Relation of the protein glycation, oxidation and nitration to the osteocalcin level in obese subjects*

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ABSTRACT

Carboxylated osteocalcin (Gla-OC) contributes to the bone formation, whereas its undercarboxylated form (Glu-OC) takes part in the energy metabolism. In vitro studies had shown that treatment of osteoblast-like cells with advanced glycation end product-modified bovine serum resulted in reduced synthesis of collagen 1 and osteocalcin. The aim of this study was to find association between Gla-OC and markers of protein glycation, oxidation and nitrination, as well as pro-inflammatory and anti-inflammatory defense markers in obese subjects. Non-obese (body mass index (BMI)<30 kg/m²; n=34) and obese subjects (30<BMI <40 kg/m²; n=98), both sexes, aged 25 to 65 years, were included in this study. Urinary glycation, oxidation and nitrination adduct concentrations were determined by stable isotopic dilution analysis liquid chromatography and mass spectrometry, and normalized to creatinine. Obese subjects had lower Gla-OC serum levels when compared to the non-obese controls. Obese subjects had increased serum concentrations of insulin, C reactive protein, interleukin 6, leptin and insulin resistance index (HOMA-IR). Urinary early glycation and advanced glycation end product (AGE) free products, Ne-fructosyl-lysine and 3-deoxyglucosone-derived hydroimidazolone, respectively, and oxidative damage marker, N-formylkynurenine free adduct, were increased in the obese compared to the non-obese subjects. Serum Gla-OC was negatively correlated with urinary methylglyoxal-derived AGE, hydroimidazolone MG-H1, and N-formylkynurenine free adducts. The Gla-OC/Glu-OC index negatively correlated with the MG-H1 free adduct, and correlated positively with the antioxidant defense marker – the glutathione peroxidase activity. Our results suggest that increased AGEs and protein oxidative damage markers in the course of obesity may contribute to decreased Gla-OC level and, consequently, future risk of decreased bone formation.

Key words: advanced glycation end products, oxidative stress, obesity, osteocalcin

INTRODUCTION

Recent studies have shown that bone cells take part not only in the skeletal remodeling but are also involved in the adipose tissue metabolism. In turn, the adipose tissue could also influence bone remodeling by releasing biologically active substances. Several mechanisms have been described to explain the relationship between adipose tissue energy metabolism and bone remodeling.

Osteocalcin (OC) is one of secretory products from osteoblasts which regulates glucose and lipid metabolism (Hauschka et al., 1989; Lee et al., 2007; Kanazawa et al., 2011). Osteocalcin is a non-collagenous protein of the bone, which is released into circulation when a new bone is formed (Price et al., 1994). Recent studies have shown that carboxylated osteocalcin (Gla-OC) interacts with hydroxyapatite crystals and modulates their growth. It contributes to bone formation, calcium ion homeostasis and is considered a marker of bone turnover (Dowd et al., 2003; Kruger et al., 2006). Bone resorption leads to decarboxylation of osteocalcin and releases of its advanced glycation end product-modified form, Glu-OC, which participates in the glucose and lipid metabolism (Lee et al., 2007; Ferron et al., 2014; Wei et al., 2014). Mice with osteocalcin deficiency are hyperglycemic, hypoinsulinemic, have low pancreatic β-cell mass, decreased insulin sensitivity, increased fat mass and decreased energy expenditure (Pi et al., 2008; Wei et al., 2014). Insulin signaling in osteoblasts stimulates decarboxylation of Gla-OC (Glu-OC formation), whereas leptin secreted by adipocytes inhibits OC activation indirectly, leading to inhibition of insulin secretion and causing glucose intolerance (Ducy et al., 2000; Takeda et al., 2002; Ferron et al., 2010). Both osteocalcins (Gla-OC and Glu-OC) are detectable in circulation. Bone tissue can regulate glucose metabolism through an endocrine cross-talk between osteoblasts, adipocytes, and other organs (Kanazawa, 2015). Fat mass could in turn increase bone resorption through upregulating proinflammatory cytokines, such as for example IL-6 and TNF-α. These cytokines could induce osteoclast activity through regulation of the RANKL/RANK/OPG pathway (Kaneshiro et al., 2014; Osta et al., 2014).

Furthermore, in obesity, inflammation is associated with increased oxidative stress. Obese subjects displayed markers of oxidative stress, elevated levels of reactive...
oxygen species (ROS) (Keaney et al., 2003), and diminished antioxidant defense resulting from lower antioxidant enzyme activity, such as glutathione peroxidase (Olusi, 2002). It is also reported that obesity is connected with increased amounts of protein advanced glycation endproducts (AGEs) in the body (Unoki et al., 2010; Gaens et al., 2014).

