blood advances

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Cytokine profiling in 128 patients with transient abnormal myelopoiesis: a report from the JPLSG TAM-10 trial

Tracking no: ADV-2023-011628R2

Genki Yamato (Gunma University Graduate School of Medicine, Japan) Yusuke Tsumura (Nagoya University Graduate School of Medicine, Japan) Hideki Muramatsu (Nagoya University Graduate School of Medicine, Japan) Akira Shimada (Jichi Medical University, Japan) Takahiro Imaizumi (Nagoya University Hospital, Japan) Hiroyuki Tsukagoshi (Gunma Prefectural Institute of Public Health and Environmental Sciences, Japan) Taeko Kaburagi (Gunma Children's Medical Center, Japan) Norio Shiba (Yokohama City University Graduate School of Medicine, Japan) Yoshiyuki Yamada (Gunma Children's Medical Center, Japan) Takao Deguchi (National Center For Child Health and Development, Japan) Tomoko Kawai (National Research Institute for Child Health and Development, Japan) Kiminori Terui (Hirosaki University Graduate School of Medicine, Japan) Etsuro Ito (Hirosaki University Graduate School of Medicine, Japan) Kenichiro Watanabe (Shizuoka Children's Hospital, Japan) Yasuhide Hayashi (Jobu University, Japan)

Abstract:
Transient abnormal myelopoiesis (TAM) occurs in 10% of neonates with Down syndrome (DS). Although most patients show spontaneous resolution of TAM, early death occurs in approximately 20% of cases. Therefore, new biomarkers are needed to predict early death and determine therapeutic interventions. This study aimed to determine the association between clinical characteristics and cytokine levels in patients with TAM. A total of 128 patients with DS with TAM enrolled in the TAM-10 study conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group were included in this study. Five cytokine levels [interleukin (IL)-1b, IL-1 receptor agonist, IL-6, IL-8, and IL-13] were significantly higher in patients with early death than in those with non-early death. Cumulative incidence rates (CIR) of early death were significantly associated with high levels of the five cytokines. Based on unsupervised consensus clustering, patients were classified into three cytokine groups: hot-1 (n = 37), hot-2 (n = 42), and cold (n = 49). The CIR of early death was significantly different between the cytokine groups [hot-1/2 (n = 79); cold (n = 49); CIR (95% confidence interval [CI]) = 16.5% (7.9%-24.2%); 2.0% (0.0%-5.9%), P = 0.013]. Furthermore, cytokine groups (hot-1/2 vs. cold) were independent poor prognostic factors in the multivariable analysis for early death [hazard ratio (95% CI) = 19.25 (2.056-180.3), P = 0.010]. These results provide valuable information that cytokine level measurement was useful in predicting early death in patients with TAM and might help to determine the need for therapeutic interventions.

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: G.Y. conducted the study, analyzed the data, and wrote the paper. Y.H. designed and conducted the study, led the project, and wrote the paper. Y.T. and H.M. wrote the paper and analyzed the data. A.S., N.S., T.Kaburagi, T.D., T.Kawai, and Y.Y. analyzed the data. T.I. performed statistical analyses. H.T. performed the research and bioinformatics analysis. Y.T. wrote the paper. K.T. and E.I. performed the GATA1 mutation analysis. K.W. collected clinical samples and data. All authors critically reviewed and revised the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Data Availability Statement Requests for data sharing may be submitted to Yasuhide Hayashi (hayashiy@jobu.ac.jp).

Clinical trial registration information (if any):

Regular Article

Cytokine profiling in 128 patients with transient abnormal myelopoiesis: a report from the JPLSG TAM-10 trial

Genki Yamato[1,2], Yusuke Tsumura[3], Hideki Muramatsu[3], Akira Shimada[4], Takahiro Imaizumi[5], Hiroyuki Tsukagoshi[6], Taeko Kaburagi[1], Norio Shiba[7], Yoshiyuki Yamada[1,8], Takao Deguchi[9], Tomoko Kawai[10], Kiminori Terui[11], Etsuro Ito[11], Kenichiro Watanabe[12], Yasuhide Hayashi[1,13]

1. Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Japan.
2. Department of Pediatrics, Gunma University Graduate School of Medicine, Maebashi, Japan.
3. Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan
4. Department of Pediatrics, Jichi Medical University, Shimotsuke, Japan.
5. Department of Advanced Medicine, Nagoya University Hospital, Nagoya, Japan.
6. Department of Research Group, Gunma Prefectural Institute of Public Health and Environmental Sciences, Maebashi, Japan
7. Department of Pediatrics, Yokohama City University Hospital, Yokohama,

Japan.

8. Department of Pediatrics, Tokai University School of Medicine, Isehara, Japan.

9. Children's Cancer Center, National Center for Child Health and Development, Tokyo, Japan.

10. Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development, Tokyo, Japan

11. Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

12. Department of Hematology and Oncology, Shizuoka Children's Hospital, Shizuoka, Japan

13. Institute of Physiology and Medicine, Jobu University, Takasaki, Japan.

Corresponding author: Yasuhide Hayashi, MD, PhD

Department of Hematology/Oncology, Gunma Children's Medical Center, 779, Shimohakoda, Hokkitsu, Shibukawa, 377-8577 Gunma, Japan.

Tel.: +81-274-20-2115, Fax: +81-274-42-5204

E-mail address: hayashiy@jobu.ac.jp

Running title: Cytokine profiling in TAM

Word count: 2854 words

Abstract count: 247 words

Figures/Tables count: 3 Figures, 4 Tables

Supplemental files: 1 file

Reference count: 25

Keywords: transient leukemia, transient abnormal myelopoiesis, transient myeloproliferative disorder, cytokine, early death, Down syndrome

Data Availability Statement

Requests for data sharing may be submitted to Yasuhide Hayashi (hayashiy@jobu.ac.jp).

Key Points

Unsupervised consensus clustering of cytokine profiles in 128 patients with TAM identified groups at higher risk for early death.

Measurement of levels of cytokine provides valuable information for patients with TAM that may help determine therapeutic interventions.

