

# Changes in the blood antioxidant defense of advanced age people

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**Introduction:** Since 1956 there have been numerous scientific articles about free radical theory of aging which both confirm and deny the theory. Due to oxygen metabolism, there are relatively high concentrations of molecular oxygen in human cells, especially in mitochondria. Under normal physiological conditions, a small fraction of oxygen is constantly converted to ROS, such as superoxide radical ( $O_2^{\cdot-}$ ),  $H_2O_2$ , and related metabolites.

**Aim of the study:** The aim of this work was to show the relation between the activity of main antioxidative enzymes and the age of the examined patients.

**Materials and methods:** The analysis of antioxidant defense was performed on the blood samples from 184 “aged” individuals aged 65–90+ years, and compared to the blood samples of 37 individuals just about at the beginning of aging, aged 55–59 years.

**Results:** The statistically significant decreases of Zn,Cu-superoxide dismutase (SOD-1), catalase (CAT), and glutathione peroxidase (GSH-Px) activities were observed in elderly people in comparison with the control group. Moreover, an inverse correlation between the activities of SOD-1, CAT, and GSH-Px and the age of the examined persons was found. No age-related changes in glutathione reductase activities and malondialdehyde concentrations were observed.

**Conclusion:** Lower activities of fundamental antioxidant enzymes in the erythrocytes of elderly people, which indicate the impairment of antioxidant defense in the aging organism and the intensity of peroxidative lipid structures, were observed.

**Keywords:** aging, antioxidant enzymes, free radicals, oxidative stress

## Introduction

One of the main global problems is the aging of society which is supposed to be a combined result of low fertility, low immigration, and prolonged lifespan.<sup>1,2</sup> In order to tackle this issue it is necessary to learn more about aging at the biochemical and molecular levels, as well as about the biological, medical, and social consequences of this process. A 30-year gain in life expectancy has been recorded in highly developed countries during the 20<sup>th</sup> century.<sup>2–4</sup> This tendency has also been noticeable in Poland since 1990. According to the projection of Polish Central Statistical Office, the life expectancy in Poland may increase from 70.0 years in 2007 to 77.1 years in 2035 for males and from 79.7–82.9 for females, respectively. In this period of time, the percentage of Polish population at post-working age (60+ years for women and 65+ years for men) may change from 16%–26.7%.

An integral part of aerobic metabolism is ROS generation which should be analyzed according to its two main functions. On the one hand, ROS plays an important role in biomodulating and regulating many cellular functions, such as

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defense against pathogens, signal transduction processes during transmission of intercellular information, and activation of specific transcription factors.<sup>5-9</sup> On the other hand, an excessive quantity of ROS has a deleterious effect on cells, reacting with a variety of molecules and thereby interfering with cellular functions.<sup>10,11</sup> To cope with the elevated generation of ROS, ROS-scavenging biochemical pathways have been developed in aerobic cells.<sup>12-16</sup>

One of the more serious objections against free radical theory of aging are results of research performed on mtDNA mutator mice. They have a mutation in exonucleasic catalytic domain of mitochondrial subunit of gamma polymerases.

A consequence of this mutation is an increased expression of proteins which do not have recovery properties. This leads to accumulation of mtDNA mutations.<sup>17</sup> The examined mice have a series of symptoms which resemble the natural process of aging, such as kyphosis, anemia, alopecia, greying of the hair, loss of hearing, cardiomyopathy, decreased fertility, loss of weight, and shortened life expectancy. Mutator mice have lack of ROS production and oxidative damages, even though the respiratory chain remains functioning. This observation contradicts the concept in which ROS and mtDNA mutation fuel one another and results in gradual deterioration of mitochondrial function. In the examined mice, an increased level of ROS may cause extension of life expectancy, which is a contradiction of the free radical theory of aging. This leads to a conclusion that the hypothesis of this theory is incorrect.<sup>18-20</sup> However, the results from numerous scientific results about the increase of oxidative stress with age suggest the unclear relation between the free radical theory and the aging process.

