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*ORIGINAL ARTICLE*

# Antioxidant enzymes activities and plasma levels of oxidative stress markers in B-chronic lymphocytic leukemia patients

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## Summary

***Purpose:*** *Overproduction of reactive oxygen species (ROS) intermediates above the functional capability of cellular antioxidants may result in instability of important macromolecules and represents the molecular basis of many diseases including inflammation processes, cardiovascular alterations, cancer etc. The purpose of this study was to determine plasma level of superoxide anion, hydrogen-peroxide and malondialdehyde (MDA) as markers of oxidative stress and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as antioxidant enzymes in B-chronic lymphocytic leukemia (B-CLL) patients.*

***Methods:*** *The study included 29 untreated B-CLL patients in stage A, and 21 in stages B and C, classified according to the Binet system; 31 healthy volunteers formed the control group. After centrifugation of heparinized peripheral blood, plasma levels of all investigated parameters were determined using spectrophotometric methods.*

***Results:*** *Plasma CAT activity was increased in B-CLL patients compared with control subjects; also, progression of disease was related with significantly higher plasma activity of CAT. Also, B-CLL patients showed significantly higher plasma concentration of MDA compared with controls. No statistically significant differences of superoxide anion and hydrogen peroxide as well as plasma activity of SOD and GPx between the tested groups were noted.*

***Conclusion:*** *Increase of CAT activity in B-CLL patients indicates that there is stimulation of the antioxidant enzyme system, while the increase of MDA concentration shows increased lipid peroxidation level. According to these results it could be concluded that an imbalance exists between oxidants and antioxidants in the plasma of B-CLL patients.*

**Key words:** catalase, chronic lymphocytic leukemia, glutathione peroxidase, malondialdehyde, reactive oxygen species, superoxide dismutase

## Introduction

CLL is a predominantly clonal B cell neoplasm of small, resting, long-living B-cells. Despite recent advances in the understanding of the genetics [1], biology [2], clinical behavior [3] and treatment [4], there is no established treatment for CLL and its progression and outcome are highly unpredictable. Expansion of malignant cells leads to their accumulation in the peripheral blood, bone marrow and many tissues. These cells are functionally defective and immunologically distinct from normal B cells [5]. The clinical course of B-CLL is highly heterogeneous, ranging from less than 2 years in symptomatic patients with advanced disease to more than 20 years for patients with an early stage and non-progressive disease [6]. Although the pathogenesis of B-CLL is not fully elucidated, the progressive increase of lymphocyte count coupled with the very low proportion of proliferating cells has led to the notion that B-CLL may be determined by defective apoptosis [7]. The precise mechanisms underlying apoptosis still remain largely unknown. Dysregulation of p53, c-myc and bcl-2 oncogenes can be a cause of defective apoptosis in B-CLL [8]. And, even though the B-CLL cells molecular alterations involving different oncogenes and tumor suppressor genes have been established, the role of oxidative stress in the pathogenesis of this disease is poorly understood and remains a matter of research [9].

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An increasing body of evidence shows that oxidative stress may be involved in both initiation and promotion of multistage carcinogenesis [10]. Overproduction of ROS intermediates above the functional capability of cellular antioxidants may result in instability of important macromolecules, and represents the molecular basis of many diseases including inflammation processes, cardiovascular alterations and cancer [11]. ROS are chemically active molecules generated endogenously during various cellular metabolic activities. In mammalian cells, mitochondria are the major intracellular source of ROS generation [11]. Overproduction of ROS can result in detrimental cellular damage including lipid peroxidation, DNA adduct formation, protein oxidation and enzyme inactivation that ultimately lead to cell death. Diverse cellular ROS, such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^{\cdot}$), have long been thought as harmful by-products of life of an aerobic environment. ROS are highly reactive and potentially toxic compounds, capable of modifying and damaging several types of cellular macromolecules including lipids, proteins and DNA, ultimately leading to cytotoxicity and mutagenesis [11]. Therefore, cells have evolved elaborate defense systems to counteract the toxic effects of ROS. These include both nonenzymatic (glutathione, pyridine nucleotides, ascorbate, retinoic acid, thioredoxin and tocopherol) and enzymatic (such as SOD, CAT, GPx and peroxyredoxin) pathways that limit the rate of oxidation and thereby protect cells from oxidative stress [12,13]. Notwithstanding, evidence is emerging that ROS also act as signals or mediators in many cellular processes, such as cell proliferation, differentiation, apoptosis and senescence [14]. The redox environment of a cell may alter the balance between apoptosis and mitosis by affecting gene expression and enzyme activity [15]. Consequently, cellular redox state is increasingly accepted as key mediator of multiple metabolic, signaling and transcriptional pathways essential for normal cellular function, cell survival and apoptosis [16].

