Oxidative Stress and BCR-ABL1 Transcript Levels in Chronic Myeloid Leukemia: an Intricate Relationship

EMILIA GEORGIANA PASCU1, MIHNEA ALEXANDRU GAMAN2*, CORNEL MOISA3, AMELIA MARIA GAMAN1,4
1 University of Medicine and Pharmacy of Craiova, Department of Pathophysiology, 2 Petru Rares Str., 200349, Craiova, Romania
2 Carol Davila University of Medicine and Pharmacy, 8 Eroii Sanitari Blvd., 050474, Bucharest, Romania
3 County Emergency Hospital Slatina, Department of Haematology, 5 Crisan Str., 230008, Slatina, Romania
4 Clinic of Haematology, Filantropia City Hospital, 1 Filantropiei Str., 200143, Craiova, Romania

Chronic myeloid leukaemia (CML) is a chronic myeloproliferative neoplasm characterized by the presence of the Philadelphia chromosome. Oxidative stress is involved in CML etiopathogenesis and disease progression, as well as the response to tyrosine kinase inhibitors (TKI) treatment. We evaluated oxidative stress levels in 47 CML patients vs. controls. The total antioxidant capacity (TAC) was measured using a FLUOstar Omega microplate reader (reagents from Sigma-Aldrich). Cellular reactive oxygen species (ROS) were evaluated using a CyFlow SPACE Sysmex flow-cytometer (reagents from Abcam). Oxidative stress levels were higher in CML patients vs. controls. The maximum TAC value and the minimum ROS value were recorded in CML patients with a BCR-ABL1 transcript value of 0.1-1%, suggesting that the production of plasma antioxidants progressively increases as a compensatory mechanism in CML patients undergoing TKI treatment in order to annihilate ROS. The pseudonormalization of the cell redox status observed in these patients could be an alarm signal prior to the development of resistance to TKI treatment or disease progression.

Keywords: oxidative stress, chronic myeloid leukaemia, tyrosine kinase inhibitors, BCR-ABL1 transcript

Oxidative stress reflects an imbalance between the overproduction of reactive oxygen species (ROS) and the cellular antioxidant defense systems [1-3]. At low concentrations, ROS are involved in several physiological processes, i.e. cell signaling, enzyme activation, gene expression, apoptosis, antimicrobial defense and phagocytosis, but increased levels of oxidative stress generate structural and functional cellular alterations [4-9]. High levels of oxidative stress can act as triggers for some cellular signaling pathways responsible for cellular damage by generating mutagenic compounds, initiating and maintaining inflammatory response and overexpressing oncogenes [10-12]. These cellular changes can lead to the development of neoplasms (including myeloproliferative and lymphoproliferative diseases), cardiovascular, neurological or renal disorders, diabetes or atherosclerosis [13-16]. ROS are involved in hematopoietic cellular processes under both physiological (cell cycle progression, cell motility, intracellular signaling) and pathophysiological conditions (etiopathogenesis and evolution of acute and chronic myeloproliferative and lymphoproliferative disorders) [17-20].

Chronic myeloid leukaemia (CML) is a chronic myeloproliferative neoplasm characterized by the presence of the Philadelphia (Ph) chromosome, resulting from a reciprocal translocation between chromosomes 9 and 22, and the BCR-ABL1 oncogene, a product of the Ph chromosome. The BCR-ABL1 oncogene encodes a chimeric BCR-ABL1 protein, the p210 oncoprotein, with constitutively active ABL1 tyrosine kinase activity, and activates signalling pathways such as MAPK, PI3K, JAK/STAT or Hedgehog, that are involved in leukemogenesis, clonal instability and disease progression [21-24]. Activation of the PI3K/mTOR and PI3K/AKT pathways by BCR-ABL1 contributes to the enhanced production of ROS [25-26]. CML evolves in three phases: chronic, accelerated and blastic phase. CML treatment is based on tyrosine kinase inhibitors (TKI) [27]. Imatinib (1st generation TKI), dasatinib and nilotinib (2nd generation TKI) are approved for the first and second line therapy of CML patients, while bosutinib and ponatinib are reserved for patients who developed resistance or were intolerant to the aforementioned TKI [27-28]. The greatest challenge of targeted CML therapy is the development of resistance to TKI [29]. Resistance to TKI can be either primary (failure to obtain complete cytogenetic response/complete haematological response) or secondary (treatment failure or disease progression by losing the complete cytogenetic response/complete haematological response that was initially reached using TKI treatment) [27, 30]. Several BCR-ABL1-dependent resistance mechanisms have been described: BCR-ABL1 amplification, kinase mutations and genomic instability. The involvement of oxidative stress in such mechanisms has been already documented [31-33]. Resistance to TKI also develops by BCR-ABL1-independent mechanisms, but little research has been conducted to clarify their occurrence [34-35].