Protein AGEs are end-stage adducts formed in a non-enzymatic reaction of proteins with saccharides and related metabolites. Glucose reacts with proteins to mainly form the early-stage glycation adduct, N\textsubscript{-}fructosyl-lysine (FL) residues. FL residues degrade to form AGEs, such as N\textsubscript{-}carboxyethyl-lysine (CEL) residues. AGEs may also be formed by direct reaction of reactive dicarbonyl metabolites, methylglyoxal (MG) and 3-deoxyglucosone (3-DG) with proteins. The major AGE formed by MG is the hydromidazolone N\textsubscript{\textdegree}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (MG-H\textsubscript{\textdegree}) residue, with formation of N\textsubscript{-}carboxyethyl-lysine (CEL) and other minor AGE residues as well. The major AGE formed by 3-DG is hydromidazolone N\textsubscript{\textdegree}-(5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl) ornithine (3DG-H) residue and related isomers. AGE-modified proteins undergo proteolysis to form related glycated amino acids called AGE free adducts. AGE free adducts, when released into plasma, have high renal clearance and are excreted in urine. There are also minor contributions to AGE free adducts by direct glycation of amino acids and absorption after digestion of AGE-modified proteins in food. Similarly, protein oxidation forms methionine sulfoxide (MetSO) and N\textsubscript{-}formylkynurenine (NFK) residues, and protein nitrination forms 3-nitrotyrosine (3-NT) residues; and after cellular proteolysis, related oxidation and nitrination free adducts are excreted in urine. Urinary excretion of glycation, oxidation and nitrination free adducts are approximate measures of whole body fluxes of protein glycation, oxidation and nitration, respectively (Thorlalley & Rabbani, 2014; Rabbani & Thornalley, 2012). AGE-modified proteins are dysfunctional or functionally inactivated. They have been implicated in pathogenesis of obesity and related metabolic and vascular complications. Examples of AGE-modified proteins are: MG-modified collagen-IV, LDL and HDL (Dobler et al., 2006; Rabbani et al., 2011; Godfrey et al., 2014). Increased formation of AGEs has been linked to dysglycaemia, insulin resistance and vascular inflammation in overweight and obese subjects (Xue et al., 2016) – as recently reviewed (Rabbani et al., 2016). AGEs have been proposed to bind to cell surface receptors and induce production of reactive oxygen species, inflammatory cytokines, such as tumour necrosis-factor alpha (TNF\text{-}\alpha), and activation of NF\text{-}\kappaB leading to bone remodeling disorder, but there is doubt if this occurs or is functional in vivo (Ramasamy et al., 2012; Rabbani et al., 2016). In vitro studies with osteoblastic cell cultures demonstrated that AGEs could affect osteoblast proliferation and differentiation by modification of collagen (Alikhani et al., 2007; Mercer et al., 2007; Franke et al., 2011), as well as could induce apoptosis in bone cells through MAPK, p38, caspase-8, and caspase-9 signaling pathways (Alikhani et al., 2007; Weinberg et al., 2014; Tanaka et al., 2015). Yamamoto and coworkers have shown that treatment with AGE-modified bovine serum of osteoblast-like cells resulted in a reduced synthesis of collagen I and osteocalcin (Yamamoto et al., 2001). Other investigators had found contrary evidence and suggested that AGE binding in vitro may be non-productive since based on the normally found level of AGEs, the best characterized receptor, the receptor for AGEs (RAGE), would be predicted to be always saturated with the AGE protein ligands (Buettner et al., 2008; Rabbani et al., 2016).

Studies in humans concerning correlation between Gla-OC and AGEs are still lacking. Therefore the aim of this study was to find a correlation between Gla-OC and protein glycation, oxidation and nitrination products, as well as pro-inflammatory and antioxidant defense markers in obese subjects, who were characterised in our previous paper (Razny et al., 2017). In this study, we use some biochemical parameters estimated before in the group of obese participants (Razny et al., 2017), and also determine urinary excretion of protein glycation, oxidation and nitrination free adducts – FL, CML, MG-H\textdegree, 3DG-H, CEL, MetSO, NFK and 3-NT (Thorlalley & Rabbani, 2014).

MATERIALS AND METHODS

Study population. The study was approved by the Bioethics Committee of the Jagiellonian University in Cracow, Poland (opinion No. KBET/82/B/2009) and all subjects gave written informed consent. Volunteers were recruited from patients of the Out-patient Clinic: the Clinic of Obesity and Lipid Disorder Treatment at the Department of Biochemistry UJ CM in Cracow, Poland. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and with the Good Clinical Practice guidelines. The study population consisted of the same groups of volunteers described in the previous paper (Razny et al., 2017): obese (30<\text{BMI}<40 kg/m\textsuperscript{2}, n=98), and non-obese (\text{BMI}<30 kg/m\textsuperscript{2}, n=34) women and men, aged 25–65 yrs. The subjects with diseases that could affect the metabolism of glucose and lipids (diabetes mellitus, pregnancy, endocrine disorders, kidney or liver dysfunction and other chronic diseases) were disqualified from attendance in the study. Subjects included in the study did not take any medication except for hypotensive drugs (metabolically neutral). All participants enrolled in this study were asked to follow an isocaloric diet with low amount of polyunsaturated fatty acids, anti-oxidative vitamins and alcohol for 2 weeks before the study began. The percentage of the fat tissue in the body was estimated with the bioelectrical impedance method using Segmental Body Composition Analysers TANITA BC 418 MA (Tanita, Tokyo, Japan).