Abstract

Transient abnormal myelopoiesis (TAM) occurs in 10% of neonates with Down syndrome (DS). Although most patients show spontaneous resolution of TAM, early death occurs in approximately 20% of cases. Therefore, new biomarkers are needed to predict early death and determine therapeutic interventions. This study aimed to determine the association between clinical characteristics and cytokine levels in patients with TAM. A total of 128 patients with DS with TAM enrolled in the TAM-10 study conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group were included in this study. Five cytokine levels [interleukin (IL)-1b, IL-1 receptor agonist, IL-6, IL-8, and IL-13] were significantly higher in patients with early death than in those with non-early death. Cumulative incidence rates (CIR) of early death were significantly associated with high levels of the five cytokines. Based on unsupervised consensus clustering, patients were classified into three cytokine groups: hot-1 (n = 37), hot-2 (n = 42), and cold (n = 49). The CIR of early death was significantly different between the cytokine groups [hot-1/2 (n = 79); cold (n = 49); CIR (95% confidence interval [CI]) = 16.5% (7.9%–24.2%); 2.0% (0.0%–5.9%), *P* = 0.013]. Furthermore, cytokine groups (hot-1/2 vs. cold) were independent poor prognostic factors in the multivariable analysis for early death [hazard ratio (95% CI) = 19.25 (2.056–180.3), *P* = 0.010]. These results provide valuable information that cytokine level measurement was useful in predicting early death in patients with TAM and might help to determine the need for therapeutic interventions.

Introduction

Transient abnormal myelopoiesis (TAM), also known as transient leukemia or transient myeloproliferative disorder, is a unique clonal myeloproliferation characterized by immature megakaryoblasts. It occurs in 10% of neonates with Down syndrome (DS).[1] Although most patients show spontaneous resolution of TAM without therapeutic interventions, approximately 20% of TAM cases result in early death (death within nine months), and approximately 20% of the survivors develop acute megakaryoblastic leukemia within four years.[2-6] Our previous reports showed that high white blood cell (WBC) count (≥100 × 10^9/L), systemic edema, low birth weight, preterm birth at <37 weeks of gestational age, and elevated direct bilirubin level >5 mg/dL were associated with early death.[2-6] Low-dose cytarabine (LDAC) is a common therapy for TAM. It has been reported that LDAC should be considered for patients with life-threatening symptoms and risk factors associated with early death.[7] Additionally, it has been reported that the LDAC intervention rate was adversely associated with the early death rate.[2] However, further studies are needed to determine the criteria for consensus therapeutic intervention.

Previous reports showed that cytokine levels are associated with liver failure, which is a cause of early death in patients with TAM.[8] Thus, cytokine levels can be new biomarkers to predict early death in patients with TAM. However, no large cohort data are available for cytokine analyses in patients with TAM. Thus, this study aimed to determine the association between clinical characteristics and cytokine levels in patients with TAM by analyzing 128 patients with DS with TAM enrolled in the TAM-10 prospective observational

study conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG).

Methods

Patients

A total of 167 neonates diagnosed with TAM were prospectively registered in the TAM-10 study between May 2011 and February 2014 conducted by the JPLSG of the Japan Children's Cancer Group. The TAM-10 study was registered with the UMIN Clinical Trials Registry (UMIN-CTR, URL: http://www.umin.ac.jp/ctr/index.htm, number UMIN000005418). The details of the eligibility criteria and the Central Review System, including the *GATA1* mutation analysis, are shown in the previous report.[2] Clinical data and sample collections in the clinical trials were approved by the Institutional Review Boards of each participating institution. Written informed consent was obtained from all patients' parents/guardians. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethical Review Board of the JPLSG. Cytokine levels were analyzed in 128 of the 167 patients for whom serum samples were available in the early postnatal period (days 0–8). A comparison of clinical characteristics between 128 and 39 patients with or without available samples is shown in *Supplemental Table 1*.

Cytokine analysis

Serum concentrations of the following 27 cytokines were determined using the Bio-Prex cytokine assay (Bio-Rad, Hercules, CA, USA), measured using a

Luminex System (Austin, TX, USA), and quantified using Bio-Plex software (Bio-Rad). The details of 27 cytokines are described in *Supplemental Table 2*. Serum samples were frozen at −80°C immediately after collection and stored until analysis. Each sample was analyzed twice. The mean values of measurements were used as representative values for each subject.

Statistical analysis

Optimal cutoff values for biomarkers were determined using the Youden index of the receiver operator characteristic (ROC) curve based on logistic regression analyses. The association between the covariates and early death (<9 months of age) was evaluated in univariable and multivariable Cox proportional hazard models using the stepwise Akaike information criterion method. Between-group comparisons were performed using the Mann–Whitney U test or Fisher's exact test, as appropriate. Differences in cytokine levels between groups were determined using the Mann–Whitney U test. A correction for multiple testing was performed using the Benjamini–Hochberg method with the threshold *P*-value set at <0.05.

Cluster analysis was performed by two-step unsupervised consensus clustering of 27 cytokine variables. Five cytokines (IL-2, IL-12, IL-15, IL-17, and RANTES) with missing values in ≥10 patients were excluded from subsequent analyses (*Supplemental Figure 1A*). Details of missing values for the remaining 22 cytokines are shown in *Supplemental Figures 1B and 1C*. Missing values of 22 cytokines were imputed using the random forest-based algorithm, missForest.[10] The features were log-standardized for data preprocessing. For

sensitivity analyses, cluster analyses were also performed on the dataset complemented with different imputation methods based on the k-nearest neighbor (kNN) and principal component analysis (PCA) (*Supplemental Figure 1D, E*)[11]. In addition, complete data analysis was performed on 43 patients without missing data for all 27 cytokines.

All statistical analyses were performed using EZR software version 1.36 (Saitama Medical Center, Jichi Medical University, Saitama, Japan)[12] and R Version 4.3.2. with "naniar" (https://github.com/njtierney/naniar), "ConsensusClusterPlus"[13], "ComplexHeatmap" (https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html), and "ggplog2" (https://ggplot2.tidyverse.org/) packages. A two-tailed *P*-value <0.05 was considered statistically significant. Details and other information on statistical analysis are described in *Supplemental Methods*.