The aim of our study was to evaluate oxidative stress levels in CML patients and to assess the molecular response to TKI treatment via the BCR-ABL1 transcript.

Experimental part

Patients, materials and methods

The study group included 47 patients diagnosed with CML according to the ELN/WHO criteria, registered in the Haematology Clinic, Filantropia City Hospital, Craiova, Romania, and a control group of 20 healthy subjects free of any condition that could alter their redox status. Informed consent was obtained from all subjects involved in this study. All procedures were carried out in accordance with the ethical standards specified in the Declaration of Helsinki and had the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania (approval no. 74/23.02.2017). The group of patients with CML was divided into subgroups according to gender, age and type of treatment. ROS levels and the total antioxidant...
capacity (TAC) were measured both in the CML group and in the control group. Molecular response was evaluated in CML patients by the BCR-ABL1 transcript by RQ-PCR. Statistical analysis of data was performed and a p-value ≤0.05 was considered statistically significant.

Equipment and reagents
A FLUOstar Omega microplate reader and a Sigma-Aldrich antioxidant kit (CS0790) were used for TAC determination. A CyFlow SPACE Sysmex flow-cytometer (cell sorter function included) and an Abcam kit for quantitative measurement of cellular ROS (ab113851) were employed for ROS assessment. In addition, an Eppendorf 5702R cooling laboratory centrifuge and an incubator (able to ensure a constant temperature of 37°C for at least 4 h) were also used for the physical processing of blood samples.

Processing of blood samples
Blood samples (2 x 9mL) in EDTA test tubes were processed by repeated cycles of centrifugation - cell washing in order to obtain the plasma required for TAC measurement and the leukocytes required for cellular ROS measurement. Blood samples were centrifuged (2600 G; 15 min; 4°C), plasma was collected, and the sediment was transferred into a single tube out of which 8 mL were collected, treated with 27 mL of phosphate buffered saline (PBS) 1x and gently homogenized. The diluted sediment was carefully added over 15 mL of Biocoll solution and the tubes were centrifuged (400 G; 40 min; 21°C, SOFT). The supernatant was removed, the leukocytes were collected and treated with 30 mL PBS 1x. The tubes were centrifuged again (400 G; 10 min; 21°C), the supernatant was removed, and the leukocyte sediment was collected and treated with 200µL PBS 1x.

TAC evaluation
The principle of the test consists in the formation of the ferromyoglobin radical of metmyoglobin and hydrogen peroxide that oxidize 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and produce a green soluble chromogen which can be spectrophotometrically read at a wavelength of 405 nm using a microplate reader. 96 wells colourless flat bottom microplates were used to perform the determinations. Antioxidants in the sample (plasma) suppress the production of the cation radical in a concentration-dependent manner, so the colour intensity of the chromogen decreases proportionally to the plasma TAC concentration. For determining the standard curve, the kit includes Trolox, a soluble vitamin E analogue.

