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Original Paper

Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue

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Key Words
N-acetylcysteine • Alpha-lipoic acid • Oxidative stress • Matrix metalloproteinase-2 • Matrix metalloproteinase-9

Abstract
Background/Aims: The high-fat diet (HFD) regime causes obesity and contributes to the development of oxidative stress in the cells by the production of reactive oxygen species and the occurrence and progress of inflammation. Despite years of studies, there is no data explaining the mechanism of action of N-acetylcysteine (NAC) or alpha-lipoic acid (ALA) on matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) in visceral and subcutaneous adipose tissue of HFD-fed rats. Our experiment aimed to evaluate for the first time the influence of chronic antioxidants administration on MMPs biology after an HFD regime as a potential therapeutic strategy for obesity-related complications prevention. ***Methods:*** Male Wistar rats were fed a standard rodent chow or an HFD with intragastric administration of NAC or ALA for ten weeks. The collected samples were subjected to pathohistological evaluation. Real-time PCR and western blot approaches were used to check whether NAC or ALA impacts MMP2/9 expression. ***Results:*** Antioxidant supplementation markedly reduced the number of circulating inflammatory cytokines, and tissue macrophage infiltration. Moreover, NAC and ALA have a divergent impact on MMP2 and MMP9 expression in different adipose tissue localization. ***Conclusion:*** Based on our results, we speculate that NAC and ALA have a prominent effect on the MMP2/9 functions under obesity conditions.

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Introduction

Nowadays, overweight and obesity are considered global epidemics and are the leading causes of death (roughly 2.8 million people die each year because of the conditions) [1]. Obesity is the main factor predisposing to the development of cardiovascular, respiratory, neurological, gastrointestinal, hepatic, endocrine, skeletal, and renal complications, as well as to a significant psychosocial burden or an increased incidence of cancer [2, 3]. The risk of obesity-related complications is associated with the amount of adipose tissue, its location (abdominal obesity or gluteal-femoral obesity), and the duration of the underlying disease [3].

Recent studies have demonstrated that matrix metalloproteinases (MMPs) are involved in processes taking place in adipose tissue, such as adipogenesis and angiogenesis. The sources of MMPs include adipocytes, preadipocytes, fibroblasts, endothelial cells, as well as immune cells [4]. MMPs are involved in physiological and pathological complications of obesity through the degradation and remodeling of the extracellular matrix (ECM) molecules [5]. In this research, we were focused on metalloproteinases 2 (MMP2) and 9 (MMP9), otherwise known as gelatinases A and B, which belong to type IV collagenases. The main function of the gelatinases is the degradation of type IV collagen, which is the main component of the vascular basement membrane. Damage to this barrier facilitates the migration of leukocytes to adipose tissue, thus enhancing its inflammation. The activation of the leukocytes causes the release of pro-inflammatory cytokines and MMPs, the above drives the vicious circle even further [6].

A high-fat diet (HFD) regime, and thus a positive energy balance, causes obesity and contributes to the development of oxidative stress in the cells, the production of reactive oxygen species (ROS), and the progress of inflammation [7, 8]. The increase in the amount of ROS in adipose tissue leads to impaired adipogenesis, recruitment, and activation of macrophages, secretion of inflammatory adipokines, or damage to the tissue's biological structures. The coexistence of oxidative stress and inflammation in adipose tissue contributes to the subsequent development of obesity and the formation of a vicious circle [9]. It was proved that antioxidants supplementation, e.g. with N-acetylcysteine (NAC) or alpha-lipoic acid (ALA), can reduce oxidative stress, the number of inflammatory cytokines, macrophage infiltration, and ultimately other complications of obesity [9, 10]. Hence, alteration in MMPs metabolism (e.g., MMP2 and MMP9) may be a therapeutic strategy for obesity and its complication. Thus, the goal of this study was to elucidate changes in adipose tissue matrix metalloproteinases, especially MMP2 and MMP9 after chronic antioxidants administration (NAC and ALA) after an HFD regime as a potential therapeutic strategy for obesity-related complications prevention.

Materials and Methods

Animals and Study Design

After six days of adaptation to the conditions in an animal facility, male Wistar rats were divided into the following four groups – control (CTRL), high-fat diet (HFD), a high-fat diet supplemented with N-acetylcysteine (HFD+NAC), and a high-fat diet supplemented with α-lipoic acid (HFD+ALA) (10 rats in each group). The CTRL group was formulated as the control for HFD, HFD+NAC, and HFD+ALA. Forty male Wistar rats were housed under standard conditions (21°C ± 2°C, 12h reverse light/dark cycle) with ad libitum access to a control standard chow (LSM, Agropol, Motycz, Poland; containing 10.3% fat, 24.2% protein, and 65.5% carbohydrate) or a high-fat diet (Research Diet, USA, catalog number D12492; containing 59.8% fat, 20.1% protein, 20.1% carbohydrate; 279.6 mg/kg of the cholesterol) from weaning until sacrifice. Wistar rats were fed a high-fat diet not isocaloric relative to control animals. After six weeks of the experiment, once daily, each morning between 8 and 9 am, the animals in the HFD+NAC group received N-acetylcysteine at a dose of 500 mg/kg body weight, whereas the rats from the HFD+ALA group received α-lipoic acid solution at a dose of 30 mg/kg body weight (once a day, every day for the consecutive 4 weeks). The solutions of NAC (Sigma-

Aldrich, catalog number: A9165) and ALA (Sigma-Aldrich, catalog number: PHR2561-1G) were prepared by dissolving the substances in the saline solution and immediately applied intragastrically by gastric gavage to rats from appropriate groups. The doses were based on the literature analysis [11, 12]. We decided to use 500 mg/kg body weight of NAC and 30 mg/kg body weight of ALA as those are the most frequently used, non-toxic doses with proven antioxidative effects [11–13]. Similarly, saline (2 ml/kg body weight) was administered to the CTRL and HFD-fed rats. Body weight was monitored every day and the amount of NAC, ALA, or saline solution was adjusted accordingly. The intragastric administration of antioxidants ensured that rats obtained a full dose calculated for their body weight. According to each rat's body weight, which was controlled every two days, the dose of antioxidants administration was adjusted. After ten weeks, the rats fasted twelve hours and were anesthetized by intraperitoneal phenobarbital injection (80 mg/kg body weight). The rats were placed lying down on a heating pad (37°C). Blood was drawn from the abdominal aorta and was immediately centrifuged to obtain plasma. Samples of adipose tissue (visceral and subcutaneous) were taken from the abdominal area. The harvested tissues were immediately frozen using aluminum forceps precooled with liquid nitrogen. All the obtained samples (adipose tissue and plasma) were stored at -80^{0}C until further analysis. All the experimental procedures were approved by the Ethical Committee for Animal Experiments at the Medical University of Bialystok, Poland.