Biochemical measurements. After two weeks of diet standardization, venous blood samples were drawn for biochemical analysis after 12 hrs of overnight fasting. Samples were centrifuged at 4000 rpm for 10 min to obtain serum and plasma, which were stored at –80°C for further processing.

Plasma glucose, total cholesterol, HDL-cholesterol and triglycerides were measured by enzymatic colorimetric methods (Allmed, Krakow, Poland) using the MaxMat Analyzer (MaxMat S.A., Montpellier, France). The intra and inter-assay coefficients of variation were as follows: 2.3% and 3.5% (glucose), 1.4% and 3.4% (triglycerides), 1.4% and 3.8% (total cholesterol), 2.1% and 2.8% (HDL-cholesterol), LDL-cholesterol was calculated using the Friedewald formula. Insulin in serum was assayed by an immunoradiometric method (DIA-source, ImmunoAssays, Louvain-la-Neuve, Belgium) using a gamma counter (LKB Instruments, Mount Waverley, Australia). The intra and inter-assay coefficients of variation were 2.1% and 6.5%, respectively. Basal insulin resistance was determined by a homeostasis model of assessment (HOMA-IR) (Marti et al., 2001). Free fatty acids were determined by gas-liquid chromatography (GLC; Shimadzu, Japan). Plasma urea and creatinine levels were assayed using an automated biochemical analyser, Randox Laboratories (B River Road, Crumlin, County Antrim, Northern Ireland). Plasma testosterone and SHBG concentrations were measured using a solid-phase chemiluminescence immunoassay (IKA Monarch, Germany) on the Immulite 2000 analyser (Siemens, Munich, Germany). The intra and inter-assay coefficients of variation were 3.7% and 4.2%, respectively.

Intrahepatic triglyceride concentration was measured with a commercial kit (Roche Diagnostics, Germany). The intra and inter-assay coefficients of variation were 2.0% and 4.0%, respectively. Insulin sensitivity was assessed with the homeostasis model assessment (HOMA-IR) (Marti et al., 2001). Free fatty acids were determined by gas-liquid chromatography (GLC; Shimadzu, Japan). Plasma urea and creatinine levels were assayed using a solid-phase chemiluminescence immunoassay (IKA Monarch, Germany) on the Immulite 2000 analyser (Siemens, Munich, Germany). The intra and inter-assay coefficients of variation were 3.7% and 4.2%, respectively.
acid (FFA) level was measured in non-frozen plasma by an enzymatic colorimetric method (Roche Diagnostics GmbH, Mannheim, Germany). CRP was determined by the highly sensitive immunoturbidimetric method (APTEC Diagnostics nv, Sint-Niklaas, Belgium). Within- and between-run imprecision CVs were 1.66% and 2.08%, respectively. Visfatin (Nampt/PBEF) was assayed by ELISA (BioVendor, Prague, Czech Republic). Within-run and between-run imprecision CVs were 6% and 7%, respectively. Gla-OC and Glu-OC were determined in serum by ELISA (Takara, Kyoto, Japan). Within-run and between-run imprecision coefficients of variation were: <4.8% and <2.4% (Gla-OC), and <6.66% and <9.87% (Glu-OC), respectively. Total osteocalcin level was calculated as the sum of Gla-OC and Glu-OC. Leptin, adiponectin (Adipocyte complement-related protein of 30 kDa – Acrp 30), resistin, and IL-6 were measured in serum using ELISA (R&D Systems Europe, Ltd., Minneapolis, USA). Within-run and between-run imprecision CVs were 3% and 4% for leptin, 4% and 6% for adiponectin, 5.3% and 8.2% for resistin, 6% and 7% for IL-6, respectively. Antioxidant defense markers (total antioxidant status of plasma (FRAP, ferric reducing ability of plasma), activity of glutathione peroxidase (GPx), activity of glutathione reductase (GR) were determined in plasma by automated enzymatic colorimetric methods (Allmed, Kraków, Poland) using the MaxMat Analyzer (MaxMat S.A., Montpellier, France). Within-run and between-run imprecision CVs were as follows: 3.2% and 7% (FRAP), 4.2%, and 8.5% (GPx), 3.8% and 8% (glutathione reductase), respectively.

