

# The Mechanism of the Osteoprotective Action of a Polyphenol-Rich *Aronia melanocarpa* Extract during Chronic Exposure to Cadmium is Mediated by the Oxidative Defense System

## Authors

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## Key words

- *Aronia melanocarpa*
- Rosaceae
- cadmium
- polyphenol-rich chokeberry extract
- bone tissue
- oxidative/antioxidative status
- oxidative damage
- protection

## Abstract

Recently, we demonstrated in a rat model that consumption of a polyphenol-rich extract obtained from the berries of *Aronia melanocarpa* could protect from cadmium-induced disorders in bone turnover and changes in bone mineral status. The aim of this study was to investigate whether the osteoprotective effect of this extract is mediated by the oxidative defense system. Enzymatic and nonenzymatic antioxidants, total antioxidative and oxidative status, hydrogen peroxide, and markers of oxidative protein, lipid, and DNA damage were determined in bone tissue at the distal femoral epiphysis of female Wistar rats receiving 0.1% aqueous *A. melanocarpa* extract (prepared from the lyophilized commercial extract containing 65.74% of polyphenols) as the only drinking fluid and/or cadmium in the diet (1 and 5 mg/kg) for 3, 10, 17, and 24 months. The total oxidative and antioxidative status of the serum was also evaluated. The administration of *A. melanocarpa* extract provided significant protection from cadmium-induced oxidative stress in the bone and serum, and from lipid peroxidation and oxidative damage to the protein and DNA in the bone tissue. Numerous correlations were noted between indices of the oxidative/antioxidative bone status and markers of bone metabolism previously assayed in the animals receiving *A. melanocarpa* extract. The results allow the conclusion that the ability of *A. melanocarpa* extract to mediate the oxidative defense system and prevent oxidative modifications of protein, lipid, and DNA in the bone tissue plays an important role in its osteoprotective action under exposure to cadmium. The findings provide further evidence supporting our suggestion that chokeberry may be a promising natural agent for protection against the toxic action of cadmium in women chronically exposed to this metal.

## Abbreviations

ALP:	alkaline phosphatase
AME:	extract from the berries of <i>Aronia melanocarpa</i>
BMD:	bone mineral density
Ca/AW:	the ratio of calcium content in bone and the bone ash weight
Ca/DW:	the ratio of calcium content in bone and the bone dry weight
CAT:	catalase
CTX:	carboxy-terminal cross-linking telopeptides of type I collagen
GPx:	glutathione peroxidase
GR:	glutathione reductase
GSH:	reduced glutathione
LPO:	lipid peroxides
OC:	osteocalcin
OPG:	osteoprotegerin
OSI:	oxidative stress index
PC:	protein carbonyl groups
RANK:	receptor activator of nuclear factor- $\kappa$ B
RANKL:	receptor activator of nuclear factor- $\kappa$ B ligand
ROS:	reactive oxygen species
sRANKL:	soluble receptor activator of nuclear factor- $\kappa$ B ligand
sRANKL/OPG:	the ratio of sRANKL and OPG
SOD:	superoxide dismutase
TAS:	total antioxidative status
TOS:	total oxidative status
T-SH:	total thiol groups
% mineral comp.:	percentage content of mineral components in bone tissue
8-isoP:	8-isoprostanes
8-OHdG:	8-hydroxy-2'-deoxyguanosine

**Supporting information** available online at <http://www.thieme-connect.de/products>

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## Introduction

Recently, the possibility of using natural plant ingredients for protection against the dangerous action of xenobiotics to which humans are exposed throughout their lifetime, including Cd, which is one of the most toxic heavy metals, has been of special interest to researchers [1–9]. Evidence that even relatively low exposure to Cd contributes to osteoporosis and bone fractures [10–12] together with forecasts that the general population's exposure to this element will increase [2] are reasons that growing attention has been focused on searching for an effective means of protection from the unfavorable action of this xenobiotic, including damage to the skeleton.

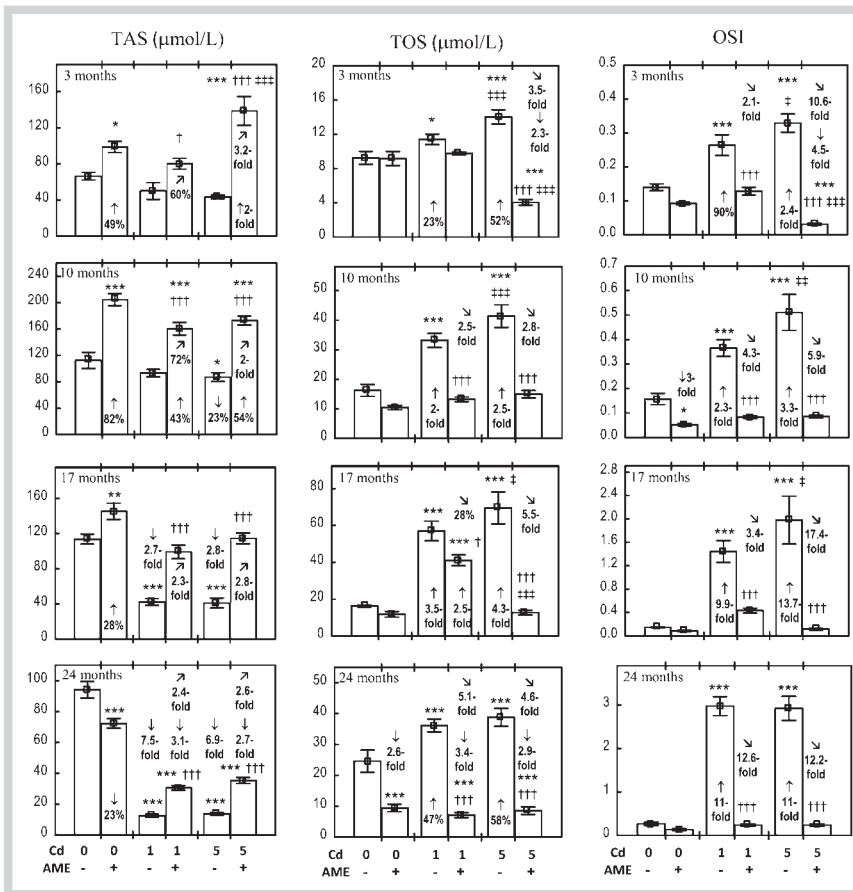
It seems that polyphenols, being the most abundant bioactive components in our diet [13,14], are a promising group of compounds playing a role in this protection [3–9,15]. Owing to the large number of -OH groups, these compounds are capable of chelating metal ions, including Cd<sup>2+</sup> [4,6,14]. It has been revealed that some food products abundant in polyphenolic compounds, including especially green tea and soybeans, may offer protection against various effects of exposure to Cd; however, the data is very sparse and refers only to chosen compounds [3,5–9,15–17]. One of the richest sources of polyphenols is the chokeberry [*Aronia melanocarpa* (Michx.) Elliott; Rosaceae]. Nowadays, the consumption of aronia berries is widely recommended as one of the most abundant sources of compounds having a beneficial influence on the organism, and the richest source among plant materials of anthocyanins, which are characterized by strong antioxidative properties [13,14,18–21]. Data provided by Kowalczyk et al. [15] and our recent findings [6,22,23] seem to indicate that the consumption of chokeberries and their products may prevent Cd accumulation in the body and its toxic action. Using a female rat model of low and moderate lifetime human exposure to Cd (1 and 5 mg Cd/kg diets, respectively, for up to 24 months), we have revealed that a polyphenol-rich AME may decrease the gastrointestinal absorption and body burden of this xenobiotic (including its accumulation in the liver, kidneys, and bone tissue) [22], as well as significantly prevent disturbances induced by this heavy metal in the bone turnover, and changes in the bone mineral status [23].