The TAM-10 study was registered with the UMIN Clinical Trials Registry (UMIN-CTR, URL: http://www.umin.ac.jp/ctr/index.htm, number UMIN000005418) and was conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) of the Japan Children's Cancer Group. Clinical data and sample collections in the clinical trials were approved by the Institutional Review Boards of each participating institution. Written informed consent was obtained from all patients' parents/guardians. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethical Review Board of the JPLSG.

Results

Patient characteristics

Table 1 shows the clinical characteristics and laboratory findings of 128 patients with TAM. The median (range) values of gestational age, body weight at birth, WBC count, and percentage of blasts at diagnosis were 37 (29–40) weeks, 2,588 (1,438–3,714) g, 48.3 (4.7–478.7) × 10^9/L, and 41% (1%–96%), respectively. Of the 128 patients, 87 (68%) had congenital heart disease, and 14 (11%) had other congenital abnormalities. Trisomy 21 was observed in 126 patients (98%), trisomy 21 mosaicism in 1 patient (1%), and a normal karyotype in 1 patient (1%). Systemic edema was observed in 26 patients (20%) and organ hemorrhage in 12 patients (9%). Somatic *GATA1* gene mutations were confirmed in 127 patients (99%) using Sanger and/or next-generation sequencing. One patient with undetectable *GATA1* mutations had flow cytometry markers (CD7+/CD117+/CD56+), consistent with a TAM phenotype. The expression type of *GATA1* mutations was determined based on a previous report.[2] High-expression mutations were observed in 57 patients (45%), whereas low-expression mutations were observed in 58 patients (45%). Of the 128 patients, 46 (36%) received LDAC.

Of the 128 patients, 20 (16%) died, and early death (<9 months of age) occurred in 14 (11%). The causes of early death were as follows: multiple organ failure (5), liver failure (1), respiratory failure (3), sepsis (1), congenital heart disease (1), and other reasons (3) (*Supplemental Table 3*). The cumulative incidence rate (CIR) of early death at 9 months was 11.0% [95% confidence interval (CI): 5.3%–16.2%], and the leukemia development rate at 4 years was

20.9% (95% CI: 12.9%–28.2%) (*Supplemental Figure 2*). The early death group had a significantly lower gestational age (P = 0.003), lower birth weight (P = 0.035), higher WBC counts (P = 0.006), higher rate of organ hemorrhage (P = 0.012), and higher rate of systemic edema (P<0.001) than the non-early death group, which are poor prognostic factors associated with early death (Table 1). The median values and ranges of the 27 cytokines and the number of subjects for each cytokine are shown in *Supplemental Table 2*. Five cytokines (IL-2, IL-12, IL-15, IL-17, and RANTES) with missing values in ≥10 patients were excluded from subsequent analyses (*Supplemental Figure 1A*).

Relation between cytokine levels and clinical characteristics

The comparison between 29 patients with a high WBC count (≥100 × 10^9 cells/L, a poor prognostic factor in patients with TAM) and 99 patients without a high WBC count for 22 cytokine levels showed that the levels of six cytokines (IL-1b, IL-6, IL-7, IL-8, IL-13, and MCP-1b) were significantly higher in patients with high WBC counts (Table 2). The association between expression types of *GATA1* mutations and 22 cytokine levels is shown in *Supplemental Table 4***.** Six cytokines (IL-4, Eotaxin, PDGF-bb, basic FGF, MIP-1β, and TNF-α) were significantly higher in the high *GATA1* expression group than in the low *GATA1* expression group.

Furthermore, the correlation between cytokine levels and hepatomegaly and serum markers of liver fibrosis (procollagen type III peptide, type IV collagen, and hyaluronic acid) was evaluated (*Supplemental Table 5*). The serum levels of several cytokines, especially IL-13 (r = 0.35, P<0.05) and Eotaxin (r = 0.35,

P<0.05), were correlated with hepatomegaly. Additionally, five cytokines (IL-6, IL-9, Eotaxin, IP-10, and MIP-1β) were positively correlated with two liver fibrosis markers. Cytokine levels were compared in patients with or without leukemia development (n = 23 vs. n = 105), and no cytokine showed significant differences between the two groups (*Supplemental Table 6*).

Strong association between cytokine levels and early death

Cytokine levels were compared between the early death (n = 14) and non-early death groups (n = 114). The levels of five cytokines [IL-1b (*P* = 0.031), IL-1ra (*P* = 0.007), IL-6 (*P* = 0.022), IL-8 (*P* = 0.004), and IL-13 (*P* = 0.037)] were significantly higher in the early death group than those in the non-early death group (Table 3). The optimal cytokine cutoff points of IL-1b, IL-1ra, IL-6, IL-8, and IL-13 were determined as 2.9 pg/mL, 256.0 pg/mL, 141.0 pg/mL, 102.0 pg/mL, and 9.2 pg/mL, respectively, to predict early death using ROC curves, which yielded the highest sum of sensitivity and specificity (*Supplemental Figure 3*). The CIR of early death was significantly associated with higher levels of these five cytokines, respectively (Figure 1). Additionally, in a subgroup analysis restricted to 99 patients with low WBC counts (<100 × 10^9 cells/L), high levels of these five cytokines were significantly associated with early death (*Supplemental Figure 4*).

An unsupervised clustering analysis was performed using the values of 22 cytokines. Missing values (1.1%; *Supplemental Figure 1B*) were computationally imputed using the missForest method. The patients were divided into three groups: hot-1 (n = 37), hot-2 (n = 42), and cold (n = 49) (Figure

2A). The hot-1 group showed high inflammatory cytokine levels, including IL-8, IL-6, and IL-1β (Figure 2B). The hot-2 group was characterized by elevated IL-5 levels (Figure 2C). The cold group did not show any significant cytokine elevation (Figure 2D). The clinical characteristics of the three groups are described in *Supplemental Table 7*. The CIR of early death was significantly different between the cytokine groups [hot-1/2 (n = 79); cold (n = 49); CIR (95% confidence interval [CI]) = 16.5% (7.9–24.2%); 2.0% (0.0–5.9%), *P* = 0.013]. The cytokine hot-1/2 groups showed significantly higher early mortality compared to the cytokine cold group (Figure 3 and *Supplemental Figure 5*). For sensitivity analyses, cluster analyses were conducted on datasets complemented using other imputation methods: kNN and PCA (*Supplemental Figure 6A,B*). In addition, complete data analysis was performed for 43 patients without missing data for 27 cytokines (*Supplemental Figure 6C*). The reproducibility of the three identified clusters was high while using the missForest-imputed dataset as a reference; the concordance rates with the kNN, PCA, and complete data analysis were 0.94, 0.99, and 0.95, respectively (*Supplemental Figure 1D, E*).

