Canadian Journal of Physiology and Pharmacology

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2019-0340.R1
Manuscript Type:	Article
Date Submitted by the Author:	13-Aug-2019
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Is the invited manuscript for consideration in a Special Issue:	Current Trends in Physiological Sciences Serbia Meeting
Keyword:	visceral adipose tissue, mesenteric arteries, oxidative stress, endothelial dysfunction

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VITAMIN D ALLEVIATES OXIDATIVE STRESS IN ADIPOSE TISSUE AND MESENTERIC VESSELS FROM OBESE PATIENTS WITH SUBCLINICAL INFLAMMATION

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Abstract

Obesity is an age-independent, life-style triggered, pandemic disease associated with both endothelial and visceral adipose tissue (VAT) dysfunction leading to cardiometabolic complications mediated via increased oxidative stress and persistent chronic inflammation. The present study was aimed to assess the oxidative stress in VAT and vascular samples and the effect of *in vitro* administration of vitamin D. VAT and mesenteric artery branches were harvested during abdominal surgery performed in patients referred for general surgery (n = 30) that were randomized into 2 subgroups: non-obese and obese. Serum levels of C reactive protein (CRP) and vitamin D were measured. Tissue samples were treated or not with the active form of vitamin D - 1,25(OH)₂D₃ (100 nM, 12 h). The main findings are that in obese patients: i) a low vitamin D status was associated with increased inflammatory markers and reactive oxygen species generation in VAT and vascular samples and, ii) *in vitro* incubation with vitamin D alleviated oxidative stress in VAT and vascular preparations and also, improved the vascular function. We report here that serum level of vitamin D inversely correlated with the magnitude of oxidative stress in the adipose tissue. *Ex vivo* treatment with active vitamin D mitigated obesity-related oxidative stress.

Key words: visceral adipose tissue, mesenteric arteries, oxidative stress, endothelial dysfunction.

Introduction

Obesity is an age-independent, life-style triggered, rapidly expanding pandemic disease (Bhupathiraju and Hu 2016) characterized by chronic systemic and adipose tissue inflammation as major pathomechanism (Goran and Alderete 2012; Ha and Bauer 2018). Dysfunction of the abdominal visceral adipose tissue (VAT), the most representative ectopic fat, has been causally linked to the deleterious effects of obesity on cardiovascular and metabolic health (Fuster et al. 2016; Lim and Meigs 2013). Obesity related-white adipose tissue inflammatory dysfunction has been suggested to be caused by a failure of proper expansion in order to accommodate the surplus of triglycerides (leading to local and systemic lipotoxicity), adverse adipokines and pro-inflammatory cytokines profiles, increased oxidative stress, extracellular matrix fibrosis and mitochondrial dysfunction (Boudina and Graham 2014; Engin 2017; Vegiopoulos et al. 2017). All these central obesity-related pathomechanisms result over the time in insulin resistance and increased susceptibility for type 2 diabetes mellitus (Burhans et al. 2018; Kusminski et al. 2016).

Obesity is associated with both systemic (Manna and Jain 2015) and local adipose oxidative stress via the activation of both classical sources of reactive oxygen species (ROS) namely the dysfunctional mitochondrial respiratory chain, NADPH (NOX) oxidases (in particular NOX 4 in adipocytes and NOX 2 in infiltrating macrophages), xanthin oxidase, and uncoupled nitric oxide synthases (Boudina and Graham 2014; Jankovic et al. 2015) and novel ROS sources, such as monoamine oxidases (MAOs) (Sturza et al. 2019a). These sources might potentiate each other according to the "kindling radicals" concept (Muntean et al. 2016) thus leading to the perpetuation of the pro-inflammatory status and progressive insulin resistance (McMurray et al. 2016).

Vascular endothelial dysfunction is currently acknowledged as the earliest event in

atherosclerosis and a critical component of obesity-induced cardio-metabolic pathologies (Pi et al. 2018). Endothelial dysfunction has been classically defined as the impaired vasodilation mainly due to decreased nitric oxide (NO) availability (Symons and Abel 2013). In the setting of obesity both adipose tissue and vasculature (including the microcirculation) are major sources of oxidative stress and low-grade inflammation that synergistically aggravate endothelial dysfunction (Iantorno et al. 2014; Viridis et al. 2019). The contribution of vascular dysregulation to the development of metabolic disorders has been recently reviewed (Pi et al. 2018) as well as the role of perivascular adipose tissue in regulating the vascular redox status (Oikonomou and Antoniades 2018).