Due to the peculiar metabolism, the red blood cells are vulnerable to oxidative stress.<sup>21</sup> The main source of free radicals in erythrocytes is the process of auto-oxidation of hemoglobin which results in methemoglobin. Furthermore, it may lead to cross-reactions between globin chains which precipitate as so-called Heinz bodies. What is observed is the protein breakdown, creation of large protein complexes, damage of glycoprotein at the surface of blood cells, and disturbance of transport through membrane and membrane potential.<sup>22,23</sup> These processes lead to the loss of function and viability of red blood cells. In erythrocytes, there is an exhaustion of glutathione stocks which additionally causes the decrease of antioxidative enzymes' activity, increase peroxidation of cell membrane lipids and oxidative damages of hemoglobin. This may lead to hemolysis of erythrocytes and the release of heme iron and

intensification of reaction of free radical among the endothelium.<sup>21,24-27</sup>

The oxidative stress theory is one of the most popular and controversial explanations of aging pathomechanisms. The free radical theory of aging, proposed in 1956 by Harman, assumed the endogenous generation of oxygen free radicals from normal oxygen-utilizing metabolic processes and their essential role in the aging processes.<sup>28</sup> Later, Harman and other researchers modified this theory, in accordance with the findings in this area.<sup>29,30</sup> According to this hypothesis, the loss of balance between pro-oxidants and antioxidants leads to accumulation of oxidative damage in macromolecules with age, which results in the disturbances in functional cellular processes and development of aging.<sup>31-33</sup> In recent years there have been a lot of studies supporting the role of ROS in molecular aging mechanisms.<sup>34,35</sup> The confirmation of oxidative stress increase with age of diverse organisms, and the generation of transgenic invertebrates overexpressing the antioxidant enzymes with increased lifespan were among the most important results of these studies.<sup>36-39</sup> Nevertheless, there were no alterations in the lifespan in most of the examined mouse models, which under- or overexpressed a wide variety of genes coding for antioxidant enzymes.<sup>40-42</sup> Thus, the role of oxidative stress in aging mammals is not fully understood and still demands further inquiries.<sup>43,44</sup>

## Materials and methods

The study was carried out on 221 persons (100 males, 121 females), divided into seven age subgroups (55-59, 65-69, 70-74, 75-79, 80-84, 85-89, and 90+ years). The subgroup of 55-59 year-old persons was the control group. The participants were recruited according to a multi-stage procedure designed for the study performed in the Department and Clinic of Geriatrics in order to choose a representative sample of elderly people.

The selected anthropometric parameters such as growth, weight, and total cholesterol had a normal distribution. Regarding these parameters, the results of examined patients did not show statistically significant differences (Table 1).

Blood samples were collected in the morning (08:00 am) after 12 hours of fasting from the cubital vein in heparinized tubes (3 mL) to obtain erythrocytes. All samples were centrifuged (2,500 g for 10 minutes). After plasma removal, the hemolysate was prepared by threefold freezing and thawing the washed erythrocytes, suspended in ultrapure water. The hemolysate was used to determine the parameters of oxidative stress and antioxidative defense.

**Table 1** Anthropometric parameters of the examined groups (mean  $\pm$  SD)

Parameter	55–59 years	65–69 years	70–74 years	75–79 years	80–84 years	85–89 years	90+ years
Body mass index	24.2 $\pm$ 4.2	24.1 $\pm$ 3.1	24.8 $\pm$ 2.5	22.30 $\pm$ 2.5	24.70 $\pm$ 3.7	22.4 $\pm$ 2.1	21.1 $\pm$ 2.3
Body mass (kg)	72.2 $\pm$ 7.5	73.1 $\pm$ 4.5	75.4 $\pm$ 6.5	74.7 $\pm$ 4.5	69.8 $\pm$ 5.5	71.2 $\pm$ 3.5	69.2 $\pm$ 7.5
Body height (cm)	170 $\pm$ 4.3	172 $\pm$ 3.3	173 $\pm$ 4.3	169 $\pm$ 5.3	168 $\pm$ 2.3	169 $\pm$ 5.3	167 $\pm$ 6.3
Total cholesterol (mg/dL)	175 $\pm$ 8	182 $\pm$ 4.8	168 $\pm$ 5.2	164 $\pm$ 3.1	172 $\pm$ 6.2	161 $\pm$ 7.1	171 $\pm$ 3.8