The univariable analysis showed that the following covariates were correlated with early death: cytokine group, gestational age, organ hemorrhage, systemic edema, congenital heart disease, high WBC counts in peripheral blood, systemic steroid therapy, and hepatomegaly (*Supplemental Table 8*). The multivariable analysis was performed in two models using factors extracted using the stepwise Akaike information criterion method, which were identified as significantly different in univariable analysis. The multivariable analysis (Model 1), without incorporating cytokine group, identified the following independent risk

factors for early death: high WBC counts [hazard ratio (HR) (95% CI) = 3.450 (1.127–10.56), *P* = 0.030], systemic edema [HR (95% CI) = 13.76 (3.784–50.06), *P*<0.001], hepatomegaly [HR (95% CI) = 3.375 (1.108–10.28), *P* = 0.032], and congenital heart disease [HR (95% CI) = 0.294 (0.096–0.903), *P* = 0.033], and the multivariable analysis (Model 2), incorporating cytokine group, showed that cytokine hot-1/2 group was an independent prognostic factor [HR (95% CI) = 15.53 (1.434–168.3), *P* = 0.024] (Table 4).

Discussion

A total of 22 cytokine levels were analyzed in 128 patients with DS with TAM who were enrolled in the TAM-10 prospective observational study to determine the association between cytokine levels and clinical characteristics. Five cytokines (IL-1b, IL-1ra, IL-6, IL-8, and IL-13) were significantly associated with early death. Furthermore, an unsupervised clustering analysis based on the 22 cytokine levels generated three groups (cytokine hot-1, hot-2, and cold). The cytokine hot-1/2 groups showed significantly higher early death rates than the cytokine cold group.

The univariable analysis showed a strong association between the cytokine hot-1/2 group and early death (HR [95% CI] = 8.509 [1.113–65.05]), and a multivariable model incorporating cytokine group (Model 2) identified the cytokine hot-1/2 group as an independent prognostic factor. These findings indicate that the cytokine group is a potent prognostic factor for TAM and may outperform the traditional clinical prognostic factor, WBC count.

The IL-1 family consists of proinflammatory cytokines such as IL-1b and

anti-inflammatory cytokines such as IL-1ra.[14] IL-1b is a potent proinflammatory cytokine, originally identified as an endogenous thermogenic agent, and IL-1ra is an acute phase protein secreted by the liver in response to inflammatory stimuli and can inhibit signal transduction.[15] It has been reported that patients with TAM who died early had significantly elevated levels of both IL-1b and IL-1ra. However, IL-1ra is considered much less effective than agonists, requiring up to 1000-fold excess IL-1ra to inhibit IL-1 signaling.[16] These findings suggest that the observed IL-1ra elevation is a secondary event, and IL-1 signaling is activated in patients with severe TAM. IL-6 promotes B and T lymphocyte differentiation and IgG production.[17,18] Furthermore, IL-6 has been reported to be involved in cancer cell proliferation via STAT3 activation[19] and promote cancer cell migration and invasion.[20-22] Shitara *et al.*[23] reported a case of severe TAM that showed IL-6 elevation in the pericardial fluid. Targeted therapy with cytokine antagonists, such as anakinra and canakinumab, which inhibit IL-1 signaling, and tocilizumab, which inhibits IL-6, have already been approved and demonstrated clinical efficacy for the treatment of hypercytokinemia in various diseases. These cytokine antagonists are expected to be evaluated in clinical studies as a potential future treatment for hypercytokinemia in severe TAM.

The correlation between cytokine levels and other clinical features was evaluated, except for early death. This study revealed that no cytokine levels were associated with leukemia development. Only the flow cytometric minimal residual disease positivity has been reported to be a valuable marker for predicting leukemia development.[2,24] These results implied that it might be difficult to predict leukemia development from any data at the time of diagnosis.

Furthermore, the association between cytokine levels and *GATA1* expression type was investigated. The results showed that seven cytokine levels were significantly associated with the *GATA1* expression type. All seven cytokine levels were higher in patients in the *GATA1* high-expression group than in those in the *GATA1* low-expression group. Kanezaki[25] reported that the mutation types of *GATA1* affected the amount of the mutant, and the *GATA1* expression type significantly affected the TAM phenotype. The study findings might imply that the *GATA1* high-expression type caused high levels of their cytokines.

This study has several limitations. First, this study included patients enrolled in the JPLSG TAM-10 study, and clinical samples immediately after diagnosis for cytokine measurement in 23% (39 patients) were unavailable and could not be included in the analysis. Most clinical characteristics did not show significant differences between patients with and without cytokine information; however, WBC counts at diagnosis, blast rates, and percentage of patients receiving LDAC were significantly higher in cases with cytokine information (Supplemental Table 1). Second, of the 27 cytokines measured, the percentage of deficient values for 22 cytokines used in the analysis was only 1.1% (*Supplemental Figure 1B*); however, 5 cytokines were deficient in >10% of cases and had to be excluded from subsequent analyses. Moreover, we performed a complete data analysis of 43 cases for which we had data for all 27 cytokines and found consistent results (*Supplemental Figure 1D, E*). Third, the dosage and intervention criteria of LDAC were not standardized, although a relatively high percentage (36%) of patients were treated with LDAC as per the policy of the participating centers. Fourth, the clinical significance of cytokine profiling

analysis has not been validated due to the absence of a validation cohort. This limitation is largely unavoidable given the rarity of TAM and the scarcity of international prospective studies in this field. However, we plan to re-evaluate the cytokine profiling analysis in the future using clinical samples from patients enrolled in our ongoing prospective clinical trial (jRCTs041190063).