Adipose tissue Histopathology

The samples of visceral and subcutaneous adipose tissue of the rats were taken and immediately fixed in 10% buffered formalin and processed routinely for embedding in paraffin. The paraffin blocks were cut into 4 μm sections, attached to positively charged glass slides (Superfrost Plus; Menzel Gläser, Braunschweig, Germany), and stained with hematoxylin and eosin.

Paraffin-embedded sections were deparaffined and hydrated in pure alcohols.

Toluidine Blue Stain Kit (no cat. SS057 BioGenex; 49026 Milmont Drive, Fremont, CA 94538 USA) was used to stain mast cells. The kit included: potassium permanganate, potassium metabisulphite, Scott's solution, Toluidine Blue Solution. The staining was done by following the manufacturer's instructions attached to the kit.

Immunostaining was performed by the following protocol: for antigen retrieval, the sections were subjected to pretreatment in a pressure chamber and heated using Target Retrieval Solution (S 1699 Agilent Technologies, Inc. 5301 Stevens Creek Blvd Santa Clara, CA 95051, USA). After cooling down to room temperature, the sections were incubated with Peroxidase-Blocking Solution for 10 minutes to block endogenous peroxidase activity.

The sections with the monoclonal mouse primary antibody (Agilent Technologies, Inc.), CD68 (M0876), were incubated for 1 hour at RT in a humidified chamber. The antiserum was previously diluted in Antibody Diluent, Background Reducing, Ready-to-use diluent (S 3022 Agilent Technologies, Inc.) in a ratio of 1:50 for CD68.

The procedure was followed by incubation (30 minutes) with secondary antibody (EnVision FLEX, High pH (Link), HRP. Rabbit/Mouse. (K800021-2 Agilent Technologies, Inc. 5301 Stevens Creek Blvd Santa Clara, CA 95051, USA). The bound antibody was visualized by 1-min incubation with DAB Flex chromogen. Finally, the sections were counterstained in hematoxylin QS (H-3404, Vector Laboratories; Burlingame, CA), mounted, and evaluated under the light microscope. Appropriate washing with Wash Buffer (S 3006 Agilent Technologies, Inc.) was performed between each step (3 times for 2 minutes). Sections were dehydrated with absolute alcohol followed by xylene, and coverslipped with Entellan (Merck). The specificity of the antibody was confirmed using a negative control, which involved replacing the antibody with the Antibody Diluent (no staining), and positive control, which involved staining a human tonsil with CD68.

The assessment of the cell size and number of CD68 was performed by two experienced histologists (independent from each other) and analyzed with ImageJ software (The National Institutes of Health, MD, USA). Images were converted to 8-bit greyscale, and the background was subtracted. Then, the binary threshold function was adjusted to separate the cells from background staining. The total cells area was calculated as the total number of pixels in images with a set threshold [14, 15]. Results were plotted in GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Results are depicted as mean ± standard deviations. The statistical significance level was set as $p<0.05$.

Table 1. Primers sequences for real-time PCR analysis

Gene	Primer Sequence		Annealing temperature
	Forward	Reverse	
β-actin	5'-ACGGTCAGGTCATCACTATCG-3'	5'-GGCATAGAGGTCTTTACGGATG-3'	58°C
MMP2	5'-AAAGGAGGGCTGCATTGTGAA-3'	5'-CTGGGGAAGGACGTGAAGAGG-3'	58°C
MMP9	5'-AGGTGCCTCGGATGGTTATCG-3'	5'-TGCTTGCCCAGGAAGACGAA-3'	59°C

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the rats' visceral and subcutaneous adipose tissue using the TriRreagent RNA Isolation Reagent according to the manufacturer's protocol (Sigma-Aldrich). The total RNA amount was determined by spectrophotometry and RNA quality was verified by measuring the sample's absorbencies at 260 and 280 nm [16]. The synthesis of the complementary DNA was done using the EvoScript universal cDNA master kit (Roche Molecular Systems, Boston, MA, USA). Specific primers used in this study are presented in Table 1. Real-time PCR was carried out using the LightCycler 96 System Real-Time thermal cycler with FastStart Essential DNA Green Master (Roche Molecular Systems). Cycling conditions were: 15s denaturation at 95°C, 15s annealing at 58°C for β-actin, MMP2, and 59°C for MMP9, and 15s extension at 72°C for 45 cycles. Melting curve analysis was performed before each reaction to verify PCR product specificity. The mRNA levels of the target genes were normalized to the rat's β-actin and calculated according to the Pfaffl method [17].

Proteins Analysis

To investigate the protein expression of various MMPs in visceral and subcutaneous adipose tissue extracts, we used Western blot analysis. The samples were homogenized in an ice-cold RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The total protein concentration was determined using the BCA method with bovine serum albumin (BSA) as a standard. Next, homogenates (20 µg of the total protein) were reconstituted in Laemmli buffer, separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated overnight with antibodies i.e., MMP2 (1:2500, cat. no. ab92536, Abcam, Cambridge, UK), MMP9 (1:10000, cat. no. ab76003, Abcam, Cambridge, UK), and β-actin (1:200, cat. no. ab115777, Abcam, Cambridge, UK). Thereafter, the PVDF membranes were incubated with secondary antibodies conjugated with anti-rabbit IgG conjugated to horseradish peroxidase (cat. no. 7074S, Cell Signaling). Protein bands were visualized using an enhanced chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA) and quantified densitometrically (ChemiDoc visualization system EQ, Bio-Rad Systems). Equal protein concentrations were loaded in each lane, which was confirmed by Ponceau S staining. Protein expression (Optical Density Arbitrary Units) was normalized to β-actin expression. Finally, the control was set to 100, and the experimental groups were expressed relative to the control.

