## Significance of apoptosis and autophagy of leukemic blasts for the outcomes of acute myeloid leukemia patients

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#### **Abstract**

**Background.** Cytostatic treatment induces apoptosis or other types of cell death like autophagy, necrosis, mitotic catastrophe, etc. Autophagy can play a role in the drug resistance of neoplastic cells, allowing the survival of blast cells under stressful conditions, such as the use of cytostatics. Studies on apoptosis and autophagy 12–24 h after the start of treatment have not been conducted until now.

**Objectives.** The study aimed to investigate the predictive and prognostic significance of autophagy and apoptosis in patients with acute myeloid leukemia (AML).

**Material and methods.** The study included 38 patients. Blood was collected before and 12–24 h after the start of treatment, since at that time point, the appropriate blast cell count was still available. Autophagy was measured with the expression of the ATG5, MAP1L3, LC3–I, and LC3–II proteins. The percentage of mononuclear cells in early and late apoptosis was evaluated with flow cytometry, using the annexin V and propidium iodide (PI) binding assay.

**Results.** The percentage of apoptotic blast cells before treatment was not associated with the response. However, in the remission group, the overall percentage of apoptotic cells measured 12–24 h after the start of treatment was higher than in non-remission patients, which was statistically significant. In neither group we found any difference in the level of autophagy before and 12–24 h after the start of treatment. Nevertheless, we observed an increasing tendency of the MAP1LC3 protein expression (not statistically significant) in the remission group 12–24 h after the start of treatment. Patients with a higher percentage of blast cells in apoptosis and with a higher expression of MAP1LC3 protein measured 12–24 h after the start of the therapy had longer overall survival (OS).

**Conclusions.** A higher percentage of apoptotic as well as autophagic blast cells measured 12–24 h after the start of the chemotherapy is an independent factor associated with better outcomes.

Key words: apoptosis, acute myeloid leukemia, autophagy

#### Introduction

The results of several studies have shown that chemotherapeutic drugs can induce non-apoptotic types of cancer cell death. These processes include autophagy, mitotic catastrophe and necrosis as well as ageing.<sup>1–4</sup>

Apoptosis is a programmed cell death, which under physiological conditions makes it possible to maintain tissue homeostasis, both in embryonic development and in adult life, by preserving a sufficient number of cells in the developing organism and by eliminating abnormal damaged cells. It is involved in the tissue regeneration process, but also in the organ involution process (thymus atrophy). The induction of apoptosis can occur under the influence of intrinsic factors (derived from other cells) or extrinsic factors, such as ultraviolet radiation, ionizing radiation, high temperature, cytostatics, and free radicals. 8–10

Autophagy, unlike other processes, does not always lead to cell death. It may be one of the mechanisms of cancer cell death, but on the other hand, it can be an adaptive process that allows the survival of tumor cells under stressful conditions, such as the use of cytostatics. In such situations, it can allow cells to adapt to stressors and is a survival strategy for them. There are few reports on the role of autophagy in the treatment of acute leukemia. It seems that autophagy is the most common mechanism of cancer cell proliferation through generating resistance to the chemotherapy. The results of the studies are ambiguous – some point to its cytoprotective role for leukemia cells, while others show its antitumor role. At present, we can observe constant attempts to investigate the effects of modifying autophagy in various types of cancers, including hematologic malignancies. 11–15

The few reports on the role of apoptosis and autophagy in the treatment of patients with acute myeloid leukemia (AML) prompted us to investigate their role in the induction therapy.

This study investigated the percentage of early and late apoptotic cells. Autophagy was measured with the expression of the products of 2 important genes (*ATG5* and *MAPILC3B*) – ATG5 and LC3B proteins. <sup>16</sup> The ATG5 protein is the part of the ATG12-ATG5-ATG16L complex, which is essential for the phagophore elongation. The oligomerization of this complex causes the lengthening of the phagophore. In the next stage, the phagophore closes, which leads to the formation of the autophagosome.

