Osteocalcin is independently associated with C-reactive protein during lifestyle-induced weight loss in metabolic syndrome

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List of abbreviations

ALAT	Alanine aminotransferase
ASAT	Aspartate aminotransferase
BMI	Body mass index
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
DXA	Dual Energy X Ray-Absorptiometry
ELISA	Enzyme linked immunosorbent assay
HbA1c	Hemoglobin A1c
HDL	High density lipoprotein
HOMA	Homeostasis model assessment
IL6	Interleukin 6
LDL	Low density lipoprotein
LH-RH	Luteinizing hormone releasing hormone
MetS	Metabolic syndrome
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
T2DM	Type 2 Diabetes mellitus
TG	Triglycerides
TGFβ1	Transforming growth factor β1
TNFα	Tumor necrosis factor α

Short summary

Bone-derived osteocalcin has been suggested to be a metabolic regulator, potentially improving insulin sensitivity. To scrutinize the relation between osteocalcin and peripheral insulin sensitivity, I analyzed changes of serum osteocalcin relative to changes in insulin sensitivity, low-grade inflammation and bone mineral density following lifestyle-induced weight loss in individuals with metabolic syndrome (MetS). 74 nonsmoking men (45–55 yr) with MetS were randomized to a lifestyle-induced weight loss program (supervision via telemonitoring) or to a control group. Before and after the 6 months intervention period clinical and laboratory parameters and serum osteocalcin levels were determined in fasting blood samples. Lifestyle-induced changes of body composition were analyzed by Dual Energy X Ray-Absorptiometry (DXA). 30 participants in the control and 33 participants in the intervention group completed the study and were included in the data analysis. In participants of the intervention group, weight loss resulted in markedly improved insulin sensitivity and amelioration of low-grade inflammation. Increased serum levels of osteocalcin correlated inversely with BMI ($r= -0.63$; $p< 0.001$), total fat mass ($r= -0.58$, $p< 0.001$), total lean mass ($r= -0.45$, $p< 0.001$), C-reactive protein (CRP) ($r= -0.37$; $p< 0.01$), insulin ($r= -0.4$; $p< 0.001$), leptin ($r= -0.53$; $p< 0.001$), triglycerides ($r= -0.42$; $p< 0.001$) and alanine aminotransferase (ALAT) ($r=-0.52$; $p< 0.001$). Regression analysis revealed that osteocalcin was associated with changes in CRP but not with changes in insulin concentration, adipose tissue mass or bone mineral density. These results illustrate that the weight loss-induced higher serum osteocalcin is primarily associated with reduced inflammation.
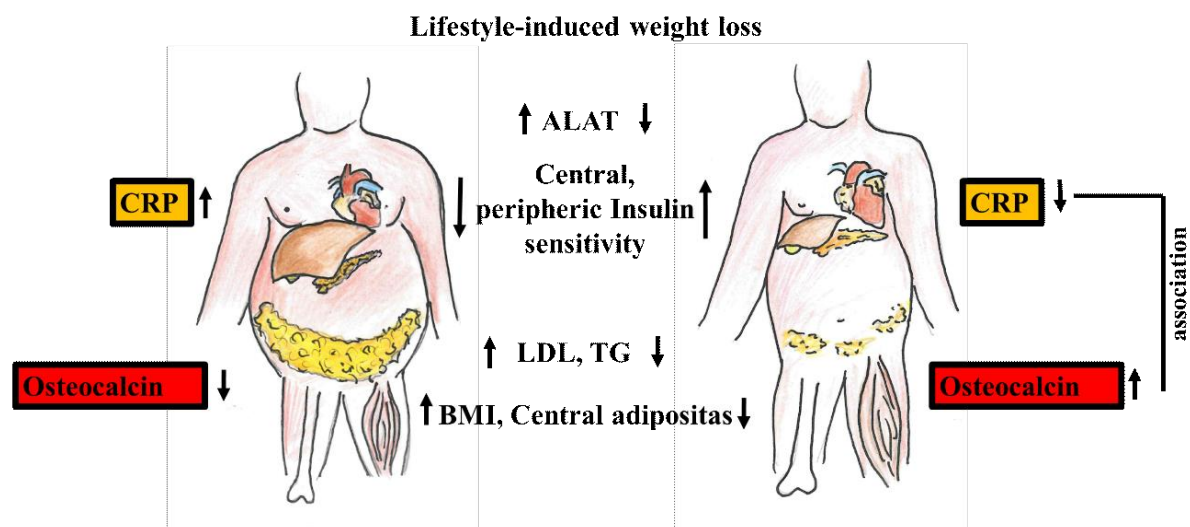
Graphical abstract

Figure 1: Short graphic summary of the research project: The cluster of raised fasting plasma glucose, abdominal adiposity, high triglycerides and high blood pressure is known as the metabolic syndrome (MetS). Lifestyle-induced weight loss is regarded an efficient therapy to reverse insulin resistance, to prevent T2DM and to reduce low-grade inflammation and CVD in patients with MetS (*1, 2*). A link between energy and bone metabolism has been proposed (*3–5*). Interestingly, osteocalcin knock out mice are characterized by an increase in visceral fat mass, hyperglycemia, hypoinsulinemia and reduced β-cell mass (*6*), indicating that osteocalcin regulates glucose and insulin metabolism (*7–9*). Additionally to osteocalcin`s effect on insulin, osteocalcin is known to suppress proinflammatory cytokine secretion *in vitro* (*10*).

Weight loss (intervention group) in individuals with MetS lead to an increase of osteocalcin, lowering of liver enzymes (transaminases), increased insulin sensitivity, increased bone mass, lowering of the inflammation markers CRP and IL6, lowering of the BMI as well as triglycerides and LDL cholesterol. Surprisingly, weight loss- induced changes in osteocalcin levels (intervention group) in individuals with MetS are associated with an improvement in low-grade inflammation, but not with changes in metabolism. These data suggest that osteocalcin reflects changes in insulin sensitivity only indirectly, possibly due to comcomitantly improved low-grade inflammation.

2 Introduction

The clustering of abdominal adiposity, elevated fasting glucose, elevated fasting triglycerides, reduced HDL cholesterol and high blood pressure, all established cardiovascular risk factors, is known as metabolic syndrome (MetS). Its increasing prevalence places a great burden on the health care system, as people with MetS have twice the risk of developing cardiovascular disease (CVD) and a five-fold increased risk of type 2 diabetes (*11*). In addition, the risk of CVD and type 2 diabetes increases with the number of MetS components (*12*). So far, lifestyle-induced weight loss has been considered an efficient therapy to reverse the MetS and prevent type 2 diabetes and CVD in people with MetS (*1, 2*). A link between (i) bone and (ii) energy metabolism and glucose homeostasis has been proposed (*3–5*). Of the different bone turnover markers, reduced osteocalcin levels are associated with overweight and MetS parameters that include higher waist circumference, higher triglyceride and glucose levels, increased blood pressure and lower HDL-cholesterol (*13–15*). Of note, osteocalcin levels are increased following 12 weeks exercise training and correlations between changes in BMI, HOMA, adipose tissue derived adiponectin and osteocalcin were observed, suggesting a functional interaction of osteocalcin with metabolic pathways upon weight loss (*16*). The measurement of serum osteocalcin appears to be a reliable index of bone formation, provided that vitamin D status and kidney function are normal (*17*). Interestingly, osteocalcin knockout mice are characterized by an increase in visceral fat mass as well as hyperglycemia, hypoinsulinemia and reduced β-cell mass (*6*), supporting the notion that osteocalcin regulates glucose metabolism and insulin metabolism in mice, although the underlying mechanism remains poorly defined (*7–9*). Furthermore, administration of osteocalcin has been shown to improve insulin sensitivity and to decrease the severity of adiposity and T2DM in mice fed with a high-fat diet (*8*). A relationship between reduced osteocalcin levels and adiposity has also been identified in apparently healthy children independent of their pubertal development (*18*). In addition to its effects on metabolism, osteocalcin has also been shown to suppress proinflammatory cytokine secretion, while stimulating the anti-inflammatory interleukin-10 in whole organ adipose tissue culture (*10*). Weight loss in MetS subjects leads to a reduction in low-grade inflammation, a reduction in body weight, and an improvement in glucose metabolism (*19–21*). However, the relationship between osteocalcin changes and systemic inflammation, body composition and metabolic function in MetS in weight loss is heretofore unknown.

To address this question, we determined the changes and interrelations of osteocalcin with clinical, metabolic and inflammatory parameters following lifestyle-induced weight loss in 74 well-defined individuals with MetS in a prospective study.

2a The metabolic syndrome

Based on the consensus definition from 2009, the MetS is defined as a cluster of metabolic risk factors that combine three out of five criteria. These criteria include abdominal adiposity (waist circumference >102 cm or BMI >30 kg/m^2), fasting triglyceride concentration $\geq$ 1.7 mmol/L; high-density lipoprotein (HDL) cholesterol <1.00 mmol/L; fasting glucose $\geq$ 5.6 mmol/L; blood pressure $\geq$ 130/85 mmHg or treatment for hypertension.

The MetS serves as precursor and may predict development of diabetes and CVD. Data of the NHANES public-use datasets show that the prevalence of the MetS increased between 1988–1994 and 1999–2006 among U.S. adults from 27.9% to 34.1% (22). This increase is associated with a concomitant increase in comorbidities and associated medical costs. Of note, total costs increase by an average of 24% per additional risk factor (23). Thus, identification and treatment of patients with MetS reduces the risk for progression to diabetes or CVD and reduces the medical care costs of public health care systems.

The rising prevalence of the MetS is supposed to be caused by the worldwide doubling in adiposity rates since 1980 (24). Accordingly, the risk of developing MetS and associated insulin resistance is closely linked to adiposity as well as lack of physical activity.

The prevalence of MetS is continuously increasing; in the US about a third of the population suffers from MetS (25–29). A high calorie diet combined with reduced physical activity is associated with impaired metabolic consequences such as decreased insulin sensitivity and increased abdominal fat accumulation (30, 31). So far, lifestyle-induced weight loss is regarded an efficient therapy to reverse MetS and to prevent type 2 diabetes and CVD in individuals with MetS, since effective lifestyle changes may reduce all of the metabolic risk factors (2). Studies show that low calorie diet reduces the risk of acquiring MetS (32) and furthermore can decrease the mortality in MetS (33, 34). The beneficial impact of healthy diet in MetS is further underlined by the fact that unsaturated fatty acids may decrease CVD risk (35). Exercise represents another important factor affecting the risk of MetS, as any type of physical activity has been shown to decrease the risk of developing MetS (32), of developing CVD (36–38) and of CVD mortality (39, 40). In addition, physical activity decreases the risk of developing T2DM (41, 42). Although lifestyle-induced weight loss therapy constitutes as first-line intervention, information about the molecular pathways that promote reversibility of MetS is scarce at best.