For determination of biomarkers of protein glycation, oxidation and nitration, urine samples were collected during second urination of the day (mid-stream). The samples were centrifuged (4°C, 10 min, 1000 g) and frozen at –80°C for further measurements. Protein glycation, oxidation and nitration free products (glycated, oxidized and nitrated amino acids) were determined by assay of analytes in the ultrltraltricate (12 kDa filter cut-off, 50 µl aliquot) of urine. The assays were assayed by stable isotopic dilution analysis liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS), calibrated by reference to authentic standards and normalized to the urine creatinine level. Urine creatinine was measured using colorimetric assay based on the Jaffe method (Roche Diagnostics GmbH, Mannheim, Germany). The determined analytes were the following: glycation products Fl, MG-H1, CEL, Nε-carboxymethyl-lysine (CML); oxidation products: MetSO and NFK, and nitration adduct 3-nitrotyrosine (3NT) (Thornalley & Rabbani, 2014; Rabbani et al., 2017).

**Statistical analyses.** Statistical analyses were performed with the Statistica software (StatSoft). Nominal data were analyzed by χ² test. To assess the normality of data, the Shapiro-Wilk test was used. Continuous variables were log transformed if required. Normally distributed data are presented as mean ± S.E.M. or otherwise as median and quartile range 25–75%. Differences between the two studied groups were analyzed by unpaired t-test or U-Mann Whitney test (for non-normally distributed data). Comparison of results between multiple groups was performed by one way ANOVA, followed by post hoc Tukey test or Kruskal-Wallis test and Dunn’s test (for non-normally distributed data). To find a relation between variables, the Spearman rank correlation was used. The differences between variables with the P value less than 0.05 were considered to be significant.

### RESULTS

**Characteristics of participants**

Ninety-eight obese subjects and thirty-four non-obese subjects were recruited for this study. Clinical characteristics of these study groups are given in Table 1. Obese subjects had increased BMI (34.0 ± 28.4 kg/m²; P<0.001), adipose tissue mass (40.8 ± 34.5 kg; P<0.001), waist circumference (119 ± 89 cm), and blood pressure when compared to the non-obese subjects. They also had increased plasma leptin levels, fasting insulin and HOMA-IR index. There were no differences in the plasma total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides.

**Table 1. Characteristics of subjects participating in the study** (Razny et al., 2017)

|                   | Non obese (n=34) | Obese (n=98) | P*   |
|-------------------|-----------------|-------------|------|
| Age (years)       | 48.1±1.9        | 46.7±1.2    | 0.816|
| Sex, female (%)   | 79              | 73          | 0.391|
| BMI (kg/m²)       | 28.4 (27.4–29.1) | 34.0 (32.0–36.5) | <0.001|
| Waist circumference (cm) | 46 (107–110) | 116 (110–119) | <0.001|
| Adipose tissue mass (%) | 80%           | 85%         | 0.010|
| Systolic BP (mm Hg) | 120 (116–130) | 130 (120–140) | 0.020|
| Diastolic BP (mm Hg) | 80 (70–86)    | 85 (80–90)  | 0.010|
| Total Cholesterol (mmol/l) | 5.36±0.15    | 5.54±0.11   | 0.986|
| HDL Cholesterol (mmol/l) | 1.30±0.03    | 1.31±0.02   | 0.997|
| LDL Cholesterol (mmol/l) | 3.49±0.14    | 3.55±0.09   | 0.765|
| NEFA (mmol/l)      | 0.69±0.03      | 0.76±0.02   | 0.141|
| Triglycerides (mmol/l) | 1.31±0.11    | 1.51±0.07   | 0.201|
| Glucose (mmol/l)   | 5.22±0.08      | 5.24±0.05   | 0.488|
| Insulin (IU/ml)    | 12.4±1.48      | 16.7±0.78   | <0.001|
| HOMA-IR            | 2.06 (1.85–2.50) | 3.45 (2.64–4.66) | <0.001|
| Total OC (ng/ml)   | 15.92±0.96     | 15.17±0.47  | 0.100|
| Glu-OC (ng/ml)     | 12.68±0.90     | 11.36±0.39  | 0.048|
| Glu-OC/Glu-OC (ng/ml) | 3.68±0.81   | 3.83±0.22   | 0.281|

BMI, body mass index; BP, blood pressure; Gla-OC, carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment; LDL, low density lipoprotein; NEFA, non-esterified fatty acids; OC, osteocalcin; WHR, waist to hip ratio. *The same groups of subjects were described in a previous paper (Razny et al., 2017) Significant difference between non-obese and obese group (unpaired t-test or Mann-Whitney U-test for non-normally distributed variables) P<0.05, †Median, 25–75% in parentheses; ‡Mean ± S.E.M.
triglycerides and FFA between the study subject groups. Obese subjects had lower Gla-OC level, whereas serum Glu-OC and total serum osteocalcin were unchanged, which was also reported in a previous paper (Razny et al., 2017). In case of cytokines and adipokines, the obese subjects had increased serum hs CRP and IL-6 as well, when compared to the non-obese subjects (Table 2).