The autophagosome closes the part of the cytoplasm with components to be digested. During the autophagosome formation, the LC3 protein undergoes a lipid-mediated transformation of the cytosolic form (LC3-I form), thereby producing a lipid form (LC3-II), which is equivalent to the production of autophagosomes in the cell, and the expression of this protein reflects the number of produced autophagosomes.  $^{17-20}\,$ 

Thus, it was investigated whether cytostatic treatment activated the transcription of both genes as well as the post-translational transformation of the LC3B protein, required for the production of autophagosomes.

#### **Objectives**

The primary objective of the study was to investigate the predictive and prognostic significance of the autophagy and apoptosis of blast cells in patients with AML – before and 12–24 h after the start of the induction therapy. In this study, we attempted to determine the potential differences in the percentage of apoptotic cells and the expression of markers such as ATG5, MAP1LC3, LC3-IB, and LC3-IIB measured before (spontaneous apoptosis) and after the start of the treatment, depending on the response to the therapy. The study also investigated the correlation between the percentage of apoptotic cells, the level of expression of the *MAP1LC3* and *ATG5* genes before and after the start of the induction therapy, and overall survival (OS).

#### **Material and methods**

#### **Patients**

In total, this study enrolled 38 patients with AML (20 men and 18 women): 29 patients with de novo diagnosis, 2 patients with AML relapse, and 7 with AML secondary to myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD).

Depending on the remission status achieved after the induction therapy, 2 groups of patients were identified:

Group I: 20 patients (aged 18–67 years; median: 36 years) who achieved remission, including 18 patients with de novo diagnosis, 1 patient with a relapse (22 months after the completion of the treatment) and 1 with the blast crisis during MPD; the DAC (daunorubicin, cytosine arabinoside, cladribine) regimen was used in 8 patients and the DA (daunorubicin, cytosine arabinoside) regimen in 12 patients, without a reduction in the scheduled doses (Table 1 summarizes the DAC and DA regimens);

Group II: 18 patients (aged 35–77 years; median: 55.5 years) who did not obtain remission after the induction treatment, including 11 patients with de novo diagnosis, 6 patients with AML secondary to MDS or MPD and 1 patient with a relapse 3 years after the completion

**Table 1.** Protocols of the induction chemotherapy used in patients with acute myeloid leukemia (AML)

Age of patients [years]	Induction chemotherapy
<60	DA daunorubicin: 60 mg/m² iv. (day 1–3) cytarabine: 200 mg/m², continuous infusion (day 1–7) DAC daunorubicin: 60 mg/m² iv. (day 1–3) cytarabine: 200 mg/m², continuous infusion (day 1–7) cladribine 5 mg/m² (day 1–5)
>60	DA (Cancer and Leukemia Group B – CALGB) daunorubicin: 45 mg/m² iv. (day 1–3) cytarabine: 100 mg/m², continuous infusion (day 1–7)

 $\mathsf{D}-\mathsf{daunorubicine}; \mathsf{A}-\mathsf{cytarabine}; \mathsf{C}-\mathsf{cladribine}; \mathsf{iv.}-\mathsf{intravenously}.$ 

men was used in 17 patients and the DAC regimen in 1 patient. In 5 patients, it was necessary to reduce the dose of the drugs for various reasons: old age (n = 2), pregnancy (n = 1), venous thrombosis (n = 1), and prevoius use of low doses of cytosine arabinoside (20 mg/m<sup>2</sup> over 10 days) (n = 1).

In all patients, blood was collected before and 12-24 h after the start of the induction chemotherapy into 9-milliliter tubes to isolate mononuclear cells. The data is presented in Tables 1-3.