Lipids Analysis

The content of plasma lipids (FFA, DAG, TAG, and PL) was measured using gas-liquid chromatography as described previously [18, 19]. The selected lipid fractions were extracted using Bligh and Dyer's method [20]. Then, the lipids were separated by thin-layer chromatography (TLC) into specific fractions. Next, they were fractionated on Silica Gel Plates (silica plate 60, 0.25 mm; Merck).

Individual fatty acid methyl esters (FAMEs) present in each fraction were identified and quantified according to the retention times of the standards by gas-liquid chromatography (Hewlett-Packard5890 Series II gas chromatograph, HP-INNOWax capillary column; Agilent Technologies, Santa Clara, CA, USA). The total amount of FFA, DAG, TAG, and PL was estimated as the sum of individual fatty acid species in the evaluated fraction and expressed in nanomoles per mg of protein.

Statistical Analysis

The obtained results were analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Briefly, in the first step, the assumptions of the statistical methods were checked. The normality of the results' distribution was tested using the Shapiro-Wilk test. Analysis of variance (ANOVA) followed by post-hoc pairwise Student's *t*-tests was carried out to determine the existence of differences between the studied groups. For all analyses, p values < 0.05 were considered to be statistically significant. The results are expressed as mean ± SD.

Results

Supplementation with antioxidants during a high-fat diet regime affects body weight and plasma metabolic parameters

In the present study, high-fat diet feeding was associated with greater body mass and metabolic parameters. To determine the effects of the antioxidants supplementation during the HFD regime, we evaluated weekly weight gain, glucose and insulin levels, as well as HOMA-IR value, and also a plasma lipids content in male Wistar rats, under the nutritional conditions (CTRL and HFD) and in response to the antioxidants supplementation during the HFD regime (HFD+NAC and HFD+ALA). The animals' body weight was significantly increased after HFD alone (+22%, p<0.0001, HFD vs. control group; Fig. 1a, b) as well as in the rats at HFD regime with NAC or ALA (+24%, p<0.0001; +18%, p<0.0001; HFD+NAC and HFD+ALA vs. control group; respectively; Fig. 1a, b). Our previous study established that the glucose and insulin levels, as well as HOMA-IR values, were significantly increased in HFD groups (+26%, p=0.014, HFD; +123%, p=0.0010, HFD+NAC; +8-fold, p=0.0021, HFD+ALA; vs. control group; respectively) [21]. Glucose and insulin levels, as well as HOMA-IR values were lower in HFD+NAC (-28%, p=0.0016; -51%, p=0.0042; -87%, p=0.0023; HFD+NAC vs. HFD group; respectively) and in HFD+ ALA groups (-19%, p=0.0210; -48%, p=0.0024; -88%, p=0.0022; HFD+ALA vs. HFD groups; respectively) [21]. Interestingly, we also observed significant differences in glucose level between the groups treated with the antioxidants (+12%, p=0.0172, HFD+ALA vs. HFD+NAC) [21]. As expected, the HFD caused an increase in the plasma phospholipid content (+31%, p=0.0009, vs. control group; Fig. 1d). There was a relevant decrease in the plasma diacylglycerols and triglycerides content in the NAC-treated group (-50%, p<0.0001, Fig. 1e; -45%, p=0.0009, Fig. 1f; respectively; vs. control group). Moreover, comparison between HFD and HFD+NAC revealed a pronounced decrease in the plasma phospholipids, diacylglycerols, and triglycerides content in the latter group (-23%, p=0.0040, Fig. 1d; -37%, p=0.0045, Fig. 1e; -53%, p=0.0080, Fig. 1f; respectively; vs. HFD

