is indicated by upward arrows (↑) and downward arrows (↓), respectively.

Molecular Function

	GO:0004930 - G-protein coupled receptor activity; p-value 0.00002; 36 genes		GO:0004984 - Olfactory receptor activity; p-value 0.000024; 26 genes
↑	G protein-coupled receptor 132(GPR132)	↓	olfactory receptor family 1 subfamily D member 4 (gene/pseudogene)(OR1D4)
↑	G protein-coupled receptor 139(GPR139)	↓	olfactory receptor family 1 subfamily J member 2(OR1J2)
↓	G protein-coupled receptor 176(GPR176)	↓	olfactory receptor family 1 subfamily L member 8(OR1L8)
↑	G protein-coupled receptor 183(GPR183)	↑	olfactory receptor family 10 subfamily G member 3(OR10G3)
↑	G protein-coupled receptor 34(GPR34)	↓	olfactory receptor family 12 subfamily D member 3(OR12D3)
↓	G protein-coupled receptor class C group 5 member C(GPRC5C)	↓	olfactory receptor family 2 subfamily A member 4(OR2A4)
↓	G protein-coupled receptor class C group 6 member A(GPRC6A)	↓	olfactory receptor family 2 subfamily A member 42(OR2A42)
↓	cadherin EGF LAG seven-pass G-type receptor 1(CELSR1)	↓	olfactory receptor family 2 subfamily G member 3(OR2G3)
↓	frizzled class receptor 7(FZD7)	↓	olfactory receptor family 2 subfamily M member 4(OR2M4)
↓	olfactory receptor family 1 subfamily D member 4 (gene/pseudogene)(OR1D4)	↓	olfactory receptor family 2 subfamily T member 11 (gene/pseudogene)(OR2T11)
↓	olfactory receptor family 1 subfamily J member 2(OR1J2)	↓	olfactory receptor family 2 subfamily T member 12(OR2T12)
↓	olfactory receptor family 1 subfamily L member 8(OR1L8)	↑	olfactory receptor family 3 subfamily A member 3(OR3A3)
↑	olfactory receptor family 10 subfamily G member 3(OR10G3)	↑	olfactory receptor family 4 subfamily C member 16 (gene/pseudogene)(OR4C16)
↓	olfactory receptor family 12 subfamily D member 3(OR12D3)	↓	olfactory receptor family 4 subfamily C member 3(OR4C3)
↓	olfactory receptor family 2 subfamily A member 4(OR2A4)	↓	olfactory receptor family 4 subfamily C member 45(OR4C45)
↓	olfactory receptor family 2 subfamily A member 42(OR2A42)	↓	olfactory receptor family 4 subfamily F member 29(OR4F29)
↓	olfactory receptor family 2 subfamily G member 3(OR2G3)	↑	olfactory receptor family 5 subfamily AU member 1(OR5AU1)
↓	olfactory receptor family 2 subfamily M member 4(OR2M4)	↑	olfactory receptor family 5 subfamily H member 15(OR5H15)
↓	olfactory receptor family 2 subfamily T member 11 (gene/pseudogene)(OR2T11)	↑	olfactory receptor family 5 subfamily L member 1 (gene/pseudogene)(OR5L1)
↓	olfactory receptor family 2 subfamily T member 12(OR2T12)	↓	olfactory receptor family 51 subfamily G member 2(OR51G2)
↑	olfactory receptor family 3 subfamily A member 3(OR3A3)	↓	olfactory receptor family 52 subfamily L member 1(OR52L1)
↑	olfactory receptor family 4 subfamily C member 16 (gene/pseudogene)(OR4C16)	↑	olfactory receptor family 6 subfamily Y member 1(OR6Y1)
↓	olfactory receptor family 4 subfamily C member 3(OR4C3)	↓	olfactory receptor family 8 subfamily D member 4(OR8D4)
↓	olfactory receptor family 4 subfamily C member 45(OR4C45)	↓	olfactory receptor family 8 subfamily G member 2(OR8G2)
↓	olfactory receptor family 4 subfamily F member 29(OR4F29)	↑	olfactory receptor family 8 subfamily J member 1(OR8J1)
↑	olfactory receptor family 5 subfamily AU member 1(OR5AU1)	↑	olfactory receptor family 9 subfamily I member 1(OR9I1)

↑	olfactory receptor family 5 subfamily H member 15(OR5H15)
↑	olfactory receptor family 5 subfamily L member 1 (gene/pseudogene)(OR5L1)
↓	olfactory receptor family 51 subfamily G member 2(OR51G2)
↓	olfactory receptor family 52 subfamily L member 1(OR52L1)
↑	olfactory receptor family 6 subfamily Y member 1(OR6Y1)
↓	olfactory receptor family 8 subfamily D member 4(OR8D4)
↓	olfactory receptor family 8 subfamily G member 2(OR8G2)
↑	olfactory receptor family 8 subfamily J member 1(OR8J1)
↑	olfactory receptor family 9 subfamily I member 1(OR9I1)
↑	relaxin/insulin like family peptide receptor 1(RXFP1)

Table S3. The GO-term showing significant differences when comparing AML cell populations for patients with and without clonal heterogeneity; the comparison being based on analysis of Biological Processes. The genes included in the analysis were identified by the comparison of 12 AML patient samples with and 27 samples without evidence for clonal heterogeneity when analyzing the PI3K-Akt-mTOR pathway. Two GO-terms were then identified based; G-protein coupled receptor signaling pathway (GO:0007186; *p*-value 0.0000078; 43 genes) and Detection of chemical stimulus involved in sensory perception of smell (GO:0050911; *p*-value 0.000015; 26 genes). Several genes were included in both these terms; genes that did not overlap between these two terms are shaded in grey. Increased or decreased gene expression for patients with detectable subpopulations when analyzing constitutive PI3K-Akt-mTOR inhibition is indicated by upward arrows (↑) and downward arrows (↓), respectively.

Biological Processes			
GO:0007186 - G-protein coupled receptor signaling pathway; p-value 0.0000078; 43 genes		**GO:0050911 - Detection of chemical stimulus involved in sensory perception of smell; p-value 0,000015; 26 genes**	
↓	C-C motif chemokine ligand 15(CCL15)	↓	olfactory receptor family 1 subfamily D member 4 (gene/pseudogene)(OR1D4)
↑	G protein-coupled receptor 132(GPR132)	↓	olfactory receptor family 1 subfamily J member 2(OR1J2)
↑	G protein-coupled receptor 139(GPR139)	↓	olfactory receptor family 1 subfamily L member 8(OR1L8)
↓	G protein-coupled receptor 176(GPR176)	↑	olfactory receptor family 10 subfamily G member 3(OR10G3)
↑	G protein-coupled receptor 183(GPR183)	↓	olfactory receptor family 12 subfamily D member 3(OR12D3)
↑	G protein-coupled receptor 34(GPR34)	↓	olfactory receptor family 2 subfamily A member 4(OR2A4)
↓	G protein-coupled receptor class C group 5 member C(GPRC5C)	↓	olfactory receptor family 2 subfamily A member 42(OR2A42)
↓	G protein-coupled receptor class C group 6 member A(GPRC6A)	↓	olfactory receptor family 2 subfamily G member 3(OR2G3)
	NLR family pyrin domain containing 6(NLRP6)	↓	olfactory receptor family 2 subfamily M member 4(OR2M4)

↓ apolipoprotein C3(APOC3)	↓ olfactory receptor family 2 subfamily T member 11 (gene/pseudogene)(OR2T11)
↓ cadherin EGF LAG seven-pass G-type receptor 1(CELSR1)	↓ olfactory receptor family 2 subfamily T member 12(OR2T12)
↓ frizzled class receptor 7(FZD7)	↑ olfactory receptor family 3 subfamily A member 3(OR3A3)
↑ natriuretic peptide receptor 1(NPR1)	↑ olfactory receptor family 4 subfamily C member 16 (gene/pseudogene)(OR4C16)
↓ olfactory receptor family 1 subfamily D member 4 (gene/pseudogene)(OR1D4)	↓ olfactory receptor family 4 subfamily C member 3(OR4C3)
↓ olfactory receptor family 1 subfamily J member 2(OR1J2)	↓ olfactory receptor family 4 subfamily C member 45(OR4C45)
↓ olfactory receptor family 1 subfamily L member 8(OR1L8)	↓ olfactory receptor family 4 subfamily F member 29(OR4F29)
↑ olfactory receptor family 10 subfamily G member 3(OR10G3)	↑ olfactory receptor family 5 subfamily AU member 1(OR5AU1)
↓ olfactory receptor family 12 subfamily D member 3(OR12D3)	↑ olfactory receptor family 5 subfamily H member 15(OR5H15)
↓ olfactory receptor family 2 subfamily A member 4(OR2A4)	↑ olfactory receptor family 5 subfamily L member 1 (gene/pseudogene)(OR5L1)
↓ olfactory receptor family 2 subfamily A member 42(OR2A42)	↓ olfactory receptor family 51 subfamily G member 2(OR51G2)
olfactory receptor family 2 subfamily G member 3(OR2G3)	↓ olfactory receptor family 52 subfamily L member 1(OR52L1)
↓ olfactory receptor family 2 subfamily M member 4(OR2M4)	↑ olfactory receptor family 6 subfamily Y member 1(OR6Y1)
↓ olfactory receptor family 2 subfamily T member 11 (gene/pseudogene)(OR2T11)	↓ olfactory receptor family 8 subfamily D member 4(OR8D4)
↓ olfactory receptor family 2 subfamily T member 12(OR2T12)	↓ olfactory receptor family 8 subfamily G member 2(OR8G2)
↑ olfactory receptor family 3 subfamily A member 3(OR3A3)	↑ olfactory receptor family 8 subfamily J member 1(OR8J1)
↑ olfactory receptor family 4 subfamily C member 16 (gene/pseudogene)(OR4C16)	↑ olfactory receptor family 9 subfamily I member 1(OR9I1)
↓ olfactory receptor family 4 subfamily C member 3(OR4C3)	
↓ olfactory receptor family 4 subfamily C member 45(OR4C45)	
↓ olfactory receptor family 4 subfamily F member 29(OR4F29)	
↑ olfactory receptor family 5 subfamily AU member 1(OR5AU1)	
↑ olfactory receptor family 5 subfamily H member 15(OR5H15)	
↑ olfactory receptor family 5 subfamily L member 1 (gene/pseudogene)(OR5L1)	
↓ olfactory receptor family 51 subfamily G member 2(OR51G2)	
↓ olfactory receptor family 52 subfamily L member 1(OR52L1)	
↑ olfactory receptor family 6 subfamily Y member 1(OR6Y1)	

↓	olfactory receptor family 8 subfamily D member 4(OR8D4)
↓	olfactory receptor family 8 subfamily G member 2(OR8G2)
↑	olfactory receptor family 8 subfamily J member 1(OR8J1)
↑	olfactory receptor family 9 subfamily I member 1(OR9I1)
↑	platelet derived growth factor receptor like(PDGFRL)
↑	prostaglandin E receptor 1(PTGER1)
↓	regulator of G-protein signaling 13(RGS13)
↑	vomeronasal 1 receptor 5 (gene/pseudogene)(VN1R5)

Table S4. Monoclonal antibodies used in the flow-cytometric studies.

Antibody	Manufacturer	Specificity
Alexa Fluor® 647 Mouse anti-PTEN	BD Biosciences	Recognizes PTEN, regardless of phosphorylation status
Alexa Fluor® 647 Mouse anti-PDPK1 (pS241)	BD Biosciences	Recognizes the phosphorylated S241 in the activation loop of human PDPK1
Alexa Fluor® 647 Mouse anti-PKCα	BD Biosciences	Recognizes PKCα , regardless of phosphorylation status
Alexa Fluor® 647 Mouse anti-PKCα (pT497)	BD Biosciences	Recognizes the phosphorylated T497 in the kinase domain of human PKC a
PE Mouse anti-Akt1	BD Biosciences	Recognizes Akt1, regardless of phosphorylation status
Alexa Fluor® 647 Mouse anti-Akt (pS473)	BD Biosciences	Recognizes Akt phosphorylated at S473
PE Mouse Anti-Akt (pT308)	BD Biosciences	Recognizes Akt phosphorylated at T308
Anti-RHEB antibody	Abcam	Specifically detects Rheb, regardless of phosphorylation status
Anti-Tuberin antibody	Abcam	Specifically detects Tuberin/TSC2
mTOR (7C10) Rabbit mAb (Alexa Fluor® 647 Conjugate)	Cell Signaling Technology	Detects endogenous levels of total mTOR protein
PE Mouse Anti-mTOR (pS2448)	BD Biosciences	Recognizes mTOR that is phosphorylated at S2448
Anti-Raptor antibody	Abcam	Specifically detects raptor, regardless of phosphorylation status
Anti-FKBP38 antibody	Abcam	Specifically detects FKBP38, regardless of phosphorylation status
Alexa Fluor® 647 Mouse anti-4EBP1 (pT36/pT45)	BD Biosciences	Recognizes the phosphorylated T36 and T45 of activated human 4EBP1
Alexa Fluor® 647 Mouse anti-elF4E (pS209)	BD Biosciences	Recognizes the phosphorylated S209 (pS209) of eIF4E
Alexa Fluor® 647 Mouse anti-S6 (pS244)	BD Biosciences	Specifically detects the S6 ribosomal protein phosphorylated at S244
PE Mouse anti-S6 (pS240)	BD Biosciences	Specifically detects the S6 ribosomal protein phosphorylated at S240
V450 Mouse anti-S6 (pS235/pS236)	BD Biosciences	Specifically detects the S6 ribosomal protein phosphorylated at S235 and S236

Table S5. The complete gene list from patients with dual AML cell populations. We compared the global gene expression profiles for 12 AML samples with and 27 samples without dual AML cell populations. A feature subset selection (FSS) analysis was performed for identification of the most discriminative genes between the two groups, and 1209 genes were then identified (i.e., p-value <0.05).