#### Measurement of early and late apoptosis

Simultaneous administration of propidium iodine (PI) and annexin makes it possible to distinguish between healthy cells and necrotic cells as well as early and late apoptotic cells. The percentage of mononuclear cells in early and late apoptosis and their viability was evaluated with flow cytometry before and 12-24 h after the start of the induction therapy with the use of the annexin V and PI (Becton Dickinson, Franklin Lakes, USA) binding assay. Half a million cells were incubated in 100 µL of the annexin V binding buffer (10 mM Hepes/NaOH, pH 7.4, 0.14 M NaCl, 2.5 mM CaCl<sub>2</sub>) with 5 µL of bound annexin V-FITC (fluorescein isothiocyanate) and 5 µL of PI at room temperature for 15 min. At the end of incubation 1 mL of the buffer was added and the cytometry analysis was performed (flow cytometer Partec PAS (Particle Analysing System), Partec GmbH, Münster, Germany). Next, the percentage of annexin V-binding cells corresponding to early apoptosis, not stained with PI, was measured. The annexin-binding and red-stained (PI) cells showed late apoptosis. The total percentage of apoptotic cells was expressed as the sum of early apoptosis and late apoptosis.

#### Molecular studies

#### Ribonucleic acid isolation

Total ribonucleic acid (RNA) was isolated from the mononuclear cells obtained from the peripheral blood of patients diagnosed with AML, using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A  $10 \times 10^6$  cell pellet was MDS – myelodysplastic syndrome.

of the maintenance treatment; the DA regi- Table 2. Clinical characteristics of patients with remission after the start of the induction therapy

Clinical characteristics of patients with remission after the start of the induction therapy	n	М	min	max	25Q	75Q
AML de novo AML secondary to MPD Recurrence of AML	18 1 1			-		
Age [years]	20	36.0	18.0	67.0	26.0	56.5
Sex female male	9 11			_		
FAB M0 M1 M2 M4 M5 M6	0 4 9 4 3 0			-		
White blood cells [10°/L]	20	47.4	4.7	225.6	19.4	134.1
Hemoglobin [g%]	20	9.60	6.40	14.10	8.65	10.70
Platelets [10 <sup>9</sup> /L]	20	44.5	17.0	292.0	30.0	89.0
Percentage of blasts in blood at diagnosis	16	85.0	7.0	96.0	37.0	89.5
Percentage of blasts in bone marrow at diagnosis	20	76.0	25.0	93.0	49.0	87.0
Unfavorable cytogenetic or molecular abnormalities	3			-		

AML – acute myeloid leukemia; FAB – French-American-British classification; M – median; MPD – myeloproliferative disease; n – number of patients; 25Q – upper quartile; 75Q – lower quartile

Table 3. Clinical characteristics of patients without remission after the induction therapy

Clinical characteristics of patients without remission after the induction therapy	n	M	min	max	25Q	75Q
AML de novo AML secondary to MDS AML secondary to MPD Recurrence of AML	11 4 2 1			-		
Age [years]	18	55.5	35.0	77.0	46.0	64.0
Sex female male	9 9			-		
FAB M0 M1 M2 M4 M5 M6 bilinear	0 5 3 5 4 0			-		
White blood cells [109/L]	18	23.9	1.5	308.5	4.2	40.9
Hemoglobin [g%]	18	8.90	6.60	14.40	8.20	10.60
Platelets [10 <sup>9</sup> /L]	18	57.0	14.0	224.0	28.0	125.0
Percentage of blasts in blood at diagnosis	18	68.5	23.0	96.0	40.5	81.0
Percentage of blasts in bone marrow at diagnosis	17	26.0	1.0	88.0	16.0	67.0
Unfavorable cytogenetic or molecular abnormalities	3					

lysed in 600  $\mu L$  of the RLT buffer with the addition of 1  $\mu L$  of 6-mercaptoethanol and homogenized by passaging several times through a syringe needle. After centrifugation (3 min, 13,400 rpm), the supernatant was transferred into new Eppendorf tubes. One volume of 70% ethanol was added to the supernatant and the sample was thoroughly mixed. The obtained solution was applied in 700- $\mu L$  aliquots on a nucleic acid-binding silica column, mounted in 2-mL tubes, followed by rinsing with high ionic strength buffers supplied by the manufacturer, and rotating for 15 s, 10,000 rpm. The bound RNA was eluted into fresh tubes collecting RNase-free water with a volume of 30  $\mu L$  (1 min, 10,000 rpm).