ROS detection in leukocytes
The Abcam DCFDA ROS detection kit uses 2',7'-dichlorofluorescein diacetate reagent (DCFDA), a fluorogenic dye measuring hydroxyl, peroxyl and other intracellular ROS. Following cell diffusion, DCFDA is fluorogenic dye measuring hydroxyl, peroxyl and other intracellular ROS. Following cell diffusion, DCFDA is deacetylated by cellular esterases to produce a non-fluorescent compound that will be oxidized by ROS to 2',7'-dichlorofluorescein (DCF). DCF is a strong fluorescent compound that can be detected by fluorescence spectroscopy at 495 nm and 529 nm spectra, respectively. The Abcam DCFDA kit includes: 20 mM DCFDA, 10x Buffer, 55 mM tert-butyl hydrogen peroxide (TBHP) solution. Additional materials are also required: bidistilled water and fetal bovine serum. After separation of leukocytes, (leukocytes from 2 x 9 mL whole blood in 200µL PBS 1x), flow-cytometer samples are prepared. For each patient sample/control sample, three determinations were made:

- negative control (56 µL leukocytes suspension + 644µL PBS = 700µL total reaction volume), basal (56 µL leukocytes suspension + 644µL Buffer 1x + 0.7µL DCFDA) and positive control (56 µL leukocytes suspension + 644 µL Supplemented Buffer 1x + 2.6µL TBHP solution + 644 µL DCFDA). Negative control is read immediately after preparation. Basal and positive control are incubated at 37°C (30 min for basal and 4 h for positive control). CyFlow acquisition used for sample reading was FSC: 248; SSC: 280; FITC: 720.

Results and discussions
The study group included 47 CML patients: 23 females (48.94%) and 24 males (51.06%). The mean age of the study group was 59.9 years and the age range was 22-87 years. The control group consisted of 20 healthy subjects free of any conditions that could alter their oxidative status. The mean age of the control group was 55 years and the age range was 20-70 years.

We noticed that the mean age of both male and female CML patients was around 60 years. The sixth decade of life is cited in literature as the age group predominantly affected by CML [32]. Analysing the mean TAC and ROS values and their corresponding age groups, it is surprising to note that both TAC and ROS values progressively increased in the CML age groups (20-40 years: TAC 0.19 mM/L, ROS 9.95 mM/L; 40-60 years: TAC 0.28 mM/L, ROS 9.93 mM/L; 60-80 years: TAC 0.28 mM/L, ROS 10.77 mM/L; >80 years: TAC 0.48 mM/L, ROS 11.57 mM/L). Several studies have shown that oxidative stress is normally involved in cellular aging processes and is responsible for the depletion of antioxidant reserves and, consequently, for increasing ROS activity in the cell [35-36]. Surprisingly, in the present study, we noticed that both TAC and ROS values progressively increased in CML age groups, reaching the maximum in the age group of >80 years. This may be due to complicated mechanisms generated by the interaction between the chronic myelogenous disease, the aging processes and treatment. It is possible that, in CML, the production of ROS is more accelerated vs. the natural aging process and that oxidative stress levels vary also due to the therapy employed, as in other haematological malignancies [37-38]. In these conditions, compensatory pathophysiological mechanisms might lead to an increased synthesis of antioxidants and TAC elevations.

There were no significant differences in oxidative status between males and females in the CML group. Analysing the gender distribution of the CML group, we noticed that the mean age of the women subgroup was 60.8 years. In this subgroup, we recorded the following: mean BCR-ABL1 transcript value = 8.9% mean TAC value = 0.27 mM/L and mean ROS value = 10.25 mM/L. The mean age of CML male subgroup was 59 years, the mean BCR-ABL1 transcript value = 5.3%, the mean TAC value = 0.28 mM/L, and the mean ROS value = 10.58 mM/L. Statistical analysis of the data based on gender distribution of patients did not return any statistically significant results with regards to oxidative stress levels and BCR-ABL1 transcript value (p = 0.12). Analysing the mean TAC value and the mean ROS value in the CML group vs. the control group, we recorded statistically significant results (p = 0.006): the mean TAC value was higher and the mean ROS value = 10.58 mM/L. The control group consisted of 20 healthy subjects free of any conditions that could alter their oxidative status. The mean age of the control group was 55 years and the age range was 20-70 years.
expression of the BCR-ABL1 gene induces ROS production [33].

At the time of enrolment, the patients in the study group were in CML chronic phase, but at the time of TAC and ROS assessment, CML patients were in different phases of disease (chronic or blastic transformation) and in treatment with different generations of TKI or hydroxyurea.