**Urinary protein oxidation, nitration and glycation free adducts and antioxidant defense markers**

Urine levels of protein glycation markers, FL and 3DG-H free adducts, and protein oxidation marker, NFK free adduct, were increased in obese subjects with respect to the non-obese subjects. Urinary levels of other glycation free adducts, MG-H1, CML, CEL, oxidation free adduct, MetSO, and nitration free adduct, 3-NT, were unchanged between study subject groups. Blood antioxidant defense markers, FRAP, GPx and GR, were also not significantly different between the two study groups (Table 2).

**Correlation of protein oxidation, nitration and glycation products with osteocalcin**

There were weak negative correlations of Gla-OC with urinary MG-H1 (r = –0.205, P = 0.020) and NFK free adducts (r = –0.249, P = 0.005) (Table 3). With the strong negative correlation of urinary NFK free adduct with Gla-OC, the subjects were classified by Gla-OC quartiles and urinary NFK free adduct levels were compared between these groups. In quartile 2 of serum Gla-OC, there is a trend for higher urinary NFK free adduct levels with respect to quartile 4 (Fig. 1). In addition, the Gla-OC cor-

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**Table 2. Serum cytokines and adipokines, urinary protein glycation, oxidation and nitration free adducts and antioxidant defense markers in obese and non-obese subjects**

| Cytokines and adipokines | Non-obese (n=34) | Obese (n=98) | P*  |
|--------------------------|------------------|--------------|-----|
| hs CRP (mg/l)            | 0.80 (0.42–1.52) | 2.22 (0.96–3.88) | 0.001 |
| IL-6 (pg/ml)             | 1.07±0.12        | 1.63±0.10     | 0.001 |
| IL-8 (pg/ml)             | 2.13 (1.58–2.89) | 2.21 (1.59–3.14) | 0.767 |
| TNFα (pg/ml)             | 4.8 (3.53–5.91)  | 5.94 (4.41–7.52) | 0.096 |
| Leptin (ng/ml)           | 27.80±1.74       | 40.66±2.44    | <0.001 |
| Adiponectin (μg/ml)      | 6.72 (4.42–9.46) | 6.14 (4.31–8.59) | 0.441 |
| Resistin (ng/ml)         | 9.78±0.52        | 10.11±0.40    | 0.730 |
| Visfatin (ng/ml)         | 1.09±0.16        | 1.17±0.08     | 0.237 |

**Urinary excretion of protein glycation, oxidation and nitration free adducts (nmol/mg creatinine)**

| FL                        | 4.18±0.488       | 7.679±1.176   | 0.017 |
| MG-H1                    | 2.548±0.345      | 3.364±0.286   | 0.140 |
| 3DG-H                    | 0.357±0.052      | 0.549±0.053   | 0.029 |
| CML                      | 6.364±0.605      | 8.789±0.837   | 0.165 |
| CEL                      | 0.476 (0.29–1.09) | 0.720 (0.39–1.39) | 0.106 |
| 3NT                      | 0.003 (0.002–0.005) | 0.003 (0.002–0.006) | 0.081 |
| NFK                      | 0.010 (0.004–0.029) | 0.028 (0.008–0.067) | 0.036 |
| MetSO                    | 0.050±0.008      | 0.050±0.004   | 0.984 |

**Antioxidant defense markers**

| FRAP (mmol/l)            | 0.990±0.038       | 1.046±0.020   | 0.126 |
| GPx (U/l)                | 520.967±40.703    | 476.825±16.266 | 0.230 |
| GR (U/l)                 | 66.168±2.966      | 66.664±1.400  | 0.676 |

CEL, Nε-carboxyethyl-lysine; CML, Nε-carboxymethyl-lysine; 3DG-H, hydroimidazolones derived from 3-deoxyglucosone; FL, fructosyl-lysine; FRAP, ferric reducing ability of plasma; GPx, glutathione peroxidase; GR, glutathione reductase; hs CRP, high sensitivity C reactive protein; IL-6, interleukin 6; IL-8, interleukin 8; MetSO, methionine sulfoxide; MG-H1, Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; NFK, N-formylkynurenine; 3NT, 3-nitrotyrosine; TNFα, tumor necrosis factor α. *Significant difference between non-obese and obese group (unpaired t-test or Mann-Whitney U-test for non-normally distributed variables) P<0.05, **Median, 25–75% in parentheses; Mean ± S.E.M.
Table 3. Spearman rank correlation between protein glycation, oxidation and nitration free adducts in urine (nmol/mg creatinine) and Gla-OC, Gla-OC/Glu-OC index as well as leptin and hs CRP. Correlations with \( P < 0.05 \) were considered significant.