People who develop MetS have to commit to strict lifestyle change in order to avoid long-term complications, such as CVD or T2DM. This lifestyle change includes increased physical exercise combined with reduced calorie intake and avoidance of food components with high glycaemic index aiming to become accustomed to fewer calories through weight loss and to adjust metabolism accordingly (43). Hence, individuals with MetS are taught to eat low-calorie, healthy, conscious, low-sugar and low-fat foods and exercise regularly. This requires great adherence and willpower and often the lifestyle change can only be made temporarily. Long term weight loss is extremely challenging due to interactions between the biology, behavior, and the obesogenic environment (44). Processed food

and industrialization of the food system contribute largely to unsuccessful stable weight loss (*45*). In a meta-analysis of 29 long-term weight loss studies, more than 50 % of the lost weight was regained within two years and by five years more than 80 % of lost weight was regained (*46*). Hypercaloric nutrition and lack of exercise play an important role in the development of the MetS, and are therefore also the primary therapeutic targets. Since stable weight loss, in particular persistent weight loss, remains challenging, we need better insights into the mechanisms endangering weight loss and leading to weight gain again. One important aspect may be low-grade inflammation, which is associated with adiposity.

Chronic low-grade inflammation within the adipose tissue has been identified as a major component of the MetS (*47–49*). There is increasing evidence showing that chronic low-grade inflammation is associated with metabolic dysfunction. CRP has been shown to be linked to components of MetS and CRP levels in MetS have been shown to predict cardiovascular events (*50–52*). The interrelations of metabolic changes and chronic low-grade inflammation in MetS are highly connected. Nunn et al. (*53*) postulated that chronic subclinical inflammation associated with the MetS could be one reason for the continued physical inactivity, supporting a central role of low-grade inflammation. The rationale for this assumption is the well described behavioral change associated with injury and infection, which is commonly known as "inflammatory-induced sickness behavior" (*54*). Such observations suggest a complex crosstalk between adipose tissue, inflammation and impact on activity. Furthermore, many features of the MetS are associated with insulin resistance. The insulin resistance can be estimated by the homeostasis model of assessment index (HOMA index), which is calculated from fasting glucose and fasting insulin. Unfortunately, insulin assays are not standardized yet and therefore not suitable for diagnostic purposes. New approaches to detect individuals with MetS, e.g. based on inflammation or associated changes in other organs such as the liver, may be clinically useful.

Individuals with MetS commonly have an increased risk of nonalcoholic fatty liver (NAFL) that may lead to nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD) (*55–57*). NAFLD is generally diagnosed by increased transaminase levels and sonographic evidence of fat accumulation in the liver. Laboratories can routinely determine the NAFLD score, which estimates liver scarring based on age, BMI, impaired fasting glucose, alanine aminotransferase (ALAT; formerly also glutamate pyruvate transaminase, GPT), aspartate aminotransferase (ASAT, formerly also glutamate pyruvate transaminase, GOT), platelet count and albumin concentration. ALAT is specifically produced by hepatocytes. Whereas ALAT is only present in the cytoplasm and increases earlier and more dynamically in less severe hepatitis, ASAT is present in the cytosolic and mitochondrial compartment and increased ASAT levels predominate in more fulminant cases of hepatitis and liver cell destruction. Hence, determination of ALAT together with ASAT, provides valuable information about the affected cell compartment and the severity of the liver affection. ALAT was first characterized in 1950 by Arthur

Karmen (*58*). It catalyzes two parts of the alanine cycle, precisely the transfer of an amino group from L-alanine to α-ketoglutarate. This reversible transamination results in pyruvate and L-glutamate. The half-life of ALAT is approximately 47 hours. Histologically, NAFL presents as small or large fat droplets in the liver parenchyma (Figure 2). A major risk is the progression into NASH, which manifests itself as decreased performance and upper abdominal discomfort and histologically with inflammation, ballooning and cell death of the hepatocytes. An association between ALAT level and chronic inflammation, thought to reflect the associated low-grade hepatic inflammation, is known (*59*). Vanni et al. summarized the link between NAFLD (the hepatic manifestation of the metabolic syndrome, with insulin resistance as the main pathogenetic mechanism) and metabolic syndrome (*60*), indicating that hyperinsulinemia may be the consequence of NAFLD rather than vice versa. The free fatty acids in the serum (product of visceral adipose tissue lipolysis) represent the major source of triglycerides in NAFLD. The lipid overload overwhelms the oxidative capacity and reactive oxygen species lead to lipid peroxidation and cytokines and eventually fibrogenesis.
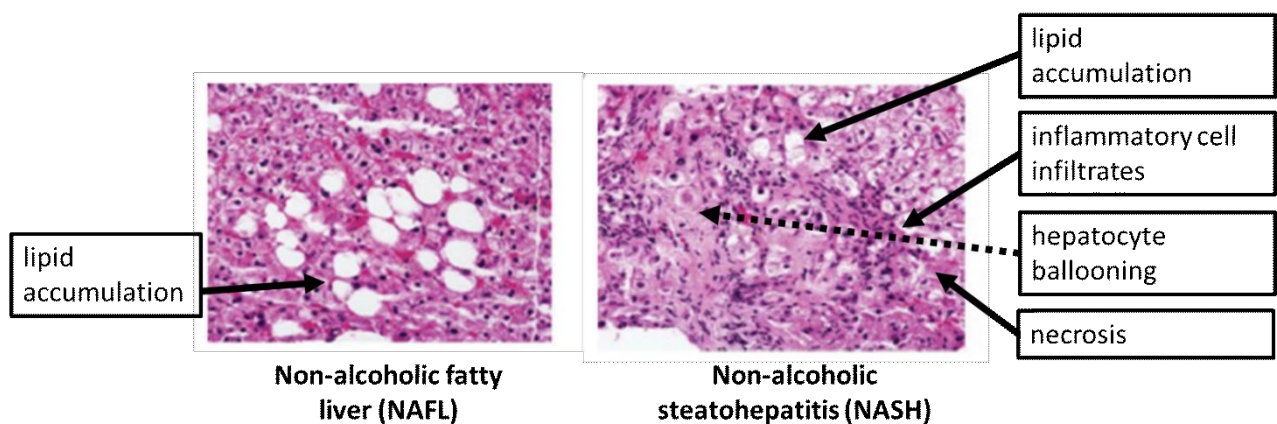

Figure 2: Typical histology of fatty liver disease: NAFL (left) and NASH (right) (*61*). NAFL and NASH are combined by the generic term NAFLD ("non-alcoholic fatty liver disease"). NAFL ('nonalcoholic fatty liver') describes a simple or bland fatty liver disease, in which small or large droplets of fat can be detected histologically, but not inflammatory changes. NASH ("nonalcoholic steatohepatitis") is characterized by an inflammatory reaction with hepatocyte damage such as ballooning and necroapoptosis with or without fibrosis (*62*).

The form of body fat distribution in MetS is characterized by fat accumulation mainly in the abdominal region, while the rest of the body remains comparatively slim, refers to the "apple shape (*63*)" which is associated with an increased risk of developing CVD (*64–68*). On the other hand, the form of body fat distribution, with fat accumulating mainly in the hip region (refers to as "pear shape (*63*)"), is associated with a lower risk of developing MetS complications (*69, 70*). To analyze body composition, precisely the amount of lean mass, bone mass and fat mass, Dexa scan technique can be used. This technique was originally intended for the evaluation and assessment of osteoporosis.

Interestingly, adiposity protects mammals from osteoporosis and fracture risk, suggesting that adipose tissue derived adipokines promote bone remodeling and bone mass density. Adiposity may influence bone remodelling through secretion of cytokines that directly target the bone. For example, adipose tissue secretes cytokines such as tumour necrosis factor-a (TNF-a), interleukin (IL)-1 and IL-6 regulate bone remodelling by triggering bone resorption or suppressing bone formation. In addition, adipose tissue derived leptin and adiponectin can similarly influence bone remodelling by endocrine actions or through direct influence on the hypothalamus regulating the sympathetic tone. Hence, adipokines such as adiponectin that influence the central nervous system may alter the communication of nerve impulses to the bone and paracrine influences on the skeletal cells (*71*). Moreover, the sympathetic stimulus can then inhibit the osteoblast differentiation. Another important aspect in the fat tissue- bone crosstalk is the fact, that adipocytes are an integral part of the bone marrow microenvironment, thus influencing skeletal function as well as hematopoiesis. The question arises as to whether the bone represents an independent endocrine organ that influences and can regulate the body's energy consumption.
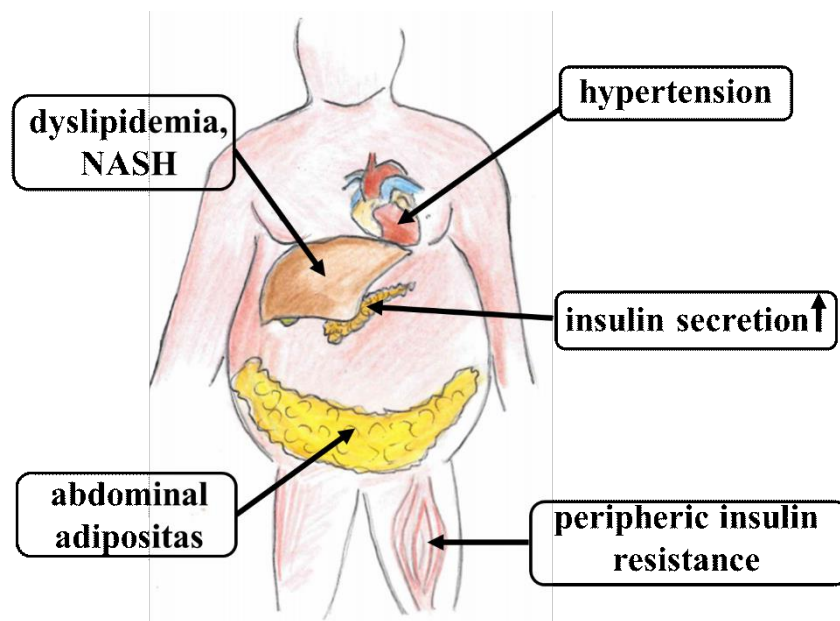

Figure 3: The MetS is a cluster of conditions that increase cardiovascular risk (*72–74*). This syndrome is mainly characterized by the simultaneous occurrence of dyslipidemia, increased blood pressure, central adiposity and peripheral insulin resistance or disturbed glucose tolerance.

2b C-reactive protein (CRP) and its role in MetS

CRP is a circular, pentameric protein found in blood plasma. The concentration of CRP increases after an inflammatory stimulus, being an acute phase protein derived primarily from the liver and being stimulated by IL-6 secreted from T lymphocytes or macrophages. Physiologically, it binds to lysophosphatidylcholine, which is expressed on dead or dying cells. This activates the complement system via C1q. The CRP was the first pattern recognition receptor to be identified (*75, 76*). It was discovered by Tillett and Francis in 1930 (*77*) and got its name because it reacted with the capsular polysaccharide (C-polysaccharide) of pneumococcus in patients with acute inflammation. The gene of CRP is located on chromosome 1 (1q23.2) (*76*), whereas CRP has 224 amino acids and a molecular mass of 25.106 Da.

CRP is elevated in a variety of diseases (*78*), as it increases as a result of acute or chronic inflammatory reactions (*79, 80*) of various origins, such as bacterial (*81–83*), viral (*84, 85*), fungal infections (*86*), rheumatoid diseases (*87, 88*), malignancies (*89, 90*), tissue damage (*91, 92*) or necrosis (*93*). TGFβ1 or TNFα can also increase CRP.