In conclusion, this study showed that cytokine profiling provides supportive information along with previous clinical prognostic factors such as WBC count as a biomarker for predicting early death and may contribute to precision medicine for patients with TAM.

Acknowledgements

The authors thank Enago (https://www.enago.jp) for the English language review. This work was supported by Health and Labor Sciences Research Grants (201128038B and 201324122A) and the Japan Agency for Medical Research and Development (AMED) (Innovative Cancer Medical Practice Research Project, 15Aek0109055h0002 and 18ck0106435h0001).

Author Contributions

G.Y. conducted the study, analyzed the data, and wrote the paper. Y.H. designed and conducted the study, led the project, and wrote the paper. Y.T. and H.M. wrote the paper and analyzed the data. A.S., N.S., T.Kaburagi, T.D., T.Kawai, and Y.Y. analyzed the data. T.I. performed statistical analyses. H.T. performed the research and bioinformatics analysis. Y.T. wrote the paper. K.T. and E.I. performed the *GATA1* mutation analysis. K.W. collected clinical samples and

data. All authors critically reviewed and revised the manuscript.

Competing Interests: The authors have no conflicts of interest to declare.

References

1. Pine SR, Guo Q, Yin C, Jayabose S, Druschel CM, Sandoval C. Incidence and clinical implications of GATA1 mutations in newborns with Down syndrome. Blood. 2007;110(6):2128-2131.
2. Yamato G, Deguchi T, Terui K, et al. Predictive factors for the development of leukemia in patients with transient abnormal myelopoiesis and Down syndrome. Leukemia. 2021;35(5):1480-1484.
3. Gamis AS, Alonzo TA, Gerbing RB, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. Blood. 2011;118(26):6752-6759.
4. Klusmann JH, Creutzig U, Zimmermann M, et al. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. Blood. 2008;111(6):2991-2998.
5. Massey GV, Zipursky A, Chang MN, et al. Children's Oncology Group (COG). A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's Oncology Group (COG) study POG-9481. Blood. 2006;107(12):4606-4613.
6. Muramatsu H, Kato K, Watanabe N, et al. Risk factors for early death in neonates with Down syndrome and transient leukaemia. Br J Haematol. 2008; 142(4):610-615.
7. Tunstall O, Bhatnagar N, James B, Wright M; British Society for Haematology. Guidelines for the investigation and management of Transient Leukaemia of Down Syndrome. Br J Haematol. 2018;182(2):200-211.

8. Kinjo T, Inoue H, Kusuda T, et al. Chemokine levels predict progressive liver disease in Down syndrome patients with transient abnormal myelopoiesis. Pediatr Neonatol. 2019;60(4):382-388.
9. Kato M, Tsukagoshi H, Yoshizumi M, et al. Different cytokine profile and eosinophil activation are involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing. Pediatr Allergy Immunol 2011;22(1 Pt 2):e87-e94.
10. Stekhoven DJ, BuehlmannP. MissForest - nonparametric missing value imputation for mixed-type data. Bioinformatics. 2012;28(1):112-118.
11. Josse J, Husson F. missMDA: A Package for Handling Missing Values in Multivariate Data Analysis. Journal of Statistical Software. 2016;70(1):1–31.
12. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48(3):452-458.
13. Wilkerson MD, Hayes ND. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. Bioinformatics. 2010;26(12):1572-1573.
14. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta. 2014;1843(11):2563-2582.
15. Boraschi D, Lucchesi D, Hainzl S, et al. IL-37: a new anti-inflammatory cytokine of the IL-1 family. Eur. Cytokine Netw. 2011;22(3):127-147.
16. Gabay C, Lamacchia C, Palmer G. Palmer. Il-1 pathways in inflammation and human diseases. Nat. Rev. Rheumatol. 2010;6(4):232-41.
17. Muraguchi A, Kishimoto T, Miki Y, et al. T cell-replacing factor-(TRF) induced

IgG secretion in a human B blastoid cell line and demonstration of acceptors for TRF. J Immunol. 1981;127(2):412–6.

18. Lotz M, Jirink F, Kabouridis P, et al. B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. J Exp Med. 1988;167(3):1253–8.

19. Ara T, Declerck YA. Interleukin-6 in bone metastasis and cancer progression. Eur J Cancer. 2010;46(7):1223–31.

20. Walter M, Liang S, Ghosh S, Hornsby PJ, Li R. Interleukin 6 secreted fromadipose stromal cells promotes migration and invasion of breast cancer cells. Oncogene. 2009;28(30):2745–55.

21. Foran E, Garrity-Park MM, Mureau C, et al. Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6. Mol Cancer Res. 2010;8(4):471–81.

22. Mano Y, Aishima S, Fujita N, et al. Tumor-associated macrophage promotes tumor progression via STAT3 signaling inhepatocellular carcinoma. Pathobiology. 2013;80(3):146–54.

23. Shitara Y, Takahashi N, Aoki Y, Kato M, Nishimura R, Tsuchida S. Cytokine Profiles in Pericardial Effusion in a Down Syndrome Infant with Transient Abnormal Myelopoiesis. Tohoku J Exp Med. 2017;241(2):149-153.

24. Flasinski M, Scheibke K, Zimmermann M, et al. Low-dose cytarabine to prevent myeloid leukemia in children with Down syndrome: TMD Prevention 2007 study. Blood Adv. 2018;2(13):1532-1540.

25. Kanezaki R, Toki T, Terui K, et al. Down syndrome and GATA1 mutations in

transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia. Blood. 2010;116(22):4631-4638.