Taking into account the pro-oxidative action of Cd and oxidative stress involvement in the mechanisms of this metal's toxicity, including osteotoxic action [3,5,24–28], and the strong antioxidative properties of chokeberry [13,14,18–20], we have hypothesized that the osteoprotective effect reported by us [23] of AME during chronic exposure to Cd may be mediated by the oxidative defense system (the system of defense from oxidative processes and oxidative stress). The aim of the present paper was to investigate this hypothesis. For this purpose, numerous indices of the oxidative/antioxidative bone status and markers of oxidative protein, lipid, and DNA damage were determined in the bone tissue, and mutual dependences between these parameters, indices of bone turnover (Table 1S, Supporting Information), and bone mineral status (Table 2S, Supporting Information) previously determined in these animals [23] as well as Cd concentration in the bone tissue (Table 3S, Supporting Information) [22] were evaluated.

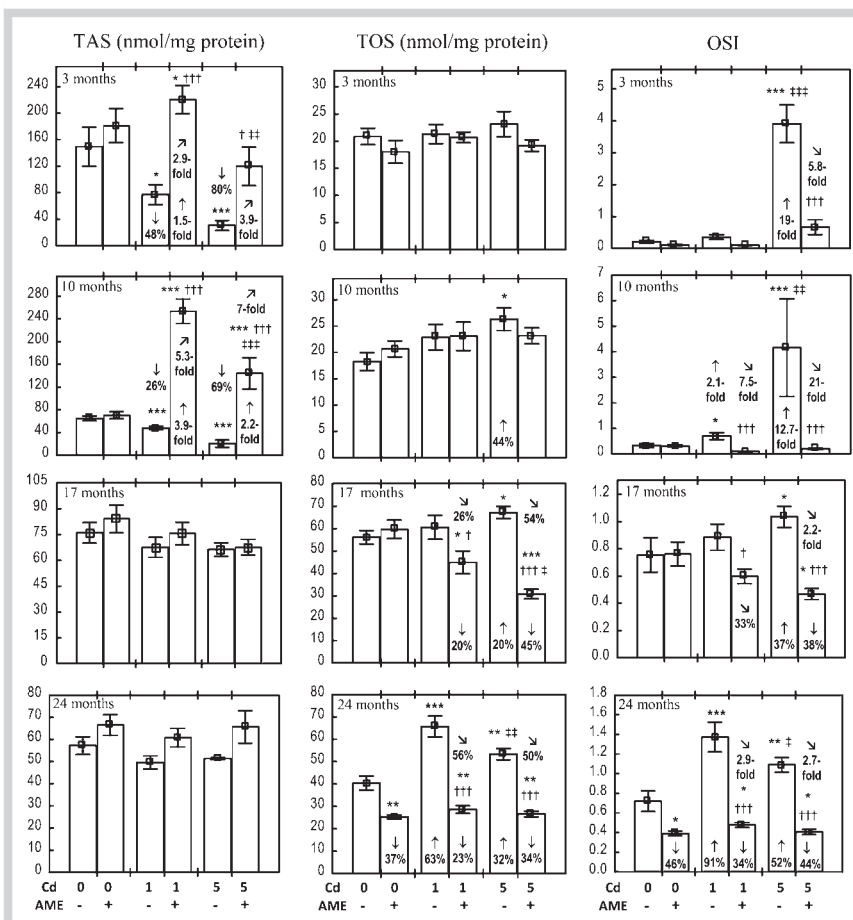
## Results

The administration of AME alone for 3, 10, and 17 months increased the TAS of the serum and had no influence on its TOS or OSI, except for a decrease in OSI after 10 months. After 24 months, TAS and TOS were lower, but the OSI was unchanged, compared to the control group (Fig. 1). In the animals treated with the 1 mg Cd/kg diet, TAS decreased from the 17th month, whereas at the higher exposure, it decreased from the 10th month (Fig. 1). TOS and OSI were dose- and exposure duration-dependently increased compared to the control group (Fig. 1). The level of oxidative stress in the serum (expressed as OSI) at both levels of Cd treatment gradually increased throughout the whole experiment. The Cd-induced disturbances of the oxidative/antioxidative balance in the serum after 3, 10, and 17 months were more advanced at the higher exposure, but after 24 months, they were similar at the 1 and 5 mg Cd/kg diets (Fig. 1). The administration of AME at both levels of Cd exposure markedly (partially or completely) improved the antioxidative status of the serum (Fig. 1). The serum TAS was higher in all groups receiving AME under Cd exposure, whereas TOS and OSI were lower compared to the respective groups treated with Cd alone, except for a lack of difference in TOS between the Cd<sub>1</sub> and Cd<sub>1</sub> + AME groups after 3 months. However, the fact that the serum TOS in the last group at this time point did not differ compared to the control group indicates a beneficial impact of AME (Fig. 1).

The administration of AME alone for up to 24 months had no impact on TAS or any of the determined indices of the enzymatic (GPx, GR, SOD, and CAT) and nonenzymatic (GSH, T-SH) status of the bone tissue, except for an increase in the activities of GPx after 3 months and SOD after 17 months (Figs. 2–4, Table 1). The exposure to the 1 and 5 mg Cd/kg diets for 3 and 10 months decreased the bone tissue TAS; however, this effect was not observed after the longer exposure (Fig. 2). As is evident from the data presented in Figs. 3 and 4, the bone activities of GPx, SOD, and CAT were unchanged or decreased, depending on the exposure duration. The activity of GR was unchanged after 3, 10, and 17 months, except for an increase in GR activity in the Cd<sub>5</sub> group after 3 months, but increased after 24 months. The bone concentration of GSH was unchanged due to the exposure to Cd, except for its decrease (by about 45%) in the Cd<sub>1</sub> and Cd<sub>5</sub> groups after 10 months (Table 1). The exposure to the 1 and 5 mg Cd/kg diets also resulted in a decrease in the concentration of T-SH in the bone tissue after 3 (by 47% and 62%, respectively) and 24 months (by 39% and 46%, respectively). The Cd-induced changes in the antioxidative status of the bone tissue were similar at both levels of exposure; however, at some time points, the activities of SOD and CAT were lower, whereas the activities of GPx and GR were higher in the Cd<sub>5</sub> group than in the Cd<sub>1</sub> group (Figs. 2–4, Table 1). Consumption of AME under exposure to Cd not only completely prevented this metal-induced decrease in the bone tissue TAS, but even enhanced the value of this parameter (Cd<sub>1</sub> + AME group after 3 and 10 months, and Cd<sub>5</sub> + AME group after 10 months) compared to the control group (Fig. 2). AME administration under exposure to Cd improved the enzymatic and nonenzymatic antioxidative barrier. In most cases, it completely prevented the Cd-induced decrease in GPx, SOD, and CAT, except for GPx activity after 24 months and CAT activity after 10 months in the Cd<sub>1</sub> + AME group. Moreover, AME coadministration under the whole treatment with the 5 mg Cd/kg diet decreased GR activity, and after 24 months, this effect was also observed at the lower