In line with these facts the aim of our study was to determine plasma levels of superoxide anion, hydrogenperoxide and MDA as markers of the oxidative stress in B-CLL patients and also to determine potential alterations in the antioxidant enzymatic system that are believed to play a central role in this process.

## Methods

*Patients and controls*

The local ethics committee approved the study and, prior to initiation, written informed consent was obtained from all subjects according to the Declaration of Helsinki. B-CLL was diagnosed by established clinical criteria and confirmed by immunophenotypic analysis for the expression of CD5, CD19 and monoclonal immunoglobulin in accordance with NCI Working Group Guidelines [17]. The clinical staging was based on the Binet system [18]. Twenty-nine stage A B-CLL patients (12 females and 17 males, median age 65.8 years, range 53-82), 21 B and C stage B-CLL patients (9 females and 12 males, median age 64.7 years, range 55-79) and 31 healthy control subjects (13 females and 18 males, median age 65.3 years, range 51-81) were included into the study. Most of the patients were newly diagnosed cases. Previously diagnosed patients had not received antileukemic treatment at least 6 months prior to the investigation. The controls were healthy volunteers without known acute and chronic diseases. Exclusion criteria for all subjects were positive parameters of systemic inflammation (erythrocyte sedimentation rate, serum fibrinogen and C-reactive protein levels) due to other aetiologies or positive anamnestic data for other illnesses that might have influenced the investigated parameters (e.g. autoimmune diseases, acute and chronic infections, systemic and local inflammations, etc). All subjects (patients and controls) were nonsmokers, without alcohol abuse problems, and none of them performed regular exercise other than daily activities. Also, nobody was receiving any systemic and topical treatment (corticosteroids, cyclosporine A, etc) within 6 months prior to the initiation of the investigation.

*Blood sampling*

All blood samples were obtained in the morning and collected in polystyrene tubes. Heparinized peripheral blood (10 ml) was centrifuged at 400 g for 10 min to separate plasma and cells. The plasma aliquots were stored at -80° C until analysis. Hemolyzed samples were excluded.

*Determination of antioxidant enzymes*

Superoxide dismutase (Cu-Zn SOD) and GPx activities were estimated using Ransod and Ransel kits, supplied by Randox Laboratories, Ardmore, Northern Ireland, UK. The samples were processed differently for the two enzymes according to the instructions of the manufacturer.

*Determination of Cu-Zn SOD*

This assay employs xanthine and xanthine oxidase to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazoli-

um chloride (INT) to form red formazan dye [19]. SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD activity is defined as the amount of enzyme that causes a 50% inhibition of the rate of reduction of INT observed in the blank. Reagent blank was assayed with 0.01 M phosphate buffer pH 7.0. The standard curve was prepared using serial dilutions of 4.0 U/mL SOD. SOD activity was then measured at 37° C at 505 nm (Olympus AU 600, Tokyo, Japan) and expressed as enzyme activity (kU/L).

*Determination of GPx*

The assay was performed according to the method of Paglia and Valentine [20]. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and nucleotide adenine dinucleotide phosphate (NADPH), the oxidized glutathione is immediately converted to the reduced form with oxidation of NADPH to $NADP^+$ and the decrease in absorbance was read at 340 nm (Olympus AU 600, Tokyo, Japan). One unit of GPx activity is defined as the amount of enzyme that causes oxidation of 1 nmol/L NADPH per min in the presence of the specific substrate. GPx activity was expressed as enzyme activity (U/L).

