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BASELINE SERUM LEVELS OF MULTIPLE CYTOKINES AND ADHESION MOLECULES IN PATIENTS WITH ACUTE MYELOID LEUKEMIA: RESULTS OF A PIVOTAL TRIAL

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***Aim*: Evaluation of serum levels of 17 cytokines and 5 adhesion molecules in patients with newly diagnosed acute myeloid leukemia (AML) using biochip array technology. We searched for links between baseline levels and age, hyperleukocytosis, secondary origin of AML, resistance to induction therapy with cytarabine and daunorubicin and standard risk stratification according to cytogenetics and molecular genetics. *Methods*: We evaluated the sera of 51 consecutive patients. Serum samples were analyzed by biochip based immunoassays on the Evidence Investigator analyzer. T-tests were used for statistical analysis. *Results*: We found that higher age is associated with lower levels of interleukin (IL)-12. Patients with secondary disease were older, had higher levels of EGF and IL-7, and lower levels of E-selectin, IL-12 and IL-13. In hyperleukocytosis, the levels of IL-1β, IL-2, TNF-α, VCAM-1, ICAM-1, E-selectin and L-selectin were increased, whereas levels of IFN-γ and MCP-1 were decreased. In patients who failed to achieve complete remission after induction therapy, we found lower E-selectin and P-selectin levels. High risk patients had lower levels of IFN-γ. *Conclusion*: Some leukemic cell subpopulations have the ability to produce cytokines that modulate the microenvironment by inducing inflammation. This causes endothelial cells to be activated and overexpress adhesion molecules. Hyperleukocytosis and secondary origin of the disease are the major factors influencing the cytokine and adhesion molecule profile in newly diagnosed AML patients.**

***Key Words*: cytokines, adhesion molecules, biochip array, acute myeloid leukemia.**

Acute myeloid leukemia (AML) shows a high degree of heterogeneity owing to a variety of mutations and the mechanisms involved in leukemogenesis. Cytokines and adhesion molecules have been studied as markers of immune system activation in various diseases including hematologic malignancies and AML [1, 2]. Cytokines are soluble molecules carrying specific information for target cells. Acting through a surface receptor, they provide target cells with specific information about conditions inside the organism and cause a specific response. The response may be, e.g. stimulating and activating in the case of inflammation or in the case of tissue damage, causing proliferation or apoptosis. Under abnormal conditions, this physiological role of cytokines is maladaptive. The effect of inflammation and altered cytokine signalling on oncogenesis leading to tumor progression, has been documented [3, 4]. Blood cells and their marrow-based progenitors are exquisitely responsive to their environment, and cytokines are an essential part of it. On binding to cytokine receptor, signal transduction pathways (STP) are activated and abnormalities in signalling through STP are common in AML. Cytokines play a role in leukemogenesis, AML cell persistence, treatment outcome and allogeneic transplantation-related phenomena [5]. Cytokine-related mechanisms of leukemogenesis, AML cell persistence and resistance to chemotherapy are complex. Modulation of the cytokine network can disrupt signalling pathway activation and overcome the high resistance to treatment. It may also increase the selectivity of AML treatment, reduce the overall treatment-related toxicity and improve outcomes of AML treatment in all age groups of patients. Adhesive interactions also trigger signal transduction pathway activation and this prevents the apoptosis of both normal and malignant cells. A correlation between expression of defined adhesion molecules and patient outcome has been found for several malignant diseases including AML.