**Fig. 1** Effect of AME on the oxidative/antioxidative balance in the serum of rats chronically exposed to Cd. The rats received Cd in their diet at concentrations of 0, 1, and 5 mg/kg and 0.1% aqueous AME or not (“+” and “–”, respectively). Data are represented as mean ± SE for 8 rats, except for 7 animals in the AME group, and the groups exposed to the 1 and 5 mg Cd/kg diets alone after 24 months. Statistically significant differences (Anova, Duncan’s multiple range test): \**p* < 0.05, \*\*\**p* < 0.001 vs. control group; †*p* < 0.05, †††*p* < 0.001 vs. respective group receiving Cd alone; ‡*p* < 0.05, ‡‡‡*p* < 0.001 vs. respective group receiving 1 mg Cd/kg diet (alone or with AME). Numerical values in bars or above the bars indicate the percentage change or a factor of change compared to the control group (↓ decrease; ↑ increase) or the respective group receiving Cd alone (↘ decrease; ↗ increase).



**Fig. 2** Effect of AME on TAS and TOS status and OSI in the bone tissue of rats chronically exposed to Cd. The rats received Cd in their diet at concentrations of 0, 1, and 5 mg/kg and 0.1% aqueous AME or not (“+” and “–”, respectively). Data are represented as mean ± SE for 8 rats, except for 7 animals in the AME group, and the groups exposed to the 1 and 5 mg Cd/kg diets alone after 24 months. Statistically significant differences (Anova, Duncan’s multiple range test): \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. control group; †*p* < 0.05, †††*p* < 0.001 vs. respective group receiving Cd alone; ‡*p* < 0.05, ‡‡‡*p* < 0.001 vs. respective group receiving 1 mg Cd/kg diet (alone or with AME). Numerical values in bars or above the bars indicate the percentage change or a factor of change compared to the control group (↓ decrease; ↑ increase) or the respective group receiving Cd alone (↘ decrease; ↗ increase).

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**Table 1** Effect of AME on GSH and T-SH concentration in the bone tissue at the distal femoral epiphysis of rats exposed to Cd.

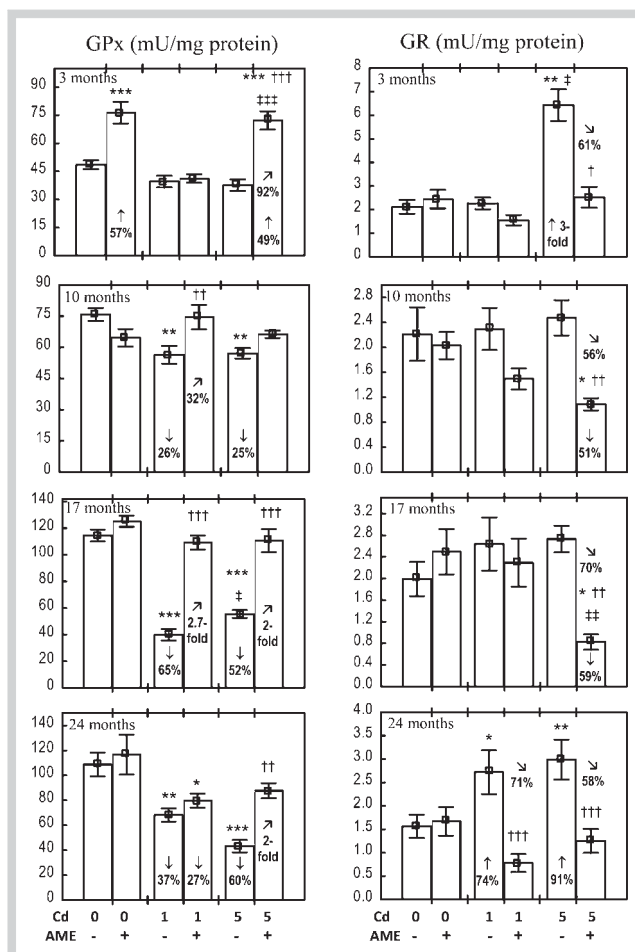
Group	Experiment duration			
	3 months	10 months	17 months	24 months
GSH (nmol/mg protein)				
Control	1.628 ± 0.130	2.308 ± 0.429	2.507 ± 0.331	2.437 ± 0.224
AME	2.163 ± 0.377	1.564 ± 0.236	1.945 ± 0.238	2.241 ± 0.298
Cd <sub>1</sub>	1.426 ± 0.163	1.258 ± 0.073*	2.480 ± 0.327	2.680 ± 0.199
Cd <sub>1</sub> + AME	1.386 ± 0.214	1.902 ± 0.246	2.702 ± 0.390	2.314 ± 0.177
Cd <sub>5</sub>	1.862 ± 0.270	1.300 ± 0.226*	2.972 ± 0.309	2.477 ± 0.269
Cd <sub>5</sub> + AME	1.438 ± 0.244	1.562 ± 0.216	2.189 ± 0.240	2.301 ± 0.203
T-SH (nmol/mg protein)				
Control	48.12 ± 6.89	44.39 ± 5.45	70.65 ± 6.29	64.97 ± 12.17
AME	44.83 ± 3.27	37.94 ± 3.57	69.74 ± 6.56	73.95 ± 8.50
Cd <sub>1</sub>	25.26 ± 3.54***	30.76 ± 3.39	69.03 ± 6.06	39.80 ± 4.04*
Cd <sub>1</sub> + AME	31.15 ± 2.46**	37.02 ± 5.06	59.26 ± 11.43	52.57 ± 4.98
Cd <sub>5</sub>	18.07 ± 1.53***	29.25 ± 2.78	61.54 ± 5.24	34.77 ± 2.27**
Cd <sub>5</sub> + AME	26.17 ± 2.81***	40.47 ± 7.61	52.36 ± 6.27	63.11 ± 5.62†

Data are represented as mean ± SE for 8 rats, except for 7 animals in the AME, Cd<sub>1</sub>, and Cd<sub>5</sub> groups after 24 months. Statistically significant differences (Anova, Duncan's multiple range test) compared to the control group (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001) and respective group receiving Cd alone (†p < 0.05) are marked

exposure (● Fig. 3). The consumption of AME provided complete protection from the 10-month exposure to the 1 and 5 mg Cd/kg diets that caused a decrease in the bone tissue GSH concentration and from the decrease in the concentration of T-SH after 24 months, but not after 3 months (● Table 1). Numerous correlations between the assayed indices of antioxidative bone status were noted (Table 4S, Supporting Information).