Initially, CRP was measured by the quellung reaction and thus only gave qualitative results. The quellung reaction, also called the Neufeld reaction, is a biochemical reaction in which antibodies bind to the bacterial capsule of among others Streptococcus pneumoniae, Klebsiella pneumoniae, Neisseria meningitides and Bacillus anthracis. CRP has been described as a substance in the serum of the patients with an acute inflammatory reaction which reacts with the C-polysaccarid on pneumococcus. The binding can then be visualized under the microscope; the positive reaction demonstrating opaque and enlarged capsules.

In healthy people, the CRP ranges between 0.8 and 3.0 mg/L. CRP increases with age (*94*) and shows seasonal fluctuations (*95*). Metabolic low-grade inflammation is assumed, when CRP varies between 2 and 10 mg/L. Higher values are observed in pregnancy, mild infections and viral infections (10-40 mg/L), bacterial infections (40-200 mg/L), severe bacterial infection and burns (> 200 mg/L).

Today, CRP is routinely measured in dynamic methods that use CRP-specific antibodies. Modern tests today are much more sensitive than the former quellung reaction. A high-sensitive CRP method quantifies CRP levels in the range of 0.5-10 mg/L, enabling detection of low-grade inflammation, e.g. associated with metabolic diseases. Among others, it can be used to monitor the cardiovascular risk (*96*).

Study results show that patients with higher basal CRP values live with a higher risk of developing diabetes mellitus, high blood pressure and cardiovascular diseases (*97*). Other studies have shown that

CRP can exacerbate ischemic necrosis (complement dependent). In animal models CRP inhibition can be an effective therapy for myocardial and cerebral infarcts (*98*). Chronic low- grade inflammation is a major component of the MetS (*47–49*). Case–control and cross-sectional studies have shown elevated CRP already in children and adolescents with components of the MetS (*99–106*).

Since we measured increased osteocalcin levels after weight loss in our study, we investigated the association of the latter with inflammatory parameters IL6 and CRP in order to elucidate their interrelations in MetS in lifestyle- induced weight loss.

2c Bone-derived osteocalcin and inflammation

Osteocalcin or "bone γ-carboxylglutamic acid-containing protein" ("BGP"; gene: BGLAP) is a peptide hormone discovered in 1975 in most vertebrates. Its synthesis is dependent on vitamin K due to the Gla domains. Osteocalcin is a non-collagenous protein found in the bone matrix, which has a molecular weight of 5800 Da and contains 49 amino acids. It is synthesized uniquely in the bone by osteoblasts (*107*) and plays an important role in bone metabolism and in the regulation of bone mineralization. Studies indicate that the amount of circulating osteocalcin reflects the rate of bone formation (*108–111*). Osteocalcin has various hormone-like properties: It is a cell-specific molecule that is synthesized as a premolecule, carboxylated at three amino acids and then secreted into the circulation (*112, 113*). It acts like a systemic hormone that signals to the pancreas, fat, muscles, testes and brain (*114*). Analyzes of osteocalcin-deficient mice show an increase in visceral fat mass, hyperglycaemia, hypoinsulinemia and reduced β-cell mass (*6*). After production it is partially incorporated into the bone; the rest is found freely circulating in the blood. The exact physiological function is still unclear (*115–117*). Clinically, osteocalcin is determined in the plasma for the identification of women at risk for osteoporosis, monitoring of bone metabolism in menopause, during hormone replacement therapy, treatment with LH-RH agonists and monitoring of bone metabolism in patients with growth hormone deficiency, hypothyroidism, hyperthyroidism and chronic kidney disease.

Furthermore, a link between osteocalcin and inflammation has been suggested: osteocalcin can suppress pro-inflammatory cytokine secretion, while stimulating the anti-inflammatory interleukin-10 in whole organ adipose tissue culture (10). In mice, osteocalcin knockout increases the visceral fat mass. In our study, we investigated inflammatory parameters such as IL6 and CRP in order to elucidate the association of bone-derived osteocalcin changes and low-grade inflammation in MetS in a lifestyle-induced weight loss. A link between osteocalcin and inflammation has been suggested: osteocalcin can suppress pro-inflammatory cytokine secretion. Low levels of CRP characterize MetS (*118–122*) and can be reduced in weight loss (*123–125*).

Osteocalcin can trigger adipocytes to secrete adipokines and thus represents an important link to metabolism and inflammation. Osteocalcin seems to play a major role in bone metabolism and has interrelations to fat tissue and inflammation. Hence, bone-derived osteocalcin has been suggested to be a metabolic regulator (*8, 126, 127*), providing a link between energy metabolism and glucose homeostasis (*3–5*). Intriguingly, a therapeutical application of osteocalcin to mice fed with a high-fat diet could improve insulin sensitivity and fat content in T2DM (*8*), suggesting that osteocalcin is – somehow – mechanistically linked to metabolism and the underlying mechanism may provide insights into new therapeutic approaches to adiposity.
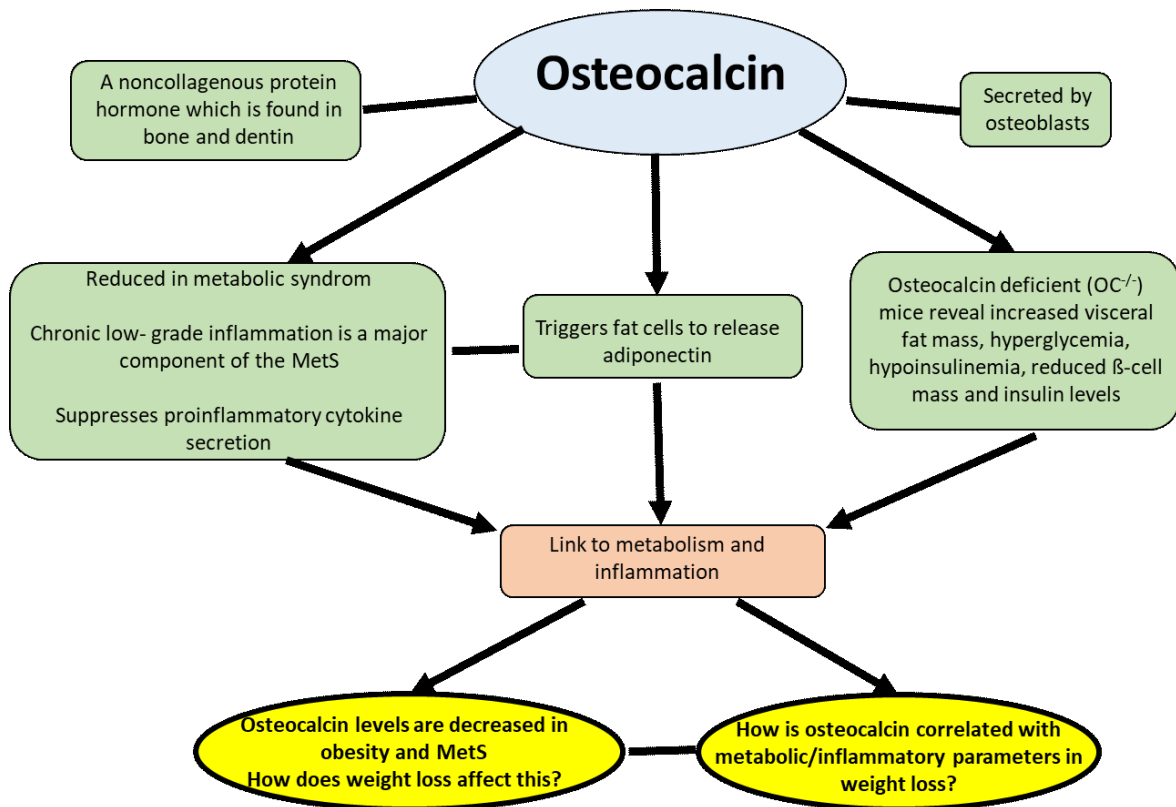
Figure 4: Scheme summarizing the functions of osteocalcin. Osteocalcin is a non-collagenous protein of bone and dentin. It has anti-diabetic and anti-inflammatory properties and is lowered in MetS. Osteocalcin can trigger adipocytes to secrete adipokines and thus represents an important link to metabolism and inflammation. Osteocalcin-deficient mice have increased fat mass and are prone to diabetes.

Aim of the study

The relationship between changes in osteocalcin, body composition, metabolic parameters and systemic low-grade inflammation in MetS remains unknown. To address this question, we determined changes and interrelations of osteocalcin with clinical, metabolic and inflammatory parameters following lifestyle-induced weight loss in 74 well-defined individuals with MetS in a prospective study.

2d Study design

The study is embedded in a prospective, two-armed, controlled, mono-centric, randomized, 6-months intervention trial to identify changes in clinical and laboratory parameters in individuals with MetS following lifestyle-induced weight loss. For this purpose, paired blood samples and subcutaneous adipose tissue biopsies were collected at the Institute of Clinical Chemistry and Pathobiochemistry, Otto-von-Guericke University, Magdeburg, Germany. The trial included non-smoking, non-diabetic men aged between 45 and 55 years with MetS as defined by the consensus guidelines 2009 (*11*) (three out of five criteria): abdominal adiposity (waist circumference >102 cm or BMI >30 kg/m^2), fasting triglyceride concentration ≥1.7 mmol/L (or drug treatment); high-density lipoprotein (HDL) cholesterol <1.00 mmol/L; fasting glucose ≥5.6 mmol/L (or drug treatment); blood pressure ≥ 130/85mmHg or treatment for hypertension. 74 well-defined individuals with MetS were included in the study and underwent a structured education about diet and the importance of physical activity. Individuals were randomly assigned to a 6-month lasting telemonitored lifestyle-induced weight loss program or a control group by a web-based randomisation tool using permuted block randomisation with stratification on BMI (Randomisation In Treatment Arms (RITA); University of Lübeck, Germany). Participants of the intervention arm were instructed to reduce calorie intake, and perform a low-carbohydrate diet with preference for low glycemic index carbohydrates. The subjects were advised to increase their usual daily physical activity, like walking or cycling, rather than to engage in particular sports. The recommendation was to perform these activities moderately but steadily, slowly enough to be able to talk at the same time, and to keep the pulse below 120/min (*128*). Participants of the intervention arm received accelerometers measuring their daily activity and instructions for daily data transmission of body weight, physical activity and approximated caloric intake. Clinical and laboratory parameters, osteocalcin concentrations and body composition were determined before and after the six-month intervention period.
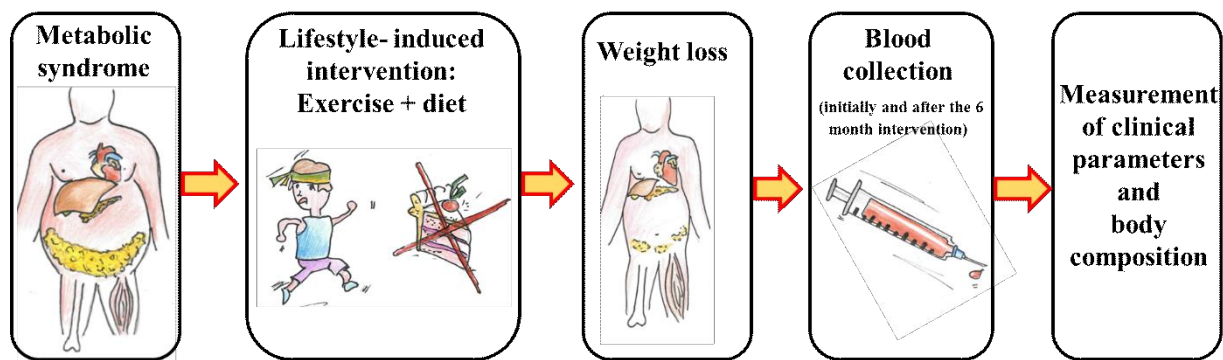
Figure 5: Schematic of study design. We aimed to determine the changes and interrelations of osteocalcin with clinical, metabolic and inflammatory parameters following lifestyle- induced weight loss with exercise in a well-defined MetS cohort. Participants with MetS underwent a structured lifestyle-induced intervention (exercise and diet). Clinical and laboratory parameters and osteocalcin levels were measured initially and after the 6-month study period. The study is embedded in a two-armed, controlled, monocentric, randomized, 6-month intervention trial. 30 participants in the control and 33 participants in the intervention group completed the study and were included in the data analysis. The study population did not differ in the distribution of age, sex and parameters of the MetS.