|                    | All (n=132) | Non-obese (n=34) | Obese (n=98) |
|--------------------|-------------|-----------------|-------------|
|                    | \( r \)     | \( P \)         | \( r \)     | \( P \)         | \( r \)     | \( P \)         |
| Gla-OC             |             |                 |             |                 |             |                 |
| MG-H1 & GLA-OC     | -0.205      | 0.020           | -0.085      | 0.640           | -0.219      | 0.033           |
| CEL & GLA-OC       | -0.160      | 0.070           | 0.150       | 0.403           | -0.251      | 0.014           |
| NFK & GLA-OC       | -0.249      | 0.005           | -0.316      | 0.073           | -0.204      | 0.047           |
| GPX & GLA-OC       | 0.079       | 0.374           | 0.349       | 0.046           | -0.041      | 0.689           |
| GLA-OC/Glu-OC      | -0.165      | 0.062           | -0.016      | 0.928           | -0.207      | 0.044           |
| FL & GLA-OC/Glu-OC | -0.204      | 0.021           | 0.001       | 0.997           | -0.271      | 0.008           |
| MG-H1 & GLA-OC/Glu-OC | -0.171  | 0.054           | 0.140       | 0.437           | -0.272      | 0.008           |
| GPX & GLA-OC/Glu-OC | 0.188       | 0.032           | 0.268       | 0.132           | 0.141       | 0.170           |
| Leptin             |             |                 |             |                 |             |                 |
| 3DG-H & Leptin     | 0.222       | 0.012           | 0.040       | 0.826           | 0.163       | 0.114           |
| CML & Leptin       | 0.190       | 0.031           | -0.130      | 0.471           | 0.204       | 0.047           |
| FRAP & Leptin      | -0.161      | 0.067           | -0.235      | 0.188           | -0.200      | 0.050           |
| hs CRP             |             |                 |             |                 |             |                 |
| CML & hs CRP       | 0.262       | 0.003           | 0.016       | 0.929           | 0.262       | 0.010           |
| FRAP & hs CRP      | -0.113      | 0.201           | -0.165      | 0.359           | -0.201      | 0.049           |

CEL, N\text{-}carboxyethyl-lysine; CML, N\text{-}carboxymethyl-lysine; 3DG-H, hydroimidazolones derived from 3-deoxyglucosone; FL, fructosyl-lysine; FRAP, ferric reducing ability of plasma; GPx, glutathione peroxidase; hs CRP, high sensitivity C reactive protein; MetSO, methionine sulfoxide; MG-H1, N\text{-}5-(hydro-5-methyl-4-imidazolon-2-yl)-ornithine; NFK, N\text{-}formylkynurenine; 3NT, 3\text{-}nitrotyrosine

Relation of AGEs to osteocalcin in obesity

In our study, obese subjects had decreased serum carboxylated osteocalcin Gla-OC and increased urinary FL, 3DG-H and NFK free adducts when compared to the non-obese controls. The level of Gla-OC was correlated negatively with urinary NFK free adduct in obese subjects \((r=–0.251, P=0.015)\). In the non-obese subjects, Gla-OC correlated positively with GPx \((r=0.349, P=0.046)\) and total osteocalcin correlated negatively with urinary NFK free adduct \((r=–0.345, P=0.049)\). In the non-obese and obese subject groups combined, Gla-OC/Glu-OC index correlated negatively with urinary MG-H1 free adduct \((r=–0.209, P=0.021)\) and correlated positively with GPx \((r=0.188, P=0.030)\). Gla-OC/Glu-OC index also correlated negatively with urinary MG-H1 free adduct \((r=–0.271, P=0.008)\) in the obese subject group (Table 3). With the strong negative correlation of urinary MG-H1 free adduct and Gla-OC/Glu-OC index, obese subjects were re-classified by Gla-OC/Glu-OC quartiles and urinary MG-H1 free adduct levels were compared between quartiles. Urinary MG-H1 free adduct levels were lower in the obese subjects in quartile 4 of Gla-OC/Glu-OC, with respect to quartile 1 \((P<0.05, Fig. 2)\). In obese subjects, the urinary CEL and FL free adduct levels also correlated negatively with Gla-OC/Glu-OC index \((r=–0.272, P=0.008)\) and \((r=–0.207, P=0.044)\), respectively. In the non-obese and obese subject groups combined, serum leptin positively correlated with urinary 3DG-H and CML free adduct levels \((P=0.012)\) and \((r=0.190, P=0.031)\), respectively. In the non-obese subjects, serum leptin correlated negatively with FRAP \((r=–0.200, P=0.05)\). In the non-obese and obese subject groups combined, urinary CML free adduct correlated positively with hs CRP \((r=0.262, P=0.005)\). In the obese subjects only, urinary CML free adduct correlated negatively with FRAP \((r=–0.201, P=0.049)\).