Alterations in the interacting functional network of cytokines and adhesion molecules may have direct effect on the malignant cells or have indirect effect on leukemogenesis through altered functions of bone marrow stromal elements [6, 7]. Further knowledge gained from multi-analytical determination of cytokines and adhesion molecules could allow better diagnosis and management of hematologic malignancies, since cytokines or their receptors may also represent a target for specific anticancer therapy at the molecular level. Recently, some studies reported the possible diagnostic and prognostic use of cytokine levels in newly diagnosed AML and myelodysplastic syndromes [8–10]. The aim of this study was to evaluate baseline serum levels of cytokines and adhesion molecules in patients treated for AML. We evaluated changes in cytokine and adhesion molecule levels as-

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Abbreviations used: AML – acute myeloid leukemia; CR – complete remission; EGF – epidermal growth factor; ICAM – intercellular adhesion molecule; IL – interleukin; IFN – interferon; MCP – monocyte chemotactic protein; MDS – myelodysplastic syndrome; STP – signal transduction pathways; TNF – tumor necrosis factor; VCAM – vascular cell adhesion molecule; VEGF – vascular endothelial growth factor.

sociated with age, hyperleukocytosis and secondary AML origin. Age-related changes were suspected in several studies and older patients have often been excluded from analyses. On the other hand, age need not correlate with biological age or performance status. Based on our own experience with good results using a curative approach in patients aged 65–70 with ECOG performance status 0–2, we decided to search for age-related alterations in cytokine and adhesion molecule levels. A secondary AML usually evolves from previous myelodysplasia or myelofibrosis and is more frequent in older patients. Nevertheless, due to progress in the treatment of malignancies in younger patients (e.g. Hodgkin's lymphoma, testicular, early breast and prostate cancer) and curative treatment, some younger patients may also suffer from secondary AML. Hyperleukocytosis may be associated with life threatening leukostasis, which is based on capillary obstruction by adherent activated myeloblasts in endothelia. There were two reasons why we evaluated hyperleukocytosis separately. First, we expected increased levels of cytokines directly produced by myeloblasts in the blood flow. Second, we anticipated high levels of soluble adhesion molecules as leukostasis and extravascular leukemic cell dissemination is exquisitely dependent on adhesive interactions [11].

Further, we aimed to determine the prognostic value of baseline cytokine and adhesion molecule levels. We divided patients according to standard risk stratification based on cytogenetic and molecular genetic examination into low-risk, intermediate-1 and -2 and high risk subgroups to determine whether these subgroups differed in cytokine and/or adhesion molecule levels. We then took a detailed look at those patients who failed to achieve complete remission (CR) after induction chemotherapy combining cytarabine and daunorubicin. Failure of induction therapy has direct therapeutic consequences. Irrespective of standard prognostic indicators, these patients suffered from aggressive disease and were reinduced by salvage regimen and allotransplanted if a suitable donor was available. Early identification of these patients would allow treatment to be more personalized which is the basic aim of modern medicine.

PATIENTS AND METHODS

Patients. A total of 51 newly diagnosed AML patients, 19 males and 32 females, mean age 52.5 ± 13.4, median 56.2 years, were studied. According to cytogenetic and molecular genetic evaluation, 11 patients were classified as low risk, 9 — intermediate-1 risk, 12 — intermediate-2 risk and 19 — high risk disease. In 24 patients, normal karyotype was present. Of these, 5 had NPM-1 mutated (Nucleophosmin-1), 8 patients had both NPM-1 and FLT3-ITD (Famosin-like tyrosin kinase 3 — internal tandem duplications), 3 patients had solely FLT3-ITD and 8 patients had various mutations including CEBPa (CCAT enhancer binding protein alpha) and mutations including MLL (Multi-lineage leukemia) gene. In 27 patients at least 1 cytogenetic abnormality was present. Of these, 4 patients had CBF (core binding factor) positive disease, all 4 patients with acute promyelocytic leukemia had typical translocation present and 6 patients had complex changes including 3 or more translocations. According to FAB classification, 2 patients had AML M0, 10 patients — AML M1, 25 patients — AML M2, 4 patients — AML M3, 6 patients — AML M4, 3 patients — AML M5 and 1 patient — AML M7. Hyperleukocytosis requiring urgent leukapheresis was present in 12 patients. All patients were induced with "3+7" induction chemotherapy consisting of cytarabine 100 mg/m^2 per day for 7 consecutive days and daunorubicin 90 mg/m^2 for the first 3 days of therapy in younger patients. In patients aged 65 or more, daunorubicin 45–60 mg/m^2 was administered. This was followed by consolidation with high-dose cytarabine. In those patients who failed to achieve CR after "3+7" induction regimen, the salvage chemotherapy "FIAG-Ida" was administered. A total of 31 patients who had intermediate or high risk disease or failed to achieve CR after "3+7" induction regimen had suitable donors for allogeneic stem cell transplantation and were able to undergo the procedure. These patients were allotransplanted. The study was approved by the local Ethics Committee and all patients gave written consent.