Malondialdehyde (MDA) concentration in erythrocytes, as well as erythrocytic activities of Cu-Zn superoxide dismutase (SOD-1; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), cellular glutathione peroxidase (GSH-Px; EC 1.11.1.9), and glutathione reductase (GR; EC 1.6.4.2) were assayed by the methods of Placer et al, Misra and Fridovich, Beers and Sizer, Paglia and Valentine, and Flohe and Gunzler, respectively.<sup>45–49</sup> MDA level was expressed as a concentration of thiobarbituric acid reactive substances, read at 532 nm. SOD-1 activity was determined at 37°C by recording the increase in absorbance at 480 nm following the auto-oxidation of adrenaline, inhibited by SOD-1. CAT activity was measured at 25°C by recording H<sub>2</sub>O<sub>2</sub> decomposition at 240 nm. GSH-Px activity was determined at 25°C by recording the decrease in absorbance at 340 nm following the oxidation of NADPH in the presence of tert-butyl hydroperoxide as a substrate, GSH, yeast GR, and NaN<sub>3</sub> as a CAT inhibitor. GR activity was determined at 37°C by recording the decrease in absorbance at 340 nm following the oxidation of NADPH in the presence of oxidized glutathione. The hemoglobin concentration in the hemolytate was estimated after conversion into cyanmethemoglobin form using a commercial reagent (Biomed, Lublin, Poland), read at 540 nm.

## Statistical analysis

All results were expressed as mean  $\pm$  SD. The one-way analysis of variance followed by the Tukey post hoc test was performed to determine the statistical significance of differences. The Pearson's correlation coefficient was used to quantify the relationship between the measured parameters. The level of significance was set at  $P < 0.05$ .

## Results

All results of measured biochemical parameters of oxidative stress were shown in Table 2. Figure 1–5 present parameters in particular age groups together with the trend line showing the tendency of change with age.

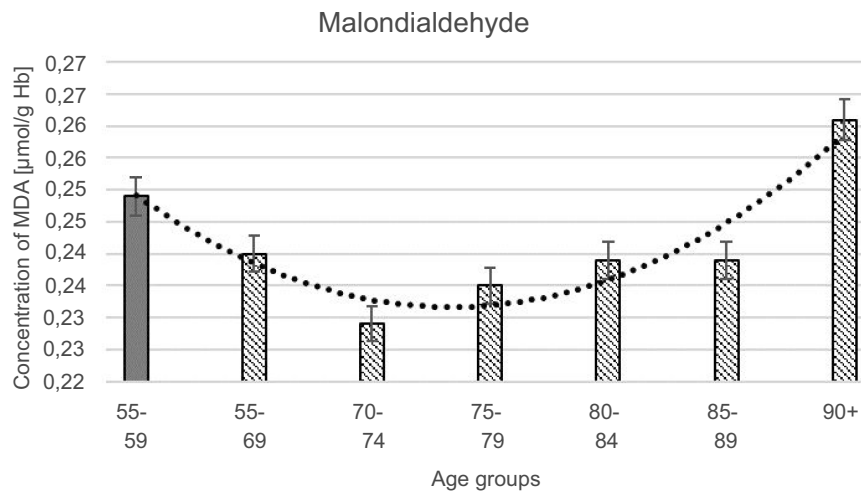
The progressive decrease of antioxidant enzymatic defense in the erythrocytes during aging was observed in this study. The activities of SOD-1 were lowered by 17% in elderly subjects over 90 years of age in comparison with the persons aged 55–59 years, whereas the activities of CAT and GSH-Px were reduced by 20% and 27%, respectively. Only GR activities remained unchanged during aging in this study.