Gene ID	Symbol	Search Key	Illumina (ILM)-gene	P-value
127099	LOC127099	XM_060328.1	LOC127099	5.4429588162369E-5
100133469	LOC100133469	XM_001724642.1	LOC100133469	1.7669808120755886E-4
70	ACTC1	NM_005159.3	ACTC1	1.8889853780927157E-4
441762	LOC441762	XR_000268.2	LOC441762	1.9046862375469861E-4
29798	C2orf27A	NM_013310.3	C2ORF27A	2.295635289463627E-4
256302	C17orf103	NM_152914.1	C17ORF103	2.488848990283393E-4
100132215	LOC100132215	XM_001725462.1	LOC100132215	3.06887290218517E-4
100134277	LOC100134277	XM_001715852.1	LOC100134277	5.030423315792432E-4
402562	LOC402562	XM_939955.1	LOC402562	5.296271780355028E-4
92293	TMEM132C	XM_941994.1	TMEM132C	6.06081218427997E-4
100134261	LOC100134261	XM_001720725.1	LOC100134261	8.150840426564979E-4
125704	C18orf51	XM_945013.1	C18ORF51	8.157639850416456E-4
		Hs.582068	HS.582068	0.0010039973861543901
2857	GPR34	NM_001033513.1	GPR34	0.0011522345386179183
5602	MAPK10	NM_138980.1	MAPK10	0.0012104478969770386
1390	CREM	NM_001881.2	CREM	0.0012766444909731805
23213	SULF1	NM_015170.1	SULF1	0.0013817061034787775
127845	GOLT1A	NM_198447.1	GOLT1A	0.0014992835786023498
4143	MAT1A	NM_000429.2	MAT1A	0.00159068162706494
766	CA7	NM_005182.2	CA7	0.001644218788577064
647645	LOC647645	XM_942915.1	LOC647645	0.0019364782100578552
54801	HAUS6	NM_017645.3	HAUS6	0.0021784295379641723
64897	C12orf43	NM_022895.1	C12ORF43	0.0022888948062138475
645333	LOC645333	XM_932822.1	LOC645333	0.002298417112688192
100129122	LOC100129122	XM_001714405.1	LOC100129122	0.002358840674516944
650686	LOC650686	XM_001714190.1	LOC650686	0.002402213696133287
100130453	LOC100130453	XM_001717928.1	LOC100130453	0.0024686861240564824
728534	LOC728534	XM_496481.2	LOC728534	0.0024810022011861103
		Hs.544400	HS.544400	0.002570670446831798
		Hs.90866	HS.90866	0.002622155947935263
9578	CDC42BPB	NM_006035.2	CDC42BPB	0.0026464265370225127
390561	LOC390561	XM_931039.1	LOC390561	0.0030045102560638933
126006	PCP2	NM_174895.1	PCP2	0.003152601601927658
9994	CASP8AP2	NM_012115.2	CASP8AP2	0.003163468273950473
2266	FGG	NM_000509.4	FGG	0.003165510367807606
647307	LOC647307	XR_039752.1	LOC647307	0.0031975215144755143
100134549	LOC100134549	XM_001724842.1	LOC100134549	0.0032950797679523972
6812	STXBP1	NM_001032221.1	STXBP1	0.003339918394712154
643441	LOC643441	XM_933117.1	LOC643441	0.003445040137023792
1004	CDH6	NM_004932.2	CDH6	0.003606641166778987

646201	LOC646201	XR_038827.1	LOC646201	0.003735695619295815
124274	GPR139	NM_001002911.1	GPR139	0.0038673555386653483
		Hs.541889	HS.541889	0.003928735303692297
51557	LGSN	NM_016571.1	LGSN	0.00396746217456975
100129027	LOC100129027	XM_001726404.1	LOC100129027	0.004310105288322697
64241	ABCG8	NM_022437.2	ABCG8	0.004340420679539069
200576	PIP5K3	NM_152671.2	PIP5K3	0.004391969194981006
284359	IZUMO1	NM_182575.1	IZUMO1	0.004395986667519091
72	ACTG2	NM_001615.3	ACTG2	0.004571774525431297
		Hs.308351	HS.308351	0.004593524046106714
441873	LOC441873	XM_497653.2	LOC441873	0.004867506422327089
9247	GCM2	NM_004752.1	GCM2	0.004942484701647254
		Hs.564111	HS.564111	0.004988788091118323
148203	LOC148203	XM_938387.1	LOC148203	0.005112562979819541
441177	LOC441177	NM_001013720.1	LOC441177	0.0051398015983046535
9217	VAPB	NM_004738.3	VAPB	0.005175783078796158
391112	OR6Y1	NM_001005189.1	OR6Y1	0.0052210678682481125
338785	KRT79	NM_175834.2	KRT79	0.005267242199597024
10675	CSPG5	NM_006574.2	CSPG5	0.005338716851169746
4837	NNMT	NM_006169.2	NNMT	0.005340018415067846
731605	LOC731605	XM_001732887.1	LOC731605	0.0053742561572059306
158062	LCN6	NM_198946.2	LCN6	0.00551050561678674
		Hs.550619	HS.550619	0.005615448366994092
5903	RANBP2	NM_006267.3	RANBP2	0.005944117799023746
100134560	LOC100134560	XM_001718995.1	LOC100134560	0.00603271039077236
81575	APOLD1	NM_030817.1	APOLD1	0.0061665469488665905
55607	PPP1R9A	XM_934519.1	PPP1R9A	0.006209093089838259
51233	C22orf43	NM_016449.2	C22ORF43	0.006251254964463802
729731	LOC729731	XM_001131140.1	LOC729731	0.006443489149451054
641746	LOC641746	XR_036993.1	LOC641746	0.0064734119737271095
401399	LOC401399	XM_938040.1	LOC401399	0.006503933510587002
91445	RNF185	NM_152267.2	RNF185	0.006643879293476746
343069	HNRNPCL1	NM_001013631.1	HNRNPCL1	0.00668848680455546
653342	LOC653342	XM_926986.1	LOC653342	0.006704451522523867
		Hs.130971	HS.130971	0.006835062263307346
90326	THAP3	NM_138350.2	THAP3	0.006845201780484901
406993	MIR211	NR_029624.1	MIR211	0.007000712087657615
27253	PCDH17	NM_014459.2	PCDH17	0.007157587685755881
317705	VN1R5	NM_173858.1	VN1R5	0.007308537801323615
642698	LOC642698	XM_926147.1	LOC642698	0.007500827909900659
647591	LOC647591	XM_936642.1	LOC647591	0.00768313187287398
100129999	LOC100129999	XM_001723251.1	LOC100129999	0.007978078516773852
652874	LOC652874	XM_942590.1	LOC652874	0.008058888114695309
100133191	LOC100133191	XM_001720224.1	LOC100133191	0.008172412960470546
6792	CDKL5	NM_003159.2	CDKL5	0.008262129917024259
387267	KRTAP5-4	NM_001012709.1	KRTAP5-4	0.00833750183090909

400941	FLJ42418	NM_001001695.1	FLJ42418	0.008346757855315017
57611	ISLR2	NM_020851.1	ISLR2	0.008393630774620991
23269	MGA	NM_001080541.1	MGA	0.008679894079816232
85376	RIMBP3	NM_015672.1	RIMBP3	0.008898642569320844
284323	ZNF780A	NM_001010880.1	ZNF780A	0.008978682929154574
653539	LOC653539	XM_934566.1	LOC653539	0.009019103245916179
386681	KRTAP10-8	NM_198695.1	KRTAP10-8	0.009021269079951547
3800	KIF5C	NM_004522.1	KIF5C	0.009118444043063216
652882	LOC652882	XM_942599.1	LOC652882	0.009171572198060862
641955	LOC641955	XM_935721.1	LOC641955	0.0093460168089686
3620	IDO1	NM_002164.4	IDO1	0.009363909447106255
378884	NHLRC1	NM_198586.2	NHLRC1	0.0094580006051593
652737	LOC652737	XM_001718112.1	LOC652737	0.009546427444424247
54778	RNF111	NM_017610.6	RNF111	0.00968125336763508
100129083	LOC100129083	XM_001724757.1	LOC100129083	0.009689938019977624
51364	ZMYND10	NM_015896.2	ZMYND10	0.009907589492135787
		Hs.542544	HS.542544	0.009956858921022441
649909	LOC649909	XM_943982.1	LOC649909	0.010006376036609718
1325	CORT	NM_001302.3	CORT	0.010217548361631685
		Hs.563373	HS.563373	0.010407261517864428
59350	RXFP1	NM_021634.1	RXFP1	0.010545283382671588
7321	UBE2D1	NM_003338.3	UBE2D1	0.010566544072874764
84083	ZRANB3	NM_032143.2	ZRANB3	0.010841513823416579
158763	FLJ30058	NM_144967.2	FLJ30058	0.010930058649637631
10537	UBD	NM_006398.2	UBD	0.011172926856343891
54462	FAM190B	NM_018999.2	FAM190B	0.011271251153973216
650154	LOC650154	XM_944071.1	LOC650154	0.01129310117726207
11218	DDX20	NM_007204.3	DDX20	0.011344211025161022
644116	LOC644116	XM_933284.1	LOC644116	0.011369913354189182
4103	MAGEA4	NM_001011550.1	MAGEA4	0.011459719768653004
8085	MLL2	NM_003482.1	MLL2	0.011495307726533902
3709	ITPR2	NM_002223.1	ITPR2	0.011520969086767587
340481	ZDHHC21	NM_178566.2	ZDHHC21	0.011700678983183417
441332	FAM90A12	XM_496961.2	FAM90A12	0.011926926139427045
		Hs.27048	HS.27048	0.012139723893189567
100132839	LOC100132839	XM_001725950.1	LOC100132839	0.012251193131297656
643338	LOC643338	XM_927997.1	LOC643338	0.012309378127665593
		Hs.522935	HS.522935	0.012366037463090873
100313774	MIR302E	NR_031683.1	MIR302E	0.012466799678927403
		Hs.560698	HS.560698	0.012548076535386902
131831	FAM194A	NM_152394.2	FAM194A	0.012617196044206526
30818	KCNIP3	NM_013434.3	KCNIP3	0.012677315030705984
285311	C3orf56	NM_001007534.1	C3ORF56	0.01269820706499609
402381	SOHLH1	NM_001012415.1	SOHLH1	0.012733956612552355
643038	LOC643038	XM_926407.1	LOC643038	0.012838978911910548
		Hs.542481	HS.542481	0.013035512837795495

100302274	MIR1178	NR_031589.1	MIR1178	0.01305870627028648
		Hs.538176	HS.538176	0.013288378164829525
728747	LOC728747	XM_928873.1	LOC728747	0.013678378485791646
		Hs.565086	HS.565086	0.013681184497987222
9249	DHRS3	NM_004753.4	DHRS3	0.013702070752348021
100129808	LOC100129808	XR_039762.1	LOC100129808	0.013741295789919082
100133577	LOC100133577	XM_001716788.1	LOC100133577	0.013790216364984282
649991	LOC649991	XM_939078.1	LOC649991	0.01379355474644613
	ERCC-00098	ERCC-00098	ERCC-00098	0.013910035983225006
100134057	LOC100134057	XM_001718124.1	LOC100134057	0.01407780501664926
283687	C15orf37	NM_175898.2	C15ORF37	0.014124185960867468
3626	INHBC	NM_005538.2	INHBC	0.014353200725974444
644419	LOC644419	XM_932175.1	LOC644419	0.014357581612702485
2255	FGF10	NM_004465.1	FGF10	0.014382194667132114
100129583	FAM47E	NM_001136570.1	FAM47E	0.014423022504089493
		Hs.523127	HS.523127	0.01443578466046612
441956	LOC441956	XM_498859.2	LOC441956	0.01450209839706341
415	ARSE	NM_000047.1	ARSE	0.014587512708243683
406905	MIR1-2	NR_029662.1	MIR1-2	0.014600747058617292
9366	RAB9P1	NR_000039.1	RAB9P1	0.014848160505048023
6853	SYN1	NM_006950.2	SYN1	0.01507960250070801
727944	LOC727944	Hs.444961	LOC727944	0.015091515444061319
202459	LOC202459	NM_145303.1	LOC202459	0.015143355361588198
150244	FLJ31568	NM_152509.1	FLJ31568	0.015402338394228391
389633	LOC389633	XM_372030.4	LOC389633	0.015490223100747054
100132117	LOC100132117	XM_001725240.1	LOC100132117	0.015580093268710318
391670	LOC391670	XR_019025.1	LOC391670	0.015722796465197728
400156	RPS26L	NR_002225.2	RPS26L	0.01576965248812111
266697	POM121L4P	NR_024592.1	POM121L4P	0.01579271983878519
		Hs.560984	HS.560984	0.015800344711278804
254173	TTLL10	NM_153254.1	TTLL10	0.015812652787782506
6326	SCN2A	NM_021007.2	SCN2A	0.015940809007239015
653275	LOC653275	XM_932990.1	LOC653275	0.016001770436982435
		Hs.259386	HS.259386	0.016004345473892765
650975	LOC650975	XM_940080.1	LOC650975	0.016029588990556298
652258	LOC652258	XM_941668.1	LOC652258	0.01608013134347099
63974	NEUROD6	NM_022728.2	NEUROD6	0.016150373017089045
285643	KIF4B	NM_001099293.1	KIF4B	0.016248333616922687
		Hs.159049	HS.159049	0.016366558208216923
653424	LOC653424	XM_932020.1	LOC653424	0.01637344608415435
646269	LOC646269	XR_019325.2	LOC646269	0.016470839817159313
		Hs.390407	HS.390407	0.016512944520498406
8601	RGS20	NM_170587.1	RGS20	0.016542861227450488
51430	C1orf9	NM_014283.2	C1ORF9	0.01685998003402229
652798	LOC652798	XM_942459.1	LOC652798	0.016879126997924807
164684	WBP2NL	NM_152613.1	WBP2NL	0.016883078047378892

