High VEGFC expression is associated with unique gene expression profiles and predicts adverse prognosis in pediatric and adult acute myeloid leukemia

Hendrik J.M. de Jonge¹, Peter J.M. Valk², Nic J.G.M. Veeger³, Arja ter Elst¹, Monique L. den Boer⁴, Jacqueline Cloos⁵,⁶, Valérie de Haas⁶, Marry M. van den Heuvel-Eibrink⁴,⁶, Gertjan J.L. Kaspers⁵,⁶, Christian M. Zwaan⁴,⁶, Willem A. Kamps¹,⁶, Bob Löwenberg² and Eveline S.J.M. de Bont¹,⁶

1. Division of Pediatric Oncology/Hematology, Department of Pediatrics, Beatrix Children's Hospital, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.
2. Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands.
3. Department of Epidemiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.
4. Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands.
5. Division of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands.

Short running title: VEGFC mRNA associates with poor outcome in AML.

Scientific category: Myeloid Neoplasia

Corresponding author: Eveline SJM de Bont, MD, PhD. Division of Pediatric Oncology/Hematology, Department of Pediatrics, Beatrix Children's Hospital, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. P.O. Box 30.001, 9700 RB Groningen, the Netherlands. e-mail: e.de.bont@bkk.umcg.nl, tel: 0031503614146, fax: 0031503611671.
ABSTRACT

High VEGFC mRNA expression of AML blasts is related to increased in vitro and in vivo drug resistance. The prognostic significance of VEGFC on long-term outcome and its associated gene expression profiles remain to be defined. We studied the effect of VEGFC on treatment outcome and investigated gene expression profiles associated with VEGFC using microarray data of 525 adult and 100 pediatric AML patients. High VEGFC expression appeared strongly associated with reduced complete remission rate ($P = .004$), reduced overall and event-free survival (OS and EFS) in adult AML ($P = .002$ and $P < .001$ respectively). Multivariable analysis established high VEGFC as prognostic indicator independent of cytogenetic risk, FLT3-ITD, NPM1, CEBPA, age and WBC ($P = .038$ for OS and $P = .006$ for EFS). Also in pediatric AML high VEGFC was related to reduced OS ($P = .041$). A unique series of differentially expressed genes was identified that distinguished AML with high VEGFC from AML with low VEGFC, i.e., 331 upregulated genes (representative of proliferation, VEGF-receptor activity, signal transduction) and 44 downregulated genes (e.g. related to apoptosis) consistent with a role in enhanced chemoresistance.

In conclusion, high VEGFC predicts adverse long-term prognosis and provides prognostic information in addition to well-known prognostic factors.
INTRODUCTION

Vascular endothelial growth factor-C (VEGFC) is a (lymph)angiogenic growth factor and signals through kinase insert domain receptor (KDR, i.e. VEGF Receptor-2) and fms-related tyrosine kinase 4 receptor (FLT4, i.e. VEGF Receptor-3).\(^1\)\(^2\) In general, by VEGF stimulation, VEGFRs become phosphorylated and transmit intracellular signals resulting in cell proliferation and survival.\(^3\)\(^4\)

Acute myeloid leukemic (AML) blasts express VEGFC and its receptors KDR and FLT4.\(^5\) Exogenously added VEGFC promotes \textit{in vitro} cell survival via activation of the FLT4 and KDR heterodimeric receptor, as shown in two AML cell lines and in five cases of primary AML.\(^6\) Recently, we described that \textit{endogenous} VEGFC mRNA expression levels of primary AML cells were related to increased \textit{in vitro} resistance for six AML-related drugs.\(^7\) In addition, a relation was demonstrated between high VEGFC mRNA and slow AML blast disappearance during induction treatment \textit{in vivo}. This was apparent from the higher blast counts in the bone marrow on day 15 after start of induction chemotherapy and a prolonged time to achieve complete remission.\(^7\) Currently, insight into molecular mechanisms responsible for the unfavorable treatment response and possible relationships of VEGFC levels with other biological factors with prognostic significance is lacking. Furthermore, to date, the effect of VEGFC on long-term outcome has not been assessed in a large series of cases. We set out to study the effect of VEGFC on overall and event free survival (OS and EFS) both in pediatric as well as in adult AML and considered the impact of VEGFC in relation to other established cytogenetic and gene mutation prognostic markers. Finally, we investigated gene expression profiles associated with VEGFC mRNA expression by using Affymetrix HGU133Plus2.0 gene expression data of 525 adult and 100 pediatric AML cases.
METHODS