Moreover, the strong negative correlations between age and SOD-1, CAT, and GSH-Px activities in the erythrocytes were observed.

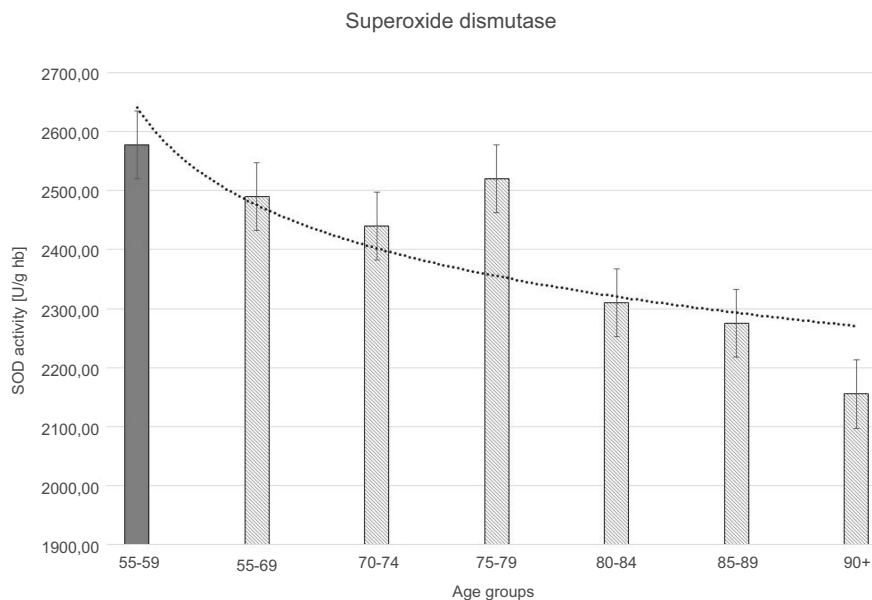
**Table 2** Selected parameters of pro- and antioxidant balance in the blood of the examined groups (mean  $\pm$  SD)

Age groups (years)	Number of subjects (male/female)	Malondialdehyde [ $\mu$ mol/g Hb]	Zn,Cu-superoxide dismutase [U/g Hb]	Catalase [BU/g Hb]	Glutathione peroxidase [U/g Hb]	Glutathione reductase [U/g Hb]
55–59 (control)	37 (17/20)	0.249 $\pm$ 0.031	2578 $\pm$ 170.8*	24.54 $\pm$ 1.76*	14.95 $\pm$ 3.17*	58.55 $\pm$ 7.13
65–69	28 (13/15)	0.240 $\pm$ 0.026	2490 $\pm$ 220.0	24.17 $\pm$ 2.11	13.36 $\pm$ 2.29	59.89 $\pm$ 12.76
70–74	29 (12/17)	0.229 $\pm$ 0.0350	2440 $\pm$ 206.2	24.37 $\pm$ 2.46	14.10 $\pm$ 2.39	56.51 $\pm$ 12.71
75–79	29 (14/15)	0.235 $\pm$ 0.0329	2520 $\pm$ 203.3	23.10 $\pm$ 1.86	13.18 $\pm$ 2.57	57.87 $\pm$ 10.69
80–84	32 (16/16)	0.239 $\pm$ 0.036	2310 $\pm$ 192.7	23.09 $\pm$ 2.12	12.86 $\pm$ 2.56	54.75 $\pm$ 15.89
85–89	32 (12/20)	0.239 $\pm$ 0.0327	2275 $\pm$ 169.9	22.39 $\pm$ 2.29	13.09 $\pm$ 2.14	62.97 $\pm$ 12.27
90+	34 (16/18)	0.261 $\pm$ 0.0346	2155 $\pm$ 156.8	20.47 $\pm$ 1.27	11.01 $\pm$ 1.71	58.42 $\pm$ 18.41

**Note:** \*Statistically significant differences between control group (55–59 years) and elderly persons,  $P < 0.001$ .