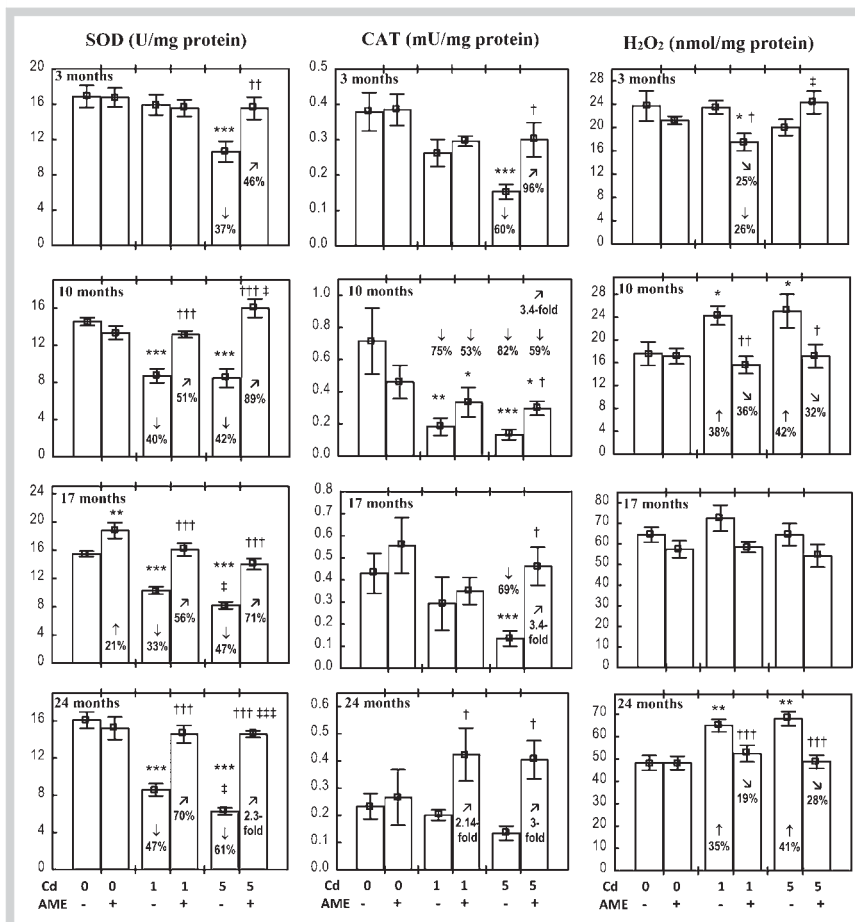
The administration of AME alone for 24 months, but not for shorter periods, decreased the TOS of the bone tissue and the level of oxidative stress expressed as OSI (● Fig. 2). Moreover, the consumption of AME alone throughout the experiment had no impact on the H<sub>2</sub>O<sub>2</sub> concentration in the bone tissue (● Fig. 4). In the rats exposed to the 5 mg Cd/kg diet, the TOS of the bone tissue was increased from the 10th month of the experiment, whereas at the lower treatment, this effect was observed only after 24 months (● Fig. 2). Treatment with the 5 mg Cd/kg diet induced oxidative stress (expressed as OSI) in the bone tissue already after 3 months and the effect was more markedly advanced after 3 and 10 months than after 17 and 24 months. In the animals intoxicated with the 1 mg Cd/kg diet, the OSI was increased after 10 and 24 months. At both levels of Cd exposure, the concentration of H<sub>2</sub>O<sub>2</sub> in the bone tissue was increased after 10 and 24 months (● Fig. 4). The administration of AME under the exposure to the 1 and 5 mg Cd/kg diets completely prevented this heavy metal-induced increase in the bone concentration of H<sub>2</sub>O<sub>2</sub> (● Fig. 4), TOS, and OSI (● Fig. 2). After 17 and 24 months, TOS and OSI in the Cd<sub>1</sub> + AME and Cd<sub>5</sub> + AME groups, except for OSI in the Cd<sub>1</sub> + AME group after 17 months, were even lower than the respective control values (● Fig. 2).

The administration of AME alone for 3, 17, and 24 months decreased the bone concentration of PC (● Fig. 5). After 24 months, the bone concentrations of 8-isoP, LPO, and 8-OHdG also decreased (● Figs. 5 and 6). The exposure to 1 and 5 mg Cd/kg diets for 10, 17, and 24 months increased the bone tissue concentration of PC, whereas concomitant AME administration provided partial (after 10 and 17 months) or complete (after 24 months) protection from this change. Moreover, PC concentration in the Cd<sub>1</sub> + AME and Cd<sub>5</sub> + AME groups after 3 months was lower com-



**Fig. 3** Effect of AME on the activities of GPx and GR in the bone tissue of rats chronically exposed to Cd. The rats received Cd in their diet at concentrations of 0, 1, and 5 mg/kg and 0.1% aqueous AME or not ("+" and "-", respectively). Data are represented as mean ± SE for 8 rats, except for 7 animals in the AME group, and the groups exposed to the 1 and 5 mg Cd/kg diets alone after 24 months. Statistically significant differences (Anova, Duncan's multiple range test): \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. control group; †p < 0.05, ††p < 0.01, †††p < 0.001 vs. respective group receiving Cd alone; ‡p < 0.05, ‡‡p < 0.01, ‡‡‡p < 0.001 vs. respective group receiving 1 mg Cd/kg diet (alone or with AME). Numerical values in bars or above the bars indicate the percentage change or a factor of change compared to the control group (↓ decrease; ↑ increase) or the respective group receiving Cd alone (↘ decrease; ↗ increase).

pared to the Cd<sub>1</sub> and Cd<sub>5</sub> groups, respectively (● Fig. 5). The bone concentration of 8-isoP in the animals exposed to the 1 and 5 mg Cd/kg diets alone was increased throughout the experiment, except for the Cd<sub>5</sub> group after 3 months. The concomitant administration of AME completely prevented this impact of Cd. Moreover, the bone 8-isoP concentration in the Cd<sub>1</sub> + AME group after 17 months, and in the Cd<sub>5</sub> + AME group after 24 months, was even lower compared to the control group (● Fig. 6). The exposure to Cd alone had no impact on the bone concentration of LPO, except for its increase in the Cd<sub>5</sub> group after 24 months (● Fig. 6). The bone tissue concentration of LPO in the Cd<sub>1</sub> + AME group after 3 and 24 months, and in the Cd<sub>5</sub> + AME group after 17 and 24 months, was lower than in the control group, providing clear evidence of the protective impact of AME (● Fig. 6). The exposure to the 1 mg Cd/kg diet for up to 24 months had no impact on the bone tissue concentration of 8-OHdG, whereas at the higher



**Fig. 4** Effect of AME on the activities of SOD and CAT and H<sub>2</sub>O<sub>2</sub> concentration in the bone tissue of rats chronically exposed to Cd. The rats received Cd in their diet at concentrations of 0, 1, and 5 mg/kg and 0.1% aqueous AME or not (“+” and “-”, respectively). Data are represented as mean ± SE for 8 rats, except for 7 animals in the AME group, and the groups exposed to the 1 and 5 mg Cd/kg diets alone after 24 months. Statistically significant differences (Anova, Duncan’s multiple range test): \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. control group; †*p* < 0.05, ††*p* < 0.01, †††*p* < 0.001 vs. respective group receiving Cd alone; ‡*p* < 0.05, ‡‡*p* < 0.001 vs. respective group receiving 1 mg Cd/kg diet (alone or with AME). Numerical values in bars or above the bars indicate the percentage change or a factor of change compared to the control group (↓ decrease; ↑ increase) or the respective group receiving Cd alone (↘ decrease; ↗ increase).

treatment, the value of this marker of oxidative DNA damage was increased after 17 and 24 months (● Fig. 5). AME not only provided complete protection from the Cd-induced increase in 8-OHdG, but also after 24 months at both levels of this metal treatment, the value of this parameter was even lower compared to the control group. Moreover, the beneficial effect of AME consumption, under intoxication with the 1 and 5 mg Cd/kg diets in terms oxidative DNA damage, was evident already after 10 months, as the concentration of 8-OHdG in the Cd<sub>1</sub> + AME and Cd<sub>5</sub> + AME groups was lower compared to the respective groups treated with Cd alone (● Fig. 5).