Patients

Gene expression profiling (GEP) has been performed on 525 consecutive adult AML patients who have been treated according to sequential HOVON/SAKK AML-04, -04A, -29, -32, -42, -43 protocols (available at http://www.hovon.nl). The adult and pediatric AML patients have been included in previous gene expression profiling studies. An independent second series of cell specimens from 100 newly diagnosed pediatric patients with AML -who have been treated according to subsequent Dutch Childhood Oncology Group (DCOG) AML DCOG-BFM-87, DCOG-92/94, DCOG-97 protocols (available at http://www.skion.nl), was used for validation of the data of the adult AML cohort. All adult as well as pediatric patients in this study were newly diagnosed with AML and established according to WHO criteria. All patients provided written informed consent in accordance with the Declaration of Helsinki, and the study was approved by all participating institutional review boards. Cell specimens were collected at the time of diagnosis. All subjects provided written informed consent. Cytogenetic risk group distinction (favorable, intermediate, and unfavorable) is according to HOVON/SAKK and DCOG protocols. Favorable cytogenetic risk was defined as t(8;21)(q22;q22), inv(16)(p13.1;q22), or t(16;16)(p13.1;q22) and t(15;17). Unfavorable cytogenetic risk was defined as: complex cytogenetic abnormalities (i.e. three or more distinct clonal abnormalities), -7, -5, del 5q or del 7q, abnormalities of the long arm of chromosome 3 (abn 3q), t(6;9)(q23;q34), t(9;22)(q34;q11), or abnormalities of the long arm of chromosome 11 (abn 11q23). All other cytogenetic abnormalities as well as AML without cytogenetic abnormalities were considered to indicate an intermediate cytogenetic risk. In
pediatric AML 11q23 and t(6;9)(q23;q34) abnormalities were considered as intermediate cytogenetic risk.

Isolation and Quality Control of RNA, Gene Expression Profiling and Quality Control

The samples for gene expression profiling were obtained and analyzed as described previously. Detailed clinical, cytogenetic, and molecular information is available at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession number GSE6891 for adult AML patients and GSE22056 for pediatric AML patients).

Class comparison

Differentially expressed probes were identified for 262 AML samples with high VEGFC mRNA (i.e. above the median VEGFC mRNA level) vs. 262 AML samples with low VEGFC (i.e. below the median VEGFC mRNA level) mRNA expression levels using a multivariable permutation test in Biometric Research Branch ArrayTools (BRB ArrayTools) version 3.8.0. BRB ArrayTools has been developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differential expression was considered significant at $P < .0001$. A random variance t-test was selected to permit the sharing of information among probe sets within class variation without assuming that all of the probe sets possess the same variance.

Gene ontology analysis

To investigate the biological significance of the gene lists, we used GO (http://www.geneontology.org). After mapping each gene to the GO tree structure, the
number of genes was determined at or below any given node in the GO hierarchy and the amount of statistically enrichment (Fisher exact test and FDR adjustment) for each GO node relative to chance observation, using a previously developed procedure (GeneTrail).17

Statistical Analysis
Statistical analyses were performed with SPSS software, release 16.0. Actuarial probabilities of overall survival (OS) (with death due to any cause) as well as event-free survival (EFS, with failure in case of no complete remission [CR1] or relapse or death) were estimated according to the Kaplan–Meier method. For quantitative parameters overall differences between the cohorts were evaluated using an F-test (or Student-t in case of two groups) for normally distributed variables or a Kruskal-Wallis test (or Mann-Whitney-U test in case of two groups) for skewed distributed variables. For qualitative parameters, overall group differences were evaluated using a Chi-square test (or Fisher exact in 2x2 setting). Correlations were calculated with the Spearman rank correlation coefficient (rho). The association between VEGFC and OS and EFS was tested in univariate Cox models. Cox regression analysis was applied to determine the association of VEGFC expression and OS/EFS with adjustment for risk factors such as age, white blood cell count, cytogenetic risk group (i.e. favorable, intermediate or unfavorable), Fms-like tyrosine kinase 3 gene internal tandem duplication (FLT3-ITD), mutations in genes encoding nucleophosmin 1 (NPM1) and the transcription factor CCAAT/enhancer binding protein α (CEBPA). The proportional hazard assumption was checked using log-log survivor functions (parallel curves). In addition the presence of time-dependence indicating violation of
the proportional hazard assumption was assessed. All tests were 2 tailed, and a $P$ value of less than .05 was considered statistically significant.
RESULTS

High VEGFC expression level is related to reduced OS and EFS in adult AML.

Five hundred and twenty-five adult AML patients were analyzed for mRNA expression of VEGFC by gene-expression profiling. Clinicopathologic, demographic, and molecular data including age at diagnosis, baseline cytogenetics, initial white blood cell count, percentage blasts, platelet count, FLT3-ITD, NPM1 and CEBPA mutation status are included in Table 1. The median OS for the total cohort was 16.3 months, and the median follow-up for survivors was 61.4 months.

Firstly, we tested the association of VEGFC expression as a continuous variable with patient survival. Higher expression levels of VEGFC were significantly associated with reduced OS and EFS (HR: 1.66, 95% CI: 1.16-2.37, \(P = .006\) for OS; HR: 1.66, 95% CI: 1.19-2.32, \(P = .003\) for EFS). We then defined patient subgroups on the basis of high and low VEGFC expression. In an initial step, within the entire cohort of 525 patients, the association between different levels of VEGFC expression and survival was evaluated using quartiles of VEGFC expression to assess appropriateness of VEGFC expression as a continuous variable (Supplementary Figure 1). As the risks in first and second quartile were highly comparable and significantly different from third and fourth quartile, the median VEGFC level was chosen as the appropriate cut off point (hereafter referred to as high and low VEGFC). The latter cut off point corresponds with the threshold used in most previous studies on VEGFC mRNA/protein in various malignancies including AML.\(^7,18-20\) Table 1 lists the clinical characteristics of the entire cohort as well as of patient subgroups distinguished by high versus low mRNA expression of VEGFC. No significant differences in VEGFC mRNA expression were observed among different cytogenetic risk groups nor the genotypic NPM1/FLT3-ITD subgroups or CEBPA
mutant subsets. Also age at diagnosis, white blood cell count, percentage of bone marrow blasts, platelets and the number of patients who had received an allogeneic stem cell transplantation were not different among patients with high vs low VEGFC.