728603	FRMPD2L2	XM_931737.1	FRMPD2L2	0.016916786673588584
644710	LOC644710	XM_932320.1	LOC644710	0.017266395442179817
116966	WDR17	NM_170710.3	WDR17	0.017375785759740654
22801	ITGA11	NM_012211.3	ITGA11	0.01749005821141641
100133718	LOC100133718	XM_001713866.1	LOC100133718	0.01757902065841129
64493	C16orf10	NR_024121.1	C16ORF10	0.017662612203510786
653140	LOC653140	XM_931412.1	LOC653140	0.017685876567057807
375567	VWC2	NM_198570.1	VWC2	0.017809725623884182
100134592	LOC100134592	XR_039069.1	LOC100134592	0.01784849213198316
100133431	LOC100133431	XM_001713711.1	LOC100133431	0.018033690902546494
390507	LOC390507	XM_941221.1	LOC390507	0.018059490429512676
791115	PWRN2	NR_026647.1	PWRN2	0.01809236729739171
652128	LOC652128	XM_941465.1	LOC652128	0.018097957782505475
100302139	MIR1537	NR_031718.1	MIR1537	0.01813555205320322
		Hs.577082	HS.577082	0.018400209049827254
5176	SERPINF1	NM_002615.4	SERPINF1	0.018577222684230937
350	APOH	NM_000042.1	APOH	0.018690631761723663
10551	AGR2	NM_006408.3	AGR2	0.018848439149668654
323	APBB2	NM_173075.3	APBB2	0.01892954217116504
653419	LOC653419	XM_934475.1	LOC653419	0.018976474616645995
147650	LOC147650	NM_207324.1	LOC147650	0.018986756554713668
11096	ADAMTS5	NM_007038.2	ADAMTS5	0.01913768724155538
150368	FAM109B	NM_001002034.1	FAM109B	0.019167605385620224
406887	MIRLET7E	NR_029482.1	MIRLET7E	0.019214688371754666
64506	CPEB1	NM_030594.3	CPEB1	0.01924155328879387
		Hs.131656	HS.131656	0.01967564250413684
644090	LOC644090	XM_945887.1	LOC644090	0.019790031059694815
652071	LOC652071	XM_930113.1	LOC652071	0.019830966384617423
641852	LOC641852	XM_935593.1	LOC641852	0.019928268073840807
85360	SYDE1	NM_033025.4	SYDE1	0.020251423488305664
401101	LOC401101	XM_379234.3	LOC401101	0.020290327540105495
4008	LMO7	NM_005358.3	LMO7	0.02032681405922698
246213	SLC17A8	NM_139319.1	SLC17A8	0.02040938433833191
		Hs.450953	HS.450953	0.020425896992517793
100133118	LOC100133118	XM_001719352.1	LOC100133118	0.020561847635159287
3913	LAMB2	NM_002292.2	LAMB2	0.020727864885455943
		Hs.523508	HS.523508	0.02072946020971049
5345	SERPINF2	NM_000934.1	SERPINF2	0.02075928219921853
22844	FRMPD1	NM_014907.1	FRMPD1	0.02094698274076949
		Hs.577845	HS.577845	0.020961537623100948
4666	NACA	NM_005594.2	NACA	0.02100119453837278
255426	RASGEF1C	NM_001031799.1	RASGEF1C	0.021191749833611283
255626	HIST1H2BA	NM_170610.2	HIST1H2BA	0.021376189511903815
650965	LOC650965	XM_944440.1	LOC650965	0.0214008969373685
643534	LOC643534	XM_936822.2	LOC643534	0.021559336520668215
140738	TMEM37	NM_183240.1	TMEM37	0.021599174420979573

148170	CDC42EP5	NM_145057.2	CDC42EP5	0.021608582355112586
8139	GAN	NM_022041.2	GAN	0.02170980675855108
642441	LOC642441	XM_930678.3	LOC642441	0.021736426354992913
139189	DGKK	NM_001013742.1	DGKK	0.021767396571142218
650180	LOC650180	XM_939263.1	LOC650180	0.02182579988548141
933	CD22	NM_001771.1	CD22	0.021869730561595586
442251	LOC442251	XM_498135.2	LOC442251	0.02187831310995123
644037	LOC644037	XR_017337.2	LOC644037	0.022220250277086427
337967	KRTAP6-2	NM_181604.1	KRTAP6-2	0.02222310695794649
644841	LOC644841	XM_927936.1	LOC644841	0.022422888467140092
		Hs.578887	HS.578887	0.02243599378753856
284541	CYP4A22	NM_001010969.2	CYP4A22	0.022461197895480303
		Hs.544737	HS.544737	0.022511129189707275
81555	YIPF5	NM_030799.6	YIPF5	0.022539229264113298
649501	LOC649501	XM_938580.1	LOC649501	0.022567620190517213
29933	GPR132	NM_013345.2	GPR132	0.022575922292977182
729732	LOC729732	XM_932827.1	LOC729732	0.022685263572146966
1946	EFNA5	NM_001962.1	EFNA5	0.022870521985390184
9957	HS3ST1	NM_005114.2	HS3ST1	0.023096833903532978
100131294	LOC100131294	XM_001718622.1	LOC100131294	0.02335883403484874
219437	OR5L1	NM_001004738.1	OR5L1	0.023421551832223223
728753	FAM90A19	XM_001129368.2	FAM90A19	0.02348762306281699
83999	KREMEN1	NM_032045.3	KREMEN1	0.023749641111062317
55871	CBWD1	NM_018491.3	CBWD1	0.0237621427097727
81930	KIF18A	NM_031217.2	KIF18A	0.023886877809522717
730077	LOC730077	XM_929823.1	LOC730077	0.023982448377878662
55554	KLK15	NM_017509.2	KLK15	0.0240493655076375
27290	SPINK4	NM_014471.1	SPINK4	0.02408277086088165
349114	LOC349114	XR_040229.1	LOC349114	0.024269512235205234
100131223	LOC100131223	XR_039276.1	LOC100131223	0.02448826957591335
79750	ZNF385D	NM_024697.1	ZNF385D	0.02449260284148093
652608	LOC652608	XM_942140.1	LOC652608	0.024538555779626832
390445	OR5AU1	NM_001004731.1	OR5AU1	0.024666882109893585
1446	CSN1S1	NM_001890.1	CSN1S1	0.0247003630519056
574436	MIR485	NR_030160.1	MIR485	0.024719706991961146
653453	LOC653453	XM_933338.1	LOC653453	0.02494463989225702
85508	SCRT2	NM_033129.1	SCRT2	0.025139068866499865
645649	LOC645649	XM_928663.1	LOC645649	0.025223184090347517
171389	NLRP6	NM_138329.1	NLRP6	0.025317308419384465
133482	SLCO6A1	NM_173488.2	SLCO6A1	0.025329794343735376
		Hs.132448	HS.132448	0.02553378780006922
80818	ZNF436	NM_030634.1	ZNF436	0.0255551614087837
80031	SEMA6D	NM_024966.2	SEMA6D	0.025558717309230792
92340	C17orf72	NR_015354.2	C17ORF72	0.025633877058255945
		Hs.399823	HS.399823	0.025670833890299852
100133067	LOC100133067	XM_001725464.1	LOC100133067	0.02589841479039926

23262	HISPPD1	NM_015216.2	HISPPD1	0.025898595112993666
		Hs.537971	HS.537971	0.026063789227305127
54822	TRPM7	NM_017672.2	TRPM7	0.0261330076463797
100132088	LOC100132088	XR_037760.1	LOC100132088	0.026147330213606424
10846	PDE10A	NM_006661.1	PDE10A	0.02617993149900035
		Hs.494628	HS.494628	0.026203085945303402
100128328	LOC100128328	XM_001715053.1	LOC100128328	0.026391195653177747
284912	LOC284912	NM_203375.1	LOC284912	0.02645942324817142
100133959	LOC100133959	XM_001720421.1	LOC100133959	0.026649547761975846
5205	ATP8B1	NM_005603.2	ATP8B1	0.026998467102858623
		Hs.164254	HS.164254	0.02708058345318273
645870	LOC645870	XM_930479.1	LOC645870	0.02708664365272622
5681	PSKH1	NM_006742.1	PSKH1	0.027094411579846493
644264	LOC644264	XM_927442.1	LOC644264	0.027203704934803792
23414	ZFPM2	NM_012082.2	ZFPM2	0.027380423257296505
219954	OR9I1	NM_001005211.1	OR9I1	0.027405531858696788
100129461	LOC100129461	XM_001719437.1	LOC100129461	0.027424973554395375
54477	PLEKHA5	NM_019012.2	PLEKHA5	0.027462476844466694
29785	CYP2S1	NM_030622.5	CYP2S1	0.027469637841125567
57045	TWSG1	NM_020648.3	TWSG1	0.027547802917928584
10869	USP19	NM_006677.1	USP19	0.027715324591564602
100133911	LOC100133911	XM_001714502.1	LOC100133911	0.02782298055874275
100132029	LOC100132029	XM_001719312.1	LOC100132029	0.027832907304590242
651125	LOC651125	XM_940255.1	LOC651125	0.02787994837746972
257396	LOC257396	XM_001715443.1	LOC257396	0.027909105492990874
730389	LOC730389	XM_001726418.1	LOC730389	0.02802181380683962
649151	LOC649151	XM_944782.1	LOC649151	0.028035977164727922
57084	SLC17A6	NM_020346.1	SLC17A6	0.02808767449264285
10417	SPON2	NM_012445.1	SPON2	0.02809670267559562
729826	LOC729826	XM_932003.1	LOC729826	0.02810333996192765
138715	ARID3C	NM_001017363.1	ARID3C	0.028113477207784367
		Hs.543971	HS.543971	0.02817707262444036
400844	FLJ42133	NM_001001690.1	FLJ42133	0.02820580226917168
		Hs.444411	HS.444411	0.028238626580358715
401648	LOC401648	XR_038983.1	LOC401648	0.028314991818208114
650428	LOC650428	XM_939516.1	LOC650428	0.028702941205273995
100133896	LOC100133896	XM_001723682.1	LOC100133896	0.028715684876214117
401491	FLJ35024	NR_015375.1	FLJ35024	0.028811076040184593
221468	TMEM217	XM_929843.1	TMEM217	0.029022603350601032
11185	INMT	NM_006774.4	INMT	0.0291037479385151
100129159	LOC100129159	XM_001719666.1	LOC100129159	0.029374739907140807
389671	LOC389671	XM_374275.2	LOC389671	0.029484891395720376
154288	C6orf221	NM_001017361.2	C6ORF221	0.029593828182104785
647154	LOC647154	XM_934436.1	LOC647154	0.029829653041726342
386724	AMIGO3	NM_198722.1	AMIGO3	0.029951202277859936
89796	NAV1	NM_020443.2	NAV1	0.02999043524271502