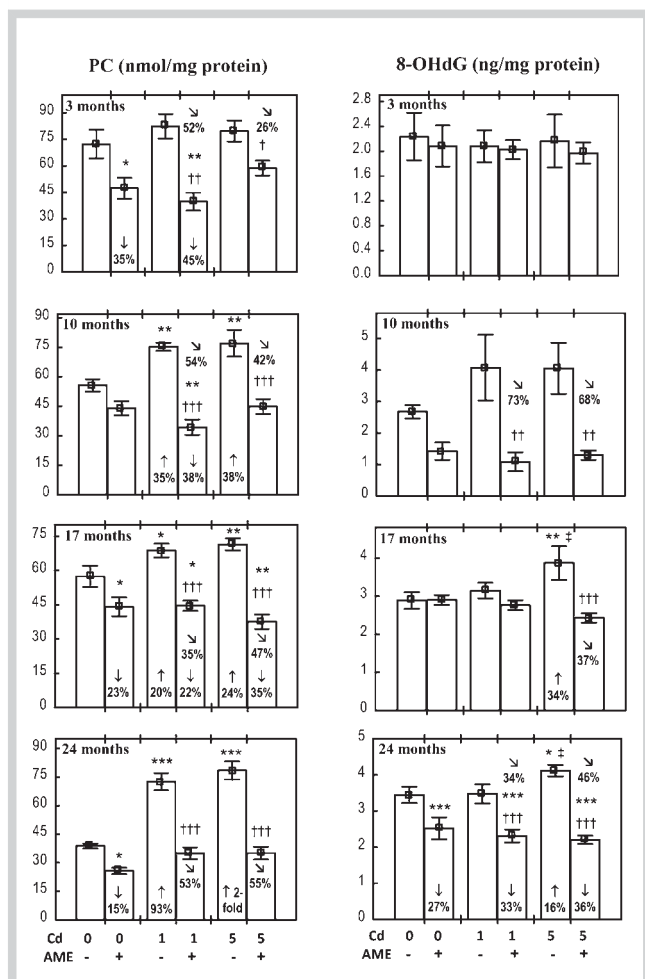
The results of the ANOVA/MANOVA analysis suggest that the improvement of the oxidative/antioxidative status of the serum and bone, and protection from oxidative changes in the bone tissue due to AME administration under the exposure to Cd, was the result of both an independent action of the extract and an interaction of its ingredients with Cd; however, the independent effect of AME seems to be stronger (Tables 55–75, Supporting Information).

Numerous positive dependences occurred between indices of oxidative bone status and markers of oxidative protein, lipid, and DNA damage in the bone tissue of rats receiving AME (alone and with Cd; Table 85, Supporting Information). Moreover, a close relationship existed between the oxidative/antioxidative status of the serum and bone (Table 95, Supporting Information). In the rats administered AME, alone and under Cd exposure, numerous correlations were noted between indices of the oxidative/antioxidative bone status and markers of bone turnover in the serum (concentrations of OC and CTX) and bone tissue (activity of ALP, concentrations of sRANKL and OPG and the ratio of

sRANKL/OPG; ● Table 2) as well as the femur BMD and indices of bone mineral status at the femoral distal epiphysis (% mineral comp., Ca/AW, and Ca/DW; ● Table 3) previously determined in these animals [23]. TAS and some other indices of the antioxidative bone status positively correlated with the markers of bone formation and indices of the bone mineral status, and negatively with chosen markers of bone resorption. Moreover, TOS, OSI, and numerous other indices of oxidative bone status, including markers of oxidative modifications of lipid, protein, and DNA, negatively correlated with the indices of bone formation, and positively with bone resorption markers (● Tables 2 and 3). In the animals receiving or not receiving AME under the exposure to Cd, negative correlations were noted between this heavy metal concentration in the bone at the distal femoral epiphysis and TAS ( $r = -0.232$ ,  $p < 0.01$ ), the activities of GPx, SOD, and CAT ( $r = -0.303$ ,  $r = -0.485$ ,  $r = -0.268$ , respectively;  $p < 0.001$ ), as well as the T-SH concentration ( $r = -0.231$ ,  $p < 0.01$ ). Moreover, the bone Cd concentration positively correlated ( $p < 0.001$ ) with OSI ( $r = 0.262$ ) and the concentrations of PC ( $r = 0.248$ ) and 8-isoP ( $r = 0.297$ ).

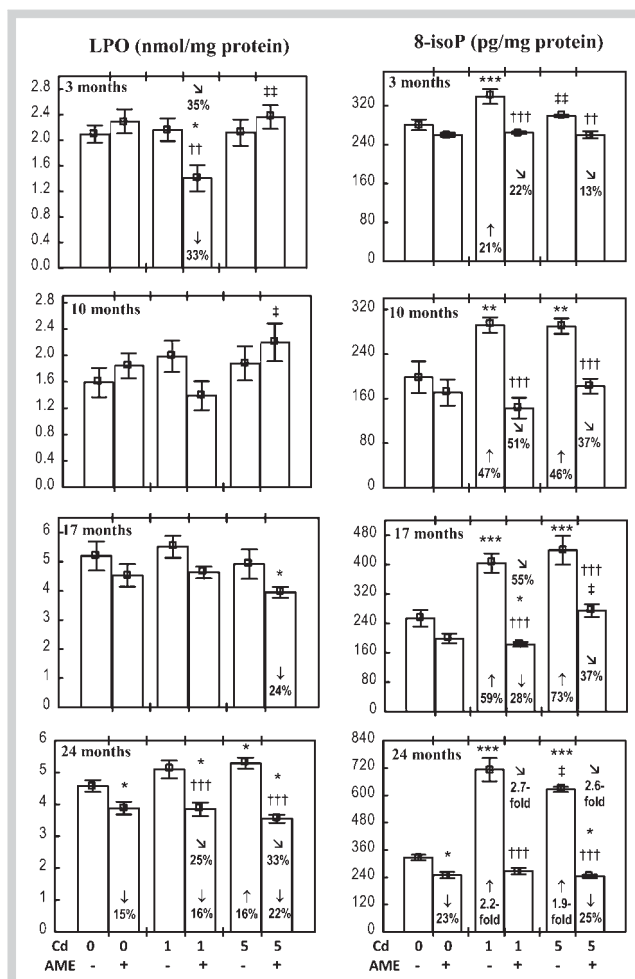
## Discussion

The present paper has been focused on the involvement of the oxidative defense system in the mechanisms of the osteoprotective impact of a polyphenol-rich chokeberry extract under exposure to Cd. Oxidative-reductive processes are an integral component of continuously ongoing bone remodelling, and the bone tis-