The complete remission rate was significantly lower among AML patients with high VEGFC as compared to those with low VEGFC (Table 1, \( P = .004 \)). Interestingly, the patients with high VEGFC expression levels showed a significantly reduced OS (Figure 1A, \( P = .002 \)) and a reduced EFS (Figure 1B, \( P < .001 \)). Patients with high and low VEGFC transcript levels had median OS of 13 and 19 months and estimated 5-year OS rates of 35% and 47%, respectively. In addition, we specifically evaluated the effect of VEGFC on OS and EFS among cytogenetically intermediate risk AML representing the largest prognostically distinct subgroup (63% of cases studied). Firstly, we tested the association of VEGFC expression as a continuous variable with patient survival in AML cases with intermediate cytogenetic risk. Higher expression levels of VEGFC tend to associate with reduced OS and EFS (HR: 1.45, 95% CI: .95-2.22, \( P = .085 \) for OS; HR: 1.40, 95% CI: .94-2.08, \( P = .100 \) for EFS). Furthermore, within the cohort of intermediate cytogenetic risk cases, patients with low VEGFC compared favorably with high VEGFC in terms of OS (\( P = .034 \)) and EFS (\( P = .083 \)) (Figure 1C and 1D). Although numbers were relatively small, we next studied the effect of VEGFC on OS and EFS within specific cytogenetic subgroups (Supplementary Figure 2). No difference in OS was found among core binding factor (CBF) AMLs (i.e. t(8;21) and inv16)) with low versus high VEGFC (\( P = .193 \)). Interestingly, CBF AMLs with high VEGFC showed a significantly reduced EFS (\( P = .017 \)). In addition, patients harboring t(15;17) with high VEGFC expression showed no difference in OS (\( P = .582 \)) but, interestingly, tend to show a reduced EFS (\( P = .084 \)). Hereafter, the two largest specific cytogenetic subgroups of AML patients with
poor risk cytogenetics within our cohort (i.e. complex cytogenetics and -5(q)/-7(q) abnormalities) were compared for OS and EFS among patients with low versus high VEGFC. In patients with complex cytogenetics no effect of VEGFC on OS and EFS was evident ($P = .676$ and $P = .851$, respectively). Finally, patients with -5(q)/-7(q) cytogenetic abnormalities and high VEGFC tend to show a reduced OS ($P = .067$), but no effect was evident on EFS ($P = .320$).

**Prognostic value of VEGFC expression level in the context of other risk factors in adult AML.**

Univariate analysis demonstrated that besides high VEGFC expression values (HR: 1.41, 95% CI: 1.13-1.76, $P = .003$ for OS and HR: 1.44, 95% CI: 1.17-1.78, $P = .001$ for EFS), also age, white blood cell count, NPM1 mutation, FLT3-ITD, CEBPA and cytogenetic risk group (i.e. unfavorable risk, intermediate and favorable risk) significantly affected OS and EFS (data not shown). When we subsequently, considered the latter variables in a multivariable analysis high VEGFC maintained its independent prognostic value for both OS as well as EFS: (HR: 1.29, 95% CI: 1.02-1.63, $P = .038$ for OS; HR: 1.38, 95% CI: 1.09-1.71, $P = .006$ for EFS) (details in Table 2).

Moreover, we evaluated AML patients with intermediate risk cytogenetics and considered VEGFC transcript level, FLT3-ITD and NPM1 gene mutation status separately. Patients with low VEGFC expression in the absence of a FLT3-ITD constituted a favorable subset of patients with a significantly improved OS and EFS compared to the other three groups combined ($P = .026$ for OS and $P = .032$ for EFS, Figure 2A and 2B). For patients with low VEGFC levels and absent FLT3-ITD, the estimated 5-year OS was 50% and the estimated EFS at 5 years was 40%. In
contrast, the outcome of patients with both high VEGFC expression and FLT3-ITD were worse, as the 5-year OS and EFS rates were only 30% and 26%, respectively. Of note, also within cytogenetically normal karyotype AML, patients with low VEGFC in the absence of a FLT3-ITD tend to have a better prognosis as compared with the other cytogenetically normal karyotype AML patients (P = .11 for OS and P = .04 for EFS, Supplementary Figure 3A and 3B respectively). Furthermore, patients with the combination of a high VEGFC level without a NPM1 mutation showed a nonsignificant trend as regards a reduced OS (P = .098, Supplementary Figure 4A) and a significantly reduced EFS compared to the other three groups combined (P = .012, Supplementary Figure 4B).

**Consistency of VEGFC as a prognostic factor in pediatric AML.**

We wished to study the effect of VEGFC on outcome using an independent cohort of pediatric AML patients (Table 3) and defined patient subgroups on the basis of high and low VEGFC expression using the identical cut off point (i.e. median) as applied to the above adult series of AML. As in adult AML patients, the complete remission rate was significantly reduced in pediatric AML patients with high VEGFC (Table 3, P = .048). Univariate analysis demonstrated that high VEGFC significantly affected OS in pediatric AML (HR: 1.81, 95% CI: 1.02-3.21, P = .044). For EFS a non significant trend was seen (HR: 1.57, 95% CI: 0.94-2.62, P = .087). Figure 3 shows the Kaplan-Meier plots for pediatric AML patients with high versus low VEGFC with regard to OS (Figure 3A, P = .041) and EFS (P = .084, Figure 3B). Thus, the VEGFC prognostic data in pediatric AML appear in general agreement with those in adult AML.
Distinct gene expression profiles between AML samples with high versus low VEGFC expression level.