**Figure 1** Malodddialdehyde (MDA) concentration with the trend line showing the tendency of change with age.



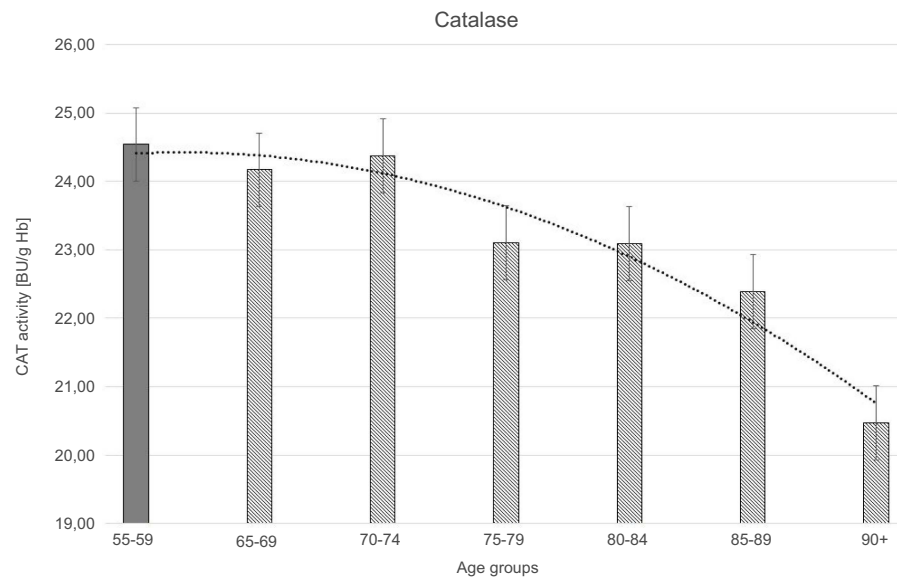
**Figure 2** Superoxide dismutase (SOD) activity with the trend line showing the tendency of change with age.

The statistically significant decreases of erythrocytic SOD-1 ( $P < 0.00001$ ), CAT ( $P < 0.00001$ ), and GSH-Px ( $P < 0.001$ ) activities were observed in elderly groups in comparison with the control group. Moreover, strong negative correlations between the activities of SOD-1 ( $r = -0,6341$ ; Figure 6A), CAT ( $r = -0,6316$ ; Figure 6B), and GSH-Px ( $r = -0,4475$ ; Figure 6C) and the age of the examined persons were found. No age-related changes in GR activities and MDA concentrations were noticed.

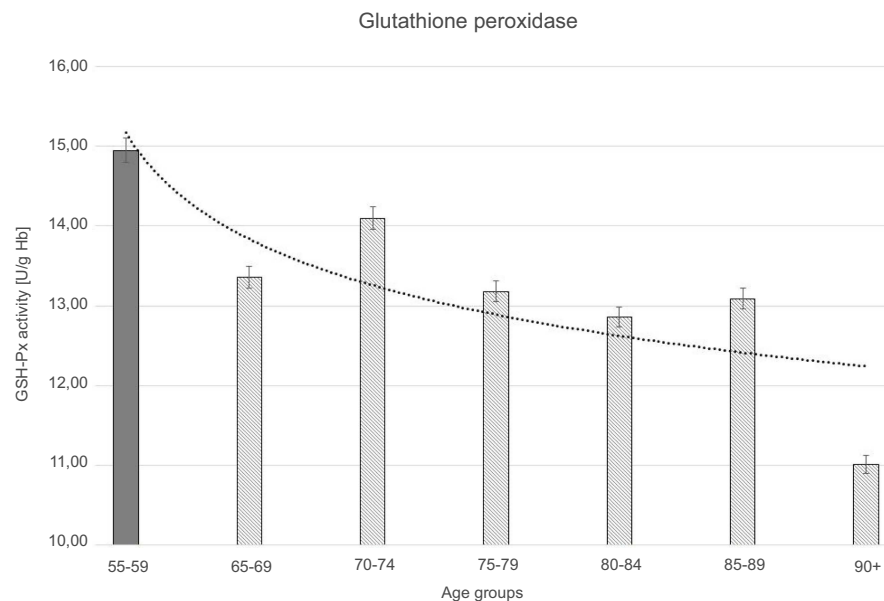
## Discussion

ROS is not only a toxic side product of aerobic metabolism but it may also play an important role as signaling molecules.