In the present study, we noticed that the level of oxidative stress markers (the type of response to treatment) at the moment of TAC and ROS evaluation, we identified 5 subgroups: subgroup 1 (BCR-ABL1 transcript 0% - undetectable - deep molecular response) = 34%, subgroup 2 (BCR-ABL1 transcript 0-1% - major molecular response) = 19.1%, subgroup 3 (BCR-ABL1 transcript 0.1-1%) = 14.89%, subgroup 4 (BCR-ABL1 transcript 1-10% - suboptimal response) = 14.89%, and subgroup 5 (BCR-ABL1 transcript >10% - therapeutic failure/blastic transformation) = 17%.

We observed that the minimum TAC value was recorded in CML patients with major molecular response (subgroup 2), followed by CML patients with deep molecular response (subgroup 1). We also noted that the mean age of subgroup 1 was lower than the other subgroups. The maximum TAC value, as well as the minimum ROS value, were obtained for subgroup 3. We noted that, except for subgroup 3 who recorded a higher TAC value and a lower ROS value vs. the control group, all the other subgroups recorded lower TAC values and higher ROS values than those of the control group. These results are depicted in Table 1.

We noted that a clear correlation cannot be established between the TAC value and the ROS value obtained for each of the CML subgroups. For the first two subgroups, TAC values tend to decrease as the ROS values increase, but the situation is inconsistent with the following 2 subgroups (subgroup 3 - low ROS values and high TAC values, subgroup 4 - increased ROS values, increased TAC values, but lower vs. controls). For the subgroup 5 (therapeutic failure/blastic transformation), the same situation as for the first two subgroups was described (slightly reduced TAC value vs. controls, slightly increased ROS values vs. controls).

We noticed that the maximum TAC value and the minimum ROS value in the study group were recorded in the subgroup of patients with BCR-ABL1 transcript value ranging from 0.1-1%. All the other subgroups registered lower TAC results than the mean value of the control group and higher ROS values higher than the mean value of the control group. We raise the suspicion that the production of plasma antioxidants progressively increases as a compensatory mechanism in CML patients undergoing TKI treatment in order to annihilate ROS (produced by BCR-ABL1-independent mechanisms or by TKI therapy itself) until the stage of major molecular response is reached [30, 33, 41]. Subsequently, antioxidant reserves are exhausted, leading to a significant increase in the level of cellular ROS that maintains the BCR-ABL1 oncogene activity and triggers its auto-mutagenesis process and the development of mutant clones. These conditions lead to progression of the disease despite the initial favourable response to treatment [42-43].

Conclusions

In the present study, we noticed that the level of oxidative stress is higher in patients with CML compared to healthy subjects. We did not find a correlation between TAC values, ROS values and the age of the patients, possibly due to the

| BCR-ABL1 Transcript | Mean age (years) | Mean BCR-ABL (%) | Mean TAC (mM/L) | Mean ROS (mM/L) |
|---------------------|-----------------|-----------------|----------------|----------------|
| BCR-ABL1 0 %        | 55.63           | 0.00            | 0.27           | 10.43          |
| BCR-ABL1 0.1-1%     | 65.11           | 0.01            | 0.23           | 10.54          |
| BCR-ABL1 0.1-1%     | 58.71           | 0.38            | 0.36           | 9.25           |
| BCR-ABL1 >10%       | 67.43           | 3.35            | 0.29           | 11.14          |
| Control group       | 55.00           | -               | 0.35           | 10.07          |

Table 1

OXIDATIVE STRESS MARKERS AND DISEASE MARKERS IN CML PATIENTS
intricate metabolic pathways that drive oxidative stress and are related to the aging process.

An imbalance of the redox status was observed in all CML subgroups (decrease in TAC values, increase in ROS values) regardless of the type of administered therapy (first generation TKI, second generation TKI or hydroxyurea). It is possible that the pseudo-normalization of cellular redox status observed in the subgroup of patients with BCR-ABL1 transcript value of 0.1-1% (TAC value > mean control value, ROS value < mean control value) could be an alarm signal prior to the development of resistance to treatment/disease progression. It is necessary to monitor the redox status of these patients during treatment in order to obtain conclusive results in this regard.