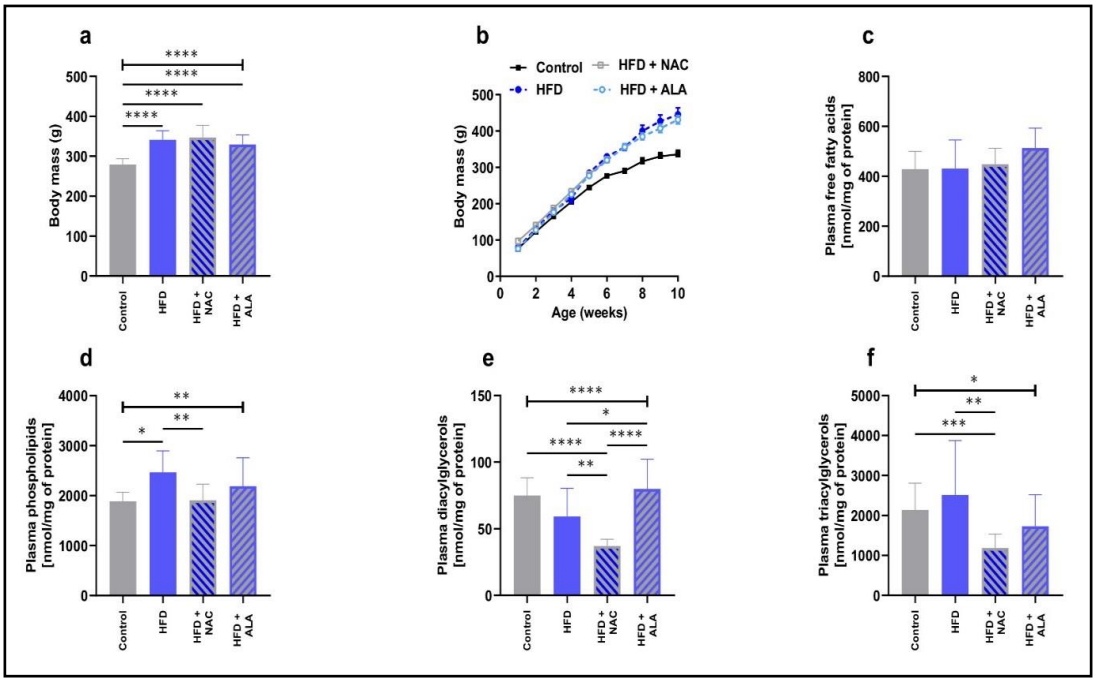

Fig. 1. The effects of N-acetylcysteine (NAC) and α-lipoic acid (ALA) supplementation on body mass, glucose and insulin levels, as well as HOMA-IR value, and plasma metabolic parameters. Control – control rats; HFD – high-fat diet-fed rats; HFD+NAC – high-fat diet-fed rats that received N-acetylcysteine; HFD+ALA – high-fat diet-fed rats that received α-lipoic acid; HOMA-IR – homeostatic model assessment of insulin resistance; *p<0.05 vs. control; #p<0.05 vs. HFD; ^p<0.05 vs. HFD+NAC.

group). On the other hand, we observed significant changes in the plasma diacylglycerols content in the NAC+ALA group (+35%, p=0.0491, vs. HFD group, Fig. 1e) as well as between the two groups with antioxidants treatment (+115%, p<0.0001, HFD+ALA vs. HFD+NAC group, Fig. 1e). Thus, the antioxidants supplementation during the HFD regime affects body weight, plasma glucose, and insulin levels, as well as HOMA-IR value, and plasma lipids content.

Histological changes in adipose tissue

Histological examination of the adipose tissue sections in the HFD group revealed significantly larger dimensions of the fat cells, especially in SAT (Fig. 2b; p<0.0001, HFD V vs. Control V, Fig. 4a; p=0.0165, HFD S vs. Control S, Fig. 4b), when compared to the control. Immunohistochemical analyses in this group showed also an increase in the number of CD68 immunopositive cells (HFD S vs Ctrl S, Fig. 3; p=0.0061, 4d). However, the greatest number of infiltrating macrophages was found in VAT, where they formed large clusters (Fig. 3a). The adipocytes in the VAT and SAT of the HFD+NAC group were slightly smaller than the fat cells of the HFD group, and their cell membrane had a thin rim of folded cytoplasm (Fig. 2, 4a, and b). There was a lower number of macrophages observed in HFD+NAC when compared to HFD (Fig. 3), with more CD68 immunopositive cells compared to VAT (Fig. 3a; p=0.0358, 4c). The shape of the fat cells in the HFD+ALA group was similar to that found in the control group, while the size of the adipocytes was slightly smaller than in the HFD (Fig. 2; p=0.0003, HFD+ALA V vs. HFD V, Fig. 4a; p=0.0209, HFD+ALA S vs. HFD S, Fig. 4b). The number of CD68 immunopositive cells was higher in both VAT and SAT of HFD+ALA compared to HFD (Fig. 3; p=0.0088, HFD+ALA V vs. HFD V, Fig. 4c; Fig. 4d). In the HFD+ALA group, a slightly higher number of macrophages was found in the adipose tissue sections, from both the locations (Fig. 2) compared to the respective control groups (Fig. 2). The number of CD68 immunopositive cells in the HFD+ALA group was higher in both types of adipose tissue compared to HFD+NAC (Fig. 3; p=0.0358, HFD+ALA V vs. HFD+NAC V, Fig. 4c; Fig. 4d). On the other hand, in HFD+ALA the size of the adipocytes, in VAT and SAT, was slightly smaller compared to HFD+NAC (Fig. 2; p=0.0010, HFD+ALA V vs. HFD+NAC V, Fig. 4a; Fig. 4b).

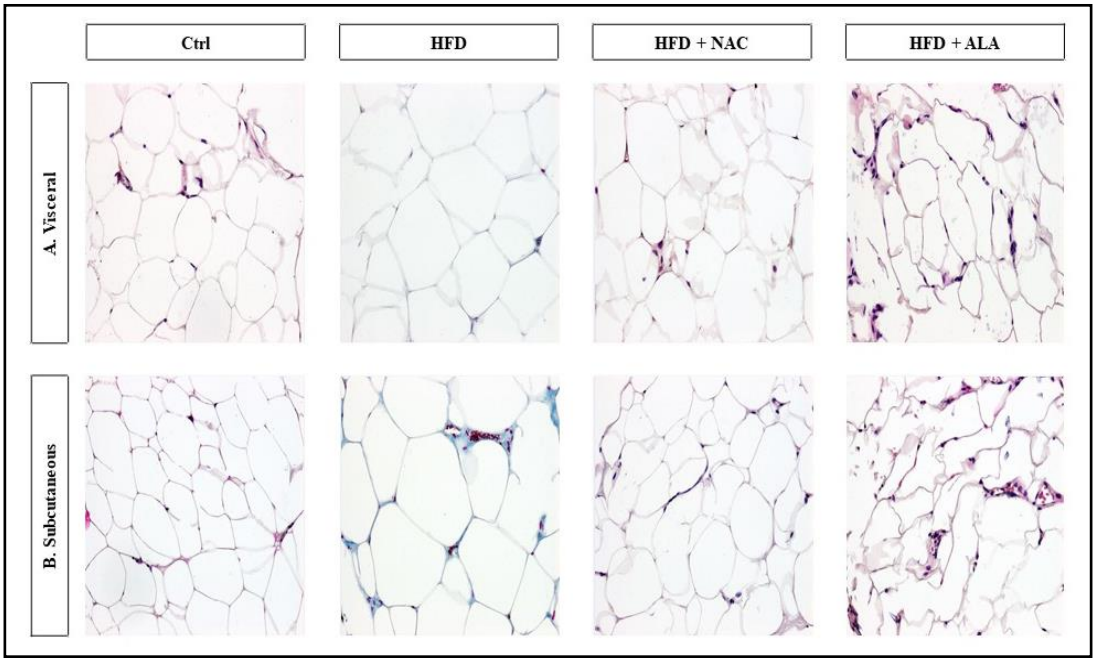

Fig. 2. Representative microphotographs of visceral and subcutaneous adipose tissue. The samples were stained with hematoxylin and eosin (H&E). Magnification: 200x. Ctrl – control rats; HFD – high-fat diet-fed rats; HFD+NAC – high-fat diet-fed rats that received N-acetylcysteine; HFD+ALA – high-fat diet-fed rats that received α-lipoic acid.