The most current research confirms the possibility that ROS takes part in different physiological processes as signaling molecules of stress in response to cellular damage.<sup>50</sup> Therefore, the physiological level of ROS is most probably the main important issue for maintaining cellular homeostasis, whereas the increased production of ROS at a certain level has a detrimental influence on physiology of the cells. Among model organisms not showing an increase in the level of oxidative damage or cases of life extension among organisms with a high level of oxidative stress, suggest that the free radical theory of aging may be incorrect.<sup>51,52</sup> Undoubtedly, mitochondria play an important role in the aging process and the accumulation of mutations and decreasing with age



**Figure 3** Catalase (CAT) activity with the trend line showing the tendency of change with age.

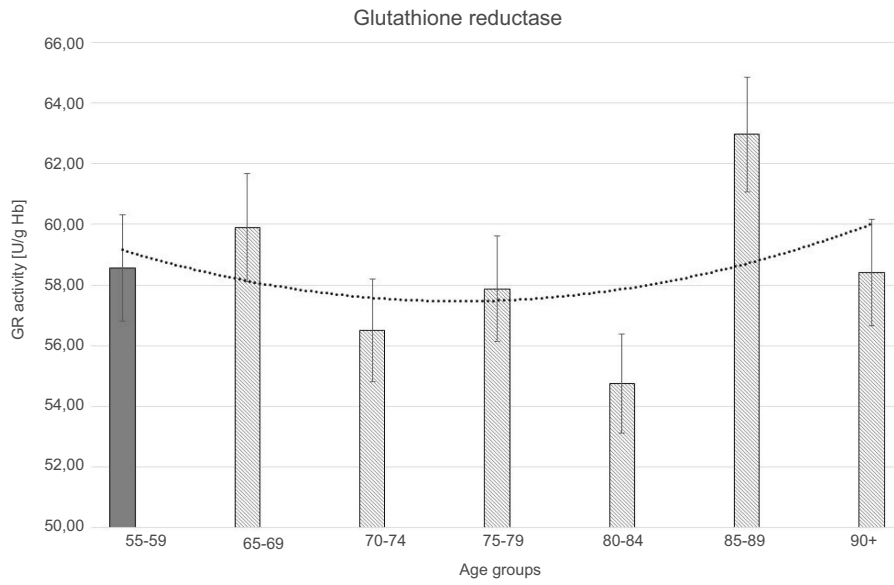


**Figure 4** Glutathione peroxidase (GSH-Px) activity with the trend line showing the tendency of change with age.

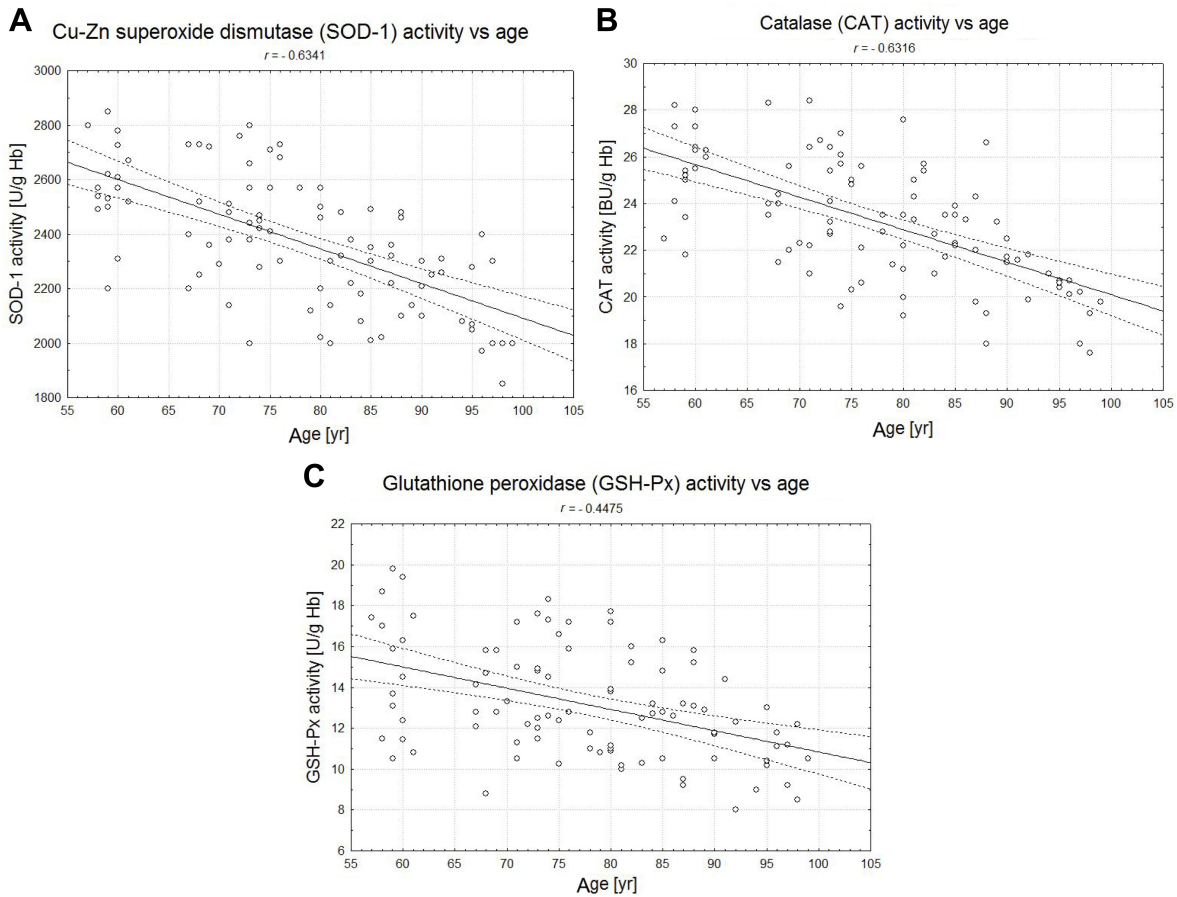
mitochondrial function irreversibly leads to cellular dysfunction. In the context of increasing research on the role of ROS in signaling and cellular regulation, one should not completely reject the role of free radicals in the process of aging.<sup>53</sup>