#### **Reverse transcription**

Complementary deoxyribonucleic acid (cDNA) was obtained on the matrix of the isolated RNA, using the High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Foster City, USA) as recommended by the manufacturer. The reaction was performed at  $37^{\circ}$ C for 120 min in a DNA Engine® Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, USA). Reverse transcriptase was thermally inactivated by heating the samples at  $85^{\circ}$ C for 5 min. The obtained cDNA was stored at  $-20^{\circ}$ C until polymerase chain reaction (PCR) was performed.

#### Real-time polymerase chain reaction

We assessed the expression of the ATG5 and MAP1LC3 genes with the use of real-time quantitative PCR (RQ-PCR). The primers and probes of Life Technologies (Carlsbad, USA), TaqMan® Real-Time PCR Assay, Hs00169468\_m1 and Hs99999908\_m1 were used for MAP1LC3 and  $\beta$ -glucuronidase reference gene (GUSB), respectively. The reaction was performed in a Universal Master Mix (Life Technologies) on a 7500 Real-Time PCR System from Applied Biosystems. We used the following universal protocol: 45 cycles of 95°C for 15 s, 60°C for 1 min, with previous incubation at 50°C for 2 min, and 95°C for 10 min (the polymerase activation). Each assay was repeated 3 times. For each assay, the difference Ct was determined for the test and reference genes ( $\Delta$ Ct), and  $2^{-\Delta Ct}$  was calculated. The value obtained was the measure of the expression of the test gene.

#### Western blot

#### Preparation of cell lysates

The cell lysis was performed at 4°C for 30 min after the suspension of the cell pellet in 60  $\mu$ L of radioimmuno-precipitation assay (RIPA) lysis buffer supplemented with 1mM of phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, USA). Then, the samples were centrifuged at 4°C for 10 min at 8000  $\times$  g and the supernatant was transferred into new tubes. After adding a sufficient

quantity of the loading buffer (4 times concentrated), the samples were denatured by placing them in a boiling water bath for 5 min.

### Electrophoresis of proteins in denaturing conditions (sodium dodecyl sulfate-polyacrylamide gel electrophoresis - SDS-PAGE)

After the denaturation in a boiling water bath, the protein samples were briefly centrifuged and then placed into 15% polyacrylamide gel wells. The quantity of lysate in the gel well corresponded to 40  $\mu$ g of protein. The separation was held in the electrophoresis buffer, initially at 80 V and in the later phase at 110 V. The electrophoresis was conducted for approx. 2 h and was terminated when the loading buffer reached the lower gel layer.

#### Detection of the LC3-I and LC3-II proteins using western blot

Upon completion of gel electrophoresis, the gel protein was transferred to the nitrocellulose membrane by electroblocking. The transfer was carried out in the electrolytic buffer at 350 mA for 60 min. After the blocking step (1 h at room temperature) in a 5% solution of skimmed milk in TBST (tris-buffered saline, Tween 20) (Sigma-Aldrich), the membrane was incubated with mouse monoclonal antibody against human protein LC3B (MB-M186-3 anti-LC3; MBL International Corporation, Woburn, USA) in TBST with 0.5% skimmed milk. After overnight incubation at 4°C, the membrane was washed 4 times with TBST at room temperature and then incubated with secondary antibodies against Fc fragment of murine immunoglobulin G (IgG) conjugated with horseradish peroxidase (Dako A/S, Glostrup, Denmark), dissolved in TBST with 0.5% skimmed milk. After 4-fold membrane rinsing with TBST, the protein bands, bound by the antibody conjugated to the enzyme, were detected using the chemiluminescence assay. For this purpose, the West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, USA) reagent was used. This reagent contains luminol, which, under the influence of horseradish peroxidase, emits light. The solution of this reagent, prepared according to the manufacturer's instructions, was spot on the membrane and then the membrane was inserted into the plastic foil. After exposure to photographic film, the film was developed using the reagents Kodak® GBX developer and Kodak GBX fixer (Sigma-Aldrich).