*Determination of CAT activity*

The method of Goth [21] was used to spectrophotometrically estimate CAT activity in the samples. Determination of CAT activity is based on the ability of CAT to break two molecules of hydrogen peroxide into two molecules of water and oxygen. Hydrogen peroxide is a substrate of CAT. If there is as an assumption that the activity of CAT is directly proportional to the amount of the hydrogen peroxide used in the reaction over a period of time (min), then the difference in the amount of hydrogen peroxide in the reaction before and after the addition of the enzyme (CAT) represents the activity of CAT. The concentration of hydrogen peroxide can be determined spectrophotometrically in the presence of ammonium molybdate. Reaction of hydrogen peroxide with ammonium molybdate generates a yellow, stable and complex compound, the absorbance of which is read at 405 nm wavelength (LKB Biochrom Ultrospec 4050, Cambridge, England). The amount of hydrogen peroxide used in the catalyst reaction was presented as difference in the concentrations of hydrogen peroxide in the reaction before and after CAT was introduced in the reaction mixture. The unit for CAT activity was expressed as the amount of CAT that decomposes 1 μmol of hydrogen peroxide (substrate) per min under standard conditions (25° C, pH 7.0). CAT activity was expressed as enzyme activity (kU/L).

*Superoxide anion and hydrogen peroxide determination*

The plasma values of superoxide anion and hydrogen peroxide were measured using sprectrophotometric methods [22,23]. Absorbances were registered using spectrophotometer (LKB Biochrom Ultrospec 4050, Cambridge, England). Concentrations of both ROS were calculated using mathematic formulas [22, 23] and results were expressed as nmol/mL.

*MDA determination*

The lipid peroxidation product MDA concentration in plasma was determined by thiobarbituric acid assay according to the protocol of Ohkawa et al. [24] using spectrophotometer LKB Biochrom Ultrospec 4050, Cambridge, England. The concentration of thiobarbituric acid reactans (TBARs) was calculated according to the mathematic formula described by Ohkawa et al. [24] and the results were expressed as nmol/mL.

*Statistical analysis*

The results were expressed as mean ± standard deviation (SD). Statistical analysis was done using the SPSS package (Statistical Package for the Social Sciences version 11.0. for Windows). Statistical significance was assessed by the Student's t test. The mean ± SD values of all parameters between the groups were compared using analysis of variance (ANOVA). The differences were consider to be significant when p-value was less than 0.05 and highly significant when p-value was less than 0.01.

## Results

No statistically significant differences of SOD plasma activity between all tested groups were noted (stage A B-CLL: 6.52 ± 0.50 kU/L, B+C stages B-CLL: 6.52 ± 0.38 kU/L, control 6.68 ± 0.47 kU/L, $p > 0.05$; Figure 1). Also no significant differences were seen between plasma GPx activity of the control group (674.1 ± 165.0 U/L) and both B-CLL patients groups (stage A: 628.3 ± 177.0 U/L and B+C stage: 669.5 ± 196.1 U/L; Figure 2). Significant changes of plasma CAT activity among the investigated groups were registered (control vs. B+C stages: $p < 0.01$ and A vs. B+C stages: $p < 0.01$). Namely, the lowest value of CAT activity was detected in the control group (19.9 ± 3.2 kU/L), higher value was seen in the A stage B-

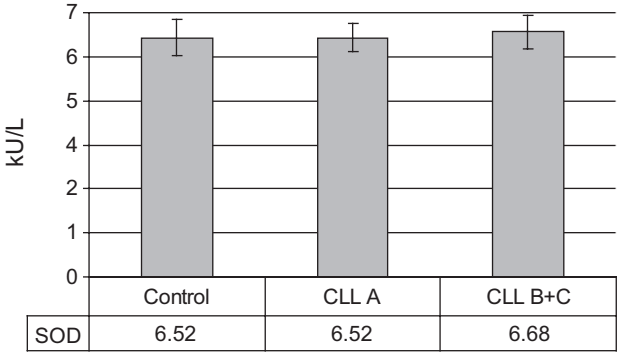


**Figure 1.** Activity of superoxide dismutase (SOD) in plasma of stages A and B+C CLL patients and control subjects are represented by mean ± SD. Control group vs. both CLL patient groups, p >0.05.