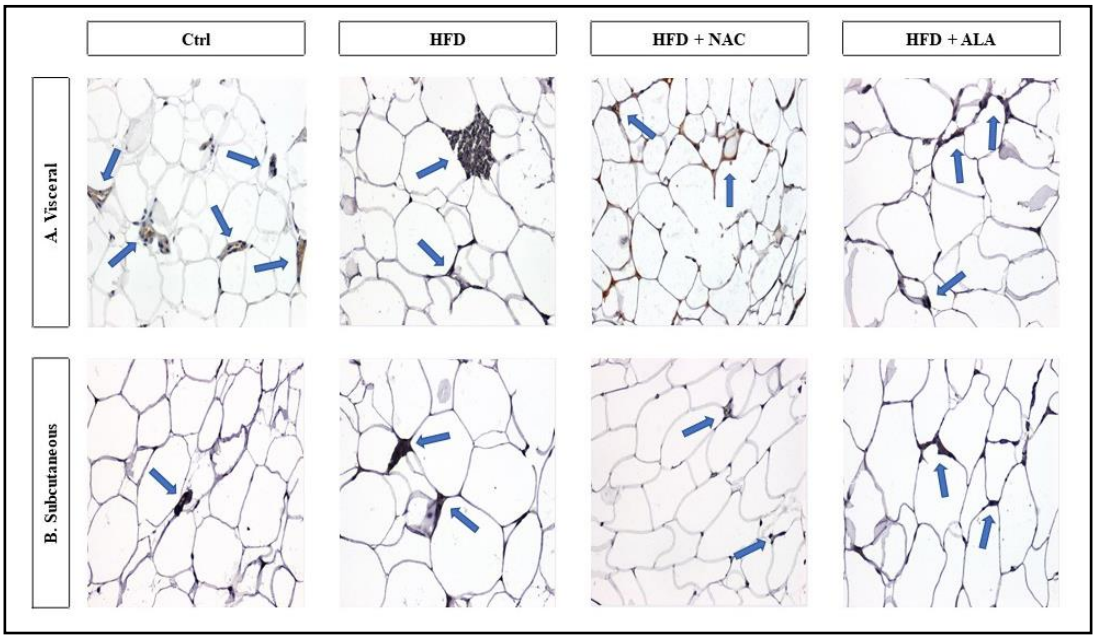

Fig. 3. Representative microphotographs of visceral and subcutaneous adipose tissue. The samples were stained with the pro-inflammatory marker (CD68). Magnification: 200x. Ctrl – control rats; HFD – rats fed a high-fat diet; HFD + NAC - rats fed high-fat diet + N-acetylcysteine; HFD + ALA – rats fed high-fat diet + α-lipoic acid.

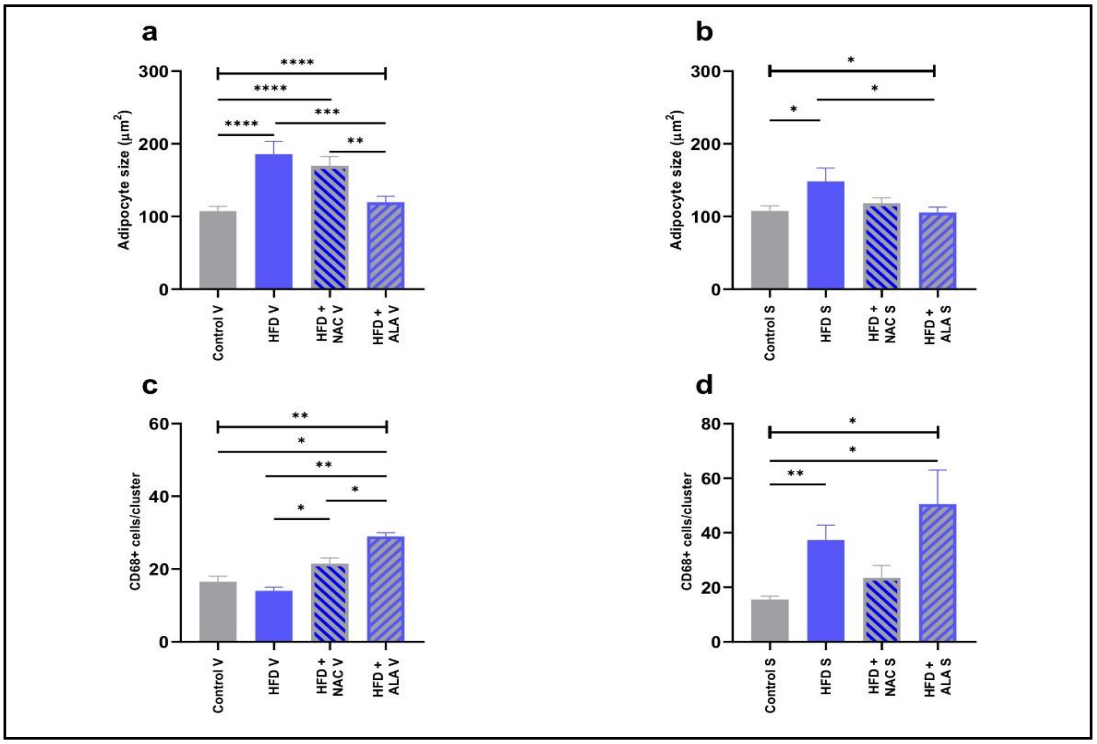

Fig. 4. The effects of NAC and ALA on: (a, b) adipocyte size and (c, d) CD68 cells number in adipose tissue. Control V—control rats, visceral adipose tissue; HFD V—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC V—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA V—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; Control S—control rats, subcutaneous adipose tissue; HFD S—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC S—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA S—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