Vitamin D is a lipophilic hormone that accumulates in adipose tissue and presents a wide range of functions, including regulation of calcium homeostasis, anti-inflammatory effect and modulation of adipogenesis (Ding et al. 2012; Mutt et al. 2014; Pannu et al. 2016). Obesity has been associated with low vitamin D status (Wimalawansa 2018), defined as decreased concentrations of the serum 25-hydroxyvitamin D, without a consensus on whether this deficiency represents a cause or consequence of VAT dysfunction (Di Nisio et al. 2017).

The present study was aimed to assess the: i) serum vitamin D status and levels of circulating inflammatory markers in correlation with local oxidative stress in VAT and vascular samples harvested from obese vs. non-obese patients, and ii) *in vitro* effects of 1,25(OH)₂-D₃, the active vitamin D, on ROS production in both adipose tissue and vascular samples and vasomotor function in mesenteric arteries, respectively.

Material and Methods

Samples of VAT from omentum and mesentery and branches of mesenteric arteries were harvested from consecutive patients referred for abdominal surgery (n = 30), randomized into two

groups: obese (OB, n=17, 10 men and 7 women) and non-obese patients (non-OB, n=13, 7 men and 6 women) patients. The study protocol was reviewed and approved by the Ethics Committee of the University for Medicine and Pharmacy of Timisoara nr. 11/31.03.2017. The study was conducted according to the criteria stipulated by the Declaration of Helsinki and each participant signed the written informed consent before participating in the study. Obesity was defined as a body mass index (BMI) higher than 30 kg/m². Patients' characteristics are summarized in Table 1.

In the obese group (n=17), the diagnostics were as follows: abdominal eventration (2), cholelithiasis (3), Crohn disease (1), epigastric hernia (2), umbilical hernia (2), intestinal fistula (1), sigmoidian diverticulosis (1), gastric cancer (1), colon cancer (3), retroperitoneal tumor (1). As for the non-obese patients (n=13), they were referred to the Surgery Clinic for the following diagnostics: abdominal eventration (3), cholelithiasis (2), umbilical hernia (2), vesico-sigmoid fistula (1), gastric cancer (1), colon cancer (2), rectal cancer (1), cecum cancer (1).

Oxidative Stress Assessment by Means of the Ferrous Iron Xylenol Orange Oxidation (FOX) Assay

VAT and vascular samples were harvested during abdominal surgery in Hanks solution on ice and immediately transferred to the laboratory, cleaned, and incubated at 37°C in endothelial cell growth basal medium (EBM, containing 0.1% BSA), in the presence or absence of the active form of vitamin D - 1,25(OH)₂D₃ (100 nM, 12 h incubation). Hydrogen peroxide (H₂O₂) production was assessed using the Ferrous iron xylenol orange OXidation (FOX) assay (PeroxiDetect Kit, Sigma Aldrich) as previously described (Sturza et al. 2013). The principle of the assay is that hydrogen peroxide oxidizes Fe²⁺ to Fe³⁺ ions at acidic pH. The Fe³⁺ ion forms a

colored adduct with xylene orange, which is measured spectrophotometrically at 560 nm. Subsequently, the H_2O_2 production was calculated using a standard curve. The results are expressed in nmols of H_2O_2 /h/mg tissue.

In order to investigate the antioxidant capacity of vitamin D we assessed whether it act as H_2O_2 scavenger. In this regard, a volume of 100 μl of $1,25(\text{OH})_2\text{D}_3$ solution (100 nM) plus 100 μl of H_2O_2 solution (100 μM) were mixed with 2 ml of the working color reagent and incubated at room temperature (22-25°C) for ~30 minutes. PEG-catalase (100 U/mL, SigmaAldrich) was used as positive control since is a classic H_2O_2 scavenger. The results were expressed as % of H_2O_2 inhibition.