3 Original peer-reviewed publication: "Osteocalcin Is Independently Associated with C-Reactive Protein during Lifestyle-Induced Weight Loss in Metabolic Syndrome"

Article

Osteocalcin Is Independently Associated with C-Reactive Protein during Lifestyle-Induced Weight Loss in Metabolic Syndrome

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Abstract: Bone-derived osteocalcin has been suggested to be a metabolic regulator. To scrutinize the relation between osteocalcin and peripheral insulin sensitivity, we analyzed changes in serum osteocalcin relative to changes in insulin sensitivity, low-grade inflammation, and bone mineral density following lifestyle-induced weight loss in individuals with metabolic syndrome (MetS). Participants with MetS were randomized to a weight loss program or to a control group. Before and after the 6-month intervention period, clinical and laboratory parameters and serum osteocalcin levels were determined. Changes in body composition were analyzed by dual-energy X-ray absorptiometry (DXA). In participants of the intervention group, weight loss resulted in improved insulin sensitivity and amelioration of inflammation. Increased serum levels of osteocalcin correlated inversely with BMI (r = −0.63; p < 0.001), total fat mass (r = −0.58, *p* < 0.001), total lean mass (r = −0.45, *p* < 0.001), C-reactive protein (CRP) (r = −0.37; *p* < 0.01), insulin (r = −0.4; *p* < 0.001), leptin (r = −0.53; *p* < 0.001), triglycerides (r = −0.42; *p* < 0.001), and alanine aminotransferase (ALAT) (r = −0.52; *p* < 0.001). Regression analysis revealed that osteocalcin was independently associated with changes in CRP but not with changes in insulin concentration, fat mass, or bone mineral density, suggesting that weight loss-induced higher serum osteocalcin is primarily associated with reduced inflammation.

Keywords: metabolic syndrome; osteocalcin; lifestyle-induced weight loss

1. Introduction

The cluster of raised fasting plasma glucose, abdominal obesity, high triglycerides, and high blood pressure—all well-established cardiovascular risk factors—is commonly referred to as metabolic syndrome (MetS). Its increasing prevalence represents a major public health burden, as individuals with MetS have twice the risk of developing cardiovascular disease (CVD) and a five times elevated risk for type 2 diabetes mellitus (T2DM) [1]. In MetS, lifestyle-induced weight loss is regarded an effective therapy to reverse insulin resistance, to prevent T2DM, and to reduce low-grade inflammation and CVD [2,3].

A link between (I) bone and (II) energy metabolism and glucose homeostasis has been proposed [4–6]. Of the different bone turnover markers, reduced osteocalcin levels are associated with overweight and MetS parameters that include higher waist circumference, higher triglyceride and glucose levels, increased blood pressure, and lower HDL-

cholesterol [7–9]. Of note, in one study, osteocalcin levels were increased following 12 weeks of exercise training and correlations between changes in BMI, HOMA, adipose tissue-derived adiponectin, and osteocalcin were observed [10].

Osteocalcin is a noncollagenous protein secreted by osteoblasts, reflecting osteoblast activity [11]. Interestingly, osteocalcin knockout mice are characterized by an increase in visceral fat mass, as well as hyperglycemia, hypoinsulinemia, and reduced β-cell mass [12], indicating that osteocalcin regulates glucose metabolism and insulin secretion in mice [13–15]. Furthermore, administration of osteocalcin was shown to improve insulin sensitivity and to decrease the severity of obesity and T2DM in mice fed with a high-fat diet [14]. A relationship between reduced osteocalcin levels and obesity was also identified in apparently healthy children, independent of their pubertal development [16]. In addition to its effects on insulin, osteocalcin has also been shown to suppress proinflammatory cytokine secretion, while stimulating the anti-inflammatory interleukin 10 in whole organ adipose tissue culture [17]. However, the relationship between changes in osteocalcin, body composition, metabolic parameters, and systemic low-grade inflammation following lifestyle-induced weight loss in MetS remains unknown. To address this question, we determined the changes and interrelations of osteocalcin with clinical, metabolic, and inflammatory parameters following lifestyle-induced weight loss in 74 well-defined individuals with MetS in a prospective study.

2. Results

2.1. Clinical and Laboratory Parameters

We analyzed serum osteocalcin levels before and after lifestyle-induced weight loss in individuals with metabolic syndrome (MetS). Seventy-four nonsmoking men (45–55 years old) with MetS were randomized to a lifestyle-induced weight loss program (supervised via telemonitoring) or to a control group. Clinical and laboratory parameters and osteocalcin concentrations were determined in fasting blood samples before and after the six-month weight loss intervention (Figure 1, Supplementary Table S1). Thirty participants in the control and 33 participants in the intervention group completed the study and were included in the data analysis. Two participants of the intervention group and four of the control group did not follow the study protocol, and three participants of the control group left the study because they were not selected for the intervention group. The remaining two dropouts of the intervention group declined to continue for undisclosed reasons. The study populations did not differ in regard to the distribution of age, sex, or MetS parameters. Participants of the intervention arm reduced their individual body weight by at least 5%. Notably, 76% reduced their body weight by at least 10%, and 40% reduced their body weight by at least 15%, similar to previous reports [18] (Figure 2). To address the question of whether weight loss affects osteocalcin levels in individuals with MetS, and whether osteocalcin is associated with metabolic parameters, body composition, or inflammation, we determined the changes and interrelations of predefined parameters before and after lifestyle-induced weight loss (Figure 3, Supplementary Table S1). In participants of the intervention group, lifestyle-induced weight loss resulted in reduced levels of insulin, leptin, LDL cholesterol, triglycerides, C-reactive protein (CRP), and alanine aminotransferase (ALAT) (Figure 3a).
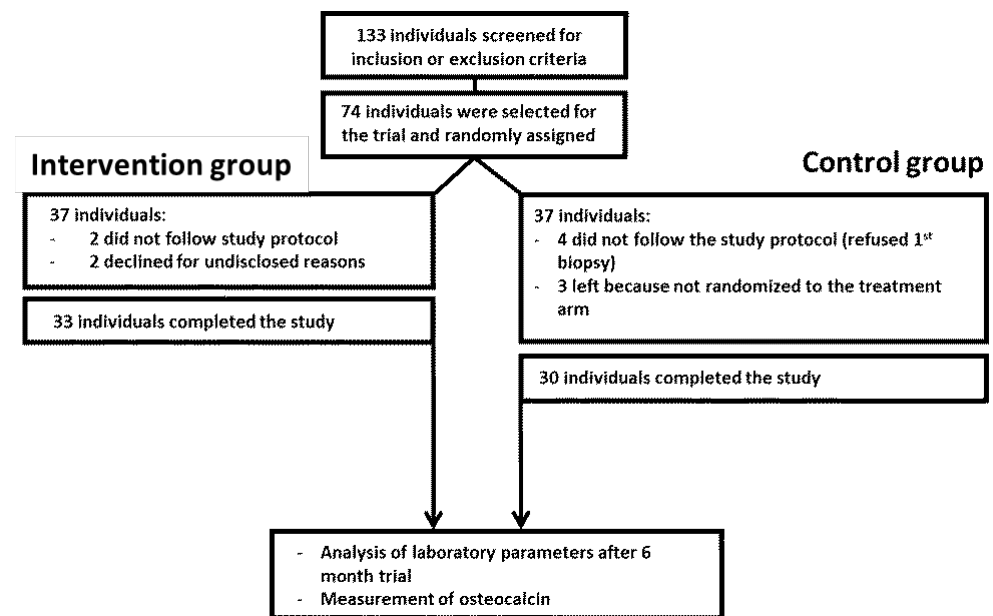
Figure 1. Schematic of study design. The study was embedded within a two-armed, controlled, monocentric, randomized, 6-month intervention period. Paired blood samples were collected before and after the 6-month intervention period. Thirty participants in the control group and 33 in the intervention group completed the study and were included in the data analysis. Two participants of the intervention group and four of the control group did not follow the study protocol, and three participants of the control group left the study due to not being included in the weight loss program (intervention group). The remaining two dropouts of the intervention group declined to continue for undisclosed reasons.
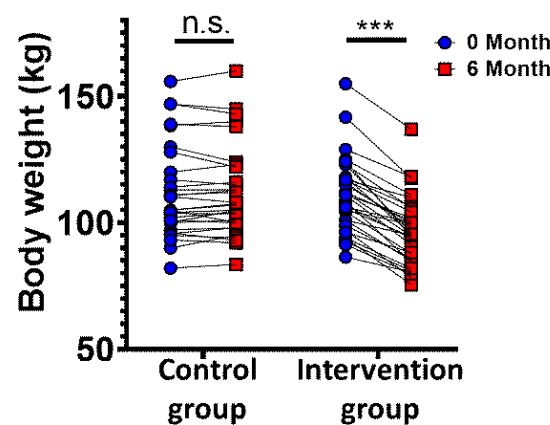
Figure 2. Line graph of total weight loss in both groups. Data is presented as total body weight in kg. The Wilcoxon signed-rank test was used to analyze differences between paired samples with *n* = 33 intervention, *n* = 30 control, *** $p < 0.001$.
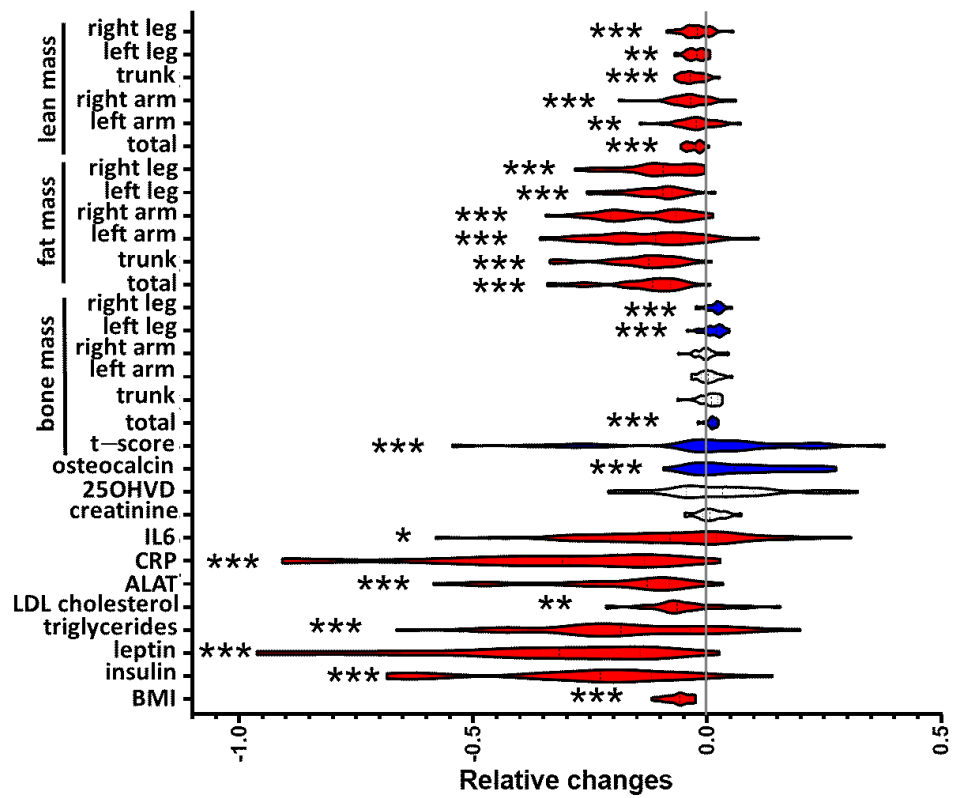
(**a**)
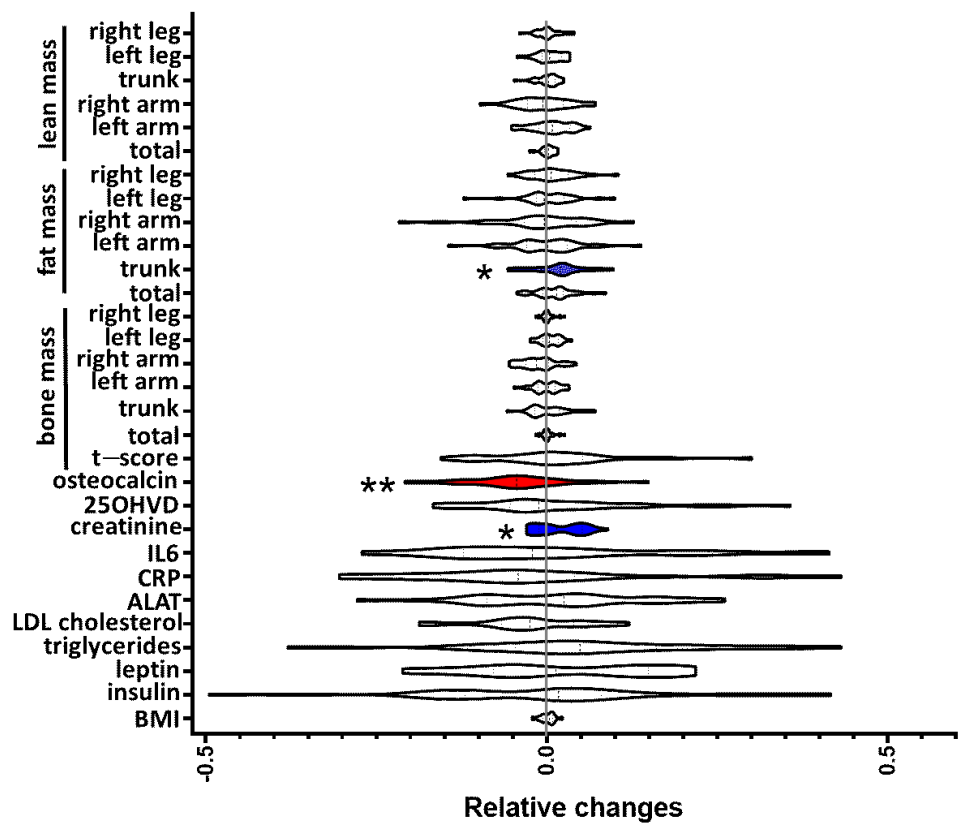
(**b**)