Serum collection. Peripheral blood was collected in serum separating tubes, immediately transported to the laboratory and processed within 2 hours of collection by centrifugation at 1500 rpm × 5 min. All sera were collected before leukapheresis, administration of hydroxyurea or induction therapy. If specimens were not to be analysed immediately, they were stored frozen in small aliquots at –20 °C as recommended by the Cytokine Array manufacturer. Repeat freeze/thaw cycles were avoided.

Methods. All analytes were measured by biochip array technology using chemiluminescent sandwich immunoassays applied to the Evidence Investigator Analyzer (Randox Laboratories Ltd., Crumlin, UK). The Evidence Investigator Biochip Array technology is used to perform simultaneous quantitative detection of multiple analytes from a single patient sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilised antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay is employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labelled with horseradish peroxidase (HRP) and thus an increase in the chemiluminescent signal emitted. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and compared to that from a stored calibration curve. The concentration of analyte present in the sample is calculated from the calibration curve.

We evaluated circulating levels of the following 17 cytokines and 5 soluble adhesion molecules: interleukins (IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-23), vascular

endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), epidermal growth factor (EGF), monocyte chemotactic protein-1 (MCP-1), E-selectin, L-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The results are expressed in nanograms per litre (ng/L) for cytokines and micrograms per litre (μg/L) for adhesion molecules.

Statistical analysis. Statistical analysis was performed with the "Statistica" software using two-tailed t-tests. The values were expressed as means ± standard deviation. Probability values $p < 0.05$ were considered statistically significant.

RESULTS

Age dependent differences. Originally, we analysed 3 subgroups of patients divided according to age. The first subgroup consisted of younger patients aged less than 55 years (n = 25), the second subgroup included patients aged 55–65 years (n = 11) and the third subgroup included patients 65 and older (n = 15). Because there were no significant differences between younger and 55–65 year old patients, we analysed both subgroups together against patients older than 65 years.

In patients aged 65 or more, we found a significant decrease in IL-12 levels (0.95 ± 1.14 ng/L *vs*. 4.11 ± 3.76 ng/L, $p = 0.025$).

Secondary AML. The group with secondary AML consisted of 15 patients with a previous history of MDS (myelodysplastic syndrome). Two patients progressed to AML from chronic myelomonocytic leukemia and two patients from a previous myeloproliferative disorder. They were much older than those with primary disease (62.9 ± 6.7 *vs*. 46.6 ± 13.3 years, $p = 0.00008$). In secondary AML, we found higher IL-7 (6.13 ± 4.42 ng/L *vs*. 3.59 ± 1.97 ng/L, $p = 0.047$) and EGF levels (26.64 ± 26.58 μg/L *vs*. 7.49 ± 8.05 μg/L; $p = 0.004$) (Fig. 1). The levels of IL-12 (1.26 ± 1.46 ng/L *vs*. 4.32 ± 3.87 ng/L; $p = 0.021$), IL-13 (2.16 ± 3.02 ng/L *vs*. 5.09 ± 4.47 ng/L, $p = 0.049$) and E-selectin (14.85 ± 10.36 μg/L *vs*. 28.84 ± 18.16 μg/L; $p = 0.018$) were decreased in secondary AML (Fig. 2, 3). The leukocyte counts had a trend to lower counts in the secondary AML group which were significant after exclusion of patients with acute promyelocytic leukemia (15.93 ± 15.99 • 10^9/L *vs*. 53.35 ± 54.44 • 10^9/L, $p = 0.048$). We also found trends towards higher MCP-1 (246.14 ± 128.35 ng/L *vs*. 167.56 ± 101.96 ng/L, $p = 0.055$) and lower IL-10 levels (1.53 ± 1.16 ng/L *vs*. 4.99 ± 4.86 ng/L, $p = 0.075$) in secondary AML.