To extend the observation that clinical parameters differ between AML samples with high vs. low VEGFC, the transcriptome of 262 adult AML samples with high VEGFC was compared with the transcriptome of 262 adult AML samples with low VEGFC. This comparison revealed that 459 unique genes (represented by 778 probe sets) were higher expressed (“Up-with-VEGFC” group), and that 192 unique genes (represented by 250 probe sets) were lower expressed (“Down-with-VEGFC” group) with elevated VEGFC at the significance level of \( P \leq 1.0 \times 10^{-7} \) (Supplementary Table 1). With regard to the differential expression of VEGFC itself, -as expected- the VEGFC gene was the highest ranked among the list of differentially expressed genes. Off note, when a more extreme cut off point for VEGFC was chosen (i.e. first vs fourth quartile) a highly comparable list of differentially expressed genes was found; 648/651 genes were similar between both methods.

Validation of VEGFC dependent differences in gene expression profiles.

In order to validate the list of differently expressed genes between AML samples with high vs. low VEGFC, two independent gene expression profiling cohorts were used; i.e. the pediatric AML cohort described above (\( n = 100 \)) and a publicly available adult AML gene expression profiling cohort (\( n = 180 \))\(^{21} \). The 459 “Up-with-VEGFC” genes and the 192 “Down-with-VEGFC” genes were individually validated as a continuous variable dependent on VEGFC. Hence, 345/459 (75%) “Up-with-VEGFC” as well as 59/192 (31%) “Down-with-VEGFC” genes could be confirmed (i.e. ‘validation step 1’ in Figure 4A) in the pediatric AML set. Subsequently, a third independent adult AML cohort described by Tomasson et al. (\( n=180 \))\(^{21} \) was used for an additional validation
(i.e. ‘validation step 2’ in Figure 4A); 331/345 “Up-with-VEGFC” and 44/59 “Down-with-VEGFC” were found. In summary, of the differentially expressed genes upon VEGFC in the original dataset, 331 “Up-with-VEGFC” genes and 44 “Down-with-VEGFC” genes could be confirmed to be significantly differently expressed as a continuous variable depending on VEGFC in two independent AML cohorts (Figure 4A). The entire list of differentially expressed genes after two validation steps is presented in Supplementary Table 1.

**Biological processes enriched for “up and down with-VEGFC” genes.**

Then biological processes (represented by GO-ontologies) enriched among AML samples with high vs. low VEGFC were analyzed. For this analysis, those genes which were significantly upregulated or downregulated after two validation steps, were used. This 331 (“Up-with-VEGFC”) and 44 (“Down-with-VEGFC”) differentially expressed genes consisted of 105 significantly “Up-with-VEGFC” and 56 significantly “Down-with-VEGFC” GO-ontologies (Supplementary Table 2). This analysis revealed for instance that genes involved in biological processes as proliferation, signal transduction (e.g. PAK3 and SOS2 which are both upstream activators of MEK1/ERK\(^{22-25}\) and PDK1 which is a direct activator of Akt/PKB\(^{26,27}\)), cell adhesion, vascular endothelial growth factor receptor activity (e.g. KDR, PDGFR\(\alpha\) and NRP1), angiogenesis and wnt-protein binding were upregulated with increasing VEGFC. Genes involved in biological processes as apoptosis (TIA1 and ANP32A), cellular metabolic process, cell communication and DNA dependent transcription were found to be downregulated with increasing VEGFC (Figure 4B).

**DISCUSSION**
In this study we show that adult as well as pediatric AML patients with a high VEGFC transcript level have an inferior disease outcome. To further improve the insight into the molecular mechanisms associated and possibly responsible for the differences in VEGFC related poor outcome in AML, we have analyzed expression profiles of 525 AML patients in relation to VEGFC. This analysis revealed, after validation in two independent AML cohorts of 100 children and 180 adults, distinct gene expression profiles in AML with high VEGFC versus low VEGFC expression.

VEGFC is predominantly known for its ability to promote the formation of new lymphatic vessels by inducing proliferation, migration and sprout formation of existing lymphatic endothelial cells (i.e. lymphangiogenesis). The spread of cancer cells from a primary tumor to the lymphatics and blood stream is now understood to be due to active recruitment of new lymphatics by tumor-derived VEGFC. However, several studies reported other functions for VEGFC, which are independent of lymphangiogenesis, and instead are important for cancer progression. For example, an autocrine VEGFC loop promoting the invasion and metastasis of lung, breast and gastric cancer cells and stimulating survival of Kaposi’s sarcoma, malignant mesothelioma and prostate cancer cells has been described. Furthermore, in vivo, several studies have been linked VEGFC to the progression of diverse cancer types (e.g. lung adenocarcinoma, head and neck carcinomas, breast, prostate and colorectal cancer).