Oxidative Stress Assessment by Means of Immunofluorescence

The total amount of ROS was determined using the DHE (dihydroethidium) probe according to a previously described technique (Duicu et al. 2016). Briefly, samples were embedded in optimal cutting temperature compound (OCT) and snap-frozen. The frozen fragments were cut in 8 μm thick cryosections and put on glass slides. After 3 washes with phosphate buffered saline (PBS), 5 minutes each, the cryosections were incubated in the dark with DHE for 30 minutes at room temperature. Excess DHE was removed by 3 additional washes with PBS. The slides were mounted with Vectashield (Vector Laboratories) and immediately analyzed in confocal microscope (Olympus Fluoview FV1000). Images were obtained using laser excitation at 488 nm. Image analysis was performed using Icy Bioimage Analysis software (de Chaumont et al. 2012).

Vascular Reactivity Studies

Organ bath experiments were performed in mesenteric arteries branches rings using the myograph system (DMT). The concentration of phenylephrine, used for precontraction, was adjusted to obtain an precontraction level of 80% of the contraction elicited by KCl (80 mmol/L). Endothelium-dependent relaxation to increasing concentrations of acetylcholine (Ach 10^{-9} M - 10^{-4} M) was recorded after 12 h of *in vitro* incubation with the active form of vitamin D, calcitriol $1,25(\text{OH})_2\text{D}_3$ (100 nM).

Chemicals

All reagents were purchased from Sigma Aldrich (Merck).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.5.0 Software, SUA. Descriptive analysis of continuous data is presented as mean $\pm$ standard error of the mean (SEM) values. Paired and unpaired Student t test were used for group comparison of continuous variables. Pearson correlation coefficient (r) was computed as a measure of the linear association between quantitative variables. Statistical significance was set at a value of $P < 0.05$. All reported probability values were two-tailed. A 0.05 level of significance was considered, while marking the highly significant values as well.

Results

Obesity Is Associated with Low Serum Vitamin D, Increased Systemic Inflammatory Markers and Augmented ROS Generation in VAT

The characteristics of patients included in this pilot study are summarized in Table 1. Correlations among the clinical and laboratory parameters in the obese group are depicted in Fig. 1. As shown, obese patients presented a significantly low serum vitamin D together with a high plasma level of C-reactive protein (CRP), as well as a trend for increase in the erythrocyte sedimentation rate (ESR). Vitamin D deficiency was found in the obese patients (serum levels < 20 ng/mL); however, a degree of insufficiency was also present in the non-obese group (Table 1). Also, ionized (free) calcium level (but not total calcium) was lower in the obese vs. non-obese patients ($p=0.04$). Serum vitamin D level was inversely associated with BMI ($r= -0.476$, $p<0.05$). In the same line, a comparable negative and even more significant correlation was found between ionized calcium and BMI ($r= -0.451$, $p<0.01$). Of note, the free calcium level was also strongly associated with the inflammatory markers, ESR ($r= -0.551$, $p<0.01$) and CRP ($r= -0.401$, $p<0.05$) as well as with the blood glucose level ($r= -0.432$, $p<0.05$) - Fig. 1.

Another important observation is that the magnitude of oxidative stress measured in VAT correlated with both the degree of obesity and inflammatory status. Thus, a strong positive correlation ($r=0.69$, $p<0.01$) has been observed between H_2O_2 production in adipose tissue (FOX assay) and BMI. Similarly, the local ROS generation positively correlated with the CRP values ($r=0.476$, $p<0.01$) (Fig. 1).

Vitamin D Mitigated Oxidative Stress in VAT Isolated from Obese Patients

In the presence of low serum vitamin D in both obese and non-obese patients (but mainly in the obese group with subclinical inflammation), we thought to investigate whether acute incubation with the active form of vitamin D, $1,25(OH)_2D_3$ or calcitriol (100 nM), can modulate the level of local oxidative stress. We assessed the level of hydrogen peroxide by FOX assay and we quantified the total ROS level by immunofluorescence (IF), respectively. As shown

in Fig. 2A (left panels) and 2B oxidative stress was significantly higher in the OB vs the non-OB group. Incubation for 12 hours with the active vitamin D mitigated the amount of total ROS in IF (Fig. 2A) and H_2O_2 assessed with FOX assay (Fig. 2B), suggesting that vitamin D is able to improve the oxidative status of the white adipose tissue. It has to be mentioned the anti-oxidant effect of vitamin D was significant in the obese group, but not in the non-obese one, thus confirming the need for reducing the level of ROS in adipose tissue in the setting of obesity.