The effects of NAC and ALA on the mRNA levels of MMP2 and MMP9 in the adipose tissues of HFD-fed rats

We determined MMP2 and MMP9 mRNA expression levels in VAT and SAT of the rats fed with HFD with/without antioxidants supplementation. The NAC treatment resulted in the decreased MMP9 mRNA expression in VAT and SAT (-66%, p=0.0254, Fig. 5d; -73%, p=0.0009, Fig. 5e; vs. control group; respectively). Interestingly, four-week-long administration of NAC alongside the HFD regime resulted in a significant decrease in the MMP9 mRNA expression in VAT and SAT when compared to HFD alone (-70%, p=0.0009, Fig. 5d; -92%, p=0.0150, Fig. 5e; vs. HFD group; respectively). A comparison between HFD+NAC and HFD+ALA showed a significantly higher MMP9 mRNA expression level in the SAT of the latter group (+6-fold, p=0.0162, Fig. 5e). Overall, we found greater MMP2 and MMP9 mRNA expressions in SAT compared to VAT (+97%, p<0.0001, Fig. 5c; +2-fold, p=0.0089, Fig. 5f; HFD S vs. HFD V; respectively). There was also a significant difference between the tissues in HFD+ALA with respect to MMP9 mRNA expression (+1.5-fold, p=0.0349, HFD+ALA S vs. HFD+ALA V, Fig. 5f).

The effects of NAC and ALA on protein levels of MMP2 and MMP9 in the adipose tissue of HFD-fed rats

We measured the total expression of MMP2 and MMP9 proteins in VAT and SAT in the obese rats after an HFD with/without antioxidants supplementation. In comparison to the control, the administration of the HFD resulted in an increased protein expression of MMP2 in VAT, and MMP9 in VAT and SAT (+2-fold, p<0.0001, Fig. 6a; +3-fold, p=0.0018, Fig. 6c; +2-fold, p=0.0052, Fig. 6d; vs. control group; respectively). Four-week-long administration of

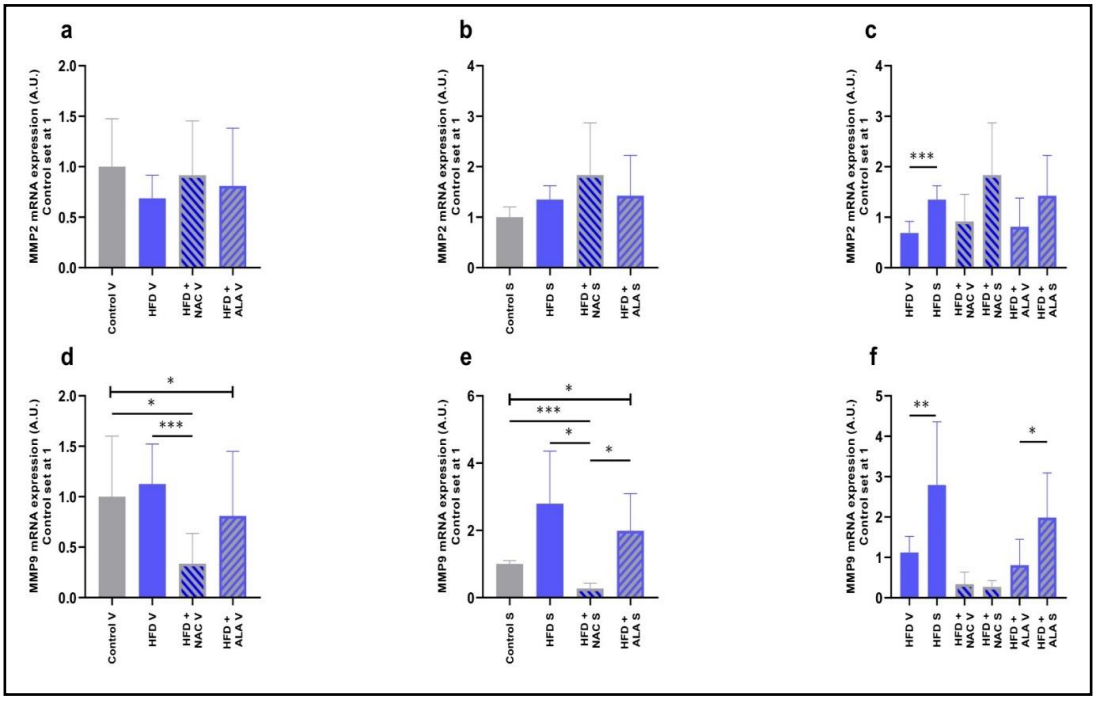

Fig. 5. The effects of NAC and ALA on: (a, b) MMP2 and (c, d) MMP9 mRNA levels in adipose tissue. The effect of NAC and ALA between tissue levels of: (c) MMP2 and (f) MMP9 mRNA. Control V – control rats, visceral adipose tissue; HFD V – high-fat diet-fed rats, visceral adipose tissue; HFD+NAC V – high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA V – high-fat diet +fed rats that received α-lipoic acid, visceral adipose tissue; Control S - control rats, subcutaneous adipose tissue; HFD S – high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC S – high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA S – high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue; * p<0.05; ** p<0.01; *** p<0.001.