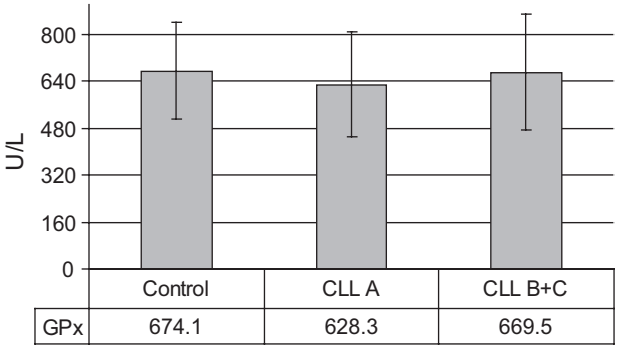


**Figure 2.** Activity of glutathione peroxidase (GPx) in plasma of stages A and B+C CLL patients and control subjects are represented by mean ± SD. Control group vs. both CLL patients groups, p >0.05.

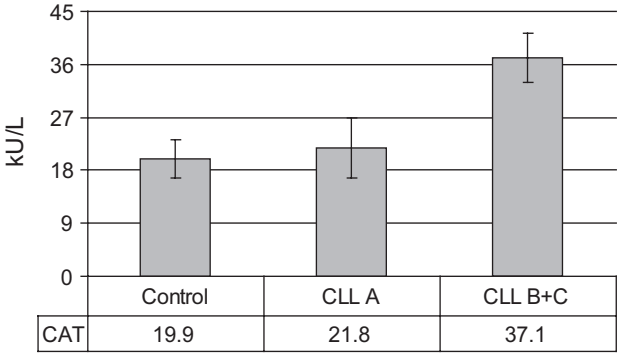


**Figure 3.** Activity of catalase (CAT) in plasma of stages A and B+C CLL patients and control subjects are represented by mean ± SD. Control group vs. B+C stages, p<0.01. A vs. B+C stages, p <0.01.

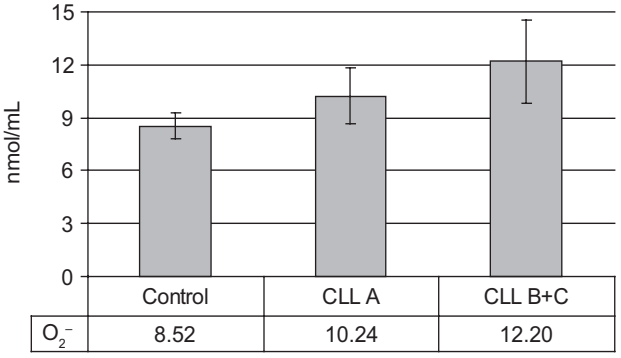


**Figure 4.** Concentration of superoxide anion radical ($O_2^-$) in plasma of A and B+C stage CLL patients and control subjects are represented by mean ± SD. Control vs. both patient groups, p >0.05.

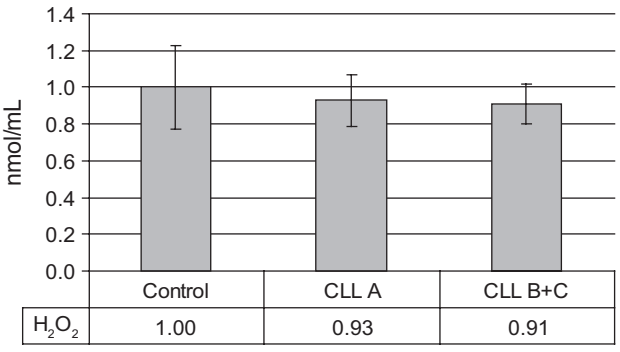


**Figure 5.** Concentration of hydrogen peroxide ($H_2O_2$) in plasma of stage A and B+C CLL patients and control subjects are represented by mean ± SD. Control group vs. both patient groups, p >0.05.