A possible role for VEGFC in the pathogenesis of AML was for the first time suggested by Fiedler et al. who detected VEGFC and KDR/FLT-4 expression of AML blasts at protein and/or mRNA levels. In addition, VEGFC/FLT4 expression was found to be significantly higher in AML patients compared to normal controls using immunohistochemical staining. In vitro studies of Dias et al. identified a role for
exogenously added VEGFC (as a model for paracrine signaling) in survival and proliferation of leukemic cells and protection against chemotherapy-induced apoptosis via blc-2 induction through FLT4 signaling\textsuperscript{6}. Furthermore, AML cells are also able to express VEGFRs themselves and therefore autocrine signaling has been described in AML\textsuperscript{45,46}. We found \textit{KDR}, but not \textit{FLT4}, to be upregulated with \textit{VEGFC} in this study. The autocrine VEGF signaling is found to be primarily mediated via \textit{KDR}\textsuperscript{45,46}. To our knowledge, autocrine VEGFC signaling via \textit{FLT4} has not been described in AML. However, this clearly needs further studies.

Interestingly, in a relatively small cohort of 90 adult AML patients, subgroup analysis suggested that patients with a low \textit{VEGFC} expression level (in combination with a high \textit{ANG2} expression level) had a significantly better long-term prognosis compared to patients with a high \textit{VEGFC} expression level.\textsuperscript{47} Our study shows for the first time that a high \textit{VEGFC} expression level is associated with reduced complete remission rate, reduced OS as well as EFS in AML. The prognostic impact of \textit{VEGFC} expression was also apparent in the subset of AML with intermediate prognostic risk cytogenetics. Furthermore, within the subgroup of AML patients with intermediate cytogenetic risk, it appeared that patients with low \textit{VEGFC} without \textit{FLT3}-ITD had a better prognosis. Of note, comparable results were found among cytogenetically normal karyotype AML patients with low \textit{VEGFC} and without \textit{FLT3}-ITD in terms of treatment outcome. Although numbers were relatively small, our results suggest that favorable cytogenetic risk AML patients with high \textit{VEGFC} showed reduced EFS. Finally, patients with -5(q)/-7(q) cytogenetic abnormalities with high \textit{VEGFC} tend to show reduced OS. However, further and larger studies are needed to generate more conclusive results in (these) specific cytogenetic subgroups regarding \textit{VEGFC} expression and treatment outcome.
Most of the patients of this study were fully characterized with regard to FLT3-ITD, NPM1, CEBPA, cytogenetics and clinical characteristics as age and WBC, which thus allowed consideration of a comprehensive panel of gene mutation, cytogenetic and clinical prognostic markers. In multivariable analyses, high VEGFC was identified as an independent risk indicator for both OS as well as EFS. Of note, our analysis relied on relative expression differences between samples and not on absolute expression levels. Nonetheless, before quantitative measures of VEGFC expression can be used for clinical decision making, additional standardization of the methods used to determine VEGFC expression levels in combination with prospective trials is necessary.

Gene expression profiling revealed a unique signature associated with (high) VEGFC. As one might expect, the biological process VEGFR activity (representing genes as KDR, PDGFRA and NRP1) was elevated with high VEGFC. Notably, no correlation was found between VEGFA and VEGFC. Furthermore, the GEP signature revealed genes involved in proliferation (e.g. PAK3 and SOS2, known activators of the MEK1/ERK pathway\textsuperscript{22-25} and PDK1 which is a direct activator of Akt/PKB\textsuperscript{26,27}) to be up-regulated with high VEGFC. In endothelial cells the ERK and Akt pathways are reported to be activated by VEGFC and to be important for proliferation and survival signals respectively\textsuperscript{28,32}. Of note, it has been suggested by Su and colleagues that signal transduction in response to VEGFC varies with cell type, since VEGFC did not activate the ERK pathway in lung adenocarcinoma cells\textsuperscript{43}.

The GEP signature revealed up-regulation of a set of genes whose overexpression has been found to negatively correlate with prognosis of AML; e.g. ABCC3 (also known as MRP3) gene and certain other members of the ABC-family (i.e. ABCB9, ABCC8 and CFTR) were found to be associated with elevated VEGFC. Many ABC-
family members have been shown to be able to efflux cytostatic drugs.\textsuperscript{48,49} In AML as well as in Acute Lymphoblastic Leukemia (ALL), \textit{ABCC3} is associated with a lower chance of survival, both in adults as well as children.\textsuperscript{50,51} \textit{In vitro}, it was demonstrated that MRP3 causes resistance against etoposide, teniposide, and methotrexate.\textsuperscript{52,53} In conclusion, our study provides evidence to indicate that \textit{VEGFC} expression predicts outcome in both pediatric as well as adult patients with AML. In multivariable analysis, high \textit{VEGFC} emerged as a prognostic indicator that independently predicted shorter survival. Finally, GEP revealed a unique signature associated with elevated \textit{VEGFC}, which we established in three independent AML cohorts. The interruption of \textit{VEGFC} signaling in AML might offer potential therapeutic targets for antileukemic treatment interventions.
ACKNOWLEDGEMENTS

This work was partially supported by the Foundation for Pediatric Oncology Research Groningen (HJM de J). ESJM de B is supported by a grant of the Dutch Cancer Society (Grant number 3661).
Authorship contributions:

Hendrik J.M. de Jonge designed research, analyzed and interpreted data, performed statistical analysis, wrote the manuscript

Peter J.M. Valk analyzed and interpreted data, collected data, wrote the manuscript

Nic J.G.M. Veeger analyzed and interpreted data, performed statistical analysis

Arja ter Elst analyzed and interpreted data

Monique L. den Boer collected data

Jacqueline Cloos collected data

Valérie de Haas collected data

Marry M. van den Heuvel-Eibrink collected data

Gertjan J.L. Kaspers collected data

Christian M. Zwaan collected data

Willem A. Kamps collected data

Bob Löwenberg analyzed and interpreted data, collected data, wrote the manuscript

Eveline S.J.M. de Bont collected data, designed research, analyzed and interpreted data, wrote the manuscript

All authors approved the final version of the manuscript.