These pathways involve a complex antioxidant defense system that is composed of both low molecular weight antioxidants (reduced glutathione [GSH], ascorbic acid, tocopherols, etc) and antioxidant enzymes. The first line in enzymatic antioxidant defense is SOD, which catalyses dismutation of  $O_2^{\cdot -}$  into  $H_2O_2$ . Subsequently,  $H_2O_2$  may be

dismutated into  $H_2O$  by CAT. Differently from CAT, GSH-Px may catalyse the reduction of both  $H_2O_2$  and organic peroxides, using GSH as a hydrogen donor whereby GSH is oxidized to glutathione disulfide (GSSG). GR can reduce GSSG into GSH, just providing with this molecule for antioxidant actions. The activities of antioxidant enzymes, as well as the levels of low molecular weight antioxidants have been observed to be altered in old organisms, which may lead to the intensification of oxidative stress and in this way to the development of aging. Several



**Figure 5** Glutathione reductase (GR) activity with the trend line showing the tendency of change with age.



**Figure 6** The correlations between the erythrocytic Zn-Cu superoxide dismutase (SOD-1) (A), catalase (CAT) (B), and glutathione peroxidase (GSH-Px) (C) activities and age of examined persons ( $P < 0.05$ ).

reports, as well as our own previous results, confirm the weakening of the enzymatic antioxidant defense during the organism's aging.<sup>54,55</sup>