56751	BARHL1	NM_020064.2	BARHL1	0.030022852681444435
133619	PRRC1	NM_130809.2	PRRC1	0.030187721912594043
64122	FN3K	NM_022158.2	FN3K	0.03021107536473889
56254	RNF20	NM_019592.5	RNF20	0.03033470528345383
8771	TNFRSF6B	NM_032945.2	TNFRSF6B	0.03035914723145836
8392	OR3A3	NM_012373.1	OR3A3	0.03053809988231804
		Hs.133261	HS.133261	0.030568099022101985
100131124	LOC100131124	XR_038480.1	LOC100131124	0.03061359552459085
655	BMP7	NM_001719.1	BMP7	0.030651295946450993
145447	ABHD12B	NM_181533.3	ABHD12B	0.030664450871831905
114902	C1QTNF5	NM_015645.2	C1QTNF5	0.0307441372846035
1272	CNTN1	NM_001843.2	CNTN1	0.030973923100136438
100134397	LOC100134397	XM_001716222.1	LOC100134397	0.03102925073273608
4747	NEFL	NM_006158.1	NEFL	0.031244890324453
51201	ZDHHC2	NM_016353.2	ZDHHC2	0.03130797889576718
645038	LOC645038	XM_932599.1	LOC645038	0.03134188336365769
100131507	LOC100131507	XR_038156.1	LOC100131507	0.031346082065930175
		Hs.195035	HS.195035	0.031634632331781154
654346	LGALS9C	NM_001040078.2	LGALS9C	0.031764887661290764
8563	THOC5	NM_001002878.1	THOC5	0.03191725864259395
100132705	LOC100132705	XM_001715205.1	LOC100132705	0.03192907747211482
55545	MSX2P1	NR_002307.1	MSX2P1	0.0319808871078602
219428	OR4C16	NM_001004701.1	OR4C16	0.03203493369424288
647247	LOC647247	XM_934568.1	LOC647247	0.032152819934808
56849	TCEAL7	NM_152278.1	TCEAL7	0.03222680725793716
57175	CORO1B	NM_020441.2	CORO1B	0.03222783672970617
641768	LOC641768	XM_935907.3	LOC641768	0.0322664317396529
197407	ZNF48	NM_152652.1	ZNF48	0.03229426087776541
100302163	MIR1278	NR_031691.1	MIR1278	0.032440391913297596
55679	LIMS2	NM_017980.2	LIMS2	0.032517058083104546
5834	PYGB	NM_002862.3	PYGB	0.032539078867889336
644934	LOC644934	XM_934677.1	LOC644934	0.0325617138291315
1302	COL11A2	NM_080681.1	COL11A2	0.03256917573410545
		Hs.156178	HS.156178	0.03263352286288794
725	C4BPB	NM_001017367.1	C4BPB	0.03290258407073522
3741	KCNA5	NM_002234.2	KCNA5	0.033000791233888896
441233	LOC441233	NM_001013724.1	LOC441233	0.03308686516536579
651008	LOC651008	XM_940120.1	LOC651008	0.03331768852877934
440243	LOC440243	XM_496041.3	LOC440243	0.033377254112434064
653876	LOC653876	XM_936221.1	LOC653876	0.033379693490334726
79677	SMC6	NM_024624.3	SMC6	0.03343033375558616
401957	LOC401957	XM_496379.2	LOC401957	0.03343759911919372
114815	SORCS1	NM_052918.3	SORCS1	0.03385617247917471
100128404	LOC100128404	XM_001713786.1	LOC100128404	0.03386855319301855
652286	LOC652286	XM_941705.1	LOC652286	0.033962860994664016
399706	LOC399706	NM_001010910.1	LOC399706	0.034007211184900424

1413	CRYBA4	NM_001886.1	CRYBA4	0.03401175055796066
441502	RPS26P11	NR_002309.1	RPS26P11	0.03409019967664746
		Hs.207074	HS.207074	0.034157092547502425
11281	POU6F2	NM_007252.2	POU6F2	0.0342334160199717
25903	OLFML2B	NM_015441.1	OLFML2B	0.03426761739469739
911	CD1C	NM_001765.1	CD1C	0.03437678703029185
201181	ZNF385C	NM_001013624.1	ZNF385C	0.03442394727482257
		Hs.61151	HS.61151	0.03450872404206222
		Hs.572889	HS.572889	0.034602714666213466
283824	LOC283824	XM_936476.1	LOC283824	0.03469855579099817
389320	C5orf48	NM_207408.1	C5ORF48	0.03480603097892593
643943	LOC643943	XM_934575.1	LOC643943	0.03492449423224515
84140	FLJ13305	NM_032180.1	FLJ13305	0.034924817386345224
653149	NBPF6	XM_926213.3	NBPF6	0.034948186249392776
83755	KRTAP4-12	NM_031854.2	KRTAP4-12	0.03495961113206711
644222	LOC644222	XM_932052.1	LOC644222	0.03502179995398587
403274	OR5H15	NM_001005515.1	OR5H15	0.03528865792443498
642302	LOC642302	XM_925847.1	LOC642302	0.03532679261216908
5757	PTMA	NM_002823.2	PTMA	0.03533048715758714
85449	KIAA1755	NM_001029864.1	KIAA1755	0.03550092188291261
280658	SSX7	NM_173358.2	SSX7	0.035527787941814014
		Hs.559234	HS.559234	0.035570714704618304
8894	EIF2S2	NM_003908.3	EIF2S2	0.03570589969817165
647123	LOC647123	XM_930141.1	LOC647123	0.035816525085092685
353140	LCE2C	NM_178429.2	LCE2C	0.035934579863750746
677801	SNORA14A	NR_002955.1	SNORA14A	0.0361854163552442
125058	TBC1D16	NM_019020.2	TBC1D16	0.03626812263100942
652469	LOC652469	XM_941921.1	LOC652469	0.0362927530207742
		Hs.66072	HS.66072	0.03637146426565126
7128	TNFAIP3	NM_006290.2	TNFAIP3	0.03639524499582533
84667	HES7	NM_032580.1	HES7	0.0364117990492306
644928	LOC644928	NM_001093732.1	LOC644928	0.036419987574674156
7809	BSND	NM_057176.2	BSND	0.03653091396146062
440792	LOC440792	XM_496493.2	LOC440792	0.03654208725894107
492303	LOC492303	NR_002830.1	LOC492303	0.03660139461851856
401281	FLJ27255	NM_207501.1	FLJ27255	0.03662507602211143
391767	LOC391767	XM_936313.1	LOC391767	0.036632788973511736
645141	LOC645141	XM_932673.1	LOC645141	0.036680323689545984
375607	C7orf52	NM_198571.1	C7ORF52	0.03678010633215606
400169	DKFZp451A211	NM_001003399.1	DKFZP451A211	0.037087843662292584
441257	LOC441257	NM_001023562.1	LOC441257	0.03748984854102287
5998	RGS3	NM_130795.2	RGS3	0.03750498381085232
30845	EHD3	NM_014600.1	EHD3	0.03752440328750746
128209	KLF17	NM_173484.2	KLF17	0.03753163799650583
86614	HSFY1	NM_001001871.1	HSFY1	0.03756517173064518
643717	LOC643717	XM_931762.1	LOC643717	0.03759956434879695

3185	HNRNPF	NM_004966.2	HNRNPF	0.03766736732960908
60482	SLC5A7	NM_021815.2	SLC5A7	0.037777752067761614
84775	ZNF607	NM_032689.3	ZNF607	0.037833110045205016
646982	LOC646982	XM_929953.1	LOC646982	0.037858020157582745
26533	OR10G3	NM_001005465.1	OR10G3	0.03787398889924781
100132994	LOC100132994	XM_001716545.1	LOC100132994	0.0381880201170454
51454	GULP1	NM_016315.2	GULP1	0.03823336785815221
147670	LOC147670	XM_938354.1	LOC147670	0.038271133361022
100133667	LOC100133667	XM_001716955.1	LOC100133667	0.038353586825590834
441377	LOC441377	XM_938599.1	LOC441377	0.038425188952630224
		Hs.576557	HS.576557	0.03845300174614561
27190	IL17B	NM_014443.2	IL17B	0.03851235934753757
1612	DAPK1	NM_004938.1	DAPK1	0.038513267889527844
406981	MIR19B2	NR_029491.1	MIR19B2	0.03865875848007942
100129489	LOC100129489	XM_001725742.1	LOC100129489	0.0388785449116513
389043	LOC389043	XM_374012.2	LOC389043	0.038894805019877444
2326	FMO1	NM_002021.1	FMO1	0.0389169991967331
643369	LOC643369	XM_926699.1	LOC643369	0.03894931459555177
646688	LOC646688	XR_038043.1	LOC646688	0.03904764703794127
407009	MIR224	NR_029638.1	MIR224	0.039111411385302795
9991	ROD1	NM_005156.4	ROD1	0.03912009402141927
55901	THSD1	NM_018676.2	THSD1	0.0391402794320996
387742	FAM99A	NM_001014374.1	FAM99A	0.039529403362823914
504188	LOC504188	NM_001013404.1	LOC504188	0.03960475777464169
55748	CNDP2	NM_018235.1	CNDP2	0.03969157665674203
641515	LOC641515	XR_041510.1	LOC641515	0.0397689232251305
80318	GKAP1	NM_025211.2	GKAP1	0.03986648588847759
389376	SFTA2	NM_205854.1	SFTA2	0.03999423728853999
648533	LOC648533	XM_937587.1	LOC648533	0.04031944639793983
10694	CCT8	NM_006585.2	CCT8	0.0403292788968682
6098	ROS1	NM_002944.2	ROS1	0.04033312843536948
6670	SP3	NM_003111.3	SP3	0.04042713037654128
1404	HAPLN1	NM_001884.2	HAPLN1	0.04048525841710001
4881	NPR1	NM_000906.2	NPR1	0.040502565677173176
649801	LOC649801	XM_938871.1	LOC649801	0.04050521442632797
650144	LOC650144	XM_939226.1	LOC650144	0.04054045691347192
4887	NPY2R	NM_000910.2	NPY2R	0.04079826941342508
60439	TTTY2	NR_001536.1	TTTY2	0.04092232641545795
652517	LOC652517	XM_941993.1	LOC652517	0.041006153896141075
		Hs.575322	HS.575322	0.04100760502736633
644421	LOC644421	XM_932176.1	LOC644421	0.04115702923750378
		Hs.567469	HS.567469	0.04119812979787987
644150	LOC644150	XM_933686.1	LOC644150	0.04121231842909383
26301	GBGT1	NM_021996.3	GBGT1	0.04133282944557231
56947	MFF	NM_020194.4	MFF	0.04134914870555508
124790	HEXIM2	NM_144608.1	HEXIM2	0.0414099130711993

4781	NFIB	NM_005596.1	NFIB	0.04142927296674448
652667	LOC652667	XM_942241.1	LOC652667	0.04145856823506089
		Hs.550320	HS.550320	0.041472016762589686
728353	LOC728353	XR_041035.1	LOC728353	0.04148634552345762
		Hs.538064	HS.538064	0.041536830424488996
100130648	LOC100130648	XM_001719568.1	LOC100130648	0.04156307760807585
100302138	MIR1292	NR_031699.1	MIR1292	0.041597253865857386
54842	MFSD6	NM_017694.2	MFSD6	0.04168319322226114
100129722	LOC100129722	XM_001723078.1	LOC100129722	0.04178887578344652
728897	LOC728897	XM_001714242.1	LOC728897	0.04179921046302966
		Hs.570505	HS.570505	0.04186242156445938
642828	LOC642828	XR_016385.2	LOC642828	0.04189952262354723
128876	FAM83C	NM_178468.2	FAM83C	0.0420309294383992
7350	UCP1	NM_021833.3	UCP1	0.04209019401793435
57703	CWC22	NM_020943.1	CWC22	0.042102050543425557
2162	F13A1	NM_000129.2	F13A1	0.042112733355483295
8463	TEAD2	NM_003598.1	TEAD2	0.04212361026047233
123624	AGBL1	Hs.569517	AGBL1	0.042251663296841206
51155	HN1	NM_016185.2	HN1	0.04254373110656518
654085	LOC654085	XM_942123.1	LOC654085	0.04257358154415917
		Hs.547985	HS.547985	0.04260022423480719
4780	NFE2L2	NM_006164.2	NFE2L2	0.04267011054111266
728612	LOC728612	XR_015601.1	LOC728612	0.0427617681836858
100131909	LOC100131909	XM_001722362.1	LOC100131909	0.04290454348425088
23284	LPHN3	NM_015236.3	LPHN3	0.04330834214616471
85443	DCLK3	NM_033403.1	DCLK3	0.043353620219200605
9840	KIAA0748	XM_934138.1	KIAA0748	0.04336354602898456
650646	LOC650646	XM_930416.1	LOC650646	0.04349481866826087
26168	SENP3	NM_015670.3	SENP3	0.043643046874244765
441655	LOC441655	XM_497366.2	LOC441655	0.043662954044794984
653051	LOC653051	XM_925795.1	LOC653051	0.04369705229509728
651465	LOC651465	XM_944690.1	LOC651465	0.04376632172718305
642712	LOC642712	XM_926777.1	LOC642712	0.043815420015324714
9154	SLC28A1	NM_201651.1	SLC28A1	0.043841747366748285
100128746	LOC100128746	XM_001726588.1	LOC100128746	0.043874905167078086
646566	LOC646566	XM_933706.1	LOC646566	0.04390382362985182
729356	LOC729356	XR_036863.1	LOC729356	0.04392491941625278
169834	LOC169834	XM_936297.1	LOC169834	0.04406591788604947
647836	LOC647836	XM_936893.1	LOC647836	0.044074300034243834
79158	GNPTAB	NM_024312.3	GNPTAB	0.044177209332562146
		Hs.551358	HS.551358	0.04429590120343183
55511	SAGE1	NM_018666.1	SAGE1	0.04454669580107023
114088	TRIM9	NM_052978.3	TRIM9	0.044713601155745256
1880	GPR183	NM_004951.4	GPR183	0.044766978044226774
58525	WIZ	NM_021241.2	WIZ	0.04487145485387742
		Hs.570535	HS.570535	0.0448985342130216