**Fig. 5** Effect of AME on oxidative protein and DNA damage in the bone tissue of rats chronically exposed to Cd. The rats received Cd in their diet at concentrations of 0, 1, and 5 mg/kg and 0.1% aqueous AME or not ("+" and "-", respectively). Data are represented as mean  $\pm$  SE for 8 rats, except for 7 animals in the AME group, and the groups exposed to the 1 and 5 mg Cd/kg diets alone after 24 months. Statistically significant differences (Anova, Duncan's multiple range test): \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control group; † $p < 0.05$ , †† $p < 0.01$ , ††† $p < 0.001$  vs. respective group receiving Cd alone; ‡ $p < 0.05$  vs. respective group receiving 1 mg Cd/kg diet (alone or with AME). Numerical values in bars or above the bars indicate the percentage change or a factor of change compared to the control group ( $\downarrow$  decrease;  $\uparrow$  increase) or the respective group receiving Cd alone ( $\searrow$  decrease).

sue oxidative/antioxidative status plays an important role in its physiology and pathology [24–27]. Oxidative stress has been recognized as one of the mechanisms of osteotoxic Cd action [24, 26, 27]; however, the exact mechanisms of this metal's involvement in the development of bone damage have not been explained until now. Based on the available data [24, 26, 27], it seems that Cd-induced ROS, which cannot be removed due to their excessive generation, and insufficient antioxidative protection may cause oxidative damage to the macromolecules in the bone tissue and influence the bone turnover. Excessive production and accumulation of ROS has a detrimental impact on bone metabolism via influencing the RANK/RANKL/OPG system [24–26]. ROS (particularly  $H_2O_2$ ) increases the number of osteoclasts and activates their function, leading to a loss of bone mass and a decrease in bone formation via inhibiting osteoblasts generation from osteo-



**Fig. 6** Effect of AME on lipid peroxidation in the bone tissue of rats chronically exposed to Cd. The rats received Cd in their diet at concentrations of 0, 1, and 5 mg/kg and 0.1% aqueous AME or not ("+" and "-", respectively). Data are represented as mean  $\pm$  SE for 8 rats, except for 7 animals in the AME group, and the groups exposed to the 1 and 5 mg Cd/kg diets alone after 24 months. Statistically significant differences (Anova, Duncan's multiple range test): \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control group; † $p < 0.05$ , †† $p < 0.01$ , ††† $p < 0.001$  vs. respective group receiving Cd alone; ‡ $p < 0.05$ , †† $p < 0.01$ , ††† $p < 0.001$  vs. respective group receiving 1 mg Cd/kg diet (alone or with AME). Numerical values in bars or above the bars indicate the percentage change or a factor of change compared to the control group ( $\downarrow$  decrease;  $\uparrow$  increase) or the respective group receiving Cd alone ( $\searrow$  decrease).

progenitor cells and shortening their survival [25]. The possible mechanisms of pro-oxidative Cd action in the bone tissue and oxidative stress involvement in the development of damage to the skeleton have been previously reported by us [24, 27] and thus they are not discussed in detail in this paper.

The measurements performed in the present study not only confirmed our previous findings, but they also revealed that this heavy metal deregulates the oxidative-reductive processes, leading to the development of oxidative stress with oxidative lipid and protein modifications in the bone tissue, even at lower exposure (1 mg Cd/kg diet) and lower bone concentration ( $0.0425 \pm 0.0036 \mu\text{g/g}$  dry wt.) than had previously been revealed [24, 27]. The fact that TAS was unchanged due to the 24-month exposure to the 1 and 5 mg Cd/kg diets in spite of the decreased activities of GPx and SOD may be explained by the increased GR activity,

**Table 2** Relationships between markers of bone turnover and indices of the oxidative/antioxidative status of the bone tissue in rats receiving AME alone and under exposure to Cd.

	Serum		Bone tissue at the distal femoral diaphysis			
	OC	CTX	ALP	OPG	sRANKL	sRANKL/OPG
<b>Indices of antioxidative status</b>						
TAS	0.311***	NS	0.318***	NS	NS	-0.206**
GPx	0.430***	-0.399***	-0.211**	0.524***	-0.230***	-0.522***
GR	-0.252***	0.323***	NS	-0.263***	0.328***	0.351***
SOD	0.504***	NS	0.378***	0.436***	-0.270***	-0.507***
CAT	0.265***	-0.164*	0.228**	0.301***	-0.301***	-0.421***
GSH	0.166*	NS	-0.394***	0.223**	0.208**	NS
T-SH	0.261***	NS	-0.334***	0.356***	NS	-0.266***
<b>Indices of oxidative status</b>						
TOS	NS	0.143*	-0.634***	NS	0.491***	0.207**
OSI	-0.171*	0.235**	-0.437***	NS	0.496***	0.390***
H <sub>2</sub> O <sub>2</sub>	NS	0.173*	-0.673***	0.205**	0.440***	NS
PC	-0.370***	0.423***	NS	-0.369***	0.377***	0.495***
8-isoP	-0.375***	0.397***	-0.349***	-0.372***	0.509***	0.581***
LPO	NS	0.169*	-0.654***	0.213***	0.436***	NS
8-OHdG	-0.175*	0.152*	-0.485***	NS	0.338***	0.193**

Data are represented as a correlation coefficient (r) and the level of statistical significance (p); \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; NS: not statistically significant (p > 0.05)

which might be a defensive response to the decreased GPx activity. The increased bone concentration of H<sub>2</sub>O<sub>2</sub> might result from both its insufficient detoxification due to the decreased activity of GPx and/or CAT, and increased production. It seems possible that Cd might stimulate ROS formation, including H<sub>2</sub>O<sub>2</sub>, and affect their detoxification via influencing (directly and indirectly) the activity of the bone cells [24,26].

The present paper is the first report providing evidence that the consumption of chokeberry extract under chronic exposure to Cd may offer protection from oxidative stress and oxidative modifications of protein, lipid, and DNA in the bone tissue, and that its protective action against Cd-induced bone damage is mediated by the oxidative defense system. The protective effect of AME can be explained by its high content of polyphenolic compounds, especially including anthocyanins, which possess strong antioxidative potential [13,14,18–20]. Polyphenols act as direct free radical scavengers [20], inhibit the activities of pro-oxidative enzymes [20], and increase antioxidative enzyme activities [3,8,9] as well as play a role in the regeneration of antioxidative vitamins (vitamin C and E) [29,30]. The observations made in the animals receiving AME alone revealed the effectiveness of the extract in the improvement of the antioxidative system of the bone tissue. Moreover, other ingredients of *A. melanocarpa* berries, including vitamin C, vitamin E, β-carotene, and bioelements, which influence the activity of antioxidative enzymes such as manganese, zinc, copper, selenium, and iron [14], might also contribute to the favorable impact of AME. Owing to the relationship between the level of ROS and the rate of bone turnover [24–27], it should be taken into account that the direct beneficial impact of AME on the oxidative/antioxidative bone status under Cd exposure might also be related, at least partly, to the influence of some ingredients of the extract, including polyphenols and zinc, on the activity of osteoclasts and the process of bone formation [27,31,32]. Chlorogenic acid has also been reported to inhibit osteoclasts differentiation and bone resorption by the downregulation of RANKL [31], whereas quercetin and zinc have been noted to stimulate bone formation [27,32–34]. Zinc not only stimulates osteoblasts proliferation and differentiation, but it also suppresses osteoclasts differentiation by, among others, antagonizing NF-κB

**Table 3** Relationships between indices of bone mineral status and oxidative/antioxidative bone status in rats receiving AME alone and under exposure to Cd.