Importantly, $1,25(\text{OH})_2\text{D}_3$ did not inhibit the H_2O_2 production as compared to catalase, the classical H_2O_2 scavenger, which argues against the scavenger-type antioxidant activity of the vitamin - as depicted in Fig. 2C.

We also plotted the BMI against the total amount of H_2O_2 generated in VAT samples harvested from both groups in the presence and the absence of active vitamin D, respectively; the results are presented in Fig. 2D. As depicted in Fig. 2D (left), in the absence of vitamin D, a double clusterisation pattern became apparent, i.e., in patients with a $\text{BMI} < 30 \text{ kg/m}^2$ a maximum of 4 nM $\text{H}_2\text{O}_2/\text{h/mg}$ tissue was obtained at FOX assay, whereas for a $\text{BMI} \geq 30 \text{ kg/m}^2$ the values ranged between 4 and 7 nM $\text{H}_2\text{O}_2/\text{h/mg}$ tissue. Overnight incubation with the active vitamin D elicited the 'aggregation' of all values in a single cluster, below 4.5 nM $\text{H}_2\text{O}_2/\text{h/mg}$ tissue (Fig. 2D, right). Important, similar results were obtained for vascular samples in the presence vs. the absence of vitamin D administration (as showed in Fig. 3D - see below).

Vitamin D Reduced the Oxidative Stress and Improved Vascular Reactivity of Mesenteric Arteries Branches Isolated from Obese Patients

We further assessed the effects of incubation with $1,25(\text{OH})_2\text{D}_3$ on oxidative stress and endothelial function in mesenteric arteries branches isolated from the two subgroups of patients.

After 12 h of *in vitro* treatment with $1,25(\text{OH})_2\text{D}_3$ the magnitude of overall oxidative stress (assessed in IF, Fig. 3A) and the generation of hydrogen peroxide (FOX assay, Fig. 3B) were significantly reduced in vascular samples, particularly in those from obese patients.

As revealed by the myograph studies of vascular function, a significant rightward shift of the concentration–response curve to Phe was found in the arteries isolated from OB patients as compared to the non-OB ones (Fig. 3C - left). Subsequently, we measured the endothelium-dependent relaxation in response to cumulative doses of ACh in the presence *vs.* the absence of $1,25(\text{OH})_2\text{D}_3$ (100 nM). As depicted in Fig. 3C (right) endothelial-dependent relaxation was significantly decreased in the OB *vs.* the non-OB group ($p < 0.05$). Moreover, a significant improve in the vascular relaxation response to ACh after incubation with vitamin D of vascular preparations from obese (but not from non-obese) patients was obtained.

Discussion

Obesity has been largely associated with low serum levels of vitamin D, oxidative stress, and systemic inflammation. Accordingly, obesity is an important risk factor for vitamin D deficiency in both adults and children (Pramono et al. 2019; Zakharova et al. 2019). Vitamin D deficiency is currently defined by serum $25(\text{OH})\text{D}_3$ levels < 20 ng/mL (50 nmol/L) while insufficiency is diagnosed for values ranging between 20-30 ng/mL (50-75 nmol/L) (Holick et al. 2011). The laboratory parameter $25(\text{OH})\text{D}_3$ (also known as calcidiol) is largely accepted as the main indicator of vitamin D status since is the predominant circulating metabolite and has a longer half-life (weeks) as compared to the one of the active vitamin D ($1,25(\text{OH})_2\text{D}_3$ or calcitriol) which is in the range of hours (Zerwekh 2008). In the present pilot study that recruited consecutive patients referred for general surgery, this classical marker was used for the assessment of vitamin D status. We identified both vitamin D deficiency in the non-obese group

and insufficiency in the obese one, respectively, observations that support the increased prevalence of the low vitamin D status in the elderly Romanian population. Moreover, the low vitamin D was inversely associated with the BMI; these results are in line with a recent meta-analysis that found an overall reverse relationship between BMI and serum vitamin D in diabetic and non-diabetic subjects (Rafiq and Jeppesen 2018).