Figure 3. (**a**) Changes in body composition and clinical and laboratory parameters in participants of the intervention group. Violin plots of log ratios of given parameters before and after the 6-month intervention period. The Wilcoxon signed-rank test was used to analyze differences between paired

samples; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The grey line denotes baseline levels. The red color indicates a significant reduction while the blue color reflects a significant increase in the indicated parameter after the intervention period. (**b**) Changes in body composition and clinical and laboratory parameters in participants of the control group. Violin plots of log ratios of given parameters before and after the 6-month study period. The Wilcoxon signed-rank test was used to analyze differences between paired samples; * $p < 0.05$; ** $p < 0.01$. The grey line denotes baseline levels. The red color indicates a significant reduction while the blue color reflects a significant increase in the indicated parameter after the study period.

Reduction in body weight was mainly attributable to a significant reduction in individual body fat mass, as measured by DXA (−23,57%, $p < 0.001$; Figure 3a). In controls, BMI and other parameters of body composition remained unchanged, and trunk fat mass even increased by 4.78% [18] (Figure 3b). In both groups, no progression to overt type 2 diabetes was observed. We observed an increase ($p = 0.01$) in bone-derived osteocalcin levels in participants of the intervention group following the 6-month weight loss trial (Figure 3a). Meanwhile, in participants of the control group (Figure 3b), we observed a slight decrease in osteocalcin after the 6-month trial period. DXA was performed at baseline and after the 6-month trial period in order to evaluate lifestyle-induced changes in body composition. Participants in the intervention group showed an increased t-score and increased total bone mass, mainly due to an increase in bone mass in the legs (Figure 3a). In addition, we observed a reduction in body fat mass in all analyzed regions (total, trunk, both arms, both legs) in the intervention group. Similarly, lean mass was reduced in all measured body regions after weight loss. In the control group, a slight increase in trunk fat mass, increased creatinine levels (Figure 3b), and decreased GFR levels (Supplementary Table S1) were observed in addition to the decreased osteocalcin, while other parameters remained unchanged (Figure 3b).

2.2. Correlation Analysis of Osteocalcin and Parameters of the Metabolic Syndrome

We calculated the correlation between changes in osteocalcin, metabolic markers, body composition, and inflammation. We found correlations of the relative (log ratios) changes in osteocalcin with BMI ($r = −0.63$; $p < 0.001$), leptin ($r = −0.53$; $p < 0.001$), ALAT ($r = −0.52$; $p < 0.001$), CRP ($r = −0.37$; $p < 0.01$), insulin ($r = −0.4$; $p < 0.001$), and triglycerides ($r = −0.42$; $p < 0.001$) (Figure 4). Hence, an increase in osteocalcin was found to be associated with lifestyle-induced weight loss and the associated metabolic and inflammatory improvements. Interestingly, while a correlation was found between osteocalcin and total fat mass ($r = −0.58$; $p < 0.001$) and between osteocalcin and total lean mass ($r = −0.45$; $p < 0.001$), we found no correlation between osteocalcin and total bone mass ($r = 0.08$).

The dendrogram (Figure 4, Supplementary Figure S1) shows that osteocalcin connects closely to bone mass changes (clade ι). GFR and osteocalcin (chunk 11 and 12) join together first in the branching diagram and are closely connected to changes in bone mass (chunk 10). Of note, this clade (clade ι) is clearly separated from the others. Body composition parameters BMI, total fat mass, leptin, and total lean mass are linked (clades α, β) and more distinctly related to changes in inflammatory and liver-injury markers CRP and ALAT, respectively (clade δ). Insulin level changes and triglyceride level changes were strongly similar (clade ζ), and on a higher level connected to all previously mentioned parameter changes in inflammation and body composition (clade ε) (Figure 4).
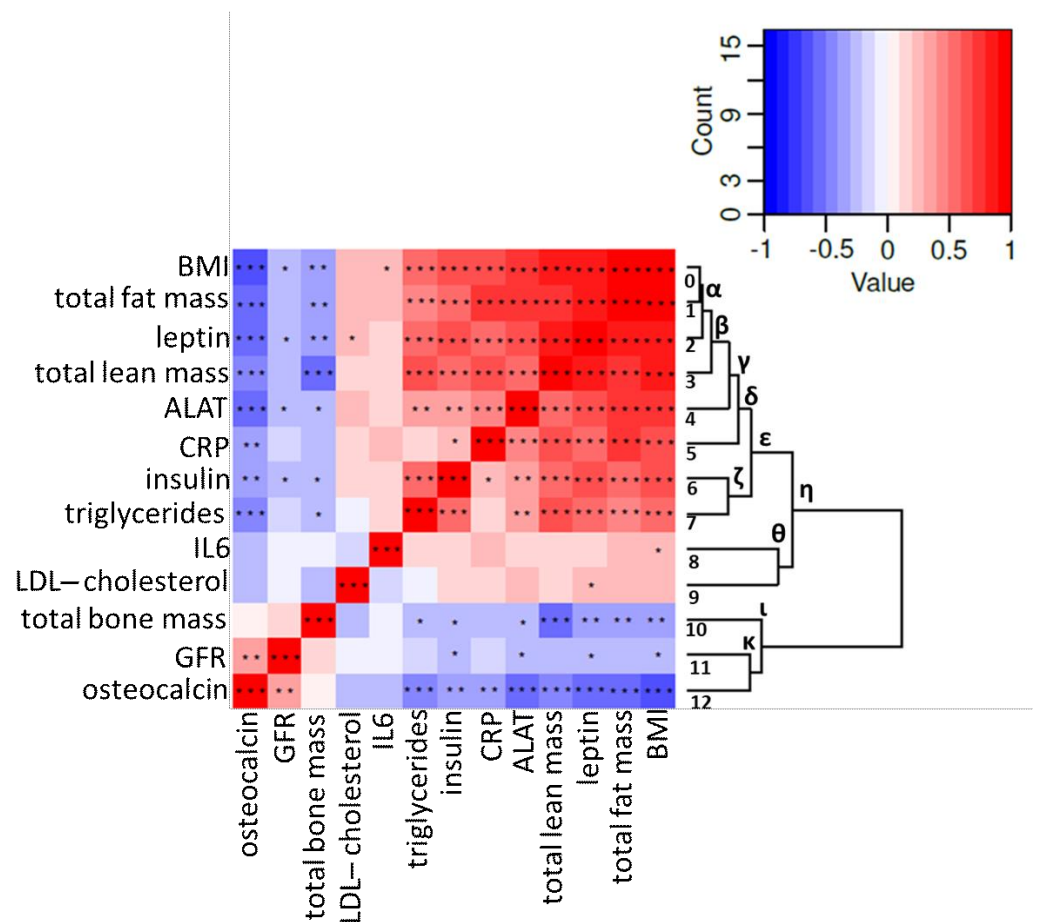
Figure 4. Correlation between OC, metabolic markers, body composition, and inflammation during the 6-month study in both groups. The heatmap (left) represents Spearman correlations between relative changes in variables. Colors indicate the correlation coefficient, ranging from blue (−1,0) to white (0,0) to red (1,0). Stars represent significant *p*-values: * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. The dendrogram (right) indicates the hierarchical relationship between variables. Variables with similar patterns of correlation are clustered together. Numbers indicate chunks, Greek letters indicate clades.