455 **Table 1. Clinical characteristics of 128 TAM patients**

	All patients N = 128	Patients with early death n = 14	Patients without early death n = 114	*P*–value*
Sex (male:female)	64:64	9:5	55:59	0.396
Median gestational age, week (range)	37 (29–40)	34 (29–38)	37 (31-40)	**0.003**
Median birth weight, gram (range)	2588 (1438–3714)	2249 (1438–3044)	2624 (1598-3714)	**0.035**
Median age at diagnosis, day (range)	0 (0–8)	0 (0–8)	0 (0–8)	0.854
Congenital heart disease, n (%)	87 (68)	5 (36)	82 (72)	**0.012**
Other congenital abnormally, n (%)	14 (11)	2 (14)	12 (11)	0.651
Chromosomal status, n				
Trisomy 21: Mosaic trisomy 21: Normal	126:1:1	14:0:0	112:1:1	1.000
Median WBC count at diagnosis, x 10^9/L (range)	48.3 (4.7–478.7)	157.3 (14.3–238.5)	44.3 (4.7–478.7)	**0.006**
Median blasts percentage in PB at diagnosis, % (range)	41 (1–96)	60 (5–95)	37 (1–96)	0.057
Direct Bilirubin, mg/dL, median (range)	0.8 (0–12.3)	1.0 (0.3–5.6)	0.7 (0–12.3)	0.134
Hepatomegaly, cm, median (range)**	3 (0–8)	5 (0–8)	2 (0–7)	**0.043**
Systemic edema, n (%)	26 (20)	11 (79)	15 (13)	**<0.001**
Organ hemorrhage, n (%)	12 (9)	4 (29)	8 (7)	**0.027**
Therapeutic interventions, n (%)	60 (47)	11 (79)	49 (43)	**0.021**
Low dose cytarabine, n (%)	46 (36)	6 (43)	40 (35)	0.586
Exchange blood transfusion, n (%)	16 (13)	4 (29)	12 (11)	0.076
Systemic steroid therapy, n (%)	24 (19)	8 (57)	16 (14)	**<0.001**
Classification of *GATA1* mutation				
High expression type mutation, n (%)	57 (45)	6 (43)	51 (45)	1.000
Low expression type mutation, n (%)	58 (45)	6 (43)	52 (46)	1.000
Unclassified mutation, n (%)	12 (9)	2 (14)	10 (9)	0.620
Negative, n (%)	1 (1)	0	1 (1)	1.000
Events***, n (%)	42 (33)	14 (100)	28 (25)	**<0.001**
Early deaths (<9 months of age), n (%)	14 (11)	14 (100)	0 (0)	**<0.001**
Later phase deaths (after 9 months), n (%)	5 (4)	0 (0)	5 (4)	1.000
Leukemia development, n (%)	23 (18)	0 (0)	23 (20)	0.073

PB, peripheral blood

*P-value was evaluated between patients with early death vs. patients without non-early death using Fisher's exact test or Mann-Whitney U test.

**Under costal margin, ***Events were defined by death or leukemia development

Table 2. Serum concentrations (pg/mL) of cytokines between TAM patients with or without a high WBC count

	Patients with a high WBC count*, n = 29	Patients without a high WBC count, n = 99	*P*–value**
IL-1b, median (range)	3.13 (1.42-325.2)	2.49 (0.63-3662)	**0.005**
IL-1ra	211.6 (19.71-868.1)	116.0 (8.85-9285)	0.064
IL-4	4.06 (1.69-10.43)	3.79 (0.89-32.97)	0.698
IL-5	2.295 (0.11-16.44)	2.47 (0.06-20.88)	0.625
IL-6	117.9 (8.49-1537.9)	33.70 (1.75-6851)	**0.003**
IL-7	31.67 (4.91-613.2)	12.10 (0.97-184.5)	**0.007**
IL-8	93.43 (19.42-8350)	44.97 (8.81-37418)	**0.023**
IL-9	26.71 (2.46-130.8)	21.89 (1.26-250.6)	0.225
IL-10	14.69 (5.36-260.5)	11.62 (1.56-170.6)	0.066
IL-13	19.84 (1.01-123.0)	8.980 (0.58-199.2)	**0.029**
Eotaxin	176.7 (48.34-2265)	148.4 (12.50-892.0)	0.090
PDGF-bb	5610 (300.0-18523)	4459 (61.75-18489)	0.060
basic FGF	60.79 (16.31-935.3)	47.42 (6.77-254.3)	0.079
G-CSF	65.73 (25.02-7232)	55.71 (9.39-1770)	0.078
GM-CSF	173.8 (47.04-851.1)	132.0 (6.05-1836)	0.081
IFN-r	93.31 (16.52-597.9)	74.79 (10.22-6328)	0.606
IP-10	2011 (296.0-18554)	1742 (70.84-18686)	0.602
MCP-1(MCAF)	617.2 (86.33-4051)	195.8 (31.28-10398)	**0.020**
MIP-1a	7.710 (0.53-759.1)	6.640 (0.71-565.6)	0.460
MIP-1b	380.7 (121.7-4387)	280.1 (59.98-50908)	0.229
TNF-a	50.40 (21.43-362.8)	40.81 (10.23-1029)	0.216
VEGF	112.6 (12.17-1974)	76.85 (8.27-4490)	0.083

*a high WBC count was defined as over 100×10^9 cells/L.

**P-value was evaluated using the Mann-Whitney U test followed by the Benjamini and Hochberg

Table 3. Serum concentrations (pg/mL) of cytokines between TAM patients with or without early death

	Patients with early death n = 14	Patients without early death n = 114	*P*–value*
IL-1b, median (range)	4.435 (1.94-351.1)	2.630 (0.63-3662)	**0.031**
IL-1ra	372.1 (101.9-1544)	122.0 (8.85-9285)	**0.007**
IL-4	4.690 (1.69-9.35)	3.725 (0.89-32.97)	0.178
IL-5	5.210 (0.26-20.88)	2.140 (0.06-17.35)	0.232
IL-6	222.9 (23.55-1537)	37.43 (1.75-6851)	**0.022**
IL-7	31.30 (4.43-613.2)	12.86 (0.97-301.1)	0.081
IL-8	217.1 (28.92-8350)	45.87 (8.81-37418)	**0.004**
IL-9	34.92 (2.46-110.2)	23.43 (1.26-250.6)	0.335
IL-10	28.38 (1.85-260.5)	11.71 (1.56-170.6)	0.069
IL-13	22.80 (3.52-173.5)	9.045 (0.58-199.2)	**0.037**
Eotaxin	178.2 (46.17-2265)	150.4 (12.50-892.0)	0.424
PDGF-bb	5755 (300.0-18523)	4568 (61.75-18489)	0.434
basic FGF	60.28 (19.26-935.3)	49.75 (6.77-254.3)	0.261
G-CSF	58.34 (25.63-7232)	56.40 (9.39-1770)	0.354
GM-CSF	141.7 (59.65-851.1)	138.1 (6.05-1836)	0.750
IFN-r	106.4(26.36-597.9)	74.79 (10.22-6328)	0.329
IP-10	1360 (195.7-17268)	1857 (70.84-18686)	0.604
MCP-1(MCAF)	658.6 (93.37-4051)	292.3 (31.28-10398)	0.222
MIP-1a	7.79 (2.63-759.1)	6.660 (0.53-565.6)	0.347
MIP-1b	454.5 (116.8-50908)	283.8 (59.98-40790)	0.185
TNF-a	63.46 (21.97-362.8)	40.66 (10.23-1029)	0.065
VEGF	105.7 (12.17-1974)	76.85 (8.27-4490)	0.275