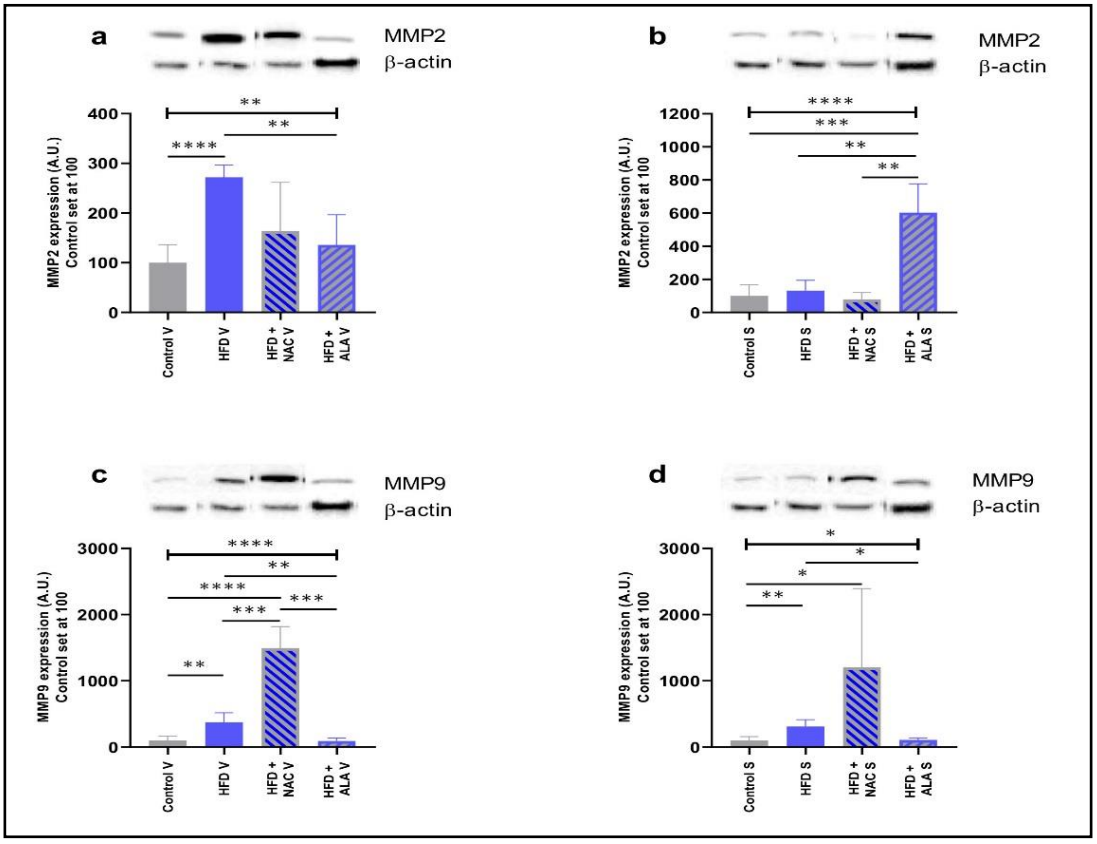

Fig. 6. The effects of NAC and ALA on (a, b) MMP2 and (c, d) MMP9 protein expressions in adipose tissue during the HFD regime. Control V - control rats, visceral adipose tissue; HFD V – high-fat diet-fed rats, visceral adipose tissue; HFD+NAC V – high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA V – high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; Control S - control rats, subcutaneous adipose tissue; HFD S – high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC S – high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA S – high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

NAC alongside the HFD regime resulted in significant increases in the expression of MMP9 proteins in VAT and SAT when contrasted with the control (+14-fold, p<0.0001, Fig. 6c; +11-fold, p=0.0469, Fig. 6d; vs. control group; respectively). On the other hand, only MMP2 protein expression was greater in SAT when juxtaposing HFD+ALA with the control (+5-fold, p=0.0002, HFD+ALA vs. control, Fig. 6b). The administration of NAC alongside the HFD regime also resulted in a significant increase in the expression of MMP9 proteins in VAT when compared to HFD alone (+3-fold, p=0.0004, vs. HFD, Fig. 6c). Interestingly, the addition of ALA during the HFD regime led to significant changes in MMP2 (VAT: -50%, p=0.0059, Fig. 6a; SAT: +4-fold, p=0.0071, Fig. 6b; vs. HFD; respectively) and MMP9 (VAT: -76%, p=0.0061, Fig. 6c; SAT: -66%, p=0.0126, Fig. 6d; vs. HFD; respectively) protein expression. The juxtaposition of the NAC treatment with the ALA application showed a higher protein expression of MMP2 in SAT and a lower protein expression of MMP9 in VAT, of the latter group (+7-fold, p=0.011, Fig. 6b; -94%, p=0.0003, Fig. 6c; HFD+NAC vs. HFD+ALA; respectively).

Discussion

In the present study we examined, presumably for the first time in the literature, the effects of two anti-oxidative agents (N-acetylcysteine and α-lipoic acid) supplementation on matrix metalloproteinase-2 and matrix metalloproteinase-9 expression in the visceral and subcutaneous adipose tissue of the rats with obesity induced by a high-fat diet. Herein, we found that the anti-oxidants affect the matrix metalloproteinases expressions (MMP2 and MMP9), as confirmed by measurements of the genes' mRNA and protein expression levels. This indicates that NAC and ALA may promote changes in the structure of adipose tissue. One of the most remarkable findings of our research is that different anti-oxidative molecules may have a dissimilar impact on adipose tissue's phenotype, as reflected in a specter of parameters ranging from histological images to the proteins (MMP2 and MMP9) expression levels.

Chronic HFD feeding leads to the development of obesity, which is a health burden itself, but also contributes to the development of many life-threatening comorbidities, including inflammation, oxidative stress, type 2 diabetes mellitus, or insulin resistance [22]. Therefore, unsurprisingly our 10-week high-fat diet regime resulted in a significant increase in the body weight of the rats, changes in their plasma lipids content, and decreased whole-body insulin sensitivity. The existence of the last phenomenon was confirmed by significantly higher blood insulin and glucose levels as well as the HOMA-IR index found in the HFD-fed rats as compared to the control animals. NAC and ALA affect body mass, plasma glucose, insulin, and lipid profile, therefore they might become potential therapeutic agents against the onset of oxidative stress and its complications induced by HFD-feeding [23, 24]. Interestingly, the body masses of the rats from the HFD groups (with or without the antioxidants) did not differ. Still, we observed a reduction in the levels of hyperglycemia, hyperinsulinemia, and HOMA-IR in the groups with antioxidants when compared to the HFD alone. The addition of NAC and ALA restored the values of the above-mentioned parameters to the range observed in the control group.