Hyperleukocytosis. Hyperleukocytosis in AML is defined as a blood count containing at least 50 • 10^9/L leukocytes. Patients with initial hyperleukocytosis (n = 12) had higher levels of IL-1β (4.64 ± 4.44 ng/L *vs*. 0.84 ± 0.49 ng/L, $p = 0.0006$), IL-2 (13.74 ± 15.77 ng/L *vs*. 3.48 ± 2.85 ng/L, $p = 0.009$), TNF-α (4.79 ± 3.39 ng/L *vs*. 2.14 ± 1.03 ng/L, $p = 0.002$) (Fig. 4). We also found lower levels of MCP-1 (102.38 ± 51.29 ng/L *vs*. 225.89 ± 114.87 ng/L, $p = 0.007$) and IFN-γ (0.42 ± 0.49 ng/L *vs*. 2.03 ± 1.47 ng/L, $p = 0.005$), in these patients (Fig. 5, 6).

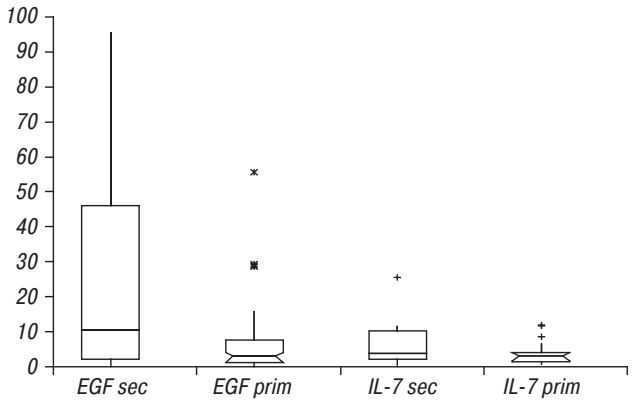

Fig. 1. Serum levels of EGF and IL-7 in primary and secondary AML. EGF — epidermal growth factor; IL-7 — interleukin 7; sec — secondary AML; prim — *de novo* AML

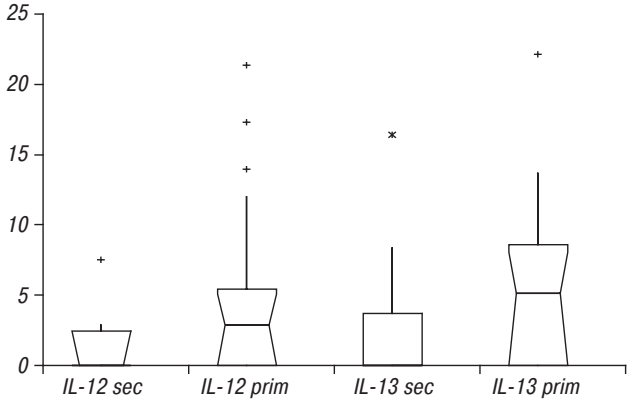

Fig. 2. Serum levels of IL-12 and IL-13 in primary and secondary AML. IL-12 — interleukin 12; IL-13 — interleukin 13; sec — secondary AML; prim — *de novo* AML

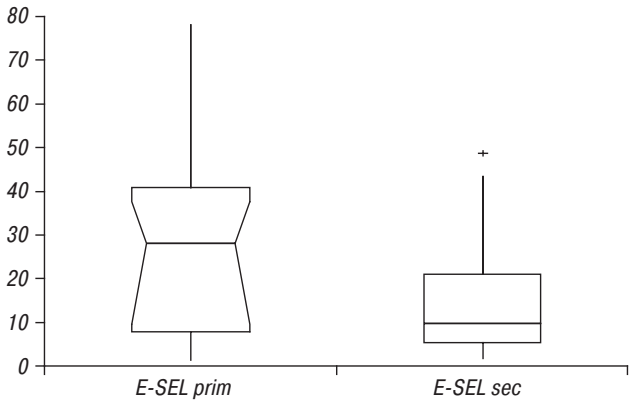

Fig. 3. Serum levels of E-selectin primary and secondary AML. E-SEL — E-selectin; sec — secondary AML; prim — *de novo* AML