Disclosure of conflicts of interest: None of the authors have a conflict of interest to disclose.
REFERENCES


25. Reynolds LF, de Bettignies C, Norton T, et al. Vav1 transduces T cell receptor 
signals to the activation of the Ras/ERK pathway via LAT, Sos, and RasGRP1. 

26. Mora A, Komander D, van Aalten DM, et al. PDK1, the master regulator of 

27. Storz P, Toker A. 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-
kinase signaling. *Front Biosci* 2002; 7:d886-d902.

28. Alitalo K, Carmeliet P. Molecular mechanisms of lymphangiogenesis in health 


of expression with VEGFR-3 suggests a role in lymphatic vascular 

lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 

32. Karkkainen MJ, Makinen T, Alitalo K. Lymphatic endothelium: a new frontier of 

stimulates progression of human gastric cancer via both autocrine and 

stimulates the migration and proliferation of Kaposi's sarcoma cells. *J Biol 

by agents that target the VEGF and VEGF-C autocrine loops. *Int J Cancer* 
2003; 104(5):603-610.

protects prostate cancer cells from oxidative stress by the activation of 
mammalian target of rapamycin complex-2 and AKT-1. *Cancer Res* 2009; 
69(15):6042-6048.

and its receptor VEGFR-3 in non-small-cell lung cancer: concurrent 
expression in cancer cells from primary tumour and metastatic lymph node. 

38. Su JL, Yen CJ, Chen PS, et al. The role of the VEGF-C/VEGFR-3 axis in 


51. Steinbach D, Wittig S, Cario G, et al. The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may


### TABLE 1. ADULT AML PATIENT CHARACTERISTICS.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients</th>
<th>Low VEGFC</th>
<th>High VEGFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>525</td>
<td>262</td>
<td>262</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>46.6 (15.2-77.2)</td>
<td>43.7 (15.2-75.5)</td>
<td>49.7 (15.8-77.2)</td>
</tr>
<tr>
<td>Median WBC x10^9/l (range)</td>
<td>26 (0.3-510)</td>
<td>34 (0.6-510)</td>
<td>20 (0.3-349)</td>
</tr>
<tr>
<td>Median blasts, % (range)</td>
<td>65 (1-99)</td>
<td>70 (2-99)</td>
<td>62 (1-99)</td>
</tr>
<tr>
<td>Median platelets x10^9/l (range)</td>
<td>56 (3-998)</td>
<td>53 (6-494)</td>
<td>59 (3-998)</td>
</tr>
</tbody>
</table>

**Cytogenetic risk**

<table>
<thead>
<tr>
<th><strong>Favorable</strong></th>
<th>89 (17%)</th>
<th>57 (22%)</th>
<th>32 (12%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;21)</td>
<td>34</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>20</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>inv16</td>
<td>35</td>
<td>22</td>
<td>13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Intermediate</strong></th>
<th>331 (63%)</th>
<th>164 (63%)</th>
<th>166 (63%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal karyotype</td>
<td>218</td>
<td>111</td>
<td>107</td>
</tr>
<tr>
<td>+ 8</td>
<td>25</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>-9q</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>81</td>
<td>41</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Unfavorable</strong></th>
<th>85 (16%)</th>
<th>34 (13%)</th>
<th>51 (19%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11q23</td>
<td>11</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>complex</td>
<td>20</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>-5(q)/-7(q)</td>
<td>42</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>abn(3q)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>t(6;9)</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>t(9;22)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>other</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Not available</strong></th>
<th>20 (4%)</th>
<th>7 (3%)</th>
<th>13 (5%)</th>
</tr>
</thead>
</table>

| FLT3-ITD vs no FLT3-TD | 143/382 | 68/194 | 74/188 |
| NPM1 wild type vs mutant | 366/159 | 184/78 | 181/81 |
| CEBPA wild type vs mutant $ | 486/38 | 240/22 | 245/16 |