**DISCUSSION**

In our study, obese subjects had decreased serum carboxylated osteocalcin Gla-OC and increased urinary FL, 3DG-H and NFK free adducts when compared to the non-obese controls. The level of Gla-OC was correlated negatively with urinary CEL and NFK free adducts.

Osteocalcin is a marker of bone turnover. It contains three glutamate residues, one of which is \(\gamma\)-carboxylated. This \(\gamma\) residue mediates the binding of calcium and hydroxyapatite to osteocalcin (Dowd et al., 2003). The endocrine function of osteocalcin is mediated by its under-
carboxylated form (Glu-OC) (Pi et al., 2011, Ferron et al., 2012). Glu-OC does not bind Ca\(^{2+}\) and does not require elevated Ca\(^{2+}\) concentration to fold into a helical structure (Hauschka et al., 1982; Dowd et al., 2001). It acts on pancreatic \(\beta\)-cells to increase insulin secretion, and on muscle and white adipose tissue to promote glucose homeostasis. Both osteocalcin forms are detectable in circulation. We have deduced the Gla-OC/Glu-OC ratio index and employed this in a correlation analysis. The Gla-OC/Glu-OC index correlated negatively with urinary MG-H1 and CEL free adducts and positively with the GPx activity.

Our results are in agreement with recent findings which indicated that obesity is connected with increased amounts of AGE in the body (Unoki et al., 2010; Andrade et al., 2015, Li et al., 2005; Gaens et al., 2014). However, studies are not clear to what extent AGEs reflect hyperglycemia or contribute to the progression of diabetes. It is well documented that excessive consumption of saturated fat and glucose can also promote advanced glycation (Beisswenger et al., 2005; Sandu et al., 2005; Forbes et al., 2013).

In the study presented here, the obese subjects with higher fasting insulin and insulin resistance (higher HOMA-IR index) without increased fasting plasma glucose, had increased urinary FL and 3DG-H free adducts when compared to the non-obese controls. Urinary FL free adduct are mainly derived from proteolysis of the FL residues of proteins glycated by glucose. In turn, urinary 3DG-H free adduct is formed mostly from proteolysis of 3DG-H residues of proteins glycated by 3-deoxyglucosone, which is mainly formed by degradation of FL. They are formed non-oxidatively (Thornalley et al., 1999). Urinary FL and 3DG-H free products principally originate from proteolysis of glycated proteins in tissues, with a minor contribution from glycated proteins found in food (Erbersdobler & Faist, 2001; Rabbani et al., 2014; Thornalley & Rabbani, 2014). Levels of urinary FL and 3DG-H free adducts relate to protein glycation in vascular and tissue compartments of the body in both, postprandial and fasting stages, and may be more responsive to metabolic dysfunction in obesity than measurements of fasting plasma glucose. Also, they may be considered as better markers than glycated albumin which likely suffers interference from the effects of change in albumin transcapillary escape rate and increased dwell time of albumin in interstitial fluid in obesity (Masania et al., 2016). Several AGE receptors are linked to increased inflammation, in case of the AGES’ action on bone cells, alternatively, AGE binding to its receptors can induce the production of inflammatory cytokines and reactive oxygen species (ROS), which is in agreement with results of our study. Namely, we have found positive correlation between AGES: 3DGHH and CML with proinflammatory leptin, as well as CML with hsCRP, which is elevated in obese subjects of the study presented here. Leptin, which is elevated in obese individuals, plays a key role in mediating a pro-inflammatory state in obesity and can induce oxidative stress (Wannamethee et al., 2007; Korda et al., 2008), which in consequence could lead to depletion of antioxidant defense markers (Niedowicz et al., 2005).

In our study, we have observed not statistically significant differences for ferric reducing ability of plasma (FRAP), glutathione reductase (GR) and glutathione peroxidase activity (GPx) in obesity, when compared with the non-obese subjects. GPx activity in serum of these patients was slightly lower (not statistically significant) than in controls. However, it has been shown that plasma GPx is mainly of the renal origin and decreases in GPx could indicate changes in renal biochemistry and binding of GPx to target cell membranes, rather than reflect the whole body response to oxidative stress. Moreover, other features of antioxidant defenses in obesity could be impaired (Molnar et al., 2004; Matusik et al., 2015). However, results of our study show a relation between inflammation, and antioxidative capacity in obese patients. We have found inverse correlation between leptin and ferric reducing ability of plasma (FRAP), as well as between hsCRP and FRAP. Slightly elevated level of FRAP and GR in obese subjects and association of FRAP with proinflammatory cytokines could suggest that the antioxidant defense tries to compensate for an enhanced production of ROS, but probably cannot compensate for it fully, which results in oxidative protein modification by reactions with amino acid residues which was also observed in our study. The correlation between generated ROS and certain oxidative modifications of individual amino-acids has been reported (Cai & Yan, 2013). In the study presented here, the obese subjects had increased urinary NFK free adduct, when compared to the non-obese subjects. NFK is a major product of oxidative damage to tryptophan. Urinary NFK free adduct correlated negatively with Glu-OC. Studies in humans concerning the effect of oxidation protein products on bone formation and osteocalcin level are still lacking. Experiments performed on osteoblast cell lines by Zhong (Zhong et al., 2009) reported that exposure of rat osteoblast cells to oxidation protein products down-regulated the expression of osteocalcin mRNA and protein, as well as inhibited proliferation of the cells. So far, the mechanism of oxidative modification of proteins in bone cells is not fully known. It was postulated that oxidative modification of proteins could inhibit proliferation and differentiation of the osteoblast cells through the ROS-dependent NF-κB pathway (Zhong et al., 2009).