References
1.KURUTAS, E. B., Nutr. J., 15, nr. 1, 2016, p. 71.
2.BREITENBACH, M., ECKL, P., Biomolecules, 5, nr. 2, 2015, p. 1169.
3.GAMAN, M. A., EPINGLEAC, M. E., GAMAN, A. M., Rev. Chim. (Bucharest), 70, no. 3, 2019, p. 977.
4.ZHU, Q. S., XIA, L., MILLIS, G. G., LOWELL, C. A., TOUW, I. P., COREY, S. J., Blood, 107, nr. 5, 2006, p. 1847.
5.SIES, H., Redox Biol., 11, 2017, p. 613.
6.SHOKOLENKO, I. N., WILSON, G. L., ALEXEYEY, M. F., World J. Exp. Med., 4, nr. 4, 2014, p. 46.
7.MAROCCHI, I., ALTIERI, F., PELUSO, I., Oxid. Med. Cell. Longev., 2017, nr. 2017, 2017, p. 6501046.
8.GAMAN, A. M., BUGA, A. M., GAMAN, M. A., POPA-WAGNER, A., Oxid. Med. Cell. Longev., 2014, nr. 2014, 2014, p. 158135. doi: 10.1155/2014/158135.
9.EPINGLEAC, M. E., DIACONU, C. C., GAMAN, A. M., Rev. Chim. (Bucharest), 93, nr. 12, 2013, p. 71.
10.KALMANTI, L., SAUSSELE, S., LAUSEKER, M., Oncotarget, 53, nr. 4, 2017, p. 1169.
11.RASOOL, F. V., GAYMES, T. J., OMDIVAR, N., BRADY, N., BEURREL, S., PLA, M., REBOUL, M., LEA, N., CHOMIENNE, C., THOMASON, N. S., MUFTI, G. J., PADUA, R. A., Cancer Res., 67, nr. 2017, p. 8762.
12.AMINA, A. A., DAVID, J. M. E., KRISTIAN, M. B., STUART, A. R., Cancer Sci., 2015, 2015, 2015, p. 454659.
13.LUIS GOMES, A., DIMITROVA TCHEKALAROVA, J., ATANASOVA, M., DO CONCEICAO MACHADO, K., DE SOUSA RIOS, M. A., PAZ, F. C. J., GAMAN, A. M., YELE, S., SHILL, M. C., KHAN, I. N., ISLAM, M. A., ALJ, E. S., MISHRA, S. K., ISLAM, M. T., MUBARAK, M. S., DA SILVA LOPES, L., DE CARVALHO MELO-CAVALCANTE, A. A., Biomed. Pharmacother., 106, nr. 2018, 2018, p. 1686. doi: 10.1016/j.biopha.2018.07.121.
14.NIENLES, M. H., IRVINE, H., VEDELE, S., RAUNGAARD, B., BECK-NIELSEN, H., HANDBERG, A., Oxid. Med. Cell. Longev., 2016, nr. 2016, 2016, p. 2492858.
15.YUAN, T., YANG, T., CHEN, H., FU, D., HU, Y., YANG, J., QIN, Y., YU, X., HU, W., XIE, X., Redox Biol., 20, nr. 2019, 2019, p. 247.
16.GAMAN, M. A., DOBRICA, E. C., PASCU, E. G., KOZMA, M. A., EPINGLEAC, M. E., GAMAN, A. M., PANTEA, S. A., BRATU, O. G., DIACONU, C. C., J Med Sci., 6, nr. 1, 2019, p. 157. DOI: 10.22534/7674.61.P157161.
17.BATTISTI, V., Maders, L. D., Bagatini, M. D., Santos, K. F., Spanevello, R.V., Maldonado, P.A., Brule, A. O., Araujo o MDO, C., Schetinger, M. R., Morsch, V. M., Clin. Biochem., 41, nr. 7-8, 2008, p. 511.
18.FARQUHAR, M. J., BOWEN, D. T., Int. J. Hematol., 77, nr. 4, 2003, p. 342.