646040	LOC646040	XM_933354.1	LOC646040	0.044910321983449665
100130663	LOC100130663	XM_001719837.1	LOC100130663	0.0449171581004836
10156	RASA4	NM_006989.3	RASA4	0.0449172733642023
100129412	LOC100129412	XM_001724449.1	LOC100129412	0.04509846942017952
693142	MIR557	NR_030284.1	MIR557	0.04522478913460713
83898	KRTAP4-8	XM_927495.1	KRTAP4-8	0.04533959100938478
8440	NCK2	NM_003581.2	NCK2	0.045390805618902325
26582	DUX3	NM_012148.2	DUX3	0.045444015891660465
51338	MS4A4A	NM_148975.1	MS4A4A	0.045824373203041946
23090	ZNF423	NM_015069.2	ZNF423	0.0458380449604099
112399	EGLN3	NM_022073.2	EGLN3	0.04586641476579686
645450	LOC645450	XM_928482.1	LOC645450	0.04587792597520967
		Hs.565704	HS.565704	0.04604524295035083
727994	LOC727994	XM_001717645.1	LOC727994	0.04616846813419221
5157	PDGFRL	NM_006207.1	PDGFRL	0.046193739473114004
282969	C10orf125	NM_198472.1	C10ORF125	0.04621714785670731
647983	LOC647983	XM_937039.1	LOC647983	0.046354241561175194
79031	PDCL3	XM_929879.1	PDCL3	0.04641754842007101
100313938	MIR548G	NR_031662.1	MIR548G	0.04655543373008615
1611	DAP	NM_004394.1	DAP	0.04656125580018756
391241	LOC391241	XM_372864.3	LOC391241	0.046576699058196815
150921	TCF23	NM_175769.1	TCF23	0.04661232178619408
619554	MIR486	NR_030161.1	MIR486	0.046612985679481606
100132979	LOC100132979	XM_001723476.1	LOC100132979	0.046630454056350454
651362	LOC651362	XM_940505.1	LOC651362	0.04669258782808066
8125	ANP32A	NM_006305.2	ANP32A	0.046783736391782925
2887	GRB10	NM_005311.3	GRB10	0.046952512945653696
26235	FBXL4	NM_012160.3	FBXL4	0.04696753087023498
644714	LOC644714	XM_934514.1	LOC644714	0.04703281511457221
727910	LOC727910	XM_001718130.1	LOC727910	0.047078199178451074
		Hs.571403	HS.571403	0.04708841921734624
100134805	LOC100134805	XM_001719203.1	LOC100134805	0.04712513232533933
745	C11orf9	NM_013279.1	C11ORF9	0.04715254550434623
160492	IFLTD1	NM_152590.1	IFLTD1	0.04717322013598507
7015	TERT	NM_198253.2	TERT	0.04718613531279058
		Hs.545952	HS.545952	0.04722315518419996
8624	PSMG1	NM_203433.1	PSMG1	0.04737253754741517
100129271	C1orf68	NM_001024679.2	C1ORF68	0.04737332589684549
219477	OR8J1	NM_001005205.1	OR8J1	0.04737755389151637
3872	KRT17	NM_000422.1	KRT17	0.047425982292438607
643493	LOC643493	XM_931578.1	LOC643493	0.047431793499921646
2710	GK	NM_000167.3	GK	0.04747795359305717
8528	DDO	NM_003649.2	DDO	0.04759894764627622
54868	TMEM104	NM_017728.2	TMEM104	0.04763931569476485
64786	TBC1D15	NM_022771.3	TBC1D15	0.04776252741607036
100302129	MIR1915	NR_031736.1	MIR1915	0.04780680859532939

729475	RAD51AP2	Hs.515802	RAD51AP2	0.04782097794386552
		Hs.544828	HS.544828	0.048024235371097825
		Hs.580655	HS.580655	0.04817920321578044
652845	LOC652845	XM_942543.1	LOC652845	0.04829554628170876
	ERCC-00111	ERCC-00111	ERCC-00111	0.04833840947152641
		Hs.260074	HS.260074	0.04835079602446493
3231	HOXD1	NM_024501.1	HOXD1	0.048401910255265405
64377	CHST8	NM_022467.3	CHST8	0.04858975405102501
		Hs.560387	HS.560387	0.04860858415283664
100134121	LOC100134121	XM_001714675.1	LOC100134121	0.04867882754541401
10053	AP1M2	NM_005498.3	AP1M2	0.048839436915587696
100132649	LOC100132649	XM_001718045.1	LOC100132649	0.04887013981366635
6169	RPL38	NM_001035258.1	RPL38	0.04897774040010492
652512	LOC652512	XM_941983.1	LOC652512	0.04917845930977059
8819	SAP30	NM_003864.1	SAP30	0.04921232319667151
57507	ZNF608	NM_020747.1	ZNF608	0.0493426107426408
2658	GDF2	NM_016204.1	GDF2	0.04942968746423412

Table S5. The complete gene list from patients without dual AML cell populations. We compared the global gene expression profiles for 12 AML samples with and 27 samples without dual AML cell populations. A feature subset selection (FSS) analysis was performed for identification of the most discriminative genes between the two groups, and 1209 genes were then identified (i.e., p-value <0.05).

Gene ID	Symbol	Search Key	Illumina (ILM)-gene	P-value
642528	LOC642528	XM_926022.1	LOC642528	1.817595639495555E-4
100130508	LOC100130508	XM_001716255.1	LOC100130508	4.447421112487998E-4
		Hs.567094	HS.567094	6.800822576455169E-4
643857	LOC643857	XM_927130.1	LOC643857	7.808779365329758E-4
653111	LOC653111	XM_926073.2	LOC653111	8.070077698455909E-4
401498	LOC401498	NM_212558.1	LOC401498	8.296802641247719E-4
		Hs.313888	HS.313888	9.294829916806852E-4
203414	CXorf24	XM_926772.1	CXORF24	9.756606904733263E-4
		Hs.535372	HS.535372	0.001000817614127855
23555	TSPAN15	NM_012339.3	TSPAN15	0.001079624881794884
642553	LOC642553	XM_926041.1	LOC642553	0.0011128384035927249
254225	RNF169	XM_940188.1	RNF169	0.0011286947961539869
284293	HMSD	NM_001123366.1	HMSD	0.0012888164659033512
9182	RASSF9	NM_005447.2	RASSF9	0.0013483219634245417
574446	MIR511-2	NR_030168.1	MIR511-2	0.0013825423617592363
100134461	LOC100134461	XR_039702.1	LOC100134461	0.0013886851670043363
643464	LOC643464	XM_931563.1	LOC643464	0.001403925102163556
		Hs.561144	HS.561144	0.0014203934909458207
127150	LOC127150	XM_497717.2	LOC127150	0.0014787996671713956
100134368	LOC100134368	NR_024453.1	LOC100134368	0.0014831756022624565
644451	LOC644451	XR_037207.1	LOC644451	0.0017513812633183805
100133232	LOC100133232	XM_001720154.1	LOC100133232	0.0018682046476840581
642608	LOC642608	XM_930949.1	LOC642608	0.0018893903351726647

		Hs.563292	HS.563292	0.0019857574463121937
649189	LOC649189	XM_943611.1	LOC649189	0.0020192891414445874
388572	LOC388572	XM_001722169.1	LOC388572	0.0020738138287287465
646334	LOC646334	XM_929268.1	LOC646334	0.0020862534372304044
		Hs.232517	HS.232517	0.0021399762832749383
654209	LOC654209	XM_941064.1	LOC654209	0.0022286393784698762
288	ANK3	NM_020987.2	ANK3	0.0022312829691701246
344148	NCKAP5	NM_207363.2	NCKAP5	0.002265105586918537
653796	LOC653796	XM_929960.1	LOC653796	0.0023010467263336162
84931	FLJ14816	XR_017947.2	FLJ14816	0.002610085693546829
647219	LOC647219	XM_930260.1	LOC647219	0.0026196262039302792
652355	LOC652355	XM_939668.1	LOC652355	0.00274036261086637
1583	CYP11A1	NM_000781.1	CYP11A1	0.002750373015735045
130	ADH6	NM_000672.2	ADH6	0.0028473581005427464
693235	MIR92B	NR_030281.1	MIR92B	0.0028723933716857998
768239	PSAPL1	NM_001085382.1	PSAPL1	0.002904197951432086
118425	GDEP	NR_026555.1	GDEP	0.0029104742057124177
		Hs.162932	HS.162932	0.0031252062777078807
		Hs.559820	HS.559820	0.003222428963051623
100133034	LOC100133034	XR_036886.1	LOC100133034	0.0032667673919331147
		Hs.561960	HS.561960	0.003384888778334856
642412	LOC642412	XM_925931.1	LOC642412	0.0035227020471639255
100134821	LOC100134821	XM_001722581.1	LOC100134821	0.003624282268300971
340970	LOC340970	XR_038494.1	LOC340970	0.003737685755019992
		Hs.114286	HS.114286	0.003932674181302355
768222	MIR770	NR_030528.1	MIR770	0.003986096141096122
100131897	LOC100131897	XM_001725433.1	LOC100131897	0.004047894840157547
158825	LOC158825	XM_944306.1	LOC158825	0.004123734367948094
		Hs.541921	HS.541921	0.00413122853488739
729291	LOC729291	XR_041493.1	LOC729291	0.0041748180800655005
286310	LOC286310	XM_938575.1	LOC286310	0.004247249772839345
649021	LOC649021	XM_938105.1	LOC649021	0.0043309613155418465
6339	SCNN1D	NM_002978.2	SCNN1D	0.004384104476889884
9148	NEURL	NM_004210.3	NEURL	0.004404169604086631
100129272	LOC100129272	XR_038735.1	LOC100129272	0.004489042421780521
646686	LOC646686	XM_929633.1	LOC646686	0.0045888199572226215
100134245	LOC100134245	XM_001723619.1	LOC100134245	0.004651492454884095
729759	OR4F29	NM_001005221.2	OR4F29	0.004687841476130208
		Hs.579243	HS.579243	0.004778268630255688
400804	LOC400804	NR_024236.1	LOC400804	0.004843756848718989
	ERCC-00019	ERCC-00019	ERCC-00019	0.004868380227138507
		Hs.555115	HS.555115	0.0048744360081739
		Hs.545650	HS.545650	0.004878823751515827
		Hs.560651	HS.560651	0.004922635228871089
643195	LOC643195	XM_926563.1	LOC643195	0.004978023243253226
647323	LOC647323	XM_930391.1	LOC647323	0.005000769305957843

345	APOC3	NM_000040.1	APOC3	0.005101164742546303
642384	LOC642384	XM_926149.1	LOC642384	0.005173983519467021
56134	PCDHAC2	NM_031883.2	PCDHAC2	0.005303901459912222
100132515	LOC100132515	XM_001719495.1	LOC100132515	0.0053358302717896695
729623	LOC729623	XM_001716155.1	LOC729623	0.005393654110446185
127077	OR2T11	NM_001001964.1	OR2T11	0.005415934852160404
651007	LOC651007	XM_940119.1	LOC651007	0.005429676243793426
81931	ZNF93	NM_031218.2	ZNF93	0.005455575321176081
651481	LOC651481	XM_944705.1	LOC651481	0.0055417632881616195
100133612	LOC100133612	NR_024455.1	LOC100133612	0.005662336973006867
		Hs.129329	HS.129329	0.005768818496994594
84141	FAM176A	NM_001135032.1	FAM176A	0.005810056386037936
652264	LOC652264	XM_941675.1	LOC652264	0.005858098708048516
729459	LOC729459	XM_001720731.1	LOC729459	0.005911584917413475
		Hs.290834	HS.290834	0.005924131281531853
100132318	LOC100132318	XM_001718904.1	LOC100132318	0.0059987630165777885
389730	FAM75A6	XR_041507.1	FAM75A6	0.006002113715609757
		Hs.147725	HS.147725	0.006082745949588517
402160	LOC402160	XM_938047.1	LOC402160	0.00618058117796696
54550	NECAB2	NM_019065.2	NECAB2	0.006269455344883447
401898	ZNF833	NM_001013691.1	ZNF833	0.006362520312844321
10655	DMRT2	NM_181872.1	DMRT2	0.006384440067591239
139542	LOC139542	XM_066752.1	LOC139542	0.0064241876819909885
		Hs.505855	HS.505855	0.006434400433945601
650439	LOC650439	XM_944199.1	LOC650439	0.006508297260284593
1112	FOXN3	XM_929939.1	FOXN3	0.00660337190070171
407021	MIR29A	NR_029503.1	MIR29A	0.006642280164138524
		Hs.540289	HS.540289	0.006958649946345595
100134638	LOC100134638	XM_001719382.1	LOC100134638	0.0069592213315213935
339906	PRSS42	NM_182702.1	PRSS42	0.007160306544423903
121599	SPIC	NM_152323.1	SPIC	0.007388445760845789
649385	LOC649385	XM_938457.1	LOC649385	0.007497483289329452
23443	SLC35A3	NM_012243.1	SLC35A3	0.007553044335781477
442147	LOC442147	XM_498029.2	LOC442147	0.007648291765228071
100130701	LOC100130701	XM_001717180.1	LOC100130701	0.007827397078180115
653053	LOC653053	XM_925853.1	LOC653053	0.007900204294587978
29986	SLC39A2	NM_014579.1	SLC39A2	0.007999477533355752
8521	GCM1	NM_003643.2	GCM1	0.00812388029222538
122183	FLJ40296	XM_939203.1	FLJ40296	0.008183868937692471
		Hs.560728	HS.560728	0.008286770271927719
9837	GINS1	NM_021067.2	GINS1	0.008296728324984135
100131243	LOC100131243	XR_042510.1	LOC100131243	0.008446767342929243
693219	MIR634	NR_030364.1	MIR634	0.008456173083631108
100313772	MIR548M	NR_031667.1	MIR548M	0.00849958265815995
		Hs.551062	HS.551062	0.008645163460421596
		Hs.516420	HS.516420	0.00889648558077197