	Indices of bone mineral status			
	Femur BMD	Bone tissue at the distal femoral epiphysis % mineral comp.	Ca/DW	Ca/AW
<b>Indices of antioxidative status</b>				
TAS	0.302***	0.421***	0.331***	NS
GPx	0.561***	NS	0.250***	0.331***
GR	-0.297***	NS	NS	NS
SOD	0.480***	0.503***	0.268***	NS
CAT	0.270***	0.292***	0.275***	0.189*
GSH	0.162*	-0.210**	NS	NS
T-SH	0.365***	NS	NS	NS
<b>Indices of oxidative status</b>				
TOS	NS	-0.524***	-0.144*	NS
OSI	-0.276***	-0.541***	-0.260***	NS
H <sub>2</sub> O <sub>2</sub>	NS	-0.517***	-0.254***	NS
PC	-0.612***	-0.184*	-0.149*	-0.201*
8-isoP	-0.430***	-0.271***	-0.536***	-0.506***
LPO	NS	-0.455***	-0.259***	NS
8-OHdG	-0.198**	-0.433***	-0.232**	NS

Data are represented as a correlation coefficient (r) and the level of statistical significance (p); \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; NS: not statistically significant (p > 0.05)

activation [27,33,34]. Polyphenols, due to their ability to bind Cd<sup>2+</sup> [4], may decrease the body burden of Cd and thus protect against the effects of its direct and indirect action, resulting in the improvement of the oxidative/antioxidative balance and bone turnover [6,15,22,23]. It is also necessary to take into consideration that the molecular mechanisms of the beneficial impact of polyphenols on bone tissue metabolism might be related to the ability of these compounds to enhance the production of nitric oxide, which is essential for the induction of new bone formation [35], as well as to induce the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant gene expressions and thereby to decrease the oxidative status [7].

We have previously reported that AME administration under exposure to the 1 mg Cd/kg diet had only a very slight protective impact against this metal's accumulation in the organism, whereas its application under treatment with the 5 mg Cd/kg diet significantly decreased the apparent absorption and retention in the body and increased urinary concentration of this heavy metal, resulting in its lower concentration in the blood and lower accumulation in soft tissues (mainly in the liver and kidneys) and bone tissue. Due to the lower Cd transportation into the sites of its toxic action, including bone tissue, and faster elimination from the organism of the absorbed metal, the amounts of Cd at the sites of its action, including oxidative action, were lower, and thus the effects of its toxicity were less intensified or even completely counteracted by AME administration [22]. Since Cd, even at low concentrations, may directly influence bone tissue cell activity and oxidative/antioxidative balance, it seems reasonable to assume that the beneficial impact of AME on the oxidative/antioxidative status of the bone tissue under the higher of the investigated levels of Cd treatment was, at least to some extent, the result of the lower bone concentration of this metal. Thus, via decreasing Cd accumulation in the bone and kidneys, AME might provide protection from the direct and/or indirect impact of this xenobiotic (depending on the exposure level) on the skeleton [22, 23, 36]. Numerous correlations noted between Cd concentration in the bone tissue and the measured markers of the bone oxidative/antioxidative status in the animals receiving, or not, AME under the treatment with this metal confirm the possibility of such a mechanism. The fact that AME administration under exposure to the 1 mg Cd/kg diet improved the oxidative/antioxidative bone status without influencing the bone concentration of this metal shows the direct protective impact of this extract's ingredients on the oxidative/antioxidative balance in bone tissue. Because the possible protective impact of AME on the bone tissue under and without exposure to Cd has not been investigated until now by other authors, a wider discussion of our results is impossible. Moreover, it should be taken into consideration that the improvement of the oxidative/antioxidative status of the serum (reflecting the whole body oxidative/antioxidative status) and bone tissue by AME administration under physiological conditions and exposure to Cd might be related to the ability of polyphenols to penetrate into tissues (including bone). These compounds have been reported to be present in various organs of rats, including target organs for Cd, such as kidneys and liver [37]; however, their penetration into the bone tissue has not been investigated, but it cannot be excluded. The available data indicating that consumption of polyphenols improves bone turnover and BMD in both human [21] and experimental animals [16, 23] confirm the possibility of the direct impact of these compounds on bone status.

We are aware that our study does not allow for explaining the exact mechanisms of the oxidative defense system's involvement in the protective action of the chokeberry extract against Cd-induced disorders in bone metabolism. The next limitation of this study is conducting the experiment only in a female rat model. Because sex hormones influence Cd metabolism and toxicity, and the female skeleton is more susceptible to the damaging impact of this heavy metal [38–40], the conclusion on the beneficial impact of AME on bone oxidative/antioxidative status and the involvement of the extract's antioxidative potential in the mechanisms of osteoprotective influence refers only to the female skeleton.

In summary, it has been revealed for the first time that consumption of the chokeberry extract under low and moderate chronic exposure to Cd exerts an osteoprotective impact via mediating the oxidative defense system and preventing oxidative modifications of protein, lipid, and DNA in the bone tissue. This is the most important and practically useful finding of the present study. Based on the results, it seems possible that the chokeberry may be a promising natural plant origin product for preventing oxidative stress-related bone damage in women chronically exposed to Cd. The results support the potential of *A. melanocarpa* berries, recently reported by us [6, 22, 23], to be an effective natural product for protection against Cd toxicity. However, the possible prophylactic use of the chokeberry under environmental exposure to Cd needs further investigation involving epidemiological studies in both men and women.

## Materials and Methods



### Chemicals

All chemicals and reagents were of the highest grade purity or analytical purity. Most of the assays were performed with the use of commercial kits. Ultrapure water was used in all measurements.

### Cadmium diets

Homogeneous in terms of Cd content diets, containing 1 and 5 mg Cd/kg, were prepared by Label Food "Morawski" by the addition of cadmium chloride ( $\text{CdCl}_2 \times 2\frac{1}{2} \text{H}_2\text{O}$ ; POCh) into the ingredients of the standard Labofeed H diet (breeding diet) and Labofeed B diet (maintenance diet) [22]. The mean Cd concentration determined in the diets reached  $1.09 \pm 0.13$  mg/kg and  $4.92 \pm 0.53$  mg/kg, respectively, whereas its concentration in the standard Labofeed diets was  $0.0584 \pm 0.0049$  mg/kg (mean  $\pm$  SD).

### *Aronia melanocarpa* extract

A certified (Certificate KJ 4/2010) lyophilized AME, containing 65.74% of polyphenols (18.65% of anthocyanins), was supplied by Adamed Consumer Healthcare. The polyphenolic profile of *A. melanocarpa* berries is well known and widely reported [13, 14, 18]; however, the phytochemical profile of AME was estimated (Fig. 1S, Supporting Information) and quantified by us (Table 10S, Supporting Information) [23]. The total polyphenols content in the lyophilized AME and the compounds concentration in its 0.1% aqueous solution reached  $61.24 \pm 0.33\%$  and  $0.612 \pm 0.003$  mg/mL (mean  $\pm$  SE), respectively [23].

According to the producer's declaration and literature data [13, 14], AME also contains other components such as sugar, pectins, sugar alcohols (sorbitol, parasorboside), phytosterols, triterpenes, and carotenoids as well as minerals and vitamins.

### Animals

One hundred and ninety-two young (3–4 weeks old) female Wistar rats [CrI:WI (Han)] purchased from the certified Laboratory Animal House in Brwinów were used. The animals were kept in controlled conventional conditions (temperature  $22 \pm 2^\circ\text{C}$ , relative humidity  $50 \pm 10\%$ , 12-h light/dark cycle). They had free access to drinking water and food. Throughout the first three months, all animals were maintained on the Labofeed H diet and thereafter they received the Labofeed B diet.