The quantity of protein in individual polyacrylamide gel pathways was normalized using  $\beta$ -actin reference protein. For this purpose, the complexes of anti-LC3 Abs with secondary antibodies conjugated with the membrane were removed. Next, the membrane was incubated overnight with anti- $\beta$ -actin murine Ab. After overnight incubation, the membrane was washed and then incubated with an antibody conjugated with horseradish

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Total percentage of apoptotic cells in the early Μ 250 p-value\* of patients and late apoptotic phases after induction therapy Percentage of apoptotic cells in the early and late 7.86 0.82 23.66 4.27 12.91 apoptotic phases after the induction therapy (n = 18)0.0078 Percentage of apoptotic cells in the early and late 0.8 62.2 6.7 47.4 26.3 apoptotic phases after the induction therapy (n = 20)

Table 4. Total percentage of apoptotic cells in the early and late apoptotic phases, determined after the induction therapy, depending on the remission status

peroxidase. The detection of chemiluminescence after spotting the substrate for horseradish peroxidase was similar to that of LC3. The LC3-II protein migrates faster than LC3-I in western blot.

Due to the differences in the migration rates of the LC3-I (18 kD) and LC3-II (16 kD) proteins, 2 separate bands were obtained in the western blot procedure.

#### Statistical methods

In each group, the median values (M), range (min-max), and lower and upper quartiles (25Q-75Q) of the continuous parameters were calculated. Statistical significance between the means for different groups was calculated with the nonparametrical Mann-Whitney U test, because the number of cases in the control group was small and the variances in the groups were not homogeneous (the homogeneity of variance was determined with Bartlett's test). Statistical significance between the means for dependent groups was calculated with the non-parametrical Wilcoxon parity test, because the number of cases in the control group was small and the variances in groups were not homogeneous (the homogeneity of variance was determined with Bartlett's test). Since the expected value in the cell was below 5, the Fisher test was used for discrete parameters to analyze the incidence of features in the study groups.

For the chosen pairs of parameters, a correlation analysis was performed and Pearson's correlation factor r was calculated. The p-value  $\leq 0.05$  was considered statistically significant.

#### Results

# Evaluation of the total percentage of apoptotic cells (in the early and late apoptotic phase) determined 12–24 h after the start of the induction therapy, depending on the remission status

A statistically significant intergroup difference was found in the total percentage of apoptotic cells (in the early and late apoptotic phase) after the start of the induction therapy between the non-remission (n=18) and remission (n=20) groups (p=0.0078). In the remission group, the overall

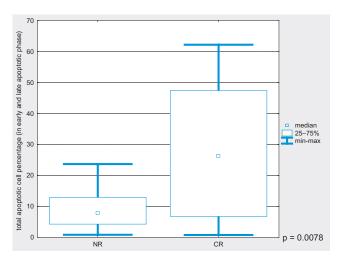


Fig. 1. Total percentage of apoptotic cells (in the early and late apoptotic phases) determined after the induction therapy, depending on the remission status

percentage of apoptotic cells after the start of the induction treatment was higher compared to non-remission patients. The data is presented in Table 4 and Fig. 1.

# Comparison of the total percentage of apoptotic cells (in the early and late apoptotic phase) in patients with remission, before and after the start of the induction therapy

After the start of the induction therapy, a statistically significant increase in the total percentage of apoptotic cells (in the early and late apoptotic phase) was observed in the remission group compared to the values before treatment (p = 0.00059). The data is presented in Table 5 and Fig. 2.