Histological examination has shown that the HFD regime leads to an increase in the size of adipocytes (especially in SAT), an increase in the number of CD68 immunopositive cells, and an increase in the number of macrophages, especially in VAT. This is reflected in the research results obtained also by Gollisch et al. [25]. These changes are caused by adipose tissue remodeling and the penetration of immune cells into the tissue [26]. Consequently, it leads to the development of obesity, accompanied by adipocyte hypertrophy and hyperplasia [27–29]. Previous studies also reported that HFD feeding was able to induce obesity in rodents within 8 weeks [30]. A positive energy balance accompanying the development of obesity contributes to stress in the cell, production of reactive oxygen species (ROS), and inflammation [7]. Oxidative stress leads to the development of obesity by stimulating the deposition of white adipose tissue and changing the amount of food intake [31]. Studies conducted by two independent teams have shown that oxidative stress contributes to an increase in preadipocyte proliferation, adipocyte differentiation, and influences the size of mature adipocytes [32, 33]. It has also been shown that adipocytes produce huge amounts of reactive oxygen species that damage cells and their inner biological structures. Thus, they contribute to the aggravation of the already existing inflammation, creating a vicious circle of the inflammatory response and further growth of adipose tissue [34]. The use of antioxidant supplementation during the HFD regime contributed to a reduction in the size of adipocytes, an increase in the number of CD68 immunopositive cells, and an increase in the number of macrophages in VAT and SAT, compared to the HFD group, which is confirmed also by the results of other studies [23, 35–37]. Additionally, we observed histological differences between the groups with antioxidant supplementation. After ALA supplementation, we observed fewer CD68 immunopositive cells and the adipocytes were larger in comparison to the results obtained from the NAC group. Our data show that both NAC and ALA supplementation is capable of diminishing lipids accumulation in both VAT

and SAT. Interestingly, the results show that ALA supplementation has a greater therapeutic effect on the morphology of adipose tissue in both localizations.

The quantitative real-time polymerase chain reaction revealed that the NAC supplementation during application of the HFD regime led to a decreased MMP9 mRNA expression in VAT and SAT when compared to the HFD alone. We also observed significant differences in the effects of NAC and ALA supplementation during HFD on MMP9 mRNA expression in SAT. In contrast, we found no significant changes (between any of the examined groups) in MMP2 mRNA expression in VAT or SAT. Still, inter-tissue comparison of HFD revealed that VAT adipocytes had greater levels of MMP2 and MMP9 mRNA than their SAT counterparts. This is also reflected in the histological results, where we noted that in SAT, of the HFD group, adipocytes had greater size and the number of CD68 immunopositive cells (macrophages), in relation to VAT. Our findings are in agreement with previous studies [38]. Tomita et al. showed that there is a correlation between oxidative stress and VAT or MMP9 mRNA in HFD-feed rats. They also observed a lipid deposition, immunostaining of CD68, and MMP9 mRNA expression was observed in the aorta's intima in the HFD-feed group [38]. Supplementation with NAC and ALA enhanced adipocyte differentiation and expansion, showing that antioxidants may play a role in downregulating pathways associated with collagen accumulation and abnormal adipocyte growth to attenuate the pathogenic "obesity phenotype".

In many biological systems, protein expression does not follow its mRNA levels. The difference in the obtained results may also be due to the greater sensitivity of the real-time PCR method than the Western blot. Application of the HFD regime led to an increase in MMP2 protein expression in VAT, and MMP9 protein expression in VAT and SAT. Despite the application of the HFD, NAC and ALA were able to increase MMP2 and MMP9 protein levels in AT. Despite that, each of the antioxidants, NAC or ALA, supplemented during the HFD regime has various influences on MMP2 and MMP9 protein expression in VAT and SAT, compared to respective HFD groups.

Unfortunately, so far, no study results have been published, where scientists check the direct activity of MMP2 and MMP9, in the treatment's context of ALA or NAC, in an obesity model. However, Uemura et al. proved, by using the gelatin zymography and Western blot, that the activity and expression of MMP9, but not MMP2 were significantly increased in vascular tissue and plasma of two distinct rodent models of diabetes mellitus. Enhanced MMP-9 activity was significantly reduced by treatment with the NAC [39]. In other research, Liu et al. investigated the role of MMP2 in pancreatic β-cell injury induced by oxidative stress. Intracellular MMP2 expression and activity were determined by real-time PCR, Western blotting, and zymography. They proved that NAC treatment inhibited MMP2 expression and activity, and partially reversed cell apoptosis and insulin secretion [40]. Bogani et al. sought to determine whether the antioxidants as NAC or ALA affect gelatinase production and secretion. The results show that thiol compounds affect MMPs' expression and activity in different ways. MMP2 activity is directly inhibited by NAC, while ALA is ineffective. On the other hand, MMP9 expression is inhibited by ALA at a pretrascriptional level [41].

Conclusion

In the current study, we presented presumably the first report about the effects of NAC and ALA supplementation on the MMP2 and MMP9 levels in visceral and subcutaneous adipose tissue of the rats with high-fat diet-induced obesity. Moreover, we believe our study to be the first one that compares the influence of the two antioxidants on the matrix metalloproteinases expression in white adipose tissue. Our data illustrate a potential role for NAC and ALA supplementation in the modulation of matrix metalloproteinases expression in visceral and subcutaneous adipose tissue of the animals fed with an HFD. The antioxidants treatment can help to protect adipose tissue against oxidative stress by regulating MMP2 and MMP9 expression.

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Author Contributions

Conceptualization, M.W., A.C.; data curation, M.W., B.L., I.K., A.C.; formal analysis, M.W., A.C.; funding acquisition, M.W.; investigation, M.W., I.K.; methodology, M.W., A.C.; material collection, M.W.; supervision, A.C., B.L.; validation, M.W., B.L., A.C.; visualization, M.W.; writing—original draft, M.W.; writing—review and editing, A.C., B.L..

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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