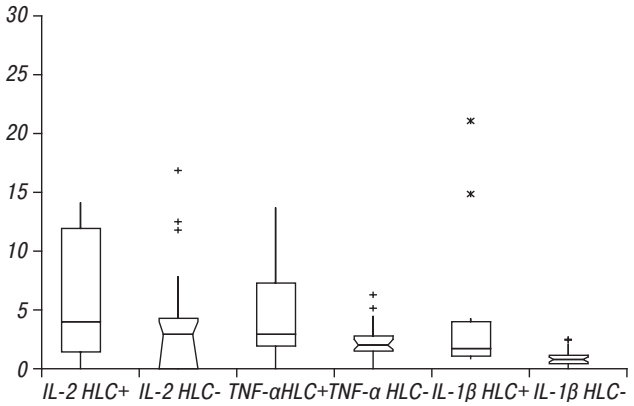

Fig. 4. Serum levels of IL-2, TNF-α and IL-1β in patients with and without hyperleukocytosis. IL-2 — interleukin 2; TNF-α — tumor necrosis factor-alpha; IL-1β — interleukin 1β; HLC+ — hyperleukocytosis; HLC− — without hyperleukocytosis

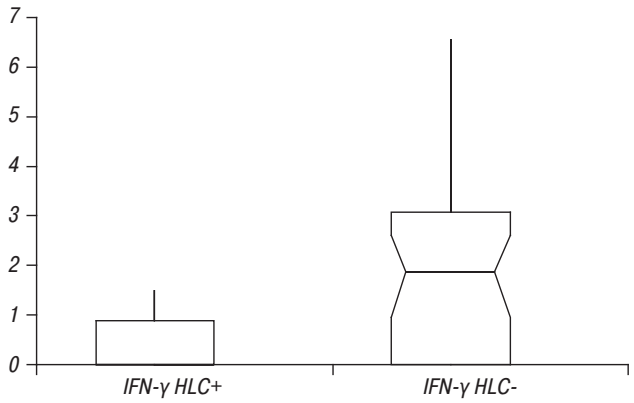

Fig. 5. Serum levels of IFN-γ in patients with and without hyperleukocytosis. IFN-γ — interferon-gamma; HLC+ — hyperleukocytosis; HLC– — without hyperleukocytosis

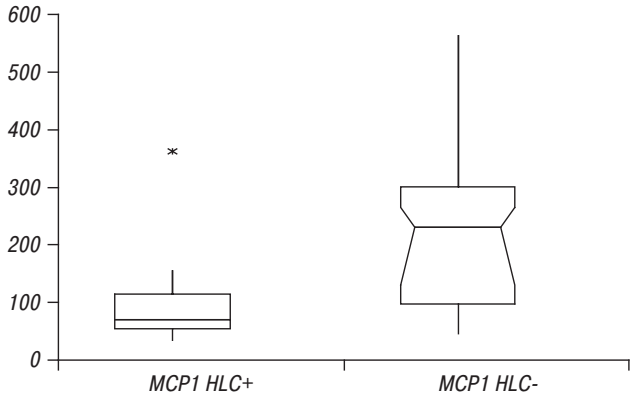

Fig. 6. Serum levels of MCP-1 in patients with and without hyperleukocytosis. MCP1 — monocyte chemotactic protein-1; HLC+ — hyperleukocytosis; HLC- — without hyperleukocytosis