## Comparison of the MAP1LC3 protein expression in the remission group before and after the start of the induction therapy

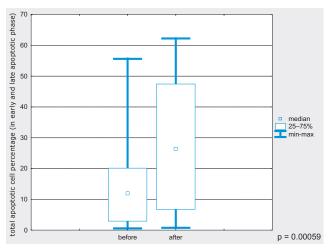
In the remission group, the comparison of the MAP1LC3 protein expression before and after the start of the induction therapy demonstrated an increasing trend, although without statistical significance (p = 0.0930). The data is presented in Table 6 and Fig. 3.

 $<sup>\</sup>hbox{* Mann-Whitney U test; I-non-remission group of patients; II-remission group of patients.}\\$ 

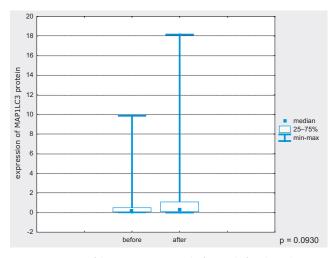
Percentage of apoptotic cells in the early and late apoptotic phases, determined in patients with remission, before and after the start of the induction therapy	Group of patients	М	min	max	25Q	75Q	p-value*	
Percentage of apoptotic cells in the early and late apoptotic phase before the induction therapy	(n = 20)	11.9	0.6	55.6	2.9	20.1	0.00050	
Percentage of apoptotic cells in the early and late apoptotic phase after the induction therapy	(n = 20)	26.3	0.8	62.2	6.7	47.4	0.00059	

**Table 5.** Percentage of apoptotic cells in the early and late apoptotic phases, determined in patients with remission, before and after the start of the induction therapy

<sup>\*</sup> Wilcoxon test; II – remission group of patients.



**Fig. 2.** Total apoptotic cell percentage (the early and late apoptotic phases) in the remission group before and after the start of the induction therapy



**Fig. 3.** Expression of the MAP1LC3 protein before and after the induction therapy in patients with remission

## Results of the LC3-I and LC3-II protein expression assay performed with the use of the western blot procedure

In the remission group, cell pellets were tested in 16 of 20 patients, while in the non-remission group in 16 of 18 subjects. This was due to the inability to obtain a sufficient number of mononuclear cells in the precipitate in some patients (patients with leukopenia at diagnosis).

The differences in the speed of the migration of the LC3-I and LC3-II proteins resulted in 2 separate bands in the western blot procedure. LC3-II (16 kD) migrates faster than LC3-I (18 kD). The level of the LC3-II protein directly correlates with the quantity of autophagosomes. In the remission and non-remission groups, differences in the LC3-I and LC3-II expression before and after the start of the chemotherapy were not reported. The data is presented in Fig. 4.

### Correlation between selected parameters and overall survival of patients

The longer OS was related to the percentage of cells in the late stages of apoptosis and to the total percentage of apoptotic cells (in early and late apoptosis) after the start of the induction therapy. The data is presented in Table 7.

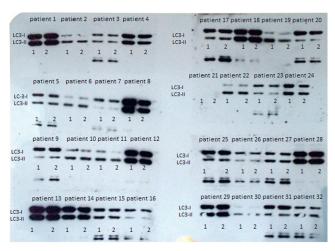
#### **Discussion**

This study investigated whether the percentage of apoptotic cells as well as the expression of selected autophagic markers in mononuclear populations, before and after the start of the induction chemotherapy, may impact the therapeutic response in AML patients.

Table 6. Expression of the MAP1LC3 protein in patients with remission before and after the start of the induction therapy

Expression of the MAP1LC3 protein in patients with remission before and after the start of the induction therapy	Group of patients	М	min	max	25Q	75Q	p-value*
Expression of the LC3 protein before the induction chemotherapy	(n = 20)	0.115	0.033	9.872	0.058	0.498	0.0020
Expression of the LC3 protein after the induction chemotherapy	l (n = 20)	0.54	0.00	18.15	0.10	1.19	0.0930

<sup>\*</sup> Wilcoxon test; II – remission group of patients.