## Experimental protocol

The study was approved by the Local Ethics Committee for Animal Experiments in Białystok (Poland; approval number 60/2009 on 21 September 2009). All procedures with the animals were performed according to the ethical principles and institutional guidelines and International Guide for the Use of Animals in Biomedical Research.

After a five-day acclimatization, the rats were randomly allocated into six experimental groups, each containing 32 animals (mean body weight about 65 g). One group received AME alone (AME group), two groups were treated with Cd alone via the diet containing 1 and 5 mg Cd/kg (Cd<sub>1</sub> and Cd<sub>5</sub> groups), and the next two groups received AME during the exposure to Cd (Cd<sub>1</sub> + AME and Cd<sub>5</sub> + AME groups) for 3, 10, 17, and 24 months. The last group, maintained on redistilled water (<0.05 µg Cd/L) and standard Labofeed diet (without AME and Cd), served as a control. AME was administered as a 0.1% aqueous solution of the powdered extract, used as the only drinking fluid. The solution was prepared daily (stable for at least 24 h) and contained <0.05 µg Cd/L. The concentration of the aqueous solution of AME was chosen based on the available literature data [15,41] to reach polyphenol intake markedly higher than the recommended daily consumption, but not too high.

In our study, there was no positive control since, until now, there was no effective chelating therapy for Cd. Moreover, the synthetic compounds used for this purpose are strong chelators of bioelements and, thus, they cannot be administered for long periods [6].

The daily intake of Cd throughout the 24-month exposure to the 1 and 5 mg Cd/kg diets reached 37.50–84.88 µg/kg b.wt and 196.69–404.76 µg/kg b.wt, respectively, whereas polyphenol intake reached 41.5–104.6 mg/kg b.wt, irrespective of whether these substances were administered alone or in conjunction [22, 23]. There were no clinical signs of morbidity during the study; however, three cases of spontaneous death (in the AME, Cd<sub>1</sub>, and Cd<sub>5</sub> groups) were noted between the 17th and 24th month. The experimental protocol has been presented in detail in our previous reports [22,23]. Since females are more vulnerable to Cd toxicity, including bone damage, than males [38–40], the study was conducted in females.

At termination, the animals were anesthetized with barbiturate (Morbital, 30 mg/kg b.wt, i. p.) and whole blood was collected by cardiac puncture in tubes with and without anticoagulant (heparin). Next, different organs and tissues, including both femurs and tibias, were dissected. A portion of the whole blood collected without anticoagulant was centrifuged after coagulation and the serum was separated. The bones, after cleaning of the surrounding muscles and tissues, were weighted. The biological material not used immediately was stored frozen at –70 °C or –20 °C until all measurements were performed.

Samples of the serum and bone slices from the distal femoral epiphysis (trabecular bone region) were used in this study. To evaluate the oxidative/antioxidative bone status, numerous indices of the enzymatic (GPx, GR, SOD, and CAT) and nonenzymatic (GSH and T-SH) antioxidative barrier, TAS (a marker of total antioxidative potential), TOS (a marker of total oxidative status), and concentration of H<sub>2</sub>O<sub>2</sub> were measured in the bone tissue. TAS and TOS were also determined in the serum, and the OSI (a marker of oxidative stress intensity) was calculated (OSI = TOS/TAS). In order to evaluate the extent of oxidative changes in the bone tissue, sensitive markers of oxidative modifications of protein and DNA,

such as PC and 8-OHdG, respectively, as well as LPO and 8-isoP, as markers of lipid peroxidation, were determined.

## Analytical procedures

**Preparation of bone tissue homogenates:** With the aim of evaluating all indices of the oxidative/reductive bone status, except for 8-OHdG, 10% homogenates of the bone from the distal femoral epiphysis in a cold potassium phosphate buffer (50 mM, pH = 7.4) with the addition of butyl-hydroxytoluene were prepared using a high-performance homogenizer (Ultra-Turrax T25; IKA), as previously reported [24]. Each homogenate was divided into two portions – one was centrifuged (MPW-350R centrifugator, Medical Instruments) at 700 × g for 20 min (for CAT, TAS, TOS, H<sub>2</sub>O<sub>2</sub>, LPO, and PC measurements) and the other at 20000 × g for 30 min (for GPx, GR, and SOD) at 4 °C, and the aliquots were collected for measurements [42].

**Assays:** TAS and TOS were determined with the use of ImAnOx (TAS) ELISA kit and PerOx (TOS) ELISA kit by Immundiagnostik AG. The assay of TAS is based on the reaction of antioxidants present in the analyzed sample with a defined amount of exogenously provided H<sub>2</sub>O<sub>2</sub> and photometrical determination of the residual H<sub>2</sub>O<sub>2</sub>, while under the assay of TOS, total lipid peroxides are measured in the reaction with peroxidase.

The activities of GPx, GR, and SOD were determined with the use of a Bioxytech GPx-340 kit (OxisResearch), Bioxytech GR-340 kit, and kit by the Cayman Chemical Company, respectively, whereas the CAT assay was performed according to the spectrophotometric method by Aebi [43]. The concentration of GSH was measured using the Bioxytech GSH-400 kit (OxisResearch) and that of T-SH by the modified Ellman's method [44]. H<sub>2</sub>O<sub>2</sub> was determined according to the method described in the Bioxytech H<sub>2</sub>O<sub>2</sub> – 560 kit by Oxis. PCs were determined with the spectrophotometric method based on the reaction of PC with 2,4-dinitrophenylhydrazine [45]. 8-isoP and LPO were assayed using an 8-isoP EIA kit by the Cayman Chemical Company and a Bioxytech LPO-586 kit by Oxis. The concentration of 8-OHdG was determined using an ELISA kit by Percipio Biosciences after DNA isolation from the bone with the use of the Nuclear Extraction Kit by the Cayman Chemical Company.

All assays with the use of commercial kits were performed as described in the manufacturers' instructions, and the measured parameters were adjusted for protein concentration (determined with the use of an EMAPOL kit). Analytical quality of all measurements was checked and is available, together with the main principles of the performed assays (if not described in this section), as Supporting Information.

An automated microplate washer (Thermolabsystems Wellwash 4, Labsystems), Thermo Scientific Multiskan GO spectrophotometer, ELISA universal microplate reader (BIO-TEK INSTRUMENTS INC, ELX<sub>800</sub>), and Hitachi U-3010 spectrophotometer were employed.

## Statistical analysis

The Statistica 10 package (StatSoft) was used to analyze the data. A one-way analysis of variance (Anova) with Duncan's multiple range post hoc test was conducted for comparisons between individual groups and to determine which two means differed statistically significantly ( $p < 0.05$ ). To discern the possible interactions between Cd and AME, a two-way analysis of variance (ANOVA/MANOVA, test F) was conducted. F values having  $p < 0.05$  were considered statistically significant. Spearman rank

correlation analysis was performed to investigate mutual relationships among variables.

### Supporting information

Details on the effects of AME and Cd, the UPLC polyphenolic profile of the extract, and the main principles of the assays of the indices of the oxidative/antioxidative status and analytical quality of these measurements are available as Supporting Information.

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### Conflict of Interest

The authors declare that there are no conflicts of interest.

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