Experiments conducted in vitro indicated inhibition of osteogenesis (downregulation of osteocalcin level) by AGES. Results of our studies have shown that the MG-H1 free adduct correlated negatively with serum osteocalcin and the Gla-OC/Glu-OC index. In obese subjects, urinary CEL free adduct also correlated negatively with Gla-OC and the Gla-OC/Glu-OC index. Our findings seem to be in agreement with previous in vivo data. Yamamoto (Yamamoto et al., 2001) had reported that treatment of osteoblast-like cells with AGES-modified bovine serum resulted in a reduced synthesis of collagen I and osteocalcin in response to stimulation of calcitriol. Treatment of osteoblast-like cells with AGE-modified RANKL increased osteoclastogenic potential. AGEs may enhance apoptosis in osteoblasts to form normal bone and increase osteoclasts and osteoblasts by inhibiting pro-oprototic cytokines. AGEs could also affect bone formation by inhibiting the osteoclast differentiation of stromal cells which is related to suppression of endoplasmic reticulum stress sensors and accumulation of abnormal proteins in the cells (Tanaka et al., 2013). Osteoblasts are known to synthesize proteins...
and to secrete them into the bone matrix during their differentiation. Unfolded proteins are removed through ER stress response. AGEs suppressed the levels of endoplasmic reticulum (ER) stress sensors such as IRE1α, ATF6 and thus contributed to inhibition of osteocalcin mRNA.

Besides ER stress, the cells could destroy damaged substances and organelles in the process known as autophagy, whose deficiency can cause increased oxidative stress in osteoblasts, secretion of receptor activator for nuclear factor-κB ligand (RANKL), and decreased mineralization. On the other hand, excessive autophagy is harmful to cells and leads to damage or death of cells (Alva et al., 2004; Platini et al., 2010). Studies by Meng (Meng et al., 2015) had indicated that short term effects of AGE-BSA included increased osteogenic function (increase in osteocalcin and alkaline phosphatase function) and decreased osteoclastogenic function (inhibition of RANKL and osteoprotegerin expression), which are likely mediated by autophagy and the RAGE/Raf/MEK/ERK signal pathway. However, increased treatment resulted in an opposite effect.

The study presented here has potential limitations. Namely, an unequal number of both sexes and the small number of subjects in each category. Another limitation of the study concerns participants enrolled in the control group of the study who were non-obese (BMI < 30 kg/m²) but did not display normal weight. The small number of the control group participants, in comparison to the obese subjects, could have also been a significant limitation. However, both groups of subjects did not differ statistically in regards of sex and age. A further potential limitation of this study is also the fact that protein oxidation, nitration and glycation free adducts were measured in urine of the study population. Subjects did not differ in regards of eGFR (not shown) and the results were normalized according to the creatinine level in urine. Urinary AGE free adduct levels may not reflect plasma protein AGEs, although it was shown recently that urinary MG-H1 free adduct level was a more sensitive marker of changes in insulin resistance than plasma protein AGEs (Xue et al., 2016).

In the study presented here, we have found higher level of urinary protein glycation and oxidation free adducts, FL, 3DG-H and NFK in obese subjects, when compared to the non-obese controls. This may indicate an increased modification of proteins in the state of insulin resistance and oxidative stress. We have also found negative correlation of some urinary protein glycation and oxidation free adducts with Gla-OC - a marker of bone formation. Previous studies had shown that Gla-OC was correlated negatively with inflammatory markers, such as hs CRP (Razny et al., 2017). Thus, decreased Gla-OC level, which may reflect defects in bone formation, could be a result of obesity associated inflammation, oxidative stress or the effect of AGEs. The mechanism of the AGEs’ effect on Gla-OC should be elucidated. Therefore, we postulate that the results of our study could be the basis for further studies explaining the mechanism of the effect of AGEs on osteocalcin action in larger groups of subjects. Our results argue in favor of the suggestion that increased formation of AGEs and protein oxidation products in insulin resistance in obesity could contribute to decreased Gla-OC level and in consequence lead to inhibition of bone formation.

Disclosure statement

There are no conflicts of interest.

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