**Fig. 4.** Expression of the LC3-I and LC3-II proteins in patients diagnosed with acute myeloid leukemia (AML) determined with the western blot assay

Patients 1–16 – non-remission after the treatment; patients 18–32 – remission after the induction therapy; number: 1 – expression of the LC3-I and LC3-II proteins before the chemotherapy; 2 – expression of the LC3-I and LC3-II proteins after the chemotherapy.

**Table 7.** Correlation between overall survival (OS) and selected parameters

Parameter	OS
Difference in the percentage of cells in the late-stage apoptosis before and after the chemotherapy [%] <sup>1</sup>	r = 0.36 p = 0.027
Difference in the total percentage of cells in early and late apoptosis before and after the chemotherapy [%] <sup>2</sup>	r = 0.37 p = 0.024
Difference in the expression of the MAP1LC3 protein before and after the chemotherapy	r = 0.32 p = 0.054

<sup>1</sup> cell percentage in the late-stage apoptosis after the chemotherapy – percentage of cells in the late apoptotic phase before the chemotherapy/percentage of cells in the late apoptosis phase before the chemotherapy; <sup>2</sup> total percentage of cells in apoptosis after the chemotherapy – total percentage of cells in apoptosis before the chemotherapy/percentage of cells in the late apoptosis phase before the chemotherapy.

A small number of reports confirm the importance of apoptosis as a prognostic factor in the treatment of acute leukemia. A study by Smith et al. demonstrated that the percentage of blast cells in the spontaneous phase of apoptosis correlated with a good response following an intravenous (iv.) cytarabine administration. The median proportion of blasts in the apoptosis phase in the blood of patients achieving complete remission (CR) after the treatment (after 24 h of culture) and in non-remission (NCR) subjects was 19.5% (range: 3.6-64%) and 4.2% (range: 1.8-7.0%), respectively. This intergroup difference was statistically significant (p = 0.0007).

Other reports have also pointed at the importance of apoptosis as a prognostic factor in the treatment of acute leukemia. Its role has been confirmed through the measurement of apoptosis markers, such as the presence of Fas receptors and tumor necrosis factors (TNFs), the Smac/DIABLO expression levels, and the caspase-3 activity. It has been found that a loss of receptors for

ligands like Fas or TNF before the induction therapy resulted in resistance to daunorubicin-induced apoptosis of the test Jurkat or U937 cell lines. Other studies have demonstrated that a higher expression of Smac/DIABLO is an independent predictor of greater overall remission and longer OS (p < 0.001 and p = 0.003, respectively), while a reduced caspase-3 activity correlated with resistance to apoptosis.  $^{23,24}$ 

The results of our study did not show any statistically significant differences in the percentage of apoptotic cells before the induction therapy (cells in the spontaneous phase of apoptosis), depending on the achievement of remission after the treatment. There are few studies on the effects of cytostatic treatment on apoptosis, regarding its level before treatment and the remission status after therapy.<sup>21–24</sup> One study examined mononuclear cells isolated from the bone marrow of 31 patients diagnosed with de novo acute leukemia. The study demonstrated that the abovementioned cytostatics induced caspasedependent apoptosis. Moreover, spontaneous apoptosis did not correlate with treatment-induced apoptosis, while induced apoptosis was significantly higher in patients who achieved CR after cytostatic treatment. Tests performed after the induction therapy showed that the percentage of cells in the apoptotic phase was significantly higher in those patients compared to the NCR group (p = 0.0130and p = 0.0078, respectively).<sup>25</sup>

In our study, a significant increase in the percentage of apoptotic cells after the induction therapy in the remission group was crucial. However, the non-remission group patients did not obtain such a result.