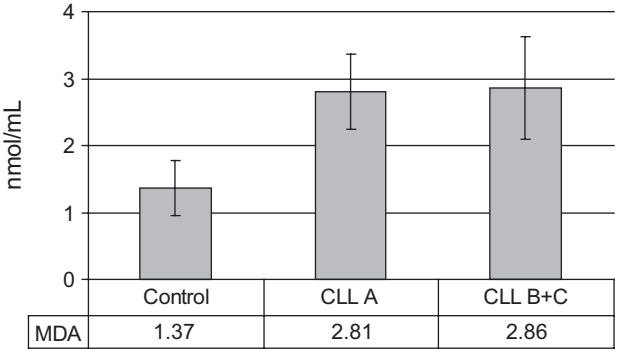


**Figure 6.** Concentration of malondialdehyde (MDA) in plasma of stage A and B+C CLL patients and control subjects are represented by mean ± SD. Control group vs. stage A, p <0.01 and control group vs. stages B+C, p <0.001.

CLL patients (21.8 ± 5.2 kU/L), while the highest value of CAT activity was detected in the plasma of the B + C stages of disease (37.1 ± 4.1 kU/L; Figure 3).

During this research we determined the markers of oxidative stress damage. The control group plasma levels of superoxide anion (8.52 ± 0.74 nmol/mL) was less than in both groups of B-CLL patients (stage A: 10.24 ± 1.58 nmol/mL and stages B + C: 12.2 ± 2.36 nmol/mL) but without statistical significance (p > 0.05, Figure 4). Interestingly, progression of disease was followed with increase of superoxide anion plasma levels, but also without significant differences (stage A vs. B stages+C, p > 0.05). Although our investigation showed that control subjects had higher plasma levels of hydrogen peroxide

(1.00 ± 0.23 nmol/mL) than both investigated groups of B-CLL patients (stage A: 0.93 ± 0.14 nmol/mL and stages B+C: 0.91 ± 0.11 nmol/mL), no statistical significance was noted ($p > 0.05$, Figure 5). Figure 6 illustrates significantly higher plasma concentration of MDA in all groups of B-CLL patients compared with healthy controls (control: 1.37 ± 0.41 nmol/mL, stage A: 2.81 ± 0.56 nmol/mL, stages B+C: 2.86 ± 0.77 nmol/mL; control vs. stage A, $p < 0.01$ and control vs. stages B+C, $p < 0.001$).

## Discussion

A small amount of reactive oxygen radicals is constantly produced in the body as a consequence of aerobic metabolism. The chemical reactions of the radicals with unsaturated fatty acids of lipids, proteins or amino acid chains, remainings of both base and sugar nucleotides and nucleic acids are directly responsible for the excessive production of free radicals, resulting in oxidative damage of different cells. A disturbance of oxidative metabolism is a common feature of transformed tumor cells [25]. Both the alterations of antioxidant enzymes and increase in the production of ROS have been described to contribute to tumorigenesis [26].

Our results indicate that oxidative stress and lipid peroxidation are accelerated in patients with B-CLL. Also, in the present study we investigated the SOD, CAT and GPx activities in the plasma of CLL patients in order to obtain a comprehensive view of the cancer patient antioxidant enzyme machinery.

There is great heterogeneity in lymphocytic production of superoxide anion among patients of different clinical stages. The most likely explanation of this phenomenon lies in the fact that B-CLL cells of patients of different clinical stages have different metabolic activities. There is evidence that B-CLL cells produce a larger amount of superoxide anion than the cells of healthy subjects [27], which is in accordance with our results. Namely, we detected higher levels of superoxide anion in the plasma of B-CLL patients than in the control group with increasing plasma levels with progression of disease. Monitoring the activity of SOD in plasma of B-CLL patients and comparing the values of the control group we demonstrated that there are no statistically significant differences between these tested groups. This finding may be an additional confirmation that the plasma concentration of superoxide anion of patients primarily depends on the metabolic activities of B-CLL lymphocytes, but not so much on the activity of SOD which metabolizes superoxide anion in known enzymatic antioxidative cascades. Unlike us, a group of Turkish researchers [28] has shown that in the serum of patients with B-CLL there was a statistically significant decrease in the activity not only of SOD but also of GPx, as well as an increase in the MDA concentration.