Soluble adhesion molecules were greatly increased in hyperleukocytosis. We found VCAM-1 (1113.65 ± 519.71 μg/L *vs*. 599.59 ± 180.40 μg/L, $p = 0.0002$), ICAM-1 (437.51 ± 177.07 μg/L *vs*. 296.56 ± 80.99 μg/L, $p = 0.004$) and E-selectin (40.65 ± 13.90 μg/L *vs*. 18.39 ± 14.11 μg/L, $p = 0.0006$) (Fig. 7, 8). The levels of L-selectin were at the upper limit of the Array sensitivity in the vast majority of patients with hyperleukocytosis, similar to several patients without hyperleukocytosis. Hence, the results are probably affected. However, we measured increased levels of L-selectin (3262.18 ± 265.10 μg/L *vs*. 2307.96 ± 1010.81 μg/L, $p = 0.006$) in hyperleukocytosis (data not shown).

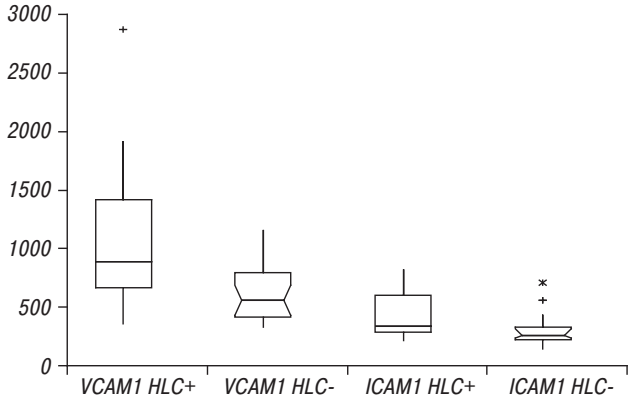

Fig. 7. Serum levels of VCAM-1 and ICAM-1 in patients with and without hyperleukocytosis. VCAM1 — vascular cell adhesion molecule-1; ICAM1 — intercellular adhesion molecule-1; HLC+ — hyperleukocytosis; HLC– — without hyperleukocytosis

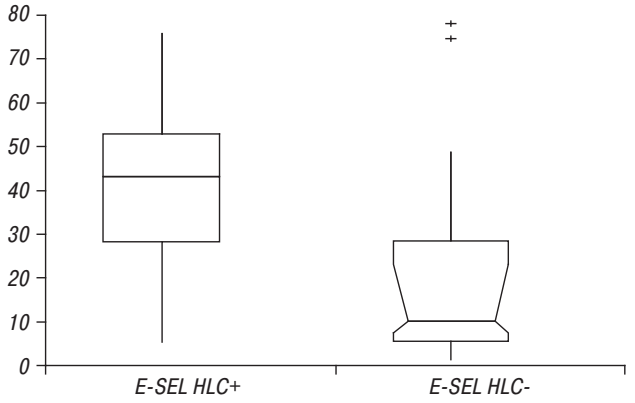

Fig. 8. Serum levels of E-selectin in patients with and without hyperleukocytosis. E-SEL — E-selectin; HLC+ — hyperleukocytosis; HLC– — without hyperleukocytosis

Resistance to induction therapy. Those who had not achieved CR after induction chemotherapy and were reinduced with the salvage regimen (n = 11), had lower E-selectin (11.63 ± 7.34 μg/L *vs*. 26.93 ± 17.81 μg/L, $p = 0.028$) and P-selectin levels (76.19 ± 23.29 μg/L *vs*. 136.04 ± 64.74 μg/L, $p = 0.022$). On the other hand, those who failed to achieve CR had significantly higher CRP (C-reactive protein) levels (63.4 ± 54.8 mg/L *vs*. 27.4 ± 26.0 mg/L, $p = 0.017$) (Fig. 9, 10).