2.3. Multiple Regression Analysis between Osteocalcin and Parameters of the Metabolic Syndrome

Considering all variables that were significantly correlated with osteocalcin (Figure 4: BMI, total fat mass, leptin, total lean mass, ALAT, CRP, insulin, GFR, and triglycerides) as potential independent determinant variables, we performed a Bayesian multiple linear regression analysis to identify which of these variables are predictive for changes in osteocalcin (Figure 5). To avoid multicollinearity in the regression, we excluded leptin from the independent variables, since it was highly associated with insulin and glucose (not shown), as well as 'total fat mass', due to it being highly associated with BMI (not shown). The regression analysis was performed with data of both groups. In summary, we fit the regression model to the data and obtained a posterior distribution that describes the relation between the independent and dependent variables. The regression showed that CRP level change is the most promising variable to predict osteocalcin level change in lifestyle-induced weight loss. At a confidence interval (CI) of 90%, the probability mass of beta-CRP does not contain zero, while the other variables do (mean beta-CRP = −0.09, with a standard deviation of 0.05, ranging from −0.18 to −0.01 with a 90% CI, Figure 5).
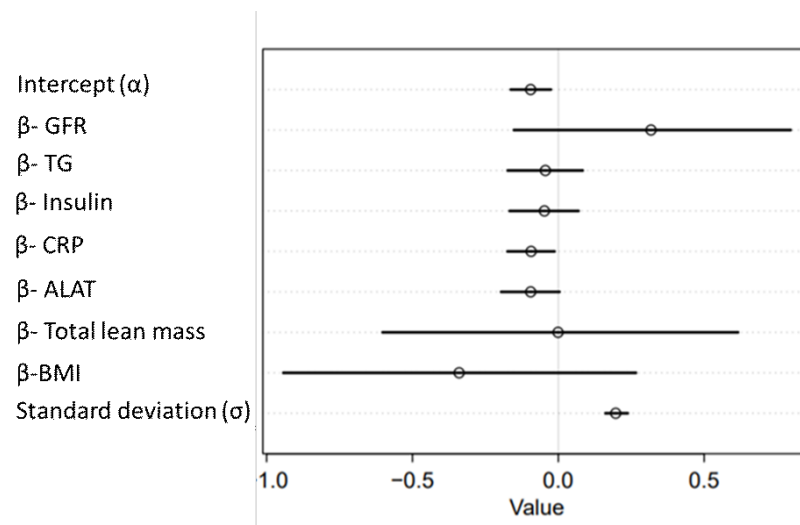
Figure 5. Multiple linear regression analysis between osteocalcin (dependent) and metabolic, inflammatory, and body composition variables (independent). *X*-axis, confidence intervals (CIs) of the strength of correlation to osteocalcin change; *y*-axis, parameters of the regression: slope coefficients of each variable (beta), intercept (alpha), and standard deviation (sigma). Considering all slope coefficients, beta-CRP is the only parameter with a completely negative CI, indicating that CRP level change is associated with OC level change in lifestyle-induced weight loss. All other slope CIs range from negative to positive values, indicating that their variables alone cannot predict OC level change. TG, triglycerides; CRP, C-reactive protein; ALAT, alanine aminotransferase; BMI, body mass index; GFR, glomerular filtration rate.

3. Discussion

To examine the effect of weight loss-induced changes in body composition and metabolic parameters on osteocalcin levels, we determined osteocalcin serum levels, body composition, and metabolic parameters in a prospective, blinded, randomized weight loss study. Multiple linear regression analysis revealed that changes in insulin, leptin, bone mass, and adipose tissue did not show relevant associations with increased serum osteocalcin levels following weight loss. The only association we found to be independently related to osteocalcin was that of C-reactive protein (CRP). This raises the question as to whether the previously reported associations of osteocalcin with metabolic parameters are, in fact, indirect, instead reflecting the subchronic inflammatory state associated with MetS.

The correlation between osteocalcin and inflammation in our study is in agreement with observations in nontraumatic fractures in which osteocalcin was inversely associated with CRP, independently of metabolic, cardiovascular, or chronic diseases [19,20]. Congruently, Riquelme-Gallego et al. identified an association between reduced osteocalcin levels and increased cardiovascular risk in MetS patients without diabetes mellitus type 2 [21]. Furthermore, osteocalcin has been associated with IL6 and CRP in T2DM [22,23]. Likewise, we observed a decrease in IL6; yet this association did not reach significance, possibly due to the low number of participants that were enrolled in our study. In support of a close interaction between osteocalcin and CRP, osteocalcin has been shown to suppress proinflammatory cytokine secretion (TNF-alpha and IL6), while stimulating anti-inflammatory interleukin–10 in vitro [17]. The latter finding raises the intriguing possibility that osteocalcin is not associated with, but actually modulates, low-grade inflammation in the setting of MetS. As our study was not designed to determine causality, future studies are needed to address this possibility.

The observed increase in circulating osteocalcin levels following lifestyle-induced weight loss is congruent with cross-sectional observations showing reduced osteocalcin in patients with obesity or MetS [24–27].

Given the predominant production and secretion of osteocalcin by osteoblasts, we initially expected that an increase in osteocalcin would be related to changes in bone mass. Contrary to our assumption, however, osteocalcin was not correlated with changes in bone mass. Instead, it was correlated with reduced adipose tissue mass, reduced lean mass, and changes in metabolic and inflammatory parameters that include fasting insulin levels, leptin, triglycerides, CRP, and ALAT. However, regression analysis revealed that the only parameter independently associated with osteocalcin was CRP. Of note, vitamin D and glomerular filtration rate did not change following lifestyle-induced weight loss, excluding the hypothesis that observed changes in osteocalcin and bone mass were influenced by kidney function or vitamin D deficiency in our study [28,29]. Taken together, these findings imply that increased osteocalcin levels following lifestyle-induced weight loss are primarily associated with reduced low-grade inflammation, while improved peripheral insulin sensitivity and liver parameters may be secondary to these changes.

Strengths of the current study include a well-characterized study population with no differences at baseline, a prospective study design, and a high compliance of participants as a result of daily telemonitoring and weekly letters commenting on individual weight progress. All laboratory measurements were performed following standard operating protocols and laboratory technicians were blinded. We used dual-energy x-ray absorptiometry, which is the standard method of measuring bone mineral density in clinical and research settings, to analyze body composition before and after lifestyle-induced weight loss. In order to generate a homogenous study group, only middle-aged Caucasian males with MetS were included in this study. While this increased homogeneity among participants, the results therefore cannot be generalized to other ethnical groups, genders, or individuals without MetS. An additional limitation is the relatively small sample size. The observational period was 6 months, precluding conclusions on the long-term effects of weight loss on osteocalcin. In the controls, we observed decreased osteocalcin levels following the 6-month study period. As the study started in May and ended in November, a possible reason for reduced osteocalcin levels in the control group are seasonal effects. Assuming a comparable seasonal effect in the intervention group, correction of serum osteocalcin levels for a potential seasonal effect in the weight loss group would potentially result in even higher serum osteocalcin levels. As we did not observe changes in vitamin D levels, vitamin D-driven seasonal effects on bone metabolism and osteocalcin can be excluded. In addition, our study does not allow us to deduce any causal relationship between osteocalcin and CRP. Further research is necessary to study the mechanistic interaction between osteocalcin and CRP and, potentially, other inflammatory markers.

In summary, 6 months of controlled lifestyle-induced weight loss led to an increase in bone-derived osteocalcin levels, which are associated with reduced inflammation, represented by CRP measurements, in MetS. Interestingly, the increased osteocalcin serum levels were not correlated to changes in bone mineral density. Since inflammation and insulin sensitivity are linked, our study suggests that osteocalcin reflects changes in insulin sensitivity only indirectly, presumably due to concomitantly improved inflammation.

4. Materials and Methods

4.1. Research Design

The study was embedded in a prospective, two-armed, controlled, monocentric, randomized, 6-month intervention trial to identify changes in clinical and laboratory parameters in individuals with MetS following lifestyle-induced weight loss. For this purpose, paired blood samples and subcutaneous adipose tissue biopsies were collected at the Institute of Clinical Chemistry and Pathobiochemistry, Otto von Guericke University, Magdeburg, Germany. The trial was registered at the German Clinical Trials Register (ICTRP Trial Number: U1111-1158-3672) in July 2014. The trial included nonsmoking, nondiabetic men aged between 45 and 55 years with MetS, as defined by the consensus definition in

2009 [1]. Three out of five criteria needed to be met: abdominal obesity (waist circumference > 102 cm or BMI > 30 kg/m²); fasting triglyceride concentration ≥ 1.7 mmol/L (or pharmaceutical intervention); high-density lipoprotein (HDL) cholesterol < 1.00 mmol/L; fasting glucose ≥ 5.6 mmol/L (or pharmaceutical intervention); and blood pressure ≥ 130/85 mmHg or treatment for hypertension. Exclusion criteria were smoking, diabetes mellitus type 2, surgical procedure for weight loss within the previous 6 months, severe renal dysfunction (creatinine concentration >2.0 mg/dl), active liver disease, obesity of known endocrine origin, or inability to walk at least 30 min per day. Participants were recruited by an advertisement in a regional newspaper. Out of 133 individuals screened for inclusion or exclusion criteria from May 2012 to August 2012, 74 individuals were selected for the trial. All participants of the study were instructed to reduce calorie intake and maintain a low-carbohydrate diet with preference for low-glycemic index carbohydrates via structured education. The subjects were advised to increase their usual daily physical activity, such as walking or cycling, rather than to engage in particular sports. The recommendation was to perform these activities moderately but steadily, slowly enough to be able to talk at the same time, and to keep the pulse below 120/min. The key difference between the two groups was a daily telemetric report by the study participant and weekly written feedback from a doctor in the intervention arm, while the control group was only instructed once at the beginning of the study [30]. Beyond these instructions, no special diet or recommendations, e.g., for physical activities, were given. Individuals were randomly assigned to a 6-month lasting telemonitored lifestyle-induced weight loss program or a control arm by a web-based randomization tool using permuted block randomization with stratification on BMI (Randomization in Treatment Arms (RITA); University of Lübeck, Germany). A blinded investigator, who did not have any interaction with the participants during the screening and enrolment process, managed group allocation assignments. Participants of both arms were linked to an identification number and samples were collected in sequentially numbered containers. Hence, laboratory technicians were blinded regarding the origin and allocation of the samples. Participants of the intervention arm received accelerometers measuring their daily activity and instructions for daily data transmission of body weight, physical activity, and approximate caloric intake [30]. Participants of the intervention arm received regular feedback by weekly letters commenting on their individual weight progress and daily exercise-related energy expenditure. At baseline and after 6 months, subcutaneous adipose tissue biopsies and peripheral blood samples were obtained from participants of both arms. All study participants were examined after 3 months and screened for fasting blood glucose and glycated hemoglobin (HbA1c) to prevent complications secondary to progression to overt type 2 diabetes. The expression profile of microRNAs in paired blood and subcutaneous adipose tissue will be analyzed as a primary outcome (not part of this report).

4.2. Dual Energy X-ray Absorptiometry

We applied the DXA method (dual-energy X-ray absorptiometry) to determination of the bone density on the femoral neck. The relevant measurement region (ROI, region of interest) was determined and the measured radiation absorption in grams per square centimeter (g/cm²) was calculated according to an area measurement. When measuring the spine, L1–L4 are used as standard. Densitometric measurement results of an individual are compared with an age- and gender-specific control group. A normative database, as implemented by the device manufacturers in the operating software, is indispensable for the interpretation of patient results. The measured bone mineral density is given as a t value (t-score). The t-score refers to the peak bone mass divided by the standard deviation of this mean. The value is given as a unit in standard deviations. Evaluation of the bone density should only be carried out primarily on the femoral neck. DXA requires low radiation exposure, easy standardization, and availability of the method. The t-score is defined as the difference between a measured bone mineral density (BMD) and the expected normal value divided by the population standard deviation.