*P-value was evaluated using the Mann-Whitney U test followed by the Benjamini and Hochberg

458

Table 4. Multivariable Cox regression analyses of early death

Covariates		Number	Multivariable analysis-Model 1 without incorporating cytokine group		Multivariable analysis-Model 2 incorporating cytokine group	
			HR (95% CI)	*P-value*	HR (95% CI)	*P-value*
Cytokine group	cold	49	Exclusion		(1)	**0.024**
	hot-1/2	79			15.53 (1.434–168.3)	
Systemic edema	No	102	(1)	**<0.001**	(1)	**<0.001**
	Yes	26	13.76 (3.784–50.06)		19.24 (4.787–77.30)	
Congenital heart disease	No	41	(1)	**0.033**	(1)	**0.012**
	Yes	87	0.294 (0.096–0.903)		0.174 (0.044–0.681)	
WBC	$<100x10^9$ /L	99	(1)	**0.030**	(1)	0.607
	$\geq 100x10^9$ /L	29	3.450 (1.127–10.56)		1.383 (0.401–4.770)	
Hepatomegaly	<5 cm	100	(1)	**0.032**	(1)	**0.006**
	≥5 cm	28	3.375 (1.108–10.28)		5.839 (1.639–20.80)	
Akaike's information Criterion			102.4		96.17	
Likelihood ratio			40.00 ($P < 0.001$)		48.21 ($P < 0.001$)	

CI, confidence interval; HR, hazard ratio; Int, intermediate

Figure legends

Figure 1. Cytokine levels are valuable markers for predicting early death in patients with TAM. (A) Cumulative incidence rates (CIR) of early death between patients with TAM with high and low levels of IL-1b [high, n = 64; low, n = 64; CIR (95% CI) = 20.3% (9.8%–29.6%) vs. 1.6% (0.0%–4.6%), *P*<0.001], (B) high and low levels of IL-1ra [high, n = 36; low, n = 90; CIR (95% CI) = 30.6% (13.8%–44.1%) vs. 3.4% (0.0%–7.0%), *P*<0.001], (C) high and low levels of IL-6 [high, n = 28; low, n = 98; CIR (95% CI), 32.1% (12.4%–47.4%) vs. 4.1% (0.0%–8.0%), *P*<0.001], (D) high and low levels of IL-8 [high, n = 35; low, n = 90; CIR (95% CI), 31.4% (14.2%–45.2%) vs. 2.2% (0.0%–5.3%), *P*<0.001], and (E) high and low levels of IL-13 [high, n = 69; low, n = 59; CIR (95% CI), 18.8% (9.1%–27.6%) vs. 1.7% (0.0%–4.9%), *P*<0.001].

Figure 2. Total 128 patients with TAM are classified into three groups by an unsupervised **consensus clustering analysis based on cytokine profiling.** (A) Based on unsupervised clustering, patients were classified into three cytokine groups [hot-1 (n = 37), hot-2 (n = 42), and cold (n = 49) groups]. Missing data (1.1%) in 22 cytokines were imputed using the missForest method. Black boxes indicate each clinical feature. Gray boxes indicate patients with no data. (B–D) The mean cytokine differences (X-axis) and the negative log10-transformed statistical *P*-values (Y-axis) between (B) hot-1 group and other groups, (C) hot-2 group and other groups, (D) cold group and other groups are shown in the volcano plot.

Figure 3. Cytokine group is significantly associated with the early death rate in patients with TAM. The CIR of early death in patients with TAM between

cytokine hot-1/2 and cold groups [hot-1/2, n = 79; cold, n = 49; CIR (95% CI) = 16.5% (7.9%–24.2%); 2.0% (0.0%–5.9%), *P* = 0.013].