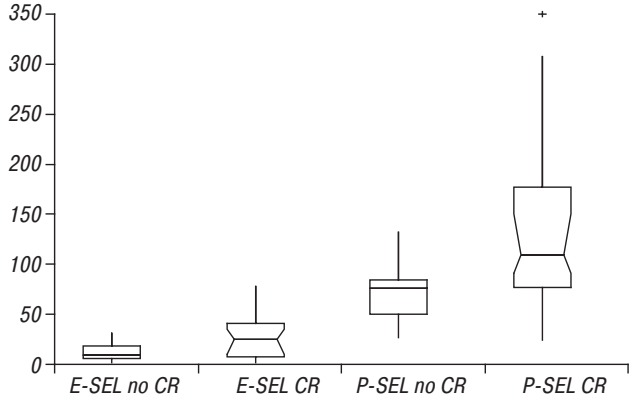

Fig. 9. Serum E-selectin and P-selectin levels in patients who failed to reach CR after induction therapy. E-SEL — E-selectin; P-SEL — P-selectin; no CR — failed to reach CR after induction therapy; CR — attained CR after induction therapy

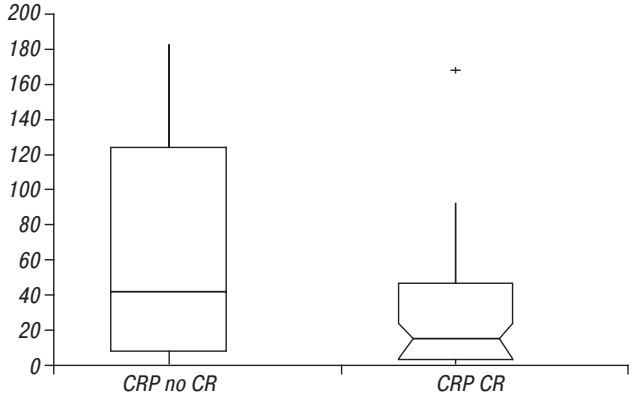

Fig. 10. Serum CRP levels in patients who failed to reach CR after induction therapy. CRP — C-reactive protein; no CR — failed to reach CR after induction therapy; CR — attained CR after induction therapy

Risk stratification and cytokine levels. Finally, we analysed subgroups based on cytogenetic and molecular genetic risk stratification. There was no significant difference between the groups but when

we analysed the high risk subgroup (n = 19) against the 3 other prognostic subgroups (n = 32) we found decreased IFN-γ levels (1.02 ± 1.11 ng/L *vs*. 2.03 ± 1.49 ng/L, $p = 0.049$) in high risk patients (Fig. 11). No other significant differences were found in the levels of other evaluated cytokines or adhesion molecules in any analysis.

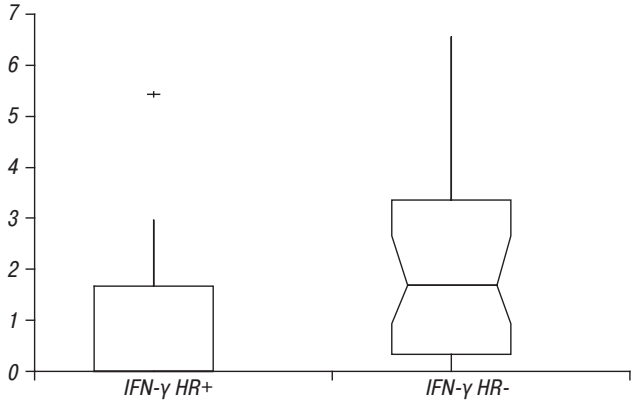

Fig. 11. Serum levels of IFN-γ in high risk patients. IFN-γ — interferon-gamma; HR+ — high risk; HR– — other risk

DISCUSSION

Altered levels of cytokines and adhesion molecules have been found in many pathological states and have been linked to autoimmune diseases, allergies and cancer, including AML [3–5, 12–14]. Cytokines and adhesion molecules form an unique interacting functional network. This led us to study both systems together.