4.3. Clinical and Laboratory Parameters

Osteocalcin levels were measured at baseline and at the end of the study (after 6 months) in paired serum samples using a commercially available sandwich enzyme-linked immunosorbent assay (Human osteocalcin ELISA, Cat. No. RIS002, BioVendor Research and Diagnostic Products GmbH) at the Institute for Clinical Chemistry and Pathobiochemistry, Otto von Guericke University, Magdeburg. The intra-assay coefficients of variation (CVs) ranged from 3.1% to 4.7%, the interassay CVs ranged from 3.5% to 5.6%, and the lower limit of detection was 0.1 ng/mL, as according to the manufacturer. Body weight, height, waist circumference, and blood pressure were measured by qualified medical personnel according to standard operating protocols at baseline and after 6 months. Body composition was analyzed by dual-energy X-ray absorptiometry [18]. All blood samples were collected in the morning (8 a.m. to 9 a.m.) from the antecubital vein after a 12-hour overnight fast. All laboratory measurements were performed at the Institute of Clinical Chemistry and Pathobiochemistry, OvGU, Magdeburg, Germany. Fasting blood glucose, triglycerides, ALAT, ASAT, low-density lipoprotein (LDL) cholesterol, HDL cholesterol, and total cholesterol were analyzed by commercial enzymatic methods using a random-access analyzer (Modular, Roche Diagnostics, Mannheim, Germany). Lipoprotein fractions were analyzed by ultracentrifugation. Glucose was determined in sodium fluoride plasma. HbA1c was determined by high-performance liquid chromatography. Insulin was determined by 125I-radioimmunoassay (RIA) according to the manufacturer's instructions (INSULIN-CT, CIS bio, Berlin, Germany).

4.4. Statistical Analysis

Data are given as median and interquartile range (IQR). Differences between independent samples (intervention vs. control at baseline or 6 months) were analyzed by Mann–Whitney U test. Paired samples were analyzed by Wilcoxon signed-rank test. Correlations between relative changes in parameters before and after the trial were assessed by Spearman's rank correlation.

Calculations were performed using IBM SPSS Statistics, version 22.0 (IBM Corporation, Armonk, NY, USA). Results were considered significant at $p < 0.05$. The data analysis was performed using GraphPad Prism, version 8.0, R version 4.0.3., and RStudio version 1.3.959.

In order to identify which variables may influence osteocalcin metabolism, a Bayesian multiple linear regression was performed. Based on the low sample size, we decided to use Bayesian multiple regression analysis, which is a robust model. The posterior distribution of the linear regression was approximated using Markov chain Monte Carlo using the rethinking R package. The input variables were the ratio of relative changes (log ratio) between parameters before and after the trial period. The input variables were those that were significantly correlated to osteocalcin in the study (Figure 4, Supplementary Figure S1): 'BMI-change', 'Leptin-change', 'Total Muscle Mass-change', 'ALAT-change', 'CRP-change', 'Insulin-change', 'TG-change', 'GFR-change', and 'Total fat mass-change'. To avoid multicollinearity, 'Leptin-change' was removed, since it was highly associated to insulin and glucose (not shown). Similarly, 'Total fat mass' was also removed, since it is highly associated with BMI (not shown). Both control and intervention groups were used as input. The likelihood of 'osteocalcin-change' follows a normal distribution, with the mean 'osteocalcin-change' and a standard deviation. The linear model is: osteocalcin $\sim N(\alpha + \sum \beta_i x_i, \sigma)$, with parameters being: α, the intercept of the regression, which represents the change in OC when all other parameters have changes equal to zero; β_i, the slope of each variable; x_i, which represents the rate of change in the dependent variable, "osteocalcin-change", when the independent parameter changes by 1 unit. In addition, the following priors were used for the calculations: $\alpha \sim N(0,0.5)$, $\beta \sim N(0,0.4)$, and $\sigma \sim Expo(1)$. The hyperparameters were chosen to conform to a vague prior.

Supplementary Materials:

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4 Summary

Dissertation to obtain the academic degree

Dr. med.

Title: Osteocalcin is independently associated with C-reactive protein during lifestyle-induced weight loss in metabolic syndrome

submitted by:

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prepared in:

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To examine the relationship between osteocalcin, body composition and metabolic parameters during lifestyle induced weight loss, we performed a multiple linear regression analysis with BMI, lean mass, ALAT, CRP, insulin and triglycerides as independent variables using data from a weight loss trial in individuals with MetS. Our results revealed that changes in insulin, bone mass, and adipose tissue did not show independent associations with increased serum osteocalcin levels following weight loss. The only association we found to be independently related to osteocalcin was with CRP. This raises the question as to whether the previously reported associations of osteocalcin with metabolic parameters are indirect, depending primarily on the interaction of osteocalcin with inflammation. Our finding is in agreement with studies finding a link between inflammation and bone metabolism (*129–131*) and osteocalcin dependent suppression of pro-inflammatory cytokine secretion (TNF-alpha and IL6) and stimulation of anti-inflammatory compounds (*10*).

While our study strengthens the interaction between low-grade inflammation, reflected by elevated CRP levels, and osteocalcin, it was not desinged to address the causal relationship between these two

parameters. Future studies are requied to determine whether osteocalcin can regulate low-grade inflammation directly or vice versa and whether the association of osteocalcin with systemic metabolism depends on an anti-inflammatory effect.

Our finding is supported by previous studies. Thus, in a population-based study to assess the risk of non-traumatic fractures, osteocalcin as a marker of bone turnover was inversely correlated with CRP (*132*). Hence, the current observation corroborates the suspected relationship between inflammation and osteocalcin.

One possible explanation for the increased osteocalcin levels is increased physical activity, which was an important part of the 6-month weight loss intervention study. While weight loss induced by calorie restriction leads to a decrease in bone mineral density, exercise induced weight loss prevents demineralization of the bone (*133*). Given that osteocalcin is exclusively produced and secreted by osteoblasts, it could be assumed that the increase in osteocalcin is related to weight loss-induced changes in the bone. We performed DXA scan analysis to assess body composition before and after lifestyle-induced weight loss and to elucidate a possible relationship between bone mass and osteocalcin. In line with other weight loss studies where exercise is an integral part (*134*), we observed increased bone mineral density along with reduced fat and lean tissue mass after lifestyle-induced weight loss. Given the increased bone mass after lifestyle-induced weight loss, we expected to find an association between changes of bone mass and changes of osteocalcin levels. However, the only independent association with osteocalcin was with CRP, thereby paving the way for a novel interrelation of bone metabolism and low-grade inflammation in MetS.

Since the fat tissue is highly related and interconnected with chronic low-grade inflammation in MetS by contributing to cytokine secretion, we assumed a major relationship between fat tissue and osteocalcin levels (*135–137*). The mechanisms linking adiposity to low osteocalcin levels are not fully understood. Leptin-deficient ob/ob and leptin-resistant db/db mice have elevated osteocalcin levels (*138*). Next to its inflammatory action, leptin has been shown to regulate bone remodeling in osteoblasts (*139*). Interestingly, leptin does not only derive from adipose tissue, but also from the bone itself (*140, 141*). In adult human bone, leptin is expressed in chondrocytes, stromal cells and bone lining cells (*142*). Leptin appears to be expressed only in the cells of the osteogenic lineage, which are permanent and inactive. Those cells belog to the terminology of the "bone basic cellular system (BBCS)", which is primarily involved in regulating mechanical strains as well as in managing bone remodeling processes. Therefore, it appears likely, that leptin is able to modulate those processes.

Hence, a complex communication network between bone and adipose tissue in metabolic disorders has been suggested. It has been reported that increased bone mineral density and increased osteocalcin are correlated with decreased insulin levels in obese children after weight loss, suggesting a regulatory hormonal loop including osteocalcin and insulin (*143*). Lee et al. recently demonstrated in a murine

model that bone regulates the insulin-glucose signal axis and energy metabolism by secreting osteocalcin, indicating a link between osteocalcin and insulin metabolism and blood sugar (*3*). Analysis of osteocalcin$^{-/-}$ mice revealed an increase in visceral fat mass as well as hyperglycaemia and hypoinsulinemia and reduced β-cell mass (*6*). Similar information was obtained recently in a human cross-sectional study. Circulating osteocalcin was linked to insulin sensitivity and to insulin metabolism. Post weight loss serum osteocalcin levels were associated with both insulin sensitivity and fasting triglycerides in this non-diabetic weight loss cohort. Hence, circulating osteocalcin levels were suggested to reflect the metabolic role of the bone during adiposity (*144*). However, our findings imply that increased osteocalcin levels following lifestyle-induced weight loss are primarily associated with reduced low-grade inflammation, while improved peripheral insulin sensitivity and liver parameters may be secondary to these changes. Perspectively, we can envision examining low osteocalcin as a potential biomarker in MetS. It is expected to increase upon successful weight loss, as osteocalcin also increases after bariatric surgery (*145*).

A role of low osteocalcin concentrations as a biomarker for MetS and low-grade inflammation has been suggested (*146*). Recent studies have reported possible associations between osteocalcin and adiposity and glucose metabolism (*147, 148*). Therapeutic application of osteocalcin in a mouse model improves components of the MetS (*8*). Daily injections of osteocalcin significantly improved glucose tolerance, insulin sensitivity, β-cell mass and insulin metabolism in mice fed a normal diet. Additionally, these mice showed increase of muscle mitochondria, increased energy expenditure, showed healed hepatic steatosis and were less obese. These studies suggest a prospective therapeutic role of osteocalcin.

Our finding, that osteocalcin is not primarily associated with metabolic parameters but with inflammation in MetS, challenges the current understanding that osteocalcin has a direct impact on metabolism. Based on our current finding we propose that osteocalcin and inflammation are primarily linked, which in turn has an impact on metabolism. Future studies, including analyses of genetically modified mice, are needed to dissect the causality and the underlying mechanisms.

Strengths of our study include a well-characterized study population with no differences at baseline, a prospective study design, and a high compliance of participants as a result of daily telemonitoring and weekly letters commenting on individual weight progress. In order to generate a homogenous study group, only middle-aged Caucasian males with MetS were included in this study. All laboratory measurements were performed following standard operating protocols and laboratory technicians were blinded. We used dual-energy x-ray absorptiometry, which is the standard method of measuring bone mineral density in clinical and research settings, to analyze body composition before and after lifestyle-induced weight loss. The observational period was 6 months, precluding conclusions on the long-term effects of weight loss on osteocalcin. In the controls, we observed decreased osteocalcin levels following the 6-month study period. As the study started in May and ended in November, a possible reason for

reduced osteocalcin levels in the control group are seasonal effects. Assuming a comparable seasonal effect in the intervention group, correction of serum osteocalcin levels for a possible seasonal effect in the weight loss group would potentially result in even higher serum osteocalcin levels. As we did not observe changes in vitamin D levels, vitamin D-driven seasonal effects on bone metabolism and osteocalcin can be excluded. In addition, our study does not allow us to deduce any causal relationship between osteocalcin and CRP. Further research is necessary to study the mechanistic interaction between osteocalcin and CRP and, potentially, other inflammatory markers.