56834	GPR137	NM_020155.2	GPR137	0.008938805140316653
199699	DAND5	NM_152654.2	DAND5	0.008942142967801789
84467	FBN3	NM_032447.3	FBN3	0.00898446467340792
652665	LOC652665	XM_001719814.1	LOC652665	0.009055477529238248
728654	LOC728654	Hs.578787	LOC728654	0.00905675591396397
654117	LOC654117	XM_939660.1	LOC654117	0.009082287421969534
148738	HFE2	NM_213653.2	HFE2	0.0091178306291845
6340	SCNN1G	NM_001039.2	SCNN1G	0.00911900427873428
5017	OVOL1	NM_004561.2	OVOL1	0.009159824466742588
		Hs.582355	HS.582355	0.009196764808675737
		Hs.581967	HS.581967	0.009287204096533039
649094	LOC649094	XM_945141.1	LOC649094	0.009407504706926069
5100	PCDH8	NM_002590.2	PCDH8	0.009772616370694803
645039	LOC645039	XM_928095.1	LOC645039	0.009787421976979783
2277	FIGF	NM_004469.2	FIGF	0.009833563822276537
9104	RGN	XM_937211.1	RGN	0.009849569716599147
113146	AHNAK2	NM_138420.2	AHNAK2	0.009922939294713589
120227	CYP2R1	NM_024514.4	CYP2R1	0.01001708226445753
652505	LOC652505	XM_941974.1	LOC652505	0.010040278019144642
		Hs.540000	HS.540000	0.010058940716288397
284618	C1orf104	NM_001039517.1	C1ORF104	0.010193965306880588
130612	TMEM198	NM_001005209.1	TMEM198	0.010292867538476691
		Hs.582338	HS.582338	0.010306764806367223
114784	CSMD2	NM_052896.2	CSMD2	0.010320462285929569
653623	LOC653623	XM_932876.1	LOC653623	0.010339962262382542
648993	LOC648993	XM_932049.1	LOC648993	0.010645850219767923
389400	GFRAL	NM_207410.1	GFRAL	0.01069802979537872
5651	PRSS7	NM_002772.1	PRSS7	0.01074816405737597
401551	WDR38	XM_941914.1	WDR38	0.010901481221334513
378948	RBMY1B	NM_001006121.1	RBMY1B	0.010973341854028786
27127	SMC1B	NM_148674.3	SMC1B	0.01098107373278998
100130808	LOC100130808	XM_001719331.1	LOC100130808	0.011022411175326036
8324	FZD7	NM_003507.1	FZD7	0.011119660122521448
100129055	LOC100129055	NR_024524.1	LOC100129055	0.011190951965361953
644311	LOC644311	XM_933386.1	LOC644311	0.011245518853591773
81469	OR2G3	NM_001001914.1	OR2G3	0.01125014975576129
	ERCC-00112	ERCC-00112	ERCC-00112	0.011616489420920842
642468	LOC642468	XM_931123.1	LOC642468	0.011628890636355619
642273	FAM110C	NM_001077710.1	FAM110C	0.01165792905924736
		Hs.520328	HS.520328	0.011873977183376822
		Hs.504674	HS.504674	0.011964393260100225
645126	LOC645126	XM_928158.1	LOC645126	0.011969802328208998
431704	RGS21	NM_001039152.2	RGS21	0.012066177424885915
653799	LOC653799	XM_930003.1	LOC653799	0.012084751428363714
		Hs.561963	HS.561963	0.01211700673958969
		Hs.519022	HS.519022	0.012308192274700338

2671	GFER	NM_005262.2	GFER	0.012358644008364584
27445	PCLO	NM_033026.5	PCLO	0.012435428150906784
374900	ZNF568	NM_198539.2	ZNF568	0.01251107186987248
51666	ASB4	NM_145872.1	ASB4	0.012561012785904813
728701	LOC728701	XM_001732848.1	LOC728701	0.01272238204011093
26257	NKX2-8	NM_014360.2	NKX2-8	0.012760908430736332
652148	LOC652148	XM_945224.1	LOC652148	0.012772870119896178
100130592	LOC100130592	XM_001725751.1	LOC100130592	0.012811240982860801
		Hs.539714	HS.539714	0.012840912412962124
57569	ARHGAP20	NM_020809.2	ARHGAP20	0.013118470760808389
653665	LOC653665	XM_928758.1	LOC653665	0.013128469298894354
4710	NDUFB4	NM_004547.4	NDUFB4	0.013222649280353434
401940	PRAMEF3	XM_001713666.1	PRAMEF3	0.013249479371121564
654466	KGFLP2	NR_003670.1	KGFLP2	0.013290672798568059
402217	LOC402217	XM_926343.1	LOC402217	0.013309286116595792
		Hs.529442	HS.529442	0.013367123475068342
84626	KIAA1862	NM_032534.1	KIAA1862	0.013422970670634833
		Hs.222909	HS.222909	0.013500050558039321
		Hs.278303	HS.278303	0.013528025823074474
79541	OR2A4	NM_030908.1	OR2A4	0.013535774011872056
23629	BRD7P3	NR_002730.2	BRD7P3	0.0136581493075215
642655	LOC642655	XM_926114.1	LOC642655	0.01368705768083874
222545	GPRC6A	NM_148963.1	GPRC6A	0.013711233775200095
401224	AACSL	XM_376454.3	AACSL	0.01371367905687376
6276	S100A5	NM_002962.1	S100A5	0.01379519022527498
90987	ZNF251	XM_937814.1	ZNF251	0.013899350375122767
285962	FLJ40852	NM_173677.1	FLJ40852	0.014060434770464255
728181	LOC728181	Hs.577605	LOC728181	0.0141347940546377
81543	LRRC3	NM_030891.3	LRRC3	0.014323459527928824
26254	OPTC	NM_014359.3	OPTC	0.014352304331036903
		Hs.553088	HS.553088	0.014374538074530693
406890	MIRLET7G	NR_029660.1	MIRLET7G	0.01440899484424025
646698	LOC646698	XM_929644.1	LOC646698	0.01483732667353354
147687	ZNF417	NM_152475.1	ZNF417	0.01493098172105505
574537	UGT2A2	NM_001105677.1	UGT2A2	0.01503029185309763
644452	LOC644452	XM_927592.1	LOC644452	0.015181770414054904
647527	LOC647527	XM_942881.1	LOC647527	0.015201527808483101
440337	LOC440337	NM_001013705.1	LOC440337	0.015425289593572807
768212	MIR758	NR_030406.1	MIR758	0.015508613507342983
727894	LOC727894	XM_001720317.1	LOC727894	0.015543022881295037
650433	LOC650433	XM_928063.1	LOC650433	0.015552972852532355
3375	IAPP	NM_000415.1	IAPP	0.015621056486094994
648684	LOC648684	XM_937754.1	LOC648684	0.01565313143237827
128239	IQGAP3	NM_178229.3	IQGAP3	0.015658724162056693
8120	AP3B2	NM_004644.3	AP3B2	0.0156638685342592
100127886	LOC100127886	XM_001721104.1	LOC100127886	0.01569002017637976

100129520	LOC100129520	XM_001720546.1	LOC100129520	0.015726804775336712
81797	OR12D3	NM_030959.2	OR12D3	0.015879234124184034
442204	LOC442204	XM_941158.1	LOC442204	0.015897567172426633
648879	LOC648879	XM_937958.1	LOC648879	0.01599569702802101
653392	LOC653392	XM_929544.1	LOC653392	0.016044495811424156
		Hs.454935	HS.454935	0.016147535401897353
		Hs.169896	HS.169896	0.016207540339586237
9469	CHST3	NM_004273.2	CHST3	0.016243901989474365
		Hs.552917	HS.552917	0.0162697160542665
653759	LOC653759	XM_933689.1	LOC653759	0.016371098321163312
645769	LOC645769	XM_928769.1	LOC645769	0.016510185576198752
7781	SLC30A3	NM_003459.4	SLC30A3	0.016511028193686883
80144	FRAS1	NM_206841.1	FRAS1	0.016523873196868023
664617	MIR542	NR_030399.1	MIR542	0.016613714909317213
100302134	MIR1289-2	NR_031621.1	MIR1289-2	0.01662531022456159
4134	MAP4	NM_002375.3	MAP4	0.016775584222864293
677775	SCARNA5	NR_003008.2	SCARNA5	0.016785208842328966
100133602	LOC100133602	XR_036856.1	LOC100133602	0.01683666670821988
400352	LOC400352	XR_016578.2	LOC400352	0.017079739864692106
653803	LOC653803	XM_376278.3	LOC653803	0.017127976599654482
645643	LOC645643	XM_933022.1	LOC645643	0.017150239866511565
441488	LOC441488	XM_929997.1	LOC441488	0.017215380981883834
644076	GLYCAM1	XM_945144.1	GLYCAM1	0.01722458438549089
646399	LOC646399	XM_929326.1	LOC646399	0.01754998407582004
652760	LOC652760	XM_942393.1	LOC652760	0.017582158167762218
100133056	LOC100133056	XM_001723741.1	LOC100133056	0.017614278666529242
653513	LOC653513	XM_927826.1	LOC653513	0.01764358092608569
100133638	LOC100133638	XM_001714231.1	LOC100133638	0.01773533627268364
29113	C6orf15	NM_014070.1	C6ORF15	0.017803645740275556
653149	LOC653149	XM_926213.1	LOC653149	0.01787715309412106
653941	LOC653941	XM_943151.1	LOC653941	0.018263229409780438
8385	OR1D4	NM_003552.2	OR1D4	0.018307855268222265
649280	LOC649280	XM_941486.1	LOC649280	0.018391792044690356
83856	FSD1L	NM_031919.1	FSD1L	0.01846573577669781
		Hs.579259	HS.579259	0.018467485733116545
		Hs.581657	HS.581657	0.018606211149496413
		Hs.133257	HS.133257	0.018767528532055853
100048912	ANRIL	Hs.556821	ANRIL	0.01879524479320668
692200	SNORD85	NR_003066.1	SNORD85	0.018976701195394474
		Hs.581645	HS.581645	0.01899843200332198
79710	MORC4	NM_024657.2	MORC4	0.01910590570428638
122038	LOC122038	XM_062912.1	LOC122038	0.019196424317865713
100128838	LOC100128838	XM_001724524.1	LOC100128838	0.01922384593465647
		Hs.242159	HS.242159	0.019287226676571085
729528	PRAMEF14	NM_001099854.1	PRAMEF14	0.019416555497664207
406902	MIR10A	NR_029608.1	MIR10A	0.019425208689276614

650977	LOC650977	XM_935578.1	LOC650977	0.01956314166256176
		Hs.541600	HS.541600	0.019629559223353542
2525	FUT3	NM_000149.1	FUT3	0.019700089768871422
729393	LOC729393	XM_935556.1	LOC729393	0.019865272887790345
100132209	LOC100132209	XM_001714087.1	LOC100132209	0.020221726568477035
652575	LOC652575	XM_942082.1	LOC652575	0.02028866877821377
100313884	MIR548H4	NR_031680.1	MIR548H4	0.020330775601036345
653806	LOC653806	XM_930050.1	LOC653806	0.020337837904343084
		Hs.563518	HS.563518	0.020396989331975476
10060	ABCC9	NM_020298.2	ABCC9	0.02040799559747524
646574	LOC646574	XM_933715.1	LOC646574	0.02062140321117068
730196	LOC730196	XM_930127.1	LOC730196	0.020624550982535607
650721	LOC650721	XM_944342.1	LOC650721	0.02062949543418754
642692	LOC642692	XM_926143.1	LOC642692	0.020863646281524408
		Hs.571515	HS.571515	0.020882015379294242
55908	LOC55908	NM_018687.3	LOC55908	0.02090164536976935
		Hs.534913	HS.534913	0.021066473132055688
643922	LOC643922	XM_927182.1	LOC643922	0.02111492999640619
441795	LOC441795	XM_937553.1	LOC441795	0.02113572373628669
		Hs.550218	HS.550218	0.02113799599016684
649041	LOC649041	XM_938124.1	LOC649041	0.021141231059488303
284417	TMEM150B	XM_209187.5	TMEM150B	0.02123943361465546
462	SERPINC1	NM_000488.2	SERPINC1	0.021275751939699005
441016	LOC441016	XM_936464.1	LOC441016	0.021327771230071232
56675	NRIP3	NM_020645.1	NRIP3	0.021359967827102422
		Hs.409403	HS.409403	0.021368339724236827
10512	SEMA3C	NM_006379.2	SEMA3C	0.021410594450011266
65082	VPS33A	NM_022916.3	VPS33A	0.021667463211417614
344558	SH3MD4	XM_293090.5	SH3MD4	0.02171773623940785
219557	MGC26647	NM_152706.2	MGC26647	0.02173921534576355
92105	INTS4	XM_941208.1	INTS4	0.02174200017972866
286557	RBMY1A3P	NR_001547.1	RBMY1A3P	0.02176637253887792
729240	PRR20C	NM_001130405.1	PRR20C	0.021780497537751933
158852	CT45-2	NM_152582.3	CT45-2	0.02184468944229154
646823	LOC646823	XM_933959.1	LOC646823	0.021954614523768918
51804	SIX4	NM_017420.2	SIX4	0.02196686864294639
100131696	LOC100131696	XM_001719335.1	LOC100131696	0.021973367354500152
		Hs.576474	HS.576474	0.021982121554966757
643937	LOC643937	XM_927195.1	LOC643937	0.02199470986446304
100131999	LOC100131999	XM_001714361.1	LOC100131999	0.02210884720074493
		Hs.543340	HS.543340	0.02221949537497687
728022	LOC728022	XM_001720082.1	LOC728022	0.022536695804648364
100134292	LOC100134292	XM_001717524.1	LOC100134292	0.022562678768112604
85302	FBF1	XM_946190.1	FBF1	0.02267592886449689
643062	LOC643062	XM_926433.1	LOC643062	0.02270477174650738
256144	OR4C3	NM_001004702.1	OR4C3	0.02274452747329593