Figure 1

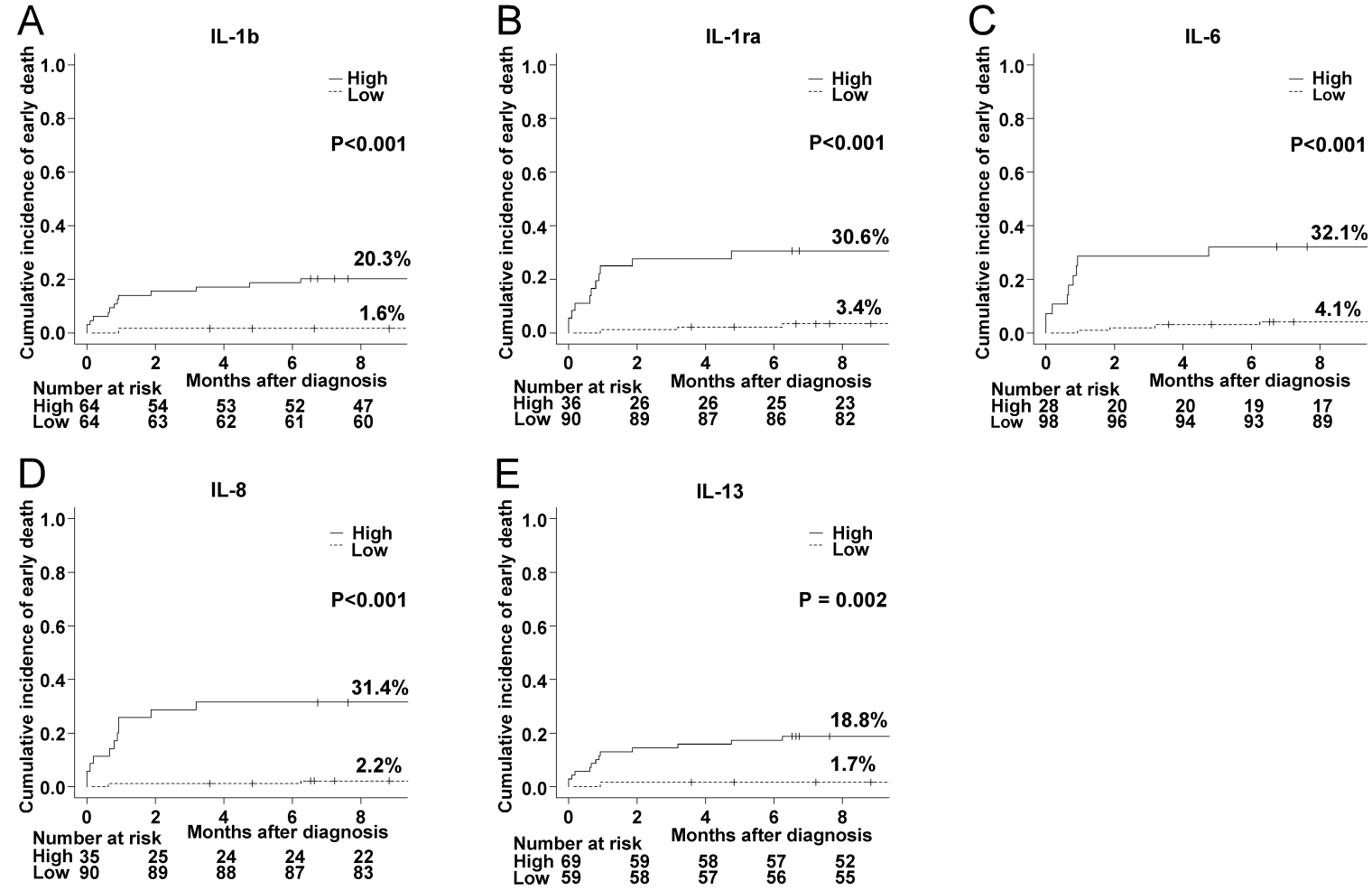

Figure 2

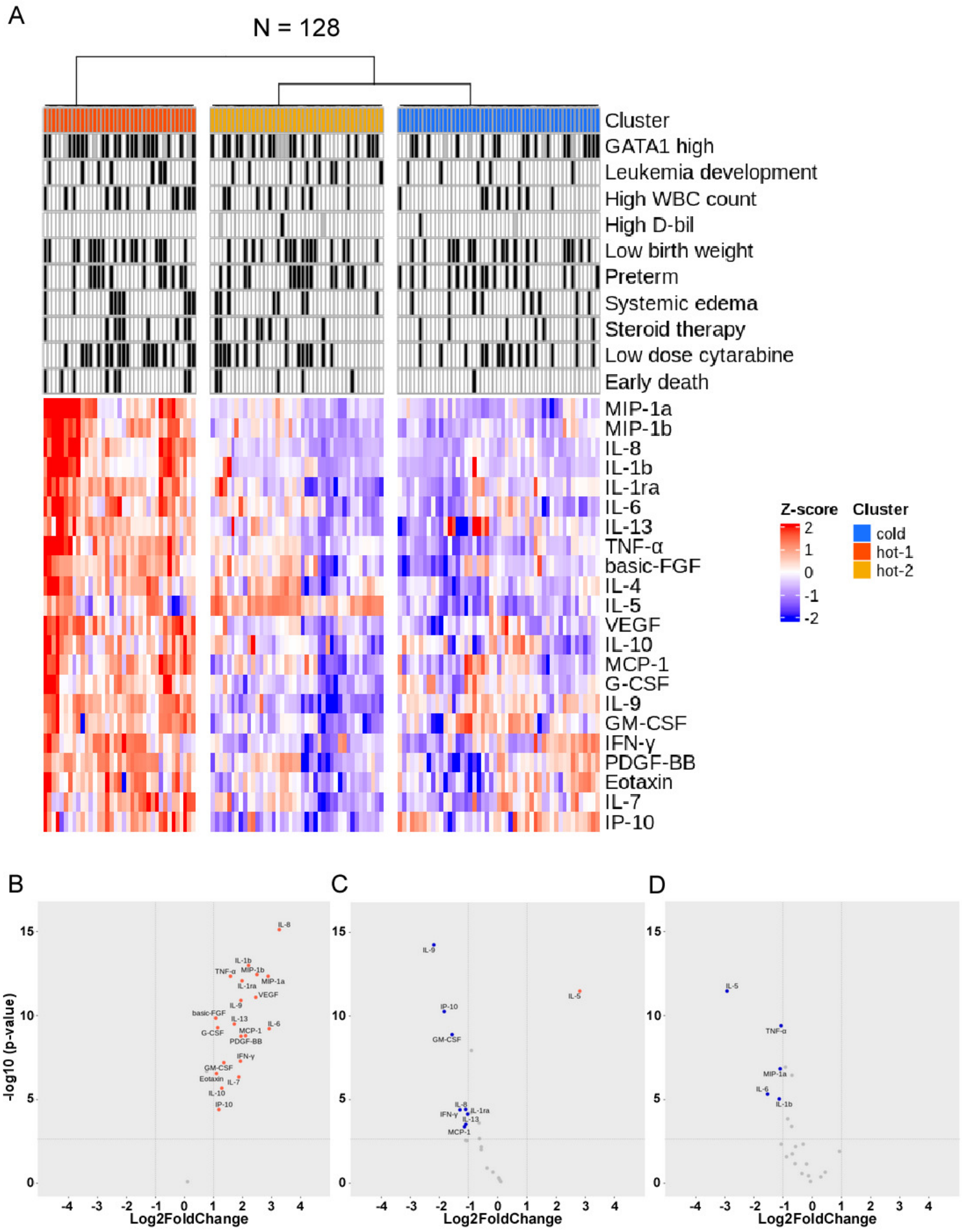

Figure 3