In summary, 6 months of controlled weight loss study resulted in an increase in bone-derived osteocalcin levels associated with the reduced inflammation represented by CRP measurements in individuals with MetS. Interestingly, osteocalcin changes were not correlated with changes in bone mineral density and in a linear regression analysis osteocalcin was no longer associated with metabolic markers, while the association with low-grade inflammation remained. Since inflammation and insulin sensitivity are linked, our study suggests that osteocalcin reflects changes in insulin sensitivity only indirectly, presumably due to concomitantly improved inflammation.

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6 Supplementary data

2.4. Supplemental Figures

Supplementary Table S1. Clinical parameters of participants in the control and intervention groups before and after the study period. Data are presented as median (interquartile range). The Wilcoxon signed-rank test was used to analyze differences between paired samples with *n* = 33 intervention, *n* = 30 control, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001. No differences were found between treatment group vs. control group at baseline, analyzed by Mann–Whitney U test. Some parameters (indicated by [1]) were published in a previous study [18]. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; HbA1c, hemoglobin A1c; CRP, C-reactive protein; IL6, interleukin 6; ALAT, alanine aminotransferase; 25OHVD, 25-hydroxyvitamin D; GFR, glomerular filtration rate.

	Control Group		Intervention Group	
	0 Month	**6 Month**	**0 Month**	**6 Month**
Age (years) [1]	48 (43,75–51,25)		48 (45–51,5) n.s.	
BMI [1]	33,4 (31,7–37,1)	33,6 (32,4–35,8) n.s.	33,0 (31,2–35,8)	29,0 (27,3–31,4) ***
Waist circumference (cm)	114 (111–126,25)	114 (105,5–123) n.s.	116 (107,5–123,5)	105 (97–110) ***
LDL cholesterol (mmol/L) [1]	3,4 (3,0–4,4)	3,2 (2,7–4,1) n.s.	3,7 (3,1–4,7)	3,4 (2,4–4,1) **
HDL cholesterol (mmol/L) [1]	1,3 (1,1–1,5)	1,4 (1,1–1,5) n.s.	1,2 (1,0–1,4)	1,4 (1,2–1,5) ***
TG (mmol/L) [1]	2,0 (1,5–2,6)	2,0 (1,5–3,6) n.s.	2,1 (1,4–3,9)	1,4 (1,1–2,0) ***
HbA1c (%)	5,6 (5,3–5,9)	5,6 (5,3–5,8) n.s.	5,6 (5,3–6,05)	5,4 (5,2–5,6) ***
Insulin (pmol/L) [1]	62,5 (50–88,5)	61 (47–90,25) n.s.	88 (59–134,5)	47 (25–70) ***
Leptin (ng/mL)	10,9 (8,3–20,2)	13,4 (8,1–18,3) n.s.	12,5 (8,2–17,0)	5,6 (3,0–8,4) ***
CRP (mg/L)	3,0 (1,3–5,5)	2,5 (0,8–4,6) n.s.	3,0 (1,6–5,9)	1,1 (0,6–2,3) ***
IL6 (pg/mL)	2,1 (1,8–3,0)	2,5 (1,7–3,5) n.s.	2,6 (1,9–4,0)	2,0 (1,6–2,4) *
ALAT (μmol/s*L)	0,7 (0,5–1,1)	0,7 (0,5–1,1) n.s.	0,6 (0,5–1,0)	0,4 (0,3–0,5) ***
25OHVD (ng/mL)	22,3 (17,3–28,8)	23,6 (21,2–27,7) n.s.	22,4 (17,4–30,4)	24,2 (20–32,9) n.s.
GFR (mL/min/1.73m²)	95,0 (83,75–102,25)	89,5 (80,0–98,25) *	100,0 (93,0–106,0)	97,0 (92,5–104,5) n.s.
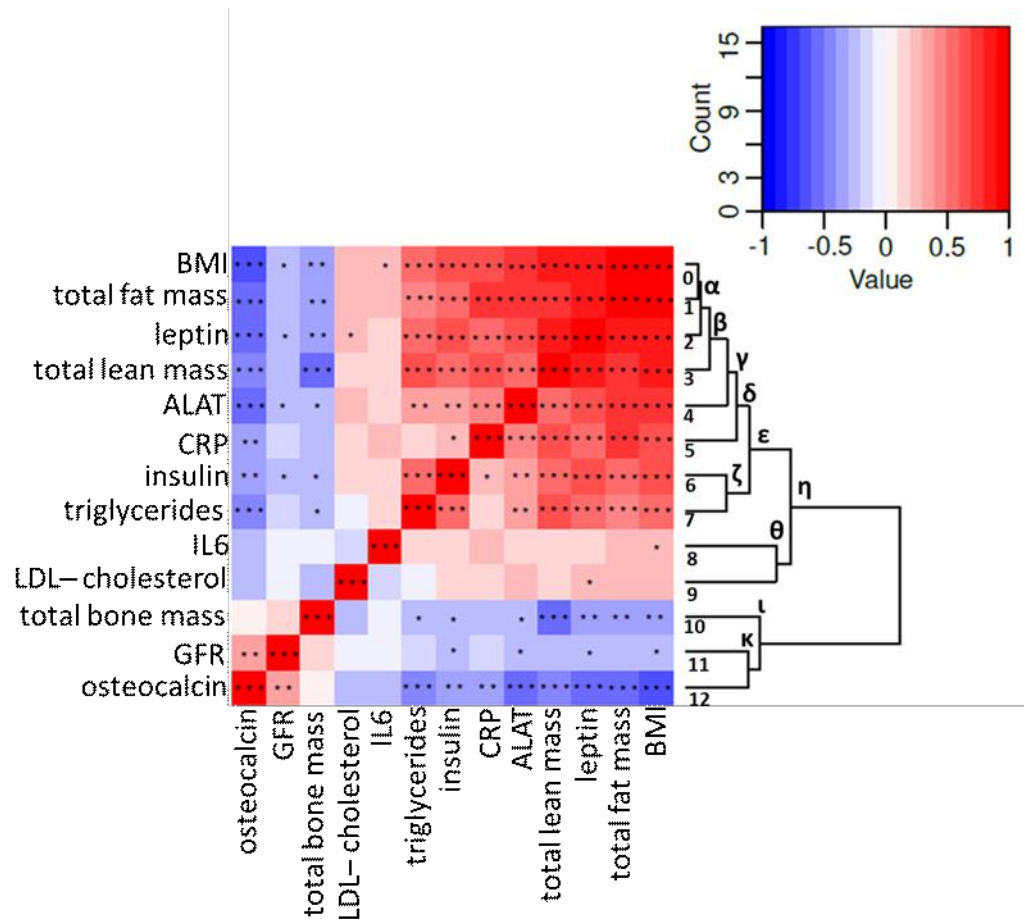
Supplementary Figure S1. Correlation between OC, metabolic markers, body composition, and inflammation during the 6-month intervention in both groups. The dendrogram is a branching diagram that represents the relationships of similarity among a group of entities. Each branch is called a clade (α–κ). The terminal end of each clade is called a leaf. Clades can have just one leaf (these are called simplicifolious) or they can have more than one. Two-leaved clades are bifolious. The arrangement of the clades explains which leaves (parameters) are most similar to each other. The height of the branch points indicates how similar or different they are from each other: the greater the height, the greater the difference. The term chunk represents each segment of the dendrogram at the parameter level, here 0–12. CRP, C-reactive protein; ALAT, alanine aminotransferase; BMI, body mass index; IL6, interleukin 6; LDL, low-density lipoprotein; GFR, glomerular filtration rate.

7 Declaration about the independent preparation of the work

Declaration about the independent preparation of the work.

I hereby declare that I have completed the present work independently and without undue assistance or using other aids than those specified. I guarantee that no third parties neither directly nor indirectly benefit from a remuneration or monetary benefits in connection with the content of the submitted dissertation and that the submitted work has not been submitted elsewhere, neither in Germany nor abroad in the same or similar form of another examination authority for the purpose of a doctorate or another examination procedure. Material adopted from other sources and from other people that were used in the work or are directly related, has been marked as such. In particular all people who were directly involved in the creation of the present work were mentioned. The current legal requirements with regard to the approval of clinical studies, the provisions of the Animal Welfare Act, the provisions of the Genetic Engineering Act and the general Data protection regulations have been complied with. I also assure that I have received the regulations of the Leipzig University's statutes to ensure good scientific practice and have complied with them.

8 Presentation of own contribution

Silke Zimmermann is the first author of the original publication, which has been published in the peer-reviewed journal "Metabolites". She is responsible for the data analysis and data interpretation. She was instrumental in the compilation of the results and the manuscript involved.

I agree with the submission of the thesis and included publication according to the doctoral regulations of the university Leipzig, Faculty of Medicine. I hereby confirm the marked contribution of the doctoral student.

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9 Curriculum vitae

The curriculum vitae is taken out for publication on the publication server of the University of Leipzig. The author is following the recommendation of the Leipzig University Library.

If masculine personal names are used in the dissertation, they always refer to male and female persons.

10 Publications

- **Zimmermann S**; Walter Costa MB; Mathew A; Krishnan S; Schneider JG; Roomp K; Isermann B; Biemann R. Osteocalcin Is Independently Associated with C-Reactive Protein during Lifestyle-Induced Weight Loss in Metabolic Syndrome. Metabolites 2021, 11, 526. https://doi.org/10.3390/metabo11080526
- **Zimmermann S**; Federbusch M; Isermann B; Kohli S. Vaccine induced thrombotic thrombocytopenia: Insights from blood smear (submitted in Thrombosis and Haemostasis, revised)
- Perner C, Perner F, Gaur N, **Zimmermann S,** Witte OW, Heidel FH, Grosskreutz J, Prell T. Plasma VCAM1 levels correlate with disease severity in Parkinson's disease. J Neuroinflammation. 2019 May 8;16(1):94
- Madhusudhan T, Ghosh S, Wang H, Dong W, Gupta D, Elwakiel A, Stoyanov S, Al-Dabet MM, Krishnan S, Biemann R, Nazir S, **Zimmermann S**, Mathew A, Gadi I, Rana R, Zeng-Brouwers J, Moeller MJ, Schaefer L, Esmon CT, Kohli S, Reiser J, Rezaie AR, Ruf W, Isermann B. Podocyte Integrin-β_3 and Activated Protein C Coordinately Restrict RhoA Signaling and Ameliorate Diabetic Nephropathy. J Am Soc Nephrol. 2020 Aug;31(8):1762-1780
- Reinicke M, **Zimmermann S,** Begcevic Brkovic I, Wassermann C, Landmann J, Leyh J, Bechmann I, Ceglarek U. Obesity and diet-related influences of cerebral phytosterol concentrations on inflammatory microglial response (submitted)
- Al-Dabet M, Shahzad K, Elwakiel A, Sulaj A, Kopf S, Bock F, Gadi I, **Zimmermann S**, Rana R, Krishnan S, Gupta D, Nazir S, Baber R, Scholz M, Geffers R, Mertens PR, Nawroth PP, Griffin J, Dockendorff C, Kohli S, Isermann B. Reversal of hyperglycemic memory by targeting epigenetically sustained renal p21 expression; (submitted in Nature communications)

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