388468	POTEC	NM_001137671.1	POTEC	0.022857498615990433
284467	FAM19A3	NM_001004440.1	FAM19A3	0.022993737812894953
5549	PRELP	NM_002725.3	PRELP	0.023129210919753312
643365	FLJ44054	NR_024609.1	FLJ44054	0.023168962721932574
51090	PLLP	NM_015993.1	PLLP	0.023229776752251536
55025	FLJ20712	XM_929740.1	FLJ20712	0.02332271643085656
55890	GPRC5C	NM_022036.2	GPRC5C	0.023339187030995644
5731	PTGER1	NM_000955.2	PTGER1	0.02334109658809124
100134687	LOC100134687	XM_001725648.1	LOC100134687	0.023364433029817847
6534	SLC6A7	NM_014228.2	SLC6A7	0.02351409087980999
153218	SPINK5L3	XM_376433.2	SPINK5L3	0.023561990750792117
389396	C6orf140	XM_001717222.1	C6ORF140	0.02380808919026948
148766	LOC148766	XM_944043.1	LOC148766	0.023814290697377855
161357	MDGA2	NM_182830.2	MDGA2	0.0239783457626738
100128074	LOC100128074	XM_001716711.1	LOC100128074	0.024034324339671117
389888	LOC389888	XM_372248.3	LOC389888	0.02420778796700878
3854	KRT6B	NM_005554.2	KRT6B	0.024242414229249387
253650	FLJ35740	NM_147195.1	FLJ35740	0.024248226617046308
9620	CELSR1	NM_014246.1	CELSR1	0.02442422597179391
650689	LOC650689	XM_944331.1	LOC650689	0.024620891735010586
650851	LOC650851	XM_939939.1	LOC650851	0.02470620850525972
		Hs.445179	HS.445179	0.024783874247643473
100131060	LOC100131060	XM_001726569.1	LOC100131060	0.024789556725427153
79837	PIP4K2C	NM_024779.3	PIP4K2C	0.024790272965445812
643431	LOC643431	XM_928128.1	LOC643431	0.0248750546433254
389365	LOC389365	XM_371797.2	LOC389365	0.02499211911817448
727884	LOC727884	XR_037459.1	LOC727884	0.02512510114659658
149708	WFDC5	NM_145652.2	WFDC5	0.025211307697428416
644054	FAM25C	NM_001137548.1	FAM25C	0.025286841884306046
206338	FLJ90650	NM_173800.3	FLJ90650	0.025331849080773603
345274	SLC10A6	NM_197965.1	SLC10A6	0.025539266030957946
574508	MIR505	NR_030230.1	MIR505	0.025542229322075727
677830	SNORA50	NR_002980.1	SNORA50	0.025648115404393816
		Hs.538367	HS.538367	0.0258033480835266
644316	LOC644316	XM_927484.1	LOC644316	0.0258203783413274
100128717	LOC100128717	XR_037607.1	LOC100128717	0.02594124537372537
93659	CGB5	NM_033043.1	CGB5	0.02604889505318224
		Hs.548213	HS.548213	0.026209602842411143
643402	LOC643402	XM_926737.1	LOC643402	0.02622565874116249
117531	TMC1	NM_138691.2	TMC1	0.026310984504829084
5303	PIN4	NM_006223.2	PIN4	0.026349367250758066
767610	SNORD114-29	NR_003222.1	SNORD114-29	0.02636118469746321
393076	LOC393076	XM_935699.1	LOC393076	0.02642569718895187
644098	LOC644098	XM_931998.1	LOC644098	0.026472465676084144
391819	KRT18P42	XR_039234.1	KRT18P42	0.02653184971257089
100129376	LOC100129376	XM_001714929.1	LOC100129376	0.026682345819321992

157724	SLC7A13	NM_138817.2	SLC7A13	0.026698560476787658
29931	LOH3CR2A	NM_013343.1	LOH3CR2A	0.026792621726054573
56122	PCDHB14	NM_018934.2	PCDHB14	0.026817705068609882
100131183	LOC100131183	XR_039203.1	LOC100131183	0.02688306877348543
		Hs.353831	HS.353831	0.026919042069835364
5080	PAX6	NM_000280.2	PAX6	0.027014121380323875
144402	CPNE8	NM_153634.2	CPNE8	0.027083771991721642
		Hs.580251	HS.580251	0.027115028419016078
728656	LOC728656	XM_932849.1	LOC728656	0.02719021068000494
652326	LOC652326	XM_941750.1	LOC652326	0.027209804441805464
648064	LOC648064	XM_937115.1	LOC648064	0.027538570723816908
		Hs.564082	HS.564082	0.02763550270793093
245973	ATP6V1C2	NM_144583.3	ATP6V1C2	0.027694136772679557
130120	REG3G	NM_001008387.1	REG3G	0.027932711689056387
340561	MGC42638	XM_926284.1	MGC42638	0.028277961439309544
3714	JAG2	NM_145159.1	JAG2	0.02841786844924962
		Hs.542618	HS.542618	0.028503835503501194
26492	OR8G2	NM_001007249.1	OR8G2	0.0285351290547875
		Hs.221951	HS.221951	0.028565419239745177
		Hs.538525	HS.538525	0.028632798570462893
574493	MIR520H	NR_030215.1	MIR520H	0.02865016994231157
9180	OSMR	NM_003999.1	OSMR	0.029033257307868314
		Hs.529590	HS.529590	0.029095528736048856
100129264	LOC100129264	XM_001725448.1	LOC100129264	0.029164465075395617
		Hs.232520	HS.232520	0.02932739677614784
79823	C2orf34	NM_024766.1	C2ORF34	0.02943581684894508
84530	SRRM4	NM_194286.2	SRRM4	0.029557850541321092
390880	LOC390880	XM_372707.3	LOC390880	0.029577098030507207
		Hs.544707	HS.544707	0.029829129270451688
348801	LNP1	XM_379203.2	LNP1	0.02985157069630891
		Hs.571245	HS.571245	0.029888463512216464
403257	OR4C45	NM_001005513.1	OR4C45	0.029893456666116217
100131573	LOC100131573	XM_001716335.1	LOC100131573	0.029921462243786185
642867	LOC642867	XM_931130.1	LOC642867	0.02995179201679189
574460	MIR498	NR_030182.1	MIR498	0.02999828744136964
5130	PCYT1A	NM_005017.2	PCYT1A	0.03001622031954481
6359	CCL15	NM_004167.3	CCL15	0.030086532136797735
693191	MIR606	NR_030337.1	MIR606	0.030092216058631844
130951	C2orf65	NM_138804.2	C2ORF65	0.030141646111764182
8542	APOL1	NM_145344.1	APOL1	0.03014373088034522
114038	C21orf84	NM_153752.1	C21ORF84	0.030270306908501375
		Hs.576698	HS.576698	0.030320096011271686
402066	LOC402066	XM_377725.3	LOC402066	0.030385264422877218
220032	GDPD4	NM_182833.1	GDPD4	0.030427690838538468
11245	GPR176	NM_007223.1	GPR176	0.030603100342009137
389442	LOC389442	XM_945400.1	LOC389442	0.030698112698738667

148811	PM20D1	NM_152491.3	PM20D1	0.030930813297524267
		Hs.568434	HS.568434	0.031003115105758768
391002	PRAMEF8	NM_001012276.1	PRAMEF8	0.031078148393666006
647954	LOC647954	XM_938141.1	LOC647954	0.03115141005922734
6003	RGS13	NM_144766.1	RGS13	0.03115524736341413
390616	ANKRD34C	XM_930512.1	ANKRD34C	0.03126601674428487
283585	LOC283585	XM_375099.2	LOC283585	0.03126958784080985
651787	LOC651787	XM_944941.1	LOC651787	0.03128112310360932
		Hs.46693	HS.46693	0.03129196190008781
		Hs.537983	HS.537983	0.03139569345078746
645848	LOC645848	XM_933202.1	LOC645848	0.03145673574418213
645201	LOC645201	XM_928236.1	LOC645201	0.031545488210135406
643446	LOC643446	XM_932755.1	LOC643446	0.0315889226603109
1014	CDH16	NM_004062.2	CDH16	0.0317473861882028
100129536	LOC100129536	XR_038075.1	LOC100129536	0.031820383098682216
441250	TYW1B	XR_015176.2	TYW1B	0.03183031116099876
644669	LOC644669	XM_927774.1	LOC644669	0.0318303814739753
		Hs.546079	HS.546079	0.03191419153433756
643799	LOC643799	XM_929213.1	LOC643799	0.031949486116225896
340895	C10orf112	XM_001716843.1	C10ORF112	0.032093992948235735
651923	LOC651923	XM_945017.1	LOC651923	0.0321414742003836
650632	LOC650632	XM_939721.1	LOC650632	0.03219295571905057
100302229	MIR1250	NR_031652.1	MIR1250	0.03222536563546908
648898	LOC648898	XM_944772.1	LOC648898	0.03224153084769567
677840	SNORA71D	NR_003018.2	SNORA71D	0.03228973382905253
		Hs.566864	HS.566864	0.032351696758506805
387755	INSC	NM_001031853.2	INSC	0.032364003360592905
127064	OR2T12	NM_001004692.1	OR2T12	0.032808211355858825
114771	PGLYRP3	NM_052891.1	PGLYRP3	0.032837605539625184
		Hs.581365	HS.581365	0.032875995067218604
11260	XPOT	NM_007235.3	XPOT	0.03293121754836928
440026	TMEM41B	NM_015012.1	TMEM41B	0.033056690688610224
400958	LOC400958	XM_379100.3	LOC400958	0.03307097076308331
100129000	LOC100129000	XM_001719583.1	LOC100129000	0.033091916558510376
652833	LOC652833	XM_942519.1	LOC652833	0.03321298535418646
149775	GNASAS	NR_002785.2	GNASAS	0.03344199943180014
653781	LOC653781	XM_929706.1	LOC653781	0.03347996462830669
222183	FLJ37078	NM_153043.3	FLJ37078	0.03354247547865309
100134372	LOC100134372	XM_001714877.1	LOC100134372	0.03355176427687743
643435	LOC643435	XM_931545.1	LOC643435	0.03357003327282396
5874	RAB27B	NM_004163.3	RAB27B	0.033575194307435996
79954	NOL10	NM_024894.1	NOL10	0.0335990779953308
84793	MGC12982	NR_026878.1	MGC12982	0.03361970373140142
286075	ZNF707	NM_173831.2	ZNF707	0.03371320701560756
100131320	LOC100131320	XM_001723688.1	LOC100131320	0.03385445543039937
283025	C10orf40	NR_024340.1	C10ORF40	0.03394751501046586

5599	MAPK8	NM_002750.2	MAPK8	0.03399866452797381
644079	LOC644079	XM_001720206.1	LOC644079	0.0341912882375524
368	ABCC6	NM_001079528.1	ABCC6	0.03431493026050804
100132767	LOC100132767	XM_001720251.1	LOC100132767	0.03440080915221909
138881	OR1L8	NM_001004454.1	OR1L8	0.03456597396989249
6584	SLC22A5	NM_003060.2	SLC22A5	0.03460459391804023
677829	SNORA49	NR_002979.2	SNORA49	0.03463335854931575
		Hs.574252	HS.574252	0.03477897443857523
728876	LOC728876	Hs.207162	LOC728876	0.03482274997843915
119467	CLRN3	NM_152311.1	CLRN3	0.035018728475164886
79822	ARHGAP28	NM_030672.2	ARHGAP28	0.03505231068410014
		Hs.562032	HS.562032	0.03508937672895057
7767	ZNF224	NM_013398.1	ZNF224	0.03512398245050982
5916	RARG	NM_000966.3	RARG	0.035124813290261965
100128537	LOC100128537	XM_001714326.1	LOC100128537	0.03517421150909691
		Hs.542875	HS.542875	0.03517734679208625
		Hs.18849	HS.18849	0.03527114019290617
100132972	LOC100132972	XR_039130.1	LOC100132972	0.035333362585726155
		Hs.549487	HS.549487	0.0354015803965153
93986	FOXP2	NM_148898.1	FOXP2	0.03550743036656206
284697	BTBD8	NM_183242.1	BTBD8	0.03557651653273978
81629	TSSK3	NM_052841.3	TSSK3	0.03559026459232305
728193	LOC728193	XR_001254.1	LOC728193	0.03560005450194582
55214	LEPREL1	NM_018192.2	LEPREL1	0.03561315135191362
100132963	LOC100132963	XM_001714657.1	LOC100132963	0.035694192240537256
	ERCC-00017	ERCC-00017	ERCC-00017	0.035822810531763706
10143	CLEC3A	NM_005752.2	CLEC3A	0.0358258784540761
100131673	LOC100131673	XM_001723856.1	LOC100131673	0.03586836841115992
692108	SNORD67	NR_003056.1	SNORD67	0.035873412006882904
643927	LOC643927	XM_927188.1	LOC643927	0.03590837992065841
729643	LOC729643	XM_001130893.1	LOC729643	0.03611177583217427
654101	LOC654101	XM_939354.1	LOC654101	0.03611818282844728
641311	RPL31P11	XR_000997.1	RPL31P11	0.03615679052967315
		Hs.545364	HS.545364	0.036224827502454135
646764	LOC646764	XM_929715.1	LOC646764	0.036249539928655795
727997	LOC727997	XM_001127849.1	LOC727997	0.03635395411914439
		Hs.542667	HS.542667	0.03639857127404468
		Hs.541092	HS.541092	0.03644836231965023
728924	LOC728924	XM_001132979.1	LOC728924	0.036626538623253165
652335	LOC652335	XM_941764.1	LOC652335	0.03689505668917005
26583	DUX2	NM_012147.2	DUX2	0.03698973758707169
344382	LOC344382	XM_293026.5	LOC344382	0.03700152462800427
100133053	LOC100133053	XM_001720766.1	LOC100133053	0.037011044365361706
642018	LOC642018	XM_936088.1	LOC642018	0.037069802753291296
643549	FLJ40606	XR_041973.1	FLJ40606	0.03716768958347956
401317	LOC401317	XM_938320.1	LOC401317	0.037207267570032204