Our results are in agreement with the general finding that patients with secondary AML are usually older than those with primary disease. The decrease in IL-12 levels found in elderly patients is probably attributed to secondary AML. We venture to suggest that the origin of AML is a more important factor for cytokine and adhesion molecule levels than age *per se*. The finding of lower IL-13 levels in secondary AML is in agreement with previous results, where MDS patients had lower IL-13 levels than AML [9]. Further, the trend to lower IL-10 levels in secondary disease may explain the recently published negative prognostic impact of low IL-10 levels [10].

We found evidence that hyperleukocytosis has direct impact on levels of several cytokines and soluble adhesion molecules. We think that increased levels of IL-1 beta and IL-2 reflect production of these cytokines by myeloblasts in the blood circulation. The IL-1, IL-2 and b-chemokine of the CC subgroup CCL-3 (CC-ligand-3) were shown to stimulate leukemic cell proliferation. IL-1β is the predominant secreted form of IL-1 [15]. Possibly, the myeloblasts trigger autocrine inflammatory loops enhancing their own proliferation which causes hyperleukocytosis and TNF-α inflammatory overproduction. E-selectin is expressed by endothelia. Its expression is regulated on the transcriptional level and is induced by TNF-α, IL-1 and oncostatin M. In response to myeloid growth factors, endothelia increase expression of E-selectin, VCAM-1 and ICAM-1 and promote augmented leukocyte adhesion in a p38 MAPK dependent manner. Soluble adhesion molecules originate from proteolytic cleavage of surface-expressed adhesion molecules. In lymphoma-bearing mice, the lymphoma cells are the major source of soluble L-selectins [16–18]. We speculate that in hyperleukocytosis the soluble L-selectin molecules originate from myeloblasts, whereas increased levels of VCAM-1, ICAM-1 and E-selectin originate from inflammatory activated endothelia.

In those patients who failed to reach CR after "3+7" induction therapy, the levels of E- and P- selectins were found to be decreased. Further, the higher CRP level in these patients indicates an activated inflammatory response. It is unclear whether these high CRP levels were caused by systemic infection or reflect the activity of highly aggressive disease. From previous studies, we know that cytokine levels do not correlate with infection [9], but we lacked data for adhesion molecules. Decreased levels of selectin adhesion molecules seem to be in contradiction with the findings in hyperleukocytosis, as one would expect inferior outcomes in patients with hyperleukocytosis which was found to increase E-selectin levels. What we have to take into account is the ability of leukemic blasts to bind to soluble adhesion molecules. In KG-1, AML cell line interactions with both P- and E- selectins under flow have been documented. Upon adhesion of myeloblasts to selectin adhesion molecules, the adherent myeloblasts may sustain toxic concentrations of chemotherapeutic agents and thus be rescued from death [19, 20]. This mechanism suggests how adhesive interactions protect AML cells during chemotherapy. For this reason, possibly, the levels of E- and P-selectins in patients resistant to induction chemotherapy were low because the soluble adhesion molecules were attached to leukemic blasts and thus not measured. The origin of the MCP-1 and IFN-γ decrease remains unclear and is worth further investigation.

The risk stratification of AML is exactly defined by cytogenetics and molecular genetics. We attempted to match standard risk stratification with specific alterations in cytokine or adhesion molecule levels. We found decreased IFN-γ in the high risk subgroup which was not described previously [9]. On critical reappraisal however, the high risk subgroup in our analysis included both patients with normal karyotype and those with high risk molecular genetics, the same as patients with high risk cytogenetics and complex karyotype. Thus, the high risk group was not homogenous and this may have been reflected in the results.

Based on analysis of patient sera and healthy blood donors as a non-leukemic control, we previously reported that changes in cytokine and adhesion molecule levels are related to disease activity [21]. Our results indicate that serum levels of specific cytokines and adhesion molecules are significantly altered in AML patients and this enables us to further understand the mechanisms of disease progression and resistance to treatment. We highlight the importance of E- and P-selectin levels in AML and to the best of our knowledge this is the first published evidence *in vivo*. Further studies in a larger number of patients

will be needed to confirm our data and define the potential role of these and additional markers in the risk stratification and therapy of AML patients.

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: None declared.

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