There are diverse assumptions about the causes of observed age-dependent decline in antioxidant enzymatic defense. The oxidative modifications of enzymatic proteins caused by ROS are supposed to be one of the possible mechanisms of this phenomenon.<sup>56</sup> Malnutrition in elderly people, resulting from poor nutritional habits, loss of appetite, or intestinal malabsorption in this group of people, may be the other probable explanation. As a result of malnutrition, deficiencies of some trace elements may occur, such as Zn<sup>2+</sup> ions, essential for SOD-1 activity or selenium, essential for the synthesis of selenoenzyme GSH-Px.<sup>57,58</sup> Taking into account this explanation, the unchanged activity of GR during aging in this study may point to the sufficient supply of riboflavin in the diet of examined elderly subjects. Nevertheless, among the most interesting hypotheses, there is a possible link between the age-dependent decrease of antioxidant enzymes' activities and the lowered levels of pineal hormone melatonin, which is observed in elderly people. Melatonin may both regulate the expression of genes coding for antioxidant enzymes such as SOD, GSH-Px, and GR and directly influence their activities.<sup>59</sup> The amplitude of melatonin production declines with aging until almost total disappearance of its specific day-night cycle in many persons over 65 years of age.<sup>60,61</sup> Thus, the decrease of antioxidant enzymes' activities during aging may be at least partly due to the failure of melatonin secretion in elderly subjects.

On the contrary, some authors found an increase of different antioxidant enzymes' activities in elderly subjects, which may implicate the compensatory effect of augmented oxidative stress in aging organisms.<sup>62-64</sup> Among the most interesting studies, there are results obtained by Kłapcińska et al, in the examination of oxidative stress parameters in healthy Polish centenarians.<sup>65</sup> There were significantly higher erythrocytic CAT and GR activities found in centenarians as compared to young healthy adults in Kłapcińska et al's study. These results indicate that increased capacity for antioxidant defense may contribute to human longevity.

It is still not fully understood whether the augmentation of oxidative stress with advancing age is due to a decrease of antioxidant defense system or an increase in endogenous reactive oxygen production. The oxidative stress intensity may be approximately estimated by the measurement of erythrocytic concentrations of MDA formed from the breakdown of lipid hydroperoxides. Several authors demonstrated the increased MDA concentrations in elderly subjects.<sup>54,55</sup> The

elevated MDA levels correspond to the intensified rate of lipid peroxidation during aging. Lipid peroxidation processes result in impaired membrane fluidity, increased non-specific membrane permeability, and inactivation of membrane enzymes, which may contribute to the damage occurring in aging organisms.<sup>66-70</sup> Surprisingly, there were no age-dependent changes in MDA levels during aging in the present study. It is worth mentioning that some authors found decreased MDA concentrations in healthy centenarians as compared to the aged subjects or even to younger adults.<sup>65,68,71</sup> These results may support the hypothesis that reduced oxidative stress may be related to the increase in lifespan of human beings.

## Conclusion

Summing up, there are many reports supporting the role of oxidative stress in development of aging processes in human organisms. However, these data are still not consistent and further studies are necessary to confirm the existing hypotheses. Our preliminary study revealed some interesting links between antioxidant enzymatic defense capacity and aging. The subsequent investigations in the framework of this study may lead us to some answers for the questions about the role of oxidative stress in aging processes.

The obtained results clearly show the decrease of crucial activity of antioxidative enzymes with the simultaneous intensity of peroxidative lipids. In this context, it makes sense to continue the research aiming at thoroughly explaining the role of this phenomenon in the aging process. Based on the results of research on mice, it may be stated that free radical reactions may not be the only reason for intensity of the aging process but they certainly have an influence on it.

## Data sharing statement

The datasets generated during and/or analyzed during the current study can be obtained from the corresponding author upon request from other scientists.

## Ethics statement

This study was approved by the Bioethics Commission of Nicolaus Copernicus University in Torun. All participants gave their written informed consent and the study was conducted in accordance with the Declaration of Helsinki.

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## Disclosure

The authors report no conflicts of interest in this work.

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