As concerning the systemic inflammatory markers, in the present study, a significant increase was found only for CRP ($p=0.02$) and not for ESR in the obese vs. non-obese patients. However, whether the low serum vitamin D in the setting of obesity is a cause or a consequence of the impaired metabolism in the adipose tissue and/or of the pro-inflammatory status still remains an open question (Migliaccio et al. 2019).

Another important finding of the present study is the association of the increased local oxidative stress in VAT with the severity of obesity and the systemic inflammation, respectively. The magnitude of *local* H_2O_2 production was positively correlated with BMI and plasma CRP; this observation lend support to the literature data reporting the association of obesity with *systemic* oxidative stress and low-grade inflammation (Filgueiras et al. 2018). Moreover, the local oxidative stress in VAT was negatively correlated with the serum vitamin D. The observation that VAT is the fat deposit most strongly associated with 25(OH)D concentrations has been recently reported in cross-sectional cohort study performed in Netherlands (Rafiq et al. 2018), these authors reported that serum 25-hydroxyvitamin D concentrations were inversely related with VAT (but not with subcutaneous adipose tissue) in both men and women with a mean age of 56 (± 6) years which is comparable to the one in our obese group. Despite the fact that vitamin D deficiency was not addressed in this study, this observation is suggestive for the critical role of VAT in modulating the serum level of vitamin D.

Vitamin D is a major regulator of calcemia by increasing its intestinal absorption yet less attention has been paid in the literature to the systemic calcium balance in conditions associated with low vitamin D status, such as obesity. In the present study, both total and ionized calcium were within the normal range yet the latter parameter was lower in the serum of OB vs. non-OB patients ($p=0.04$). Interestingly, a strong negative correlation was found between the level of ionized calcium and BMI. This observation was previously reported in a pioneering study by Andersen et al in a pilot study of patients with morbid obesity. These authors noticed a significantly low level of ionized calcium in obese patients as compared to normal weight individuals, with total calcium displayed similar values in both groups; the degree of overweight was negatively correlated with the ionized calcium value (Andersen 1986). The observation was further confirmed by the work of Lind *et al*, which also reported a significant negative correlation between BMI and plasma ionized calcium. In their elegant study, the authors demonstrated that the shift from the ionized to the non-ionized form of serum calcium was associated with abdominal fat distribution (and not obesity *per se*) (Lind et al. 1993). One putative mechanism might be related to an increased release of free fatty acids from the abdominal adipocytes with the subsequent increase of calcium binding to plasma albumins; the process could be potentiated by the elevation of blood catecholamines that occur in obesity related-activation of the sympathetic nervous system (Kotsis et al. 2018) and have been earlier reported to lower the ionized calcium fraction (Joborn et al. 1990). Important, in patients with morbid obesity changes in serum ionized calcium were reversible along with weight loss (Andersen et al. 1988).

Of note, in our study the serum ionized calcium was also negatively correlated with the inflammatory markers (ESR and CRP) and glycemia, respectively; these observations require further investigation to elucidate the underlying pathomechanisms.

It has to be mentioned that certain cancers, including the gastro-intestinal ones were associated with vitamin D deficiency (recently reviewed by Goyal et al. 2019). In our study a comparable number of patients were hospitalized for surgical cure of colorectal and gastric cancers in both OB and the non-OB groups. The vitamin D deficit was more severe in the obese patients, an observation that is suggestive for the contribution of the dysfunctional white adipose tissue to the impaired vitamin D status. However, we admit as a limitation of the present study the lack of assessment of vitamin D in the VAT samples.

Interestingly, no changes in lipid profile were found in the obese group where normal values (or values close to the upper limit) for triglycerides, total cholesterol, LDL-cholesterol and mean values above 45 mg/dl for HDL-cholesterol were found (Table 1). A possible explanation for this lipid profile could be the existence of metabolically „healthy” obese patients within the study group (of note, only 3 obese patients were treated with statins, whereas in the remaining patients the lipid profile was not controlled). It is tempting to speculate that in the non-treated patients with a BMI over 30 kg/m² adipose tissue expansion has adapted to accommodate the excessive energy substrate without causing VAT dysfunction and thus limited modification of biological parameters (Ctoi et al. 2018).