There are few reports on the role of autophagy in the treatment of acute leukemia, and the results of the available studies are ambiguous. Some point to its cytoprotective role for leukemia cells, while others show its anticancer role. Modification of the autophagy process can also influence the proliferation of leukemia cells.

Bosnjak et al. demonstrated that cytarabine used in leukemia test cell lines inhibited the mammalian target of rapamycin (mTOR) kinase phosphorylation by inhibiting AKT kinase, and thus stimulated autophagy. 11 Adding autophagy inhibitors such as bafilomycin or chloroquine stimulated the apoptosis of leukemia cells. 11 This fact may indicate the cytoprotective role of autophagy in leukemia cells. In another paper, treatment with daunorubicin induced autophagy by stimulating ERK1/2 kinase.<sup>12</sup> The inhibition of this process by the use of the chloroquine inhibitor of the kinase and small interfering RNA (siRNA), which mute the expression of the ATG5 and ATG7 genes, reduced the viability of leukemia cells.<sup>12</sup> This may also indicate the cytoprotective role of autophagy in these cells. Palmeira dos Santos et al. showed that autophagy suppression may initially increase the antiproliferative effect of cytarabine on leukemia cells. After 24 h, however, this process may, quite to the contrary, promote the resistance of leukemia cells to treatment.<sup>13</sup>

One study evaluated the combination of daunorubicin and rapamycin in the therapy of patients with acute lymphoblastic leukemia (Ph+). The synergistic effect of inhibiting the leukemia cell proliferation while using the abovementioned drugs was associated with mTOR blockade, autophagy increase and cell cycle arrest in the G1 phase. In mice implanted with myeloblasts, Willems et al. demonstrated that the inhibition of the mTORC1 and mTORC2 complex improved survival by reducing the tumor mass without generating visible toxicity effects. The inhibitor stimulated apoptosis in leukemic cells without inducing that effect in normal immature CD34+ cells, and also intensively stimulated autophagy.

In our studies, the expression of the ATG5 and MAP1LC3 proteins showed no intergroup difference, both before and after the treatment between the remission and non-remission groups. In the remission group, however, an upward trend in the expression of the MAP1LC3 protein after the start of the induction therapy is noteworthy (p = 0.0930).

Moreover, we did not observe any differences in the LC3-I and LC3-II protein expression, before and after the chemotherapy, between the remission and non-remission groups.

In the remission group, the observed that the increase in the MAP1LC3 expression after the start of the induction therapy was not associated with the change in the LC3-IB and LC3-IIB protein levels measured with western blot assay. It may, therefore, be suspected that the expression of the MAP1LC3 protein is further regulated by another factor, e.g., micro-ribonucleic acid (miRNA), which acts on the level of post-transcriptional regulation of gene expression. In the remission group, the increased expression of the MAP1LC3 protein after the start of the treatment may indicate higher susceptibility of mononuclear cells to enter a pathway of autophagy. Nevertheless, the negative factor acting at the level of post-transcriptional gene regulation eventually changes the course of this process.

Cytostatics used in the induction regimen potentially stimulate apoptosis, provided that an adequate supply of blasts to the chemotherapy is maintained. Blast cell sensitivity depends not only on cytogenetic prognosis, but also on the genesis of the disease (de novo vs secondary leukemia) and the intensity of the cytostatic treatment.

In our studies, longer OS was related to the percentage of cells in the late stages of apoptosis and to the total percentage of apoptotic cells (in early and late apoptosis) after the start of the induction therapy.

#### **Conclusions**

Our results indicate that patients with a good therapeutic response experience more intense cytostatic-induced apoptosis compared to non-remission subjects. Following the above, it is concluded that apoptosis disturbances are one of the causes of chemotherapy failure.

Based on the presented results, we can assume that apoptosis activity is a useful indicator of good prognosis in AML. At present, we need further studies to confirm the impact of autophagy on the therapeutic response of AML patients, to evaluate the possibility of modification of this process and to assess the effect of this change on patients' response.

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