| Allogeneic SCT | 140* | 66* | 74* |
| Autologous SCT | 68 | 40 | 28 |

<table>
<thead>
<tr>
<th>Cycles to CR</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;3</th>
<th>no CR</th>
<th>Relapse</th>
<th>Death vs. Alive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>297 (57%)</td>
<td>111 (21%)</td>
<td>8 (2%)</td>
<td>5 (1%)</td>
<td>104 (20%)</td>
<td>202 (39%)</td>
<td>316/209</td>
</tr>
<tr>
<td>2</td>
<td>149 (57%)</td>
<td>67 (25%)</td>
<td>6 (2%)</td>
<td>2 (1%)</td>
<td>38* (15%)</td>
<td>97 (37%)</td>
<td>141/121*</td>
</tr>
<tr>
<td>3</td>
<td>147 (56%)</td>
<td>44 (17%)</td>
<td>2 (1%)</td>
<td>3 (1%)</td>
<td>66* (25%)</td>
<td>105 (40%)</td>
<td>174/88*</td>
</tr>
</tbody>
</table>
### TABLE 2. MULTIVARIABLE ANALYSIS OF VEGFC AS PROGNOSTIC MARKER FOR OVERALL AND EVENT-FREE SURVIVAL.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th>Event-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>VEGFC$</td>
<td>1.29 (1.02-1.63)</td>
<td>.038</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2.14 (1.43-3.20)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Poor*</td>
<td>3.79 (2.46-5.84)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age, decades</td>
<td>1.12 (1.03-1.22)</td>
<td>.008</td>
</tr>
<tr>
<td>WBC¶</td>
<td>1.35 (1.06-1.73)</td>
<td>.016</td>
</tr>
<tr>
<td>FLT3 ITD#</td>
<td>1.74 (1.32-2.28)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>NPM1 mutation§</td>
<td>.57 (.43-.77)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CEBPA mutation^</td>
<td>.51 (.30-.84)</td>
<td>.008</td>
</tr>
<tr>
<td>Characteristics</td>
<td>All patients</td>
<td>Low VEGFC</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>No. of patients</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>9.0 (0.0-16.7)</td>
<td>10 (0.0-15.0)</td>
</tr>
<tr>
<td>Median WBC x10^9/l (range)</td>
<td>36.5 (2.3-483)</td>
<td>33.8 (2.5-320)</td>
</tr>
<tr>
<td>Cytogenetic risk</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Favorable</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(8;21)</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>inv16</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td><strong>Intermediate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal karyotype</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>other</td>
<td>39</td>
<td>16</td>
</tr>
<tr>
<td>+8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Unfavorable</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complex</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>-5(q)/-7(q)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><strong>Not available</strong></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>CR vs. no CR</td>
<td>79/21</td>
<td>44/6*</td>
</tr>
<tr>
<td>Relapse</td>
<td>40</td>
<td>23</td>
</tr>
<tr>
<td>Death vs. Alive</td>
<td>48/52</td>
<td>20/30</td>
</tr>
</tbody>
</table>
TABLE AND FIGURE LEGENDS

TABLE 1
“Low VEGFC” indicates a VEGFC mRNA expression level below the median VEGFC level and “High VEGFC” indicates a VEGFC mRNA expression level above the median VEGFC level. Abbreviations: WBC, white blood cell count; NPM1 indicates nucleophosmin 1; FLT3, fms-related tyrosine kinase 3, ITD, internal tandem duplication; NPM1, nucleophosmin 1, CEBPA, CCAAT/enhancer binding protein α; CR, complete remission. Cytogenetic risk group distinction (favorable, intermediate, and unfavorable) is described in Methods. $, For one patient the CEBPA status is unknown. # indicates 12/140 patients with an allogeneic stem cell transplantation (SCT) after non-myeloablative conditioning. ^ indicates 4/66 patients with an allogeneic SCT after non-myeloablative conditioning. ˚ indicates 8/74 patients with an allogeneic SCT after non-myeloablative conditioning. * indicates a significant difference when AML patients with a low vs. high VEGFC expression level were compared ($P < .0.1$).

TABLE 2
HR indicates hazard ratio; CI, confidence interval, intermediate refers to intermediate cytogenetic risk as defined in Methods, poor refers to poor cytogenetic risk as defined in Methods, WBC, white blood cell count; FLT3; fms-related tyrosine kinase 3, ITD, internal tandem duplication; NPM1, nucleophosmin 1 and CEBPA, CCAAT/enhancer binding protein α.
$High VEGFC$ (i.e. above the median VEGFC level) vs $low VEGFC$ (i.e. below the median VEGFC level).
*Cytogenetic risk versus good cytogenetic risk.
WBC greater than 20 x 10^9/L.

# FLT3-ITD versus no FLT3-ITD.

§ NPM1 mutation versus no NPM1 mutation.

^ CEBPA mutation versus no CEBPA mutation.

TABLE 3

“Low VEGFC” indicates a VEGFC mRNA expression level below the median VEGFC level and “High VEGFC” indicates a VEGFC mRNA expression level above the median VEGFC level. CR indicates complete remission. Cytogenetic risk group distinction (favorable, intermediate, and unfavorable) is described in Methods. * indicates a significant difference when AML patients with a low vs. high VEGFC expression level were compared (P <.01).

FIGURE 1

Kaplan-Meier plots show the overall survival (OS) (A) and event-free survival (EFS) (B) of adult AML patient subgroups with high (n= 262) versus low (n= 262) VEGFC expression.

(C+D) Evaluation of the effect of VEGFC on OS and EFS among cytogenetically intermediate risk AML patients (n= 331). Firstly, within the 331 cytogenetically intermediate risk AML patients the association between VEGFC expression and survival was evaluated using tertiles of VEGFC expression. As the risks in second and third tertile were comparable (data not shown), the first versus the combination of the second and third VEGFC tertile were compared with regard to OS and EFS. Kaplan-Meier plots show the OS (C) and EFS (D) in cytogenetically intermediate risk AML patients with high (i.e. second and third tertile combined) versus low (i.e. first
tertile) transcript levels of VEGFC. Intermediate cytogenetic risk is defined in Methods.