Adipose tissue expansion is known to occur through various degrees of hypertrophy and hyperplasia. Hypertrophy generates abnormally large adipocytes by lipid-overload that causes impairment of cell function, cytokines secretion, low-grade inflammation, and ultimately cell death; hyperplasia is represented by a generation of new adipocytes from progenitor cells, maintaining normal size, shape and function during the stage of healthy adaptation (Fuster et al. 2016). However, obese patients presented a significantly different inflammatory profile, with higher values of CRP as compared to the control group (Table 1). Albeit not statistically significant, ESR values recorded a similar change with higher values in the obese individuals.

The inflammatory status associated with obesity might be viewed as the earliest change in metabolic health since the lipid profile which is generally modified in obesity, showed no significant changes in the obese group. Markers of inflammation such as IL-6, CRP and TNF- α have been reported to be increased in overweight and obese individuals, while the abdominal visceral fat being recognized as an important source for IL-6 production (Fuster et al. 2016). In this respect, a potential limitation of our study is that the pro-inflammatory cytokines profile was not assessed. Indeed, in the setting of obesity, a theory of a vicious cycle of excess body fat, insulin resistance, and chronic, low-grade systemic inflammation has been recently proposed (Park et al. 2014): the excess of adipocytes represents an important source of inflammatory cytokines that can alter glucose metabolism, contributing thus to insulin resistance, which will further exacerbates adiposity by influencing glucose disposal and fat storage (Jiao and Xu 2008; McArdle et al. 2013).

In the present study adipose oxidative stress was mitigated after acute *ex vivo* incubation with the active vitamin D, as assessed both by IH and FOX assay. These results in humans are in agreement with a study performed in rats with high-fat diet-induced obesity that have been chronically treated with vitamin D (500 IU/kg/day) in oral administration for 5 weeks (Farhangi et al. 2017).

Vascular dysfunction is at the core of pathologies associated with obesity and hypertension, conditions that share as combinatorial pathomechanisms the inflammatory status and the increased oxidative stress. The obesity-related vascular abnormalities include the impairment of the endothelial-dependent relaxation, an increased contractile response to vasoconstrictors prior to the occurrence of structural remodeling (Reho and Rahmouni 2017).

Vitamin D has emerged in the past decades as important vasculo-protective compound in animal models and patients; indeed, several trials reported the association between low 25(OH)D₃

levels and cardiovascular risk factors (such as obesity) and cardiovascular (and cerebral) events (Christakos et al. 2016). In this translational study we report the occurrence of functional vascular abnormalities in mesenteric artery branches harvested from the obese group that were partially reversible after *ex vivo* incubation of with the active vitamin D, as previously reported for diabetic rat aortic rings (Sturza et al. 2019b). Of note, in this experimental study *ex vivo* incubation of rat aortic segments with the active form of vitamin D significantly reduced the expression of MAO-A isoform, an emerging vascular source of oxidative stress in cardio-metabolic pathologies. Whether this holds true in humans warrants further investigations. Additional experiments are equally required in order to elucidate the signaling pathways underlying the antioxidant effect of vitamin D.

Conclusions

In conclusion, systemic inflammation correlates with the severity of local oxidative stress in the adipose tissue in obese patients with low vitamin D status. *In vitro* treatment with the active form of vitamin D significantly reduced oxidative stress in both adipose tissue and vascular preparations; in the latter an improved vascular reactivity (reduced contractility and increased endothelium-dependent relaxation) was also reported. The promising findings that obesity-induced vascular and adipose tissue dysfunctions are, at least in part, reversible, with drugs and/or weight loss, are crucial for the design of therapeutic strategies aimed preventing the vascular and metabolic complications of obesity.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This study was financially supported by the university grant PIII-C5-PCFI-2017/2018-01.

We are grateful to Dr. Norbert Pop for providing the adipose tissue and vascular samples for the period he worked as resident in the 1st Clinic of Surgery, Faculty of Medicine, “Victor Babeş” University of Medicine and Pharmacy, Timișoara, Romania.