During this research we measured the plasma level of hydrogen peroxide. We detected insignificant higher value of plasma hydrogen peroxide in the control group than in the groups of B-CLL patients with gradually decreased levels with disease progression. There are several possible explanations of this phenomenon. In plasma, hydrogen peroxide originates not only from B lymphocytes but also from other cells. Moreover, it is known that plasma albumin performs detoxification of many free radicals, among them hydrogen peroxide, and this process is more intense in B-CLL patients than in healthy subjects [29]. The mechanism of this phenomenon is still not known. In addition, B-CLL lymphocytes express and release CAT as the most important enzyme in detoxication of hydrogen peroxide more than healthy lymphocytes [29]. In our study we showed that there was a significant increase of CAT activity in plasma of B-CLL patients compared with control subjects. This enzyme, localized in cell peroxisomes, catalyzes the disintegration of two molecules of hydrogen peroxide in molecules of water and oxygen. Because of its primarily peroxisomal localization it is not involved in the regulation of cell redox potential [30]. However, the enzyme could also have an extracellular localization [31] and proved to act as a potential antioxidant. According to these facts, the decrease of hydrogen peroxide level in the plasma of B-CLL patients, shown in our study, might be caused by increased activity of CAT.

GPx is a selenoprotein which reduces lipidic or nonlipidic hydroperoxides, as well as hydrogen peroxide, while oxidizing glutathione. In our study we showed that there was a mild trend of decreasing GPx plasma activity in B-CLL patients but comparison found that the differences between the groups were not statistically significant. The results of other studies are in accordance with our results. Namely, Bakan et al. [28] also showed decreased serum activity of GPx in patients with CLL. Similar findings were obtained in some different types of tumors. Arivazhagan et al. have shown decreased GPx activity in the serum of gastric cancer patients [32].

The process of lipid peroxidation is one of oxidative conversion of polyunsaturated fatty acids which are important for normal function of most mammalian cells. MDA is one of the end-products of lipid peroxidation induced by ROS and well correlated with the degree of lipid peroxidation [33]. Lipid peroxidation of cellular structure may play an important role in the pathogenesis of many pathological processes such as carcinogen-

esis. There are different results of serum MDA level in B-CLL patients in the literature. Devi et al. [34] showed that plasma lipid peroxidation products in untreated leukemia patients (such as B-CLL) were within normal range. Our results are in concordance with some other studies [28,35]. Increasing MDA concentrations in the plasma of B-CLL patients, which was shown in our study, might not be only a consequence of increased production but also a failure of the antioxidative defense system. Namely, the serum activity of glutathione-S-transferase, glutathione reductase, Cu-Zn SOD were reduced in the B-CLL patients, which may be one mechanism caused for the increased concentration of MDA in plasma [28]. Also MDA values were higher in B-CLL lymphocytes than in lymphocytes of healthy subjects, mainly due to a decrease in the antioxidative enzyme activities of the intracellular protection enzymes such as SOD and CAT, indicating that the intracellular level of MDA is a marker of disease evolution [35]. Reduced activities of SOD and GPx can cause accumulation of superoxide anion and hydrogen peroxide in tumor cells. In addition, lipid peroxidation, as one of the main effects of oxidative stress in cells, can cause increased concentration of MDA. Due to reduced activity of antioxidant enzymes in the lymphocytes lysates, especially GPx, lipid peroxidation is increased in malignant cells, which might lead to increased concentration of MDA in plasma [36,37]. This extensive lipid peroxidation in malignant B-cells, as indicated by increased MDA values in these cells, might be in concordance with different forms of DNA base lesions [35]. Several authors claim that MDA acts as tumor promoter and co-carcinogenic agent due to its high cytotoxicity and inhibitory effect on protective enzymes [38,39].

## Conclusion

Our findings suggest that disbalance between extensive oxidative stress and antioxidative enzyme system may be related to the pathogenesis and evolution of CLL. The identification of adequate oxidative markers for tumor cell metabolism may be useful for early diagnosis and for assessment of tumor progression. Understanding of endogenous mechanisms of carcinogenesis by serious oxidative stress and molecular action of carcinogens must be further elucidated.

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