FIGURE 2
Evaluation of the combination of Fms-like tyrosine kinase 3 gene internal tandem duplication (FLT3-ITD) and VEGFC expression level among AML patients with intermediate risk cytogenetics. (A) Overall survival (OS) and event free survival (EFS) (B) are shown for the four subgroups divided by FLT3-ITD and VEGFC status. “High VEGFC” indicates a VEGFC expression level above the median VEGFC level in the specific patient group, whereas “low VEGFC” indicates a VEGFC expression level below the median VEGFC level in the specific patient group. The “low and high VEGFC without FLT3-ITD” subgroups consist of 108 patients per group and the “low and high VEGFC with FLT3-ITD” subgroups consist of 57 patients per group. P-values are given for the overall comparison across all four groups.

FIGURE 3
The 100 pediatric AML patients were split in a group with high VEGFC (i.e. above the median VEGFC level, n = 50) and a group with low VEGFC (i.e. below the median VEGFC level, n = 50) mRNA expression levels. Kaplan-Meier plots show the overall survival (A) and event-free survival (B) of pediatric AML patient subgroups with high versus low transcript levels of VEGFC.

FIGURE 4
(A) The transcriptome of 262 adult AML samples with high VEGFC was compared with the transcriptome of 262 adult AML samples with low VEGFC mRNA expression.
level. This comparison revealed 459 unique genes that were higher expressed in the high VEGFC group (“Up-with-VEGFC” group), and 192 unique genes that were lower expressed in the high VEGFC group (“Down-with-VEGFC” group) at the significance level of $P \leq 1.0 \times 10^{-7}$. The gene expression profiling cohort of 100 pediatric AML samples was used to validate the differentially expressed unique genes between samples with high vs low VEGFC (i.e. validation step 1). Hereafter an independent publicly available cohort of 180 adult AML samples was used (i.e. validation step 2).\textsuperscript{20} After two validation steps 331 unique genes were found to be higher expressed in AML patients with high compared low VEGFC and 44 unique genes were found to be lower expressed in AML patients with high VEGFC compared to AML patients with low VEGFC.

(B) Biological processes (represented by Go-ontologies) enriched among the 331 (“Up-with-VEGFC”) and 44 (“Down-with-VEGFC”) differentially expressed unique genes revealed 105 significantly “Up-with-VEGFC” and 56 significantly “Down-with-VEGFC” Go-ontologies at the significance level of $P <.05$. 
FIGURE 1. VEGFC IN RELATION TO OVERALL AND EVENT FREE SURVIVAL IN ADULT AML.
C

Cum Survival

P = .034

Overall survival (months)

D

Cum Survival

P = .083

Event Free Survival (months)
FIGURE 2. EVALUATION OF THE COMBINATION OF FLT3-ITD AND VEGFC EXPRESSION AMONG AML WITH INTERMEDIATE RISK CYTOGENETICS.

A

B
FIGURE 3. VEGFC IN RELATION TO OVERALL AND EVENT FREE SURVIVAL IN PEDIATRIC AML.
FIGURE 4. GENES AND GO-ONTOLOGIES DISTINGUISHING AML SAMPLES WITH A HIGH VS LOW VEGFC mRNA EXPRESSION LEVEL.

A

- n = 459 unique genes "Up-with-VEGFC"
- n = 346/459 unique genes "Up-with-VEGFC"
- n = 331/345 unique genes "Up-with-VEGFC"

525 AML gene arrays

Validation step 1

n = 192 unique genes "Down-with-VEGFC"
- n = 58/192 unique genes "Down-with-VEGFC"

Validation step 2

n = 44/49 unique genes "Down-with-VEGFC"

Gene Symbol | Description
--- | ---
PDGFRA | platelet derived growth factor receptor alpha
KDR | kinase insert domain receptor (type III receptor, ty. kin.)
NRP1 | neuregulin 1
SEM65 | sema, transmembrane and cytoplasmic domain 6D
ABCC3 | ATP-binding cassette, sub-family C, member 3
RUNX1 | runt-related transcription factor 1
PYH4 | pyruvate kinase 4
PLXDC1 | pleckstrin domain containing 1
NRAF2 | nuclear receptor subfamily 2, group F, member 2
HEY1 | hairy enhancer of split related with YRPW motif 1
FK1 | pyruvate dehydrogenase kinases, isoform 1
RASSF1 | ras protein-specific guanine nucleotide-releasing factor 1
PAK5 | p21-activated kinase 5
SOS2 | son of sevenless homolog 2 (Drosophila)

Gene Symbol | Description
--- | ---
ANP32A | acidic nuclear phosphoprot. 32 fam. memb. a
AKAP9 | a kinase (PKA) anchor protein (cyclic) 9
TAF11 | TAFI11 RNA polymerase II, TAFI box binding protein
PIM1 | PIM1 kinase, related to PIM family
TIA1 | TIA1 cytoplasmic granule-associated, RNA binding protein
CDK8 | cyclin-dependent kinase 8
TGF1 | TGFα-induced factor homeobox 1

B

- n = 105 Go-ontologies "Up-with-VEGFC"

Biological processes

- "Up-with-VEGFC"

- "Down-with-VEGFC"

Go-ontologies

- Cell adhesion
- Cell proliferation
- Vascular endothelial growth factor receptor activity
- Cell surface receptor-linked signal transduction
- Signal transduction
- Angiogenesis
- Rho protein signal transduction

Go-ontologies

- Nucleic acid binding
- DNA binding
- Cellular metabolic process
- Cell communication
- Metabolic process
- Transcription, DNA dependent
- Apoptosis
High VEGFC expression is associated with unique gene expression profiles and predicts adverse prognosis in pediatric and adult acute myeloid leukemia