This article is based upon work from COST Action EU-CARDIOPROTECTION CA16225 supported by COST (European Cooperation in Science and Technology).

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Table 1. Characteristics of the study groups.

Parameter	Non-OBESE (n=13)	OBESE (n=17)	<i>p</i>
Age (years)	64.5±8.4	57.9±8.5	<i>n.s.</i>
Sex (M/F)	7 / 6	10 / 7	<i>n.s.</i>
Body Mass Index (kg/m²)	25.4±2.3	34.4±3.9*	<0.001
Erythrocyte Sedimentation Rate (mm/h)	18.5±15.4	34.5±23.5	<i>n.s.</i>
C reactive protein (mg/L)	8.1±6.8	44.5±39.1*	0.02
Triglycerides (mg/dL)	113.0±31.2	132.9±84.2	<i>n.s.</i>
Total Cholesterol (mg/dL)	180.1±37.3	190.0±41.7	<i>n.s.</i>
HDL-Cholesterol (mg/dL)	48.6±15.1	45.8±15.2	<i>n.s.</i>
LDL-Cholesterol (mg/dL)	111.2±29.8	110.6±33.9	<i>n.s.</i>
Fasting plasma glucose (mg/dL)	99.7±14.4	111.4±26.2	<i>n.s.</i>
Diastolic blood pressure (mmHg)	75.1±7.3	77.5±9.4	<i>n.s.</i>
Systolic blood pressure (mmHg)	125.3±9.0	133.5±19.7	<i>n.s.</i>
Urea (mg/dL)	43.3±19.5	36.8±11.1	<i>n.s.</i>
Creatinine (mg/dL)	1.0±0.3	0.9±0.3	<i>n.s.</i>
Uric acid (mg/dL)	6.1±1.8	5.8±1.3	<i>n.s.</i>
Total calcium (mg/dL)	9.3±0.7	9.0±0.7	<i>n.s.</i>
Ionized calcium (mg/dL)	4.1±0.2	3.9±0.3*	0.04
Serum phosphate (mg/dL)	3.8±0.5	3.5±0.6	<i>n.s.</i>
25(OH)-D₃ (ng/mL)	24.8±17.7	11.7±4.3*	0.03

Values are means ± SD, **p* <0.05.

FIGURE LEGEND

Figure 1. Correlation matrix between various laboratory parameters and adipose tissue oxidative stress (H_2O_2 production assessed by FOX assay). The type of correlation was defined by a positive (blue shades) or negative (red shades) r value. The strength of correlation was considered moderate for r values between 0.3 and 0.39, strong for r values between 0.4 and 0.69, respectively very strong for r values of 0.7 and above. $*p$ – correlation is significant at the 0.05 level (2-tailed), $**p$ – correlation is significant at the 0.01 level (2-tailed). BMI - Body Mass Index, ESR - Erythrocyte Sedimentation Rate, CRP - C Reactive Protein.

Figure 2. The effects of $1,25(\text{OH})_2\text{D}_3$ (100 nM, 12h incubation) on VAT samples in obese (OB) vs. non-obese (nonOB) patients. A. DHE staining; B. FOX assay, $n=30$, $***p<0.001$; C. Assessment of the antioxidant (H_2O_2 scavenger) effect of vitamin D (100 nM); catalase (100 U/mL) served as positive control; D. Correlative scatter plot between BMI and hydrogen peroxide production (FOX assay) with and without vitamin D. DHE - Dihydroethidium, FOX - Ferrous Iron Xylenol Orange Oxidation.

Figure 3. The effects of $1,25(\text{OH})_2\text{D}_3$ (100 nM, 12h incubation) on vascular samples in obese (OB) vs. non-obese (nonOB) patients. A. DHE staining; B. FOX assay, $n=30$, $***p<0.001$; C. Phenylephrine-induced contraction and acetylcholine-induced relaxation, $n=30$, $*p<0.05$ OB vs nonOB, #OB vs OB+ $1,25(\text{OH})_2\text{D}_3$; D. Correlative scatter plot between BMI and hydrogen peroxide production (FOX assay) with and without vitamin D. DHE - Dihydroethidium, FOX - Ferrous Iron Xylenol Orange Oxidation.

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190x253mm (300 x 300 DPI)

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