100128485	LOC100128485	XM_001715311.1	LOC100128485	0.037552837763810396
		Hs.545648	HS.545648	0.03783155531545804
642194	LOC642194	XM_942778.1	LOC642194	0.03789201700212872
654032	LOC654032	XM_939494.1	LOC654032	0.03813728856527247
643414	LIPL2	XM_937207.1	LIPL2	0.03817870804042538
692086	SNORD17	NR_003045.1	SNORD17	0.038200153388503036
647080	LOC647080	XM_934321.1	LOC647080	0.03830985050841766
	ERCC-00053	ERCC-00053	ERCC-00053	0.03834869514975889
		Hs.534809	HS.534809	0.03834879151926415
57683	KIAA1571	XM_937234.1	KIAA1571	0.038358552981103154
729041	LOC729041	XM_001717661.1	LOC729041	0.03836846629500259
100127887	LOC100127887	XM_001723461.1	LOC100127887	0.03853325132203725
6048	RNF5	NM_006913.2	RNF5	0.03860009093862527
286464	CXorf59	NM_173695.1	CXORF59	0.038680235450205415
340549	LOC340549	XM_293332.1	LOC340549	0.038917475446315276
255411	LOC255411	XM_932014.1	LOC255411	0.03895963998255315
391365	SULT6B1	NM_001032377.1	SULT6B1	0.03911302437449217
651763	LOC651763	XM_940981.1	LOC651763	0.039244630984538384
389031	LOC389031	XM_940482.1	LOC389031	0.03926998584035779
338662	OR8D4	NM_001005197.1	OR8D4	0.0393256779732204
256281	NUDT14	NM_177533.2	NUDT14	0.03936138517869517
338751	OR52L1	NM_001005173.1	OR52L1	0.03952706246953922
55037	PTCD3	NM_017952.4	PTCD3	0.03965831209140964
730050	LOC730050	XM_942380.1	LOC730050	0.03970261977275519
649469	LOC649469	XM_938551.1	LOC649469	0.039726795675571205
647651	LOC647651	XM_936697.1	LOC647651	0.03976741733921677
100133418	LOC100133418	XM_001714459.1	LOC100133418	0.039798961546514564
		Hs.143909	HS.143909	0.03987953435966447
		Hs.541752	HS.541752	0.04004451312984737
147945	NLRP4	NM_134444.3	NLRP4	0.04005249679157248
6288	SAA1	NM_000331.2	SAA1	0.040065832203235015
		Hs.547175	HS.547175	0.040153296946881016
8074	FGF23	NM_020638.2	FGF23	0.04016990512673844
441381	LRRC24	NM_001024678.1	LRRC24	0.04029216676542084
728343	LOC728343	XM_939774.1	LOC728343	0.040324070434557116
		Hs.475950	HS.475950	0.0403536263781617
375010	LOC375010	XM_927556.1	LOC375010	0.040404601611003954
4656	MYOG	NM_002479.3	MYOG	0.04050658101039191
		Hs.437179	HS.437179	0.04052765359085568
116449	CLNK	NM_052964.2	CLNK	0.0405367112071025
3060	HCRT	NM_001524.1	HCRT	0.04065663865807545
26740	OR1J2	NM_054107.1	OR1J2	0.04079535607325396
146177	LOC146177	XM_370944.4	LOC146177	0.04082276324038739
400965	LOC400965	NM_001013677.1	LOC400965	0.040887347474745844
		Hs.580126	HS.580126	0.0410471418128887
		Hs.545887	HS.545887	0.04111865623111575

727827	LOC727827	XM_001127359.2	LOC727827	0.04112645889694715
135656	DPCR1	NM_080870.2	DPCR1	0.04113050080090478
647515	LOC647515	XM_001718676.1	LOC647515	0.04116426887393105
100133348	LOC100133348	XM_001716396.1	LOC100133348	0.04126059807045334
1447	CSN2	NM_001891.1	CSN2	0.04130424464274184
647197	LOC647197	XM_934499.1	LOC647197	0.04144851834200355
		Hs.157621	HS.157621	0.041452897550665085
285479	LOC285479	XM_211908.5	LOC285479	0.04162774662655042
23430	TPSD1	NM_012217.2	TPSD1	0.04162903602256395
100130123	LOC100130123	XM_001726844.1	LOC100130123	0.04178163825145163
		Hs.545230	HS.545230	0.0418944246284238
641978	LOC641978	XM_935752.1	LOC641978	0.04193526858697818
		Hs.583274	HS.583274	0.0419771000983454
284099	C17orf78	NM_173625.3	C17ORF78	0.0420299255226024
284451	ODF3L2	NM_182577.1	ODF3L2	0.042116097623423256
		Hs.569313	HS.569313	0.04213676251389568
148645	MGC40168	NM_153709.1	MGC40168	0.042325609228867814
50945	TBX22	NM_016954.2	TBX22	0.042337420790947905
		Hs.560736	HS.560736	0.042560307781693854
100129343	LOC100129343	XM_001724611.1	LOC100129343	0.0426296711237734
100129514	LOC100129514	XM_001724518.1	LOC100129514	0.042757097797527295
645218	LOC645218	XM_945368.1	LOC645218	0.042789979520314755
83481	EPPK1	XM_926068.1	EPPK1	0.0428175486064293
3547	IGSF1	NM_001555.2	IGSF1	0.04284432572336439
258010	SVIP	NM_148893.1	SVIP	0.042926970724047234
646280	LOC646280	XM_929223.1	LOC646280	0.043143966073033654
51802	ACCN5	NM_017419.1	ACCN5	0.043346582974570357
		Hs.538535	HS.538535	0.04341198215012938
		Hs.562701	HS.562701	0.04362372092389725
441501	FLJ46446	XM_001715413.1	FLJ46446	0.04370004734540954
54985	HCFC1R1	NM_001002017.1	HCFC1R1	0.0437394289594447
653060	LOC653060	XM_925779.1	LOC653060	0.043789209896504436
		Hs.135587	HS.135587	0.04379133238179812
		Hs.40289	HS.40289	0.04400621524649907
652762	LOC652762	XM_945940.1	LOC652762	0.044008620289967326
100132966	LOC100132966	XM_001714155.1	LOC100132966	0.04407882716170898
692057	SNORD12	NR_003030.1	SNORD12	0.044299546532056915
26812	SNORD37	NR_002602.1	SNORD37	0.04433768044660433
728116	LOC728116	NM_144621.2	LOC728116	0.04442996576954535
55049	C19orf60	NM_001100419.1	C19ORF60	0.04453832178515606
100128356	LOC100128356	XM_001719204.1	LOC100128356	0.04462339465347257
100124542	SNORA11E	NR_003712.1	SNORA11E	0.04473602678130252
100129119	LOC100129119	XM_001718523.1	LOC100129119	0.04499965944222805
81282	OR51G2	NM_001005238.1	OR51G2	0.04508569441179958
		Hs.582113	HS.582113	0.0451131786661592
		Hs.537150	HS.537150	0.04516681361170144

51555	PEX5L	NM_016559.1	PEX5L	0.04524256321740555
100130370	LOC100130370	XM_001715409.1	LOC100130370	0.045377009541867616
		Hs.442504	HS.442504	0.04540920889661434
442425	FOXB2	NM_001013735.1	FOXB2	0.04542397134263472
644152	LOC644152	XM_927360.1	LOC644152	0.04542523508126548
		Hs.563147	HS.563147	0.04546159300769052
		Hs.537608	HS.537608	0.04562692161699765
653434	LOC653434	XM_933966.1	LOC653434	0.04568770829349333
100124540	SNORA11C	NR_003710.1	SNORA11C	0.04576946351814093
		Hs.555208	HS.555208	0.045770937282462607
26245	OR2M4	NM_017504.1	OR2M4	0.04578649557143999
401109	FLJ25996	NM_001001699.1	FLJ25996	0.04589285003980043
100130141	LOC100130141	XR_038164.1	LOC100130141	0.046055871031270804
126868	C1orf161	NM_152367.1	C1ORF161	0.04609199594523736
285512	FAM13AOS	NR_002806.2	FAM13AOS	0.046145373406548514
402317	OR2A42	NM_001001802.1	OR2A42	0.04616272697550872
		Hs.541191	HS.541191	0.04625642254718605
374739	TEPP	NM_199046.1	TEPP	0.04625830589891997
285697	LOC285697	XM_941117.1	LOC285697	0.04632282358450559
		Hs.566890	HS.566890	0.04635531669736749
5155	PDGFB	NM_033016.1	PDGFB	0.04650986869654247
727836	LOC727836	XR_038572.1	LOC727836	0.04652114694423314
389730	LOC389730	XR_000527.1	LOC389730	0.046547519642591115
374378	GALNTL4	NM_198516.1	GALNTL4	0.046735817907034405
643037	LOC643037	XM_936787.1	LOC643037	0.04679494780096214
100302130	MIR1302-4	NR_031633.1	MIR1302-4	0.04686041799156694
649186	LOC649186	XM_938252.1	LOC649186	0.04686938946073637
		Hs.561430	HS.561430	0.046901961073004754
		Hs.302412	HS.302412	0.04694635264572553
8330	HIST1H2AK	NM_003510.2	HIST1H2AK	0.046960800231884994
		Hs.544495	HS.544495	0.04696506376407463
		Hs.574587	HS.574587	0.0469684078568494
100131244	LOC100131244	XM_001724055.1	LOC100131244	0.046981434770345706
		Hs.546135	HS.546135	0.046996587342102505
651177	LOC651177	XM_940301.1	LOC651177	0.04700075868907026
340602	CXorf67	NM_203407.1	CXORF67	0.047001076847879825
646570	LOC646570	XM_933711.1	LOC646570	0.04703739710584047
		Hs.550042	HS.550042	0.047115038681197076
1297	COL9A1	NM_001851.3	COL9A1	0.04714323250208604
10014	HDAC5	NM_001015053.1	HDAC5	0.04722777454918724
641995	LOC641995	XM_935775.1	LOC641995	0.047228437613621325
729374	LOC729374	XR_038569.1	LOC729374	0.04724643599108605
653786	LOC653786	NR_003676.2	LOC653786	0.04725527473065633
26780	SNORA68	NR_000012.1	SNORA68	0.047285746510851566
400019	LOC400019	XM_378349.3	LOC400019	0.047393136010094226
140691	TRIM69	NM_080745.3	TRIM69	0.0474119722132346

647234	LOC647234	XM_934553.1	LOC647234	0.047439423733171025
56163	RNF17	NM_031994.1	RNF17	0.04767407512859314
89932	PAPLN	NM_173462.2	PAPLN	0.04768950584794584
403314	APOBEC4	NM_203454.1	APOBEC4	0.04793759494161416
4842	NOS1	NM_000620.1	NOS1	0.048026600103485875
100126319	MIR216B	NR_030623.1	MIR216B	0.048043630203721495
		Hs.58089	HS.58089	0.048076218570047326
646358	LOC646358	XM_929287.1	LOC646358	0.048107642962684015
439941	C8orf54	XR_000579.1	C8ORF54	0.04814616528597775
1826	DSCAM	NM_206887.1	DSCAM	0.04833964991785896
651711	LOC651711	XM_940923.1	LOC651711	0.0483643092356878
		Hs.582594	HS.582594	0.0484084727751594
84809	CROCCL1	XR_039823.1	CROCCL1	0.048508335050976716
36	ACADSB	NM_001609.2	ACADSB	0.04852330528721858
149465	WDR65	NM_152498.1	WDR65	0.04853470869428911
652510	LOC652510	XM_941981.1	LOC652510	0.04863040057616678
1800	DPEP1	NM_004413.1	DPEP1	0.048648356765277206
5279	PIGC	NM_153747.1	PIGC	0.04869033866821056
645651	LOC645651	XM_928665.1	LOC645651	0.048761572597092004
644415	LOC644415	XR_018194.2	LOC644415	0.04887709279665332
154288	ECAT1	NM_001017361.1	ECAT1	0.04888972575962395
646187	LOC646187	XR_016991.2	LOC646187	0.04903448172268005
645196	LOC645196	XM_928233.1	LOC645196	0.04918479829182512
81607	PVRL4	NM_030916.1	PVRL4	0.0493173128212757
285193	DUSP28	NM_001033575.1	DUSP28	0.0493731738586513

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Grafisk design: Kommunikasjonsavdelingen, UiB / Trykk: Skipnes Kommunikasjon AS

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ISBN: 978-82-308-3529-6